# PROLACTIN AND REPRODUCTION

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Submission for the Degree of D.Sc.,

Prolactin and Reproduction

Secretary to the University of Edinburgh.

Dear Sir,

I wish to submit the enclosed thesis for consideration for the degree of D.Sc. These publications have not been submitted in whole or in part for any other degree.

I certify that the works in the thesis are my own. My own part in collaborative research projects are indicated in each section.

Yours sincerely,

Alan S. McNeilly.

19.8.83.

PROLACTIN AND REPRODUCTION

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# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

*Name of Candidate* DR. ALAN SWAIN McNEILLY

*Address* ..

*Degree* D.Sc., *Date* 6.7.83.

*Title of Thesis* Prolactin and Reproduction


This thesis covers papers published over an 11 year period from 1971-1983. Research was undertaken to determine whether (i) prolactin was essential for normal ovarian and testicular function and (ii) high levels of prolactin that occurred as a clinical pathological state and physiologically in breast feeding and seasonal anoestrous were implicated in the associated suppression of reproductive function.

To enable this research to proceed required reliable assay methods for prolactin and gonadotrophins in all species studied and investigations into the control of gonadotrophin secretion and ovarian function to provide the basic background in which to explore the role of prolactin. The radioimmunoassays I developed for prolactin and gonadotrophins have been used in all investigations reported in this thesis and have been utilized world-wide.

In man it was thought that LHRH released both LH and FSH and it was used, subsequently, in the treatment of hypogonadal states. The essential role of LHRH in maintaining gonadotrophin secretion was shown by immunoneutralization studies in the ewe while studies on the gonadotrophin subunits showed the common $\alpha$ subunit to always be present in both pituitary and plasma while LHRH appeared to control $\beta$ subunit formation. The essential role of pulsatile LH secretion in controlling ovulation was also established and confirmed when ovulation was induced by pulsatile hormone administration in anovulatory women and sheep.

Both in vivo and in vitro studies indicated in women and other species that prolactin was essential, with LH and FSH, for normal ovarian function. Hyperprolactinaemia induced in the male rat suppressed gonadotrophin secretion by increasing hypothalamic sensitivity to the negative feedback effects of gonadal steroids. A similar increased sensitivity was shown in breast feeding women and led to a major study on the causes of lactational infertility in women.

This was the first large scale longitudinal study in breast feeding women and it established that suppression of ovulation post partum was dependent on both suckling frequency and duration. Resumption of ovulation only occurred when either or both suckling parameters were reduced. Parallel studies showed the importance of suckling patterns and the milk ejection reflex in establishing and maintaining lactation and emphasized the need for on-going support for breast feeding mothers.

Introduction

This thesis covers the majority of my research papers published over an eleven year period from 1972-1983. Papers published during this time but not included in this thesis have been listed in an appendix.

After completing my Ph.D., in 1971 under the late Professor S.J. Folley at the NIRD, Reading, U.K., my interest in the role of prolactin in the control of gonadotrophin secretion and ovarian and testicular function was stimulated when I was in the Department of Obstetrics and Gynaecology, St. Bartholomew's Hospital, London. Before starting these investigations I had to establish reliable radioimmunoassay methods for the measurement of human LH, FSH and prolactin. I then collaborated with several clinical colleagues in investigations into the role of prolactin both in physiological and pathological situations. As a scientist it was clearly not possible for me to be actively involved in patient management but in all other aspects I provided the only scientific input. I carried out several studies on the role of prolactin in the menstrual cycle, ovarian function and pregnancy and initiated and collaborated in a series of studies on the role of gonadotrophin subunits.

I was able to continue research in animals with other colleagues into the more basic aspects of prolactin and gonadotrophin secretion.

To further these investigations I spent one year with Dr Henry Friesen in Winnipeg, Canada where I developed techniques for measuring prolactin and gonadotrophin receptors in the ovary and mammary gland.

With this background I joined the staff of the MRC Reproductive Biology Unit, Edinburgh, under the Director, Professor Roger Short, in 1976 and have continued both basic research in animals and clinical research into the interactions between prolactin and the gonadotrophins. Much of the clinical research has been carried out in collaboration with my clinical colleagues Professors David Baird and Peter Howie. I have concentrated, in particular, on the mechanisms controlling ovarian function in the breeding season and anoestrus in the ewe and the effects of breast feeding on the

resumption of fertility post partum in women.

I have been involved personally in all the studies reported in this thesis and have collaborated with a number of people whose names are acknowledged in the authorship of the papers. The principal author whose name occurs first in the list of authors was normally responsible for the organization of the project and the writing of the manuscript. In several clinical papers, however, the principal author was the clinician in charge of the patients involved in the study. In these papers I was always involved in, if not the instigator, of the research protocols and responsible for the analysis of the results.

I have indicated the role I played in the studies reported in the following sections.

SECTION 1: DEVELOPMENT OF METHODS

The study of endocrinology depends on the availability of specific, sensitive and reliable methods for the measurement of hormones both at the site of secretion and in the circulation. In most instances the method of choice is radioimmunoassay. Many of the studies I have undertaken have depended on the development of specific radioimmunoassays and in one instance it was required that I first prepared pure hormones before assay development.

a) Assays for human prolactin, gonadotrophins and gonadotrophin subunits

Human prolactin was first purified in 1970 and was available only in extremely limited quantities in 1971. Following a visit to Dr. Henry Friesen's Laboratory in Montreal, Canada in 1971, I developed a reliable radioimmunoassay for human prolactin based on antiserum I had raised (1,2). At the same time I developed reliable radioimmunoassays for human LH and human FSH (2) which, together with the human prolactin assay have been used throughout all my studies on the role of prolactin in human reproduction. More recently I have adapted the method for human LH to give a rapid assay essential for the detection of the preovulatory LH surge in women (3).

To enable studies on the role of gonadotrophin subunits (see Section 3b)) Dr. Claus Hagen and I developed specific radioimmunoassays for the common $\alpha$ subunit and for the hormone specific $\beta$ subunits of LH and hCG (4,5). A specific bioassay for LH was also developed in collaboration with Drs. Kramer, Holdaway and Rees while I was at St. Bartholomew's Hospital (6,7,8).

b) Assays for prolactin and gonadotrophins in other species

To allow studies on prolactin in the goat Dr. Pat Andrews and I first purified goat prolactin and then showed it to be immunologically similar to ovine prolactin (9). Goat growth hormone purified at the same time as goat prolactin (9) was used to develop a specific goat growth hormone radioimmunoassay (10).

To investigate the interactions between prolactin and the gonadotrophins in sheep it became essential to measure FSH. As no reliable method was available Dr. Judy McNeilly and I developed a

specific and sensitive heterologous radioimmunoassay for ovine FSH (11). This assay is now used extensively throughout the world to measure FSH not only in sheep but in many other species including my own work in the cow (12) and elephant (13,14).

A similar situation arose when I was investigating changes in ovarian and mammary prolactin receptors in the rabbit. No method was available to measure rabbit prolactin. Hence I developed a heterologous radioimmunoassay which proved suitable not only for the rabbit (15) but also for the measurement of prolactin in the marmoset (16), red deer (17) and elephant (13). After initial work on my behalf this assay has also been applied to and is the only method available for measuring prolactin in many other species.

I also developed a similar radioimmunoassay for avian prolactin (18,19) which is also the only method available to other workers for measuring prolactin in a large spectrum of avian species.

SECTION 2: PROLACTIN

a) Control of secretion

With the development of reliable assay methods as detailed previously I collaborated in several clinical and animal studies which investigated the nature of the control of prolactin secretion. Previous reports had indicated that a synthetic ergot derivative, 2 bromergocryptine, CB154, made by Sandoz, Switzerland, inhibited prolactin secretion in the rat. Working with Professor Michael Besser at St. Bartholomew's Hospital, London, in 1971, we showed that patients with galactorrhoea and amenorrhoea had elevated plasma levels of prolactin as measured by my radioimmunoassay and that treatment with CB154 effected a cure with a reduction in prolactin, cessation of galactorrhoea and resumption of menstrual cycles (20,21). Subsequent studies have shown that CB154 is a dopamine agonist which is effective in all species investigated including my own studies in the rabbit (15) and marmoset (16) and that dopamine is the principal inhibitor of prolactin secretion.

In the course of other clinical studies I discovered that metoclopramide was a potent releaser of prolactin (22). This has subsequently been identified as a dopamine receptor antagonist and has been used both as a method of assessing pituitary prolactin reserve and to induce long-term hyperprolactinaemia as an investigative tool. The degree of hyperprolactinaemia induced does not correlate directly with the dose of drug given and this was explained to a major extent when we showed in studies using chlorpromazine that it was correlated to the blood level of the drug (23).

However, although hypothalamic inhibition appears to be a major control of prolactin secretion, it was also clear that other factors, in particular stimulators or releasing factors were also involved. In the short-term, blood levels of prolactin in both men (24) and women (25) showed minute by minute changes which, although related to sleep did not appear to correlate with changes in other hormones (26). Other workers had shown that Thyrotrophin Releasing Hormone (TRH) caused the release of prolactin as well as Thyroid Stimulating Hormone (TSH) and might be a

physiological prolactin releasing factor. In collaboration with Drs. Mortimer and Besser we confirmed that both single injections and infusions of TRH would release prolactin in both men (27,28,29) and women (2) independently of stimulators of the other anterior pituitary hormones (27). This latter observation led to the development of the combined test of total pituitary function using TRH, LHRH and insulin hypoglycaemia as a single test. Subsequently I showed in collaboration with Dr. Gilmore that the prolactin releasing activity in the human foetal hypothalamus could not be accounted for by TRH (30). This stimulated a long-term study with Dr. Fraser where we actively immunized ewes against TRH to induce immunoneutralization. High titre antibodies were induced leading to a substantial reduction in thyroid hormone secretion and clearly indicating that TRH was important in the control of thyroid secretion (31). However, there was little or no effect on prolactin secretion at any stage of the reproductive cycle in the ewe suggesting that TRH probably forms only a minor component of the overall control of prolactin secretion (32) and does not explain the increase in prolactin levels occurring in response to light (33).

Increased prolactin secretion has been shown during epidural anaesthesia (34), in patients with renal failure (35,36) and during pregnancy in women with pre-eclampsia (37) while lower than normal levels of prolactin were found in patients with anorexia nervosa (38,39). On the other hand, we failed to show any alteration in prolactin secretion in patients with breast cancer (40).

b) Prolactin and ovarian function

Although prolactin in essential for normal luteal function in rodents, the role of prolactin in ovarian function in other species remains unclear. In one of the earliest studies I failed to demonstrate any significant changes in plasma levels of prolactin during the menstrual cycle (41,42,43,44) although we showed an inverse relationship between prolactin and progesterone levels in follicular fluid of both human (45,46) and bovine (47) ovarian follicles implying that prolactin was involved in the control of steroidogenesis in both the human and cow. This was subsequently confirmed both by *in vitro* experiments with

human granulosa cells (45,48,49) and the identification of specific receptors for prolactin on the human corpus luteum (50). Recently we have shown conclusively that there is a significant increase in prolactin associated with the preovulatory surge of LH (49) indicating altered hypothalamic catecholamine secretion associated with LHRH secretion at midcycle in women.

These data on changes in prolactin in the normal menstrual cycle proved the basis for understanding the effects of hyperprolactinaemia occurring either pathologically or as a result of breastfeeding. The latter is dealt with in a later section (Section 4c)). While hyperprolactinaemia is clearly associated with amenorrhoea (20,21,51) and increased secretion of adrenal androgen secretion (52) we showed that CB154 therapy was effective in restoring normoprolactinaemia and fertility (21). Since the precise role of prolactin in this syndrome remained unclear, I began a series of studies using the sheep in which we had previously demonstrated that the period of anoestrus was also associated with hyperprolactinaemia (53). However, CB154 treatment, while successfully lowering prolactin levels, did not restore normal ovulation nor ovarian activity in anoestrous ewes (54,55,56). Pulsatile LH secretion was also reduced in anoestrous but was essential for normal follicular development and ovulation in the breeding season (57,58). Thus we induced ovulation in anoestrous by giving pulsatile injections of physiological amounts of LH (59). These results have led me to suggest that the increase in prolactin is only incidental to the cessation of ovarian activity which results primarily from a suppresion of hypothalamic LHRH secretion (60). This may also be the case in the majority of situations where high levels of prolactin are associated with reduced or absent ovarian function (also see Section 4c)). Such a conclusion is supported by studies in subordinate marmoset monkeys which have normal levels of prolactin but remain infertile (61).

c) Prolactin in the male

While sheep proved an excellent model for studying the role of prolactin and ovarian function, it was not suitable at that time for more acute studies on the possible effects of hyperprolactinaemia on hypothalamo-pituitary function. Accordingly I initiated a series of

studies using as a model the adult male rat with donar pituitary transplants under the kidney capsule to induce chronic long term hyperprolactinaemia. Hyperprolactinaemia induced in this way induced a prolonged suppression of gonadotrophin secretion (62) which was dependent on the presence of the testes but not the adrenal glands (63). The clear reduction in plasma and pituitary levels of LH and FSH resulted from a presumptive decrease in hypothalamic LHRH secretion (62) resulting in a decrease in pituitary LHRH receptors (64). Subsequently I showed that the chronically raised levels of prolactin increased hypothalamic sensitivity to the negative feedback effects of testosterone (65) by a mechanism independent of the pineal (66).

In studies on testicular function in these rats we showed that prolactin increased testicular LH receptors (67,68) it did not prevent LH-induced down regulation of these receptors (69,70). It was clear, however, that normal levels of prolactin formed part of the complex controlling steroidogenesis and spermatogenesis in the rat (71,72).

SECTION 3: GONADOTROPHINS

a) Control of secretion

The identification in 1970 of the hypothalamic peptide LHRH which controlled LH secretion stimulated a series of clinical studies at St. Bartholomew's Hospital. I was the only scientific member of the team involved. Initially, we showed that LHRH was a potent releaser of FSH as well as LH i.e. that there was only one gonadotrophin releasing hormone which did not release any other hormones (73). Subsequently a simple LHRH test was developed which is now in routine use for the investigation of pituitary reserve in the investigation of infertility (2,74,75) which we have subsequently extended in Edinburgh by the use of a long-acting analogue of LHRH (76,77). Absent or poor LH and FSH responses to the LHRH test indicated a specific deficiency in hypothalamic LHRH secretion in patients with hypogonadotrophic hypogonadism (28,38) with an absence of normal pulsatile secretion (77). This led us to use pulsed injections of large doses of LHRH to induce resumption of testicular function in men and ovulation in women (78,79,80,81) some years before the present therapies using more frequent lower dose injections of LHRH (82).

In parallel with the investigations using LHRH, studies were continued on the normal control of gonadotrophin secretion. Using an immature rat model we demonstrated that an increase in adrenal progesterone secretion was essential in contrast to other species, for the induction of a normal preovulatory surge in the rat (83,84). In both the sheep (57) and women (44) we showed that the increase in follicular phase oestrogen levels occurred in response to an increase in the pulsatile secretion of LH which ultimately resulted in the preovulatory surge (3,85). These results were essential in our understanding of the absence of sustained follicular development in periods of ovarian inactivity both clinically and during anoestrous in the ewe and allowed the development of treatments to induce ovulation based on the physiological changes (e.g. 59,82). Immunoneutralization studies in sheep confirmed that pulsatile secretion of LH was due to secretion of hypothalamic LHRH (58,85,86) and that the preovulatory surge in the ewe was also totally dependent

on LHRH (86,87). These studies have led to the development of important concepts on the regulation of LHRH secretion and the implications for control of reproduction (see 88,89).

b) Studies on gonadotrophin subunits

Studies on the chemical composition on the glycoprotein hormones Thyroid stimulating hormone (TSH), LH and FSH from the pituitary and human chorionic gonadotrophin (hCG) showed them to consist of a common $\alpha$ subunit and a hormone specific $\beta$ subunit. While both the pituitary and placenta had previously been shown to contain free subunit as well as the intact hormones (see 90) almost nothing was known about the control of secretion or the circulating levels, if any of these subunits. After developing specific radioimmunoassays for the intact hormones and the subunits (see Section 1) Claus Hagen and I identified only the free $\alpha$ subunit in plasma and ovarian follicular fluid during the menstrual cycle (91) the levels of which changed in parallel with LH and FSH. However during pregnancy, levels of free $\alpha$ subunit were substantially increased in both maternal and foetal plasma (92,93,94) the former being derived from the placenta (94) and the latter from the foetal pituitary (92). While foetal pituitaries from early gestation contained substantial amounts of free subunit but little intact hormone, towards term the proportion of intact LH and FSH to $\alpha$ subunit increased to be equivalent to that in the adult pituitary (92,93) this change occurring in association with the appearance of LHRH in the foetal hypothalamus (95,96). We suggested from these results that LHRH acted on the pituitary gonadotrophins to modulate LH and FSH secretion by altering the synthesis of the B subunits of LH and FSH (90,97).

SECTION 4: LACTATION

The principal aim of this area of research was to understand the mechanism whereby suckling induced infertility postpartum in women and in particular to investigate the role of prolactin. However in addition to suppressing ovarian activity (see 98) suckling also caused the release of prolactin to maintain milk production and oxytocin to cause milk ejection and make the milk available to the baby (see 99). It became apparent that there was a severe lack of knowledge in the clinical situation in all three areas. Since lactational infertility was entirely dependent on the maintenance of lactation it became essential to also investigate the role of both prolactin in milk production and oxytocin in milk ejection. Throughout these studies I was the principal scientific investigator.

a) Prolactin and milk production

Since the control of milk production and mammary physiology is best understood in animals and, in particular the rabbit, I began studies in the rabbit by first developing a radioimmunoassay suitable for measuring prolactin in rabbit plasma as an essential pre-requisite since no method was then available (see Section 1). High prolactin levels following conception fell towards mid pregnancy and only increased again at term when mammary development was almost complete (100). Prolactin receptors were detected early in mammary development but the numbers only increased after parturition (101). During lactation we were able to show that measurement of prolactin binding milk fat globule membranes from milk allowed a non-invasive assessment of mammary prolactin binding applicable to the clinical situation (102). These studies suggested that mammary development occurred principally in response to steroid and not prolactin or placental lactogen stimulation during pregnancy whereas postpartum prolactin was essential for milk production by increasing prolactin receptors on the mammary gland.

In women, in contrast to the rabbit, prolactin levels increase progressively throughout pregnancy (103) and are high at the time of delivery. However lactogenesis only begins postpartum after the decline in placental steroid levels, with a significant increase in

milk production occurring by day 3 post delivery (104). Although breast-engorgement occurs at this time in the majority non breast feeding mothers, we found no hormonal correlates to explain the varying degrees of severity of engorgement (105). The volume of milk obtained by the baby during suckling in the first few days postpartum was independent of the amount of prolactin released during suckling (104) but was dependent on the baby itself (106). Having developed a simple test weighing procedure to measure milk volume intake which has now been adopted world-wide (107) we also showed that mothers giving high milk volumes in the first week of lactation continued to breast feed for longer than mothers who gave smaller amounts (108). Larger milk volumes were associated with more frequent and longer duration suckling and with higher social class mothers (108).

b) Oxytocin and milk ejection

In the late 1960's it was thought that the oxytocin released in response to suckling was responsible in part for the concommitant release of prolactin. However, while confirming that oxytocin was released during partuition in goats (109,110) and guinea-pigs (111), prolactin release was totally independent of these changes in oxytocin (112). Not only did we show that the mid brain neural pathway for oxytocin release in the goat was independent of the prolactin pathway (113), CB 154 induced blockade of the prolactin response to suckling in the rat did not effect oxytocin release (114). These results confirmed that oxytocin and prolactin secretion normally occurred independently of one another.

We have subsequently confirmed this in women by showing that substantial oxytocin release will occur in response to stimuli from the baby before the start of suckling in the absence of any change in prolactin (115). We also observed that periodic spontaneous milk ejection can occur during lactation independently of any specific suckling or baby derived stimuli (116). Since oxytocin release is easily inhibited by stress, our results were of immediate clinical importance emphasizing that breast feeding mothers should be exposed to the minimum of stressful stimuli before suckling.

The results of our studies show that physiologically and

endocrinologically all mothers are capable of breast feeding for a prolonged period but that social factors play an over-riding part in the maintenance of breast feeding. Our data on the endocrinology of milk production both in terms of prolactin and milk production and oxytocin and milk ejection have laid the foundation for proper advice to be given to help breast feeding mothers, while studies on the influence of social class on the continuation of breast feeding have already had a significant impact on clinical practice.

c) Lactational Infertility

In the early 1970's there was ample epidemiological evidence that breast feeding delayed the resumption of fertility in women. However, the mechanisms involved were not understood (see 98). In an early short term study we showed that suckling effectively suppressed ovarian oestrogen secretion (117) and prevented the positive feedback action of oestrogen in inducing the preovulatory surge of LH up to 100 days of lactation (118). These studies suggested possible mechanisms whereby suckling effected ovarian function which were further evaluated in a cross sectional study in breast feeding African women (119).

It was clear, however, that no true understanding of the effects of suckling could be achieved without a proper evaluation of the suckling and ovarian activity on a daily or weekly basis in individual subjects followed from delivery through to the resumption of normal menstrual cyclicity. On the arrival of Dr. Peter Howie, a consultant Obstetrician and Gynaecologist in the MRC Unit of Reproductive Biology, we initiated a series of longitudinal studies in breast feeding women, Peter providing the clinical input while I undertook all the scientific aspects of the research. The methods we have developed have been adopted world wide.

Our results show that breast feeding can suppress fertility and maintain prolonged periods of amenorrhoea (120). Suppression is dependent on the maintenance of both the suckling frequency and, as we discovered, the suckling duration (120,121) and any reduction in either parameter of suckling, caused, for instance, by introduction of supplementary food, precipitated a resumption of ovarian activity

(120,121,122,123). Resumption of ovarian activity was associated with a series of inadequate luteal phases (120,124) explaining the reduced fertility of breast feeding mothers who resume menstruation during lactation. Pregnancy only occurred in women when luteal function returned to normal following a significant reduction in breast feeding (125). Studies on the endocrinology of the suckling-induced suppression of ovarian activity have suggested that there is a significant reduction in normal pulsatile LH secretion as a consequence of reduced hypothalamic LHRH release (89) and results of these studies will be published shortly.

The unique feature of our studies has been its longitudinal nature in individuals. This has allowed a proper understanding of why breast feeding is fully effective in some individuals and not in others. Our results have stimulated world wide interest and our study procedures are presently being utilized in large studies in many parts of the world. We have reviewed the implications of our work (126).

While the major part of my studies have involved lactational infertility in women, I have also shown that in the marmoset, a new world monkey which does not have a period of suckling-induced lactational infertility, (127) nevertheless, has raised plasma levels of prolactin throughout lactation (16). This implies that suckling may act independently of prolactin in inhibiting reproduction (128). This was further emphasized by studies in red deer which showed that the level of nutrition in lactation effected the availability of milk which in turn altered suckling activity of the young and, as a consequence, prolactin secretion (17), a new concept with important implications in the clinical area.

Our work in lactation has provided a physiological and endocrinological basis for the rational treatment of breast feeding mothers, both in terms of optimizing milk production itself and in controlling fertility postpartum.

SECTION 5: REVIEWS

During the course of my research I have written several reviews in which I have been able to place my own research in the context of other published work. I have referred to some of these as appropriate in the previous sections (60,88,89,98,99,126).

# PUBLICATIONS

## SECTION 1 DEVELOPMENT OF METHODS

### a) Assays for human prolactin, gonadotrophins and gonadotrophin subunits.


1. McNEILLY, A.S. (1973)
   Radioimmunoassay for human prolactin.
   Proc. Roy. Soc. Med. 66, 863-864.
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   The prolactin LH, FSH and TSH response to a combined LHRH/TRH test in normal male subjects and during the menstrual cycle.
   Clin. Endocr. 3, 427-435.
3. DJAHANBAKHCH, O., McNEILLY, A.S., HOBSON, B.M. and TEMPLETON, A.A. (1981) A rapid luteinizing hormone radioimmunoassay for the prediction of ovulation.
   Br. J. Obstet. Gynaecol. 88, 1016-1020.
4. HAGEN, C. and McNEILLY, A.S. (1975)
   The specificity and application of a radioimmunoassay for the $\alpha$ subunit of luteinising hormone in man.
   Acta Endocr. 78, 664-674.
5. HAGEN, C. and McNEILLY, A.S. (1976)
   Evaluation of the specificity of a luteinizing hormone $\beta$ and $\alpha$ subunit assay.
   Clinica Chemica Acta, 69, 193.
6. REES, L.H., HOLDAWAY, I.M., KRAMER, R., McNEILLY, A.S. and CHARD, T. (1973) New bioassay for luteinising hormone.
   Acta Endocr. Suppl. 177, 167.
7. KRAMER, R.M., HOLDAWAY, I.M., REES, L.H., McNEILLY, AS. and CHARD, T. (1974) Technical aspects of the redox bioassay for LH.
   Clin. Endocr. 3, 375-382.
8. HOLDAWAY, I.M., KRAMER, R.M., McNEILLY, A.S., REES, L.H. and CHARD, T (1974) Application of the redox bioassay for luteinising hormone.
   Clin. Endocr. 3, 383-388.

b) Assays for prolactin and gonadotrophins in other species.

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Purification and characterisation of caprine prolactin.
J. Endocr. 60, 359-367.

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Radioimmunoassay for ovine and caprine growth hormone: its application to the measurement of basal circulating levels of growth hormone in the goat.
Horm. Metab. Res. 7, 34-40.

11. McNEILLY, J.R., McNEILLY, A.S., WALTON, J.S. and CUNNINGHAM, F.J. (1976)
Development and application of a heterologous radioimmunoassay for ovine follicle-stimulating hormone.
J. Endocr. 70, 69-79.

12. BASS, J.J., McNEILLY, A.S. and MORETON, H.E. (1979)
Plasma concentrations of FSH and LH in entire and castrated prepubertal bull calves treated with Gn-RH.
J. Reprod. Fert. 57, 219-222.

13. McNEILLY, A.S., MARTIN, R.D., HODGES, J.K. and SMUTS, G.L. (1983)
Blood concentrations of gonadotrophins, prolactin and gonadal steroids in males and in non-pregnant and pregnant female African Elephants (Loxodonta africana).
J. Reprod. Fert. 67, 113-120.

14. HODGES, J.K., HENDERSON, C. and McNEILLY, A.S. (1983)
Blood concentrations of oestrogens during pregnancy in the African Elephant (Loxodonta Africana).
J. Reprod. Fert. 67, 73-87.

15. McNEILLY, A.S. and FRIESEN, H.G. (1978)
Heterologous radioimmunoassay for rabbit prolactin.
Endocrinology 102, 1539-1547.

16. McNEILLY, A.S., ABBOTT, D.H., LUNN, S.F., CHAMBERS, P. and HEARN, J.P. (1981) Changes in plasma levels of prolactin during the cycle, pregnancy and lactation and their relationship to return of fertility post partum in the common marmoset (Callithrix jacchus).
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Nature, 302, 145-147.

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Acta Endocr. 89, 60-69.

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Poultry Sci. 58, 963-970.

SECTION 2: PROLACTIN

a) Control of Secretion

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21. THORNER, M.O., McNEILLY, A.S., HAGEN, C. and BESSER, G.M. (1974)
Long-term treatment of galactorrhoea and hypogonadism with Bromocriptine.
Br. Med. J. 4, 499-506.

22. McNEILLY, A.S., THORNER, M.O., VOLANS, G. and BESSER, G.M. (1974)
Metaclopramide and prolactin.
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**Radioimmunoassay of Human Prolactin**

Only recently have immunological and other techniques demonstrated that prolactin is a separate entity from human growth hormone (HGH). For a routine radioimmunoassay three requirements must be met: first, an adequate supply of purified antigen for standard, labelling and immunization; secondly, a supply of specific antisera; and thirdly, a method for the separation of antibody-bound and free hormone. At present, the major problem with the radioimmunoassay of human prolactin is that the purified hormone is only available in very limited quantities. A number of workers have partly overcome this by the use of assays based on prolactin from other species which may show a partial cross-reaction with human material (Bryant *et al.* 1971, Guyda & Friesen 1971, Hwang *et al.* 1971, Jacobs *et al.* 1971, L'Hermite *et al.* 1972). These assays are suitable for qualitative and in some cases quantitative estimations of circulating hormones. Only one truly homologous radioimmunoassay using purified prolactin for standard, labelling and immunization has been published (Hwang *et al.* 1971).

The studies in our laboratory have been directed towards the establishment of a homologous radioimmunoassay for human prolactin designed for routine clinical use. Purified human prolactin was kindly supplied by Dr H Friesen and used for labelling and standard. Another preparation, described by Mr Davies & Dr Hartree (see p 862) was used for immunization. Antibody-bound and free hormone were separated using a second antibody system. Labelled hormone ($^{125}$I-human prolactin) was prepared by a modification of the chloramine-T method of Greenwood *et al.* (1963). Various antisera were screened for use in the assay, including 22 to sheep prolactin, 11 to HPL and 18 to Raben HGH. All could bind labelled hormone but the resulting assays were not sufficiently sensitive to measure basal circulating levels of prolactin.

However, using an antiserum to the prolactin prepared by Mr Davies, a specific and sensitive radioimmunoassay for prolactin has been developed. In this system, HGH has only 0.01% of the activity of prolactin, and other hormones tested show no cross-reaction. The minimum detectable level of prolactin in serum is 0.5 ng/ml and the assay has been applied to the measurement of prolactin in various physiological and clinical situations.

In normal females throughout the menstrual cycle levels varied between 6 and 50 ng/ml serum; no consistent pattern of release could be observed. The normal range in adult males was 6–25 ng/ml serum. During pregnancy there is a rise in circulating levels of prolactin in maternal serum (20–340 ng/ml) and high levels of prolactin (0.3–8 $\mu$g/ml) can be found in amniotic fluid. Prolactin release has been shown to occur after both suckling and injection of thyrotrophin-releasing hormone, with an increase of between two and twenty-fold over basal levels. In a series of 22 patients with galactorrhœa, serum levels varied between 35 and 1150 ng/ml; in no case was there a significant elevation of HGH levels. In 5 of these cases the levels fell during treatment with 2-Br-α-ergocryptine (CB 154) from 260–1000 to 8–40 ng/ml.

These results confirm that human prolactin can be measured independently of HGH. The present method is sufficiently sensitive to measure levels of prolactin in all clinical and physiological situations so far investigated. However, routine establishment of the technique still requires that larger supplies of purified human prolactin are made available.

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# PROLACTIN, TSH, LH AND FSH RESPONSES TO A COMBINED LHRH/TRH TEST AT DIFFERENT STAGES OF THE MENSTRUAL CYCLE

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## SUMMARY

A combined test with LHRH and TRH was investigated in the normal female subject during the menstrual cycle. LH and FSH responses were not affected by raised prolactin or TSH levels after TRH.

No correlation was seen between either basal levels or responses of prolactin and TSH after TRH, and no difference in responses on days 4 or 24 were observed. The increments in prolactin and TSH were significantly greater in female than in male subjects.

Although FSH responses to LHRH+TRH were not significantly different, LH responses on day 24 were greater than on day 4. A significant linear correlation between FSH and LH responses to LHRH was seen.

The results indicate that prolactin and TSH responses to TRH are greater in female than male subjects and that changes in LH and FSH after LHRH do affect these responses. Normal ranges for hormone responses after LHRH and TRH are defined.

## INTRODUCTION

The availability of synthetic preparations of gonadotrophin releasing hormone (LHRH) and thyrotropin releasing hormone (TRH) has allowed the use of these materials in assessment of pituitary function and reserve for luteinizing hormone (LH), follicle stimulating hormone (FSH), thyrotrophin stimulating hormone (TSH) and prolactin.

It has been shown in males that LHRH and TRH may be given at the same time without affecting the responses of any of the hormones involved (Mortimer *et al.*, 1973a; Harsoulis *et al.*, 1973). This combined test has the advantage that it reduces the number of samples taken and the time required for assessment of pituitary function.

No information is available on such a test in females. The present study was undertaken to provide normal ranges of responses of female subjects to the combined test through the

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menstrual cycle and to compare the responses with those after LHRH alone in the female and with TRH and LHRH either separately or combined when given to normal male subjects.

## METHODS

*Study and subjects*

Studies were carried out on eleven women (ages 25–35 years) with a regular menstrual history for at least 1 year before the tests, which were performed on day 4 and day 24 of the same cycle in all subjects. In a further five female subjects LHRH tests were performed without TRH on day 4 and day 24. In addition, these responses were compared to TRH and LHRH tests alone carried out in thirteen normal male subjects, and combined TRH/LHRH tests performed on a further six male subjects.

An antecubital vein was cannulated 60 min before the start of the tests, and LHRH (100 $\mu$g) and TRH (200 $\mu$g) were injected i.v. simultaneously as described by Mortimer *et al.* (1973a). Blood samples were withdrawn at 0, 20 and 60 min after injection, serum separated, and stored at $-20°C$ until assay. All samples were assayed for LH, FSH, prolactin and TSH.

*Assay methods*

Serum LH and FSH were measured by double antibody radioimmunoassay. To facilitate the routine running of these assays in our laboratory, the assay buffer, incubation times and volumes of reagents used in the assays were the same.

LH (IRC 2/69 supplied by Dr A. S. Hartree) and FSH (CPD S 6 supplied by Dr W. R. Butt) were labelled with $^{125}I$ to a specific activity of 80–140 $\mu$Ci/$\mu$g using the chloramine-T method of Greenwood *et al.* (1963). After iodination, unreacted $^{125}I$ was removed by column chromatography on Sephadex G-75 (10 ml column) and the fractions containing immunoreactive hormone combined and stored at $-20°C$. Before addition to the assay, both labelled LH and FSH were purified by adsorption on to Whatman CF-11 ion-exchange resin. Highly immunoreactive LH and FSH were eluted with buffer containing 50% horse serum (Hunter, 1969). With inclusion of this purification step, labelled preparations of both LH and FSH retained their immunological activity for at least 3 months at $-20°C$.

Medical Research Council (MRC) preparations LH 68/40 (assuming 77 i.u. per ampoule) and FSH 68/39 (assuming 33 i.u. per ampoule) were used as standards, and diluted in neat horse serum. No loss in immunological activity of either of these standards was observed after 14 months of storage in aliquots at $-20°C$.

After addition of standard or sample (100 $\mu$l) to assay buffer (400 $\mu$l 0·1 M phosphate, pH 7·6, containing 2% horse serum (Wellcome Reagents Ltd)), 50 $\mu$l of antiserum was added (MRC GP 70/229 guinea-pig anti-LH final dilution 1:130,000, or rabbit anti-FSH supplied by Dr W. R. Butt, final dilution 1:60,000). After incubation for 2–3 days at 4°C, 200–500 pg (50 $\mu$l) of $^{125}I$-LH or $^{125}I$-FSH were added and incubation continued for 1–3 days at 4°C. Separation of antibody-bound and free hormone was achieved by second antibody. For LH, 50 $\mu$l anti guinea-pig $\gamma$-globulin precipitating serum (Wellcome RD 18) and 50 $\mu$l normal guinea-pig serum diluted 1:5 and 1:40 respectively were added to each tube while for FSH anti-rabbit $\gamma$-globulin precipitating serum (Wellcome RD 17) (50 $\mu$l, 1:10) and normal rabbit serum (50 $\mu$l, 1:80) were added. After incubation for 16–24 hr

at 4°C tubes were centrifuged, the supernatant removed and the precipitate containing bound hormone counted in an automatic gamma counter (Wallac).

Serum prolactin was measured by a double antibody radioimmunoassay employing highly purified human prolactin (kindly supplied by Dr H. Friesen) for standard and iodination, and an antiserum raised against an impure preparation of human prolactin (McNeilly, 1973).

Prolactin was iodinated to a specific activity of 40–80 $\mu$Ci/$\mu$g by a modification of the chloramine-T method (Greenwood *et al.*, 1963), and purified by column chromatography on Sephadex G-100 (40 × 2·2 cm). The immunoreactive fractions were combined and stored in aliquots at −20°C. Appreciable loss of immunoreactivity occurred after storage of $^{125}$I-prolactin for more than 4 weeks and little improvement was achieved by re-purification of the labelled preparation.

In view of the shortage of pure human prolactin, doubling dilutions of a pool of serum containing a high prolactin concentration from a patient with galactorrhoea were standard-

TABLE 1. Details of the radioimmunoassays for LH, FSH and prolactin

| | Radioimmunoassays for: | | |
|---|---|---|---|
| | LH | FSH | Prolactin |
| Antibody | Guinea-pig anti-LH (MRC) | Rabbit anti-FSH (Butt) | Rabbit anti-prolactin (McNeilly) |
| Iodination material | LH IRC 2/69 (Hartree) | FSH CPDS 6 (Butt) | Prolactin (Friesen) |
| Standard | MRC 68/40 | MRC 68/39 | Serum standardized against Friesen prolactin and MRC 71/222 (10 mU/amp)[7] |
| Maximum sensitivity[1] | 0·6 mU | 0·1 mU | 1·6 ng |
| Cross reaction[2] | | | |
| LH (68/40) | — | 400 mU | >1 u |
| FSH (CPDS 6)[5] | 80 mU | — | >1 u |
| TSH (NPA TSH)[6] | 23 ng | 15 ng | 100 ng |
| HGH (MRC) | >100 $\mu$g | >100 $\mu$g | >100 $\mu$g |
| HPL (Lederle) | >1 mg | >1 mg | >1 mg |
| Prolactin (Friesen) | >8 $\mu$g | >8 $\mu$g | — |
| Other hormones[3] | >10 $\mu$g | >10 $\mu$g | >10 $\mu$g |
| Interassay variation[4] | 7% | 10% | 8% |
| Intraassay variation[4] | 4% | 6% | 6% |

[1] Expressed as least detectable amount of hormone per ml serum.
[2] Cross-reaction indicates amount of hormone/ml serum which would register in the assay.
[3] Includes ACTH, insulin, oxytocin and vasopressin.
[4] Percent coefficient of variation of ten replicates each at three dose levels per assay.
[5] CPDS 6 expressed in terms of MRC 68/39 FSH.
[6] 1 mg NPA TSH = 7 U MRC 68/38 TSH.
[7] 1 mU MRC prolactin standard A (71/222) = 54 ng Friesen or Lewis prolactin.

ized against preparations of human prolactin supplied by Dr H. Friesen and Dr J. Lewis, and against MRC Research standard A (71/222, assuming 10 mU per ampoule).

For assay, standard or sample (50 $\mu$l), assay diluent (450 $\mu$l, 0·05 M barbitone, pH 8·6, containing 2·5% BSA (BDH)) and antiserum (50 $\mu$l, final dilution 1:12,000) were incubated for 24 hr at 4°C. After addition of $^{125}$I-prolactin (50 $\mu$l; 200–500 pg) incubation was continued for 24–48 hr at 4°C, when antibody bound and free hormone were separated by second antibody, as described for FSH.

Serum TSH was measured by a heterologous double antibody radioimmunoassay using highly purified human TSH for iodination, MRC 68/38 TSH as standard, and an anti-porcine TSH antiserum raised in a rabbit. The methods for this assay are described by Merrett & McNeilly (1974).

Details of the assay systems used including reagents, sensitivity, specificity and precision are given in Table 1. All results were compared using the Student's '*t*' test.

## RESULTS

### *Prolactin and TSH*

No significant differences were seen between either basal levels of prolactin and TSH or the responses to TRH on days 4 and 24 of the cycle (Table 2). In addition, there was no

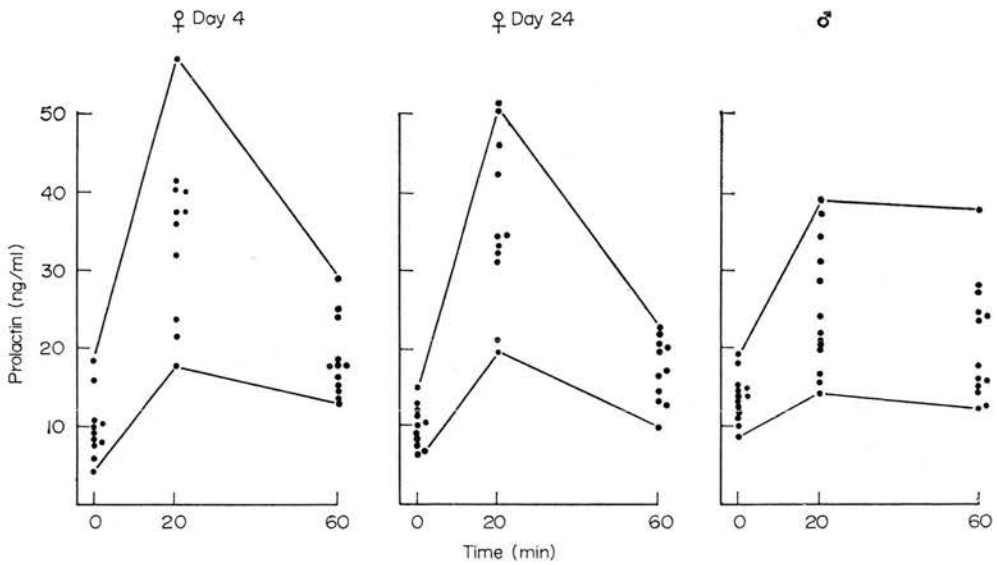



FIG. 1. Prolactin response to TRH+LHRH in normal male subjects and female subjects on days 4 and 24 of the menstrual cycle. Individual levels are shown at each time period and range of response is shown by the closed lines. Note that responses on days 4 and 24 in the female are the same, but both are greater than in the male.

linear correlation ($r<0·4$) between the increments in prolactin and TSH in either female or male subjects. The responses of both hormones to TRH in female subjects were greater than male subjects (Figs. 1 and 2). The TSH and prolactin responses to TRH alone or TRH+

TABLE 2. Ranges of basal levels and increments of prolactin and TSH after TRH alone, or TRH+LHRH in normal male and female subjects

| Group | Test | *n* | Prolactin (ng/ml) | | | TSH ($\mu$u/ml) | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 min | 0–20 min* | 0–60 min* | 0 min | 0–20 min* | 0–60 min* |
| MALE | | | | | | | | |
| | TRH | 13 | 9·0–19·1 | 2·1–26·0 | 1·1–20·0 | 0·4–3·0 | 4·0–13·1 | 2·5–10·7 |
| | TRH+LHRH | 6 | 12·0–18·3 | 2·2–26·0 | 3·8–26·0 | 0·4–2·7 | 4·2–11·9 | 2·6–8·3 |
| FEMALE | | | | | | | | |
| Day 4 | TRH+LHRH | 11 | 6·0–19·4 | 9·1–40·8 | 3·9–19·9 | 0·8–4·0 | 0·3–18·3 | 0·8–14·4 |
| Day 24 | TRH+LHRH | 11 | 6·2–15·4 | 3·2–40·7 | 3·0–14·1 | 0·8–3·9 | 2·9–18·3 | 1·3–12·2 |

* Increment between times stated.

TABLE 3. Ranges of basal levels and increments of LH and FSH after LHRH alone, or LHRH+TRH in normal male and female subjects

| Group | Test | *n* | LH (mU/ml) | | | FSH (mU/ml) | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 min | 0–20 min* | 0–60 min* | 0 min | 0–20 min* | 0–60 min* |
| MALE | | | | | | | | |
| | LHRH | 18 | 0·8–13·3 | 3·5–47·9 | 3·3–19·3 | 0·4–5·8 | 0·6–6·5 | 0·9–2·8 |
| | LHRH+TRH | 6 | 3·7–8·7 | 10·4–23·9 | 10·2–20·0 | 0·2–1·3 | 2·4–4·6 | 1·5–5·0 |
| FEMALE | | | | | | | | |
| Day 4 | LHRH | 5 | 1·4–9·8 | 1·2–25·6 | 3·1–16·2 | 2·0–3·2 | 0·4–5·7 | 0·0–4·3 |
| Day 24 | LHRH | 5 | 2·7–7·3 | 13·9–90·9 | 9·4–37·0 | 1·1–2·2 | 3·6–6·9 | 0·1–6·0 |
| Day 4 | LHRH+TRH | 11 | 1·5–9·6 | 6·7–20·0 | 1·2–13·3 | 1·4–6·1 | 2·5–6·0 | 1·7–10·2 |
| Day 24 | LHRH+TRH | 11 | 1·9–7·9 | 13·7–48·9 | 8·7–46·8 | 0·5–2·9 | 2·6–2·9 | 1·9–5·6 |

* Increment between times stated.

LHRH in male subjects were not significantly different ($P<0{\cdot}01$). Differences in basal levels of LH or FSH did not affect either the basal levels or increments of prolactin and TSH after TRH in males and females.

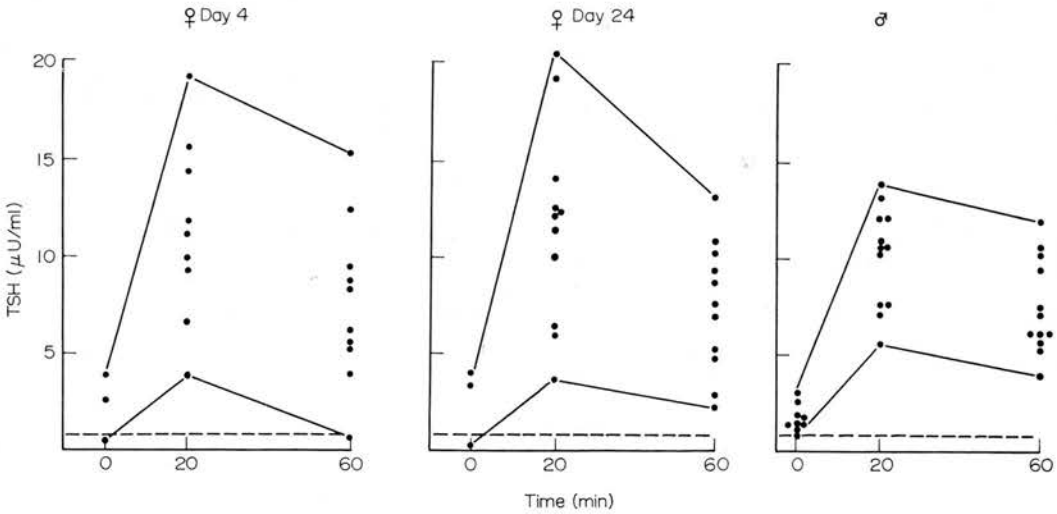



FIG. 2. TSH response to TRH+LHRH in normal male subjects and female subjects on days 4 and 24 of the menstrual cycle. Individual levels are shown at each time period and range of response is shown by the closed lines.

*LH and FSH*

In female subjects, basal levels of LH and FSH on day 4 and day 24 were not significantly different. The increments of LH and FSH to the combined test of LHRH and TRH were unrelated to either the basal levels or increments of either prolactin or TSH and fell within the same range as responses to LHRH alone. This indicated no significant effect of either prolactin or TSH levels on LH and FSH responses. Although in ten of the eleven subjects the LH response on day 24 was greater than on day 4 (Table 3, Fig. 3), no difference in FSH response was seen.

Subjects with the lowest and highest basal levels of FSH responded with the least and greatest increment of FSH respectively. The same was not true for LH.

There was a significant linear correlation ($P<0{\cdot}001$) between the increments ($\Delta$) of LH and FSH to LHRH on days 4 and 24 (Fig. 4).

## DISCUSSION

The present report defines the normal ranges of response for prolactin, TSH, LH and FSH after LHRH+TRH during the menstrual cycle and confirms that in the female as well as the male responses to the two releasing hormones are unaffected when they are given together.

The results demonstrate that there is no difference in the TSH/prolactin response to

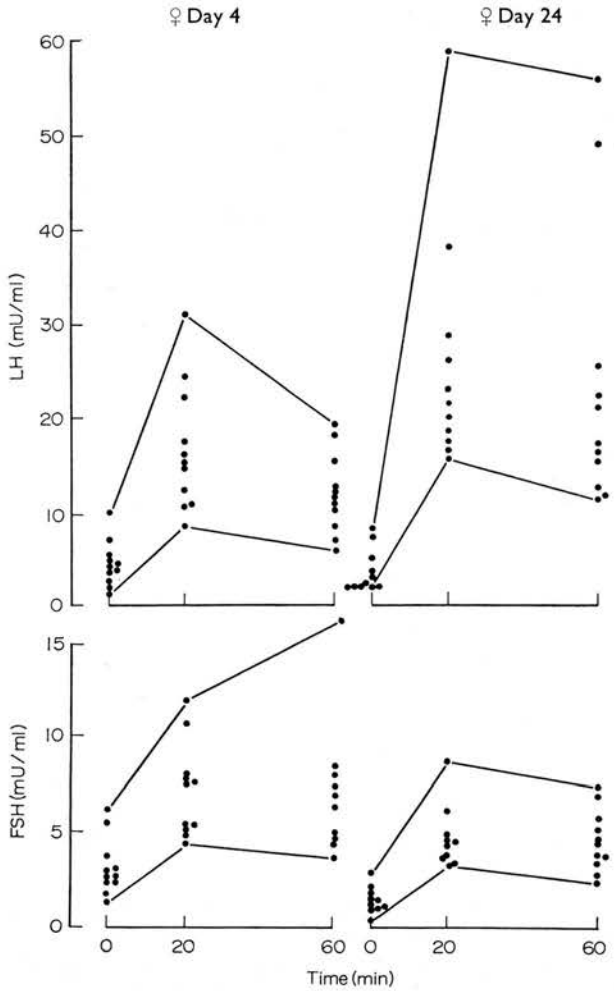



FIG. 3. LH and FSH responses to LHRH+TRH in female subjects on days 4 and 24 of the menstrual cycle. Individual levels are shown at each time period and range of response is shown by the closed lines.

TRH during the early follicular or luteal phases of the menstrual cycle, a finding similar to that of Tyson & Friesen (1973). This suggests that high levels of oestrogen and progesterone do not interact at pituitary level in the female to alter the sensitivity of either prolactin or TSH cells to TRH. In addition, there is no correlation between the basal levels and the response of prolactin or TSH, and no relationship between the levels of gonadotrophin and the TSH/prolactin response.

The response of both hormones were greater in the female than in the male, in agreement with previous findings for TSH (Ormston, 1972; Snyder & Utiger, 1972) and prolactin (Bowers *et al.*, 1972; Foley *et al.*, 1972; Jacobs *et al.*, 1973).

The increased release of LH but not FSH after LHRH in the luteal phase of the cycle

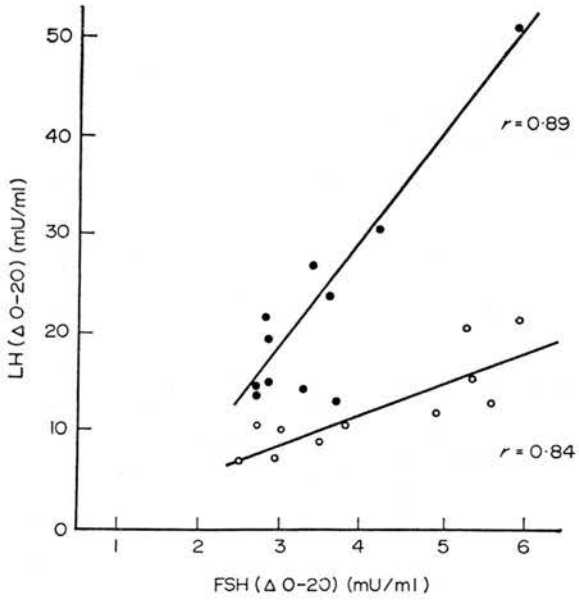



FIG. 4. Correlation between increments of LH and FSH after LHRH on days 4 (○) and 24 (●) of the menstrual cycle.

confirms previous reports (Nillius & Wide, 1972; Yen *et al.*, 1972). It has been suggested that oestrogen and progesterone may exert a synergistic or additive effect on the action of LHRH on the pituitary (Yen *et al.*, 1972). However, in males, treatment with oestrogen reduces the LH and FSH response to LHRH (Mortimer *et al.*, 1973b).

From the results presented in this paper, however, it may be concluded that the combined LHRH+TRH test is applicable to the female as well as male subject.

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# A RAPID LUTEINIZING HORMONE RADIOIMMUNOASSAY FOR THE PREDICTION OF OVULATION

BY

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**Summary**

A rapid luteinizing hormone (LH) radioimmunoassay (total time 4 h) has been developed and used to measure the preovulatory LH surge in 22 patients. Ovulation, assessed by laparoscopy or mini laparotomy, did not occur until at least 32 h after the start of the LH surge. This rapid LH radioimmunoassay provides a simple method of predicting ovulation for the correct timing of oocyte collection or artificial insemination.

CURRENT research in *in vitro* fertilization and the increased demand for artificial insemination requires considerable accuracy in the timing of ovulation. While development of the growing follicle before ovulation may be monitored by ultrasound or by measuring oestrogen in plasma or urine, the most accurate method would seem to be the detection of the ovulation-inducing surge of luteinizing hormone (LH) which occurs in both urine and plasma before ovulation. Although some groups have used a commercially available kit (Hi-Gonavis, Mercia Brocades) to measure LH in urine to predict ovulation (Brandau, 1978; Edwards *et al*, 1980), we have found this method to be unreliable in some patients (Djahanbakhch *et al*, 1980). While the routine LH radioimmunoassays are accurate and precise, they require a total time of at least 24 hours for completion, but for *in vitro* fertilization or insemination work it is necessary to have a result within 3–4 hours. We have developed a fast, accurate and sensitive LH radioimmunoassay which would be suitable for monitoring the pre-ovulatory LH surge. We now describe the assay and assess its use in predicting ovulation.

## MATERIALS AND METHODS

### *Radioimmunoassay for LH*

The radioimmunoassay is based on the routine radioimmunoassay used in this centre

which has been described in detail previously (McNeilly and Hagen, 1974; Hunter and Bennie, 1979). All assay constituents were made up in assay diluent which consisted of sodium phosphate solution (0·05 mol/l), pH 7·5, containing 2% bovine serum albumin (BSA, Sigma) and sodium azide (0·1%). The method uses a rabbit antiserum raised against human LH (WRB/F87; Lynch and Shirley, 1975) supplied by Professor W. R. Butt, purified human LH for iodination (NIBSC 76/569 or LH × S4 from Dr S. S. Lynch and Prof W. R. Butt) and NIBSC LH preparation 68/40 designated as containing 77 IU/ampoule.

The $^{125}$I-labelled human LH was prepared by the chloramine-T method described previously (McNeilly and Hagen, 1974) and after iodination was purified on a Sephadex G–100 column (1 × 24 cm) eluted with assay diluent and stored in aliquots at 4°C; it was useable for up to four weeks after iodination. Specific activities calculated by isotope recovery from the Sephadex columns ranged between 60 and 90 Ci/g (n = 17).

For assay 50 µl of antibody diluted 1:100 000 was added to duplicate 100 µl aliquots of standard (200 to 1·6 IU/l) or sample followed by 50 µl of $^{125}$I-labelled human LH (approx 200 pg, 20 000 cpm). After incubation for 2·5 h at room temperature (18 to 22°C), 50 µl of normal rabbit serum (1:50) and 50 µl of Donkey anti rabbit γ-globulin (1:8, SA 4090); were added and incubation continued at room temperature for a further 30 min.

At the end of this incubation, the tubes were centrifuged at 600 *g* for 30 min at 4°C, the supernatant was removed and the precipitate containing bound hormone was counted in either an automatic (LKB, Wallac) or a multihead (NE 600, Nuclear Enterprises) gamma counter.

Aliquots from pooled plasma samples from normal men and pre- and postmenopausal women were included routinely as quality control sera at 27, 49 and 78% B/Bo. In addition for the first 50 radioimmunoassays in which results were required on the same day, the patient's sample from the previous day was also included as an additional control. Initially samples (range 4 to 70 IU/l) were assayed in both the short and normal incubation radioimmunoassays to compare results.

*Steroid assays*

Urinary excretion of total oestrogen was measured fluorimetrically (Brown *et al*, 1968) and urinary pregnanediol was determined by gas-liquid chromatography (Chamberlain and Contractor, 1968). Plasma progesterone was measured using a specific radioimmunoassay described previously (Scaramuzzi *et al*, 1975).

*Patients and collection of samples*

Twenty two patients (ages 24–36 years) with spontaneous ovulatory cycles ranging from 26–29 days were studied; 12 women were being investigated for infertility of non-endocrine origin and ten were undergoing sterilization. Of the latter group, 4 women had been fitted with an IUCD which was removed two days before operation; the remaining 6 women were studied during the second menstrual cycle after stopping oral contraceptives and had used barrier contraceptives in the month before study.

From day 10 after the beginning of the last menstrual period (LMP) the total urine passed in 24 hours was collected daily and blood samples (15 ml) were collected every 8 hours until one day after the end of the pre-ovulatory LH surge. Thereafter daily blood samples were obtained for estimation of progesterone.

Urinary excretions of total oestrogens and pregnanediol were estimated daily and LH was estimated each day using the short LH RIA. Progesterone levels were also estimated in the plasma samples stored at −20°C at a later date. In 14 patients, the ovaries were visualized by laparoscopy or mini laparotomy at different times after the first rise in plasma levels of LH and the oocyte recovered by suction from the dominant follicle.

## Results

*Radioimmunoassay of LH*

The rapid radioimmunoassay has been in use daily for more than 300 days and has proved totally reliable. In routine use the sensitivity of the assay is 3 IU/l and the standard curve is linear up to 200 IU/l. The correlation between plasma levels of LH estimated by the rapid and routine RIA's in 124 samples was $r = 0{\cdot}913$ ($y = 0{\cdot}87\ x + 2{\cdot}92$). The intra- and interassay coefficients of variation (as %) for the rapid LH

RIA were 11 and 16%, 4 and 10% and 9 and 16% for the quality control sera (n = 82) at levels of 6 (78% B/Bo), 29 (49% B/Bo) and 56 (27% B/Bo) IU/l respectively. Results were available within four hours of receipt of the sample.

*Measurement of the preovulatory LH surge*

Using the rapid LH RIA the pre-ovulatory LH surge was detected in all 22 patients studied and occurred between days 11 and 16 (mean = 13 days) post LMP. Basal levels of LH prior to the surge were 6 to 13 IU/l and peak values ranged between 50 and 200 IU/l. The duration of the surge was very variable ranging between 16 and 48 hours (mean 30·5 hours; Fig. 1). Similar variation was seen in the time interval from the start of the surge of LH (defined as a rise of more than 100% of the previous value followed by a continued increase in levels) and the peak of the preovulatory rise in LH (range 8 to 24 hours; mean 14·1 hours).

A significant rise ($p < 0{\cdot}001$) in the concentration of progesterone levels was seen to accompany the first rise in plasma LH levels in all 22 patients, and this rise in progesterone continued throughout the period following the start of the LH surge in 8 of the 12 women investigated for infertility (Fig. 2).

*Prediction of ovulation*

Laparoscopy or mini-laparotomy in 14 patients showed that no ovulations had occurred in the 8 women examined up to 32 hours after the first rise in plasma LH levels (Fig. 3). On the other hand when ovulations were assessed in terms of time from the peak in levels of LH much more variation was seen.

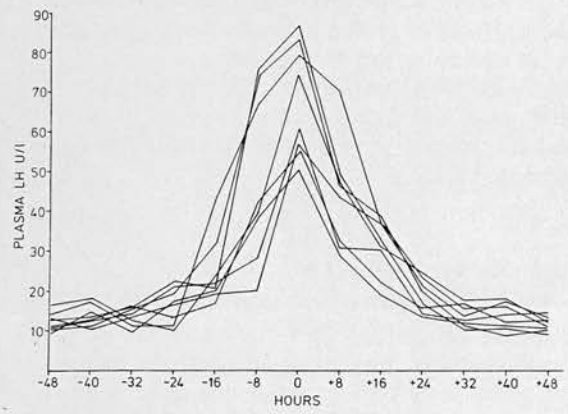



FIG. 1

Changes in plasma levels of LH during the pre-ovulatory surge in eight subjects. Blood samples were collected every eight hours and results are centred around the peak value of LH in each subject.

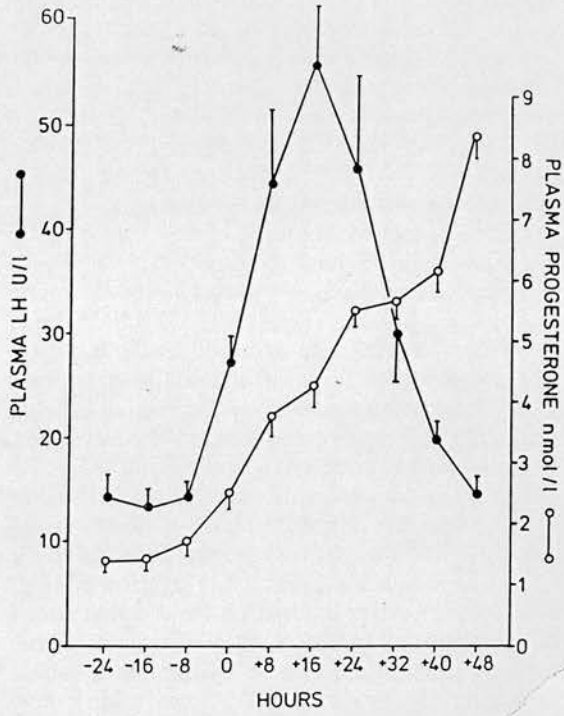



FIG. 2

Plasma levels (Mean ± SEM) of LH and progesterone centred around the start of the pre-ovulatory surge of LH in eight subjects.

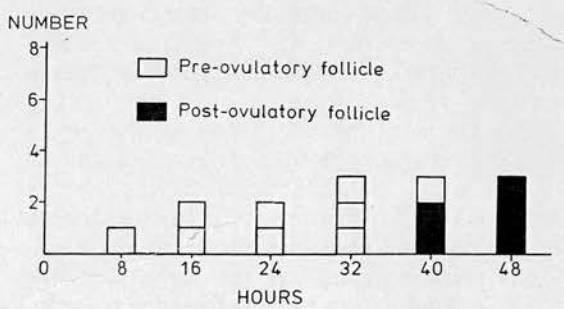



FIG. 3

Number of subjects with pre- or postovulatory follicles in relation to the time from the onset of the preovulatory LH surge.

## Discussion

The rapid radioimmunoassay for plasma LH reported here has proved reliable in clinical use for the accurate and reliable measurement of the preovulatory LH surge. It is equally satisfactory for measurement of LH in urine (Djahanbakhch *et al*, 1980; Boyle, Hall and McNeilly, unpublished observations) and the results obtained with both the rapid and the routine LH radioimmunoassays correlate well.

The increase in plasma levels of progesterone during the preovulatory surge of LH is in accord with previous observations (Thorneycroft *et al*, 1974; Laborde *et al*, 1976; Langren *et al*, 1977). This parallel increase in both progesterone and LH suggests that luteinization of granulosa cells within the follicle occurs in response to the increase in LH levels and clearly suggests that the radioimmunoassay for LH is detecting biologically active LH. Indeed in a previous study there was no change in plasma progesterone levels when LH estimated by radioimmunoassay remained low even though the Hi-Gonavis kit measured an apparent increase in LH (Djahanbakhch and McNeilly, unpublished observations).

The large variation in the duration of, and the time to the peak level of LH during the preovulatory surge together with the variation in peak levels of LH makes the use of these parameters difficult in predicting the time to ovulation. On the other hand ovulation appeared to occur consistently 33 h or more after the start of the LH surge. This has been defined as an increase in LH levels of more than 100% of the previous value which is followed by a further increase in LH levels. This definition prevents the false assumption of the beginning of the LH surge when a rise in the level of LH seen in one sample is followed by a fall, the beginning of the actual preovulatory surge occuring some hours later (Fig. 4).

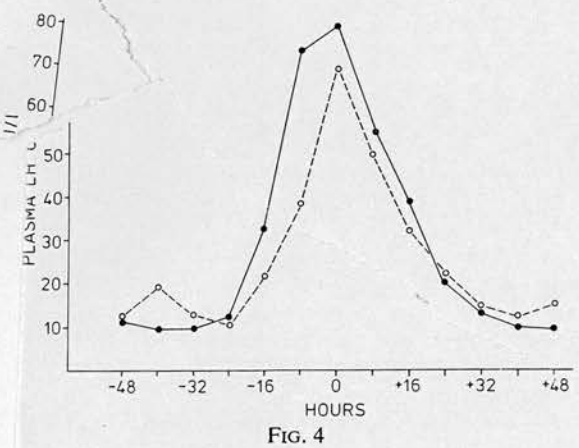



FIG. 4

Changes in plasma levels of LH during the preovulatory surge in two individual subjects. Results are centred around the peak value of LH.

Urinary oestrogens and ultrasonic visualization of the pre-ovulatory follicle can be used successfully to monitor follicular development (Brown *et al*, 1968; Hackeloer *et al*, 1979), but because of individual variation in both the magnitude of the pre-ovulatory peak of oestrogen and in follicle diameter, these measurements cannot be used reliably to time ovulation (Djahanbakhch, Young, Lees, and Scott, unpublished observations).

Similarly the assessment of changes in cervical mucus while indicating increased oestrogen production does not give any guide to the precise time of ovulation (France and Boyer, 1975).

The present study confirms previous reports that detection of the preovulatory surge of LH either in urine or plasma (Edwards *et al*, 1980; Djahanbakhch *et al*, 1980; Kerin *et al*, 1980) is a reliable method for timing ovulation. It may be concluded from this study that to detect the onset of the preovulatory surge of LH frequent measurements of LH (at least 8-hourly) is required. This should begin when a significant rise (two times the basal level) in urinary oestrogen occurs or when ultrasonic visualization has confirmed the presence of a developing follicle, but in practice these latter assessments are only necessary to reduce the time period over which frequent samples need be collected and are otherwise not essential.

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# THE SPECIFICITY AND APPLICATION OF A RADIOIMMUNOASSAY FOR THE α-SUBUNIT OF LUTEINIZING HORMONE IN MAN

By

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## ABSTRACT

A sensitive radioimmunoassay for the α-subunit of LH has been developed. Differences in the immunopotency of LH, FSH and subunit preparations were found and are described in detail. The assay has been applied to the measurement of circulating levels of α-subunit; higher levels of α-subunit were found in post-menopausal than in pre-menopausal women, or men, and a rise in α-subunit was seen after LH-RH but not TRH administration. Elevated levels of free α-subunit were found in foetal cord blood at term.

These results suggest that the α-subunit circulates independently of the intact pituitary and placental glycoprotein hormones.

The glycoprotein hormones (LH, FSH, HCG and TSH) consist of two non-identical non-covalently bound subunits (*Swaminathan & Bahl* 1970; *Hartree et al.* 1971; *Saxena & Rathman* 1971; *Pierce* 1971), an α-subunit which is common to all, and a hormone specific β-subunit (*Reichert et al.* 1973; *Hartree et al.* 1971). Both *in vivo* and *in vitro* studies have demonstrated that neither subunit possesses biological activity when compared with the intact hormone (*Kammerman & Canfield* 1972; *Catt et al.* 1973). It has recently been reported (*Franchimont et al.* 1972; *Laburthe et al.* 1973) that the α-subunit may circulate independently of the intact hormone. In the present study the specificity of a radioimmunoassay for the luteinizing hormone α-subunit (LHα) has been investigated, and blood levels of LHα subunit measured under different physiological situations in the human.

## MATERIALS AND METHODS

### *Hormone preparations*

Human LH$\alpha$ (4.1.72) kindly supplied by Dr. A. S. Hartree, was used for both iodination and standard. The following hormone preparations were used to assess the specificity of the radioimmunoassay system; human luteinizing hormone $\beta$-subunit, LH$\beta$ supplied by Dr. R. M. Lequin (LH$\beta$ (Q)), and Dr. A. S. Hartree (LH$\beta$ (AS)) and the Medical Research Council (71/342); human LH$\alpha$ supplied by the Medical Research Council (27/20); human luteinizing hormone supplied by Dr. A. S. Hartree (LH IRC 2/69) and the Medical Research Council (LH 68/40); human follicle stimulating hormone (FSH; CPDS 3 and CPDS 6) supplied by Dr. W. R. Butt; human chorionic gonadotrophin (HCG, CR 115); human chorionic gonadotrophin $\alpha$-subunit (HCG$\alpha$, CR 115) and human chorionic gonadotrophin $\beta$-subunit (HCG$\beta$, CR 115) all supplied by Professor R. E. Canfield; human thyroid stimulating hormone (TSH, NPA) from National Institutes of Health, Bethesda, Maryland, USA; human placental lactogen (HPL 71/69), human growth hormone (HGH 69/76), and human adrenocorticotrophin (ACTH 73/548) from the Medical Research Council; and human prolactin supplied by Professor H. Friesen.

### *Antiserum*

The antiserum used in the assay (rabbit anti-FSH, $C_3$) was initially raised against FSH (CM 1 preparation, Dr. A. S. Hartree) by sc injections of 200 $\mu$g FSH in Freund's complete adjuvant to rabbits. After three injections at monthly intervals, the animal was bled, serum separated and stored at –20°C.

### *Iodination*

The $\alpha$-subunit was iodinated by a modification of the chloramine-T method (*Greenwood et al.* 1963) to specific activities of 150–300 $\mu$Ci/$\mu$g. After iodination, the labelled subunit was purified by gel filtration on Sephadex G-100 (0.5 × 30 cm).

The typical elution pattern after iodination of LH$\alpha$ showed three peaks. The first contained either aggregated subunit or intact LH. The second consisted of the subunit, and the most immunoreactive material was found on the descending part of this peak. The third peak consisted of free iodide. The most immunoreactive fractions, determined by binding in antibody excess, were combined, aliquoted and stored at –20°C.

### *Assay methods*

Serum LH$\alpha$ was measured by double antibody radioimmunoassay. The standard was diluted and stored at –20°C in horse serum (No. 3 Wellcomes Reagents Ltd.). All other dilutions were made with 0.05 M phosphate buffer, pH 7.6, containing 2 % horse serum. The plasma or standard (100 $\mu$l) of varying concentrations were added to 0.4 ml of the diluent in the incubation tube: this was followed by the addition of antiserum (50 $\mu$l), at a final dilution of 1:9600.

After incubation for 24 h at 4°C, 50 $\mu$l (25–100 pg) of $^{125}$I-LH was added and incubation continued for 2–3 days at 4°C.

Separation of antibody bound and free hormone was achieved by second antibody. Fifty $\mu$l anti-rabbit $\gamma$-globulin precipitating serum (Wellcome RD 17, 1:10) and normal rabbit serum (50 $\mu$, 1:80) were added to each tube. After incubation for 16–24 h at 4°C, tubes were centrifuged, the supernatant removed and the precipitate containing bound hormone counted in an automatic gamma counter (Wallac GTL-500). The accuracy of the assay was assessed by determining the recovery of LH-$\alpha$ added to

normal human serum. Intra- and inter-assay variation was expressed as the coefficient of variation of repeated assay of quality control sera.

Details of the methods of measuring serum immunoreactive LH, FSH and TSH have been reported elsewhere (*McNeilly & Hagen* 1974).

### *Column chromatography*

A 90.0 × 1.5 cm column of Sephadex G-100 (Pharmacia, London) was equilibrated with 0.05 M phosphate buffer, pH 7.4, containing 10 μg/ml of bovine serum albumin (Armour Pharmaceutical Company). The flow rate of the column was adjusted to 6 ml per h and 3 ml fractions were collected at 4°C for all studies, LH (IRC 2/69) and LHα (Hartree) were chromatographed and LH and LHα were assayed in each fraction.

### *Serum concentrations*

Basal α-subunit concentrations were measured in 43 normal male subjects, 13 pre- and 22 post-menopausal female subjects, and in 6 foetal arterial and venous umbilical cord blood samples. In addition, the subunit response at 0, 20 and 60 min after either LH-RH (100 μg) or TRH (200 μg) were studied in 12 normal men. In all cases blood samples were withdrawn by antecubital venepuncture, serum separated and stored at –20°C until assayed.

*Table 1.*
Details of the radioimmunoassay for LHα-subunit.

| | |
|---|---|
| Antibody | Rabbit anti-FSH |
| Iodination material | LHα – 4.1.72 (Hartree) |
| Maximum sensitivity[1] | 0.2 ng |
| Cross-reaction[2] | |
| LHβ (Lequin) | 1 μg |
| LH( IRC 2/69)[5] | 24 ng |
| Repurified LH ($LH_9$)[6] | 64 ng |
| FSH (CPDS 6)[7] | 250 ng |
| TSH (NPA)[8] | 50 ng |
| HCG (CR 115) | 100 ng* |
| HCGα (CR 115) | 6 ng |
| HCGβ (CR 115) | > 1 μg* |
| Other hormones[3] | > 1 μg |
| Intra-assay variation[4] | 8 % |
| Inter-assay variation[4] | 12 % |

1) Expressed as least detectable amount of hormone per ml serum.
2) Cross-reaction indicates amount of hormone which would produce 50 % inhibition in the assay.
3) Includes ACTH, HGH, prolactin and HPL.
4) Per cent coefficient of variation of 6 replicates each at three dose levels per assay.
5) 1 ng LH (IRC 2/69) = 2 mU LH (MRC 68/40 assuming 40 IU/amp).
6) Peak fractions of LH from the column chromatography of LH (IRC 2/69).
7) 1 ng FSH (CPDS 6) = 2 mU FSH (MRC 69/104, assuming 10 IU/amp).
8) 1 mg NPA TSH = 7 U MRC 68/38 TSH.
* Indicates non-parallel inhibition.

## RESULTS

The specificity and precision of the $\alpha$-subunit assay system developed is given in Table 1. All gonadotrophin hormones and subunits with the exception of HCG and HCG$\beta$ gave inhibition parellel to that of the LH$\alpha$ standard (Fig. 4). HCG$\alpha$ was equipotent to LH$\alpha$ and dilutions of human serum gave a parallel response in the assay. LH$\alpha$ was recovered quantitatively from serum with a recovery of 95 $\pm$ 14 % (mean $\pm$ SD).

All LH and FSH preparations gave inhibition parallel to that of the LH$\alpha$ standard (Fig. 2). On a weight basis, FSH (CPDS 3) was 20 times more potent than FSH (CPDS 6). Repurified LH ($LH_2$) (see below) was 3 times less potent

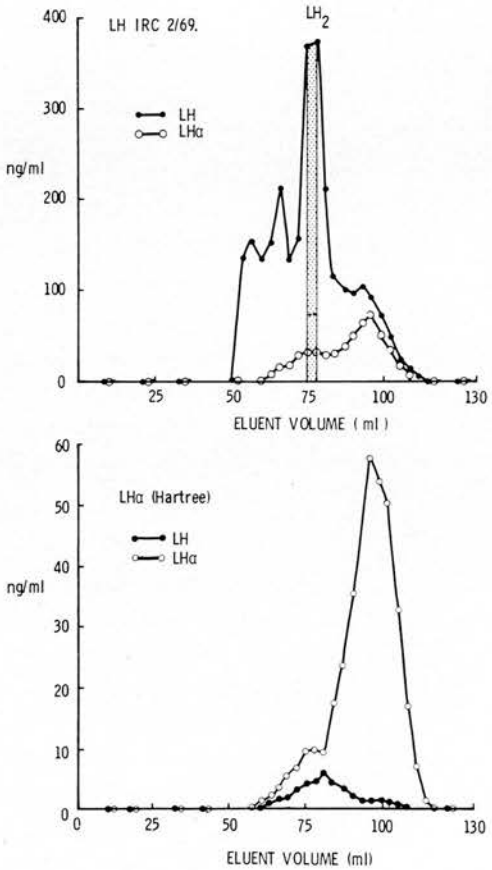



*Fig. 1.*
Column chromatography of LH and LH$\alpha$. Elution patterns determined by radioimmunoassay. The LH peak ($LH_2$) was used for cross-reaction study in the LH$\alpha$ assay.

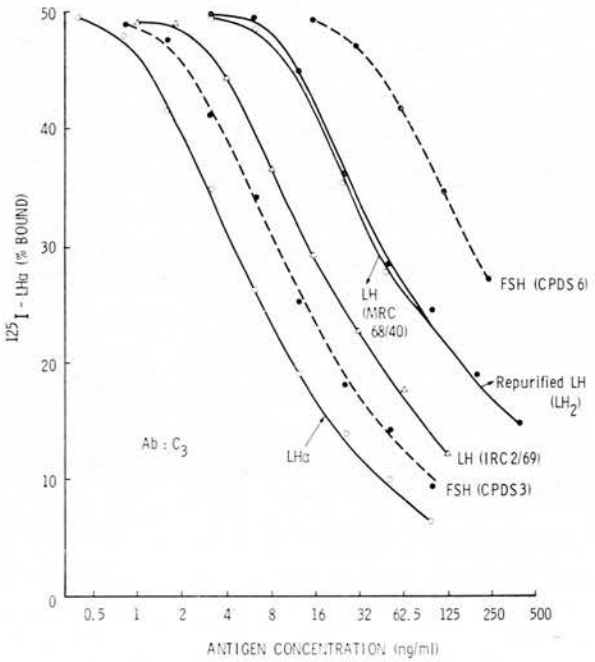



*Fig. 2.*
Cross-reaction of the different LH and FSH preparations in the LH$\alpha$-subunit assay.

than LH IRC 2/69 preparation and equipotent to LH 68/40 (assuming 40 IU/amp). The LH$\beta$ preparation obtained from Dr. Hartree and the MRC were equipotent but 10 times less potent than the LH$\alpha$ standard (Fig. 3). In contrast, LH$\beta$ (Lequin) was 50 times less potent than the other two LH$\beta$ preparations.

*Column chromatography*

The LH preparation (LH IRC 2/69) contained immunoreactive material which both physically and immunologically behaved like LH (Ve/Vo = 1.51) but also contained at least one peak with a molecular weight higher than LH (Ve/Vo = 1.21) and one peak of a molecular species similar to the LH$\alpha$ subunit (Ve/Vo = 1.78) (Fig. 1). The peak fractions of LH ($LH_2$) which gave parallel inhibition in the LH assay were pooled and used for cross-reaction studies. The LH$\alpha$ preparation showed only one major peak (Ve/Vo = 1.78).

*Serum concentrations*

Post-menopausal women had significantly higher values of $\alpha$-subunit than pre-menopausal women and men ($P < 0.01$) (Fig. 5). Intravenous administra-

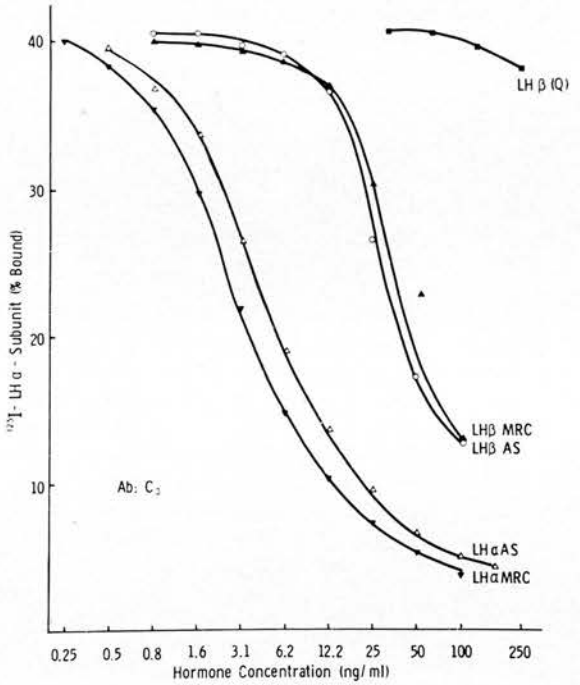



*Fig. 3.*

Cross-reaction of the different LH$\alpha$ and LH$\beta$ subunit preparations in the LH$\alpha$ assay. Note that LH$\beta$ (Lequin) is 50 times less potent than LH$\beta$ (Hartree).

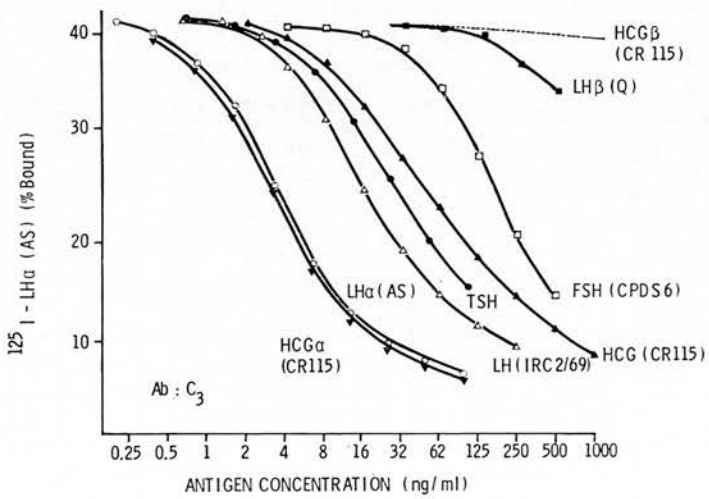



*Fig. 4.*

Cross-reaction of the intact glycoprotein hormones and their subunits in the LH$\alpha$ subunit assay.

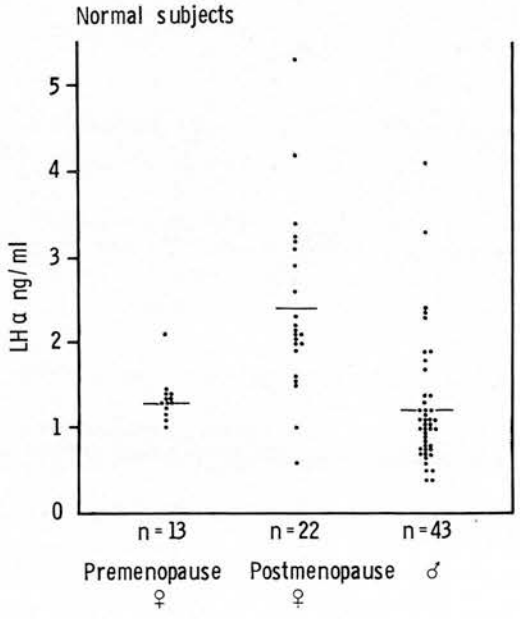



*Fig. 5.*

LH$\alpha$ subunit concentration in serum in normal pre-menopausal, post menopausal women, and men. Individual levels are shown for each group.
The straight line (——) indicates the mean for each group.

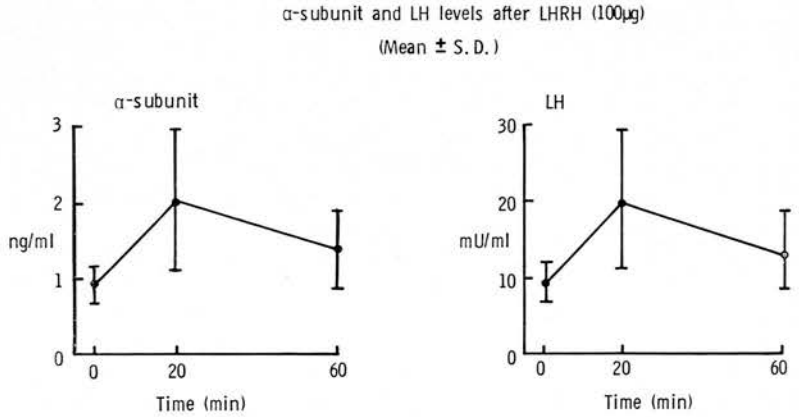



*Fig. 6.*

Mean (± SD) responses of LH and $\alpha$-subunit to LH-RH (100 $\mu$g) in 12 normal male subjects. (LH expressed as mU 68/40/ml).

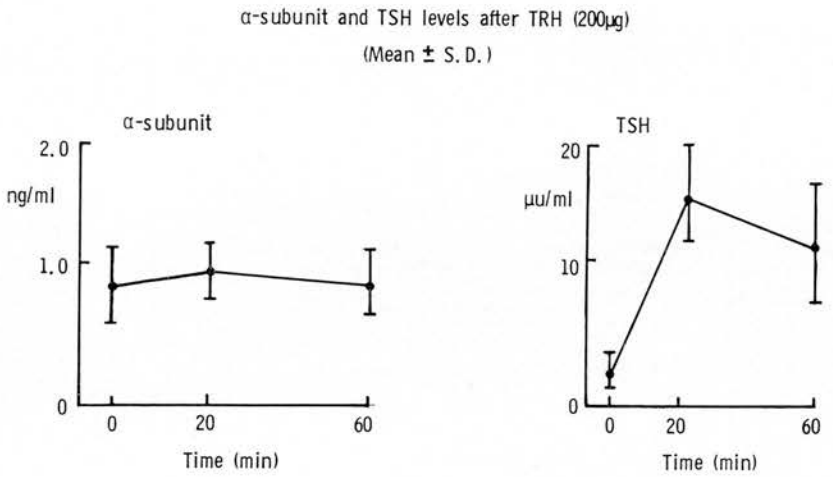



*Fig. 7.*
Mean (± SD) responses of TSH and α-subunit to TRH (200 μg) in 8 of the 12 normal men shown in Fig. 7.

tion of LH-RH (100 μg) in 12 normal men (Fig. 6) resulted in a significant ($P < 0.01$) rise in serum LHα-like activity after 20 min. In contrast, no significant responses were seen after intravenous administration of TRH (200 μg) in 6 normal men (Fig. 7).

In the foetus no significant ($P > 0.05$) difference was seen between arterial and venous cord levels of intact hormone or α-subunit, but in all cases levels of α-subunit (40–60 ng/ml) were at least two times greater than those of intact LH (24–69 ng/ml), or FSH (0.9–1.5 ng/ml).

## DISCUSSION

The interpretation of specificity of the subunit assay is difficult, since the reactivity of intact LH, FSH and the LHβ subunit from different sources showed considerable variation (Figs. 2 and 3). In addition, the LHα assay appears to detect LH IRC 2/69 at a level of 2 ng/ml or greater. As this is equivalent to 4 mU/ml of LH (MRC 68/40 assuming 40 IU/amp) in serum, it would be expected that all LH levels in excess of 4 mU/ml would be detectable and measured as LHα-subunit. This would indicate that the LHα subunit assay is not sufficiently specific in terms of LH to measure subunit alone, since the normal values of LH for men (up to 10 mU/ml) and women (up to 15 mU/ml) are in excess of the apparent cross-reaction with LH. However, chromatographic analysis of LH IRC 2/69 (Fig. 1) revealed some 10 %

*Table 2.*
The specificity* of different α-subunit assays.

| LHα assay | LH | FSH | TSH | HCG | HCGα | FSHα | HCGβ | FSHβ | LHβ |
|---|---|---|---|---|---|---|---|---|---|
| *Vaitukaitis et al.* (1972) | – | – | – | 2 %<br>Canfield | 100 %<br>Canfield | 75 %<br>Saxena | – | – | – |
| *Hagen & McNeilly* (in press) | 8 %<br>MRC 68/40 | 1 %<br>CPDS 6 | 25 %<br>NPA | 2 %<br>CR 115 | 100 %<br>CR 115 | – | 0.01 %<br>CR 115 | – | 0.01 %<br>Lequin |
| HCGα assay | LH | FSH | TSH | HCG | LHα | FSHα | HCGβ | FSHβ | LHβ |
| *Laburthe et al.* (1973) | 50 %<br>Hartree | 25 %<br>LER 869 | – | – | – | – | – | – | – |
| *Rabinowitz et al.* (1973) | 2 %<br> | 5 %<br>LER 1366 | – | 2 %<br>CR 117 | 100 % | 10 %<br>LER 1661 | 2 %<br>CR 117 | 50 % | – |
| *Vaitukaitis & Ross* (1972) | 7 %<br>LER 960 | 2 %<br>LER 1366 | 1 %<br>Pierce | 1 %<br>CR 100 | 2 %<br>Reichert | 5 %<br>Saxena | 0.01 %<br>CR 100 | 1 %<br>Saxena | 0.5 %<br>Reichert |

* Expressed as the potency of the hormone or subunit in percentage of the material to which the assay is directed. Notice that different authors have used different hormone and subunit preparations to assess the specificity.

contamination with a molecule indistinguishable from the $\alpha$-subunit, repurified LH ($LH_2$) did cross-react in the subunit assay at levels up to 10 ng/ml, 20 mU 68/40/ml. Thus is appears that in most situations the circulating levels of intact LH will not interfere in the assay.

In the subunit assay equivalent inhibition was seen with both LH$\alpha$ and HCG$\alpha$. As the $\alpha$-subunits of the glycoprotein hormones appear to be immunologically similar (*Reichert et al.* 1973), the exact nature of the immunoreactive material measured in the LH$\alpha$ subunit assay remains to be determined. The same non-specificity is seen in other $\alpha$-subunit assays with the exception of that reported by *Vaitukaitis & Ross* (1972), (Table 2).

Application of the $\alpha$-subunit assay to the measurement of circulating levels in normal male and pre- and post-menopausal female subjects indicated that it may circulate independently of the intact hormones, since the amount of subunit measured in these sera and after LH-RH administration was greater than could be explained by the levels of LH, FSH and TSH. The absence of detectable $\alpha$-subunit release after TRH may reflect either no release of $\alpha$-subunit or lack of cross-reaction of the TSH $\alpha$-subunit.

The high levels of $\alpha$-subunit present in the foetal cord sera were of considerable interest since *Franchimont & Pasteels* (1972) have demonstrated independent release of $\alpha$-subunit from the foetal pituitary *in vitro*. However, since the LH$\alpha$ and HCG$\alpha$ subunits cross-react equally in the assay our present results do not indicate the origin of this subunnit and a placental origin cannot be ruled out.

The present report confirms the results obtained by *Laburthe et al.* (1973), that the $\alpha$-subunit circulates in "free" form, and that it may be secreted independently of LH. The LH$\alpha$ radioimmunoassay described is sufficiently specific and sensitive to measure basal circulating levels of the subunit and further work is required to determine whether situations occur in which the subunit is secreted at different rates to intact hormone.

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We thank Dr. T. Chard for helpful discussions and Drs. W. R. Butt, R. E. Canfield, H. G. Friesen, A. S. Hartree, R. M. Lequin, the MRC and NPA for reagents used in the radioimmunoassays.

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# EVALUATION OF THE SPECIFICITY OF A LUTEINIZING HORMONE β AND α SUBUNIT ASSAY

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## Summary

A non-specific antihuman FSH antiserum which is able to bind $^{125}$I-labelled FSH, LH, LHβ and LHα subunits, has been shown to contain at least three different antibody populations directed against different parts of the LH and FSH molecule. This antiserum has been used to develop a rather specific radioimmunoassay for determination of the common α subunit. Further, by gel filtration it is shown that the LH IRC2/69 preparation contains about 30% of intact LH and about 10% of α and LHβ subunit, respectively. In contrast, the LH 68/40 preparation contains about 96% of intact LH and only small amounts of α and LHβ subunit. Also, a considerable difference in the content of the α subunit was found between two different FSH preparations. The contamination of the preparations of the intact hormones is shown to have a marked influence on the cross-reactivity of the substances in an LHβ and α subunit radioimmunoassay.

## Introduction

The three pituitary glycoprotein hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), and human chorionic gonadotrophin (HCG) of placental origin consist of two non-covalently bound subunits, a common α and a hormone-specific β subunit [1—4].

The structural relationship and complexity of these hormones and subunits has made it difficult to obtain chemically pure materials for raising specific antibodies and for standards. Therefore, the development of specific radioimmunoassays for measurement of these substances may encounter considerable

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problems of immunological cross-reactivity in which structurally related substances may exhibit significant activity in the assays [5,6].

Beside these general problems a more specific problem concerning the structure of the glycoprotein hormones exists due to the common antigenic sites on the α subunits and on the different β subunits of these hormones [7—10].

The present study shows that a non-specific antiserum, containing at least three different antibody populations directed against different parts of the gonadotrophin hormones, can be used in a rather specific radioimmunoassay directed against the common α subunit. Further, it is shown that the interpretation of the specificity of the α and an LHβ subunit radioimmunoassay is difficult, since the cross-reactivity of intact LH and FSH from different sources showed considerable differences.

## Materials and methods

### *Hormones and subunits*

The biological and immunological activities of the hormone and subunit preparations used are shown in Table I.

Because the same biological system and the same antibody have not been used for the determination of all LH and all FSH activities, respectively, the biological and immunological potency of the different preparations are not strictly comparable [11].

The LH MRC 68/40 preparation was supplied by the Medical Research Council, London. LH IRC2/69, LHα 4.1.1972 and LHβ 18.10.1972 subunit preparations were supplied by Dr. A. Stockell Hartree, Cambridge. The FSH CPDS3 and FSH CPDS6 preparations were supplied by Dr. W.R. Butt, Birming-

TABLE I

THE BIOLOGICAL AND IMMUNOLOGICAL POTENCIES OF HORMONE AND SUBUNIT PREPARATIONS

All values are expressed in I.U. of the 2nd International Reference Preparation of Human Menopausal Gonadotrophin per mg of hormone or subunit. For the conversion between different standard preparations see refs. 27 and 28.

| Preparation | Biological potency | | Immunological potency | | Reference No. |
|---|---|---|---|---|---|
| | LH | FSH | LH | FSH | |
| LH MRC 68/40 * | 7000 | — | 35 000 | <82 | 12,13 |
| LH IRC2/69 ** | 7500 | — | 10 000 | <37 | 12,13 |
| FSH CPS3 | — | 5000 | 6 580 | 13 080 | 14 |
| FSH CPDS6 ** | — | 7500 | 895 | 20 579 | 14 |
| LHβ ** | 425 | — | 13 140 §§ | <20 §§ | 15 |
| LHα ** | 555 | — | 2 190 | <20 §§ | 15 |
| FSHα N-611-C | — | <30 | — | 20 | 10 |
| TSHα N-745-B | — | — | — | <20 §§ | — |
| HCGα CR 115 | 115 § | — | Non parallel §§ | <20 §§ | 16 |

* Assuming 77 I.U. LH and <0.9 I.U. FSH biological activity per ampoule (1 ampoule contaning 11 μg pituiary protein).

** Used for iodination and as reference preparations in the respective assays.

§ HCG activity using the 2nd International HCG standard.

§§ Unpubslished observation by the authors.

ham; FSHα and TSHα were from Dr. A.F. Parlow, California; and HCGα was from Dr. R.E. Canfield, New York. In the studies of antiserum $C_3$, FSH CPDS6, LH IRC2/69, LHβ and LHα subunit were used as reference preparations, respectively.

*Iodination*

Human FSH CPDS6, LH IRC2/69, LHβ and LHα subunits were iodinated by a modification of the chloramine-T method [17—19].

*Antisera*

The antisera evaluated for the LHα assay (rabbit anti-FSH) was raised against FSH (CM1 preparation, Dr. A. Stockell-Hartree) by subcutaneous injections of 200 μg FSH in Freund's adjuvant into 16 rabbits. After three injections at monthly intervals, the animals were bled, the serum separated and stored at −20°C. All antisera showed binding to $^{125}$I-labelled LHα subunit with titres (50% binding of 50 pg [$^{125}$I] LHα) from 1 : 3000 to 1 : 24 000. The most sensitive and most specific antiserum when used in an LHα assay in terms of LH IRC2/69, FSH CPDS6 and LHβ subunit was used for further studies (antiserum $C_3$).

The anitserum used in the LHβ assay (rabbit anti-LHβ subunit; AS 146) was raised against human LHβ subunit (from Dr. A. Stockell-Hartree) and was kindly supplied by Dr. H.S. Jacobs, London.

*Assay conditions*

The LH, FSH, LHβ and α subunit radioimmunoassays were performed as previously described [17—19]. Working antiserum $C_3$ were selected from standard antiserum dilution curves to give a binding of $^{125}$I- labelled hormone or subunit (50 pg) to the antiserum of about 50%. The dilutions were as follows, 1 : 100 000 for $^{125}$I-labelled FSH CPDS6; 1 : 5000 for $^{125}$I-labelled LH IRC2/69; 1 : 3600 for $^{125}$I-labelled LHβ AS subunit and 1 : 12000 for $^{125}$I-labelled LHα subunit.

*Column chromatography*

250 mg of LH IRC2/69 or FSH CPDS6, 500 mg of FSH CPDS or 1000 mU of LH 68/40 were diluted in 3 ml of 0.05 M phosphate buffer, pH 7.4 and the solution applied to a 1.5 cm × 90 cm Sephadex G-100 column. The protein was eluted with 0.05 M diluent buffer containing 10 mg/ml of bovine serum albumin (Armour). The flow rate of the column was adjusted to 6 ml/h and 3-ml fractions were collected, at 4°C. The void volume of the column was estimated to be 51 ml by blue dextran-2000 (Pharmacia Ltd., U.K.) and the hormone and subunit concentration in each fraction was measured by the LH, FSH, LHβ and α subunit radioimmunoassays [17—19].

## Results

*Testing antiserum $C_3$ for more than one antibody population*

By using antiserum $C_3$ and $^{125}$I-labelled FSH CPDS6, the straight parts of the inhibition curves of LH IRC2/69 and LHα were parallel to that of the FSH

CPDS6 standard (Fig. 1A). On a weight basis the LHα subunit was equipotent to the FSH standard up to 6.2 ng/ml where the standard curve showed a sharp break. The LHβ subunit was, on a weight basis, 50 times less potent than the FSH standard.

In the "LH" and "LHβ" assay systems where $^{125}$I-labelled LH IRC2/69 and LHβ subunit were used (Figs. 1B and 2A) the inhibition curves of LH IRC2/69 and LHβ were parallel to the standard. However, non parallelism was seen for LHα and FSH CPDS6.

By using $^{125}$I-labelled LHα (Fig. 2B) the displacement curves for LH IRC2/69, FSH CPDS6 and LHβ subunits were parallel to the LHα standard. On a weight basis LH IRC2/69, LHβ and FSH CPDS6 were 4, 12 and 70 times less potent than the LHα standard.

*Specificity of the LHα subunit assay*

The LHα, FSHα, TSHα and HCGα subunit preparations were all equipotent in the LHα assay (Fig. 2B). The LH 68/40 preparation was 3 times less potent than the LH IRC2/69 preparation and on a weight basis FSH CPDS3 was 20 times more potent than FSH CPDS6.

*Specificity of the LHβ subunit assay*

In contrast to the equipotency of the four different α subunit preparations in the LHα assay, different potencies of these subunits were seen in the LHβ subunit assay (Fig. 3A). The inhibition curves of TSHα, LHα and FSHα subunits were parallel to the LHβ standard. In concentrations up to 100 ng/ml the HCGα preparation was not able to displace labelled LHβ subunit from the antibody. As in the LHα subunit assay a difference in potency between LH IRC2/69 and LH 68/40, and FSH CPDS3 and FSH CPDS6 could be demonstrated. For a similar effect, the concentration of LH IRC2/69 has to be increased by a

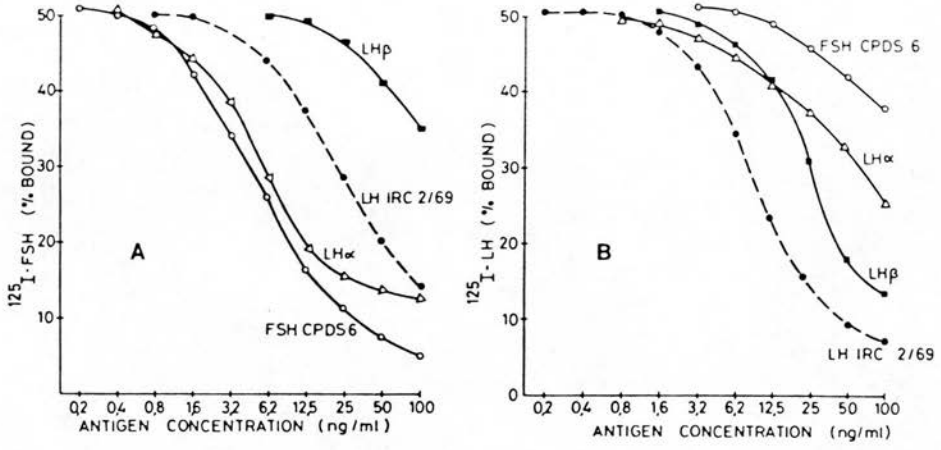



Fig. 1. Competitive binding curves of FSH CPDS6 (○———○), LHα (△———△), LH IRC2/69 (●- - - - -●) and LHβ (■———■) in an "FSH assay" system (A) using antiserum $C_3$ (dilution 1 : 100 000) and $^{125}$I-labelled FSH CPDS6, and in an "LH assay" system (B) using antiserum $C_3$ (dilution 1 : 5000) and $^{125}$I-labelled LH IRC2/69.

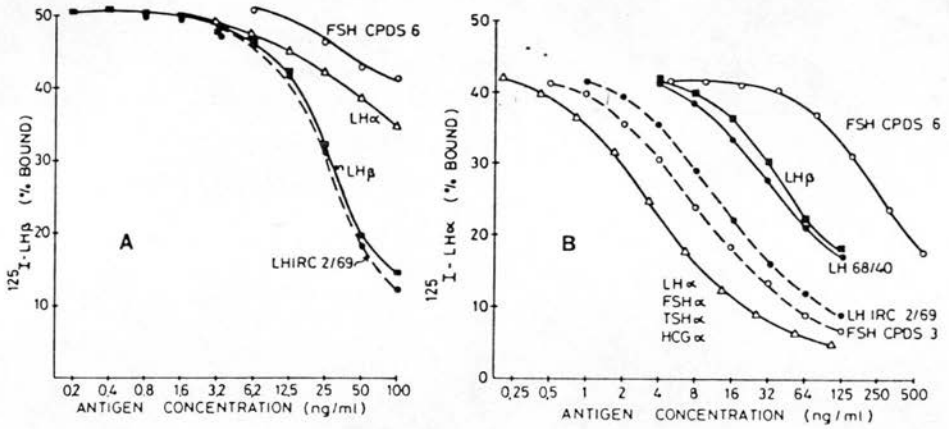



Fig. 2. Competitive binding curves of LHβ (■———■), LH IRC2/69 (●- - - - -●), LHα (△———△) and FSH CPDS6 (○———○) in an "LHβ assay" system (A) using antiserum $C_3$ (dilution 1 : 3600) and $^{125}$I-labelled LHβ subunit, and of LHα (△) FSHα (△), TSHα (△), HCGα (△), FSH CPDS3 (○- - - - -○), FSH CPDS6 (○———○), LH IRC2/69 (●- - - - -●) LH 68/40 (●———●) and LHβ (■———■) in an "LHα assay" (B) using antiserum $C_3$ (dilution 1 : 12 000) and $^{125}$I-labelled LHα subunit.

factor of 2 compared to that of LH 68/40 and FSH CPDS3 by a factor of 16 compared to that of FSH CPDS6.

*Gel filtration*

The peak fractions of LH and FSH were eluted around 75 ml while the peak fractions for both α and LHβ subunits were eluted around 100 ml (Figs. 4 and 5) [19].

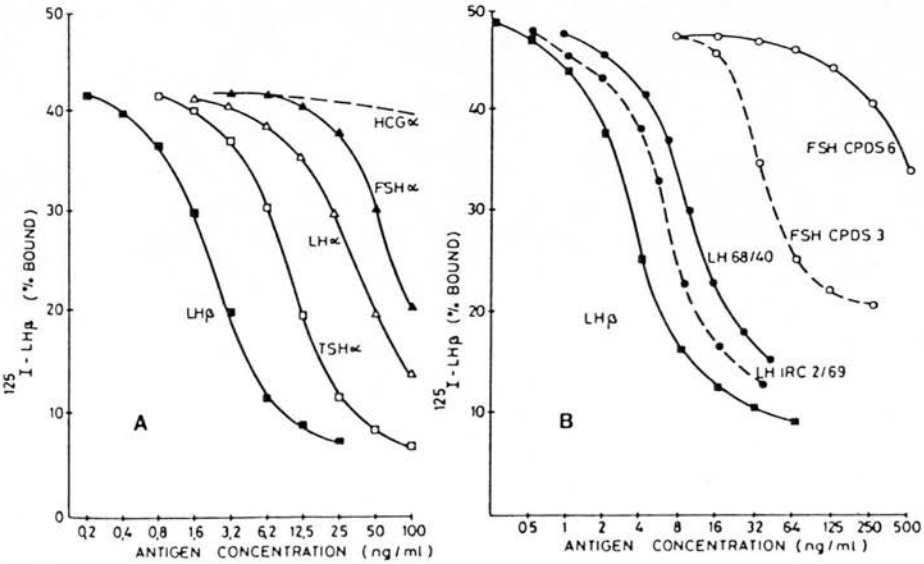



Fig. 3. Competitive binding curves of LHβ (■———■), TSHα (□———□), LHα (△———△), FSHα (▲———▲), and HCGα (- - - - -) in an "LHβ assay" (A) using antiserum AS 146 (dilution 1 : 60 000) and $^{125}$I-labelled LHβ subunit, and of LHβ (■———■), LH IRC2/69 (●- - - - -●), LH 68/40 (●———●), FSH CPDS3 (○- - - - -○) and FSH CPDS6 (○———○) in an "LHβ assay" (B) using antiserum AS 146 (dilution 1 : 60 000) and $^{125}$I-labelled LHβ subunit.

*LH preparations*

The LH IRC2/69 preparation (Fig. 4A) showed an elution pattern compatible with a content of about 30% of intact LH which was eluted at 75 ml, but also about 10% of molecular species which both physically and immunologically were recognised as α and LHβ subunits, respectively. In addition, at least two peaks of molecular species larger than LH could be demonstrated. These presumably represent different aggregates of LH and its subunits. The LH 68/40 preparation (Fig. 4B) showed one major peak which was eluted at 75 ml and only small amounts of other molecular species. These other species were the α subunit (2%) and the LHβ subunit (1.5%). The molecular species eluted at 75 ml but recognised both in the α and LHβ subunit assays presumably represents LH which cross-reacts in these assays (Figs. 3 and 4).

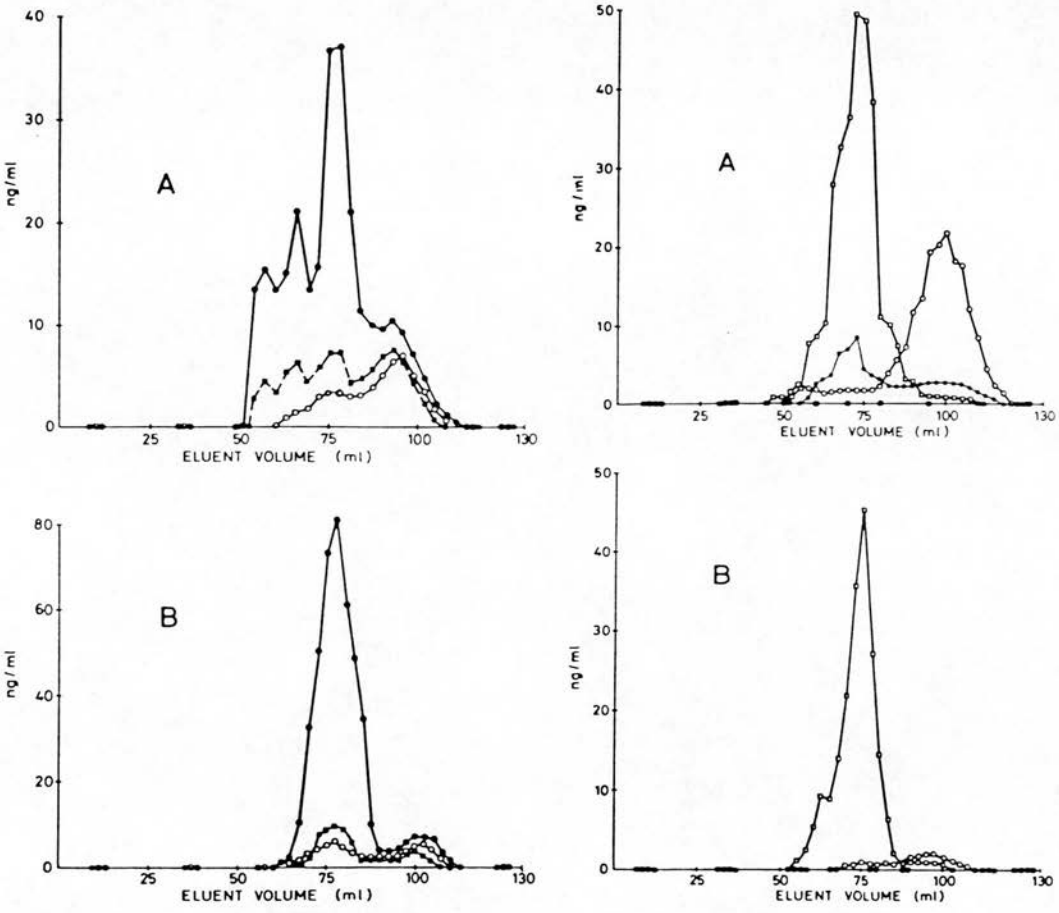



Fig. 4. Gel filtration of LH IRC2/69 (A) and LH 68/40 (B) on Sephadex G-100. The elution pattern is determined by LH (●——●), LHβ (■- - - - -■) and α subunit (○——○) radioimmunoassays.

Fig. 5. Gel filtration of FSH CPDS3 (A) and FSH CPDS6 (B) on Sephadex G-100. The elution pattern is determined by FSH (□——□), LH (●——●), LHβ (■——■) and α subunit (○——○) radioimmunoassays.

*FSH preparations*

The FSH CPDS3 preparation showed a very heterogenous picture (Fig. 5A). Only 32% of this preparation was recognised as immunoreactive FSH eluted at 75 ml where the immunoreactive LH (9%) also was found. No immunoreactive LHβ subunit could be detected but the common α subunit eluted at 100 ml formed about 50% of this preparation. In contrast, the FSH CPDS6 preparation was almost homogenous (Fig. 5B). No immunoreactive LH or LHβ subunit could be detected and the common α subunit eluted at 100 ml formed only 3% of this preparation.

## Discussion

The LHα subunit assay described is a double antibody radioimmunoassay, where the first antibody is a non-specific antiserum ($C_3$) raised against impure FSH (CM 1) containing both LH and FSH activity [13]. As expected, the antiserum contained at least three different antibody populations directed against different parts of the glycoprotein hormones. The antibodies binding labelled LH and LHβ subunits seemed to be the same (Figs. 1B and 2A), presumably directed against the specific β subunit of LH, but different from those binding FSH and LHα subunits (Figs. 1A and 2B). In the "FSH" assay system using labelled FSH as tracer, the sharp break in the LHα inhibition curve indicates interaction with a subset of lower affinity antibodies, which were readily saturated with smaller amounts of antigen. Hence, in agreement with other studies [20] it seems likely that FSH has at least two different antigenic sites.

The structural and immunological similarities between different α subunits [20—24] are shown by the equipotency of all α subunit preparations in the LHα assay (Fig. 2B); the same non-specificity has been described for other α subunit assays [9,23,24]. However, in the LHβ assay (Fig. 3A) the same α subunits behaved differently and showed different dose-response curves. This may not be true cross-reaction due to intrinsic immunologic differences; it is more likely to show the different degrees of contamination of these subunit preparations.

The percentage contamination of the LHα preparation calculated from the degree of inhibition in the LHβ assay (6%) (Fig. 3A) and from the gel filtration study [18] agrees well with the bioassay values obtained (Dr. A. Stockell-Hartree, personal communication); which showed about 5% contamination with LHβ subunit or 10% contamination with intact LH.

The degree of cross-reaction of the LHα preparation in the LHβ assay (Fig. 3A) could be explained from the content of LHβ subunit [18]. This suggests that the anti LHβ antiserum (AS 146) recognises antigenic sites on the LHβ subunit, which are not available on the LHα subunit. The fact that the HCGα preparation shows less than 0.5% cross-reaction in the LHβ assay supports this finding

Both the LH IRC2/69 and FSH CPDS3 preparations have been found to contain more than one molecular species [25,26]. In contrast, the LH 68/40 and FSH CPDS6 preparations have shown a high degree of purity [13,14]. This agrees with the 2% contamination with α subunit and 1.5% contamination with LHβ subunit found in the LH 68/40 preparation in this study (Fig. 4B). The

potency of LH 68/40 in the LHα subunit assay could be explained if one assumed that the cross-reaction is multifactorially caused, as much by intrinsic antigenic similarities as by the degree of contamination.

The FSH CPDS3 preparation is very impure, being shown both by gel filtration and by cross-reaction studies to contain about 50% α subunit and 10% LH (Figs. 2B, 3B and 5A). In contrast, the FSH CPDS6 preparation has been shown by both methods (Figs. 2B, 3B and 5B) to contain only 3% α subunit and 97% FSH.

The present study has shown that a non-specific antihuman FSH antiserum which is able to bind $^{125}$I-labelled FSH, LH, LHβ and α subunits and which contains at least three different antibody populations can be used in a rather specific radioimmunoassay for the determination of the common α subunit. It has also been shown that the structural similarities and complexity of the glycoprotein hormones and subunits make it difficult to evaluate the specificity of the radioimmunoassays against these substances.

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# New Bioassay for Luteinizing Hormone

UNTIL now it has not been possible to measure, by biological assay, the basal circulating concentrations of luteinizing hormone (LH). As a result, all information on the physiological role of this critically important pituitary glycoprotein is based either on indirect evidence, or on the use of immunological assays which do not necessarily measure biologically active hormone. We now describe a specific biological assay for LH which is more sensitive than radioimmunoassay, and several orders of magnitude more sensitive than other biological assays. The principle of the new method is based on a combination of the ovarian ascorbic acid depletion (OAAD) technique of Parlow[1], and the histochemical method of measuring ACTH described by Chayen *et al.*[2]. Reducing activity in ovarian slices treated with LH is estimated by microdensitometry after staining with Prussian blue.

Female weanling Wistar rats (21–25 d old) received a subcutaneous injection of 50 IU of pregnant mares' serum gonadotrophin ('Gestyl', Organon) followed 50–60 h later by 25 IU of human chorionic gonadotrophin ('Pregnyl', Organon). Seven to 10 d after the first injection the rats were killed and the ovaries removed, stripped of surrounding fat and each cut into three equal pieces. Each portion was placed on defatted lens paper on a stainless steel mesh grid in a vitreosil dish containing Trowell's T-8 medium (*p*H 7.6) enriched with $10^{-3}$ M sodium ascorbate. The dishes were placed in an air-tight perspex culture chamber and incubated for 5 h at 37° C in 95% oxygen and 5% $CO_2$. The medium was then removed and replaced with T-8 medium containing human LH or dilutions of plasma samples. After exposure for 4 min to LH or plasma the tissue was removed and chilled rapidly by immersion in *n*-hexane

maintained at −65° C by a mixture of solid carbon dioxide and industrial spirit. After freezing, the ovarian tissue was placed in a cold glass tube and stored at −70° C. Sections of tissue 12 μm thick were prepared in a cryostat at −30° C. After drying, the sections were stained by immersion in a mixture of 2.4% ammonium ferric sulphate (3 vols) and 0.1% potassium ferricyanide (1 vol). Fresh stain was added three times at intervals of 7 min to give a total reaction time of 21 min. The intensity of colour produced by the formation of ferric ferricyanide (Prussian blue) in the theca lutein cells, which is a function of the redox potential of the tissue, was then measured by scanning and integrating microdensitometry using the 'Vickers' M85 microdensitometer. The integrated extinction of light in ten areas (approximately twenty cells per area) of luteinized tissue was measured at 640 nm and again at 460 nm to correct for non-specific absorption of light by the section ; the difference between the two readings was calculated and the result multiplied by 100 for convenience.

A graph of the integrated extinction (times 100) against the concentration of LH (MRC 68/40) revealed an inverse linear correlation between the intensity of the stain in the theca lutein cells and the logarithm of the concentration of LH over the range 5–0.005 mU $ml^{-1}$ (Fig. 1). The mean index of precision calculated from sixteen consecutive assays performed by two assayists was 0.12. The mean slope of the regression line for sixteen assays was 1.96, that is, for each ten-fold change in LH concentration a change of 1.96 units of extinction occurred. The minimum detectable quantity of LH (MRC 68/40) was 0.005 mU $ml^{-1}$ of medium.

A variety of natural and synthetic protein and peptide hormones was studied to assess the specificity of the assay (Table 1). These were purified human FSH (CPDS/3), ovine prolactin (NIH-P-S8), purified porcine ACTH (third IWS), synthetic α (1–24) ACTH ('Synacthen', Ciba), synthetic AVP (Ferring AB) and purified human TSH (MRC DE 323). All

**Table 1** Cross-reactivity of Various Peptide Hormones in the LH Redox Bioassay

| Peptide | Percentage cross-reactivity |
|---|---|
| FSH (CPDS/3) | 1.0 |
| Ovine prolactin (NIH-P-S8) | 1–2 |
| ACTH (third IWS) | 0.3 |
| ACTH ('Synacthen') | <0.01 |
| AVP (synthetic) | <0.01 |
| TSH (DE 323) | 1.0 |

All hormones were assayed at two concentrations, 100 and 10 ng $ml^{-1}$, against LH IRC 2/64.

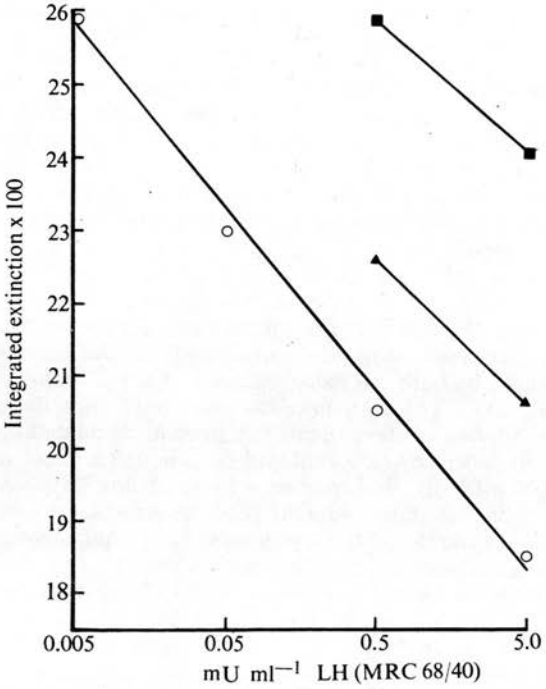



**Fig. 1** LH redox standard curve: ○, standard LH; ▲, normal male; plasma diluted 1 : 10 and 1 : 100; ■, isolated gonadotrophin deficiency; plasma diluted 1 : 2.3 and 1 : 23.

were tested at two concentrations of 100 and 10 ng $ml^{-1}$ and compared with human LH (IRC 2/64) on a weight basis. This LH preparation was chosen for these comparisons because of its high degree of purity and low content of FSH and TSH as determined by radioimmunoassay. The FSH and ovine prolactin preparations cross-reacted to the extent of 1–2%, which could be caused by contamination with LH as a similar cross-reaction was observed in a specific radioimmunoassay. Similarly, LH contamination may account for the small degree of cross-reactivity (0.3%) of purified

**Table 2** Comparison of Plasma LH Concentrations (mU $ml^{-1}$, MRC 68/40) Measured by the Redox Bioassay and Radioimmunoassay

| Diagnosis | Redox bioassay | Radio-immunoassay |
|---|---|---|
| Normal male | 5.1 | 2.9 |
| Post-menopausal female | 23 | 20.2 |
| Isolated gonadotrophin deficiency | 0.07 | <0.4 |
| Normal male after LHRH | 40 | 44.1 |

porcine ACTH (third IWS), as biologically active synthetic $\alpha$ (1–24) ACTH gave no cross-reaction (<0.01%).

A comparison between two different preparations of LH showed that MRC LH 69/104 was approximately twice as potent as MRC LH 68/40, assuming a content of 25 IU of 69/104 and 40 IU 68/40 per ampoule. This is within the limits of reported potency estimates for these materials[3]. The preparation LH IRC2/64, used in the specificity studies, gave a potency estimate of 4,000 IU $mg^{-1}$ when assayed against MRC 68/40.

Plasma LH concentrations in a normal male before and after administration of 100 $\mu$g luteinizing hormone-releasing hormone (LHRH), a post-menopausal female, and a man with apparent isolated gonadotrophin deficiency were measured by both the redox bioassay and radioimmunoassay (Table 2). The radioimmunoassay could not detect LH in the plasma of the patient with presumed isolated gonadotrophin deficiency (<0.4 mU $ml^{-1}$), although a concentration of 0.07 mU $ml^{-1}$ was measured by the redox bioassay. The other concentrations were in good agreement.

The specificity of this assay is at least equivalent to that of any existing system for measurement of LH. The sensitivity is better than that of a typical radioimmunoassay, and vastly superior to that of the best current bioassays. Concentrations of LH can be estimated which have been previously undetectable by other assay techniques, and it will be possible for the first time to determine biologically active hormone throughout the menstrual cycle. It thus appears that the type of histochemical assay, initially described by Chayen and his colleagues for ACTH, is applicable to other hormones and may represent a new dimension in endocrinological investigation.

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# TECHNICAL ASPECTS OF THE REDOX BIOASSAY FOR LUTEINIZING HORMONE

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## INTRODUCTION

The cytochemical approach to hormone assay described by Chayen *et al.* (1972) has recently been applied to the measurement of luteinizing hormone (LH), providing a specific and sensitive assay for this material (Rees *et al.*, 1973). This system has a sensitivity comparable to most radioimmunoassays, and is adequate for the measurement of circulating levels of LH. However, our initial experience has revealed several problems which need further evaluation before the technique can be widely used. Three main areas of difficulty exist. First, the sample capacity is low and attempts have been made to improve this by several modifications. Second, there is considerable variation in both assay sensitivity and precision due in part to variation of response between individual animals. Finally, the response to LH occasionally shows a biphasic pattern. This paper describes a number of modifications designed to overcome these difficulties.

## ROUTINE ASSAY PROCEDURE

A simplified flow diagram of the assay is shown in Fig. 1. The standard LH used throughout these studies has been MRC LH 68/40 (assuming a content of 40 units per ampoule). The precision, sensitivity and specificity of the assay have been described (Rees *et al.*, 1973) and are further discussed in this symposium (Holdaway *et al.*, 1974). Preliminary data suggest that the change in redox state observed in ovarian segments after exposure to LH is reflected in increased progesterone output. Culture medium from segments exposed to a high dose (50 mU/ml) of LH over 4 min showed a significant increment of progesterone (720 and 140 pg/ml in two animals) compared to segments exposed to a low hormone concentration (0·05 mU/ml).

## IMPROVEMENT OF ASSAY OUTPUT

(a) *Increase in ovarian size*

Since the assay is performed by exposing segments of luteinized ovary from a single

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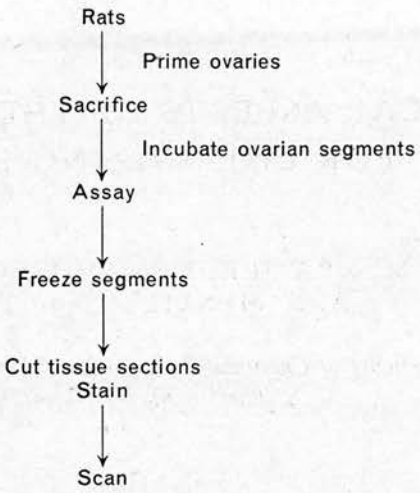



FIG. 1. Simplified flow diagram of the redox bioassay for LH.

animal to dilutions of standard LH or unknown sample, it would be an obvious advantage to increase the number of segments available. Fig. 2 shows the ovarian weight at different times after priming animals with gonadotrophins. This pattern was the same whether the response was expressed as absolute ovarian weight or ovarian weight per unit body weight. The usual time of sacrifice (112 hr) did not coincide with the time of maximum ovarian weight (64 hr). However, histological examination showed that between 16 and 64 hr after priming, ovaries consisted mainly of fluid-filled follicles with very little luteinized tissue available for scanning. By 112 hr full luteinization had occurred. At the same time, it was found that doubling the priming dose of human chorionic gonadotrophin resulted in a significant

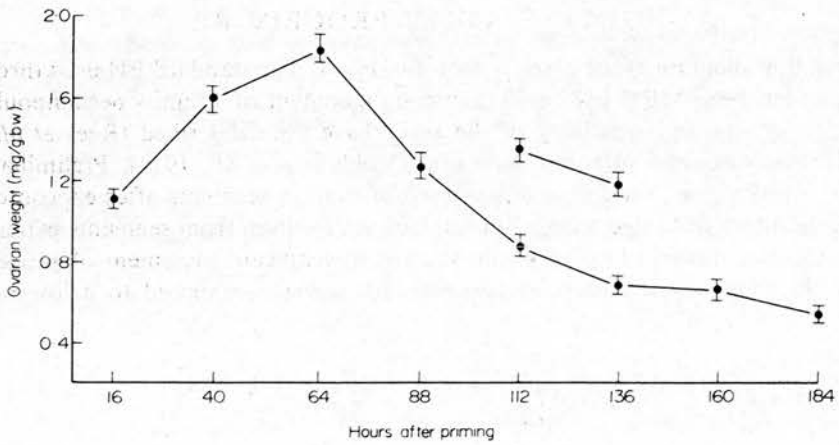



FIG. 2. The pattern of ovarian weight increase after priming rats with 50 I.U. pregnant mares' serum (PMS) and 25 I.U. HCG (mean ±SD, $n=3$). Short trace is ovarian weight after 50 I.U. PMS and 50 I.U. HCG (mean ±SD, $n=4$).

increase in ovarian weight at 112 hr and 136 hr (Fig. 2), providing sufficient segments to allow measurement of two or three dilutions of up to three unknown samples per assay.

(b) *Reduction of scanning time*

Most histochemical procedures involving quantitation by microdensitometry compensate for non-specific absorption (light scattering) by the stained tissue (Chayen *et al.*, 1972). Thus, in the redox assay of LH, as originally described, readings of stain intensity were made at a wavelength of 640 nm and a second reading at 460 nm, was subtracted as a non-specific blank. However, a review of fifty-five assays showed that the subtraction of a blank reading

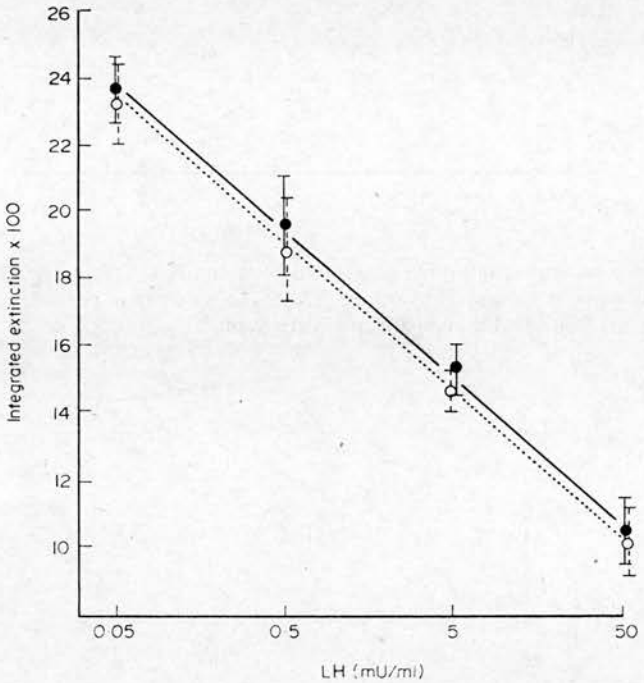



FIG. 3. Relationship between standard curves from one animal plotted with (interrupted line) and without (solid line) blank subtraction (mean ±SD of duplicate determinations).

did not affect either the slope or precision of the standard curve (Fig. 3) so that this correction has now been eliminated. Similarly, a 50% reduction in the area scanned did not affect the precision of the assay (Fig. 4). These two modifications have reduced the time spent in scanning by 75% and a further saving has been made by improvements in the mechanics of the microdensitometer.

## VARIATIONS IN ASSAY SENSITIVITY

Using a highly purified pituitary LH preparation (IRC2/64, kindly donated by Dr A. Hartree), the initial sensitivity of the redox assay was reported as 10 pg LH/ml (Rees *et al.*, 1973). When this preparation was replaced by MRC LH 68/40, the assay sensitivity was

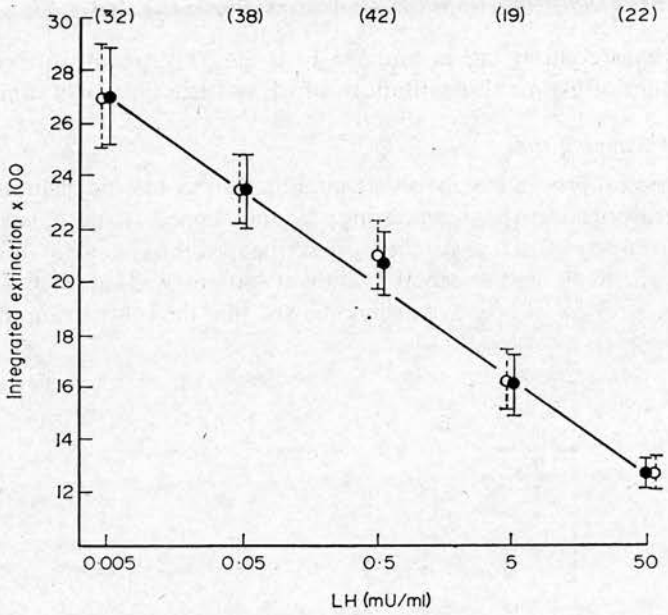



FIG. 4. Standard curve measuring five areas per tissue section ($10 \times 10^4$ $\mu m^2$) (●) and ten areas per tissue section ($20 \times 10^4$ $\mu m^2$) (○) (mean ± SE). The numbers in parentheses refer to the number of assays available for comparison at each point.

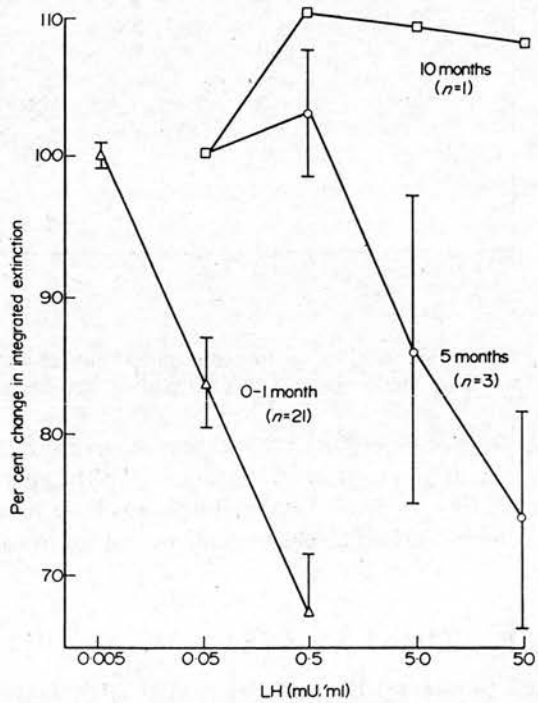



FIG. 5. Standard curves obtained from LH (MRC 68/40) stored in solution (10 I.U./ml) at −15°C for different lengths of time (mean ± SD).

TABLE 1. Variation in redox bioassay dose response curves in six Wistar and one Sprague-Dawley strain of rat

| Strain | *n* | Slope mean | Mean sensitivity (mU/ml LH 68/40) | Slope C of V (%) (mean) |
|---|---|---|---|---|
| Wistar, Barts | 4 | −1·50 | 0·05 | 58·6 |
| Wistar, Nottingham SPF SB | 4 | −0·87 | 5·0 | 24·4 |
| Wistar, WAG | 3 | −0·54 | 5·0 | 49·2 |
| Wistar, AGUS | 3 | −1·58 | 0·05 | 49·9 |
| Wistar, CFHB | 4 | −1·59 | 0·5 | 47·4 |
| Wistar, CFY | 4 | −0·76 | 5·0 | 90·5 |
| Sprague-Dawley | 4 | −1·0 | 0·5 | 68·5 |

found to vary from 0·5 to 0·005 mU/ml (approximately 100–1 pg/ml). At least some of this variation is due to instability of this preparation in frozen solution (Fig. 5); prolonged storage at −15°C causes a marked loss of biological activity.

Variation in assay sensitivity also appears to result from variation of response to LH between different animals. Since the strain of animal used may influence the results in a bioassay (Sakiz & Guilleman, 1963), seven strains of rat were compared in the LH redox assay to determine whether one strain would consistently provide uniform and sensitive dose response curves (Table 1). Considerable differences in the slope, sensitivity and precision of standard curves were observed both between strains, and within the same strain. No strain appeared to offer a significant advantage over the albino Wistar in current use.

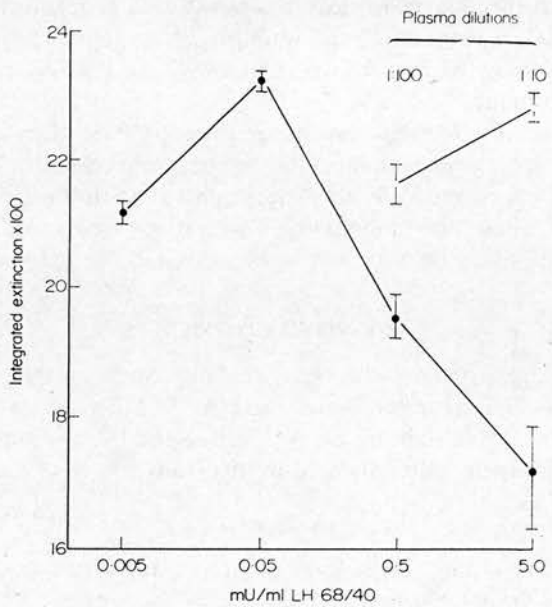



FIG. 6. Biphasic response of standard curve and parallel response of plasma sample (mean of duplicate measurements of integrated extinction).

## BIPHASIC STANDARD CURVES

Some 15% of standard curves show a change from a negative to positive slope between 0·05 and 0·005 mU/ml (Fig. 6), and dilutions of plasma shown to have a low LH content by radioimmunoassay may behave in an identical manner. However, most plasma dilutions fall within the portion of the curve with a negative slope.

## DISCUSSION

The redox bioassay of LH provides a sensitive and specific method for the measurement of LH which, however, has some disadvantages in its present form. Notable among these is a limited assay throughput; initially, only three or four samples could be assayed in a working week but the modifications outlined above have more than doubled this throughput without compromising assay sensitivity or precision. If the recently described modification of the corticotrophin redox assay (Alaghband-Zadeh *et al.*, 1974), in which multiple sections of tissue are exposed to hormone, can also be applied to the LH system, most of the problems of sample capacity will be resolved.

There are several explanations for the variation observed in assay sensitivity and precision. The preparation chosen for assay standardization has been shown to lose bioactivity on storage in solution at −15°C and more suitable or improved storage conditions are now being sought. There is also considerable variation in sensitivity between different animals, so that a small percentage of assays are too insensitive to permit precise measurement of unknown samples. Unfortunately, no animal strain with a consistently sensitive response has yet been found.

The cause of the biphasic response to LH observed in 10–15% of assays has not been determined. However, this change in slope has not caused problems in the measurement of normal plasma levels. In contrast to the multiphasic response reported with the ovarian cholesterol depletion assay (Skosey & Goldstein, 1964), the biphasic response does not seem to invalidate this technique.

It is particularly encouraging that the change in redox state of ovarian tissue induced by LH is reflected by appropriate changes in progesterone output. This correlation with recognized physiological events within the ovary contributes to the overall validation of the procedure. However, these are preliminary observations and more data are required to explore fully the relationship between steroidogenesis and the redox change.

## ACKNOWLEDGMENTS

I. M. Holdaway is supported by the New Zealand Medical Research Council, R. M. Kramer by the Medical Research Council, and A. S. McNeilly by the Wellcome Trust. We wish to thank Dr D. B. Crighton, Dr A. Hartree and the Division of Biological Standards (M.R.C.) for supplying materials used in this study.

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*Clinical Endocrinology* (1974) **3**, 383–388.

# APPLICATIONS OF THE REDOX BIOASSAY FOR LUTEINIZING HORMONE

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The redox assay provides a highly sensitive biological method for the measurement of luteinizing hormone (LH) (Rees *et al.*, 1973; Kramer *et al.*, 1974). Most current bioassays for gonadotrophins are limited in sensitivity, and can only measure high concentrations of hormone such as those in the pituitary or in extracts of bulk urine collections. As a result, most information on the physiological role of LH is based on indirect evidence or the measurement of plasma levels by radioimmunoassay; the latter is subject to the possibility of dissociation between immunological and biological activity (Hunter *et al.*, 1973; Peckham *et al.*, 1973). In the present studies we have examined the plasma levels of LH under various conditions, using both radioimmunoassay and the redox bioassay.

*Assay of plasma LH*

The technique of the assay has already been described in detail (Rees *et al.*, 1973) and subsequent modifications have led to an increased sample throughput (Kramer *et al.*, 1974). The sensitivity with respect to LH MRC 68/40 (40 units per ampoule) is such that a plasma level of 0·5 mU/ml can usually be detected using 1 ml of plasma at a starting dilution of 1:10. The between-assay variation for successive determinations on a plasma pool in six assays was 30%. Up to ten samples can be measured in a working week.

Radioimmunoassay of LH was carried out using LH MRC 68/40 as standard (40 units per ampoule), a specific antiserum to LH (MRC GP 70/229), and separation of bound and free hormone by the addition of a second antibody.

*Stability of endogenous LH in plasma*

There was no apparent difference in the biological activity of plasma assayed immediately after collection, and aliquots which were frozen and thawed once prior to assay. However, repeated freezing and thawing of plasma prior to assay caused a loss of activity (Fig. 1). Samples for assay are now aliquotted and frozen after separation of plasma and the assay is carried out immediately after thawing.

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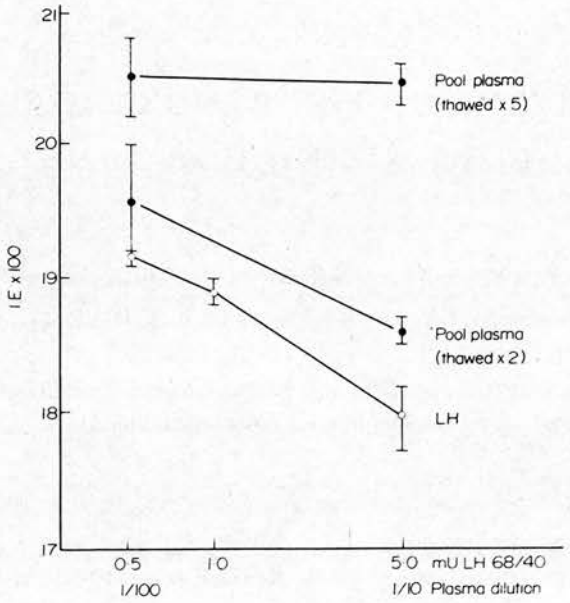



FIG. 1. Influence of repeated freeze-thawing of aliquots of a plasma sample on the bioactive LH content. Immunoactive LH content of this sample was 15·5 mU/ml. Each point represents the mean of duplicate measurements of integrated extinction.

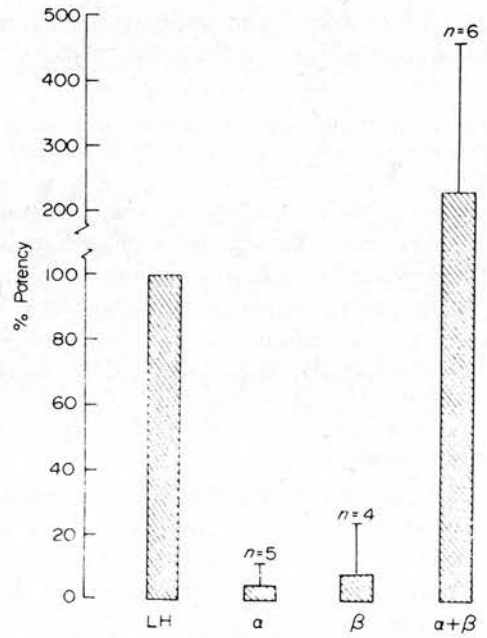



FIG. 2. Potency (mean ±SD) of LH subunits before and after recombination compared with equimolar amounts of LH MRC 68/40.

*Assay of LH subunits*

The $\alpha$ and $\beta$ subunits of LH (kindly supplied by the Medical Research Council, Division of Biological Standards) had low activity when compared with intact hormone in the redox system (Fig. 2). After incubation of the two subunits in 0·05 M phosphate buffer, pH 7·5, at 4°C for 78 hr, activity was much increased. Indeed, the recombined preparation apparently exceeded LH MRC 68/40 in potency; this probably reflects the use of stored LH 68/40 in this comparison, since the biopotency of this material decreases with prolonged storage in solution at −15°C (Kramer *et al.*, 1974).

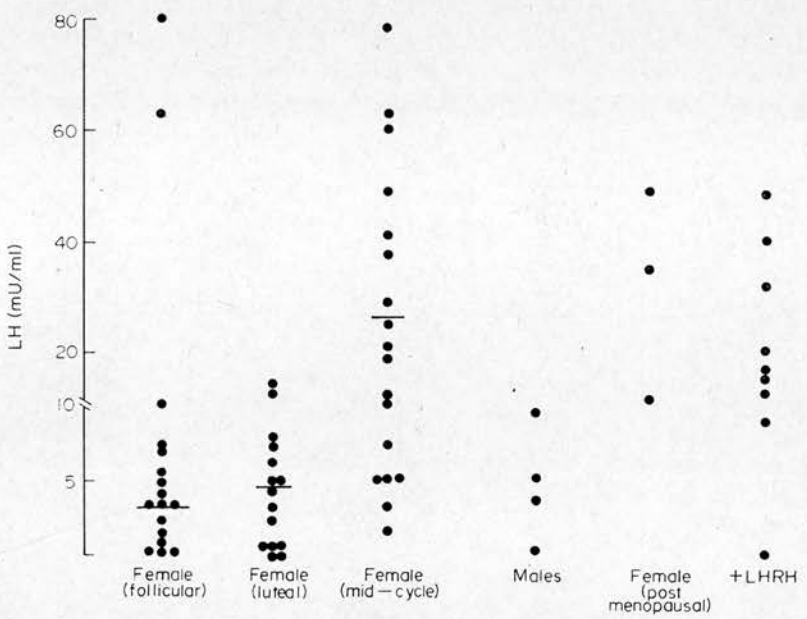



FIG. 3. Plasma LH concentrations (mU/ml MRC 68/40) measured by redox bioassay. Horizontal bars represent mean levels, excluding two highest measurements in female (follicular) group. +LHRH = levels 20 or 60 min after injection of synthetic gonadotrophin-releasing-hormone.

*Measurement of LH levels in plasma*

Dilutions of plasma usually give dose response curves parallel to standard hormone (Rees *et al.*, 1973). Significant non-parallelism between plasma and standard has been observed with 12% of plasma samples, but whether non-parallelism results from technical factors, specific or non-specific interference in the assay, or a difference between pituitary and plasma LH remains to be ascertained.

Plasma concentrations of LH have been measured in a variety of situations (Fig. 3). At the lower levels of LH in normal males and females, there was in general good agreement between the results of radioimmunoassay and biological assay. However, at higher levels, such as those encountered in females at mid-cycle, or in subjects who had received an injection of gonadotrophin-releasing hormone (Gn-RH), considerable discrepancies were observed.

In three subjects from whom serial plasma samples were available the mid-cycle rise in levels of immunoreactive LH was not accompanied by a rise in material active in the redox assay (Fig. 4). In two of these subjects there was an increase in biologically active material on the day immediately preceding the peak of immunoreactive LH, and in all three subjects there was a broad peak of bioactive material in the early part of the luteal phase which was not reflected in the results of immunoassay.

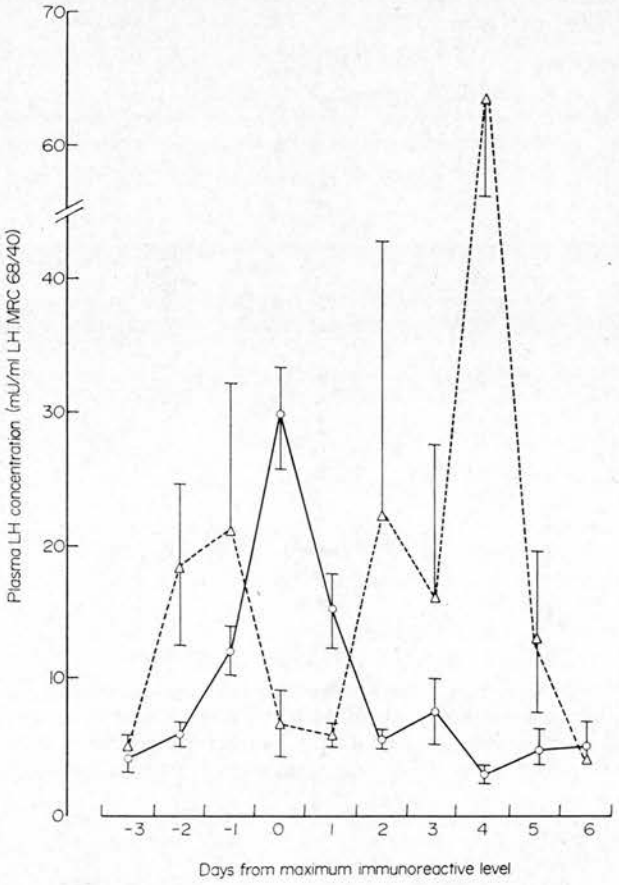



FIG. 4. Plasma LH concentrations (mean ±SEM) in three normal female subjects around mid-cycle measured by radioimmunoassay and redox bioassay. ○, Immunoreactive; △, bioactive.

Measurements of plasma LH activity in five subjects 20 min after administration of gonadotrophin-releasing hormone are shown in Fig. 5. Of three subjects with a normal pituitary–gonadal axis (subjects 1, 2 and 3, Fig. 5), the increase in biologically active LH was greater than that of immunoreactive LH, both in increment and absolute level, in two. A patient with impotence (subject 4, Fig. 5) had a normal response of immunoreactive LH but a poor response of bioactive hormone. A male patient with apparent idiopathic gonadotrophin deficiency (subject 5, Fig. 5) showed no rise using either assay. All measurements

included in Figs. 4 and 5 were made on samples that had been thawed and refrozen at least once prior to bioassay.

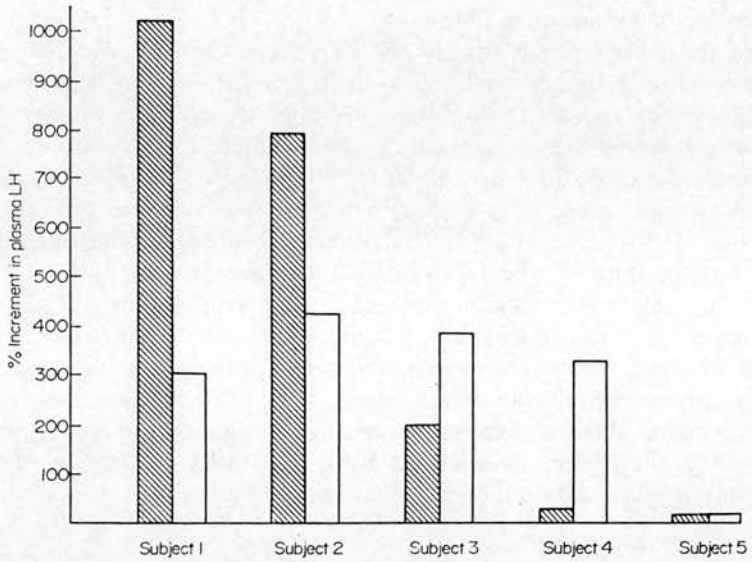



FIG. 5. Percentage increment over basal plasma LH measurements in samples obtained 20 min after intravenous administration of gonadotrophin-releasing-hormone to five subjects. Shaded columns: bioactive LH; open columns: immunoactive LH.

## DISCUSSION

The results obtained so far by the application of the redox assay to the measurement of plasma LH have been encouraging in some respects and disquieting in others. Thus, the behaviour of the subunits of LH in the assay is consistent with other reports of the bioactivity of these materials (Pierce, 1971), and the results obtained with measurement of basal circulating levels of LH are, in most circumstances, in good agreement with those obtained by radioimmunoassay. However, there is a large apparent discrepancy between immunological and biological LH measurements in plasma after administration of gonadotrophin-releasing hormone and at the time of midcycle in ovulating women. In the latter instance the pattern observed was consistent in the three subjects studied. The cause of this discrepancy is unknown and cannot, for instance, be readily explained on the basis of a difference in the *in vivo* half-times of bioactive and immunoreactive LH. However, it should be noted that these samples had been thawed and refrozen at least once, and in some instances several times, before assay. Thus caution is necessary in the interpretation of these data until the results have been repeated in a large number of subjects using adequate precautions against degradation of hormone in plasma samples. It is interesting to note that previous measurements of bioactive LH in plasma at midcycle showed a broad peak of LH activity (Watson, 1972), the duration of the rise being much greater than that found with radioimmunoassay. The precise relationship of the material measured by Watson to other

hormonal events is uncertain, since levels of immunoreactive LH and progesterone were not reported. Studies on the urinary excretion of bioactive gonadotrophins also reveal a broad and often variable peak at midcycle (Stevens, 1969), usually poorly related to the sharp rise in circulating immunoreactive LH.

In principle, the redox bioassay for LH offers several advantages over existing bioassays. It is sufficiently sensitive to measure basal levels in 1 ml or less of unextracted plasma. The precision of measurement of LH in plasma, although not as good as that which can be obtained with radioimmunoassay, appears to be satisfactory for a bioassay system. The assay is reasonably specific for LH (Rees *et al.*, 1973), with only HCG causing important cross-reaction among a range of glycoprotein and pituitary hormones so far tested. However, in its present form the assay suffers from disadvantages. The throughput is very limited, although this is likely to be improved by further development of the method (Kramer *et al.*, 1974). In contrast to radioimmunoassay, the conditions for sample handling are critical and require further investigation. But the most important problem is validation of the procedure. Existing results indicate striking discrepancies between this assay and a radioimmunoassay, particularly during measurement of elevated hormone levels; it is not at present clear whether these discrepancies are random and due to unidentified technical factors, or whether they reflect a new aspect of the physiology of LH. Considerable further work is necessary in the validation of this assay before any firm conclusions can be drawn.

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# PURIFICATION AND CHARACTERIZATION OF CAPRINE PROLACTIN

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SUMMARY

Prolactin was isolated from frozen goat pituitary glands by a simple procedure involving gel filtration and chromatography on DEAE-cellulose. The major product (yield, 2·5 mg/g pituitary tissue) had high pigeon crop sac-stimulating activity (27 i.u./mg) and was free of growth hormone and other pituitary hormones. The molecular weight was similar to that of ovine prolactin.

Caprine prolactin was immunologically indistinguishable from ovine prolactin in radioimmunoassays, in which ovine prolactin antiserum and either ovine or caprine prolactin labelled with $^{125}I$ were used.

The results indicate that caprine and ovine prolactin are closely related and that radioimmunoassay for ovine prolactin may be used to estimate caprine prolactin in serum.

## INTRODUCTION

Radioimmunoassay methods for prolactin have been applied to the measurement of blood levels of prolactin in the goat (Bryant & Greenwood, 1968; Johke, 1969; Hart, 1972) but ovine prolactin, because of its availability, has been used as the standard in each case. A test of the assumption that the ovine and caprine hormones have no significant immunological differences was clearly required. For this purpose we have isolated prolactin from goat pituitaries and studied its cross-reaction with ovine prolactin in a radioimmunoassay system.

## MATERIALS AND METHODS

### *Pituitaries*

Pituitary glands were excised from seven castrated male goats immediately after slaughter. Extraneous tissue was removed and the glands (total wt 4·34 g) were rapidly frozen and stored at −20 °C.

### *Preparation of pituitary extract*

The glands were homogenized in a small rotating-blade homogenizer (Folley & Watson, 1948) with 0·2 M-ammonium hydrogen carbonate (40 ml). After being stirred

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for 1 h at 5 °C the homogenate was centrifuged at 10000 *g* for 45 min, yielding a clear extract (38 ml, pH 8·5) containing about 31 mg protein/ml [estimated from absorbance at 260 and 280 nm (Warburg & Christian, 1942) by using the Calbiochem nomograph (Calbiochem Ltd, London, W. 1)].

*Gel filtration procedures*

For gel filtration on Sephadex G-100, the gel (fine grade; Pharmacia (Great Britain) Ltd, London, W. 5) was swollen in 0·2 M-ammonium hydrogen carbonate, packed into a column (2·5 × 50 cm) and equilibrated with the same buffer at 5 °C. The column was run at a hydrostatic pressure sufficient to give a flow-rate of 25 ml/h. The effluent was collected in 3 ml fractions.

Bio-Gel P-100 (100–200 mesh; Bio-Rad Laboratories Ltd, St Albans, Herts.) was swollen in 0·05 M-Tris–hydrochloride buffer, pH 7·8, and a column (2·5 × 42 cm) was packed with the gel in the cold and equilibrated with the same buffer. The flow-rate at a small hydrostatic pressure was 5 ml/h and the effluent was collected in 3 ml fractions.

*Ion-exchange chromatography*

DEAE-cellulose (DE-52; Whatman Biochemicals Ltd, Maidstone, Kent) was washed with 0·3 M-NaOH, water, 0·3 M-HCl and water again, and then equilibrated with 0·05 M-Tris–hydrochloride buffer, pH 7·8. Chromatography was carried out with a column (1 × 16 cm) of the ion-exchanger at 5 °C, the effluent being collected in 3 ml fractions. Proteins retained on the column in the presence of equilibrating buffer were eluted with an NaCl gradient, produced by pumping 0·15 M-NaCl dissolved in the equilibrating buffer into 200 ml of the same buffer, stirring continuously, and running the resultant solution through the column at the same rate (20 ml/h). An LKB Perpex pump (LKB Instruments Ltd, Croydon, Surrey) was used.

*Isolation of protein fractions*

The protein solutions from ion exchange chromatography were freed of non-volatile salts by membrane ultrafiltration in a Diaflo model 50 ultrafiltration cell containing a UM-10 membrane (Amicon N.V., Oosterhout N.B., Holland) and immersed in ice. Each solution was concentrated to 10 ml; dilution to 60 ml with 0·05 M-ammonium hydrogen carbonate and concentration again to 10 ml was repeated six times. The final solutions were lyophilized.

*Electrophoresis in polyacrylamide gels*

The homogeneity of isolated protein fractions was investigated by disc electrophoresis in 7% polyacrylamide gels at pH 8·9 as described by Davis (1964). The running gels measured 0·5 × 5·5 cm and electrophoresis at 4 mA/gel was continued until the dye front had nearly reached the bottom of the gel. Gels were stained with Naphthalene Black 10B (Amido Black 10B) (Raymond A. Lamb, Alperton, Middlesex) and de-stained electrolytically.

The protein fractions were also examined by disc electrophoresis under denaturing conditions to estimate the molecular weights of the constituent polypeptide chains (Weber & Osborn, 1969; Reynolds & Tanford, 1970) by the method of Anderson,

Cheeseman, Knight & Shipe (1972) modified for columns of gel as above instead of for plates. The gels, prepared in 0·05 M-sodium phosphate buffer, pH 8·0, contained 5 % acrylamide, 0·25 % bisacrylamide, 0·1 % sodium dodecyl sulphate (all w/v), 5 mM-EDTA and 5 mM-$\beta$-mercaptoethanol. Proteins were dissolved in 0·05 M-sodium phosphate buffer, pH 7·0, containing 40 % (w/v) sucrose, 0·1 % (w/v) sodium dodecyl sulphate, 5 mM-EDTA and 5 mM-$\beta$-mercaptoethanol to give concentrations of 5 mg/ml, and the solutions kept at room temperature for at least 1 h before use. The gels were pre-run at 2·5 mA/gel before application of samples (5 $\mu$l), then at the same current for another 3·5 h at room temperature. The gels were stained with Coomassie Brilliant Blue R (Raymond A. Lamb, Alperton, Middlesex) and de-stained in acetic acid–acetone mixtures. The following bovine proteins were used as molecular weight markers: $\alpha$-lactalbumin (mol. wt 14200), $\beta$-lactoglobulin (subunit mol. wt 18400), $\beta$-casein (monomer mol. wt 25000) and serum albumin (mol. wt 67000). The serum albumin was obtained from Sigma (London) Chemical Co. Ltd, Kingston-upon-Thames, Surrey, and the other proteins were prepared in this Institute.

### *Bioassay*

The biological activity of the isolated prolactin fractions was assessed by Dr I. A. Forsyth using the local pigeon crop sac method of Nicoll (1967). In preliminary bioassays NIH-P-S-6 prolactin (25 i.u./mg) was used as standard and in definitive bioassays of fraction $P_1$ with the balanced incomplete block design described by Forsyth & Hosking (1969) the 2nd International Standard prolactin (22 i.u./mg) was used for comparison.

### *Radioimmunoassay*

Caprine prolactin fractions $P_1$, $P_2$ and $P_3$ were compared with ovine prolactin NIH-P-S-6 (25 i.u./mg) in a specific ovine prolactin radioimmunoassay. Antiserum was raised in a rabbit by the subcutaneous injection of 500 $\mu$g ovine prolactin (NIH-P-S-6) in Freund's complete adjuvant (Difco). Booster injections were given 6 weeks and 4 months after the initial injection and the animal was bled 10 days after the last injection. Serum was separated and stored in portions at $-20$ °C.

Both ovine (NIH-P-S-6) and caprine (fraction $P_1$) prolactins were iodinated with $Na^{125}I$ (Radiochemical Centre, Amersham, Bucks) by a modification of the chloramine-T method of Greenwood, Hunter & Glover (1963) and purified as described by Hart (1972).

To determine a suitable antibody dilution for standard radioimmunoassay curves, the antiserum was diluted with assay buffer (0·05 M-sodium phosphate, pH 7·5, containing 1 % egg albumin and 0·01 M-sodium chloride) and 200 $\mu$l portions were mixed with one or other of the labelled hormones (0·5 ng) in 50 $\mu$l of assay buffer. After incubation of the mixture for 24 h at 4 °C, 50 $\mu$l of a second antibody (donkey anti-rabbit $\gamma$-globulin RD 17, Wellcome Reagents Ltd, Beckenham, Kent) diluted 1:10 with assay buffer and 50 $\mu$l normal rabbit serum diluted 1:80 with assay buffer, were added. Incubation at 4 °C was continued for another 16 h, then antibody-bound and free hormone were separated by centrifugation at 700 ***g*** for 30 min at 4 °C. Radioactivity in the precipitate was determined in a well-type gamma scintillation counter (LKB-Wallac, Sweden).

To compare the three caprine prolactin fractions with ovine prolactin NIH-P-S-6, samples of each prolactin preparation in 50 $\mu$l of assay buffer were mixed with 200 $\mu$l assay buffer and 50 $\mu$l anti-ovine prolactin anti-serum at a final dilution of 1:600000. Then, after incubation at 4 °C for 24 h, 0·5 ng of either $^{125}$I-labelled ovine prolactin or $^{125}$I-labelled prolactin $P_1$ in 50 $\mu$l assay buffer was added and incubation at 4 °C continued for another 24 h. Antibody-bound and free hormone were separated and the radioactivity of the bound hormone determined as above.

### *Assay for other pituitary hormones*

The isolated caprine prolactin fractions were assessed for contamination by other pituitary hormones with specific radioimmunoassays and iodinated hormones as follows: for growth hormone (GH), with caprine GH (see Results section) as standard, by Dr I. C. Hart, Reading; for luteinizing hormone (LH), with ovine LH as standard, by Dr R. Short and Dr V. A. Box, Cambridge; for thyrotrophin (TSH) with ovine TSH as standard, by Dr T. Merrett, St Bartholomew's Hospital, London and for corticotrophin (ACTH) by Dr J. C. Ratcliffe, St Bartholomew's Hospital, London.

## RESULTS

### *Isolation procedure*

The pituitary extract, divided into two portions each containing about 590 mg protein, was fractionated by gel filtration on Sephadex G-100 (Fig. 1). Fractions containing low molecular weight proteins from the two runs were combined as indicated in the diagram and lyophilized, yielding 82 mg material. When this lyophilized material was suspended in 0·05 M-Tris–hydrochloride buffer, pH 7·8, not all of it dissolved, even when the pH was raised to 9 by adding 2 M-$NH_4OH$. The suspension was, therefore, centrifuged at 10000 ***g*** for 15 min and the clear supernatant applied to the Bio-Gel P-100 column. The pink colour of the solution indicated the presence of haemoglobin and this showed clearly on the elution diagram (Fig. 2) when absorption at 410 nm was measured. Fractions corresponding to those which contained ovine prolactin in trial runs were combined as indicated, giving 51 ml of solution containing about 54 mg protein (estimated from absorbance at 260 and 280 nm).

The protein solution from P-100 chromatography was allowed to percolate through the DEAE column, then elution was continued with equilibration buffer until the protein concentration in the effluent was decreasing sharply as indicated by extinction at 230 nm. Elution was then continued with the NaCl gradient. The separation obtained is shown in Fig. 3. Fractions were combined as indicated and the protein isolated.

Fraction GH (14 mg) contained growth hormone and was retained for further study (I. C. Hart, D. S. Flux, P. Andrews & A. S. McNeilly, unpublished observations). It was used in radioimmunoassays to test the caprine prolactin for the presence of GH. Fractions $P_1$, $P_2$ and $P_3$ weighed 11, 4 and 8 mg respectively and were investigated further as prolactin fractions.

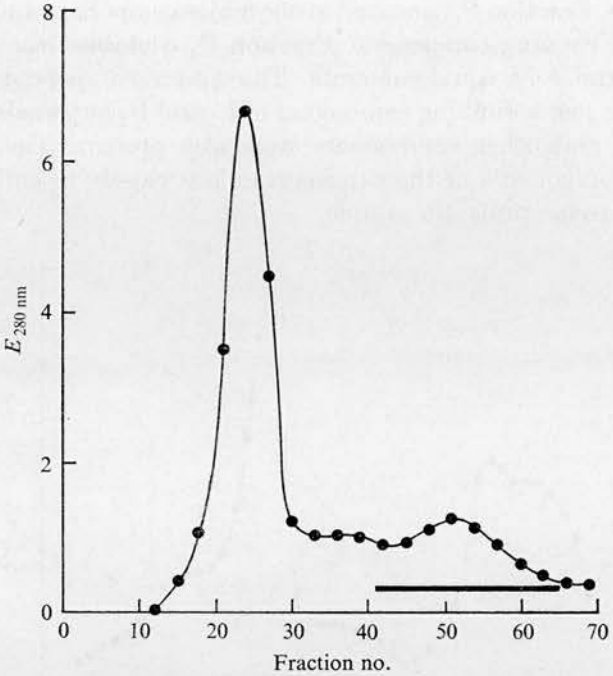



Fig. 1. Gel filtration of caprine pituitary extract (19 ml containing about 590 mg protein) on a column of Sephadex G-100 (2·5 × 50 cm) in 0·2 M-ammonium hydrogen carbonate at 5 °C. The effluent was collected in 3 ml fractions. Fractions comprising the peak of low-molecular-weight proteins were combined as indicated by the bar.

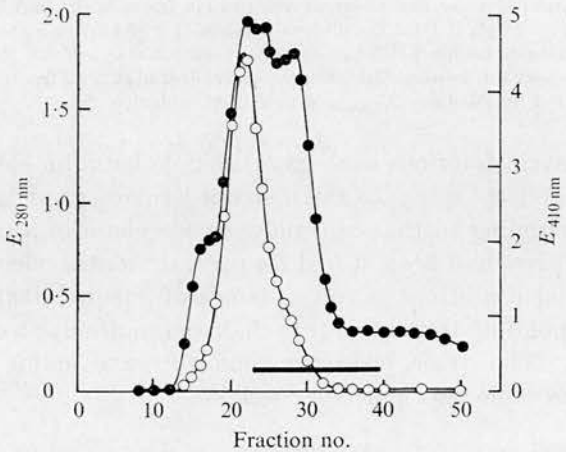



Fig. 2. Gel filtration of the low-molecular-weight protein fraction (about 80 mg) from Sephadex G-100 chromatography (Fig. 1) on a column of Bio-Gel P-100 (2·5 × 42 cm) in 0·05 M-Tris–hydrochloride buffer, pH 7·8, at 5 °C. The effluent was collected in 3 ml fractions. Fractions were combined as indicated by the bar. $E_{280\,nm}$, ●; $E_{410\,nm}$, ○.

### *Electrophoresis*

Electrophoresis of the caprine prolactin fractions and a sample of ovine prolactin (NIH-P-S-9) in polyacrylamide gels at pH 8·9 gave the results shown on the Plate. The bands were compared by relating their migration rates with those of the corre-

sponding dye fronts. Fraction $P_1$ consisted of one major component and a much smaller amount of a faster-running component. Fraction $P_2$ contained the same two components in approximately equal amounts. The major component of fraction $P_3$ corresponded to the faster-running component of $P_1$ and $P_2$ but small amounts of the slower component and other components were also present. The electrophoretic behaviour of the components of the caprine fractions closely resembled that of the components of the ovine prolactin sample.

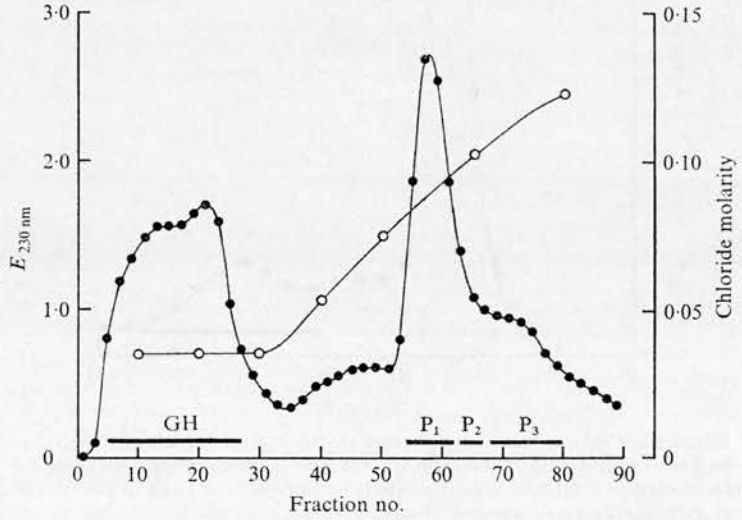



Fig. 3. Fractionation of the low-molecular-weight protein fraction (54 mg) from Bio-Gel P-100 chromatography (Fig. 2) on a DEAE-cellulose column (1 × 16 cm) equilibrated initially with 0·05 M-Tris–hydrochloride buffer, pH 7·8. Subsequent elution was with an NaCl gradient in the same buffer (see the text for details). The effluent was collected in 3 ml fractions. Fractions were combined as indicated by the bars. $E_{230\,nm}$, ●; chloride molarity, ○.

The caprine prolactin fractions each gave a single band on electrophoresis in the presence of sodium dodecyl sulphate and mercaptoethanol at pH 8, with the mobility in each case corresponding to that of a polypeptide chain of a molecular weight of about 20000. After they had been stored for several months, electrophoresis of each fraction under similar conditions gave two bands of unequal density, the major and minor bands corresponding to polypeptide chains of molecular weights of 20000 and 40000, respectively. The ovine prolactin similarly gave major and minor bands, corresponding to the same two molecular weights.

### *Biological activity*

Preliminary bioassays indicated that fractions $P_1$, $P_2$ and $P_3$ had good pigeon crop sac-stimulating activity. A definitive bioassay of fraction $P_1$ gave a potency of 27·3 (16·8–52·5) i.u./mg.

### *Radioimmunoassay*

Antibody dilution curves obtained with $^{125}$I-labelled ovine and caprine prolactins were identical and the antiserum to ovine prolactin bound 50 % of either labelled hormone at a final antibody dilution of 1:600000. Standard radioimmunoassay

curves obtained at this antibody dilution are shown in Fig. 4. In both systems used, i.e. anti-ovine prolactin antiserum and either $^{125}$I-labelled ovine prolactin (Fig. 4*a*) or $^{125}$I-labelled caprine prolactin (Fig. 4*b*), the standard curves for ovine and all three fractions of caprine prolactin were parallel. On a weight basis the ovine prolactin standard NIH-P-S-6 and caprine prolactin fraction $P_1$ were of equal potency in both systems, whereas caprine prolactin fractions $P_2$ and $P_3$ were respectively 80 % and 60 % as active as $P_1$.

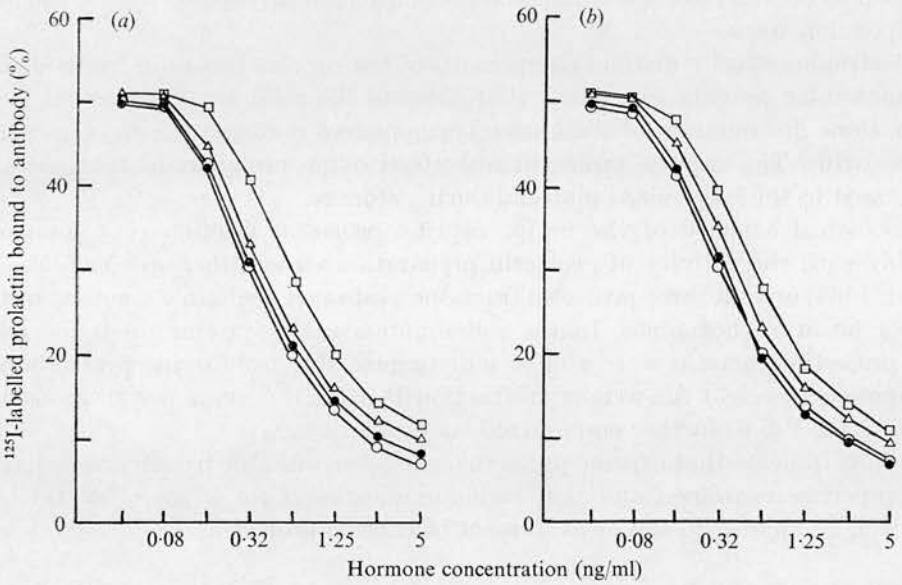



Fig. 4. Standard inhibition curves obtained by using ovine prolactin antiserum, different caprine or ovine prolactin preparations as indicated and either (*a*) $^{125}$I-labelled ovine prolactin or (*b*) $^{125}$I-labelled caprine prolactin. ○, ovine prolactin NIH-P-S-6; ●, caprine prolactin $P_1$; △, caprine prolactin $P_2$; □, caprine prolactin $P_3$.

No inhibition was seen in either system in the presence of caprine GH (see *Isolation procedure*), ovine GH (NIH-GH-S-9), ovine LH (NIH-LH-S-17), ovine FSH (NIH-FSH-S-9), ovine TSH (NIH-TSH-S-6) or ACTH (Synacthen, CIBA Laboratories Ltd, Horsham, Sussex).

Assays of ten different goat and sheep plasma samples in each radioimmunoassay system with either ovine or caprine fraction $P_1$ prolactin as standard yielded similar results (A. S. McNeilly and J. R. McNeilly, unpublished observations).

### *Assay for other pituitary hormones*

All three fractions of caprine prolactin contained less than 0·2 % by weight of GH, LH, TSH and ACTH.

## DISCUSSION

Fractionation of goat pituitary extracts for prolactin and GH was based on the assumption that both hormones have molecular weights in the region of 20000, similar to those of the corresponding hormones from the sheep and other mammalian species (Andrews, 1966). Trial experiments with ovine prolactin and other proteins

showed that gel filtration in Bio-Gel P-100 columns gave very good resolution of clean mixtures of proteins in the required molecular weight range but the columns were less satisfactory for fractionating crude extracts or samples containing much salt. Accordingly an initial fractionation of the pituitary extract was done with Sephadex G-100 and the product refractionated in Bio-Gel. About half the material isolated by gel filtration was prolactin and about a quarter was GH. The buffers used for chromatography were chosen to facilitate transfer of material from one stage of the preparation to the next and the small amount of material involved obviated the need for precipitation steps.

The electrophoretically distinct components of the caprine prolactin fractions had similar molecular weights suggesting that, despite the mild conditions used in its isolation, some deamidation of the native hormone had occurred (Lewis, Cheever & Hopkins, 1970). The caprine prolactin resembled ovine prolactin in that a stable dimer formed in the lyophilized material during storage.

The biological activity of the major caprine prolactin fraction ($P_1$) compared favourably with the activity of prolactin preparations from other species (Jiang & Wilhelmi, 1965) and all three prolactin fractions contained negligible amounts of GH and other pituitary hormones. In the radioimmunoassay systems used, the three caprine prolactin fractions were almost indistinguishable from ovine prolactin and the equipotency, weight for weight, of fraction $P_1$ with the ovine prolactin used as standard (NIH-P-S-6) further emphasized the similarity.

Our results indicate that caprine prolactin is indistinguishable from ovine prolactin in the properties examined and that radioimmunoassays for ovine prolactin may equally well be applied to the measurement of caprine prolactin.

We thank Dr I. A. Forsyth for the prolactin bioassays, Drs V. A. Box, I. C. Hart, T. Merrett, J. G. Ratcliffe and R. Short for the radioimmunoassays for other pituitary hormones and Dr M. Anderson for the molecular weight estimations. A. S. McNeilly is grateful to the Wellcome Foundation for financial support.

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## DESCRIPTION OF PLATE

Polyacrylamide gel electrophoresis of caprine and ovine prolactins at pH 8·9. 1, 2 and 3 are caprine prolactin fractions $P_1$, $P_2$ and $P_3$ respectively, and 4 is ovine prolactin (NIH-P-S-9).

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# Radioimmunoassay for Ovine and Caprine Growth Hormone: Its Application to the Measurement of Basal Circulating Levels of Growth Hormone in the Goat

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Summary

A specific double antibody radioimmunoassay for ovine and caprine growth hormone (GH) is described in which a comparison is made between the results obtained with purified preparations of ovine and caprine GH as the labelled antigens. Measurement of the circulating levels of GH in plasma taken from four lactating female, four castrated male and four virgin female goats showed no significant differences between the levels of GH in lactating and non-lactating animals. Marked variations in plasma GH were found between samples from different goats and between samples from the same goat. Possible reasons for these variations are discussed.

**Key-Words:** *Growth Hormone – Radioimmunoassay – Ovine – Caprine – Basal Levels*

## Introduction

Injection of growth hormone (GH) into normal lactating cows during various stages of lactation may result in an increase in milk production (*Machlin* 1973) and hormonal replacement studies in hypophysectomized lactating goats have shown that GH is one of a group of hormones necessary for complete restoration and maintenance of milk yield (*Cowie, Knaggs and Tindal* 1964, *Cowie* 1969). The development of radioimmunoassay techniques has enabled workers to investigate circulating levels of GH in lactating cows (*Hove and Blom* 1971, *Koprowski, Tucker and Convey* 1972), but as yet no study has been carried out with ruminants to compare diurnal levels of GH in lactating and non-lactating animals.

Attempts to measure caprine GH in plasma by using a homologous radioimmunoassay for ovine GH in the manner now generally adopted for caprine prolactin (*Hart* 1972, *McNeilly and Andrews* 1974) showed that, with the antiserum used, cross reaction between the ovine GH standard and the goat plasma sample was incomplete. In an attempt to find a valid radioimmunoassay for caprine GH we have compared the results obtained when purified preparations of ovine and caprine GH were used as standards in the assay.

## Materials and Methods

*Ovine GH*

Specific radioimmunoassays for ovine prolactin (*Hart* 1972, 1973) indicated that the ovine GH preparations NIH-GH-S9 and NIH-GH-S10 both contained prolactin and data issued with these hormones (Endocrinology Study Section, National Institute of Arthritis and Metabolism and Digestive Diseases, Bethesda, Maryland 20014, USA) showed that NIH-GH-S9 also contained small quantities of LH and TSH. An ovine GH preparation suitable for use in radioimmunoassay procedures was obtained from NIH-GH-S9 by removing contaminating hormones by ion-exchange chromatography on DEAE-cellulose as follows.

Ovine GH 1.8 g (NIH-GH-S9) was dissolved in 360 ml of 0.05 M-Tris-hydrochloride buffer, pH 9 and the solution dialysed for 48 hr at 4°C against two changes of five volumes of 0.05 M-Tris-HCl buffer, pH 8.6. It was then filtered to remove undissolved material and applied to a column (3 cm x 15 cm) of DEAE-cellulose (DE-52; Whatman Biochemical Ltd., Maidstone, Kent, England) which had been equilibrated with 0.05 M-Tris-HCl buffer, pH 8.6. The column was eluted initially with 75 ml of the equilibration buffer, then with a NaCl gradient in 500 ml of equilibration buffer and finally with 150 ml of 0.5 M-NaCl in the same buffer. The column effluent was collected in 10 ml fractions, commencing when the GH solution was first applied to the column. The chromatography was carried out at 4°C.

Selected effluent fractions were examined by disc electrophoresis in 7% polyacrylamide gel at pH 8.9 (*Davis* 1964) and used as a guide for combining the effluent fractions containing GH as follows: fraction A, effluent fractions 11-60; fraction B, effluent fractions 61-100. Fractions A and B were both adjusted to pH 7.0 with M-HCl and ammonium sulphate was added to 70% saturation to precipitate the protein. The precipitates were dissolved in 0.1 M-ammonium hydrogen carbonate, pH 8.5, and the solutions dialysed for 24 hr against distilled water at 4°C. After centrifugation the solutions were passed through a column (2.4 cm x 30 cm) of Sephadex G-25 equilibrated with water, to remove the remaining salts. Fraction A was also submitted to gel filtration on a column (2.4 cm x 50 cm) of Sephadex G-100, equilibrated with water, from which it emerged as a single peak in the effluent. The final solutions were lyophilized, yielding fraction A, 51.0 mg; fraction B, 88.0 mg. The remainder of the GH applied to the DEAE-cellulose column, still contaminated with prolactin and small amounts of other proteins, was recovered in a similar manner from the later effluent fractions (1.003 g).

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*Caprine GH*

Growth hormone was prepared from goat pituitaries as described by *McNeilly and Andrews* (1974). Electrophoresis in 7% polyacrylamide gel at pH 8.9 indicated slight contamination with haemoglobin.

*Bioassay and biological purity*

The relative potencies of the ovine (fraction A) and caprine GH preparations were determined against the International Standard for growth hormone (Medical Research Council, Division of Biological Standards, National Institute for Medical Research, Hampstead Laboratories, Holly Hill, London, NW3 6RB) by the rat tibia test for bioassay of GH (*Papkoff and Li* 1962). The ovine (fractions A and B) and caprine GH preparations were also assessed for contamination by other anterior pituitary hormones with specific radioimmunoassays as follows: for prolactin as described by *Hart* (1973); for LH and TSH by Mr. G. Jenkins, ARC Institute of Animal Physiology, Babraham, Cambridge and for ACTH by Dr. A.P. Scott, Department of Chemical Pathology, St. Bartholomew's Hospital, London, EC1.

*Antisera*

No attempt was made to raise antisera to caprine GH as only 14 mg of this material was available (*McNeilly and Andrews* 1974). Antisera to ovine GH were raised in guinea-pigs by injection of 0.5 mg ovine GH (fractions A and B) in Freund's complete adjuvant at 2-3 week intervals for 5 months, or injection of similar doses of GH, conjugated with rabbit serum albumin at similar intervals for 2-3 months. Antisera were stored at -20°C without preservative.

*Iodination of ovine and caprine GH*

Caprine GH was iodinated by the chloramine T method (*Greenwood, Hunter and Glover* 1963) and purified as described by *Hart* (1972) for prolactin. The average uptake of $^{125}$I on to caprine GH was 55.2 ± 4.1% (mean ± SEM, n = 10) and the specific activity of the labelled preparation ranged from 55.8 to 139.0 μCi/μg.

Both the chloramine T and the lactoperoxidase (*Morrison and Boyse* 1970) methods were used in attempts to iodinate ovine GH (fraction A and NIH-GH-S10) but the specific activities of the resulting iodinated preparations were too low to achieve adequate sensitivity in the radioimmunoassay. However, when ovine GH was dissolved in 2 M-urea and the solution kept at room temperature for 45 min before the chloramine T iodination, immunoreactive $^{125}$I-ovine GH of high specific activity (130-140 μCi/μg) was obtained.

*Radioimmunoassay*

Diluent buffer consisted of 0.25% egg albumin (Sigma Chemical Company, St. Louis, USA) in 0.04 M-phospho-saline buffer, pH 7.3, containing 0.01 M-EDTA and 0.1% sodium azide. The reagents were added to disposable polystyrene tubes (6 cm x 1 cm; Luckhams Ltd., Victoria Gardens, Burgess Hill, England) in the following order: 100 μl of hypophysectomized goat plasma or 100 μl of sample (diluted in hypophysectomized goat plasma); 100 μl of a solution of standard GH in diluent buffer or 100 μl of diluent buffer; 50 μl of guinea-pig antiserum to ovine GH (final dilution 1:100,000). The diluted antiserum bound 35-45% of the added $^{125}$I-GH when no competing unlabelled GH was present in the system. The reagents were mixed and incubated at 4°C. After 48 hr, 200-500 pg of $^{125}$I-GH in 50 μl of diluent buffer was added to each tube and incubation continued. After another 24 hr, 50 μl of rabbit antiserum to guinea-pig γ-globulin (1:16, Wellcome Reagents Ltd., Beckenham, England) plus 50 μl of normal guinea-pig serum (1:160) were added and incubation continued. After a further 24 hr the reaction tubes were centrifuged at 950 g for 20 min and the supernatants, which contained free $^{125}$I-GH, were discarded. The radioactivity remaining in the precipitate (bound $^{125}$I-GH) was determined in an automatic gamma counter (Panax Gamma-160). All assays were performed in duplicate.

*Experimental animals*

Blood samples (10 ml) were taken at 30 min intervals throughout a 24 hr period from three groups of pedigree British Saanen goats – four castrated males, four anoestrous virgin females and four anoestrous lactating females – on the following dates respectively: 13 July, 26 July and 9 August 1971. The method used has been described by *Hart* (1973) for prolactin assay.

## Results

*Bioassay and purity of the GH preparations*

Bioassay results for the GH preparations are given in Table 1.

Table 1. The relative potencies of ovine (fraction A) and caprine GH as determined by the rat tibia test

| GH preparation | i.u./mg | 95% limits |
|---|---|---|
| Ovine GH (fraction A) | 0.47 | 0.1-0.73 |
| Caprine GH | 0.63 | 0.32-0.90 |

Radioimmunoassays showed that neither ovine GH, fractions A and B, nor caprine GH contained detectable amounts of other anterior pituitary hormones, as follows: prolactin, < 0.01%; LH, < 0.2%; TSH, < 0.2%; ACTH, < 0.07%.

*Specificity*

The specificity of the antiserum routinely used in the assay was investigated with $^{125}$I-caprine GH as the labelled antigen (Fig. 1). No cross reaction was found with caprine prolactin, ovine prolactin (NIH-P-S6), ovine follicle stimulating hormone (NIH-FSH-S9) or ovine luteinizing hormone (NIH-LH-S17). At concentrations above 400 ng/ml, ovine thyroid stimulating hormone (NIH-TSH-S6) inhibited the binding of $^{125}$I-caprine GH to the antiserum.

Slopes of the regression lines given by different bovine, caprine and ovine GH preparations depended on whether $^{125}$I-caprine GH or $^{125}$I-ovine GH (fraction A) was used as the labelled antigen (Table 2). When $^{125}$I-ovine GH was used the slope given by the ovine GH preparations differed significantly ($P > 0.0$ from that given by the ovine and caprine GH preparations. When $^{125}$I-caprine GH was used, however, no significant difference ($P > 0.1$) was found between the slopes given by the three GH preparations tested. Furthermore, when $^{125}$I-caprine GH was used as the labelled preparation the slopes of the regression line given by goat plasma samples P1 and P2 (b = -1.281 and -1.172 respectively) were not significantly different from those given by bovine, caprine and ovine G

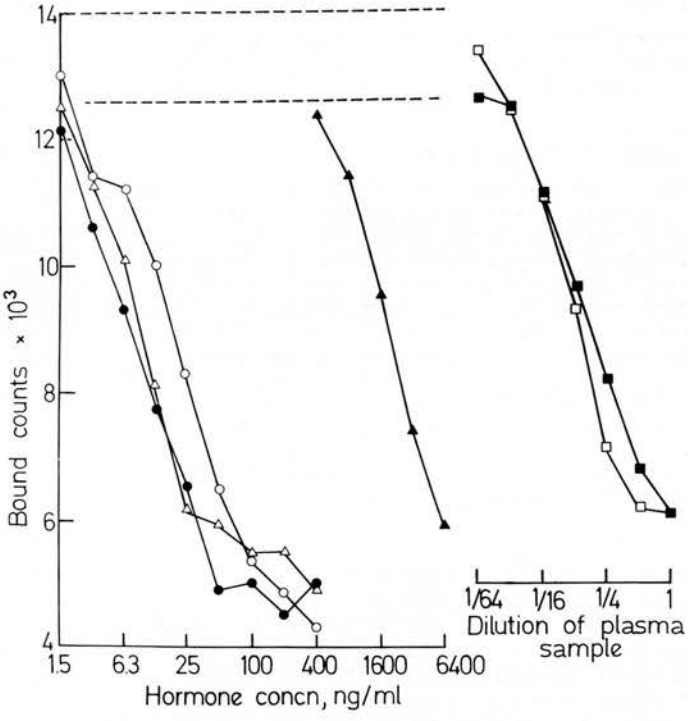



Fig. 1. Inhibition curves obtained when equal quantities of different hormone preparations were used to inhibit the binding between $^{125}$I-caprine GH and antiserum to ovine GH. ○------○ caprine GH; ●—● bovine GH (NIH-GH-B9); △—△ ovine GH (NIH-GH-S10); ▲—▲ ovine TSH (NIH-TSH-S6); □—□ and ■—■ goat plasma samples P1 and P2 respectively. The results for caprine prolactin, ovine prolactin (NIH-P-S6), ovine FSH (NIH-FSH-S9) and ovine LH (NIH-LH-S17) were contained between the broken lines.

*Sensitivity*

With $^{125}$I-caprine GH as the iodinated antigen, the following levels of ovine GH (NIH-GH-S10) were used in the assay as standards: 0, 0.25, 0.5, 1.0, 2.0 and 4.0 ng/ml. The percentage of labelled hormone bound at each GH concentration was respectively: 35.4 ± 0.62; 35.6 ± 0.77; 34.1 ± 0.42; 32.6 ± 0.29; 29.6 ± 0.46 and 27.0 ± 0.63 (mean ± SEM, n = 10). The values obtained for 0 and 0.25 ng/ml of GH were not significantly different, but a signifikant ($P < 0.02$) fall occurred at 0.5 ng/ml. The minimum detectable level of ovine GH in the assay is therefore between 0.25 and 0.5 ng/ml.

*Recovery*

Recovery was determined by assaying samples of hypophysectomized goat plasma to which known amounts of unlabelled ovine GH (NIH-GH-S10) had been added. Ovine GH (fraction A) was used as the iodinated hormone and ovine GH (NIH-GH-S10) as

Table 2. Slopes of regression lines obtained with different preparations of GH, when $^{125}$I-ovine GH or $^{125}$I-caprine GH were used in the radioimmunoassay

| Labelled hormone | GH preparation | Regression slope (b) | Significance | |
|---|---|---|---|---|
| $^{125}$I-ovine GH | Ovine GH (fraction A) | −1.378 | No significant difference, $P > 0.1$ | Significantly different, $P < 0.01$ |
| | Ovine GH (NIH-GH-S10) | −1.181 | | |
| | Bovine GH (NIH-GH-B9) | −0.919 | No significant difference, $P > 0.1$ | |
| | Bovine GH (NIH-GH-B15) | −0.924 | | |
| | Caprine GH | −0.869 | | |
| $^{125}$I-caprine GH | Ovine GH (NIH-GH-S10) | −1.360 | No significant difference, $P > 0.1$ | |
| | Bovine GH (NIH-GH-B9) | −1.141 | | |
| | Caprine GH | −1.203 | | |

Table 3. Recovery of added ovine GH (NIH-GH-S10) from hypophysectomized goat plasma determined by radioimmunoassay

| Amount of added GH (ng/ml) | Amount of GH found (ng/ml; mean ± SEM) | Mean % recovery of GH (n = 20) |
|---|---|---|
| 0 | 2.0 | – |
| 400 | 368 ± 4.8 | 92.2 |
| 200 | 182 ± 3.9 | 91.3 |
| 100 | 91.7 ± 1.2 | 91.7 |
| 50 | 46.0 ± 0.8 | 92.0 |
| 25 | 23.9 ± 0.5 | 95.6 |
| 12.5 | 11.8 ± 0.2 | 94.4 |
| 6.25 | 5.9 ± 0.1 | 95.2 |

the standard. Each sample was assayed at three dilutions, each dilution being assayed in duplicate. The recoveries (Table 3) were regarded as satisfactory.

*Growth hormone in goat plasma*

In all three groups of animals the concentration of GH in the blood varied from one goat to another (Fig. 2) and no significant difference was found between the average levels of GH determined in blood samples taken throughout the day from lactating female goats and those taken from non-lactating animals. Although the circulating level of hormone remained relatively stable in some animals (Goats 382, 404, 488 and 500) marked fluctutations occurred in others especially between 20.00 h and 08.00 h. An appreciable increase in the level of GH was also noted in some of the goats (Goats 281, 351, 476, 504, 521 and 4690) during this period.

## Discussion

*Radioimmunoassay*

In radioimmunoassay the absolute levels of hormone estimated in plasma are affected by the particular preparation used as a standard, and the results indicated that there were important differences among those tested in these experiments. Ovine GH (NIH-GH-S9) had a potency of 1.01 i.u./mg (95% limits, 0.71-1.43; *Reichert and Wilhelmi* 1973) but the fraction A, prepared from it, though free from other anterior pituitary hormones had an estimated potency of 0.47 i.u./mg (95% limits, 0.1-0.73) when tested against the international standard. The reason for this drop is no known but it is possible that the first GH eluted by the salt gradient from DEAE cellulose contained mol cular forms of GH, with respect to secondary and tertiary structure, that differed from the bulk of the GH added. The biological activity of the caprine GH (0.63 i.u./mg, 95% limits 0.32-0.90) was higher than that of ovine GH (fraction A), but not as high as ovine NIH-GH-S10 (0.89 i.u./mg) and bovine NIH-GH-B9 (0.86 i.u./mg). Although the two are not necessarily associated the lower biological activity of caprine GH was accompanied by a lower immunological activity in the radioimmunoassay (Fig. 1). Use of this preparation as a standard in earlier determinations of GH in goat plasma, with fraction A from ovine GH as the labelled antigen, therefore resulted

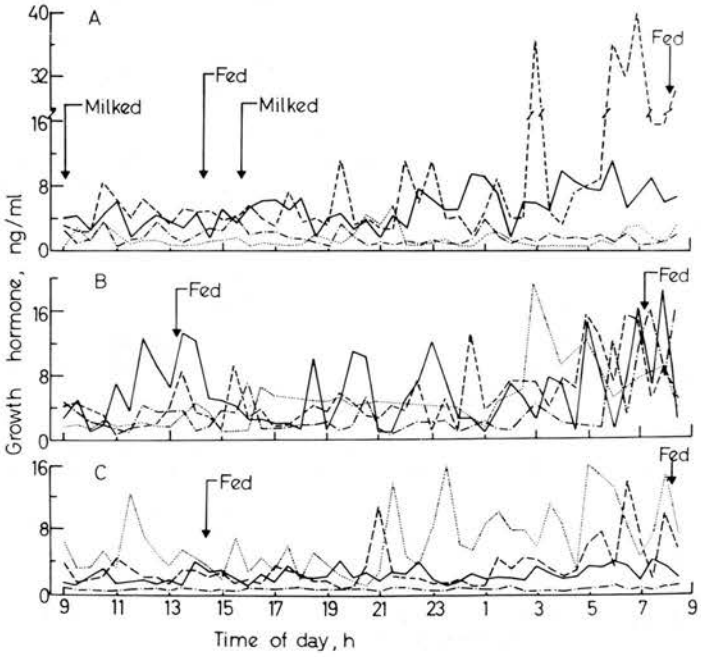



Fig. 2. Basal circulating levels of GH in the goat, found in blood samples taken at 30 min intervals throughout a 24 hr period. (A) anoestrous lactating females ——, - - - -, - . -, Goats 281, 357, 404 and 382. (B) Castrated males ——, - - - -, - . -, . . . . Goats 471, 476, 504 and 481. (C) Virgin females ——, - - - -, - . -, . . . . Goats 500, 4690, 488 and 521

in overestimates of GH concentrations (*Hart and Flux* 1973). To overcome this problem, the purified ovine and caprine GH preparations have now been used only as the iodinated hormones, in assays for ovine and caprine GH respectively, and ovine GH (NIH-GH-S10) used as the standard.

The double antibody radioimmunoassay is specific and sensitive enough to measure basal circulating levels of GH in goats. The cross reaction with high concentrations of TSH (NIH-TSH-S6) is similar to that reported by *Davis* (1972) which is thought to be due to contamination of this TSH preparation with GH. However, even if the cross reaction was with TSH itself, the circulating levels of TSH in ruminants are so low (*Freychet, Pelletier and Rosselin* 1969) that such a cross reaction would probably not interfere with the radioimmunoassay of GH.

*Growth hormone in goat plasma*

The considerable variation in plasma levels of GH between different goats of the same group and the fluctuations in hormone concentration which often occurred between samples taken consecutively from the same animal are in agreement with results obtained in cows and heifers (*McAtee and Trenkle* 1971, *Koprowski, Tucker and Convey* 1972, *Hove and Blom* 1971). Although changes in the blood levels of metabolites (e.g. amino acids, free fatty acids and glucose) can affect GH levels in ruminants (*Hertelendy, Takahashi, Machlin and Kipnis* 1970, *McAtee and Trenkle* 1971, *Davis* 1972, *Hertelendy and Kipnis* 1973) there is some disagreement as to whether or not such changes in hormone concentration can be caused by feeding and fasting (*Machlin, Takahashi, Horino, Hertelendy, Gordon and Kipnis* 1968, *McAtee and Trenkle* 1971, *Trenkle* 1971, *Hove and Blom* 1971, *Bassett* 1972). *Hove and Blom* (1973) have noted however that the circulating level of GH in moderately underfed cows was significantly higher than that in adequately fed animals. Furthermore, although the hormone levels varied considerably in adequately fed cows the levels fluctuated even more in those which were underfed. The relatively high, widely fluctuating GH levels found in some of our goats may therefore have been caused by a metabolic deficit, although it is difficult to understand how such a deficit might have arisen as all the animals had constant access to hay. On the other hand the significant hormonal increase in some of the goats (Goats 281, 357, 476, 504, 521 and 4690) during the night and early morning might more reasonably be explained by a fall in metabolite concentration as, during this period, the goats ate very little and remained lying down.

In man the onset of typical slow-wave sleep stimulates an increase in the blood level of GH (*Honda, Takahashi, Azumi, Irie, Sakuma, Tsushima and Shizumi* 1969) and this release is inhibited by the occurrence of paradoxical sleep. *Klemm* (1966) has shown that the goat will experience as many as six typical sleep periods, of about 5 min duration, during the daylight hours and each of these is followed by a similar period of paradoxical sleep. He speculated that the frequency of the sleep periods might increase at night. A relationship might therefore exist in goats between the frequency of the sleep periods, the fluctutations in GH level found in the individual animals and the rise in hormone level which occurs during the night and early morning.

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We are indebted to the Endocrinology Study Section, National Institutes of Health, USA, for generous supplies of ovine and bovine anterior pituitary hormones and to Mrs. K.A.M. Danby and Mr. R. Huggins for skilled technical assistance. We are grateful to Mr. S.C. Watson for hypophysectomizing the rats used in the bioassay of GH and to Drs. A.T. Cowie and H.L. Buttle for the hypophysectomy of the goats from which GH-free plasma was obtained. One of us (ICH) was supported by a Science Research Council Studentship.

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# Effect of Short Periods of Fasting and Time of Day on Serum Levels of Gonadotropins, Testosterone and Glucose in Male Rats*

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Summary

In two experiments, food was withheld from male rats beginning at 12:00 p.m. Fasted rats and fed controls were killed 8, 16 and 24 hours later. Rats were 4 and 6 months old in experiments 1 and 2, respectively. Fasted rats had lower ($P < 0.01$) serum levels of LH, FSH and glucose than did fed rats; serum levels were independent of the duration of the fast. Serum levels of testosterone were not significantly affected by fasting. Serum levels of glucose and testosterone varied with time of day (hour, $P < 0.01$) and were highest at 12:00 p.m. and 4:00 a.m. respectively. Fasting had a greater effect on serum glucose levels in experiment 1 than in experiment 2 (experiment x fasting, $P < 0.01$), and the pattern of change in FSH levels over time of day differed in the two experiments (experiment x hour, $P < 0.01$).

**Key-Words:** *Follicle Stimulating Hormone – Luteinizing Hormone – Testosterone – Serum Glucose – Fasting – Circadian Rhythm*

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## Introduction

Underfeeding in male rats leads to reduced accessory sex gland and testicular weights and sterility, presumably due to reduced gonadotropin secretion (*Mulinos and Pomerantz* 1941). Starvation likewise results in atrophy of accessory sex glands (*Negro-Vilar, Dickerman and Meites* 1971, *Howland and Skinner* 1973) and in reductions in the serum levels of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (*Howland and Skinner* 1973, *Root and Russ* 1972). Reduced levels of hypothalamic FSH-releasing factor in starved rats (*Negro-Vilar, Dickerman and Meites* 1971) suggest that altered pituitary function is mediated by neural mechanisms.

The present study was designed to determine if changes in LH and FSH levels could be demonstrated during acute fasting and if changes in gonadotropin levels were associated with changes in serum levels of glucos or testosterone (T).

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# DEVELOPMENT AND APPLICATION OF A HETEROLOGOUS RADIOIMMUNOASSAY FOR OVINE FOLLICLE-STIMULATING HORMONE

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SUMMARY

A highly specific and sensitive heterologous double antibody radioimmunoassay for ovine follicle-stimulating hormone (oFSH) is described in detail. The assay using a rabbit antiserum to human FSH and either $^{125}$I-labelled rat FSH or $^{125}$I-labelled oFSH as tracer is specific for FSH. A maximum cross-reaction (B/Bo = 50 %) of 0·1 % was observed with other ovine, rat or human pituitary hormones or human chorionic gonadotrophin.

Serum levels of oFSH in samples collected daily throughout the oestrous cycle showed large individual variations. In five out of nine animals a peak of FSH was observed on the day of oestrus.

## INTRODUCTION

The full understanding of the hormonal control of gonadal function in sheep is limited at present by the lack of information on the dynamics of secretion and levels of follicle-stimulating hormone (FSH) in the circulation. Despite the availability of highly purified ovine FSH (oFSH; Jutisz, 1965; Hashimoto, McShan & Meyer, 1966; Papkoff, Gospodarowicz & Li, 1967; Papkoff & Ekblad, 1970; Sherwood, Grimek & McShan, 1970) only one sufficiently specific and sensitive homologous radioimmunoassay for oFSH using unabsorbed antiserum has been described (Kragt & Cons, 1973).

The difficulty has been to raise an antiserum to oFSH which does not cross-react with ovine luteinizing hormone (oLH) or ovine thyroid-stimulating hormone (oTSH). In many instances absorption of these non-specific antisera with oLH or oTSH did not yield sufficient increase in specificity to allow accurate measurement of FSH in serum (Bailly du Bois, Kerdelhué & Jutisz, 1970; Kerdelhué, Kann & Jutisz, 1972; L'Hermite, Niswender, Reichert & Midgley, 1972; Cunningham & Hebert, 1973; Hopkinson & Pant, 1973; Salamonsen, Jonas, Burger, Buckmaster, Chamley, Cumming, Findlay & Goding, 1973). To circumvent problems of specificity, heterologous radioimmunoassays using either oFSH antisera and $^{125}$I-labelled human FSH tracer ($^{125}$I-labelled hFSH) (L'Hermite *et al.* 1972; Hopkinson & Pant, 1973; Salamonsen *et al.* 1973) or hFSH antiserum and $^{125}$I-labelled oFSH tracer (Salamonsen *et al.* 1973) have been developed. These methods are sufficiently specific and sensitive to measure levels of FSH in the circulation of the ewe.

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The present paper describes in detail the development of a highly specific and sensitive heterologous radioimmunoassay for oFSH and its application to the measurement of serum levels of FSH in the sheep with particular reference to changes in blood levels of FSH during the sheep oestrous cycle.

## MATERIALS AND METHODS

### *Antiserum*

Two antisera (M91 and M94) were raised against hFSH in rabbits by the multiple site injection technique (Ross, Vaitukaitis & Robbins, 1971; Butt, Lynch & Shirley, 1974). Details of the production of these antisera have been described in detail elsewhere (Lynch & Shirley, 1975).

### *Iodination of FSH preparations*

Ovine FSH (Papkoff G4-5DC), rat FSH (rFSH, NIAMDD Rat-I-1) and hFSH (CPDS 13) were iodinated with Na $^{125}$I (Radiochemical Centre, Amersham, Bucks) by a modification (McNeilly & Hagen, 1974) of the chloramine T method of Greenwood, Hunter & Glover (1963). Each $^{125}$I-labelled FSH preparation was purified by column chromatography on Sephadex G-100 and the most immunoreactive fractions were stored at −20 °C. $^{125}$I-labelled hFSH was added to assays without further purification while $^{125}$I-labelled oFSH and rFSH were first repurified using Whatman CF11 ion exchange resin as described by Hunter (1969) and modified by McNeilly & Hagen (1974).

The range of specific activities for $^{125}$I-labelled hFSH, rFSH and oFSH were 85–160 ($n$ = 20), 45–100 ($n$ = 14) and 60–90 $\mu$Ci/$\mu$g ($n$ = 5) respectively.

### *Assay procedure*

All dilutions were made in 0·04 M-sodium phosphate buffer, pH 7·0 containing 0·15 M-NaCl, 0·01 M-EDTA, 0·5 % egg albumin and 0·1 % sodium azide (radioimmunoassay diluent; RIAD).

To determine the ability of the two antisera to bind iodinated rat, human and ovine FSH preparations, each antiserum was double diluted with RIAD from an initial dilution of 1:400 and 50 $\mu$l of each dilution were added in duplicate to tubes containing 200 $\mu$l RIAD and 50 $\mu$l normal rabbit serum (1:200). After addition of 50 $\mu$l of the appropriate $^{125}$I-labelled FSH tracer (18000 c.p.m.; approx. 150 pg), the tubes were incubated for 24 h at 4 °C. Separation of antibody-bound and free hormone was achieved by the addition of 50 $\mu$l of a second antibody (donkey anti-rabbit-$\gamma$-globulin; RD 17, Wellcome, 1:20). Incubation was continued for 16 h at 4 °C then antibody-bound and free hormone were separated by centrifugation at 600 $g$, 4 °C for 30 min. The antibody-bound $^{125}$I-labelled FSH in the precipitate was measured in a well-type Gamma scintillation counter (Panax).

To assess the specificity of the antisera, 200 $\mu$l of doubling dilutions of oFSH (Papkoff G4-5DC), oLH (Papkoff G3-222B) and oTSH (NIH TSH-S6) (starting concentration 1 $\mu$g/ml for each standard preparation) were incubated for 48 h at 4 °C with 50 $\mu$l of antiserum diluted to give 20 % binding of the appropriate $^{125}$I-labelled FSH added. After addition of 50 $\mu$l of FSH tracer, incubation was continued for 48 h at 4 °C. Bound and free hormone were separated and the bound fraction was determined as described above.

### *Specificity of the heterologous FSH assays*

The hormone preparations used to assess the specificity of the FSH assay are listed in Table 1. In addition, the cross-reaction of the $\alpha$- and $\beta$-subunits of hFSH ($\alpha$; N 611C and $\beta$ N 596C, Dr A. F. Parlow) and $\beta$-subunits of hLH ($\beta$ Q; Dr R. M. Lequin), hTSH (N 615C,

Dr A. F. Parlow) and human chorionic gonadotrophin (HCG) ($\beta$ CR 115, Dr R. Canfield) were tested in the heterologous oFSH assays using anti-hFSH antiserum and $^{125}$I-labelled rFSH and oFSH tracers.

The parallelism between dose–response curves for oFSH standard (Reichert) diluted in buffer or hypophysectomized sheep and goat sera (kindly donated by Dr H. L. Buttle, NIRD, Reading) and dilutions of ovine pituitary extract and serum samples containing endogenous oFSH from oestrous and anoestrous ewes and wethers was assessed to validate the assay for measurement of FSH in sheep serum.

*Precision and accuracy of the heterologous FSH radioimmunoassay*

The accuracy of the assay was determined by the measurement of known amounts of standard oFSH (NIH FSH S8) added to hypophysectomized sheep and goat sera.

The precision of the assay expressed as the intra- and inter-assay coefficients of variation (%) was assessed by repeated assay of three pools of sheep sera from anoestrous and cyclic ewes both within and between assays.

*Comparison between heterologous and homologous assays for FSH*

The final heterologous assay developed for ovine FSH utilized the antiserum against hFSH and either $^{125}$I-labelled rFSH or $^{125}$I-labelled oFSH as tracer and was also suitable for the measurement of hFSH and rFSH. To provide additional validation that this assay measured FSH in the serum of each of these species, human and rat serum samples selected at random were assayed in the heterologous FSH assay using $^{125}$I-labelled rFSH tracer. These results were compared with the levels estimated by the relevant homologous FSH assay. The homologous hFSH assay has been described in detail elsewhere (McNeilly & Hagen, 1974); rFSH was estimated using homologous reagents from the NIAMDD, Bethesda, Maryland, U.S.A.

In addition a comparison was made between levels of oFSH in 45 serum samples estimated by the heterologous assay using either $^{125}$I-labelled rFSH or $^{125}$I-labelled oFSH tracers.

*Measurement of blood levels of FSH during the oestrous cycle of the ewe*

Daily blood samples were collected by jugular venepuncture throughout the oestrous cycle in nine Clun-Forest ewes which had previously had a normal oestrus. Serum was separated and stored at $-20$ °C until assayed. Measurement of both LH and progesterone confirmed that all cycles were normal.

## RESULTS

### *Heterologous assay for ovine FSH*

*Final form of assay*

Anti-hFSH antisera M91 and M94 bound 25–45 % $^{125}$I-labelled rFSH and oFSH tracers at final dilutions of 1:100000 and 1:50000 respectively. At these dilutions both antisera gave assays of sufficient sensitivity (minimum detectable concentration (B/Bo = 90 %) 15 ng NIH FSH S8/ml) to measure levels of FSH in the circulation of sheep using either tracer. For the routine assay antiserum M91 was used.

*Specificity of assay*

Table 1 shows the cross-reactivity of all the standards investigated in the heterologous assays using both $^{125}$I-labelled rFSH and oFSH tracers.

*Sheep hormones*

Using $^{125}$I-labelled rFSH tracer (Fig. 1*a*), all the NIH FSH preparations showed inhibition curves parallel to the purer FSH preparations (Papkoff and LER 1491 with biological

potencies of 60 and 40 × NIH FSH-S1 respectively). Cross-reaction with all other sheep hormone preparations examined was < 0·1 % (Table 1).

When $^{125}$I-labelled oFSH tracer was used (Fig. 1*b*) all FSH preparations were again parallel although LER oFSH showed greater potency than Papkoff oFSH (2·2:1). Papkoff LH showed no significant inhibition (< 0·01 %) while NIH TSH S6 showed slightly greater cross-reactivity (0·2 %) than with $^{125}$I-labelled rFSH tracer. All other hormones tested showed < 0·01 % cross-reactivity (Table 1).

Table 1. *Cross-reactions of ovine, rat and human pituitary hormone preparations in the heterologous FSH radioimmunoassay using either $^{125}$I-labelled rat or ovine FSH tracers and anti-human FSH antiserum (M91)*

| | | Cross-reaction (%) | |
|---|---|---|---|
| | Hormone | $^{125}$I-labelled rat FSH | $^{125}$I-labelled ovine FSH |
| Ovine | | | |
| FSH | G4 5DC (Papkoff) | 100 | 100 |
| | LER 1491 (Reichert) | 40 | 220 |
| | NIH FSH S3 | 1·54 | 1·18 |
| | NIH FSH S8 | 0·88 | 0·77 |
| | NIH FSH S9 | 4·17 | — |
| LH | G3 222 B (Papkoff) | 0·07 | < 0·01 |
| | NIH LH S11 | 0·04 | < 0·01 |
| | NIH LH S15 | 0·06 | < 0·01 |
| | NIH LH S17 | < 0·01 | < 0·01 |
| TSH | NIH TSH S6 | 0·07 | 0·2 |
| GH | NIH GH-S10 | 0·08 | < 0·01 |
| Prolactin | NIH P-S6 | < 0·01 | < 0·01 |
| Rat | | | |
| FSH | NIAMDD Rat-FSH-2 | 75 | 76 |
| LH | NIAMDD Rat-LH-I-1 | < 0·01 | < 0·01 |
| TSH | NIAMDD Rat-GH-I-1 | < 0·01 | < 0·01 |
| GH | NIAMDD Rat-GH-I-2 | < 0·01 | < 0·01 |
| Prolactin | NIAMDD Rat-PRL-I-2 | < 0·01 | < 0·01 |
| Human | | | |
| FSH | CPDS 13 (Butt) | 100 | 400 |
| LH | IRC2/69 (Stockell-Hartree) | 0·1 | < 0·01 |
| TSH | NPA | 0·68 | 0·25 |
| GH | B/L4 (Lowry) | < 0·01 | < 0·01 |
| Prolactin | S/L1 (Lowry) | < 0·01 | < 0·01 |
| HCG | CR 117 (Canfield) | 1·18 | < 0·01 |

Cross-reaction was calculated as the amount of hormone (w/w) giving 50 % inhibition in the radioimmunoassay.

### *Rat hormones*

Rat FSH showed immunological potencies of 75 and 76 % of the Papkoff oFSH preparation using $^{125}$I-labelled rFSH and oFSH tracers respectively. No significant cross-reaction (< 0·01 %) occurred with rat LH, TSH, growth hormone (GH) or prolactin (Table 1).

### *Human hormones*

The hFSH preparation (CPDS 13) showed an immunological potency identical to that of the Papkoff sheep FSH preparation using the $^{125}$I-labelled rFSH tracer (Table 1; Fig. 2*a*). Intact TSH (NPA) and HCG (CR 117) gave cross-reactivities of 0·68 and 1·18 % respectively

using the same tracer whereas the LH (IRC 2/69) showed a cross-reactivity of 0·1 %. Both the GH and prolactin preparations investigated showed no inhibition at a concentration of 1 $\mu$g/ml (cross-reactivity of $<0{\cdot}01$ %).

Using $^{125}$I-labelled oFSH tracer, a more specific assay was achieved (Fig. 2*b*). However, hFSH (CPDS 13) in this system was four times more potent than the Papkoff FSH preparation. TSH gave a cross-reaction of 0·25 % while HCG, LH, prolactin and GH showed insignificant cross-reaction ($<0{\cdot}01$ %).

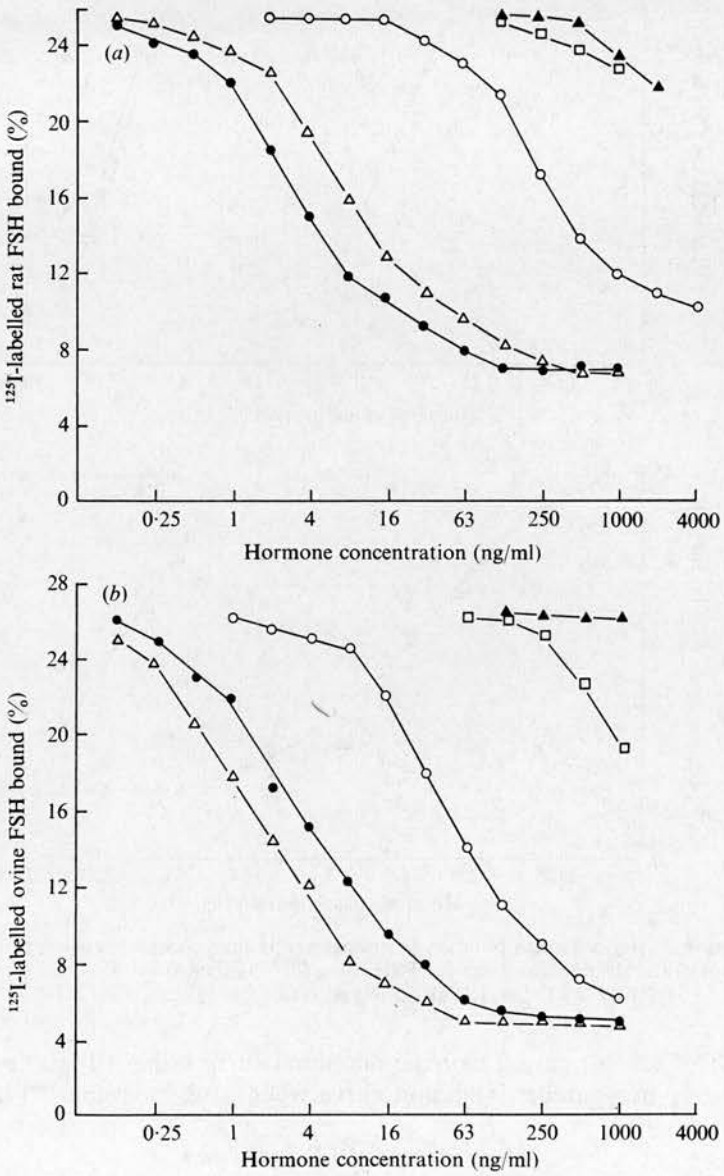



Fig. 1. Cross-reaction of ovine pituitary hormone preparations in the heterologous radioimmunoassay for FSH using (*a*) $^{125}$I-labelled rat FSH and (*b*) $^{125}$I-labelled ovine FSH. ●, Papkoff FSH; △, Reichert FSH; ○, NIH FSH S8; □, NIH TSH S6; ▲, Papkoff LH.

Using either $^{125}$I-labelled rat or ovine tracers, no significant inhibition (<0·03 %) was seen with HCG$\beta$, hLH$\beta$ and hTSH$\beta$ subunit preparations (Fig. 3). Human FSH $\alpha$-subunit showed parallel inhibition with hFSH with cross-reactivities of 0·08 and 1·5 % using $^{125}$I-labelled rFSH and oFSH tracers.

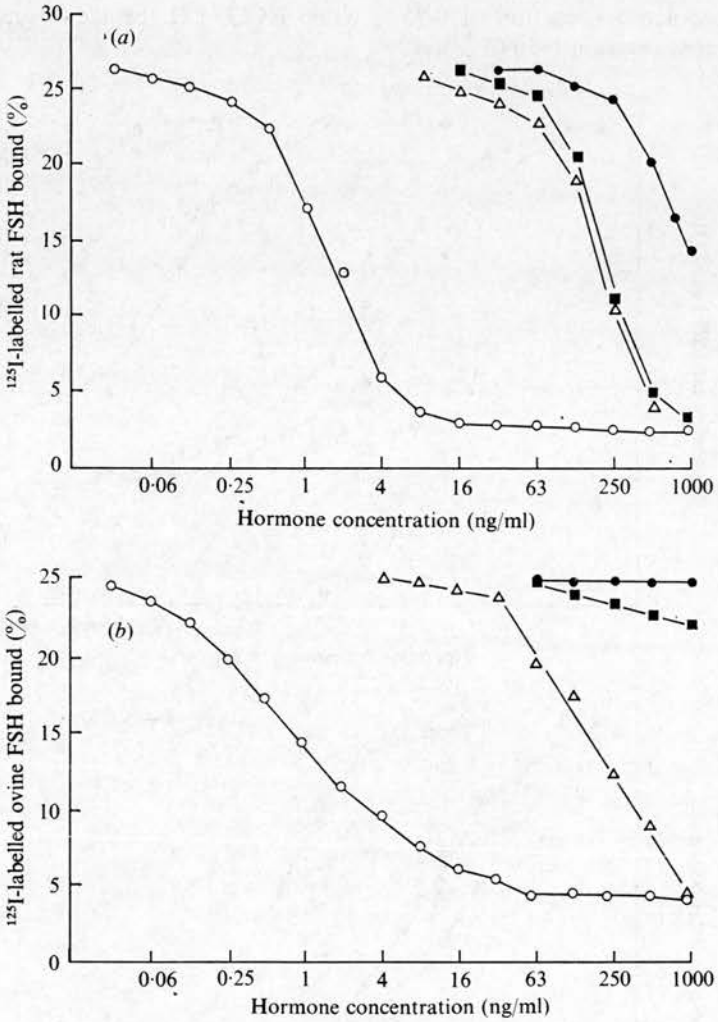



Fig. 2. Cross-reactivity of human pituitary hormones and human chorionic gonadotrophin (HCG) in the heterologous radioimmunoassay for FSH using (*a*) $^{125}$I-labelled rat FSH and (*b*) $^{125}$I-labelled ovine FSH. ○, FSH CPDS 13; ●, LH IRC2/69; ■, HCG CR 117; △, TSH NPA.

Human FSH $\beta$-subunit gave a biphasic inhibition curve using $^{125}$I-labelled rFSH tracer and a completely non-parallel inhibition curve when assessed using $^{125}$I-labelled oFSH tracer (Fig. 3).

### *Measurement of FSH in serum*

*Parallelism*

Serial dilutions of sera from an anoestrous ewe and a castrated ram, and a crude pituitary extract were parallel to the inhibition curves obtained with NIH FSH S8 oFSH standards

and other oFSH preparations using $^{125}$I-labelled rFSH as tracer (Fig. 4). Addition of hypophysectomized sheep serum to the standards did not affect parallelism. Similar data were obtained using dilutions of rat sera or human sera which were parallel to the rFSH and hFSH standards respectively.

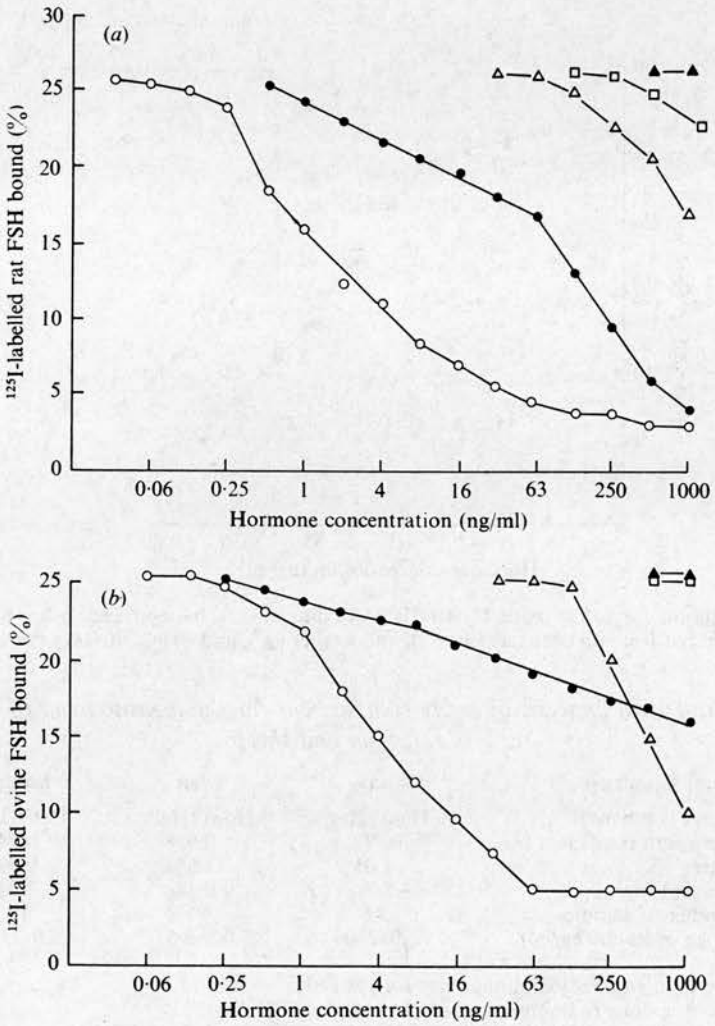



Fig. 3. Cross-reactivity of subunit preparations of human pituitary and placental glycoprotein hormones using (*a*) $^{125}$I-labelled rat FSH and (*b*) $^{125}$I-labelled ovine FSH. ○, ovine FSH (Papkoff); △, human FSH$\alpha$; ●, human FSH$\beta$; □, human TSH$\beta$; ▲, human LH$\beta$ and HCG$\beta$.

*Accuracy*

The accuracy, assessed by recovery of oFSH standard (10 $\mu$g/ml to 39 ng/ml) added to serum from hypophysectomized sheep or goats was $98 \pm 4$ (S.E.M.) % ($n = 28$) and $96 \pm 6$ % ($n = 24$) respectively.

*Precision*

Intra-assay and inter-assay variation (expressed as the coefficient of variation) of results on replicate samples was 5 % ($n$ = 30) and 9 % ($n$ = 38) respectively.

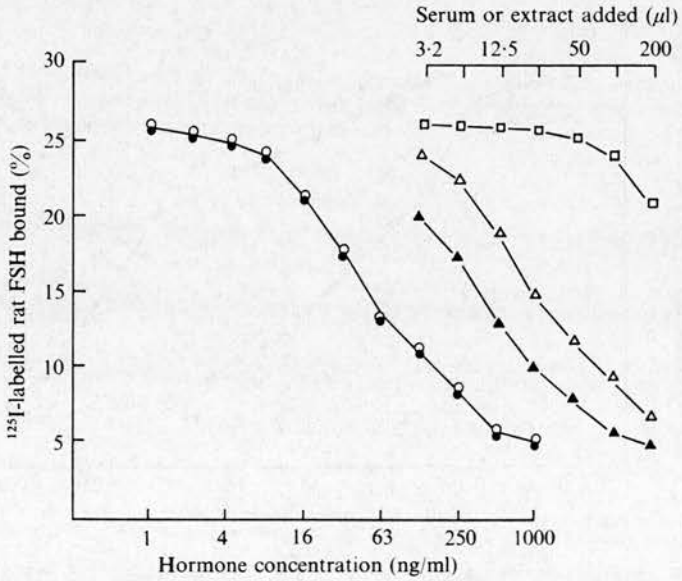



Fig. 4. Inhibition curves for ovine FSH NIH S8 in diluent (○), hypophysectomized sheep plasma (●), plasma from an oestrous ewe (□), and wether (▲), and ovine pituitary extract (△).

Table 2. *Statistical analysis of assay comparisons for the measurement of serum FSH in rat, man and sheep*

| Serum samples from ... | Rat | Man | Sheep |
|---|---|---|---|
| Assays compared ... | Hom[1]/Het[3] | Hom[2]/Het[3] | Het[3]/Het[4] |
| Regression coefficient ($r$) | 0·965 | 0·958 | 0·948 |
| Slope | 1·03 | 1·05 | 0·89 |
| Intercept (ng/ml) | −5·7 | +0·14 | +1·91 |
| Number of samples | 30 | 40 | 45 |
| Range of levels (ng/ml) | 220–2300 | 0·5–9·5 | 20–118 |

[1] Homologous radioimmunoassay for rat FSH.
[2] Homologous radioimmunoassay for human FSH.
[3] Heterologous FSH radioimmunoassay using $^{125}$I-labelled rat FSH tracer.
[4] Heterologous FSH radioimmunoassay using $^{125}$I-labelled ovine FSH tracer.

*Comparison of assays*

A summary of the results of comparing serum FSH levels in the rat and man estimated by the relevant homologous FSH radioimmunoassay and the heterologous FSH radioimmunoassay using $^{125}$I-labelled rFSH tracer are shown in Table 2. In both cases results showed highly significant linear correlations ($P < 0{\cdot}001$) and a slope approaching unity (Table 2). Serum levels of oFSH estimated by the two heterologous radioimmunoassays also showed a highly significant linear correlation ($P < 0{\cdot}001$, Table 2) with an intercept of 1·91 ng/ml related to $^{125}$I-labelled rFSH tracer system.

*Blood levels of FSH during the oestrous cycle in the ewe*

No major changes in serum levels of FSH were noted during the oestrous cycle on daily sampling in nine ewes (Fig. 5). Variations in blood levels of up to fivefold were seen in individual cases (Fig. 5), and in four ewes, levels were higher and in five ewes levels were lower on the day of oestrus than on the days immediately preceding and following oestrus (days −3 to +3).

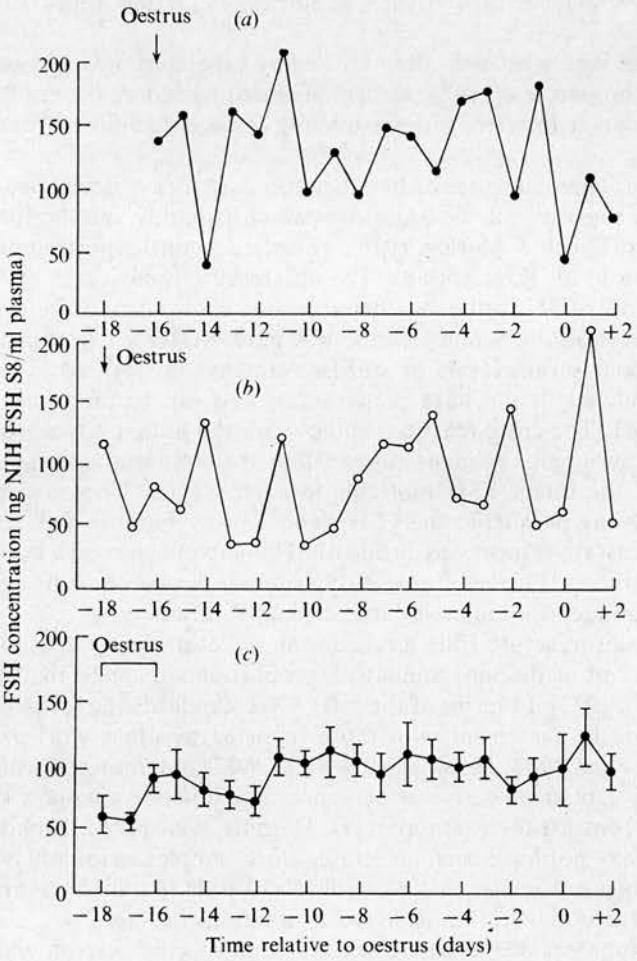



Fig. 5. Plasma concentrations of FSH (NIH FSH S8) in two individual ewes (*a*) and (*b*) and mean levels (±S.D.) in nine ewes (*c*) during the oestrous cycle.

## DISCUSSION

The present paper describes in detail the characteristics of two highly specific and sensitive double antibody radioimmunoassays for FSH. In view of the heterologous nature of the assays, i.e. using an hFSH antiserum and either rFSH or oFSH tracer, the method was equally applicable to the measurement of ovine, rat or human FSH. This was demonstrated by the highly significant correlations between estimates of serum FSH made by the heterologous and those of the relevant homologous FSH radioimmunoassays in man and rat. In

addition, results were nearly identical when FSH levels in sheep serum samples were estimated using the hFSH antiserum with either rFSH or oFSH tracer.

Cross-reaction studies with the purified pituitary hormone preparations of sheep, rat or human origin showed that the assay was completely specific for FSH with a maximum of 0·1 % cross-reaction with any of the hormones tested (Table 1), except hTSH and HCG. The TSH showed 0·7 and 0·3 % cross-reaction with rFSH and oFSH tracer assays respectively (Table 1). This level of cross-reaction is similar to that seen in the homologous hFSH assay (McNeilly & Hagen, 1974; Lynch & Shirley, 1975) and appears to reflect FSH contamination.

No interference was seen with albumin or any other serum component (see Kerdelhué *et al.* 1972; Salamonsen *et al.* 1973) as demonstrated by failure of sera from hypophysectomized sheep or goats to interfere with the binding of tracer to antibody or standard inhibition curves.

The very similar characteristics of both heterologous assays developed using either rFSH or oFSH tracers suggest that the antiserum which is highly specific for hFSH when used with hFSH tracer (Lynch & Shirley, 1975) is directed against a portion of the FSH molecule which is common in all three species. The difference in activity of the Reichert oFSH in relation to Papkoff oFSH in the two heterologous assays cannot be explained at present. However, the similarity in potency of the less pure NIH FSH preparations and the close correlation between serum levels of oFSH estimated in both assays suggest that these differences are related to the pure preparations and not to more crude preparations or endogenous oFSH. The cross-reaction studies with the human glycoprotein hormone subunits in both heterologous systems suggest that this common site may be related to the conformation of the intact FSH molecule in each species. The cross-reaction of human $\alpha$-subunit while being parallel to the FSH standard was minimal, 0·08 and 1·5 % for rFSH and oFSH tracer assays respectively, while hFSH $\beta$-subunit showed a non-parallel inhibition curve with both tracers. Further studies using subunit preparations from both rat and ovine glycoprotein hormones are underway to elucidate this finding.

The measurement of serum FSH levels during the oestrous cycle showed a peak of FSH at oestrus in five out of the nine animals, a proportion similar to that previously reported (Salamonsen *et al.* 1973). In terms of the NIH FSH standards the range of levels between 20 and 230 ng/ml are in agreement with those reported by other workers (Kerdelhué *et al.* 1972; L'Hermite *et al.* 1972; Hopkinson & Pant, 1973; Salamonsen *et al.* 1973) with higher levels during the late than early luteal phase. No obvious post-oestrous peak could be demonstrated in contrast to other reports (L'Hermite *et al.* 1972; Hopkinson & Pant, 1973) but any change may not have been detectable since samples were only obtained once daily. The results do suggest however that blood levels of FSH in individual animals vary erratically throughout the cycle with up to fivefold variations in levels.

With the development of the highly sensitive and specific heterologous double antibody radioimmunoassay described in this paper, the control of secretion and role of FSH in the sheep is now under investigation.

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# Plasma concentrations of FSH and LH in entire and castrated prepubertal bull calves treated with Gn-RH

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**Summary.** In bulls there was no increase in plasma FSH and only a small increase in LH over the first 14 weeks of age. In steers (castrated) plasma LH and FSH were unchanged for the first 3 weeks but increased significantly at 7 and 14 weeks. After 100 μg Gn-RH, LH release in bulls was minimal until 7 and 14 weeks and there was no comparable rise for FSH. LH and FSH responded to Gn-RH throughout the trial in the steers. The neonatal calf testes selectively inhibited the release of FSH from the pituitary even when challenged with Gn-RH.

## Introduction

Plasma concentrations of luteinizing hormone (LH) in entire and castrated bulls fall immediately after birth and then gradually rise until 28 days when the values of plasma LH in the steers increase rapidly above those of entire bulls (Bass, Peterson, Payne & Jarnet, 1977). LH is released in prepubertal bulls in response to the administration of synthetic gonadotrophin-releasing hormone (Gn-RH) (Mongkonpunya, Hafs, Convey, Tucker & Oxender, 1975; Kesler & Garverick, 1977) but the effect of castration on release of LH and follicle-stimulating hormone (FSH) in response to Gn-RH stimulation in prepubertal bull calves has not been reported. The present study was undertaken to investigate the effect of castration of prepubertal bulls on plasma concentrations of LH and FSH and the response of these hormones to Gn-RH.

## Materials and Methods

Two British Friesian bull calves castrated at birth (steers) and two entire bull calves (bulls) were reared in individual pens under identical management conditions. The birth weights (41·5–46·0 kg) and final liveweights (61–64 kg) were similar for the steers and bulls.

At 1, 2, 3, 7 and 14 weeks of age, between 09:00 and 10:00 h, jugular vein blood was collected by venepuncture at 40, 30, 20 min and immediately before the intravenous administration of 100 μg Gn-RH (Gonadorelin: Ayerst). Samples were then collected at 30, 60 and 90 min after the GN-RH injection. After centrifugation the plasma was stored at −20°C until analysis.

The concentrations of LH and FSH in the plasma were measured using the radioimmunoassays described by Scaramuzzi, Caldwell & Moor (1970) and McNeilly, McNeilly, Walton & Cunningham (1976) respectively. These assays, originally developed for measuring ovine LH and FSH, were tested for specificity by using the following bovine pituitary hormone preparations: LH (NIH-LH-B8); FSH (CH-1-76; potency 164 × NIH-FSH-S1); TSH (30

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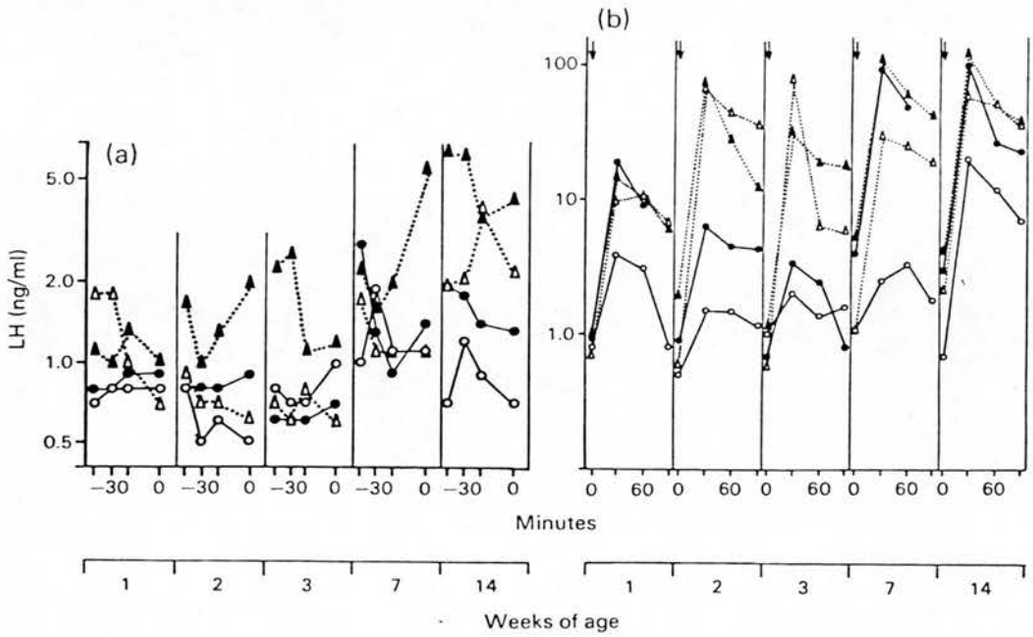
i.u./mg, Pierce); prolactin (NIH-P-B12); and GH (NIH-GH-B15). Each hormone preparation was tested in double dilution from 5 μg/ml. The LH assay was specific for LH and all other hormone preparations tested showed <0·1% cross-reaction (w/w at 50% B/Bo). Similarly, the FSH assay was specific for FSH and cross-reaction with all other hormone preparations was also <0·1% (w/w at 50% B/Bo). It had been established previously that no serum interference occurred in either radioimmunoassay (Scaramuzzi *et al.*, 1970; McNeilly *et al.*, 1976). Dilution curves of plasma from cows and bulls were parallel to the respective standards in each assay. Recovery of added hormone to plasma and intra-assay variation as coefficient of variation was 98 ± 4% and 7% ($n$ = 30), and 101 ± 5% and 8% ($n$ = 40) in the LH and FSH assays respectively. All samples were assayed at two dilutions (1:2 and 1:10) in one assay to reduce variability. Results are expressed in terms of NIH-LH-B8 and NIH-FSH-B1 for LH and FSH respectively.

Differences in hormone concentrations within individuals were assessed by obtaining the hormone concentrations within age before and after Gn-RH injections. Statistical significance was examined by using the F test on logarithmically transformed data.

## Results

### *Preinjection plasma concentrations and the response to 100 μg Gn-RH*

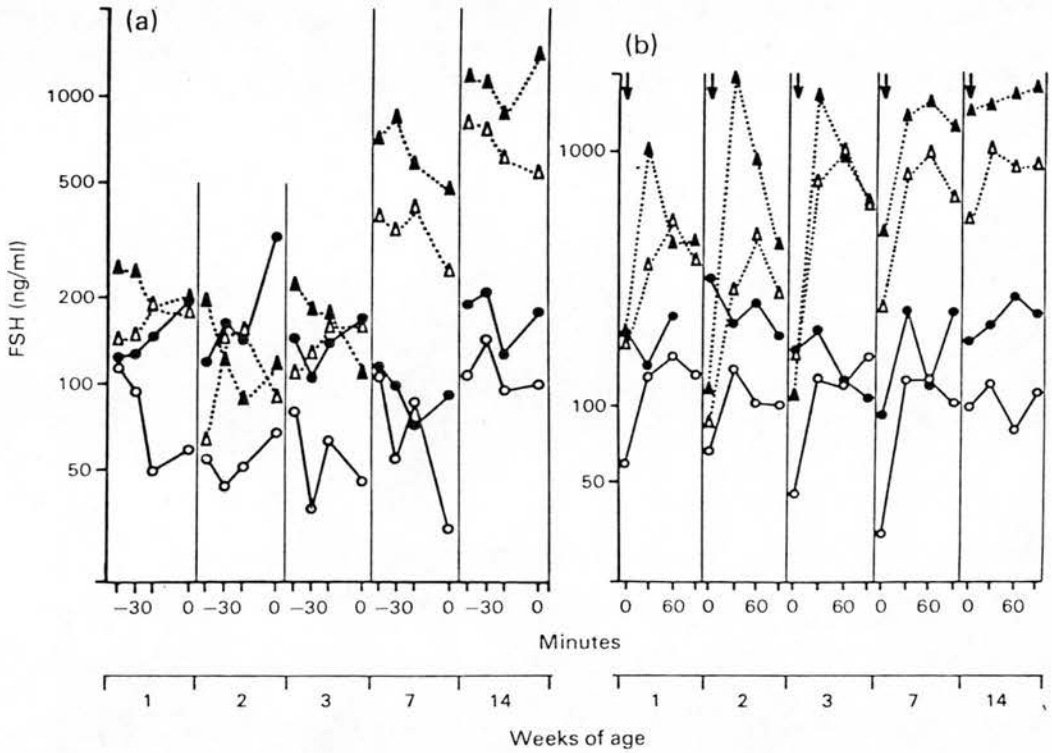
*LH.* The plasma concentrations of LH in the bulls and steers were similar for the first 7 weeks (Text-fig. 1a), but by 14 weeks of age values were relatively higher in the steers. When the preinjection samples at each age were combined for individual animals, Bull B (0·85 to 1·60), Steer C (1·23 to 2·45) and Steer D (1·09 to 4·89) had significantly ($P < 0·01$) higher LH plasma concentrations (ng/ml) at 14 weeks (proportional s.e. × 100 = 13·4%) than at 1 week (proportional s.e. × 100 = 14·1%) of age.




**Text-fig. 1.** Plasma LH concentrations in Bulls A (○) and B (●) and Steers C (△) and D (▲) before (a) and after (b) injections of 100 μg Gn-RH (arrows) at different ages after birth.

The LH response to Gn-RH increased from Weeks 1 to 14 in both bulls and steers ($P < 0{\cdot}01$) (Text-fig. 1b) although the dose fell from 2·3 to 1·6 μg per kg liveweight.

*FSH.* The preinjection plasma concentrations of FSH in both bulls and steers were similar and remained below 300 ng/ml until at least 3 weeks of age (Text-fig. 2a). The levels in the bulls remained at these low concentrations throughout the trial whereas the steers showed markedly higher concentrations ($P < 0{\cdot}05$) at 14 weeks than at 1 week of age. The combined individual concentrations of FSH before Gn-RH showed that there was a significant ($P < 0{\cdot}001$) rise from 1 to 14 weeks of age in steers but not in bulls. The administration of Gn-RH had no significant effect on the concentration of FSH in bulls (<300 ng/ml) but had a marked ($P < 0{\cdot}05$) effect in steers (Text-fig. 2b), mean plasma concentrations reaching values greater than 1300 ng/ml. There were significant differences ($P < 0{\cdot}01$) between the combined individual data for FSH after Gn-RH from Weeks 1 to 14 for the steers but no differences for the bulls. There was also a significant difference ($P < 0{\cdot}1$) in relative increase to Gn-RH between bulls and steers at 1 week of age.




**Text-fig. 2.** Plasma FSH concentrations in Bulls A (○) and B (●) and Steers C (△) and D (▲) before (a) and after (b) injections of 100 μg Gn-RH (arrows) at different ages after birth.

## Discussion

Although only 4 animals were involved in this study significant differences were found between the steers and bulls in response to Gn-RH. In agreement with earlier reports (Mongkonpunya *et al.*, 1975; Karg *et al.*, 1976) we found no increase in FSH levels and only a slight increase in LH levels in bulls during the neonatal period. Castration caused no marked response in plasma concentrations of LH and FSH during the first 3 weeks of age. A similar refractory period has been reported for LH (Bass *et al.*, 1977).

The small release of LH by the pituitary in response to a Gn-RH challenge in the first 3 weeks of life agrees with the data of Kesler & Garverick (1977) who found no change in response to Gn-RH during the first 25 days. The LH increase at 7 and 14 weeks of age to Gn-RH is in agreement with work on ram lambs (Galloway & Pelletier, 1974) but not with that on bull calves (Mongkonpunya *et al.*, 1975).

The FSH concentrations in the bulls remained low throughout the experiment and were not stimulated by Gn-RH, but in the steers there was a marked increase in plasma FSH and a significant increase in relative response to Gn-RH at 1 week of age. Our results suggest that the presence of the testes inhibits the release of FSH from the pituitary when challenged with Gn-RH during the neonatal period.

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# Blood concentrations of gonadotrophins, prolactin and gonadal steroids in males and in non-pregnant and pregnant female African elephants (*Loxodonta africana*)

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**Summary.** No seasonal variation in any of the hormones measured was apparent in males or females. Testosterone levels in males increased around puberty (10–11 years) and remained significantly higher in adult than prepubertal males. This was not accompanied by any significant change in levels of LH, FSH or prolactin.

In non-pregnant females there was no apparent difference in levels of LH, FSH or prolactin with age. There was a significant increase in progesterone around puberty (12 years) but there was considerable overlap in values between prepubertal and adult females.

During pregnancy, progesterone levels were significantly higher than in non-pregnant females with maximum levels occurring at mid-pregnancy (9–12 months). However, there was considerable overlap in values between non-pregnancy and pregnancy. Concentrations of LH and FSH decreased significantly during mid-pregnancy while prolactin levels increased dramatically during pregnancy; after 7 months of gestation until term levels were always at least 8 ng/ml greater than in any non-pregnant female. It is suggested that this consistent increase in plasma/serum levels of prolactin can be used to diagnose pregnancy in the elephant.

## Introduction

Very little is known about the reproductive endocrinology of the African elephant (*Loxodonta africana*), but such information is essential to develop controlled breeding programmes in captivity. In view of the long gestation period of the elephant (approximately 22 months) a reliable method for the diagnosis of pregnancy is also important.

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been identified, by biological and immunological assays, in pituitary glands from African elephants (Carr, 1969). Plasma levels of LH increased around oestrus and after the injection of LH-RH in two female Asiatic elephants (Chappel & Schmidt, 1979) and gonadotrophin-like activity has also been measured in the urine of a pregnant Asiatic elephant (Fujimoto, Koto, Imori & Nakama, 1970).

Plasma levels of unconjugated oestrogens and progesterone (Plotka, Seal, Schobert & Schmoller, 1975) and urinary levels of oestrone and oestradiol (Ramsay, Lasley & Stabenfeldt, 1981) are low in female elephants in comparison with most other species. The corpora lutea have a

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low content of and secretory capacity for progesterone (Smith, Hanks & Short, 1969; Ogle, Braach & Buss, 1973) and although a modest increase in plasma concentrations of progesterone may occur during pregnancy (Plotka *et al.*, 1975) this needs to be confirmed. Circulating levels of testosterone in male Indian elephants are generally low, but increase dramatically during the period of increased aggressive and sexual behaviour associated with the phenomenon known as 'musth' (Jainudeen, Katongole & Short, 1972). 'Musth' has only recently been recognized in African elephants (Poole & Moss, 1981) and although testicular testosterone levels are extremely variable (Buss & Johnson, 1967; Short, Mann & Hay, 1967) no data are available on plasma testosterone values in male African elephants.

Likewise, there is no information on changes in plasma levels of LH in the male or on FSH or prolactin in the male or the female. With the development of heterologous radioimmunoassays for LH, FSH and prolactin and steroid radioimmunoassays of improved sensitivity and specificity, we have examined the changes in blood concentrations of these hormones in male and female African elephants of different ages and reproductive status.

## Materials and Methods

*Animals and blood samples.* Blood samples kindly made available by Dr R. C. Malpas (Department of Applied Biology, University of Cambridge, U.K.) were obtained from 27 male and 90 female (55 non-pregnant; 35 pregnant) elephants shot between September 1973 and April 1974 in the Rwenzori National Park and in the Kabalega Falls National Park both north (Kabalega North) and south (Kabalega South) of the river Nile in Uganda. The period of collection covered the wet and dry seasons in all three areas (Malpas, 1977). A further series of blood samples was obtained from 2 male and 39 females (8 non-pregnant; 31 pregnant) elephants shot between August 1977 and April 1978 in the Central District of Kruger National Park, South Africa. After collection of blood samples, serum (Uganda collections) or plasma (South African collections) was separated, frozen as soon as possible (1–6 h) and stored at −20°C until hormonal analysis at the M.R.C. Unit of Reproductive Biology, Edinburgh.

The age of the animals was estimated by the criteria of Laws (1966). All female elephants of reproductive age (> 10 years old) were examined for evidence of pregnancy and/or lactation. Stage of pregnancy was determined by reference to fetal weight and crown–rump length (Perry, 1953; Laws, 1966). In some instances the ovaries were examined and the number and state of any corpora lutea recorded.

### *Radioimmunoassays of LH, FSH and prolactin*

LH, FSH and prolactin concentrations were measured by the heterologous radioimmunoassays described in detail previously (LH: Welschen *et al.*, 1975; FSH: McNeilly, McNeilly, Walton & Cunningham, 1976; prolactin: McNeilly & Friesen, 1978). Reagents, buffers, volumes and times of incubation were exactly as described.

*Specificity.* Since no purified preparations of elephant LH, FSH or prolactin were available a crude pituitary extract was prepared from acetone-dried pituitary glands collected from African elephants and kindly provided by Mr W. R. Carr, Animal Breeding Research Organization, Roslin, Midlothian (Carr, 1969). Approximately 100 mg amounts (n = 4) of this powder were extracted in 4 ml 0·01 N-ammonium bicarbonate, pH 9·0. After shaking for 2 h at room temperature the extract was centrifuged at 600 *g* for 1 h at 4°C and the supernatant stored at −20°C until assayed.

Parallelism between the ovine hormone standards used and dilutions of the elephant pituitary extract and plasma and serum samples from male and female elephants was then assessed. The slopes of the dose–response curves were not significantly different in each of the radioimmuno-

assays for LH, FSH and prolactin. Therefore all samples were assayed against, and are reported in terms of, ng of the appropriate ovine pituitary standard (NIH-LH-S14, NIH-FSH-S10 and NIH-P-S6).

Since there was no effect of plasma or serum in these assays, the hormone measurements in each fluid were taken as comparable and results are expressed in terms of plasma/serum.

*Accuracy and precision.* Accuracy of the assays assessed by measurement of known amounts of ovine standard or diluted elephant pituitary extract added to elephant plasma and serum was 95 ± 3 (s.e.m.), 102 ± 4 and 98 ± 4% for LH, FSH and prolactin respectively ($n$ = 7). The precision, expressed as the intra- and inter-assay coefficients of variation (%) assessed by repeated assays of 3 pools of elephant plasma with binding in the 3 assays over the range 32–83% B/Bo, were 10 and 14% ($n$ = 13) respectively for LH, 9 and 12% for FSH and 8 and 13% for prolactin.

*Identification of hormones measured.* To assess the form of the immunologically active elephant hormone measured by each radioimmunoassay, elephant pituitary extract was fractionated at 4°C by gel filtration on Sephadex G-100 (2 × 50 cm), eluted with 0·25 M-Tris–HCl (pH 7·6) containing 0·1% bovine serum albumin (BSA). Fractions (1 ml) were collected and analysed for LH, FSH and prolactin by radioimmunoassay and by radioreceptor assay (LH: Cheng, 1976; FSH: Cheng, 1975; prolactin: Shiu, Kelly & Friesen, 1973).

After gel filtration of elephant pituitary extract, single peaks of radioimmunoassayable LH, FSH and prolactin were found eluting in positions similar to those for the comparable ovine $^{125}$I-labelled hormones. For each hormone the fractions containing immunoreactivity corresponded with those displaying radioreceptor activity. Because there was no suitable elephant pituitary standard it was not possible to make a quantitative comparison of the radioimmuno- and radioreceptor assay estimates of activity.

*Radioimmunoassay of steroids*

Plasma or serum levels of testosterone and progesterone were measured by the specific radioimmunoassays described by Corker & Davidson (1978) and Scaramuzzi, Corker, Young & Baird (1975) respectively. All samples were measured in duplicate. Procedural losses during extraction were monitored individually by the addition of tracer amounts of [$^3$H]testosterone or [$^3$H]-progesterone (sp. act. 60 Ci/mmol and 90 Ci/mmol, respectively). Mean recovery values were 82 ± 3% for testosterone ($n$ = 29) and 85 ± 4% for progesterone ($n$ = 20) and results were corrected accordingly. The accuracy of each assay, assessed by recovery of known amounts of pure unlabelled testosterone or progesterone (100–10 000 pg) added to plasma or serum samples from various elephants, was 96 ± 3% ($n$ = 20) and 97 ± 5% ($n$ = 20) respectively. The sensitivity and intra- and interassay precision (as coefficient of variation) were 10 pg/ml, 10·4% and 11·0% for testosterone and 50 pg/ml, 9·3% and 12·6% for progesterone.

In addition, progesterone concentrations (range 375–3738 pg/ml) in plasma samples from 2 non-pregnant and 4 pregnant elephants were measured before and after celite chromatography (Hodges, Gulick, Czekala & Lasley, 1981) using a different specific progesterone radioimmunoassay (Hodges, Eastman & Jenkins, 1982). The results obtained by the three different procedures were not significantly different ($P > 0·05$, paired $t$ test).

Statistical analysis was by one- or two-way analysis of variation and by Student's $t$ test.

## Results

*Hormone levels in male elephants*

Plasma/serum samples were obtained from a total of 29 male elephants aged between 1 and 30 years of age. No significant changes associated with age were seen in concentrations of LH (overall mean ± s.e.m.: 15 ± 4 ng/ml; range 8–23 ng/ml), FSH (72 ± 8 ng/ml; range 10–194 ng/ml), pro-

lactin (0·9 ± 0·1 ng/ml; range 0·2–3·7 ng/ml) and progesterone (604 ± 118 pg/ml; range 177–3125 pg/ml). In contrast, there was a significant difference ($P < 0·01$) between testosterone values in animals less than (0·43 ± 0·09 ng/ml, N = 10) or greater than (2·57 ± 0·52 ng/ml, N = 17) 12 years of age, the time at which sexual maturity is first recognized (Perry, 1953). None of the animals in the younger group had levels > 1 ng/ml (range 0·14–0·98 ng/ml) whereas 12 of the 17 animals in the older group had levels exceeding this value (1·13–6·5 ng/ml).

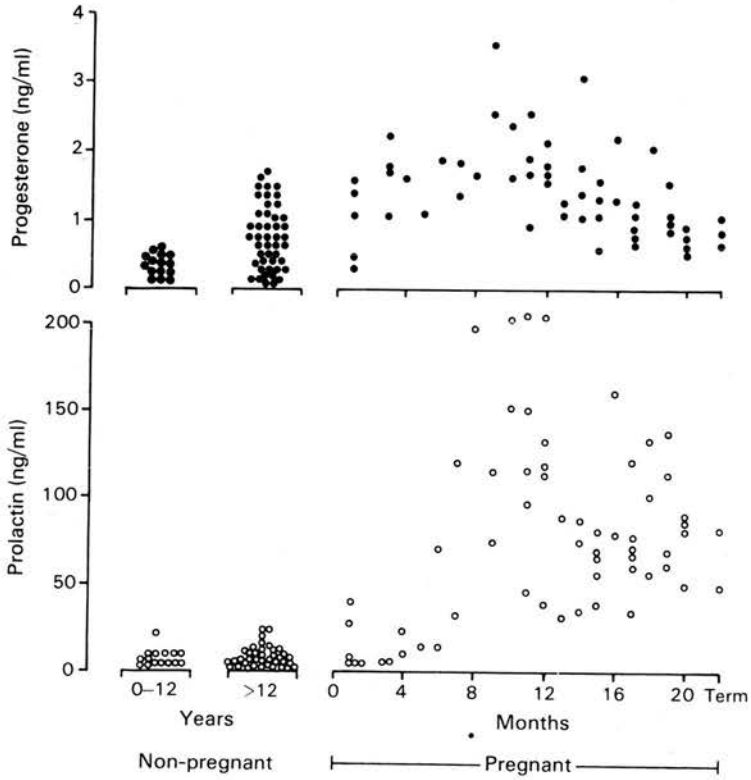
*Hormone levels in non-pregnant female elephants*

Blood samples were obtained from 63 non-pregnant females 1–60 years of age. There was no significant change associated with age for mean ± s.e.m. concentrations of LH (22 ± 1·4 ng/ml, range 8–52 ng/ml), FSH (95 ± 9 ng/ml; range 30–256 ng/ml) or prolactin (6·9 ± 0·7; range 0·8–22·4 ng/ml). However, progesterone values in 15 females < 13 years of age (316 ± 52 pg/ml; range 120–595 pg/ml) were significantly lower ($P < 0·01$) than in the older females (707 ± 77 pg/ml; range 92–1495 pg/ml, N = 48) although there was considerable overlap between the two groups (Text-fig. 1).

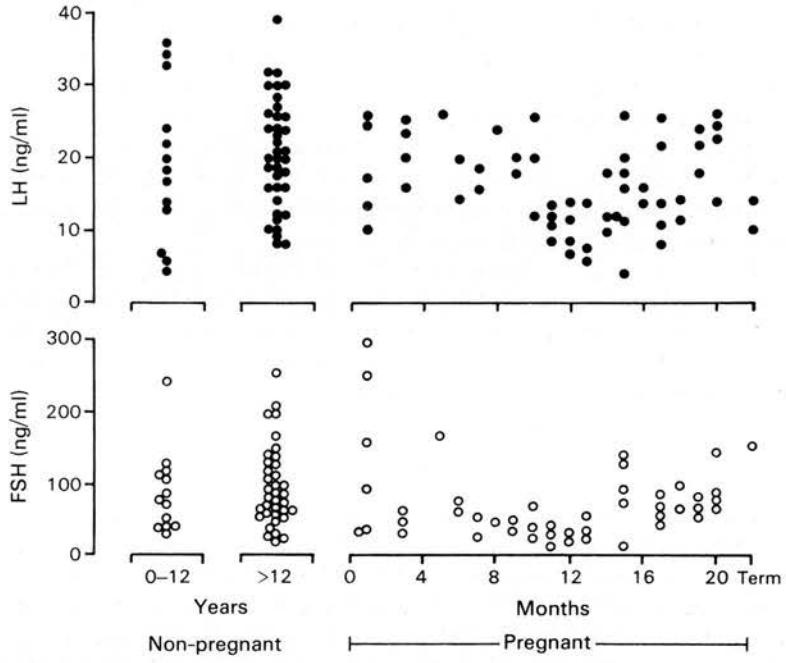
*Hormone levels in pregnant female elephants*

Blood samples were obtained from 66 pregnant animals 13–49 years old of which 49 (74%) were lactating. Of the 37 females that were > 13 months pregnant, only 2 were not lactating.

*LH and FSH.* The levels of LH and FSH in plasma/serum throughout pregnancy are shown in Text-fig. 2. For statistical analysis, levels were grouped at 4 monthly intervals, i.e. 1–4, 5–8, 9–12 months etc. Plasma/serum levels of LH tended to be lower throughout pregnancy than those in non-




**Text-fig. 1.** Blood concentrations of progesterone and prolactin in female African elephants at different ages and stages of pregnancy.




**Text-fig. 2.** Blood concentrations of LH (●) and FSH (○) in female African elephants at different ages and stages of pregnancy.

pregnant females but this was only significant ($P < 0{\cdot}05$) between 9 and 16 months of pregnancy (Text-fig. 2). After an initial non-significant increase during the first 2 months of pregnancy, FSH values were lower than in non-pregnant animals throughout the rest of gestation, although this was only significant ($P < 0{\cdot}05$) between 9 and 12 months of gestation (Text-fig. 2).

*Progesterone*. Progesterone concentrations during pregnancy (1414 ± 92 pg/ml; N = 53) were significantly higher ($P < 0{\cdot}01$) than those in mature (> 12 years) non-pregnant females

**Table 1.** Plasma concentrations of progesterone in individual female African elephants in relation to the number and physiological state of the corpora lutea (CL) or corpora albicantia (CA) in both ovaries of elephants shot in the Central District of the Kruger National Park, South Africa in August 1977

| Reproductive status | Animal no. | Age (years) | CL or CA | No. | Plasma progesterone (pg/ml) |
|---|---|---|---|---|---|
| Anoestrous | | | | | |
| | 1341 | 38 | CA | Numerous | 242 |
| | 1348 | 14 | CA | Numerous | 310 |
| | 1340 | 26 | CA | Numerous | 365 |
| Oestrous | | | | | |
| | 1349 | 21 | CL | 1 | 747 |
| | 1359 | 20 | CL | 2 | 684 |
| | 1358 | 14 | CL | 3 | 1300 |
| | 1353 | 16 | CL | 6 | 1065 |
| Pregnant | | | | | |
| | 1350 | 15 | CL | 12 | 1762 |
| | 1352 | 14 | CL | 14 | 1832 |
| | 1355 | 20 | CL | 13 | 800 |
| | 1354 | 30 | CL | 12 | 522 |

(707 ± 77 pg/ml, N = 48). However, there was considerable overlap between values in the two groups. The highest levels of progesterone occurred during mid-pregnancy and levels declined towards term (Text-fig. 1). From 8 months of gestation until term only 10 of the 44 progesterone values were above the maximum level seen in non-pregnant females (Text-fig. 1).

In 7 non-pregnant and 4 pregnant elephants the ovaries were examined and the number and appearance of the corpora lutea were recorded. An increase in the number of corpora lutea was associated with increased plasma levels of progesterone except for values for 2 pregnant elephants at 19 and 20 months gestation which were low (Table 1).

*Prolactin.* There was a significant increase ($P < 0{\cdot}001$) in prolactin values during pregnancy (50 ± 7 ng/ml, N = 63) compared with those in non-pregnant females (6·9 ± 0·7 ng/ml; N = 61) (Text-fig. 1). After 7 months of pregnancy all prolactin concentrations were at least 8 ng/ml greater than the maximum level found in any non-pregnant female (Text-fig. 1). There was no significant difference in prolactin values between lactating and non-lactating pregnant animals.

## Discussion

The data presented in this paper examine for the first time in some detail the changes in reproductive hormones in male and female African elephants in relation to age and pregnancy.

The heterologous radioimmunoassays for LH, FSH and prolactin have been validated for several different species and have always been shown to be specific (LH: Welschen *et al.*, 1975; Hodges, 1978; FSH: McNeilly *et al.*, 1976; Bass, McNeilly & Moreton, 1979; Fraser, 1981; prolactin; McNeilly & Friesen, 1978; McNeilly, Abbott, Lunn, Chambers & Hearn, 1981; Hinds & Tyndale-Biscoe, 1982). In the absence of purified elephant pituitary hormones, validation for each hormone radioimmunoassay was confirmed by the parallelism between standard hormone preparations, plasma and serum samples and dilutions of elephant pituitary extract. Recovery of added hormone to elephant plasma or serum samples showed that the radioimmunoassays were applicable in quantitative terms and the activity measured in each appeared as single separate peaks on Sephadex gel filtration. In the circumstances it is felt that each of these hormone assays has been validated for use in the elephant, although final confirmation must await the availability of purified preparations of elephant LH, FSH and prolactin.

The failure to detect any differences between LH, FSH and prolactin levels in male or non-pregnant females at any stage in samples collected in Uganda and South Africa and at different times of the year (between August and April) suggests that any seasonal change in the levels of these hormones is likely to be small. However, a decrease in thyroxine and triiodothyronine uptake values was found in the dry compared to wet seasons in elephants in the Kabalega National Park, Uganda (Brown, White & Malpas, 1978). The lack of differences between pre- and post-pubertal animals is probably due to the relatively small number of samples collected around this time.

The similarity of prolactin values in lactating and non-lactating females is not too surprising because it was not possible to record when last suckling occurred and the age of calf was not recorded. Since both suckling and time *post partum* are the most important contributors to the levels of prolactin *post partum* in mammals that have been adequately studied (Cowie, Forsyth & Hart, 1980), the role of prolactin in lactation in the elephant clearly needs more detailed investigation.

The increase in testosterone levels after 11 years of age in male elephants coincides with the onset of puberty (Perry, 1953). The levels are similar to those reported for male Asian elephants but no values as high as those observed during the musth in male Asian elephants (Jainudeen *et al.*, 1972) were obtained.

In the female there was a clear increase in progesterone values after puberty (> 10 years of age; Perry, 1953) although there was considerable overlap in levels between prepubertal and mature females. The levels agree with those reported by Plotka *et al.* (1975) but are higher than those suggested originally by Hanks & Short (1972). The higher than expected levels of progesterone in prepubertal animals may have been due to an unidentified progestagen cross-reacting in the radio-

immunoassay used, but the progesterone values were confirmed by using a different radioimmunoassay for progesterone with and without celite chromatography. As in the hyrax, the low levels of progesterone during pregnancy in the elephant may be due to rapid conversion of progesterone to 20α-dihydroprogesterone by erythrocytes (Heap, Gombe & Sale, 1975). However, metabolism of progesterone in fresh samples of blood in the elephant was less than 2% after a 1-h incubation period (A. J. Hill & J. K. Hodges, unpublished observations). In addition, elephant serum levels of 20α- and 17α-dihydroprogesterone, the main products of erythrocyte metabolism, are similar to those of progesterone (J. K. Hodges, unpublished observations).

There appeared to be an increase in progesterone concentrations in relation to an increase in the number of corpora lutea present in the ovaries. This is of interest since it has been suggested that, because of the small capacity of individual corpora lutea to secrete progesterone, a certain critical mass of luteal tissue may be required to maintain plasma levels of progesterone at a level compatible with pregnancy (Perry, 1953; Hanks & Short, 1972) although this view has been questioned (Ogle *et al.*, 1973; Smith & Buss, 1975). During pregnancy plasma levels were significantly higher than in non pregnant females. However, there was considerable overlap in levels of progesterone, indicating that measurement of progesterone alone would not be suitable to diagnose pregnancy in the elephant. In the second half of pregnancy, there was a decline in progesterone values towards term, when there is histological evidence of a reduction in corpus luteum function (Smith & Buss, 1975).

The decline in plasma levels of LH and FSH between 10 and 14 months of pregnancy occurs when there is also a repression of follicular development (Smith & Buss, 1975), probably due to the negative feedback effects of gonadal and placental steroids.

The dramatic increase in prolactin values during pregnancy could be used for pregnancy diagnosis in the elephant. The reason for the increase in the elephant is not known, but in other species such an increase occurs in relation to the raised circulating oestrogen values associated with pregnancy (McNeilly, 1980; Cowie *et al.*, 1980). Since a similar rise and change in ratio of oestrogens also occurs during pregnancy in the elephant, and coincides with the time when prolactin levels are also increased (Hodges, Henderson & McNeilly, 1983) it is possible that, as in other species, oestrogens in the elephant act directly to increase prolactin secretion. It is also possible that the prolactin is placental in origin although placental lactogens from other species do not cross-react in the radioimmunoassay used in the present study (McNeilly & Friesen, 1978).

While no relationship between lactation and prolactin was apparent in non-pregnant females, when prolactin levels were high in the second half of pregnancy almost all elephants were lactating as judged by the presence of milk in the mammary gland. Perry (1953) also found that in a majority of animals pregnancy was accompanied by lactation.

The present results indicate that measurement of plasma or serum levels of prolactin can be used to diagnose pregnancy at least beyond 7 months of gestation, a finding of considerable advantage to those interested in breeding programmes for the elephant.

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# Circulating oestrogen concentrations during pregnancy in the African elephant (*Loxodonta africana*)

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**Summary.** Oestrone, oestradiol-17β and oestriol were measured in plasma samples from non-pregnant and pregnant African elephants shot in the wild. Enzymic hydrolysis of plasma showed that approximately 90 and 96% of the total (i.e. conjugated plus unconjugated) concentrations of oestrone and oestradiol-17β, respectively were represented by conjugated hormones. Unconjugated oestrogens remained low (<50 pg/ml) in all samples, with no distinction between non-pregnant and pregnant animals. Levels of total oestrone during pregnancy varied between 160 and 594 pg/ml but were not significantly different from non-pregnant values. Total oestradiol-17β concentrations were significantly elevated during pregnancy ($P < 0{\cdot}01$) and, despite considerable individual variation (193–1428 pg/ml), were consistently higher than non-pregnant values after 6 months of gestation. The elevated levels of oestradiol-17β resulted in a reversal of the total oestradiol-17β : oestrone concentration ratio at about 6 months of pregnancy. Concentrations of total oestriol did not exceed 103 pg/ml. An indirect method of measurement indicated that oestradiol-17β sulphate was probably the most abundant circulating oestrogen during pregnancy in the African elephant.

## Introduction

There are very few data on the hormonal characteristics of pregnancy in the Asian or African elephant. Those which do exist have indicated unusually low levels of steroids in luteal tissue (Smith, Hanks & Short, 1969; Hanks & Short, 1972) and in the blood (Plotka, Seal, Schobert & Schmoller, 1975) compared with other mammals, and have been unable to provide any clear endocrine distinction between the pregnant and non-pregnant condition. However, McNeilly, Martin, Hodges & Smuts (1983) have now reported a modest, but significant, elevation in blood progesterone and a marked increase in prolactin levels during pregnancy in the African elephant. In this paper the circulating oestrogen concentrations during pregnancy are described.

## Materials and Methods

### *Sample collection*

The plasma samples used in this study were from 22 pregnant and 5 non-pregnant African elephants killed by shooting in the Kruger National Park, South Africa, in August 1977 and in February and April 1978. Details of blood collection and the preparation, storage and transport of plasma are described by McNeilly *et al.* (1983). Non-pregnant elephants were judged to be sexually

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mature by age determination (range 15–32 years; Laws, 1966) and from evidence of previous ovulation (i.e. presence of corpora lutea or corpora albicantia). Large follicles were present in the ovaries from 3 of the 5 non-pregnant animals. Stage of pregnancy was estimated from measurement of fetal weight and crown–rump length according to the method of Perry (1953) and Laws & Parker (1968). The ages of pregnant animals were 9–36 years.

*Hormone assays*

Unconjugated oestrone, oestradiol-17β and oestriol in plasma (1·0 ml) were measured by radioimmunoassay procedures previously described (Hodges, Brand, Henderson & Kelly, 1983). The concentration of total oestrogen (i.e. conjugated plus unconjugated) was measured by incorporating an enzymic hydrolysis step before assay (Hodges *et al.*, 1983). Plasma (0·3 ml) was hydrolysed by adding 0·005 ml β-glucuronidase arylsulphatase (Sigma Chemical Co., Poole, England) in 0·6 ml phosphate-saline buffer (PBS, pH 5·0) or 0·015 ml β-glucuronidase (Pasteur Institute, Paris, France) in 0·6 ml PBS (pH 7·0) and incubation for 20 h at 37°C. The volumes of each enzyme preparation were chosen to contain equivalent glucuronidase activities (600 Fishman units) under optimum conditions; the Sigma preparation contained an additional 16·4 units of sulphatase activity per 0·005 ml (1 unit hydrolyses 1 μM nitrocatechol sulphate/h at pH 5·0 and 37°C). No appreciable sulphatase activity was found in the glucuronidase preparation. All total hormone concentrations reported here, with the exception of those indicated in Table 1, are after hydrolysis with glucuronidase arylsulphatase.

Tracer amounts of [$^3$H]oestrone and [$^3$H]oestradiol-17β (sp. act. 92 Ci/mmol and 100 Ci/mmol respectively) or [$^3$H]oestriol (sp. act. 114 Ci/mmol; Radiochemical Centre, Amersham) were added to hydrolysed and non-hydrolysed samples before extraction with re-distilled diethyl ether. The extractants were dried under nitrogen, reconstituted in the appropriate organic solvents and subjected to celite column chromatography (Hodges, Gulick, Czekala & Lasley, 1981). Selected fractions were evaporated to dryness, reconstituted in assay buffer and separate aliquots taken for assay (in duplicate) and recovery determination. The mean ± s.e.m. recovery values for the oestrone, oestradiol-17β and oestriol assays were 67·6 ± 1·1% ($n$ = 64), 61·8 ± 0·6% ($n$ = 78) and 73·4 ± 3·0% ($n$ = 10), respectively.

The origins and specificities of the antisera used here have been described (Hodges *et al.*, 1983). The sensitivity of each assay, defined as the minimum hormone concentration detectable/ml elephant plasma was 21 pg oestrone, 20 pg oestradiol-17β and 12 pg oestriol for unconjugated hormone and 86 pg oestrone, 83 pg oestradiol-17β and 40 pg oestriol for total hormone (after hydrolysis). Buffer and hydrolysis enzyme blanks which were extracted and run through celite for each assay gave values less than the sensitivities of the respective assays. Interassay precision, expressed as the coefficient of variation for replicate determinations of a low and a high value plasma pool, was 6·7 and 11·2% ($n$ = 5) for oestrone and 10·7 and 11·6% ($n$ = 5) for oestradiol. Oestriol measurements were performed in a single assay. Intra-assay precision was <8% for each hormone.

Validation of the measurement of oestrogens was achieved by performing celite column co-chromatography and comparing the profile of immunoreactivity with that of pure tritiated steroid across the appropriate elution fractions at 0·5 ml intervals (Hodges *et al.*, 1983). Text-figure 1(a) illustrates this procedure for oestrone and oestradiol-17β measured in a randomly selected plasma sample after hydrolysis.

Continuous elution reverse-phase high-pressure liquid chromatography was also performed on one sample from mid-pregnancy to determine whether immunoreactive oestrogens other than oestrone, oestradiol-17β and oestriol were present in significant amounts. Details of the procedure have been previously reported (Hodges *et al.*, 1981). Oestrogen immunoreactivity in each 0·5 ml fraction eluted from the column was measured using an oestriol trisuccinyl-directed antibody non-specific for oestrogens (Tulchinsky & Abraham, 1971). Tracer amounts of [$^3$H]oestrone,

[$^3$H]oestradiol-17β and [$^3$H]oestriol were added to the sample before extraction and chromatography to indicate their elution positions on the column.

The results from all measurements are reported. Unconjugated oestrogens and total oestriol were not measured in every sample due to insufficient volume of plasma.

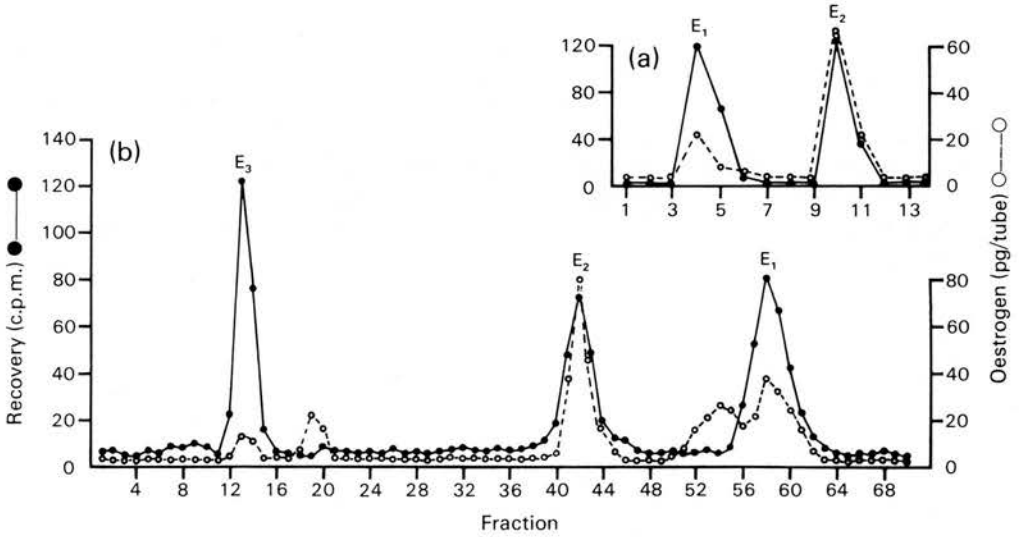
*Statistical analysis*

Oestrogen concentrations in non-pregnant and pregnant animals were compared by Wilcoxons rank sum test for unpaired data. Concentration ratios were compared by Student's *t* test.

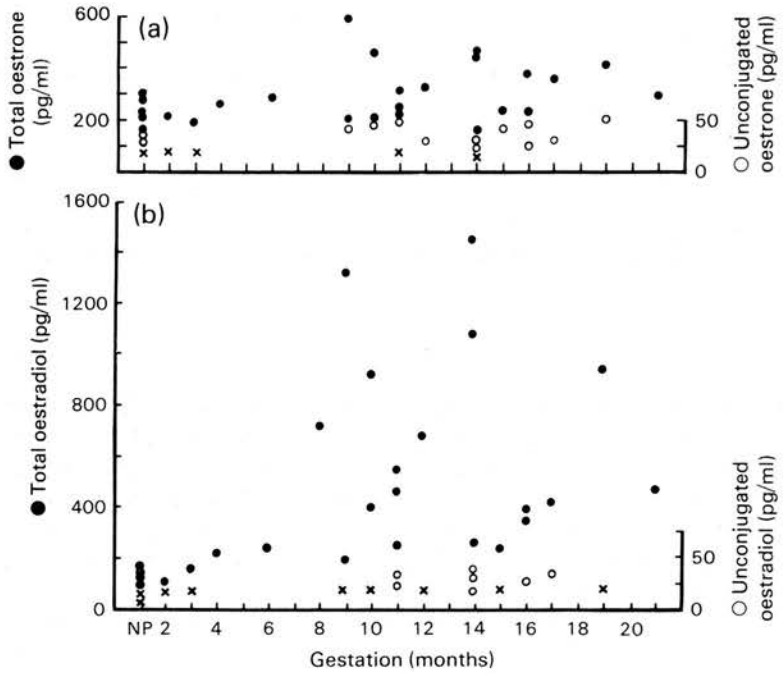
## Results

Co-chromatographic analysis of pure tritiated oestrone and oestradiol-17β and plasma oestrogen immunoreactivity in the fractions taken to assay is shown in Text-fig. 1(a). The similarity between the profiles of immunoreactivity and radioactivity was taken to indicate the absence of any substantial contamination from cross-reacting substances. Continuous elution reverse-phase HPLC (Text-fig. 1(b)) shows the relative levels of various component oestrogens in a plasma sample from mid pregnancy. Oestradiol-17β and oestrone accounted for most of the oestrogen present although additional immunoreactivity was found in fractions 19–20 and 52–56.

Circulating concentrations of unconjugated and total oestrone in pregnant and non-pregnant animals are shown in Text-fig. 2(a). Levels of unconjugated oestrone were extremely low in all samples and there was no obvious distinction between values for non-pregnant (<21–35 pg/ml) and pregnant (<21–53 pg/ml) animals. Concentrations were undetectable (<21 pg/ml) in 1 of 3 samples from non-pregnant and 5 of 15 samples from pregnant elephants. Concentrations of total




**Text-fig. 1.** Chromatography of plasma oestrogens of the African elephant. (**a**) Celite column co-chromatography of oestrone ($E_1$) and oestradiol-17β ($E_2$) in hydrolysed plasma. Oestrogen immunoreactivity measured by specific oestrone and oestradiol-17β antibodies is compared with recovery of pure tritiated hormone in 0·5 ml fractions across the entire eluate taken for assay (3·5 ml for each hormone). (**b**) High-pressure liquid chromatography separation of ether-extracted, hydrolysed plasma from an elephant 12 months pregnant. The profile of oestrogen immunoreactivity measured by non-specific oestrogen antibody is compared with the recovery of [$^3$H]oestrone ($E_1$), [$^3$H]oestradiol ($E_2$) and [$^3$H]oestriol ($E_3$).




**Text-fig. 2.** Plasma concentrations of unconjugated and total oestrone (a) and oestradiol-17β (b) in non-pregnant (NP) and pregnant elephants. Values below the limit of the assay are indicated by ×.

oestrone were much higher, 160–290 pg/ml for non-pregnant animals and 161–594 pg/ml throughout gestation. The highest values were measured after 9 months of gestation although about 50% of the values during this period remained in the non-pregnant range. There was no significant difference between total oestrone concentrations in non-pregnant and pregnant animals ($P > 0{\cdot}05$). In samples in which unconjugated oestrone could be detected, 88·9 ± 1·9% of the value for total oestrone could be accounted for by conjugated hormone (mean ± s.e.m., $n = 11$).

Concentrations of unconjugated oestradiol-17β also remained low during pregnancy (<20–41 pg/ml, pregnant; <20–32 pg/ml, non-pregnant) as shown in Text-fig. 2(b). Values in 2 of 3 non-pregnant and 7 of 14 pregnant animals were undetectable (<20 pg/ml). However, the concentrations of total oestradiol-17β during pregnancy (mean ± s.e.m., 498·9 ± 72·3 pg/ml, $n = 22$; range 116–1428 pg/ml) were significantly higher than those in samples from non-pregnant animals (mean ± s.e.m., 157 ± 6 pg/ml, $n = 5$; range 120–186 pg/ml) ($P < 0{\cdot}01$). Furthermore, despite considerable individual variation, concentrations of total oestradiol-17β after 6 months gestation were consistently higher than values in non-pregnant animals. In 7 samples in which the levels of unconjugated oestradiol-17β were detectable, 95·8 ± 1·0% of the value for total oestradiol-17β was accounted for by conjugated hormone.

Levels of unconjugated oestriol were undetectable (<12 pg/ml) in all samples measured. The concentration of total oestriol in 1 non-pregnant elephant was 89 pg/ml, whereas levels in 4 pregnant animals (7–16 months) varied between 52 and 103 pg/ml.

Comparison between concentrations of unconjugated oestrone and oestradiol-17β is difficult because of the large proportion of undetectable values. However, in those samples with detectable levels of both unconjugated hormones the mean ± s.e.m. concentration ratio of oestradiol-17β : oestrone was 0·86 ± 0·1 ($n = 7$). Concentrations of total oestradiol-17β were lower than those of total oestrone in all samples from non-pregnant animals ($n = 5$) and pregnant animals up to 6

**Table 1.** Circulating concentrations of oestrone and oestradiol-17β, measured after hydrolysis with two different enzyme preparations in non-pregnant and pregnant elephants

| Condition | Oestrone (pg/ml) | | Oestradiol-17β (pg/ml) | |
|---|---|---|---|---|
| | Glucuronidase | Glucuronidase arylsulphatase | Glucuronidase | Glucuronidase arylsulphatase |
| Not pregnant | 98 | 206 | 34 | 85 |
| | 95 | 170 | 47 | 106 |
| Pregnant—10 months | 149 | 386 | 85 | 544 |
| —14 months | 104 | 443 | 52 | 960 |
| —22 months | 151 | 348 | 45 | 329 |

months of gestation ($n = 4$). In contrast, in 17 of 18 samples from animals beyond 6 months of gestation, values for total oestradiol-17β exceeded those of total oestrone. Furthermore, the total oestradiol-17β : oestrone concentration ratio during pregnancy (mean ± s.e.m., 1·50 ± 0·16, $n = 22$) was significantly greater than that in non-pregnant animals (mean ± s.e.m., 0·73 ± 0·1, $n = 5$) ($P < 0·05$).

The effect of hydrolysis using enzyme preparations with and without additional sulphatase activity on levels of oestrone and oestradiol-17β in selected samples is shown in Table 1. Whilst glucuronidase alone increased the concentrations of both hormones (compare with unconjugated levels in Text-figs 2a & b), most of the increase in oestrogen immunoreactivity after hydrolysis was associated with the sulphatase enzyme. The difference between the effects of the two enzymes was greater for oestradiol than for oestrone.

## Discussion

This paper provides the first detailed account of circulating levels of oestrone and oestradiol-17β during pregnancy in the African elephant. The pattern of low levels of unconjugated oestrogens confirms a previous report (Plotka *et al.*, 1975) describing oestrogen concentrations of 12–37 pg/ml (measured by non-specific oestradiol antibody) in 8 samples from Asian and African elephants in various reproductive states. In agreement with the present results there was no evidence for an increase in unconjugated oestrogen levels associated with pregnancy. The only other report of circulating oestrogens in the elephant is by Chappel & Schmidt (1979) who measured concentrations of oestradiol-17β of up to 13 pg/ml during oestrus in a single Asian elephant.

Although the concentrations of unconjugated oestrone and oestradiol in the elephant remain low during pregnancy, other unconjugated oestrogens may be more abundant. Nevertheless, the levels of oestrogen reported here compare with those found throughout much of gestation in several domestic species including sheep (Challis, 1971; Liggins, Fairclough, Grieves, Forster & Knox, 1977) and cattle (Wettemann & Hafs, 1973; Hoffman, Wagner, Rattenberger & Schmidt, 1977). It is not known whether there is an increase in unconjugated oestrogen concentrations during late pregnancy in the elephant comparable with that described for the sheep and cow (Challis, 1971; Hoffman *et al.*, 1977).

The present results demonstrate that most of the circulating oestrone and oestradiol-17β in the African elephant is in conjugated form. In many other species in which elevated concentrations of conjugated oestrogens are a feature of pregnancy, oestrone sulphate is present in greatest quantities (Loriaux, Ruder & Lipsett, 1971; Robertson & King, 1974; Tsang, Hackett & Turner, 1975; Tsang & Hackett, 1979). Based on an indirect method of estimation, the present data suggest that the most abundant oestrogen during pregnancy in the African elephant is oestradiol-17β sulphate. Direct measurement of individual oestrogen conjugates is needed to confirm this. The HPLC data suggest

that conjugates of oestrone and oestradiol-17β account for most of the overall oestrogen immunoreactivity in the sample at 12 months of gestation. However, the proportion of 'total' oestrogen represented by oestrone and oestradiol-17β at other stages of pregnancy is not necessarily the same, and it cannot be assumed that the sample is representative of the oestrogen concentrations in other elephants.

The preferential increase in conjugated oestradiol-17β during pregnancy results in a reversal of the total oestradiol-17β : oestrone concentration ratio. Although this ratio is significantly higher in pregnant than in non-pregnant animals when all samples are included, total oestrone continues to be more abundant than oestradiol-17β during the early stages of pregnancy, and the reversal of the concentration ratio coincides with the appearance of elevated concentrations of conjugated oestradiol-17β at about 6 months gestation. McNeilly *et al.* (1983) have shown that prolactin levels also begin to increase at about this time and have suggested that the two events are related, as indeed they appear to be in several other species during pregnancy (Cowie, Forsyth & Hart, 1980).

The significance of the increase in total oestradiol-17β concentrations in relation to the control of pregnancy in the elephant is obscure, particularly in view of the considerable variation in levels between individuals. Circulating concentrations of hormones do not necessarily reflect their secretion and nothing is known of the production or metabolism of oestrogens in the elephant. Similarly, the relative contribution of the ovary and conceptus to circulating oestrogen concentrations is not known. An ovarian follicular source is unlikely as follicular development is repressed between 5 and 16 months of pregnancy (Smith & Buss, 1975) and there is a decline in LH and FSH levels over a similar period (McNeilly *et al.*, 1983). Histologically, the corpus luteum appears most steroidogenically active between 2 and 14 months of pregnancy (Smith & Buss, 1975). Although there is evidence to suggest that plasma levels of progesterone increase during pregnancy in relation to an increase in the number of corpora lutea (McNeilly *et al.*, 1983), such a relationship in terms of oestrogen concentrations has yet to be examined.

From a practical point of view, diagnosis of pregnancy in the elephant presents considerable difficulties. Until recently, the development of and appearance of milk in the mammary glands in the second half of gestation together with an absence of cyclic oestrous behaviour were the only reliable criteria for use in pregnancy diagnosis. McNeilly *et al.* (1983) have described the potential for pregnancy diagnosis at 7 months using the measurement of plasma prolactin. The present study suggests that measurement of conjugated oestradiol-17β or the concentration ratio of oestradiol-17β : oestrone may also be informative by this stage in pregnancy, although combined analysis of prolactin and oestrogen would allow a more confident diagnosis. Urinary oestrogens are measurable during the oestrous cycle of the Asian elephant (Ramsay, Lasley & Stabenfeldt, 1981) but their levels during pregnancy have not been reported. The identification of the appropriate oestrogen metabolite in urine would be of much greater practical value for pregnancy diagnosis of elephants both in captivity and in the wild.

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# Heterologous Radioimmunoassay for Rabbit Prolactin*

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**ABSTRACT.** A highly specific heterologous double-antibody RIA has been developed to measure rabbit PRL by using guinea pig antiserum to human PRL and ovine [$^{125}$I]iodo-PRL. Rabbit pituitary PRL and serum give parallel dose-response curves in the assay and no cross-reaction (<0.1%) occurs with GH, placental lactogens, LH, FSH, or TSH from several different species. The assay is suitable for the measurement of human, ovine, bovine, caprine, and canine PRL in addition to rabbit PRL, but shows no cross-reaction with rat PRL. Reproducibility and precision of the assay are within acceptable limits.

Gel filtration of rabbit pituitary PRL and rabbit serum on Sephadex G-100 revealed coincident peaks of activity measured by RIA and by PRL radioreceptor assay. The molecular weight of rabbit PRL appeared similar to that of ovine PRL.

Serum PRL levels increased after the injection of both TRH and chlorpromazine and were reduced by CB154 (Bromocriptine). Venepuncture stress caused an increase in PRL in nonpregnant or postpartum non-suckled animals, but small or no increases were seen in lactating female rabbits. (*Endocrinology* **102:** 1539, 1978)

IN RECENT years the rabbit has been utilized extensively for the study of the action of PRL on both lactation (1) and ovarian function (2). Changes in the pituitary content of PRL have been recorded under these conditions (3, 4).

However, no data are available on the blood levels of PRL during the different stages of reproduction. At the present time, rabbit PRL has not been purified and is not available for the development of a homologous RIA. To overcome this difficulty we have developed a heterologous RIA based on the inhibition by rabbit PRL preparations of the binding of $^{125}$I-labeled ovine PRL to guinea pig antiserum to human PRL (hPRL).

This paper describes the development of this assay and the measurement of serum levels of PRL after TRH, chlorpromazine, stress, and Bromocriptine (CB154) treatment.

## Materials and Methods

### *Materials*

Bovine serum albumin (fraction V) was obtained from Miles Laboratory, lactoperoxidase was from Calbiochem, La Jolla, CA. Na$^{125}$I (carrier-free) was purchased from New England Nuclear, Boston, MA. $H_2O_2$ (30% vol/vol) was obtained from Fisher Scientific, Fair Lawn, NJ. All other reagents and chemicals were reagent grade.

No rabbit PRL standard is presently available. A laboratory standard rabbit PRL preparation, therefore, was prepared. Twelve female rabbit pituitaries (270 mg) were extracted in 10 ml 0.01 N ammonium bicarbonate, pH 9.0, for 30 min at room temperature. The extract was centrifuged at 15,000 × *g* for 40 min at 4 C. The supernatant was diluted 1/10 with 0.025 M Tris-HCl, pH 7.6, containing 0.1% bovine serum albumin (BSA), aliquoted, and stored at −20 C until required. One aliquot was immediately assayed for PRL content in the PRL radioreceptor assay (RRA) by using the rabbit mammary gland receptor as described in detail previously (5). By using ovine PRL (oPRL, NIH-P-S-10, 26 IU/mg) as reference standard, the pituitary extract (76-1-MC) assayed at 2.6 μg oPRL Eq/mg. This extract was used as the working standard and serum levels are reported in terms of ng 76-1-MC rabbit PRL/ml.

### *Antisera*

To overcome the problems of separation of bound and free hormone by second antibody precipitation when measuring PRL in rabbit serum samples, only PRL antisera raised in guinea pigs were investigated. Twenty-six antisera raised against hPRL in 10 different guinea pigs were as-

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sessed. In some cases, only a single bleeding from one animal was available, but in others up to six serial bleedings were available from one animal. All animals were immunized sc with 100 μg hPRL in Freund's complete adjuvant and received four booster injections at 2-week intervals. All animals were bled by cardiac puncture under ether anesthesia and after the serum was separated, it was stored at −20 C until assessed.

*Radioiodination of PRL*

oPRL (NIH P-S-10) and rat PRL (rPRL; NIAMDD RP-1) were used as hormones for radioiodination. Iodination of oPRL was carried out by the lactoperoxidase method (6) at room temperature, exactly as described previously (5). The labeled hormone (ovine [$^{125}$I]iodo-PRL) was separated from aggregate hormone and $^{125}$I by gel filtration on Sephadex G-100 (2 × 50 cm) with 0.025 M Tris-HCl buffer, pH 7.4, containing 0.1% BSA (wt/vol) as solvent. Radioiodination of rPRL was carried out by the chloramine-T method (7) and the product was purified as described above for ovine [$^{125}$I]iodo-PRL. Both rat and ovine [$^{125}$I]iodo-PRLs were stored at −20 C until used for assay. The specific activity, expressed as microcuries per μg hormone, was assessed by trichloracetic acid precipitation and ranged between 84–165 (n = 16) and 60–125 (n = 10) μCi/μg for ovine [$^{125}$I]iodo-PRL and rat [$^{125}$I]iodo-PRL, respectively.

*Assay procedure*

All dilutions were made in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 2.0% BSA, and 0.1% sodium azide (RIA, diluent, RIAD). After initial assessment by antibody dilution curves, one antiserum (33-9) which bound more than 60% of both rat [$^{125}$I]iodo-PRL and ovine [$^{125}$I]iodo-PRL at a dilution of 1/10,000 was selected for full evaluation. Assays for rabbit PRL were carried out by a double antibody procedure similar to those previously described by oPRL (8) and oFSH (9). The reaction mixture consisted of antiserum suitably diluted to bind 30–50% of counts (final dilution of 1/20,000), RIAD, and standards or samples to be assayed. After incubation of the mixture at 4 C for 24 h, iodinated oPRL or rPRL was added and the incubation was continued at 4 C for 48 h. Rabbit or sheep anti-guinea pig γ-globulin was then added and incubation continued at 4 C for another 16 h before bound and free hormone were separated by centrifugation. The antibody-bound iodinated PRL in the precipitate was measured in an automatic γ-counter (LKB).

*Specificity of the heterologous PRL assays*

The hormone preparations used to assess the specificity of the PRL assays are listed in Table 1. Each hormone preparation was added in a volume of 100 μl, starting at a concentration of at least 1 μg/ml, and incubated as described above. The percentage of cross-reactivity was calculated as the ratio of the amount of hPRL to the amount of hormone required to produce a 50% ($B/B_0$) inhibition of the binding of tracer to the antiserum, times 100.

The parallelism between the dose-response curves for rabbit pituitary PRL standard diluted in RIAD and serum from a rabbit treated with CB154 (Bromocriptine; Sandoz) which contained <1 ng PRL/ml by RRA or human serum containing <1 ng/ml PRL by homologous RIA was assessed. At the same time, dose-response curves for sera from normal male and nonpregnant, pregnant, and lactating female rabbits were diluted and assayed against the rabbit pituitary standard in RIAD.

*Precision and accuracy of the heterologous PRL assay*

The accuracy of the assay was determined by the measurement of known amounts of rabbit PRL standard added to rabbit serum containing low levels of PRL. The precision of the assay expressed as the intra- and interassay coefficients of variation (%) was assessed by the repeated assay of four pools of rabbit sera containing PRL levels from 10–165 ng/ml, both within and between assays.

*Identification of the rabbit PRL measured*

To assess the form of the immunologically active PRL measured by the RIA, the rabbit pituitary PRL standard was assessed by both isoelectric focusing and gel filtration on Sephadex G-100. Analytical thin layer polyacrylamide isoelectric focusing was performed by using an LKB Multiphor as recommended in the manufacturer's instruction manual (10), with carrier ampholytes with a pH range from 3.5–10.0. Isoelectric focussing was continued for 3 h, after which the gels were cut serially at 1 mm and each segment was eluted overnight in 1 ml 0.025 M Tris-HCl, pH 7.6, containing 0.1% BSA. All fractions were assayed immediately for PRL by RIA and RRA (5) and for GH by RRA (11). Both the rabbit pituitary PRL standard (76-1-MC) and the samples of serum from rabbits after

chlorpromazine or TRH treatment were fractionated at 4 C by gel filtration on Sephadex G-100 (2 × 5 cm), eluted with 0.25 M Tris-HCl, pH 7.6, containing 0.1% BSA. One milliliter fractions were collected and analyzed for PRL by RIA and RRA (5) and for GH by RRA (11). Protein concentrations were estimated by measuring absorbance at 280 nm.

*Comparison between heterologous and homologous assays for PRL*

Two heterologous assays for rabbit PRL, using either ovine [$^{125}$I]iodo-PRL or rat [$^{125}$I]iodo-PRL, and different guinea pig antisera to hPRL were developed and measured both rabbit and human PRLs. To provide additional validation that these assays measured PRL in serum in each of these two species, human and rabbit sera were selected at random and assayed in both heterologous RIAs for PRL. Rabbit PRL standard (76-1-MC) was used when measuring rabbit sera, and hPRL standard was used for human sera. The hPRL concentrations in the human sera were also measured against the same hPRL standard by using a homologous hPRL RIA as described previously (12).

*Measurement of serum PRL levels in the rabbit*

To determine whether the heterologous rabbit PRL RIA would measure changes in PRL levels *in vivo* in response to agents known to alter blood PRL levels in other species, blood samples were collected in each of four female rabbits before and after the injection of TRH (Hoechst; 25 μg iv), Chlorpromazine (May and Baker, England; 12.5 mg im), and CB154 (Bromocriptine, Sandoz; 0.6 mg iv). To overcome any effects of stress, samples (2 ml blood) were collected by an indwelling silastic catheter (0.6 mm od) implanted in the ear artery 1 h before injection of the test substances. Blood was collected from −30 to 60 min, allowed to clot for 3 h at 4 C, and the serum was removed after centrifugation and stored at −20 C until assayed.

Stress, in particular that associated with venepuncture, is known to cause the release of PRL in several species. Thus, in consideration of future experiments requiring regular daily venepuncture sampling from the marginal ear vein of the rabbit, blood samples were also obtained before and 5 and 10 min after a venepuncture stress (venepuncture repeated four times within 30 sec), in each of three nonpregnant, nonlactating female rabbits, three postpartum nonlactating female rabbits and three postpartum lactating female rabbits.

## Results

*Heterologous assay for rabbit PRL*

Of the 26 antisera tested, eight bound more than 45% ovine [$^{125}$I]iodo-PRL or rat [$^{125}$I]-iodo-PRL. One antiserum (33-9), using ovine [$^{125}$I]iodo-PRL, was available in adequate quantity and showed acceptable standard curves with sufficient sensitivity to measure basal levels of PRL in all of the sera examined. Thus, only antiserum 33-9 was tested fully for specificity by using ovine [$^{125}$I]iodo-PRL. For comparative purposes only, antiserum AGP4-1, using rat [$^{125}$I]iodo-PRL was also used, as the RIA established with this system had characteristics very similar to those of antiserum 33-9 with ovine [$^{125}$I]iodo-PRL.

*Specificity of the heterologous assay for rabbit PRL*

The binding of ovine [$^{125}$I]iodo-PRL to antiserum 33-9 was displaced in a parallel manner by PRL preparations of various species except the rat (Fig. 1), but not by other pituitary hormones or placental lactogen (Table 1). With the cross-reaction of hPRL defined as 100%, rabbit PRL showed only 17% cross-reaction. Under routine assay conditions the assay was sensitive to 2 ng rabbit PRL/ml with a sensitivity to hPRL of 0.3 ng/ml. Sensitivity was determined as the amount of hormone which could be detected at 90% B/Bo.

Serial dilutions of sera from normal male and nonpregnant, pregnant, or lactating fe-

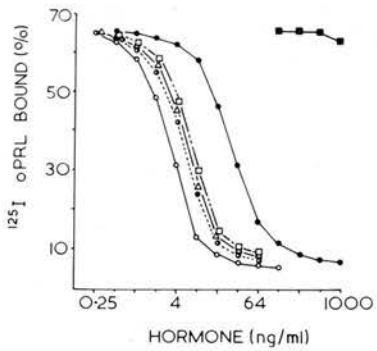



FIG. 1. Inhibition curves for PRLs of several species in the heterologous PRL RIA. hPRL (○——○), oPRL and bPRL (◑——◑), pPRL (△——△), dog PRL (□——□), rabbit PRL (●——●), and rPRL (■——■).

TABLE 1. Cross-reaction of guinea pig antiserum to hPRL (33-9) with ovine [$^{125}$I]iodo-PRL tracer with hormones of several different species

| Hormone | Species | Source | Cross-reaction (%)[a] |
|---|---|---|---|
| PRL | Human | Friesen 75-7-28 | 100 |
| | Sheep | NIH P-S10 | 86 |
| | Steer | NIH P-B3 | 83 |
| | Pig | NIH SP162C | 80 |
| | Rat | NIAMDD P-I-1 | <0.1 |
| | Dog | Schering | 70 |
| | Rabbit | McNeilly 76-1-MC | 17 |
| GH | Human | NIH HS 1146 | |
| | Monkey | NIH M 945 A | |
| | Sheep | NIH RIA 074313 | |
| | Steer | NIH RIA 1003 A | |
| | Pig | NIH P 526B | |
| | Rat | NIAMDD GH-I-2 | |
| | Mouse | NIAMDD AFP 689-B | |
| | Dog | Wilhelmi D100A | |
| | Rabbit | McNeilly 76-2 | |
| Placental lactogen | Human | Friesen 201 | |
| | Sheep | Chan-Friesen 20 | |
| | Goat | Currie-Friesen 2009 | <0.1 |
| | Steer | Roy-Friesen 75-2 | |
| | Rat | Deegan-Friesen 75-1001 | |
| | Monkey | Friesen DE4 | |
| LH | Human | NIH LER-960 | |
| | Sheep | NIH LH-S18 | |
| | Rat | NIAMDD LH-I-1 | |
| | Rabbit | McNeilly-Cheng 76-4 | |
| FSH | Human | NIH LER-1575 C | |
| | Steer | Chan-bF-1 | |
| | Rat | NIAMDD FSH-1-2 | |
| | Rabbit | McNeilly-Cheng 76-5 | |

[a] Cross-reaction was calculated as the ratio of the amount of hPRL to the amount of hormone required to produce a 50% inhibition in the binding of the tracer to the antiserum, × 100.

male rabbits or human sera were parallel to the inhibition curves obtained with rabbit PRL standards (Fig. 2). Addition of sera from hypophysectomized human patients or rabbits treated with Bromocriptine to the rabbit PRL standard did not affect parallelism and no inhibition of binding occurred with the sera from Bromocriptine-treated rabbits in which RRA PRL levels were <1 ng PRL/ml (Fig. 2).

*Accuracy and precision of the assay*

The accuracy, assessed by the recovery of rabbit PRL standard (0.8–200 ng/ml) added to serum from a normal male rabbit previously assayed at 2.6 ng/ml was 105 ± 2% (SEM) (n = 16). The coefficients of variation determined from the results of assays on replicate samples of each of four pools of rabbit sera containing PRL levels of 10, 35, 84, and 165 ng/ml were 6.4%, 6.7%, 7.1%, and 7.6%, respectively (n = 20 for each pool), within assays, and 10.4%, 11.0%, 11.1%, and 12.6%, respectively (n = 19 for each pool), between assays.

*Characterization of rabbit PRL*

Two peaks of immunoassayable PRL with pI of 6.0 and 7.8 were found after isoelectric focusing (Fig. 3). Both were active in the PRL RRA but absolute values for PRL for comparison with the RIA estimates could not be made due to the high nonspecific binding in the RRA associated with these samples.

A single peak of radioimmuno- and radioreceptor-assayable PRL was found after gel filtration of the rabbit pituitary PRL. This peak eluted at the same position as ovine [$^{125}$I]iodo-PRL used as a marker and suggests a molecular weight of rabbit PRL similar to oPRL (*viz* 23,000; Fig. 4a). Gel filtration of rabbit sera taken after chlorpromazine or TRH treatment revealed RIA PRL which eluted with the same elution volume as the rabbit pituitary PRL (Fig. 4b).

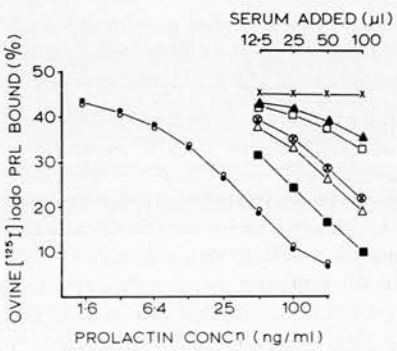



FIG. 2. Inhibition curves in the heterologous PRL RIA for rabbit PRL for serum from a rabbit treated with CB154 (X), a nonpregnant (△), pregnant (▲), and lactating (■) female rabbit, a normal male rabbit (□) and a normal human male (⊗). Comparison is made with the rabbit PRL standard (76-1-MC) in either diluent (○) or in serum from the CB154-treated rabbit (●).

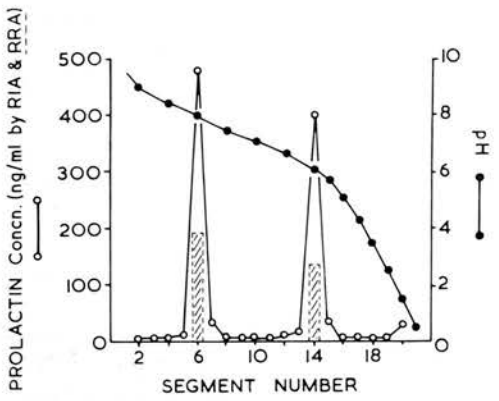



FIG. 3. Isoelectric focusing patterns of rabbit pituitary PRL extract. The anode was placed at segment 22 while the cathode was on segment 1. Eluates of gel segments (1 mm) were assayed and the distribution of rabbit PRL was determined by RIA (O——O) and RRA (bars).

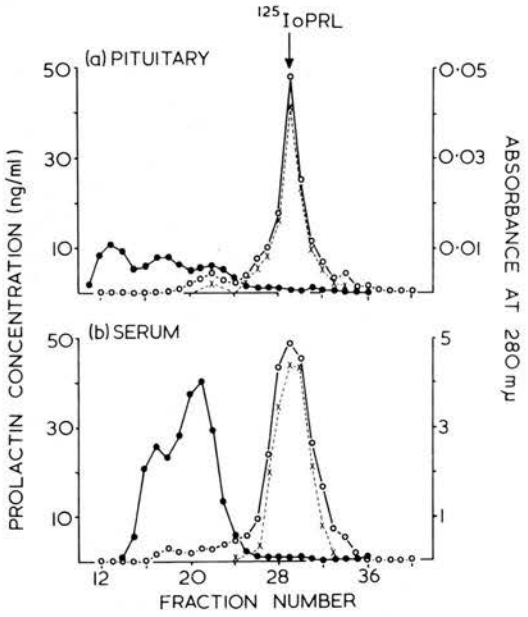



FIG. 4. Gel filtration on a Sephadex G-100 column (2 × 50 cm) of a rabbit pituitary PRL preparation (a) and a rabbit serum sample taken 60 min after chlorpromazine injection (b). One-milliliter fractions were collected and assayed by RIA (O) and RRA (X) for PRL. The arrow represents the point at which ovine [$^{125}$I]iodo-PRL is eluted on this column.

### *Comparison between heterologous and homologous PRL RIAs*

Highly significant linear correlations ($P < 0.001$) were found between PRL levels estimated by all the RIA systems. Serum PRL levels in the rabbit estimated by both heterologous RIAs by using ovine [$^{125}$I]iodo-PRL with antiserum 33-9 and rat [$^{125}$I]iodo-PRL with antiserum A4-1 showed a highly significant linear correlation ($r = 0.873$; $P < 0.001$) with an intercept of 0.80 ng/ml related to the ovine [$^{125}$I]iodo-PRL system. PRL levels in human sera ($n = 30$) estimated by the homologous hPRL RIA and the heterologous RIA by using ovine [$^{125}$I]iodo-PRL and antiserum 33-9 also showed a highly significant linear correlation ($r = 0.975$; $P < 0.001$) with an intercept of −2.0 ng/ml related to the heterologous RIA.

### *Measurement of serum PRL levels in the rabbit*

*Stress.* An increase in PRL levels in two of three nonpregnant, nonlactating female rabbits and in two of three postpartum nonlactating female rabbits was seen within 5 min of a venepuncture stress (Fig. 5, A and B). One animal in each of these two groups showed no response. Only one postpartum lactating female rabbit showed an increase in PRL levels after venepuncture stress and this increment was modest (8 ng/ml). No change was seen in the other two lactating females (Fig. 5C).

*TRH.* All five of the rabbits showed an increase in PRL levels 10 min after the iv injection of 25 μg TRH (Fig. 6). Peak values (2.5–15.0 times baseline; increment of 28–290 ng/ml) were seen between 10 and 30 min after injection and returned to near preinjection levels by 60 min. One rabbit had extremely high basal levels of PRL (280–300 ng/ml), but showed a similar response after TRH (1.3-fold increase; increment of 90 ng/ml).

*Chlorpromazine.* A 9-fold increase in PRL levels occurred after the im injection of 12.5 mg chlorpromazine in three out of four rabbits. The increase in the fourth rabbit was less dramatic (1.5-fold; Fig. 7) but occurred in the face of greatly elevated basal levels of PRL (250–290 ng/ml). In all of the rabbits, an increase in PRL levels occurred within 15 min of injection and peak levels were achieved between 15 and 30 min. PRL levels remained elevated during the whole period of sampling (140 min; Fig. 7) and all rabbits became

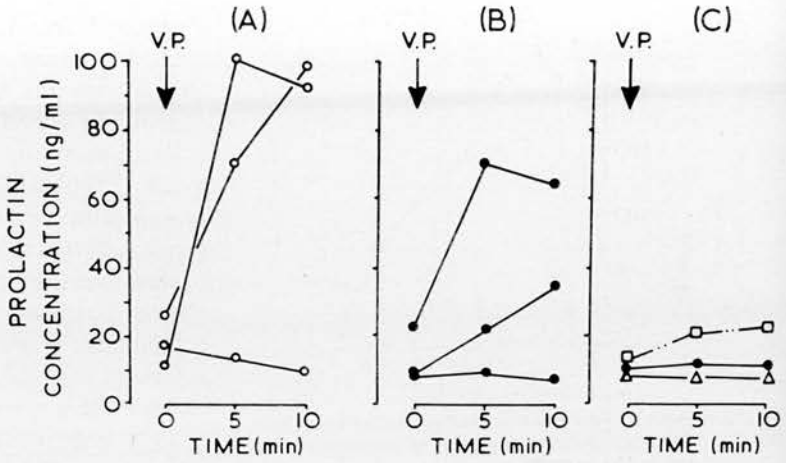



FIG. 5. Effect of venepuncture (V.P.) stress on serum levels of PRL in nonpregnant, nonlactating (A); postpartum, nonlactating (B); and postpartum lactating (C) female rabbits.

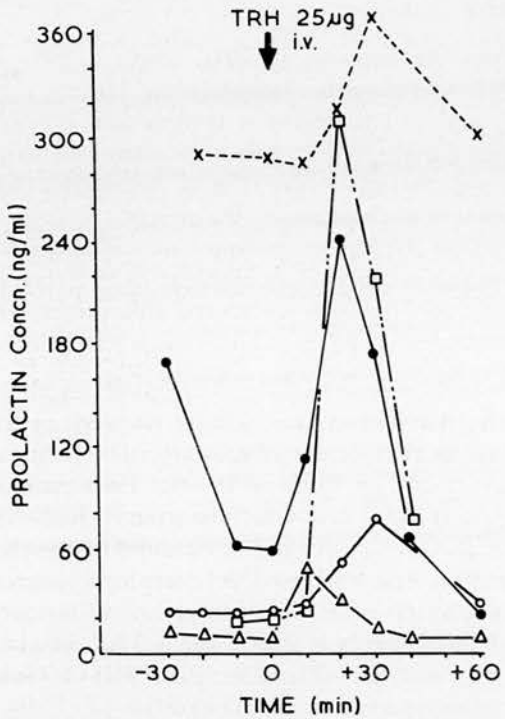



FIG. 6. Effect of iv injection of TRH (25 µg) on serum PRL levels in five female rabbits.

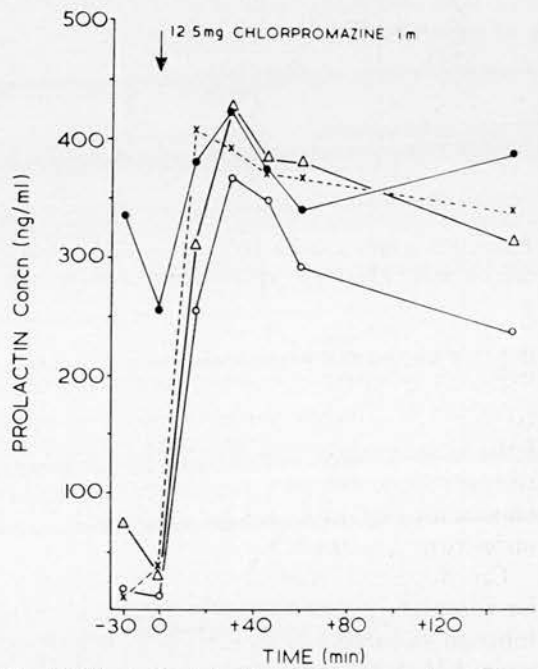



FIG. 7. Effect of im injection of chlorpromazine (12.5 mg) on serum PRL levels in four female rabbits.

drowsy and remained so for 4–6 h.

*Bromocriptine (CB154).* PRL levels decreased within 10 min of the iv injection of 0.6 mg CB154 in all four of the rabbits (Fig. 8). Blood levels of PRL declined over the 60 min after injection and showed a 70–78% decrease in the three rabbits with normal basal blood levels (8–12 ng/ml). The fourth rabbit, which in the previous test with TRH and chlorpromazine also had elevated blood levels of PRL, showed a 91% decrease in PRL levels from starting levels of 220 ng/ml (Fig. 8).

## Discussion

This paper describes in detail the development of a highly specific heterologous RIA suitable for the measurement of PRL in the rabbit. Two systems have been described us-

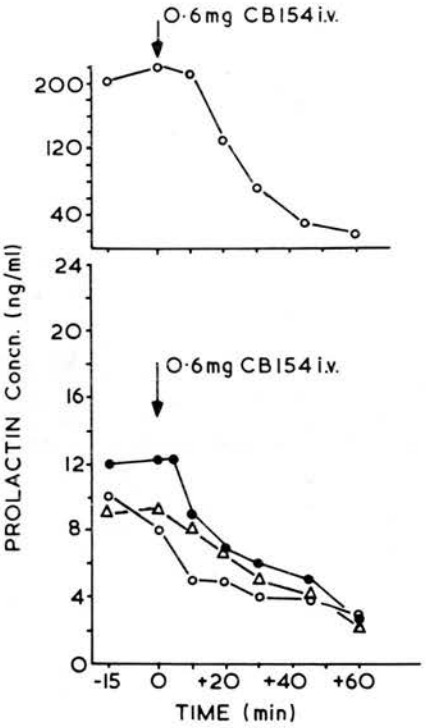



FIG. 8. Effect of iv injection of Bromocriptine (CB154, 0.6 mg) on serum PRL levels in four female rabbits.

ing guinea pig antiserum to hPRL and either $^{125}$I-labeled oPRL or rPRL. Although both RIAs were suitable for the measurement of PRL in several species, the system using antiserum 33-9 and ovine [$^{125}$I]iodo-PRL was chosen for full validation because more of this antiserum was available.

Cross-reaction studies showed this assay to be completely specific for PRLs, with no inhibition occurring with GH, placental lactogens, LH, FSH, or TSH of several different species. The degree of cross-reactivity of the PRLs varied with the species of origin but was maximal with hPRL (100%). Ovine, bovine, and porcine PRL showed almost equal activity to each other (80–86% cross-reaction), although, surprisingly, rPRL showed no cross-reaction at all (<0.1%). The great sensitivity of the assay for hPRL (0.3 ng/ml) allowed the measurement of rabbit PRL despite showing only a 17% cross-reaction. Thus, the sensitivity of the assay for rabbit PRL (2 ng/ml) was more than adequate to measure serum levels of PRL in all situations studied, because minimum normal serum levels ranged upwards from 4 ng/ml.

No serum interference in the assay was seen, as confirmed by the absence of effect from the addition of either human serum from a hypophysectomized patient (containing <1 ng/ml by homologous hPRL RIA) or serum containing <1 ng PRL/ml by RRA, obtained from a rabbit after treatment with Bromocriptine. All rabbit sera tested showed displacement curves parallel with the rabbit PRL standard.

Further evidence that the heterologous assay measured rabbit PRL was gained by Sephadex gel filtration of the rabbit PRL standard and rabbit serum. In both cases, the immunological PRL activity eluted coincidentally with a $V_e/V_o$ identical to that of the ovine [$^{125}$I]iodo-PRL used as a marker. The biological PRL activity of the serum PRL assessed by RRA was identical with the RIA PRL activity. After isoelectric focusing of the rabbit PRL standard, bio-(RRA) and immunological (RIA) activity resolved into two components, revealing a heterogeneity of rabbit PRL similar to that seen with PRLs of other species (8).

In view of the heterologous nature of the PRL RIA, the method was equally applicable to the measurement of PRL in other species. Evidence that the assay gave an accurate and precise measurement of PRL in serum was obtained by the demonstration of highly significant correlations between estimates of human serum PRL by the heterologous and homologous hPRL assays and of rabbit serum PRL by the two heterologous PRL assays.

Treatment of rabbits with TRH caused a rapid increase in serum PRL levels similar to that demonstrated in other species (13–15), with peak values occurring 10–30 min after injection and returning to preinjection levels by 60 min. A more sustained increase in PRL levels occurred after the injection of chlorpromazine. Peak values occurred at 15–30 min and were maintained for at least 140 min. This pattern of PRL release is similar to that seen in other species (see Ref. 16).

Bromocriptine (CB154) is a potent inhibitor of PRL secretion in all mammalian species so far investigated (17–19) and the rabbit is no

exception. Treatment with 0.6 mg CB154 iv caused an immediate reduction in serum PRL levels which remained suppressed for at least 60 min post injection. In view of the dependence of lactation on PRL secretion in the rabbit and the present demonstration that serum PRL levels are suppressed by CB154 treatment, it seems reasonable to conclude that, as inferred by Taylor and Peaker (20), the suppression of lactation in the rabbit by CB154 treatment, as in other species (17–19), is caused by a reduction in the circulating PRL level.

PRL release in response to specific or nonspecific stress has been reported in several species (21–23), one such stress being venepuncture. In consideration of several experiments planned in rabbits requiring repeated daily collection of blood samples from the marginal ear vein, it was necessary to determine the effect of venepuncture on serum PRL levels in the rabbit in order to develop criteria for the rejection of samples as a result of sampling stress. The PRL response to venepuncture was variable. Any increase in PRL levels occurred within 5 min of venepuncture. Thus, only the sample obtained at first venepuncture was used in subsequent studies. It was of interest that the same venepuncture stress applied to lactating female rabbits was almost without effect on the serum PRL levels.

The present paper reports the development, validation, and application of a heterologous RIA for rabbit PRL. It is specific and sufficiently sensitive for the measurement of PRL in a variety of physiological situations so far investigated in the rabbit (McNeilly and Friesen, unpublished observation). It is hoped that the measurement of serum PRL levels in the rabbit will shed new light on the already carefully investigated role of PRL action on the ovary (2) and mammary gland (1) in this species.

## Acknowledgments

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# Plasma prolactin concentrations during the ovarian cycle and lactation and their relationship to return of fertility *post partum* in the common marmoset (*Callithrix jacchus*)

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**Summary.** A heterologous double-antibody radioimmunoassay was used to measure plasma prolactin concentrations in 27 marmosets. The assay was valid for the marmoset because plasma levels of prolactin were increased in response to TRH and metoclopramide and suppressed in response to bromocriptine treatment.

During the cycle there were no consistent changes in plasma prolactin concentrations. During lactation mothers suckling single or twin infants had higher prolactin levels than did non-suckling females and levels were highest with twins. No statistically significant delay in the resumption of ovulation *post partum* was observed for the suckling and non-suckling females; conception occurred in all but one marmoset by 70 days *post partum*.

These results show that neither the suckling stimulus nor high levels of prolactin *post partum* delay the return of ovulation and fertility in the common marmoset, a result in contrast to that for all other primate species so far investigated.

## Introduction

Lactation in women is associated with amenorrhoea and infertility (see McNeilly, 1979, for references). Both the suckling stimulus and high levels of prolactin associated with lactation have been implicated as causal agents, but which of these two components is the more important has yet to be resolved. Lactation is also associated with a period of infertility in other primates, e.g. the rhesus monkey (Weiss *et al.*, 1973), Savannah baboon (Attmann, Attmann & Hausfater, 1978), chimpanzee (Tutin, 1980) and gorilla (Harcourt, Fossey, Stewart & Watts, 1980). In contrast, lactation in the common marmoset monkey is not associated with any apparent post-partum infertility, in spite of continued sucking by the infant and its complete dependence on maternal milk for up to 100 days (Lunn & Hearn, 1978; Chambers & Hearn, 1979; Lunn & McNeilly, 1981). We have therefore validated a heterologous radioimmunoassay for prolactin in the marmoset and studied the changes in plasma prolactin concentrations during the ovarian cycle (approximately 17 days) and lactation.

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## Materials and Methods

### *Animals*

The 27 marmoset monkeys used (285–360 g body wt) were sexually mature. Full details of their management have been published previously (Hearn, Lunn, Burden & Pilcher, 1975). Animals were exposed to natural light with additional illumination between 06:00 and 19:00 h.

### *Experimental procedures*

To assess the changes in plasma levels of prolactin during the ovarian cycle, 10 cyclic female marmosets housed individually with vasectomized males were bled (0·5 ml) every 3rd day for 30 days. Normal ovarian cyclicity was determined by measurement of the levels of progesterone in the same samples (Hearn & Lunn, 1975).

During pregnancy and lactation, 13 marmosets were bled (0·5 ml) between 1 and 14 days before the expected date of delivery and then every 3 or 4 days up to 90 days *post partum*. After parturition mothers and their surviving infants remained in the mother's peer group (Abbott & Hearn, 1978).

To obtain blood samples the animals were held in a restraining device (Hearn, 1977), and blood samples were taken between 09:00 and 12:00 h without anaesthesia from the femoral vein with a heparinized syringe and placed on ice. The blood was centrifuged in the syringe barrel at 500 ***g*** for 20 min at 4°C and the plasma samples were stored at −20°C until assayed for prolactin.

### *Measurement of prolactin*

Marmoset prolactin was measured by a heterologous double-antibody radioimmunoassay described in detail previously (McNeilly & Friesen, 1978). The assay uses a guinea-pig anti-human prolactin antiserum (No. 33–9) with $^{125}$I-labelled ovine prolactin as tracer and ovine prolactin (NIH-P-S6, 26 i.u./mg) as standard.

*Specificity*. Previous studies with a wide range of hormones from several species had shown this assay to be specific for prolactin (McNeilly & Friesen, 1978). Since no purified marmoset prolactin standard was available, the parallelism between the ovine standard used, dilutions of marmoset plasma and a crude extract of marmoset pituitary was assessed. The pituitary extract was prepared by homogenization of 4 pituitaries (25 mg) in 2 ml 0·01 M-ammonium bicarbonate, pH 9·0. After shaking for 30 min at room temperature the extract was centrifuged at 600 ***g*** for 1 h at 4°C and the supernatant stored at −20°C until assayed.

*Accuracy and precision.* Accuracy of the assay was assessed by measurement of known amounts of ovine prolactin standard (0·3–200 ng/ml) or diluted marmoset pituitary extract (1 : 5 to 1 : 1000) added to marmoset plasma containing low levels of prolactin (samples taken after treatment with bromocriptine (CB154: Sandoz, Basle, Switzerland)). The precision of the assay, expressed as the intra- and inter-assay coefficient of variation (%), was assessed by repeated assays of three pools of plasma containing 4, 25 and 45 ng prolactin/ml (approximately 80, 40 and 25% B/Bo).

*Biological validation.* To provide biological validation of the assay, marmosets were treated with agents known to raise (thyrotrophin releasing hormone (TRH: Hoechst AG, Frankfurt, Germany) and metoclopramide (Maxalon: Beechams, Betchworth, Surrey)) or lower (bromocriptine) prolactin levels in other species. To evaluate cross-reaction with LH and FSH marmosets were also treated with LH-RH (Hoechst AG, Frankfurt, Germany).

Five cyclic female marmosets were injected with saline (9 g NaCl/l, 0·5 ml i.v.), bromocriptine (2·5 mg, i.m.), metoclopramide (2·0 mg, i.m.), TRH (25 μg, i.v.) or LH-RH (2 μg, i.v.). Blood samples were withdrawn as described below 30 min before and at 0, 30, 60 and 90 min after injection; plasma was separated and stored at −20°C until assayed for prolactin.

*Measurement of progesterone*

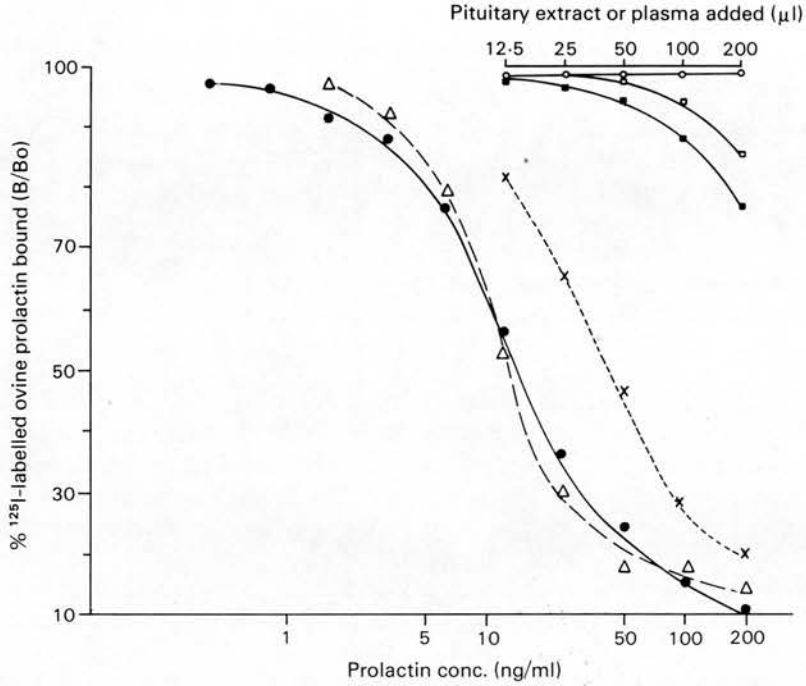
Plasma progesterone was measured by a specific radioimmunoassay exactly as previously described in detail and validated for the marmoset (Chambers & Hearn, 1979). The sensitivity of the assay, defined as the mass of hormone required to suppress the binding of the labelled hormone to 90% of the binding achieved with no hormone added (B/Bo), was 1·7 ng/ml. The mean (± s.d.) recovery of labelled progesterone from plasma samples was 69 ± 3% ($n = 95$) and was used to correct for procedural losses during the extraction procedure. Intra- and inter-assay coefficients of variation for replicate determinations of a pool of marmoset plasma were 5% ($n = 10$) and 9% ($n = 6$) respectively.

Statistical evaluation of results was by analysis of variance and Student's $t$ test.

## Results

*Validation of the prolactin assay*

The comparison of inhibition curves for ovine prolactin standard and marmoset plasma and pituitary extracts are shown in Text-fig. 1. The slopes of the dose–response curves were not significantly different. Therefore all samples were assayed against, and are reported in terms of, ng ovine prolactin/ml plasma.



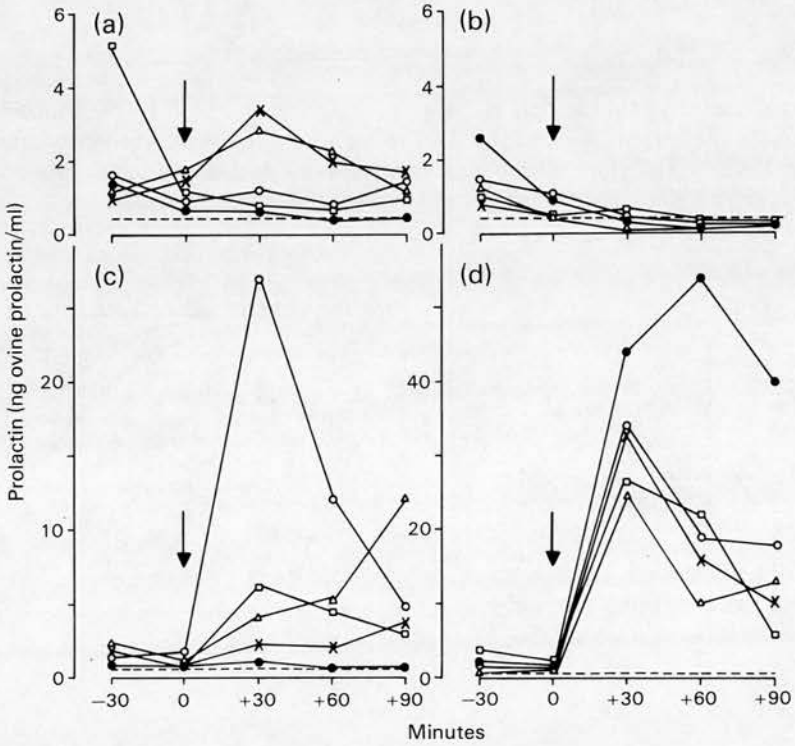

**Text-fig. 1.** Standard curve of ovine prolactin (●, NIH-P-S6) and dilution curves of (a) a marmoset pituitary extract (△); (b) plasma taken from a female marmoset after metoclopramide stimulation (×); (c) normal female marmoset plasma (□); (d) male marmoset plasma (■) and (e) female marmoset plasma taken after administration of bromocriptine (○).

*Accuracy and precision.* The accuracy assessed by recovery of ovine prolactin standard and marmoset pituitary extract added to marmoset plasma previously assayed at 1·5 ng prolactin/ml was 97 ± 4 (s.e.m.) % and 104 ± 8% respectively ($n = 4$). The precision of the assay, as

coefficients of variation, was 7% ($n = 16$) and 11% ($n = 10$) for intra- and inter-assay variation respectively.

*Biological validation.* As shown in Text-fig. 2, saline had no significant effect on plasma prolactin concentrations which ranged between 0·4 and 5 ng/ml. The injection of bromocriptine caused suppression of plasma values to below the sensitivity of the assay (i.e. <0·2 ng/ml compared with the preinjection value of 0·8 ± 0·1 ng/ml).

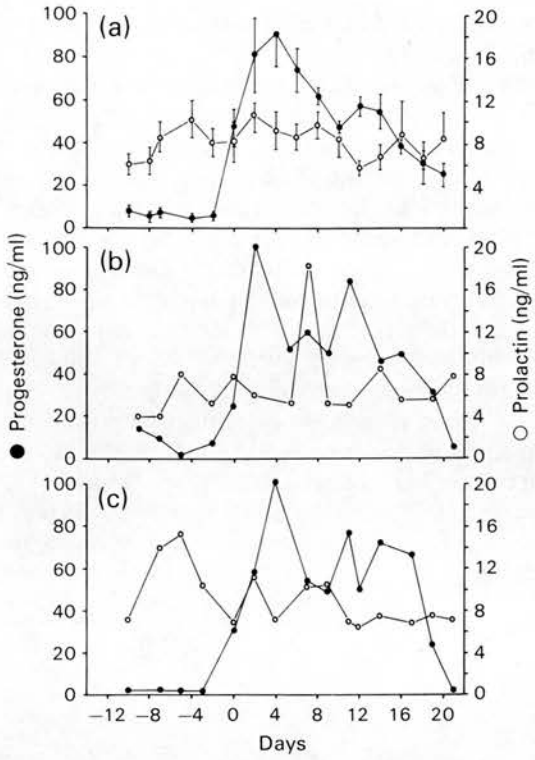
Both TRH and metoclopramide caused a significant ($P < 0.001$) increase in prolactin concentrations by 30 min after injection, the effect of metoclopramide (32·4 ± 3·1 ng/ml) being significantly greater ($P < 0.001$) than that of TRH (9·7 ± 4·3 ng/ml). Injection of LH-RH had no significant effect on plasma levels of prolactin which ranged between 0·8 and 4·7 ng/ml.




**Text-fig. 2.** Plasma levels of prolactin in female marmosets after the injection (↓) of (a) saline, (b) bromocriptine (2·5 mg i.m.), (c) TRH (25 μg i.v.) or (d) metoclopramide (2·0 mg i.m.). Five animals were used (●, ○, □, △, ×) and each was given all tests. The broken line indicates the sensitivity of the assay.

*Prolactin levels during the cycle*

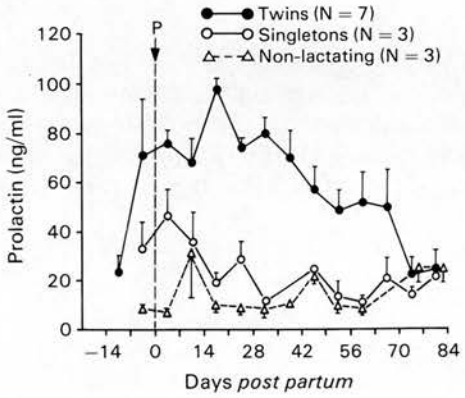
The changes in plasma levels of prolactin were synchronized around the first day that plasma progesterone concentrations were >20 ng/ml (=Day 0), indicative of the start of the luteal phase (Hearn & Lunn, 1975). No significant differences were apparent in mean levels of prolactin (Text-fig. 3a) although individual animals showed increased values in the follicular (Text-fig. 3b) and/or luteal phases of the cycle (Text-fig. 3c).




**Text-fig. 3.** Changes in the plasma levels (mean ± s.e.m.) of prolactin (○) and progesterone (●) during the cycle in 10 female marmoset monkeys (a) and in two individual females (b and c). The data have been synchronized around the first day progesterone values were > 20 ng/ml (Day 0).

*Prolactin levels during lactation*

Concentrations of prolactin during the last 12 days of pregnancy and during lactation are shown in Text-fig. 4. Of the 13 marmosets being bled, 5 gave birth to triplets, 7 to twins and 1 had a single infant. By Day 5 *post partum* all the infants of 3 mothers (one set of triplets, two sets of twins) had died, and these females were considered to be non-lactating. Two infants died




**Text-fig. 4.** Changes in mean ± s.e.m. plasma prolactin concentration during lactation in marmosets suckling one (○; N = 3) or two (●; N = 7) young, or not lactating (△; N = 3). P = parturition.

in each of two pairs of triplets born, leaving a group of 3 mothers with single infants. Seven mothers raised twins, two pairs of which resulted from a death in two sets of triplets. There was no relationship between the pre-partum plasma levels of prolactin and the number of young born.

In non-lactating females plasma prolactin concentrations remained low (< 22 ng/ml), within the normal range for cyclic marmosets for the duration of the study (up to Days 70–77 *post partum*). However, between Days 8 and 14 an increase in mean plasma values occurred because of the transiently elevated prolactin levels observed in 1 of the 3 females.

Prolactin concentrations were higher in mothers suckling single infants compared to non-lactating mothers but this only achieved significance ($P < 0{\cdot}05$) immediately after parturition. In contrast plasma levels of prolactin immediately *post partum*, and throughout lactation until around Day 65 *post partum*, were significantly higher ($P < 0{\cdot}05$) in mothers suckling twins than in both other groups.

Ovulation followed by a rise in plasma progesterone levels occurred in all 13 animals *post partum* (Table 1). Plasma levels of progesterone and the duration of the luteal phase were not significantly different from those in the normal non-pregnant female.

**Table 1.** Days *post partum* to first ovulation and conception, the number of cycles to and the plasma levels of prolactin around the time of conception (Days −7 to +7) in marmosets suckling no, one or two young

| | No. of young suckled | | |
|---|---|---|---|
| | 0 | 1 | 2 |
| Days to: | | | |
| First ovulation | 18 ± 4 (3) | 15 ± 1 (3) | 29 ± 9 (7) |
| Conception | 28 + 8 (3) | 57 + 10 (3) | 56 + 11 (6) |
| No. of females conceiving in successive cycles *post partum* | | | |
| 1st | 2 | 0 | 3 |
| 2nd | 1 | 3 | 3 |
| Other | — | — | 1* |
| Plasma prolactin at conception (ng/ml) | 8 ± 1 (5) | 23 ± 8 (4) | 40 ± 18 (6) |

Values are mean ± s.e.m. for the no. of observations in parentheses.
* Conception did not occur during the observation period.

Twelve animals became pregnant during the observation period, 5 conceiving during the first and the remainder during the second cycle *post partum*. The time to first ovulation assessed by progesterone levels > 20 ng/ml was not significantly different between the three groups but time of conception was significantly delayed ($P < 0{\cdot}05$) in both groups of lactating mothers compared to the non-lactating females (Table 1). Prolactin concentrations around conception were also significantly higher ($P < 0{\cdot}05$) in the lactating females than in those not lactating (Table 1).

## Discussion

The present results for prolactin concentrations in marmosets clearly show that high levels of prolactin and a maintained suckling stimulus do not necessarily inhibit the resumption of ovarian activity and return of fertility *post partum*.

The application of the heterologous radioimmunoassay to the measurement of marmoset

prolactin appears valid since dilutions of plasma and crude pituitary extract are parallel to the ovine prolactin standard and the assay is precise. The high percentage recovery of both ovine prolactin standard and marmoset pituitary prolactin added to marmoset plasma, together with the absence of plasma interference when prolactin levels are suppressed below the detection limit of the assay, clearly indicate that marmoset plasma does not show any non-specific interference in the assay. Although purified marmoset pituitary hormone preparations are not available, it is reasonable to presume that the assay is measuring prolactin since: (1) injection of bromocriptine, a specific inhibitor of prolactin secretion (Flückiger, 1972), suppressed plasma levels of prolactin; (2) TRH caused a significant rise in prolactin similar in time course to that seen in other species (Bowers, Friesen, Hwang, Guyda & Folkers, 1971; Davis & Borger, 1972; Convey, Tucker, Smith & Zolman, 1972); (3) metoclopramide, a dopamine receptor antagonist, caused a highly significant increase in prolactin levels similar to the specific rise in serum levels of prolactin seen in man (McNeilly, Thorner, Volans & Besser, 1974); and (4) injection of LH-RH, which results in a large increase in circulating levels of LH in the marmoset (Hodges, 1978), did not affect the plasma concentrations of prolactin. It is felt that the radioimmunoassay has been adequately validated to allow measurement of prolactin in the plasma of the common marmoset.

No consistent changes in plasma levels of prolactin were seen in the follicular or the luteal phase of the cycle in the marmoset. This observation is similar to those for other primates (rhesus monkey: Butler, Krey, Lu, Peckham & Knobil, 1975; chimpanzee: Reyes, Winter, Faiman & Hobson, 1975; gorilla: Nadler, Graham, Collins & Gould, 1979; man: McNeilly & Chard, 1974). It remains to be determined whether prolactin plays any role in the normal cycle of the common marmoset.

Lactation in the marmoset was associated with elevated plasma concentrations of prolactin similar to those in the rhesus monkey (Butler *et al.*, 1975) and in women (Rolland, Lequin, Schellekens & de Jong, 1975), but unlike the responses of these primates, suckling and its associated hyperprolactinaemia in marmosets were not associated with any delay in the return to ovulation *post partum*. Similarly, the luteal phase associated with first ovulation was of normal duration and progesterone levels were normal. The first post-partum cycle was also capable of leading to a pregnancy unlike the situation in breast-feeding women in which luteal function after first ovulation is usually inadequate (Delvoye, Badawi, Demaegd & Robyn, 1978; McNeilly, 1979, 1980; Duchen & McNeilly, 1980; McNeilly, Howie & Houston, 1981). In this particular study, the time to conception was delayed in lactating marmosets (Table 1). Nevertheless, whether conception occurred in the first or second cycles *post partum* appeared to be a random event since 3 marmosets suckling twins conceived at the first ovulatory cycle. Considering birth intervals in the colony as a whole ($n = 250$), lactation *per se* does not influence the time of resumption of ovulatory cycles or conception *post partum* (Lunn & McNeilly, 1981).

The reason for the differences in the pattern of return of fertility in lactating marmosets compared to other primates is uncertain. Primates with a long period of infant dependancy on the mother and who are immature at birth, e.g. man, chimpanzee and gorilla, have long lactation-induced birth intervals (Short, 1980). In the marmoset gestation is relatively long for such a small mammal, the young are well developed at birth, grow rapidly and are relatively independent of the mother by the time the next young are born, approximately 160 days later (Lunn & McNeilly, 1981). It appears therefore that the marmoset has no need of a lactation-induced delay in the resumption of fertility. Nevertheless, the present results clearly show that high levels of prolactin and a maintained suckling stimulus are associated with lactation in the marmoset, and that the animal is not susceptible to the inhibitory influences of these stimuli on reproductive function, a feature unique in primates studied so far.

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# Nutrition and lactational control of fertility in red deer

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**In many mammals, including man, lactation is associated with a delay in return of fertility. The pattern of suckling activity, particularly suckling frequency, is known to be important in determining the length of this anovulatory phase[1,2]. In a seasonally breeding ungulate such as red deer, lactation coincides with a photoperiodically controlled period of seasonal anoestrus[3]. Nevertheless, lactation may still exert an important effect on the duration of infertility as lactating red deer hinds on poor quality hill pasture resume oestrus at a later date than non-lactating hinds and frequently fail to ovulate in the year following the birth of a calf[4,5]. It has been proposed that this reproductive failure is due to the effect of both lactation and poor planes of nutrition on maternal body condition[6-8]. As a result of our recent studies on the influence of plane of nutrition on milk yield and suckling behaviour in red deer we now propose an alternative hypothesis. We suggest that the principal influence of low planes of nutrition is to increase the suckling frequency of the calf in response to a decrease in availability of milk. It is this increase in suckling frequency, and perhaps the associated increase in plasma levels of prolactin, which is the major determinant of the reproductive failure and is due to the influence of plane of nutrition on the production of milk by the mother rather than on maternal body condition. This hypothesis may have important implications for our understanding of the role of nutrition in determining the length of lactational infertility in many species including man.**

Seventeen red deer hinds (8 or 9 yr old; 75–90 kg body weight) which had been kept on a hill pasture with supplementary hay throughout pregnancy were randomly allocated to one of two treatment groups at the time of calving. Nine hinds were placed in a paddock of permanent grass maintained in a vegetative state at 1,800 kg dry matter per hectare and with a mean sward digestibility of 69%. The other eight hinds were placed in a paddock of hill pasture dominated by dwarf shrubs and indigenous grasses of low density and quality with a mean digestibility of ~60%. This pasture had a complex effect on the grazing activity of the lactating hinds which resulted in a significant reduction in overall food intake[9]. Milk yields were estimated over 24-h periods at 10–14-day intervals throughout lactation as described in Fig. 1 legend.

**Table 1** Changes in body weight (mean ± s.e.m.) of red deer hinds and calves throughout lactation for group on indigenous hill ($n = 8$) or permanent grass pasture ($n = 9$)

| Stage of lactation (days) | Body weight (kg) Hind* | | Calf | |
|---|---|---|---|---|
| | Hill pasture | Permanent pasture | Hill pasture | Permanent pasture |
| 0 | — | — | 7.9 ± 0.4 | 7.8 ± 0.3 |
| 10 | 84.9 ± 1.3 | 83.5 ± 2.2 | 11.7 ± 0.5 | 11.6 ± 0.3 |
| 20 | 83.4 ± 2.4 | 82.3 ± 1.6 | 15.4 ± 0.8 | 15.8 ± 0.4 |
| 30 | 83.5 ± 2.6 | 85.2 ± 2.1 | 18.3 ± 0.9 | 19.0 ± 0.5 |
| 40 | 84.5 ± 2.6 | 88.4 ± 2.6 | 21.1 ± 0.9 | 22.6 ± 0.6 |
| 50 | 85.6 ± 2.4 | 87.5 ± 2.1 | 23.6 ± 0.9 | 26.5 ± 0.7 |
| 60 | 85.6 ± 2.6 | 87.3 ± 2.1 | 26.3 ± 1.0 | 29.9 ± 0.7 |
| 70 | 86.7 ± 2.6 | 89.4 ± 2.4 | 29.1 ± 1.0 | 33.6 ± 0.7 |
| 80 | 87.4 ± 2.7 | 91.2 ± 2.3 | 31.6 ± 1.1 | 37.6 ± 0.8 |
| 90 | 87.3 ± 2.7 | 92.0 ± 2.1 | 34.1 ± 1.2 | 41.4 ± 0.9 |
| 100 | 88.7 ± 2.8 | 92.8 ± 2.2 | 36.2 ± 2.1 | 44.7 ± 0.9 |

* No measurements were made on the day of calving.

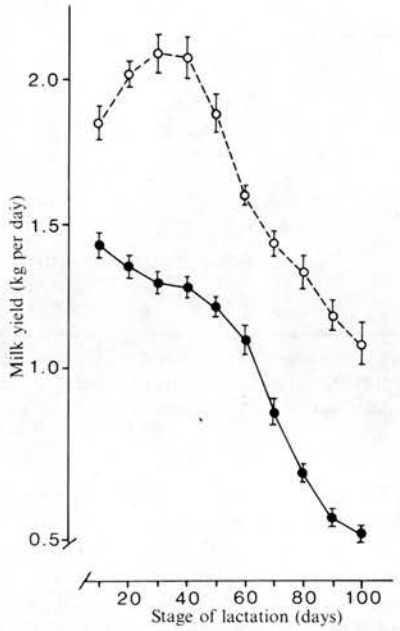



**Fig. 1** Mean milk yields (±s.e.m.) of red deer hinds on hill (●) and permanent grass (○) throughout lactation. Hinds were collected from the pasture, separated from their calves, and a blood sample (15 ml by jugular venipuncture) collected from manually restrained hinds within 30 min of the gathering. Subsequently, milk yields were estimated by the weight difference (±5 g) of the calf before and after suckling at 6-hourly intervals over a 24-h period. Plasma samples were stored at −20 °C until assayed using a heterologous radioimmunoassay described in detail previously[21]. Both groups of hinds were separated from their calves on 15 September at ~110 days of lactation and placed in a single group for breeding with one stag.

Hinds on the permanent grass pasture had significantly higher (about 1.6 times) milk yields than those of hinds on hill pasture (Fig. 1). Despite these differences in lactational performance, there were no significant differences in body weight of the hinds (Table 1). However, the reduced milk yields of the hinds on hill pasture was associated with a significant reduction in the weight gain of the calves throughout lactation (Table 1).

Plasma levels of prolactin were significantly higher in the hinds on the hill pasture although in both groups mean prolactin levels declined throughout lactation in response to declining daylength (Fig. 2) as shown previously[10]. Further, while prolactin levels in each group were significantly correlated with milk yield (Fig. 2) it was clear that for any given level of milk yield, hinds on improved pasture had significantly lower prolactin

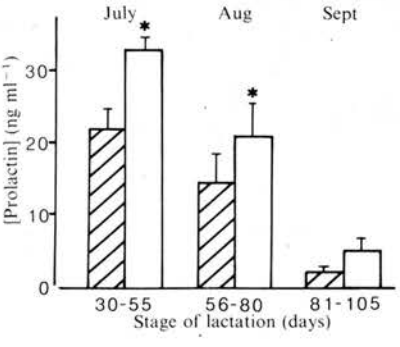



**Fig. 2** Relationship between plasma levels of prolactin (mean ± s.e.m.) and stage of lactation for red deer hinds on hill (□) and permanent grass (▨) pastures.

**Table 2** Pattern of suckling activity by calves of red deer hinds on hill and permanent grass pasture at different stages of lactation

| | Stage of lactation (days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 40–60 | | | 61–80 | | | 81–100 | | |
| Group | No. of suckling bouts | No. of rejects | Suckling time per day(s) | No. of bouts | No. of rejects | Suckling time per day(s) | No. of bouts | No. of rejects | Suckling time per day(s) |
| Hill pasture | 4.2 ± 0.7 | 5.0 ± 0.6 | 304 ± 27 | 4.1 ± 0.9 | 2.4 ± 0.7 | 275 ± 62 | 3.6 ± 0.8 | 3.0 ± 0.6 | 201 ± 49 |
| Permanent grass pasture | 1.9 ± 0.3 | 0 | 837 ± 42 | 1.5 ± 0.5 | 0.6 ± 0.1 | 808 ± 51 | 1.4 ± 0.7 | 0 | 623 ± 77 |

Results include the number of successful suckling attempts and their total daily duration, and the number of attempted sucklings which were rejected by the mother. All observations reported were made between 05.00 and 22.00. A limited number of observations made between 22.01 and 04.59 using an image intensifier suggested that suckling during the hours of darkness is very uncommon.

levels than hinds on hill pasture (Fig. 3). These differences in prolactin levels were completely unexpected and are in contrast with results in non-lactating cattle and sheep where animals on low planes of nutrition have significantly lower plasma levels of prolactin than those on high planes of nutrition[11].

As the level of prolactin in lactation is related to the frequency and intensity of the suckling stimulus, with high suckling frequencies inducing increased prolactin secretion[2], our prolactin results suggested that the plane of nutrition might be affecting the suckling pattern of the calves in the two groups. Observational data confirmed this and showed that the differences in milk yields were associated with a dramatically different pattern of suckling activity by the calves in the two experimental groups. Not only did calves on low quality hill pasture suckle more frequently for short periods but they frequently attempted to suckle and were rejected by their mothers throughout lactation. In contrast, calves on improved pasture suckled infrequently but for longer, and suckling attempts were rarely rejected by their mothers (Table 2). Thus, although the overall pattern of prolactin release throughout the year is principally influenced by daylength in red deer[10], our data show that suckling frequency has an additional modifying influence on prolactin levels even when these are increased in seasonal anoestrus (Fig. 2).

These results provoke the question, why did the calves of low yielding mothers suckle more frequently? One important reason may be the amount of milk available in the mammary gland at any one suckling time and this is dependent on the fill rate of the mammary gland. Sedation and machine milking[12] of our hinds at 4-hourly intervals indicated that the mammary glands of the hill pasture group filled at half the rate of those of hinds on the permanent pasture ($18.0 \pm 3.2\ g\ h^{-1}$ compared with $39.6 \pm 2.7\ g\ h^{-1}$ (mean ± s.e.m.), $P < 0.01$; at mid-September). Thus, at any one time less milk is available to the suckling calf on hill pastures and, in an attempt to obtain adequate milk intake to maintain growth at its genetic potential, the calf returns to the mother at frequent intervals. Indeed, the calves returned more frequently than mothers would allow them to suckle therefore the rejection rate per attempted suckling was considerably higher than in the permanent pasture group with higher milk yields (Table 1).

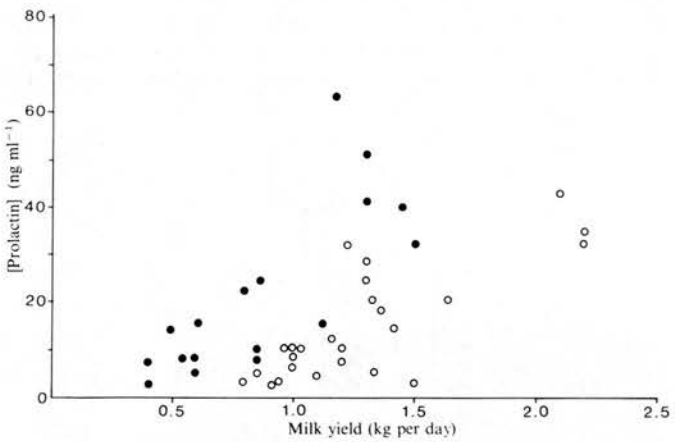



**Fig. 3** Relationship between milk yield and plasma levels of prolactin for red deer hinds on hill (●) and permanent grass (○) pastures throughout lactation. Regression equations: hill pasture group, $y = 0.039x - 11.92$, $r = 0.79$, $P < 0.01$; permanent grass pasture group, $y = 0.023x - 14.70$, $r = 0.79$, $P < 0.01$.

All the available evidence suggests that suckling frequency is of major importance in maintaining increased prolactin secretion and the control of the duration of infertility associated with lactation[1,2,13]. Suckling duration only becomes important as suckling frequency declines[13]. In our study the hinds in the improved pasture group returned to oestrus significantly earlier than the hill group (+6.2 days, $P < 0.05$) suggesting that the pattern of suckling activity by their offspring had an important modifying influence on the resumption of breeding activity following seasonal anoestrus. It is interesting that in the study of wild red deer on Rhum, hinds which supported male calves with a higher suckling frequency returned to oestrus 11 days later than those suckling female calves[14].

The implications of our studies may also be important with regard to breast feeding and the human birth interval. Whether prolactin *per se* is ultimately involved in the control of fertility in any species remains unknown[2,15]. However, it is clear that in women the duration of lactational infertility and amenorrhoea is directly related to the intensity of the suckling stimulus[2,15–17] which in turn controls the level of prolactin. Fertility returns post partum when the suckling stimulus diminishes[15,17]. The influence of nutrition on the duration of lactational amenorrhoea in women, and hence the interbirth interval, is also a matter of conjecture[18,19]. However, most evidence suggests that overt malnutrition has only a minor role in maintaining lactational amenorrhoea[18]. Significantly, however, it has been shown that improving the maternal diet by giving a high protein biscuit to the mother resulted in a reduction in plasma prolactin levels and an earlier resumption of fertility in breast-feeding mothers in the Gambia[20]. However, although this study suggests that the improved maternal nutrition did not affect the pattern of suckling in these women, this has not been fully evaluated (R. Whitehead, personal communication). Our data suggest that, within a species, the pattern of suckling activity by the offspring is highly variable and suckling frequency depends not only on the requirements of the suckling offspring but also on the availability of milk, which, in turn, depends on the nutritional state of the mother. Thus in both seasonal and non-seasonal breeding mammals, including man, it is possible that the influence of maternal nutritional state on the resumption of fertility during lactation is more directly related to its impact on the suckling activity of the offspring than to the maternal nutritional status *per se*.

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# A HETEROLOGOUS RADIOIMMUNOASSAY FOR AVIAN PROLACTIN: APPLICATION TO THE MEASUREMENT OF PROLACTIN IN THE TURKEY

By

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## ABSTRACT

A specific heterologous double-antibody radioimmunoassay has been developed to measure turkey prolactin (PRL) using a guinea pig anti-hPRL antiserum and $^{125}$I-labelled ovine PRL [$^{125}$I]oPRL. Turkey pituitary prolactin and serum give parallel dose-response curves and no cross-reaction is seen with turkey growth hormone, LH or FSH, or mammalian LH, FSH, TSH, GH or placental lactogens. The RIA is accurate and precise and is sufficiently sensitive to measure PRL in all physiological situations investigated in the turkey. The RIA will measure PRL in several avian species including the chicken, duck, goose, pheasant, pheasant × chicken $F_1$ hybrid, pigeon, quail and rook.

Plasma PRL concentrations in laying and broody turkey hens were not significantly different (46.5 ± 2.5 vs. 39.7 ± 3.8 ng/ml) but both were significantly higher ($P < 0.001$) than in non-laying turkey hens (4.6 ± 0.7 ng/ml).

Oestradiol injection into laying hens did not alter PRL levels while the same injection in non-laying hens caused a significant three-fold increase in plasma PRL levels.

Heterologous radioimmunoassays have been described in which antibodies raised against mammalian hormones cross-react with avian pituitary hor-

mones. Such radioimmunoassay systems have been described for avian GH (*Hayashida* 1970; *Farmer et al.* 1974) and avian FSH (*Croix et al.* 1974; *Follett* 1976).

Only one homologous radioimmunoassay for chicken prolactin has been described (*Scanes et al.* 1976) and this shows only limited cross-reaction with avian species other than the chicken. It was the purpose of this investigation to develop a heterologous radioimmunoassay for prolactin suitable for use in several avian species, in particular the turkey.

## MATERIALS AND METHODS

### *Materials*

Bovine serum albumin (Fraction V) was obtained from Miles Laboratory; lactoperoxidase from Calbiochem, La Jolla, California; Na[$^{125}$I] (carrier free) from New England Nuclear, Boston, Mass., and $H_2O_2$ (30 %, v. v.) from Fisher Scientific, Fair Lawn, N. J. All other reagents and chemicals were of reagent grade.

Purified hormones from several mammalian species were used to assess the initial specificity of the assay. These were hPRL (Friesen 75–7–28); oPRL (NIH P–S10); bPRL (NIH–P–B3); pPRL (NIH SP 162C); rPRL (NIAMDD P–I–1); dogPRL (Schering); hGH (NIH HS1146); mGH (NIH M945 A); pGH (NIH RIA 073313); bGH (NIH RIA 1003A); pGH (NIH P 526 B); rGH (NIAMDDGH–I–2); mouseGH (NIAMDD AFP 689–B); dogGH (Wilhelmi D100 A); hLH (NIH LER 960); oLH (NIH LH–S18); rLH (NIAMDD–LH–I–1); hFSH (NIH LER–1575 C); bFSH (CH–bF–1); rFSH (NIAMDD FSH–I–2). TurkeyGH (W 27CD) was kindly supplied by Drs. Farmer and Papkoff; turkeyFSH, turkeyLH and two turkeyPRL preparations (M SER–I and HS–1, II) were kindly supplied by Dr. K. W. Cheng, Winnipeg. ChickenLH (IRC–2) was supplied by Dr. F. J. Cunningham, Reading.

During the development of this RIA no turkeyPRL standard was available. A laboratory standard turkeyPRL (T2–76) was therefore prepared. Seventeen turkey pituitaries (343 mg) were extracted in 4 × 0.5 ml 0.2 M ammonium bicarbonate pH 7.8 for 3 min at 4°C and the extract was centrifuged at 10 000 *g* for 45 min at 4°C. The supernatant obtained was diluted 1:10 in 0.025 M TRIS–HCl buffer 7.4 containing 0.1 % BSA, aliquoted and stored at –70°C until assayed. These conditions were optimal for the extraction of bio- and immunoassayable PRL (*McNeilly & Etches*, unpublished observation). The prolactin content was measured in the prolactin radioreceptor assay (RRA) using the mammary gland receptor as described in detail previously (*Shiu et al.* 1973). Ovine prolactin (oPRL, NIH P-S10, 26 IU/mg) was used as standard and the amount of prolactin in the pituitary extract (T2–76) was assessed and used as standard in all the RIA studies.

### *Antiserum and iodination of prolactin*

During the development of heterologous radioimmunoassay systems for the measurement of prolactin in several mammalian species (*McNeilly & Friesen* 1978), it became apparent that one system was suitable for the measurement of prolactin in several avian species.

The antiserum used (AGP–4) was raised in a guinea-pig against human prolactin and the preparation has been described elsewhere (*McNeilly & Friesen* 1978).

Ovine prolactin (NIH P-S10, 26 IU/mg), generously provided by the NIH, Bethesda, Maryland, was used as labelled hormone. Iodination of oPRL was carried out by the lactoperoxidase method (*Thorell & Johansson* 1971) at room temperature exactly as described previously (*McNeilly & Friesen* 1978). $^{125}$I-labelled hormone were purified by gel filtration on Sephadex G-100 (2 × 50 cm) eluted with 0.025 M TRIS–HCl buffer pH 7.4 containing 0.1 % bovine serum albumin (BSA). The [$^{125}$I]oPRL was stored at –20°C until used for assay.

### *Assay procedure*

Maximum binding of [$^{125}$I]oPRL of around 94 % could be achieved with a final antibody dilution of 1:1000 and a working dilution (final dilution 1:30 000) estimated by antibody dilution curves, was chosen which would bind 30–40 % of the added tracer.

Assays were carried out using 0.01 M sodium phosphate buffer pH 7.4 containing 0.15 M NaCl, 2.0 % BSA and 0.1 % sodium azide (radioimmunoassay diluent, RIAD). The exact radioimmunoassay method has been described in detail elsewhere (*McNeilly & Andrews* 1974; *McNeilly & Hagen* 1974; *McNeilly & Friesen* 1978).

### *Specificity, precision and accuracy of the assay*

The hormones used to assess the specificity of the RIA are listed in the Materials section above. Each hormone preparation was added at a starting concentration of at least 1 μg/ml. Cross-reactivity was calculated as the amount of hormone (w/w) giving 50 % (B/Bo) inhibition in the radioimmunoassay.

Extracts of several avian species were prepared as described above for the preparation of the turkey pituitary prolactin standard and stored in aliquots at –20°C until assayed. The prolactin content in these pituitary extracts was assessed using both the RIA and the prolactin RRA (*Shiu et al.* 1973). Serum samples from these same species were also obtained and together with the pituitary extracts were double diluted and assayed to assess cross-reactivity and parallelism. The avian species assessed in this way were: domestic duck (*Anas platyrhynchos*), domestic pigeon (*Columbia livia*), domestic goose (*Anser anser*), ring-necked pheasant (*Phasianus colchicus*), domestic chicken (*Gallus domesticus*), pheasant × chicken $F_1$ hybrid, Japanese quail (*Coturnix coturnix*), rook (*Corvus frugilegus*).

The parellelism between dose-response curves for turkey prolactin standard diluted in RIAD, serum from hypophysectomised chicken, quail and rat or human serum containing < 1 ng hPRL/ml by homologous RIA was assessed to determine whether any plasma or serum interference occurred in the RIA. At the same time sera from laying, non-laying and broody female turkeys were diluted and assayed against the turkey PRL standard diluted in RIAD.

The accuracy of the RIA was determined by the measurement of known amounts of turkeyPRL standard added to turkey serum containing low levels of PRL and to plasma from a hypophysectomized chicken. The precision of the assay expressed as the intra- and inter-assay co-efficients of variation (%) were assessed by the repeated assay of three pools of turkey sera containing PRL levels of 1, 15 and 36 ng/ml both within and between assays.

*Sephadex chromatography of turkey prolactin standard*

To assess the form of the immunologically active prolactin measured by the radioimmunoassay the turkey pituitary prolactin standard (T2–76) and turkey sera containing high or low levels of PRL were fractionated at 4°C by gel filtration on Sephadex G-100 (2 × 50 cm) eluted with 0.025 M TRIS HCl pH 7.6 containing 0.1 % BSA. One ml fractions were collected and analysed for PRL by RIA and RRA (*Shiu et al.* 1973) and for GH by RRA (*Tsushima & Friesen* 1973). Protein concentrations were estimated by measuring absorbance at 280 nM.

*Plasma prolactin in mature turkey hens and the effects of oestrogen*

To determine the relationship between reproductive condition and plasma prolactin, blood samples were removed from 31 laying hens, 27 broody hens and 24 non-laying hens. These were Nicholas Large White hens which were provided by and housed at Cuddy Farms Ltd., Strathroy, Ontario.

To determine the effects of oestradiol on plasma prolactin, four laying and five non-laying hens were injected subcutaneously with 1.0 mg of oestradiol in ethanol:saline (1:1, v/v). Blood samples (5 ml) were removed 30 min before injection, immediately before injection and at 15, 30, 60 and 120 min after injection, centrifuged to remove plasma which was stored at –20°C until assay. Statistical analysis was by analysis of variance or Student's *t*-test.

*Table 1.*

Cross-reaction of guinea-pig anti-human prolactin antiserum (AGP4–1) with [$^{125}$I]oPRL with prolactin preparations from different species and available avian hormones.

| Species | Scurces | Cross-reaction (%)* |
|---|---|---|
| turkeyPRL | a) McNeilly-Etches ($T_2$–76) | 100 |
| | b) Cheng HS–1 G-100 II | 36 |
| | c) Cheng M–SER G-100 I | 17 |
| dPRL | Schering | 100 |
| rPRL | NIAMDD P–I–1 | 27 |
| hPRL | Friesen 75–7–28 | 126 |
| pPRL | NIH SP162C | 55 |
| oPRL | NIH P–S–10 | 94 |
| bPRL | NIH P–B–3 | 82 |
| turkeyGH | W27CD Farmer & Papkoff | < 0.01 |
| turkeyLH | Cheng TLH | < 0.01 |
| turkeyFSH | Cheng TFSH | < 0.01 |
| chickenLH | LH IRC 2 | < 0.01 |

## RESULTS

*Heterologous assay for turkey PRL*

The heterologous RIA developed was specific for prolactins and no cross-reaction ($< 0.1$ %) was seen with GH, LH, FSH, TSH or placental lactogens from those species, both mammalian and avian which were tested. The cross-reactions of the prolactins from different species is shown in Table 1 and for the turkey preparations in Fig. 1. All prolactin preparations tested showed parallel inhibition curves, while the most potent preparation was hPRL.

Standard curves of turkeyPRL reference standard in RIAD, or hypophysectomized human or chicken serum were identical and these same sera failed to cause any inhibition in the RIA (Fig. 2). Dilutions of turkey plasma and serum samples were parallel to the turkeyPRL (Fig. 2). The accuracy of the assay assessed by the recovery of turkeyPRL standard (50 ng/ml to 1 ng/ml)

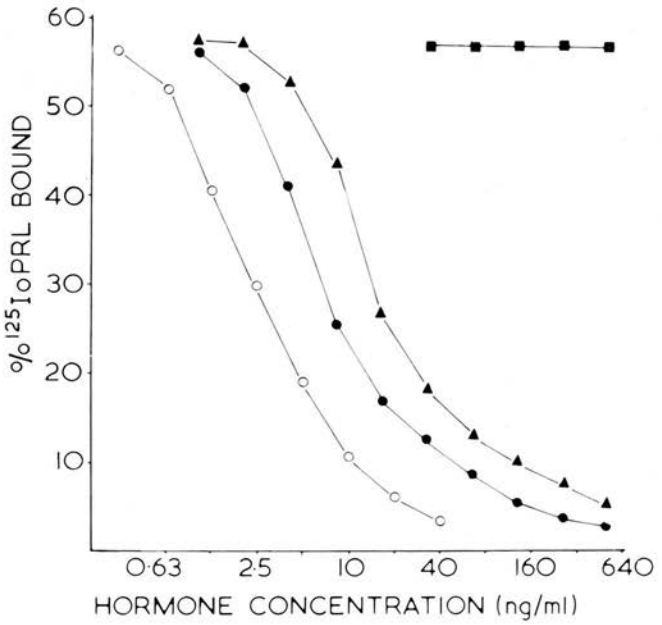



*Fig. 1.*

Inhibition curves for turkey pituitary hormone preparations in the heterologous RIA for avian PRL. PRL reference standard $T_2$-76 (○—○); PRL preparations HS-1 G100 II (Cheng; ●—●); PRL preparation M-SER G100 I (Cheng; ▲—▲) and turkeyGH (W27CD, Farmer & Papkoff), turkeyLH (Cheng TLH) and turkeyFSH (Cheng TFSH) (■—■).

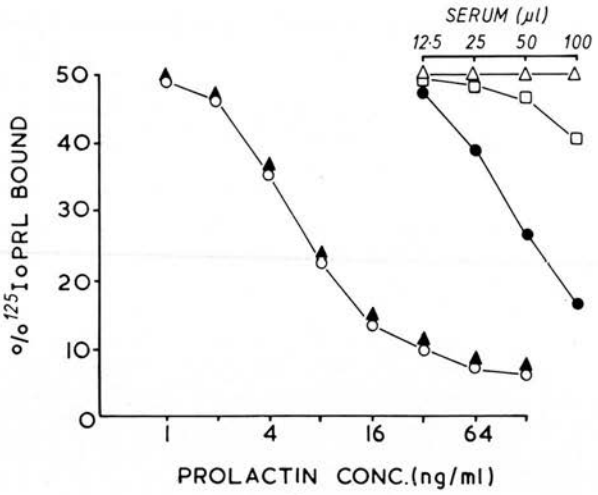



*Fig. 2.*

Inhibition curves for turkeyPRL reference standard ($T_2$–76) in diluent (○) or plasma from a hypophysectomized chicken (▲) and dilutions of plasma from a laying (●) and non-laying (□) turkey hen and a hypophysectomized chicken (△) in the heterologous avian PRL RIA.

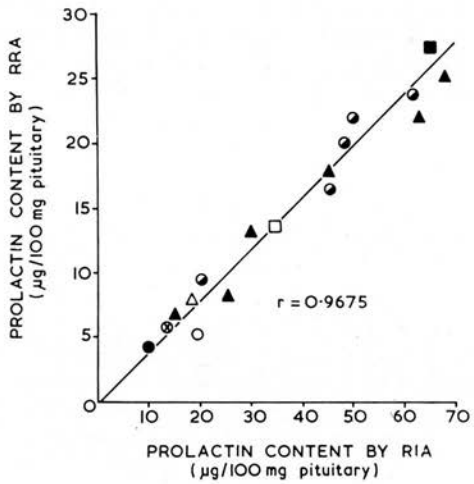



*Fig. 3.*

Correlation between RIA and RRA estimates of pituitary prolactin content in several avian species (duck, ○; pigeon, ●; goose, ⊗; pheasant, △; pheasant × chicken, □; quail, ■; turkey, ▲; chicken, ◑).

added to turkey serum containing 1.7 ng/ml PRL was 98 ± 4 (SEM) % (n = 38). Intra- and inter-assay variation (expressed as the coefficient of variation) of results on replicate samples was 8.9 % (n = 38) and 13.7 % (n = 24), respectively.

Pituitary extracts from all eight avian species studied were parallel to the turkey PRL standard in the RIA. Comparison of the PRL levels obtained for these pituitary extracts by RIA and by the prolactin RRA showed a highly significant ($P = < 0.001$) linear correlation (Fig. 3). In all these species the assay was of sufficient sensitivity to measure PRL in the sera, and serum dilutions from each avian species were parallel to the standards.

*Sephadex chromatography of turkey PRL*

A single peak of radioimmuno- and radioreceptor assayable PRL was found after gel filtration on Sephadex G-100 of the turkey pituitary prolactin standard (Fig. 4). While no attempt was made to establish an accurate molecular weight for this activity the elution volume would suggest a molecular weight approximately 32 000.

The concentrations of prolactin in Nicholas Large White turkey hens varied according to the reproductive state of the bird. The plasma concentrations of prolactin in laying, broody and non-laying hens were 46.5 ± 2.5 ng/ml (mean

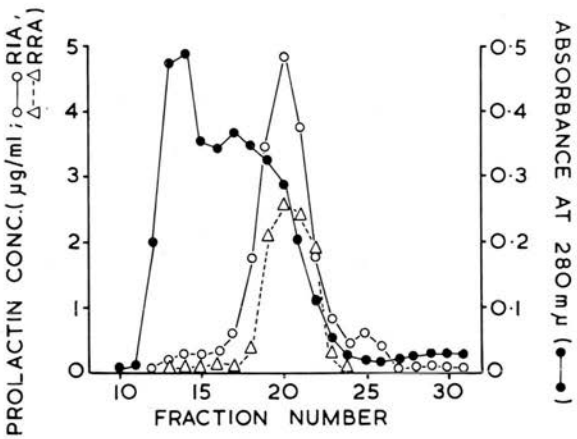



*Fig. 4.*
Gel filtration on Sephadex G-100 (50 × 2.5 cm) of a turkeyPRL preparation. One ml fractions were collected and assayed for PRL by RIA (○) and RRA (△).

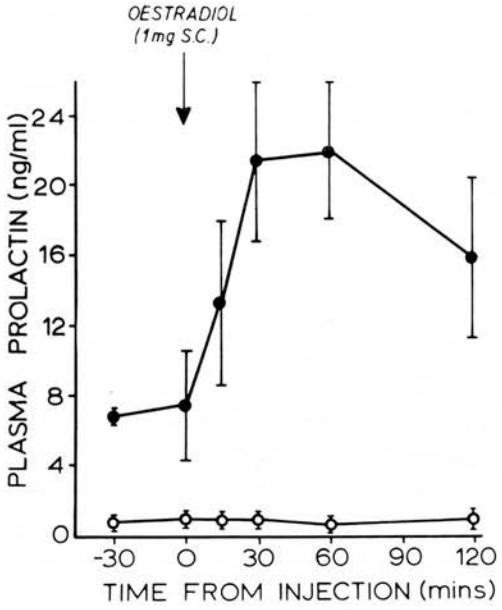



*Fig. 5.*
Plasma concentrations (ng/ml) of prolactin following injections of 1 mg of oestradiol into four laying hens (○—○) and five non-laying hens (●—●).
Results are mean ± SD.

± SEM), 39.7 ± 3.8 ng/ml and 4.6 ± 0.7 ng/ml, respectively. The difference between broody and laying hens was not significantly different ($P > 0.05$) but both groups were significantly higher ($P < 0.001$) than in the non-laying hens.

The injection of oestradiol (Fig. 5) into laying hens did not alter the concentration of prolactin ($t = 0.48$, $P > 0.05$), whereas the same injection given to non-laying hens was followed by a three-fold increase ($t = 4.18$, $P < 0.01$) in plasma prolactin.

## DISCUSSION

The heterologous radioimmunoassay described in this paper is specific for prolactins in all species investigated, is precise, accurate and reproducible. No non-specific interference is seen in the RIA, and plasma or serum from hypo-

physectomised avian and mammalian donors do not cause any displacement in the assay. This is in marked contrast to the report of *Nicoll* (1975). The binding achieved during routine assay (30–40 % $^{B}$/T) is of the same order as that in homologous RIAs and the slope of the inhibition curve is sufficient to ensure accuracy and precision. This is further demonstrated by the recovery data (98 ± 4 %) and the intra- and inter-assay variation (8.9 and 13.7 %, respectively).

Evidence that the RIA is measuring prolactin in the avian species is gained from the gel filtration of the turkey prolactin standard. Both the immunoassayable and bioassayable (RRA) activities coincided suggesting that both assays are detecting the same activity. The estimated molecular weight of approximately 32 000 is similar to that of purified turkeyPRL (*Cheng*, personal communication). The lower cross-reactivity of semipure preparations of turkeyPRL (Table 1; Fig. 1) compared to the house standard preparation of turkeyPRL (T2–76) merely reflects the fact that the house preparation is a crude pituitary homogenate which was assigned a potency per ml of extract in terms of RRA activity. The relative potencies of the two semipure turkeyPRL preparations supplied by Dr. K. W. Cheng (i.e. TPRL HS–1 G-100–II = 2 × potency of TPRL M–SER G-100 T) estimated by the heterologous PRL RIA described here (Table 1; Fig. 1) is in agreement with the relative biological potencies of these preparations as estimated by RRA (*Cheng*, personal communication).

The heterologous RIA may be used to estimate PRL in other avian species. This was demonstrated by the high correlation between the PRL activity in the avian pituitary extracts determined by the RIA and the RRA. That the correlation is with the PRL content of the pituitaries is emphasized by the lack of cross-reaction of GH in the RRA for PRL (*Shiu et al.* 1973) and the presence of a very low GH content in the pituitary extracts and total lack of correlation between the GH content and the PRL content as estimated by either RIA or RRA. Dilutions of serum in each of these avian species were parallel to the pituitary extracts dilutions and to the purified PRL preparations. These results indicate that the heterologous RIA described here is applicable to the measurement of PRL in other avian species. Before finally accepting this observation for other avian species however, it is suggested that a lack of non-specific interference of plasma or serum should be demonstrated for each avian species investigated.

Absence of non-specific serum interference in this RIA is further emphasized by the good agreement between serum levels of PRL samples from cow, sheep, rat and human as measured by this RIA and the relevant homologous RIAs (*McNeilly*, unpublished observations).

Injection of oestradiol into non-laying hens resulted in a highly significant increase in PRL levels which were maximal between 30 and 60 min after the

injection. By contrast, no changes were observed after the same injection into laying hens. Similar increases in prolactin following the administration of oestradiol have been observed in man (*Frantz* 1973), rats (*Neill & Smith* 1974) and cows (*Schams et al.* 1974). The increase in prolactin following oestradiol correlates well with plasma concentrations of prolactin in broody, laying and non-laying Nicholas Large White hens where large differences were observed between non-laying or broody hens.

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# Plasma Concentrations of Prolactin during the Reproductive Cycle of the Domestic Turkey (*Meleagris gallopavo*)

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*ABSTRACT* Plasma concentrations of prolactin were measured by a heterologous double antibody radioimmunoassay during the reproductive cycle of Large White turkey hens. In all hens, there was a large increase in the plasma concentration of prolactin as laying commenced. Six weeks after illumination, the concentration of prolactin reached a maximum of 300 to 400 ng/ml and thereafter the plasma values declined. By 15 weeks after illumination, plasma prolactin had declined to baseline values of less than 10 ng/ml. There was no apparent relationship between the concentration of prolactin and egg production, and there were no differences between the changes in prolactin in plasma from broody and non-broody hens. Broodiness invariably was observed after the large increase in plasma prolactin, but the large increase in plasma prolactin was not invariably associated with broodiness. It was suggested that selective breeding for egg production has favored the retention of a genotype which is insensitive to the antigonadal effects of prolactin.

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## INTRODUCTION

A constant aggravation to the turkey producer is the occurrence of broodiness in the breeder hen. Although many empirical methods of disrupting broody behavior have been attempted, none has provided a consistently successful means of altering this aspect of maternal behavior (Nestor *et al.*, 1971). In part, this lack of success can be attributed to the lack of an adequate understanding of the physiological mechanisms which control broodiness in turkeys and other gallinaceous birds.

It is generally accepted that the pituitary hormone prolactin is responsible for broody behavior (Sturkie, 1976). Indeed, this would appear to be a logical conclusion since injections of prolactin will elicit several types of maternal behavior in a variety of birds. In the domestic fowl, Riddle *et al.* (1935) showed that injections of prolactin would induce hens to incubate a nest of eggs. Nalbandov (1945) and Nalbandov and Card (1945) showed that roosters, which are normally aggressive towards day-old chicks, would provide maternal care following injections of prolactin. Prolactin would also appear to be involved in the development of accessory structures for incubation, since exogenous prolactin either alone or in combination with estrogens and androgens resulted in the development of a brood patch in various passerine species, California quail, domestic chickens, and phalaropes (see Drent, 1975 for review).

In addition to demonstrating that exogenous prolactin induces broodiness in domestic hens, Bates *et al.* (1935) also showed that prolactin caused regression of the ovary or testes and this observation has been substantiated by Breneman (1942) and Nalbandov (1945). Similar anti-gonadal responses to prolactin have been observed in the pigeon (Bates *et al.*, 1937), the Japanese quail (Camper and Burke, 1977a) and various passerine species (Bailey, 1950; Lofts and Marshall, 1956; Thapliyal and Saxena, 1964; Meier and Dusseau, 1968; and Meier, 1969). Eisner (1960) has cited a number of instances where injections of prolactin or presumably high concentrations of endogenous prolactin correlate with the loss of secondary sexual characteristics which are dependent upon a functional gonad. Further evidence of the antigonadal actions of prolactin is provided by Tanaka *et al.* (1971) who showed that appropriately timed injections of prolactin will inhibit ovulation. Camper and Burke (1977b) extended these observations by demonstrating that injections of prolactin will abolish the gonadotrophin-induced rise in serum estradiol and progesterone in the turkey.

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While each of these reports lends credence to the hypothesis that prolactin is responsible for broody behavior and that prolactin is antigonadal in birds, a variety of investigators have found prolactin to be neither antigonadal nor responsible for broodiness. Eisner (1960) has cast some doubt on the validity of a general conclusion regarding the involvement of prolactin in broodiness since the induction of all aspects of parental behavior by prolactin is not a ubiquitous response in the class Aves. In doves, Lehrman and Brody (1961) have reported evidence which suggests that progesterone rather than prolactin may be responsible for incubation behavior. Juhn and Harris (1956) were unable to confirm that prolactin was anti-gonadal in chickens and similar results were observed by Shani *et al.* (1973). In both the California quail (Jones, 1969) and the Japanese quail (Alexander and Wolfson, 1970; Renzoni, 1970) prolactin failed to induce regression of the gonads. Similarly, an anti-gonadal effect of prolactin was not demonstrated in three species of migrating passerines (Laws and Farner, 1960; Meier and Dusseau, 1968).

Recently, it has been recognized that prolactin can affect a wide variety of biological systems (see Bern and Nicoll, 1968; Nicoll and Bern, 1972 for review). For example, exogenous prolactin can induce premigratory fattening in sparrows (Meier and Farner, 1964), stimulate hepatic lipogenesis in pigeons (Goodridge and Ball, 1968a,b), and alter the osmoregulatory system of marine birds (Ensor, 1975). In addition, there is some evidence which indicates a role for avian prolactin in moulting (Juhn and Harris, 1958; Payne, 1972).

Although this multitude of actions has been reported and contradicted, circulating concentrations of prolactin have not been measured during any of the physiological situations from which the information has been derived or to which the data has been extrapolated. This absence has been due to the lack of a suitable method for measuring circulating concentrations of prolactin. A radioimmunoassay for prolactin from a variety of avian species has recently been developed by McNeilly *et al.* (1978) and this assay has been used to assess the relationship of plasma prolactin to various aspects of parental and migratory behavior. In snow geese, Campbell *et al.* (1978) showed a peak of prolactin at the time of incubation and were unable to show a peak at any other time of the year. In ruffed grouse, plasma prolactin rose as the number of eggs in the nest increased, reached a maximum when laying terminated and incubation commenced, and these maximal values were maintained throughout incubation (Etches *et al.*, 1979). These data support the proposition that prolactin is both anti-gonadal and involved in the regulation of parental behavior in at least two orders of the class Aves. This report describes the changes in plasma prolactin in both broody and non-broody hens during the reproductive cycle of the domestic turkey.

## MATERIALS AND METHODS

One hundred Nicholas Large White turkey hens, generously provided by Cuddy Farms, Ltd., Strathroy, Ontario, were housed in an environmentally controlled building with a 14L: 10D photoperiod at 30 weeks of age. This photoperiod was maintained throughout the experiment and feed and water were provided *ad libitum*. The hens were trap-nested daily and observed for indications of broodiness during five days per week. Hens were judged as broody if they repeatedly entered into and were reluctant to leave the nest for a period of 24 hr or more. A 5 ml blood sample was removed from each hen at weekly intervals throughout the experiment and prolactin was measured in this sample by the method of McNeilly *et al.* (1978). All blood samples were removed betweeen 0900 and 1100 hr.

The hens were housed in 16 floor pens in groups of 6 or 7. Twenty-six of the 100 hens laid their eggs on the floor either continuously or intermittently. Neither plasma prolactin nor egg production was quantitated for these hens although the occurrence of broodiness was recorded in all 100 hens. Due to the large number of samples which were collected during the 34 weeks of production, not all samples were assayed for prolactin. To accomplish the aims of this experiment, the hens for analysis were selected, first, so that a large group of broody and non-broody hens could be compared, and, second so that the plasma concentrations of prolactin in hens could be correlated with varying rates of egg production. Plasma prolactin was, therefore, measured in 1440 samples from 44 hens, 23 of which were broody. Within each of the subclasses for comparison, the hens were selected at random.

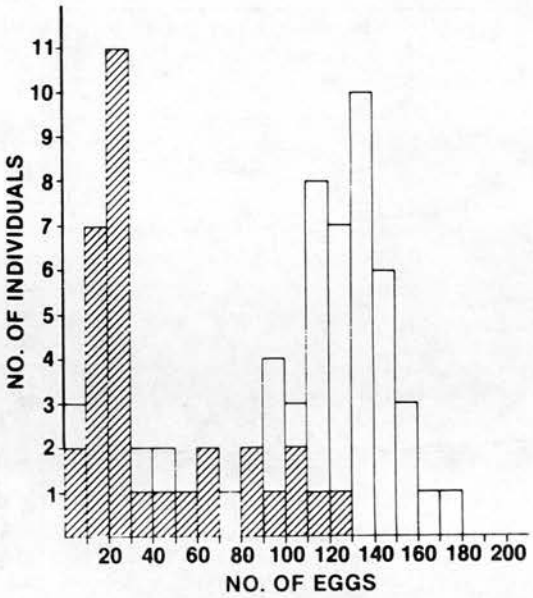



FIG. 1. The distribution of egg production within the flock of Nicholas Large White turkey hens. The open bars indicate the number of hens which were not broody and the cross hatched bars indicate the number of hens which exhibited broody behavior.

## RESULTS

The distribution of total egg production for this flock of hens is illustrated in Figure 1. The distribution was distinctly bimodal with one group of hens laying between 10 and 30 eggs while the second group of hens laid between 80 and 170 eggs.

The relationship between egg production and plasma concentrations of prolactin in the low producing hens is illustrated in Groups 1 to 4 of Figure 2 and the same relationship is illustrated for the high producing hens in Groups 5 to 8 of Figure 3. These groups were arbitrarily created by combining the data from 5 or 6 hens of comparable productivity. Essentially the same changes in the plasma concentration of prolactin were observed in all groups of hens regardless of the number of eggs which they laid during the experimental period. A large increase in plasma prolactin was observed in the first two weeks after the photoperiod was extended to 14L:10D. During the third week, prolactin returned to baseline concentrations and remained low until the sixth week at which time a period of rapid increase in the plasma concentration of prolactin began. Egg production was initiated in all birds during the fourth week, when plasma concentrations of prolactin were low. High concentrations of prolactin were observed between the sixth and twelfth week of production, regardless of the number of eggs produced and gradually declined thereafter until the thirty-fourth week.

Of the 100 hens which were studied, 32 were designated as broody during the experimental period. The distribution of these individuals within the total population is shown in Figure 1. Those hens which were classified as broody laid 43.3 ± 6.1 eggs (mean ± SEM) whereas those hens which were not classified as broody laid 129.0 ± 6.5 eggs (mean ± SEM). This difference was statistically significant using student's t-test ($t = 9.18$, $P < .01$).

Broodiness was first observed during the sixth week following photo-stimulation and was not observed after the seventeenth week. Broodiness occurred most frequently during the seventh week (Fig. 4). At this time both plasma prolactin and egg production were at their maximum. In individual hens, the maximum concentration of prolactin occurred within the week prior to the onset of broodiness. While there were significant differences in egg production between broody and non-broody hens no obvious differences existed in the concentrations of prolactin between these two groups (Fig. 4).

## DISCUSSION

It is interesting that the distribution in egg production in this flock of hens was distinctly bimodal and that those hens in the subclass which produced fewer eggs were generally broody (Fig. 1). This observation supports the general hypothesis that broodiness in turkeys is negatively correlated with egg production. In this flock of hens, the reduction in egg production associated with broodiness was much more devastating then that which was reported previously by Haller and Cherms (1961). In the latter study, egg production from broody hens was 54% of that from non-broody hens whereas in the present study, egg production was reduced to 33% of the non-broody level. This difference may have been due to the absence of a broody control program or to the higher productivity of non-broody hens which was observed in the present investigation.

Although there is substantial evidence which suggests that prolactin is antigonadal, the data presented in Figures 2 and 3 indicate that a quantitative relationship between egg production and plasma concentrations of prolactin

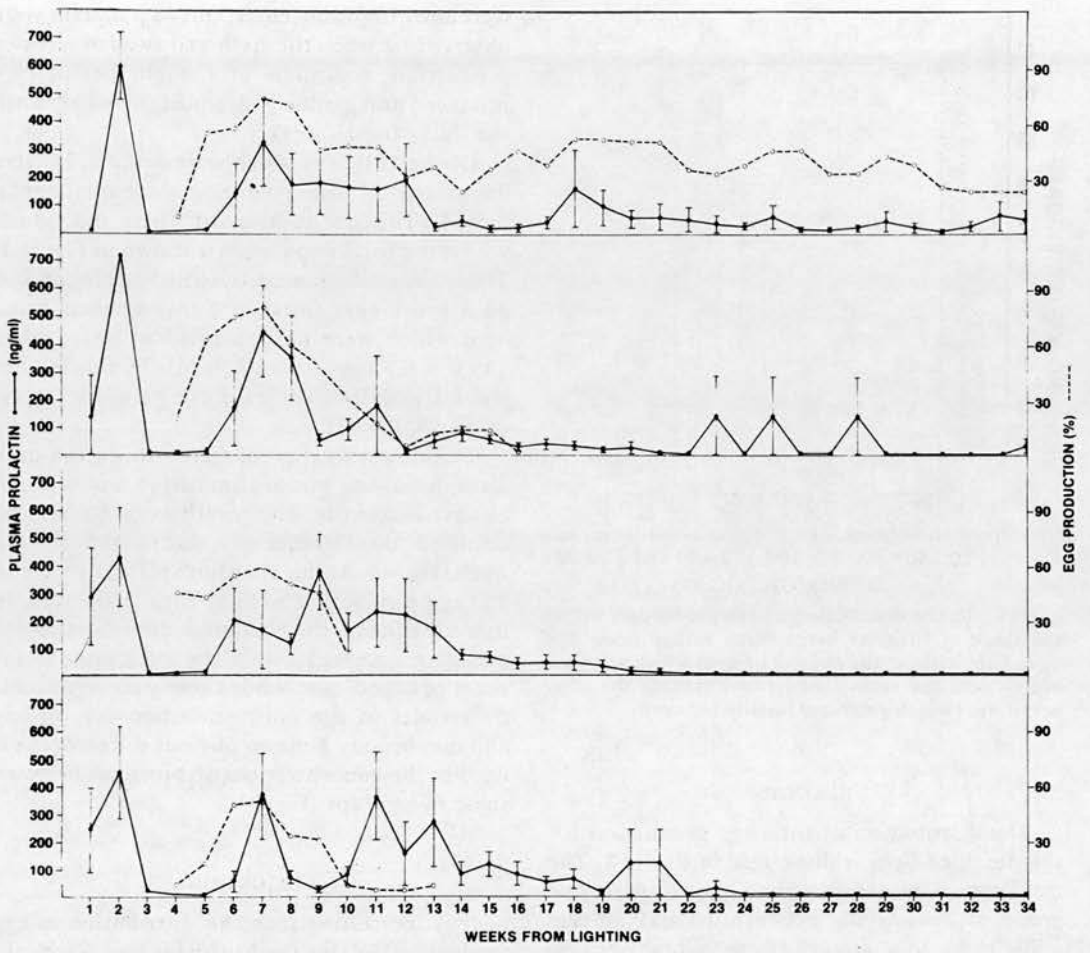



FIG. 2. Plasma concentrations (mean ± SEM) of prolactin (●——●) and hen-day egg production (○-----○) in hens laying 10 to 18 eggs (Group 1, n = 5, lower panel), 20 to 25 eggs (Group 2, n = 6, lower middle panel), 26 to 41 eggs (Group 3, n = 6, upper middle panel) and 70 to 102 eggs (Group 4, n = 6, upper panel).

does not exist. Regardless of the number of eggs produced the profile of prolactin during the reproductive cycle is similar. In addition, there were no obvious differences between the plasma concentrations of prolactin in birds which became broody and those which did not exhibit a desire to incubate their eggs (Fig. 4). A slight difference in the concentrations of prolactin in broody and non-broody hens was noted at 12 and 13 weeks after lighting, although it is difficult to relate this small change to the physiological events which accompany broodiness and egg production.

In previous studies, the involvement of prolactin in the endocrine control of broodiness was investigated by quantitating the pituitary content of prolactin using the pigeon crop-sac bioassay. Using this method, it was established that the onset of broodiness was accompanied by high concentrations of pituitary prolactin in the hen (Burrows and Byerly, 1936; Saeki and Tanabe, 1955; Nakajo and Tanaka, 1956), the pigeon (Schooley, 1937), the pheasant (Breitenbach and Meyer, 1959), and the turkey (Cherms *et al.*, 1962). Assuming that increases in pituitary content of a hormone cause a concommitant increase in the plasma concentration of that hormone, the changes in the plasma concentration of prolactin preceding the onset of broodiness (Fig. 4) substantiate the previous reports. Plasma prolactin was much higher prior to the onset of broodiness than at any other stage of egg production in the broody hen and, therefore, the decline in egg production which was observed after week 7 could be attributed to the anti-gonadal effects of the high concen-

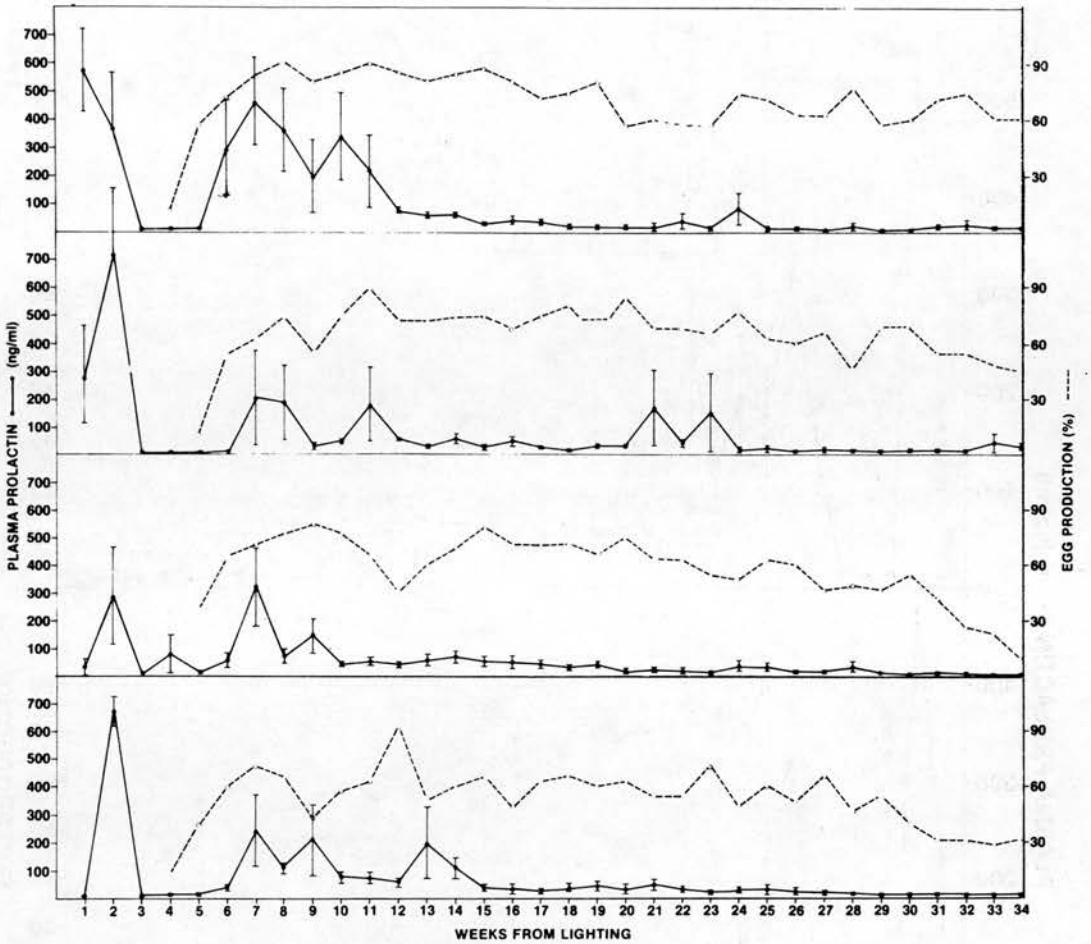



FIG. 3. Plasma concentrations (mean ± SEM) of prolactin (●——●) and hen-day egg production (○——○) in hens laying 104 to 115 eggs (Group 5, n = 5, lower panel), 118 to 133 eggs (Group 6, n = 6, lower middle panel), 134 to 147 eggs (Group 7, n = 5, upper middle panel), and 150 to 173 eggs (Group 8, n = 5, upper panel).

tration of prolactin observed at this time. This interpretation of the data is in agreement with both the anti-gonadal effects and the maternal behavior effects of exogenous prolactin which have been observed many times in a variety of avian taxa (see Introduction for review).

The plasma concentrations of prolactin in laying hens differ substantially from the previous reports of the pituitary content of prolactin during egg production. While high concentrations of prolactin were not observed in the pituitaries of hens, pigeons, pheasants, or turkeys during the egg production stage of the reproductive cycle (Burrows and Byerly, 1936; Schooley, 1937; Saeki and Tanabe, 1955; Nakajo and Tanaka, 1956; Breitenbach and Meyer, 1959; Cherms *et al.*, 1962), a peak in the concentration of prolactin was observed in the plasma of laying hens. This peak occurred at the same time and was of similar magnitude to the peak which was observed prior to the onset of broodiness (Fig. 4). It is evident that major changes in the plasma concentration of prolactin occur very rapidly after photostimulation and the onset of lay and that the duration of the peak is approximately 10 to 14 days. Therefore, unless the ages of the hens which were used in the comparisons of pituitary content were precisely synchronized, spurious differences in prolactin content might be observed.

The changes in plasma prolactin during the egg production cycle of broody turkey hens (Fig. 4) is in good agreement with the large body of evidence which implicates prolactin in

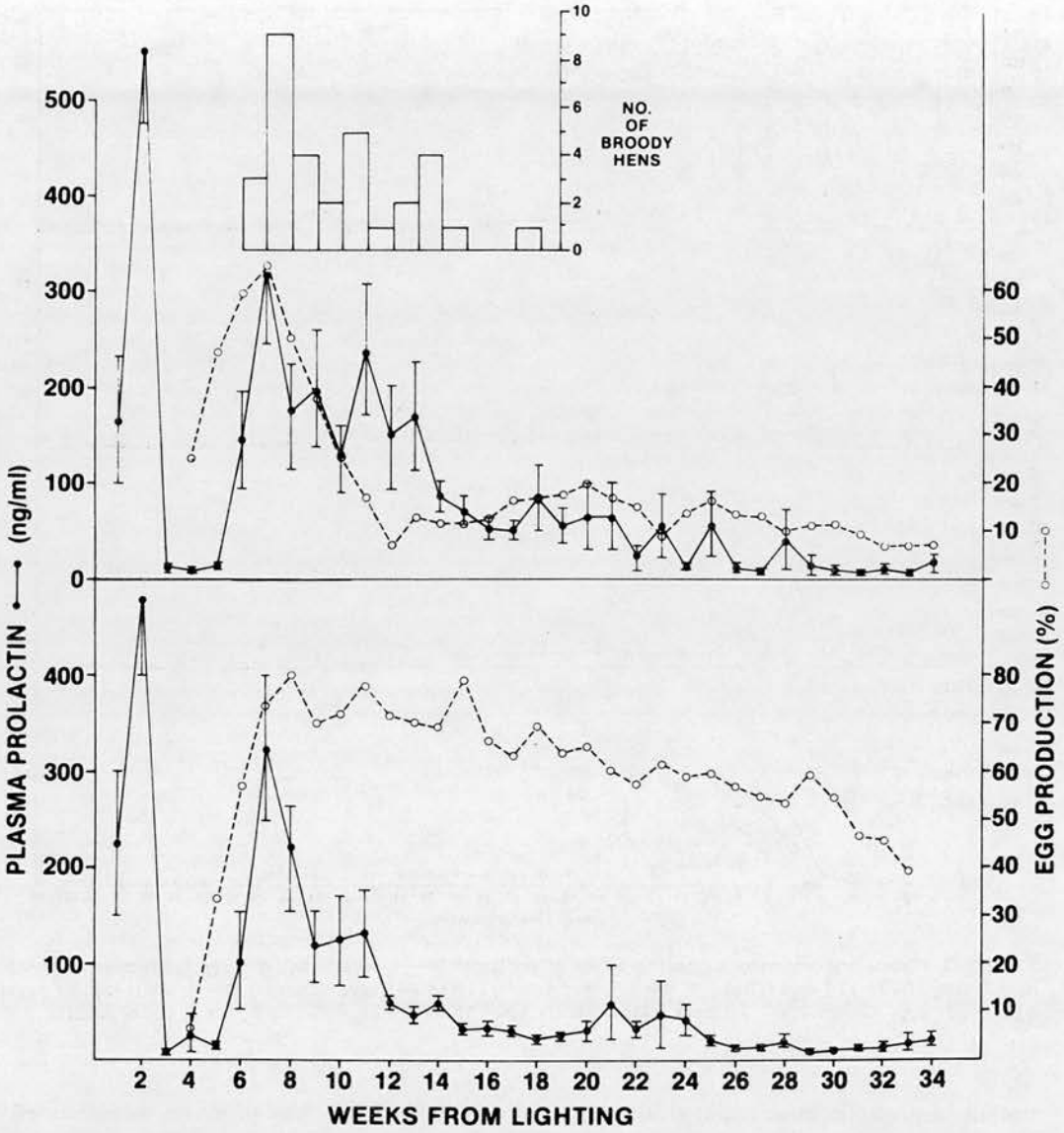



FIG. 4. Plasma concentrations (mean ± SEM) of prolactin (●——●) and hen-day egg production (○----○) in broody (top panel) and non-broody (bottom panel) hens. The frequency of the onset of broodiness is indicated by the histogram in the upper panel. Twenty-three broody hens and 20 non-broody hens are included in the prolactin and egg production data.

the regression of the gonad and the initiation of maternal behavior in birds. However, a similar large peak exists in high producing turkey hens which continue to lay large numbers of eggs. The large number of eggs which these hens lay is an enigma of domestication and genetic selection for which an adequate physiological explanation has not been elucidated. It is possible that a prerequisite for high egg production is a refractoriness or insensitivity to the antigonadal effects of prolactin and that genetic selection has favored the retention of this genotype.

From the data in Figures 2, 3, and 4 it is evident that a large peak in prolactin precedes the onset of lay. This peak coincides with the extension of the photoperiod from 6L:18D to 14L:10D and, therefore, this increase in prolactin may be a photoperiodic response. In the

Pekin duck (Gourdji and Tixier-Vidal, 1966) and the Japanese Quail (Gourdji, 1970; Alexander and Wolfson, 1970) a similar increase in the prolactin content of the pituitary gland was observed following experimental photostimulation. Tixier-Vidal and Assenmacher (1962) noted an increase in the activity of the presumptive prolactin secreting cells after exposing male ducks to long days. However, in the present study, the increase in prolactin during weeks 1 and 2 cannot be attributed to a photoperiodic effect with certainty, since the hens were transported a distance of approximately 80 miles immediately before the first sample was removed. Therefore, the change in plasma prolactin may be similar to the stress-induced increases in prolactin which have been observed in several mammalian species by Johke (1970), Raud *et al.* (1971), and Frantz (1973).

## ACKNOWLEDGMENTS

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...A. Robin, and the staff of the obstetrics and gynaecology ...partment of the Baragwanath Hospital for allowing us to study ...atients under their care; and Dr. C. Kniep for permission to

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# Galactorrhoea: Successful Treatment with Reduction of Plasma Prolactin Levels by Brom-ergocryptine

J. M. BESSER, LYNNE PARKE, C. R. W. EDWARDS, ISABEL A. FORSYTH, A. S. McNEILLY

...ritish Medical Journal, 1972, 3, 669–672

## Summary

**...n five patients with inappropriate lactation and amen...rrhoea or impotence brom-ergocryptine was found to ...uppress the lactation and to diminish the raised plasma ...rolactin levels. The responses to treatment suggest that ...here may be an inverse relationship between prolactin ...ecretion and gonadotrophin secretion in man.**

## ...ntroduction

In most patients inappropriate lactation is accompanied by ...aised plasma levels of prolactin. Inappropriate lactation may ...e due to hypothalamic or pituitary disease, or to the action of psychoactive drugs (Forsyth *et al.*, 1971; Kleinberg and Frantz, 1971; Besser and Edwards, 1972). This hyperprolactinaemia is usually accompanied by reduced plasma gonadotrophin levels and amenorrhoea in women and impotence in men. There may be a reciprocal mechanism controlling secretion of prolactin and the gonadotrophins (Ben-David *et al.*, 1971; Kamberi, *et al.*, 1971a, 1971b). Pathological galactorrhoea is difficult to treat, though if it follows medication with the contraceptive pill it may occasionally respond to clomiphene. We now report the use of the ergot alkaloid 2-Br-alpha-ergocryptine (referred to in this paper as brom-ergocryptine) in five patients with galactorrhoea and show that it rapidly lowers plasma prolactin levels leading to cessation of lactation and, with the exception of a patient who had undergone partial hypophysectomy, to resumption of normal gonadal function with menstruation or potency. Lutterbeck *et al.* (1971) previously reported preliminary clinical studies and termination of galactorrhoea in three non-puerperal women on brom-ergocryptine, and Varga *et al.* (1972) showed that it inhibits puerperal lactation.

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## Methods

*Prolactin Bioassay.*—The lactogenic response obtained in cultured mammary tissue of pseudopregnant rabbits was used

as an index of the lactogenic activity in the plasma samples. Details of the methods were given by Forsyth and Myres (1971) and Forsyth *et al.* (1971). This assay responds only to prolactin, placental lactogen, and primate growth hormone. In every plasma sample assayed for prolactin the immunoreactive growth hormone levels were also measured and in each case were shown to be too low to register in the prolactin bioassay. The lactogenic activity of the plasma samples was therefore due to their prolactin content. The assay is only semiquantitative and the plasma prolactin concentration was estimated by comparison with the activities of known amounts of standard sheep prolactin (NH-P-S6, 25 IU/mg); results are expressed in ng/ml ovine prolactin equivalent. The sensitivity of this assay in plasma is about 50 ng/ml, and plasma from normal control subjects shows no activity (Forsyth *et al.* 1971). Brom-ergocryptine itself and any metabolites contained in plasma from patients on long-term therapy were shown not to interfere with the prolactin bioassay.

*Prolactin Radioimmunoassay.*—This assay was performed as described by Hwang *et al.* (1971) using antiserum raised against human growth hormone and $^{125}$I-labelled human prolactin. Growth hormone concentrations less than 1,000 ng/ml do not cross-react in this system. The minimum detectable level of prolactin in plasma was 22 ng/ml, and in normal subjects concentrations are below this level.

*Other Hormone Assays.*—Plasma fluorogenic corticosteroids, serum immunoreactive growth hormone (GH), thyrotrophin (TSH), luteinizing hormone (LH), protein bound iodine (P.B.I.), and T-3 uptake were measured by standard techniques (details of the methods used were given by Hall *et al.*, 1972, and Besser *et al.*, 1972) and plasma 17-hydroxyandrogens (17-OHA) as a measure of testosterone concentration by the technique of Anderson (1970).

*Clomiphene Stimulation Tests.*—Doses of 150 or 200 mg per day of clomiphene were given and the plasma LH response was followed over 10 days. The lower dose was used in the female patients. In normal subjects the LH levels rise outside the normal range during clomiphene administration (Anderson *et al.*, 1972). In this LH assay the normal range is 0·8-4·5 mU/ml in males and in females during the follicular phase of their menstrual cycle.

*Insulin tolerance tests* were performed using 0·15 U/kg soluble insulin intravenously, with measurement of the GH, plasma corticosteroid, and blood sugar responses. In each case the blood sugar fell to less than 40 mg/100 ml and the patient was seen to sweat.

*Thyrotrophin-releasing hormone (TRH)* tests were performed according to Ormston *et al.* (1971), sampling for serum TSH at 0, 20, and 60 minutes after the intravenous injection of 200 μg TRH.

## Patients

No patient had evidence of hepatic or renal disease and none gave a history of taking a drug likely to cause galactorrhoea (Besser and Edwards, 1972). All patients were euthyroid, had normal visual fields, and in all the serum GH level was suppressed to less than 5 ng/ml during an oral glucose tolerance test, thus excluding a diagnosis of acromegaly. No patient had clinical or biochemical evidence of Cushing's syndrome. The major clinical and endocrine findings before treatment are shown in Table I. Additional features are given below.

*Case 1.*— A man with bilateral mild gynaecomastia and copious lactation, a female distribution of body fat, and normal body hair. P.B.I. 4·7 μg/100 ml, T-3 resin 1·01, plasma 17-OHA 8·5 ng/ml (normal range 4·9-21·5 ng/ml). TRH test: serum TSH level at 0 min 2·9, 20 min 15·0, 60 min 10·6 μU/ml (normal response). Urine total oestrogen excretion (Searle's) 5 and 7 μg/24 hr (normal), air encephalogram normal.

*Case 2.*—Nulliparous woman. P.B.I. 4·9 μg/100 ml, T-3 resin 1·09, urinary total oestrogens 11 and 29 μg/24 hr. TRH test: serum TSH level at 0 min 2·1, 20 min 12·0, 60 min 6·8 μU/ml (normal response). Air encephalogram normal.

*Case 3.*—A woman with two past pregnancies, five and six years before. She had received an oral contraceptive for three years but the galactorrhoea and amenorrhoea did not start until 11 months after this had been stopped. P.B.I. 7·0 μg/100 ml, T-3 resin 1·02 TRH test: serum TSH at 0 min 1·9, 20 min 7·5, 60 min 6·0 μU/ml (normal response). Urinary total oestrogens 10 and 14 μg/24 hr.

*Case 4.*—A man with profuse bilateral galactorrhoea and mild gynaecomastia which developed three months after partial hypophysectomy for a chromophobe adenoma of the pituitary. He was taking oral hydrocortisone replacement (30 mg/day). P.B.I. 4·7 μg/100 ml, T-3 resin 1·01, plasma LH 0·6 mU/ml, plasma 17-OHA 5·3 ng/ml. TRH test: serum TSH at 0 min 1·0, 20 min 3·3, 60 min 2·7 μU/ml (impaired), fasting serum GH<2 ng/ml on several occasions. He had been impotent for 18 months before operation and did not need to shave.

*Case 5.*—Nulliparous woman with irregular periods for one year after stopping contraceptive pill followed by four years' complete amenorrhoea. Bilateral galactorrhoea noticed two months after stopping Ortho-Novin. P.B.I. 6·0 μg/100 ml, resin T-3 1·12, urinary total oestrogens 5 and 14 μg/24 hr, plasma 17-OHA 0·9 ng/ml (normal female level).

*Prolactin Levels.*—Plasma prolactin levels measured by bioassay and radioimmunoassay before treatment were raised in each patient (Figs. 1-3).

*Treatment.*—Treatment with brom-ergocryptine was started with 3 mg/day increasing after two days to 6 mg/day. In cases 4 and 5 the dose was further increased to 9 mg/day after six and two weeks respectively as the galactorrhoea had not completely subsided.

## Results

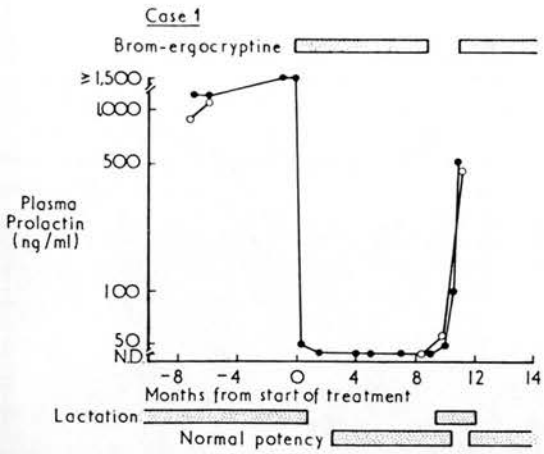
*Side Effects.*—One patient complained of heartburn and one of anorexia. These symptoms disappeared when the capsules were taken with food. No other side effects were seen and there were no changes in haemoglobin, leucocytes, platelets, liver function tests, blood urea, or electrolytes.

*Effects on Galactorrhoea and Amenorrhoea.*—The milk flow lessened within two weeks of treatment in each case and had completely disappeared after one to 12 weeks. Regular menstruation resumed in the women between three and six weeks after starting brom-ergocryptine and was maintained throughout

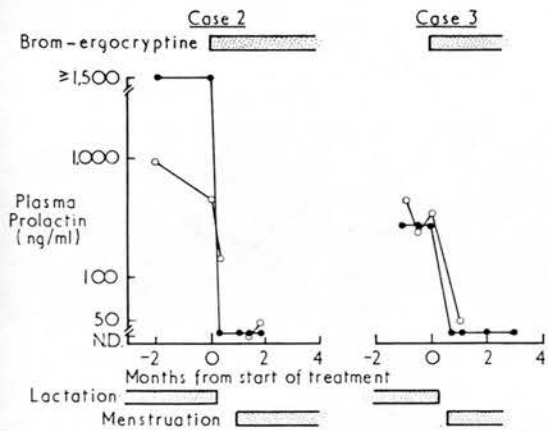
TABLE I—*Major Clinical and Endocrine Findings before Treatment*

| Case No. | Sex and Age | Duration of Disorder | | | Plasma LH Response to Clomiphene* (mU/ml) | Response to Hypoglycaemia | | Pituitary Fossa on Radiography |
|---|---|---|---|---|---|---|---|---|
| | | Galactorrhoea | Amenorrhoea | Impotence | | Plasma Corticosteroids† (μg/100 ml) | GH† (ng/ml) | |
| 1 | M. 25 | 18 months | — | 18 months | Impaired, 2·4 rising to 3·4 | Normal, 8 rising to 25 | Impaired maximum level 11 | Abnormal‡ |
| 2 | F. 25 | 6 months | 6 years | — | Normal, 1·5 rising to 6·2 | Normal, 10 rising to 29 | Impaired maximum level 17 | Enlarged |
| 3 | F. 25 | 12 months | 11 months | — | Impaired, 2·0 rising to 3·5 | Normal, 18 rising to 26 | Impaired maximum level 12 | Enlarged |
| 4 | M. 36 | 3 months | — | 2 years | — | On replacement | — | Enlarged |
| 5 | F. 27 | 5 years | 4 years | — | Normal, 4·4 rising to 5·3 | Normal, 17 rising to 29 | Normal maximum level 43 | Normal |

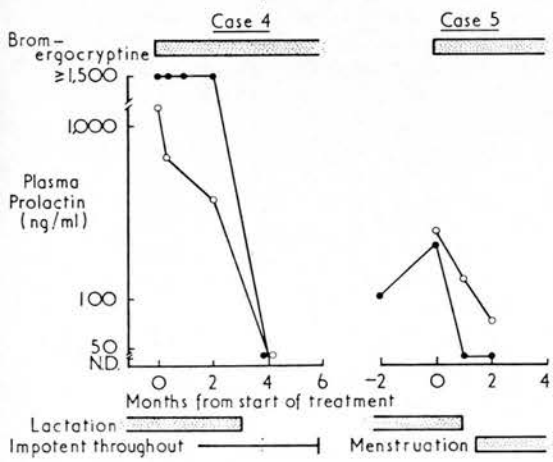
* Normal range for serum LH : 0·8 to 4·5 mU/ml in males and females in follicular phase of cycle, rising outside the normal range after 7 to 10 days of clomiphene (standard LH; M.R.C. 68/40).
† Normal response during insulin tolerance test (0·15 U/kg) : plasma corticosteroids rise to above 21 μg/100 ml, serum GH to above 20 ng/ml (standard GH; M.R.C. 69/46).
‡ Assymetric fossa suggestive of tumour but not frankly enlarged.




-Plasma prolactin concentrations measured by bioassay (●) and nunoassay (○) in a man with galactorrhoea during treatment with gocryptine. Changes in lactation and potency are also shown. Not detectable in assay—for example, prolactin levels < 50 ng/ml say, <22 ug/ml by radioimmunoassay).




-Plasma prolactin concentrations measured by bioassay (●) and radio-assay (○) in two women with galactorrhoea and enlarged pituitary during treatment with brom-ergocryptine. Changes in lactation and uation are also shown. N.D. = Not detectable in assay.




-Plasma prolactin concentrations measured by bioassay (●) and radio-assay (○) during treatment with brom-ergocryptine in a partially ysectomized man with galactorrhoea (case 4) and a woman who ed galactorrhoea and amenorrhoea after oral contraceptive medica-ase 5). The changes in lactation and menstruation are shown; the nained impotent. N.D. = Not detectable in assay.

ent. In the men potency returned in Case 1 in four weeks, ase 4 remained impotent throughout treatment. Details changes are given in Table II.

*sma prolactin levels* fell rapidly during treatment in each nd remained low as long as treatment was continued (Figs. 1-3). Further details on the response to treatment in the patients are given below.

TABLE II—*Effects of Treatment with Brom-ergocryptine*

| Case No. | Weeks to Resumption of Normal | | Weeks to Disappearance of Galactorrhoea | Maintenance Dose of Brom-ergocryptine (mg/day) |
|---|---|---|---|---|
| | Menses | Potency | | |
| 1 | — | 4* | 8 | 6 |
| 2 | 6 | — | 1 | 6 |
| 3 | 3 | — | 1 | 6 |
| 4 | — | Absent | 12 | 9 |
| 5 | 6 | — | 4 | 9 |

* Treatment continued 10 months then stopped; mild galactorrhoea returned within 2 weeks but became copious with return of impotence by 8 weeks.

*Case 1.*—During the 10 months of treatment with brom-ergocryptine the patient's breast tissue atrophied and the body fat was redistributed in a male pattern. The plasma 17-OHA rose from 8·5 to 15·9 mg/ml. Treatment (6 mg/day) was continued for 10 months then stopped. Mild galactorrhoea had returned two weeks later and was copious by two months, when impotence had also returned. During this time plasma prolactin levels rose (Fig. 1). Within three weeks of restarting brom-ergocryptine galactorrhoea had stopped and potency had returned.

*Case 2 and 3.*—Regular menstruation without galactorrhoea continued after four and six months respectively.

*Case 4.*—This man's breast tissue atrophied, was non-secreting, and the body fat adopted a male distribution during the six-months of therapy. Nevertheless, he remained impotent, with low plasma 17-OHA levels (3·7 ng/ml).

*Case 5.*—This patient had two menstrual periods and then stopped brom-ergocryptine. Menstruation had not returned during the next three months but the breasts remained non-secreting.

## Discussion

A raised plasma prolactin level appears to be the feature common to the symptoms of galactorrhoea and amenorrhoea or impotence whether due to a pituitary or hypothalamic tumour or to exposure to tranquillizers or oral contraceptives (Forsyth *et al.*, 1971; Kleinberg and Frantz, 1971; Besser and Edwards, 1972). Brom-ergocryptine suppresses pathological and puerperal lactation (Lutterbeck *et al.*, 1971; Varga *et al.*, 1972); we have confirmed the former observations and also now shown that the treatment is associated with suppression of the raised plasma prolactin levels whether measured by bioassay or radioimmuno-assay. It is of great interest that this cessation of galactorrhoea and suppression of prolactin levels was accompanied by regular menstruation in all the women and return of potency in the man who had not had a hypophysectomy (Case 1). When this man's therapy was stopped lactation and impotence promptly returned, together with a rise in the plasma prolactin levels. The condition remitted with resumption of treatment. Menstruation continued in the patient with the post-oral-contraceptive galactorrhoea-amenorrhoea syndrome only during the two months of treatment (Case 5).

This study allowed a direct comparison of the assay of plasma prolactin concentrations by bioassay and radioimmunoassay. As the figures show, there is good agreement between the results of the two techniques in the lower ranges of values. When the levels are greatly raised, above 400 ng/ml, the results of bioassay are much higher than the immunoreactive levels in some patients. The source of the dissociation is not clear.

The responses to treatment in this study suggest that there may be an inverse relation between prolactin secretion and gonadotrophin secretion in man. When serum prolactin levels are high gonadotrophin secretion is reduced, but normal gonadotrophin secretion can be achieved when the prolactin levels fall. There is evidence of a similar relation in rats (Ben-David *et al.*, 1971; Kamberi *et al.*, 1971a, 1971b).

Brom-ergocryptine and related compounds appear to act directly on the pituitary cells in mammals to suppress prolactin secretion (Flückiger and Wagner, 1968; Yanai and Nagasawa,

1970; Pasteels *et al.*, 1971; Billiter and Flückiger, 1971; Hoekfelt and Fuxe, 1972), and there is no evidence that it affects other pituitary hormones. The studies reported here show that it suppresses plasma prolactin levels in man, and it is possible that the drug is acting directly on the pituitary, since it was effective in the patient with a partial hypophysectomy whose pituitary remnant was presumably out of functional contact with the hypothalamus. The drug is effective in suppressing abnormal prolactin-dependent galactorrhoea without side effects in the doses used and at the same time it allows the return of normal menstruation or potency. While the value of oestrogen therapy to suppress puerperal lactation remains controversial it is general experience that it is ineffective in pathological lactation. Levodopa has been used, but while it has appreciable acute effects in lowering the plasma prolactin levels, its effects on the galactorrhoea are inconsistent and may not be sustained (Kleinberg *et al.*, 1971; Friesen *et al.*, 1972; Malarkey *et al.*, 1971; Turkington, 1972).

Treatment with brom-ergocryptine appears to offer a definite advance in the management of patients with galactorrhoea.

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# Long-term Treatment of Galactorrhoea and Hypogonadism with Bromocriptine

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## Summary

**Seventeen women and four men with galactorrhoea and associated hypogonadism have been treated with bromocriptine for 2 to 28 months. In 18 patients the gonadal status became normal as the galactorrhoea improved. The gonadally unresponsive patients had either pituitary tumours or premature menopause. Prolactin levels fell with treatment; withdrawal of the drug was associated with an increase in serum prolactin and a recurrence of the galactorrhoea and hypogonadism. Two patients tried to become pregnant on treatment and both succeeded. Raised prolactin levels appear to block the actions of the gonadotrophins at a gonadal level rather than prevent their synthesis or release; lowering prolactin secretion with bromocriptine allows resumption of normal gonadal function. Bromocriptine appears to be the treatment of choice for inappropriate lactation in association with hypogonadism on a long-term basis.**


## Introduction

The syndrome of inappropriate lactation has been shown usually to be associated with increased circulating prolactin levels (Frantz and Kleinberg, 1970; Forsyth *et al.*, 1971; Hwang *et al.*, 1971). Bromocriptine (2-brom-α-ergocryptine, CB 154) specifically reduces prolactin secretion by an action at the pituitary level and has been shown to be effective in the suppression of lactation whether it be puerperal (Varga *et al.*, 1972; Rolland and Shellekens, 1973) or pathological, and this is associated with a reduction in circulating prolactin levels (Lutterbeck *et al.*, 1971; Besser *et al.*, 1972 a; Leutenegger *et al.*, 1972; Del Pozo *et al.*, 1973; Varga *et al.*, 1973). Inappropriate lactation with high prolactin levels is usually associated with hypogonadism whether this be menstrual irregularity or amenorrhoea in women or impotence in men; early reports indicated that the gonadal function returns to normal as the hyperprolactinaemia and galactorrhoea abate during the early phases of bromocriptine therapy (Lutterbeck *et al.*, 1971; Besser *et al.*, 1972 a; Leutenegger *et al.*, 1972; Varga *et al.*, 1973).

We report our experience with long-term treatment of the galactorrhoea-hypogonadism syndrome with bromocriptine in 17 women and four men.

## Patients

The 21 patients (tables I and II) were first seen at St. Bartholomew's Hospital between 1969 and 1973. Preliminary accounts of five of them have been given elsewhere (cases 1, 4, 10, 18, and 19; Besser *et al.*, 1972 a). No patient had evidence of hepatic or renal disease and none gave a history of taking a drug likely to cause galactorrhoea, except for oestrogen and progestogen therapy in four cases. Six of the patients had previously been pregnant. Only one had evidence of acromegaly; there was no evidence of thyroid deficiency, and only in case 2 (with radiological evidence of a pituitary tumour) and case 18 was there evidence of adrenocortical deficiency.

*Galactorrhoea.*—In the women the galactorrhoea had been present for eight months to 10 years before presentation but in two it was found only on examination; in two others it had started in the post-partum period (see table III). Galactorrhoea in the men had been present for 3 to 15 months before presentation (see table IV).

*Gonadal Status.*—Thirteen women had had amenorrhoea for between six months and six years, preceded in two cases by irregular periods. Periods were irregular without amenorrhoea in four patients. Two patients had evidence of the polycystic ovary syndrome on gynaecography, and one was hirsute. Two amenorrhoeic women initially appeared to be suffering from premature menopause but this was confirmed by ovarian biopsy in only one, the ovary from the other patient containing normal but undeveloped ovarian follicles. The four men all had absent or reduced potency.

*Oestrogen-Progestogen Therapy.*—In four women galactorrhoea was preceded by treatment with an oestrogen-progestogen oral contraceptive preparation prescribed for irregular menstruation. In one patient the milk appeared during treatment and in the others it occurred afterwards.

*Pituitary Fossa Radiography.*—Seven patients (six women and one man) were shown to have abnormal fossae, and in two of the men the appearance was equivocal in view of a double contour to the floor of the fossa.

## Methods

Details of the methods for measuring serum immunoreactive prolactin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and growth hormone (GH) have been given elsewhere (McNeilly, 1973; Mortimer *et al.*, 1973; McNeilly and Hagan, 1974). Plasma corticosteroids (Mattingly, 1962) and total urinary oestrogen (Brown *et al.*, 1968, Searle) were measured fluorometrically. Plasma 17β-hydroxyandrogens (17-OHA) as a measure of testosterone and dihydrotestosterone concentrations were assayed by the technique of Anderson (1970).

In the assays used the normal ranges for serum LH (M.R.C. standard 68/40, 77 IU/ampoule) in male subjects was 0·8-13·3 U/l. and in female subjects in the follicular phase 1·4-9·8 U/l.; for FSH (M.R.C. standard 69/104, 10 IU/ampoule) the normal range in male subjects was 0·1-3·5 U/l. and in female subjects in the follicular phase 0·8-3·2 U/l. Plasma 17-OHA levels in male subjects normally range from 4·9 to 21·0 ng/ml.

*Clomiphene Stimulation Test.*—A dose of 3 mg/kg/day up to a maximum of 200 mg/day was given and the changes in serum gonadotrophin were followed for up to 10 days. In normal male subjects serum LH has been shown usually to rise above the normal range in this time (Anderson *et al.*, 1972). The normal range for menstruating women varies with the stage of the cycle (Vanden-Berg and Yen, 1973) and therefore comparisons of the results of the test in amenorrhoeic women with results in normal women is difficult.

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TABLE I—*Basal Data on Women with Galactorrhoea*

| Case No. | Age (Years) | Aetiology† | Parity | Serum Prolactin (ng/ml)‡ | | Serum LH (U/l.) | Serum FSH (U/l.) | 24-hr Urine Oestrogens (μg/Day)‖ | Reserve of: 1 | | Pituitary Fossa on Radiography | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Before Treatment | On Bromocriptine | | | | ACTH | GH | | |
| 1* | 26 | S.P.T. | 2 | 296 | | 4·8 | | 14 | N | I | Enlarged | |
| 2* | 36 | „ | 0 | 203 | 18 | 1·8 | 0·9 | 27 | I | N | „ | |
| 3* | 26 | „ | 2 | | | 7·0§ | | 23 | N | I | „ | |
| 4* | 26 | „ | 0 | 88 | 10·3 | 0·8 | | 11 | N | I | „ | |
| 5 | 19 | „ | 0 | >100 | 14·4 | <0·8§ | 0·1§ | 13 | N | I | „ | Chromophobe adenoma at operation |
| 6 | 39 | „ | 1 | | | 2 | 1·4 | | N | N | „ | Acromegaly—post irradiation |
| 7 | 27 | A.O.C. | 0 | 102 | | 0·8 | 1·1 | 11 | N | N | Normal | |
| 8 | 42 | „ | 0 | 22 | | 4·8§ | 12·5§ | 26 | N | N | „ | Dilated cerebral ventricles |
| 9 | 29 | „ | 0 | 94 | 14·4 | 10·4§ | 6·0§ | 25 | N | N | „ | |
| 10 | 27 | „ | 0 | 235 | | 4·4 | | 14 | N | N | „ | |
| 11 | 24 | Unknown | 0 | | | 1·8 | | 15 | N | N | „ | Polycystic ovary syndrome |
| 12 | 30 | „ | 2 | 15 | | 6 | 0·8 | 26 | N | N | „ | Polycystic ovary syndrome. Post-partum galactorrhoea |
| 13 | 36 | „ | 3 | | 11·6 | 21§ | | 12 | N | I | „ | Premature ovarian failure |
| 14 | 38 | „ | 1 | 8·2 | | 22 | | | | | „ | Post-partum galactorrhoea |
| 15 | 34 | „ | 0 | 23 | 10·8 | 31§ | 11§ | 13 | N | N | „ | Unknown |
| 16 | 27 | „ | 0 | 44 | 12 | 1·8 | | 14 | N | N | „ | Unknown |
| 17 | 34 | „ | 0 | 13 | | 1·6§ | | 24 | N | N | „ | Unknown |

*Presumed pituitary tumour from radiographical evidence.
†S.P.T. = Suspected pituitary tumour. A.O.C. = After oral contraceptive.
‡Normal range 6-25 ng/ml.
§Excessive response to LH/FSH-RH.
‖Normal range (follicular phase) 7-30 μg/day.
¶Based on responses to insulin tolerance test. N = Normal. I = Impaired. (Hall *et al.*, 1972.)

TABLE II—*Basal Data on Men with Galactorrhoea*

| Case No. | Age (Years) | Aetiology† | Serum Prolactin (ng/ml)‡ | | Serum LH (U/l.) | Serum FSH (U/l.) | Plasma 17-OHA (ng/ml)§ | Reserve of:1 | | Pituitary Fossa on Radiography | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Before Treatment | On Bromocriptine | | | | ACTH | GH | | |
| 18 | 36 | S.P.T. | 1,572 | 7·1 | 3·2 | | 5·3 | I | | Enlarged | Chromophobe adenoma at operation |
| 19* | 26 | „ | 899 | <22 | 4·8 | | 8·5 | N | I | Equivocal | |
| 20* | 20 | „ | 73 | 13·8 | 5·6 | 1·7 | 10·7 | N | N | Equivocal | |
| 21 | 28 | Unknown | 30 | 13·9 | 2·4 | 0·75 | 4·8 | N | N | Normal | |

*Presumed pituitary tumour from radiographical evidence.
†S.P.T. = Suspected pituitary tumour.
‡Normal range 6-25 ng/ml.
§Normal range 4·9-21·0 ng/ml.
¶Based on responses to insulin tolerance test. N = Normal. I = Impaired. (Hall *et al.*, 1972.)

*Gonadotrophin-releasing hormone tests* were performed using 100 μg gonadotrophin-releasing hormone (LH/FSH-RH, Hoechst) intravenously as a bolus with measurement of serum LH and FSH at 0, 20, and 60 minutes (Besser *et al.*, 1972 b; Mortimer *et al.*, 1973).

*Bromocriptine Therapy.*—Treatment was usually started with a dose of 2·5 mg twice daily with food and increased after two months if full clinical improvement, as indicated by cessation of galactorrhoea and return of normal gonadal function, had not been attained. Treatment was arbitrarily stopped after 2 to 28 months in nine patients.

## Results

The clinical and biochemical responses to treatment are shown in tables III and IV.

*Galactorrhoea* was improved in all the patients. The improvement usually became noticeable within one week and ceased completely in 12 cases between one week and three months after starting therapy (tables III and IV). In five cases a trace of breast milk could still be expressed but the breasts did not discharge spontaneously and did not inconvenience the patient. In one patient with premature ovarian failure (case 13) the galactorrhoea was initially reduced but was still troublesome and could not be further influenced with oestrogen. In six of the patients in whom treatment was withdrawn after one year or more the galactorrhoea recurred within three days to two weeks but responded again on resumption of bromocriptine. Two other patients had treatment stopped when they became pregnant.

*Gonadal Status.*—In 11 of the 13 patients with amenorrhoea (table III) menstruation was restored from 3 to 24 weeks after starting therapy with bromocriptine. The three patients whose gonadal function did not change had the following associated conditions: hypophysectomy and pituitary irradiation, acromegaly, and premature ovarian failure. Three patients previously had irregular or heavy periods but not amenorrhoea with the galactorrhoea uncomplicated by pituitary disease, and these were restored to normal. Two patients attempted to become pregnant and both succeeded within two months; one was delivered of a normal child and the other was still pregnant. All four male patients (table IV) regained normal potency within six months. Therapy was withdrawn from nine responsive patients with the following results (tables III and IV): amenorrhoea recurred in four patients, periods became irregular in one, one was pregnant, and one man became impotent again.

*Prolactin levels* were measured basally in 17 patients and were raised in 12 (tables I and II). In response to bromocriptine serum prolactin was reduced to normal in each of the 11 patients in whom it was measured during treatment. In three patients prolactin levels were measured again after stopping treatment and they were again raised (tables III and IV).

*Gonadotrophins.*—Serum LH and FSH levels were detectable and normal in all patients before treatment except for cases 9, 13, and 15, in which they were raised (table I). When treated with clomiphene before bromocriptine therapy (table V) the eight women with normal basal gonadotrophin levels showed a definite rise in gonadotrophins but in none did the level exceed the normal range for the follicular phase of the cycle, and this response was probably impaired. The responses to clomiphene were impaired in all three men tested. Seven out of 11 women but neither male tested with LH/FSH-RH had excessive gonadotrophin responses. Despite these abnormal gonadotrophin findings 18 of the 21 patients with disordered gonadal function reverted to apparently normal gonadal status with abolition of the galactorrhoea on bromocriptine therapy.

*Dose of Bromocriptine.*—Maintenance doses of 5 to 32 mg/day were used but the usual dose was 7·5 mg/day. The duration of the treatment was 2 to 28 months.

*Side Effects.*—The only side effects were dyspepsia and nausea in five patients, and these occurrred about two hous after drug administration. The symptoms were particularly severe when treatment was initiated and when taken on an empty stomach; they could be avoided in most cases by taking the capsules in the middle of a meal. When symptoms continued the dose was reduced to 1 mg twice daily for a time, after which it could be gradually increased without recurrence of symptoms. In no case was it necessary to withhold treatment because of side effects. Though bromocriptine is an ergot alkaloid there were no symptoms or signs of ergotism or digital vasospasm. Five patients lost from 3·5 to 18 kg in weight over 3 to 18 months in spite of no apparent change in appetite or food intake. They had all gained this weight during the time that they had been galactorrhoeic.

## Discussion

Altogether 20 of the 21 patients with galactorrhoea responded to bromocriptine with reduction or cessation of galactorrhoea, and gonadal function was restored to normal in 14 of the 17 women and in all four men. When serum prolactin levels were measured during treatment they had in all cases been reduced to normal. When treatment was withdrawn galactorrhoea returned in all but one case and each patient again became hypogonadal but the symptoms abated again when bromocriptine therapy was resumed. Side effects were not a serious problem and were present in only a quarter of the patients, and only as therapy started. The early side effects of dyspepsia and nausea were avoided either by taking the drug with food or by reducing the dose for a short while; it was never necessary to withhold treatment because of side effects. These results confirm our preliminary findings (Besser *et al.*, 1972 a) and indicate that this compound may be used for continuous therapy without any escape of prolactin secretion or recurrence of galactorrhoea while on bromocriptine; prolonged treatment, up to 28 months in two cases, was devoid of adverse effects. We have no evidence, however, that the underlying pathological process is altered by bromocriptine therapy.

TABLE III—*Women. Duration of Galactorrhoea and Amenorrhoea and Effects of Treatment*

| Case No. | Aetiolog | Duration of Galactorrhoea (Months) | Duration of Amenorrhoea (Months) | Bromocriptine Dose (mg/Day) | Interval to Cessation of Galactorrhoea (Months) | Interval to Cessation of Amenorrhoea (Months) | Duration of Treatment (Months) | Effects of Stopping Treatment |
|---|---|---|---|---|---|---|---|---|
| 1 | S.P.T. | 14 | 12 | 6·0 | 0·75 | 1 | 16 | Milk back in 3 days. Prolactin 50 ng/ml after withdrawal of bromocriptine |
| 2 | „ | 11 | 15 | 5·0 | 2 | 6 | 11 | Immediate amenorrhoea |
| 3 | „ | E. | 24 | 5·0 | 1 | 1 | 15 | Not stopped |
| 4 | „ | 8 | 72 | 6·0 | 0·25 | 1·5 | 18 | Immediate amenorrhoea. Galactorrhoea in 2 weeks. Prolactin 88 ng/ml after withdrawal of bromocriptine |
| 5 | „ | E. | 24 | 15·0 | >4† | >4† | 4 | Not stopped |
| 6 | „ | 120 | 12* | 5·0 | 1‡ | N.C. | 8 | Not stopped |
| 7 | A.O.C. | 48 | 68 | 7·5 | 0·5‡ | 1·5 | 2 | Not stopped |
| 8 | „ | 120 | 200* | 7·5-10·0 | 3 | N. | 8 | Not stopped |
| 9 | „ | 84 | 6 | 7·5 | 0·5 | 1 | 2 | Not stopped |
| 10 | „ | 60 | 48 | 9·0 | 1 | 1·5 | 2 | Breasts remained dry. Amenorrhoea returned |
| 11 | Unknown | 18 | 24 | 6·0 | 0·3 | 2 | 6·5 | Immediate galactorrhoea and amenorrhoea |
| 12 | „ | 84 | 6 | 7·5 | 0·75‡ | 2 | 2 | Galactorrhoea in 8 weeks |
| 13 | „ | 108 | 36 | 15·0 | N.C. | N.C. | 12 | None |
| 14 | „ | 18 | 48* | 5·0 | 0·5‡ | N. | 6 | Periods irregular |
| 15 | „ | 18 | 6 | 7·5 | 1 | 2·5 | 3 | Not stopped |
| 16 | „ | 18 | 24 | 6·0 | 1·5 | 0·75 | 11 | Post-partum breast engorgement. Bromocriptine stopped when became pregnant. Galactorrheoa returned within a few days |
| 17 | „ | 54 | 60* | 7·5 | 0·75‡ | 1 (N.) | 3 | Unknown |

*Dysfunctional bleeding (irregular, often heavy menstruation).
†Had not stopped.
‡Trace of milk persisted.
E = Found only on examination. N.C. = No change. N = Periods normalized.

TABLE IV—*Men. Duration of Galactorrhoea and Impotence and Effects of Treatment*

| Case No. | Aetiology | Duration of Galactorrhoea (Months) | Duration of Impotence (Months) | Bromocriptine Dose (mg/Day) | Interval to Cessation of Galactorrhoea (Months) | Interval to Cessation of Impotence (Months) | Duration of Treatment (Months) | Effects of Stopping Treatment |
|---|---|---|---|---|---|---|---|---|
| 18 | S.P.T. | 3 | 24 | 9·0 | 3 | 6 | 28 | Not stopped |
| 19 | „ | 18 | 18 | 6·0 | 2 | 1 | 28 | Impotence with milk in 1 week. Prolactin 500 ng/ml after withdrawal of bromocriptine |
| 20 | „ | 4 | 36 | 32·0 | 3* | 2 | 4 | Not stopped |
| 21 | Unknown | 15 | ? | 5·0 | 0·5† | ? | 8·5 | Not stopped |

*Had not stopped.
†Trace of milk persisted.

TABLE V—*Gonadotrophin Responses to Clomiphene on Days 0, 5, 7, and 10*

| Case No. | Serum LH (U/l.) | | | | Serum FSH (U/l.) | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 7 | 10 | 0 | 5 | 7 | 10 |
| | | | | *Women* | | | | |
| 1 | 4·8 | 3·0 | 6·6 | 4·2 | | | | |
| 2 | 1·8 | 2·4 | | | | | | |
| 4 | 0·8 | 11·1 | 10·4 | 9·6 | | | | |
| 5 | <0·8 | | 1·5 | 1·1 | 0·1 | | 1·2 | 1·2 |
| 7 | 0·8 | 4·0 | 5·0 | 5·6 | 1·1 | 3·3 | 8·0 | 2·8 |
| 10 | 8·6 | | 9·4 | 10·6 | | | | |
| 11 | 1·8 | 7·8 | 7·2 | 13·4 | | | | |
| 13 | 21·4 | 20·8 | | 22·2 | | | | |
| 15 | 30·8 | 42·4 | 26·2 | 20·4 | 10·9 | >25 | >25 | >25 |
| 16 | 1·8 | 6·8 | 8·6 | 8·8 | | | | |
| | | | | *Men* | | | | |
| 19 | 4·8 | 4·8 | 5·2 | 6·2 | | | | |
| 20 | <0·8 | 4·4 | | 8·6 | <0·1 | 0·7 | | 1·7 |
| 21 | 2·4 | 5·6 | 6·4 | 9·8 | | | | |

Bromocriptine is a potent suppressor of prolactin secretion, and this has been confirmed both clinically and by measuring the circulating prolactin levels. Levodopa also suppresses prolactin secretion but has the disadvantage that its duration of action is short (Malarkey *et al.*, 1971), the incidence of side effects is high with doses necessary for adequate suppression, and it does not consistently improve galactorrhoea (Copinschi *et al.*, 1973). Recent work indicates that bromocriptine is a dopaminergic agonist (Corrodi *et al.*, 1973), and it is possible that its suppression of prolactin is through this mechanism but with a more prolonged length of action than levodopa. The therapeutic actions as well as the side effects would be compatible with this as they are very similar to those of levodopa.

Before treatment all the patients had either menstrual irregularities or amenorrhoea or impotence. Nevertheless, basal gonadotrophin levels were normal in all patients but two, both of whom had high rather than low levels. Excessive responses to LH/FSH-RH were seen in seven out of 13 patients tested but there was no correlation between gonadotrophin levels and the levels of circulating immunoreactive prolactin, and this excessive response to LH/FSH-RH confirms our previous report (Mortimer *et al.*, 1973). A normal response may also occur but we have not seen an impaired response in this context, in contrast to the experience of others (Zarate *et al.*, 1973).

In the galactorrhoea syndromes the gonadal function is usually impaired irrespective of whether immunoreactive prolactin levels are raised. When these patients are treated with bromocriptine and prolactin levels fall the gonadal function usually returns to normal. The only patients in whom this did not occur in the present series were two with pituitary tumour and one with a premature menopause. In five patients the gonadal function became normal with cessation of galactorrhoea even though radiographical evidence of a pituitary tumour was present. The hypogonadism which exists in association with the pretreatment galactorrhoea does not appear to be due to inadequate gonadotrophin synthesis or reserve as the responses to LH/FSH-RH are not impaired; however, the responses to clomiphene, which normally acts through the hypothalamus, are impaired. This situation is analogous to that in post-partum women in whom gonadotrophin secretion occurs early during lactation but does not cycle normally for some time and oestrogen secretion is low (Reyes *et al.*, 1972). During puerperal lactation the ovaries appear to be refractory to exogenous gonadotrophin during the time that prolactin secretion is increased (Zarate *et al.*, 1972). It appears, therefore, that the raised prolactin levels in some way have antigonadotrophic actions at the gonadal level.

It seems likely that the hypogonadism in patients with pathological lactation is secondary to an inappropriately raised prolactin level, since the hypogonadism as well as the galactorrhoea respond to bromocriptine, which specifically blocks prolactin secretion. The reduction in prolactin appears to allow the circulating gonadotrophin levels to act on the gonad to stimulate steroidogenesis. The sex steroids then may feed back on the hypothalamus and pituitary to allow normal release of gonadotrophins, leading to a return of normal gonadal function, ovulation, and spermatogenesis.

One of the patients with persistently raised gonadotrophin levels, galactorrhoea, and amenorrhoea was of particular interest. It was initially tempting to ascribe a diagnosis of a premature menopause to her; however, ovarian biopsy showed the presence of follicles and treatment with bromocriptine resulted in cessation of the galactorrhoea with regular menstruation. Similarly two patients had galactorrhoea with the polycystic ovary syndrome and both responded well. This association was described by Forbes *et al.* (1954), and a number of their patients had hirsuties and seborrhoea as well as galactorrhoea and amenorrhoea or irregular periods. They also noted urinary 17-oxosteroid excretion at the upper limit of normal, or above, and we recorded this in six of our women and one of the men. It seems possible that prolactin may alter adrenocortical androgen production. The amenorrhoea associated with oral contraceptive medication also responds well to bromocriptine if galactorrhoea is present as well. A trial of this drug in post-oral contraceptive amenorrhoea without galactorrhoea is currently taking place.

Bromocriptine is an effective, safe, and satisfactory long-term treatment for galactorrhoea and it reverses the associated hypogonadism in both men and women. It appears to be the treatment of choice for this condition, since it is effective and devoid of adverse effects.

We are grateful to Dr. R. Evans, of Sandoz Products Ltd., for the provision of bromocriptine, to the Peel Medical Trust, the Joint Research Board of St. Bartholomew's Hospital, and the Wellcome Trust for financial support, and the physicians who referred the patients.

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British Medical Journal 3, 729 (1974).

## Metaclopramide and Prolactin

SIR,—Dr. M. Shaklai and others (18 May, p. 385), reporting the occurrence of cardiac arrhythmia during the administration of metaclopramide, make reference to the fact that this drug is considered to be free of side effects in adults. In recent studies we have discovered that metaclopramide is a potent stimulator of prolactin release in both men and women when given intramuscularly, intravenously, or orally in a dose of 10 mg. Levels increased from 3- to 8-fold within five minutes of either an intramuscular or intravenous injection and within 60 minutes after oral administration; these levels remained elevated for at least eight hours. Long-term administration of metaclopramide has occasionally been associated with galactorrhoea.

Metaclopramide is a derivative of procainamide and we have previously reported[1] that a related compound, sulpuride, is also a very potent releaser of prolactin in man. In view of a recent report on the effects of prolactin on the rat heart in vitro, causing changes in both heart rate and amplitude of contraction and inducing dysrhythmia,[2] it would seem possible that the effects observed by Dr. Shaklai and his colleagues in their patient may be related to the elevation in the circulating prolactin. We are at present investigating the site of action of metaclopramide and the effects of other procainamide derivatives on prolactin secretion in man.—We are, etc.,

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1 Thorner, M. O., *et al.*, *Journal of Endocrinology*, 1974, **61**, 132.
2 Nassar, B. A., *et al.*, *British Medical Journal*, 1974, **2**, 27.

# CORRELATION BETWEEN PLASMA LEVELS OF PROLACTIN AND CHLORPROMAZINE IN PSYCHIATRIC PATIENTS

BY

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PRELIMINARY COMMUNICATION

# Correlation between plasma levels of prolactin and chlorpromazine in psychiatric patients[1]

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SYNOPSIS Plasma levels of chlorpromazine (CPZ) and prolactin were measured repeatedly in 14 psychiatric patients throughout CPZ treatment. Mean prolactin level was elevated in 11 subjects (all six women and five of eight men). Mean plasma prolactin correlated significantly with mean plasma CPZ but not with the dose of the drug. Only patients with mean plasma prolactin above 35 ng/ml developed Parkinsonian side-effects.

There is evidence that a single dose of chlorpromazine (CPZ) produces a rise of plasma prolactin in human subjects (Kleinberg *et al.*, 1971; Friesen *et al.*, 1972) and that circulating levels of prolactin are elevated in psychiatric patients during treatment with phenothiazines and other neuroleptics (Frantz *et al.*, 1972; Beumont *et al.*, 1974). These findings suggest two questions: (1) is the prolactin response related either to the daily dose or to the plasma concentration of the drug, and (2) is it related to any of the clinical effects of the neuroleptics?

## METHODS

### SUBJECTS

We measured plasma levels of prolactin and CPZ in 14 schizophrenic patients throughout chlorpromazine treatment which in all but two subjects lasted for at least four weeks. CPZ doses ranged from 225 to 600 mg per day. Five patients had received phenothiazine drugs in the previous three months; one had received amitriptyline and tranylcypromine (Parstelin). The rest had no psychotropic drugs in this period. Blood samples were collected weekly, both before and two hours after the morning dose of the drug, starting from the fourth or seventh day of the treatment. Clinical response was assessed on the days of blood sampling using the Brief Psychiatric Rating Scale (Overall and Gorkham, 1962) for the mental state and check-lists for the side effects of Parkinsonian (Simpson and Angus, 1970), and autonomic side effects (devised for the investigation). Plasma prolactin was measured by a specific double antibody radioimmunoassay (McNeilly and Hagen, 1974). Plasma CPZ was determined by a modification of Curry's gas-chromatographic method (Curry, 1968) which allows the measurement of the parent drug and three of its metabolites (SO-CPZ, 70H-CPZ, and $Nor_1CPZ$) and which uses an internal standard to correct for variability of extraction (MacKay *et al.*, 1974). Further detail of the method is available on request, and will be published in the final report.

[1]This work was supported by grants from the Medical Research Council and the Oxford Regional Hospital Board.
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## RESULTS

In all subjects plasma levels of prolactin and CPZ fluctuated erratically from week to week. There was no consistent difference between the initial and the later weeks of treatment. Mean levels of prolactin, measured both before and after the morning dose of the drug, were above the range of normal values in all six female patients—that is, above 20 ng/ml—and in five of the eight male patients—that is, above 20 ng/ml. In all these subjects plasma prolactin was elevated from the end of the first week of CPZ administration throughout the treatment period. Mean prolactin levels of the women patients both before and after the morning dose—$41 \pm 13$ ng/ml and $42 \pm 11$ ng/ml respectively—were higher than the corresponding values from men—$24 \pm 10$ ng/ml and $30 \pm 12$ ng/ml (these

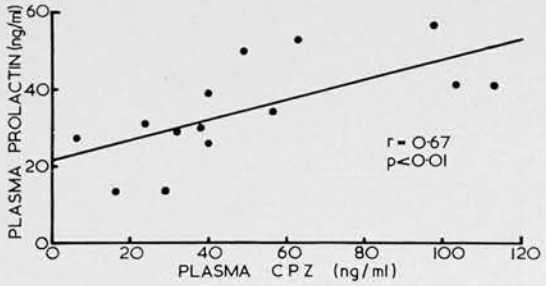



FIG. 1. *Correlation between plasma levels of prolactin and CPZ in 14 subjects. Individual means from samples taken two hours after the morning dose of the drug.*

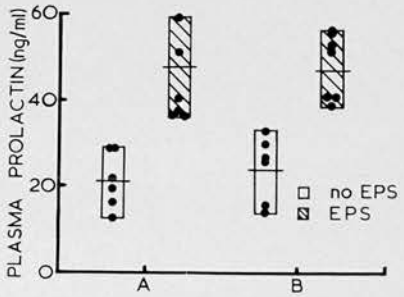



FIG. 2. *Prolactin level and extrapyramidal side-effects (EPS) in 12 patients.* A: *individual means of morning plasma prolactin.* B: *individual means of plasma prolactin two hours after the morning dose of CPZ.*

and the corresponding data that follow are expressed as means and standard deviations). Only in two male patients was there a rise in plasma prolactin after every morning dose of CPZ; in the remaining subjects the changes in plasma prolactin after the morning dose were inconsistent.

Means of weekly estimations were used because prolactin levels fluctuate from hour to hour; thus the sustained, or average, level is more likely to be relevant to the effects of CPZ. However, correlations calculated using every pair of values were also significant: between prolactin and chlorpromazine $P<0.001$ both from samples taken before and two hours after the morning dose of CPZ.

Mean plasma prolactin levels of individual patients were not related to the daily dose of the drug. There was, however, a significant positive correlation between prolactin level and plasma CPZ concentration. Figure 1 shows the relation between the levels of plasma prolactin and plasma CPZ after the morning dose of the drug ($r=.67$; $P<0.01$). The correlation between the corresponding levels from samples taken before the morning dose was also significant ($r=.69$; $P<0.01$).

The relationship between prolactin levels and symptoms of Parkinsonian was examined in 12 patients—two were omitted who presented with extrapyramidal symptoms before chlorpromazine treatment began. None of the patients whose mean plasma prolactin remained below 35 ng/ml before or after the morning dose of the drug showed extrapyramidal symptoms, while all patients with higher levels developed mild or moderate Parkinsonism (Fig. 2). Mean levels of CPZ both before and after the morning dose were also higher in patients with extrapyramidal side-effects ($37\pm27$ ng/ml and $74\pm30$ ng/ml) than in subjects free from these symptoms ($11\pm7$ ng/ml and $24\pm12$ ng/ml). Neither the therapeutic effect of CPZ nor the presence of autonomic side-effects was related to prolactin levels.

## COMMENT

Effects of phenothiazines on the central nervous system are difficult to measure in man. The findings that plasma levels of prolactin are related to those of CPZ and that there is a relationship between high prolactin and Parkinsonian side-effects suggest that prolactin levels provide one index of the effect of phenothiazines on the central nervous system. Both excessive prolactin release (De Wied and De Jong, 1974) and a Parkinsonian syndrome appear to be produced by functional dopamine deficiency (in the hypothalamus and basal ganglia respectively). It is not surprising, therefore, that they occur in the same individuals during administration of CPZ which is thought to block dopamine receptors.

We are grateful to Dr. P. MacKinnon and Dr. B. M. Mandelbrote for their help and advice; to Dr. F. J. Letemendia and Dr. A. D. Harris for allowing us to study patients under their care; and to Mrs. E. Green and Mrs. E. Kuht for nursing assistance.

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# SHORT COMMUNICATIONS

## SHORT-TERM VARIATION IN BLOOD LEVELS OF PROLACTIN, LUTEINIZING HORMONE AND FOLLICLE-STIMULATING HORMONE IN NORMAL MEN THROUGHOUT THE DAY

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(*Received* 12 *November* 1973)

The release of prolactin may be related to that of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Bern & Nicoll, 1968). The present study was designed to determine whether there is any relationship between the short-term variation in blood LH and FSH levels (Naftolin, Yen & Tsai, 1972; Nankin & Troen, 1972), and that of prolactin levels in the blood.

A total of 230 blood samples were obtained from five normal men (age 24–29) at 15-min intervals between 09.00 and 16.30 h. All subjects continued their normal daily routine during the experiment. Samples were collected through an indwelling Polythene cannula, and the plasma was separated and stored at −20 °C. Prolactin levels were determined by radioimmunoassay (McNeilly, 1973). The results are expressed as ng standard human pituitary prolactin (kindly provided by Dr H. Friesen)/ml. Serum LH and FSH were measured by double-antibody radioimmunoassay as described by McNeilly & Chard (1974). Results were expressed in mu. LH standard (MRC 68/40)/ml and mu. FSH standard (MRC 68/39)/ml, assuming a unitage per ampoule of 40 and 33 units of LH and FSH respectively. All samples from each individual were measured in the same assay to reduce inter-assay variation.

Episodic release of LH was seen in all five subjects, with peaks occurring at intervals of 140 to 200 min, and an overall coefficient of variation of 40·6%. Levels of FSH showed similar variation (29·2%). Prolactin levels showed less overall variation (18·1%), but there were striking random fluctuations; the peaks did not correlate with those of LH (Fig. 1).

The pulsatile release of LH observed in this study was very similar to that described previously by Nankin & Troen (1971) and Naftolin *et al.* (1972), and was not accompanied by any change in FSH levels. The release of prolactin showed considerable fluctuation, similar to that seen in normal female subjects at different stages of the menstrual cycle (McNeilly & Chard, 1974). There was no correlation with the variation in LH levels, indicating that the release of prolactin and LH are unrelated in the normal man.

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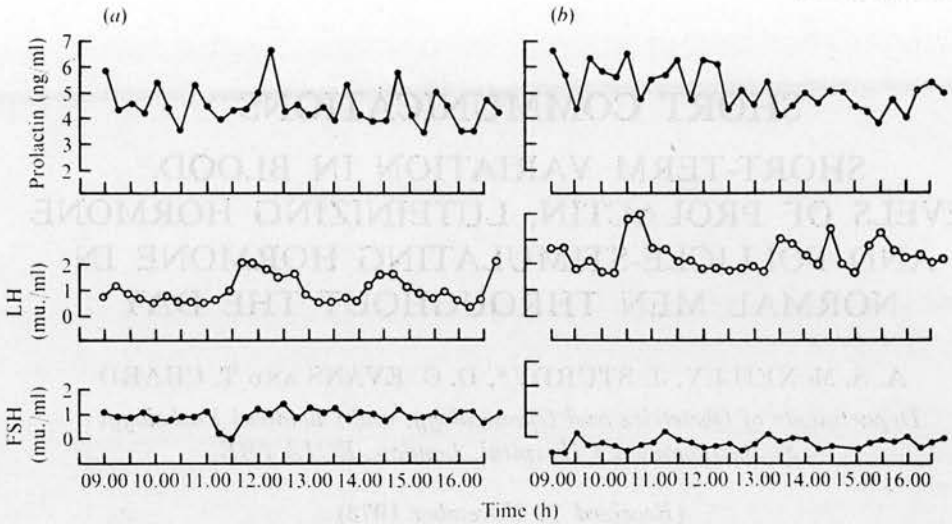



Fig. 1. Serum prolactin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels at 15-min intervals between 09.00 and 16.30 h in two normal men (*a*) and (*b*).

A. S. McNeilly is in receipt of a grant from the Wellcome Trust. We would also like to thank Miss J. Hook for technical assistance.

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# SHORT-TERM VARIATION IN BLOOD LEVELS OF PROLACTIN IN WOMEN

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Levels of prolactin in the circulation show wide short-term fluctuations (McNeilly & Chard, 1974; McNeilly, Sturdy, Evans & Chard, 1974) suggesting episodic release. Prolactin levels were measured in closely spaced serial samples in an attempt to estimate the frequency of release.

Informed consent was obtained from eight pregnant women, five women in the first week after delivery, and four non-pregnant women. Blood was collected through a Polythene cannula inserted 30 min before sampling. Three sampling schedules were used: (1) every 20 s for 10 min and then every minute for a further 10 min; (2) every 30 s for 15 min and then every minute for 15 min; (3) every minute for 30 min. Plasma prolactin was measured by a specific double antibody radioimmunoassay (McNeilly, 1973; McNeilly & Hagen, 1974) with a within assay variation of ± 6 %. All samples from one patient were estimated in a single assay.

In every case the variation of prolactin levels in serial samples was greater than the assay variation (Table 1). Taking an increment of 12 % or greater in successive samples as evidence of release, the time intervals between 'peaks' were irregular (Fig. 1), and the mean interval varied with the sampling schedule: for samples every 60 s it was 4·5 min, at 30 s, 2·3 min, and at 20 s, 1·3 min.

Episodic release has been observed with other pituitary hormones including luteinizing hormone (Santen & Bardin, 1973), adrenocorticotrophic hormone (Kreiger, 1974), and oxytocin (Chard, 1975), and is now clearly shown for prolactin. The estimated half-life of prolactin in the circulation is about 15 min (Friesen, Hwang, Guyda, Tolis, Tyson & Myers, 1972); this is compatible with the fluctuations observed here if the entry of the hormone into the circulation is in the form of a bolus (Gibbens, Boyd, Crocker, Baumber & Chard, 1972). Finally, since there was no difference in the frequency of release between groups with high and low levels it seems likely that amplitude rather than frequency is the main controlling factor of the mean levels.

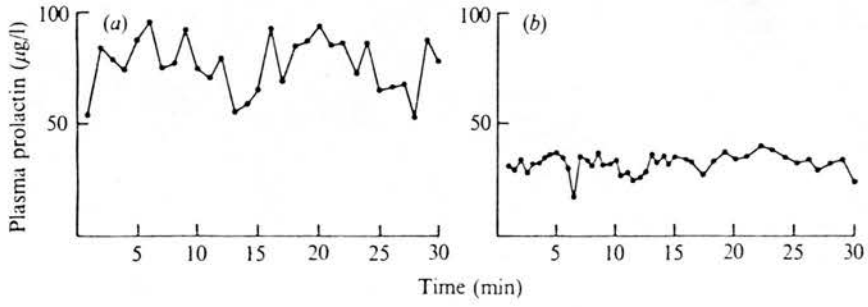



Fig. 1. Plasma prolactin in closely spaced samples from two women: (*a*) 34 weeks of pregnancy; (*b*) 3 days after delivery, lactation established.

Table 1. *Variation of serial prolactin levels in pregnant and lactating women and in normally menstruating women*

| Clinical status | Sampling schedule* | Coefficient of variation for all samples (%) | Mean interval between peaks (min) | | |
|---|---|---|---|---|---|
| | | | 20 s sampling | 30 s sampling | 60 s sampling |
| Pregnant | | | | | |
| Week of gestation | | | | | |
| 32 | 2 | 6·9 | – | 1·6 | 6·0 |
| 34 | 3 | 15·7 | – | – | 4·0 |
| 36 | 3 | 24·0 | – | – | 4·0 |
| 36 | 1 | 8·0 | 0·7 | – | 12·0 |
| 36 | 1 | 40·7 | 0·9 | – | 2·3 |
| 39 | 1 | 18·8 | 2·3 | – | 6·5 |
| 32† | 2 | 21·3 | – | 1·4 | 5·0 |
| 36† | 2 | 25·2 | – | 2·3 | 4·0 |
| Lactation established: not suckling during sampling | | | | | |
| Days since delivery | | | | | |
| 3 | 2 | 13·1 | – | 5·5 | 4·3 |
| 5 | 2 | 15·5 | – | 1·8 | 6·0 |
| 6 | 2 | 38·0 | – | 1·7 | 2·5 |
| 7 | 3 | 19·5 | – | – | 2·8 |
| Post-partum, 7 h after suction termination of pregnancy at 8 weeks | 2 | 31·6 | – | 2·3 | 2·5 |
| Normal menstruating | | | | | |
| Day of cycle | | | | | |
| 14 | 2 | 18·6 | – | 2·0 | 5·0 |
| 23 | 3 | 18·2 | – | – | 4·8 |
| Unknown | 1 | 27·1 | 1·2 | – | 3·0 |
| Amenorrhoea (anorexia nervosa) | 3 | 36·6 | – | – | 2·0 |

*See text for details of sampling. †Twin pregnancy.

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# THE RELATIONSHIP BETWEEN CIRCADIAN VARIATIONS IN CIRCULATING THYROTROPHIN, THYROID HORMONES AND PROLACTIN

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SUMMARY

Half-hourly blood samples were taken from six clinically euthyroid men over a continuous period of 24 h. Their concentrations of total thyroxine (T4), total triiodothyronine (T3), thyrotrophin (TSH) and prolactin (PRL) were assessed together with the degree of unsaturation of thyroid hormone binding proteins as determined by the thyroid hormone uptake test (THUT). Both T3 and T4 were also measured in urine samples collected serially during the same 24 h period. Significant circadian changes in serum TSH, THUT, serum and urine T4 and serum PRL were demonstrated in all subjects. TSH showed a reciprocal pattern to serum T4, with higher levels during the evening and at night than the daytime. This TSH pattern did not coincide with PRL secretion. Further studies on the same subjects did not show any significant effect of posture, corticosteroid or T4 administration upon circadian changes in TSH. There appeared to be no consistent circadian changes in serum or urinary T3. It seems likely that the TSH circadian rhythm is centrally determined and that free T3 levels are maintained more or less constant by variation in peripheral conversion from T4.

Although the circadian pattern of hormones of the hypothalamic-pituitary-adrenal axis are well-recognized, there is still controversy regarding the existence of such a pattern for the hormones of the hypothalamic-pituitary-thyroid axis. Several investigations have failed to demonstrate any rhythmic change in TSH levels in man (Utiger, 1965; Odell *et al.*, 1967; Hershman & Pittman, 1971; Webster *et al.*, 1972), while other groups who have reported circadian variations do not agree as to the time of day at which TSH concentrations are at their maximum (Nicoloff *et al.*, 1970; Patel *et al.*, 1972; Vanhaelst *et al.*, 1972; Weeke, 1973). Conflicting results have also been reported regarding the circadian changes in protein-bound iodine (PBI), total serum T4 and free T4 (FT4) (Lemarchand-Beraud & Vanotti, 1969; De Costre *et al.*, 1971).

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The lack of sensitivity in the hormone assays may account for these discrepant findings. With the development of highly sensitive and precise radioimmunoassays (RIA), a more extensive study was carried out to investigate the circadian variations in serum TSH, T3, T4 and PRL secretions, the T3 and T4 urinary pattern, and the effect of posture, corticosteroid and T4 administration upon these circadian patterns.

## SUBJECTS, MATERIALS AND METHODS

Six healthy male volunteers, aged 25-35, were studied. They were not taking any drugs. 5 ml of blood were obtained every half-hour via an indwelling forearm venous cannula over a 24 h period, together with serial 2 hourly urine collections during the day and 4 hourly collections at night. The subjects carried out their normal daily activities, went to sleep around midnight, and were rarely disturbed by the withdrawal of blood. In addition, two of the same subjects were bled as above from 09.00-20.00 h and then were recumbent (without falling asleep) from 12.00-18.00 h following an otherwise identical protocol. Another subject from the original group was given 0.5 mg of dexamethasone every 6 h starting at 09.00 h on the morning preceding this study. He was then bled at intervals from 18.00 to 09.00 h (the following morning). A different subject was given 200 ug of T4 by mouth daily for 48 h and he went through the same protocol again.

In all blood samples the serum was separated within 2 h and stored frozen until required for assay. When urine collections were made, individual volumes and time of collections were recorded. To avoid interassay variability, all samples from a single subject were run in one assay. Total T4 concentrations in 20 $\mu$l of whole serum were determined by RIA, using 8-anilino-1-naphthalene sulphonic acid (200 $\mu$g) to inhibit T4 binding to endogenous thyroxine binding globulin (TBG) and charcoal to separate antibody bound and free hormone fractions. The anti-T4 serum was raised in rabbits by multiple intra-dermal injections of T4-bovine serum albumin conjugate. Total T3 concentrations were measured in 50 $\mu$l of whole serum by a similar RIA procedure (Chan *et al.*, 1975), using a goat anti-T3 serum which showed 0.0125% cross-reactivity with T4. THUT (thyroid hormone uptake test) value was determined using the Thyopac-3 Kit (Radiochemical Centre, Amersham). As the radioactivity in the supernate is measured, values fall below 90% in hyperthyroidism and rise above 120% in hypothyroidism, compared with a control serum. Circulating TSH was measured by the double antibody method of Hall and his colleagues (1971), and the results expressed as mU/l of the MRC standard 68/38. The antiserum was raised in rabbits against human TSH and was highly specific, showing no cross-reactivity with human chorionic gonadotrophin (HCG) and, therefore, no HCG was added to the incubation mixtures. Circulating PRL was determined by RIA (McNeilly & Hagen, 1974). Urinary T3 and T4 excretions were measured as described elsewhere (Chan & Landon, 1972; Chan *et al.*, 1975; Chan, 1974) and the results expressed as ng of hormone excreted per min. Urinary creatinine was measured by a standard method using the autoanalyser (Technicon handbook number N-11b).

## RESULTS

The sensitivity and within-assay precision of the assays used are outlined in Table 1. The coefficients of variation were calculated from ten analyses of three serum samples with hormone levels in the low, normal and high range respectively.

Table 1. Characteristics of the hormone assays

| Test | Sample volume (μl) | Detection limit | The within-assay coefficient of variation (%) for 3 different samples | | |
|---|---|---|---|---|---|
| | | | Low | Normal | High |
| *Serum* | | | | | |
| TSH (RIA) | 100 | 0.1 mU/l | 8.7 | 5.0 | 7.4 |
| T4 (RIA) | 20 | 6.4 nM/l | 8.9 | 7.0 | 8.1 |
| T3 (RIA) | 50 | 0.04 nM/l | 7.5 | 2.5 | 4.5 |
| PRL (RIA) | 50 | 0.5 μg/l | 7.2 | 7.0 | 8.3 |
| THUT | 100 | – | 2.2 | 2.0 | 1.6 |
| *Urine* | | | | | |
| T4 (CPBA*) | 1000 | 25 ng/dl | 8.6 | 4.6 | 8.6 |
| T3 (RIA) | 100 | 25 ng/dl | 8.0 | 3.5 | 7.5 |

* Competitive protein binding assay.

Absolute mean day and night circulating hormone concentrations in all subjects are presented in Table 2. Daytime represents the period between 07.30 and 24.00 h and nighttime is the interval between 00.00 and 07.30 h.

Fig. 1 shows the mean changes in serum TSH, T4, T3 and THUT in all subjects expressed in the manner of Orth & Island (1969); each value is represented as per cent deviation from the subject's own mean hormone concentration during that particular study day and the mean values for the six subjects are shown. The representative sem. is shown on the graphs, and the ranges of the SEM for all points on the graphs are presented in Table 3.

*Changes in circulating TSH levels.* A well-defined TSH circadian pattern was evident in all subjects, with lower levels during the daytime, but significantly increased values at night. The increase began approximately 4 h prior to the onset of sleep, which was at around midnight. In addition, episodic TSH fluctuations were observed throughout the 24 h. One subject, in particular, had exaggerated fluctuations and a more marked circadian variation. He showed a mean TSH concentration for that day of 5mU/l, with maximum and minimum concentrations of 20 and 3.4 mU/l respectively. Further investigations showed that this healthy and clinically euthyroid volunteer had normal serum total T3 and T4 concentrations, and no microsomal or thyroglobulin antibodies as determined by immunofluorescence techniques (Dr W. J. Irvine).

*Changes in circulating thyroid hormones levels.* Serum total T4 concentrations also showed a distinct circadian pattern, with minimum concentrations between midnight and 08.00 h. Table 2 shows, in all subjects, statistically significant differences between these concentrations and those recorded during the daytime. Total serum T3 concentrations did not show such a definite circadian pattern although non-significantly decreased concentrations were found between 02.00 and 08.00 h.

*Changes in urinary T4 and T3 excretions.* The mean of all subjects are shown in Fig. 2. It can be seen that the urinary T4 excretion was characterized by a rise in the morning and a fall at night, but remained constant during the waking hours. The timing varied slightly between individuals, with peak values between 08.00 and 12.00 h and nadir values in the

Table 2. Mean day and night circulating hormone concentrations ± SEM in all six subjects studied*

| Subjects | TSH (mU/l) | | THUT (%) | | T4 (nM/l) | | T3 (ng/dl) | | PRL (μg/l) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day† | Night‡ | Day† | Night‡ | Day† | Night‡ | Day† | Night‡ | Day† | Night‡ |
| 1 | 1.55 ± 0.07 | 1.73 ± 0.10¶ | 106 ± 0.31 | 99 ± 0.70** | 116.49 ± 2.45 | 96.41 ± 1.67§ | 2.54 ± 0.03 | 2.09 ± 0.03§ | 9.31 ± 0.22 | 14.02± 1.11§ |
| 2 | 1.79 ± 0.09 | 2.78 ± 0.22§ | 103 ± 0.41 | 97 ± 0.58§ | 104.00 ± 1.67 | 84.05 ± 1.16§ | 1.94 ± 0.03 | 1.79 ± 0.05** | 9.30 ± 0.43 | 13.16 ± 0.82§ |
| 3 | 1.28 ± 0.05 | 1.49 ± 0.06†† | 88 ± 0.41 | 81 ± 0.47§ | 81.74 ± 1.80 | 68.73 ± 2.96¶ | 2.00 ± 0.03 | 1.80 ± 0.02§ | 5.05 ± 1.68 | 6.69 ± 1.48‖ |
| 4 | 5.47 ± 0.40 | 8.84 ± 1.10¶ | 103 ± 0.46 | 96 ± 0.54§ | 97.18 ± 1.93 | 84.95 ± 1.93§ | 2.01 ± 0.03 | 2.04 ± 0.10 | 13.10 ± 0.30 | 15.36 ± 0.71‖ |
| 5 | 2.65 ± 0.17 | 3.39 ± 0.22** | 100 ± 0.43 | 96 ± 0.82§ | 91.78 ± 1.54 | 84.82 ± 1.67¶ | 1.39 ± 0.03 | NS<br>1.40 ± 0.17 | 12.70 ± 0.24 | 15.51 ± 0.99§ |
| 6 | 2.27 ± 0.13 | 2.92 ± 0.16¶ | 105 ± 0.32 | 101 ± 0.45§ | 106.19 ± 1.93 | 88.69 ± 2.32§ | 2.44 ± 0.03 | NS<br>2.31 ± 0.04¶ | 10.47 ± 0.36 | 12.33 ± 0.68†† |

* Probability values for comparison between mean day and night hormone levels using student *t*-test (2 tailed).

† Day = 07.30–24.00 h.
‡ Night = 00.00–07.30.
§ = $P < 0.001$.
‖ = $P < 0.005$.
¶ = $P < 0.01$.
** = $P < 0.05$.
†† = $P < 0.02$.
NS = Not significant, $P > 0.05$.

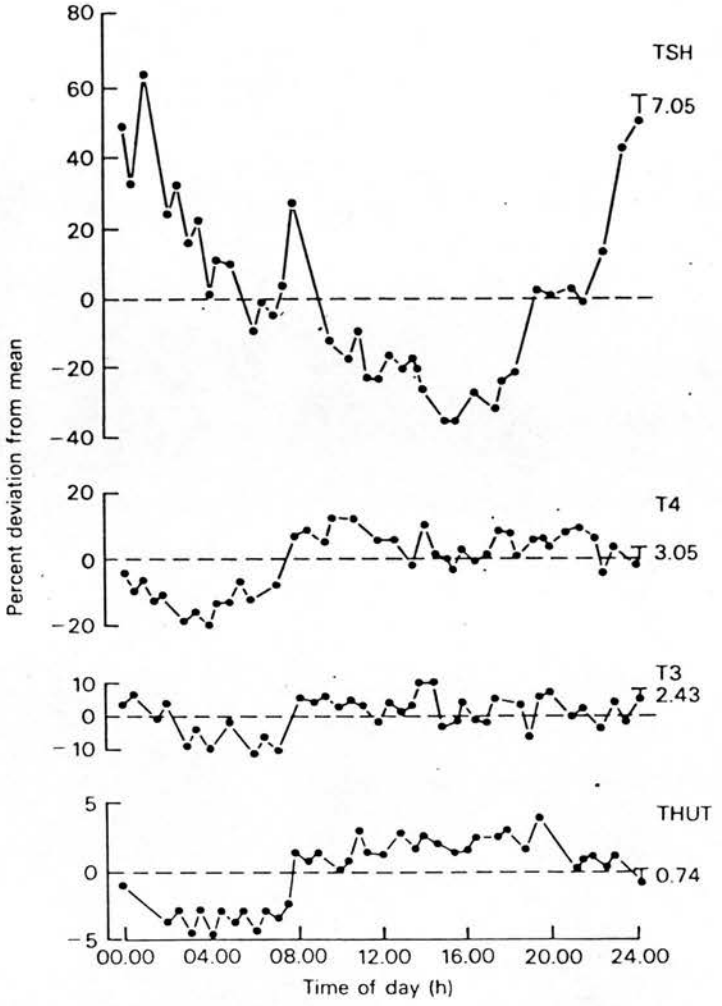



Fig. 1. Mean circadian changes in serum TSH, T4, T3 and THUT in all six subjects, presented as the percent deviation from the subjects' own mean hormone levels for that particular day. – – – individual mean hormone level; (Ŧ) mean + SEM for the variable.

Table 3. Absolute ranges of SEM for all points on Fig. 1

| Hormone | SEM range | Mean ± SD |
|---|---|---|
| TSH | 18.09 – 1.89 | 7.05 ± 3.82 |
| T4 | 6.03 – 0.60 | 3.03 ± 1.21 |
| T3 | 5.21 – 0.21 | 2.43 ± 1.14 |
| THUT | 1.53 – 0.21 | 0.74 ± 0.30 |

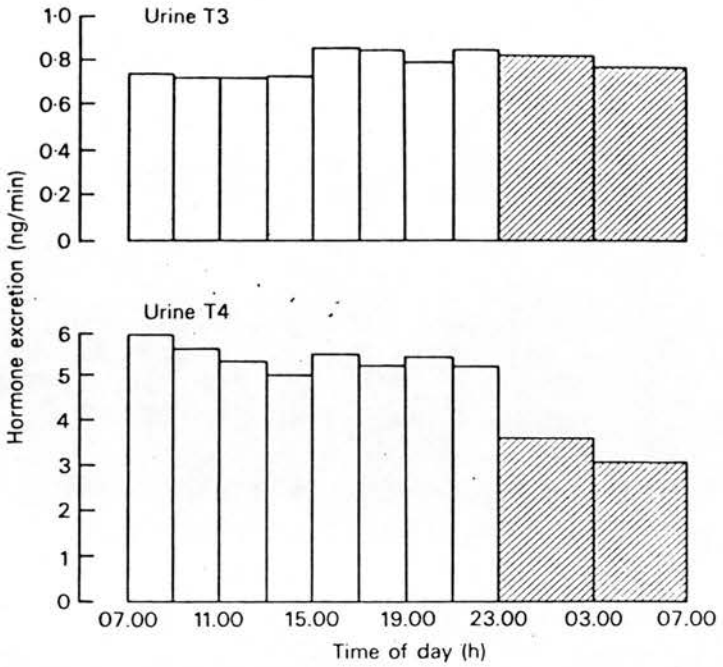



Fig. 2. Mean urinary T3 and T4 excretion in six subjects over a 24 h period (shaded areas indicate urinary hormone excretion during night-time).

second half of the night (between 04.00 and 08.00 h). In contrast, urinary T3 excretion remained constant throughout the 24 h period, that is within ± 0.1 ng/min of the mean. Urinary creatinine excretion did not show any circadian fluctuation. The mean excretion was 6.42 ± 0.54 nM/min.

*Changes in THUT value.* As shown in Fig. 1, it is evident that the degree of unsaturation of the thyroid hormone binding proteins, as reflected by the THUT value, shows a well-defined circadian pattern which is almost identical to the one found for serum T4.

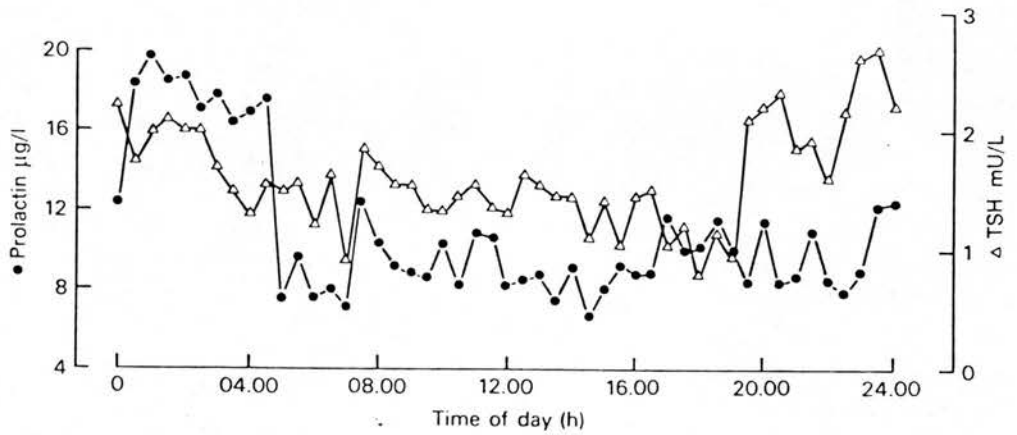



Fig. 3. Circadian changes in concentration of serum PRL (solid symbols) and TSH (open symbols) in one subject shown as absolute values.

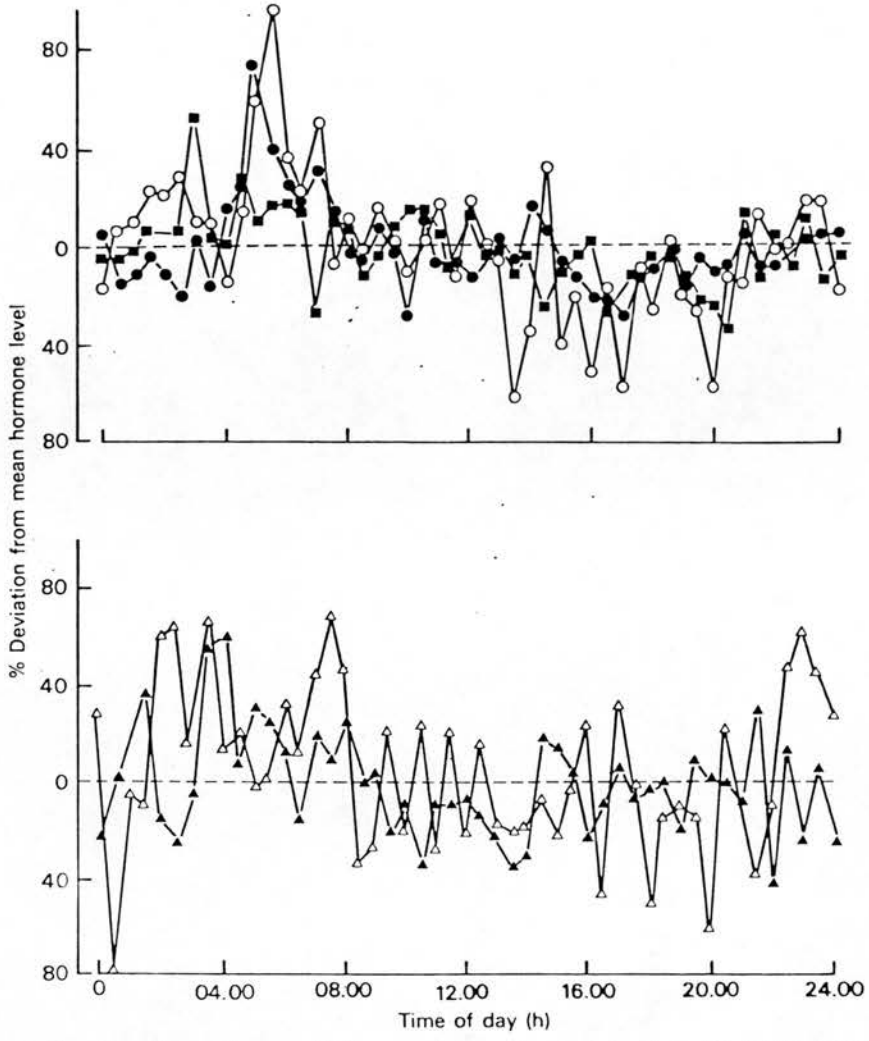



Fig. 4. Circadian changes in serum PRL concentrations in remaining five subjects (the values are expressed as percentage deviation from each subject's own mean daily concentration).

*Changes in circulating PRL.* Fig. 3 shows the relationship between the circadian changes in circulating PRL and TSH concentrations in one representative subject, although all six subjects studied showed the same relationship. The mean day and night serum PRL concentrations are given in Table 2. The PRL patterns were characterized by episodic fluctuations, with marked increases at night. The period of maximal PRL secretion varied in different individuals, either from midnight to 04.00 or 04.00 to 07.00 h (Fig. 4). It always occurred after the TSH peak.

*Effect of posture.* Effects of changes in posture were studied in two subjects. There was no significant variation in the TSH circadian pattern in either subject, whether in a vertical or horizontal position (Fig. 5). The circulating T4 and THUT circadian patterns also remained unchanged (see Appendix).

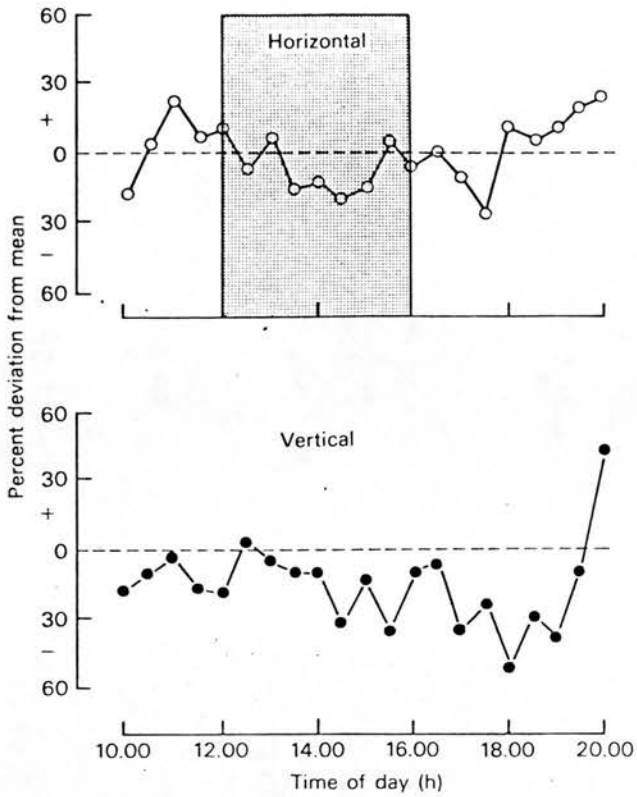



Fig. 5. Effect of posture on circadian changes in serum TSH concentrations in one subject (the shaded area indicates the period when the subject was recumbent; the lower graph shows the TSH circadian pattern, in the same subject, during a normal day).

*Effect of corticosteroid.* The circadian variation in circulating endogenous corticosteroids was abolished by dexamethasone, to non-cycling levels of less than 55 nmol/l. Although the mean concentrations of serum TSH, T3, and PRL were significantly lowered compared with predexamethasone treatment ($P < 0.05$, $< 0.005$ and $< 0.05$ respectively), their circadian patterns remained very much the same (Fig. 6).

*Effect of T4 administration.* Fig. 7 shows the circadian TSH pattern in one subject either basally or after T4 administration. No significant change was seen although the mean TSH concentration was lowered from 2.9 to 0.9 mU/l.

## DISCUSSION

Using highly specific, sensitive and precise RIA, it has been possible to measure with confidence, small changes in the concentrations of circulating hormones. Thus, we have been able to measure, throughout a continuous period of 24 h, simultaneous variations in the serum concentration of TSH, T3 and T4, making it easier to interpret the possible role of each of these hormones in the feedback mechanisms controlling the hypothalamic-pituitary-thyroid axis. In addition, since only small volumes of serum are required in the measurement

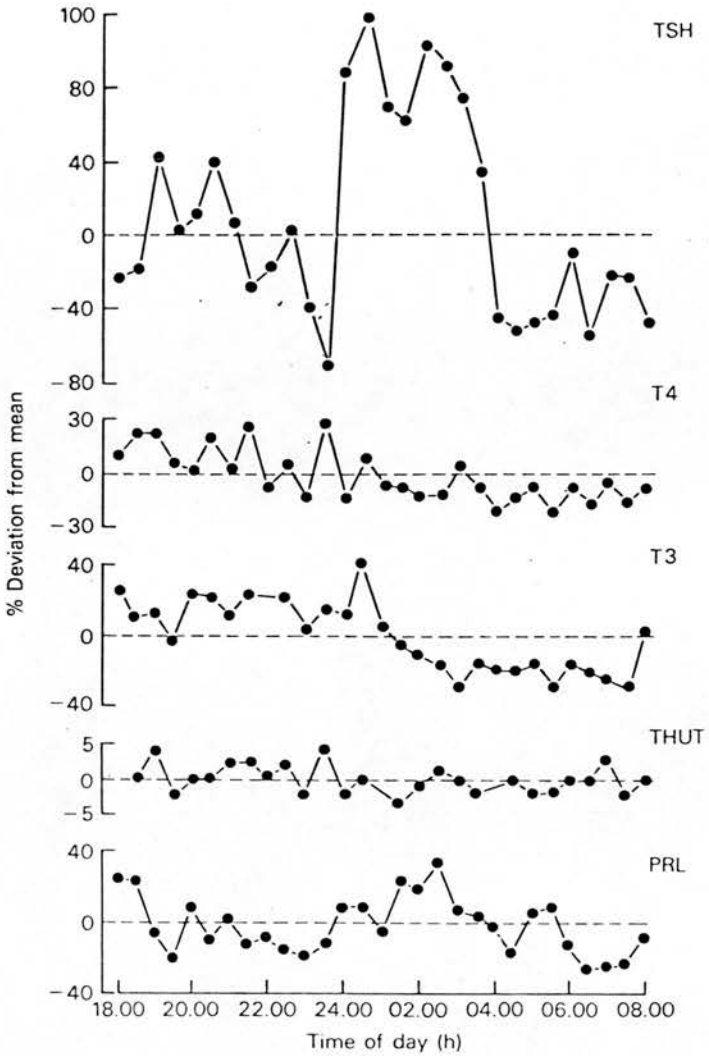



Fig. 6. Circadian changes in TSH, T4, T3. THUT and PRL in one subject given dexamethasone (0.5 mg 6 hourly) starting 9 h before beginning sampling at 18.00 h.

of THUT and PRL, we were able to include them in this study, keeping the total amount of individual blood loss down to a reasonable minimum of approximately 240 ml over a period of 24 h, which represents about 4.9 ± 0.5% of the estimated total blood volume of 4.99 ± 0.55 l (Gray & Frank, 1953). This loss would be insufficient to account for the circadian changes found, since differences between maximal and minimal hormone concentrations of up to 140% were observed. This study clearly confirms the existence of circadian patterns for serum TSH, PRL, T4 and THUT whilst there is no evidence of any rhythmic variations in the serum concentration of total T3. The circadian pattern of serum TSH is in agreement with the findings of Patel and his colleagues (1972) and Weeke (1973) who reported higher TSH levels at night in all their subjects, while Vanhaelst and coworkers (1972) found higher

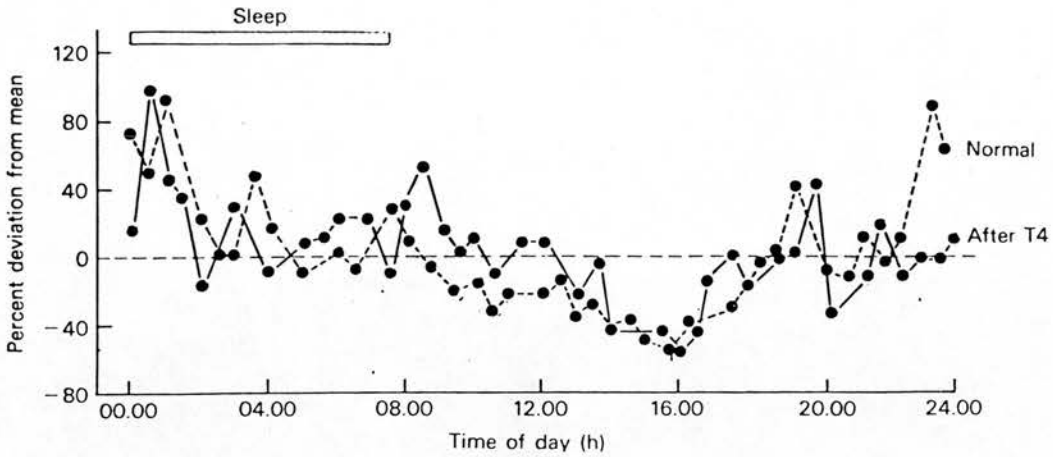



Fig. 7. Circadian changes in serum TSH concentrations, before and after thyroid suppression by T4 administration (200 μg/day for 48 h) in one subject.

nocturnal TSH levels in women, but not in men. Present observations showed that the increase in TSH occurred approximately 4 h prior to the onset of sleep, and maximum concentrations were attained between 23.00 and 02.00 h. Weeke (1973) found higher TSH levels at night, between 20.00 and 08.00 h, but was able to detect a high TSH peak in only one subject (between 07.00 and 09.00 h). All our subjects showed a well-defined circadian pattern for serum total T4 and THUT. These patterns were similar to each other, but reciprocal to TSH. Lemarchand-Beraud and Vanotti (1969) showed the same inverse relationship between TSH and free T4 patterns. We have confirmed the findings of Robyn and coworkers (Robyn *et al.*, 1973; Vanhaelst *et al.*, 1973) that TSH and PRL release are not concomitant and may well be unrelated. Changes in posture during daytime, from the vertical to horizontal position, have no apparent effect on the circadian variations in any of the thyroid hormones, TSH or PRL. In contrast, DeCostre and his colleagues (1971) found that a reversed circadian pattern of serum total T4 was produced as soon as the patient's sleep-wake cycle was reversed. Alterations in hepatic clearance or haemoconcentration are not likely to explain the circadian variations in TSH, since the changes in plasma proteins, haemoglobin and haematocrit are such that the lowest values occur at night (Renbourn, 1947), whereas TSH is at its maximum at this time.

Nicoloff *et al.* (1970) demonstrated a circadian pattern of thyroidal iodine release with maximum levels around 04.00 h and postulated the presence of a negative feedback regulation of TSH by circulating corticosteroids and this concept received some support from the work of Patel *et al.* (1974), who reported abolition of the circadian rhythm of TSH by pharmacological amounts of cortisol (200 mg/24 h). However, it is at variance with our finding that the abolition of the circadian rhythm of endogenous plasma corticosteroids with 2 mg per day of dexamethasone for 24 h did not alter the circadian pattern of TSH.

It seemed possible that the primary event was a fall in T4 due to altered peripheral clearance of the hormone and that the TSH changes were secondary to this. We have attempted to exclude this by showing that administration of exogenous T4, maintaining the lowered serum T4 concentrations constant, did not abolish the TSH circadian rhythm. Similarly posture was not responsible, either through central TSH or peripheral T4 effects

since acute changes in posture during daytime, have no apparent effect on the circadian variations observed.

Finally, in our studies the TSH circadian pattern was not dependent on the corticosteroid circadian rhythm, as abolition of this with dexamethasone did not alter the changes in TSH levels. The practical clinical consequences of these circadian studies is that the collection of blood for PRL, TSH and other thyroid hormone estimations should be performed at a particular time of day (e.g. between 09.00 and noon) when hormone concentrations have attained the mean basal level. It would be wise to bear in mind the possible circadian changes of hormone concentrations when interpreting results of any in vivo test performed over a period of several hours.

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See Appendix on page 349.

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# INTERACTION BETWEEN SECRETION OF THE GONADOTROPHINS, PROLACTIN, GROWTH HORMONE, THYROTROPHIN AND CORTICOSTEROIDS IN MAN: THE EFFECTS OF LH/FSH-RH, TRH AND HYPOGLYCAEMIA ALONE AND IN COMBINATION

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SUMMARY

The interaction between the mechanisms involved in the LH, FSH, growth hormone, prolactin, ACTH and TSH responses to the synthetic LH and FSH releasing hormone, thyrotrophin releasing hormone and insulin induced hypoglycaemia was studied in twelve normal male volunteers. Each subject acted as his own control and the test procedures were performed individually and in combination. The simultaneous administration of one releasing hormone with another or with insulin in no way modified the hormonal responses to either releasing hormone or to hypoglycaemia. Clinical testing with these procedures may therefore be performed simultaneously, so that the pituitary reserve for the five anterior pituitary hormones may be assessed together in under 2 hr. In addition it has been shown that TRH releases a small amount of FSH but not LH in male subjects.

## INTRODUCTION

The recent synthesis of hypothalamic releasing hormones for thyrotrophin (TSH) and prolactin, TRH, and luteinizing hormone (LH) and follicle stimulating hormone (FSH), LH/FSH-RH, has allowed investigation of the pituitary reserve of these hormones under physiological and pathological conditions. Clinically it is frequently necessary to assess the reserve of all the anterior pituitary hormones and it is therefore valuable to know whether releasing hormones can be administered simultaneously with other pituitary func-

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tion test procedures, without any interference with secretion of one hormone by concomitant release of another. Not only would this information permit simultaneous testing of the pituitary reserve capacity for different hormones, thus saving time, but would also provide physiological information concerning any competition between the mechanisms involved in their release.

It has already been shown that in man there is no interaction between the release of TSH after intravenous TRH and growth hormone (GH), and corticotrophin (ACTH) secretion in response to insulin induced hypoglycaemia (Besser *et al.*, 1971), and therefore that TRH and insulin tests can be carried out simultaneously. The present paper reports the effects on circulating levels of anterior pituitary hormones after (a) simultaneous administration of TRH and LH/FSH-RH and (b) LH/FSH-RH given during insulin-induced hypoglycaemia. Changes in the blood levels of immunoreactive LH, FSH, GH, prolactin, TSH and of fluorogenic corticosteroids, as an index of ACTH secretion, have been assessed. In addition an unexpected alteration in serum FSH has been found after TRH administration.

## SUBJECTS AND METHODS

(A) Twelve healthy male volunteers, aged 26–41 years, were divided into two equal treatment groups. Each subject participated in three experiments at intervals of 7 days. The experiments were performed single (subject) blind and their order was randomized according to Latin square designs such that each subject acted as his own control. The test procedures performed in the two groups were as follows:

*Group* 1: *Hypoglycaemia–LH/FSH-RH interaction* (*six subjects*)

(a) i.v. insulin + LH/FSH-RH.
(b) 0·9% saline + LH/FSH-RH.
(c) i.v. insulin + 0·9% saline.

*Group* 2: *TRH–LH/FSH-RH interaction* (*six subjects*)

(a) i.v. TRH + LH/FSH–RH
(b) 0·9% saline + LH/FSH-RH
(c) i.v. TRH + 0·9% saline.

In both groups circulating levels of immunoreactive LH, FSH, prolactin, GH, and TSH, fluorogenic corticosteroids and blood sugar were measured on all blood samples.

The doses of the drugs used were soluble insulin 0·15 units/kg body weight, LH/FSH-RH (Hoechst) 100 μg in 4 ml water, TRH (Roche) 200 μg in 2 ml 0·9% saline, and 0·9% saline 2 ml, and each injection was given as a bolus.

All subjects fasted from midnight on the day of testing, and a forearm venous cannula was inserted between 08.00 and 09.00 hours. After 30 min, basal blood samples were obtained and then further samples were withdrawn via the cannula each 15 min for up to 105 min (group 1) or 75 min (group 2). In group 2 a 20 min sample was obtained instead of the one at 15 min to keep the study in line with the standard TRH test (Ormston *et al.*, 1971).

*Group* 1 *subjects*: after the basal blood sample was obtained intravenous insulin or its saline control was given and after 15 min the LH/FSH-RH or its saline control. The second injection was delayed, since it is known that ACTH does not start to rise after insulin administration until about 15 min have elapsed (Besser *et al.*, 1971) and it was hoped to stimulate ACTH, GH, prolactin, LH and FSH secretion approximately simultaneously.

*Group* 2 *subjects*: the TRH, LH/FSH-RH or saline controls were given immediately after

the basal samples were obtained, although the TRH and LH/FSH-RH were not mixed in the same syringes since it was not known whether they were compatible.

(B) Six further healthy male volunteers aged 23–32 years were fasted from midnight on the day of testing and a forearm venous cannula was inserted at 08.30 hours. After 30 min basal blood samples were taken for serum FSH and TSH. This was then followed immediately by 2 ml of 0·9% saline or 200 μg TRH in 2 ml of saline, three subjects receiving the control injection and three the TRH, as a single bolus. Sampling continued at 20, 30, 45, 60 and 75 min following the injection as in group 2 above. There was a cross over of experiments 1 week later. The maximum increments in serum FSH after the saline and TRH injection were statistically compared on a within-subject basis.

In all the studies, serum or plasma was rapidly separated and stored at −20°C until assayed; all samples from each subject were always assayed in the same batch. Serum LH and FSH were measured in duplicate by a double-antibody radioimmunoassay as described for assays 1 in Besser *et al.* (1972a) except that a different rabbit anti-FSH antiserum was used (kindly provided by Professor Butt, Birmingham). Results were expressed in milliunits (mU)/ml of LH standard MRC 68/40 and FSH standard MRC 68/39, taking the contents of each ampoule as 39·8 and 32·8 units respectively.

Serum TSH was measured by a double-antibody immunoassay (Hall *et al.*, 1971) and the results were expressed in μU/ml of MRC standard 68/38, and serum GH by the method of Hartog *et al.* (1964) expressed in ng/ml of MRC standard A. Serum prolactin was assayed using a specific double-antibody technique and an anti-human prolactin antiserum raised in rabbits (McNeilly, 1973). The results are expressed as ng/ml of a standard human pituitary prolactin (kindly provided by Dr H. Friesen). This was also used for iodination. Plasma fluorogenic corticosteroids were measured by the method of Mattingly (1962) and blood sugar by a ferro-ferricyanide method (Technicon). There was no crossreactivity between any of the radioimmunoassays at levels many times greater than those encountered in these

TABLE 1. Sensitivities and crossreactivity characteristics of the different radioimmunoassays

| | Radioimmunoassays for: | | |
|---|---|---|---|
| | LH (mU/ml) | FSH (mU/ml) | TSH (μU/ml) |
| Minimum detectable blood levels | 0·4 | 0·2 | 0·4 |
| Crossreactivity with:* | | | |
| LH (mU/ml) | | 80 | >1000 |
| FSH (mU/ml) | 60 | | 450 |
| TSH (μU/ml) | 600 | 250 | |

* Greater than the amount stated would just be detected in the assay.

experiments (Table 1), the minimum detectable blood levels in the assays are shown in the Table. Adequate hypoglycaemia was achieved in all subjects after insulin in that each subject sweated and in each the blood sugar fell to less than 40 mg/100 ml. Within-subject

comparisons of the changes in hormone levels after the various treatments were made using Student's '*t*' test (two-tailed).

## RESULTS

### (A) *Group* 1: *Hypoglycaemia–LH/FSH-RH interaction*

The changes in circulating LH and FSH levels after LH/FSH-RH, and those in GH, prolactin, corticosteroids and blood sugar after insulin-induced hypoglycaemia were the same whether insulin and LH/FSH-RH were given separately or together (Figs. 1–5). There was one subject who failed to show a plasma prolactin response after either insulin alone or in combination with LH/FSH-RH despite a blood sugar fall to less than 20 mg/100 ml on both occasions and his values have been omitted from Fig. 5. LH/FSH-RH alone did not release prolactin, growth hormone, TSH or corticosteroids; hypoglycaemia alone was not associated with changes in blood levels of TSH, LH or FSH.

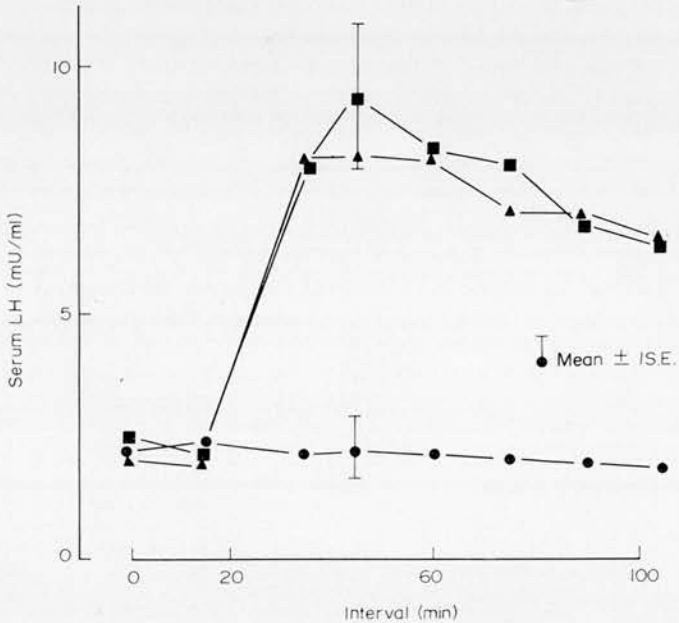



FIG. 1. The influence of insulin-induced hypoglycaemia on serum LH in response to 100 $\mu$g LH/FSH-RH in six normal subjects. ●, Insulin+saline; ■, insulin+LHRH; ▲, saline+LHRH.

### *Group* 2: *TRH–LH/FSH-RH interaction*

The changes in circulating TSH and prolactin levels after TRH, and those in LH and FSH after LH/FSH-RH were the same whether the two releasing hormones were given separately or together (Figs. 6–9). In two of the subjects, the experiment was repeated giving the LH/FSH-RH 20 min after the TRH dose, at which time serum prolactin levels were at

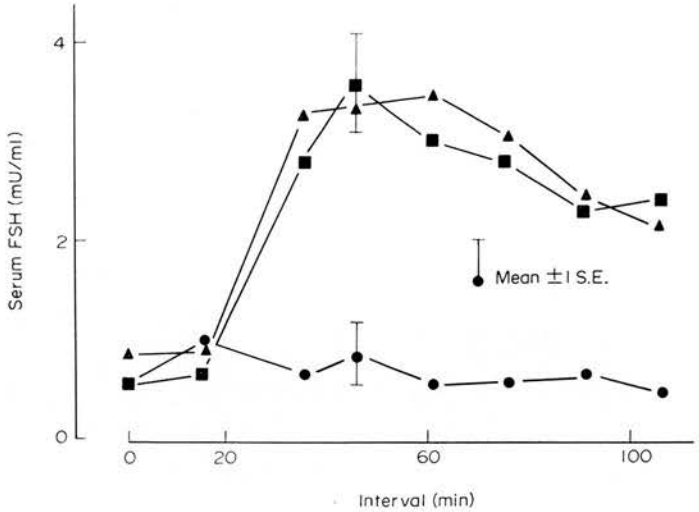



FIG. 2. The influence of insulin-induced hypoglycaemia on serum FSH in response to 100 $\mu$g LH/FSH-RH in six normal subjects. ●, Insulin+saline; ■, insulin+LHRH; ▲, saline+LHRH.

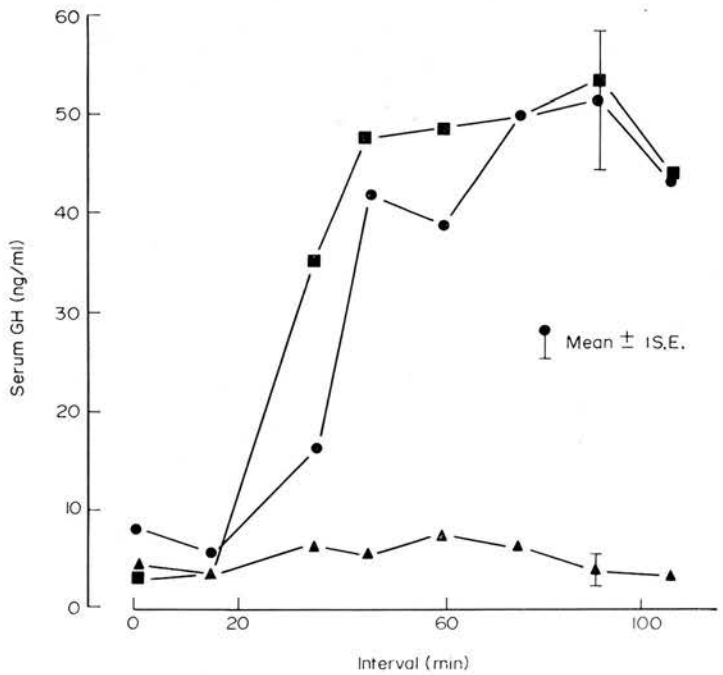



FIG. 3. The influence of 100 $\mu$g LH/FSH-RH on serum GH in response to insulin-induced hypoglycaemia in six normal subjects. ●, Insulin+saline; ■, insulin+LHRH; ▲, saline+LHRH.

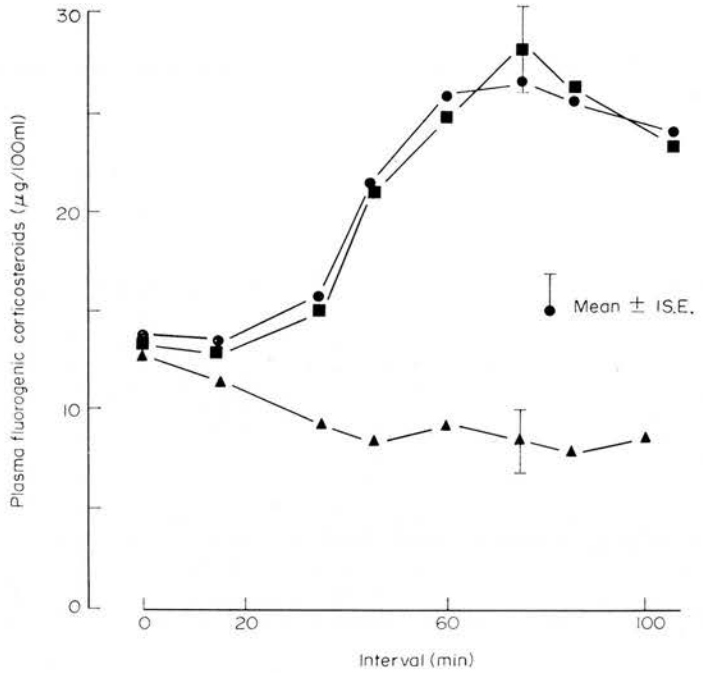



FIG. 4. The influence of 100 μg LH/FSH-RH on plasma fluorogenic corticosteroids in response to insulin-induced hypoglycaemia in six normal subjects. ●, Insulin+saline; ■, insulin+LHRH; ▲, saline+LHRH.

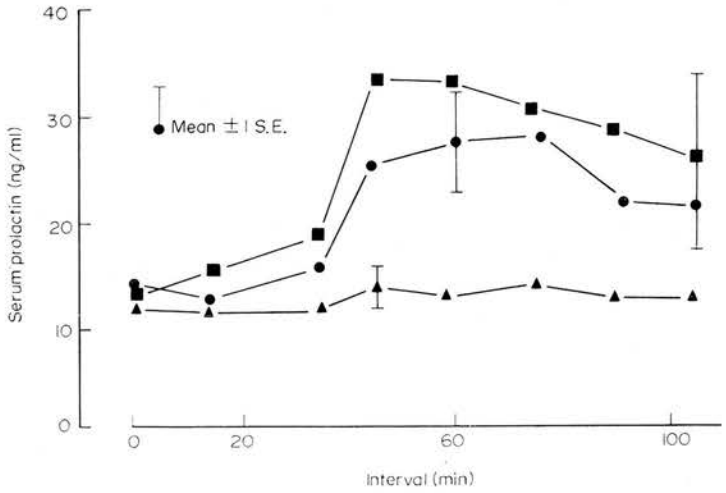



FIG. 5. The influence of 100 μg LH/FSH-RH on serum prolactin in response to insulin-induced hypoglycaemia in five normal subjects. ●, Insulin+saline; ■, insulin+LHRH; ▲, saline+LHRH.

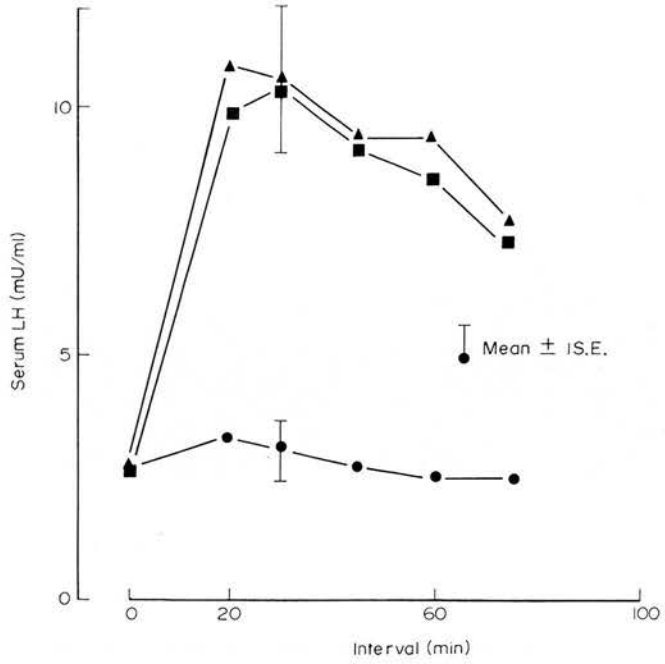



FIG. 6. The influence of 200 $\mu$g TRH on serum LH response to 100 $\mu$g LH/FSH-RH in six normal subjects. ●, Saline+TRH; ■, saline+LHRH; ▲, LHRH+TRH.

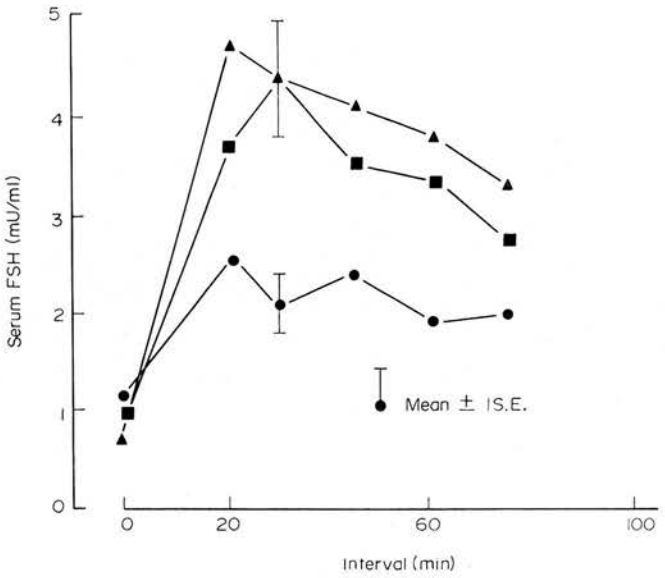



FIG. 7. The influence of 200 $\mu$g TRH on serum FSH response to 100 $\mu$g LH/FSH-RH in six normal subjects. ●, Saline+TRH; ■, saline+LHRH; ▲, TRH+LHRH.

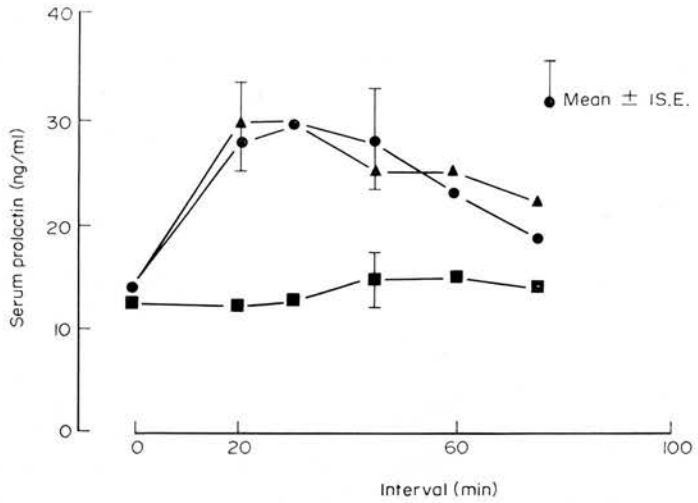



FIG. 8. The influence of 100 $\mu$g LH/FSH–RH on serum prolactin response to 200 $\mu$g TRH in six normal subjects. ●, Saline+TRH; ■, saline+LHRH; ▲, LHRH+TRH.

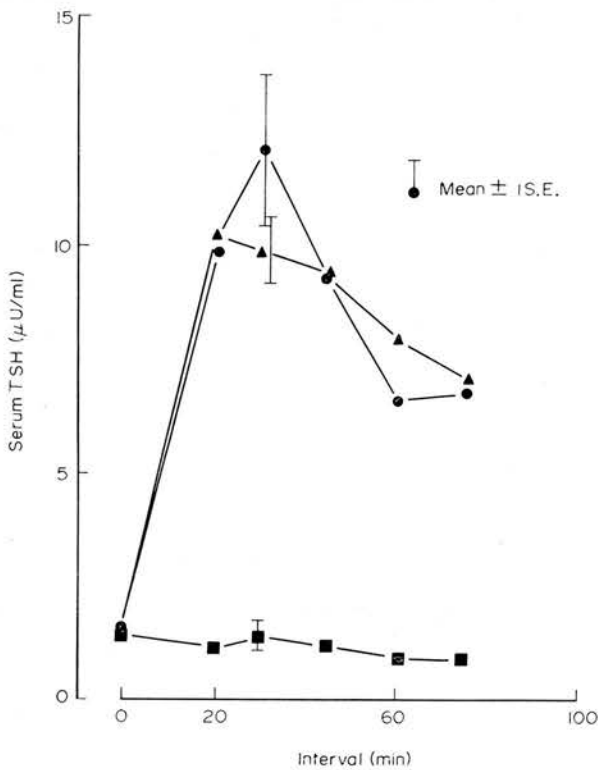



FIG. 9. The influence of 200 $\mu$g TRH on serum TSH response to 100 $\mu$g LH/FSH-RH in six normal subjects. ●, Saline+TRH; ■, saline+LHRH; ▲, LHRH+TRH.

their maximum. The serum LH and FSH changes were again unaffected. TRH alone was not associated with any changes in GH, corticosteroids or LH. Unexpectedly in each of the six subjects a small increase in serum FSH was seen after TRH with a peak level between 20 and 45 min (Fig. 7). The change was significant ($P<0{\cdot}01$). For this reason further studies were carried out (B).

(B) The effect of a bolus of TRH 200 $\mu$g in 2 ml of saline was compared with 2 ml of normal saline in a further six normal subjects. The mean maximum increment in serum FSH following saline was $0{\cdot}1 \pm 0{\cdot}15$ (SE) mU/ml while that after TRH was $1{\cdot}7 \pm 0{\cdot}43$ mU/ml. This difference was significant ($P<0{\cdot}01$).

## DISCUSSION

We have previously shown that the synthetic decapeptide LH/FSH-RH causes a rise in circulating levels of both LH and FSH in man (Besser *et al.*, 1972a) and now report that this effect is not influenced by concomitant release of prolactin and TSH in response to TRH, or of prolactin, GH and ACTH (as reflected by corticosteroid secretion) during hypoglycaemia. There does not appear to be any interaction or competition between the mechanisms for release of any of these hormones under the conditions studied. It is of particular interest to note the lack of any interference with gonadotrophin release when prolactin secretion is stimulated since there is clinical evidence in man (Besser *et al.*, 1972b) as well as evidence from animal experiments (Kamberi *et al.*, 1971; Ben-David *et al.*, 1971) that when prolactin secretion is active gonadotrophin secretion is reduced. It remains to be seen whether in man prolactin secretion will impair a stimulus to gonadotrophin release at the hypothalamic rather than at the pituitary level. Prolactin secretion in response to either TRH or hypoglycaemia was uninfluenced by concomitant gonadotrophin release after LH/FSH-RH.

The hypothalamic hormones are usually regarded as relatively specific in their actions. Although the release of prolactin as well as TSH in response to TRH is well documented (Jacobs *et al.*, 1971), no changes in other pituitary hormones have been consistently described. However, Franchimont (1972) reported finding occasional females who showed some LH release when given intravenous TRH at about the time of the mid-cycle luteal peak. We have now found a consistent but small increase in serum FSH in males after a single bolus injection of TRH. We have subsequently also demonstrated this effect with TRH infusions and shown that the response is suppressed by small doses of oestrogen (Mortimer *et al.*, 1973). We have confirmed in this study that in males LH/FSH-RH does not cause release of prolactin, GH, ACTH or TSH, and that TRH does not release LH, GH or ACTH.

In clinical endocrine practice it is desirable to obtain maximum information about the pituitary status of patients in the shortest possible time and with the least inconvenience to the patient. We have previously shown that TSH, GH and ACTH reserve can be assessed simultaneously by performing an insulin-hypoglycaemia test and TRH test at the same time (Besser *et al.*, 1971). We now show that LH/FSH-RH can also be administered simultaneously. It is now our routine practice to give 100 $\mu$g LH/FSH-RH and 200 $\mu$g TRH intravenously (from separate syringes) at the start of a standard insulin tolerance test, and to monitor the changes in circulating LH, FSH, prolactin TSH, GH, corticosteroids and blood sugar over the next 90 min. This combined test of the pituitary reserve capacity for the five hormones can be performed on outpatients who are then allowed home after lunch.

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# Isolated Pituitary Gonadotrophin Deficiency: Gonadotrophin Secretion after Synthetic Luteinizing Hormone and Follicle Stimulating Hormone-releasing Hormone

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## Summary

**The responses of serum immunoreactive luteinizing hormone (LH) and follicle stimulating hormone (FSH) after intravenous injection of 100 μg of synthetic LH/FSH-RH have been studied in 14 patients with the syndrome of isolated pituitary gonadotrophin deficiency. Nine of the patients showed a rise of both hormones, two a small rise of FSH only, and three were unresponsive. In two of the unresponsive patients injection of a 500-μg dose produced a small rise of LH only. Of the patients who responded, four had LH and FSH responses within the normal adult range, while in the others the responses were smaller and delayed. It is suggested that this syndrome is due to a lack of the hypothalamic-releasing hormone itself, rather than to a pituitary deficiency. However, repeat assessment after prolonged administration of the releasing hormone will be necessary before a pituitary disorder can be excluded in all patients. The synthetic LH/FSH-RH, preferably as a depot preparation, may provide a means of treating these patients to induce the development of puberty and subsequent fertility.**


## Introduction

With the availability of sensitive radioimmunoassays for luteinizing hormone (LH) and follicle stimulating hormone (FSH) the condition of isolated pituitary gonadotrophin deficiency (hypogonadotrophic hypogonadism) has been increasingly recognized as a cause of partial or complete failure of puberty. The condition may occur alone or be associated with other developmental abnormalities such as anosmia, harelip, cleft palate, and craniofacial asymmetry (Kallman *et al.*, 1944). These patients have low or low-normal serum gonadotrophin levels which do not rise after clomiphene administration. Pituitary function is otherwise normal, though blunted growth hormone responses to hypoglycaemia have been described (Odell *et al.*, 1967; Hornichter *et al.*, 1968; Bardin *et al.*, 1969; Anderson *et al.*, 1972).

It remains unclear whether the primary defect in this condition is at a hypothalamic or pituitary level, though the former site has been more often postulated, and histological abnormalities in the hypothalamus have been described (De Morsier and Gauthier, 1963). This view is supported also by the recent report of Naftolin *et al.*, (1971) who found a small increase in serum LH in two patients after administration of a purified ovine hypothalamic extract.

The hypothalamic-releasing hormone for LH/FSH has recently been isolated and shown to be a decapeptide (Schally *et al.*, 1971). The decapeptide has now been synthesized, and by using this material Besser *et al.* (1972) have shown that it releases LH and to a lesser extent FSH in normal men and women.

The aim of the present study was to establish whether patients with isolated gonadotrophin deficiency were able to secrete LH or FSH from the pituitary in response to an injection of LH/FSH-RH, and to assess the potential therapeutic value of the releasing hormone in such patients.

## Patients and Methods

Fourteen patients (10 men and four women) were studied, and details of clinical features, previous therapy, and basal investigations are shown in the Table. The patients presented with complete or partial failure of puberty and the women with primary amenorrhoea. All had a eunuchoid habitus, a normal chromosome karyotype, and normal pituitary fossa radiographs. By using the criteria suggested by Hall *et al.* (1972), all the patients had normal pituitary function with respect to growth hormone and adrenocorticotrophic hormone (assessed by responses to insulin-induced hypoglycaemia) and thyroid stimulating hormone as judged by protein bound iodine and $^{131}I$ uptake. Case 4, however, had an impaired growth hormone response, peak value 14 ng/ml (M.R.C. standard A HGH). Basal LH levels (mean of three to four estimations) were low in all patients and serum FSH was low in all but two (Cases 3 and 7). None of the subjects showed a rise of serum LH or FSH during a clomiphene test confirming the gonadotrophin deficiency, and all the men had low basal plasma 17β-hydroxyandrogen levels which rose during a human chorionic gonadotrophin stimulation test, indicating the presence of testicular Leydig cells (Anderson *et al.*, 1972).

*Assay Systems.*—Serum LH and serum FSH were each measured in triplicate in two different radioimmunoassay systems, details of which have been given previously (Besser *et al.*, 1972; Marshall *et al.*, 1972). Results in the different assays for each hormone were essentially the same, and for clarity values from one system only are presented here (assay 2, Besser *et al.*, 1972). For comparison purposes the same standard preparation M.R.C. 69/104 (derived from LER 907) was used

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for both the LH and FSH assays and results expressed as mIU/ml. For LH, 1 mI M.R.C. research standard A = 2 mIU M.R.C. 69/104. In the assays a change of greater than 0·5 mIU/ml for either LH or FSH was significant. When using this standard the normal ranges for adult subjects were: men, LH 3·6-8·2; FSH 1·5-8·4 mIU/ml; women (follicular phase), LH 3·6-9·0; FSH 2·0-8·2 mIU/ml. Plasma 17β-hydroxyandrogens were measured in the men by the method of Anderson (1970). The normal range for men at 9 a.m. was 4·9-21·5 ng/ml.

*Test Procedure.*—A 100-μg sample of synthetic LH/FSH-RH (Hoechst) in 4 ml of sterile water was given to the recumbent, non-fasted patients between 9 a.m. and 10 a.m. by rapid intravenous injection. Samples for LH and FSH assay were taken 15 minutes and immediately before the injection, and at frequent intervals during the subsequent two hours. In two patients a dosage of 500 μg intravenously was also given. Previous therapy had been discontinued for at least two months before administration of LH/FSH-RH with the exception of Case 2, when the interval was four weeks. All the men had noted a reduction in libido after treatment was stopped.

*Control Subjects.*—These were 18 normal men, and five women during the follicular phase of the menstrual cycle. Details of the LH and FSH responses are given elsewhere (Besser *et al.*, 1972). Informed consent was given by all patients and control subjects.

## Results

Serum LH and FSH levels before and after injection of 100 μg LH/FSH-RH in the 14 patients are shown in Fig. 1. Nine patients showed a rise of both LH and FSH, two (Cases 2 and 4) showed an FSH rise only, and three (Cases 5, 8, and 10) showed no change in the levels of either hormone during the test period.

In four patients (Cases 1, 3, 7, and 9) responses of both hormones were within the range seen in normal adult subjects,

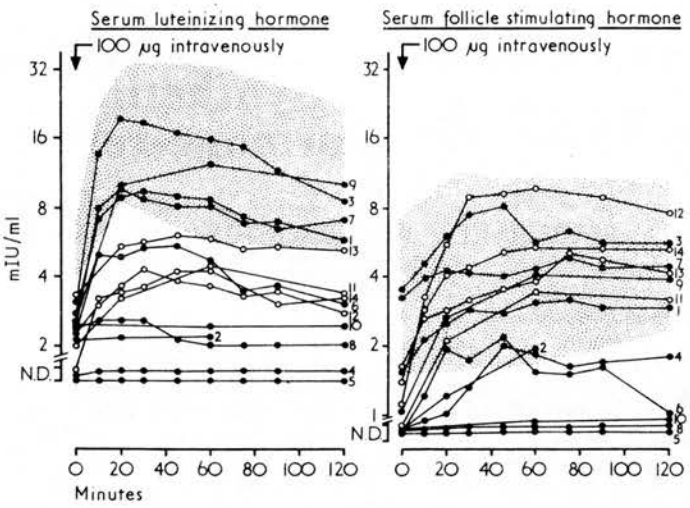



FIG. 1—Responses of serum LH and FSH in 14 patients after intravenous injection of 100 μg LH/FSH-RH. Case numbers as listed in Table. Shaded areas indicate range of response seen in normal subjects. ● = Males. ○ = Females. Mean of the − 15 and 0-minute values is shown. ND = Not detectable in the assays used. A logarithmic scale of gonadotrophin levels is used for clarity.

*Clinical Details of Patients Studied*

| Case No. | At Presentation | | Testicular Size (cm) | Bone Age | Testicular Biopsy | | Basal Hormone Levels | | | Therapy Before Presentation | Therapy before Administration of LH/FSH-RH† |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age | Clinical Features* | | | Leydig Cells | Spermatozoa | LH (mIU/ml) | FSH mI (U/ml) | 17β-hydroxy-androgen (ng/ml) | | |
| Men: | | | | | | | | | | | |
| 1 .. | 26 | Prepubertal | 2·5 × 1·5 × 1·0 | 15 | Occasional | None | <2·0 | <1·0 | 1·6 | Nil | HCG 1,000 IU for 17 months |
| 2 .. | 21 | Stage II puberty | 1·5 × 1·0 × 1·0 | 15 | — | — | 2·1 | <1·0 | 3·2 | Nil | HCG 500 IU for 2 months |
| 3 .. | 26 | Stage III puberty | 3·5 × 2·0 × 2·5 | 17 | None | Occasional | 3·4 | 3·0 | 3·9 | Nil | HCG 1,000 IU for 18 months |
| 4 | 25 | Stage II puberty | 2·0 × 2·0 × 1·5 | 15 | None | None | <2·0 | <1·0 | 1·4 | HCG for 6 weeks, intramuscular testosterone for 6 months | HCG 1,000 IU for 19 months |
| 5 .. | 19 | Prepubertal | 2·0 × 2·0 × 1·5 | — | None | None | <2·0 | <1·0 | 1·0 | Nil | HCG 2,000 IU for 12 months |
| 6 .. | 19 | Prepubertal | 2·0 × 1·0 × 1·0 | 15 | — | — | 2·6 | <1·0 | 1·3 | Nil | HCG 2,000 IU for 6 months |
| 7 .. | 18 | Prepubertal | 2·0 × 1·0 × 1·0 | 12 | None | None | 2·4 | 2·7 | 0·8 | Nil | HCG 2,000 IU for 12 months |
| 8 .. | 30 | Normal male hair. Voice broken | 2·5 × 1·5 × 1·0 | Adult | None | None | 2·0 | <1·0 | 2·5 | Intramuscular testosterone for 10 years | None for 2 months |
| 9 .. | 21 | Stage III puberty | 2·0 × 1·0 × 1·0 | 17 | None | None | 2·2 | <1·0 | 1·3 | HCG for 2 months, Intramuscular testosterone for 7 months | None for 3 months |
| 10 .. | 25 | Stage II puberty | 1·5 × 0·5 × 0·5 | 16 | None | None | 2·6 | <1·0 | 1·7 | Intermittent testosterone for 2 years | HCG 500 IU, Pergonal for 12 months 300 IU |
| Women: | | | | | | | | | | | |
| 11 | 26 | 1° amenorrhoea. Small uterus. Normal pubic hair. Small breasts | — | 17 | — | — | 2·0 | <1·0 | — | Oral oestrogens for 2 years | None for 8 years |
| 12 .. | 22 | 1° amenorrhoea. Small uterus. Normal pubic hair. Small breasts | — | 18 | — | — | <2·0 | <1·0 | — | Oral oestrogens for 5 months | Minovlar for 18 months |
| 13 .. | 22 | 1° amenorrhoea. Small uterus. Normal pubic hair and breasts | — | 18 | — | — | <2·0 | 1·2 | — | Oral oestrogens for 1 year | Minovlar for 6 months |
| 14 .. | 24 | 1° amenorrhoea. Small uterus. Normal pubic hair and breasts. Anosmic | — | Adult | — | — | <2·0 | <1·0 | — | Oral oestrogens for 18 months | Volidan 21 for 4 months |

*Puberty stages according to Tanner (1962).
†This therapy was discontinued for at least two months before administration of LH/FSH-RH, except in Case 2 when the interval was four weeks.
HCG = Human chorionic gonadotrophin. For therapy the dose of HCG indicated was given twice weekly and Pergonal three times a week.

though basal values were low. In five (Cases 6, 11, 12, 13, and 14) who had low basal values and an LH response, the LH rise was smaller and delayed, maximum values being seen 45-60 minutes after the injection. In general, the patients with a good LH response showed a noticeable FSH rise. In the four women patients, however, an appreciable increase in FSH was seen, while the LH rise was small and delayed.

*Repeat Tests.*—Repeat injections with the same dose of LH/FSH-RH were given to four patients (Cases 1, 12, 13, and 14) between three and six weeks after the first injection. In all four the LH and FSH responses were reproducible, being of similar magnitude and time course to those seen after the first injection. LH and FSH responses during these tests in Case 12 are shown in Fig. 2. No progressive increase in the response of

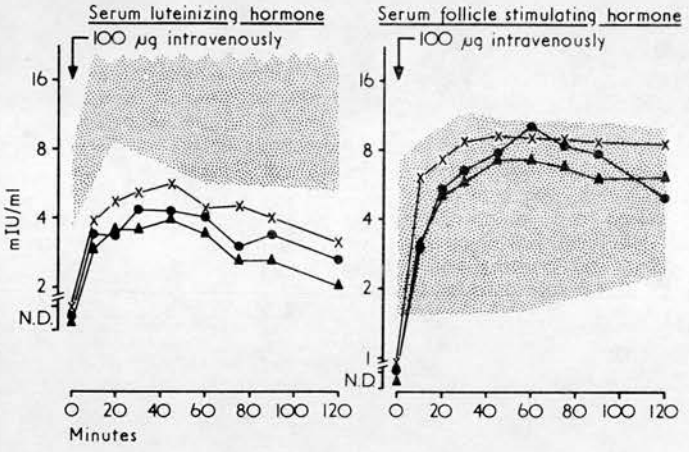



FIG. 2—Responses of serum LH and FSH to three intravenous injections of 100 μg LH/FSH-RH in one patient (Case 12). ● = first injection. × = second injection (three weeks later). ▲ = third injection (five weeks later). Shaded areas indicate range of responses seen in normal subjects.

either hormone was seen during the later two tests. In two patients (Cases 8 and 10) who did not have a hormone response after the first injection, a repeat test with 500 μg LH/FSH-RH was performed. In both a small rise of serum LH was seen (Case 8 <2·0-2·6, Case 10 <2·0-3·0 mIU/ml) but neither patient showed an FSH response (all values <1·0 mIU/ml).

## Discussion

The failure of these patients to achieve normal adult development is due to a lack of adequate circulating levels of one or both pituitary gonadotrophins. Hitherto it has not been clear whether this results from a deficiency of releasing hormone secretion by the hypothalamus, or from the inability of the anterior pituitary gland to secrete gonadotrophins. In this study nine of 14 patients showed a response of both LH and FSH after injection of 100 μg LH/FSH-RH, and in another two a small LH rise was seen after the larger dose. This shows that the anterior pituitary cells in these patients are capable of secreting gonadotrophins, and suggests that their hypogonadism results from deficient gonadotrophin-releasing hormone secretion. The degree of response varied, some patients showing a normal rise from low basal values, whereas others had smaller, delayed gonadotrophin response.

Some of the variation in the hormonal responses might be accounted for by the degree of previous stimulation of the pituitary by endogenous releasing hormone. Thus, two of the four patients who had normal hormonal responses to LH/FSH-RH had clinical evidence of partial spontaneous puberty, suggesting that the pituitaries of these patients may have been exposed to some endogenous releasing hormone and that their deficiency was only partial. These patients had also shown a noticeable and early 17β-hydroxyandrogen rise after human chorionic gonadotrophin, suggesting previous stimulation of the testis by endogenous gonadotrophin. In those with absent or small hormonal responses to the releasing hormone, it is not possible at present to decide whether their hypogonadism is simply due to chronic releasing hormone deficiency or whether the pituitary itself is unable to respond. This might be resolved after repeated stimulation with the releasing hormone. If a normal gonadotrophin response develops this would confirm that the pituitary cells had merely been atrophied. Repeat tests with 100 μg in four of the present patients did not show an increased response, but this may be due to the long time interval between injections.

Interpretation of the FSH responses in these patients is difficult, as in normal adults FSH release does not always occur after injection of LH/FSH-RH (Besser *et al.*, 1972). It is of interest to note, however, that in each of the women patients a normal FSH response occurred in the presence of a small delayed LH rise. This may represent a partial failure of the pituitary LH secreting cells only. Alternatively, there could be two rather than one releasing hormone—one each for LH and FSH. While much evidence is available that the synthetic decapeptide releases both LH and FSH, doubt exists as to whether this is the only gonadotrophin-releasing hormone, or whether another, predominantly releasing FSH, is present in the hypothalamus. If this is the case then these subjects could be suffering from a deficiency of the LH-releasing hormone only.

These results indicate that in most cases of isolated pituitary gonadotrophin deficiency there is a failure of hypothalamic-releasing hormone secretion, though results after prolonged administration of the releasing hormone are needed to exclude degrees of pituitary impairment in some patients. Furthermore, these studies suggest that synthetic LH/FSH-RH may prove to be a satisfactory form of treatment in these patients to induce the development of puberty and subsequent fertility.

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# THE TSH, FSH AND PROLACTIN RESPONSES TO CONTINUOUS INFUSIONS OF TRH AND THE EFFECTS OF OESTROGEN ADMINISTRATION IN NORMAL MALES

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SUMMARY

Four normal males received a constant infusion of 0·9% NaCl for 1 hr followed immediately by 500 μg of TRH infused over the same period. A rise in serum TSH was observed in all subjects while in three there was also a significant FSH response. The prolactin response, unlike that of TSH, was markedly pulsatile indicating that different mechanisms exist for the release of these two hormones from the pituitary after TRH. Circulating levels of LH were unaffected.

Ethinyl oestradiol, 30 μg daily for 3 days, was administered to two of the subjects and the infusions were repeated. Both basal FSH and LH levels were depressed, as was the FSH response to the infusion of TRH. By contrast, however, the TSH response to thyrotrophin releasing hormone was enhanced after oestrogen. In one subject the basal prolactin levels were significantly higher while in both there was an augmented prolactin response to TRH, the pulsatile pattern of release being maintained.

## INTRODUCTION

Thyrotrophin releasing hormone (TRH) has been shown to release both thyrotrophin (TSH) and prolactin in humans. However, a possible role in gonadotrophin secretion has remained in doubt since radioimmunoassays for the glycoprotein hormones usually show considerable cross reaction making interpretation difficult when changes in circulating thyrotrophin (TSH), follicle stimulating hormone (FSH), and luteinizing hormone (LH) are measured simultaneously.

We now report the effects of the infusion of 500 μg of TRH on circulating levels of TSH, FSH, prolactin and LH and the alteration in these responses following oestrogen pretreatment. Assays of sufficient specificity and sensitivity to distinguish between the four pituitary hormones have been used.

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## SUBJECTS AND METHODS

Four healthy male volunteers aged 24–27 years were studied following at least 24 hr of sexual continence. Each received a constant infusion of 0·9% NaCl solution during a control period of 1 hr via a cannula placed in the right antecubital vein; this was immediately followed by a constant infusion of a solution containing 500 μg of TRH (Roche) in 0·9% NaCl over a further hour. The infusions were repeated 1 week later after the administration of ethinyl oestradiol 10 μg three times daily for 2 days and 30 μg on the morning 3–4 hr prior to the start of the experiment. Subjects were allowed to eat before the experiment but were recumbent at rest during testing. Each procedure was carried out at the same time of day on both occasions. It is known that the effect of TRH on serum TSH is the same when TRH is administered at intervals of 7 days or more (Besser *et al.*, 1971). Blood samples

TABLE 1. Sensitivities and cross reactivities of the different radioimmunoassays

| | Radioimmunoassays for: | | |
|---|---|---|---|
| | LH (mU/ml) | FSH (mU/ml) | TSH (μU/ml) |
| Minimum detectable blood levels | 0·4 | 0·2 | 0·4 |
| Cross reactivity with* | | | |
| LH (mU/ml) | — | 80 | > 1000 |
| FSH (mU/ml) | 60 | — | 450 |
| TSH (μU/ml) | 600 | 250 | — |

* Greater than the amount stated would just be detected in the assay.

were withdrawn from an indwelling venous cannula positioned in the left antecubital vein for TSH, FSH, prolactin and LH at 5 min intervals for the first 55 min, then at minute intervals for 5 min until the end of the control infusion. Minute sampling was continued during the initial 10 min of the TRH infusion and at 5 min intervals thereafter. Sera were stored at −20°C and all samples from a given subject were assayed within the same batch.

*Serum LH and FSH* were measured in duplicate by a double antibody radioimmunoassay as described before (Mortimer *et al.*, 1973a). Results were expressed in milliunits (mU/ml) of LH standard MRC 68/40 and FSH standard 68/39 taking the contents of each ampoule as 39·8 and 32·8 units respectively. The within assay coefficient of variation for LH was 5% (over the range 0·4–15 mU/ml) and 6% for FSH (over the range 0·2–10 mU/ml).

*Serum TSH* was measured by a double antibody immunoassay using an anti-TSH serum raised against porcine TSH in rabbits. Results were expressed in μU/ml of MRC Standard 68/38. The within assay coefficient of variation was 8% (over the range 0·4–20 mU/ml). There was no cross reactivity between any of the assays at the levels measured (Table 1); the specificity of the antiserum was such that it did not require prior absorption with human chorionic gonadotrophin (Merrett *et al.*, in preparation).

*Serum prolactin* was measured by a specific double antibody technique using an anti-human prolactin antiserum raised in rabbits and a purified human pituitary prolactin as standard for iodination, kindly provided by Dr H. Friesen (McNeilly, 1973a). The within assay coefficient of variation was 10% over the range studied. Basal levels do not exceed 26 ng/ml in normal males.

## RESULTS

In view of the assay variance and the known fluctuations in basal hormone levels, a significant response was considered to have occurred only when a rise 5 times the coefficient of variation or more was recorded above the maximum hormone level seen during the control infusion. For FSH this was a change of 30% and for LH 25%. Applying these criteria, three of the four subjects showed a significant FSH response to TRH (Fig. 1); the rise occurred within 6–9 min from the start of the infusion. Although the levels rose significantly they did

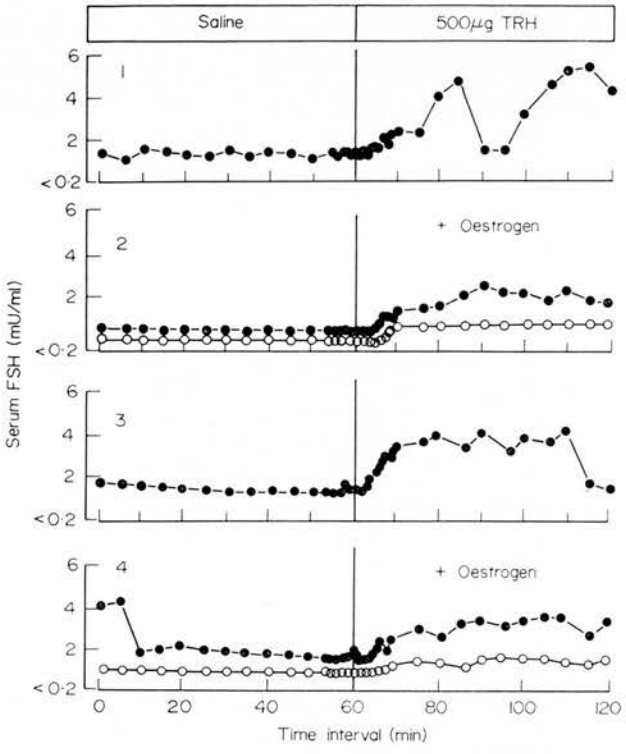



FIG. 1. The FSH response to a continuous infusion of 500 μg TRH in four normal males (●). The effect of oestrogen administration is shown in subjects 2 and 4 (○).

not exceed the limits of the normal basal male range (0·2–5·9 mU/ml). The fourth subject showed serum FSH levels between 4·0 and 4·2 mU/ml during the first 10 min of the control infusion, but levels during the remaining 45 min of saline infusion were lower, between 1·7 and 2·1 mU/ml. During the TRH infusion, the serum FSH levels rose and were maintained between 3·0 and 3·9 mU/ml for the whole period 15–60 min. However, in view of the initial high control value, the rise in FSH to TRH in this subject did not fit our criteria for a significant response (see above). In subject 1 evidence of pulsatile FSH release was observed. There was no evidence of LH release in response to TRH in any subject. Ethinyl oestradiol administration was associated with suppression of the basal FSH and LH

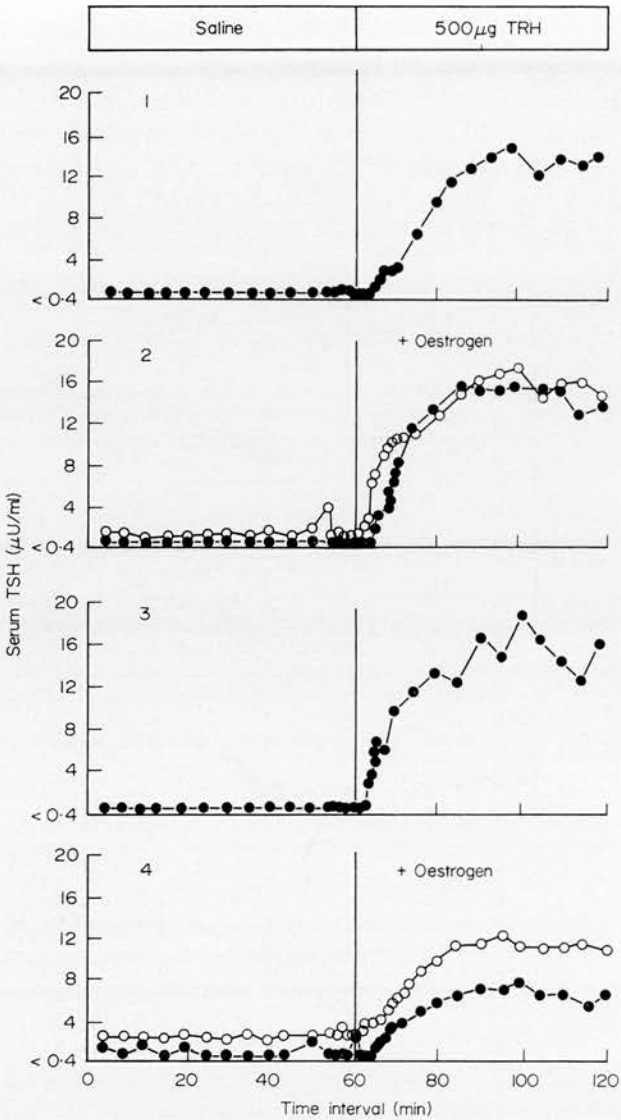



FIG. 2. The TSH response to a continuous infusion of 500 μg TRH in four normal males (●). The effect of oestrogen administration is shown in subjects 2 and 4 (○).

levels and also marked reduction in the FSH response to the infusion of TRH in both subjects to whom it was given (2 and 4, see Fig. 1).

When the changes in serum TSH were considered (Fig. 2) two of the four subjects (2 and 3) had undetectable levels of hormone during the initial saline infusion whereas the others had measurable levels for part of the control period. A response attributable to the infused material was defined as for FSH and for this hormone the response was considered to be significant when it exceeded the maximal level observed during the control infusion by 40%

or more. This occurred between 4 and 7 min from the start of TRH administration in each subject. The rise in serum TSH was initially smooth in every subject but in subject 3 fluctuating values were seen later. The maximum TSH values following TRH infusions were greater in both subjects after pretreatment with oestrogen, and basal levels were slightly raised.

Serum prolactin (Fig. 3): the basal levels fluctuated and there was a significant but small increase in subject 4 after ethinyl oestradiol. The response to the infusion of TRH was in marked contrast to that seen in TSH and FSH. Prolactin levels clearly rose in a pulsatile fashion and the response in one subject was clearly greater after pretreatment with oestrogen than before, whereas in the other the increased prolactin level was less marked although significant.

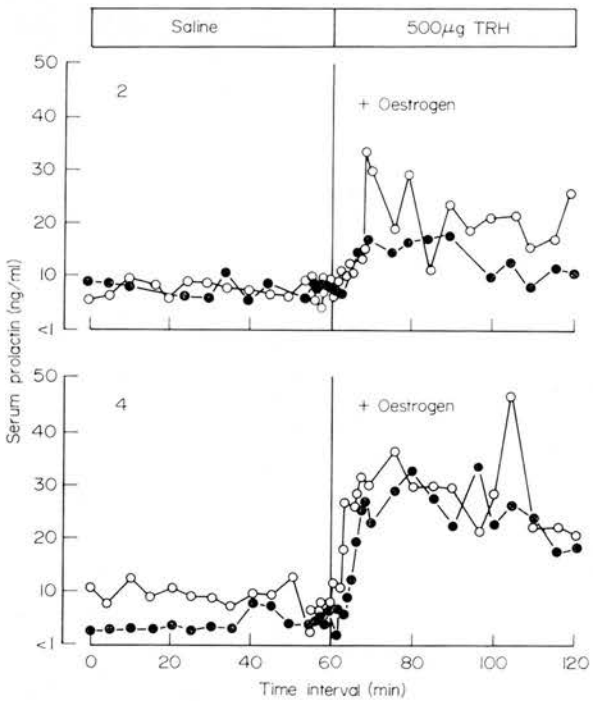



FIG. 3. The prolactin response to a continuous infusion of 500 μg TRH in two normal males (●). The effect of oestrogen administration is shown in these subjects (○).

## DISCUSSION

We have previously shown a rise in serum FSH in response to 200 μg of TRH administered as a single intravenous bolus (Mortimer *et al.*, 1973a). This effect has been confirmed in the present report which shows similar changes in serum FSH during a constant infusion of 500 μg of TRH in three out of four normal male subjects. In addition, while basal levels of both LH and FSH were reduced by the prior administration of ethinyl oestradiol the FSH response to TRH was also suppressed. There was no rise in serum LH attributable to the TRH.

In contrast to the serum FSH levels, the basal levels of TSH in both subjects and of pr lactin in one were increased by oestrogen administration and the responses to TRH we enhanced. The potentiation of TSH release from the pituitary by TRH after oestroge administration has also been described by other workers and appears to be a consiste finding (Faglia *et al.*, 1973). Oestradiol therefore appears to produce different effects on th hypothalamic–pituitary axis, with respect to the release of the gonadotrophins, thyr trophin and prolactin.

The pulsatile nature of the prolactin response provides further evidence of the dissociatio of pituitary TSH and prolactin responses to TRH. It also suggests that the pituitary pr lactin secreting cell itself is at times refractory to the action of the hypothalamic hormon A similar pattern of LH and FSH response to a continuous infusion of the gonadotroph releasing hormone (LH/FSH–RH) has also been demonstrated (Mortimer *et al.*, 1973b It is suggested that the pituitary cells have different receptor mechanisms sensitive to th same hypothalamic regulatory hormone, but associated with the release of different pituita hormones. These mechanisms, in a sense, show 'fatigue' in that release of the pituita hormone may be intermittent despite continuous delivery of hypothalamic hormone. might be that such mechanisms are partly responsible for the random fluctuations in bas prolactin and gonadotrophin levels which occur in normal men and women (McNeil *et al.*, 1974; McNeilly & Chard, 1974; Naftolin *et al.*, 1972). The amplitude and frequenc of the pulses in circulating trophic hormone levels may be important in coding the messag from the hypothalamic–pituitary systems to the target organ and may help to determine i responses.

These observations confirm the *in vitro* studies which suggested that there is no withi assay cross reactivity between these hormones or their subunits in the assays used. Howeve the physiological importance of TRH in regulation of the secretion of FSH remains unce tain and further work is needed to decide whether or not it is of biological importanc Franchimont (1972) studied the gonadotrophin responses to TRH in women and reported definite LH response, in occasional subjects, when tested around the time of the luteal pea but not at other phases of the menstrual cycle. He did not detect a rise in serum FSH. The is apparently therefore a difference between men and women in this respect and we ca confirm that the FSH response to TRH reported in males in this paper has not been seen i women. It seems possible that the higher circulating oestrogen levels in women may inhib the FSH response to TRH.

## ACKNOWLEDGMENTS

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# PROLACTIN RELEASING ACTIVITY IN THE EARLY HUMAN FOETAL HYPOTHALAMUS

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It is generally accepted that the secretion of prolactin from the mammalian pituitary gland is under inhibitory control from the hypothalamus (Meites, 1973). During studies on the development of gonadotrophin secretion in the early human foetus (Gilmore, Dobbie, McNeilly & Mortimer, 1977) it became apparent that up to week 16, the hypothalamus may exert a stimulatory influence over prolactin release which switches to the adult situation as the foetus develops towards term. This communication describes these findings in more detail.

Pituitary glands, hypothalami, and cortices were collected from foetuses delivered by hysterotomy between 10 and 19 weeks of pregnancy. The tissues were immediately frozen on solid $CO_2$ and stored at -20 °C until extracted. Blood samples were taken from each foetus together with a sample of amniotic fluid and maternal blood. Serum was separated and stored with the fluid at -20 °C until assayed for prolactin as described previously (McNeilly, 1973; McNeilly & Hagen, 1974). Each foetal pituitary gland was extracted into 1 ml 0·01 M-HCl, pH 4·0, containing $10^{-3}$ M-disopropylphosphofluoridate as enzyme inhibitor (Scott & Lowry, 1974). After homogenization for 1 min with a glass rod and sand, the extracts were centrifuged at 2000*g* for 30 min at 4 °C, the supernatant was snap frozen in a mixture of acetone–solid $CO_2$ and stored at -20 °C until assayed. Each foetal hypothalamus and cortex was extracted by homogenization in 1 ml 0·05 M-HCl (Campbell, Feuer & Harris, 1964), and the extracts were stored at -20 °C until assayed.

The prolactin releasing or inhibiting activity of the foetal hypothalamic and cortical extracts was assayed by a modification of the technique of McDonald & Gilmore (1971). Mature female Sprague-Dawley rats, maintained under controlled lighting, were ovariectomized 1 month before use. Three days before the test infusions, oestradiol benzoate (50 μg) and progesterone (25 mg) in corn oil were injected s.c. Extracts (0·9 ml) adjusted to pH 7·0 with NaOH were injected over a period of 10 s into the right carotid artery under light ether anaesthesia. Control infusions of carrier HCl neutralized (pH 7·0) with NaOH as for the extracts, thyrotrophin releasing hormone (TRH) at 200, 100, 20, 10 and 1 ng, dopamine (50 μg) or dopamine (50 μg) plus TRH (100 ng) were given under identical conditions. Blood samples (0·2 ml) were withdrawn from the iliac vein, before and 15 and 30 min after injection of the test substances. Serum was separated and stored at -20 °C until assayed for prolactin using the rat prolactin radioimmunoassay kit supplied by the NIAMDD.

The levels of TRH in 11 hypothalamic and cortical extracts were measured by Dr Jeffcoate of St Thomas's Hospital, London, using a specific radioimmunoassay (Jeffcoate, Fraser, Gunn & White, 1973). Prolactin was detectable in all foetal sera and pituitary glands; the concentration in the latter increased with foetal age (Table 1). Levels of prolactin in the amniotic fluid were low and comparable to those in the maternal and foetal circulations at week 10 but increased progressively between weeks 10 and 15 to between 4 and 100 times those in maternal or foetal sera.

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Table 1. *Total content of immunoreactive thyrotrophin releasing hormone (TRH) and biological prolactin releasing or inhibitory activity of foetal hypothalamic and cortical extracts during early pregnancy*

| Week of gestation | Foetal sex | TRH content (ng by radioimmunoassay) | | Prolactin releasing or inhibiting activity (ng TRH equivalents) | | Prolactin levels | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Foetus | | Amniotic fluid concn (ng/ml) |
| | | Hypothalamus | Cortex | Hypothalamus | Cortex | Serum (ng/ml) | Pituitary content (ng) | |
| 10 | F | 0·80 | 0·63 | 0–1 | INHIB | 20 | 40 | 8 |
| 10 | M | 1·04 | 0·70 | 0–1 | 1–10 | 8 | 34 | 20 |
| 12 | M | 1·24 | 0·50 | 1–10 | 1–10 | 10 | 26 | 65 |
| 12 | M | 1·10 | <0·10 | 0 | INHIB | 15 | 38 | 180 |
| 13 | M | 1·07 | 0·22 | 0–1 | 1–10 | 18 | 45 | 205 |
| 14 | F | 1·15 | <0·10 | 0 | 0 | 25 | 80 | 286 |
| 15 | M | 0·60 | <0·10 | 1–10 | INHIB | 30 | 200 | 1280 |
| 15·5 | F | 3·25 | 1·40 | INHIB | 0 | 38 | 76 | 1498 |
| 16 | M | 1·40 | 0·60 | INHIB | 1–10 | 84 | 89 | 3288 |
| 17 | F | 12·80 | 1·00 | INHIB | 0 | 33 | 110 | 4767 |
| 19 | F | 4·25 | 1·20 | INHIB | INHIB | 18 | 170 | 4510 |

M, male; F, female. INHIB, Inhibition of prolactin secretion.

The immunoreactive TRH content of the foetal hypothalamus was 1·3–12·8 times greater than that of the cortex and increased markedly after week 15 of pregnancy (Table 1). No correlation was seen between the content of immunoreactive TRH and the biological release of prolactin activity of either hypothalamic or cortical extracts in the rat preparation. Hypothalamic extracts up to week 15 showed either no effect ($n=2$) or prolactin releasing activity equivalent to between 0 and 1 ng ($n=3$) or 1 and 10 ng ($n=2$) TRH. All hypothalamic extracts from foetuses of 15·5 weeks or greater gestational age inhibited prolactin release ($n=4$). Cortical extracts caused release (1–10 ng TRH equivalent, $n=4$), no change ($n=4$) or inhibition ($n=4$) of prolactin secretion in the in-vivo assay. This activity was not related to hypothalamic activity or to immunoreactive TRH content.

These results suggest that up to week 16 of gestation the foetal hypothalamus is either devoid of activity or possesses only releasing activity for prolactin. After week 16, the foetal hypothalamus exerts an inhibitory influence on pituitary prolactin release similar to that in the adult. It is clear that the biological releasing activity bears no relation to TRH and the results suggest that if TRH plays any role in the control of prolactin secretion, at least in the human foetus, it is only of minor importance.

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# Effect of Chronic Immunoneutralization of Thyrotropin-Releasing Hormone on the Hypothalamic-Pituitary-Thyroid Axis, Prolactin, and Reproductive Function in the Ewe

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**ABSTRACT.** The physiological role of TRH was studied by chronic neutralization of its action by actively immunizing six adult ewes against the synthetic TRH conjugated to BSA. The animals were followed for a period of 2 yr through the various reproductive states of estrous cyclicity, anestrus, pregnancy, and lactation and were subjected to tests known to stimulate PRL release. Changes in plasma concentrations of TSH, $T_4$, $T_3$, and PRL were compared with levels in six control ewes.

All TRH-immunized ewes produced antibodies, which were maintained by booster immunizations throughout the study period. Effects on plasma contractions of immunoassayable TSH were slight and inconsistent, but there was a clear reduction in levels of $T_4$ and $T_3$ in the blood of the TRH-immunized ewes, although detectable amounts of the hormones were always present. The seasonal rises in PRL concentrations during the summer months and during pregnancy and lactation still occurred in the TRH-immunized ewes, although there was an indication that levels were slightly lower.

Stimulation of the hypothalamic-pituitary system by subjecting the animals to heat stress caused a rise in plasma PRL concentrations in both groups, but this effect was significantly less in the TRH-immunized ewes. After the administration of metoclopramide, during suckling, and during estrus, PRL levels rose in both groups. Although the mean concentration of circulating PRL seemed lower in each instance in the TRH-immunized ewes, there was not a signicant difference.

There was no difference in the time of onset of the breeding season, estrous cycle length, or the time of onset of estrous behavior in the TRH-immunized ewes. All six controls and four of the six TRH-immunized ewes became pregnant and delivered normal offspring.

The marked reductions in plasma thyroid hormone concentrations in TRH-immunized ewes confirm that TRH is an important hypothalamic stimulator of TSH and, thus, thyroid function. In contrast, the absence of any major influence of TRH immunization on PRL levels in the different physiological situations suggests that it does not have an obligatory role in the control of PRL release. However, since the thyroid hormones were still present in low amounts in TRH-immunized ewes, it remains possible that the persistence of PRL secretion resulted from the presence of small amounts of biologically active TRH. In addition, adaptation of other hypothalamic control mechanisms for PRL release could occur, minimizing the effects of TRH neutralization. This together with reduced PRL response to acute stimuli mean that the possibility that TRH plays a minor role in PRL release cannot be excluded. (*Endocrinology* **111:** 1964, 1982)

ADMINISTRATION of the hypothalamic tripeptide TRH stimulates the release of both TSH and PRL from the anterior pituitary gland (1–3). Since its isolation and synthesis over 10 yr ago, TRH has been considered the prime physiological stimulator of TSH secretion, but it is still unclear whether the stimulatory effects of PRL have physiological significance. Our understanding is complicated by the fact that PRL is primarily under negative hypothalamic control exerted via a PRL inhibitory factor, probably dopamine, while the question of a separate PRL-releasing factor remains to be resolved (4).

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In addition to examining the effects of its administration, a classic way to study the physiological role of a hormone is to inhibit its action. Perhaps the best method available of interfering with the action of TRH is neutralization by antibodies. In the present study, we have investigated the effect of neutralizing TRH over a 2-yr period in the ewe by active immunization. During this time, the ewes were followed through the various reproductive states of estrous cyclicity, anestrus, pregnancy, and lactation and were subjected to tests known to stimulate PRL release (administration of metoclopramide, heat stress, and suckling). The effects of TRH immunization were assessed by measuring circulating blood levels of TSH, $T_3$, $T_4$, and PRL.

## Materials and Methods

*Immunization*

Six mature Dam-line ewes living outside in a field station near Edinburgh were immunized against TRH, and six control ewes were immunized against BSA. TRH (a generous gift from Dr. H. Wissmann, Hoechst, Frankfurt, Germany) was conjugated to BSA by means of 1,5-difluoro-2,4-dinitrobenzol. Six milligrams of conjugate in 10 ml saline were emulsified in 16 ml Freund's complete adjuvant and injected at four sc sites under each leg in a volume of 1 ml/site in each of the six ewes. During the 2-yr period of study, six booster immunizations were administered, using the same dose of conjugate, at the intervals shown by the *arrows* in Fig. 1. Booster immunizations were given at im or sc sites in Freund's incomplete adjuvant, except for the fourth and sixth booster immunizations which were performed using complete adjuvant.

Control ewes were treated similarly, each being immunized with 1 mg BSA carrier.

*Detection and characterization of TRH antibodies*

The presence of antibodies to TRH was detected by the ability to combine with $^{125}$I-labeled TRH, prepared as described previously (5). TRH antibody titer was measured in doubling dilutions of serum from 1:100 up to between 1:3, 200 and 51,200 incubated with $^{125}$I-labeled TRH (10,000 cpm) in a final volume of 300 $\mu$l 0.01 M PBS (pH 7.4) containing 0.1% BSA. After incubation for 24 h at 4 C, antibody-bound and free labeled TRH were separated using 5 vol ice-cold ethanol, and the titer was expressed as the dilution binding 33% tracer.

*Reproductive state of ewes*

The primary immunization was performed in December during the breeding season, which lasts from November through to April. The ewes were kept with a raddled vasectomized ram during the breeding season to detect estrus.

The study was continued through the summer anestrous period and the following breeding season. In January 1979, ovulation was synchronized using progesterone pessaries. During the first week of March, the ewes were mated. They were then studied during the succeeding pregnancy, the period of lactation, and finally the return to normal estrous cycles.

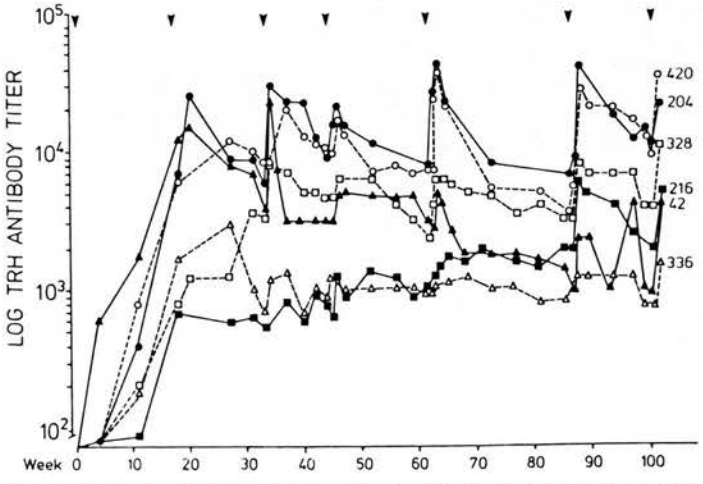



FIG. 1. Individual TRH antibody titers in six ewes immunized against TRH conjugated to BSA. *Arrows* show the time of primary immunization (week 0) and booster immunizations.

*Basal secretion of TSH, $T_3$, $T_4$, and PRL*

Blood samples were collected by vacutainer from a jugular vein at regular intervals, and plasma was stored at −20 C until required for hormone assay and antibody titer estimation. A more detailed assessment of the effects of the immunization on hormone profiles was obtained by taking sequential blood samples during February 1979. For this purpose, a cannula ending in a three-way tap was inserted into a jugular vein of three controls and four TRH-immunized ewes on the day before the study; the animals were then placed in individual holding pens in which they remained throughout the experiment. Blood samples were collected hourly between 0800 –1800 h.

*PRL concentrations at estrus*

To determine whether there was an effect of treatment on the rise in PRL at estrus, PRL concentrations were measured during an estrous period synchronized by progestagen pessaries in January. Twelve days after insertion of the pessary, the animals were fitted with cannulae, as described above, and after 2-h blood samples were collected at 30-min intervals for 5 h. The following day, two further baseline samples were collected at 0800 and 0900 h, at which time the progesterone sponge was withdrawn. Blood samples were then taken hourly until 1800 h, and between 0800 and 1800 h for the following 2 days. Beginning 1 day after sponge withdrawal and immediately before and after the blood-sampling period, the ewes were tested for estrous behavior by removing them from their holding pens and transferring them to a pen with a vasectomized ram.

*Tests of the hypothalamo-pituitary system*

In addition to the changes induced by the environment and reproductive state, the animals were subjected to tests to establish the integrity of the PRL release mechanism. For these experiments, a cannula ending in a three-way tap was inserted into a jugular vein 1 day before the study; ewes were then placed in individual holding pens.

*Metoclopramide.* Metoclopramide is a dopamine antagonist, probably exerting its major action on PRL release by preventing the inhibitory actions of dopamine at the pituitary level, thereby allowing PRL release. This test was performed in the midluteal phase of an estrous cycle in February 1979, when tonic PRL secretion was low. Three blood samples were collected at 30-min intervals before the im injection of 10 mg metoclopramide (Maxalon, Beecham Research Laboratories Brentford, United Kingdom). Blood samples were then continued for a further 150 min.

*Heat stress.* The animals, which had been living out of doors during the winter, were taken indoors to a temperature of 10 C. Blood samples were collected at time zero and 30, 60, and 120

in before transfer to a heat chamber at 43 C. Rectal temperature was taken immediately before and 30 min after transfer the heat chamber. Blood sampling ws continued at 15-min tervals for a further 45 min.

*uckling.* Between 5 and 6 weeks postdelivery, four treated and ur control ewes were each fitted with a jugular cannula as fore. During this time, the lambs were taken from their others and kept in adjacent pens. One hour after cannulation, ood samples were collected at 30-min intervals for 1.5 h. The mbs were returned to their mothers to suckle, and blood mples were collected at 5, 10, 15, 30, 45, and 60 min.

*RH test*

In November 1979, the six TRH-immunized ewes and four of e control ewes were each fitted with a cannula and placed in ns as before. On the following day, blood samples were taken and 30 min before and immediately before the iv injection of μg TRH (Roche, Santa Ana, CA) in 1 ml physiological saline. ampling was continued at regular intervals for 6 h.

*SH test*

Twenty-four hours later, the responsiveness of the thyroid to SH was tested in these ewes. Basal blood samples were taken min before and immediately before the iv injection of 500 ovine TSH (NIH TSH-S8) in 1 ml saline and at regular tervals for 7 h thereafter.

*ollection of tissues*

During 1 day in June 1979, annual shearing was performed all ewes at random by an unbiased shepherd who then eighed the wool.

One week after the TSH test in November 1979, the six RH-immunized and four control ewes were anesthetized with dium pentobarbitone and killed by cutting the jugular vein d carotid artery. After collection, blood samples were allowed clot at room temperature overnight; after centrifugation, the rum was stored at −20 C. The brain was rapidly removed and e hypothalamus, pineal gland, and similar weights of cortex d cerebellum were dissected. These tissues were placed in 2 l 0.01 M phosphate buffer, pH 7.4, for 10 min in a boiling water th. After cooling, 1 ml phosphate buffer containing 0.2% BSA as added to each sample, and the tissue were homogenized in ass homogenizers. After centrifugation at 600 × *g* for 1 h, the pernatants were collected and stored at −20 C until radioimunoassayed for TRH.

The anterior pituitary glands were weighed and bisected. ne half was placed in Bouin's fixative and, after sectioning, ained with Alcian blue periodic acid-Schiff orange G. The mainder of the anterior pituitary gland was weighed before omogenization in 5 ml 0.1 M phosphate buffer (pH 7.4) conining 0.2% BSA. After centrifugation, the supernatant was ored at −20 C before TSH and PRL assay.

Thyroid glands were weighed, fixed in Bouin's fluid, and, ter sectioning, stained with hematoxylin and eosin.

*RIAs*

PRL was measured using a specific double antibody RIA described previously (6). The sensitivity of the assay, defined as the concentration at 90% of the buffer control, was 0.5 ng PRL (NIH-P-S6) per ml. The intra- and interassay coefficients of variation were 7% and 9%, respectively. TSH was measured by a double antibody RIA developed by I. C. McMillen and J. S. Robinson (unpublished). The antiserum was raised in a guinea pig against bovine TSH (NIH-TSH-B5; 2.2 IU/mg) by Dr. J. Setchell, Oxford, United Kingdom.

$^{125}$I-Labeled bovine TSH (bTSH) was prepared by the lactoperoxidase method of iodination using highly purified bTSH (25 IU/mg) kindly supplied by Dr. J. G. Pierce, UCLA. After purification on a Sephadex G-100 column (1 × 25 cm), aliquots of [$^{125}$I]bTSH (SA, 86 and 104 μCi/μg; n = 2) were stored at 4 C until used in the assay within 4 days. The RIA procedure was similar to that for PRL (6), except that samples of 100 and 20 μl plasma were measured in duplicate. Results are expressed in terms of the purified bTSH preparation.

At a final antibody dilution of 1:240,000, 34 ± 2% (±SEM; n = 6) of [$^{125}$I]bTSH was bound. Sensitivity, defined as the concentration resulting in a response 2 SD from the buffer control, was 0.1 ng bTSH/ml. The standard curve was accurate over the range of 0.1–6 ng/ml (20% 50%, and 80% of the buffer control at 2.08, 0.70, and 0.24 ng/ml, respectively), and results were calculated after log-logit transformation of the standard curve (slope, −2.93; intercept, −0.455; quality of fit, 0.988).

Accuracy of the assay, determined by the mesaurement of bTSH standard (0.1–20 ng/ml) added to hypophysectomized fetal sheep plasma with an undetectable (<0.1 ng/ml) level of TSH was 106 ± 2.6% (n = 8) (McMillan, I. C., and J. S. Robinson, unpublished). The precision of the assay, expressed as the coefficients of variations (percentage) assessed by repeated assays of two pools of sheep plasma with TSH levels of 0.4 and 1.2 ng/ml (68% and 26% of the buffer control), was 9.7% (n = 8) within and 11.4% (n = 5) between assays.

Serial dilutions of plasma from lambs and nonpregnant, pregnant, and lactating ewes with TSH levels varying between 1.0–22 ng/ml were parallel to those of bTSH standard. There was no significant cross-reaction (<0.3%) with ovine LH (NIH-LH-S17), ovine PRL (NIH-P-56), ovine GH (NIH RIA 074313), or ovine FSH (Papkoff G4 5DC).

$T_3$ and $T_4$ were measured using solid phase RIA systems employing antisera raised in sheep (7). Sensitivities (90% of the buffer control) were 7 ng $T_4$/ml and 0.4 ng $T_3$/ml. Intra- and interassay coefficients of variation were 6% and 10% for $T_4$ and 8% and 11% for $T_3$, respectively.

RIAs for TRH were established with antisera from all six ewes using the same procedures described previously for a TRH RIA based on a rabbit antiserum (5). One of these antisera, from ewe 420, was used for the RIA of tissue contents of TRH. The specificity of this antiserum is shown in Table 1; sensitivity was 0.3 pg/tube.

Differences in response to the various tests between groups were tested by two-way analysis of variance for repeated measures using change scores (posttreatment minus pretreatment for each animal) transformed to logarithims where appropriate to equalize errors within cell. All error terms used to test for

TABLE 1. Cross-reactivity of TRH and its analogs with TRH antiserum from ewe 420

| Structure | Relative activity (%) |
|---|---|
| pGlu-His-ProNH$_2$ (TRH) | 100 |
| pGlu-Phe-ProNH$_2$ | 100 |
| pGlu-Met-ProNH$_2$ | 1.4 |
| pGlu-Lys-ProNH$_2$ | 12.5 |
| pGlu-Lys(BOC)-ProNH$_2$ | 0.26 |
| pGlu-His-ProOH | <0.005 |
| pGlu-Leu-ProOH | <0.005 |
| pGlu-Ala-ProOH | <0.005 |
| pGlu-His-Pro-propylamide | 0.03 |
| pGlu-His-TrpNH$_2$ | 0.05 |
| H.His-ProNH$_2$ | 0.004 |
| Glu(BOC)-His-ProNH$_2$ | 0.96 |
| LHRH | <0.005 |
| GH release inhibitory hormone | <0.005 |
| Oxytocin | <0.005 |

group differences or pre- *vs.* postdifferences represent the among-animal variants. All other results were analyzed by Student's *t* test.

## Results

### *Antibody formation*

All ewes immunized against TRH produced antibodies. Titers rose slowly during the first few months after immunization, reaching near-maximal levels by 20 weeks, and were maintained or stimulated further by the booster immunizations (Fig. 1). Antibody titers varied considerably among ewes, the poorest responder producing titers of around 1:1,000 and the highest with values between 1:20,000–1:50,000.

### *Reproductive state*

The time of onset of estrous cycles (first heat) was the same in both groups of ewes (last week of October during 1978 and last week of October-first week of November 1979). There was no difference in cycle lengths between groups [17 ± 0.1 days for controls and 17 ± 0.2 days for TRH-immunized ewes (mean ± SEM)]. In May 1978, one of the TRH-immunized ewes happened to become pregnant and gave birth to twins in September. After removal of progestagen pessaries on February 20th 1979, all ewes exhibited behavioral estrus 60–72 h later, and during the following cycle, all ewes mated with a fertile raddled ram. All six of the controls became pregnant and delivered normal offspring (five sets of twins and one singleton). Four of the six TRH-immunized ewes became pregnant and delivered normal offspring (three sets of twins and one singleton). All ewes delivered during the last week of July and the first week of August after 26 weeks of pregnancy. The weights of the lambs at birth and at 10 weeks of age were similar in both groups of ewes (birth weight, 3.4 ± 0.2 *vs.* 3.4 ± 0.1 kg 10 weeks, 18.4 ± 1.2 *vs.* 15.9 ± 0.9 kg for control and TRH-immunized ewes, respectively).

### *Basal concentrations of TSH, PRL, $T_3$, and $T_4$*

Basal plasma concentrations of TSH and PRL are shown in Fig. 2. Results from the ewe that became

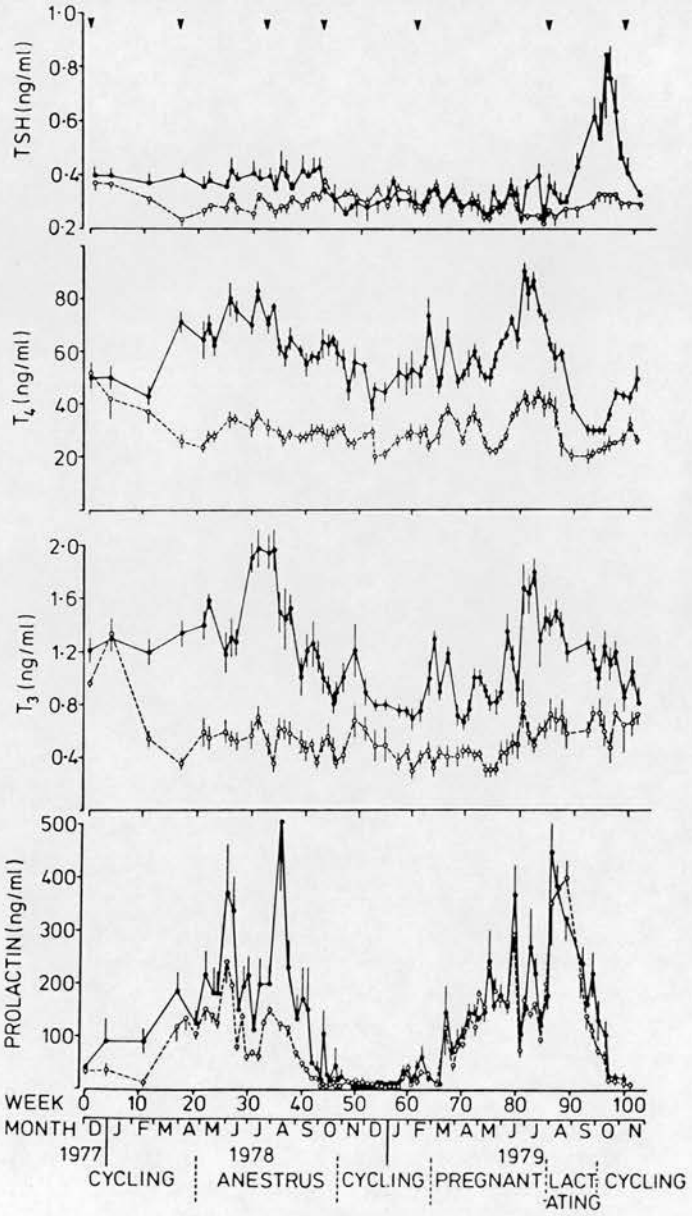



FIG. 2. Plasma concentrations of TSH, $T_4$, $T_3$, and PRL in control ewes (●) and ewes immunized against TRH (○). Values are the mean ± SEM for six animals per group, except during the anestrous and pregnancy periods when n = 5 and n = 4, respectively, for the TRH-immunized ewes (see text). *Arrows* indicate times of immunizations.

egnant during the first year of the study and the two imals that failed to become pregnant in the second ar have been excluded during the period when their productive states differed from those of the others in e group. Between weeks 17–43 of immunization, TSH vels in the TRH-immunized ewes were lower than in ntrols; values were significantly different ($P < 0.01$) on occasions. However, this difference disappeared be- een weeks 43–80, followed by a rise in TSH in the ood of the control ewes during lactation which, again, oduced significant differences ($P < 0.05$ to $P < 0.01$) tween the groups.

There were no clear differences in plasma PRL con- ntrations between the two groups. To avoid complicat- g the data, values for the estrus-induced rise in PRL in th groups of ewes have been omitted from the calcu- ions by omitting values occurring within 1 day of trus. During the early states of immunization and oc- sionally during anestrus, the mean PRL levels were wer in the TRH-immunized ewes, but this was only tistically significant on three occasions ($P < 0.05$), and e normal rises in PRL occurred during anestrus, preg- ncy, and lactation.

In the controls, there was a rise in $T_3$ and $T_4$ in the ood during the summer months. There was no marked fference between the times the ewes were in normal estrus and when they were pregnant, but the seasonal crease was more marked when the animals were lac- ing. In the TRH-immunized animals, the concentra- ns of $T_3$ and $T_4$ were clearly lower than those in ntrols by 3 months after immunization; this difference s maintained throughout the following 90 weeks of the periment. The majority of the values were lower than ose in the controls ($P < 0.001$). However, there were dications that the seasonal pattern of changes observed the controls was still apparent in the treated ewes, but a reduced level. $T_3$ and $T_4$ did not appear to be mpletely eliminated in the TRH-immunized ewes, ce plasma from a hypophysectomized goat (provided Dr. H. Buttle, NIRD, Reading, United Kingdom) gave eading below the sensitivity of the assays (<0.4 ng/ml and <7 ng/ml $T_4$).

Despite the marked differences in antibody titer among e individual TRH-immunized animals, there were no nsistent related variations in hormone concentrations. hen hormone concentrations were compared in the RH-immunized ewes that became pregnant or failed to come pregnant out of phase from their group, there s an indication of a slight rise in $T_4$ in the blood during egnancy. In ewe 204, the mean value for $T_4$ was 33 ± g/ml (n = 13) during pregnancy, while the means for e five remaining TRH-immunized ewes averaged 30 ± ng/ml. These values were not statistically different. stpartum, the mean values were similar, 27 ± 2 ng/ml (n = 11) for ewe 204 and 28 ± 1 ng/ml for the remaining ewes. When the four TRH-immunized ewes were pregnant in 1979, mean $T_4$ values were 34 ± 1.5 ng/ml (n = 21), while those for the two nonpregnant TRH-immunized ewes were significantly lower (28 ± 1.1 ng/ml; $P < 0.01$). This difference disappeared postpartum, when values were 26 ± 0.9 ng/ml (n = 18) and 25 ± 0.7 ng/ml.

When the animals were bled sequentially on a single day, a clear reduction ($P < 0.001$) in $T_3$ and $T_4$ concentrations in the blood was observed in the TRH-immunized ewes, while PRL concentrations were unaltered (Fig. 3).

*Tests of the hypothalamo-pituitary system*

PRL response to the injection of metoclopramide, heat stress, and suckling are shown in Figs. 4–6. Clearly, all of these stimuli caused an elevation of PRL in both groups of ewes. However, the TRH-immunized ewes released significantly less PRL than did the controls ($P < 0.05$) after heat stress. Similarly, when the ewes were sequentially sampled during the estrous period, a PRL surge was observed in all ewes (data not shown). The cumulative increases in PRL concentrations after these stimuli are shown in Fig. 7. The mean PRL values seemed to be lower in the TRH-immunized ewes after each of the stimuli, but the values reached significance only after heat stress.

Plasma concentrations of TSH, $T_4$, and $T_3$ were also measured during these experiments, but the treatments caused no significant changes in the values for either group. Rectal temperatures were similar in both groups of ewes before and during the exposure (room temperature, 39.2 ± 0.04 *vs.* 39.9 ± 0.1 C; heat chamber, 39.3 ± 0.3 *vs.* 39.9 ± 0.1 C for control and TRH ewes, respectively).

*TRH and TSH tests*

At the time of the TRH and TSH tests, there were no significant differences between groups in the basal plasma concentrations of TSH, PRL, $T_3$, or $T_4$. All four control ewes showed a rapid large rise in plasma levels of PRL and TSH, followed by a rise in $T_4$ and $T_3$ (Fig. 8), after TRH administration. One of the TRH-immunized ewes (no. 336) also responded to exogenous TRH with a substantial rise in TSH, while in the other immunized animals, there was little, if any, TSH response. This indicated that the antibodies in the blood of ewe 336 were insufficient to neutralize the exogenous TRH. In fact, this ewe had the lowest antibody titer throughout the study period (Fig. 1), and her hormonal profile was not included with the five other TRH-immunized ewes for the purposes of depicting the results shown in Fig. 8.

The PRL response after TRH administration was

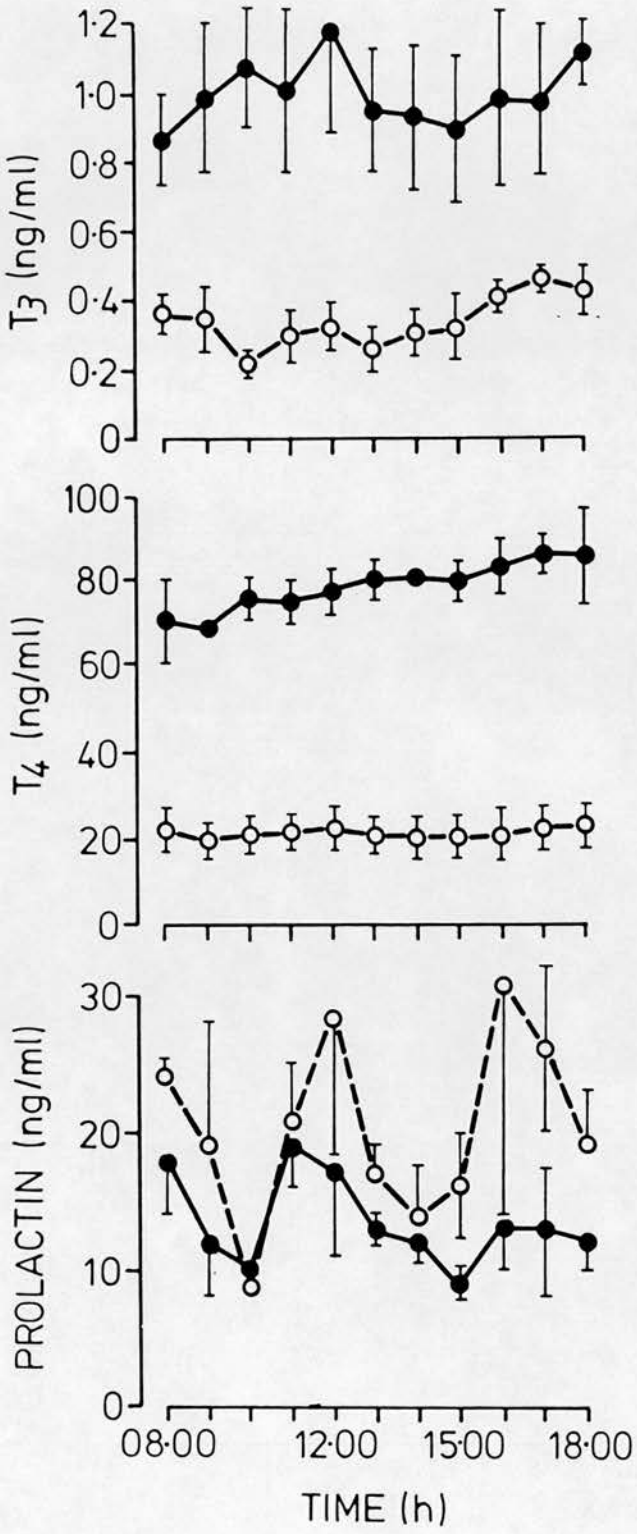



FIG. 3. Plasma concentrations of $T_3$, $T_4$, and PRL in sequential samples obtained from three control ewes (●) and four ewes immunized against TRH (○). The animals were kept in individual holding pens, and blood samples were collected from a jugular venous catheter at hourly intervals. Values are the means ± SEM. Values for $T_3$ and $T_4$ were significantly lower ($P < 0.001$) in TRH-immunized ewes.

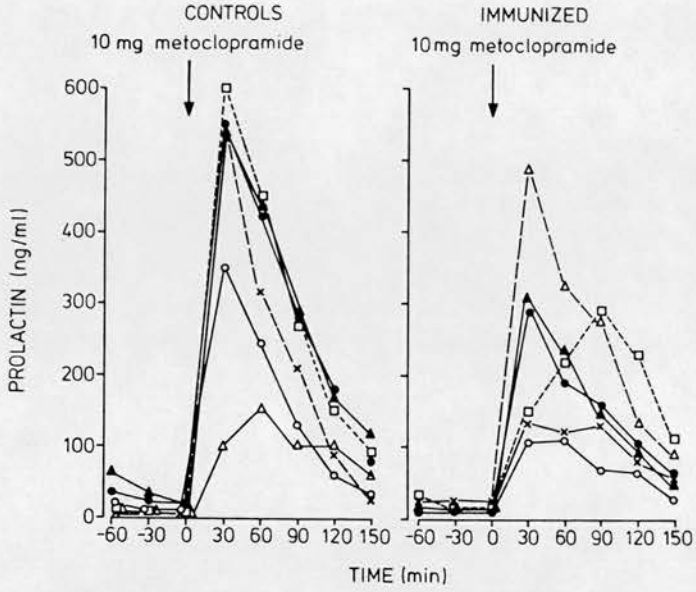



FIG. 4. Plasma PRL concentrations after im administration of 10 mg metoclopramide in six control ewes and six ewes immunized against TRH.

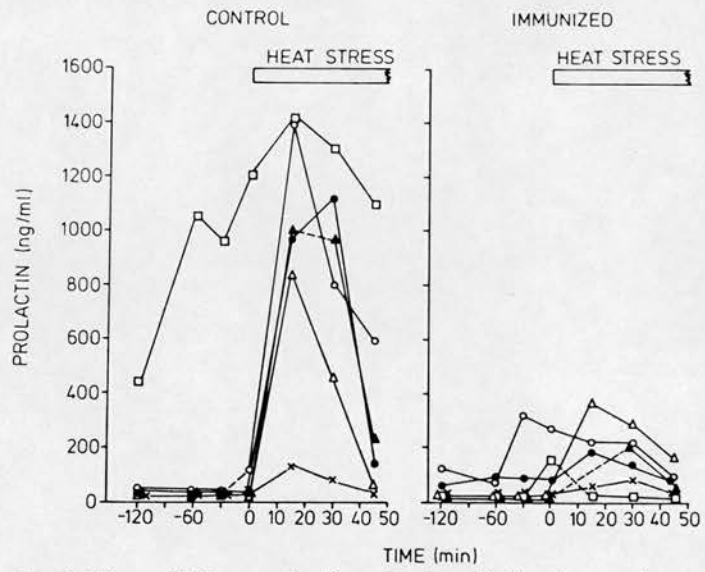
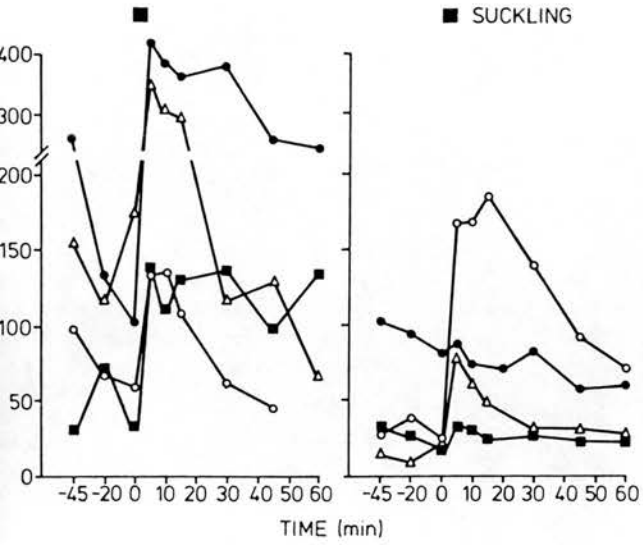
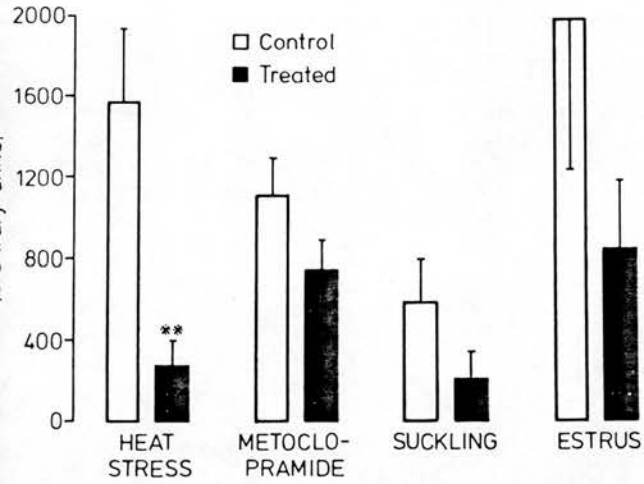



FIG. 5. Plasma PRL concentrations after transferring six control ewes and six TRH-immunized ewes from a room at 10 C to a heat chamber at 43 C.

clearly ($P < 0.01$) suppressed in the TRH-immunized ewes. The TSH response was also lower than that in the controls ($P < 0.01$) during the first 3 h after TRH administration, although the biological activity of the TRH seemed more prolonged in the immunized group. Despite the lower amount of TSH released in the TRH-immunized group, the rises in $T_4$ and $T_3$ appeared similar to those in the controls. That the TRH-immunized animals were equally, if not more, sensitive to TSH stimulation than the controls was supported by the similar




6. Plasma PRL concentrations induced by suckling in four control (*left panel*) and four TRH-immunized ewes (*right panel*).




7. Cumulative totals for increases in plasma PRL concentrations ced by heat stress, metoclopramide, and suckling and for the s-induced PRL peak (23–33 h after the withdrawal of progestagen). es are the means ± SEM (six animals per group; four animals per p for suckling experiment). **, $P < 0.05$ (control *vs.* treated).

rees of $T_3$ and $T_4$ response after the administration of H on the following day (Fig. 9).

### *sue hormone contents and histology*

There was no difference in body weight between the groups (controls, 56 ± 4.9 kg, TRH-immunized, 62.8 .3 kg), but the wool weights were significantly lower < 0.01) in the TRH-immunized ewes (controls, 3.4 ± kg; TRH-immunized, 2.7 ± 0.14 kg). There was a nificant negative correlation (r = 0.0595; $P < 0.05$) ween TRH antibody titer and wool weight. The mean ghts of the thyroid glands were not significantly al- d after TRH immunization (controls, 5.4 ± 0.4 g;

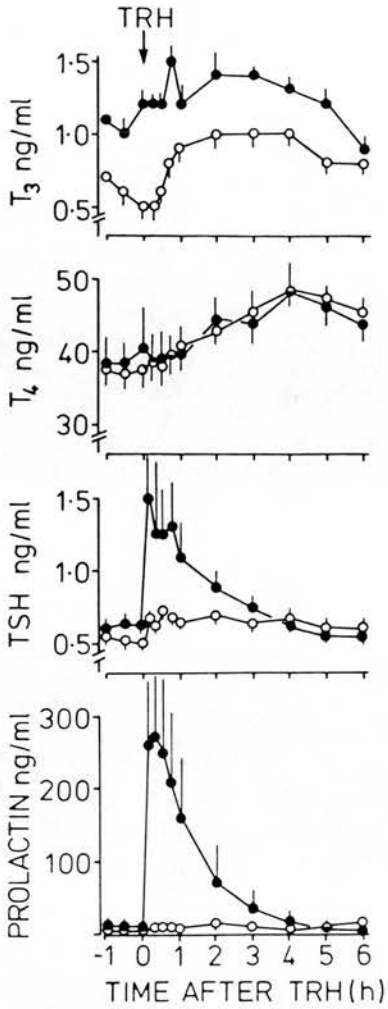



FIG. 8. Effect of iv administration of 20 μg TRH in four control ewes (●) and five TRH-immunized ewes (○) on plasma concentrations of $T_3$, $T_4$, TSH, and PRL.

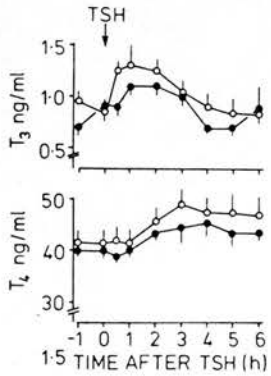



FIG. 9. Effect of iv administration of 500 μg ovine TSH (NIH TSH-S8) on plasma concentrations of $T_3$ and $T_4$ in four control (●) and five TRH-immunized ewes (○).

TRH-immunized, 4.5 ± 0.3 g), but, again, there was a significant negative correlation (r = −0.816; $P < 0.01$) between TRH antibody titer and thyroid weight.

TRH contents of the pineal gland, hypothalamus, cor-

tex, and cerebellum are shown in Table 2. The highest concentrations of TRH-like immunoreactivity were present in the hypothalamus; approximately one tenth of this concentration was found in the cerebellum, with much smaller amounts present in the cortex and pineal gland. The hypothalami of TRH-immunized ewes contained significantly less ($P < 0.05$) TRH-like immunoreactivity than did the hypothalami of control animals, while distributions in the other tissues were similar (Table 2). The high mean value for TRH in the pineal glands of the immunized animals was due to one of the ewes having a concentration 10 times that of the other animals in the group. Weights of the anterior pituitary gland were similar in both groups (0.77 ± 0.2 g in controls; 0.74 ± 0.05 g in TRH-immunized ewes).

The mean TSH content of the anterior pituitary was not significantly lower in the TRH-immunized ewes, nor did the values appear to be related to antibody titer. Pituitary PRL contents were the same in both groups (Table 2).

Histological examination of the thyroid glands consisted of assessment of follicle size and cell height. There was a tendency for a slight increase in follicle size in the TRH-immunized animals, a feature which would be consistent with reduced thyroid activity. However, this was not marked and was of insufficient degree to merit a quantitative study. Histological evaluation of the anterior pituitary glands did not reveal any marked differences between the two groups.

TABLE 2. Tissue contents of TRH, TSH, and PRL in control ewes (n = 4) and ewes immunized against TRH (n = 6)

| Tissue | Group | |
|---|---|---|
| | Controls | TRH-immunized |
| | TRH | |
| Hypothalamus | | |
| Total (ng) | 204 ± 45 | 89 ± 18[a] |
| pg/mg | 272 ± 40 | 124 ± 27[b] |
| Pineal gland | | |
| Total (ng) | 0.36 ± 0.1 | 1.22 ± 0.7 |
| pg/mg | 4.8 ± 0.6 | 14.2 ± 8.1 |
| Cerebellum | | |
| Total (ng) | 6.08 ± 1.9 | 4.98 ± 0.7 |
| pg/mg | 25.7 ± 5.9 | 22.5 ± 4.2 |
| Cortex | | |
| Total (ng) | 0.83 ± 0.2 | 1.90 ± 1.2 |
| pg/mg | 2.1 ± 0.6 | 5.7 ± 3.7 |
| | TSH | |
| Anterior pituitary gland (μg/100 mg) | 3.0 ± 0.5 | 6.1 ± 1.2 |
| | PRL | |
| Anterior pituitary gland (μg/100 mg) | 92 ± 31 | 93 ± 9 |

Values are the mean ± SEM.

[a] $P < 0.05$ *vs.* control mean.

[b] $P < 0.02$ *vs.* control mean.

## Discussion

For most of the 2-yr study period, concentrations of $T_3$ and $T_4$ in the blood of ewes actively immunized against TRH were lower than those in controls, providing further evidence that TRH is an important hypothalamic stimulator of TSH and thyroid function. The inability to demonstrate a consistent reduction of TSH in the blood of the immunized animals suggests that TSH levels in the blood of euthyroid ewes may be so low as to make measurement of any reduction difficult by the present RIA. It is reasonable to assume that the reduction of $T_4$ concentrations as observed in the immunized ewes, would normally have resulted in elevated TSH release from the pituitary (8), and this clearly failed to occur in the presence of circulating antibodies to TRH. This was illustrated when plasma thyroid hormone concentrations fell markedly in the control ewes in late summer during the latter stages of pregnancy and lactation to cause a pronounced rise in TSH output. Despite the fact that the thyroid hormones in the TRH-immunized ewes were consistently at or below these values, TSH output did not increase; when plasma $T_4$ concentrations declined further at this time, TSH concentrations rose only minimally. The seasonal fall in thyroid hormone levels which precipitated the increase in TSH release, presumably due to decreased negative feedback, appears to have been reinforced by lactation, but the mechanism for this is not clear. The reduction in wool weight in the TRH-immunized ewes probably reflects the reduction in the stimulatory effect of the thyroid hormones (9).

The seasonal increases in plasma $T_4$ and $T_3$ concentrations that occurred during the summer months in the control animals and, to a reduced extent, in the TRH-immunized ewes are likely to be caused primarily by long day length, but changes in environmental temperature and food intake may also play a role (10). Pregnancy was without major influence on TSH or thyroid hormone levels when compared to values obtained during the anestrous period of the previous year, but there was an indication that pregnancy induced a slight rise in $T_4$ concentrations from the values in those TRH-immunized ewes which were either pregnant or nonpregnant out of phase with the others in their group. These results confirm findings from other studies on thyroid hormones during pregnancy in the ewe (11) and show that the ewe is different from several other mammalian species, in-

ng the human, in not having a marked rise in the oid hormones during pregnancy. The small reduction ypothalamic content in the TRH-immunized ewes indicate an increased release of the tripeptide. Al-gh hypothalamic TRH is not changed by altering oid activity in the rat (12, 13), perhaps the prolonged ence of the TRH antibodies in this experiment ed the change.

r findings confirm and extend previous studies ing short term reduced output of TSH after passive unization against TRH in rats (14–17). Despite these inhibitory effects, we need to explain the presence tectable $T_3$ and $T_4$ in the blood of the immunized , the absence of any reduction in pituitary TSH ent, and the minimal effect on thyroid morphology. e results contrast with the effects of immunization st LHRH, which results in reductions of circulatory dal steroids to nondetectable levels, reduced pitui-content of LH and FSH, and marked reductions in dal weights and morphological activity (18). Possible ns are: 1) incomplete inhibition of TRH, 2) adap-n and increased sensitivity of the pituitary-thyroid after depletion of TRH, or 3) the existence of sec-ry factors which normally have a minor role in ulating thyroid function independently of TRH. It is ely that the circulating antibodies can neutralize all e TRH in the hypophyseal portal blood, so that a amount of biologically active TRH may still be nt. Further, hypothyroidism may extend the half-f TRH (19). The absence of a reduction in pituitary content despite deprivation of TRH may be due to val of the strong negative feedback effect of $T_4$ on hyrotrophs with a resulting increased sensitivity to RH available.

e morphology of the thyroid glands of the TRH-unized ewes seemed only marginally affected by the ment. Thus, only in the high antibody producers there signs of reduction in the size of the gland, and nsiveness to TSH was retained, as judged by the al rises in $T_4$ and $T_3$ after the administration of TSH. However, it should be noted that this part of udy was carried out during the month of November the thyroid glands of the controls were much less e than during the summer months. The results again ast with the analogous situation after active im-zation against LHRH, in which the Leydig cells of unized rats have similar sensitivity but lower respon-ess to hCG stimulation in vitro (20, 21).

ally, some $T_4$ may be produced autonomously or by factor other than TSH acting on the thyroid itself. hypothalamic dopamine and somatostatin are also cated in the control of TSH release (22–25), perhaps is a compensatory alteration in other systems when stimulation of TSH is reduced. As a consequence of $T_4$ production in the immunized ewes, $T_3$ concentrations in the blood can be produced by conversion from $T_4$ in the liver (26).

Hypothyroidism is sometimes associated with an inhibitory influence on reproductive cycles (27), but in the present study, both groups of ewes showed similar patterns in the timing of the seasonal onset and length of estrous cycles and in the timing of mating behavior when the cycles were synchronized. Further, lambs born to the TRH-immunized mothers were delivered after the normal gestation period, were of normal weight, and showed normal development.

There is disagreement in the literature over the changes in PRL after short term inhibition of TRH by passive immunization. Koch *et al.* (14) found a decrease in serum PRL in male and female rats after the administration of rabbit anti-TRH serum, but Harris *et al.* (15) failed to confirm this, despite demonstrating a fall in TSH concentrations. In our own studies, injection of 1 ml of various antisera (produced from the ewes in the present study) reduced serum TSH levels to 10% of those in controls after 1 and 24 h in male rats, but had no significant effect on PRL concentrations (Fraser, H. M., and A. S. McNeilly, unpublished observations).

Despite active immunization against TRH in the ewe, the major physiological changes in PRL in the blood throughout the periods of estrous cyclicity, anestrus, pregnancy, and lactation still occurred. The stimuli used to investigate the hypothalamic-pituitary system in the present study involved different aspects of the PRL release mechanism. Thus, metoclopramide releases PRL because of its properties as a dopamine antagonist (28), blocking the inhibitory action of dopamine on the lactotrophes and allowing spontaneous release of PRL or release of PRL by PRL-releasing factor. The effect of heat stress may involve a decreased output of dopamine from the hypothalamus, the rise in PRL brought about by suckling is thought to involve an increase in TRH release as well as a decrease in dopamine (29, 30), while the rise in PRL at estrus involves the action of estrogen both on the hypothalamus and directly on the lactotrophes (31). PRL levels in plasma rose in all of these situations despite the presence of circulating antibodies to TRH. However, the amounts of PRL released after these stimuli seemed to be lower in all cases in the TRH-immunized ewes, although this reached statistical significance only after heat stress. This suggests that when TRH is chronically neutralized by antibodies, the mechanism for the release of large quantities of PRL is reduced. This did not seem to be a function of differences in the amounts of stored hormone, since pituitary PRL contents were unaltered at autopsy. Also, it is unlikely that short term changes in TRH were involved, since there is no evidence for an increase in TRH after heat

stress, as TSH does not rise. Perhaps chronic changes in TRH exert some influence on the response of the lactotroph to dopamine.

The absence of any major influence of TRH immunization on PRL levels in the different physiological studies suggests that it is not a major hypothalamic controller of PRL release, but the effects of acute stimuli indicate that it may have a minor function in the PRL release mechanism. Further, since it appeared that the pituitary-thyroid axis of TRH-immunized ewes retained some function through adaptation or under the influence of low levels of TRH, the possibility that our results have been complicated by similar mechanisms operating for the hypothalamo-lactotroph axis must be considered.

## Acknowledgments

We are grateful to Dr. H. Wissmann for supplying the TRH conjugate, Dr. J. Sandow for providing the TRH analogs, Dr. K. McLaren for histological studies, Mrs. P. E. Warner for advice on statistics, Prof. J. S. Robinson for supplying the materials for the TSH assay, Dr. G. Beckett and the Scottish Antibody Production Unit for providing the materials for the $T_3$ and $T_4$ assays, and the NIAMDD for supplying the pituitary hormone preparations. We thank Mrs. M. Swaney and Miss N. Anderson for expert technical assistance.

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# Inhibition of Thyrotropin-Releasing Hormone by Antibodies

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Administration of the hypothalamic tripeptide thyrotrophin-releasing ormone (TRH) stimulates the release of both thyroid stimulating ormone (TSH) and prolactin from the anterior pituitary gland (3). ince its isolation and synthesis over 10 years ago, TRH has been onsidered to be the prime physiological stimulator of TSH secretion ut it is still unclear whether the stimulatory effects on prolactin ave physiological significance. Our understanding is complicated by he fact that prolactin is primarily under negative hypothalamic ontrol exerted via prolactin inhibitory factor, probably dopamine, hile the question of a separate prolactin releasing factor, peptide in ature, remains to be resolved.

In addition to the effects of its administration, a classical way of tudying the physiological role of a hormone is by its inhibition. erhaps the best method available of inhibiting TRH is by the use of RH antibodies. In the present study, we have investigated the effect f inhibiting TRH over a 2 year period in the ewe. This was achieved y active immunization, in which ewes were immunized against TRH and he antibodies generated provided continuous inhibition of the endogen- us peptide. During this time, the ewes were followed through the arious reproductive states of oestrous cyclicity, anoestrus, pregnancy nd lactation and were subjected to tests known to stimulate prolactin elease, namely administration of metaclopramide, heat stress and uckling. The effects of TRH immunization were assessed by measuring rolactin in the blood, and, in the absence of a suitable radioimmuno- ssay for ovine TSH, by measuring blood circulating levels of tri- odothyronine (T3) and thyroxine (T4).

To study the effects of immediate inhibition of TRH in the normal nimal, rats were injected with antiserum to TRH produced in the ewes passive immunization) and effects of concentrations of prolactin and RH in the blood assessed by radioimmunoassay.

## LONG TERM INHIBITION OF TRH

### Materials and Methods

Animals Six mature Dam-line ewes living outside in a field station near Edinburgh were immunized against TRH and six control ewes were immunised against serum bovine albumin (BSA).

Immunizations TRH was conjugated to BSA by means of 1,5-difluoro-2,4-dinitrobenzol and was a generous gift from Dr. H. Wissmann, Hoechst, Frankfurt, to whom we are extremely grateful. Six mg of conjugate in 10ml saline was emulsified in 16ml Freund's complete adjuvant and injected into 4 subcutaneous (s.c.) sites under each leg in a volume of 1ml per site in each of the six ewes. During the 2 year period of study, 6 booster immunizations were administered using the same dose of conjugate at intervals shown by the arrows in Fig. 1. Booster immunizations were done at intramuscular or s.c. sites in Freund's incomplete adjuvant, except for the 4th and 6th booster immunizations which were performed using complete adjuvant.

Control ewes were treated similarly, each being immunized with 1mg of the BSA carrier.

### Detection and characterisation of TRH antibodies

The presence of antibodies to TRH was detected by ability to combine with $^{125}$I-labelled TRH, prepared as described previously (4). TRH antibody titre was measured in doubling dilutions of serum from 1:100 up to between 1:3,300 and 51,200 incubated with $^{125}$I-labelled TRH (10,000cpm) in a final volume of 300µl 0.01M phosphate-buffered saline, pH 7.4, containing 0.1% BSA. After incubation for 24h at 4°C, antibody bound and free labelled TRH were separated using 5 volume of ice-cold ethanol and the titre expressed as the dilution binding 33% tracer.

Radioimmunoassays were established using antisera from all 6 ewes and the detection limits, as assessed by 10% reduction from the zero tube, ranged from 0.4 to 10pg/tube. Specificity studies were performed on 2 antisera against analogues of TRH and other brain peptides (These were a most generous gift from Dr. J. Sandow, Hoechst, Frankfurt).

### Reproductive state of ewes

The primary immunization was performed in December, during the breeding season which lasts from November through to April (Fig. 1). The study of the animals continued through the summer anoestrus period and then the following period of oestrous cycles. Towards the end of this breeding season (January 1979), the timing of ovulation was synchronized using progesterone pessaries and the ewes mated. They were then studied during the succeeding pregnancy, the period of lactation and finally, the return to normal oestrous cycles, at which time the experiment was terminated. During the study period, blood samples were collected at regular intervals (Fig. 1). and plasma stored at -20°C until required for hormone radioimmunoassay and antibody titre estimation.

### Tests of the hypothalamo-pituitary system

In addition to the changes induced by the environment and reproductive

state, the animals were also subjected to tests designed to establish the integrity of the prolactin release mechanism.

1) Metaclopramide Metaclopramide is a dopamine antagonist, probably having its major action on prolactin release by preventing the inhibitory actions of dopamine on prolactin at the pituitary level, thereby allowing spontaneous release of prolactin. This test was performed in the midluteal phase of an oestrous cycle occurring between January and February 1979, when tonic prolactin secretin is low.

A cannula ending in a 3-way tap was inserted into the jugular vein of the ewes on the day before the study and they were then placed in individual holding pens in which they remained, unstressed, throughout the experiment. Three blood samples were collected at 30 min intervals prior to an i.m. injection of 10mg maxalon. Blood samples were then continued for a further 150 mins.

2) Heat stress The animals, who had been living out of doors during the winter months, were taken indoors to a room at a temperature of 10°C and fitted with a cannula and penned as described above. Blood samples were collected at time zero and at 30, 60, and 120 min prior to the ewes being transferred to a heat chamber at 43°C. Blood sampling was continued at 15 min intervals for a further 45 min.

3) Suckling Between five and six weeks post delivery, 4 treated and 4 control ewes were fitted with a jugular cannula as before. During this time, the lambs were taken from their mothers and kept in adjacent pens. One hour after cannulation, blood samples were collected at 30 min intervals for 1½ h. The lambs were returned to their mothers to suckle and blood samples collected at 5, 10, 15, 30, 45 and 60 min .

Radioimmunoassays Prolactin, T3 and T4 was measured using established radioimmunoassays (8, 10).

Analysis of results Results were analysed using Student's t test.

## Results

All ewes immunized against TRH produced antibodies. Titres rose slowly during the first few months after immunization, reaching near-maximal production by 20 weeks. These levels of antibodies in the blood were maintained or stimulated further by the booster immunizations (e.g. Fig. 1). Antibody titres varied considerably between ewes, the poorest responder producing titres of around 1:1,000 and the highest with values between 1:20,000-1:50,000. An occasional hormone measurement indicated that these differences in TRH antibody production resulted in differential effects of T3 and T4 levels in the blood, but no consistent pattern was apparent. A typical example, taken from a ewe producing medium levels of antibody is shown along with a control ewe in Fig. 1. In the controls, there was a rise in T3 and T4 in the blood during the summer months. In the TRH-immunized animals, concentrations of T3 and T4 were clearly lower than in controls by 3 months of immunization and this difference was maintained throughout the following 90 weeks of the experiment. However, there were indications that the seasonal pattern of changes observed in the controls were still apparent in the treated ewes, but at a reduced level. T3 and T4 did not appear to be completely eliminated in the TRH-immunized ewes, since plasma from a hypophysectomized goat (provided by Dr. H. Buttle, NIRD, Reading) gave a reading of 0.3 ng/ml and 3 ng/ml in the T3 and

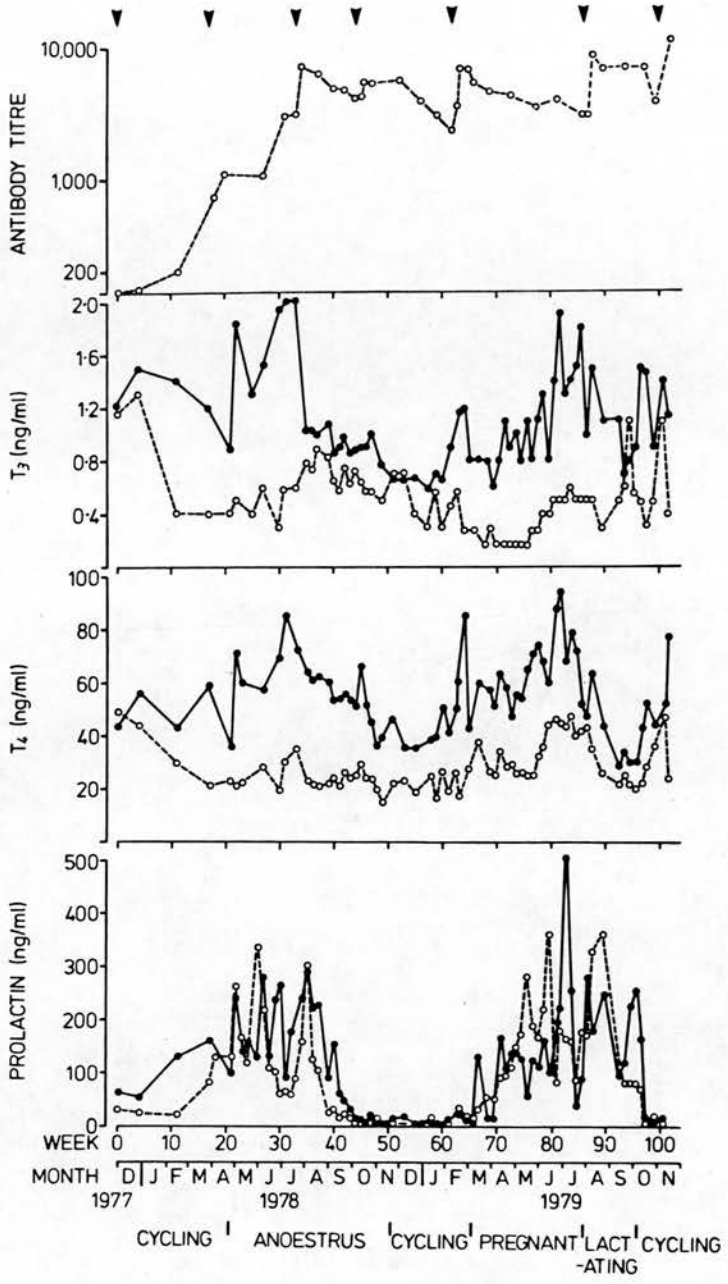



FIG. 1. Concentrations of T3, T4 and prolactin in the plasma of a BSA-immunized control ewe (●) during a 2 year period and in a ewe immunized against TRH(○) at times indicated by the arrows. The top panel shows development of TRH antibody titres in this animal (ewe no. 328).

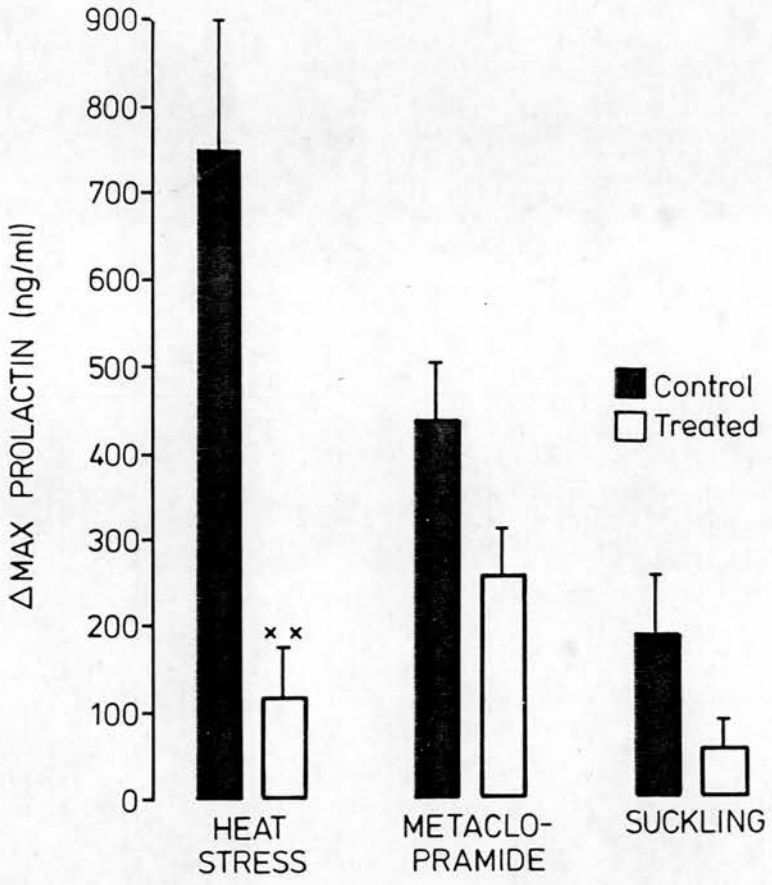



FIG. 2. Mean maximal increments in plasma prolactin concentrations, from value at time zero, after exposure to heat, injection of metaclopramide or suckling stimulation in control ewes (closed bars) in the ewes immunized against TRH(open bars). Results are the mean values ± S.E.M. for 6 animals per group (heat exposure and metaclopramide) or 4 animals per group (suckling). **P< 0.02 when compared to control. Other values not significantly different.

T4 assays respectively, values lower than those of the immunized ewes.

In contrast, there were no clear differences in plasma concentrations of prolactin between the two groups. During the early stages of immunization and occasionally during anoestrus, the mean prolactin levels were lower in the TRH-immunized ewes but this was rarely statistically significant and the normal rises in prolactin occurred during anoestrus, pregnancy and lactation.

After the ewes delivered, there were no obvious differences in the milk yield between the groups or in the development of the lambs.

Tests of the hypothalamo-pituitary system

The results of stimulating prolactin release by heat stress, metaclopramide injection and suckling are represented in Fig. 2 by the mean maximal increment in prolactin concentrations in the blood when compared

with the value at time zero. After being subjected to heat stress at 43°C, a significantly ($P < 0.02$) smaller maximal prolactin rise occurred in TRH-immunized than control ewes. Metaclopramide administration and suckling induced no significant differences between the groups, although for both cases the mean values for maximal increment in prolactin concentrations were lower in the TRH-immunized ewes.

## SHORT TERM INHIBITION OF TRH

### Materials and Methods.

Animals Adult male Sprague-Dawley rats 80-100 days old and weighing 380-420g were used. They were housed in a room at 21 ± 1°C with lights on between 05.00h and 19.00h. Food and water were available ad libitum.

Antisera TRH antisera and control antisera from the ewes were collected by venepuncture from the jugular vein, 11-16 days after booster immunizations. Between 200 and 1,000 ml blood were obtained from each ewe, allowed to clot overnight at room temperature centrifuged at 600g for 30 min at 4°C and the serum stored at -20°C until used for passive transfer experiments.

Specificity of the two antisera tested was similar and demonstrated a high specificity for TRH (Table 1).

Table 1. Cross reactivity of TRH and its analogues with TRH antiserum from ewe no. 420

| Structure | Relative Activity (%) |
|---|---|
| pGlu - His - $ProNH_2$ (TRH) | 100 |
| PGlu - Phe - $ProNH_2$ | 100 |
| pGlu - Met - $ProNH_2$ | 1.4 |
| pGlu - Lys - $ProNH_2$ | 12.5 |
| pGlu - Lys(BOC) - $ProNH_2$ | 0.26 |
| pGlu - His - ProOH | < 0.005 |
| PGlu - Leu - ProOH | < 0.005 |
| pGlu - Ala - ProOH | < 0.005 |
| pGlu - His - Pro-propylamide | 0.03 |
| pGlu - His - $TrpNH_2$ | 0.05 |
| H.His - $ProNH_2$ | 0.004 |
| Glu(BOC) - His - $ProNH_2$ | 0.96 |
| Luteinizing hormone-releasing hormone | < 0.005 |
| Growth hormone release-inhibitory hormone | < 0.005 |
| Oxytocin | < 0.005 |

Effect of antisera to TRH on prolactin and TSH

1) Dose, route and time after administration.

A high-titre TRH antiserum (from ewe no. 204) was selected and administered to 4 groups of 5 rats, at a dose of 1ml or 2ml, by intraperitoneal injection without anesthetic or intravenously via the tail vein under light ether anesthesia. Control rats were similarly treated with 1ml or 2ml antiserum to BSA. At 2 or 24h after treatment, individual cages of rats (10 rats per cage) were transferred to the operating room and, after a short exposure to carbon dioxide to induce light anesthesia, were rapidly killed by decapitation using a guillotine. Trunk blood was collected and serum stored at -20°C until assayed for prolactin and TSH.

2) Biological potency of different antisera.

Antisera from 5 of the TRH-immunized ewes representing titres in the low (1:1,000), medium (1:5,000 and 1:6,400) and high (1:16,000 and 1:20,000) range were tested for differences in their ability to inhibit TRH in vivo. 1 ml of the various antisera was injected into the tail vein of rats (5 per group) and trunk blood collected 24h later as described above and serum stored at -20°C until assayed for prolactin and TSH. Ten control rats were treated with 1ml antiserum to BSA.

Effect of antiserum to TRH on heat stress

Two hours prior to the exposure to heat stress, 10 adult male rats were injected into the tail vein with 1ml antiserum to TRH (from ewe no. 420) and 10 controls injected with 1ml antiserum to BSA. A lag time between injections and exposure to heat stress was necessary to allow any stress effect of the injection procedure to pass. The rats were divided into 2 groups. Five controls and 5 treated rats were transferred to a room at 40°C while the remaining animals were killed (time zero) and trunk blood collected as before to determine concentrations of TSH and prolactin immediately prior to the experiment. The animals transferred to the heated room were killed after 20 min.

Radioimmunoassays

Prolactin and TSH were measured using established radioimmunoassays with reagents provided by NIAMDD (9, 5). Results were analysed by Student's t test.

## Results

Effect of antisera to TRH on prolactin and TSH in the rat

1). Dose, route and time after administration.

Results are shown in Fig. 3. Since the control rats injected with either 1ml or 2ml antiserum to BSA had similar concentrations of TSH and prolactin in the blood, their results have been combined. The rats receiving an i.v. injection of anti-BSA and killed 2hr later had a lower mean TSH concentration than those injected by the i.p. route. This may have been due to the effects of ether anesthesia but was not significant.

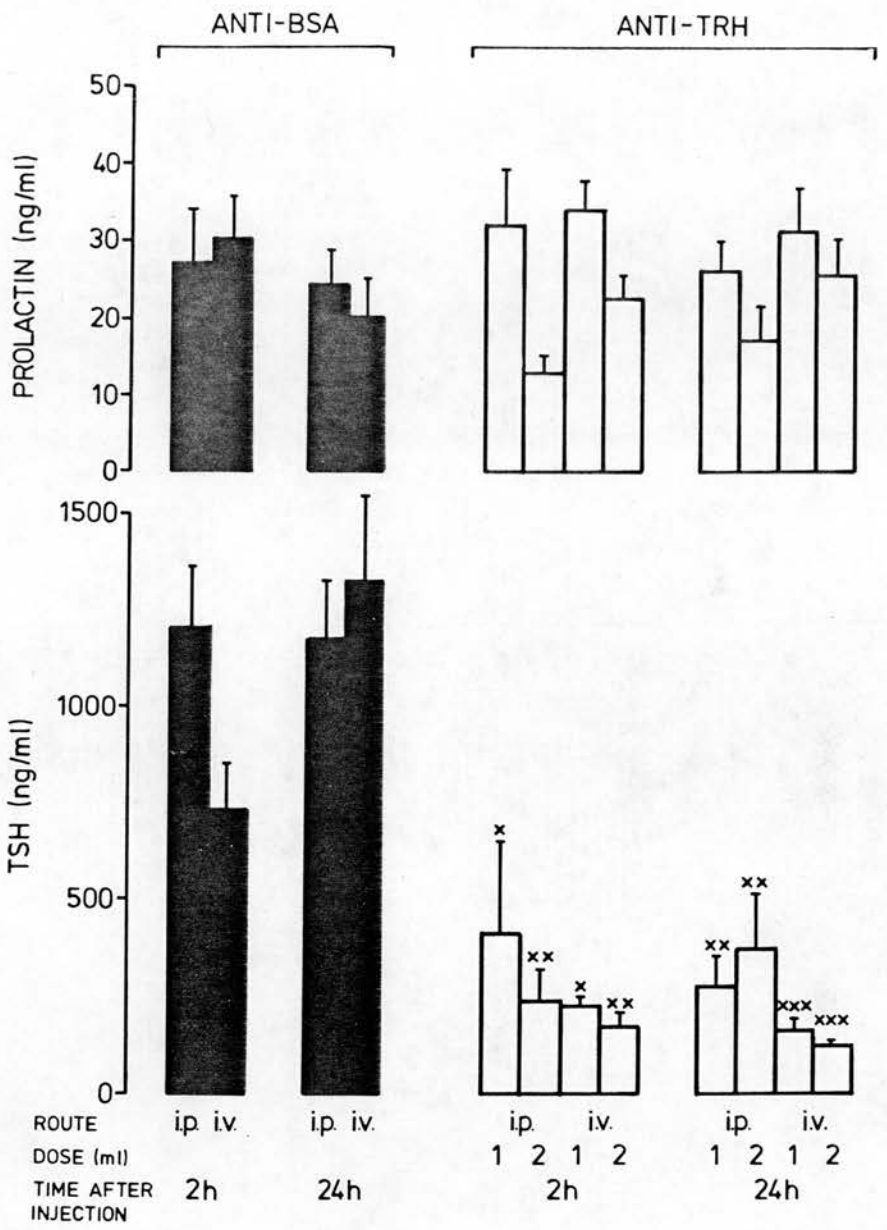



FIG. 3. Effect of an i.v. or i.p. injection of anti-BSA serum control rats (closed bars) or, an i.v. or i.p. injection of 1ml or 2ml anti-TRH serum (open bars) on serum concentrations of TSH and prolactin at 2h and 24h after treatment in adult male rats. Values are means + S.E.M. (controls, n=10; anti-TRH treated n=5 per group) *P<0.02, **P<0.01, ***P<0.001, when compared to respective controls. All other values are not significantly different from controls.

TSH concentrations were significantly reduced at both 2h and 24h after injection of antiserum to TRH. A dose of 2ml was more effective at 2h after both i.p. or i.v. injection while after 24h the 1ml dose was equally inhibitory. The higher mean values after i.p. administration were largely the results of greater individual variability because a small number in the group were much less suppressed. Perhaps the i.p. route is more succeptible to variations in uptake of the antibody.

In contrast to the inhibitory effects on TSH, there were no significant reductions in prolactin concentrations after administration of anti-TRH.

2) Effectiveness of different TRH antisera.

Fig. 4 shows how the medium- and high-titre TRH antisera caused a clear reduction in TSH concentrations in the blood while the one with low titre was without significant effect, although the mean value was lower than that of the controls. Thus, antibody titre was of value in predicting a poor effectiveness in vivo for this antiserum, but to emphasize that titre should not be used as the sole criterion, it should be noted that the medium titre antiserum no. 216 had a more marked inhibitory effect than its titre would have suggested.

Again, none of these TRH antisera had a significant effect on prolactin secretion.

Effect of heat stress.

The inhibitory action of the antiserum to TRH injected 2h prior to heat exposure was confirmed by killing a group of rats at the time the remainder were transferred to the room at 40°C. These animals had TSH concentrations in the blood of 167 $\pm$ 29 ng/ml (Mean $\pm$ S.E.M.) which was significantly lower ($P < 0.001$) than the controls (1099 $\pm$ 160 ng/ml.

In this experiment, the prolactin concentrations (16 $\pm$ 3 ng/ml) were also lower ($P < 0.05$) in this treated group compared to controls (28 $\pm$ 4 ng/ml). After heat exposure there was a dramatic rise in prolactin concentration in the controls to 120 $\pm$ 24 ng/ml. The treatment produced a similar stimulation of prolactin release in the treated animals, reaching 130 $\pm$ 17 ng/ml. TSH did not increase after heat stress.

## DISCUSSION

These results showing a consistent reduction in T3 and T4 concentrations in the blood of ewes immunized against TRH for a period of 2 years, and the dramatic fall in TSH concentrations in the blood of rats injected with antisera to TRH, provide further evidence that TRH controls TSH and thyroid function. Our results confirm and extend previous studies, which were confined to the rat, on the effect of passive transfer of antisera to TRH, (2, 6, 11, 12). The greater and more-prolonged inhibitory effect on TSH release in the present work compared with the values reported in these studies probably reflects the use of more powerful antisera to TRH in the present study.

Despite these inhibitory effects on TSH and thyroid hormones after inhibition of TRH, the presence of detectable T3 and T4 in the blood of the active-immunized ewes needs to be explained. This may be due to 1) incomplete inhibition of TRH, 2) increased sensitivity of the pituitary - thyroid axis after depletion of TRH, or 3) a secondary factor which has a minor role in stimulating thyroid function, independently of TRH.

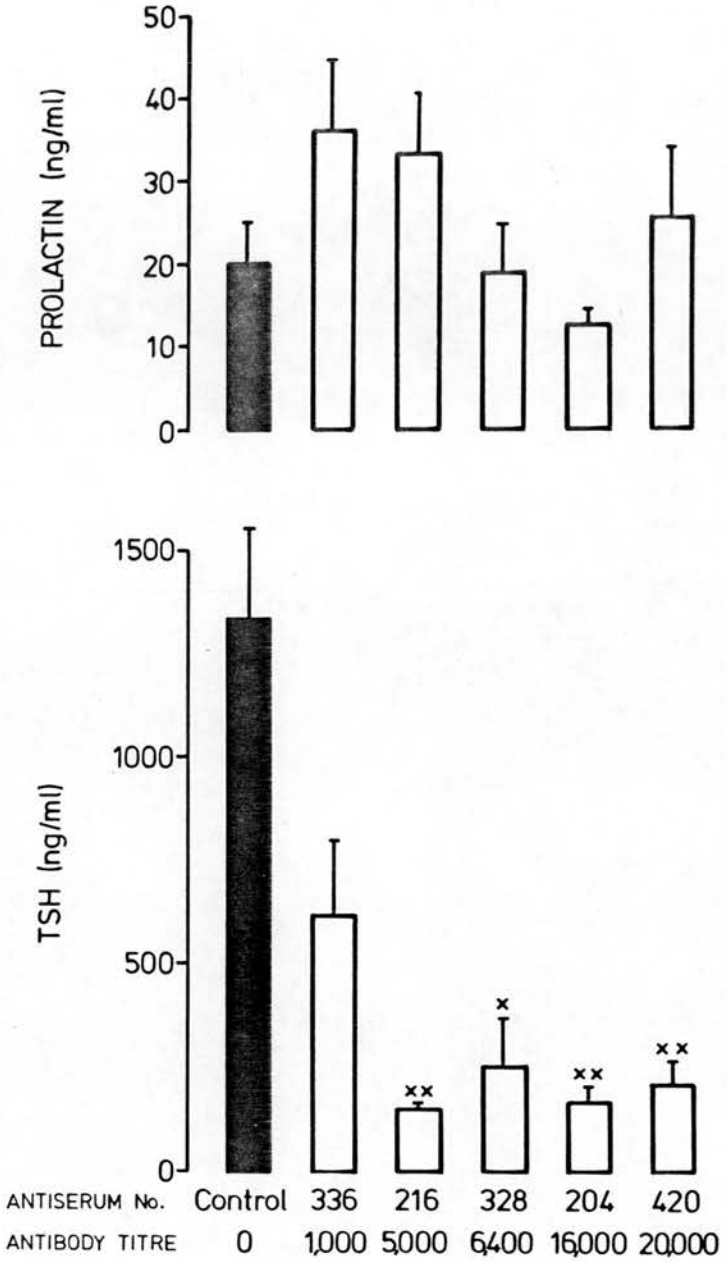



FIG. 4. Effect of an i.v. injection of 1ml of 5 different antisera to TRH with a range of antibody titres from 1:1,000 to 1:20,000 on the serum concentrations of TSH and prolactin after 24h in adult male rats (5 rats per group). The control rats (closed columns) were injected with antiserum to BSA. *$P < 0.02$, **$P < 0.01$ when compared to control group, All other values were not significantly different from controls.

The situation appears to be even more complex for prolactin. In the actively-immunized ewes, the major physiological changes in prolactin in the blood throughout the periods of oestrous cyclicity, anoestrous, pregnancy and lactation still occurred when TRH was inhibited. The animals gave a prolactin response when the inhibitory effect of dopamine was prevented by metaclopramide that was not significantly different from the controls. In addition, injection of TRH antibodies in rats had no consistent effect on prolactin concentrations in the blood.

These observations could be taken as evidence that TRH is not involved in the physiological control of prolactin release. Such a conclusion must, however, be interepreted with caution. In the ewes immunized against TRH, the prolactin response to heat stress was reduced, although this was not the case in rats injected with antibody. Also, after metaclopramide or suckling stimulus and occasionally during the 2 year period of study, although not significantly changed, the mean levels of prolactin were lower in the TRH-immunized ewes. Furthermore, Koch et al (6) reported a decrease in prolactin as well as TSH in the blood of male and female rats injected with a TRH antiserum. Perhaps our results and those of Koch et al (6) reflect a small difference in the handling of the experimental animals as prolactin release is well known to be succeptible to rapid changes in the immediate environment.

Finally, it is of interest to compare what happens to the anterior pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), when antibodies to the hypothalamic releasing hormone, luteinizing hormone-releasing hormone (LHRH) are present in the circulation. LHRH is thought to control the release of both pituitary hormones, and indeed, active or passive immunization against LHRH will cause a reduction in LH and FSH concentrations in the blood of many species (1). However, the decline in FSH is slower than for LH, for example, while injection of antibodies into rams causes an immediate cessation of LH pulses, the release of FSH is unchanged after 24h (7). These differences are not entirely due to differences in the half-lives of the hormones but are probably because there are intrinsic differences in the way two hormones can respond to the one hypothalamic releasing hormone, and subsequently show different rates of decline when the hypothalamic stimulus is removed. Another factor could be that because the inhibition of releasing hormone is unlikely to be 100 per cent effective, perhaps secretion of one pituitary hormone could be preferentially maintained by this lower level of stimulation.

Thus, while TRH seems to have a primary function in the release of pituitary TSH, we cannot yet exclude the possibility that it has a minor role in prolactin secretion, perhaps, under certain circumstances, modulating the response to the more important hypothalamic control mechanisms for this hormone.

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# The effects of a sudden decrease or increase in daylength on prolactin secretion in the ram

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**Summary.** Six adult Soay rams were preconditioned to an artificial lighting regimen of alternating 4-month periods of long (16L:8D) and short days (8L:16D) for at least 10 months before blood samples were collected at hourly intervals for 24 h at various times. The abrupt change from long to short days resulted in a progressive decrease in plasma levels of prolactin, while that from short to long days had the reverse effect; the first response to the light changes was rapid, beginning within 6 days. During the periods of high secretion there was a 24-h cycle in plasma prolactin concentrations, with a peak in both the early dark and early light phases of each day. Changes in the relative magnitude of these peaks were observed in relation to the long-term alteration in prolactin secretion. Plasma levels of FSH were also measured and a close inverse relationship between gonadotrophin and prolactin secretion was observed.

## Introduction

In the ram there are conspicuous changes in the blood levels of prolactin during the year, the highest levels occurring during the summer months and the lowest levels in the winter (Ravault, 1976). The timing of these seasonal changes can be readily modified by altering the photoperiod (Pelletier, 1973) and in the natural situation the annual variation in daylength is probably the principal environmental cue dictating the timing of the seasonal prolactin cycle. Photoperiods which favour prolactin secretion (long days) have the reverse effect on gonadotrophin secretion (Pelletier & Ortavant, 1975; Lincoln, Peet & Cunningham, 1977), and under natural conditions, therefore, there is an inverse relationship between prolactin and gonadotrophin release.

In the present study this relationship is investigated in more detail by measuring prolactin levels in blood samples collected from rams, living under artificial lighting conditions, in which gonadotrophin secretion and testicular activity have already been carefully documented (Lincoln *et al.*, 1977; Lincoln & Peet, 1977).

## General Methods

Adult rams of the Soay breed were exposed to alternating 16-week periods of 'long' days (16 h light:8 h darkness, 16L:8D) and 'short' days (8L:16D) before the initiation of experiments which investigated the effects of a sudden decrease (Exp. I) or a sudden increase (Exp. II) in daylength. Blood samples were collected from the animals by using a jugular cannula which was inserted the day before sampling. On each occasion particular care was taken not to disturb the animals, which remained in their normal pens. Faint red illumination from a torch was used to aid blood collections during the dark phase of each 24-h cycle.

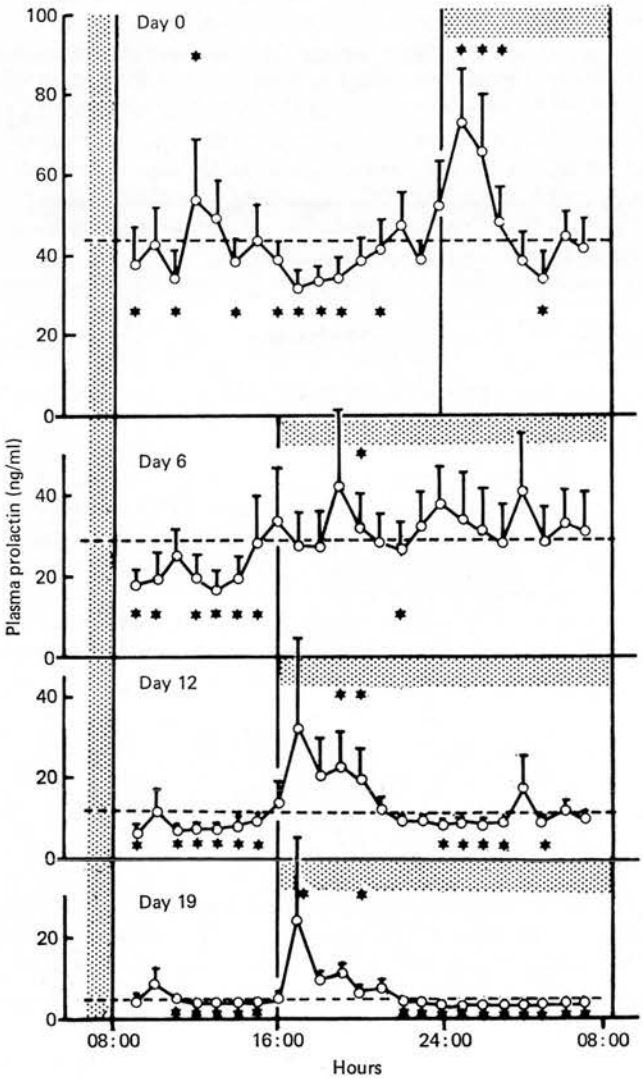
The blood samples were centrifuged immediately and the plasma stored at −20°C until assayed for ovine prolactin with a double-antibody radioimmunoassay (McNeilly & Andrews, 1974; Lamming, Moseley & McNeilly, 1974). The sensitivity of the assay was 2·0 ng/ml, with NIH-P-S9 as reference standard and all samples from one animal were measured in duplicate in a single assay.

Concentrations of LH, FSH and testosterone were also measured in these plasma samples and these results have been reported (Lincoln *et al.*, 1977). Some of the mean values for plasma FSH are given herein to allow direct comparison with the prolactin results.

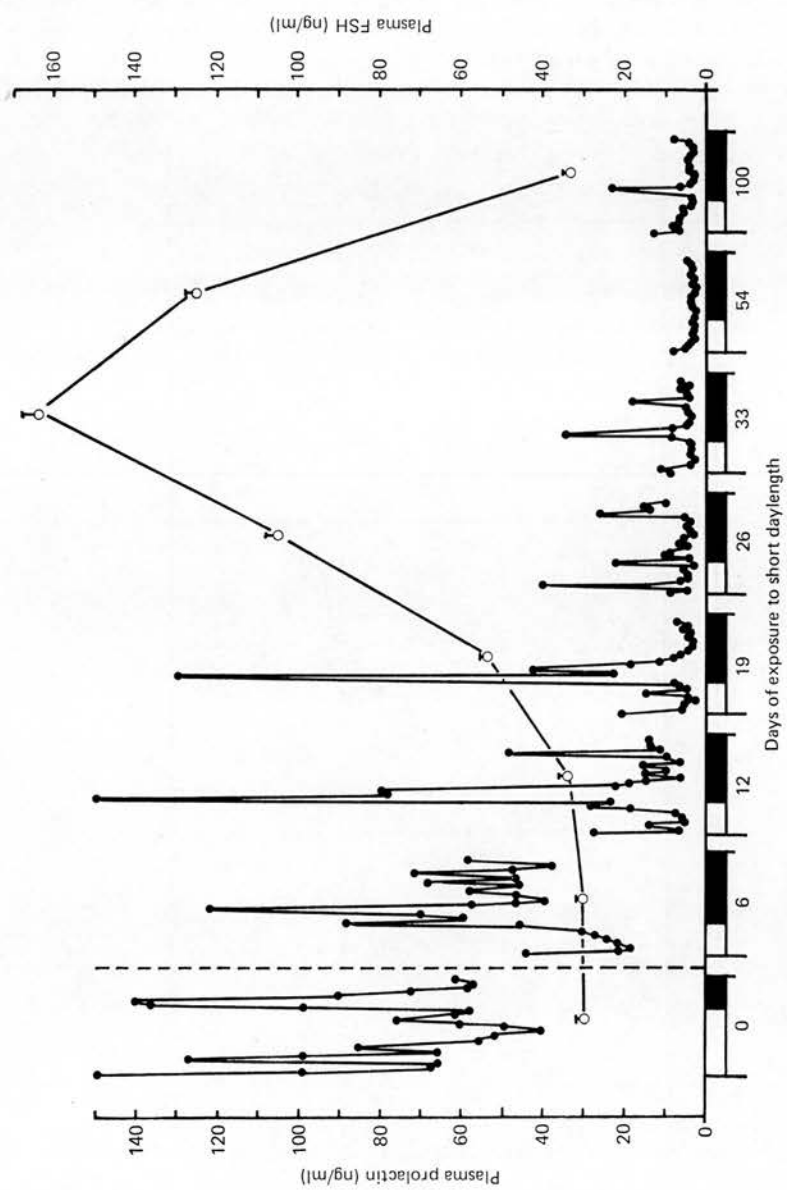
## Experiment I

*Methods*

Six rams preconditioned to the alternating 16-week photoperiod changes for 2 years were studied over a 15-week period during which the daylength was abruptly changed from long to short days by advancing the time of 'lights out' by 8 h. Blood samples were collected from each ram at hourly intervals for 24 h on Days 6, 12, 19, 26, 33, 54 and 100, as well as on Day 0 (the last day of a 16-week period of 16L:8D).




**Text-fig. 1.** Plasma levels of prolactin (ng equiv. NIH-P-S9: mean ± s.e.m.) for 6 Soay rams sampled hourly for 24 h on one occasion at the end of a 16-week period of long days (Day 0) and on 3 occasions during short days (Days 6, 12 and 19). Values marked by an asterisk (*) indicate the times during each day when at least 5 of the animals had prolactin levels above or below the daily mean of each individual. The horizonal broken line indicates the mean prolactin level for the group on that day and the stippling shows the dark phase of each 24-h cycle.



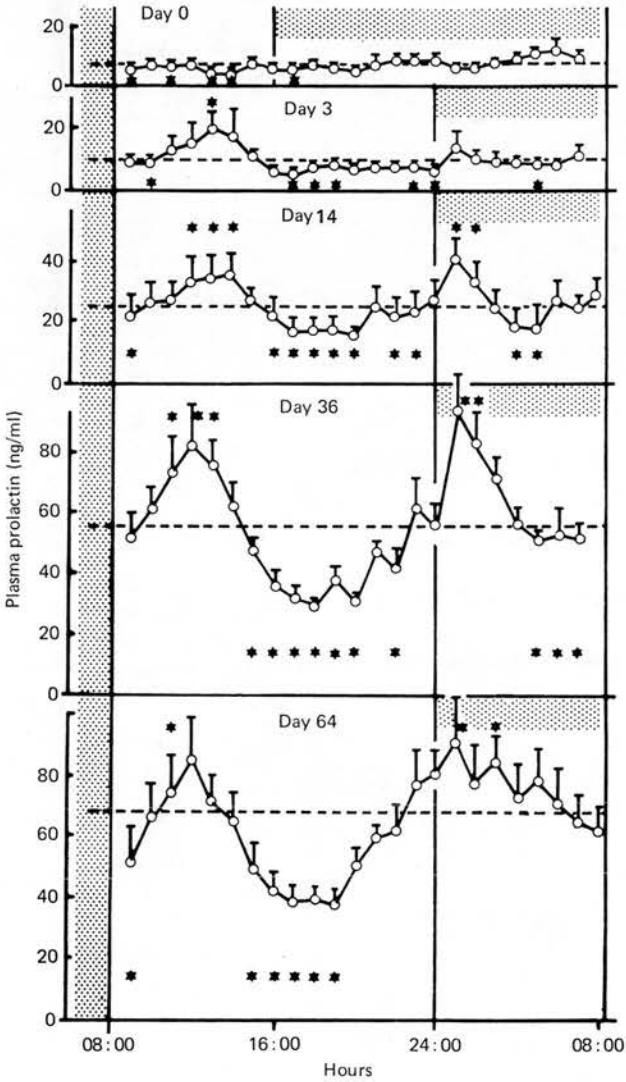

**Text-fig. 2.** Plasma levels of prolactin (●, ng equiv. NIH-P-S9) in one Soay ram sampled hourly for 24 h on 8 occasions during the study (see text). Day 0 is the last day of a 16-week period of long days. The plasma levels of FSH (○, ng equiv. NIH-FSH-S10: mean ± s.e.m.) for each 24-h period (for details see Lincoln & Peet, 1977) and the timing of the light and dark phases for each day are also shown.

*Results*

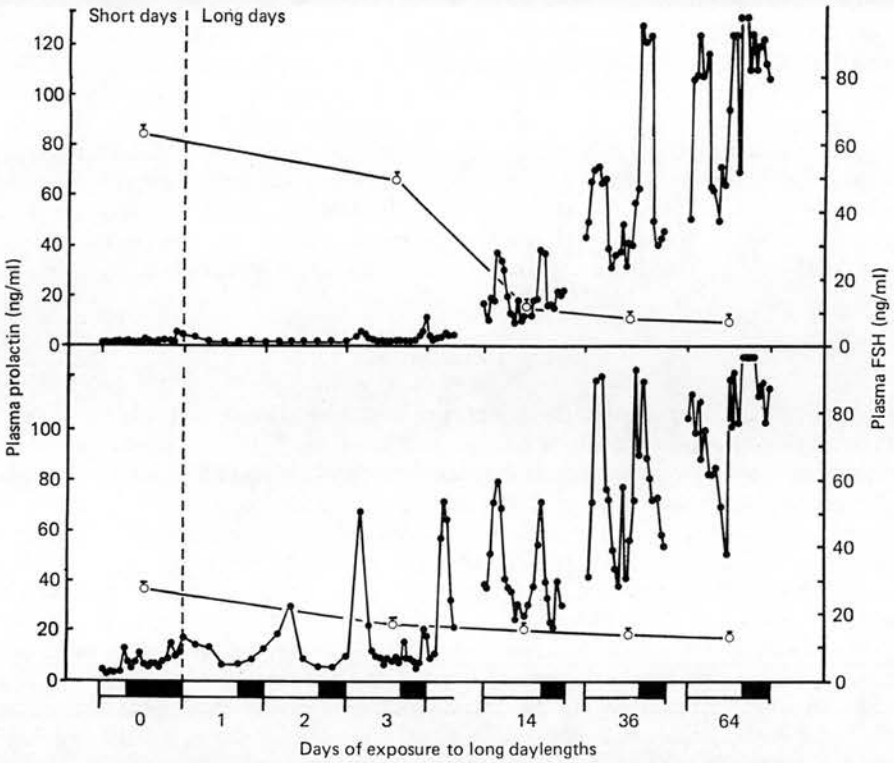
The changes in plasma prolactin concentrations for all six rams on Days 0, 6, 12 and 19 are shown in Text-fig. 1 and all results for one ram are given in Text-fig. 2.

Prolactin levels on Day 0 (long days) were universally high (24-h group mean = 43·8 ± 6·6 (s.e.m.) ng/ml). On Day 6 after the change to short days prolactin levels were significantly decreased ($P<0·01$, paired $t$ test) in all animals (24-h group mean = 28·4 ± 6·8 ng/ml), and the decline continued, the 24-h group means on Day 12 and 19 being 11·7 ± 3·4 and 5·6 ± 1·8 ng/ml respectively. The prolactin levels remained low from Day 26 (<4·0 ng/ml) onwards.

In the early part of the study when the rams were hyperprolactinaemic (Days 0, 6, 12 and 19) there




**Text-fig. 3.** Plasma levels of prolactin (ng equiv. NIH-P-S9: mean ± s.e.m.) for 6 Soay rams sampled hourly for 24 h on one occasion at the end of a 16-week period of short days (Day 0) and on 4 occasions during a period of long days (Days 3, 14, 36 and 64). Values marked by an asterisk (*) indicate the times during each day when at least 5 of the animals had prolactin levels above or below the daily mean of each individual. The horizontal broken line indicates the mean prolactin level for the group on that day, and the stippling shows the dark phase of each 24-h cycle.




**Text-fig. 4.** Plasma levels of prolactin (●, ng equiv. NIH-P-S9) in two adult Soay rams sampled on several occasions after a change from short to long days. Day 0 is the last day of a 16-week period of short days. The plasma levels of FSH (○, ng equiv. NIH-FSH-S10: mean ± s.e.m.) for each 24-h period (for details see Lincoln *et al.*, 1977) and the timing of the light and dark phases of each day are also shown.

were short-term changes in the levels of prolactin related to the light/dark cycle in each 24-h period (see Text-fig. 1): peak prolactin levels occurred during the early part of the dark phase. Since the change from long to short days was achieved by altering the timing of lights out, the nocturnal rise in prolactin levels occurred at different times of day under long and short days. In addition to the dark-phase peak, a smaller peak of prolactin occurred in the early hours of the light phase of the 24-h cycle (Text-fig. 1). This peak occurred at about the same time throughout the study until after Day 26 when it was fully depressed.

## Experiment II

### *Methods*

The same 6 rams had experienced the 16-week alternating photoperiod regimen for 10 months when they were studied for 9 weeks during a change from short (8L:16D) to long days (16L:8D). The light change was again abrupt and involved delaying 'lights out' by 8 h. Blood samples were collected at hourly intervals for 24 h on Days 3, 14, 36 and 64 following the beginning of long days, as well as on Day 0 (the last day of 16-week period of 8L:16D). Additional blood samples were also collected at 4-h intervals from Days 0 to 3.

### *Results*

The overall results for all 6 rams are shown in Text-fig. 3 and the individual results for 2 animals are given in Text-fig. 4.

Prolactin levels were low on Day 0 (end of short days; 24-h group mean = 8·8 ± 4·0 (s.e.m.) ng/ml), but within 3 days of exposure to long days prolactin levels in 4 of the 6 rams had increased significantly (group mean = 9·8 ± 3·5 ng/ml; see Text-fig. 4) and by Day 14 this increase was evident in all animals (group mean 25·6 ± 6·4 ng/ml: $P<0·01$ compared to Day 0, paired $t$ test). On Days 36 and 64 the prolactin levels were 56·0 ± 6·9 and 67·9 ± 10·9 ng/ml respectively.

During each 24-h period after the increase in prolactin levels there were short-term changes in prolactin secretion related to the light/dark cycle. Two periods of peak secretion were recognized, one early in the light phase and one early in the dark phase (Text-fig. 3). During the initial response to the change in photoperiod (Days 3 and 14) it was the daytime peak which was most conspicuous, appearing as early as Day 2 (see Text-figs 3 and 4), while later the night-time peak became more obvious.

## Discussion

The blood samples from the present studies have been assayed for LH and FSH (Lincoln & Peet, 1977; Lincoln *et al.*, 1977), and some of the FSH results are illustrated in Text-figs 2 and 4. The change from long to short days resulted in an increase in the plasma levels of LH and FSH, while the change from short to long days had the reverse effect. These changes are precisely opposite to those described for prolactin; e.g. when the rams were hyperprolactinaemic under long days gonadotrophin secretion was minimal, testicular activity was reduced and the animals were sexually inactive (Lincoln & Davidson, 1977), but when the prolactin levels decreased under the influence of short days resurgence of sexual activity occurred. These observations emphasize a very close inverse relationship between prolactin and gonadotrophin secretion, but whether this is a causal relationship, as suggested when there is infertility due to hyperprolactinaemia (Fang, Refetoff & Rosenfield, 1974; Thorner, McNeilly, Hagan & Besser, 1974), is not clear. The diurnal pattern in prolactin levels is not inversely correlated with that for gonadotrophins (see Lincoln & Peet, 1977, for diurnal rhythm in LH and FSH), and short-term fluctuations in prolactin and gonadotrophin levels are apparently independent. Similarly, after prolonged exposure to short days (Days 54 and 100, Text-fig. 2) plasma FSH levels decline markedly while prolactin levels remain low, indicating the lack of a relationship at this time. The functional significance of the changes in prolactin secretion in the ram remains obscure.

A nocturnal rise in prolactin levels similar to that seen in the ram (see also Ravault *et al.*, 1976) occurs in man (Sassin, Frantz, Kapen & Weitzman, 1973) and in the rat (Dunn, Arimura & Scheving, 1972). In man, the increase has been shown to be related to sleep, but this does not appear to be the case in the ram because the animals in the present experiment generally showed the most conspicuous signs of sleep late in the dark phase of the 24-h cycle (Lincoln *et al.*, 1977), at a time not coincident with the increased prolactin levels. In the present study the timing of the nocturnal rise in prolactin remained constant in relation to the time of 'lights' on, indicating that the transfer from light to darkness is important in determining the timing of the nocturnal rise. Similarly, the transfer from darkness to light may be important for the timing of the small daytime rise in prolactin levels, since the timing of this remained constant. These diurnal changes in prolactin levels that are lost under prolonged exposure to short days reappear when rams are transferred to long days and prolactin secretion increases again.

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# EFFECT OF EPIDURAL ANALGESIA ON THE GLYCOREGULATORY ENDOCRINE RESPONSE TO SURGERY

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SUMMARY

Plasma concentrations of glucose, insulin, glucagon, cortisol, growth hormone and prolactin were measured repeatedly in ten females undergoing abdominal hysterectomy during general anaesthesia. In addition to general anaesthesia five of the patients had continuous epidural analgesia effective for the first 26 post-operative hours. Plasma glucose was elevated during surgery and postoperatively, but not in patients having epidural analgesia. Insulin was low and unchanged in both groups. Glucagon was unchanged and similar in both groups. Cortisol was lower during surgery in the epidural group, but not postoperatively. Growth hormone increased during surgery in four of five patients receiving general anaesthesia alone, but no changes were observed in the epidural group. Prolactin was greatly elevated in all patients immediately after induction of anaesthesia and then fell rapidly during surgery, similarly in both groups. It is concluded that epidural analgesia can inhibit the hyperglycaemic response to surgical stress, but this effect cannot be uniformly correlated to changes in peripheral plasma levels of insulin, glucagon, cortisol, growth hormone or prolactin.

In response to surgical stress there is a well-documented rise in the plasma concentration of several hormones as well as of glucose. Neural impulses from the site of operation are known to play a dominant role in triggering this response. Recently, epidural analgesia, blocking neural impulses from the surgical area, has been shown to inhibit or abolish the adrenocortical and hyperglycaemic response to surgery (Bromage *et al.*, 1971; Gordon *et al.*, 1973; Cosgrove & Jenkins, 1974).

This study was undertaken to investigate in detail the glycoregulatory endocrine response to major surgery and its possible modification by epidural analgesia. The hormones considered were insulin, glucagon, cortisol, growth hormone and prolactin.

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## PATIENTS AND METHODS

Ten otherwise healthy and premenopausal women undergoing abdominal hysterectomy for metrorrhagia or cancer colli uteri in stage 0–1 were studied. Age ranged from 36 to 53 years (average 41 years in the epidural group and 43 years in the control group). The patients were selected by excluding all patients with: (1) hormonal disease, (2) hormonal contraceptives within the last 3 months, (3) other medications within 3 days prior to surgery, (4) bleeding beyond 200 ml during operation, or transfusion of blood or plasma from 6 days before the operation until after the period of examination, (5) the usual contraindications to epidural analgesia, (6) anxiety about the study. All patients gave informed consent to participate.

Routine premedication with pethidine 1 mg/kg i.m., promethazine 12·5–25 mg i.m. and atropine 0·5 mg i.m. were used in all cases. Anaesthesia was started between 08.00 and 10.00 hours. All patients received general anaesthesia induced with thiopentone and suxamethonium chloride and maintained with nitrous oxide/oxygen = 2 : 1 and enflurane (Ethrane), sometimes supplemented with pancuronium bromide (Pavulon). Respiration was controlled in a recirculating system. During the first 2 h the patients received isotonic saline 10 ml/kg/h and hereafter 1 ml/kg/h. Bleeding was substituted by $2\frac{1}{2}$ ml of isotonic saline per ml blood loss. During the 26 h postoperative period the patients received isotonic saline 1 ml/kg/h intravenously and 300 ml perorally. Glucose and drugs were prohibited, except pethidine 50 mg 4–6-hourly.

In addition to general anaesthesia five of the patients received continous epidural analgesia. It was accomplished before the general anaesthesia to confirm that analgesia extended at least to $T_8$ (three segments higher than $T_{11}$ which is the highest level of afferent impulses from the uterus). The epidural block was performed in the lateral position and an epidural catheter was inserted at $L_{1-2}$ (one case), $L_{2-3}$ (three cases), $L_{3-4}$ (one case) and extended 4–8 cm upwards in the epidural space. The analgesia was controlled continuously during the following 26 h, the level being always at least two dermatomes higher than skin incision. If pain occurred analgesia was supplemented.

For the analgesia we used bupivacaine (Marcain) 0·5% without adrenaline, 15 ml as initial dose. The duration of operation was from 80 to 165 min (average 107 min in the epidural group and 112 min in the control group).

Fourteen blood samples were taken from an intravenous catheter: two samples from 5 to 15 min before induction of anaesthesia and epidural analgesia, four samples with 20 min intervals during the first hour after skin incision and eight samples with increasing intervals during the following 26 h (Figs. 1–6).

Cortisol in plasma was measured by a competitive protein-binding technique (Kehlet *et al.*, 1974). Prolactin was measured by a specific double antibody radioimmunoassay (McNeilly, 1973). Purified human prolactin (kindly supplied by Dr H. G. Friesen) and the MRC human prolactin standard A (71/222, 10 mu/amp) were both used as standard. Growth hormone was measured by a radioimmunoassay (Hartog *et al.*, 1964) and the results expressed in ng/ml of MRC standard A. Insulin (Heding, 1972) and glucagon (Heding, 1971) were measured by radioimmunoassays. Blood for insulin and glucagon determination was immediately transferred to heparinized tubes containing 500 KIE Trasylol/ml blood and put on ice. Within 10 min blood was centrifuged at 4°C and plasma stored at −21°C until analysis. Glucagon was measured as pancreatic glucagon using a specific antiserum and the values

expressed as pg equivalents/ml. Twice crystallized pork glucagon (NOVO) was used as a standard. Glucose was measured by a routine glucose oxidase method.

Student's *t*-test was used for statistical analysis.

## RESULTS

The results are summarized in Figs. 1–6.

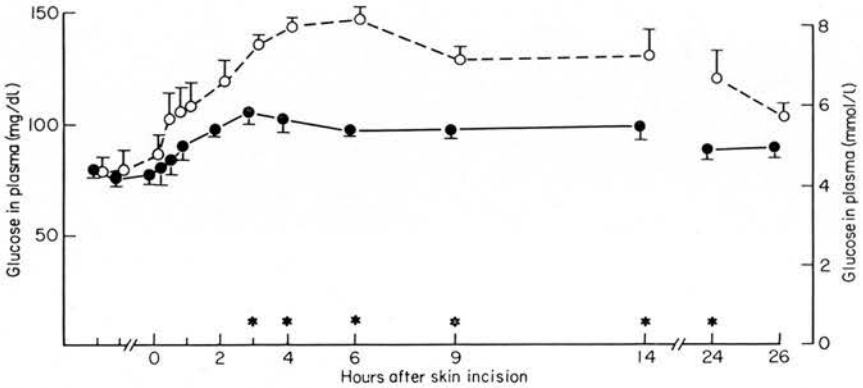



FIG. 1. Plasma glucose concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia. * $P < 0{\cdot}05$.

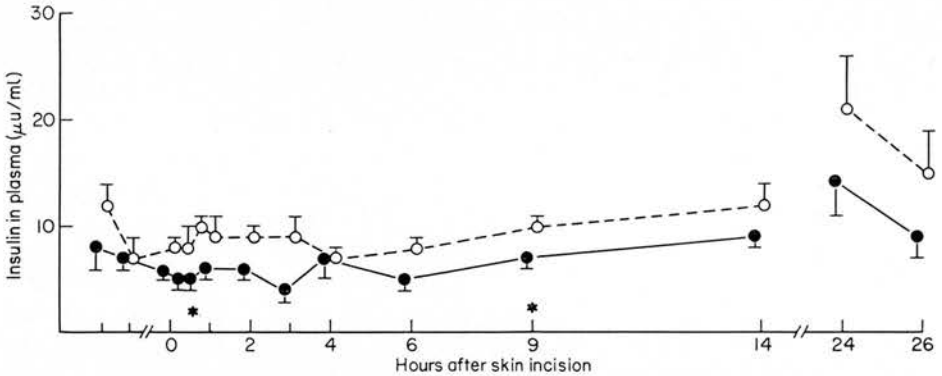



FIG. 2. Plasma insulin concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia. * $P < 0{\cdot}05$.

*Glucose* (Fig. 1) in plasma increased significantly during and after surgery under general anaesthesia. However, addition of epidural analgesia almost blocked the hyperglycaemic response to surgery.

*Insulin* (Fig. 2) in plasma was low and unchanged during surgery and postoperatively in both groups. It is evident that the insulin secretory response to the hyperglycaemic stimulus was impaired in patients receiving general anaesthesia alone.

*Glucagon* (Fig. 3) in plasma was unchanged during surgery and postoperatively and similar in the control group and the epidural group, despite hyperglycaemia in the control group.

*Cortisol* (Fig. 4) in plasma increased during and after surgery in both groups, although pre-operatively the values were significantly lower in the epidural group. Postoperatively, no difference ($P>0{\cdot}05$) could be demonstrated between the two groups.

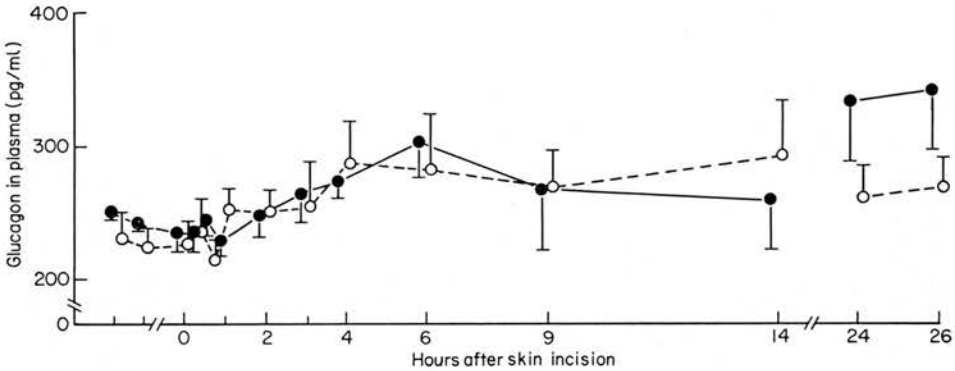



FIG. 3. Plasma glucagon concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia.

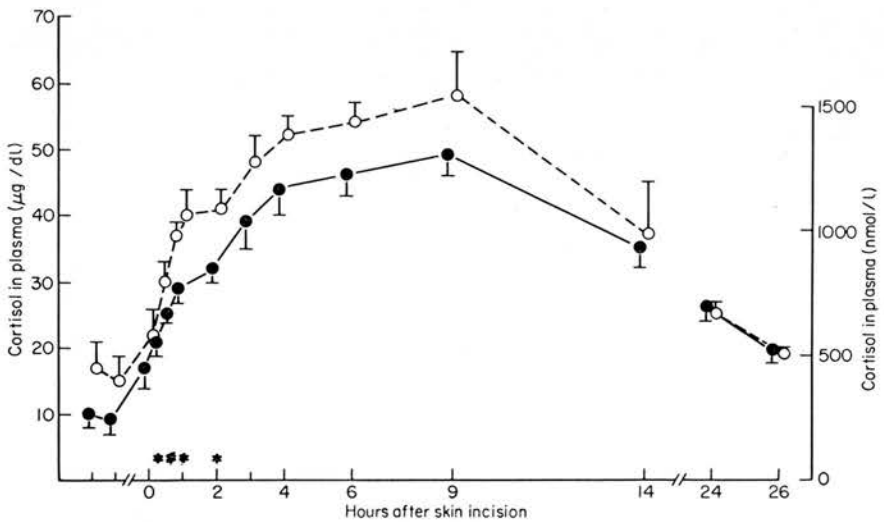



FIG. 4. Plasma cortisol concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia. * $P<0{\cdot}05$.

*Growth hormone* (Fig. 5) in plasma did not change significantly ($P>0{\cdot}05$) during surgery or postoperatively in either group, but four of five patients receiving general anaesthesia alone showed a pronounced increase during surgery. When compared to the epidural group, patients receiving general anaesthesia alone had higher growth hormone levels during surgery, but not postoperatively.

*Prolactin* (Fig. 6) in plasma showed a pronounced increase immediately after induction of anaesthesia before skin incision and then an abrupt fall early in the postoperative period without any difference between the two groups.

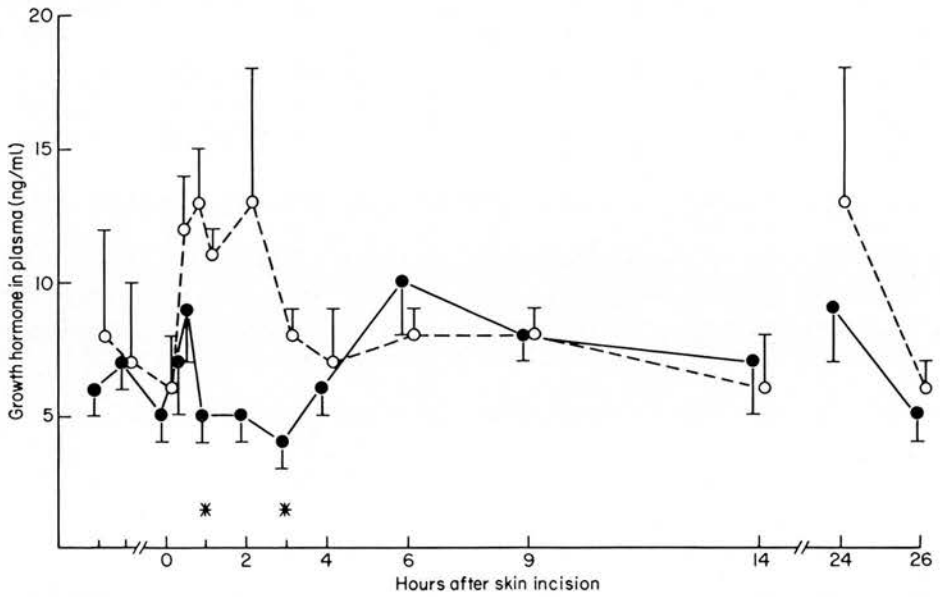



FIG. 5. Plasma growth hormone concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia. * $P<0{\cdot}05$.

None of the patients having epidural analgesia complained of abdominal pain and administration of other analgesics were not needed.

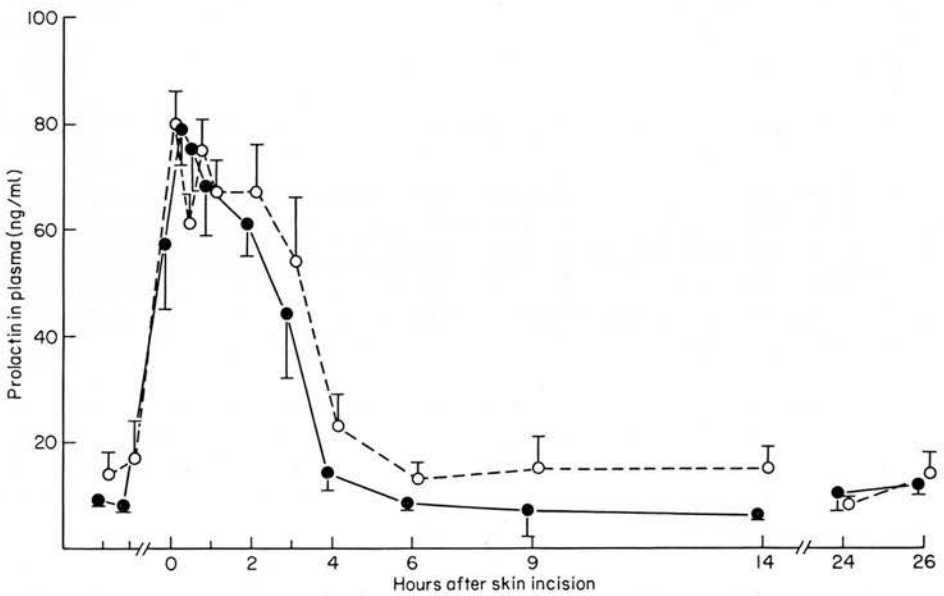



FIG. 6. Plasma prolactin concentration during and following hysterectomy in patients anaesthetized with (●) and without (○) additional epidural analgesia.

## DISCUSSION

The main observation in this study is the blocking effect of epidural analgesia on the hyperglycaemic response to surgery, thus confirming the findings of Bromage *et al.* (1971). The mechanism leading to hyperglycaemia during surgery is unknown, but apparently neurogenic stimuli from the surgical area are playing a dominant role. Therefore a battery of glycoregulatory hormones were measured during surgery with and without epidural analgesia.

Plasma insulin was low, unchanged and similar in both groups and therefore could not account for the inhibitory effect of epidural analgesia on postsurgical hyperglycaemia. However, our results clearly show that the insulin secretory response to the hyperglycaemic stimulus was inhibited during surgery under general anaesthesia, as has been demonstrated by others (Äärimaa *et al.*, 1974; Allison *et al.*, 1969).

Glucagon, known to elevate glucose in blood through glycogenolysis and glycogenesis, was investigated in detail pre- and postoperatively. Other studies during elective surgery have shown either no change (Lindsey *et al.*, 1974) or a slight pre- and late postoperative increase (Russel *et al.*, 1975). Very high glucagon levels in plasma has been found in burned patients (Wilmore *et al.*, 1974) and following major trauma (Lindsey *et al.*, 1974). Our results could not demonstrate any change in plasma glucagon during surgery, either under general anaesthesia or epidural analgesia. Thus, the blocking effect of epidural analgesia upon post-traumatic hyperglycaemia can not be explained by changes in glucagon concentration in peripheral plasma. However, our results do not exclude an altered glucagon secretion, since an increased secretion with high levels in portal plasma is not necessarily reflected by increased levels in peripheral plasma (Marliss *et al.*, 1973). Interestingly, peripheral plasma glucagon levels did not fall in response to postsurgical hyperglycaemia as observed during non stress-induced hyperglycaemia (Heding, 1971).

Cortisol in plasma showed a pronounced increase during and after surgery with a tendency to lower values under epidural analgesia as has been demonstrated by others (Bromage *et al.*, 1971; Cosgrove & Jenkins, 1974; Lush *et al.*, 1972). However, we did not in any patient succeed in a total inhibition of the adrenocortical response to surgery as has been demonstrated earlier in a few patients (Gordon *et al.*, 1973; Cosgrove & Jenkins, 1974). Furthermore, our results confirm that complete alleviation of postoperative pain by neurogenic blockade does not necessarily prevent the normal adrenocortical response to surgery (Lush *et al.*, 1972). The observed elevated plasma cortisol values in the epidural group are not caused by the concomitantly administrated general anaesthetics, since these are without stimulatory effect on hypothalamic–pituitary–adrenocortical axis (Oyama *et al.*, 1971, 1972). Neither does epidural analgesia itself elevate plasma cortisol (Oyama & Matsuki, 1971). Probably, inefficient blockade of sympathetic fibres (Brown *et al.*, 1974) or the persistent vagal neurogenic pathway (Bromage *et al.*, 1971) may explain the failure of abolishing the adrenocortical response to surgery as observed in patients with complete high trans-section of the spinal cord (Hume *et al.*, 1962). It is unlikely that the slightly lower cortisol levels observed during epidural analgesia could account for the simultaneously observed inhibition of post-traumatic hyperglycaemia, since glucocorticoids only have a slight capacity in raising plasma glucose (Marco *et al.*, 1973).

Growth hormone in plasma increased during surgery in four of five patients under general anaesthesia, but postoperatively no elevation was recorded. In contrast, growth hormone

was unchanged during surgery under epidural analgesia, an observation also made using spinal anaesthesia (Newsome & Rose, 1971). However, the blocking effect of epidural analgesia on post-traumatic hyperglycaemia does not seem to be mediated by the alleviation of the preoperative rise in plasma growth hormone, since acute elevation of growth hormone in plasma has no effect on plasma glucose (Fineberg & Merimee, 1974).

Prolactin in plasma has only been sporadically studied in connection with surgery, demonstrating elevated values (Noel *et al.*, 1972; Friesen *et al.*, 1972), but also dependent of the general anaesthetics themselves (Friesen *et al.*, 1972). Our results clearly show a pronounced increase in prolactin after induction of anaesthesia, with a rapid fall during and after surgery and unaffected by epidural analgesia. Differences in postoperative plasma glucose levels therefore can not be explained by differences in prolactin levels. Furthermore, prolactin hitherto has not been demonstrated to influence glucose metabolism.

The mechanism of the inhibitory effect of pre and postoperative epidural analgesia on the hyperglycaemia response to surgery have not been completely outlined in this study. Possibly it is mediated by an inhibition of another glycoregulatory hormonal system, the catecholamines, known to be secreted in increased amounts during sugery (Eisele *et al.*, 1974).

In conclusion, we have shown that during elective lower abdominal surgery epidural analgesia blocked the hyperglycaemic response, but this could not be uniformly correlated to a single component in the endocrine response to surgery expressed by changes in peripheral plasma levels of insulin, glucagon, cortisol, growth hormone and prolactin.

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# PITUITARY HORMONES IN WOMEN WITH CHRONIC RENAL FAILURE: THE EFFECT OF CHRONIC INTERMITTENT HAEMO- AND PERITONEAL DIALYSIS

By

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## ABSTRACT

Measurements of plasma prolactin (hPr), growth hormone (HGH), thyrotrophin (TSH), luteinizing (LH) – and follicle stimulating hormone (FSH) were performed in 20 women with chronic renal failure on regular dialysis. There was no significant difference in any of the hormone levels before and after the dialysis and no significant influence of the type of dialysis (haemodialysis and peritoneal dialysis) or the time of dialysis. Higher levels of plasma prolactin was found in the women on peritoneal dialysis than in the haemodialyzed women presumably due to the medical treatment.

In the peritoneally dialyzed group four women had irregular menstruations and normal gonadotrophic levels, but elevated hPr and it is suggested that this finding is similar to that seen in the amenorrhoea-galactorrhoea syndrome, where hPr presumably in some way have anti-gonadotrophic actions at the gonadal level.

In patients with chronic renal failure, undergoing regular haemodialysis, a variety of hormone disturbances have been found (*Schatz et al.* 1969; *Bindeballe et al.* 1973; *Gonzales-Barcena et al.* 1973; *Nagel et al.* 1973).

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Although circulating levels of pituitary hormones have not been studied extensively, elevated levels of prolactin (hPr) have been reported in these patients, which were not related to the type of renal disease or to medical treatment (*Nagel et al.* 1973). The same group found higher plasma growth hormone (HGH) before than after the dialysis, which was related to the changes in plasma glucose. However, recent reports on changes in serum thyroid stimulating hormone (TSH) levels have been conflicting. *Hershman et al.* (1972) demonstrated a significant fall in levels of TSH after haemodialysis related to the heparin induced thyroxine changes. By contrast *Nagel et al.* (1973) could not demonstrate any TSH changes. Whether these changes in the circulating levels of the pituitary hormones are directly related to treatment has not been clearly evaluated. To provide an overall picture of the changes in hormone levels we have compared the levels of the pituitary hormones hPr, HGH, TSH, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in women undergoing either chronic intermittent haemodialysis or chronic intermittent peritoneal dialysis.

## PATIENTS AND METHODS

### *Patients*

The patients for this study were selected as consecutive patients admitted for their normal dialysis over a period of 2 months.

### *Patients on chronic intermittent haemodialysis*

Blood samples were collected once a week before and after dialysis for a period of 4 weeks in 10 female patients (Table 1). The average age was 36 years ranging from 14 to 50 years. The average period of chronic intermittent haemodialysis before the investigation was 36 months ranging from 7 to 50 months. Seven of the 10 women were bilaterally nephrectomized.

Regular menstruations were seen in 4 women while 2 women had irregular menstruations. Three women had amenorrhoea for more than 24 months and one, a girl of 14 years, had never menstruated.

None of the patients had severe hepatic disease but 4 patients were chronic Australian antigen positive without other signs of hepatitis.

### *Patients on chronic intermittent peritoneal dialysis*

Ten female patients (Table 2) with an average age of 40 years ranging from 19 to 58 years were studied. The average period of dialysis before the investigation was 2 months ranging from 14 days to 4 months.

Regular menstruations were seen in 3 patients while 6 patients had irregular menstruations and 1 patient had amenorrhoea for more than 24 months.

None of the patients were bilaterally nephrectomized and none had signs of liver disease.

| Patient No. | Age (years) | Dialysis (month) | Menstruation[1] | Therapy | BUN mg/100 ml B[2] | BUN mg/100 ml A[2] | hPr ng/ml B | hPr ng/ml A | HGH ng/ml B | HGH ng/ml A | TSH μU/ml B | TSH μU/ml A | LH mU/ml B | LH mU/ml A | FSH mU/ml B | FSH mU/ml A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 14 | 7 | No | – | 31.2 | 6.1 | 12.7 | 15.5 | 6.8 | 5.5 | 1.4 | 1.1 | 0.6 | 0.9 | 1.7 | 2.1 |
| | | | | | 2.1 | 0.4 | 6.9 | 5.7 | 4.0 | 6.3 | 0.4 | 0.4 | 0.2 | 0.8 | 0.8 | 0.5 |
| 2 * ° | 20 | 41 | I | Phenytoin | 45.7 | 11.8 | 25.3 | 14.9 | 3.4 | 5.8 | 1.5 | 0.8 | 4.7 | 3.3 | 5.1 | 4.6 |
| | | | | | 3.1 | 1.0 | 16.3 | 2.8 | 2.2 | 4.4 | 0.6 | 0.4 | 1.3 | 1.6 | 0.8 | 1.2 |
| 3 * ° | 35 | 32 | R | – | 29.0 | 7.6 | 14.5 | 13.9 | 3.6 | 4.8 | 0.2 | 0.2 | 0.9 | 0.7 | 2.0 | 2.1 |
| | | | | | 0.7 | 1.1 | 3.0 | 4.6 | 2.7 | 2.4 | 0.1 | 0.1 | 0.3 | 0.5 | 1.2 | 0.5 |
| 4 * | 38 | 26 | R | – | 25.1 | 8.7 | 21.0 | 23.9 | 2.0 | 1.0 | 2.3 | 2.3 | 1.5 | 1.5 | 4.6 | 5.6 |
| | | | | | 2.4 | 0.4 | 5.0 | 3.5 | 1.7 | 0.2 | 0.1 | 0.7 | 1.4 | 1.3 | 2.6 | 2.6 |
| 5 ° | 39 | 50 | R | – | 38.2 | 8.6 | 16.7 | 25.0 | 9.0 | 5.4 | 3.0 | 2.5 | 1.4 | 1.9 | 4.6 | 5.0 |
| | | | | | 1.8 | 0.6 | 5.0 | 7.5 | 2.0 | 2.0 | 1.4 | 0.7 | 1.0 | 1.8 | 2.6 | 3.4 |
| 6 ° | 39 | 50 | I | – | 30.4 | 8.2 | 15.1 | 23.0 | 6.2 | 3.0 | 1.1 | 1.0 | 2.4 | 2.1 | 7.9 | 9.1 |
| | | | | | 1.2 | 0.5 | 2.5 | 6.4 | 1.6 | 2.0 | 0.3 | 0.6 | 1.2 | 1.2 | 2.5 | 4.1 |
| 7 * ° | 40 | 49 | P | – | 48.0 | 8.1 | 21.9 | 20.7 | 6.8 | 4.4 | 0.2 | 0.2 | 5.0 | 6.0 | 24.4 | 25.6 |
| | | | | | 3.7 | 0.3 | 2.6 | 6.0 | 0.8 | 1.3 | 0.1 | – | 0.7 | 1.2 | 4.3 | 9.8 |
| 8 ° | 44 | 44 | P | Cortisone-acetate | 34.6 | 10.3 | 28.0 | 32.9 | 4.4 | 2.6 | 0.6 | 0.4 | 21.2 | 25.5 | 50 | >50 |
| | | | | | 2.1 | 0.6 | 2.5 | 10.1 | 3.2 | 1.8 | 0.5 | 0.2 | 3.4 | 4.5 | – | – |
| 9 ° | 45 | 43 | R | – | 28.4 | 6.2 | 16.8 | 16.3 | 17.3 | 1.2 | 1.4 | 1.3 | 2.9 | 2.6 | 4.4 | 5.0 |
| | | | | | 1.1 | 1.2 | 5.4 | 6.2 | 8.3 | 0.4 | 0.8 | 0.5 | 2.1 | 2.3 | 1.4 | 1.2 |
| 10 | 50 | 20 | P | – | 25.5 | 5.7 | 31.1 | 33.0 | 8.8 | 1.3 | 3.2 | 0.4 | 13.4 | 13.6 | 50 | >50 |
| | | | | | 0.9 | 0.9 | 13.4 | 8.4 | 7.4 | 0.5 | 3.0 | 0.3 | 1.8 | 2.3 | – | – |
| Mean | 36.1 | 36.2 | | | 33.3 | 7.7 | 20.3 | 21.9 | 6.8 | 3.5 | 1.5 | 1.0 | 5.4 | 5.0 | 15.5 | 15.9 |
| ± SD | 11.8 | 14.6 | | | 8.0 | 1.8 | 6.2 | 7.0 | 4.4 | 1.9 | 1.0 | 0.8 | 6.7 | 7.9 | 19.3 | 19.2 |

1) I = Irregular menstruation, R = regular menstruation, P = post-menopausal.
2) B = before dialysis, A = after dialysis.

* Australian antigen positive.
° Bilaterally nephrectomized.

*Table 2.*
The details and plasma concentration (mean ± 1 SD) of pituitary hormones of the women on chronic peritoneal dialysis.

| Patient No. | Age (years) | Dialysis (month) | Menstruation[1] | Therapy[2] | BUN mg/100 ml B[3] | BUN mg/100 ml A[3] | hPr ng/ml B | hPr ng/ml A | HGH ng/ml B | HGH ng/ml A | TSH μU/ml B | TSH μU/ml A | LH mU/ml B | LH mU/ml A | FSH mU/ml B | FSH mU/ml A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 19 | ½ | I | α+β | 23.3 | 13.8 | 49.8 | 56.6 | 3.6 | 8.4 | 11.6 | 11.7 | 3.6 | 3.4 | 5.0 | 5.4 |
| | | | | | 3.3 | 3.1 | 13.0 | 13.3 | 3.3 | 5.0 | 8.2 | 8.5 | 2.2 | 2.7 | 1.3 | 0.3 |
| 12 | 21 | 4 | I | α+β | 34.3 | 17.8 | 133.0 | 105.0 | 12.8 | 8.5 | 2.0 | 1.4 | 0.5 | 0.7 | 3.1 | 2.8 |
| | | | | | 3.4 | 1.7 | 24.0 | 14.0 | 4.5 | 3.7 | 0.5 | 0.7 | 0.1 | 0.2 | 1.0 | 0.3 |
| 13 | 30 | 1 | R | Medical anti-conception | 24.0 | 11.3 | 14.5 | 15.8 | 5.5 | 9.0 | 2.0 | 1.4 | 0.6 | 0.8 | 2.8 | 3.9 |
| | | | | | 2.2 | 1.0 | 4.3 | 4.3 | 3.7 | 6.9 | 0.5 | 0.7 | 0.4 | 0.2 | 1.3 | 3.2 |
| 14 | 37 | 2 | I | None | 39.3 | 15.8 | 25.0 | 25.0 | 35.8 | 35.0 | 1.3 | 1.7 | 1.2 | 1.2 | 5.5 | 4.9 |
| | | | | | 2.8 | 1.7 | 12.5 | 9.0 | 15.7 | 27.5 | 0.1 | 0.8 | 0.4 | 0.4 | 1.3 | 1.9 |
| 15 | 39 | 4 | R | γ | 16.4 | 7.5 | 27.8 | 28.8 | 27.0 | 32.0 | 2.4 | 3.2 | 1.1 | 0.5 | 5.1 | 6.1 |
| | | | | | 4.3 | 1.2 | 5.7 | 7.6 | 7.6 | 12.4 | 1.0 | 0.9 | 0.3 | 0.4 | 1.2 | 0.8 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 48 | 3 | R | $\beta$ | 27.5 | 14.0 | 25.3 | 27.0 | 4.0 | – | 1.1 | 2.6 | 4.6 | 5.4 | 6.4 | 7.0 |
| | | | | | 4.2 | 2.1 | 9.8 | 6.3 | 1.5 | – | 0.4 | 1.5 | 1.5 | 1.4 | 1.2 | 1.5 |
| 17 | 49 | 2 | I | $\gamma$ + Levome- | 28.6 | 13.8 | 57.7 | 44.0 | 7.0 | 2.0 | 2.1 | 3.5 | 0.4 | 1.0 | 2.2 | 2.1 |
| | | | | promazin | 4.1 | 1.8 | 4.3 | 6.5 | 3.5 | 1.4 | 0.5 | 0.9 | 0.2 | 0.4 | 0.8 | 1.3 |
| 18 | 49 | $\frac{1}{2}$ | I | $\beta$ | 34.5 | 18.5 | 31.8 | 29.9 | 3.8 | 2.6 | 3.8 | 4.9 | 0.9 | 0.6 | 3.8 | 6.1 |
| | | | | | 4.5 | 2.4 | 8.7 | 8.0 | 2.6 | 2.6 | 1.2 | 2.6 | 0.5 | 0.2 | 1.7 | 3.8 |
| 19 | 55 | $\frac{1}{2}$ | P | Cypro- | 25.5 | 13.0 | 60.2 | 66.4 | 6.5 | 15.8 | 5.0 | 4.4 | 20.4 | 17.3 | >50 | >50 |
| | | | | heptadine | 2.7 | 2.8 | 9.0 | 15.1 | 4.0 | 7.2 | 2.0 | 3.5 | 2.1 | 4.2 | – | – |
| 20 | 58 | 1 | I | $\alpha + \beta$ | 28.5 | 16.8 | 120.0 | 83.0 | 13.8 | 9.6 | 2.5 | 2.0 | 0.8 | 0.7 | 1.7 | 1.9 |
| | | | | | 3.9 | 5.7 | 49.0 | 24.0 | 10.5 | 9.8 | 1.1 | 1.6 | 0.2 | 0.2 | 0.3 | 0.4 |
| Mean | 40.5 | 1.8 | | | 28.6 | 14.7 | 54.5 | 53.4 | 12.0 | 10.4 | 3.3 | 3.7 | 3.4 | 3.2 | 9.8 | 10.7 |
| ± SD | 13.7 | 1.4 | | | 5.9 | 3.2 | 40.9 | 35.9 | 11.0 | 10.2 | 3.2 | 3.0 | 6.1 | 5.2 | 14.8 | 15.5 |

1) I = Irregular menstruation, R = regular menstruation, P = post-menopausal.
2) $\alpha$ = Methyldopa, $\beta$ = Furosemide, $\gamma$ = Clorprothixene.
3) B = Before dialysis, A = after dialysis.

*Dialysis procedure*

*Haemodialysis.* – The patients were dialyzed with disposable parallel plate dialyzers (Gambro-Lundia or Boe-Dawids) for an average period of 10 h (range 9–11 h) twice a week and received a diet containing on average 0.8 g of protein per kg body-weight, 50 mEq. sodium, 50 mEq. potassium and about 800 ml fluid daily.

During dialysis the patients were haemodialyzed by single injections of heparin in the return line from the dialyzer under repeated control of clotting time, which varied from 15 to 60 min. The total dose of heparin per dialysis ranged from 15 000 to 25 000 IU.

*Peritoneal dialysis.* – The patients were dialyzed with either a disposable peritoneal dialysis catheter or with a permanent peritoneal dialysis catheter a. m. Tenckholff. They were dialyzed once a week for an average period of 36 h (range from 30 to 42 h). The patients received a diet containing on average 0.5 g of protein per kg body-weight.

*Blood samples.* – In all patients blood samples were collected before (9 a. m.) and after the dialysis. The study was repeated with weekly intervals for a period of 4 weeks. Details of the methods for measuring plasma immunoreactive hPr, GH, TSH, LH and FSH have been given elsewhere (*McNeilly* 1973; *Mortimer et al.* 1973; *McNeilly & Hagen* 1974).

In the assays used, the normal ranges for plasma hPr (standardized against human pituitary prolactin supplied by Dr. H. Friesen) was 5.0–20.0 ng/ml; for LH (M. R. C. standard 68/40, 77 IU/ampoule) in normal females in the follicular phase was 0.8–6.8 mU/ml; for FSH (M. R. C. standard 69/104, 10 IU/ampoule) in normal female subjects in the follicular phase was 1.6–7.4 mU/ml; for TSH (M. R. C. standard 68/38) the normal range was 0.4–3.5 $\mu$U/ml.

## RESULTS

Table 1 shows the clinical data and the peptide hormone concentration for the women on chronic intermittent haemodialysis. None of the patients showed any significant ($P > 0.01$) changes in hormone concentrations before and after the haemodialysis and no linear correlation between any of the hormones and the BUN or serum-creatinine was found. In 3 of the 10 haemodialyzed women plasma prolactin was persistently elevated ($> 20$ ng/ml), while 2 patients had plasma prolactin concentration within the normal range throughout the period of investigation and 5 patients had levels just outside or in the upper end of the normal range. Only one of the haemodialyzed patients studied (No. 9) showed levels of HGH outside the normal range ($< 10$ ng/ml) and no linear correlation was found between the increase in plasma prolactin and the levels of HGH. Plasma TSH was within the normal range in all patients. The gonadotrophic hormones LH and FSH showed an inverse relationship compared to normal pre-menopausal women with higher FSH than LH levels. Two women (No. 8 + 10) with amenorrhoea (menostasis for more than 2 years)

showed high post-menopausal values, while one woman (No. 7), who had a bilateral oophorectomy performed in 1970 only showed LH values in the upper end of the normal range.

The clinical data and the peptide hormone concentrations for the 10 women on chronic intermittent peritoneal dialysis are shown in Table 2. Neither in this group could any significant ($P > 0.05$) linear correlation between any of the hormone concentrations before and after the dialysis be demonstrated. Nine of the 10 peritoneally dialyzed women showed persistently elevated plasma prolactin levels and the mean prolactin concentration was in this group significantly ($P < 0.01$) higher than in the group of haemodialyzed women. The only patient (No. 13) with plasma prolactin concentration within normal range was a woman of the age of 30 years, who had been on peritoneal dialysis for only 1 month. However, no significant ($P > 0.05$) correlation between the period of dialysis and the elevation of plasma prolactin was found. In 4 patients (No. 12, 14, 19, 20) the concentration of HGH was above the normal range and these patients showed marked day to day variation (Fig. 1), but no correlation ($r = 0.409$, $P > 0.1$) to the elevated prolactin levels was found. TSH was persistently elevated in one (No. 11) and intermittent elevated in another 2, while 7 patients showed normal values. The gonadotrophic hormones LH and FSH showed the same inverse relationship with

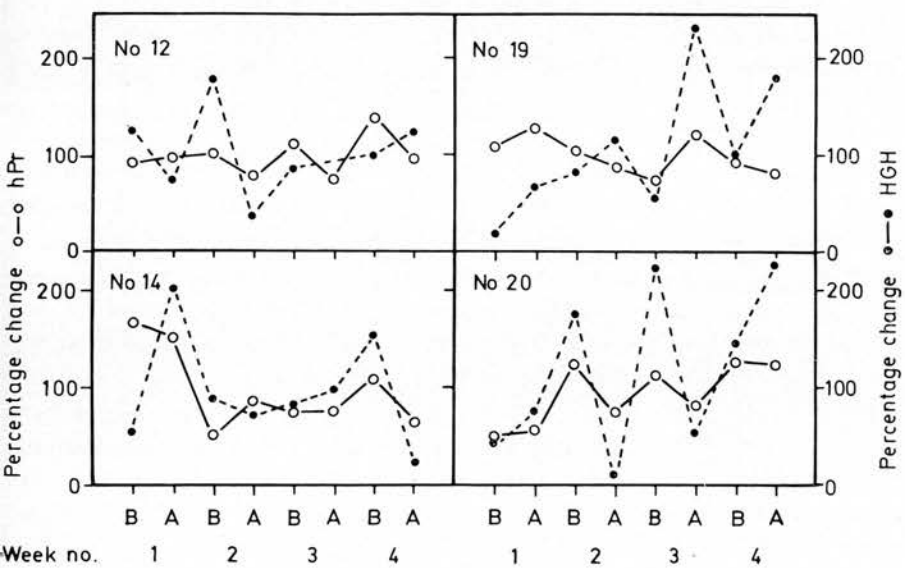



*Fig. 1.*
Percentage changes in serum prolactin and growth hormone levels before (B) and after (A) dialysis during a 4 weeks periods in patients No. 12, 14, 19 and 20.

higher values of FSH than LH as found among the haemodialyzed women. Two women (No. 19 + 20) were clinically post-menopausal, but only one (No. 19) showed high post-menopausal values. Four women (No. 12, 17, 18, 20) had irregular menstruation with normal gonadotrophin levels, but very elevated plasma hPr (31.8–133 ng/ml).

None of the groups showed significant ($P > 0.05$) linear correlation between any of the hormone concentration and the time of dialysis, and no difference in hormone concentrations between anephric patients and non-nephrectomized patients could be demonstrated.

## DISCUSSION

The present study agrees with that of *Nagel et al.* (1973) in that elevated levels of hPr occur in a high percentage (40 %) of patients undergoing chronic haemodialysis. In addition we have found that the percentage of elevated levels in patients undergoing chronic peritoneal dialysis was even higher (90 %). In contrast to *Nagel et al.* (1972) we found that the elevated hPr levels in the last group appear to be directly related to the medical treatment, metyldopa, cyproheptadin and levomepromazine. This is underlined by the facts that 5 of the 10 peritoneal-dialyzed patients treated with these groups (No. 11, 12, 17, 19, 20) showed the highest levels of hPr (44.0–133.0 μg/ml); and that the significant difference between the concentration of hPr in the haemo- and peritoneal-dialyzed group disappeared when these patients were excluded. The difference in prolactin levels in the haemo- and peritoneal-dialyzed group could also be due to the different time in chronic dialysis, 1.8 month and 36.2 month respectively in the two groups or could be due to a different stress response to the dialyzing procedure. However, the prolactin levels were not related to the time of chronic dialysis and the presumably stress induced fluctuating HGH levels were not related to the prolactin levels (Fig. 1).

The elevated levels of hPr were not manifested by galactorrhoea in any of the patients, even though these levels appeared to be permanently elevated.

Plasma HGH levels were in the upper normal range or elevated in 50 % of both haemo- and peritoneal-dialyzed patients. In 8 of the 10 haemodialyzed patients higher levels were found before than after dialysis. This is in agreement with other investigators (*Spitz et al.* 1970; *Bindeballe et al.* 1973; *Nagel et al.* 1973) who showed a rise in plasma glucose post-dialysis accompanied by the anticipated fall in HGH, and could not be explained from the known sleep related increase in HGH (*Sassin et al.* 1972; *Alford et al.* 1973), as none of the patients were sleeping during the last two hours of the dialysis. How-

ever, no significant change in HGH was seen in the peritoneal-dialyzed group before and after the dialysis.

Haemodialysis did not induce significant changes in the TSH levels. This corroborates the recent report (*Nagel et al.* 1973) but does not agree with the concept of a) heparin used in haemodialysis induces a rise in "free" thyroxine (*Schatz et al.* 1969; *Hershman et al.* 1972) or b) a fall in TSH after haemodialysis (*Hershman et al.* 1972).

In patients with severe chronic disease such as chronic renal failure, the gonadal function is usually impaired, but can return during sufficient treatment with intermittent haemodialysis (*Thaysen et al.* 1975, in press). The high FSH/LH ratio found in this study indicates impaired gonadal function in both the haemodialyzed- and peritoneally dialyzed group. In 4 patients with clinical hypogonadism, normal levels of gonadotrophins but high levels of hPr was found. This situation is analogous to that found in post-partum women in whom gonadotrophin secretion, while within normal limits, does not show cyclical release for some time and oestrogen secretion is low (*Reyes et al.* 1972), and to the situation found in amenorrhoea-galactorrhoea-syndrome with elevated prolactin levels (*Thorner et al.* 1974). It appears therefore that in some of the patients in the present study the raised hPr levels in some way have antigonadotrophic actions at the gonadal level (*McNatty et al.* 1974).

In conclusion there seems to be no difference in the pituitary hormone levels in women with chronic renal failure due to the type of chronic intermittent dialysis. There were significantly higher levels of prolactin in peritoneal- than haemodialyzed patients presumably due to the medical treatment whereas no significant difference in any of the other pituitary hormones was found.

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# PROLACTIN AND THE PITUITARY-GONADAL AXIS IN MALE URAEMIC PATIENTS ON REGULAR DIALYSIS

By

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## ABSTRACT

In 21 consecutive adult male patients with chronic renal failure on regular haemo- or peritoneal dialysis, the plasma levels of prolactin (hPr), growth hormone (HGH), thyrotrophin (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (T) and sex hormone binding globulin (SHBG) were measured.

Elevated levels of hPr were found in 16 of the patients and could not only be explained by the medicamentation. All the patients studied showed an inverse ratio of LH to FSH with higher levels of FSH than LH and 15 of the 21 patients had elevated plasma concentrations of FSH, while only 4 had elevated LH.

No significant difference in any of the hormone levels could be demonstrated before and after dialysis, and no significant correlation between the hormone levels and the time of dialysis, the type of dialysis or the age of the patient was found. However, 8 of the 21 patients showed higher levels of HGH before than after dialysis.

Impotency was found in 11 of the patients, but was not related to abnormal levels of hPr, LH, FSH, T or SHBG.

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is associated with a variety of hormonal disturbances (*Schatz et al.* 1969; *Hershman et al.* 1972; *Bindeballe et al.* 1973; *Gonzales-Barcena et al.* 1973; *Nagel et al.* 1973; *Rager et al.* 1973; *Ølgaard et al.* 1975). In male patients with chronic diseases a frequent symptom is impotency, but there appears to be no clear correlation between symptoms and hormonal disturbancies. *Rager et al.* (1973) found low values of testosterone and 5α-dihydrotestosterone in adult males with chronic renal failure, but no direct correlation with im-

*Table 1.*
Clinical data of 21 male patients on chronic dialysis.

| Patient No. | Age (years) | Dialysis (months) | Medicamentation[1] | Normal potency[2] | Gynaecomastia |
|---|---|---|---|---|---|
| *Haemodialysis* | | | | | |
| 1 | 16 | 48 | – | – | – |
| 2* Δ | 23 | 16 | β | + | – |
| 3 | 23 | 27 | – | – | – |
| 4* | 26 | 5 | – | + | – |
| 5 | 29 | 24 | – | + | – |
| 6* | 37 | 9 | – | – | + |
| 7 | 43 | 12 | – | + | – |
| 8 Δ | 54 | 12 | – | – | – |
| 9 | 55 | 38 | – | – | – |
| 10 | 55 | 21 | β | + | + |
| *Peritoneal dialysis* | | | | | |
| 11 | 27 | 1 | α,β | + | – |
| 12 | 29 | 4 | α,β | + | – |
| 13 | 32 | 3 | β | + | – |
| 14 | 35 | 3 | β prednisone | + | – |
| 15 | 39 | 4 | – | + | – |
| 16 | 41 | 1 | β | – | – |
| 17 | 43 | 1 | eltroxin | – | – |
| 18 | 43 | 3 | α,β | – | – |
| 19 | 52 | 2 | β | – | – |
| 20 | 59 | 8 | β | – | – |
| 21 | 60 | 2 | β | – | + |

* Hepatitis associated antigen positive. Δ Bilaterally nephrectomized.

1) α = methyldopa, β = furosemide.

2) – = impotency.



regained their potency, when prolactin levels were returned to normal (*Thorner et al.* 1974).

The present study was initiated to correlate clinical impotency with plasma levels of prolactin (hPr), luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (T) and sex hormone binding globulin (SHBG) in a group of male patients with chronic renal failure undergoing regular haemo- or peritoneal dialysis. For full investigation of pituitary function plasma levels of growth hormone (HGH) and thyroid stimulating hormone (TSH) were also measured.

## MATERIAL

### *Patients*

Twentyone consecutive male patients with chronic renal failure undergoing either haemo- or peritoneal dialysis were studied. The details of the patients are given in Table 1.

*Haemodialysed patients.* – The mean age of 10 haemodialysed patients was 36.1 (range 16–55) years and they had attended dialysis before the study for an average of 21.2 (range 5–48) months.

Impotency was defined as the persistent inability to develop and maintain a penile erection sufficient to conclude coitus to orgasm and ejaculation (*Hastings* 1963).

Five of the 10 patients were impotent, 3 were hepatitis-associated-antigen-positive without any other signs of liver disease, 2 were bilaterally nephrectomized and 2 had clinical gynaecomastia but none had galactorrhoea.

*Peritoneal dialysed patients.* – The mean age of the 11 peritoneal dialysed patients was 41.8 (range 27–60) years and they had attended dialysis before the study for an average of 2.9 (range 1–8) months. Six of the 11 patients were impotent. One patient had gynaecomastia but none had galactorrhoea or biochemical evidence of liver disease and none were nephrectomized.

## METHODS

### *Dialysis procedure*

*Haemodialysis.* – The patients were dialysed with disposable parallel plate dialyzers (Gambro-Lundia or Boe-Dawids) for an average period of 10 h (range 9–11 h) twice a week and received a diet containing on an average of 0.8 g of protein per kg body weight, 50 mEq. sodium, 50 mEq. potassium and about 800 ml fluid daily.

During dialysis the patients were anticoagulated by single injections of heparin in the return line from the dialyzer under repeated control of clotting time, which varied from 15 to 60 min. The total dose of heparin per dialysis ranged from 15 000 to 25 000 IU.

*Peritoneal dialysis.* – The patients were dialysed with either a disposable peritoneal dialysis catheter or with a permanent peritoneal dialysis catheter a. m. Tenckhoff.

*Table 2.*
Blood urea and hormonal levels in male patients before (B) and after (A) dialysis.

| Patient No. | Blood urea mmol/l | | hPr ng/ml | | HGH ng/ml | | TSH μU/ml | | LH mU/ml | | FSH mU/ml | | Testosterone ng/ml | | SH (10- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| *Haemodialysis* | | | | | | | | | | | | | | | |
| 1 | 26 | 6 | 19.6 | 15.4 | 8 | 8 | 1.0 | 1.2 | 4.0 | 4.5 | 2.8 | 3.4 | 6.3 | 6.6 | 11.3 |
| 2 | 33 | 3 | 14.5 | 16.6 | <2 | <2 | 0.7 | 1.6 | 2.5 | 4.6 | 10.1 | 12.1 | 7.8 | 9.8 | 3.9 |
| 3 | 29 | 10 | 22.7 | 31.4 | <2 | <2 | 1.6 | 1.2 | 2.0 | 4.0 | 11.3 | 12.6 | 0.9 | 1.2 | 5.0 |
| 4 | 28 | 10 | 17.7 | 16.5 | 14 | <2 | 1.3 | <0.4 | 2.1 | 1.8 | 12.1 | 13.0 | 9.4 | 8.2 | 2.6 |
| 5 | 33 | 10 | 21.7 | 14.6 | 7 | 3 | <0.4 | 1.9 | 1.6 | 2.2 | 6.0 | 6.2 | 1.1 | 0.7 | 4.4 |
| 6 | 20 | 10 | 20.3 | 26.0 | <2 | <2 | <0.4 | 0.9 | 14.2 | 15.3 | 18.1 | 19.3 | 8.8 | 5.8 | 3.5 |
| 7 | 34 | 15 | 16.4 | 16.2 | 18 | <2 | 0.4 | 0.8 | 0.8 | 1.5 | 7.8 | 8.0 | 3.1 | 4.4 | 2.7 |
| 8 | 30 | 10 | 24.2 | 19.2 | <2 | <2 | <0.4 | 0.6 | 17.6 | 18.2 | 19.6 | 25.9 | 3.0 | 4.4 | 3.4 |
| 9 | 18 | 5 | 24.5 | 25.0 | <2 | <2 | 1.0 | 1.1 | 8.2 | 9.6 | 12.2 | 14.1 | 5.2 | 5.9 | 4.1 |
| 10 | 35 | 10 | 26.5 | 25.1 | 7 | <2 | 2.0 | 0.9 | <0.4 | <0.4 | 2.8 | 3.1 | 2.7 | 2.9 | 3.0 |
| Mean | 28.6 | 8.9 | 20.8 | 20.6 | 6.4 | 2.6 | 0.9 | 1.0 | 5.4 | 6.2 | 10.3 | 11.7 | 4.9 | 5.0 | 4.3 |
| *Peritoneal dialysis* | | | | | | | | | | | | | | | |
| 11 | 29 | 11 | 38.5 | 40.3 | <2 | <2 | 1.8 | 3.5 | 1.4 | <0.4 | 4.2 | 4.1 | – | – | – |
| 12 | 27 | 16 | 20.3 | 16.0 | 9 | 8 | <0.4 | <0.4 | 3.1 | 4.2 | 6.5 | 6.9 | – | – | – |
| 13 | 31 | 15 | 20.1 | 23.6 | <2 | 4 | 0.8 | 1.7 | 6.2 | 3.1 | 17.7 | 14.0 | 10.9 | 9.6 | 5.6 |
| 14 | 19 | 11 | 33.6 | 34.7 | 17 | 18 | 0.4 | 1.6 | 3.5 | 3.3 | 10.9 | 9.7 | 10.4 | 14.5 | 3.9 |
| 15 | 27 | 12 | 22.2 | 18.6 | <2 | <2 | 0.6 | 1.0 | 5.1 | 5.6 | 7.8 | 9.9 | 7.2 | 9.3 | 5.4 |
| 16 | 52 | 33 | 16.8 | 15.3 | 3 | <2 | 1.2 | 0.9 | 4.4 | 4.9 | 4.0 | 4.5 | 4.6 | 5.8 | 5.0 |
| 17 | 69 | 20 | 18.2 | 13.9 | 50 | <2 | 9.5 | 8.3 | 3.7 | 4.0 | 6.5 | 6.1 | 1.4 | 2.3 | 0.6 |
| 18 | 23 | 13 | 48.8 | 45.1 | 5 | <2 | 0.4 | <0.4 | 2.9 | 2.9 | 9.3 | 9.7 | 8.0 | 8.6 | 6.4 |
| 19 | – | – | 28.0 | 30.2 | 9 | 4 | 0.4 | 0.4 | 2.8 | 3.4 | 10.4 | 10.9 | 17.8 | 15.1 | 5.8 |
| 20 | 27 | 16 | 22.7 | 17.4 | 9 | 10 | 1.4 | 1.0 | 7.1 | 6.6 | 24.2 | 21.2 | 10.5 | 5.4 | 6.5 |
| 21 | 32 | 19 | 49.8 | 43.8 | 23 | 9 | 1.1 | 1.2 | 3.5 | 3.7 | 12.7 | 14.2 | 7.4 | 2.4 | 3.3 |
| Mean | 33.6 | 16.5 | 29.0 | 27.2 | 11.9 | 5.7 | 1.8 | 1.9 | 4.0 | 3.8 | 10.4 | 10.1 | 8.6 | 8.1 | 4.7 |

a diet containing on average 0.5 g of protein per kg body weight.

*Plasma samples.* – Blood samples were taken immediately before (at 9 a. m.) and after dialysis in all patients. Plasma was then separated and stored at –20°C until assayed. All samples were assayed in the same assay.

*Radioimmunoassays.* – Details and precision of the methods for measuring plasma immunoreactive hPr, LH, FSH, TSH and HGH have been given elsewhere (*McNeilly* 1973; *Mortimer et al.* 1973; *McNeilly & Hagen* 1974). In the assays used the normal range for plasma LH (MRC standard 68/40 assuming 39.8 IU/ampoule) in male subjects was 0.4–6.7 mU/ml; for FSH (MRC standard 68/39, assuming 33 IU/ampoule) the normal range in male subjects was 0.2–7.0 mU/ml; for hPr the normal range was 5–20 ng/ml, for TSH (MRC standard 68/38, assuming 6.8 mU/ampoule) the normal range was $< 0.4$–3.0 $\mu$U/ml.

Plasma testosterone was measured by a radioimmunoassay method without a chromatographic separation (*Tyler et al.* 1973; *Williams et al.* 1974). The antiserum used was raised against testosterone-3-(O-carboxymethyl)oxime coupled to albumin (kindly provided by Dr. E. Neischlag). Only 5$\alpha$-dihydrotestosterone showed cross-reactivity (50 %) which would be of significance in plasma samples. Comparison was made with a specific method which involved chromatography on alumina columns (*Chen et al.* 1971; *Pirke* 1973), and a good correlation was obtained for male samples ($r = 0.9205$, $n = 30$). The normal range for adult male subjects was 3.5–11.0 ng/ml.

SHBG, binding-site concentration was measured by saturating the binding-sites with 5$\alpha$-dihydrotestosterone, and precipitating with ammonium sulphate (*Rosner* 1972) The normal range for adult male subjects was $2.1$–$5.1 \times 10^{-8}$ M.

### *Statistics*

Results were compared using the Mann-Whitney or Wilcoxon test.

## RESULTS

The plasma levels in both the haemo- and peritoneal dialysed patients of blood-urea, the pituitary hormones hPr, HGH, TSH, LH and FSH, T and SHBG before and after dialysis are shown in Table 2.

Plasma hPr was elevated ($> 20$ ng/ml) in 5 of the 10 patients on haemodialysis and in 7 of the 11 patients on peritoneal dialysis. All patients showed an inverse ratio of LH to FSH with higher levels of FSH than LH. Raised plasma FSH was found in 15 of the 21 patients while only 4 showed raised LH levels.

On paired analyses, the dialysis induced no significant ($P > 0.05$) change in the plasma concentrations of the pituitary hormones, T or SHBG. No significant linear correlation ($P > 0.05$) could be demonstrated between any of the hormones, SHBG or between the hormones and the age of the patients, and the month of regular dialysis before the study. In both the haemo- and



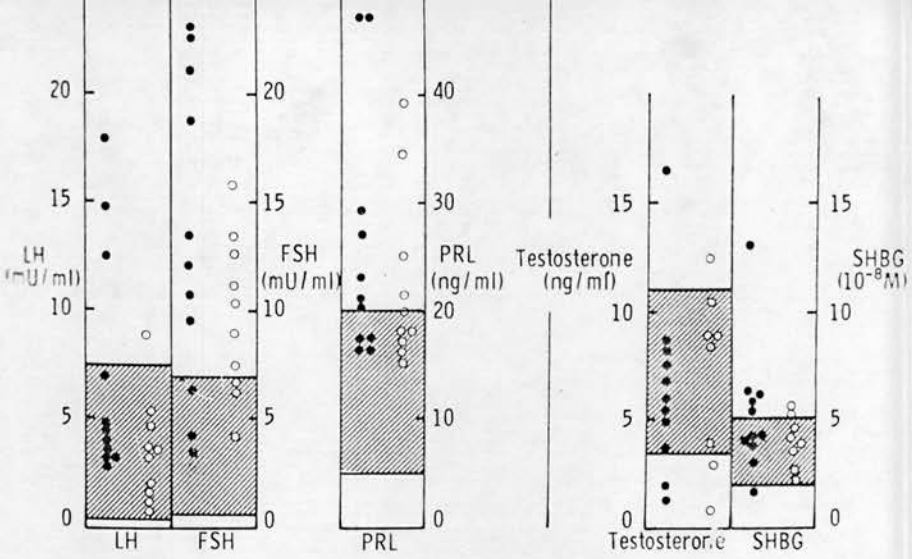



*Fig. 1.*

The mean of the serum concentrations obtained before and after dialysis of LH, FSH, hPr, T and SHBG in male patients with chronic renal failure. The hormone levels and SHBG in adult patients with impotency (●) and normal potency (o) are shown. The shaded area indicate the hormone and SHBG levels (range) in normal adult male patients.

peritoneal dialysed group the levels of TSH were within the normal range except in 1 patient (No. 17, Tables 1 and 2) who was on Eltroxin replacement therapy for hypothyroidism. Plasma HGH was elevated ($> 10$ ng/ml) in half of the patients from both groups before dialysis, while only 1 (No. 14) showed elevated levels after dialysis. As no significant difference ($P > 0.05$) in the circulating levels of the hormones or SHBG in the haemo- and peritoneal dialysed group could be found, these two groups were considered together, to investigate the relationship between hormone levels and impotency.

Lack of potency was evident in 11 of the 21 patients (Table 1). All patients had normal potency before the onset of the renal disease. However, the precise time of onset of impotency could not be documented in any of the patients. Plasma hPr was elevated ($> 20$ ng/ml) in 6 of the 11 patients with impotency and in 6 of the 10 patients with normal potency and no significant difference ($P > 0.05$) between the mean levels (before and after dialysis) of any of the hormones or SHBG in the patients with impotency and those with normal potency could be demonstrated (Fig. 1).

The T/SHBG index which indicates the unbound fraction (biologically-active) of T in plasma was within the normal range in all but 4 patients who had lower values (1 with normal potency and 3 with impotency). No significant ($P > 0.05$) linear correlation was found between the levels of LH and FSH and T or SHBG.

## DISCUSSION

The present study confirms that elevated circulating levels of hPr occurs in a high percentage of patients on regular intermittent dialysis (*Nagel et al.* 1973; *Ølgaard et al.* 1975). In contrast to female patients undergoing dialysis (*Ølgaard et al.* 1975) no significant difference in hPr levels between haemo- and peritoneal dialysed patients was found. However, treatment with methyldopa in both the male and female patients was related to elevated levels of hPr. *Nagel et al.* (1973) found no correlation between gynaecomastia and plasma hPr. In this study the 3 patients with gynaecomastia had mean levels (before and after dialysis) of hPr above normal (20.3, 25.8, and 49.8 ng/ml), but these levels were similar to those in patients without gynaecomastia.

In the galactorrhoea-hypogonadism syndrome treatment of the hyperprolactinaemia in most patients restores their gonadal function (*McNatty et al.* 1974; *Thorner et al.* 1974). However, in the present study both normal and elevated levels of hPr were found with no significant difference between those with normal potency and those with impotency. It therefore seems unlikely that prolactin *per se* is a major factor in the development of impotency in these patients. Further no significant difference in plasma LH, T or SHBG could be found between the patients with normal and those with impotency. These results support the view that hormonal as well as psychological factors may be involved in the pathogenesis of impotency (*Cooper et al.* 1970; *Lawrence & Swyer* 1974).

Normal levels of LH were found in 18 and elevated levels of FSH in 15 of the 21 male patients in contrast to the normal values of both hormones in female patients. But still an elevated FSH/LH ratio was demonstrated comparabel to the findings in female patients on regular dialysis (*Ølgaard et al.* 1975).

A circadian rhythm for HGH, hPr and T has been found, but in addition rapid variations during the day have been described (*Dray et al.* 1965; *Takahashi et al.* 1968; *Leymarie et al.* 1973; *Parker et al.* 1973). In this study none of the patients were asleep for the last 3 h period of their dialysis and no correlation between the time of the day and the hormone levels was found.



Low values of T (*Stewart-Bentley et al.* 1974) and dihydrotestosterone in haemodialysed patients with no significant change related to the circulating levels of the gonadotrophins, indicating a primary defect associated with testicular steroidogenesis have been found (*Rager et al.* 1973). In contrast, the almost normal levels of T, SHBG and LH found in this study (Fig. 1) indicate that most haemo- and peritoneal dialysed patients have an apparently normal pituitary-gonadal axis. Although the exact feedback control for LH and FSH is not fully understood, the very high levels of FSH found both in the haemo- and peritoneal dialysed group, suggests that the spermatogenesis and/or Sertoli cell function in these patients are severely affected (*Christiansen* 1972; *Kretser & Burger* 1972; *Millet et al.* 1973; *Lunglmayer et al.* 1974).

A fall in HGH related to the changes in blood glucose (*Nagel et al.* 1973) and a rise in TSH due to the heparin induced changes in thyroxin (*Hershman et al.* 1972) have been reported during haemodialysis. In contrast, in this study no significant change in either plasma HGH or TSH before and after dialysis could be demonstrated. At present no reasonable explanation can be found for these discrepancies.

In conclusion elevated levels of FSH and hPr and almost normal levels of LH, T and SHBG were found in male patients with chronic renal failure treated with either intermittent chronic haemo- or peritoneal dialysis. No correlation between abnormal hormone levels in plasma and impotency could be demonstrated.

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# Prolactin in Hypertensive Pregnancy

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## Summary

**Plasma prolactin levels were measured in 68 pregnant women with hypertension at 32 weeks gestation. They were raised in pregnancies with pre-eclamptic features, most significantly in women with a rising plasma urate level. No correlation was found between the level of the untreated blood pressure and prolactin. Proteinuria did not influence prolactin levels independently of changes in the plasma urate. The differences in prolactin levels could not be ascribed to the drugs administered.**


## Introduction

Pre-eclampsia is characterized by hypertension with increased vascular reactivity (Gant *et al.*, 1973), fluid retention, and impaired renal function usually associated with proteinuria. Excess circulating pressor or fluid-retaining agents related to the renin-angiotensin system (Weir *et al.*, 1973), deoxycorticosterone (Brown *et al.*, 1972), cortisol (Kopelman and Levitz, 1970), or aldosterone (Thomas and Flynn, 1964) have not been identified in pre-eclampsia.

Prolactin causes sodium and water retention in some vertebrates (Ensor and Ball, 1972) and may have comparable effects in mammals (Lockett, 1965; Horrobin *et al.*, 1971). Possibly some of the features of pre-eclampsia could be caused by prolactin (Friesen *et al.*, 1973; Horrobin *et al.*, 1973). We have therefore measured plasma prolactin levels in hypertensive pregnancies to determine whether raised values are associated with any features of pre-eclampsia. Because the drugs routinely used to treat pre-eclampsia (methyldopa, diazepam, barbiturates) may influence prolactin secretion part of this study was directed towards excluding this possibility.

## Patients and Methods

### EFFECT OF METHYLDOPA ON PROLACTIN LEVELS

Patients included in a trial of methyldopa for mild and moderate hypertension in pregnancy were studied at 24, 28, 32, 34, 36, and 38 weeks of gestation. They were included in the trial if on two occasions more than 24 hours apart and before 28 weeks of gestation either the systolic or diastolic pressure equalled or exceeded 140 or 90 mm Hg respectively. All blood pressures were measured with a London School of Hygiene sphygmomanometer to minimize observer bias. Patients were allocated at random to a treatment or control group.

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### RELATION BETWEEN PRE-ECLAMPSIA AND PROLACTIN

Altogether 68 patients with hypertension as defined above were studied at 32 weeks of gestation; 48 were outpatients and 20 were inpatients. Thirty-four of them were taking methyldopa. All of the inpatients were sedated with diazepam, nitrazepam, amylobarbitone sodium, or phenobarbitone. None of the patients was restricted in fluid, salt, or food intake. No patient was on diuretic therapy.

### CLINICAL

The patients were studied between 09·00 and 12·00 hours. Three blood pressure readings were taken, the first after five minutes' rest in the left lateral recumbent position, the second standing, and the third after one minute of gentle, standardized exercise, the mean of these being taken as the blood pressure for each patient. Oedema was graded by one observer on a scale 0-3 (absent to severe) in three sites, face, hands, and ankles. The sum of the three grades was used as an "oedema score." Venous blood was taken into lithium heparin as anticoagulant, and plasma separated within three hours of sampling; 2 ml was stored at −30°C for prolactin assay, and 5 ml was assayed for urate by routine automated methods (S.M.A. 12/60).

Hypertension, oedema, proteinuria, and a rising plasma urate level were the four features of pre-eclampsia studied. Significant oedema was defined as a score of 5 or more. Proteinuria was present if a midstream specimen of urine contained more than 0·3 g/l in the absence of infection. A rising plasma urate level was defined as an increase of 0·059 mmol/l (1 mg/100 ml) or more in the four weeks preceding the study.

### LABORATORY

Plasma prolactin was measured by a specific double antibody radioimmunoassay, as described (McNeilly, 1973; McNeilly and Hagen, 1974), and the results were expressed as μg of standard human pituitary prolactin per litre. Interassay and intra-assay variation as a percentage coefficient of variation of replicate serum samples was 10% and 4% respectively.

## Results

The mean plasma prolactin levels in the patients treated with methyldopa showed no significant or consistent differences from those of the untreated control group (table I). Of the 68 hypertensive women studied at 32 weeks of gestation 22 had a rising plasma urate level (table II). The prevalence of oedema was twice as high in these patients and all the cases of proteinuria were in this group. None of the 48 outpatients received any treatment other than antihypertensive therapy.

Of the 68 patients 34 were primigravid and 34 were multiparous; their mean prolactin levels were similar, 77 ± 4·6 (S.E. of mean) μg/l and 76 ± 5·3 μg/l respectively. Altogether 34 of the 68 women took no antihypertensive agents. Their mean systolic and diastolic blood pressures are shown in table III. Those with a high prolactin level, arbitrarily defined as 80 μg/l or more, had blood pressure levels identical with those of patients with a low prolactin level. Ten patients had proteinuria. They had significantly higher mean prolactin levels than the remaining 58 with no proteinuria ($P < 0.05$) (table IV). Similarly, the 12 patients with a high oedema score had higher levels of prolactin ($P < 0.05$). A much more significant difference in prolactin levels was found when the patients were classified by changes in their plasma urate levels (table V). Those with a rising urate level had a considerably higher prolactin level than those with a steady urate level ($P < 0.001$). This difference

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persisted when only outpatients were considered, in whom the unknown effects of sedative drugs could be excluded.

When the patients were divided according to the behaviour of their plasma urate levels (table VI) those with a high oedema score also had higher prolactin levels. The numbers of patients were small and the differences were not significant. The 10 patients with proteinuria all had a rising urate level. Their mean prolactin levels were similar to those of the 12 patients with a rising plasma urate level and no proteinuria (table VI).

TABLE I—*Mean Plasma Prolactin Levels (μg/l) ± S.E. of Mean in Hypertensive Pregnant Women Treated with Methyldopa and in Untreated Hypertensive Pregnant Controls. Numbers of Patients are Given in Parentheses*

| Weeks of Gestation | Hypertensive Controls | Hypertensive Treated Patients |
|---|---|---|
| 24 | 52·7 ± 5·7 (14) | 54·9 ± 4·7 (13) |
| 28 | 63·1 ± 5·2 (23) | 66·7 ± 4·8 (19) |
| 32 | 63·4 ± 4·5 (18) | 70·2 ± 5·8 (17) |
| 34 | 79·5 ± 8·0 (18) | 70·0 ± 3·2 (14) |
| 36 | 75·3 ± 4·1 (24) | 71·0 ± 3·1 (20) |
| 38 | 67·4 ± 4·8 (23) | 73·1 ± 4·8 (14) |

TABLE II—*Details of Patients Studied at 32 Weeks of Gestation*

| | No. with Rising Plasma Urate Level | No. with Steady Plasma Urate Level | Total |
|---|---|---|---|
| All patients: | 22 | 46 | 68 |
| Oedema score ⩾5 | 6 | 6 | 12 |
| Proteinuria present | 10 | 0 | 10 |
| No oedema or proteinuria | 10 | 40 | 50 |
| Antihypertensive drugs: | | | |
| Methyldopa alone | 7 | 20 | 27 |
| Methyldopa + hydrallazine | 5 | 2 | 7 |
| None | 10 | 24 | 34 |
| Outpatients | 8 | 40 | 48 |
| Inpatients | 14 | 6 | 20 |
| Inpatient drugs: | | | |
| Continuous sedation | 9 | 0 | 9 |
| Nocturnal sedation | 5 | 5 | 10 |
| Antiemetic | 0 | 1 | 1 |

TABLE III—*Untreated Women at 32 Weeks: Mean Blood Pressures ± S. E. of Mean in "High" and "Low" Prolactin Groups. Numbers of Patients are Given in Parentheses.*

| | Systolic | Diastolic |
|---|---|---|
| Prolactin ⩾80 μg/l | 129·8 ± 3·7 (15) | 72·9 ± 3·1 (15) |
| Prolactin ⩽79 μg/l | 129·1 ± 2·9 (19) | 72·4 ± 2·6 (19) |

TABLE IV—*Mean Plasma Prolactin Levels (μg/l) ± S.E. of Mean and Prevalence of Proteinuria and of High and Low Oedema Scores*

| | Prolactin Level | No. of Patients |
|---|---|---|
| Proteinuria present | 91·7 ± 9·8 | 10 |
| Proteinuria absent | 75·2 ± 3·6 | 58 |
| Significance | 0·025 < P < 0·05 | |
| Oedema score ⩾5 | 91·3 ± 3·5 | 12 |
| Oedema score ⩽4 | 74·7 ± 3·6 | 56 |
| Significance | 0·025 < P < 0·05 | |

TABLE V—*Mean Plasma Prolactin Levels (μg/l) ± S.E. of Mean in Patients with Rising or Steady Plasma Urate Levels*

| | Prolactin Level | No. of Patients |
|---|---|---|
| All patients: | | |
| Rising plasma urate | 93·8 ± 7·4 | 22 |
| Steady plasma urate | 70·0 ± 3·1 | 46 |
| Significance | P < 0·001 | |
| Outpatients: | | |
| Rising plasma urate | 103·0 ± 15·8 | 8 |
| Steady plasma urate | 68·3 ± 3·5 | 40 |
| Significance | P < 0·005 | |

TABLE VI—*Relation between Mean Plasma Prolactin Levels (μg/l) ± S.E. of Mean and Plasma Urate Behaviour, Oedema Score and Presence or Absence of Proteinuria. Numbers of Patients are Given in Parenthe es*

| | Prolactin Level | |
|---|---|---|
| | *Oedema Score >5* | *Oedema Score ⩽4* |
| Rising plasma urate | 104·2 ± 15·8* (6) | 89·9 ± 8·3* (16) |
| Steady plasma urate | 78·3 ± 6·5* (6) | 68·6 ± 3·4* (40) |
| | *Proteinuria Present* | *No Proteinuria* |
| Rising plasma urate | 91·6 ± 9·8 (10) | 95·5 ± 11·1 (12) |

*Difference between means not significant.

## Discussion

Prolactin may affect and be affected by changes in sodium and water balance in many vertebrates including some mammals (Lockett, 1965; Horrobin *et al.*, 1971; Ensor *et al.*, 1972; Buckman and Peake, 1973; Jaffe *et al.*, 1973). Prolactin levels are high in pregnancy, and our study shows that they are even higher in pregnancies with pre-eclamptic features. The most significant association was not with the evidence of fluid retention but with a rising plasma urate level; less significant associations occurred with oedema and proteinuria and no association was found with the level of the untreated blood pressure. The association with proteinuria seemed to depend on the common association with a rising urate level. The same could be true for oedema, but there were too few oedematous patients to allow this to be clarified.

Oedema of pregnancy in the absence of renal dysfunction is associated with a good prognosis (Thomson *et al.*, 1967). Proteinuria is a clearer indication of the severity of pre-eclampsia and carries the worst prognosis of the classic triad of pre-eclamptic signs (Butler and Bonham, 1963). A rising plasma urate level is an early sign of pre-eclampsia (Seitchik, 1953), correlates with the characteristic renal biopsy changes (Pollak and Nettles, 1960), and at 32 weeks of gestation is a clear indication of an impaired fetal prognosis (Beilin *et al.*, 1974).

A major problem of this study was to take account of the effect of drugs administered to the patients. Nearly all the drugs used routinely in the treatment of pre-eclampsia increase plasma prolactin levels in non-pregnant patients. Our finding that methyldopa does not increase plasma prolactin levels in pregnancy contrasts with its reported effects in non-pregnant women (Turkington, 1972 b).

In pregnancy, however, baseline plasma prolactin levels are greatly increased and if methyldopa is having any effect on prolactin the effect must be small and obscured by the much larger differences described here. Other drugs used in this study were administered to inpatients only. They included hydrallazine, used in seven patients, sedatives, and an antiemetic (dicyclomine hydrochloride) administered to one patient. The effect of hydrallazine on prolactin secretion has not been studied. The sedatives used were either barbiturates, which experimentally affect prolactin release (Wuttke *et al.*, 1971), or benzodiazepines, which may increase prolactin levels in non-pregnant patients (Fluckiger, 1972). The effects of drugs can be largely excluded by considering the 48 outpatients who took no medication other than methyldopa. In that group the association between high prolactin levels and a rising plasma urate level was still present.

To determine why the plasma prolactin was raised in these circumstances requires separate studies. The weak association with clinical oedema does not suggest that prolactin of itself is a major cause of the fluid retention of pre-eclampsia. That kind of fluid retention characteristically occurs when gross renal impairment has developed with a reduced glomerular filtration rate. The reduced renal function of pre-eclampsia is thought to be due to glomerular endothelial swelling caused by fibrin deposition in the capillary endothelium.

Non-pregnant patients with chronic renal failure have raised prolactin levels for reasons which are not clear (Frantz *et al.*, 1972; Turkington, 1972 a). Only four patients in this study

had a blood urea level above 6·6 mmol/l (40 mg/100 ml) and could be said to have a more than mild degree of renal impairment. The important renal factor in this study was renal tubular dysfunction, as shown by changes in the plasma urate levels. The hyperuricaemia of pre-eclampsia is due to diminished renal tubular excretion and not to excessive production (Chesley and Williams, 1945), and possibly this is due to lactic acidosis caused by anaerobic metabolism in the placenta (Handler, 1960). How prolactin and uric acid metabolism may be linked has yet to be determined, but the possibility that the link is a direct one, in that prolactin may control or modulate uric acid metabolism or excretion during pregnancy, should be considered.

Alternatively, pre-eclampsia may in some way interfere with prolactin clearance. If prolactin is cleared by the kidney, as are some other peptide hormones, then the early involvement of the kidney in pre-eclampsia as measured by plasma urate changes may also indicate a reduced prolactin clearance. Growth hormone (GH), however, which is structurally closely similar to prolactin, is thought to be cleared by the liver, at least in non-pregnant people (Taylor *et al.*, 1972); and the liver is not known to be involved in the early development of pre-eclampsia.

Of the other endocrine changes in pre-eclampsia the reduction in circulating plasma oestrogens (Masson, 1973) provides no explanation of the increased prolactin levels, since plasma oestrogens potentiate prolactin release (Frantz *et al.*, 1972). Human placental lactogen may also be reduced in pre-eclampsia (Letchworth and Chard, 1972) but its relation to prolactin is not known. The levels of adrenocorticotrophic hormone (ACTH) and thyroid-stimulating hormone (TSH), thought to be of pituitary and not placental origin, are raised in pre-eclampsia (Genazzani *et al.*, 1971; Mukherjee and Swyer, 1972). The same may be true of GH, but this is less certain because of the difficulty of its measurement in pregnancy (Laron *et al.*, 1967). That prolactin is also increased gives further evidence that anterior pituitary function may be generally disturbed in pre-eclampsia. TSH and prolactin are both secreted in response to thyrotrophin-releasing hormone (Bowers *et al.*, 1971), and the release of GH and ACTH are also under hypothalamic control. The results suggest that hypothalamic function could be disturbed in pre-eclampsia, and this seems the most likely explanation of these observations.

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# NOCTURNAL HORMONAL PROFILES IN MASSIVE OBESITY, ANOREXIA NERVOSA AND NORMAL FEMALES

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MOST of the major hormone systems show a 24-hr periodic secretory pattern which is functionally related to the sleep/waking cycle [1]. Aspects of hormonal activity and sleep are sensitive to diet, weight and changes in weight [2, 3]. Psychological influences in the waking state are also known to affect dietary intake and to influence directly hypothalamic–pituitary function. By examining subjects during sleep the effects of short-term influences such as physical activity, eating or anxiety can be minimized.

The deviations from normal in hormonal levels associated with chronic and stable weight disorders (anorexia nervosa, massive obesity) has been variously interpreted. For example, they might represent one component of a more general hypothalamic disorder, incorporating abnormalities of hunger and satiety or they may be secondary to the abnormal nutritional and weight status. Alternatively, psychosocial influences may be central to the generation of these disturbances. Our own working hypothesis has been that much of the hormonal abnormality observed in these conditions is an adaptation to the deviations in weight and diet, and are reversible with restoration of normal weight and nutritional habits. Psychological and social variables are of importance in the generation and maintenance of both conditions, but do not directly account for the endocrine changes; rather, the latter provide reinforcement to the former.

## SUBJECTS AND METHODS

The obese subjects were a group of 17 adult in-patients with a mean age of 38 yr (range 18–55), and a mean weight of 121 kg (range 108–71 kg). There were four males, two post-menopausal females and 11 pre-menopausal females. Two of the latter had had hysterectomies but not oophorectomies. For five days preceding the study all patients were on a standard 2400 calorie balanced diet.

Six cases of anorexia nervosa were examined, five of whom were studied within two weeks of admission. Their body weight was a mean of 30% below that of a population matched for age and height (range 22–44%). All but one had secondary amenorrhoea. Their dietary pattern was that of the 'abstaining' anorectic [4], i.e. they usually rigorously avoided carbohydrate and were not given to overeating or vomiting. One of the five had, however, begun to eat before coming into hospital and had gained 7 kg during the month before admission. The sixth case, who was not in hospital, was a 14-yr-old girl with stunted growth (height 4 ft 3 in) who had had clear-cut anorexia since the age of 10 yr and was normal in terms of weight for height. In common with the other patients she had maintained a strict avoidance of carbohydrate, but in the months preceding the study had changed her eating habits so that she was taking large amounts of protein in the form of chicken and supplementing this with cabbage; this diet satisfied her hunger and was promoting both weight gain and increase in height. Although early pubertal changes had been present since the age of 10 yr, she had not menstruated.

The control group consisted of seven normal females (nurses and students) aged 18–23 yr with apparently normal weight and menstrual function. One subject proved to have the nocturnal endocrine profile characteristics of anorexia; on further questioning it was established that she had un-

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equivocal anorexia nervosa at the time of the study and was about 9 kg under weight. Her results were therefore excluded from the analysis.

All subjects were put to bed between 22.30 and 23.00 hr following a night of adaptation in the sleep laboratory. The methods of blood collection and EEG lead placements have been described elsewhere [5]. Lights were turned out at 23.00 and blood collected at 10–20 min intervals for determination of *growth hormone* (GH), prolactin, *luteinizing hormone* (LH), *follicle stimulating hormone* (FSH), *insulin* and *thyroid stimulating hormone* (TSH). Blood for *cortisol* was collected at hourly intervals. Plasma was separated immediately and stored at − 20°C prior to analysis.

The hormone levels were determined by radioimmunoassay: growth hormone (hGH, using M.R.C. 66/217 as standard), prolactin (hP, using M.R.C. 71/222 as standard), luteinizing hormone (hLH, using M.R.C. 68/40 as standard), follicle stimulating hormone (hFSH, using M.R.C. 69/104 as standard), and thyroid stimulating hormone (hTSH, using M.R.C. 68/38 as standard). Cortisol was also determined by radioimmunoassay.

For analysis the night was divided into six equal parts of 1 hr and 20 min each. Within each of these periods each subject from each group had between three and six readings. Only the pre-menopausal female obese subjects are used for comparison between groups. These readings were averaged for each subject and the means then calculated for each group of subjects with in each time interval.*

## RESULTS

Figures 1–7 show the results for each hormone in the three groups. By comparison with the contro group, growth hormone and LH levels were lower in both the obese and anorexic subjects (Figs. 1 and 6) and insulin levels were elevated (Fig. 2). Levels of TSH (Fig. 3) are shown only for the obese and anorexic group as normal data were not available. Levels for both pathological groups were elevated when compared with the normal daytime range. (TSH + 0·8–3·4 mu/ml). Prolactin levels (Fig. 4) were lower in anorexic subjects and elevated in the obese (Fig. 4). Levels of FSH (Fig. 7) were normal in obese subjects and low in anorectics. Cortisol (Fig. 5) levels were elevated in anorectics and normal in the obese.

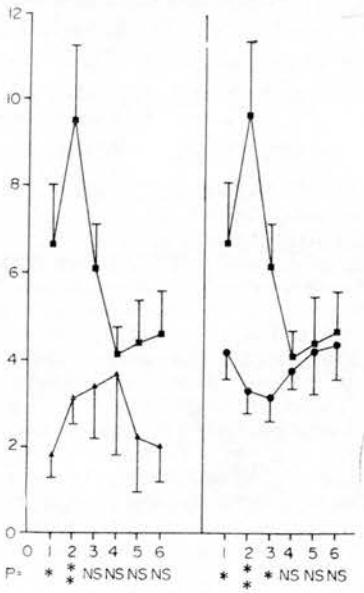



FIG. 1.—Mean GH levels in mu/ml during 6 eighty-min periods of the night. The left-handspace compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the differences (*P*) between groups is shown below each space; $P = < 0{\cdot}5$ (*), $P = < 0{\cdot}01$ (**).

*Group means were compared by the '*t*' test.

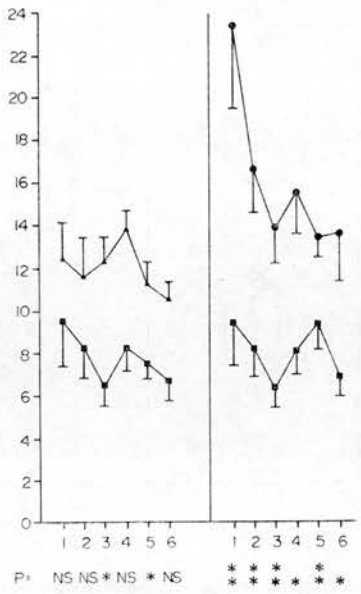



FIG. 2.—Mean insulin levels in mu/ml during 6 eighty-min periods of the night. The left-hand space compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the differences ($P$) between groups is shown below each space; $P = < 0{\cdot}5$ (*), $P = < 0{\cdot}01$ (**).

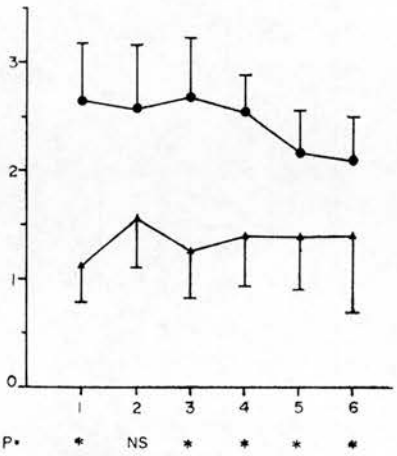



FIG. 3.—MEAN TSH levels in mu/ml during 6 eighty-min. periods of the night. The figure compares anorexia nervosa subjects (▲) with obese subjects ((●). The significances of the differences ($P$) between groups is shown below; $P = < 0{\cdot}5$ (*), $P = < 0{\cdot}01$ (**).

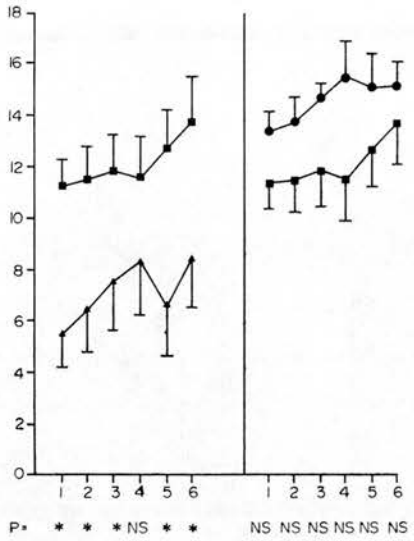



FIG. 4.—Mean prolactin levels in mu/ml during 6 eighty-min periods of the night. The left hand space compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the differences ($P$) between groups is shown below each space; $P = < 0{\cdot}5$ (*), $P = < 0{\cdot}01$ (**)

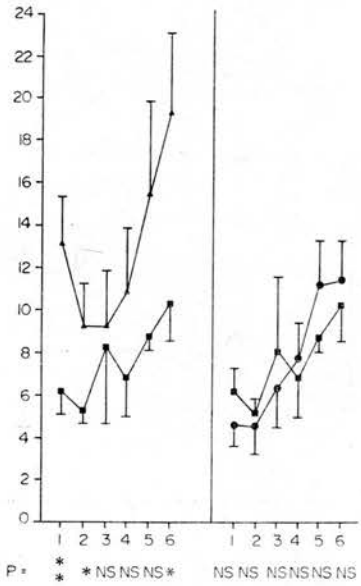



FIG. 5.—Mean cortisol levels in mu/ml during 6 eighty-min periods of the night. The left-hand space compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the difference ($P$) between groups is shown below each space; $P = < 0{\cdot}5$ (*) $t$ $P = < 0{\cdot}01$ (**)

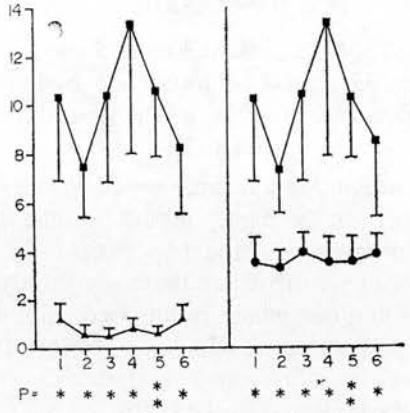



FIG. 6.—Mean LH levels in mu/ml during 6 eighty-min periods of the night. The left-hand space compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the differences (*P*) between groups is shown below each space; $P = 0{\cdot}5$ (*), $P = 0{\cdot}01$ (*).

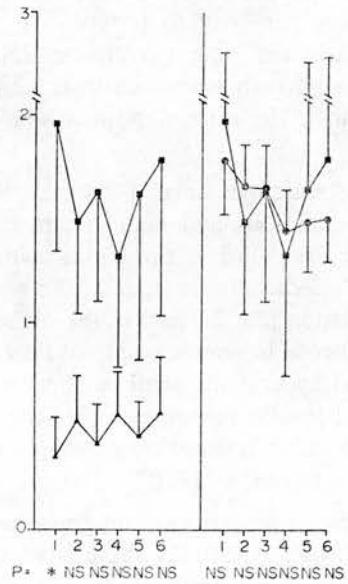



FIG. 7.—Mean FSH levels in mu/ml during 6 eighty-min periods of the night. The left-hand space compares anorexia nervosa subjects (▲) with normal subjects (■); the right-hand space obese subjects (●) with normal subjects (■). The significance of the difference (*P*) between groups is shown below each space; $P = <0{\cdot}5$ (*), $P = <0{\cdot}01$ (*).

## DISCUSSION

The hormones examined will be discussed individually.

Growth hormone results in control subjects were similar to those previously reported. All normal subjects had a surge in the first third of the night and subsequently, a number had smaller surges [6–9].

The anorexia nervosa patients, as a group, showed some evidence of a surge covering the first and second thirds of the night—a pattern quite different from that of the normals. They show a progression from two patients in whom the results were uniformly below the level of sensitivity of the assay throughout the night, to three who showed small surges and one whose results were within the normal range. This last patient had gained 7 kg prior to admission. Money [10] and others [11] have recently reported on growth hormone responses during sleep in the group of emotionally deprived, mal-nourished, sleep disturbed, stunted patients termed "psychosocial dwarfs" [12–14]. This group shows an absence of growth hormone surges and a return to a normal pattern after 4–6 weeks of hospitalization and good nourishment. At this time their sleep has returned to normal and growth has resumed. It may similarly be the case that the variation in pattern seen in our patients may be functionally and reversibly related to their dietary and weight status. In contrast to the low nocturnal levels, high resting levels have been reported during the day in anorexia nervosa [15–22]; this is also the case in other states of starvation [15, 20–23]. This has been related to decreased caloric intake and to carbohydrate restriction in particular [15–20]. Restoration of weight and normal eating patterns restored growth hormone levels to the normal range in the day [15].

In contrast with anorexic patients, the obese shows low daytime fasting plasma growth hormone and hyporesponsivity to provocative tests [25]. These deviations are reversible in some patients with weight reduction [25]. In the present study seven of the obese subjects showed growth hormone surges which were lower in amplitude, more frequent compared with the normal population, and tended to occur in the second half of the night.

The pattern of insulin secretion in normal subjects was unremarkable. Both the anorexic and the obese populations had high insulin levels; in the obese this was particularly the case in the first third of the night, coinciding with the paradoxical fall in growth hormone. Hyperinsulinism and insulin resistance is well documented in both the anorexic population [26, 27] and in the obese [28]. In the early stages of treatment, the anorectic glucose tolerance response may be attenuated and tends to become hyperglycaemic and hyperinsulinaemic with weight gain and restoration of a normal diet. The abnormal insulin response during the day time may persist for a considerable period of time after restoration of weight [27], although the impaired glucose tolerance can return to normal [29].

The relation of these responses to carbohydrate restriction and starvation in general has been discussed. [20, 21, 30–32] Some have argued that impaired insulin resistance may be an inherent constitutional factor in anorexia nervosa rather than an acquired one [20, 27, 33].

Pituitary adrenal function is usually reported to be normal in the anorexic population [20, 35–37] although some have reported increased levels of 11-hydroxycor-

ticosteroids [17, 20, 17] and have suggested that this may have diagnostic significance [20]. After treatment this also returns to the normal range.

Cortisol levels were very high in our anorectic population at the beginning and even more markedly at the end of the night. This complements the findings of Frankel *et al.* [34] who found high morning levels of plasma cortisol and a loss of the normal diurnal rhythm which reverted to normal in two out of three of our patients after treatment.

All groups showed a sleep related rise in prolactin levels towards the end of the night. [38]. Overall nocturnal levels were low in the anorectics and high in the obese. Episodic release resulting in a progressive rise in plasma concentrations [40] was seen in all three groups. Scheving and Dunn [39] have recently reviewed the literature on the factors affecting prolactin secretion; these include a circadian rhythm and other cyclical phenomena such as the menstrual cycle, oestrus and the state of fat accumulation and non-cyclical phenomena such as stress, hypoglycaemia, exercise and drugs. Many of these variables have an obvious relationship to the weight-deviant population. It should be pointed out that the anorectic population were on total bed rest and none were on phenothiazines.

By contrast, daytime levels of prolactin in anorexia nervosa have been reported as normal. [35, 41].

Nocturnal levels of TSH were elevated in both pathological groups compared with the accepted daytime range for a normal population. In the absence of control data and estimates of other thyroid hormones, it is not possible to speculate on the significance of these findings. Thyroid hormone and TSH activity in anorexia nervosa has previously been reported as normal [20, 35–37, 42].

Luteinizing hormone levels in normal subjects throughout the night were compatible with those previously described by Weitzman and Boyar [44–47, 54]. In both pathological groups the levels were very low. Low LH has consistently been reported in anorexia nervosa before treatment [2, 15, 17, 20, 35, 37, 41, 55]. The dependence of normal cyclical LH/FSH activity on weight in anorexia nervosa has already been noted and a critical menarchal weight of about 47·8 $\pm$ 4·6 kg has been identified in the general population [49]. It is possible that the secretory patterns of anorexics during sleep, which has been described as being typical of that of pre and early puberty, is also weight dependent and that the variation in results described below depend on low weight and individual differences between subjects. All our patients were well below the critical weight referred to above. When individual results are examined, three of the four normal subjects in whom it had been possible to collect 20 min samples without interruption throughout the night, secretory episodes were typical of the mature female [39, 47, 54] were found.

In both pathological groups the levels of luteinizing hormone were low. Individuals with the lowest levels of luteinizing hormone also showed the lowest levels of growth hormone.

Two of the anorectic subjects had patterns of LH similar to the "pre-pubertal" pattern described by Boyar *et al.* [47]. A further three showed much lower levels—almost uniformly below the level of sensitivity of the assay throughout the night. The two who showed evidence of a pre-pubertal pattern were slightly heavier than the other three and certainly more robust. The sixth subject, stunted, but with a normal

weight for height, showed an early pre-pubertal pattern with moderate spikes, again, similar to those described by Boyar *et al.* [47].

Of the obese patients only five (1 male and 4 premenopausal females) showed secretory episodes. The two post menopausal females had high mean levels of LH, but no secretory episodes.

It may be that the abnormalities observed in LH in the obese population may be related to their susceptibility to develop amenorrhoea during dieting.

The low nocturnal levels of FSH in anorexic patients are comparable with the findings during the day [20, 50–53, 55]; this pattern is reversible with restoration of weight and normal eating [2, 20, 37]. The wide discrepancy between the mean levels of FSH in normals and anorexics is similar to that of LH. However, FSH levels showed much greater variation, and the difference between the normal and abnormal groups was statistically marginal. FSH levels reflected no obvious secretory episodes and the values in any one individual were relatively consistent throughout the night. The more striking decrease in LH than FSH agrees with other findings [15, 52, 57, 58] and may reflect the normal pre-pubertal immature secretory pattern [59].

The discrepancy between LH and FSH levels was even more marked in the obese group, in whom FSH values were consistently within the normal range.

## SUMMARY

The nocturnal hormonal profile of growth hormone, prolactin, luteinizing hormone, follicle stimulating hormone, cortisol, insulin and thyroid stimulating hormone in groups of massively obese, anorectic and normal female subjects are compared. Chronic and stable weight disorder is associated with marked alterations in all hormone systems examined. By comparison with the control group, growth hormone and luteinizing hormone levels were lower in both the obese and anorexic subjects and insulin levels were elevated. Prolactin levels were lower in the anorexic subjects and elevated in the obese. Levels of FSH were normal in the obese subjects and lower in the anorexic. Cortisol levels were elevated in the anorexic and normal in the obese.

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# Prolactin and gonadotrophin activity in females treated for anorexia nervosa

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## Introduction

Although here is some agreement that in anorexia nervosa there is a major disturbance of function at the hypothalamic level, its precise nature remains obscure. Evidence that hypothalamic control of prolactin and gonadotrophic hormones is mediated through relatively discrete mechanisms (McNeilly, 1974) suggested that a study of these hormones during treatment of anorexia nervosa might help clarify the issue. Since phenothiazines are known to influence prolactin secretion in normal and psychiatric populations (Beumont *et al.*, 1974), these drugs were taken into account during the present investigation as a necessary aspect of treatment.

## Patients and methods

Thirteen female patients were selected on the basis of consecutive admission to hospital. Treatment, apart from psychotherapy, involved all patients in remaining in bed and consuming a normal diet until they reached their target weight, i.e. matched population mean weight (Crisp, 1967). The dose of phenothiazines was related to the patient's mental state: the more restless and distressed the patient, the higher the dosage. Ten patients were on chlorpromazine, two on perphenazine and one on both chlorpromazine and trifluoperazine.

The mean age of patients on admission to the study was 20 years 1 month; mean weight on admission to the study was 40·8 kg; and mean target weight was 53·4 kg.

Blood for hormonal assay was collected from each patient at intervals of approximately 3 weeks, usually, but not always, in the morning. The mean number of samples per patient was approximately five with a total of sixty-eight samples collected over a period of 11 months. Blood was centrifuged and the plasma frozen within 2 hr of collection. Plasma LH (luteinizing hormone) and FSH (follicle-stimulating hormone) were measured by double antibody radioimmunoassay as described by McNeilly and Hagen (1974). Prolactin was measured using the homologous antibody technique described by McNeilly (1973). Units of measurement throughout were: prolactin ng/ml; FSH mu/ml; LH mu/ml.

## Results

The mean weight of patients on leaving the study was 51·4 kg, 2·0 kg short of mean target weight and reflecting a mean weight gain of 12·6 kg. Table 1

TABLE 1. Relationship between premorbid weight, plasma prolactin and LH levels at various stages of treatment and immediate outcome

| Patent no. | Premorbid wt* | Admission | | Intermediate | | Target | | Return of menses |
|---|---|---|---|---|---|---|---|---|
| | | Prolactin (ng/ml) | LH (mu/ml) | Prolactin (ng/ml) | LH (mu/ml) | Prolactin (ng/ml) | LH (mu/ml) | |
| 1 | 95 | 8 | 5 | 2·5 | 4 | 10 | 22 | + |
| 2 | 95 | — | — | 16 | 15 | 17 | 6 | + |
| 3 | 100 | 7 | 2 | 8 | 5 | 8·5 | 20 | + |
| 4 | 100 | 15 | 1 | 12 | 2 | 9 | 4 | — |
| 5 | 105 | 8 | 9 | 7·5 | 8 | 0 | 7 | + |
| 6 | 110 | 8·5 | 0 | 14 | 0 | † | † | — |
| 7 | 110 | 11 | 0 | 24 | 0 | 7 | 7 | — |
| 8 | 110 | 9 | 5 | 9·5 | 10 | 8 | 24 | + |
| 9 | 115 | 9 | 0 | 38 | 0 | 12 | 0 | — |
| 10 | 120 | 5 | 2 | 23 | 0 | † | † | — |
| 11 | 125 | 9 | 8 | 53 | 15 | 7 | 34 | + |
| Mean | | 9·0 | 3·2 | 18·9 | 5·4 | 8·7 | 13·8 | |

* % Matched population mean weight.
† Patient left hospital before target weight reached.

shows the basic data: prolactin and LH levels reported are those within 10 days of admission to hospital; the mean of those intermediate between the admission level and the level at target weight; and those at or above target weight. Data on FSH levels are omitted here since they largely paralleled and were less complete than those on LH. Patients 12 and 13 are omitted from Table 1: 12, because she had recently had a modified leucotomy and 13, because blood was analysed only after she had reached target weight. Both subjects are referred to later.

Since previous work has suggested an association between premorbid weight of anorexia nervosa patients and whether or not LH/FSH activity is resumed on restoration of normal weight (Crisp *et al.*, 1973), this factor was again examined. Those five patients (Table 1) premorbidly less than 10% overweight showed no intermediate rise in prolactin levels whereas the six patients premorbidly 10% or more overweight shows a significant rise ($t=2{\cdot}80$, $P<0{\cdot}05$). In both groups, however, mean prolactin levels at target weight fell to levels *below* those on admission. Taking admission levels into account, the intermediate rise in prolactin levels in the more obese group is significantly different ($t=2{\cdot}45$, $P<0{\cdot}05$) from the small decrease in the less obese group.

There is a non-significant trend for menstruation to return more frequently in the less obese group (four out of five versus two out of six).

If the patients are divided into two groups according to whether or not menstruation resumed within 3 months of leaving hospital, intermediate LH levels are significantly higher in the menstruating group ($9{\cdot}5\pm2{\cdot}70$ versus $0{\cdot}4\pm0{\cdot}51$, $P<0{\cdot}005$). This trend is still apparent at target weight, but there are insufficient data for statistical analysis. There is a non-significant trend towards higher intermediate prolactin levels in the non-menstruating group.

The data in Table 1 show that there is no overall relationship between prolactin levels and weight. Table 2, however, shows a clear relationship between prolactin and phenothiazine dosage. All thirteen patients are included. Since for most patients the daily dose varied throughout treatment in relation to their emotional state, a comparison of the overall mean dose for each patient with their overall mean prolactin level would have been of little value. Instead, the table shows the highest recorded (peak) prolactin level for each patient, and the dose of phenothiazine given on that day. For those patients receiving 100 mg of less chlorpromazine (or its equivalent) per day, the mean prolactin level was 11·7 ng/ml; for those receiving more than 100 mg daily the mean prolactin level was 65·3 ng/ml. The likelihood of this association arising by chance is remote ($P<0{\cdot}05$), and there is also a consistent relationship between prolactin levels and chlorpromazine dosage ($r=0{\cdot}93$, $P<0{\cdot}001$).

TABLE 2. Relationship between chlorpromazine dosage and prolactin levels

| Patient | Daily chlorpromazine (mg) | Prolactin (ng/ml) |
|---|---|---|
| 5 | 0 | 5 |
| 13 | 0 | 9·7 |
| 3 | 50* | 9·1 |
| 8 | 75 | 10·0 |
| 4 | 75 | 10·4 |
| 2 | 100 | 17·0 |
| 1 | 100* | 21·0 |
| 10 | 150 | 37·1 |
| 9 | 225 | 55·4 |
| 7 | 250* | 36·4 |
| 11 | 300 | 47·2 |
| 6 | 400 | 30·0 |
| 12 | 800 | 175·8 |

* Approximate equivalent of other phenothiazines.

The condensed data as displayed in Table 1 fail fully to reveal the different patterns of change in prolactin and LH levels in individual patients during the intermediate period. In fact, it was possible to discern patterns. Five patients (Nos 1, 2, 3, 5 and 8) showed highly consistent and generally very low prolactin levels throughout, with a rise in LH levels as target weight approached. Only one of these was in the more obese group, and all resumed menstruation. Five patients (Nos 4, 6, 7, 9 and 10) showed inconsistent prolactin levels with peaks up to 55·4 ng/ml and no rise in LH levels as target weight approached. Two of these discharged themselves prematurely, and none resumed menstruation. Four were in the more obese group. Two patients (11 and 12) had inconsistent prolactin levels with very high peaks and a return of LH activity and menstruation. Patient 11 was premorbidly obese, 12 was not. The full data for these two are shown in Table 3.

Patient 13, for whom full data are available only around target weight, is of interest. At target weight, which she maintained for some months, she showed LH and FSH levels within normal limits, but no signs of menstruation. She had a poorly developed left breast but a normal right breast, suggesting some 'target organ resistance'.

## Discussion

The results show that premorbid obesity is significantly associated with higher prolactin levels and phenothiazine dosage during weight-gain, and more weakly associated with a failure to resume gonadotrophic and menstrual activity. The meaning of these associations may be revealed through

TABLE 3. The full data on patients 11 and 12

| Patient no. | Weight (kg) | Daily chlorpromazine | Prolactin ng/ml | LH mu/ml | FSH mu/ml |
|---|---|---|---|---|---|
| 11 (Target weight 50·0 kg) | 41·5 | 150 | 8·7 | 9·0 | 0·0 |
| | 43·2 | 150 | 87·8 | 16·0 | 0·6 |
| | 47·8 | 300 | 47·2 | 25·0 | 1·6 |
| | 49·5 | 300 | 25·4 | 0·0 | 0·0 |
| | 51·8 | 300 | 7·4 | 38·0 | 1·6 |
| 12 (Target weight 54·5 kg) | 40·5 | 75 | 7·7 | 12·8 | 0·0 |
| | 44·5 | 150 | 24·8 | 15·2 | 1·7 |
| | 49·0 | 150 | 24·4 | 5·6 | 0·0 |
| | 51·0 | 200 | 18·4 | 15·2 | 1·6 |
| | 54·0 | 200 | 18·9 | 16·0 | 0·6 |
| | 55·5 | 800 | 175·8 | 36·0 | 3·5 |

consideration of the pattern of hormone changes in the different individuals and groups.

Two main groups of patients have been defined in this study. One group requires small doses of phenothiazines, has consistently low prolactin levels, is not very obese premorbidly and more often resumes menstruation on reaching target weight or soon afterwards. The other group requires considerably more phenothiazines, has sharp peaks of prolactin activity, tends to premorbid obesity, fails to resume gonadotrophic and menstrual activity, and is more at risk for premature discharge.

One reason for failure to resume gonadotrophic activity in the more obese group might be a higher 'threshold' for the rekindling of 'pubertal' changes (Crisp, 1967) which initially occur at a mean weight of about 47 kg in the normal female population (Frisch and Revell, 1971) but which, after secondary amenorrhoea, are found to recur at a higher level (Frisch and McArthur, 1974). Furthermore, premorbidly obese subjects, who are particularly likely to adopt an anorexic posture of periodic overeating or sustained overeating and vomiting as a means of weight control, are usually the most restless and the most desperate and fearful about their capacity to maintain such control, especially when their weight reaches target levels. They, and other anorexics also, often reveal related anxiety about their emerging sexuality at this stage both in terms of its biological reality and its complex experiential effects.

However, premorbid obesity does not appear to be the only determinant of a stormy weight-gain period or return of menstruation. Patient no. 12 was premorbidly 5% below target weight, yet was chronically distressed at the experience of weight-gain and unable to sustain it after achieving it on successive occasions with intensive refeeding and psychotherapy. She was eventually advised to undergo modified leucotomy and accepted this. Her talk after operation suggested that a profound fear of sexuality had to be resolved before full recovery could occur. It is noteworthy that peak prolactin levels occurred at a time when gonadotrophic activity was also at peak. She finally killed herself, having made previous attempts earlier in her illness, about a year after leucotomy, which suggests that she had not learned to cope with those changes in her which may partly have been facilitated by the operation (Crisp and Kalucy, 1973). Patient no. 4 was not premorbidly obese, yet failed to resume gonadotrophic activity or menstruation after reaching target weight.

Presumably some factor other than an increase in menarchal weight threshold is involved in the failure to menstruate at target weight of those premorbidly obese. That such a factor may be present even in those premorbidly massively obese is suggested by the data on patient 11, who, although premorbidly very obese, resumed gonadotrophic activity well short of target weight.

This factor is unlikely to be phenothiazine dosage, since Beumont (1974) has shown clearly that phenothiazines generally have little effect on LH levels in a psychiatric population. Furthermore, the data on patient 11 suggest that a rise in prolactin *preceded* increased phenothiazine dosage. The general reciprocity between prolactin and LH levels in those patients who *fail* to menstruate is of interest here.

It may be that high arousal and any associated emotional distress can itself inhibit gonadotrophic activity, presumably operating through the cortico-hypothalamic neuronal network. Certainly, arousal is known to modulate hypothalamic functions both in animals and man. The implication is that two important factors operate in the suppression of gonadotrophic activity in anorexics, both of which are related to premorbid obesity. One appears mainly constitutional and is linked to menarchal weight thresholds and subsequent related growth, weight and fatness characteristics; the other is linked mainly to levels of arousal and emotional distress (which were often related to such factors as premorbid obesity and its psychological meaning to the patient), mediated by hypothalamic or cortico-hypothalamic

activity and the relationship of these to appetite. This latter mechanism may have been specifically interrupted by modified leucotomy in patient no. 12 and is also always a necessary focus of psychotherapy in anorexia nervosa.

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# HYPOTHALAMIC-PITUITARY-PROLACTIN AXIS IN BREAST CANCER

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***Summary*** The secretory reserve of pituitary lactophores was assessed by measuring the plasma concentration of prolactin before and 20 and 60 minutes after the intravenous injection of 200 μg. of thyrotrophin-releasing hormone (T.R.H.) in fifty women with early and fifty women with advanced cancer of the breast. Results were compared with those obtained in a group of fifty age-matched women in hospital awaiting surgery for a variety of conditions. There was no statistical difference in the mean basal prolactin levels between the three groups. Only women with advanced breast cancer exhibited a marginally greater pituitary reserve of prolactin as evidenced by a significantly higher plasma level of this hormone in response to T.R.H. stimulation. Prolactin response to T.R.H. in the early-breast-cancer group was not significantly different from that in controls. The hypothesis is put forward, based on previous experimental evidence, that a sub-optimal level of circulating thyroid hormones may abnormally sensitise mammary epithelial cells to prolactin stimulation, leading to dysplasia and eventual neoplasia.

## Introduction

PROLACTIN is essential for the promotion of growth of both spontaneous and carcinogen-induced mammary tumours in rodents,[1-3] but a role for prolactin in the pathogenesis of human breast cancer is uncertain.[4] Human prolactin has been identified separate from growth hormone, and specific radioimmunoassay

methods have been developed for the accurate measurement of its concentration in blood.[5] Early reports of the measurement of basal plasma prolactin levels in women with breast cancer are conflicting in that both normal [6] and raised [7] levels have been found. Kwa et al.[8] found a high prolactin level only in women with a family history of the disease. A dynamic test which measures the peak secretory capacity of the lactophores [9] might give a better assessment of pituitary prolactin reserve. Thyrotrophin-releasing hormone (T.R.H.), in addition to stimulating the synthesis and release of thyrotrophin (thyroid stimulating hormone, T.S.H.), is a potent prolactin secretagogue in man and many animals.[10–12] The physiological nature of T.R.H. makes it eminently suited for such a dynamic test of the functional integrity of the hypothalamic-pituitary-prolactin axis. In view of several clinical and epidemiological reports associating thyroid dysfunction with breast cancer [13,14] and the finding of a raised thyrotrophic activity in this disease,[15] it was hoped that this study would indicate whether there was any abnormality in prolactin secretion which might be mediated via the hypothalamic-pituitary-thyroid axis.

## Patients and Methods

*Patients*

The patients and controls described in Mittra and Hayward [15] participated in this study. No sedatives or tranquillisers were allowed for 48 hours before the T.R.H. test, and those patients receiving ergot alkaloids or antihypertensive drugs were excluded.

*Methods*

*Prolactin assay.*—Plasma-prolactin was measured by a specific double-antibody radioimmunoassay using an antihuman prolactin antiserum raised in rabbits and purified human pituitary prolactin as standard and for iodination [16] (kindly supplied by Dr H. Friesen).

*T.R.H. test.*—The mode of administration of T.R.H. and collection of blood is as described by Mittra and Hayward.[15]

*Statistical analysis.*—Comparison between group means was carried out using Student's $t$ test, a P value of $<0{\cdot}05$ being taken as denoting significance.

## Results

The mean basal levels of prolactin and the response to T.R.H. stimulation in early and advanced breast cancer compared to controls are given in the table. The mean basal prolactin concentrations in the early

and advanced breast-cancer groups did not differ significantly from the basal level in the controls. 20 minutes after T.R.H. stimulation plasma-prolactin in the advanced-cancer group rose to a mean concentration just significantly higher than that in the control group, but at 60 minutes the mean prolactin level in the patients with advanced breast cancer was no longer significantly different from that in the controls. 20 and 60 minute levels in the early-breast-

MEAN PROLACTIN LEVELS BEFORE AND AFTER T.R.H. STIMULATION IN BREAST-CANCER PATIENTS AND IN CONTROL SUBJECTS

| — | Prolactin (ng./ml.) | | |
|---|---|---|---|
| | Control | Early breast cancer | Advanced breast cancer |
| Basal | 14·24 ± 4·30 | 13·17 ± 2·62 | 16·10 ± 5·90 |
| 20 min. | 29·03 ± 11·41 | 30·84 ± 12·08 | 34·72 ± 16·45 ($P < 0·05$) |
| 60 min. | 21·77 ± 9·00 | 23·15 ± 11·18 | 24·58 ± 8·45 |

Mean ± S.D.
Level of significance compared to controls given in parentheses.
n = 50 in each group.

cancer group were not statistically different from control values at comparable times.

## Discussion

Using a sensitive radioimmunoassay we could find no difference between the basal plasma level of prolactin in women with breast cancer and that in age-matched controls. A dynamic test of pituitary prolactin reserve, using T.R.H. at a dose level which ensures maximal stimulation of pituitary lactotrophes,[9] demonstrated a marginally greater pituitary reserve of prolactin only in women with advanced breast cancer, while that in the early-breast-cancer group was not significantly different from controls. Since the amount of prolactin secreted by the pituitary in response to T.R.H. is inversely related to the concentration of circulating thyroid hormones,[10,11] a marginally higher T.R.H.-mediated prolactin secretion in advanced breast cancer could be explained by the concomitant finding of the highest mean T.S.H. level in these patients.[15] However, previous palliative hormone therapy might have influenced pituitary prolactin secretion in some patients in this group.

Acceleration of growth of 7,12-dimethylbenzanthracene-induced tumours in rats or induction of mam-

mary tumours in untreated animals by prolactin requires the presence of artificially raised levels of this hormone.[1–3] There is no evidence that a raised level of prolactin has any pathological effect on mammary epithelium in humans.[17] Pregnancy and lactation are two periods when a woman is subjected to significant prolactin stimulation; the former has been found to be protective against breast cancer while the latter seems unrelated to disease risk.[71] Therefore it seems not only that women with breast cancer have a normal resting blood-level of prolactin, as our results demonstrate, but also that a raised level of prolactin per se is harmless to the mammary epithelium. However, it is possible that an abnormal hormonal environment could alter the sensitivity of the mammary epithelial cells to the growth-promoting effect of prolactin which might then lead to dysplasia and eventual neoplasia. A recent finding, in animals, supports such as possibility[18]: in the absence of thyroid hormones the mammotrophic effect of normal levels of endogenous prolactin is much exaggerated, with resultant stimulation of growth of mammary epithelium in the rat. Conversely, Meites and Kragt[19] had previously observed that thyroxine suppressed prolactin-induced mammary growth round subcutaneously transplanted pituitary glands in immature hypophysectomised animals. This altered sensitivity of mammary epithelial cells to prolactin in hypothyroidism suggests another mechanism of prolactin involvement in the pathogenesis of mammary cancer.

Prolactin is generally considered to be a promoter of mammary tumour growth, whilst viral, chemical, and genetic factors have been implicated in the initiation of neoplasia.[20] However, it is also possible that, in the presence of suboptimal levels of thyroid hormones, prolactin initiates malignant transformation in cells unprimed for mammotrophic stimulation, probably in the presence of optimal concentrations of growth hormone and ovarian steroids. An observation by Hamilton and his colleagues[21] supports such a possibility. These workers were studying a radioactive halogen isotope astatine-112 which, like radioactive iodine, damages the thyroid by selective uptake.[21] In rats receiving low levels of this isotope they observed a 40% incidence of spontaneous malignant mammary tumours within a year, a finding that could not be explained on grounds of radiation effects on the mammary gland. No tumours developed in

rats receiving high doses of astatine. The disappearance of growth hormone from blood in severe hypothyroidism may have accounted for suppression of tumour growth in the latter group.[18]

A major function of prolactin at a lower level in the evolutionary scale is stimulation of somatic growth, especially in the larval stages,[22] while thyroxine is growth regulating and precipitates metamorphosis.[23] The complementary antagonism between these two hormones in amphibian development is a recent finding.[24] Neoteny of some salamanders with continued growth of larval structures in iodine-deficient areas of the world [25,26] may be due to an unabated prolactin stimulation.[22] It is conceivable that despite major functional evolution during the course of vertebrate phylogeny certain rudimentary functions persist and find expression at a higher level in the evolutionary scale. One such may be the antagonism between prolactin and thyroxine at the level of the mammary epithelium in the rat [18,19] and perhaps in man,[18] and a disturbance of this balance may exaggerate the action of one hormone and bring about a pathological change in the cells affected.

Epidemiological reports indicating a higher incidence of breast cancer in endemic goitre areas of the world [14,27] have received scant attention. Japan is a country with a low incidence of breast cancer [28] and where the dietary intake of iodine is extremely high [29]; a lower ratio of triiodothyronine to thyroxine in the thyroid glands of the Japanese compared with that reported from Europe or America [30] seems to indicate the presence of a relatively large thyroidal stable iodine pool with well-iodinated thyroglobulin in the Japanese. It is possible that a constant "normal" blood-level of thyroid hormones renders the mammary epithelial cells of Japanese women refractory to prolactin sensitisation.

I. M. thanks Prof. I. Doniach of the London Hospital for his supervision and encouragement in the preparation of a PH.D. thesis of which this study is a part. We thank Dr M. Duffus of Roche Products for supplying T.R.H. and Dr R. D. Bulbrook of the Imperial Cancer Research Fund for help in the preparation of this paper.

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# *Observations on prolactin levels during the menstrual cycle*

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The role of prolactin in the control of ovulation in the human is uncertain; studies were undertaken to determine the changes in circulating prolactin throughout the menstrual cycle.

Prolactin was determined by a specific double antibody radioimmunoassay (McNeilly, 1973) using a rabbit antibody raised against an impure preparation of human pituitary prolactin, and purified human prolactin, kindly supplied by Dr. H. Friesen, for iodination and standardisation. The results were expressed as ng Friesen prolactin/ml. Serum LH and FSH were determined by specific radioimmunoassays.

Daily blood samples were collected throughout the menstrual cycle in 9 subjects with a normal menstrual history for the previous 6 months. In a further 5 subjects blood samples were withdrawn at 15-minute intervals between 09.00 and 16.30 hours during the follicular phase (days 6 or 7), mid-cycle (days 14 or 15) and luteal phase (days 26, 27 or 28).

Samples were stored as serum at −20°C until assay. All samples from an individual were assayed together to eliminate intra-assay variation. Intra-assay variation (as % coefficient of variation) over the range of blood levels measured was 9%, 7% and 8% for prolactin, LH and FSH respectively.

The mean (± S.E.M.) blood levels of prolactin throughout the menstrual cycle, together with the overall mean ± 2 S.E.M. are shown in Fig. 1. There was a wide variation in levels (8.0–30.2 ng/ml) and although there was an apparent elevation of prolactin between days −6 and +4, on no day were the levels significantly different from those in the cycle as a whole.

In some subjects prolactin concentrations appeared to follow a cyclical pattern over periods of 2–7 days. However, sampling at 15-minute intervals showed that these apparent daily changes could be attributed to short-term variation over a period of minutes. Prolactin appears to be released in spurts occurring every 30 to 120 minutes throughout the day. There was no correlation between the pattern of prolactin secretion and either the stage of the menstrual cycle or the pulsatile releases of LH or FSH throughout the day. A similar pattern of release for prolactin has been observed in normal male subjects (McNeilly et al., 1973).

These results suggest that prolactin may not play a significant role in the menstrual cycle. However, it is equally possible that changes in the secretory pattern of prolactin may occur over very short time periods at a time when, during the present study, blood samples were not taken. This possibility is at present under investigation. No release of prolactin occurs during the menstrual cycle which matches the definitive peaks of either LH or FSH.

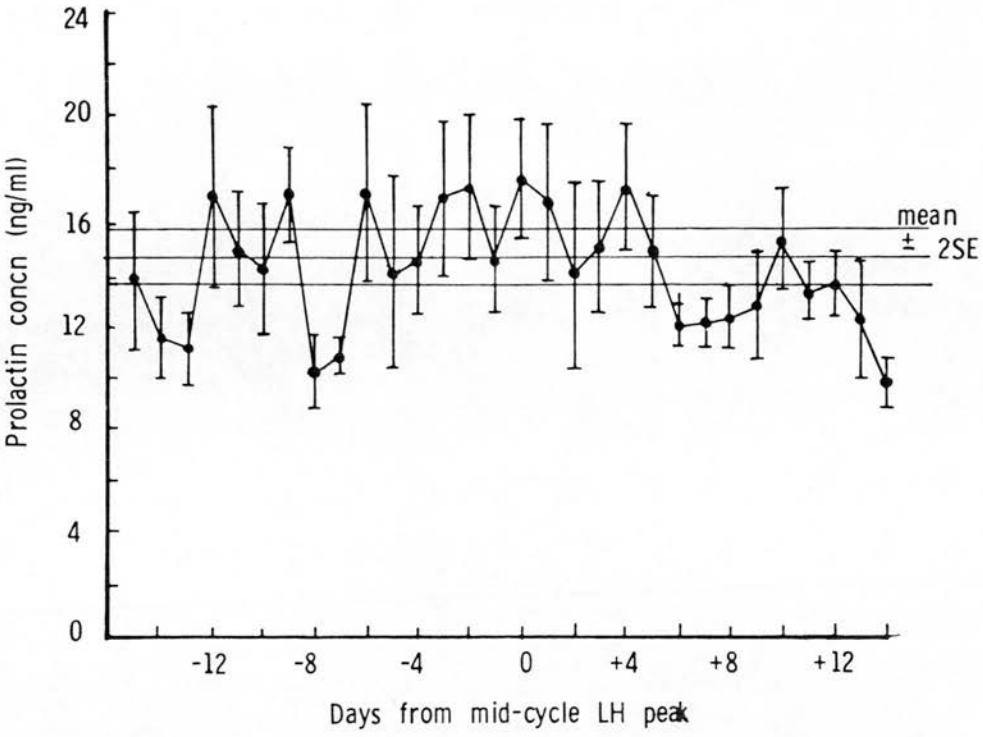



*Fig. 1. Mean blood levels of prolactin (± S.E.M.) on individual days around the mid cycle LH peak together with overall mean (± 2 S.E.M.) during nine normal menstrual cycles.*

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*Clinical Endocrinology* (1974) **3**, 105–112.

# CIRCULATING LEVELS OF PROLACTIN DURING THE MENSTRUAL CYCLE

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(*Accepted for publication* 19 *September* 1973)

## SUMMARY

In detailed studies on seventeen volunteers, it has been shown that changes in prolactin levels during the menstrual cycle are irregular and inconsistent. Some, but not all, subjects have elevated levels at mid-cycle, and higher levels in the luteal phase than in the follicular phase. At all stages of the cycle, prolactin levels may show short-term random fluctuations of as much as 110%. There was no apparent relation between levels of prolactin and those of LH, FSH, oestradiol-17$\beta$ or progesterone. It is suggested that if prolactin has any role in the control of the menstrual cycle, then the normal circulating levels are considerably in excess of the threshold requirements for its action.

## INTRODUCTION

Recent reports on changes in serum prolactin levels during the human menstrual cycle have been conflicting. Friesen and co-workers (Hwang *et al.*, 1971; Friesen *et al.*, 1972) failed to demonstrate any significant peak or difference in levels of prolactin throughout the menstrual cycle. By contrast L'Hermite and his colleagues (L'Hermite *et al.*, 1972; Robyn *et al.*, 1973) have found a peak of prolactin at mid-cycle and higher levels of prolactin during the luteal than the follicular phase of the cycle.

In an attempt to resolve this controversy, we have measured serum prolactin levels both throughout the menstrual cycle, and in short-term serial studies on specific days of the menstrual cycle.

## SUBJECTS AND METHODS

### *Subjects—study A*

A total of 320 daily samples were obtained between 08.00 and 10.30 hours during one menstrual cycle from eight women aged 19–35 years. All had had regular cycles (28–33 days) for at least 4 months prior to the study.

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*Subjects—study B*

In nine subjects, three of whom were taking oral contraceptives, a total of 698 samples were collected at 15 min intervals from 09.00 to 16.30 hours on three days of the cycle one during the follicular phase (day 6, 7 or 8), one at mid-cycle (day 14, 15 or 16) and

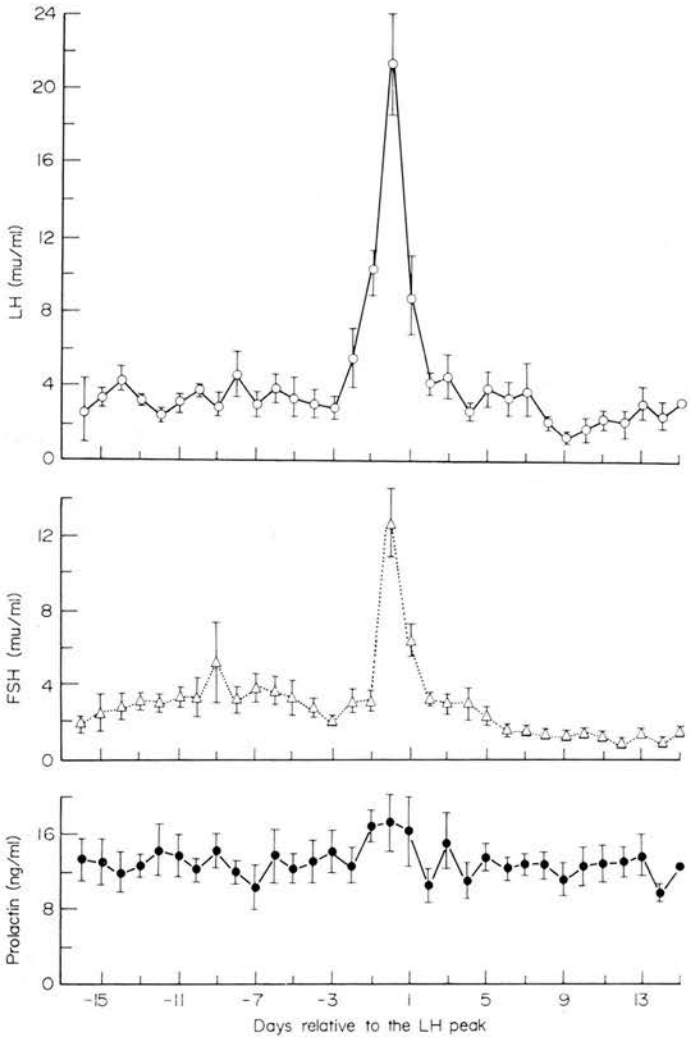



FIG. 1. Mean (± 1 SEM) levels of prolactin, LH and FSH in eight subjects during the menstrual cycle. The cycles have been synchronized around the day of the LH peak.

one during the luteal phase (day 26, 27 or 28). Of the six subjects not on the pill, retrospectiv analysis based on LH, FSH and progesterone determinations showed that the mid-cycl samples were collected either on the day before mid-cycle (one subject), or the day of mid cycle (two subjects) or the day after mid-cycle (three subjects).

*say methods*

Blood samples were allowed to clot, the serum separated by centrifugation, and stored at 20°C until assayed. All samples from an individual subject were measured in the same say.

Serum prolactin was measured by a specific double antibody radioimmunoassay as scribed previously (McNeilly, 1973). Purified human prolactin was supplied by Dr H. iesen, Montreal, Canada. Antiserum was raised in rabbits against a partially purified

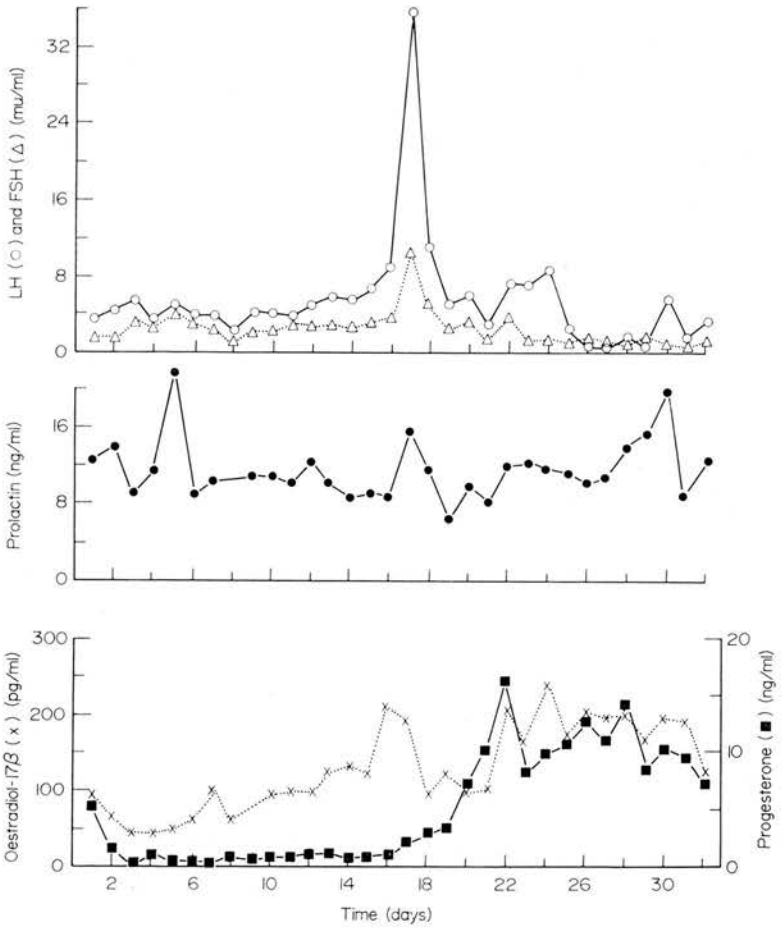



FIG. 2. Prolactin, LH, FSH, oestradiol-17$\beta$ and progesterone levels during the menstrual cycle in a single subject. Note the random peaks of prolactin, and lack of correlation with oestradiol-17$\beta$ levels.

paration of human prolactin prepared from acetone dried glands by Dr R. V. Davies, iiversity of Cambridge. Purified prolactin was labelled with $^{125}$I using the chloramine-T thod (Greenwood *et al.*, 1963) as described by Hwang *et al.* (1971). Both this preparation d the MRC Human prolactin research standard A (71/222, 10 mu/amp) were used as

standard (1 ng Friesen prolactin = 20 μU MRC 71/222). Separation of antibody-boun and free hormone was achieved by second antibody precipitation.

Serum LH was measured using a specific guinea-pig anti-LH antiserum (MRC 70/29 and LH preparations IRC 2/64 and IRC 2/69 (kindly supplied by Dr A. Stockell Hartre University of Cambridge) for iodination. Reference preparation MRC 68/40 was used a standard assuming 39·8 i.u./amp.

Serum FSH was measured using a specific rabbit anti-FSH antiserum and FSH prepa ation CPDS 3 for iodination, both materials being supplied by Dr W. R. Butt. MR preparation 68/39 was used as standard assuming 37·8 i.u./amp. Separation of antibod bound and free hormone was achieved in both the LH and FSH assays by second antibod precipitation. Oestradiol-17β and progesterone were determined by radioimmunoassay.

*Analysis of results*

The significance of the difference between means was assessed by Student's '*t*' test.

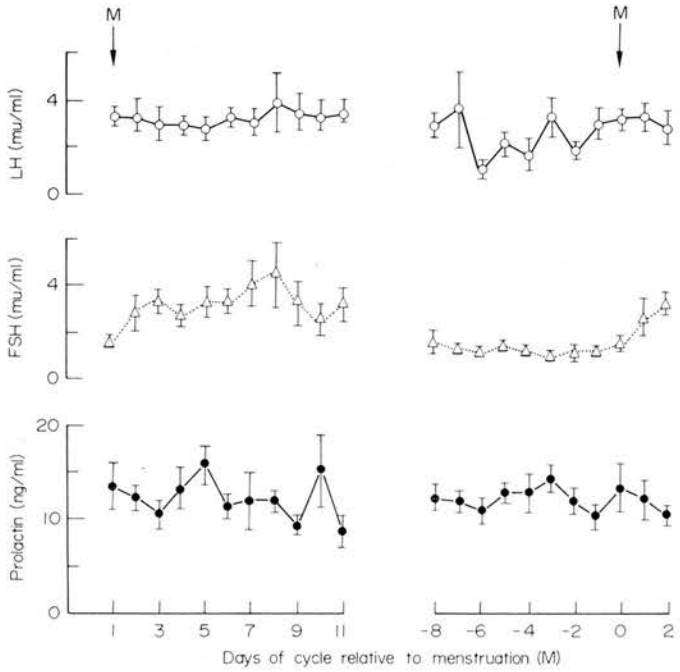



FIG. 3. Prolactin, LH and FSH levels in the 8 days before and 11 days after the onset of menstruation (M).

## RESULTS

*Study A*

The overall pattern showed a small but non-significant rise in prolactin levels in asso ation with the mid-cycle peak of LH and FSH (Fig. 1).

In three of the eight subjects there was an apparent peak of prolactin around the time

nid-cycle, but peaks of similar magnitude were also observed at other stages of the cycle Fig. 2). There was no change in prolactin levels around the time of menstruation (Fig. 3), nd no relationship between changes in the levels of prolactin and those of oestradiol-17$\beta$ r progesterone (Fig. 2).

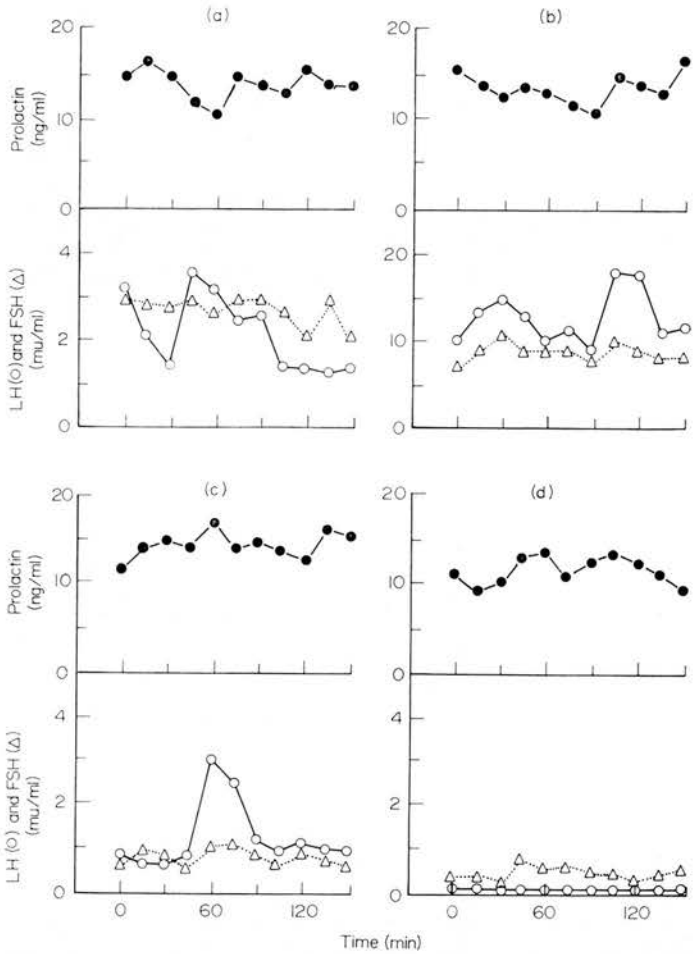



FIG. 4. Short-term changes in prolactin, LH and FSH: (a) follicular phase, subject 1, day 6; (b) mid-cycle, subject 3, day 14; (c) luteal phase, subject 1, day 26; (d) mid-cycle, subject 5, day 14, on pill. Note that prolactin levels are unrelated to either peaks of LH or basal levels of LH.

*tudy B*

Of the six subjects not on the pill, three had prolactin levels which were higher at mid-ycle than in the follicular phase, and two subjects had higher levels in the luteal phase than ı the follicular phase.

In the three subjects on the pill the levels were similar to those subjects not on the pill. here were no consistent changes in relation to the different stages of the cycle.

In all subjects, prolactin levels showed considerable random fluctuations over the time period studied; the difference between the lowest and highest levels observed in an individual was as great at 110%. The fluctuations were unrelated to the well-defined peaks of LH (Fig. 4a, b and c), or to the baseline level of LH (Fig. 5).

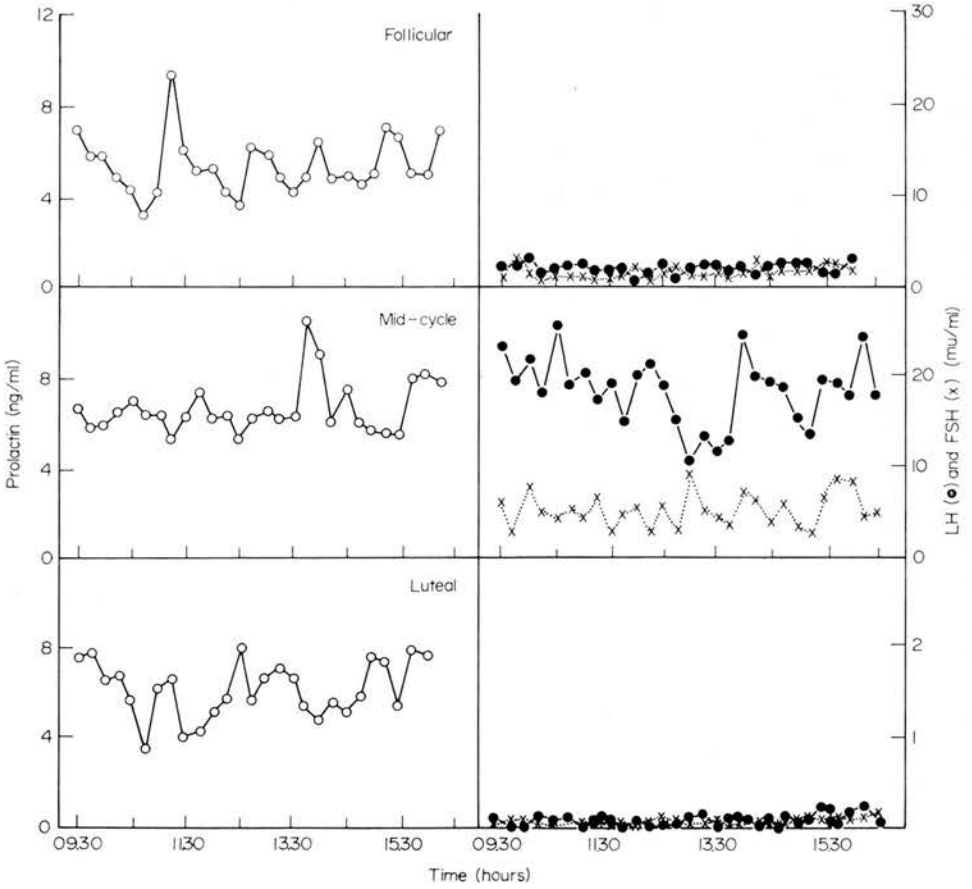



FIG. 5. Short-term changes in prolactin, LH and FSH in a single subject at different stages of the menstrual cycle. Note that in this subject the levels of prolactin are unrelated to the stage of the cycle, or to the basal levels of LH and FSH.

## DISCUSSION

The results of this study indicate that the variations in prolactin levels during the menstrua cycle are irregular and inconsistent. Thus, in some subjects (e.g. Fig. 5) no change was see at any stage of the cycle, which would agree with the observations of Friesen and his col leagues. In others, there was a significant elevation at mid-cycle, and a difference between th follicular and luteal phase, in agreement with the results of L'Hermite and his co-workers The discrepancy between the earlier workers can be attributed to two factors: first to th

differences between individuals, some of whom show specific changes while others do not; second, to the considerable random fluctuations within the same individual, an observation which has also been made in men (McNeilly, 1973) and in animals (Hart, 1973). Even in those cases where a specific change was observed at mid-cycle, it was in no sense comparable to the very large fluctuations seen in some animals at the time of oestrus (Davis *et al.*, 1971; Neill *et al.*, 1971).

There is evidence that prolactin levels may be elevated by oestrogen administration (Nicoll *et al.*, 1962; Schams & Karg, 1972). Robyn *et al.* (1973) have suggested that the variation in prolactin levels during the human menstrual cycle is directly related to changes in circulating oestrogen levels. However, in the present studies it has been shown that prolactin levels bear no relationship to the concentration of oestradiol-17$\beta$ or progesterone measured in the same sample. Furthermore, a clear-cut relationship seems unlikely in view of the inconsistent changes in the majority of cycles.

The implications of the present study are two-fold. First, the irregularity of the changes observed make it unlikely that prolactin plays any very critical role in the control of ovulation or the formation and maintenance of the corpus luteum. This postulate is further supported by the fact that pharmacological suppression of high prolactin levels, using 2$\alpha$-bromo-ergocryptine, can be associated with the return of normal menstrual cycles (Besser *et al.*, 1972). However, it cannot be excluded that the role of prolactin might be permissive, and that the levels encountered during the menstrual cycle may be considerably in excess of the threshold requirements for its action, a situation which has recently been demonstrated in prolactin-dependent breast cancer (Salih *et al.*, 1972). Secondly, it is apparent that under physiological conditions an increase or decrease in LH or FSH levels does not appear to affect either the level or the pattern of secretion of prolactin. However, high prolactin levels, such as those encountered during lactation, or in many cases of galactorrhoea, may influence the cyclical release of LH and FSH, resulting in amenorrhoea. The latter question is under investigation at the present time.

## ACKNOWLEDGMENTS

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*Clinical Endocrinology* (1975) **4**, 531–535.

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# PLASMA TESTOSTERONE AND PROLACTIN IN THE MENSTRUAL CYCLE

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(*Accepted for publication* 3 *December* 1974)

## SUMMARY

Daily hormonal studies during nine ovulatory menstrual cycles showed that plasma prolactin and testosterone concentrations fluctuated randomly and independently.

Mean plasma testosterone levels were found to be higher during the 7 days before and after the mid-cycle LH peak when compared to the premenstrual phase ($P<0{\cdot}01$). No correlation was found between daily levels of prolactin and those of LH, FSH, oestrogen or progesterone and no correlation was seen between peaks of prolactin and testosterone or mean prolactin and testosterone levels. The lack of correlation between blood levels of prolactin and testosterone during the menstrual cycle suggests that prolactin is unlikely to have any direct controlling influence on the cyclical nature of testosterone production observed during ovulatory menstrual cycles.

## INTRODUCTION

Although it is established that the ovaries secrete androgens during the normal menstrual cycle (Horton *et al.*, 1966; Rivarola *et al.*, 1967; Gandy & Peterson, 1968) studies on androgens in blood and urine collected during the cycle have given conflicting results (Ismail *et al.*, 1968; Longhino *et al.*, 1968; Dupon *et al.*, 1973). However, Judd & Yen (1973) and Abraham (1974) recently found a rise in plasma testosterone during the mid-third of the cycle with specific radioimmunoassay techniques. The purpose of the present study was to confirm or deny these findings using similar techniques. In addition, plasma prolactin was assayed to determine if its concentration correlated with that of testosterone, since the stimulus for ovarian testosterone secretion is not yet clear and there is *in vitro* evidence that prolactin may be involved in testosterone production (Evans, 1962; Ingvarsson, 1969).

## SUBJECTS AND METHODS

Nine women with a history of regular menses were studied. Their mean age was 34 years (range 25–43) and mean weight 57·2 kg (range 51·7–61·2 kg).

Correspondence: Dr M. Epstein, Endocrine Unit, Princess Margaret Hospital, Christchurch, New Zealand.

Blood was taken daily between 08.30 and 10.00 hours throughout a single menstrual cycle or from part of two consecutive cycles at the patients' homes.

Plasma testosterone was measured with a radioimmunoassay as described by Furuyama *et al.* (1970).

Plasma LH and FSH were measured with a specific double antibody radioimmunoassay modified from the method of Midgley (1966). The second International Reference Preparation–Human Menopausal Gonadotrophin (2nd IRP-HMG) was used as standard. (5 miu 2nd IRP-HMG = 1 iu MRC 68/40 LH; 1 miu 2nd IRP-HMG = 1·4 iu MRC 71/33 FSH.)

Plasma prolactin was measured by a specific double antibody radioimmunoassay as described by McNeilly (1973). The MRC human prolactin, research standard A (71/222, 10 miu/amp) was used as standard. (1 ng S/L1 or Friesen prolactin = 20 $\mu$u MRC 71/222.)

Plasma total immunoreactive oestrogens were measured by the method of Hotchkiss *et al.* (1971).

Progesterone was measured with a radioimmunoassay as described by Abraham *et al.* (1971) but without chromatographic purification of the plasma extract.

## RESULTS

All cycles were ovulatory as judged by a rise in progesterone greater than 10 ng/ml following a mid-cycle LH and FSH peak.

### *Testosterone*

There was a marked variation in individual testosterone levels from below the sensitivity of the assay (<13 ng/dl) for 20 days in one subject to levels of 50–80 ng/dl for 20 days in another. Most levels were in the 20–60 ng/dl range. Changes of >20 ng/ml were unusual between consecutive days but some subjects showed a more gradual rise or fall over several days. The changes in testosterone levels appeared independent of the fluctuations of any of the other hormones measured. Mean plasma levels were significantly higher during the 7 days before and after the mid-cycle LH peak when compared to the premenstrual phase the 4 days before menses ($P<0{\cdot}01$).

### *Prolactin*

No consistent cyclical pattern of progesterone was shown. Considerable day-to-day fluctuations occurred throughout every cycle and peaks of prolactin were seen in every phase. The prolactin values observed did not appear to be related to that of any other hormone measured. No differences were shown in the mean values obtained from all subjects during the various phases of the menstrual cycle.

### *Luteinizing hormone, follicle-stimulating hormone, oestrogen and progesterone*

The daily patterns of hormone levels observed were characteristic of those seen in normal ovulatory cycles.

Luteinizing hormone and follicle-stimulating hormone levels on the days following onset of menses were significantly higher than the levels prior to menses ($P<0{\cdot}001$). This is in direct contrast to the mean plasma oestrogen levels which fell markedly at the time of the menses.

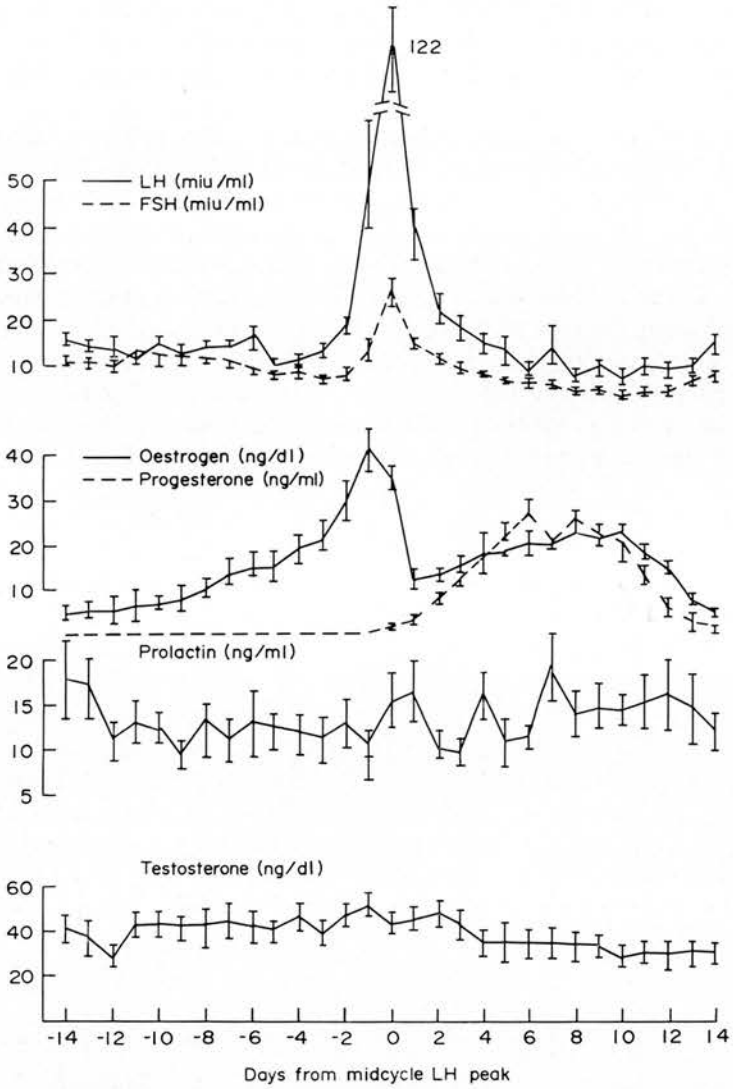



FIG. 1. Mean (±SE) plasma levels of LH, FSH, oestrogen, progesterone, prolactin and testosterone obtained from nine women during the menstrual cycle.

Daily fluctuations of these hormones appeared independent of those of prolactin or testosterone, though mean gonadotrophin levels rose together with those of testosterone in seven of the nine subjects in the post-menstrual phase. Fig. 1 shows the mean levels of the hormones studied in nine women through one cycle.

## DISCUSSION

*In vitro* data have shown the ovarian follicle, corpora lutea and stroma to be capable of producing the androgens, androstenedione and testosterone, the capacity for this synthesis

being increased around mid-cycle (Leymarie & Savard, 1968; Mikhail, 1967, 1970). Recent reports on the estimation of plasma testosterone during the menstrual cycle using a specific radioimmunoassay (Judd & Yen, 1973; Abraham, 1974) have shown that mean testosterone levels are higher during this time. Our results confirm this finding, though the significance in the individual remains uncertain because of the wide variation of levels found.

A rapid rise of LH and FSH occurring together with rising testosterone levels and prior to the maximal testosterone levels achieved, suggests that the gonadotrophins may be ultimately responsible for stimulating testosterone secretion as well as oestrogen and progesterone from the developing follicle and corpus luteum. However, there was no evidence to suggest that LH or FSH are a direct rapid stimulus to ovarian testosterone production.

Prolactin has been thought to be involved in the production of both a testosterone precursor pool and the enzymes required for testosterone metabolism. In particular, prolactin is capable of inducing activity of hydroxy-steroid dehydrogenase enzymes (Evans, 1962) in mice and rats and has been shown *in vitro* to increase testosterone secretion from guinea-pig and human adrenal tissue (Ingvarsson, 1969). As LH, FSH, oestrogen and progesterone have not been previously shown to have any correlation with testosterone secretion, prolactin was measured to see if there might be any relation between these two hormones. The results show that no such relation exists on the basis of single daily samples.

However, there is evidence that prolactin facilitates progestogen production in sheep (Denamur *et al.*, 1973; Baird *et al.*, 1969) and recently McNatty *et al.* (1974) have demonstrated a definite correlation between levels of prolactin and progesterone within human ovarian follicular fluid, although no correlation was observed between the blood levels. It may, therefore, be erroneous to dismiss a relationship between prolactin and testosterone on the basis of blood levels alone.

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# PULSATILE SECRETION OF LH, FSH, PROLACTIN, OESTRADIOL AND PROGESTERONE DURING THE HUMAN MENSTRUAL CYCLE

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SUMMARY

The pulsatile secretion of gonadotrophins and ovarian steroids was studied in normal women at different stages of the menstrual cycle. The concentration of LH, FSH, Prolactin (PRL), oestradiol and progesterone were measured in samples of plasma collected every 15 min for 6 h and the frequency and amplitude of each episodic pulse of hormone estimated. Although significant fluctuations occurred in the concentration of each hormone, LH showed the most easily identifiable pulses the frequency of which increased significantly from the early follicular to the late follicular phase of the cycle ($3{\cdot}4 \pm 0{\cdot}3$ v. $4{\cdot}4 \pm 0{\cdot}2$ pulses 6 h $P < 0{\cdot}01$). During the luteal phase the basal concentration of LH ($5{\cdot}6 \pm 0{\cdot}9$ U/l), pulse amplitude ($7{\cdot}4 \pm 1{\cdot}7$ U/l) and frequency ($1{\cdot}6 \pm 0{\cdot}2$/6 h) were much lower than at any stage of the follicular phase ($P < 0{\cdot}001$). The concentration of FSH and PRL showed a similar but less marked change to that of LH throughout the menstrual cycle with a significant decline in both basal concentration and pulse frequency in the luteal phase of the cycle. Although only 47% of all LH pulses were associated with a pulse of FSH, 70% of FSH and prolactin pulses occurred within 15 min of an LH pulse.

The basal concentration of oestradiol increased significantly from the early follicular to the late follicular phase of the cycle ($P < 0{\cdot}001$). There was evidence of episodic secretion of oestradiol with the frequency of pulses declining from a maximum of $4{\cdot}3 \pm 0{\cdot}6$/6 h in the mid-follicular phase to $1{\cdot}8 \pm 0{\cdot}5$/6 h in the luteal phase ($P < 0{\cdot}02$). In the follicular phase 74–80% of LH pulses were followed within 100 min by a significant rise in the concentration of oestradiol. It is suggested that:

**1** the secretion of LH, FSH, PRL, oestradiol and progesterone is episodic in nature;

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**2** a significant number of secretory episodes of FSH, LH and PRL are coincidental;

**3** the increased secretion of oestradiol from the pre-ovulatory follicle is associated with a rise in the frequency of episodic pulses of LH.

Although it has been known for some years that the secretion of many hormones including pituitary gonadotrophins is pulsatile in nature, it is only recently that the biological significance of this pattern of secretion has become apparent (Knobil, 1980). In the male each episodic pulse of LH is followed by a rise in the concentration of testosterone suggesting that the amount of testosterone secreted by the testis is determined by the frequency of LH pulses as well as their magnitude (Lincoln, 1974). The increased activity of the testis at puberty (Boyar *et al.*, 1972; Foster *et al.*, 1978) and at the beginning of the breeding season in sheep (Katongole *et al.*, 1974; Lincoln, 1976) is associated with an increase in the frequency of LH pulses.

In the female the change in frequency and magnitude of LH pulses at different phases of the ovarian cycle is thought to be due to the modulating effects of oestradiol and progesterone (Santen & Bardin, 1973; Yen *et al.*, 1972). In the sheep each pulse of LH is followed by an increased secretion of ovarian oestradiol and the rise in secretion of oestrogen from the preovulatory follicle is due to an increase in the frequency of LH pulses associated with a fall in the concentration of progesterone (Baird, 1978). In women the concentration of oestradiol in peripheral blood fluctuates from hour to hour (Korenman & Sherman, 1973; Lenton *et al.*, 1978). The present study was undertaken to determine whether these fluctuations in oestradiol concentration have any relationship to the pulsatile release of LH, FSH or prolactin.

## SUBJECTS AND METHODS

### *Subjects*

Eleven women aged between 21 and 38 years were recruited from laboratory staff or patients requesting sterilization. None had been taking oral contraceptives during the 3 months prior to study nor were on any medication. They had a history of regular menstrual cycles (range 27–30 days). Informed consent was obtained from each subject.

### *Design of study*

Blood samples were collected every 15 min for a 6-h period in the early follicular (EF: days 1–4), mid-follicular (MF: days 5–9) and late follicular (LF: days 10–14). In the subjects studied during the luteal phase, the day of ovulation was estimated as the day before the urinary excretion of pregnanediol exceeded 1 mg/24 h. The studies were grouped into early luteal (EL: ovulation 1–4), mid-luteal (ML: ovulation 4–10) and late luteal (LL: ovulation 10+). Because there appeared to be no difference in the hormone levels of subjects studied between the EL and ML, these groups were combined for the purpose of analysis. It was not possible to study each subject at each phase of the same cycle. Thus nineteen experiments were performed in seven subjects during the follicular phase and seven experiments in six subjects during the luteal phase.

On the morning of the experiment (about 9.00 h) following a light breakfast, an intravenous cannula was inserted into a vein in the antecubital fossa and the cannula kept

patent by flushing with a solution of 5 iu Heparin/ml of 0·9% saline. The subjects were kept supine throughout except for visits to the toilet. At approximately 12.30 h a light lunch was eaten. Beginning 60 min after insertion of the cannula, blood samples (5 ml) were collected every 15 min for 6 h into heparinized containers. The blood samples were centrifuged immediately at 2000 r/min for 30 min and the aliquots of the plasma stored at −20°C for analysis of FSH, LH, prolactin, oestradiol and progesterone.

*Analysis of data*

For the purposes of this paper a pulse was defined as occurring when the hormone concentration of two consecutive samples was greater than that of the mean of the two previous samples (*basal* value) and the value of at least one of the *peak* samples exceeded the mean basal value by more than twice the co-efficient of variation of the assay (Baird *et al.*, 1977). In the case of the first three samples of an experiment, the basal value was taken as the first sample.

Using the above definition the total number of pulses of LH, FSH, prolactin, oestradiol and progesterone were calculated. The amplitude of each pulse was measured by subtracting the basal from the peak value. Differences in the mean basal levels, pulse amplitude and frequency between the different phases of the menstrual cycle were then tested using Mann-Whitney U-test (Siegel, 1956). To determine the relationship between the pulses of each gonadotrophin the number of coinciding pulses was calculated by counting the number of pulses starting within 15 min of each other. The start of a pulse was defined as the first of the two peak values. The relationship of pulses of gonadotrophins to those of oestradiol and progesterone was analysed by determining the number of 'responsive' and 'unresponsive' pulses. As it seemed likely that there would be a latent period between the response of the gonad to gonadotrophins, a gonadotrophin pulse was regarded as 'unresponsive' only if no pulse of steroid had occurred within 100 min of the start of the gonadotrophin pulse.

The significance of any differences in the total number of pulses and the number of coinciding and responsive pulses at each phase of the cycle was tested statistically by means of $\chi^2$. In addition the concentrations of steroids and gonadotrophins were grouped around the start of each LH pulse at different phases of the cycle. As the absolute values of steroids varied widely between individuals, the values were expressed as a percentage of the mean basal value. Those pulses occurring within 90 min of the end of the sampling period were omitted from this analysis because no samples were available during the period when a rise in steroid concentration might have been expected. The significance of the rise in the concentration of hormones was determined by comparison with the mean basal value using Wilcoxon's matched pair rank test.

In some subjects, there was a significant trend in the concentration of hormones during the 6 h of experiment. In order to determine the pulsatile nature of hormone secretion and to avoid bias of this trend on the mean values, the data was 'detrended' as follows. The regression line was calculated by the method of least squares and the distance of each point from the regression line was added or substracted to the overall mean value of that experiment. Thus the 'detrended' value equals mean value of the whole experiment plus or minus the difference between the original (true) value and that obtained by extrapolation from the regression line at that time.

*Hormone assays*

Progesterone was measured in duplicates by radioimmunoassay (RIA), the details of

which have been published elsewhere (Scaramuzzi *et al.*, 1975). The intra-assay precision was 8·3% and inter-assay precision 12·2%. After extraction of the plasma with ethyl ether, oestradiol was measured by RIA similar to that previously published (Van Look *et al.*, 1977). The antiserum used was raised in goats to oestradiol 17$\beta$ 6-oxime BSA and showed 2% cross reaction to oestrone. After extraction with ether the phenolic substances were partitioned between carbon tetrachloride and 0·05 N sodium hydroxide to remove fatty substances which interfered with the binding of ligand to antibody. The intra- and inter-assay coefficients of variation were 8 and 12% respectively. All samples from the same experiment were run in the same assay. LH, FSH and prolactin were assayed using double antibody RIA techniques described earlier (McNeilly & Hagen, 1974; Vaughan Williams *et al.*, 1980). Results are expressed in terms of U/l of the appropriate standards obtained from the National Institute of Biological Standards and Control, Holly Hill, Hampstead, London (LH:68/40, 77 U/ampoule, FSH:69/104, 10 U/ampoule, Prolactin:75/504 10 mU/ampoule). The intra-assay coefficients of variation in all three assays were 10%, interassay coefficient 12%. All samples of the same experiment were assayed in duplicate in the same run.

## RESULTS

During the follicular phase the concentration of FSH, LH and oestradiol showed a pulsatile pattern in all nineteen experiments (e.g. Fig. 1). There were pulses of prolactin

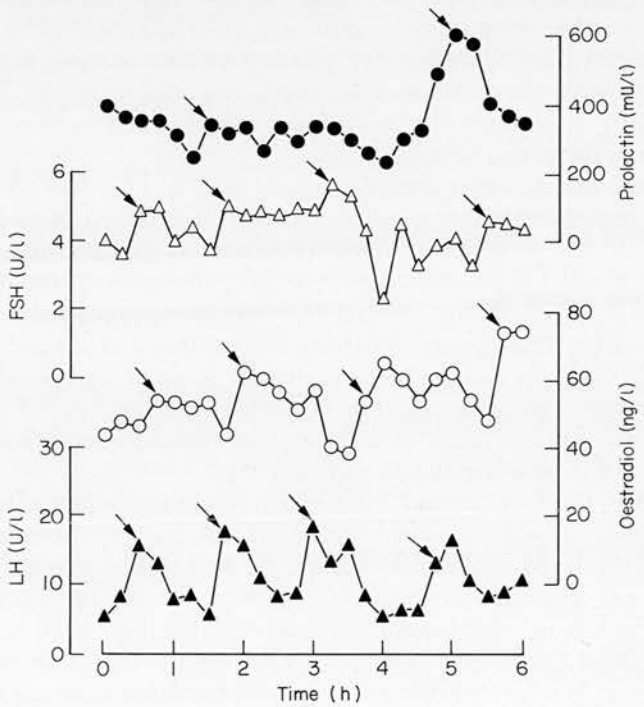



Fig. 1. The concentration of prolactin, FSH, LH and oestradiol in plasma of a 21-year-old woman on day 4 of cycle. Samples of blood were collected every 15 min from 09.30 until 15.30 h. The start of an episodic pulse of each hormone is indicated by an arrow (see text for definition). ▲ LH; ○ oestradiol; △ FSH; ● prolactin.

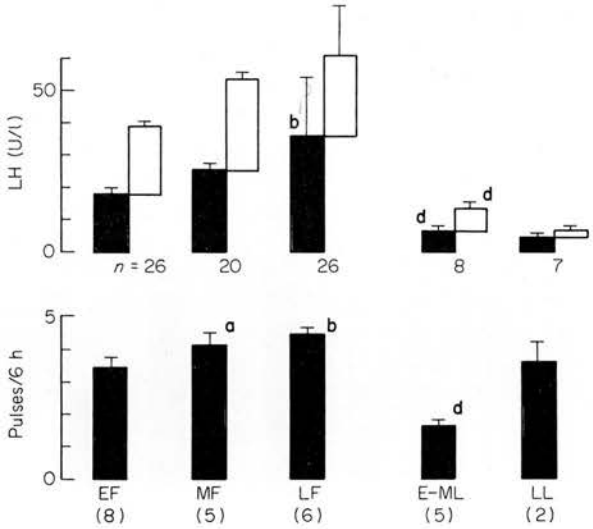



Fig. 2. The mean basal concentration (±SEM) pulse amplitude and frequency of LH at different stages of the menstrual cycle. EF = early follicular; MF = mid follicular; LF = late follicular; E-ML = early-mid luteal; LL = late luteal. $n$ = number of observations. ( ) = number of experiments. Significance of difference from EF: **a** $P<0{\cdot}05$; **b** $P<0{\cdot}01$; **d** $P<0{\cdot}001$. ■ Base; □ amplitude.

identified in fifteen of the nineteen studies during the follicular and in four of the seven studies during the luteal phase.

*LH (Fig. 2)*

During the follicular phase, the basal level and frequency of LH pulses increased significantly ($P<0{\cdot}01$) while the amplitude of each pulse remained unchanged. In the early and mid-luteal phase of the cycle there was a marked reduction in the frequency and amplitude of LH pulses as well as in the basal value as compared to the follicular phase ($P<0{\cdot}001$). In the two experiments performed during the late luteal phase the frequency of LH pulses returned to that found during the early follicular phase although the mean basal value and pulse amplitude remained low (Fig. 2).

*Oestradiol (Fig. 3)*

During the follicular phase the pattern of changes in oestradiol concentration was similar to that of LH. The basal concentration and pulse amplitude rose markedly from the early to the late follicular phase of the cycle ($P<0{\cdot}001$). Although the mean number of pulses of oestradiol during EF was similar to that of LH (3·3 and 3·4 pulses/6 h respectively) there was no further statistically significant increase in the number of oestradiol pulses as ovulation approached.

During the EML phase the mean basal concentration and pulse amplitude of oestradiol were significantly higher than during the EF phase ($P<0{\cdot}001$ and $<0{\cdot}005$ respectively). However, the frequency of pulses of oestradiol decreased in the EML compared to all follicular phases of the cycle ($P<0{\cdot}05$, $<0{\cdot}002$ and $<0{\cdot}01$ respectively).

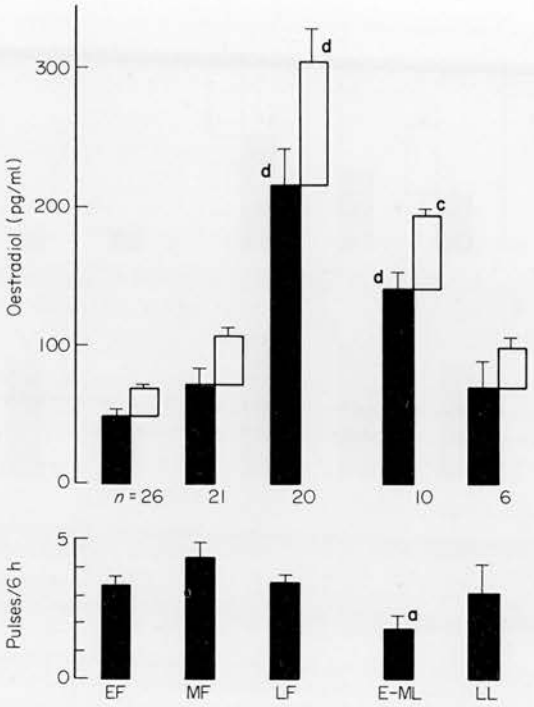



Fig. 3. The mean basal concentration (±SEM), pulse amplitude and frequency of oestradiol at different stages of the menstrual cycle (see legend of Fig. 2 for key). Significance of difference from EF: **a** $P<0{\cdot}05$; **c** $P<0{\cdot}005$; **d** $P<0{\cdot}001$. ■ Base; □ amplitude.

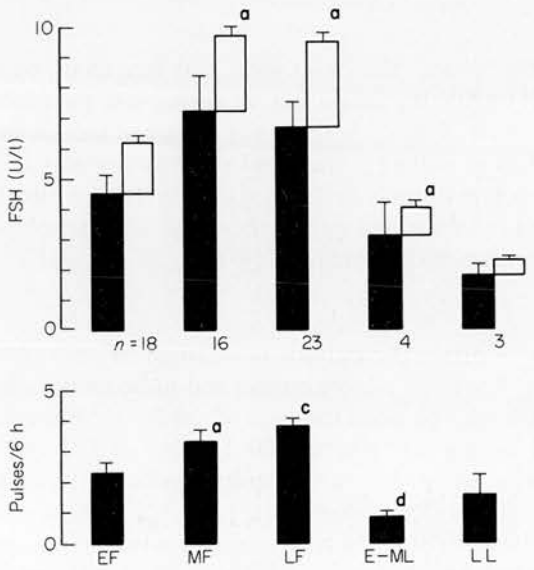



Fig. 4. The mean basal concentration (±SEM), pulse amplitude and frequency of FSH at different stages of the menstrual cycle (see legend of Fig. 2 for key). Significance of difference from EF: **a** $P<0{\cdot}05$; **c** $P<0{\cdot}005$; **d** $P<0{\cdot}001$. ■ Base; □ amplitude.

*Progesterone*

The basal concentration of progesterone rose from $<0{\cdot}1$ ng/ml in the follicular phase to $6{\cdot}0 \pm 0{\cdot}9$ ng/ml in EML ($P<0{\cdot}001$). Although there were $3{\cdot}1 \pm 0{\cdot}3$ pulses/6 h of progesterone during the EML, the amplitude of each pulse was relatively small ($2{\cdot}0 \pm 0{\cdot}4$ ng/ml).

*FSH (Fig. 4)*

The pattern of changes throughout the cycle of FSH concentration was very similar to that of LH. The number of pulses of FSH increased during the follicular phase from $2{\cdot}3 \pm 0{\cdot}3$/6 h in EF to a maximum of $3{\cdot}8 \pm 0{\cdot}3$ in LF ($P<0{\cdot}002$). Following ovulation the basal concentration fell to its lowest level in the cycle with an associated drop in both frequency ($0{\cdot}8 \pm 0{\cdot}2$ pulses/6 h $P<0{\cdot}001$) and amplitude ($0{\cdot}9 \pm 0{\cdot}2$ U/l $P<0{\cdot}025$).

In contrast to LH, the rise in the basal level of FSH from EF to LF phase of the cycle did not reach statistical significance ($P>0{\cdot}05$) although the amplitude of FSH pulses increased ($P<0{\cdot}05$).

*Prolactin (Fig. 5)*

The basal concentration of prolactin increased from the EF ($360 \pm 30$ mU/L) to reach a maximum of $520 \pm 40$ mU/L in LF ($P<0{\cdot}05$). The basal level and the frequency of pulses of PRL decreased in EML compared to the LF ($P<0{\cdot}01$ and $<0{\cdot}001$ respectively).

*Relationship between LH and steroid pulses (Fig. 6)*

The most obvious association with fluctuations in concentration of oestradiol were those of LH (Figs 1, 6 and 7). In the early and mid-follicular phase 74 to 80% of all LH

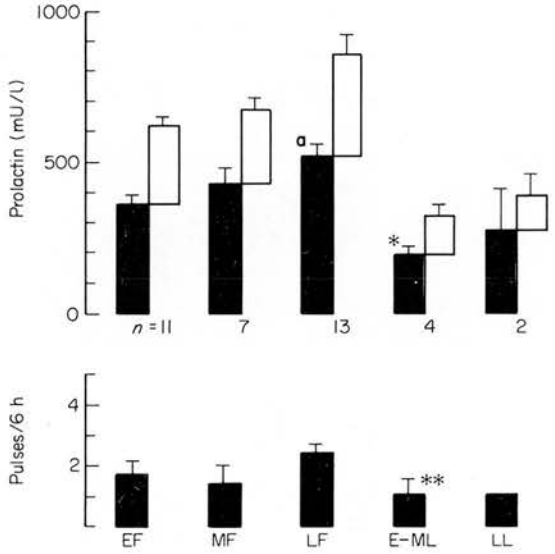



Fig. 5. The mean basal concentration ($\pm$SEM), pulse amplitude and frequency of prolactin at different stages of the menstrual cycle (see legend of Fig. 2 for key). Significance of difference from EF: **a** $P<0{\cdot}05$. Significance of difference from LF: * $P<0{\cdot}01$; ** $P<0{\cdot}001$. ■ Base; □ amplitude.

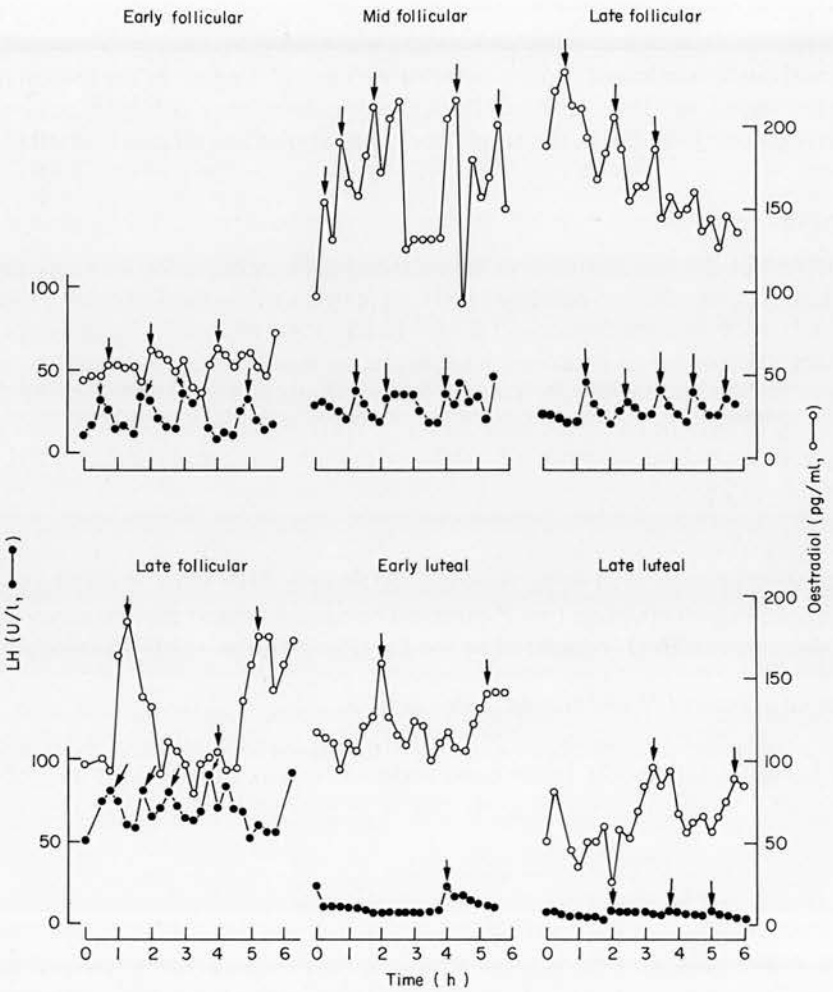



Fig. 6. Concentration of LH and oestradiol in six experiments at different stages of the menstrual cycle. Each pulse is indicated by an arrow (see text for definition).

pulses were followed within 100 min by a significant oestradiol pulse (Table 1). Although in LF and EML fewer LH pulses (63–68%) were followed by an oestradiol pulse, there was no significant change in the number of 'responsive' LH pulses in the different phases of the menstrual cycle (Table 1).

The mean time between the start of an LH pulse and an oestradiol pulse was similar at different stages of the cycle varying between 36 and 50 min (Table 1, Figure 7).

In the EML the rise in concentration of progesterone following an LH pulse occurred more rapidly than that of oestradiol. The first significant rise in progesterone concentration occurred coincidental to the start of the LH pulse with the maximum increase at 30 min. In contrast, the first significant increase in oestradiol concentration did not occur until 45 min after the LH pulse (Fig. 7).

Table 1. Number of responsive pulses of LH and FSH and the mean time between the start of a gonadotrophin and subsequent pulse of steroid

| Pulses compared | Stage of cycle | Number of subjects | Number of gonadotrophin pulses/6 h (mean ± SE) | Responsive pulses Total | % | Unresponsive* pulses/study | Time between pulses of gonadotrophin and steroid (min: mean ± SEM)† |
|---|---|---|---|---|---|---|---|
| LH:$E_2$ | EF | 8 | 3·4 ± 0·3 | 17/23 | 74 | 6/5 | 36·2 ± 3·9 |
| | MF | 5 | 4·1 ± 0·3 | 12/15 | 80 | 3/3 | 50·0 ± 6·2 |
| | LF | 6 | 4·4 ± 0·2 | 13/19 | 68 | 6/4 | 46·2 ± 2·7 |
| | EML | 5 | 1·6 ± 0·2 | 5/8 | 63 | 3/3 | 45·0 ± 12·6‡ |
| LH:Progesterone | EML | 5 | 1·6 ± 0·2 | 6/8 | 75 | 2/2 | 22·5 ± 6·4‡ |
| FSH:$E_2$ | EF | 8 | 2·3 ± 0·3 | 11/16 | 68 | 5/5 | 34·1 ± 6·7 |
| | MF | 5 | 3·3 ± 0·4 | 11/14 | 79 | 3/3 | 46·4 ± 5·9 |
| | LF | 6 | 3·8 ± 0·3 | 9/17 | 52 | 8/6 | 45·0 ± 7·5 |

* No significant difference at each stage of the cycle ($\chi^2$ test).
† No significant difference between times from LH or FSH pulses to oestradiol pulses (Mann–Whitney U-test).
‡ Progesterone pulse occurred significantly closer ($P < 0{\cdot}025$; Mann–Whitney U-test) to the LH pulse than the oestradiol pulse.

*Relationship between LH, FSH and prolactin pulses*

Although only 47% of all LH pulses were associated with a pulse of FSH, a high number (70%) of all FSH and prolactin pulses occurred within 15 min of an LH pulse. The fact, therefore, that 52–79% of FSH pulses in the follicular phase of the cycle were followed by a rise in oestradiol concentration is probably due to the coincidental pulse of LH.

## DISCUSSION

After the development of radioimmunoassays for the measurement of gonadotrophins, it was soon demonstrated in the castrate of a number of species, including man, that the concentration of LH in peripheral plasma showed marked fluctuations (rat: Gay & Midgley, 1969; monkey: Dierschke *et al.*, 1970; women: Yen *et al.*, 1972). Episodic secretion of LH also occurs in intact women (Midgley & Jaffe, 1971) with changes in amplitude and frequency occurring throughout the menstrual cycle (Santen & Bardin, 1973). The present study confirms the observations that in the luteal phase under the influence of progesterone the frequency of LH pulses is markedly reduced as compared with the follicular phase of the cycle (Fig. 2). In addition to the reduction in frequency we observed a reduction in amplitude expressed in absolute terms although the amplitude of each pulse relative to the basal level is actually increased (Yen *et al.*, 1972; Santen & Bardin, 1973).

In the present study we were able to demonstrate a significant increase in pulse frequency of LH from the early to the late follicular phase of the cycle (Fig. 2). A similar increase in pulse frequency occurs in the follicular phase of the sheep oestrous cycle (Foster *et al.*, 1975) and is the crucial factor in stimulating secretion of oestradiol from the pre-ovulatory follicle (Baird, 1978). The accuracy with which pulses of hormones can be

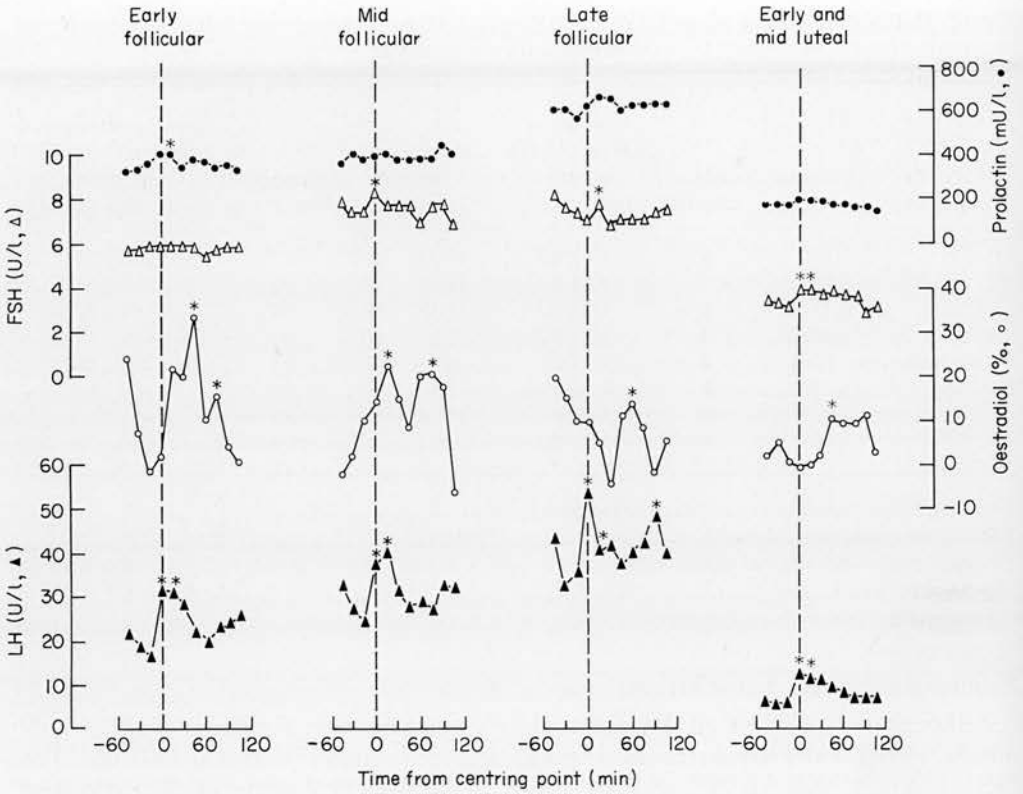



Fig. 7. The mean concentration of FSH, LH, prolactin and oestradiol at different stages of the menstrual cycle. The data have been centred relative to the start of each LH pulse. For each hormone the points which are significantly higher than the basal values are indicated by an asterisk ($P < 0{\cdot}05$). Note that the oestradiol values are expressed as percentage change from mean basal concentration.

identified depends on their half-life in plasma as well as on the precision of the assay and the frequency of sampling. With the sampling frequency used in this study (every 15 min), the maximum number of pulses of LH which could be identified in the 6-h period of sampling was approximately six. By sampling every 2–3 min, Gallo (1981) was able to demonstrate in the rat that the pre-ovulatory LH surge was composed of episodic secretion occurring every 16–23 min. Thus the LH pulse frequency in the late follicular phase of the cycle may have been underestimated in this (e.g. Fig. 6) and other studies (e.g. Santen & Bardin, 1973).

The fluctuations in the concentration of FSH were much less obvious than that of LH. Although this may be partially due to the longer half-life of FSH which makes the identification of pulses more difficult, it is likely that it represents a real difference in the regulation of FSH secretion. As LHRH is thought to be the sole hypothalamic releasing hormone for both FSH and LH, it is likely that ovarian factors (e.g. oestradiol or "inhibin") have a differential effect on the responsiveness of the anterior pituitary to LHRH (Fink, 1979; de Jong, 1979). Thus oestradiol enhances the amount of LH but reduces the amount of FSH released by pituitary cells *in vitro* in response to LHRH (rat: Schally *et al.*, 1973; sheep: Huang & Miller, 1980). This differential sensitivity of the

pituitary probably accounts for the divergence of basal levels of LH and FSH in the late follicular phase of the cycle.

A number of studies have previously reported that the concentration of oestradiol in peripheral plasma of women fluctuates markedly from hour to hour (e.g. Korenman & Sherman, 1973; Younglai *et al.*, 1975; Lenton *et al.*, 1978). The present study is the first to demonstrate a relationship between these fluctuations in steroid levels and pulsatile secretion of LH in women. Using a sampling frequency of every 20 min, Younglai *et al.* (1975) concluded that there was no evidence for the pulsatile secretion of either oestradiol or progesterone although inspection of their data from one subject on day 18 demonstrates an increased concentration of both steroids following an LH pulse. In both the ram and the ewe there is convincing evidence that the gonad responds to each pulse of LH with an increase in steroid secretion (Katongole *et al.*, 1974; Baird *et al.*, 1976). Indeed the quantity of oestradiol secreted by the ovary is determined by the frequency of LH pulses (Baird & McNeilly, 1981). Further support for the importance of pulsatile LH secretion in the development of the follicle is provided by the fact that hypogonadal states with low levels of oestradiol, e.g. hyperprolactinaemic and hypothalamic amenorrhoea, are associated with a reduced frequency of pulsatile LH secretion (Santen & Bardin, 1973; Bohnet *et al.*, 1976). Follicular development and ovulation can be induced in hypogonadotrophic monkeys (Knobil *et al.*, 1980) and women (Marshall & Kelch, 1979; Crowley & McArthur, 1980; Leyendecker *et al.*, 1980) by intermittent but not continuous administration of LHRH.

The fact that the frequency of oestradiol pulses did not increase during the follicular phase may be due to the technical difficulties of identifying a pulse of oestradiol. The increments of oestradiol concentration were modest and often close to the precision of the assay. However, three of the six subjects in the LF phase were studied at the start of the pre-ovulatory LH surge when marked changes in the pattern of steroids secreted by the ovary occur. It may be that the ovary becomes relatively refractory to the steroidogenic effect of LH at this time (Baird, 1978).

In the luteal phase the concentration of progesterone increased in response to a rise in concentration of LH (Table 1). The time interval between the rise in LH concentration and the first rise in steroid concentration was shorter for progesterone than oestradiol. In the second half of the cycle both oestradiol and progesterone are derived virtually exclusively from the corpus luteum (Baird, 1974) although the amounts secreted by each cell type within the corpus luteum remain unknown. The relatively slower response of oestradiol probably reflects the fact that this steroid is further down the biosynthetic pathway from cholesterol.

The majority of pulses of FSH were associated with a pulse of LH suggesting that a common mechanism is responsible for releasing both gonadotrophins (Fink, 1979). The concordance of pulses of prolactin with those of LH was unexpected although a recent study has demonstrated that pulsatile release of LH and PRL occurs following treatment with synthetic long acting analogue of LHRH (Casper & Yen, 1981). The fact that the secretion of prolactin is susceptible to a wide variety of factors such as stress (McNeilly, 1980) and feeding (Quigley *et al.*, 1981), probably accounts for the marked fluctuations in concentration in blood and the difficulty of identifying pulses. Nevertheless in the present study 70% of all PRL pulses occurred within 15 min of an LH pulse. Infusion of dopamine inhibits the secretion of LH as well as that of PRL (Judd *et al.*, 1978). It is possible, therefore, that the discharge of LHRH neurones is associated with a decrease in

concentration of dopamine in the hypothalamus as well as an increased synthesis of nor-adrenaline (Rance *et al.*, 1981).

The finding in the present study of significant changes in the mean concentration of prolactin throughout the menstrual cycle with a peak at mid-cycle (Fig. 5) is in agreement with some (Franchimont *et al.*, 1976; Veekemans *et al.*, 1977) but not all other studies (Hwang *et al.*, 1971; Jaffe *et al.*, 1973; McNeilly & Chad, 1974; Tyson & Friesen, 1973). The difficulty of demonstrating significant trends on daily samples due to the marked minute to minute variation in PRL concentration have already been discussed above. Although the number of subjects studied in the present study was relatively few, it was possible to demonstrate the rise at mid-cycle by analysing multiple samples from each individual. The periovulatory rise of prolactin secretion which occurs in many species (e.g. rat: Neill *et al.*, 1971; sheep: Reeves *et al.*, 1970) is probably due, in part, to the rise in the concentration of oestradiol which is known to stimulate pituitary lactotrophs (Thorner & MacLeod, 1981). In the rat at the time of the pro-oestrous LH surge the turnover of nor-adrenaline in the hypothalamus increases while there is a relative decrease in dopamine turnover (Löfström, 1977; Rance *et al.*, 1981). There is a positive correlation between the plasma concentration of oestradiol and the turnover of non-adrenaline in the pre-optic area and stria terminalis in the dioestrus rat (Löfström & Bäckström, 1978). The tubero-infundibular dopaminergic neurones projecting into the median eminence overlap extensively with LHRH terminals and it has been suggested that dopamine may normally be an inhibitory neuro-modulator of LHRH release (Fuxe *et al.*, 1973). However, the role of hypothalamic catecholamines in the control of pulsatile LH release in man remains to be determined.

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# A possible role for prolactin in control of steroid secretion by the human Graafian follicle

PROLACTIN constitutes part of the luteotrophic complex necessary for the maintenance and secretory activity of the corpus luteum in the rat[1,2], mouse[3], rabbit[4], hamster[5], ferret[6], pig[7] and sheep[8]. Recent evidence suggests, however, that prolactin may have little to do with luteal function in women[9–15]. Nevertheless, it is still possible that prolactin could play a 'permissive' role, as in other species, where alterations in peripheral blood levels, within limits, have little effect on luteal activity. In an attempt to gain more insight into the possible role of prolactin in controlling ovarian activity in women, we have studied the production of steroids by human granulosa cells growing in tissue culture, and the effects of the addition or neutralisation of human prolactin in the culture media. We report that the production of progesterone by human granulosa cells *in vitro* requires low physiological concentrations of prolactin whereas high concentrations are inhibitory.

Ovaries, endometrial biopsies and peripheral blood samples were obtained from patients aged 21–48 yr who were undergoing surgery for gynaecological disorders. The stage of the menstrual cycle was assessed from the date of the last menstrual period, the concentrations in plasma of luteinising hormone (LH), follicle stimulating hormone (FSH) and progesterone, endometrial histology, and the presence or absence of a corpus luteum. Only follicles from morphologically normal ovaries were used as the source of granulosa cells in this study.

Antral follicles were dissected out of the ovary within 2 h of ovariectomy and the fluid aspirated by syringe. The follicular fluid and peripheral plasma sample were stored at −20° C until assayed for prolactin and progesterone by specific radioimmunoassays (RIA). Prolactin concentration is expressed as ng ml$^{-1}$ Friesen prolactin of which 1 ng≡ 20 μU MRC Standard 71/222. The Friesen prolactin preparation was 90% pure and the human pituitary hormone contaminants were found by RIA to be negligible: growth hormone (HGH), HLH, HFSH, all <0.4%and the concentration of human placental lactogen (HPL) undetectable. The collapsed follicle was slit open and the granulosa cells scraped into culture medium. An aliquot of the cell suspension was counted and the viability determined[16]. The follicle wall was placed in fixative for subsequent histological examination to ensure that the basement membrane was still intact. The cells were layered on to 18 mm$^2$ coverslips and cultured for 10–14 d as previously described[17]. The culture medium was replaced every 24 h and stored at −20° C until assayed for progesterone. The variation (± s.e.m.) in the production of progesterone between replicate cultures for all treatments was found to be 6.77 ± 1.45.

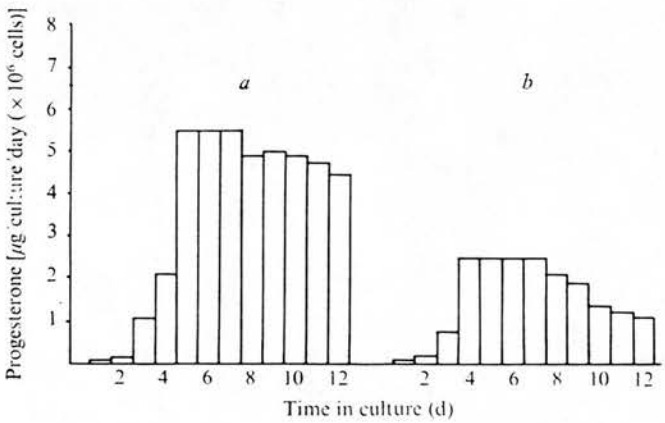



**Fig. 1** Progesterone production by luteinised granulosa cells in a culture medium where the endogenous prolactin was neutralised with anti-human prolactin serum. The cells were collected from a single late follicular phase preovulatory follicle. The concentration of endogenous prolactin in the culture medium before the addition of rabbit anti-human prolactin serum was 5.1 ng ml$^{-1}$. After precipitation of the prolactin–anti-prolactin complex the concentration was <0.2 ng ml$^{-1}$. The concentrations of progesterone in the control were determined with either normal rabbit serum, or rabbit anti-bovine serum albumin added to the culture medium at the same dilution (1/500) as the antiserum used in the anti-rabbit culture treatment. There were no significant differences in the production of progesterone between the control culture which were found to be within the precision achieved in replicate cultures. *a*, Control; *b*, anti-prolactin, $P < 0.01$.

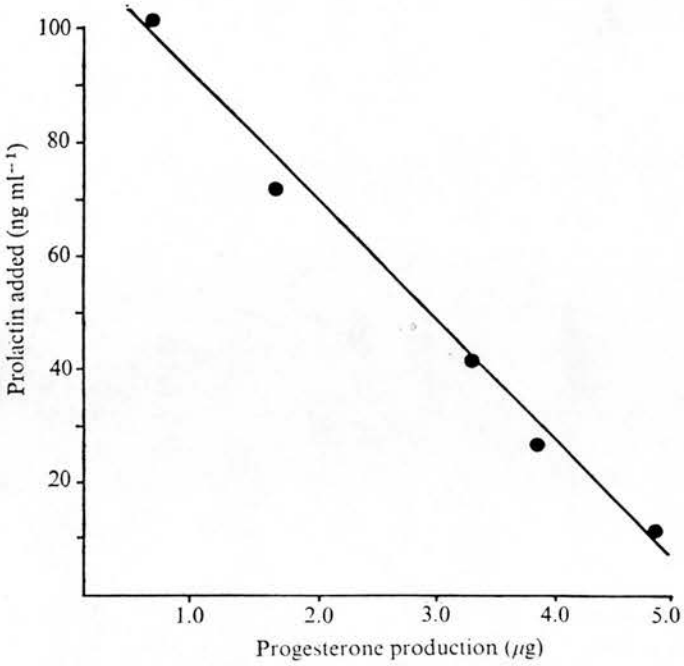



**Fig. 2** Relationship between the concentration of prolactin and the total progesterone production by human granulosa cells *in vitro*. Granulosa cells were collected from one late follicular phase preovulatory follicle. Each point is the mean result of duplicate cultures. Total progesterone production is that achieved by $10^6$ granulosa cells in 11 consecutive daily changes of culture medium.

Neutralisation of prolactin in the culture medium by the addition of rabbit anti-human prolactin serum, at a dilution which did not cross react with HPL, HGH, HLH or HFSH, caused a significant decrease in progesterone production as compared to the cultured control (Fig. 1). To confirm that free prolactin in the culture medium had been completely neutralised by the antiserum, the anti–prolactin-prolactin complex was precipitated out using a solid phase second antibody; the concentrations of LH and FSH in the medium were unaltered whereas prolactin was undetectable.

Addition of human prolactin to the culture medium had no effect on progesterone production if the final prolactin concentration did not rise above 20 ng ml$^{-1}$; the mean concentration of prolactin in the peripheral blood of women during the cycle is 15 ± 1 (± s.e.m.)[10]. When the concentrations in the culture medium were increased from 25 to 100 ng ml$^{-1}$, there was a progressive decrease in the daily progesterone production (Fig. 2). This inhibitory effect persisted, even when the LH and/or FSH concentrations were increased 50-fold, and it could be obtained with granulosa cells collected from follicles at any stage of the

menstrual cycle. In contrast the soluble non-protein fraction of the added prolactin solution did not inhibit the production of progesterone by human granulosa cells *in vitro* as compared with the controls.

Prolactin was assayed in 110 samples of follicular fluid collected at various stages of the cycle. The mean concentration (± s.e.m.) was $20 \pm 5$ ng ml$^{-1}$ for all sizes of follicle. The increase in the prolactin content of the follicle was correlated with the volume of follicular fluid (Fig. 3). The prolactin concentrations during the late follicular phase of the cycle were, however, significantly lower than at any other time of the cycle with the exception of the early luteal phase (Fig. 4).

These *in vitro* experiments suggest that high concentrations of prolactin within the follicular fluid may actually depress progesterone secretion by the granulosa cells. This might provide an explanation for the fact that galactorrhoea with high prolactin levels is commonly associated with amenorrhoea[18], and that if the prolactin levels are lowered by treatment with CB 154, ovulation and menstruation recur[19,20]. It is well recognised that post-partum lactation can inhibit ovulation in women and other animals[18,21], although the mechanism has never been explained.

Although there are now numerous descriptive accounts of the changing hormone levels in blood and urine throughout the menstrual cycle, we do not really understand what factors are responsible for the formation, maintenance and regression of the human corpus luteum. This is an area of great potential interest for the development of new forms of contraception. The *in vitro* results presented here cast doubt on the simplistic view that LH is the only gonadotrophin necessary for luteal maintenance[15], and suggest that prolactin is also involved. Furthermore, it seems probable that the gonadotrophic content of the follicular fluid may have important consequences for the secretory activity of the granulosa cells both before and after ovulation.

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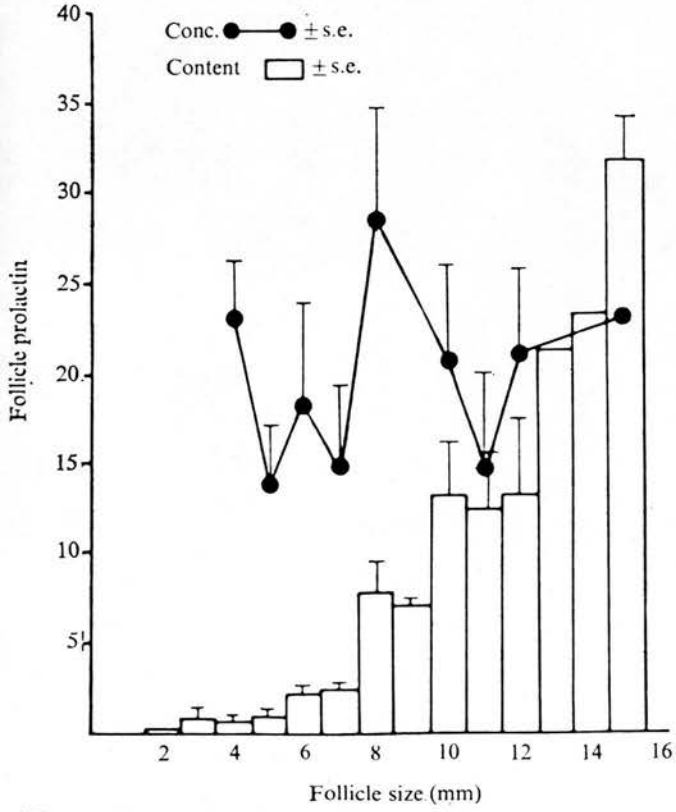



**Fig. 3** Changes in concentration and content of prolactin in follicular fluid with respect to follicle size. Vertical bars represent 1 s.e.m. Circles show prolactin concentration (ng ml$^{-1}$); bars show prolactin content (ng).

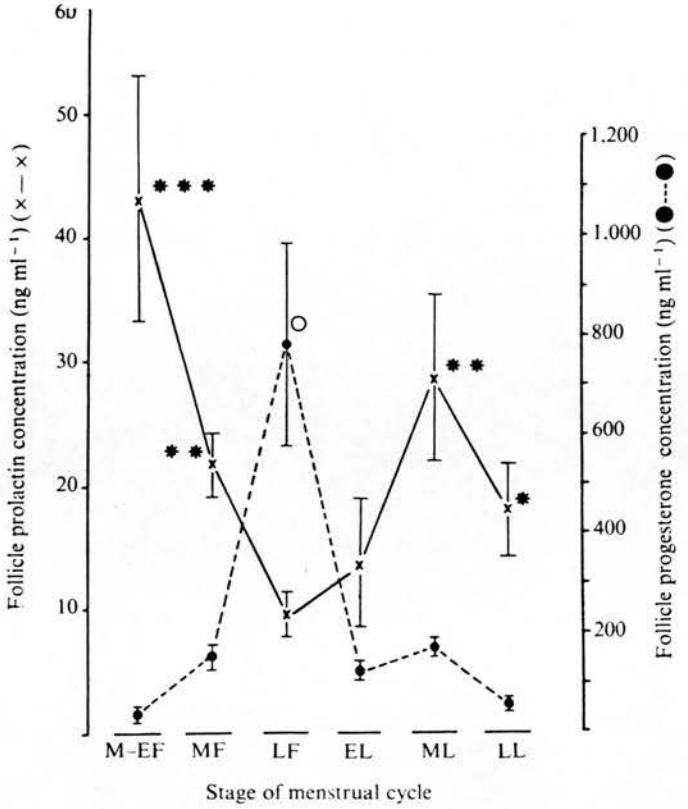



**Fig. 4** Changes in the follicular fluid concentrations of prolactin and progesterone related to the stage of the menstrual cycle. The vertical bars represent 1 s.e.m. (M-EF; MF; LL; represent menstruation-early follicular, mid follicular and late follicular phases, and EL, ML and LL represent early, mid and late luteal phases.) *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ° $P < 0.001$.

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# CHANGES IN THE CONCENTRATION OF PITUITARY AND STEROID HORMONES IN THE FOLLICULAR FLUID OF HUMAN GRAAFIAN FOLLICLES THROUGHOUT THE MENSTRUAL CYCLE

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SUMMARY

The concentrations of FSH, LH, prolactin, oestradiol and progesterone were measured in peripheral plasma and follicular fluid of women throughout the menstrual cycle. With the exception of prolactin, concentrations of pituitary and steroid hormones in follicular fluid correlated with those in peripheral plasma.

Follicle-stimulating hormone was present in a greater number of small follicles (< 8 mm) during or just after the peaks of FSH in peripheral plasma. During the mid-follicular phase the concentration of both FSH and oestradiol in fluid from large follicles (⩾ 8 mm) was high. During the late follicular phase the large follicles (⩾ 8 mm) contained high amounts of progesterone in addition to oestradiol, low physiological levels of prolactin, and concentrations of LH and FSH about 30 and 60 % respectively of those found in plasma. By contrast no large 'active' follicles (⩾ 8 mm) were found during the luteal phase although many contained both LH and FSH. Luteinizing hormone was present in a proportion of small follicles (< 8 mm) during the late follicular and early luteal but not at other stages of the menstrual cycle.

It is suggested that a precise sequence of hormonal changes occur within the microenvironment of the developing Graafian follicle; the order in which they occur may be of considerable importance for the growth of that follicle and secretory activity of the granulosa cells both before and after ovulation.

## INTRODUCTION

There is considerable evidence to suggest that pituitary gonadotrophins are responsible for the later stages of follicular growth, maturation of the oocyte and ovulation (see reviews of Baker, 1972; Biggers & Scheutz, 1972; Greep, 1973). Although it is possible to correlate changes in gonadotrophins in peripheral plasma

with the production of steroids from the ovary, the actions of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on the follicle remain obscure. It has been suggested that gonadotrophins within the follicular fluid itself may have important consequences for follicular steroidogenesis (Channing, 1970, 1972; Edwards, 1974; McNatty, Sawers & McNeilly, 1974). growth of the follicle, oocyte maturation and evolution (Edwards, 1974).

The changing concentration of peripheral plasma oestradiol during the follicular phase of the human menstrual cycle provides an index of follicle growth (Baird, 1971). Oestradiol in the peripheral plasma during this phase of the cycle is derived almost solely from the actively growing follicles (Baird & Fraser, 1974) which also contain high concentrations of oestradiol (Smith, 1960; Short & London, 1961; Giorgi, 1965; Edwards, Steptoe, Abraham, Walters, Purdy & Fotherby, 1972; Baird & Fraser, 1975; Sanyal, Berger, Thompson, Taymor & Horne, 1974). Furthermore, during the immediate preovulatory phase there is an increase in the concentration of progesterone in follicular fluid (Edwards *et al.* 1972; McNatty *et al.* 1974; Sanyal *et al.* 1974) which is also reflected in peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970).

In the present study the concentrations of LH, FSH, prolactin, oestradiol and progesterone were measured in samples of peripheral plasma and follicular fluid collected from women at varying stages of the menstrual cycle. It was hoped in this way to investigate the relationships between the concentrations of pituitary and steroid hormones in follicular fluid and to relate them to concentrations in plasma, follicle size, and the stage of the menstrual cycle.

## MATERIALS AND METHODS

### *Subjects*

Ninety-seven subjects (aged 21–48 years) who were at varying stages of the menstrual cycle were undergoing hysterectomy for various gynaecological conditions. The indications for surgery were stage 0 carcinoma of the cervix (17), menorrhagia due to fibroids (35), dysfunctional uterine bleeding (10), endometriosis (9), or chronic pelvic pains or dysmenorrhoea (26). Those with stage 0 carcinoma of the cervix had regular menstrual cycles (21–32 days) and were considered to be endocrinologically normal (Baird & Fraser, 1974). The previous menstrual cycles of the group with menorrhagia varied in length from 21–34 days and about 50 % of these subjects had ovulated in the cycle under study, as indicated by the presence of a secretory endometrium and of at least one corpus luteum at the time of the operation. The remaining subjects were in the proliferative phase as assessed by their endometrial histology and date of the last menstrual period.

### *Dating the menstrual cycle*

An endometrial biopsy and a peripheral blood sample (collected before oophorectomy) were obtained during the operation. The endometrium was examined histologically and dated according to the criteria of Noyes, Hertig & Rock (1950). The stage of the menstrual cycle was assessed in all subjects from the date of the last menstrual period, the concentrations in plasma of LH, FSH, oestradiol and pro-

gesterone, endometrial histology and the presence or absence of a corpus luteum. The menstrual cycle was divided into six phases: early follicular, still menstruating (EF); mid-follicular (MF); late follicular (LF); early luteal (EL); mid-luteal (ML) and late luteal (LL).

### *Ovarian morphology*

Ovaries were examined *in situ* to assess gross morphology and to record the presence or absence of a corpus luteum. In some cases excised ovarian specimens were examined by light microscopy after removal of the Graafian follicles to ascertain that no major ovarian pathology existed.

### *Collection of follicular fluid*

The follicular fluid analysed in this study was obtained from ovaries of the 97 subjects described above. Two different methods for the collection of the fluid were used.

In 23 subjects antral fluid was aspirated from 36 Graafian follicles through a 23G needle into a syringe during surgery. It was technically not possible to measure these follicles accurately, consequently they were classified as large ($\geqslant 8$ mm) or small ($< 8$ mm).

In 74 subjects ovarian specimens (whole ovaries or wedge biopsies) were collected into chilled Medium 199 containing Hanks' salts and HEPES buffer (20 mM) (Flow Laboratories) and all follicles which were $\geqslant 4$ mm diameter (153) were dissected out within 2 h of surgery. The diameter of each isolated follicle was measured and the antral fluid aspirated through a 27G needle into a 500 $\mu$l Hamilton syringe.

Fluid collected by either method was frozen at $-20$ °C until assayed. No attempt was made to recover the oocyte.

Follicles without a lining of granulosa cells, irrespective of size, were defined as 'cystic'. Subsequent data reported for the cystic follicles are considered separately from those obtained for normal follicles.

### *Radioimmunoassay of pituitary and steroid hormones*

#### *Luteinizing hormone and follicle-stimulating hormones*

The radioimmunoassays for LH and FSH in plasma and follicular fluid were based upon those previously described (Hunter, Edmond, Watson & McLean, 1974) with modifications for the antral fluid in order to accommodate the small volumes. The original concentrations of standards, tracer and antiserum were retained but the incubation volume was reduced to 150 $\mu$l of which 50 $\mu$l were neat (or diluted) follicular fluid or standard. The counting time was increased proportionately so that in this respect assay precision was undiminished. The following standards were used: LH, MRC 68/40 assumed 77 units/ampoule; FSH, MRC 68/39 assumed 32·8 units/ampoule (MRC National Institute for Biological Standards and Control). The concentrations of LH and FSH are expressed as mu./ml, where 1 mu. LH $\equiv$ 11·6 ng LER 907 and 1 mu. FSH $\equiv$ 44·6 ng LER 907. The assays would normally measure LH and FSH respectively over the ranges 0·8–12·8 and 0·4–6·4 mu./ml in undiluted follicular fluid. With large follicles, $\geqslant 8$ mm diameter, assays were in general carried

out in duplicate, whilst those for follicles < 8 mm diameter were single determinations. A number of LH assays were also carried out on larger volumes of pooled fluid from small follicles.

*Prolactin*

Prolactin in peripheral plasma and follicular fluid was measured using a specific double antibody radioimmunoassay (RIA) previously described (McNeilly, 1973; McNeilly & Hagen, 1974). Purified human prolactin for standards and labelling with $^{125}I$ was generously supplied by Dr H. G. Friesen, University of Manitoba, Winnipeg, Canada. The concentration of prolactin is expressed as ng Friesen prolactin/ml of which 1 ng ≡ 20 $\mu$u. MRC 71/222. The minimum detectable level of prolactin was 1·5 ng/ml for both follicular fluid and plasma. Assays were carried out in duplicate in both specimens of plasma and fluid.

*Progesterone*

Progesterone was measured in peripheral plasma using a radioimmunoassay similar to that described by Thorneycroft & Stone (1972), and in follicular fluid by the method of Neal, Baker, McNatty & Scaramuzzi (1975). Follicular fluid (2–10 $\mu$l) was diluted 100- to 1000-fold in phosphate-buffered saline (0·1 mol/l, pH 7·0) and 0·1 ml samples were assayed directly without extraction. The precision and accuracy of the assay was similar to that described by Neal *et al.* (1974). The progesterone antiserum (RI-4) was raised in a rabbit against progesterone-11$\alpha$-hemisuccinate conjugated to bovine serum albumin and the specificity was similar to that previously reported (Dighe & Hunter, 1974). The assays were conducted in duplicate and the minimum detectable level of progesterone was 300 pg/ml in plasma and 10 ng/ml in follicular fluid.

*Oestradiol*

Antiserum for the assay of oestradiol was raised in a rabbit to a conjugate of oestradiol 6-carboxymethyl–oxime and bovine serum albumin. Cross-reactions of other steroids using the routine assay conditions were: oestrone, 3%; oestriol, 0·4%; 6-oxo-oestradiol, 100%; testosterone, 0·003%; progesterone, 0·0002%.

For estimations of oestradiol in plasma an ether extract was evaporated to dryness, equal volumes of 0·05 M-NaOH and carbon tetrachloride were added and shaken together with the residue. Samples of the aqueous phase were then neutralized in a slightly acid diluent for incubation in the RIA system as described by A. Bolton and F. Rutherford (personal communication). Oestradiol in follicular fluid was assayed without prior extraction. The assays were carried out in duplicate and the minimum detectable level of oestradiol was 8 ng/ml in follicular fluid and 20 pg/ml in plasma.

## RESULTS

Concentrations of hormones in plasma and follicular fluid in subjects with stage 0 carcinoma of the cervix were compared with those of patients with menorrhagia and were not found to be significantly different. The results from all patients have therefore been pooled.

Concentrations of FSH, LH, oestradiol and progesterone in the antral fluid of

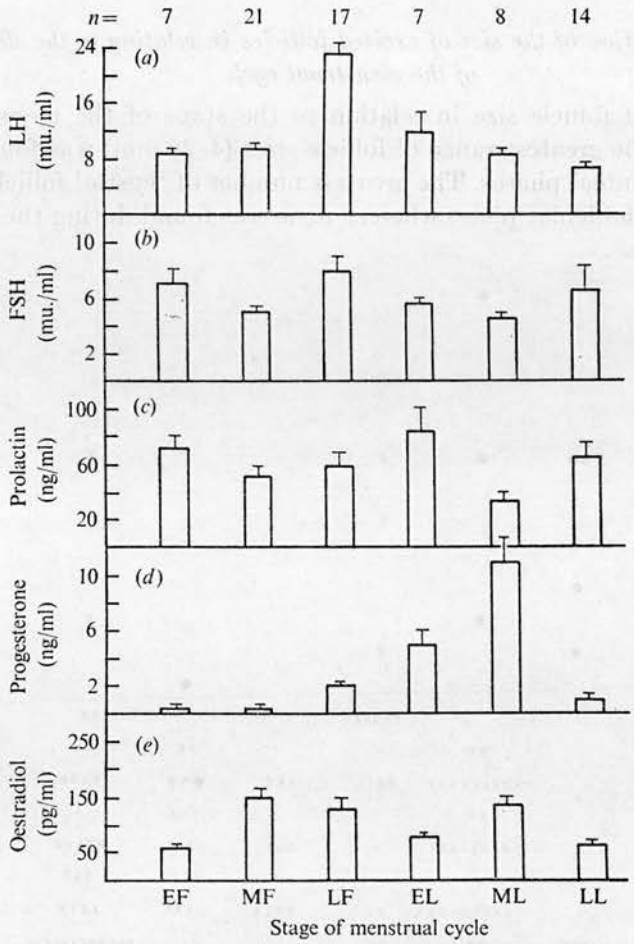



Fig. 1. Mean concentrations of (*a*) LH, (*b*), FSH (*c*) prolactin, (*d*) progesterone and (*e*) oestradiol in peripheral plasma during the menstrual cycle. Vertical lines represent + S.E.M. EF, MF and LF refer to early (still menstruating), mid- and late follicular phases respectively, while EL, ML and LL refer to early, mid- and late luteal phases respectively. Numbers of observations are given at the top of the figure.

follicles aspirated *in situ* were not significantly different from those aspirated *in vitro* ($P > 0{\cdot}2$). The results obtained for each hormone from the two methods of collection have therefore been pooled. Concentrations of prolactin in the antral fluid of follicles aspirated *in situ* are not reported. These samples were stored at $-20$ °C for at least 12 months before being assayed. The immunological and biological activity of prolactin in physiological fluids declines with prolonged storage (H. G. Friesen, personal communication).

### *Concentration of luteinizing hormone, follicle-stimulating hormone, prolactin, progesterone and oestradiol in peripheral plasma*

Mean values (± S.E.M.) obtained for the concentrations of LH, FSH, prolactin, progesterone and oestradiol in peripheral plasma with respect to the stage of the menstrual cycle are shown in Fig. 1.

*Distribution of the size of excised follicles in relation to the stage of the menstrual cycle*

A scatter plot of follicle size in relation to the stage of the menstrual cycle is shown in Fig. 2. The greatest range of follicle sizes (4–20 mm) was found in the late follicular and late luteal phases. The greatest number of 'cystic' follicles (45 %) was found in the early follicular phase whereas none was found during the late follicular or early luteal phase.

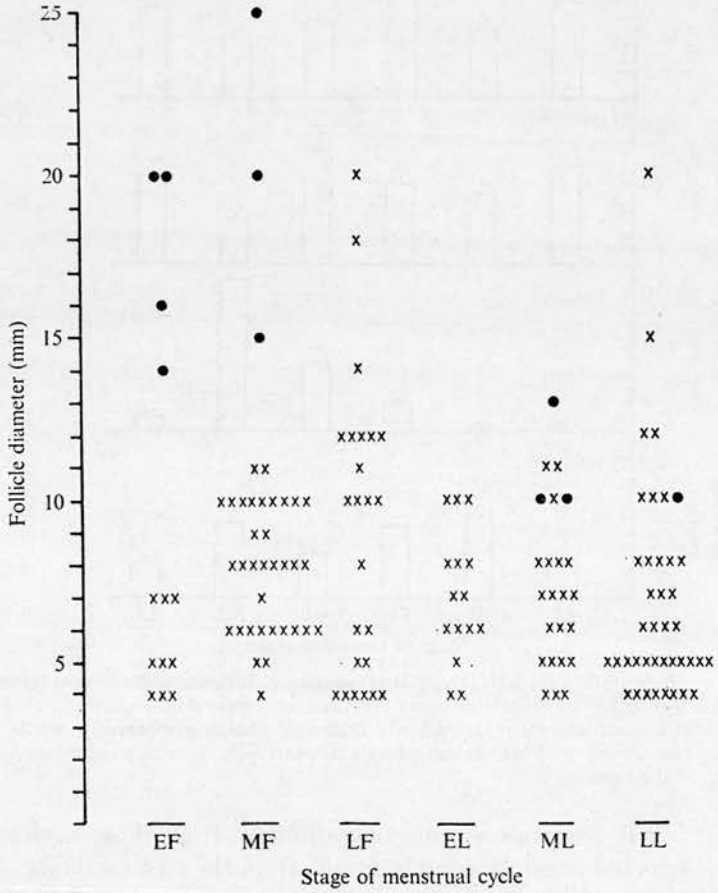



Fig. 2. Distribution of size of excised follicles in relation to the stage of the menstrual cycle. Follicles < 4 mm were not collected; ●, 'cystic' follicles; ×, 'non-cystic' follicles. See Fig. 1 for abbreviations.

*The concentrations of pituitary and steroid hormones in the follicular fluid of individual Graafian follicles*

*Follicle-stimulating hormone*

There were no significant differences in the concentration of FSH in follicular fluid from follicles aspirated *in situ* when compared with those aspirated *in vitro* ($P > 0{\cdot}2$). The mean concentration of FSH in all antral fluids in relation to size and the stage of the menstrual cycle is shown in Fig. 3*a*. The minimum detectable level of FSH in the

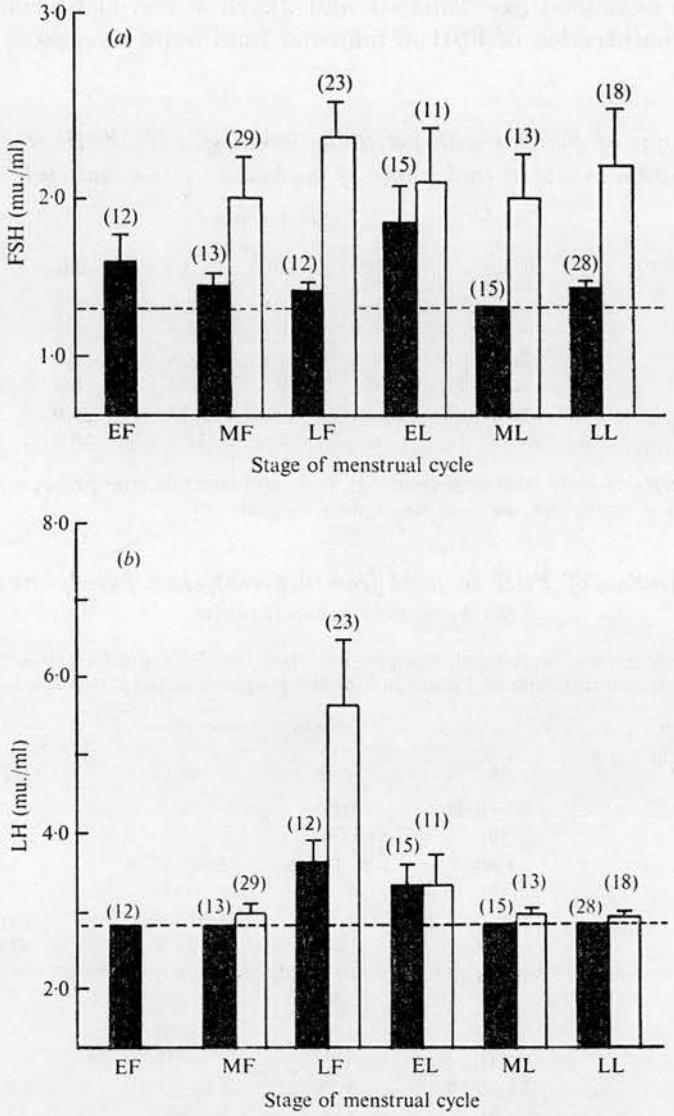



Fig. 3. Concentrations of (*a*) FSH and (*b*) LH in antral fluid in relation to follicle size and phase of the menstrual cycle. The vertical lines represent + S.E.M. White bars, mean concentration in follicles $\geqslant$ 8 mm; black bars, mean concentration in follicles < 8 mm. Broken line = minimum detectable level of (*a*) FSH (1·3 mu./ml), (*b*) LH (2·8 mu./ml). See Fig. 1 for abbreviations. Number of observations in parentheses.

smallest follicles (4 mm) was 1·3 mu./ml, which was therefore chosen as the limit of detection for all follicles. The percentage of follicles with detectable FSH at each stage of the cycle is shown in Table 1. In general, the greatest proportion of small follicles with detectable FSH are found either during or immediately after the increase in levels in the plasma (Fig. 1 and Table 1). In contrast, the concentration of FSH in some large follicles was high when the plasma concentrations were low (Fig. 1 and Fig. 3). Follicle-stimulating hormone was detectable in a proportion of the

smallest follicles examined (see Tables 1 and 2). However, at no time during the cycle was the concentration of FSH in follicular fluid more than 60 % of the levels found in plasma.

Table 1. *Percentage of follicles with detectable levels of LH, FSH or LH + FSH in relation to size at each phase of the human menstrual cycle*

| Pituitary hormone (limit of sensitivity) | Follicle size (mm) | Follicular phase | | | Luteal phase | | |
|---|---|---|---|---|---|---|---|
| | | EF | MF | LF | EL | ML | LL |
| FSH (1·3 mu./ml) | ⩾ 8 | — | 38·0 | 82·5 | 45·5 | 38·5 | 28·0 |
| | < 8 | 33·0 | 15·5 | 25·0 | 46·5 | 0 | 3·5 |
| LH (2·8 mu./ml) | ⩾ 8 | — | 11·0 | 69·5 | 25·0 | 14·0 | 22·0 |
| | < 8 | 0 | 0 | 16·5 | 16·5 | 0 | 0 |
| LH + FSH | ⩾ 8 | — | 10·0 | 69·5 | 25·0 | 14·0 | 22·0 |
| | < 8 | 0 | 0 | 16·5 | 16·5 | 0 | 0 |

EF, MF and LF refer to early (still menstruating), mid- and late follicular phase, respectively, while EL, ML and LL refer to early, mid- and late luteal phase respectively.

Table 2. *Concentration of FSH in fluid from different sized follicles at each phase of the human menstrual cycle*

(Values are means ± S.E.M. in mu./ml. Samples less than the detection limit were assumed to have a concentration of 1·3 mu./ml for the purposes of the group mean.)

| Stage of menstrual cycle* | Follicle diameter (mm) | | | |
|---|---|---|---|---|
| | 6 | 6–8 | 9–12 | 12 |
| EF | 1·50 ± 0·09 (6) | 2·30 (3) | — | — |
| MF | 1·30 (3) | 1·40 ± 0·05 (18) | 2·30 ± 0·40 (13) | — |
| LF | 1·76 ± 0·26 (8) | 1·68 (3) | 2·20 ± 0·26 (10) | 3·20 (3) |
| EL | 1·66 ± 0·23 (8) | 2·10 ± 0·50 (9) | 2·10 (3) | — |
| ML | 1·30 (7) | 1·30 (11) | 1·30 (3) | — |
| LL | 1·46 ± 0·13 (20) | 1·30 (12) | 3·00 ± 1·10 (8) | 3·28 (2) |

* For abbreviations see Table 1. Number of observations in parentheses.

*Luteinizing hormone*

The concentration of LH in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0·2$). The mean concentration of LH in all antral fluids examined with respect to follicle size and the stage of the menstrual cycle is shown in Fig. 3*b*. The percentage of follicles containing detectable concentrations of LH at various stages of the cycle is shown in Table 1; the lowest detectable level in the smallest follicles examined (4 mm) was 2·8 mu./ml, which was therefore chosen as the limit of sensitivity. There was a greater proportion of large follicles with detectable LH during the late follicular phase (Table 1), when the

concentrations were higher than at any other stage of the cycle (Fig. 3*b*). Luteinizing hormone was not detectable in any follicle of $< 8$ mm during the early and mid-follicular and mid- and late luteal phases. Fluids from 20 early and mid-follicular phase follicles were pooled to give a total volume of 50 $\mu$l, and the concentration of

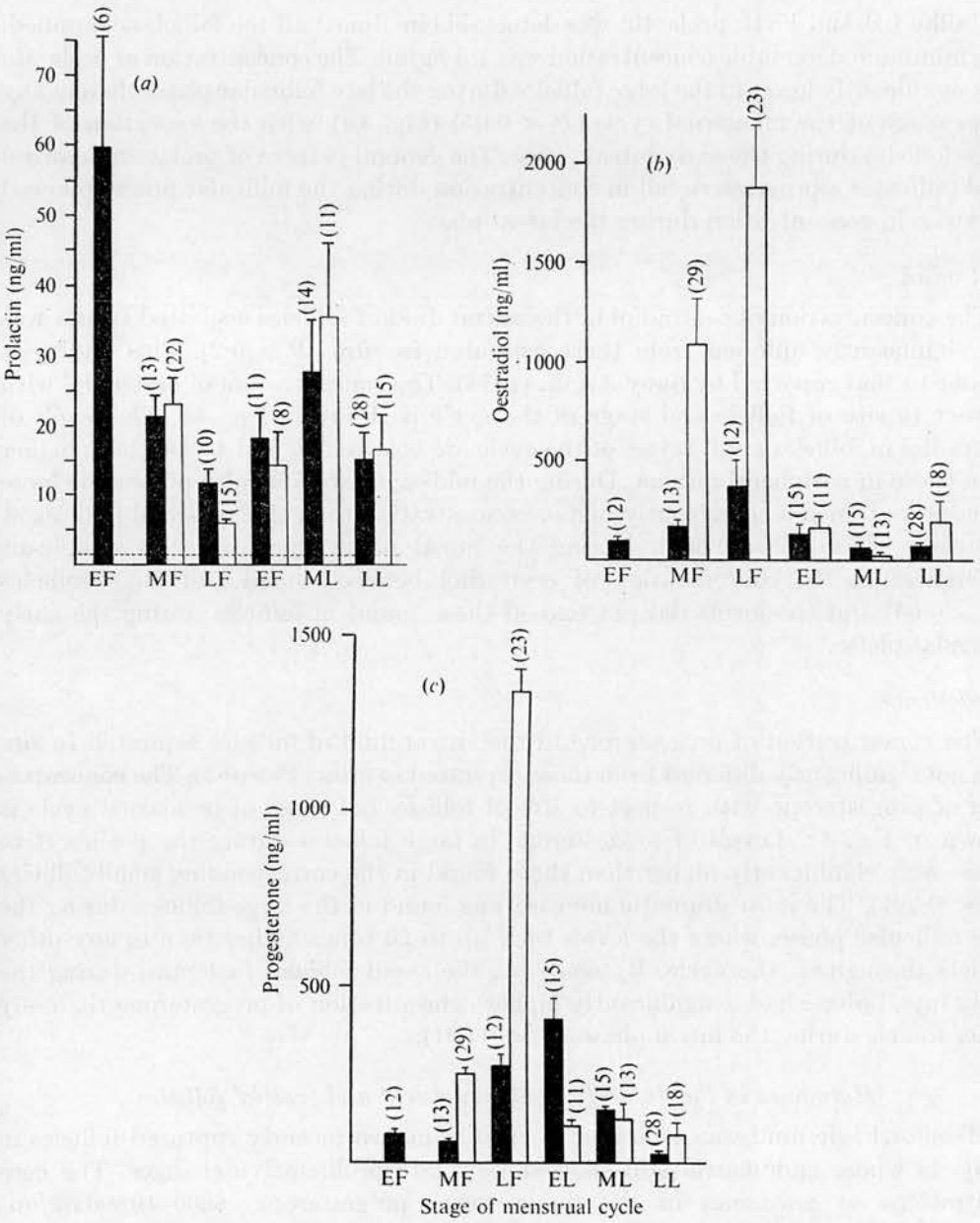



Fig. 4. Concentrations of (*a*) prolactin, (*b*) oestradiol and (*c*) progesterone in follicular fluid in relation to follicle size and stage of the cycle. Vertical lines represent + 1 S.E.M. White bars, mean concentration in follicles $\geqslant 8$ mm; black bars, mean concentration in follicles $< 8$ mm. Minimum detectable level of prolactin (1·5 ng/ml) is indicated by the broken line. See Fig. 1 for abbreviations. Numbers of observations in parentheses.

LH was 0·96 mu./ml (detection limit 0·8 mu./ml). Throughout all stages of the cycle the fluid concentration of LH was $< 30\%$ of that found in peripheral plasma. Furthermore, LH was only found in those follicles that also contained FSH (Table 1).

*Prolactin*

Unlike LH and FSH, prolactin was detectable in almost all the follicles examined. The minimum detectable concentration was 1·5 ng/ml. The concentration of prolactin was significantly lower in the large follicles during the late follicular phase than in any other stage of the menstrual cycle ($P < 0{\cdot}05$) (Fig. 4*a*), with the exception of the large follicles during the early luteal phase. The general pattern of prolactin in antral fluid indicates a progressive fall in concentration during the follicular phase followed by a rise in concentration during the luteal phase.

*Oestradiol*

The concentration of oestradiol in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0{\cdot}2$). This finding is similar to that reported by Sanyal *et al.* (1974). The concentration of oestradiol with respect to size of follicle and stage of the cycle is shown in Fig. 4*b*. The levels of oestradiol in follicles at all stages of the cycle are between 40 and 40000 times higher than those in peripheral plasma. During the mid- and late follicular phases the large follicles contained a significantly higher concentration than that in small follicles at the same phase ($P < 0{\cdot}001$). During the luteal phase there were no significant differences in the concentration of oestradiol between small and large follicles ($P > 0{\cdot}05$) and the levels did not exceed those found in follicles during the early follicular phase.

*Progesterone*

The concentration of progesterone in the antral fluid of follicles aspirated *in situ* was not significantly different from those aspirated *in vitro* ($P > 0{\cdot}2$). The concentration of progesterone with respect to size of follicle and stage of menstrual cycle is shown in Fig. 4*c*. Levels of progesterone in large follicles during the proliferative phase were significantly higher than those found in the corresponding small follicles ($P < 0{\cdot}001$). The most dramatic increase was found in the large follicles during the late follicular phase, where the levels were up to 20 times higher than in any other follicle throughout the cycle. By contrast, the small follicles ($< 8$ mm) during the early luteal phase had a significantly higher concentration of progesterone than any other follicle during the luteal phase ($P < 0{\cdot}001$).

*Hormones in the fluid of recently ruptured and 'cystic' follicles*

Haemorrhagic fluid was aspirated *in vitro* from two recently ruptured follicles in subjects whose endometria still showed very late proliferative changes. The concentrations of hormones in the fluids were: progesterone, 8600–10800 ng/ml; oestradiol, 335–210 ng/ml; LH, 9·6–7·6 mu./ml; FSH 7·9–6·8 mu./ml; prolactin, 14·6–12·1 ng/ml. The levels of LH and FSH were similar to those in peripheral plasma whereas the concentrations of prolactin in follicular fluid were about half the peripheral plasma concentrations. The steroid concentrations were 100- to 1000-fold

higher than the concentrations of oestradiol and progesterone in peripheral plasma. The concentration of progesterone in the fluid was also four to eight times higher than in late follicular phase fluid, but the concentration of oestradiol was at least ten times lower.

Table 3. *Hormone levels in human cystic follicles*

(Samples less than the detection limit were assumed to have a concentration of 1·3 mu./ml, 2·8 mu./ml, 1·5 ng/ml for FSH, LH and prolactin respectively for the purposes of the group means.)

| Stage of cycle* | Follicle size (mm) | LH (mu./ml) | FSH (mu./ml) | Prolactin (ng/ml) | Oestradiol (ng/ml) | Progesterone (ng/ml) |
|---|---|---|---|---|---|---|
| EF | 20 | 2·8 | 2·9 | 13·8 | 9 | 55 |
| | 16 | 6·0 | 1·7 | 39·0 | 205 | 20 |
| | 14 | 2·8 | 1·4 | 25·2 | 17 | 20 |
| | 20 | 2·8 | 1·2 | 47·0 | 40 | 15 |
| MF | 20 | 2·8 | 6·6 | 19·9 | 18 | 90 |
| | 15 | 12·0 | 1·3 | 23·0 | 230 | 17 |
| | 25 | 2·8 | 2·2 | 28·2 | 36 | 58 |
| ML | 10 | 2·8 | 1·3 | 23·0 | 150 | 90 |
| | 13 | 10·0 | 1·3 | 21·0 | 104 | 70 |
| | 10 | 2·8 | 1·3 | 21·0 | 30 | 80 |
| LL | 10 | 14·0 | 2·4 | 3·0 | 19 | 48 |

* For abbreviations see Table 1.

Table 4. *Concentrations (a) of oestradiol in the follicular fluid with or without FSH and (b) of progesterone in follicular fluid with or without LH (values are means* ± S.E.M. *in ng/ml)*

| | Stage of menstrual cycle* | | | | | |
|---|---|---|---|---|---|---|
| | EF | MF | LF | EL | ML | LL |
| (*a*) Oestradiol concentrations | | | | | | |
| FSH ( > 1·3 mu./ml) | 300 ± 34 (4) | 2120 ± 410 (13) | 2180 ± 310 (22) | 135 ± 40 (12) | 33 ± 26 (5) | 211 ± 200 (5) |
| FSH (⩽ 1·3 mu./ml) | 103 ± 22 (8) | 196 ± 60 (29) | 140 ± 55 (13) | 102 ± 58 (14) | 24 ± 11 (23) | 60 ± 38 (41) |
| (*b*) Progesterone concentrations | | | | | | |
| LH ( > 2·8 mu./ml) | † | 760 (2) | 1720 ± 280 (18) | 715 ± 60 (4) | 196 (2) | 320 ± 40 (4) |
| LH (⩽ 2·8 mu./ml) | 110 ± 31 (12) | 96 ± 33 (40) | 352 ± 84 (17) | 66 ± 11 (22) | 111 ± 15 (26) | 100 ± 20 (42) |

* For abbreviations see Table 1. Number of observations in parentheses. Limit of detection for FSH is 1·3 mu./ml and for LH is 2·8 mu./ml.

† No follicles containing LH.

Data relating to the hormone levels in cystic follicles are shown in Table 3. All these follicles contained low levels of oestradiol and progesterone irrespective of the stage of the cycle, suggesting that they were 'inactive' (Baird & Fraser, 1975).

*Relationship between follicular concentrations of hormones*

Those follicles which had detectable levels of FSH during the follicular phase without exception contained significantly higher concentrations of oestradiol than those with undetectable FSH (Table 4*a*) (EF, MF, LF; $P < 0{\cdot}01$, $P < 0{\cdot}001$,

$P < 0{\cdot}001$, respectively). Conversely, follicles with undetectable FSH contained low levels of oestradiol irrespective of the stage of the cycle. During the luteal phase these correlations no longer held, since follicles with or without FSH had consistently low levels of oestradiol.

A significantly higher concentration of progesterone was found in follicles containing LH (Table 4*b*) (MF, LF, EL, ML, LL; $P < 0{\cdot}001$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, $P < 0{\cdot}05$, $P < 0{\cdot}01$ respectively). All the follicles containing LH and a high concentration of progesterone were $\geqslant 8$ mm diameter and also contained FSH.

During the mid-follicular, late follicular and late luteal phase of the cycle the concentration of oestradiol in follicular fluid was correlated with that of progesterone ($r = 0{\cdot}44$, $n = 43$, $P < 0{\cdot}01$; $r = 0{\cdot}88$, $n = 33$, $P < 0{\cdot}001$; and $r = 0{\cdot}49$, $n = 37$, $P < 0{\cdot}01$, respectively).

## DISCUSSION

There was no difference in the concentration of hormones in fluid collected from follicles *in vitro* from those collected *in vivo* suggesting that there was little if any production or metabolism of hormones in the time between ovariectomy and aspiration of fluid (cf. Giorgi, Addis & Colombo, 1969; Sanyal *et al.* 1974). Although patients were undergoing surgery for a variety of reasons, the general pattern of hormones in peripheral plasma (Fig. 1) was similar to that described in normal women during the menstrual cycle (e.g. Robyn, Delvoye, Nokin, Vekemans, Badawi, Perez-Lopez & L'Hermite, 1973). However, the concentration of prolactin in peripheral plasma was up to sixfold higher than that in normal women (McNeilly, Evans & Chard, 1973; McNeilly & Chard, 1974). This is probably because the samples were collected under the stress of surgery which is known to stimulate the release of prolactin (Robyn *et al.* 1973). The overall mean concentration of prolactin in follicular fluid ($20 \pm 5$ ng/ml, $n = 189$) was much lower than that in peripheral plasma. In the absence of information about the rate of exchange of prolactin between plasma and follicular fluid *in vivo* it is impossible to determine whether this difference is of physiological importance or whether it is an artifact due to the elevated prolactin concentration in peripheral plasma as a result of stress. Since all the samples were collected under similar conditions, the striking change in follicular fluid concentration at different stages of the cycle (Fig. 4*a*) is probably not an artifact.

The distribution of follicle size in relation to the stage of the cycle is similar to that previously described in women (Block, 1951) and rhesus monkeys (Koering, 1969). Large follicles ($\geqslant 8$ mm) were much commoner during the mid- and late follicular and late luteal phases than at any other stage of the cycle. Although it is difficult to draw conclusions about the dynamics of follicle growth from these cross-sectional observations, it is tempting to speculate that these large follicles represent the end result of two waves of follicular development initiated by the peaks of secretion of FSH occurring at the onset of menses and again at mid-cycle. The minimum time necessary to develop a follicle from the antral to the mature preovulatory stage in women is probably 6–10 days (Gemzell & Johansson, 1971; Bertrand, Coleman, Crooke, Macnaughton & Mills, 1972). The concentration of FSH in small follicles reaches a maximum during the early follicular, late follicular or early luteal phases of the cycle (Table 2) which is either during or just after the peak FSH concentration in peri-

pheral blood. Furthermore, the concentration of FSH in these follicles apparently increases as they develop into mature preovulatory follicles in spite of the falling levels of FSH in peripheral blood (cf. Table 2 and Fig. 1). Some factor may increase the affinity of these follicles for FSH. Oestradiol, the concentration of which is extremely high in the mid- and late proliferative phase follicles, is known to increase the sensitivity of the ovary to FSH (Goldenberg, Vaitukaitis & Ross, 1972). The high levels of oestradiol in the large follicles during the mid- and late follicular phase are similar to those previously reported by other workers in normal (Smith, 1960; Short & London, 1961; Sanyal *et al.* 1974; Baird & Fraser, 1975) and gonadotrophin-stimulated ovaries (Short, 1964*a*; Edwards *et al.* 1972). Follicles which have detectable levels of FSH during the follicular phase also have a high concentration of oestradiol whereas those follicles with undetectable levels of FSH have significantly lower levels of oestradiol (see Fig. 3*a* and Table 4*a*).

When the concentration of FSH in peripheral plasma was high (early follicular phase and at mid-cycle) FSH was detectable in only a minority of small follicles (diameter $< 8$ mm) (Tables 1 and 2). Presumably follicles in which the concentration of FSH is high are those which are stimulated to further development (Table 4*a*). It is not known how this minority of small antral follicles are selected although it has been suggested that oocytes are 'programmed' for development in the order in which they are formed during foetal life (Henderson & Edwards, 1968).

About 17% of the small follicles (diameter $< 8$ mm) in the early luteal phase of the cycle had measurable amounts of FSH and LH (Table 1), presumably as a result of the preovulatory peaks of the gonadotrophins. In contrast to the follicular phase of the cycle, none of the follicles in the luteal phase were functionally 'active', as indicated by the persistently low concentrations of oestradiol. From the time of the LH 'surge' there is an abrupt fall in the mitotic activity of granulosa cells (Delforge, Thomas, Roux, Caneiro de Siqueiro & Ferin, 1972). Thus the presence of LH in some of these small follicles (Table 1) may interfere with their normal orderly development and consequently their steroidogenic potential (Tables 4*a* and *b*). The marked increase in cystic follicles (Fig. 2) from the mid-luteal phase is probably a consequence of this. It may be that an ordered sequence of gonadotrophins, e.g. FSH alone followed by FSH and LH, is necessary for normal follicular development.

Luteinization of the granulosa cells of the preovulatory follicle begins some 24–36 h before ovulation in response to the mid-cycle LH surge (Hertig, 1967; Delforge *et al.* 1972). After exposure to LH, the preovulatory follicle secretes increasing amounts of progesterone as indicated by the rise in concentration in ovarian (Mikhail, 1970; Lloyd, Lobotsky, Baird, McCracken, Weisz, Pupkin, Zanartu & Puga, 1971) and peripheral plasma (Johansson & Wide, 1969; Yussman & Taymor, 1970). The relatively high concentration of progesterone in follicular fluid of preovulatory follicles is similar to that found by other workers in normal (Sanyal *et al.* 1974) and gonadotrophin-stimulated ovaries (Short, 1964*a*; Edwards *et al.* 1972), and it is likely that the luteinizing granulosa cells are the source of this steroid (Channing, 1969, 1970; Edwards *et al.* 1972).

The large preovulatory follicles were characterized by a highly vascular appearance (Short, 1964*b*). The granulosa cells were very loosely attached to one another; the walls were slimy and mucoid, and the follicular fluid was bright yellow and

viscous. When the granulosa cells from large follicles collected during the pre-ovulatory phase were cultured *in vitro* they secreted maximal amounts of progesterone in response to minimal physiological concentrations of gonadotrophins in the medium (Channing, 1970; K. P. McNatty & R. S. Sawers, unpublished observations). The granulosa cells are stimulated *in vivo* by the relatively high concentrations of LH in blood and follicular fluid. Presumably the metabolic requirement of these actively secreting cells is high and may account for the relatively low oxygen tension within the follicle (Fraser, Baird & Cockburn, 1973). The relatively low concentration of prolactin may also reflect utilization or metabolism of the hormone by the follicle. It may also play a key role in controlling steroid synthesis, for the production of progesterone by human granulosa cells *in vitro* is inhibited when the concentration of prolactin in the medium exceeds 30 ng/ml (McNatty *et al.* 1974).

It is apparent that despite the presence of FSH in antral fluid the granulosa cells do not secrete progesterone in the absence of LH. Furthermore, the concentration of progesterone in follicular fluid of small follicles during the luteal phase is significantly lower than in preovulatory follicles, even though they contain both FSH and LH. The failure of the granulosa cells in small follicles to secrete substantial amounts of progesterone when exposed to LH suggests that these cells require time before they are capable of responding fully. The latter finding is consistent with the observation that granulosa cells harvested from preovulatory follicles in the pig have a significantly greater number of receptors for LH when compared with the cells harvested at other stages of the oestrous cycle (Channing & Kammerman, 1973; C. P. Channing, personal communication).

The concentration of oestradiol in the haemorrhagic fluid of recently ruptured follicles was very much lower than that found in the large preovulatory follicles. Although most of the fluid is lost at ovulation the low concentration of oestradiol in the haemorrhagic fluid is consistent with the low plasma levels during the immediate post-ovulatory period (Fig. 1) (Moor, 1974). In contrast, however, the levels of progesterone were higher than those found in the preovulatory follicle and this is consistent with the increased plasma levels indicating the growing secretory capacity of the luteinizing granulosa cells.

Steroid levels in the cystic follicles were low irrespective of the stage of the cycle and in many the pituitary gonadotrophin concentrations were high and similar to those found in plasma. These findings suggest that cystic follicles are functionally inactive and are incapable of secreting oestradiol or progesterone even in the presence of gonadotrophins.

These data suggest that a precise sequence of hormonal changes occurs within the microenvironment of the developing Graafian follicle; the order in which the changes occur may well be of considerable importance for the growth of that follicle and the secretory activity of the granulosa cells both before and after ovulation. Figure 5 shows the possible inter-relationships between the concentrations of pituitary hormones in plasma and follicular fluid with respect to the steroidogenic activity and growth of a follicle during the follicular and luteal phase of the menstrual cycle. The changes in the concentrations of pituitary hormones in follicular fluid throughout the menstrual cycle are related to those which occur in peripheral plasma. The presence of FSH in the smallest follicles appears to be important for their development. The

presence of FSH and oestradiol in the follicular phase follicles prepares them for the preovulatory production of progesterone under the influence of LH. The very low concentrations of prolactin in these follicles may be related to the upsurge in metabolic activity within the follicle during this time. The antral fluid of the preovulatory follicle contains relatively large amounts of oestradiol and progesterone, low physiological levels of prolactin, and concentrations of LH and FSH approaching 30 and

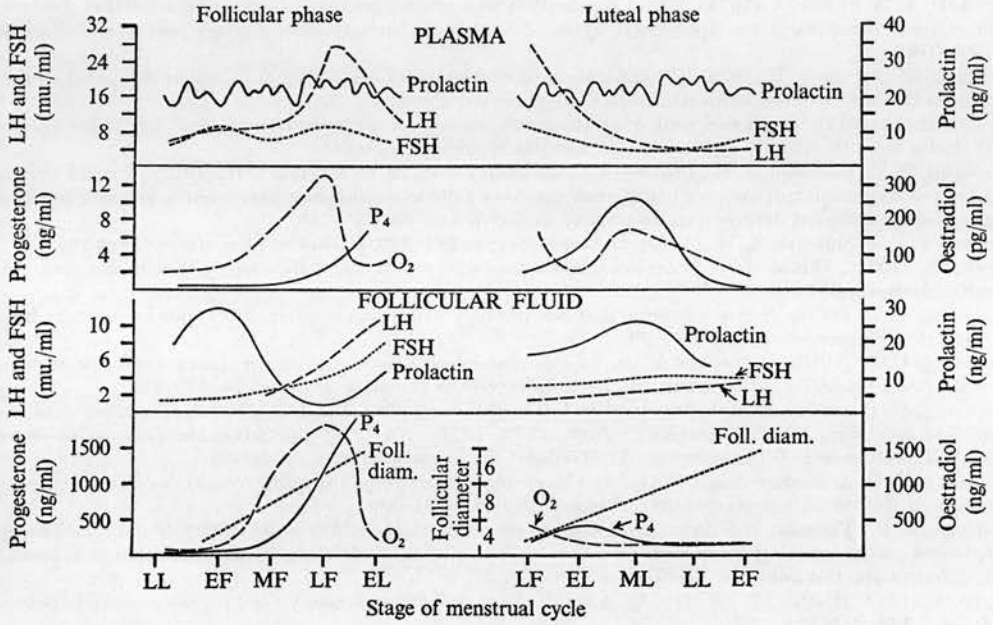



Fig. 5. The postulated relationships between the concentrations of pituitary and steroid hormones in peripheral plasma and in the follicular fluid of a developing follicle during either the follicular or luteal phase of the menstrual cycle (see text). $O_2$ = oestradiol, $P_4$ = progesterone. See Fig. 1 for abbreviations.

60 % respectively of those found in plasma. These changes in the growth of a follicle destined to ovulate are different from those proposed for the pig (Channing, 1972). Such differences that exist will possibly depend on the permeability of a Graafian follicle in different species to protein hormones (see Edwards, 1974, for review).

Furthermore, it is suggested that the large inactive follicles found during the luteal phase are a consequence of LH interference during the growth of these follicles under the influence of FSH. The consequence of these findings may prove to be important in the treatment of amenorrhoeic or anovulatory patients with exogenous gonadotrophins.

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# Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size

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**Summary.** The concentrations of LH, FSH and prolactin, and oestradiol-17β, androstenedione, testosterone and progesterone were measured in follicular fluid from small, medium and large bovine follicles. As follicle size increased, there was a significant increase in median fluid concentrations of prolactin (2-fold) and oestradiol-17β (14-fold) and a significant decrease in concentrations of LH (to 73%), androstenedione (to 30%) and testosterone (to 10%). There was no relationship between follicle size and fluid concentrations of FSH or progesterone, or between fluid concentrations of FSH and the relative concentrations of androgen and oestradiol-17β. As follicle size increased there was a significant increase in the proportion of follicles in which follicular fluid concentrations of oestradiol-17β exceeded those of androgen. There was a significant relationship between follicular fluid concentrations of prolactin and progesterone; as fluid prolactin concentrations increased, the maximum concentration of progesterone observed decreased.

## Introduction

While the concentrations of ovarian steroids and gonadotrophins present in blood throughout the oestrous cycle of the cow have been well documented (Kanchev, Dobson, Ward & Fitzpatrick, 1976; Schams, Schallenberger, Hoffmann & Karg, 1977; Ireland, Coulson & Murphree, 1979; Rahe, Owens, Fleeger, Newton & Harms, 1980), no information is available regarding the concentrations of gonadotrophins present in follicular fluid or their relationship to steroids present there. Studies in other species indicate that the relative follicular fluid concentrations of steroids and gonadotrophins vary widely between follicles, and that the intrafollicular environment of steroids and gonadotrophins may be an important regulator of follicular development (Moor, Hay, Dott & Cran, 1978; McNatty, 1978; McNatty, Gibb, Dobson, Thurley & Findlay, 1981). The purpose of this study was to determine what interrelationships existed between steroids and gonadotrophins present in bovine follicular fluid.

## Materials and Methods

### *Collection of follicular fluid*

Ovaries were obtained from 53 adult, non-pregnant cows within 1 h of their slaughter during 16 separate visits to a local Edinburgh abattoir between the months of October and February. The ovaries were transported to the laboratory in ice-chilled Eagle's Minimum Essential Medium

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(Modified) with Earle's Salts (Flow Laboratories, Irvine, Scotland). Follicular fluid from all antral follicles protruding at the surface of the ovary was aspirated individually using a syringe (1 or 2 ml) and needle (20–26 gauge). Fluid from follicles containing $\leqslant 0{\cdot}1$ ml fluid was pooled for follicles from the same ovary or each pair of ovaries. All other fluids were analysed separately. Follicles containing $<0{\cdot}3$ ml fluid were classified as small. This group consisted of 31 separate pools of follicular fluid from follicles containing $\leqslant 0{\cdot}1$ ml fluid and 15 fluids from individual follicles with $>0{\cdot}1$ ml to $<0{\cdot}3$ ml fluid. Follicles containing $\geqslant 0{\cdot}3$ ml to $<0{\cdot}8$ ml fluid and $\geqslant 0{\cdot}8$ ml fluid were classified as medium ($n = 35$) and large ($n = 37$) respectively. The median volume collected from large follicles was 1·0 ml and the greatest volume was 2·2 ml. All fluids were frozen immediately after aspiration until assayed for gonadotrophin and steroids by radioimmunoassay. The time between collecting the ovaries and freezing the aspirated follicular fluid was $<75$ min. Regression analysis on 23 individual follicles containing 0·05–1·3 ml follicular fluid indicated that follicular volume (V; μl) could be related to follicular diameter (D; mm) by the equation $V = 0{\cdot}3 \times D^3$.

*Radioimmunoassays*

*Gonadotrophins.* The concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin in follicular fluid were measured using the assays described previously by Scaramuzzi, Caldwell & Moor (1970), McNeilly, McNeilly, Walton & Cunningham (1976) and McNeilly & Andrews (1974) respectively. While these assays were originally developed to measure ovine gonadotrophins, the LH and FSH assays have been validated for use in the cow (Bass, McNeilly & Moreton, 1979). The prolactin assay was specific for bovine prolactin (NIH-P-B12), showing negligible cross-reaction ($<0{\cdot}5\%$ w/w at 50% B/Bo) with other bovine pituitary hormones: LH (NIH-LH-B8), FSH (CH-1-76 potency 164 × NIH-FSH-B1), TSH (30 i.u./mg, Pierce) and GH (NIH-GH-B15). It has been established previously that no serum interference occurred in the prolactin radioimmunoassay (McNeilly & Land, 1979). Dilution curves of plasma from cows were parallel to the bovine prolactin standard and recovery (mean ± s.e.m.) of added hormone to plasma was 97 ± 3% ($n = 30$). All the follicular fluid samples were assayed in one assay for each gonadotrophin and the intra-assay coefficient of variation was $\leqslant 6\%$ in each instance. The detection limits of the assays were 0·16 ng LH/ml, 16 ng FSH/ml and 0·5 ng prolactin/ml. Results are expressed in terms of NIH-LH-B8, NIH-FSH-B1 and NIH-P-B12 for LH, FSH and prolactin respectively.

*Steroids.* Concentrations of steroids in follicular fluid were determined using specific assays utilizing antisera and methods described previously for progesterone (Neal, Baker, McNatty & Scaramuzzi, 1975), oestradiol-17β (Van Look, Hunter, Corker & Baird, 1977), testosterone (Corker & Davidson, 1978) and androstenedione (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). Steroids were extracted from follicular fluid before assay with petroleum ether (progesterone), diethyl ether (oestradiol-17β and androstenedione) or hexane : diethyl ether (4 : 1 v/v) (testosterone). Recovery of steroids from follicular fluid was monitored by the addition of trace amounts of tritiated steroid (~1500 c.p.m.) to the samples before their extraction. The mean procedural recoveries from fluid were: progesterone 72%, oestradiol-17β 85%, androstenedione 81% and testosterone 73%. Steroids showing >1% cross-reactivity with the antisera used were: 11α-hydroxyprogesterone (35%); 11β-hydroxyprogesterone (12%); 5α-pregnane-3,20-dione (17%); and 17-hydroxyprogesterone (1·2%) with the progesterone antiserum; oestrone (5%) and oestradiol-17α (2%) with the oestradiol-17β antiserum; 5α-dihydrotestosterone (24%) with the testosterone antiserum; 11β-hydroxyandrostenedione (36%) and androsterone (43%) with the androstenedione antiserum. The limits of sensitivity of the assays were: 1·5 ng progesterone/ml, 0·2 ng oestradiol-17β/ml and 0·5 ng testosterone/ml and 0·5 ng androstenedione/ml. The intra- and inter-assay coefficients of variation were each $<10\%$ and $<16\%$ respectively for all of the assays.

*Statistics*

Non-parametric statistics were used to avoid making assumptions about the distribution of the values in the populations from which the sample data were drawn. Accordingly, sample medians are given together with 95% confidence limits calculated as described by Campbell (1967) using Nair's table. Unless otherwise stated, significant differences were determined using the Kruskal–Wallis test (Hollander & Wolfe, 1973) and comparisons of rank sums as described by Dunn (1964).

## Results

*Gonadotrophin concentrations in follicular fluid in relation to follicle size*

Table 1 shows that, as follicle size increased, FSH concentrations in follicular fluid remained unchanged, LH concentrations declined significantly and prolactin concentrations increased significantly.

**Table 1.** Concentration of gonadotrophins (ng/ml) in bovine follicular fluid in relation to follicle size

| | Follicular size (ml antral fluid/follicle) | | |
|---|---|---|---|
| | Small (<0·3) *(36, 42, 41) | Medium (≥0·3 to <0·8) *(28, 33, 33) | Large (≥0·8) *(36, 36, 37) |
| FSH | 70[a] (60–90) | 71[a] (62–82) | 65[a] (60–74) |
| LH | 4·5[a] (4·2–4·7) | 3·7[b] (3·4–4·0) | 3·3[bc] (3·0–3·5) |
| Prolactin | 14[a] (10–17) | 22[ac] (17–27) | 30[c] (24–45) |

* No. of samples assayed for FSH, LH and prolactin respectively.
Values are medians with 95% confidence limits in parentheses.
Values with different superscripts in the same row are significantly different: [ab] $P < 0.05$; [ac] $P < 0.01$.

*Steroid concentrations in follicular fluid in relation to follicle size*

Table 2 shows that, as follicle size increased, follicular fluid concentrations of oestradiol-17β rose significantly, testosterone and androstenedione concentrations fell significantly and progesterone concentrations remained unchanged. The range of concentrations of steroids in follicular fluid within each follicle size class was very large. Oestradiol-17β concentrations (ng/ml) ranged from <0·2 to 65 in small follicles, from <0·2 to 890 in medium follicles and from <0·2 to 920 in large follicles. Testosterone and androstenedione concentrations (ng/ml) ranged from 1·6 to 138 and 2·0 to 166 respectively in small follicles, 0·5 to 240 and <0·5 to 313 respectively in medium follicles and 0·7 to 121 and <0·5 to 760 respectively in large follicles. Progesterone concentrations (ng/ml) ranged from 7 to 115 in small follicles, 10 to 164 in medium follicles and 10 to 390 in large follicles.

**Table 2.** Concentration of steroids (ng/ml) in bovine follicular fluid in relation to follicle size

| | Follicle size (ml antral fluid/follicle) | | |
|---|---|---|---|
| | Small (<0·3) *(46) | Medium (⩾0·3 to <0·8) *(35) | Large (⩾0·8) *(36, 37, 35, 34) |
| Oestradiol-17β | 5[a] (1–8) | 23[c] (15–97) | 69[c] (37–142) |
| Androstenedione | 32[a] (25–41) | 18[ab] (6–30) | 10[b] (5–15) |
| Testosterone | 30[a] (19–49) | 5[c] (2–14) | 3[c] (2–6) |
| Progesterone | 35[a] (24–53) | 37[a] (21–49) | 39[a] (25–58) |

* No. of samples assayed for oestradiol-17β, androstenedione, testosterone and progesterone respectively.

Values are medians with 95% confidence limits in parentheses.

Values with different superscripts in the same row are significantly different: [ab] $P < 0{\cdot}05$; [ac] $P < 0{\cdot}01$.

*Relationship between follicle size and the ratio of oestradiol-17β to androgen concentrations in follicular fluid*

As follicle size increased, there was a significant increase ($P < 0{\cdot}001$; contingency table analysis) in the proportion of follicular fluids in which the concentration of oestradiol-17β (E) in follicular fluid exceeded that of androgen (androstenedione + testosterone; A + T). The proportion of fluids with an E:A + T ratio >1 was 3/46 for small follicles (1/31 for the pools of fluid and 2/15 for the fluids from individual follicles), 18/35 for fluids from medium sized follicles and 30/36 for large follicles.

*Relationship between FSH, oestradiol-17β and androgen concentrations in follicular fluid*

Table 3 shows that the relative concentrations of oestradiol-17β and androgen (testosterone + androstenedione) in follicular fluid were not related to those of FSH ($P > 0{\cdot}05$; Kruskal–Wallis).

**Table 3.** Relationship between bovine follicular fluid concentrations of FSH (ng/ml) and the ratio of fluid concentrations of oestradiol-17β and androgen (androstenedione + testosterone)

| | Ratio of oestradiol-17β to androgen | | | | |
|---|---|---|---|---|---|
| | $<10^{-2}$ | $\geqslant 10^{-2}$ to $<10^{-1}$ | $\geqslant 10^{-1}$ to $<10^{0}$ | $\geqslant 10^{0}$ to <10 | ⩾10 |
| No. of samples | 8 | 24 | 20 | 33 | 15 |
| FSH | 81 (52–185) | 66 (56–90) | 71 (60–96) | 64 (60–74) | 68 (60–100) |

Values are medians with 95% confidence limits of the median in parentheses.

*Relationship between prolactin and progesterone concentrations in follicular fluid*

There was a significant relationship between progesterone and prolactin concentrations in follicular fluid (Table 4): 70% of fluids examined had prolactin concentrations of ⩽30 ng/ml and progesterone concentrations in these fluids ranged from 7 to 390 ng/ml, although only 20% of these fluids had progesterone concentrations >80 ng/ml. As prolactin concentrations increased above 30 ng/ml there was a significant progressive decrease in the proportion of follicular fluids with progesterone concentrations >35 ng/ml ($P < 0{\cdot}02$, contingency table analysis).

**Table 4.** Contingency table showing the distribution of 106 follicular fluid samples with respect to follicular fluid prolactin and progesterone concentrations

| Follicular fluid prolactin conc. (ng/ml) | Follicular fluid progesterone conc. (ng/ml) | | |
|---|---|---|---|
| | ⩽35 | >35 to ⩽80 | >80 |
| ⩽30 | 30 | 30 | 15 |
| >30 to ⩽65 | 12 | 10 | 0 |
| >65 | 8 | 1 | 0 |

There was a significant relationship between follicular fluid concentrations of prolactin and those of progesterone ($P < 0{\cdot}02$; contingency table analysis).

## Discussion

The interpretation of the results of the present study in terms of possible physiological events is limited by the fact that the ovaries were obtained from animals passing through an abattoir. Consequently, no information was available on the reproductive history of the animals or of the stage of their oestrous cycle at the time of slaughter. Nevertheless, the data do demonstrate that there were significant relationships between follicle size and the relative concentrations of gonadotrophins and steroids in bovine follicular fluid.

As follicle size increased, the steroid environment of follicular fluid changed from being predominantly androgenic to predominantly oestrogenic. In only 7% of small follicles examined was the oestradiol-17β concentration in follicular fluid greater than that of the androgens while in large follicles this figure was 83%. In the cow, granulosa cells are the principal site of follicular androgen aromatization with oestradiol-17β being the major product (Lacroix, Eechaute & Leusen, 1974). An increase in granulosa cell numbers and/or aromatase activity could account for the fall in fluid concentrations of androgen and rise of oestradiol-17β concentration associated with increasing follicle size (Table 2). The sharper fall in testosterone concentrations than in those of androstenedione is consistent with previous findings that bovine granulosa cells aromatize testosterone in preference to androstenedione (Henderson & Swanston, 1978; Henderson & Moon, 1979). FSH stimulates granulosa cell aromatase activity and in this way stimulates follicular oestradiol-17β production (Hillier, 1981). In women (McNatty, 1978) and sheep (McNatty *et al.*, 1981) FSH concentrations in follicular fluid are related to the relative concentrations of oestradiol-17β and androgen present there—high FSH concentrations are associated with oestradiol-17β:androgen ratios >1 and low FSH concentrations to oestradiol-17β:androgen ratios of <1. No such relationship was evident in the present study (Table 3). Therefore, while FSH present in bovine follicular fluid may influence the aromatase activity of the follicle, it is unlikely to be the limiting factor regulating the aromatase activity

expressed by the follicle, as reflected in the relative concentrations of oestradiol-17β and androgen in follicular fluid.

While there was no significant change in follicular fluid concentrations of FSH in relation to follicle size, fluid concentrations of LH and prolactin did change significantly (Table 1). LH concentrations fell slightly, but significantly, as follicle size increased. This may reflect some utilization of the fluid LH by follicular cells, particularly granulosa cells which are bathed in the follicular fluid. In contrast to LH, prolactin concentrations in follicular fluid rose as follicle size increased, which is consistent with findings for sheep follicles (McNatty *et al.*, 1981). Fluid concentrations of prolactin appeared to limit those of progesterone. Although there was no relationship between fluid progesterone concentrations and follicle size (Table 2), analysis of individual follicles revealed that high fluid progesterone concentrations (>80 ng/ml) only occurred when those of prolactin were low ⩽30 ng/ml (Table 4). The high prolactin concentrations in follicular fluid could suppress granulosa cell progesterone production; prolactin inhibits progesterone production by human (McNatty, Sawers & McNeilly, 1974) and procine (Veldhuis, Klase & Hammond, 1980) granulosa cells *in vitro*.

While there were significant relationships between follicle size and the hormonal environment of follicular fluid, there was considerable variation, particularly in the steroid concentrations, between individual follicles of the same size. Thus, while one can generalize on how one population of follicles of a particular size is likely to differ from another, it is not possible to predict the follicular fluid environment of any individual follicle drawn from populations of follicles of defined size.

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# PROLACTIN AND PROGESTERONE SECRETION BY HUMAN GRANULOSA CELLS *IN VITRO*

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## INTRODUCTION

In primates, the role of prolactin in the control of follicular growth, corpus luteum function and ovarian steroidogenesis remains uncertain (Knobil, 1973). It has been known for some time that women with elevated levels of prolactin in plasma and apparently normal levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) commonly have amenorrhoea (see review by McNeilly, 1974) or in some cases inadequate corpus luteum function (Del Pozo, 1976). If the prolactin levels are lowered by treatment with CB-154, ovulation and menstruation recur (Besser *et al.*, 1974; Del Pozo *et al.*, 1972). One hypothesis to explain this phenomenon is that high concentrations of prolactin may have an inhibitory effect on steroidogenesis by the maturing Graafian follicle or on the newly formed corpus luteum (McNatty *et al.*, 1974).

The concentration of prolactin in the follicular fluid of preovulatory follicles is significantly lower than in follicles at any other stage of the menstrual cycle. The follicular concentrations of prolactin are not related to the concentrations in peripheral plasma (McNatty *et al.*, 1974; McNatty *et al.*, 1975a). It is possible therefore, that prolactin might have a role in regulating the biosynthetic capacity of granulosa cells.

This paper summarises the findings from collaborative studies initiated in Edinburgh: it describes the effects of the addition, removal, or neutralisation of

human prolactin in culture media on the production of progesterone by human granulosa cells maintained in tissue culture.

## MATERIALS AND METHODS

### *Patients*

Seventeen patients (aged 27 to 45 years) who were at varying stages of the menstrual cycle were undergoing hysterectomy for various gynaecological conditions.

The indications for survey were stage 0 carcinoma of the cervix (2 patients); menorrhagia due to fibroids (6), dysfunctional uterine bleeding (3) or dysmenorrhoea (6). It was not known whether the patients were originally hyperprolactinaemic; however, during anaesthesia immediately prior to hysterectomy all patients had elevated plasma levels of prolactin which ranged from 30 to 100 ng/ml.

### *Technique for Culturing Granulosa Cells*

Thirty-four follicles, varying in size from 4 to 20 mm in diameter, were isolated from the ovarian specimens.

The techniques for harvesting the cells from isolated Graafian follicles, for assessing cell viability and for maintaining the cells *in vitro* was identical to those described by McNatty *et al.* (1975a). Granulosa cells were harvested from each of the follicles and cultured separately. The cells were grown on coverslips and cultured for 10 days, and the culture medium was replaced daily and stored frozen at −20°C until assayed for progesterone. At the end of the culture, the coverslips were removed, extensively rinsed in medium without serum, and fixed with Smearfix (Raymond A. Lamb, London). The coverslips were stained with haematoxylin and eosin and the cell numbers determined by the counting technique previously described (McNatty and Sawers, 1975b).

### *Human Prolactin*

The human prolactin preparation which was added to the cultures was Friesen prolactin (1 ng = 20 μu MRC standard 71/722). The Friesen prolactin preparation was 90% pure and the human pituitary hormone contaminants were found by radioimmunoassay to be negligible: growth hormone (HGH), HLH, HFSH, all $< 0.4\%$ and the concentration of human placental lactogen was undetectable. Prolactin was dissolved in culture medium and stored at −20°C until used. After three months storage at −20°C the prolactin preparation was discarded and a fresh solution was prepared.

### *Treatment of Human Serum with Antiserum to Gonadotrophins*

Two different procedures were used to assess whether prolactin was an essential hormone for the maintenance of steroidogenesis by human granulosa cells. In the first procedure, cells were cultured in a medium containing 20% human serum, 20% human serum and normal rabbit serum or 20% human serum and either rabbit antiserum to prolactin, human growth hormone (HGH) or bovine serum albumin (BSA). Normal rabbit serum, rabbit anti-BSA, rabbit anti-HGH (W105; supplied by Dr. B. Hurn, Wellcome Laboratories, Kent, England) or rabbit anti-human prolactin (prepared by Dr. A.S. McNeilly, No. A4) which were previously dialysed extensively (4-5x) against medium 199 containing Hank's salts, Hepes Buffer (20 mM), gentamicin (50 μg/ml) and amphotericin-B (2.5 μg/ml),

in order to remove any cytotoxic preservatives, were added to the culture medium at a final dilution of 1:500 v/v. These culture media including the control with rabbit serum (1:500 v/v) were incubated at 37°C for 24 h before being used in tissue culture. After these media had been used in culture, aliquots of each daily change of culture medium were treated with an equal volume of solid phase immunosorbent, sheep anti (rabbit γ-globulin) (Organon, Oss, Holland) suspended in medium 199. The mixture was continuously slurried by gently mechanical agitation for 8 h at 4°C. After this time, the precipitated antibody-antigen complex was centrifuged at 2000 rpm for 15 min: the precipitate was discarded and the supernatant was assayed for LH, FSH and prolactin. Using this procedure it was possible to examine the progesterone content of the culture medium and relate the biological and immunological neutralisation of endogenous prolactin to changes in the daily rate of progesterone secretion.

In the second procedure granulosa cells were cultured in medium containing 20% human serum which was devoid of prolactin. The procedure for selectively removing the endogenous prolactin in the human serum was similar to that used in the neutralisation experiments and has been described in detail previously (McNatty *et al.*, 1975a).

All sera treated with antibodies, together with the control sera previously diluted to 20% (v/v) with medium 199 was then added to monolayer cultures of Hela cells (adapted for culture in human serum, Flow Laboratories, Irvine, Scotland). This procedure was used to confirm that none of the treated or control sera contained non-specific cytotoxic substances.

*Radioimmunoassays for Prolactin and Progesterone*

*Prolactin*

Prolactin in serum, follicular fluid or culture medium was measured using a specific double-antibody radioimmunoassay previously described (McNeilly, 1973; McNeilly and Hagen, 1974; McNatty *et al.*, 1975c). Purified human prolactin for standards and labelling with $^{125}I$ was generously supplied by Dr. H.G. Friesen, University of Manitoba, Winnipeg, Canada. The concentration of prolactin is expressed as ng Friesen prolactin/ml. The minimum detectable level of prolactin was 0.2 ng/ml. Assays were carried out in triplicate at the same dilution.

*Progesterone*

The technique for measuring progesterone in culture medium was a minor modification of the method described by Neal *et al.* (1975). The only change was that progesterone was extracted from the culture medium using petroleum ether (40-60 grade, 20/1, v/v): the recovery of progesterone was consistently $> 90\%$. The progesterone antiserum (R-4) was raised in a rabbit against progesterone-11α hemisuccinate conjugated to bovine serum albumin and the specificity of the assay was similar to that reported previously (Dighe and Hunter, 1974). The assays were conducted in duplicate at different dilutions and the minimum detectable level of progesterone was 90 pg/ml.

## RESULTS

*The Effect of Prolactin on the Viability and Mitotic Activity of Human Granulosa Cells in Culture*

When cells were exposed to prolactin at concentrations between 0.2 and 100 ng/ml with or without added LH+FSH there was no significant increase or de-

crease in cell numbers during the 10 days of culture when compared to untreated controls.

*The Production of Progesterone by Granulosa Cells in Culture Medium in which the Endogenous Prolactin was Neutralised in Excess Prolactin Antiserum*

Neutralisation of prolactin in the culture medium by the addition of rabbit antiserum prolactin serum (A4) (1/500, v/v, final dilution) caused a significant decrease in the production of progesterone when compared to the controls (Fig.1)

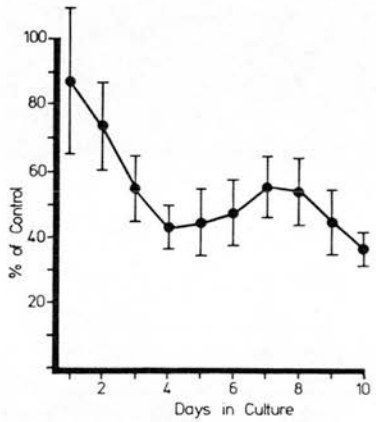



*Fig.1* The daily production of progesterone by human granulosa cells in a culture medium in which the endogenous prolactin activity was neutralised with excess rabbit anti-human prolactin serum. Results are expressed as a percentage of the controls and represent the mean of 5 duplicate cultures. Vertical bars represent ± 1 S.E.M.

($p < 0.001$, Student's t test). The controls were either untreated culture medium (5 duplicate experiments) or culture medium treated with normal rabbit serum (3), rabbit anti-BSA (3) or rabbit anti-HGH (5) all of which were present at a final dilution of 1/500 (v/v). There were no significant differences in the production of progesterone between these control cultures ($p > 0.05$) which were found to be within the precision achieved in replicate cultures. To confirm that free prolactin in the culture medium had been completely neutralised by the antiserum, the anti-prolactin-prolactin complex was precipitated out using a solid phase second antibody; the concentration of LH and FSH in the medium were unaltered whereas prolactin was undetectable ($< 0.2$ ng/ml).

*Progesterone Secretion by Granulosa Cells Grown in a Culture Medium Devoid of Prolactin*

The daily progesterone secretion by granulosa cell cultures in a medium devoid of prolactin (see Table I) is shown in Fig.2. After 24 h in either medium, the secretion of progesterone was only 60% of the controls, and from day 6 to 10 of culture the secretion rate was only 30% of the controls although the number of cells was similar in treatment and control groups. In these experiments between 80 and 125% of the cells remained after 10 days culture when compared to the number of live cells in the first day of culture.

*Table I.* Effect of adding gonadotrophin antisera on gonadotrophin concentrations in human sera.*

| Antiserum | Final dilution of antiserum | Gonadotrophin concentrations in human serum after treatment with antisera | | |
|---|---|---|---|---|
| | | LH mu/ml | FSH mu/ml | Prolactin ng/ml |
| None (concentrations before treatment) | — | 15.4 | 14.3 | 12.8 |
| Normal rabbit serum | 1:200 | 16.8 | 12.0 | 11.7 |
| Rabbit anti-BSA | 1:200 | 17.7 | 11.1 | 10.4 |
| Rabbit anti-HGH (W105) | 1:200 | 16.0 | 15.0 | 6.5 |
| Rabbit anti-human prolactin | 1:400 | 12.8 | 11.8 | < 0.2 |

* The same batch of human sera was used in all experiments. Reprinted in part from the data of K.P. McNatty *et al.* (1975a), with permission from Cambridge University Press.

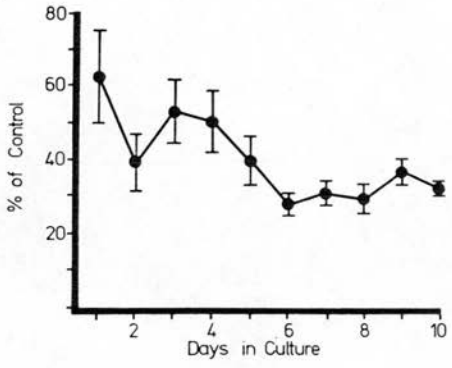



*Fig. 2* The daily production of progesterone by human granulosa cells in culture medium devoid by prolactin (5 duplicate experiments) expressed as a percentage of the controls. The vertical lines represent ± 1 S.E.M. Reprinted in part from the data of K.P. McNatty *et al.* (1975a), with permission from Cambridge University Press.

### *The Daily Production of Progesterone by Granulosa Cells in a Culture Medium Containing High Concentrations of Prolactin*

The effect of adding prolactin to culture medium on the daily production of progesterone is shown in Fig.3. There was no effect on progesterone secretion if the concentration of prolactin was between 5 and 20 ng/ml; the mean concentration of prolactin in peripheral blood of women during the menstrual cycle is 15 ± 1 (± 1 S.E.M.) (McNeilly *et al.*, 1973). When the concentrations in the culture medium were increased from 25 to 100 ng/ml, there was a progressive decrease in the daily production of progesterone. When the concentration of prolactin in the culture medium was 100 ng/ml, the total progesterone output after 10 days' culture was only 10% of that achieved in the control (Fig.3).

This inhibitory effect persisted even when the concentrations of LH (1.8 mu/ml) and FSH (1.7 mu/ml) were both increased to 50 mu/ml (Fig.4), and it could be obtained with granulosa cells collected from follicles at any stage of the menstrual cycle. Millipore filtration (0.22 μm, Millipore Corporation, Bedford, U.S.A.)

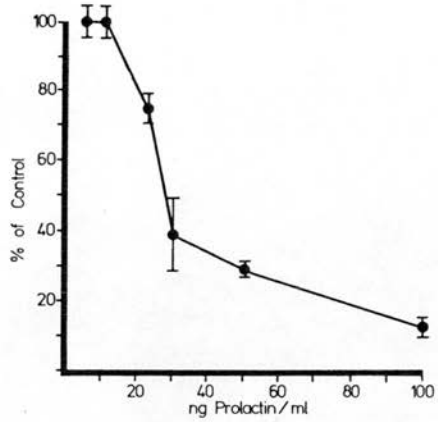



*Fig.3* Effect of varying concentrations of prolactin on the total production of progesterone by human granulosa cells during 10 days' culture when the cells were exposed to low levels of LH and FSH (each < 2 mu/ml). Each point is the mean result of 5 duplicate experiments. The vertical bars represent ± 1 S.E.M. The results are expressed as a percentage of the controls.

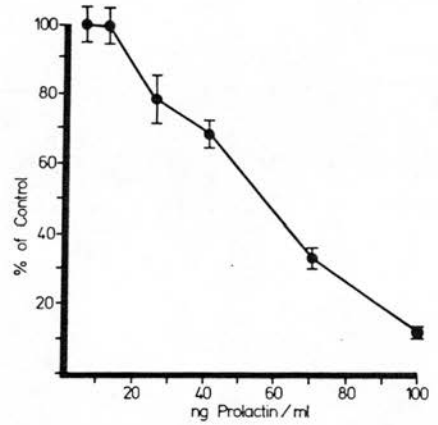



*Fig.4* Effect of varying concentrations of prolactin on the total production of progesterone by human granulosa cells during 10 days of culture when the cells were exposed to high concentrations of LH and FSH (each 50 mu/ml). Each point is the mean result of 5 replicate experiments. The vertical bars represent ± 1 S.E.M. The results are expressed as a percentage of the controls.

of prolactin dissolved in medium 199 effectively removed > 95% of the protein. The non-protein filtrate was examined for its ability to alter steroidogenesis by granulosa cells in culture and all were found to be inactive.

When the concentrations of prolactin were increased on the 5th and 6th day of culture from 10 to 100 ng/ml there was an immediate fall in the daily secretion of progesterone (Fig.5) which returned to control values when the prolactin concentrations were lowered once more.

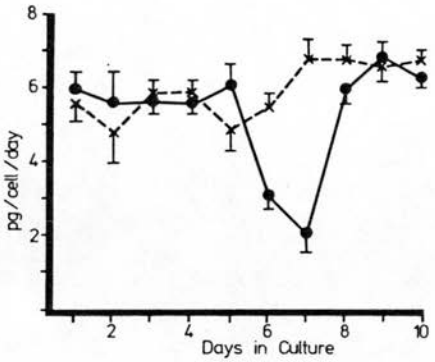



*Fig.5* Effect of increasing the concentration of prolactin in culture medium on Days 5 and 6 from 10 ng to 100 ng/ml on the daily production of progesterone by granulosa cells. x–x represents the mean daily production by cells exposed to 100 ng prolactin/ml on Days 5 and 6 (6 duplicate experiments); o–o represents the mean daily production by cells exposed to 10 ng prolactin/ml each day (6 duplicate experiments). Vertical bars represent ± 1 S.E.M.

## DISCUSSION

These studies clearly demonstrate that *in vitro*, granulosa cells are dependent on low physiological levels of prolactin for the secretion of progesterone. Furthermore the cells appear to secrete at maximum capacity when the concentration of prolactin is within the normal physiological range (i.e., 5-20 ng/ml; McNeilly *et al.*, 1973). If the concentration exceeds 20 ng/ml however, there is a marked inhibition of progesterone secretion which is not overcome by increasing the levels of LH and FSH although secretion returns to normal immediately after the levels are lowered. Since neither the viability nor the mitotic activity of the cells are affected when they are exposed to high concentrations of prolactin, the inhibitory effect is probably a consequence of changes in the biosynthetic potential of the cell. Similar results have been obtained with intact mouse follicles maintained in organ culture using human, rat or sheep prolactin (McNatty *et al.*, 1976b). In this study high concentrations of prolactin inhibited the follicle from secreting progesterone but had the opposite effect on luteal tissue. These findings confirm our earlier observations that high concentrations of prolactin may interfere with the maturation granulosa cells within the microenvironment of the Graafian follicle (McNatty *et al.*, 1974).

Prolactin is probably essential for steroidogenesis since it influences the amount of cholesterol available for metabolism to progesterone (Armstrong *et al.*, 1969; Behrman *et al.*, 1971). However, the way in which high levels of prolactin alter the biosynthetic potential of the cells remains unknown.

There is evidence to suggest that the hormonal environment of the Graafian follicle influences both the mitotic activity and biosynthetic potential of its granulosa cells so that endocrine events occurring some considerable time before ovulation may dictate the subsequent activity of the corpus luteum (McNatty and Sawers, 1975b). Changes suggestive of luteinization of the membrana granulosa can be demonstrated before rupture of the follicle in women and these are almost certainly initiated by the increase in the concentration of LH in follicular fluid of

the preovulatory follicle (Hertig, 1967; Delforge *et al.*, 1972; Baird *et al.*, 1975; McNatty *et al.*, 1975c). The timing of the various hormonal events within the follicle appears critical for granulosa cells to achieve their maximum biosynthetic capacity (McNatty and Sawers, 1975b). For example, the administration of an ovulatory dose of HCG to women, with previously regular menstrual cycles, on Days 1 to 6 after the onset of menstruation inhibited or postponed ovulation (Friedrich *et al.*, 1975). These authors also noted that there was no increase in progesterone secretion after the HCG treatment suggesting that granulosa cells were unable to luteinize fully and function normally. Similarly it seems that persistent high levels of prolactin in follicular fluid may also suppress progesterone secretion prior to ovulation thus leading to inappropriate ovarian function.

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# Prolactin and the Human Ovary

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The role of prolactin (PRL) in the control of human ovarian function has received remarkably little attention. This is, in part, due to limited supplies of normal ovarian tissue and inadequate amounts of human PRL for *in vitro* studies. Many observations have been made on the changes in ovarian activity in hyperprolactinemic states, usually pathological hyperprolactinemia. In addition, because apparently normal follicle growth and development, ovulation and corpus luteum can be induced by appropriate gonadotrophin therapy any direct effect of PRL on ovarian function has largely been dismissed. Indeed, almost all studies have concentrated on the effects of high levels of PRL on ovarian activity, and little attention has been paid to the role of PRL in the ovarian cycle in normoprolactinemic states. This chapter offers a brief but select review of some aspects of PRL and ovarian function.

## OVARIAN FUNCTION IN LACTATION

Lactation in women is associated with a period of lactational infertility including, initially, a period of amenorrhea during which time the ovary is normally quiescent with minimal follicular growth and development as judged by low levels of both plasma and urinary estrogens (20,24). This is followed in a large proportion of women by a resumption of ovarian activity (estrogen secretion) leading to ovulation and the formation of a corpus luteum. However in a large number of these cycles, corpus luteum function in terms of progesterone levels is deficient and would probably not support a pregnancy (24).

The reason for the absence of ovarian activity during lactation remains to be explained. Serum levels of follicle-stimulating hormone (FSH) throughout lactation are within the range seen in the early follicular phase of normal menstrual cycles (20). Although this does not exclude the possibility that a subtle alteration in FSH levels may occur just prior to resumption of ovarian activity, this seems unlikely. Initially, luteinizing hormone (LH) levels are low until the 4 to 6 weeks postpartum. Thereafter, basal LH levels increase, although they usually remain somewhat lower than the range seen in the

follicular phase of the normal menstrual cycle (10,20). Nevertheless, it is clear that there is no detectable change in basal LH levels around the time of resumption of ovarian follicular development (24).

During the normal menstrual cycle, LH is released in a pulsatile fashion with a pulse frequency increasing from the early follicular phase up to ovulation (1). The increasing pulse frequency of LH appears to be responsible for the increase in estradiol ($E_2$) secretion in the follicular phase of the cycle (1). In our recent studies on pulsatile secretion of LH during ovarian quiescence in lactation it is clear that in about 50% of women, pulsatile secretion of LH is reduced and increases around the time of resumption of ovarian activity (10). However, in the remaining 50% of women, pulsatile secretion of LH similar to that in the follicular phase of menstrual cycles been during lactation returns between 4 and 29 weeks before the resumption of ovarian activity (10). This suggests that some factor(s) is blocking the action of LH at a site within the ovary.

From our longitudinal studies, it is clear that resumption of ovarian activity is associated with a decrease in both suckling frequency and duration and the associated decrease in PRL levels (11,24). A similar association between hyperprolactinemia and lactational amenorrhea has been shown in cross-sectional studies in long-term lactational amenorrhea (3,7,13)). Thus, it is possible that PRL per se could be involved in blocking the action of gonadotrophins directly at the ovary as previously suggested (16,17,19).

Studies *in vitro* with granulosa cells from both rats and pigs have shown that high levels of PRL can inhibit FSH induction of aromatase activity, thus reducing $E_2$ secretion (28–30). In a single study high levels of PRL were also shown to inhibit progesterone secretion from human granulosa cells *in vitro* (16,17). Thus, there is limited evidence that hyperprolactinemia could be implicated in inhibiting steroid secretion by ovarian follicles (25).

It is clear, however, that any PRL-related block to gonadotrophin stimulation of follicle growth can be overcome if sufficient gonadotrophin is given. Thus, ovulation and normal luteal function adequate to maintain pregnancy can be induced by gonadotrophin therapy in spite of continuing hyperprolactinemia (14). This does not necessarily mean, however, that PRL in physiological (lactational) or pathological hyperprolactinemia could not provide a block sufficient to counteract endogenous levels of gonadotrophin present in these situations; i.e., the block may be more subtle than has so far been appreciated.

Many of the menstrual cycles which occur during lactation, when PRL is between 2 and 5 times higher than normal nonlactating levels, have deficient corpora lutea (24). Studies in which hyperprolactinemia has been induced in the luteal phase of normal menstrual cycles have failed to affect progesterone levels (2). This suggests that the deficient corpus luteum arises from the ovulation of an inadequately primed follicle (21). In many of these cycles the preovulatory increase in estrogen levels is within the normal limits, suggesting further that

any defect may be within the follicle itself. Indeed, McNatty (15) found that raised PRL levels in plasma and ovarian follicular fluid were associated with a reduced number of granulosa cells in the follicle and a marked reduction in intrafollicular steroidogenesis, although in most cases there was no reduction in circulating steroid levels.

The reason for the decrease in estrogen levels within the follicle may be either because of a deficient aromatase system within the granulosa cells themselves or because of a reduced secretion of androgen precursor by the theca. Which of these may occur in the in the human ovary awaits further investigation.

## PRL AND THE NORMAL OVARY DURING THE MENSTRUAL CYCLE

Few studies have investigated the role of PRL in the normal human ovary. Results from *in vitro* incubations of human granulosa cells have shown that PRL is essential for the maintenance of progesterone secretion, although there is no information on other steroids (16–18). *In vivo*, deficient luteal phases have occurred when PRL levels have been suppressed below normal in the follicular phase of the cycle in women treated with bromocriptine (CB154) (27). Although this supports the *in vitro* evidence, it is also possible that the treatment with CB154 had altered gonadotrophin secretion in a way too subtle to be detected by the blood-sampling regimens used in these studies.

Unlike many species, there appears to be no clear-cut changes in blood levels of PRL throughout the human menstrual cycle (22). Almost all observations rely on daily blood samples and either no change or increases in follicular, midcycle, or luteal phases have been reported by various groups (8,9,23,26).

Recently, we have studied methods for predicting ovulation for recovery of oocytes. This has involved taking blood samples every 8 hr around the time of ovulation; results have shown that ovulation normally occurs around 33 to 40 hr after the start of the LH surge (5,6). Associated with this rise in LH is a progressive rise in blood progesterone levels and an increase in PRL levels which begins at the start of the LH surge (4). Levels are higher at all times of the day or night on the days of the LH surge, although this increase is significant only at 4:00 p.m. and 12:00 p.m. These results explain the discrepancy in reports on changes in PRL around midcycle since, in most studies, the time of day when blood samples were taken was an uncontrolled variable. It is interesting that the main increase in PRL occurs at least 30 hr after the major increase in $E_2$ but coincides with the start of the LH surge. This supports the suggestion that there is a decrease in hypothalamic inhibition on PRL secretion, presumably a decrease in dopamine, which appears necessary to allow the increased release of hypothalamic LH releasing hormone (LHRH) causing the preovulatory LH surge (12,22).

In view of the reported effects of PRL on steroid secretion by human

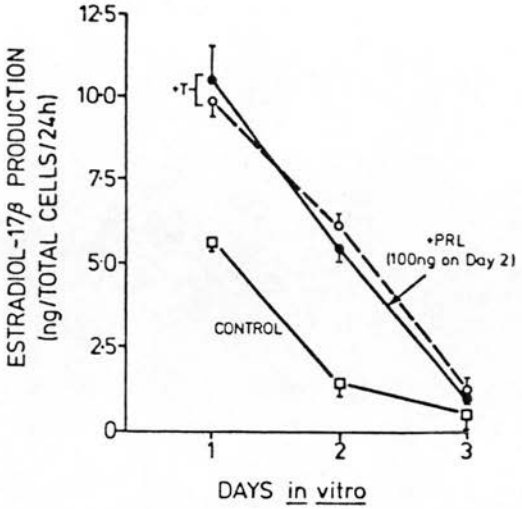



FIG. 1. Changes in the levels of 17$\beta$-$E_2$ in medium from human granulosa harvested from a preovulatory follicle 30 hr after the start of the LH surge. Cells were cultured in medium containing 5% fetal calf serum alone or in the presence of testosterone (100 ng/ml). On day 2, half of the culture dishes received human PRL (100 ng/ml) in addition to testosterone. Results are mean ± SEM ($N$=4–6).

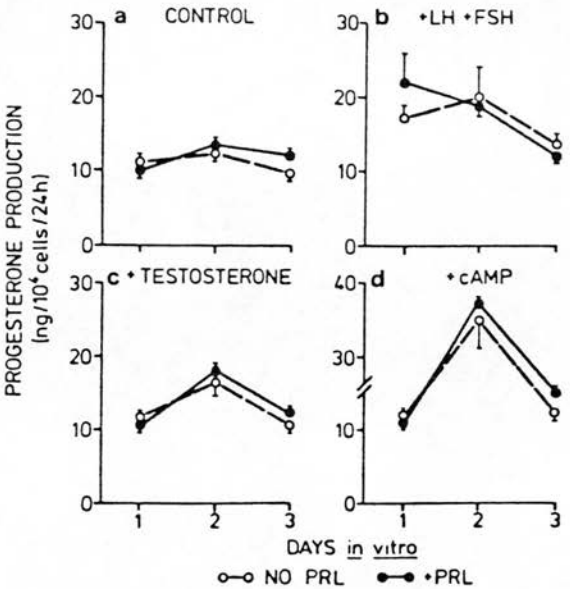



FIG. 2. Changes in the levels of progesterone in medium from human granulosa cells were harvested from a preovulatory follicle 30 hr after the start of the LH surge (see Fig.1 for details). Cells were cultured in medium alone (a), or with LH and FSH (30 ng/ml) (b), testosterone (100 ng/ml) (c), or on dibutryl cyclic AMP (0.1 mM) (d). Human

granulosa cells *in vitro*, we have reinvestigated these effects using granulosa cells aspirated from preovulatory follicles at the time of oocyte recovery in the above experiments. The follicle contents were aspirated using a laparoscopic technique (5,6) between 24 and 30 hr after the start of the LH surge; i.e., all granulosa cells had been exposed to the preovulatory surge of Lh and were beginning to luteinize. Cells were cultured for 3 days at 37°C as described previously (16,17) using medium containing 5% fetal calf serum. Cells were cultured in the presence of LH, FSH, testosterone or cyclic adenosine monophosphate (AMP) added in medium alone. The PRL level in this medium was around 2 to 3 ng (40 to 60 $\mu$U)/ml. Medium was removed daily, and on day 2 additions were made in medium containing 100 ng (2,000 $\mu$U)/ml human PRL. This addition of increased levels of PRL did not alter either basal or stimulated levels of either $E_2$ (Fig.1) or progesterone (Fig.2). The levels of PRL added were similar to those which inhibited progesterone secretion in previous studies using the same culture system (16,17). The reason for this discrepancy is unclear but requires investigation. It may be that luteinization of granulosa cells *in vitro* does not induce all the changes within the cells that occur when luteinization occurs *in vivo*. In the present study, all granulosa cells were harvested from preovulatory follicles which had luteinized *in vivo* prior to *in vitro* culture. Indeed the effects of high levels of PRL on progesterone secretion from porcine granulosa cells changes from inhibition to stimulation as these cells mature (28). It is clear that many more studies are required to clarify this situation.

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# CHANGES IN THE BINDING OF HUMAN CHORIONIC GONADOTROPHIN/LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE AND PROLACTIN TO HUMAN CORPORA LUTEA DURING THE MENSTRUAL CYCLE AND PREGNANCY

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SUMMARY

The changes in the binding of human chorionic gonadotrophin/luteinizing hormone (HCG/LH), follicle-stimulating hormone (FSH) and prolactin to 44 corpora lutea have been assessed during the luteal phase of the human menstrual cycle and early pregnancy. All corpora lutea bound HCG but out of 32 only ten bound FSH and only seven bound prolactin specifically. While binding of HCG increased to maximal levels in the mid-luteal phase, binding of FSH and prolactin was most often found in the early luteal phase. Maximum binding of HCG was associated with maximum serum levels of progesterone. Luteal regression was associated with a decrease in the binding of HCG but a causal relationship could not be established. Very low binding of HCG was found to corpora lutea of pregnancy.

These results show that (1) the changes in binding of HCG during the luteal phase of the human menstrual cycle are similar to those in other species and (2) there are specific binding sites for prolactin and FSH in the human corpus luteum.

## INTRODUCTION

The reasons for the changes in steroid content and secretory activity of the human corpus luteum throughout the luteal phase and in pregnancy are as yet unknown. Maintenance of luteal function requires the presence of minimal serum levels of luteinizing hormone (LH; Jewelewicz, Dyrenfurth, Warren, Joshi & Vande Wiele, 1974) which exerts its action through receptors which have been demonstrated by specific binding of $^{125}$I-labelled human LH or chorionic gonadotrophin (HCG) to homogenates of human corpora lutea (Cole, Weed, Schneider, Holland, Geary & Rice, 1973; Lee, Coulam, Jiang & Ryan, 1973; Wardlaw, Lauerson & Saxena, 1975; Rao, Griffin & Carman, 1977; Halme, Ikonen, Rutanen & Seppala, 1978). More information is needed about the precise changes in the binding of HCG/LH to the human corpus luteum during the luteal phase and the relationship between these changes and the binding of follicle-stimulating hormone (FSH) and prolactin. The present study was designed to relate the changes in the binding of HCG/LH, FSH and prolactin to changes in the steroid content of the corpus luteum and in levels of progesterone, oestradiol, LH, FSH and prolactin in the circulation during the luteal phase of the human menstrual cycle and pregnancy.

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## MATERIALS AND METHODS

### *Subjects*

Corpora lutea were obtained from 44 women at laparotomy for non-endocrine conditions at different stages of the menstrual cycle. Their average age was 38 ± 0·9 (S.E.M.) years and their mean cycle length was 31 ± 3 (S.E.M.) days. Informed consent for enucleation of the corpus luteum and the endometrial biopsy was obtained before surgery. Indications for surgery were as follows: menorrhagia (21), fibroids (12), tubal ligation (5), abdominal pain (3), Stage 1 carcinoma of the cervix (1) and ectopic pregnancy (2).

### *Dating of the menstrual cycle*

An endometrial biopsy and a peripheral blood sample (collected before enucleation of the corpus luteum) were obtained during operation. The stage of the luteal phase of the menstrual cycle was determined from the histology of the endometrium (Noyes, Hertig & Rock, 1950) and corpus luteum (Corner, 1956), the date of the last menstrual period and the concentrations of LH, FSH and prolactin in serum. Corpora lutea were placed into one of 5 groups: recent ovulation (<48 hours), early luteal phase (14–18 days), mid-luteal phase (19–25 days), late luteal phase (25–28 days) and corpus albicans. In addition corpora lutea were obtained from one normal pregnancy (day 37) and two ectopic pregnancies (day 69 and day 74).

### *Collection of corpora lutea*

After enucleation from the ovary, the corpus luteum was trimmed of extraneous tissue, cut into pieces and weighed on a torsion balance. One piece of corpus luteum was placed in 10% formol–saline solution for histological examination. A further piece was homogenized for 1 min on an I.L.A. homogenizer (Type × 10/20) and the steroids extracted as described in detail previously (Swanston, McNatty & Baird, 1977). The extracts (final volume 2·0 ml) were stored at −20 °C until assayed for oestradiol and progesterone. Further samples of corpus luteum (100–300 mg) for estimating the binding of HCG/LH, FSH and prolactin were placed in tubes, snap-frozen in a bath containing methanol and solid $CO_2$ within 30 min of enucleation from the ovary and stored at −20 °C for up to 8 months.

### *Oestradiol and progesterone contents of corpus luteum*

Prior to radioimmunoassay, oestradiol-17β was isolated from the extract by chromatography on a Sephadex LH-20 column using a modification of the method described by Carr, Mikhail & Flickinger (1971). Briefly, 0·2 ml of extract was evaporated to dryness and the residue dissolved in 0·1 ml benzene : methanol (15 : 85, v/v). Recovery was estimated by the addition of trace amounts (2000 d.p.m.) of [$^3$H]oestradiol in 0·02 ml ethanol. This was then applied to a Sephadex LH-20 column (14 × 1 cm) to remove non-specific interfering substance(s) and the oestradiol fraction was eluted with this solvent. The eluate was then dried, dissolved in 0·5 ml phosphate-buffered saline pH 7·0 and 0·1 ml taken as an estimate of recovery. Duplicate 0·1 ml portions from all corpora lutea were assayed in the same assay for oestradiol as described previously (Van Look, Hunter, Corker & Baird, 1977) and the results corrected for recovery.

Progesterone in the extract was assayed without further purification with the specific radioimmunoassay using sheep anti-progesterone antiserum 91920/9 described in detail previously (Scaramuzzi, Corker, Young & Baird, 1974).

### *Binding of HCG/LH, FSH and prolactin to corpora lutea*

The following hormone preparations were used for iodination and/or as standards for cross-reaction studies: HCG, CR119 (11 600 i.u./mg) and Pregnyl (Organon, Morden, Surrey, U.K.); human LH, MRC 68/40 (77 i.u./ampoule) and human LH (LH4, 5110 i.u./mg);

human FSH, CPDS 16 (5000 i.u./mg from Professor W. R. Butt, Birmingham) and MRC standard 69/104 (10 i.u. FSH, 25 i.u. LH); human prolactin, 78-5-18 (30 i.u./mg) and 77-8-12 (6 i.u./mg) both from Dr H. G. Friesen, Winnipeg, Canada; human placental lactogen (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.); human growth hormone (GH; NIH HGH iodination grade HS216OE 1·7 i.u./mg); human thyroid-stimulating hormone (TSH; Hartree De 32-3) and ovine prolactin (NIH PS-9, 30·3 i.u./mg).

In order to compare the binding activity at different stages of the luteal phase, 33 samples were processed simultaneously and initial binding studies for HCG, FSH and prolactin were performed at the same time. A further 11 corpora lutea were subsequently collected and analysed for HCG/LH binding only. Preliminary studies indicated less than 5% loss in binding activity of the corpora lutea when stored as whole pieces of tissue for periods of 8–9 months. For binding studies corpora lutea were prepared by homogenization (1/5, w/v) in 0·01 M-Tris–HCl buffer pH 7·5 containing 0·1% bovine serum albumin (BSA) at 4 °C for 20 s with a Polytron (Kinematica, Lucerne, Switzerland) at half maximum speed. The homogenate was then centrifuged at 600 *g* for 1 h at 4 °C, the supernatant fraction was removed and the precipitate was resuspended in buffer to give a concentration of 100 mg corpus luteum/ml. Because the amount of blood clot in each individual corpus luteum was variable a portion of the final homogenate was assayed for DNA (Burton, 1956) and the results of binding for each hormone are reported per μg DNA.

Preliminary experiments using each iodinated hormone preparation and the appropriate receptor preparation showed that 0·01 M-Tris–HCl containing 0·1% BSA and 0·01 M-$MgCl_2$ was suitable for assessment of the binding of all three hormones. Thus this buffer was used throughout the binding studies.

### *Human chorionic gonadotrophin*

The binding of HCG/LH was assessed with HCG CR119 labelled with $^{125}I$ by the lactoperoxidase method (Miyachi, Vaitukaitis, Nieschlag & Lipsett, 1972). Specific activities, calculated by isotope recovery after column chromatography on Sephadex G-100, were 33 and 38 Ci/g for the two tracers used in these studies. The biological activity of HCG iodinated by this method has been shown to be fully retained (Sharpe, Fraser & Sandow, 1979). Both tracers showed binding within the expected range (100–150 pg/20 mg testis) using a homogenate of adult rat testis (Sharpe, 1976; Sharpe & McNeilly, 1979).

Preliminary studies using homogenates from two corpora lutea collected during the mid-luteal phase showed that binding (1–14%, 1000–14 000 c.p.m.) was linear over a range 1–20 mg (2–40 μg DNA) of homogenate.

Binding increased to reach a plateau after 2 h incubation at 37 °C and remained constant for 4 h. At 4 °C binding achieved only 57% ($n = 4$) of that at 37 °C even after 24 h incubation. After incubation with homogenate for 3 h the unbound $^{125}I$-labelled HCG was separated from the homogenate by centrifugation and added to fresh portions of testicular homogenate. Binding of the tracer after incubation was similar to that of tracer not incubated with homogenates of human corpora lutea ($147 \pm 8$ (S.D.) *v.* $136 \pm 13$ pg $^{125}I$-labelled HCG bound/20 mg testis ($n = 6$) respectively). Therefore to assess binding in all human corpora lutea 0·1 ml of homogenate was added to 7 ml polystyrene tubes which were then incubated in duplicate or triplicate with $^{125}I$-labelled HCG (2 ng hormone) and 0·05 ml buffer for 3 h at 37 °C with shaking. Preliminary experiments indicated that these conditions gave maximum binding. Non-specific binding was assessed by the addition of 100 i.u. HCG (Pregnyl) to the buffer. Incubation was terminated by the addition of 3 ml cold buffer and the tubes were centrifuged at 1500 *g* for 30 min at 4 °C. The supernatant fluid was decanted and the precipitate counted in a well-type gamma counter (LKB Wallac, Bromma, Sweden). Cross-reaction studies were carried out subsequently on five corpora lutea homogenates showing the highest specific binding. Scatchard analysis of binding to homogenates of

corpora lutea from early ($n = 2$), mid- ($n = 2$) and late ($n = 1$) luteal phases were performed with 8 point duplicate determinations of binding of the range 0–32 mi.u. added HCG and the initial linear portion (0–16 mi.u HCG) was used to calculate the affinity constant, $K_a$.

*Follicle-stimulating hormone*

The binding of FSH was assessed with human FSH (CPDS 16) labelled with $^{125}$I by the lactoperoxidase method which retains biological activity (Cheng, 1976). Specific activity (40 Ci/g) was calculated by isotope recovery after column chromatography on Sephadex G-100. Binding activity was taken as acceptable when more than 20 pg (approximately 4000 c.p.m.) of the $^{125}$I-labelled human FSH was bound specifically to 100 μg of a homogenate of adult rat testis after incubation at 37 °C for 3 h. Since it was not possible to preselect any human corpus luteum to assess optimum incubation conditions for binding before all corpora lutea were assessed, probable optimum conditions were investigated using a homogenate of adult rat testis (100 μg wet wt/ml) prepared as described above for the human corpora lutea. Incubations with and without excess unlabelled human FSH (1 i.u. MRC human FSH standard 69/104) at 4 °C, 22 °C or 37 °C for times varying from 30 min to 24 h showed that maximum specific binding occurred after incubation for 24 h at 22 °C or 3 h at 37 °C (23–32 pg (4–7%) bound; $n = 12$). Thus to assess binding of $^{125}$I-labelled FSH to human corpora lutea incubations were carried out for 3 h at 37 °C using the same conditions, volumes of incubation and separation procedure as that employed for the HCG binding studies. Non-specific binding was assessed by the addition of 1 i.u. human FSH standard MRC 69/104, an amount five times greater than required for maximum displacement of the $^{125}$I-labelled FSH from the rat testicular homogenate.

Subsequently these incubation conditions were reassessed using two human corpora lutea which did show binding of FSH and the conditions chosen were shown to be optimum. Cross-reaction studies were carried out on the two corpus luteum preparations which showed highest specific binding.

*Prolactin*

The binding of prolactin was assessed with human prolactin (Friesen 78-5-18) labelled with $^{125}$I by the lactoperoxidase method which retains biological activity (Shiu, Kelly & Friesen, 1973). Specific activity (45 Ci/g) was calculated by isotope recovery after column chromatography on Sephadex G-100. The binding activity of the $^{125}$I-labelled human prolactin was assessed using a homogenate of late pregnant rat liver (Posner, Kelly, Shiu & Friesen, 1974) and was taken as acceptable when more than 900 pg (13 000 c.p.m.) were bound specifically to 100 μg of homogenate after incubation for 3 h at 22 °C. Non-specific binding was assessed by the addition of 5 μg ovine prolactin (NIH PS-9).

As with FSH it was not possible to preselect a human corpus luteum which would bind prolactin to assess the optimum conditions for binding before all corpora lutea were assessed. Thus preliminary studies were carried out using homogenates of ovaries from mice pretreated with pregnant mare serum gonadotrophin (10 i.u. Folligon; Intervet Laboratories Ltd, Cambridge, U.K.) followed 2 days later by HCG (10 i.u. Chorulon; Intervet Laboratories Ltd). Incubation volumes and conditions tested were the same as for FSH and results showed that optimum specific binding (approximately 320 pg (6–7%) $^{125}$I-labelled human prolactin bound/100 μg homogenate; $n = 6$) was achieved after incubation for 3 h at 22 °C. Thus these incubation conditions were adopted to assess binding to all human corpora lutea. Non-specific binding was assessed by the addition of 10 μg human prolactin (Friesen 77-8-12). This preparation was 80% pure by radioimmunoassay but had only approximately 20% (6 i.u./mg) of the expected biopotency by radioreceptor assay (I. Worsley, personal communication). The incubation conditions were reassessed using a human corpus luteum homogenate which had subsequently been shown to bind prolactin

and were found to be adequate. Cross-reaction studies were carried out on five corpus luteum preparations which showed the highest binding.

### *Radioimmunoassays*

Plasma levels of LH and FSH were determined using double-antibody radioimmunoassays utilizing antisera to LH (F87/4673) and FSH (M 93) and $^{125}$I-labelled LH (MRC 75/569) and $^{125}$I-labelled FSH (CPDS 16) respectively (McNeilly & Hagen, 1974; Hunter & Bennie, 1979). The results are expressed in terms of i.u./l of LH standard MRC 68/40 (77 i.u./ampoule) and FSH standard MRC 69/104 (10 i.u./ampoule). Plasma levels of prolactin were measured by a specific double-antibody radioimmunoassay using reagents supplied by Dr H. G. Friesen (McNeilly & Hagen, 1974; Hwang, Guyda & Friesen, 1975). Results are expressed as mi.u./l in terms of MRC prolactin standard 71/222 (10 i.u./ampoule).

Progesterone and oestradiol in plasma and extracts of corpus luteum were assayed by previously described radioimmunoassays (Swanston *et al.* 1977). All plasma samples and corpus luteum extracts were assayed in duplicate in the same assay with intra-assay coefficients of variation of 9, 7, 10, 15 and 11% for LH, FSH, prolactin, progesterone and oestradiol respectively using quality control samples reading between 15 and 80% $B/B_0$.

## RESULTS

### *Plasma levels and corpus luteum concentrations of hormones*

Mean values ($\pm$S.E.M.) obtained for the concentrations of LH, FSH, prolactin, progesterone and oestradiol in peripheral plasma at different stages of the luteal phase of the menstrual cycle are shown in Fig. 1. Preliminary analysis of these results showed no significant difference in any endocrine parameters between those patients with menorrhagia and the other patients in the study. Thus all results have been pooled. Corpus luteum weight and concentrations of progesterone and oestradiol within the corpora lutea (mean $\pm$S.E.M.) are given in Table 1. In ten cases the corpus luteum was not enucleated completely and these weights are not included. Similarly in 12 cases insufficient luteal tissue was available for estimation of the steroid content. The mean weight ($\pm$S.E.M.) of the corpus luteum increased from $1{\cdot}18 \pm 0{\cdot}32$ g within 2 days of ovulation to reach a maximum of $1{\cdot}94 \pm 0{\cdot}23$ g in the mid-luteal phase. The weights of the corpora albicantia ($1{\cdot}21 \pm 0{\cdot}23$ g) were similar to the weights of the corpora lutea in the recent ovulation phase. The corpora lutea of pregnancy were heavier than those of the normal cycle.

The concentration of progesterone within the corpus luteum was highest in the recent ovulation and early luteal phases and then declined significantly ($P < 0{\cdot}001$) in the late luteal phase and in the corpus albicans. The concentration of oestradiol within the corpus luteum did not vary significantly until the late luteal phase and in the corpora albicantia when levels were significantly lower ($P < 0{\cdot}001$) than before. The concentrations of progesterone within the corpora lutea from the normal pregnancy and one ectopic pregnancy were within the range found in the mid-luteal corpora lutea, while the level in the corpus luteum from the other ectopic pregnancy was similar to that in the corpus albicans. Oestradiol concentrations were all within the range for mid-luteal corpora lutea.

### *Binding of HCG/LH, FSH and prolactin to corpora lutea*

#### *Human chorionic gonadotrophin/luteinizing hormone*

Binding of $^{125}$I-labelled HCG occurred in all 44 corpora lutea examined and increased significantly ($P < 0{\cdot}001$) from $1{\cdot}68 \pm 0{\cdot}42$ (S.E.M.) pg/μg DNA within 2 days of ovulation to reach a maximum of $15{\cdot}06 \pm 3{\cdot}34$ pg/μg DNA in the mid-luteal phase. Binding then declined significantly ($P < 0{\cdot}001$) and in non-pregnancy was lowest ($0{\cdot}78 \pm 0{\cdot}26$ pg/μg DNA) in corpora albicantia (Fig. 2). There was no correlation between the serum levels of LH and the

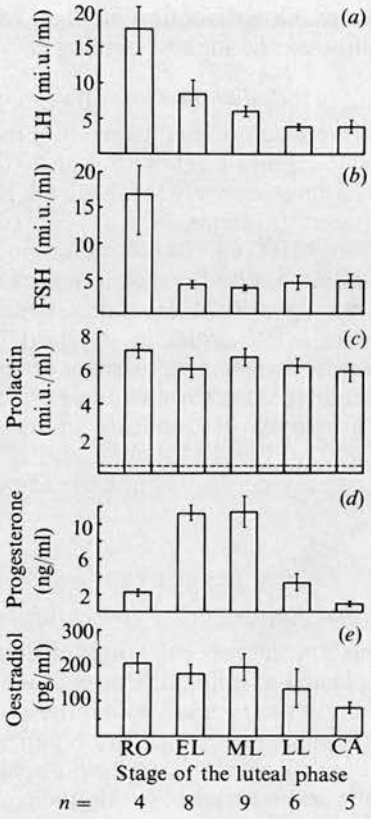



Fig. 1. Changes in the mean (±S.E.M.) plasma levels of (*a*) LH, (*b*) FSH, (*c*) prolactin, (*d*) progesterone and (*e*) oestradiol during the different stages of the luteal phase of the menstrual cycle. RO, recent ovulation; EL, early luteal phase; ML, mid-luteal phase; LL, late luteal phase; CA, corpus albicans. Numbers of observations (*n*) are given on the abscissa.

Table 1. *Weight and concentrations of oestradiol and progesterone (mean ±S.E.M) in human corpora lutea at various stages of the menstrual cycle (n, number of corpora lutea used)*

| | | | | Steroid concentrations | |
|---|---|---|---|---|---|
| | | Weight | | Progesterone | Oestradiol-17β |
| Stage | *n* | (g) | *n* | (μg/g) | (ng/g) |
| Recent ovulation | 5 | 1·18 ± 0·32 | 3 | 23·6 ± 4·7 | 131 ± 16 |
| Early luteal | 9 | 1·67 ± 0·25 | 9 | 30·5 ± 5·1 | 143 ± 38 |
| Mid-luteal | 14 | 1·94 ± 0·23 | 12 | 18·9 ± 2·3 | 190 ± 13 |
| Late luteal | 6 | 1·65 ± 0·33 | 6 | 5·0 ± 0·8* | 57 ± 15* |
| Corpus albicans | 4 | 1·21 ± 0·23 | 4 | 2·7 ± 1·0* | 36 ± 11* |
| Pregnancy | | | | | |
| Normal | 1 | 2·53 | 1 | 14·7 | 121 |
| Ectopic | 2 | 2·21 & 3·75 | 2 | 2·1 & 10·1 | 84 & 107 |

* $P < 0{\cdot}001$ compared with values for recent ovulation, early luteal and mid-luteal phases (Student's *t*-test).

binding of HCG. The lowest binding (0·06 pg/μg DNA) was found in a corpus luteum taken during the third week of a normal pregnancy. Two corpora lutea collected from women with ectopic pregnancies approximately 10 weeks after the last menstrual period bound 0·96 and 2·48 pg/μg DNA respectively. Serum levels of HCG were 3016 and 401 i.u./l respectively

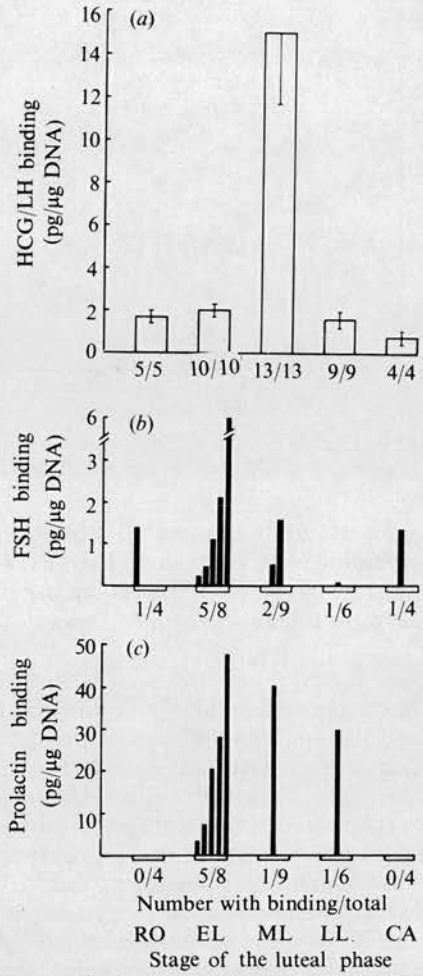



Fig. 2. Specific binding of (*a*) human chorionic gonadotrophin (HCG)/LH, (*b*) FSH and (*c*) prolactin to homogenate of human corpora lutea collected at the different stages of the luteal phase of the menstrual cycle. All corpora lutea bound HCG/LH and results are expressed as means ± S.E.M. The binding of FSH and prolactin are represented as individual results since binding did not occur to all corpus luteum preparations. The numbers of corpora lutea showing binding in relation to those examined are shown for each hormone and for each stage of the luteal phase. RO, recent ovulation; EL, early luteal phase; ML, mid-luteal phase; LL, late luteal phase; CA, corpus albicans.

and 5400 i.u./l for the normal pregnancy and did not relate to the binding of HCG to the corpus luteum.

The specificity of binding of $^{125}$I-labelled HCG was assessed in five corpora lutea and was specific for HCG and LH although HCG was 8–12 times more potent than LH. Binding was not inhibited by TSH, FSH, prolactin or GH (Fig. 3). Scatchard analysis gave a mean $K_a$ of $4{\cdot}5 \pm 1{\cdot}8$ (S.E.M) $\times 10^{-10}$ for $^{125}$I-labelled HCG ($n = 5$) and did not differ with the stage of the luteal phase.

*Follicle-stimulating hormone*

Only 10 of the 32 corpora lutea assessed bound $^{125}$I-labelled human FSH (Fig. 2). The maximum number (five out of eight) of corpora lutea showing binding occurred in the early luteal phase where binding ranged from 0·23 to 5·98 pg/μg DNA and was unrelated to

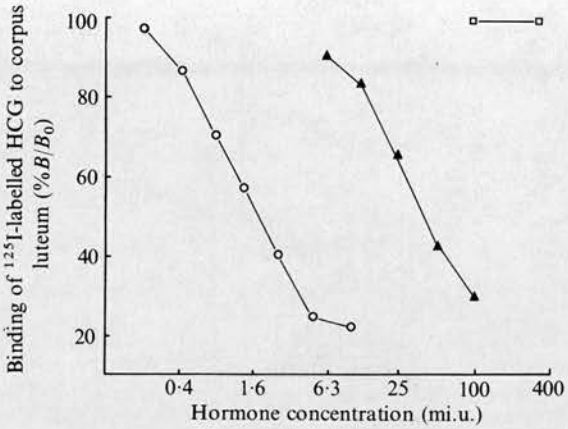



Fig. 3. Specificity of binding of $^{125}$I-labelled human chorionic gonadotrophin (HCG) to a single corpus luteum. (○) Human chorionic gonadotrophin (CR119); (▲) human LH (MRC 68/40); (□) FSH, TSH, GH and prolactin.

previous clinical history. The maximum binding in all other corpora lutea was 1·58 pg/μg DNA (mid-luteal phase). The binding of $^{125}$I-labelled FSH was specific for FSH and was not inhibited by HCG (500 mi.u.), LH (100 mi.u.), TSH (100 mi.u.), prolactin (100 mi.u.) or GH (100 mi.u.). Binding to the corpora lutea of pregnancy was not assessed.

*Prolactin*

Only 7 of the 32 corpora lutea assessed bound $^{125}$I-labelled human prolactin (Fig. 2). Binding ranged between 3·8 and 48·2 pg/μg DNA and more corpora lutea (five out of eight) bound $^{125}$I-labelled prolactin in the early luteal phase than at other stages of the luteal phase (Fig. 2), no relationship being found with previous clinical history. No binding was found in either the recent ovulation or corpora albicantia groups. Only the corpus luteum from the normal pregnancy was assessed and was found to bind 26·9 pg/μg DNA. There was no correlation between binding and the serum levels of prolactin.

The binding of $^{125}$I-labelled human prolactin was specific for prolactin ($n = 5$) and was not inhibited by FSH, LH, HCG or TSH (Fig. 4). Human GH showed minimal cross-reaction (2–8%) while ovine prolactin and human placental lactogen showed 40 ± 5% and

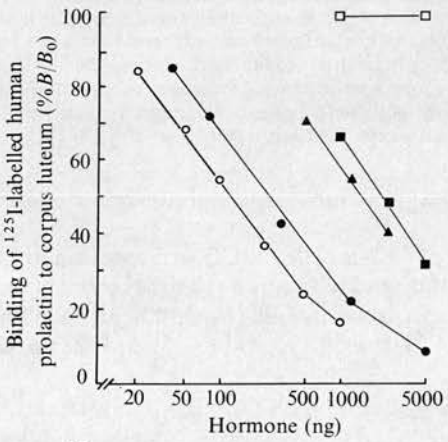



Fig. 4. Specificity of binding of $^{125}$I-labelled human prolactin to a single corpus luteum. (○) Human prolactin; (●) ovine prolactin; (▲) human GH; (■) human placental lactogen; (□) FSH, TSH, LH and human chorionic gonadotrophin.

0·9 ± 0·2% ($n = 3$) cross-reaction respectively. The low cross-reactivity of human placental lactogen was similar to that seen in comparison to ovine prolactin (0·8 ± 0·1%; $n = 2$) in the rat liver receptor assay.

## DISCUSSION

The changes in the levels of LH, FSH, progesterone and oestradiol-17β in plasma and of progesterone and oestradiol-17β in the corpus luteum during the luteal phase of the human menstrual cycle are similar to those reported previously (McNatty, Hunter, McNeilly & Sawers, 1975; Swanston *et al.* 1977).

All corpora lutea examined bound $^{125}$I-labelled HCG with a $K_a$ of $4{\cdot}5 \times 10^{-10}$ similar to that previously reported for the human corpus luteum (Cole *et al.* 1973; Lee *et al.* 1973; Wardlaw *et al.* 1975; Rao *et al.* 1977; Halme *et al.* 1978). While the binding was specific for HCG and LH, HCG was more potent confirming previous reports in the human (Rao *et al.* 1977; Halme *et al.* 1978) and other species (Rao, 1974; Papaionannou & Gospodarowicz, 1975; Stouffer, Tyrey & Schomberg, 1976). While the biopotency of the HCG preparation was twofold greater than for the human LH preparation used, the receptor activity was between 8 and 12 times greater. Thus, the reason for this difference is unclear but may relate to a lower affinity of the receptor for human LH.

The increase in binding of $^{125}$I-labelled HCG in the mid-luteal phase probably represents an absolute increase in the binding per cell since the binding is assessed per μg DNA. In addition the number of cells within the corpus luteum do not appear to increase during the luteal phase (McClellan, Diekman, Abel & Niswender, 1975). Maximal binding of HCG/LH coincided with the time of maximal progesterone secretion. A similar increase in the number of LH receptors in the corpus luteum in relation to maximum progesterone secretion has been demonstrated in the rat (Lee, Tateishi, Ryan & Jiang, 1975) and sheep (Diekman, O'Callaghan, Nett & Niswender, 1978). However, in the present study maximum binding of HCG and serum levels of progesterone were associated with a decrease in the concentration of progesterone within the corpus luteum.

The reason for the decrease in binding of HCG/LH in the late luteal phase remains obscure but is similar to results in other species (Rao *et al.* 1977; Diekman *et al.* 1978). It is unlikely that this loss in binding directly causes luteal regression since binding of HCG/LH during pregnancy is very low but progesterone secretion is maintained and enhanced. In early pregnancy, substantial amounts of HCG are found in serum only during the late luteal phase of the cycle when the decrease in HCG/LH binding has already occurred (Corker, Michie, Hobson & Parboosingh, 1976). Nevertheless, the corpus luteum remains responsive to HCG both *in vivo* and *in vitro* (Savard, Marsh & Rice, 1965; Jewelewicz *et al.* 1974).

The lowest binding of HCG/LH was found in corpora albicantia which also contain high levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Swanston *et al.* 1977). Work in other species has implicated $PGF_{2\alpha}$ in the mechanism of luteolysis and injection of $PGF_{2\alpha}$ in the rat and sheep causes a rapid decrease in progesterone secretion followed by a decrease in binding of LH (Hichens, Grinwich & Behrman, 1974; Diekman *et al.* 1978). It has been suggested (Henderson & McNatty, 1977) that as the luteal phase progresses, $PGF_{2\alpha}$ exerts maximum luteolytic action only when the biosynthetic activity of the corpus luteum is decreased. It has been shown previously (Jewelewicz *et al.* 1974) that the human corpus luteum requires a minimum but constant supply of LH to continue secreting progesterone. In the non-pregnant cycle serum levels of LH are lowest during the late luteal phase at a time when binding of HCG/LH is also decreased. This may predispose the human corpus luteum to the luteolytic effects of $PGF_{2\alpha}$, an effect which can only be overcome by a massive increase in LH stimulation provided by the increase in circulating levels of HCG occurring at this time if conception has occurred. However, the reason for the decline in binding of HCG/LH in the late luteal phase remains to be discovered.

The binding of FSH to the human corpus luteum has not been reported before. Binding was specific for FSH, but occurred in only 10 out of 32 of the corpora lutea examined. It is of considerable interest that six out of the ten corpora lutea showing binding were from the recent ovulation or early luteal phase of the cycle, a time when FSH is maximally active in stimulating the secretion of oesradiol-17β from human corpora lutea *in vitro* (M. Hunter, personal communication). The role of FSH in luteal function in the human is unknown.

Specific binding of prolactin could be demonstrated in only 7 out of 32 of the corpora lutea examined and as with FSH the majority of corpora lutea binding prolactin were found in the early luteal phase. Nevertheless only four corpora lutea bound both prolactin and FSH. All corpora lutea were collected under anaesthesia which resulted in very high plasma levels of prolactin. Therefore it is possible that these high levels of prolactin bound to the receptors in the corpora lutea and masked the receptors. A similar effect is found in the rabbit where high plasma levels of prolactin are associated with a decrease in binding of prolactin to the mammary gland and milk fat droplet membrane fractions (McNeilly & Friesen, 1977; Waters, McNeilly, Ohgo & Friesen, 1980). Prolactin appears to be essential for the maintenance of luteal function in several species, including man (McNatty, Sawers & McNeilly, 1974), although the amount of prolactin involved is minimal. For example, adequate luteal function can be maintained in women in whom prolactin levels are less than 25% of the lower limit of normal following hypophysectomy (A. S. McNeilly & D. T. Baird, unpublished observations).

The binding of HCG/LH to the human corpus luteum was low in early and newly formed corpora lutea, increased to a maximum in the mid-luteal phase, then declined dramatically in late and atretic corpora lutea. These changes were similar to those reported for HCG/LH binding during the luteal phase in other species. In contrast, FSH and prolactin binding to the human corpus luteum appears to be maximal in the early luteal phase. The physiological significance of, and the factors regulating, the changes in gonadotrophin receptors during the luteal phase of the human menstrual cycle remain to be established.

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# THE ROLE OF PROLACTIN IN SECONDARY AMENORRHOEA

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## INTRODUCTION

The presence of menstrual bleeding at regular monthly intervals is usually the bvious clinical manifestation of cyclical ovarian function. Hence the commonest resenting symptom in women with a disturbance of hypothalamic-pituitary-varian function is an alteration in menstrual pattern, e.g. amenorrhoea or menorr-agia.

Although destruction or failure of any of the components of the system ıterus, ovary, anterior pituitary or hypothalamus) may occur, by far the common-st cause of secondary amenorrhoea is thought to be a functional failure of the ypothalamic-pituitary unit (Dignam *et al.*, 1969). Cessation of menstruation due ɔ destruction of the endometrium (Asherman's syndrome), ovarian failure (pre-ıature menopause) and panhypopituitarism (e.g. Sheehan's syndrome) make up small minority of patients presenting with secondary amenorrhoea. In the re-ıainder it is presumed that ovarian inactivity is due to a lack of gonadotrophin ɛcretion secondary to a functional failure of hypothalamic function. A variety of ıctors, including stress, certain drugs (e.g. the combined contraceptive pill, reser-ine, phenothiazines), pregnancy and alteration in body weight are associated ·ith this state although in many cases no precipitating cause can be identified.

Most clinicians would agree that the minimum investigations in such patients ·ould include a full general physical and pelvic examination, haemoglobin con-ɛntration, thyroxine or protein bound iodine and an x-ray of the pituitary fossa. ı addition, measurements of urinary or serum follicle stimulating hormone will lentify those in whom there is primary ovarian failure. However, there is more ɔntroversy as to how helpful further investigations are in the clinical management



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of the individual patients. Clearly in those patients who are complaining of infertility, check of tubal patency and husband's sperm count would be necessary as well as some estimate of ovarian activity. Whether all patients with secondary amenorrhoea should have tests of hypothalamic-pituitary function will depend on the incidence in such patients of tumors and/or failure of the pituitary which could not be recognized by preliminary screening procedures. When we reviewed the literature in 1973 we were unable to find such information in a series of unselected patients with secondary amenorrhoea.

In Januay 1974 we initiated a prospective study of all patients presenting to the Gynaecological Endocrine Clinic, Royal Infirmary, Edinburgh, with the complaint of secondary amenorrhoea of at least 6 months' duration. By Novembe 1975, 47 such patients had been screened and this chapter reports the preliminary findings. We will pay particular attention as to the relevance or otherwise of the measurement of prolactin as an index of hypothalamic-pituitary disease.

## MATERIALS AND METHODS

All patients with a history of at least six months' secondary amenorrhoea referred to the Gynaecological Endocrine Clinic between January 1974 and November 1975 were screened for entry to the investigation. Preliminary screening included an initial assessment of pituitary-ovarian activity by the measurement once per week over a four to six week period of the urinary excretion of total oestrogen (Brown *et al.*, 1968), pregnanediol (Chamberlain and Contractor, 1968), luteinizing hormone (LH) and follicle stimulating hormone (FSH). This initial procedure established whether there was an absence of cyclical ovarian activity and those patients with primary ovarian failure (high urinary gonadotrophins) were excluded from the series.

A combined hypothalamic-pituitary function test was performed at the completion of the initial tracking. The patient arrived at the clinic between 8.00-9.00 a.m. fasting and a butterfly needle was placed in an ante-cubital vein. After a rest period of two and a half hours basal samples were taken at −30 and −5 mi At 0 time 200 μg thyrotropin-releasing hormone (TRH), 50 μg luteinizing hormo releasing hormone (LHRH) and 0.15 units per kilogram body weight insulin were injected intravenously through separate syringes. Further blood samples were taken at +20, +30, +60 and +90 min. Patients remained supine from arrival at the clinic until completion of the test. It has been shown previously that the release of pituitary hormones is not influenced by interaction between these combined hypothalamic (insulin) and pituitary (TRH and LHRH) stimuli (Mortimer *et al.*, 1973). Plasma samples were analysed for glucose, fluorogenic corticosteroids, and for thyroid-stimulating hormone (TSH), growth hormone (HGH), prolactin (HPr FSH, LH and 17β-oestradiol using established radioimmunoassays.

Three days later the patient was started on clomiphene (100 mg/day for 5 days) to test the integrity of the hypothalamic-pituitary system with respect to FSH and LH secretion. The concentrations of FSH and LH measured at 15 min intervals for a one and a half hour period between 10.00 and 11.30 a.m., were compared before and on the 5th day of clomiphene administration. The subsequent ovarian response was monitored by the measurement of total oestrogen an pregnanediol in 24-hour urine specimens collected once per week for a further 3 to 4 weeks. The response to clomiphene could then be classified into a) no respo

(N), b) abortive response ($A_1$) in which the total oestrogen excretion rose from less than 10 μg/24 h to between 10 and 20 μg/24 h but there was no ovulation, c) anovulatory response ($A_2$) in which the total oestrogen excretion rose above 20 μg/24 h but no ovulation occurred as indicated by the persistently low excretion of pregnanediol, and d) an ovulatory response (O) in which a rise in total oestrogen excretion was followed by a rise in the excretion of pregnanediol above 1.5 mg/24 h.

A rise in gonadotrophins during the clomiphene administration indicated the ability of the hypothalamic-pituitary system to respond to the negative feedback effect of oestrogen. The subsequent rise in oestrogen excretion reflected the development of Graafian follicles and the ability of the ovary to respond to the clomiphene-induced rise in gonadotrophins. While an ovulatory response to clomiphene (O) indicated that the positive feedback mechanism was intact; in patients in group N and $A_1$ the endogenous oestrogen stimulus was insufficient to test positive feedback. A failure of positive feedback could be deduced only in patients in group $A_2$ showing an anovulatory response.

The above test was also performed during the early follicular phase of the cycle (Days 3-5) in 8 healthy volunteers with a history of regular menstrual cycles. The early follicular phase was chosen to approximate as nearly as possible the low levels of plasma 17β-oestradiol and urinary total oestrogen excretion of the patients with secondary amenorrhoea.

## RESULTS

A classification of the 47 patients studied is illustrated in Table I. In only one patient was the presence of a pituitary tumour confirmed. Of the four patients with galactorrhoea, three followed pregnancy (Chiari-Frommel syndrome) and one did not (Argonz-del Castillo). The basal levels of prolactin were elevated above 20 ng/ml in 7 of the patients: 3 with amenorrhoea-galactorrhoea, 1 with a pituitary tumour, 1 with amenorrhoea on stopping the oral contraceptive pill and 2 with idiopathic or functional amenorrhoea. Basal prolactin concentrations were normal in all 14 cases in which the amenorrhoea was associated with weight loss with or

*Table I.* Associated features in 47 consecutive patients with secondary amenorrhoea of at least 3 months' duration.

| Associated Feature | Number of Patients | Prolactin >20 ng/ml | Galactorrhoea |
|---|---|---|---|
| Post-combined pill | 14 | 1 | 0 |
| Weight loss (>6 kg) | 8 | 0 | 0 |
| Weight loss + combined pill | 6 | 0 | 0 |
| Post-pregnancy | 3 | 2 | 3 |
| Tumour | 1 | 1 | 0 |
| Idiopathic | 15 | 3 | 1 |
| Totals | 47 | 7 | 4 |

without concomitant use of the pill and in one patient with amenorrhoea-galactorrhoea following pregnancy.

No abnormality in either the cortisol response to insulin-induced hypoglycaemia or in the TSH response to TRH was seen in any of the patients. An impaired response of HGH (maximum rise less than 10 ng/ml) to hypoglycaemia was found in two patients both of whom had hyperprolactinaemia. A pituitary adenoma was diagnosed and removed in one of these patients.

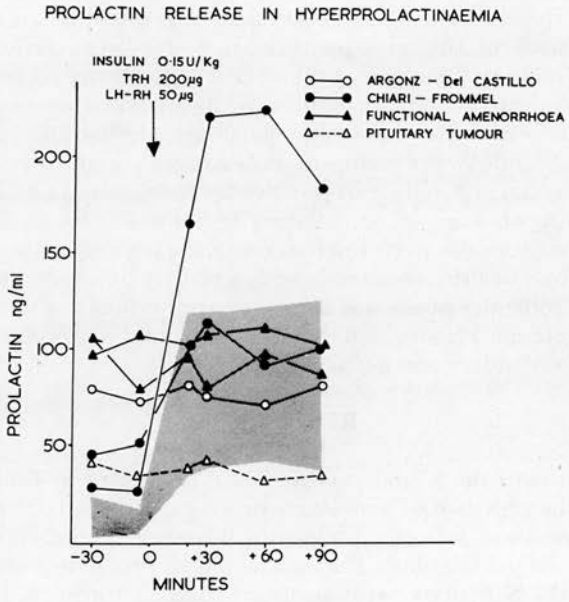



*Fig. 1* Peripheral prolactin levels (expressed in ng/ml of Friesen's human pituitary prolactin standard) before and after the intravenous injection of TRH (200 μg) and insulin (0.15 units/k body weight) in 6 women with hyperprolactinaemic secondary amenorrhoea. The shaded area represents the range observed in normal women.

Changes in peripheral prolactin levels following the injection of TRH and insulin are illustrated in Fig.1. The increase in prolactin secretion observed in the normal subjects and in the majority of patients with secondary amenorrhoea may be attributed to direct stimulation of the prolactin secreting cells of the anterior pituitary by TRH as well as to a decrease in hypothalamic secretion of prolactin release-inhibiting factor (PIF) induced by hypoglycaemia. Four of the patients with hyperprolactinaemia showed no further increase in response to TRH and insulin. Both the patients with amenorrhoea-galactorrhoea following pregnancy responded by a further increase in prolactin secretion. This may be due to the presence of an increased number of prolactin secreting cells which have persisted following pregnancy. The remaining patient (not illustrated in Fig.1) whose elevated basal levels of prolactin increased after TRH and insulin, had a normal response in respect of all other pituitary hormones and was the only patient with hyperprolactinaemia to have an ovulatory response to clomiphene. There was some technical difficulty in cannulating the vein of this patient, and it is possible that the elevated basal levels of prolactin reflect a degree of stress.

In the normoprolactinaemic patients basal levels of FSH and LH ranged from undetectable to elevated above the values found in normal women during the early follicular phase of the cycle. Peak levels of both FSH and LH after LHRH were correlated with the basal levels. The subsequent response to clomiphene was correlated to basal levels of FSH and LH although the "gonadotrophin index" (the product of the basal FSH and LH concentrations) showed the highest correlation with the response to clomiphene.

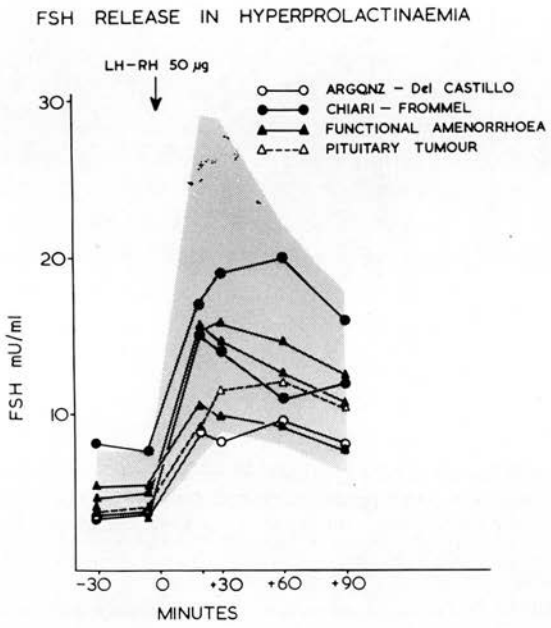



*Fig. 2* Plasma FSH levels (in mU MRC 68/39/ml) before and after the intravenous injection of 0 µg LHRH in 7 women with hyperprolactinaemic secondary amenorrhoea. The shaded area represents the range for normal women during the early follicular phase (days 3-5) of the cycle.

In patients with hyperprolactinaemia basal levels of FSH (Fig.2) were within the normal range but LH levels (Fig.3) ranged from below normal to elevated values. No correlation between peak levels of either FSH or LH following LHRH and basal levels of these hormones could be found in these patients.

The basal gonadotrophin-index in the patients with hyperprolactinaemia ranged from 4.5–30.9 (mean ± S.E.:22.0±7.0) and was statistically not significantly different from normal women (mean ± S.E.:18.7 ± 4.6). However, in contrast to normal women, only one of the hyperprolactinaemic patients had an ovulatory response to clomiphene (Table II). When compared to pretreatment values, a significant increase in gonadotrophin-index on the fifth day of clomiphene treatment was observed in 6 out of the 7 patients. No correlation could be found between basal prolactin levels and either the incremental change or absolute value of the gonadotrophin-index on the last day of clomiphene. Plasma 17$\beta$-oestradiol levels before and on the fifth day of clomiphene treatment however were significantly correlated with the corresponding gonadotrophin indices ($y = 1.16 + 1.88\ x$, where $y$ = 17$\beta$-oestradiol concentration and $x$ = gonadotrophin index; $r = 0.8868$, $n = 13$, $< 0.01$).

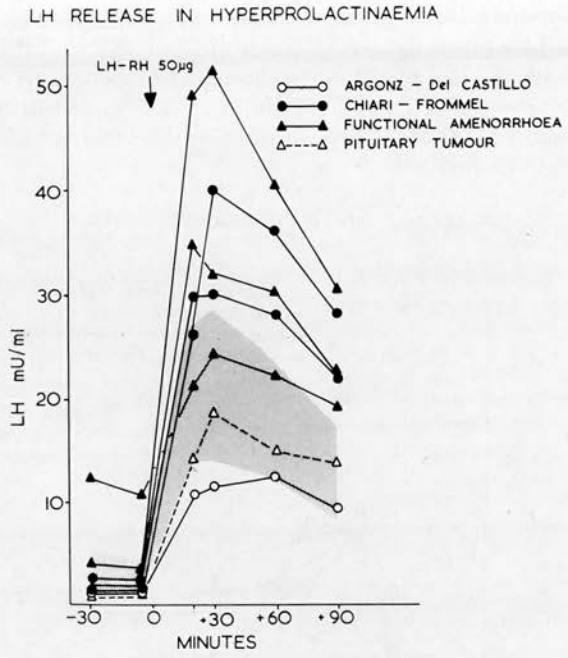



*Fig. 3* Plasma LH levels (in mU MRC 68/40/ml) before and after the intravenous injection of 50 µg LHRH in 7 women with hyperprolactinaemic secondary amenorrhoea. The shaded area represents the range for normal women during the early follicular phase (days 3-5) of the cycle

*Table II.* Classification of 47 consecutive patients with secondary amenorrhoea based on response to clomiphene.

| | | Number | Prolactin > 20 ng/ml | Galactorrhoea |
|---|---|---|---|---|
| Ovulatory | (O) | 11 | 1 | 0 |
| Anovulatory | ($A_2$) | 13 | 1 | 0 |
| Abortive | ($A_1$) | 13 | 2 | 3 |
| No response | ($N_1$) | 9 | 2 | 1 |
| Not known | | 1 | 1 | 0 |
| Totals | | 47 | 7 | 4 |

## DISCUSSION

The incidence of associated features in these patients with secondary amenorrhoea is similar to that reported in larger series (Franks *et al.*, 1975; Bohnet *et al.*, 1976). The endocrine state of patients with secondary amenorrhoea in association with stopping the combined oestrogen-gestogen pill, weight loss or stress is not unique to the precipitating factor. It would appear that hypothalamic function o these patients is liable to be upset by a variety of agents.

What is the significance of hyperprolactinaemia in patients presenting with secondary amenorrhoea (7 out of 47 in this series)? A considerable proportion of patients with non-functional pituitary tumours have elevated levels of prolactin (Jacobs and Daughaday, 1973) and measurement of prolactin may be a very useful screening procedure for this reason. In the remainder hyperprolactinaemia is presumed to be an indication of hypothalamic disease (Tolis *et al.*, 1974). The response to clomiphene was absent or anovulatory in 6 out of 7 patients with hyperprolactinaemia indicating impairment of hypothalamic function. Virtually all patients with amenorrhoea associated with hyperprolactinaemia have a failure of positive feedback indicating that in this respect hypothalamic function is abnormal (Glass *et al.*, 1975). Failure of positive feedback however is not confined to patients with hyperprolactinaemia but may occur in normoprolactinaemic secondary amenorrhoea (at least 13/47 in this series) or as an isolated deficiency in patients presenting with anovulatory dysfunctional uterine bleeding (Van Look *et al.*, 1975). It seems unlikely therefore that lack of positive feedback in hyperprolactinaemia is due solely to a direct inhibitory effect of the raised level of prolactin on the hypothalamus.

It has been suggested that the ovarian inactivity in hyperprolactinaemic states is due to a suppressive action of prolactin on ovarian activity (Reyes *et al.*, 1972). Prolactin when added to luteinized human granulosa cells in tissue culture in concentrations above 20 ng/ml suppresses progesterone production (McNatty *et al.*, 1974), and ovarian activity is minimal during suckling and lactation (Said *et al.*, 1973). In support of this concept is the fact that ovarian cyclicity is restored when levels of prolactin are lowered by 2-bromo-ergocryptine (Lutterbeck *et al.*, 1971; Besser *et al.*, 1972). However, bromocryptine is a dopamine agonist and can therefore be expected to lower prolactin not only by inhibiting its release from the pituitary cells (Friesen *et al.*, 1973) but also by increasing the synthesis and release of PIF by the hypothalamus. Since hypothalamic LHRH release is also mediated via a dopaminergic mechanism (Kamberi *et al.*, 1969) is seems not unreasonable to assume that bromocryptine may also influence hypothalamic LHRH release. Administration of bromocryptine in the post-partum period is associated with a rise in FSH (Seki *et al.*, 1974). Sulpiride on the other hand, a dopamine-antagonist which has been shown to elevate prolactin levels (L'Hermite *et al.*, 1972) reduced FSH and LH levels in postmenopausal women (Mancini *et al.*, 1975). As ovulatory cycles often return during bromocryptine administration it follows that the ability of the hypothalamus to release LH in response to oestrogen must be restored.

The fact that the post-partum ovary is relatively refractory to exogenous gonadotrophins (Zárate *et al.*, 1972) is often quoted as evidence that the high levels of prolactin associated with lactation inhibit the ovarian response to gonadotrophins. However, the ovary is still unresponsive to gonadotrophin injections even when the levels of prolactin are suppressed to normal by the administration of bromocryptine (del Pozo *et al.*, 1975), indicating that the refractoriness of the post-partum ovary is not due to the effect of prolactin alone. In the same study it was demonstrated that human menopausal gonadotrophins could induce follicular development in patients with hyperprolactinaemic secondary amenorrhoea but not in lactating women even though prolactin levels were similar in both groups. In the present study, peripheral 17$\beta$-oestradiol levels in hyperprolactinaemic patients were corre-

lated with peripheral gonadotrophin concentrations, as expressed by the gonadotrophin index, no matter what the etiology or degree of hyperprolactinaemia. This would suggest that ovarian steroid synthesis and secretion is possible even in the presence of elevated prolactin levels, provided an adequate gonadotrophin stimulus is present. Although the possibility cannot be excluded that an increased prolactin concentration may impair the follicular response to gonadotrophins, these observations make it unlikely that the absence of cyclical ovarian function in women with hyperprolactinaemic secondary amenorrhoea is due solely to an antigonadotrophic action of prolactin exerted at the gonadal level.

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# Adrenocortical Function in Hyperprolactinemic Women

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**ABSTRACT.** To study the effects of prolactin (PRL) on adrenocortical function in humans, dehydroepiandrosterone (DHA), dehydroepiandrosterone sulfate (DHAS), androstenedione (Δ) and testosterone (T) were measured in serum obtained from 35 hyperprolactinemic women with galactorrhea and amenorrhea before and after treatment with bromocriptine (39 courses). Associated with the bromocriptine-induced fall in mean PRL levels from 82 ± 8 (SE) to 14 ± 2 ng/ml (n = 39, $P < 0.0005$), DHAS fell from 322 ± 21 to 237 ± 21 μg/dl (n = 39); $P < 0.0005$), DHA fell from 492 ± 47 to 378 ± 30 ng/dl (n = 39; $P < 0.01$) while T (n = 16) and Δ(n = 13) levels were unchanged (44 ± 4 *vs.* 49 ± 4 ng/dl and 280 ± 55 *vs.* 236 ± 40 ng/dl, respectively). In addition, 4 women were infused iv with 25 μg synthetic ACTH over 4 h and serial blood samples drawn while hyperprolactinemic, and again 2–4 months later following normalization of PRL levels by bromocriptine. Although pre-infusion levels of DHAS were lower when PRL levels were normalized, no significant differences in responses of circulating DHAS, DHA, T, cortisol and 17-hydroxyprogesterone concentrations were detected between the two infusions. Since DHAS is virtually an exclusive product of the adrenal cortex, and since high PRL levels appear to inhibit ovarian steroid production, the findings suggest that hyperprolactinemia selectively stimulates adrenocortical androgen production. (*J Clin Endocrinol Metab* **45:** 973, 1977)

THE RECENT finding of prolactin (PRL) receptors on adrenal cell membrane preparations of lower mammals such as the rabbit, rat, sheep and cow (1, unpublished observations) has stimulated interest in the possibility that PRL may influence adrenocortical function. Evidence supporting this includes the finding of synergism between adrenocorticotropin (ACTH) and PRL in the stimulation of corticosterone production from isolated rat adrenal cells (2) and the finding of a stimulatory effect of PRL on aldosterone production by rat adrenal glands *in vitro* (3). In addition, experimentally-induced chronic hyperprolactinemia in male rats results in significant increases in adrenal gland weight (4).

Data suggesting an effect of PRL on adrenocortical function in man are scarce, although the occasional occurrence of hirsutism and seborrhea in women with galactorrhea and amenorrhea (5–7) has raised the possibility that hyperprolactinemia may be associated with excessive androgen production. Borderline elevated urinary 17-ketosteroids are often found in hyperprolactinemic women (5,7,8) and adrenal androgens, particularly dehydroepiandrosterone sulfate (DHAS) and dehydroepiandrosterone (DHA), are the major source of these metabolites (9). It thus appears possible that elevated PRL concentrations may increase androgen production *via* an effect on the adrenal cortex. Boyns *et al.* have provided the only direct evidence supporting this contention by demonstrating a positive effect of PRL on androstenedione and testosterone production from one human adrenal explant (10). Additionally, Giusti *et al.* recently reported that circulating DHAS concentrations in 10 women with amenorrhea and hyperprolacti-

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nemia were significantly higher than in 15 normal women (11).

The present study was undertaken in an attempt to elucidate the effects of hyperprolactinemia on adrenocortical function in man.

## Patients and Methods

*Basal steroid levels before and after bromocriptine therapy*

Thirty-five women with amenorrhea and galactorrhea associated with hyperprolactinemia gave informed consent for the study. Thirty-one (patients 1–31; see Table 1) presented following cessation of oral contraceptives. None had impairment of visual fields and only one (patient 31) showed enlargement of the sella turcica on sella tomography. This patient was studied before and after hypophysectomy and again before and after bromocriptine (2-bromo-α-ergocriptine mesylate, Sandoz) therapy, prescribed in view of continuing hyperprolactinemia post-operatively. Three patients (patients 1–3) were restudied during a second course of bromocriptine at least six months after cessation of the first course.

In all cases, 10 ml of blood were withdrawn from an antecubital vein at the first visit and again 8–10 weeks after commencement of treatment with bromocriptine in a dose of 2.5 mg bid or tid, prescribed in order to normalize the circulating PRL levels. Serum was separated and stored at −20 C until analyzed, and samples from the same patient were assayed together. PRL, DHA and DHAS were assayed in all 39 paired samples, testosterone was determined in 16 paired samples and androstenedione was determined in 13 paired samples.

*ACTH-stimulated steroid levels before and after bromocriptine therapy*

Four female patients (numbers 32–35 from Study 1) were infused *via* an indwelling catheter in an antecubital vein with 25 μg synthetic 1–24α ACTH (Cortrosyn®, Organon) dissolved in 500 ml normal saline over 4 h. The infusions were repeated two to four months later following normalization of serum PRL levels by bromocriptine 2.5 mg tid. All infusions commenced at 0830 h after an overnight fast. The repeat infusion was performed 20 days premenstrually in patient 32, 21 days premenstrually in patient 33, during menses in patient 34 and 11 days premenstrually in patient 35. Cortisol, 17-hydroxyprogesterone, DHA, DHAS, testosterone and PRL were measured in blood samples drawn at −30, −15, 0, 15, 30, 45, 60, 90, 120, 180 and 240 min from the beginning of each infusion. All samples from both infusions in each patient were analyzed in the same assay.

*Hormone analyses*

PRL (12), testosterone (13), androstenedione (13) and 17-hydroxyprogesterone (13) were measured by radioimmunoassay as previously described.

DHA was measured by radioimmunoassay utilizing an antibody raised in rabbits against DHA-7-carboxymethyl-oxime-BSA. Specificity was tested against 69 steroids listed elsewhere (13); of these the only steroids to show greater than 0.5% cross-reactivity were 16α-hydroxyandrost-5-ene-3β-ol-17-one (10.3%), 5α-androstan-3βol-17-one (1.7%), 5α-androstan-3β, 17β-diol (1.2%) and dehydroepiandrosterone sulfate (1.7%). For radioimmunoassay, test and quality control sera (0.1 ml) were diluted to 1.0 ml in water. These, along with DHA standards (0–1,000 pg in 1.0 ml 1% BSA), were extracted with 12 ml methylene chloride (DHAS remains in the aqueous phase). Ten ml of the organic extract were dried and then reconstituted in 0.1 ml 0.1M pH 7.4 phosphate buffer containing 0.1% human gamma globulin. Antiserum (0.1 ml of a 1:600 dilution) and $^{3}$H-DHA (New England Nuclear; 13,000 cpm in 0.1 ml buffer) were added. After incubation for 5 min at 37 C and 1 h at 4 C, 1.0 ml dextran-charcoal (250 mg Dextran T-80 plus 25 g Norit-A in 1,000 ml assay buffer) was added, with further incubation at 4 C for 5 min. Following centrifugation, 0.5 ml of the supernatant was counted for $^{3}$H in a PPO/POPOP/Triton X-100/toluene liquid scintillation mixture. The limit of detection of this assay was 8 pg per tube. Addition of DHA (20–100 pg) to replicate serum samples gave a consistent recovery of 94%. Within-assay coefficient of variation (C.V.) of a serum pool containing approximately 400 ng/dl was ±5.9% (n = 10). Between-assay C.V. for the same pool was ±17.5% (n = 5). Seven serum samples previously assayed by another radioimmunoassay method (14) were assayed using the present method. Comparison of the values ob-

TABLE 1. Serum PRL, DHAS, DHA, testosterone (T) and androstenedione (Δ) concentrations in 35 hyperprolactinemic women before (B) and after (A) treatment with bromocriptine for 8–10 weeks

| | PRL ng/ml | | DHAS μg/dl | | DHA ng/dl | | T ng/dl | | Δ ng/dl | |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient | B | A | B | A | B | A | B | A | B | A |
| 1 | 44 | 8 | 490 | 415 | 690 | 340 | 61 | 54 | — | — |
| 1* | 43 | 5 | 390 | 375 | 790 | 660 | 55 | 54 | — | — |
| 2 | 58 | 12 | 370 | 330 | 890 | 640 | 49 | 61 | — | — |
| 2* | 85 | 43 | 265 | 190 | 410 | 370 | — | — | — | — |
| 3 | 188 | 12 | 100 | 100 | 220 | 210 | — | — | 72 | 124 |
| 3* | 91 | 13 | 55 | 45 | 90 | 160 | 20 | 40 | — | — |
| 4 | 96 | 4 | 275 | 175 | 660 | 270 | 44 | 48 | — | — |
| 5 | 68 | 6 | 335 | 280 | 470 | 470 | — | — | 330 | 420 |
| 6 | 94 | 2 | 385 | 200 | 710 | 330 | — | — | 240 | 204 |
| 7 | 49 | 11 | 385 | 335 | 670 | 510 | 49 | 54 | — | — |
| 8 | 47 | 8 | 380 | 330 | 220 | 530 | — | — | 132 | 160 |
| 9 | 65 | 5 | 450 | 270 | 1,100 | 270 | — | — | 288 | 300 |
| 10 | 16 | 8 | 365 | 240 | 260 | 220 | — | — | — | — |
| 11 | 51 | 3 | 475 | 480 | 470 | 720 | — | — | 172 | 254 |
| 12 | 76 | 21 | 515 | 490 | 370 | 470 | — | — | — | — |
| 13 | 53 | 2 | 360 | 225 | 220 | 240 | 27 | 36 | — | — |
| 14 | 22 | 6 | 60 | 25 | 130 | 60 | — | — | — | — |
| 15 | 75 | 5 | 220 | 70 | 470 | 190 | — | — | 119 | 80 |
| 16 | 20 | 12 | 370 | 335 | 610 | 700 | — | — | — | — |
| 17 | 41 | 26 | 315 | 285 | 220 | 190 | — | — | — | — |
| 18 | 141 | 9 | 435 | 375 | 1,400 | 250 | — | — | 179 | 74 |
| 19 | 116 | 3 | 390 | 250 | 200 | 320 | — | — | 570 | 480 |
| 20 | 113 | 3 | 275 | 160 | 90 | 230 | 23 | 29 | — | — |
| 21 | 52 | 11 | 120 | 80 | 310 | 80 | — | — | — | — |
| 22 | 43 | 4 | 165 | 65 | 240 | 170 | — | — | — | — |
| 23 | 232 | 20 | 555 | 270 | 780 | 480 | 61 | 40 | — | — |
| 24 | 49 | 7 | 285 | 110 | 820 | 710 | 36 | 38 | — | — |
| 25 | 51 | 29 | 405 | 210 | 450 | 440 | — | — | — | — |
| 26 | 129 | 39 | 350 | 290 | 610 | 490 | — | — | — | — |
| 27 | 66 | 21 | 165 | 135 | 290 | 280 | — | — | — | — |
| 28 | 69 | 5 | 195 | 150 | 370 | 710 | 34 | 43 | — | — |
| 29 | 76 | 21 | 475 | 435 | 690 | 550 | 75 | 84 | — | — |
| 30 | 107 | 20 | 270 | 150 | 540 | 420 | — | — | 492 | 464 |
| 31† | 245 | 81 | 550 | 480 | 1,000 | 520 | — | — | — | — |
| 31 | 116 | 28 | 375 | 335 | 370 | 570 | — | — | — | — |
| 32 | 31 | 3 | 260 | 185 | 280 | 325 | 24 | 49 | 720 | 282 |
| 33 | 102 | 5 | 105 | 67 | 350 | 120 | 45 | 30 | 134 | 110 |
| 34 | 75 | 10 | 400 | 253 | 420 | 360 | 56 | 40 | 186 | 110 |
| 35 | 96 | 11 | 205 | 50 | 320 | 185 | 38 | 76 | — | — |
| Mean ± SE | 82 ± 8 | 14 ± 2 | 322 ± 21 | 237 ± 21 | 492 ± 47 | 378 ± 30 | 44 ± 4 | 49 ± 4 | 280 ± 55 | 236 ± 40 |

* Repeat course of bromocriptine after an interval of at least six months.
† Before and after hypophysectomy.

tained with each method gave a correlation coefficient of 0.963 over a range of values from 90 to 760 ng/dl.

DHAS was measured by a modification of the method of Buster and Abraham (15). Ten μl of test and quality control sera were diluted to 10 ml in 0.1M pH 7.4 phosphate buffer and to duplicate 0.1 ml aliquots, antiserum (0.1 ml of a 1:80,000 dilution) and $^3$H-DHA (13,000 cpm in 0.1 ml) were added. Incubation and charcoal separation procedures were performed as outlined above for the radioimmunoassay of DHA. Within assay C.V. for a pool containing approximately 400 μg/dl was ±9.1% (n = 9) and between-assay C.V. for the same pool was ±15.7% (n = 5).

Cortisol was measured by radioimmunoassay using a method similar to that for DHA described above. The antibody was raised in rabbits against cortisol-6-carboxymethyl-oxime-BSA.

Statistical analyses were performed using

TABLE 2. Comparison of mean ± SE circulating DHAS and DHA concentrations in hyperprolactinemic patients and normal subjects

| | | DHAS μg/dl | DHA ng/dl |
|---|---|---|---|
| Patients (n = 39) | B | 322 ± 21 | 492 ± 47 |
| | A | 237 ± 21 | 378 ± 30 |
| Normals (n = 14) | | 234 ± 32 | 433 ± 64 |
| Significance *vs.* normals | B | $P < 0.02$ | NS |
| | A | NS | NS |

n = Number of observations.
B = Before bromocriptine.
A = After 8–10 weeks treatment with bromocriptine.

Student's *t* test for paired samples for comparison of the data before and after bromocriptine therapy. Analysis of variance was used to assess the ACTH-infusion data and a one-tailed *t* test was used for comparison of the DHA and DHAS concentrations in the hyperprolactinemic patients and the normal women. For all analyses the values for patient 31 both before and after hypophysectomy have been included.

## Results

### *Basal steroid levels before and after bromocriptine therapy*

The PRL, DHAS, DHA, testosterone and androstenedione concentrations before and after bromocriptine are shown in Table 1, and comparisons of mean DHAS and DHA concentrations pre- and post-bromocriptine with mean values from 14 normal premenopausal women are shown in Table 2. Prior to bromocriptine therapy, only the mean serum DHAS level was significantly higher than in normal women ($P < 0.02$), in agreement with Giusti *et al.* (11). A number of the basal testosterone and androstenedione levels were above the usual range of values reported for normally cycling women: testosterone less than 60 ng/dl (8); androstenedione 120–270 ng/dl (16). However, there was no significant change in mean testosterone or androstenedione levels following bromocriptine treatment.

Each woman treated with bromocriptine exhibited marked reductions in circulating PRL levels after 8–10 weeks. However, presumably due to inadequate dosage, not all PRL concentrations had returned to the normal range (less than 15 ng/ml) by that time. Associated with this decrease in PRL in the 39 paired samples, serum DHAS concentrations fell in 35 and were unchanged in four, whereas DHA concentrations fell in 24, were unchanged in four and increased in 11 (all changes were greater than 5% of pretreatment levels).

Significant decreases in mean serum DHAS (26%) and DHA (23%) concentrations were noted after reduction of serum PRL ($P < 0.0005$ and $P < 0.01$, respectively). Overall, the PRL and DHAS concentrations were found to be significantly correlated ($r = 0.35$; $P < 0.01$) as were the PRL and DHA concentrations ($r = 0.29$; $P < 0.05$) but the correlation between PRL and either DHA or DHAS was lost when only the pre-bromocriptine or post-bromocriptine levels were considered.

Of the 35 women, only two volunteered that they considered themselves abnormally hirsute, but another 23 were considered to show a minor degree of facial hirsutism. No change in the degree of hirsutism was detected following 24 weeks treatment with bromocriptine.

### *ACTH-stimulated steroid levels before and after bromocriptine therapy*

The mean PRL concentration in the four patients at the time of the first infusion was 76 ng/ml (range 31–102) and at the repeat infusion was 7 ng/ml (range 3–11). Reduction of serum PRL was accompanied by changes in basal DHAS, DHA and testosterone levels as outlined in Table 1 (patients 32–35). Basal cortisol levels did not change (19.3 *vs.* 18.4 μg/dl; 7.8 *vs.* 5.4 μg/dl; 12.2 *vs.* 12.0 μg/dl; 15.3 *vs.* 13.8 μg/dl) whereas 17-hydroxyprogesterone levels were similar in patients 32 and 34 (55.7 *vs.* 49.7 ng/dl and 26.0 *vs.* 25.3 ng/dl, respectively) and increased in patient 33 (12 *vs.* 106 ng/dl). No

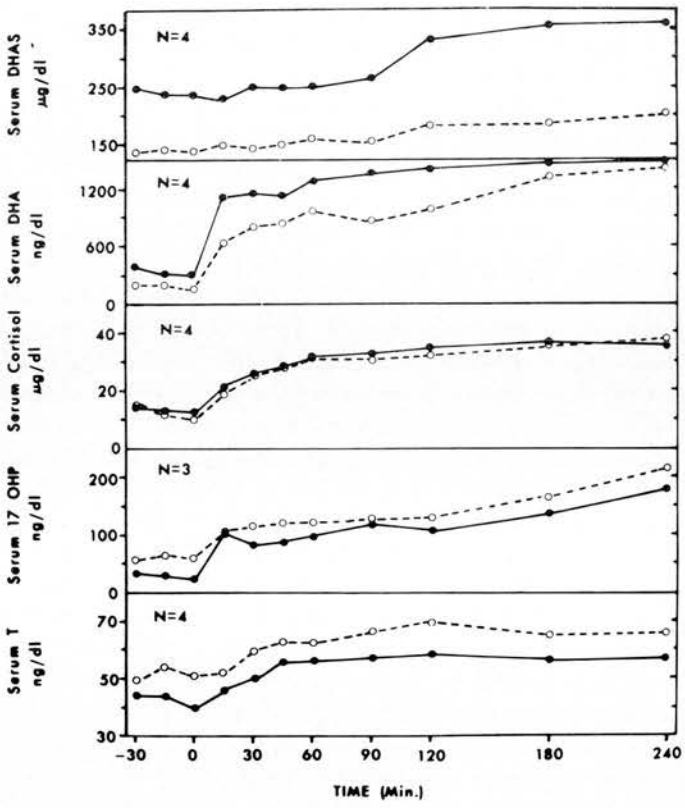



FIG. 1. Mean serum dehydroepiandrosterone sulfate (DHAS), dehydroepiandrosterone (DHA), cortisol, 17-hydroxyprogesterone (17 OHP) and testosterone (T) concentrations in four hyperprolactinemic women before and during 4 h infusions of 25 $\mu$g synthetic 1–24$\alpha$ ACTH. Closed circles refer to infusions before bromocriptine therapy; open circles refer to infusions after normalization of serum prolactin levels with bromocriptine therapy. There was no significant difference in response during the two infusions for any of the steroids.

17-hydroxyprogesterone measurements were made in patient 35. For all of the steroids studied, analysis of variance of the differences in hormone levels at each time point during the two infusions revealed no significant differences when compared with the differences of the means of the three baseline (*i.e.* pre-infusion) samples. The mean responses of the steroids during the ACTH infusions are shown in Figure 1.

## Discussion

This study shows that bromocriptine-induced reductions in circulating PRL concentrations in hyperprolactinemic women are associated with significant falls in mean circulating DHAS and DHA concentrations whereas mean testosterone, androstenedione and cortisol concentrations do not change; data from three patients suggest that there is no change in circulating 17-hydroxyprogesterone concentrations. In addition, no significant differences in steroid responses to ACTH infusions were observed following bromocriptine treatment. The DHAS findings are similar to those in three patients reported by Giusti *et al.* (11) and the lack of change of mean testosterone concentrations confirms a recent report by Seppälä *et al.* (17).

There are conflicting data in the literature regarding the site or sites of synthesis of DHAS. From analysis of adrenal and gonadal venous effluents, most investigators have concluded that DHAS is secreted solely by the adrenal gland (9,18,19). Small quantities of circulating DHAS may also be formed by peripheral conversion of steroids such as DHA and 17-hydroxypregnenolone sulfate (20–22). However, Abraham and Chakmakjian (16) detected small amounts of DHAS (less than one-tenth that found in normally menstruating women) in periph-

eral venous serum over the course of one menstrual cycle in a bilaterally adrenalectomized woman. Thus, current evidence suggests that at least 90% of the circulating DHAS in premenopausal females is derived from the adrenal cortex. Although it has been inferred that some DHAS may be secreted by the ovary (16,23) there is no direct evidence to support this. On the other hand, it is well established that DHA, androstenedione and testosterone are secreted by both the adrenal cortex and the ovary (9,19,24), with the adrenal producing about 80% of the circulating DHA and the adrenal and ovary contributing equally to the circulating androstenedione and testosterone (23). The effects of elevated PRL concentrations on production of these circulating steroids will thus reflect a balance between alterations in both gonadal and adrenal steroidogenesis.

Elevated PRL concentrations presumably suppress gonadal steroid output in view of the low circulating testosterone and estradiol levels observed in hyperprolactinemic men and women, respectively (5,25). The mechanism of this suppression has not been completely clarified, but the available evidence suggests an inhibitory effect of PRL at both the hypothalamic-pituitary (26–29), and gonadal levels (30). Although data regarding the circulating concentrations of ovarian steroids other than estradiol in hyperprolactinemic women are not available, it appears likely that ovarian production of DHA, androstenedione and testosterone would also be decreased.

Consequently, the finding of elevated DHAS levels along with the reductions in mean DHAS and DHA concentrations following lowering of circulating PRL concentrations in the present study strongly suggests that PRL exerts a stimulatory effect on adrenocortical androgen production. The cortisol and 17-hydroxyprogesterone results along with previous cortisol data in hyperprolactinemic patients (11,29) exclude a generalized stimulatory effect on the adrenal cortex. The failure to detect a change in androstenedione and testosterone levels following lowering of PRL concentrations could be explained by a balance between decreased adrenal and increased ovarian (removal of PRL inhibition) production. However, even though the mean serum DHAS concentration in the hyperprolactinemic women was significantly higher than that in normal women, a number of patients had DHAS concentrations below the normal mean suggesting that the abnormality is only partially dependent upon increased PRL concentrations. Fluctuations in serum concentrations due to pulsatile secretion are not sufficient to explain these lower values (31).

There is no evidence to suggest that bromocriptine affects adrenal or gonadal steroidogenesis directly, but it remains possible that the diminution in DHAS and DHA levels could be attributed to a mode of action of the drug other than *via* its inhibition of PRL secretion. Nevertheless, the decrease in DHAS and DHA associated with the decrease in PRL following partial hypophysectomy in patient 31 suggests that the changes noted in the other patients were due to the induced suppression of PRL.

Low dose ACTH infusions were performed in an attempt to detect subtle changes in steroid responses that may have been induced by the hyperprolactinemia. Since no significant differences in responses were noted between the infusions pre- and post-bromocriptine, particularly with respect to DHAS and DHA levels, it would appear that the presumed stimulatory effect of hyperprolactinemia on adrenal androgen production is of a mild degree. The lack of a significant correlation between the pre-bromocriptine PRL concentrations and the corresponding DHAS and DHA concentrations, coupled with the fact that the mean circulating DHAS and DHA concentrations in the hyperprolactinemic patients were only 38% and 14%, respectively, higher than the mean concentrations in the normal women, supports this contention.

The mechanism by which PRL affects

adrenal androgen production is unknown. However, there is evidence from animal experimentation that PRL causes increased accumulation of cholesterol esters in testicular tissue (32) as well as increasing the activity of the Δ4,5 isomerase-3β hydroxysteroid dehydrogenase enzyme—the enzyme responsible for conversion of DHA to androstenedione (33). If PRL exerts a similar effect in human adrenals, it seems reasonable to postulate that physiological amounts of ACTH would stimulate increased steroidogenesis from the increased amounts of cholesterol esters, and that this increased steroidogenesis would be diverted through androgenic pathways because of increased activity of the Δ4,5 isomerase-3β hydroxysteroid dehydrogenase enzyme. It is likely that preparations such as adrenal cell cultures will be necessary to elucidate fully the mechanism of action of PRL on the adrenal cortex, although data regarding the effects of PRL on production and clearance rates of adrenal androgens and on DHAS-DHA interconvertibility would also be helpful.

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# CHANGES IN CONCENTRATIONS OF FOLLICLE-STIMULATING HORMONE, LUTEINIZING HORMONE, PROLACTIN AND PROGESTERONE IN THE PLASMA OF EWES DURING THE TRANSITION FROM ANOESTRUS TO BREEDING ACTIVITY

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SUMMARY

The plasma concentrations of FSH, LH, prolactin and progesterone were measured throughout anoestrus and during the resumption of cyclic activity in two groups of ewes. Group A was maintained under conditions of natural daylength throughout the experiment, whereas Group B was exposed to 6 h of light and 18 h of darkness, the change being made abruptly on the longest day. In those ewes kept on short days, oestrus and ovulation occurred 18·3 days ($P < 0{\cdot}05$) and 23·4 days ($P < 0{\cdot}001$) earlier than in the ewes under conditions of natural daylength. Ovulation preceded oestrus by 18·6 days and 23·1 days in Groups A and B respectively.

The occurrence of ovulation was detected by the determination of plasma progesterone concentrations. In all ewes, progesterone levels were basal until the first ovulation when the pattern of secretion was typical of that seen during the oestrous cycle. In the 4 days before ovulation, the plasma progesterone concentration increased slowly to reach a maximum of $0{\cdot}66 \pm 0{\cdot}12$ (S.E.M.) ng/ml on day $-1$. The first ovulation was associated with a substantial surge of LH. Similar release of LH, thought to be related to the increased progesterone secretion, was also observed on day $-5$. Sporadic release of LH was also found before this time in some animals.

Plasma concentrations of FSH fluctuated randomly throughout anoestrus and during the transition to established oestrous cycles. These changes were not apparently related to ovulation. Throughout anoestrus, prolactin concentrations were raised but always declined before the time of ovulation. The fall in prolactin concentrations occurred sooner in the ewes on short days, pointing to a relationship with the decreased daylength.

These data suggest that the return to oestrous cycles may be brought about by the removal of an antigonadotrophic effect exerted by high concentrations of prolactin in the blood during anoestrus.

## INTRODUCTION

A major factor limiting productivity in the ewe is the long period of anoestrus which is characterized not only by a failure to exhibit oestrus but also by the absence of ovulation since the ovaries are relatively regressed and lack corpora lutea (Grant, 1934; Robinson, 1950; Hutchinson & Robertson, 1966). During anoestrus, only small and medium-sized

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follicles are found in the ovary, although the presence of some large follicles has been reported by Kammlade, Welch, Nalbandov & Norton (1952) and Hutchinson & Robertson (1966).

The ovarian regression typical of anoestrus was originally thought to be associated with a deficiency in gonadotrophin secretion. It is apparent, however, that the pituitary content of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in ewes is similar during anoestrus and the breeding season (Robertson & Hutchinson, 1962; Roche, Foster, Karsch, Cook & Dziuk, 1970). Subsequently the use of radioimmunoassays has indicated that the basal concentrations of the two hormones in the blood are also similar in the two situations (Goding, Catt, Brown, Kaltenbach, Cumming & Mole, 1969; Roche *et al.* 1970; Salamonsen, Jonas, Burger, Buckmaster, Chamley, Cumming, Findlay & Goding, 1973; Yuthasastrokosol, Palmer & Howland, 1975). Furthermore, the release of FSH and LH can easily be induced during anoestrus by the administration of either oestradiol-17$\beta$ (Jonas, Salamonsen, Burger, Chamley, Cumming, Findlay & Goding, 1973; Reeves, Beck & Nett, 1974; Walton, 1975) or gonadotrophin releasing hormone (Reeves, Tarnavsky & Chakraborty, 1974; Walton, 1975).

In view of these data, the current hypothesis that the reduced ovarian activity during anoestrus is a result of inadequate secretion of LH and FSH requires revision. A major problem in understanding the factors responsible for the onset, maintenance and end of anoestrus in the ewe is the lack of comprehensive and systematic studies on the endocrine changes during anoestrus. Only one study (Yuthasastrokosol *et al.* 1975) has evaluated the changes in oestradiol, progesterone and LH concentrations from mid and late anoestrus until the resumption of oestrous cycles.

It is well established that an increase in the level of prolactin in the blood occurs in response to an increase in daylength in both the ram (Pelletier, 1973) and the cow (Koprowski & Tucker, 1973; Karg & Schams, 1974). Since hyperprolactinaemia is associated with amenorrhoea in women (Thorner, McNeilly, Hagan & Besser, 1974), it was decided to measure changes in prolactin levels during anoestrus in the ewe.

Thus the present paper reports the changes in the levels of LH, FSH and prolactin in the blood throughout anoestrus and during the resumption of cyclic activity, and relates these changes to the levels of progesterone as an indicator of ovarian luteal function.

On the basis of these observations, a new hypothesis is proposed to explain the endocrine control of the seasonal breeding pattern of the ewe.

## MATERIALS AND METHODS

### *Animals and collection of blood samples*

Fifteen mature Clun Forest ewes were randomly divided into two groups; Group A consisted of seven ewes housed under conditions of natural daylength throughout the period February–October, whereas the remaining animals, Group B, were kept in light-proof accommodation (Ducker, Thwaites & Bowman, 1970) from 26 June and exposed to 6 h of light and 18 h of darkness (6L: 18D) until late October. Both groups were fed dried lucerne nuts and straw to maintain body weight and the incidence of oestrus was detected by active vasectomized rams equipped with marking crayons and harnesses (Radford, Watson & Wood, 1960). Ovulation was detected by measuring changes in the plasma concentrations of progesterone.

The ewes in Group A were bled thrice weekly and those in Group B were bled daily between 10.00 and 11.30 h. Blood samples were collected by jugular venipuncture into heparinized syringes and transferred to centrifuge tubes previously cooled on ice. Plasma was

separated by centrifugation at 600 *g* for 15 min at 4 °C and stored at −20 °C until assay. Vaginal smears were taken thrice weekly from all animals as part of another study.

### *Radioimmunoassays*

Progesterone was estimated by the method of Furr (1973) as modified by Glencross, Munro, Senior & Pope (1973) with some additional minor changes. In this work a different bleed of antiserum 465 (465/3) of similar specificity to the previous bleed 465/2 (Furr, 1973) was used. A single extraction of plasma with 25 vol. light petroleum (b.p. 40–60 °C) was considered sufficient since 78 ± 0·5 (S.E.M.) % ($n$ = 100) of added tritiated progesterone was recovered in the assay buffer. The same amount of tracer (20000 d.p.m. [$^3$H]progesterone) was added to standards and unknowns and 100 $\mu$l buffer were used for recovery estimates.

Plasma levels of LH were measured by a heterologous, double-antibody radioimmunoassay. The antiserum (R07/7) was raised in rabbits against NIH-LH-S16 and was used at a final dilution of 1:100000. A purified preparation of bovine LH (1·57 × NIH-LH-B7; Habich, 1972) was labelled with $^{125}$I using the method of Greenwood, Hunter & Glover (1963) and NIH-LH-S11 was used as a standard. The sensitivity of the assay was 0·8 ng NIH-LH-S11/ml plasma. The cross-reaction with a highly purified preparation of ovine FSH (LER-1491, 40 × NIH-FSH-S1, kindly provided by Dr L. E. Reichert) was 0·3 %. Cross-reactions with other pituitary hormones were < 0·01 % except for NIH-TSH-S6 which had an apparent cross-reaction of 85 %. It was considered, however, that this was caused by contamination with immunoreactive LH (Goding *et al.* 1969) since there was no change in LH levels after the administration of 200 $\mu$g thyrotrophin releasing hormone (TRH) to two anoestrous ewes. Non-specific effects caused by plasma were not observed and LH added to hypophysectomized ram plasma (provided by Dr H. Buttle) was recovered quantitatively.

Plasma levels of FSH and prolactin were measured in samples from three ewes selected at random from each group by the methods described by McNeilly, McNeilly, Walton & Cunningham (1976) and McNeilly & Andrews (1974) respectively. For any hormone all samples from one animal were processed in a single assay and coefficients of variation within and between assays did not exceed 8 % and 13 % respectively. Statistical comparisons were carried out with Student's $t$-test.

The occurrence of ovulation was inferred only when a full luteal phase pattern of progesterone concentrations was observed.

## RESULTS

### *Onset of ovulation and oestrus*

The onset of oestrus and ovulation was observed 95·7 ± 3·7 (S.E.M.) days and 77·1 ± 2·1 days after the longest day in the ewes on natural daylength (group A). In the ewes exposed to 6L:18D (group B), oestrus and ovulation occurred 18·3 days ($P$ < 0·05) and 23·4 days ($P$ < 0·001) earlier than in the ewes which continued to experience conditions of natural daylength. Ovulation preceded oestrus by 18·6 days in group A and by 23·1 days in group B since nine and 12 ovulations unaccompanied by oestrus were observed in the two groups.

### *Progesterone*

In all the ewes, the concentration of progesterone in the plasma was between 0·2 and 0·4 ng/ml when the experiment started on 26 June and remained at this low level until the time of the first ovulation (Fig. 1). After the first two ovulations, higher luteal phase levels of progesterone were apparent in the ewes of group B (Fig. 1). In both groups a small increase in the concentration of progesterone preceded what was assumed to be a rise associated with the first true ovulation. This was defined more clearly for the ewes in group B since daily

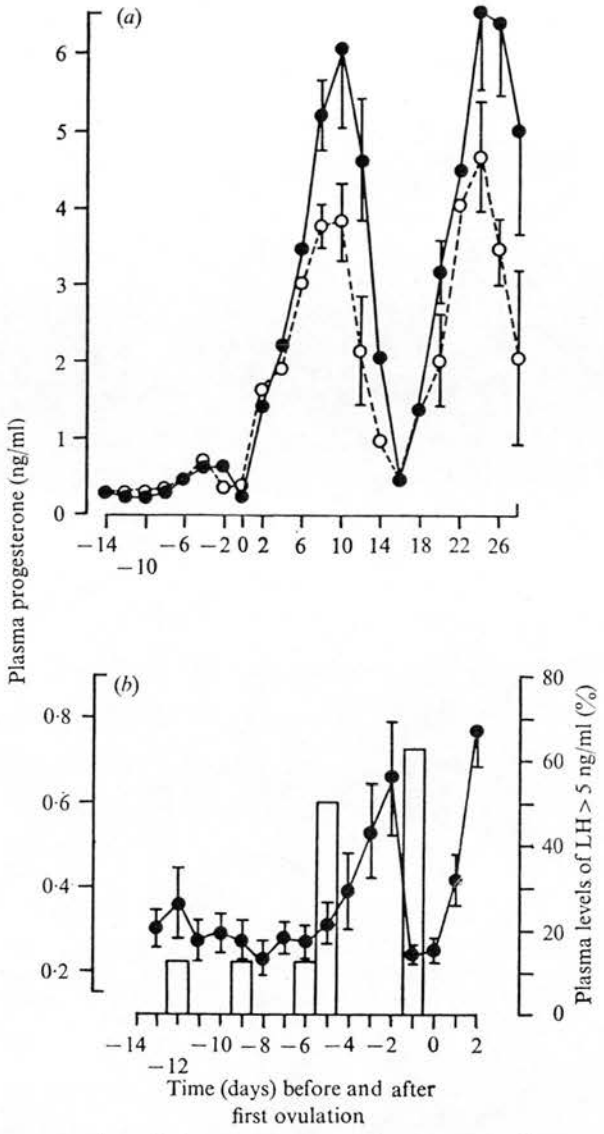



Fig. 1. (*a*) Mean concentrations of plasma progesterone in ewes exposed to natural daylength (○, $n = 7$) and ewes kept in reduced daylength (6 h light:18 h darkness) from the longest day (●, $n = 8$). The data have been arranged around the day when the first ovulation was presumed to have occurred. (*b*) Mean concentrations of plasma progesterone (●) and the incidence of raised levels of LH (open bars) around the time of first ovulation in ewes on reduced daylength ($n = 8$). Vertical lines represent ± S.E.M. On day −1 the mean concentration of LH was 16·8 ± 9·45 (S.E.M.) ng NIH-LH-S11/ml ($n = 8$) and on day −5 it was 19·1 ± 8·2 (S.E.M.) ng NIH-LH-S11/ml ($n = 8$).

estimates for progesterone were available (Fig. 1). In fact the concentration of progesterone was raised for 3 days, reaching a maximum value of 0·66 ± 0·12 (S.E.M.) ng/ml ($n = 8$). This level was significantly ($P < 0·001$) greater than that on the day that the first ovulation was assumed to have occurred (0·24 ± 0·02 ng/ml, $n = 8$).

*Luteinizing hormone*

At the start of the experiment LH concentrations in all ewes were typical of the basal values normally found during the breeding season, and ranged between 1 and 2 ng/ml (Walton, 1975). Raised levels of LH were not apparent until just before the onset of ovulation. Since an increased concentration of LH in the plasma in relation to ovulation is likely to be of short duration (< 14 h, Goding *et al.* 1969) it must be recognized that the relatively long sampling intervals used here are not the most effective for the detection of the preovulatory surge. Higher levels of LH were observed in some samples and the data obtained for group B are summarized in Fig. 1. Two periods of consistently raised concentrations of LH in the plasma were apparent. One immediately preceded the day of the first ovulation and the other occurred 5 or 6 days before this (Figs 1 and 2). In some ewes, further release of LH, apparently not associated with succeeding ovulations, was also observed (Fig. 2).

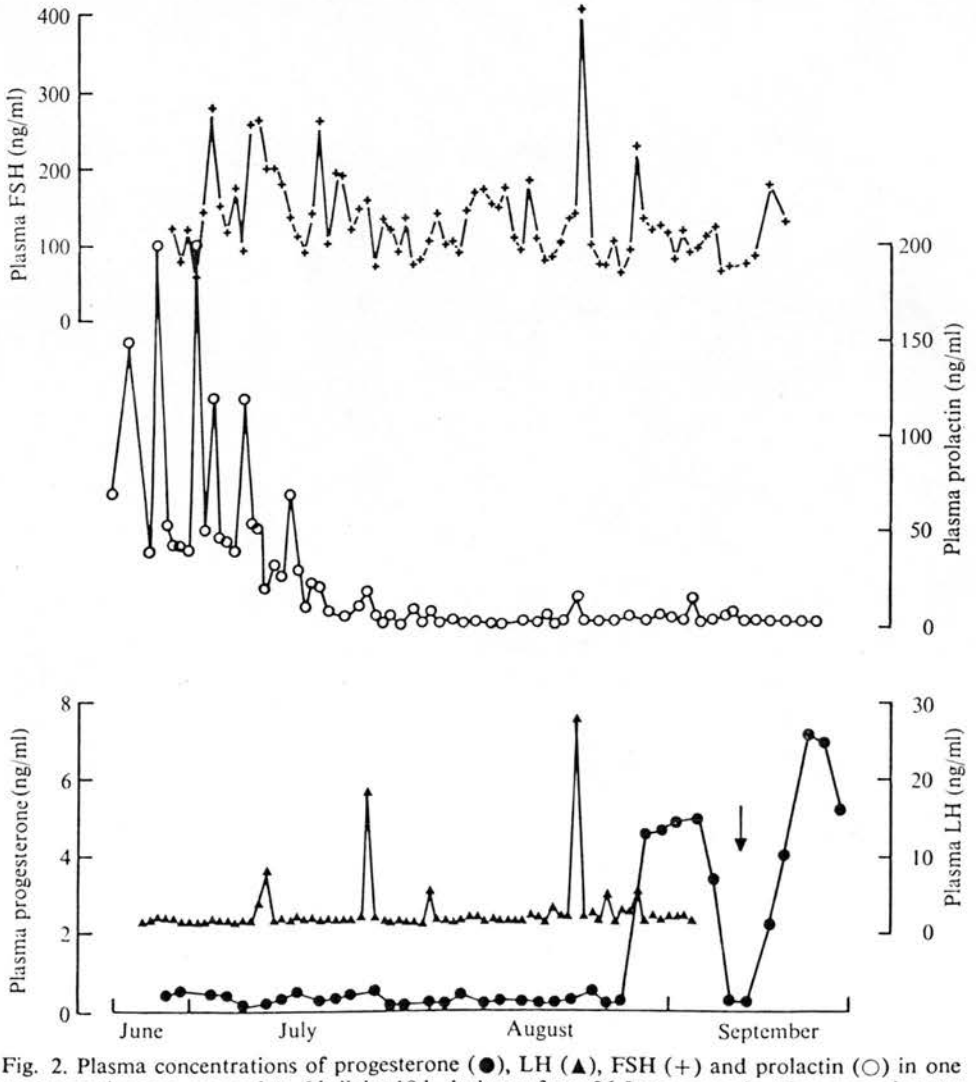



Fig. 2. Plasma concentrations of progesterone (●), LH (▲), FSH (+) and prolactin (○) in one representative ewe exposed to 6 h light:18 h darkness from 26 June onwards. The arrow denotes the occurrence of oestrus.

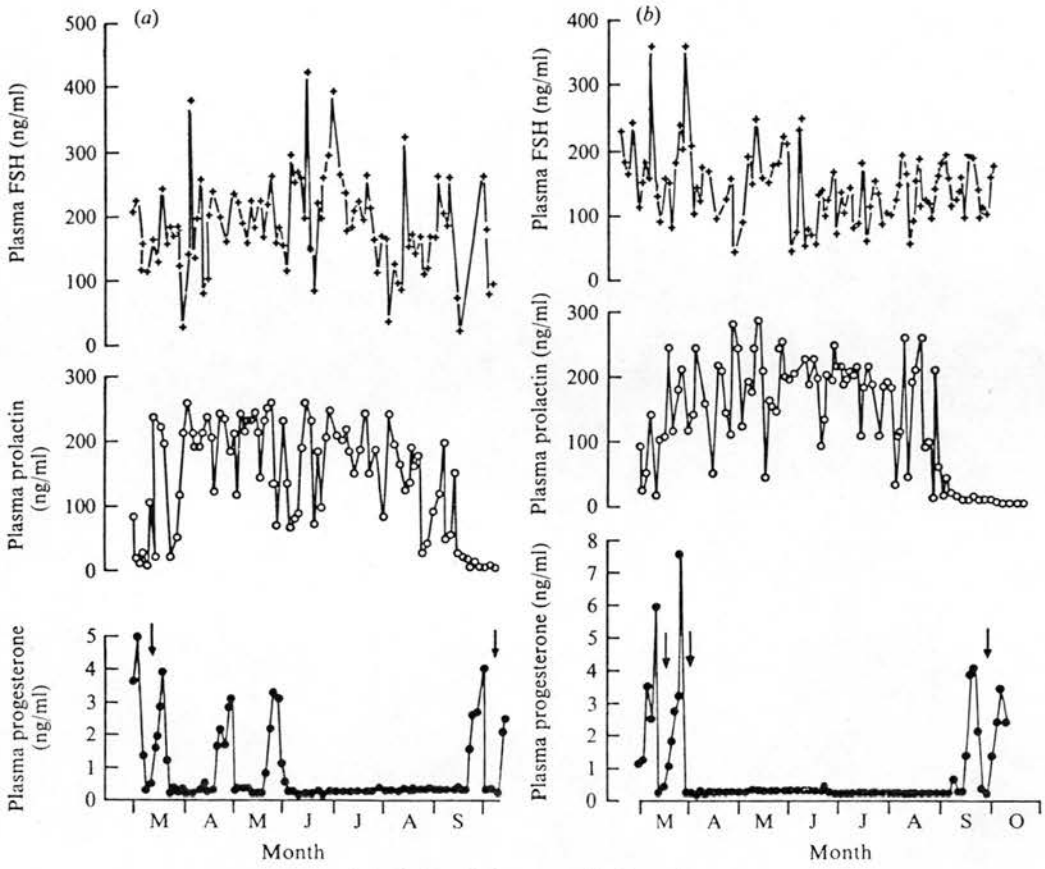



Fig. 3. See facing page for legend

Changes in plasma concentrations of prolactin and FSH were not obviously related to the time of the first ovulation and the variation was such that the data could not be pooled satisfactorily. Prolactin and FSH concentrations from representative animals of both groups are, therefore, illustrated in Figs 2 and 3.

### *Follicle-stimulating hormone*

Considerable day-to-day variation in FSH concentrations was observed (Figs 2 and 3), but there was no suggestion of an overall change in concentration during the experimental period with the exception of some high values which were associated with raised levels of LH.

### *Prolactin*

Anoestrus was characterized by high levels of prolactin in the blood (Figs 2 and 3), although it is possible that stress-induced prolactin release occurred in the ewes of both groups during the early part of the experiment. Once the animals were accustomed to blood sampling, however, the incidence of spurious high values diminished. It is evident that in all ewes there was a marked decline from the high levels of anoestrus to a lower, more stable baseline at various times before the first ovulation. This decline occurred 25–30 days before the first ovulation in the ewes of group B, but only 5–7 days before the first ovulation in the control ewes. The lower level of prolactin was then maintained during the early part of the breeding season.

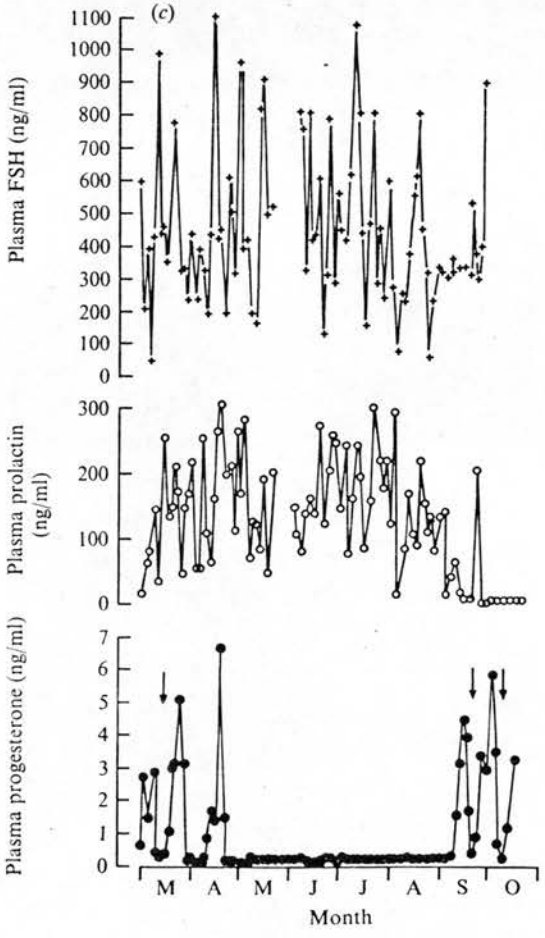



Fig. 3. Plasma concentrations of progesterone (●), FSH (+) and prolactin (○) in three individual ewes (*a*), (*b*) and (*c*) throughout anoestrus under conditions of natural daylength (group A). Arrows denote occurrence of oestrus.

## DISCUSSION

The present results confirm that after anoestrus the resumption of breeding activity occurs in response to decreasing daylength (Ducker *et al.* 1970). The first ovulations of the new breeding season, however, are likely to occur in the absence of an accompanying period of oestrous activity. For instance, in the present investigation, 13 out of the 15 ewes ovulated, as judged by a luteal phase pattern of progesterone secretion, independently of oestrus on either one or two occasions and in this respect there was no apparent effect due to treatment. The most striking effect of the abrupt reduction in daylength was the advance by approximately 20 days of the occurrence of both oestrus and ovulation. A further noticeable feature of this treatment was the higher concentration of plasma progesterone during the luteal phase. This could reflect a difference in ovulation rate and hence in the numbers of corpora lutea (Thorburn, Bassett & Smith, 1969).

In both groups there was good evidence for a small but significant increase in progesterone levels during the 4–5 days before the first ovulation. These increased progesterone levels were less than those found during the normal luteal phase. The source of this progesterone

and its physiological importance, however, are not at present clear. Almost certainly it is preceded by a release of LH similar in magnitude to the preovulatory surge (Fig. 1). There are three possible sources: first it may be of adrenal origin, secondly it may come from a corpus luteum formed as a result of an ovulation induced by the release of LH and finally it might be secreted by prematurely luteinized ovarian follicles.

An adrenal source is unlikely since progesterone secretion from the adrenal glands is low and is not stimulated by LH (Baird, McCracken & Goding, 1971). It is unlikely to be from a corpus luteum since relatively small quantities are secreted for such a short time and this would be indicative of an inadequate luteotrophic stimulus. The levels of LH and prolactin at this time are, however, within the normal ranges found during the oestrous cycle (Fig. 2). It is worth noting, however, that treatment with gonadotrophin releasing hormone during anoestrus causes release of LH and subsequent ovulation but a fully functional corpus luteum is seldom formed, with the result that only small quantities of progesterone are secreted (Haresign, Haynes & Lamming, 1973). The most likely explanation seems to be the possibility that the release of LH induced luteinization of one or more ovarian follicles. This could arise either from an insufficient release of LH or from an inherent inability of the follicles to ovulate in response to the stimulus. In favour of this explanation is the observation that the period of increased progesterone secretion corresponds to the time, i.e. 5–7 days, within which ovarian follicles grow and regress (Smeaton & Robertson, 1971; Brand & de Jong, 1973). It is encouraging that a similar pattern of progesterone secretion was also seen in cows before the first ovulation after parturition (Pope, Gupta & Munro, 1969; Donaldson, Bassett & Thorburn, 1970; Robertson, 1972).

Additional information provided by a timely laparotomy would be essential to ascertain the site of origin of this progesterone. Nevertheless, it is quite apparent that some progesterone secretion precedes the first ovulation and also that, at least in some ewes, there are releases of LH during this period which are not associated with ovulation. These results are in general agreement with those of Thorburn *et al.* (1969) and Yuthasastrokosol *et al.* (1974).

Plasma levels of FSH fluctuated randomly during anoestrus in much the same way as they did during the oestrous cycle (McNeilly *et al.* 1976). Within the limitations imposed by the sampling intervals used there were no consistent trends in FSH patterns which could be associated with cessation or resumption of ovulatory cycles.

A characteristic feature of the present observations was the raised level of prolactin throughout anoestrus (Figs 2 and 3). Previous studies have not emphasized this point (Lamming, Mosely & McNeilly, 1974). Two criticisms might be levelled at the present data. The first has already been mentioned with regard to the interpretation of the FSH and LH data and arises from the limitations imposed by a daily blood sampling technique in assessing fluctuations in hormones which can be released and cleared rapidly. The second is related to the well-known stress-induced release of prolactin. It is clear that at the end of the previous breeding season when sampling commenced the prolactin levels were generally high (Fig. 3). This might reflect either a stimulus to prolactin release caused by increasing daylength, or alternatively the fluctuations could be explained by the initial stress of sampling. At the end of anoestrus, however, the level of prolactin clearly fell in both groups of ewes to a stable base-line which suggests that by this time the animals had adjusted to the sampling routine. In this respect it is interesting that similar seasonal changes in prolactin levels have also been reported in heifers (Karg & Schams, 1974) and rams (Pelletier, 1973). In the present study the concentration of prolactin always fell before the onset of ovulation although the timing of these events varied. This variation was apparently related to the daylength since the levels fell earlier in the ewes on reduced daylength, which points to a relationship between the photoperiod and prolactin release. This is supported by studies in cattle (Bourne & Tucker, 1975) which also showed that changes in photoperiod altered serum prolactin levels.

Our data suggest that prolactin may play a role in the overall control of anoestrus in the ewe. Kann & Martinet (1975) have demonstrated that the raised levels of prolactin which occur during lactation in the ewe are directly implicated in the maintenance of lactational anoestrus. Thus higher prolactin levels reduce the release of LH in response to oestradiol but do not affect the release in response to luteinizing hormone releasing hormone (Kann, Martinet & Schirar, 1976). Suppression of the concentration of prolactin to levels occurring during normal oestrous cycles results in a return of oestrous cyclicity in lactating ewes and these authors have suggested that prolactin itself exerts an antigonadal action, probably by a direct action at the hypothalamic level (Kann *et al.* 1976). A direct inhibitory effect of prolactin on the gonads may also occur as has been described in women (Thorner *et al.* 1974) and rats (Fang, Refetoff & Rosenfield, 1974). It should be pointed out, however, that not all the present results are in accordance with this interpretation since at the end of the previous breeding season whilst ovulation was still occurring prolactin concentrations were already high. As mentioned previously this could reflect a stress response or on the other hand it may indicate that the cessation of ovulatory cycles requires a prolonged period of exposure to high concentrations of prolactin in the blood.

While the present data do not allow any suggestion of the level of interference in the hypothalamus–pituitary–gonad system, it is tempting to suggest that the raised levels of prolactin which occur during anoestrus in response to increased daylength, directly influence gonadal activity. Decreasing daylength results in a reduction of prolactin levels which is followed by a return to ovulation and a resumption of regular oestrous cycles.

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# Effect of suppression of plasma prolactin on ovulation, plasma gonadotrophins and corpus luteum function in LH-RH-treated anoestrous ewes

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**Summary.** Nineteen Scottish Blackface ewes were given LH-RH (3 × 30 μg i.v., 90-min intervals) during anoestrus when prolactin levels were elevated. Plasma levels of prolactin were suppressed with CB 154 (twice daily, i.m.) on Days −5 to 0 (N = 5), 0 to +5 (N = 5) or −5 to +5 ( N = 5) around the day of LH-RH treatment (Day 0). Control animals (N = 4) received saline on Days −5 to +5. Nine animals ovulated forming corpora lutea as judged by laparoscopy on Day +7. No difference in FSH or LH levels was found between treatments and ovulations occurred equally in all treatment groups. Progesterone levels were < 1 ng/ml in all animals up to Day 14. It is concluded that short-term suppression of prolactin does not affect the incidence of ovulation or corpus luteum progesterone production in LH-RH-treated anoestrous ewes.

## Introduction

Seasonal anoestrus in the ewe is associated with an absence of ovulation and corpus luteum (CL) function and decreased sensitivity to the positive feedback and increased sensitivity to the negative feedback effects of oestrogen on luteinizing hormone (LH) secretion (Land, Wheeler & Carr, 1976; Legan, Karsch & Foster, 1977). Serum levels of follicle-stimulating hormone (FSH) are not significantly different from levels seen during the oestrous cycle but serum levels of prolactin increase just before and remain elevated throughout seasonal anoestrus and fall before the onset of oestrous cyclicity in the autumn (Walton, McNeilly, McNeilly & Cunningham, 1977).

While ovulation has been induced by single or multiple injections or infusions of LH-RH in seasonally anoestrous ewes, the resulting CL fails to secrete normal amounts of progesterone (Crighton, Foster, Haresign, Haynes & Lamming, 1973; Crighton, Foster, Haresign & Scott, 1975; Haresign, Foster, Crighton, Haynes & Lamming, 1975; Shareha, Ward & Birchall, 1976). It was originally suggested that this was due to an inadequate release of LH at the time of ovulation (Crighton *et al.*, 1975). However, PMSG pretreatment of these ewes results in a fully functional CL, suggesting that failure of normal luteal function is probably due to inadequate gonadotrophin priming before the ovulatory surge of LH rather than to inadequacy of the surge of LH itself (Haresign & Lamming, 1978).

Nevertheless, the possibility that the elevated levels of prolactin associated with anoestrus may directly affect the function of the CL in sheep has not been examined. Lactational, pathological or pharmacologically induced hyperprolactinaemia in women is associated with absent or reduced luteal function (for references see Robyn *et al.*, 1977; McNeilly, 1979). High levels of prolactin reduce progesterone output from human granulosa cells *in vitro* (McNatty, Sawers & McNeilly, 1974) and suppress progesterone secretion and delay reactivation of the

quiescent CL of the tammar wallaby (Tyndale-Biscoe & Hawkins, 1977). Rhind, Chesworth & Robinson (1978) found a reduced output of progesterone by the CL of pregnant ewes at times when serum levels of prolactin were seasonally elevated.

In order to determine whether elevated serum levels of prolactin suppress progesterone output from CL formed during anoestrus we have assessed the effect of suppression of serum levels of prolactin on ovulation and luteal function in anoestrous ewes treated with LH-RH.

## Materials and Methods

*Animals and treatments*

Experiments were carried out in early August during anoestrus. Nineteen Scottish Blackface ewes were housed indoors under natural illumination and randomly allocated to one of four groups. All ewes were given three i.v. injections of 30 μg synthetic LH-RH (Hoechst) at 90-min intervals (10:00, 11:30 and 13:00 h) on Day 0. The 4 treatments consisted of daily injections at 09:00 and 21:00 h of saline (0·9% (w/v) NaCl) from Day −5 to Day +5, or CB 154 (100 μg 2α-bromo-ergocryptine i.m.: Sandoz) from Days −5 to 0, 0 to +5 or −5 to +5. First and last injections were given at 09:00 and 21:00 h respectively.

*Ovarian activity*

The ovaries of all ewes were examined by laparoscopy performed under barbiturate anaesthesia 6 days before LH-RH treatment; it was established that all ewes were acyclic since no CL were present. Laparoscopy was repeated 7 days after LH-RH treatment to see if any new CL had been formed.

*Blood sampling and hormone estimations*

Daily jugular venous blood samples were taken from all animals at 09:00 h on each of Days −6 to +14, and immediately before any treatment injection on that day. On Day 0, samples were collected at 30-min intervals from 1 h before to 3 h after the first injection, and subsequently every 1 or 2 h until 23 h after the first LH-RH injection. Blood samples were collected into heparinized evacuated tubes, separated by centrifugation and stored at −20°C until assayed.

Prolactin, LH, FSH and progesterone were measured in duplicate by radioimmunoassays previously described in detail (McNeilly & Andrews, 1974; Baird, Burger, Heavon-Jones & Scaramuzzi, 1974; McNeilly, McNeilly, Walton & Cunningham, 1976; Martensz, Baird, Scaramuzzi & Van Look, 1976). The sensitivities of the assays were 0·05 ng prolactin (NIH P-S6)/ml, 0·1 ng LH (NIH-LH-S14)/ml and 16 ng FSH (NIH-FSH-S10)/ml. All samples were analysed in one assay for each hormone. The intra-assay variations, as coefficient of variation (%) of three quality control sera (at 30, 55 and 80% B/Bo), were 7, 6 and 9% for prolactin, LH and FSH respectively.

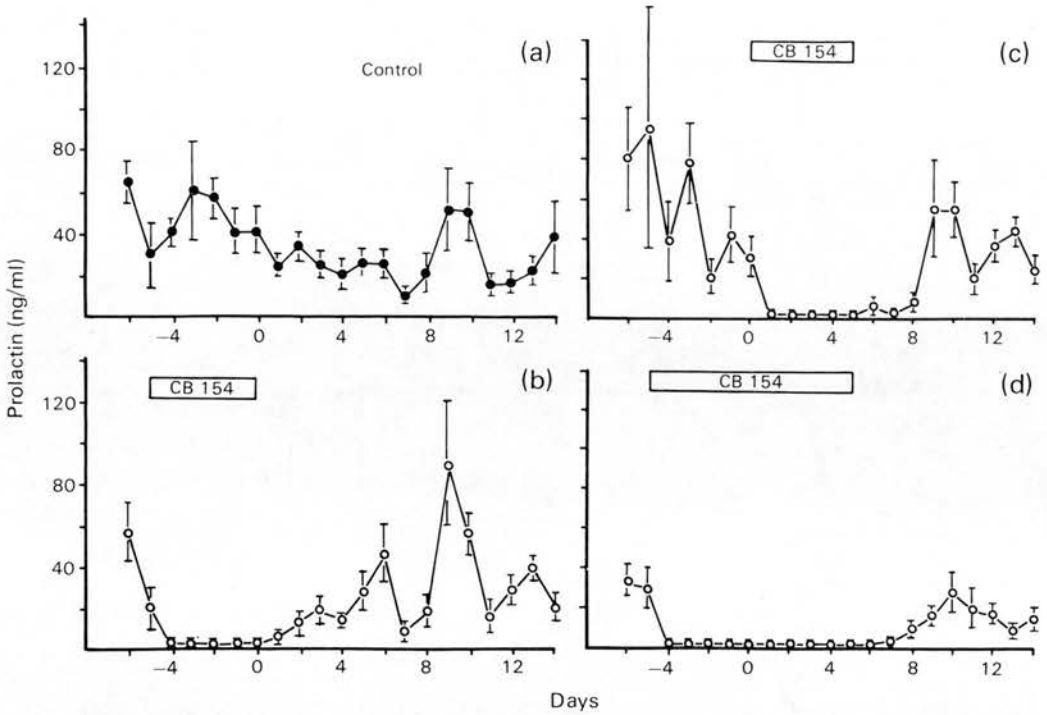
*Statistical analysis*

Results were analysed by Student's *t* test or analysis of variance.

## Results

*Effect of CB 154 on daily blood levels of hormone*

In control animals daily prolactin levels varied between 5 and 140 ng/ml (Text-fig. 1). During periods of treatment with CB 154 plasma levels of prolactin remained suppressed in all groups (range 0·5 to 2 ng/ml; Text-fig. 1). CB 154 caused an abrupt decline in plasma levels of prolactin within 30 min of injection and these low values of 0·5–1·5 ng/ml were maintained for at least 12 h.

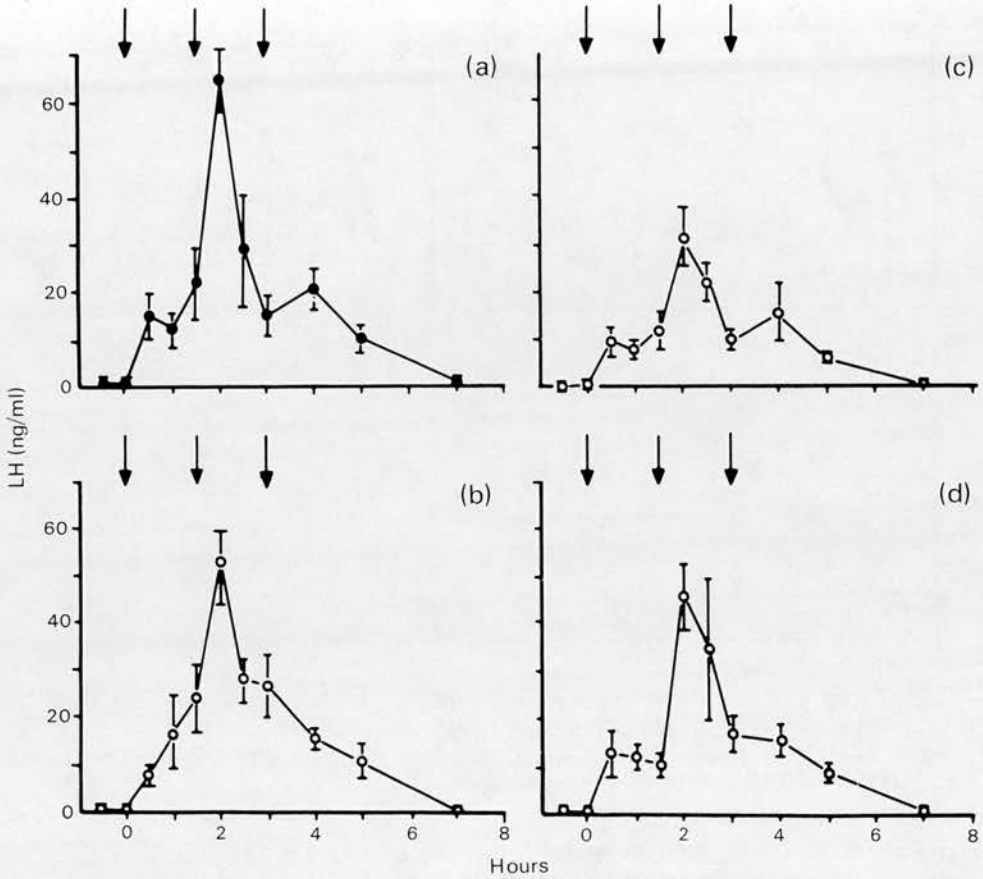



**Text-fig. 1.** Effect of CB 154, 100 μg i.m. twice daily at 09:00 and 21:00 h (horizontal bars), on mean ± s.e.m. daily plasma concentrations of prolactin in ewes before and after LH-RH injection (Day 0). (a) N = 4; (b) (c) and (d) N = 5.

During the 5-day period before LH-RH (Days −5 to −1), CB 154 had no effect on mean (± s.e.m.) daily plasma levels of LH (0·69 ± 0·10 ng/ml in untreated ewes (saline) and those treated with CB 154 from Days 0 to +5, $n = 45$, compared with 0·63 ± 0·05 ng/ml in the ewes treated with CB 154 from Days −5 to 0 and −5 to +5; $n = 50$). Daily plasma levels of FSH were also unaffacted by CB 154 (103 ± 11 ng/ml in untreated ewes and 80 ± 8 ng/ml in CB 154 treated ewes).

### *LH and FSH response to LH-RH*

*LH.* The injection of LH-RH caused an increase in the plasma concentration of LH in all animals. The pattern of response was similar in all groups (Text-fig. 2) with an initial increment of between 2 and 28 ng/ml (mean 10 ng/ml) by 30 min after the first injection of LH-RH. A much greater increase (mean increment 33 ng/ml: range 13–67 ng/ml) occurred by 30 min after the second injection of LH-RH relative to the concentration at the time of injection, while the third injection appeared ineffective, the concentration of LH increasing in only 9 of the 19 animals. The overall pattern of response was the same in those animals which ovulated and those which failed to ovulate (Text-fig. 3). A comparison of the cumulative LH release over the 8 h after the first LH-RH injection showed no significant differences between treatment groups or between those animals which did or did not ovulate (Table 1). The amount of LH released in the ewes treated with CB 154 from Days 0 to +5 was reduced compared to the control but this did not achieve significance ($P = 0·1$). The duration of response, as defined by the time taken for levels to return to preinjection values, was 7 h in 18 out of 19 animals and 9 h in the remaining animal.

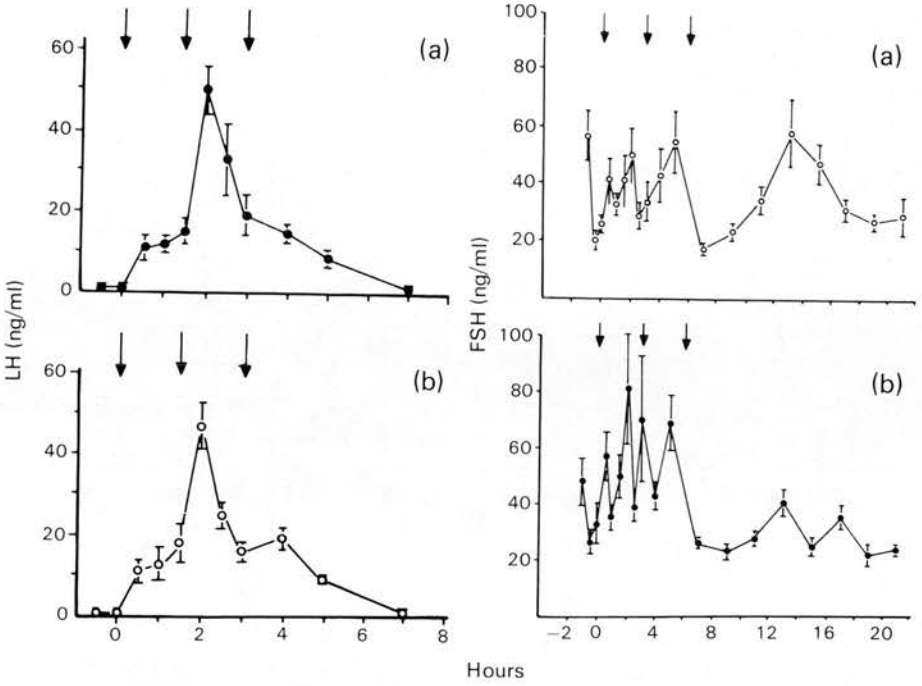



**Text-fig. 2.** Changes in the mean ± s.e.m. plasma concentration of LH in ewes after 3 i.v. injections of 30 μg LH-RH at 90-min intervals (arrows). (a) Saline-treated controls, N = 4; (b) CB 154 injected from −5 to 0 days, N = 5; (c) CB 154 injected from 0 to +5 days, N = 5; (d) CB 154 injected from −5 to +5 days, N = 5, relative to day of LH-RH treatment (Day 0).

**Table 1.** The release of LH and FSH (as cumulative totals) after 3 injections of LH-RH in anoestrous ewes treated with CB 154

| Treatment | No. of ewes | Cumulative FSH release (ng/ml) 0–7 h | 8–19 h | 0–19 h | Ratio of FSH release 0–7:8–19 | Cumulative LH release (ng/ml) (0–8 h) |
|---|---|---|---|---|---|---|
| None (controls) | 4 | 620 ± 82 | 1027 ± 49 | 1639 ± 83 | 1·79 ± 0·29 | 190 ± 33 |
| CB 154, Days −5 to 0 | 5 | 630 ± 88 | 869 ± 100 | 1500 ± 124 | 1·53 ± 0·25 | 187 ± 31 |
| CB 154, Days 0 to +5 | 5 | 509 ± 56 | 678 ± 174 | 1187 ± 185 | 1·43 ± 0·37 | 119 ± 18 |
| CB 154, Days −5 to +5 | 5 | 524 ± 72 | 691 ± 82 | 1215 ± 110 | 1·46 ± 0·32 | 159 ± 22 |
| Ovulating* | 9 | 480 ± 38 | 896 ± 94 | 1373 ± 115 | 1·94 ± 0·22 | 166 ± 24 |
| Not ovulating* | 10 | 648 ± 50 | 723 ± 78 | 1371 ± 107 | 1·23 ± 0·15 | 159 ± 16 |

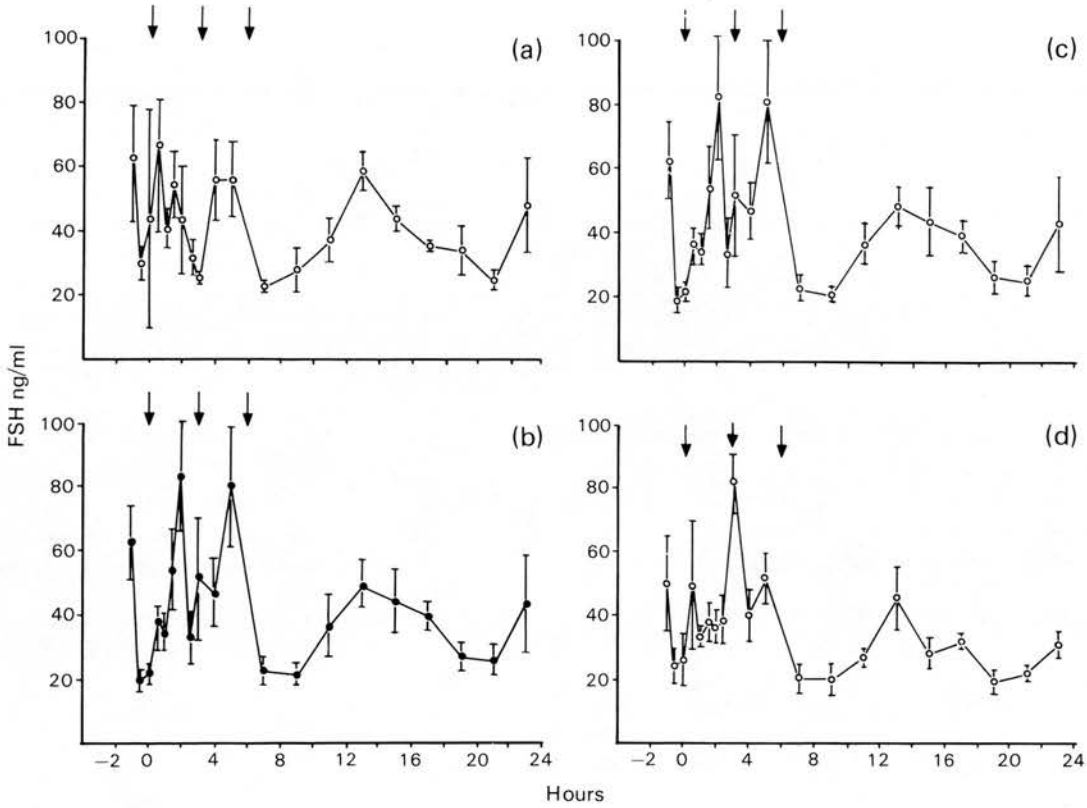
Values are mean ± s.e.m. Times are relative to first injection of LH-RH (0 h).
* Results from all 4 treatments combined.




**Text-fig. 3.** Changes in the mean ± s.e.m. plasma concentration of LH and FSH after 3 i.v. injections of 30 μg LH-RH at 90-min intervals (arrows) in animals which subsequently did (a; N = 9) or did not (b; N = 10) ovulate.

*FSH.* LH-RH caused an increase in the plasma concentration of FSH within 30 min in 17 out of 19 animals after both the first and the second injections, 1 ewe responded to the first and not the second injection while the other responded to the second and not the first injection. No immediate response was seen after the third injection of LH-RH although a rise in the plasma concentration of FSH occurred in 10 out of 19 animals 11–13 h after the first injection (5–7 h after the last injection of LH-RH; Text-fig. 4). Estimation of the amount of FSH released (as the cumulative total of serum FSH values) between 0 and 7 h after the initial injection of LH-RH showed no statistically significant differences between the four groups (Table 1, Text-fig. 4). However, animals treated with CB 154 between −5 and +5 days after LH-RH released less FSH between 8 and 19 h after the initial injection of LH-RH than did controls (691 ± 82 versus 1027 ± 49 ng/ml; $P < 0{\cdot}02$; Table 1). In addition, the overall amount of FSH released in response to LH-RH (0 to 19 h) was significantly less ($P < 0{\cdot}02$) in animals treated with CB 154 (CB 154 on Days −5 to +5 and −5 to 0) than in animals not injected with CB 154 at the time of LH-RH injection (Day 0).

The total FSH response between 0 and 19 h after the initial injection of LH-RH was the same in animals which ovulated as in those which did not (Table 1). However, significantly less ($P < 0{\cdot}02$) FSH was released in the initial response period (0–7 h) by the animals that ovulated and more FSH was released in the second response period (8–19 h) than in those which did not ovulate (Text-fig. 3b; Table 1). The ratio of the amount of FSH released between 8 and 18 h and 0 and 7 h was greater than 1·4 in 8 out of the 9 animals which ovulated. In contrast this ratio was greater than 1·4 in only 1 out of the 10 animals which failed to ovulate. There was no difference in the basal levels of FSH in the 6 days before LH-RH treatment in animals which did or did not ovulate.




**Text-fig. 4.** Changes in mean ± s.e.m. plasma concentration of FSH after 3 i.v. injections of 30 μg LH-RH at 90-min intervals (arrows). (a) Saline-treated controls, N = 4; (b) CB 154 injected from −5 to 0 days, N = 5; (c) CB 154 injected from 0 to +5 days, N = 5; (d) CB 154 injected from −5 to + 5 days, N = 5, relative to day of LH-RH treatment (Day 0).

*Ovulation and corpus luteum activity*

Treatment with LH-RH induced ovulation in 9 out of 19 ewes as judged at laparoscopy on Day 7 after LH-RH treatment. The number of animals ovulating in each group were as follows: control, 2/4; CB 154 Days −5 to 0, 3/5; CB 154 Days 0 to + 5, 2/5; CB 154 Days −5 to + 5, 2/5. No changes in plasma levels of progesterone were seen in animals which did not ovulate (progesterone levels all < 0·2 ng/ml). While plasma levels of progesterone increased within the normal range from 0 to 3 days after treatment in animals which ovulated, this increase was not sustained and in 8 out of 9 animals progesterone levels declined to basal values (< 0·1 ng/ml) by Day 9. In the 9th ewe which had been treated with CB 154 for 10 days (−5 to + 5 days) progesterone levels showed a small increase up to Day 4, became undetectable (< 0·1 ng/ml) on Day 6 and then increased progressively from Day 8 to values equivalent to those in the normal early luteal phase of the cycle (1·5 ng/ml).

## Discussion

The results of the present study confirm previous reports that LH-RH can be used to induce ovulation as judged by the presence of a CL in anoestrous ewes (by injection: Foster & Crighton, 1973; Crighton *et al.*, 1975; by infusion: Shareha *et al.*, 1976).

The results also confirm that the CL formed fails to secrete progesterone in amounts equivalent to those seen during the luteal phase of the normal oestrous cycle (Crighton *et al.*, 1973, 1975; Haresign *et al.*, 1975; Shareha *et al.*, 1976). Treatment with CB 154 with concomitant reduction in plasma levels of prolactin did not affect the basal plasma levels of LH or FSH, or the occurrence of ovulation, confirming previous observations when CB 154 was given to ewes during the oestrous cycle (Niswender, 1974) or during anoestrus when ovulation was induced by treatment with oestradiol benzoate (R. B. Land, W. R. Carr & A. S. McNeilly, unpublished observations). The failure of progesterone secretion from all CL regardless of the prolactin status does not support the hypothesis that high levels of prolactin directly inhibit progesterone secretion by the CL (Rhind *et al.*, 1978). It is possible that prolactin may directly suppress progesterone secretion from a CL formed after the follicles have been primed with gonadotrophin before ovulation. In the study by Rhind *et al.* (1978) ewes were pretreated with PMSG.

Nevertheless, in the present study in the absence of exogenous gonadotrophin priming, even when prolactin levels were suppressed in two of the groups for 5 days before ovulation was induced, luteal function was still inadequate. This suggests that if high levels of prolactin are implicated in the failure of progesterone secretion, then the effect must be directed at the early stages of follicular development. This conclusion has been suggested by studies with CB 154 and sulpiride in women (Robyn *et al.*, 1977).

It has been suggested that this failure of normal luteal function was due to an inadequate release of LH after LH-RH stimulation (Crighton *et al.*, 1973) but this does not appear to be the case. Multiple injections of LH-RH (Crighton *et al.*, 1975) and infusion of LH-RH (Shareha *et al.*, 1976) appear to release amounts of LH similar to those found during the normal LH discharge in the oestrous cycle. Further, in the present study it is apparent that the occurrence of ovulation is not dependent on the amount of LH released in response to LH-RH treatment. Ovulation occurred in one ewe releasing a cumulative total of only 78 ng LH/ml whereas no ovulation occurred in another ewe in which the cumulative total was 252 ng LH/ml. These results would support the concept that in anoestrus ovulation depends on the status of follicles within the ovary and not on the exposure of the follicle to an ovulatory surge of LH. This is supported by the observations of Hutchinson & Robertson (1966) and Haresign (1975) that in mid-anoestrus there were no follicles of a size which would normally ovulate ($> 5{\cdot}5$ mm in diameter). The present experiment was carried out in early August. Scottish Blackface ewes do not start oestrous cycles until the end of September (Wheeler & Land, 1977). Thus these animals were probably 8 weeks from spontaneous ovulation yet 9 out of 19 had follicles which ovulated in response to an LH-RH-induced surge of LH.

The difference in the pattern of FSH release in those animals which ovulated compared to those which did not is of considerable interest. While the total amount of FSH released over 19 h of observation after LH-RH injection was the same the amount of FSH released immediately after LH-RH (0 to + 7 h) was significantly less in those animals which subsequently ovulated, indicating a change in the pattern of release. The FSH response in animals which ovulated was similar to the changes in FSH seen during spontaneous oestrus in the ewe when a secondary rise in FSH occurs 12–24 h after the rise in FSH which occurs at the time of the preovulatory LH discharge (L'Hermite, Niswender, Reichert & Midgley, 1972; Pant, Hopkinson & Fitzpatrick, 1977). The basal levels of FSH in the sheep appear to decrease as oestrogen secretion increases during the period of oestrus (L'Hermite *et al.*, 1972; Pant *et al.*, 1977) and oestradiol-17β inhibits the basal output of FSH from sheep pituitary cells *in vitro* (Miller, Knight, Grimek & Gorski, 1977). The smaller initial increment in FSH after LH-RH stimulation seen in the present study may thus represent the effects of greater oestrogen secretion from a follicle which is capable of ovulating. Ovarian secretion of oestradiol is maintained during anoestrus (Martensz *et al.*, 1976) and increases in response to LH stimulation (Scaramuzzi & Baird, 1977). In the sheep oestradiol is secreted almost exclusively from the largest non-atretic follicle (Hay & Moor,

1975; Baird & Scaramuzzi, 1976) and therefore the lower initial FSH release occurring only in the animals ovulating would suggest that the difference in response pattern for FSH is mediated by ovarian steroids.

The occurrence of ovulation after LH-RH treatment is apparently not dependent on the amount of LH or FSH released at the time of ovulation but on the status of the follicles within the ovary. This conclusion supports data obtained from pretreatment of anoestrous ewes with PMSG before induced ovulation which resulted in normal luteal function (Haresign & Lamming, 1978). It further suggests that the failure of progesterone secretion from the CL which is formed when ovulation does occur is not related to a direct action of the high levels of prolactin associated with anoestrus. If prolactin is involved in the reduced progesterone secretion then this involvement must be related to an effect of elevated prolactin levels on the very early stages of follicular development, a situation similar to that in women (Robyn *et al.*, 1977).

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# Plasma FSH, LH, the positive feedback of oestrogen, ovulation and luteal function in the ewe given bromocriptine to suppress prolactin during seasonal anoestrus

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**Summary.** The effects of pharmacological reduction of the high plasma prolactin concentration typical of seasonal anoestrus in sheep were assessed with respect to positive feedback of oestrogen on LH release, ovulation, and progesterone secretion. Treatment of 16 Scottish Blackface ewes with 1 mg bromocriptine, i.m. twice daily for 12 days, reduced prolactin concentrations in peripheral plasma from 64 ± 10 ng/ml before treatment to <4 ng/ml. This treatment had no effect on the proportion of ewes discharging LH and FSH in response to 12·5 μg oestradiol benzoate (3/8 before compared with 5/16 during treatment) or the proportion of ewes ovulating in response to oestrogen treatment. Plasma progesterone concentrations remained low even in ovulating ewes.

It is concluded that treatment with bromocriptine alone is unlikely to restore oestrous cycles to ewes in seasonal anoestrus.

## Introduction

Seasonal anoestrus in sheep has been postulated to arise in part from a reduced sensitivity of the positive feedback response to oestrogen (Land, Wheeler & Carr, 1976). Since hyperprolactinaemia has also been reported to block the oestrogen-induced release of LH (Kann, Martinet & Schirar, 1978), and hyperprolactinaemia is a characteristic of anoestrous sheep (Walton, McNeilly, McNeilly & Cunningham, 1977), we have investigated the effects of reducing the prolactin concentrations in sheep during anoestrus with the dopamine agonist, bromocriptine.

## Materials and Methods

### *Animals and experiments*

The ewes were 4-year-old Scottish Blackface. The 16 animals were maintained outdoors at pasture under natural lighting at the A.B.R.O. Dryden Field Laboratory and in April 1977 a raddled vasectomized ram was introduced to check that they were anoestrous.

Reproductive activity was assessed by measuring the tonic concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in peripheral plasma, and their response to an injection of oestradiol benzoate. The occurrence of oestrus, the incidence of

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ovulation, and progesterone production by any corpora lutea were also recorded. The oestrogen-induced release of LH was assessed in only 8 of the ewes before treatment with bromocriptine so that the effects of a reduction in the concentration of prolactin could be separated from possible effects of oestrogen.

Bromocriptine (CB 154: Sandoz Ltd) was used to suppress the release of prolactin; 1 mg was given twice daily i.m. at 08:30 and 22:30 h for 12 days (Days 0–11 inclusive), each dose being made up by diluting aliquots from a stock solution of CB 154 in 70% ethanol with sterile saline (9 g NaCl/l) acidified with tartaric acid.

*Tonic concentrations of gonadotrophins in peripheral plasma*

Half of the ewes (Group A) were chosen at random and bled at 20-min intervals from 09:00 to 17:00 h on Day −4 before the start of bromocriptine treatment on Day 0. Blood was collected via an indwelling cannula placed in the jugular vein the previous day, and FSH, LH and prolactin were assayed in the plasma. This procedure was repeated in the same 8 ewes on Day 9. Comparisons of the hormone values on the 2 occasions were based on analysis of variance.

In 1 of these 8 sheep the concentration of LH on Day −4 reached 140 ng/ml on one occasion and the overall pattern was typical of a preovulatory discharge of LH. This animal was therefore excluded from the comparison of basal levels before and during bromocriptine treatment.

*Oestrogen-induced release of gonadotrophins*

The positive feedback response to oestrogen was tested in the other 8 ewes (Group B) which were injected i.m. with 12·5 μg oestradiol benzoate B.P. (Intervet) made up to 2 ml with ethyl oleate at 11:00 h on Day −4. They were bled at 0·5, 1 and 1·5 h before and every 2 h from 1 to 29 h after the oestradiol injection. Blood was collected in evacuated heparinized tubes by repeated jugular venepuncture and FSH and LH were assayed in the plasma. The same oestrogen stimulation test was conducted in all 16 ewes on Day 10.

The dose of oestradiol benzoate was chosen after a small trial in which anoestrous Blackface ewes were treated with 6·25 (4 animals), 12·5 (4 animals), 25 (4 animals) or 50 (3 animals) μg oestradiol benzoate. An LH response was obtained in 0, 1, 3 and 3 animals respectively, and 12·5 μg was therefore considered to be a suitable test dose for detecting any increase in hypothalamic sensitivity after bromocriptine treatment.

The criterion of effective positive feedback was related to comparison with the mean ± s.e. values of tonic concentrations of FSH (230 ± 50 ng/ml) and LH (1·5 ± 2·1 ng/ml). Using 3 standard deviations as a rigorous test of a deviation, this gave a criterion of 1·5 and 5 times the tonic concentration of FSH and LH respectively.

*Ovulation.* Both ovaries of all ewes were examined at laparoscopy on Days 1 and 17. The presence of a fresh (red) corpus luteum was taken as evidence of a recent ovulation. The concentration of progesterone was measured in blood samples collected daily on Days 12–18 as an index of the secretory activity of any corpora lutea.

*Oestrus.* The occurrence of oestrus was checked daily throughout the study by continuous teasing with vasectomized rams (apart from periods of blood sampling or surgery).

*Prolactin concentrations.* These were measured in samples collected daily on Days 0–11 at approximately 08:30 h, just before the next injection of bromocriptine. The mean ± s.e.m. concentration declined from 63·8 ± 10·2 ng prolactin/ml on Day 0 to 3·9 ± 0·4 ng/ml the following morning. Thereafter mean concentrations did not exceed 2·8 ng/ml, i.e. approximately 5% of normal, while bromocriptine treatment continued.

*Hormone assays*

Plasma was stored at −20°C until assayed.

*LH* was measured by the double-antibody method of Carr & Land (1975). The rabbit ovine LH antiserum validated for specificity and sensitivity by Martensz, Baird, Scaramuzzi & Van Look (1976) and a purified ovine LH for labelling (M4; Jutisz & Courte, 1968) were used in the assay and the results were computed by the A.B.R.O. Radioimmunoassay Program package based on the method of Rodbard & Lewald (1970). NIH-LH-S18 was used as the standard. The minimum detectable dose was 0·3 ng/ml and the coefficient of variation among assays was 9·6%.

*FSH* was measured in triplicate by the assay described by McNeilly, McNeilly, Walton & Cunningham (1976). All samples were measured in one assay, the sensitivity of which was 16 ng/ml (NIH-FSH-S10). The variation within the assay of 3 quality control sera (at 30, 55 and 80% B/Bo) was 9%.

*Prolactin* was measured in duplicate as described by McNeilly & Andrews (1974). All samples were measured in a single assay with a sensitivity of 0·05 ng/ml (NIH-P-56) and an intra-assay coefficient of variation of 8% for 3 quality control sera at 30, 55 and 80% B/Bo.

*Progesterone* concentrations were measured in a single-antibody solid-phase assay, similar to that described by Dighe & Hunter (1974), using a specific sheep antiprogesterone serum (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). All standards were diluted with buffer containing charcoal-treated calf plasma before extraction, so that standards and unknowns contained the same amount of plasma. A control plasma sample from a ewe with a functional corpus luteum was included in the assay, and the potency estimate was 4·5 ng/ml (95% confidence limits 3·7–5·5 ng/ml); previous experience indicated that ewes at mid-cycle or in early pregnancy had plasma progesterone levels of >2 ng/ml. The limit of sensitivity of the assay was 0·3 ng/ml.

## Results

*Tonic gonadotrophin concentrations*

*FSH.* The mean concentration of FSH was 230·8 ng/ml on Day −4, before treatment, and 228·6 ng/ml during treatment. The means for individuals ranged from 162 to 310 ng/ml before treatment and from 172 to 373 ng/ml during treatment. The analysis of variance showed that there were real differences among animals ($P < 0·001$), and the repeatability computed as the intra-class correlation was 0·63.

*LH.* Pulsatile release of LH occurred, the frequency decreasing from 1 peak/5 h before treatment to 1 peak/8 h during treatment, but the difference is not statistically significant.

As with FSH there was an approximately 2-fold range among animals, but the probability of the variation arising by chance was less than 5% only when the data were transformed to logarithms to cope with the log normal distribution of LH resulting from the pulsatile nature of its release. The highest and lowest animal means were 2·2 and 1·1 ng/ml before treatment and 2·9 and 0·4 ng/ml during treatment. The repeatability of the concentration was 3% based on the untransformed data, and 12% based on the transformed data. The arithmetic mean plasma LH concentration was 1·57 ng/ml before and 1·51 ng/ml during treatment, indicating an overall lack of effect of bromocriptine treatment on LH concentrations.

*Positive feedback*

As shown in Table 1, 3 of the 8 ewes treated with oestradiol on Day −4 discharged both FSH and LH; the rise in concentration of FSH was 2 h later than that of LH in all 3 ewes. Of the 16 animals treated with oestradiol on Day 10, LH was released by 5 (2 from Group A, 3 from

Group B). These 5 ewes also released FSH, which was again 2 h after the rise in LH concentration. Bromocriptine treatment did not therefore affect the proportion of animals releasing LH or FSH in response to oestradiol.

*Ovulation.* Oestradiol treatment was followed by ovulation in 3 of the 8 Group B animals tested on Day −4 and in 2 of the 8 Group A ewes tested on Day 10. The Group A ewe that showed a preovulatory-type LH surge on Day −4 had ovulated by the time of laparoscopy on Day 1. In none of the 16 animals was the plasma progesterone concentration on Days 12–18 above 0·7 ng/ml and it was mostly <0·3 ng/ml, indicating that the corpora lutea formed in response to oestradiol treatment were not functional. A rise in LH concentration was not detected in 2 ewes which ovulated after the oestrogen injection (Table 1). In 1 of the ewes the concentration was rising in the last 2 samples collected, but it did not do so sufficiently to meet the criteria set for positive feedback. There was no indication of an LH discharge for the other animal. Two of the 3 sheep that ovulated after the first oestradiol injection also released LH after the second oestradiol injection, further evidence that the corpora lutea were non-functional. However, neither ewe ovulated in response to the second injection. Treatment with bromocriptine did not therefore increase the proportion of ewes ovulating after injection of oestradiol benzoate.

**Table 1.** Summary of the effects of bromocriptine on the oestrogen-induced LH discharge and the incidence of ovulation in Blackface ewes during anoestrus

| | Total no. of ewes | LH response to oestradiol on Day −4 | No. with fresh CL on Day 1 | No. with (+) and without (−) LH response to oestradiol on Day 10 | No. with fresh CL on Day 17 |
|---|---|---|---|---|---|
| Oestradiol on Day 10 only (Group A) | 8 | — | 1 | 2+ | 1 |
| | | | | 6− | 1 |
| Oestradiol on Days −4 and 10 (Group B) | 8 | 3+ | 2 | 1+ | 0 |
| | | | | 2− | 0 |
| | | 5− | 1 | 2+ | 0 |
| | | | | 3− | 0 |

## Discussion

The principal object of the experiment was to examine the extent to which a reduction in the concentration of prolactin in peripheral plasma by treatment with bromocriptine might facilitate the restoration of reproductive function in anoestrous ewes. Bromocriptine treatment did reduce the concentration of prolactin dramatically but this did not lead to a detectable increase in sensitivity to positive feedback, and there was no increase in the number of animals ovulating. Small changes in the proportion of ewes discharging LH in response to the injection of 12·5 μg oestradiol could not have been detected in an experiment of this size, and with the impossibility of testing the sensitivity of intact cyclic ewes in this way the purported effect and hence the change to be detected cannot be given. Land *et al.* (1976) found that the number of ovariectomized Finnish Landrace ewes that discharged LH often varied from 4 of 12 at the time of anoestrus (the same as in the total of untreated females in the present study) to 12 of 12 in November, but no such increase was obtained in the present experiment. The results therefore do not confirm the implication of the observation by Kann *et al.* (1978) that TRH-induced hyperprolactinaemia reduced sensitivity of ovariectomized ewes to positive feedback during the breeding season, and that bromocriptine, at a dose which reduced the concentration of prolactin in the plasma of suckling ewes, increased their sensitivity to positive feedback. Despite this demonstration that high prolactin levels suppress positive feedback, the presence of high

prolactin concentrations during anoestrus, the ability of bromocriptine to suppress these concentrations, and although positive feedback may be reduced (Land *et al.*, 1976) and negative feedback may be increased (Legan & Karsch, 1979) during seasonal anoestrus, the present results indicate that insensitive positive feedback arises for reasons over and above hyperprolactinaemia. Furthermore, as reported by Haresign, Foster, Haynes, Crighton & Lamming (1975) and by McNeilly & Land (1979) for LH-RH-induced corpora lutea, the corpora lutea formed did not secrete progesterone. It may therefore be concluded from the present short-term, acute tests that unless hyperprolactinaemia has a long-term chronic effect, treatment with bromocriptine alone is unlikely to lead to a resumption of oestrous cycles. It must, however, be borne in mind that bromocriptine may have effects on reproduction beyond the suppression of prolactin release; for example, McNeilly & Land (1979) report a reduction in the LH-RH-induced release of gonadotrophins. It is also evident that individuals differ markedly; the concentration of FSH and of LH both varied 2-fold although all animals were of the same breed and all were anoestrous.

Legan & Karsch (1979) have questioned the role of changes in positive feedback in the control of anoestrus in the ewe, and propose that seasonal changes are regulated by variation in the sensitivity of the negative feedback of oestradiol on tonic LH secretion alone. Increased sensitivity during anoestrus prevents the initiation of the LH cascade which normally culminates in the preovulatory discharge. Legan & Karsch (1979) suggest that the seasonal changes in positive feedback found in long-term ovariectomized ewes by Land *et al.* (1976) may only have been detected as a result of fundamental changes in the response of the hypothalamus to oestrogen following the prolonged absence of gonadal steroids, and as such do not constitute evidence for steroid-independent change in hypothalamic activity. The magnitude of any such effect is not, however, large: 4 of 12 ewes treated with 12·5 μg oestradiol for the first time responded in the present experiment compared to 8 of 12 long-term ovariectomized ewes which responded to 50 μg in the earlier experiment. An increase in sensitivity to negative feedback and a decrease in sensitivity to positive feedback may not be alternatives but rather reflections of underlying seasonal changes in CNS activity and hence may both be characteristic of seasonal anoestrus. Other seasonal changes can be demonstrated. For example, prostaglandin F-2α leads to the release of LH during the breeding season but not during anoestrus (Roberts, Carlson & McCracken, 1976), and prolactin concentrations rise spectacularly during anoestrus (Ravault, 1976). Both of these responses may reflect a general change in spontaneous hypothalamic activity rather than a specific effect dependent upon steroids as discussed by Land & Carr (1979). Given that a decrease in hypothalamic activity would lead to both stronger negative feedback and weaker positive feedback and the known differences in feedback sensitivity and seasonality among breeds of sheep, the relative contributions of these two secondary changes to the actual cessation and resumption of cyclicity may vary among sheep and among environments.

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# Inadequate corpus luteum function after the induction of ovulation in anoestrous ewes by LH-RH or an LH-RH agonist

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**Summary.** Scottish Blackface ewes were given LH-RH (3 × 30 μg i.v., at 90 min intervals) or D-Ser-(But)$^6$-des Gly$^{10}$ LH-RH ethylamide (LH-RH agonist) as a single injection (8 or 40 μg) during anoestrus. Ovulation as judged by laparoscopy occurred in 8 of the 27 animals. Despite the fact that the LH-RH agonist induced a greater release of LH and FSH the different treatments had no effect on the number of ewes ovulating and within each treatment group there was no apparent difference in the amounts of gonadotrophins released between the ewes that did or did not ovulate. All ovulations resulted in the formation of CL associated with plasma progesterone concentrations of <1 ng/ml (1–5 ng/ml in the normal luteal phase). In comparison with CL of the normal cycle the induced CL were of lower weight and had reduced progesterone content and ability to secrete progesterone *in vitro*. However, the binding of hCG was equivalent to that of normal CL.

These results suggest that the inadequate CL formed in anoestrous ewes after a single LH-RH injection have not developed the ability to synthesize and secrete progesterone in spite of the presence of normal amounts of LH receptors.

## Introduction

While ovulation can be induced by single or multiple injections or infusions of LH-RH in seasonally anoestrous ewes, the resulting corpus luteum (CL) fails to secrete amounts of progesterone comparable to those secreted by CL during the breeding season and is therefore considered to be inadequate (Crighton, Foster, Haresign & Scott, 1975; Haresign, Foster, Haynes, Crighton & Lamming, 1975; Shareha, Ward & Birchall, 1976). Suppression of the elevated plasma levels of prolactin associated with anoestrus before induction of ovulation failed to alleviate this malfunction of the CL (McNeilly & Land, 1979; Land, Carr, McNeilly & Preece, 1980), although pretreatment of such ewes with PMSG was effective (Haresign & Lamming, 1978). These results suggest that the failure of normal luteal function in anoestrous ewes is probably due to inadequate gonadotrophin priming of the follicle before the ovulatory LH surge (McNeilly, 1980).

Nevertheless, it has been reported that ovulation followed by normal CL function can be induced in anoestrous ewes by the injection of a highly potent agonist of LH-RH (Frandle *et al.*, 1977). LH-RH agonists release a greater amount of FSH than does a single injection of LH-RH (Siddall & Crighton, 1977) and this enhanced secretion of gonadotrophin might be sufficient to

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prime the follicle before ovulation, thus allowing formation of a fully functional CL.

The present study was designed to compare CL function after ovulation had been induced by LH-RH or an LH-RH agonist. Since it was anticipated that ewes ovulating after treatment with LH-RH would have inadequate CL, the site of this inadequacy was investigated by comparing the ability of their CL to produce progesterone and bind LH *in vitro* with that of CL recovered from ewes during a normal oestrous cycle.

## Materials and Methods

### *Animals*

The experiment was carried out in July 1978. The 27 3- or 4-year-old Scottish Blackface ewes were maintained outdoors at pasture under natural lighting at the A.B.R.O. Dryden Field Laboratory. A vasectomized ram was introduced in May to check that they were anoestrous.

### *LH-RH and LH-RH agonist treatments*

Ewes were randomly allocated to 3 groups (9 ewes in each) and on Day 0 were treated as follows: Group 1, 3 i.v. injections of 30 μg synthetic LH-RH (Hoechst, Frankfurt, Germany) at 90-min intervals (10:00, 11:30 and 13:00 h); Group 2, a single i.v. injection (10:00 h) of 8 μg synthetic D-Ser-(But)$^6$-des Gly$^{10}$ LH-RH ethylamide (LH-RH agonist, Hoechst); Group 3, a single i.v. injection (10:00 h) of 40 μg LH-RH agonist.

### *Blood sampling*

Daily jugular venous blood samples were taken from all animals at 09:00 h on each of Days −5 to +18. On Day 0 samples were collected at 30-min intervals from 1 h before to 4 h after the first (Group 1) or only (Groups 2 and 3) injection and then every 1 or 2 h for a further 19 h. Blood samples were collected into heparinized evacuated tubes, and the plasma was stored at −20°C until assayed.

### *Ovarian activity and collection of CL*

The ovaries of all ewes were examined by laparoscopy performed under barbiturate anaesthesia 6 days before and 5 days after LH-RH or agonist injection. The presence of a fresh (red) CL was taken as evidence of recent ovulation. The blood sample taken on the day of the second laparoscopy (Day +5) was immediately assayed to check the progesterone secretory activity of the CL seen at laparoscopy. On Day +8 these CL were removed surgically under anaesthesia induced by barbiturate and maintained by halothane from the 8 ewes that had ovulated: 7 ewes had 1 CL each and 1 ewe had 2 CL.

After excision, each CL was trimmed of extraneous tissue, cut into pieces and weighed on a Torsion balance. Two pieces (10–200 mg depending on CL weight) were taken to estimate the binding of hCG and progesterone content, respectively; these were placed in plastic tubes, snap-frozen in a bath containing methanol and solid $CO_2$ within 30 min of removal from the ovary and stored at −20°C until analysis. The remaining pieces of CL were finely minced with two scalpel blades and washed in 50 ml Eagle's minimum essential medium containing Hepes buffer and Earle's salts (pH 7·6; EMEM) before estimation of the progesterone secretory capacity of each CL.

*In-vitro production by and content of progesterone in CL*

Equal amounts of minced luteal tissue (mean 16, range 10–27 mg in 0·1 ml EMEM) were incubated in replicate ($n$ = 2–6 depending on the amount of CL mince available) in a final volume of 2 ml EMEM alone (basal) or containing 10 i.u. hCG (Pregnyl: Organon, Morden, Surrey, U.K.). After gassing with 95% $O_2$, 5% $CO_2$ for 10 sec the tubes were sealed with Parafilm (Gallenkamp) and incubated for 3 h at 37°C in a shaking water bath. After incubation, the CL mince and medium were separated by centrifugation at 600 ***g***, and the mince was homogenized in 4 ml absolute ethanol for 1 min (I.L.A. Homogenizer, Type X10/20). The homogenate was then centrifuged and the supernatant decanted and stored at −20°C.

The initial progesterone content of the CL was assessed by extraction of duplicate amounts of CL mince before incubation. All samples were stored at −20°C until assayed for progesterone.

Progesterone production throughout the incubation was calculated by subtracting the progesterone concentration in the tissue before incubation from the mean of the total progesterone in the medium plus tissue in each incubate after incubation.

*Binding of hCG to CL*

The binding of hCG was assessed with the hCG preparation CR119 (NIH) labelled with $^{125}I$ by the lactoperoxidase method (Miyachi, Vaitukaitis, Nieschlag & Lipsett, 1972) as described in detail for human CL (McNeilly, Kerin, Swanston, Bramley & Baird, 1980). Briefly, pieces of CL (10–200 mg) were homogenized (1/5, w/v) in 0·01 M-Tris–HCl buffer, pH 7·5, containing 0·1% bovine serum albumin (BSA) at 4°C for 20 sec with a Polytron (Kinematica, Lucerne, Switzerland) at half maximum speed. The homogenate was then centrifuged at 600 ***g*** for 1 h at 4°C, the supernatant removed and the precipitate re-suspended in buffer to give a concentration of 10 mg CL tissue/ml.

To assess specific binding 0·1 ml homogenate was placed in 7-ml polystyrene tubes containing $^{125}I$-labelled hCG (2 ng hormone) and 0·05 ml buffer and incubated in triplicate for 3 h at 37°C with shaking.

Non-specific binding was assessed by the addition of 100 i.u. hCG (Pregnyl) to the buffer. Incubation was terminated by the addition of 3 ml cold buffer and the tubes were centrifuged at 1500 ***g*** for 30 min at 4°C. The supernatant was decanted and the precipitate counted in a well-type gamma counter (LKB Wallac, Bromma, Sweden). Specific binding was calculated in terms of pg hCG bound per mg CL tissue. All CL, including those of control sheep (see below), were processed at the same time.

*CL from normally cyclic sheep*

To allow comparison of the content and in-vitro production of progesterone and the binding of hCG to the CL collected after LH-RH treatment of anoestrous sheep, CL were removed on Days 5 ($n$ = 6) and 14 ($n$ = 5) of the normal oestrous cycle (November) (oestrus = Day 0) from 10 Scottish Blackface ewes. Ovulation was synchronized by the i.m. injection of a potent analogue of prostaglandin F-2α (100 μg i.m. cloprostenol) (ICI 80996: ICI, Macclesfield, Cheshire) and CL were removed and processed exactly as described above for those of anoestrous ewes.

*Hormone assays*

Prolactin, LH and FSH were measured in duplicate in specific double-antibody radio-immunoassays exactly as described in detail previously (McNeilly & Andrews, 1974; McNeilly, McNeilly, Walton & Cunningham, 1976; Martensz, Baird, Scaramuzzi & Van Look, 1976). The

sensitivities of the assays were 0·5 ng prolactin (NIH-P-S6)/ml, 0·1 ng LH (NIH-LH-S14)/ml and 8 ng FSH (NIH-FSH-S10)/ml. The intra- and interassay variations, as coefficients of variation, were 7 and 9%, 9·5 and 12% and 8 and 11% for prolactin, FSH and LH respectively.

The progesterone concentrations in plasma, extracts of corpus luteum and culture media were measured by a specific radioimmunoassay described previously for plasma (Scaramuzzi, Corker, Young & Baird, 1974). This assay has been validated for the measurement of progesterone in CL extracts (Swanston, McNatty & Baird, 1977; McNeilly *et al.*, 1980) and culture medium without extraction (Hunter, 1980). Intra- and interassay coefficients of variation (%) were 6 and 10% respectively with a sensitivity of 0·1 ng/ml.

### *Statistical analysis*

Results were analysed by Student's *t* test or analysis of variance.

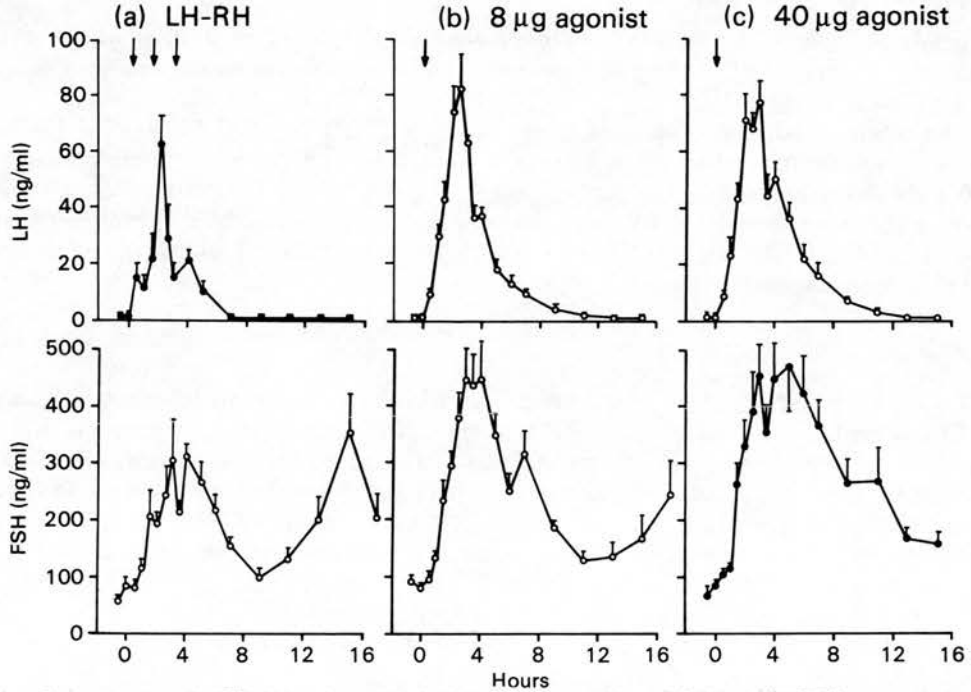
## Results

### *Ovulation*

Ovulation was induced in 8 of the 27 ewes as judged by laparoscopy on Day 5 after treatment. The numbers of animals ovulating in each group were: LH-RH, 3/9; LH-RH agonist 8 μg, 2/9: LH-RH agonist 40 μg, 3/9.

### *Plasma hormone concentrations*

*LH.* After the first injection of LH-RH there was an initial increment of 5–33 ng/ml (Text-fig. 1) by 30 min. This was followed by a much greater increment of 15–75 ng/ml 30 min




**Text-fig. 1.** Changes in the mean ± s.e.m. plasma concentrations of LH and FSH in anoestrous ewes after i.v. injection (arrows) of (a) LH-RH (30 μg at 90-min intervals) or LH-RH agonist at doses of 8 μg (b) or 40 μg (c).

after the second injection while there was little or no further change after the third injection. In contrast, after injection of LH-RH agonist plasma levels of LH increased steadily to mean (range) peak values of 92 (49–158) and 83 (50–122) ng/ml for 8 and 40 μg LH-RH agonist respectively, the peaks occurring between 2 and 4 h after injection (Text-fig. 1).

Over the 21 h after injection of LH-RH or agonist there was no significant difference in the cumulative amount of LH released between the 2 doses of agonist (Table 1) but both doses of agonist released significantly greater amounts ($P < 0{\cdot}01$) of LH than did LH-RH. There was no difference between the amounts of LH released in animals which did and those that did not ovulate in each treatment group (Table 1).

**Table 1.** The release of LH and FSH (as cumulative totals) after the injection of anoestrous ewes with LH-RH (3 × 30 μg) or LH-RH agonist (8 or 40 μg)

| Treatment | No. of ewes | Cumulative release (ng/ml) | | | |
|---|---|---|---|---|---|
| | | LH | FSH | | |
| | | (0–21 h) | 0–7 h | 8–21 h | 0–21 h |
| *LH-RH* | | | | | |
| All ewes | 9 | 196 ± 40 | 2322 ± 245 | 1455 ± 218 | 3777 ± 425 |
| Ovulating ewes | 3 | 170 ± 30 | | | 3775 ± 560 |
| Non-ovulating ewes | 6 | 168 ± 22 | | | 3778 ± 572 |
| *Agonist, 8 μg* | | | | | |
| All ewes | 9 | 365 ± 36* | 3377 ± 230* | 1354 ± 135 | 4730 ± 318 |
| Ovulating ewes | 2 | 548,229 | | | 6087,3751 |
| Non-ovulating ewes | 7 | 358 ± 33 | | | 4676 ± 330 |
| *Agonist, 40 μg* | | | | | |
| All ewes | 9 | 395 ± 120* | 3771 ± 362* | 1385 ± 140 | 5156 ± 467 |
| Ovulating ewes | 3 | 407 ± 78 | | | 4622 ± 478 |
| Non-ovulating ewes | 6 | 390 ± 46 | | | 5423 ± 561 |

Values are mean ± s.e.m. Times are relative to first injection of LH-RH or agonist (0 h).
* $P < 0{\cdot}01$ compared with value for LH-RH treated ewes (Student's *t* test).

*FSH.* All animals showed an initial peak response in plasma levels of FSH between 90 min and 4 h after injection of LH-RH or LH-RH agonist and another peak 9–21 h later (Text-fig. 1). The time to this second peak was most variable (9–17 h) in ewes injected with the high dose (40 μg) of LH-RH agonist and because of this variation a second peak is not apparent when mean values are taken. While there was an increase in the cumulative amount of FSH released over a 21-h period following injection of LH-RH agonist compared to LH-RH, this was not significant ($P = 0{\cdot}1$; Table 1). However, significantly more ($P < 0{\cdot}01$) FSH was released in the initial response period (0–7 h) after both doses of LH-RH agonist than after LH-RH although there was no difference between the two agonist groups or in the amount of FSH released during the second response period (8–21 h) in any of the groups (Table 1). Within each treatment the initial cumulative FSH response (0–7 h) was significantly correlated with the basal plasma levels of FSH before treatment (LH-RH: $r = 0{\cdot}74$, $n = 9$; LH-RH agonist 8 μg: $r = 0{\cdot}72$, $n = 9$; LH-RH agonist 40 μg: $r = 0{\cdot}84$; $n = 9$).

There was no difference in basal levels of FSH in the 4 days before treatment or in the response to LH-RH or LH-RH agonist in animals that did or did not ovulate.

*Prolactin.* In all anoestrous ewes prolactin values ranged between 56 and 230 ng/ml and none of the treatments affected these levels. During the normal luteal phase concentrations were 3–12 ng/ml and were significantly lower ($P < 0{\cdot}001$) than during anoestrus.

*Progesterone.* In the animals that did not ovulate all values remained <0·2 ng/ml regardless of treatment. In the animals that ovulated in each treatment group, progesterone concentrations

rose normally during the first 3 days after ovulation but then started to decline. Levels were significantly lower ($P < 0{\cdot}01$) on Day 8 than on Days 5 or 14 of the normal cycle (Table 2). Values on Day 14 were significantly greater ($P < 0{\cdot}01$) than on Day 5 (Table 2).

**Table 2.** Characteristics of corpora lutea collected during a normal oestrous cycle and 6 days after ovulation induced in anoestrus by LH-RH or LH-RH agonist

| | | | | Corpus luteum | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Progesterone production *in vitro* (ng/mg/3 h) | | |
| CL | No. of ewes | No. of CL | Plasma progesterone (ng/ml) | Wt (mg) | Progesterone content (ng/ml) | hCG binding (pg/ml) | Basal | + hCG | Increment |
| Normal cycle | | | | | | | | | |
| Day 5 | 6 | 6 | 1·25 ± 0·35 | 174 ± 25 | 5·7 ± 0·7 | 19·7 ± 5·1 | 29·6 ± 3·6 | 55·0 ± 7·4 | 25·4 ± 5·9 |
| Day 14 | 5 | 6 | 4·49 ± 0·80 | 379 ± 54 | 11·8 ± 0·5 | 46·9 ± 8·0 | 42·1 ± 4·1 | 58·4 ± 4·7 | 16·2 ± 5·6 |
| Anoestrus | 8 | 9 | 0·65 ± 0·07 | 90 ± 12 | 1·0 ± 0·2 | 29·4 ± 8·9 | 1·9 ± 0·7 | 2·7 ± 1·2 | 1·0 ± 0·5 |

Values are mean ± s.e.m.

*Luteal progesterone secretion* in vitro *and hCG binding.* The weight and progesterone content of the CL increased significantly ($P < 0{\cdot}02$ to $P < 0{\cdot}001$) between Days 5 and 14 of the normal cycle and values on both days were significantly greater ($P < 0{\cdot}01$ to $P < 0{\cdot}001$) than those for the CL induced during anoestrus (Table 2).

The basal production of progesterone *in vitro* was significantly greater ($P < 0{\cdot}05$) on Day 14 than Day 5 and values on these days were greater ($P < 0{\cdot}001$) than that for the CL induced during anoestrus. hCG caused a significant increase in progesterone production from Day −5 ($P < 0{\cdot}02$) and Day −14 ($P < 0{\cdot}05$) CL of the cycle but not from CL induced during anoestrus (Table 2).

The binding of hCG increased significantly ($P < 0{\cdot}02$) between Days 5 and 14 of the cycle, but binding was not significantly different at either stage from that of the CL of anoestrus (Table 2).

## Discussion

The present results confirm previous observations (Crighton, Foster, Haresign, Haynes & Lamming, 1973; Crighton *et al.*, 1975; Haresign *et al.*, 1975; McNeilly & Land, 1979) that treatment of anoestrous ewes with single injections of LH-RH induces ovulation in approximately 1 animal in 4 but that the resulting corpus luteum produces less progesterone than does the CL of a normal cycle.

It has been suggested (Crighton *et al.*, 1975; Frandle *et al.*, 1977) that poor CL function after LH-RH treatment of anoestrous ewes was due to release of reduced amounts of LH before ovulation. However, in the present study, the injection of LH-RH agonist resulted in a release of both LH and FSH which was of similar or greater magnitude and duration to that occurring before ovulation in the normal oestrous cycle in the Scottish Blackface ewe (A. S. McNeilly, unpublished observations), and in Clun Forest ewes (Siddall & Crighton, 1977). This suggests that reduced release of LH or FSH is not the primary cause of the inadequate CL.

The failure of normal CL function after LH-RH agonist injection in the present study contrasts with the results of Frandle *et al.* (1977) who induced ovulation in 8 of 13 anoestrous ewes given a single injection of LH-RH agonist and produced luteal phases similar to those of the natural cycle. The difference in results is not likely to be due to differences in the amounts of gonadotrophin release induced by the agonist since the 40 μg dose of D-Ser-(But)$^6$-des Gly$^{10}$ LH-RH ethylamide used in the present study should be as active as 100 μg of the less potent

D-leu$^6$-des Gly$^{10}$ LH-RH ethylamide used by Frandle *et al.* (1977) (Sandow, König, Geiger, Uhmann & von Rechenberg, 1978). Also, in the present study the incidence of ovulation did not appear to be influenced by the amount of LH and FSH released, and, since the CL function of the ewes that did ovulate was inadequate, it seems that the changes within the follicle which occur prior to ovulation as a prerequisite for a normal luteal phase are not induced by the LH or FSH surge alone. A period of suitable priming must therefore be required and it is unlikely that single injections of LH-RH agonist will be useful for inducing the formation of normal CL in anoestrous ewes without pretreatment. It may be that the ewes of mixed genetic background studied by Frandle *et al.* (1977) were at a more advanced stage of follicular development than were the Scottish Blackface ewes used in the present investigation.

Our results go some way to answer the question of why CL induced by LH-RH or its agonists are unable to produce much progesterone. While the CL taken during the normal cycle showed changes in weight and steroid content and were able to bind hCG and produce progesterone *in vitro* as expected (Diekman, O'Callaghan, Nett & Niswender, 1978; Sawyer, Abel, McClellan, Schmitz & Niswender, 1979), the CL taken 5 days after ovulation had been induced with LH-RH in anoestrous ewes were of reduced weight, had greatly reduced progesterone content and a negligible ability to produce progesterone *in vitro*, even after stimulation with hCG (Table 2). The plasma progesterone concentrations in these ewes were low when the corpora lutea were removed, but the binding of hCG to the luteal tissue was within the range seen during the normal luteal phase. These observations suggest that the formation of LH receptors on granulosa cells of the ovine CL are largely independent of the development of the ability to secrete progesterone. They also indicate that, even if plasma LH concentrations were raised *in vivo* after ovulation, the CL could not respond. The increased number of receptors for LH appears to be the major factor in the regulation of progesterone secretion by the corpus luteum (Diekman *et al.*, 1978) but our results suggest that there may not be a simple relationship between the binding of LH to lutein cells and the secretion of progesterone.

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# Relationship Between LH, FSH, and Prolactin Concentration and the Secretion of Androgens and Estrogens by the Preovulatory Follicle in the Ewe

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## ABSTRACT

The ovarian secretion of estradiol, androstenedione, testosterone, and progesterone and the concentration of LH, FSH, and prolactin were measured in the periovulatory period of five ewes with ovarian or utero-ovarian autotransplants. Samples of jugular and ovarian venous blood were collected every 1 or 2 h before and for 96 h after induction of luteal regression on Day 10 of the cycle by injection of 100 μg cloprostenol. Progesterone concentration in jugular vein plasma fell to less than 1 ng/ml in all ewes by 24 h, and coincidental to this decline there was a significant increase in the secretion of LH and prolactin and an associated rise in the ovarian venous concentration of estradiol, testosterone, and androstenedione. The rise in LH secretion was associated with a twofold increase in the frequency of episodic pulses each of which stimulated an increase in the secretion of estradiol. In contrast the concentration of FSH declined significantly in the 48 h after luteal regression. Shortly after the onset of estrus (48 ± 2.5 h), there was a marked rise in the concentration of prolactin, FSH, and LH which reached a peak at 61 ± 4 h. At the start of this preovulating LH surge, there was a further substantial stimulation in the secretion of estradiol, testosterone, and androstenedione, followed by a sharp fall within 3, 5, and 7 h, respectively.

In the 24 h after the preovulatory surge, there was a marked decline in the concentration of LH and prolactin as well as all ovarian steroids. However, within 12 h there was a further rise in the concentration of FSH which reached a second peak at 23 h after the LH peak.

These results suggest that the increased secretion of estradiol from the preovulatory follicle is due to stimulation by episodic pulses of LH which occur with increased frequency as the concentration of progesterone falls during luteal regression. The sustained rise in LH which occurs during the preovulatory surge stimulates and then markedly inhibits aromatase activity and eventually all steroid secretion from the follicle. Thus the final stages of development of the preovulatory follicle are determined not by FSH but by the pattern of secretion of LH.

## INTRODUCTION

The changes in the concentration of FSH, LH, prolactin, estradiol, and progesterone in jugular venous blood of the sheep in the periovulatory period have been described in a number of publications (Pant et al., 1977; Hauger et al., 1977; Bindon et al., 1979). Following regression of the corpus luteum, the dominant follicle(s) secretes increasing quantities of estradiol in response to a rise in the basal secretion of LH (Baird and Scaramuzzi, 1976a). The rise in estrogen secretion induces estrous behavior and a surge of LH which results in ovulation some 24 h later (Cumming et al. 1971). Important changes occur in the Graafian follicle at this time converting it from a structure which secretes estrogens and androgens to one which secretes mainly progesterone (Bjersing et al., 1972; Moor et al., 1975).

It has been known for some years that the sheep ovary secretes relatively large amounts of androstenedione and testosterone (Baird et al., 1968). These androgens are precursors for estrogen biosynthesis although their role in the regulation of gonadotropins and the factors controlling their secretion are unknown (Baird, 1978a). Active immunization of ewes against androstenedione and testosterone produces profound effects on ovarian function and the secretion of gonadotropins (Martenz et al., 1976; Martenz and Scaramuzzi, 1979; Scaramuzzi et al., 1980; Scaramuzzi et al., 1981).

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The present study was designed to study the secretion of androgens and estrogens from the ovary in the periovulatory period and to relate these findings to the changes in the concentration of FSH, LH, and prolactin measured simultaneously at frequent intervals. In this way it was hoped to gain insight into the factors regulating the marked change in pattern of steroids secreted by the preovulatory follicle.

## MATERIALS AND METHODS

### *Experimental Animals*

Four ewes with ovarian (Goding et al., 1967) and one ewe with a utero-ovarian autotransplant (Harrison et al., 1968) were allocated to this experiment in the middle of the breeding season (January). Ewes were either pure Tasmanian Merino (D314 and D410) or crossed with Finnish-Landrace (F × M OU57, 9U6) or Southdown (MX 16). Ewes were housed under natural lighting conditions in a covered pen at Dryden Field Station (Animal Breeding Research Organisation), Roslin, Midlothian, Scotland. The left ovary alone, or together with the oviduct, horn, and body of the uterus, cervix, and anterior vagina, had been autotransplanted to the neck at least 2 years previously. The left ovarian and uterine arteries and utero-ovarian vein were anastomosed to the carotid artery and jugular vein which were exteriorized in separate skin loops (Baird et al., 1976a). The right ovary and uterine horn were removed. The ewe with the utero-ovarian transplant had regular estrous cycles [17.5 ± 1.2 days (mean ± SD), n = 17] in contrast to the ewes with the ovarian transplants which failed to show heat due to persistence of the corpus luteum (Baird et al., 1976b). Heat was tested with a vasectomized ram at least twice per day and on some occasions more frequently (see below).

Because of the absence of cyclical ovarian function in the ewes with ovarian transplants and because of the difficulty of standardizing the timing of collections in relationship to spontaneous luteal regression, estrus, and ovulation in the utero-ovarian transplant, premature luteal regression was induced by i.m. injection (100 μg) of a potent analogue of prostaglandin $F_{2\alpha}$ ICI 80996 (cloprostenol). Luteal regression and estrus (Day 0) occurred within 72 h of this treatment (Baird and Scaramuzzi, 1975).

### *Experimental Design*

On Day 9 of the subsequent estrous cycle, both jugular veins were cannulated as described previously (Collett et al., 1973). On the side of the utero-ovarian anastomosis, a Silastic cannula (2.00 mm i.d., 3.2 mm o.d.; Dow Corning Corp., Medical Products, MI) was advanced so that the tip lay opposite the entrance of the utero-ovarian vein. Following cannulation the ewes were kept in metabolism cages in a heated room (about 15°C) until the end of the experiment.

On the morning of Day 10, 5 ml samples of jugular venous blood were collected every hour for 6 h prior to and for 60 h after a second i.m. injection of 100 μg cloprostenol. From 60–96 h the sampling frequency was reduced to every 2 h.

Samples (10 ml) of ovarian venous blood were collected every 2 h from −6 to +96 h. Samples of jugular and ovarian venous blood were collected more frequently every 10 min for two periods of 4 h during the luteal and follicular phases of the cycle, i.e., −4 to 0 h and 24 to 28 h after injection of cloprostenol. During the periods of frequent sampling, timed samples (15 ml) of ovarian blood were collected every hour and the plasma flow then calculated from the hemacrit reading. Blood was collected into sterile heparinized containers, centrifuged immediately at 4°C, the plasma aspirated, and stored at −20°C until analysis. During the 10 min sampling period the red cells were returned to the ewe via the cannula in the right jugular vein.

Commencing 36 h after the injection of cloprostenol, the ewes were tested for heat every 4 h through estrus until two successive tests were negative. The ewes were removed from the metabolism crates after the collection of the blood sample and observed in a pen for 10 min with a vasectomized ram.

### *Analytical Methods*

Progesterone, estradiol, androstenedione, and testosterone were measured in duplicate in ovarian venous plasma by methods which have been described in detail in publications from this laboratory (Scaramuzzi et al., 1975; Baird et al., 1976; Baird et al., 1974; Corker and Davidson, 1978). The precision and sensitivity of the methods are listed in Table 1.

Prolactin, LH, and FSH were measured in duplicate by radioimmunoassays described in detail by McNeilly and Andrews (1974), McNeilly et al. (1976), and Martenz et al. (1976). The sensitivities of the assays were 0.1 ng prolactin (NIH P-S6)/ml, 0.1 ng LH (NIH-LH-S14)/ml, and 16 ng FSH (NIH-FSH-S10)/ml. All samples were analyzed in one assay with coefficients of variations (%) of three quality control sera (at 27, 50, and 75% B/Bo) of 8, 9, and 7% for prolactin, LH, and FSH, respectively. High values were reassayed in dilution in assays with intraassay coefficients of variation of 7%, 8%, and 8% and interassay variations of 10%, 9%, and 12% for prolactin, LH, and FSH, respectively.

### *Statistical Analysis*

The differences between groups were analyzed statistically by two-tailed or paired Student's t test and the pulse frequency by $\chi^2$ test.

## RESULTS

### *Estrous Behavior*

All the ewes showed estrus 43–56 h after the injection of the cloprostenol (47.6 ± 2.5; mean ± SEM). As expected the duration of estrus in the F × M crosses (38 and 40 h) was longer than that in the Merino ewes (20, 25, and 32 h).

TABLE 1. Precision and sensitivity of methods used for hormone assay.

| | Antiserum | Interassay precision (%) | Limit of sensitivity (pg/ml) |
|---|---|---|---|
| Estradiol | R3 | 9.5 | 10 |
| Androstenedione | Shopman | 15.0 | 50 |
| Testosterone | E 0 1 | 11.5 | 10 |
| Progesterone | 91920/9 | 13.0 | 100 |

*Changes in Steroids and Gonadotropins*

Because of the dilution of ovarian with uterine venous blood, the concentrations of ovarian steroids in the ewe with a utero-ovarian transplant were considerably less than in the ovarian blood of those ewes with ovarian transplants, although the pattern of hormone changes was similar. The values from this ewe were excluded from the mean of the values from the four ewes with ovarian transplants which were grouped around the injection of cloprostenol and the peak concentration of LH. The LH peak occurred 60 h after the cloprostenol injection (52–68 h) so that the mean results illustrated in Figs. 1 and 2 are an accurate representation of the time scale in spite of the break in the ordinate. The results in ewe 9U6 are illustrated separately in Figs. 3 and 4 to demonstrate the exact temporal relationships between the changes in gonadotropins and steroids in an individual ewe.

There was a marked decline in the concentration of progesterone in ovarian and jugular venous blood within 6 h of the injection of cloprostenol, and functional luteolysis (progesterone concentration in jugular venous plasma <1 ng/ml) was complete in all ewes by 24 h (Figs. 1, 3). Within 6 h of the decline in the secretion of progesterone, there was a threefold rise in the concentration of LH from 0.40 ± 0.11 to 1.45 ± 0.20 ng/ml ($P<0.05$). In contrast, as the secretion of estradiol increased, the concentration of FSH fell so that by 48 h after the cloprostenol injection it was significantly lower than the preinjection value (56 ± 19 vs 92 ± 10 ng/ml; $P<0.05$, paired t test).

Six to 12 h after the cloprostenol injection there was a rise in the secretion of estradiol, androstenedione, and testosterone (Figs. 2, 4). Thereafter, there was a further marked increase in the secretion of estradiol reaching a peak of 3190 ± 1272 pg/ml at ∿54 h (44–66 h). In contrast there was no significant change in the secretion of androstenedione and testosterone so that a marked increase was found in the ratio of estradiol to both testosterone and androstenedione as the follicle reached maturity (Fig. 5).

Six hours before the peak value, the basal concentration of LH increased above 5 ng/ml. Coincidental to this rise there was a marked increase in the secretion of estradiol, androstenedione, and testosterone (Fig. 5). However, within 3 h of the start of the LH surge the secretion of estradiol fell markedly, although the secretion of testosterone and androstenedione did not reach its maximum value until some 2–3 h later. The secretion of all steroids then declined rapidly so that by 12 h after the LH peak the secretion of ovarian steroids was lower than at any other time of the estrous cycle.

Coincidental to the LH surge (61 ± 4 h) there was a fourfold rise in the concentration of FSH (Figs. 1, 3, 5) which rose from 56 ± 19 ng/ml at −12 h to a peak of 248 ± 42 ng/ml ($P<0.02$) before returning to basal values (56 ± 18 ng/ml) 6–12 h after the LH peak. Subsequently there was a rise in the concentration of FSH in all five ewes, the peak value (218 ± 70 ng/ml) occurring 16–28 h (mean 22.5 h) after the LH peak. This second FSH peak, which lasted between 18 and 32 h (mean 23 ± 4 h), was not associated with any significant change in concentration of LH (mean 0.92 ± 0.24 ng/ml). Because of slight differences in timing, the magnitude of these peaks of FSH is underestimated by the mean data illustrated in Figs. 1 and 5 in which the data are grouped relative to the LH peak.

*Pulsatile Release of Gonadotropins*

The secretion of estradiol, LH, FSH, and

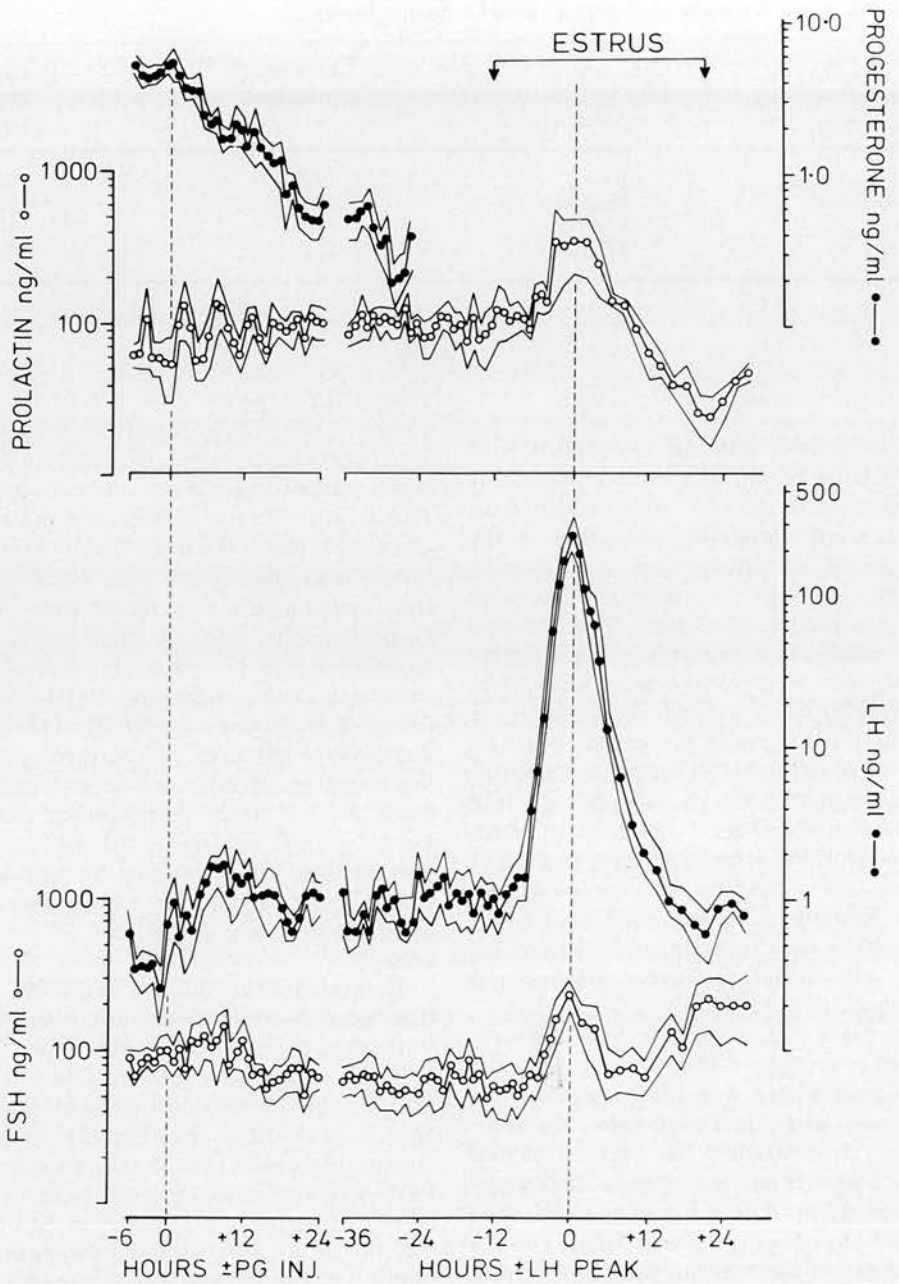



FIG. 1. Mean concentration (± SEM) of progesterone, prolactin, LH, and FSH in jugular venous plasma of four ewes with ovarian autotransplants. Results have been grouped around the injection of 100 μg cloprostenol (PG Inj.) and the peak LH value.

prolactin was measured during a period of intensive sampling at 10 min intervals for 4 h on Day 10 of the cycle (luteal) and again during the follicular phase 24–28 h after the injection of cloprostenol. The results were analyzed for each phase of the cycle in terms of the number and frequency of pulses of each hormone, a pulse being defined as occurring when two consecutive samples were higher than the two preceding samples (basal) and when the value of the highest sample (peak) exceeded the mean basal sample by at least four times the coef-

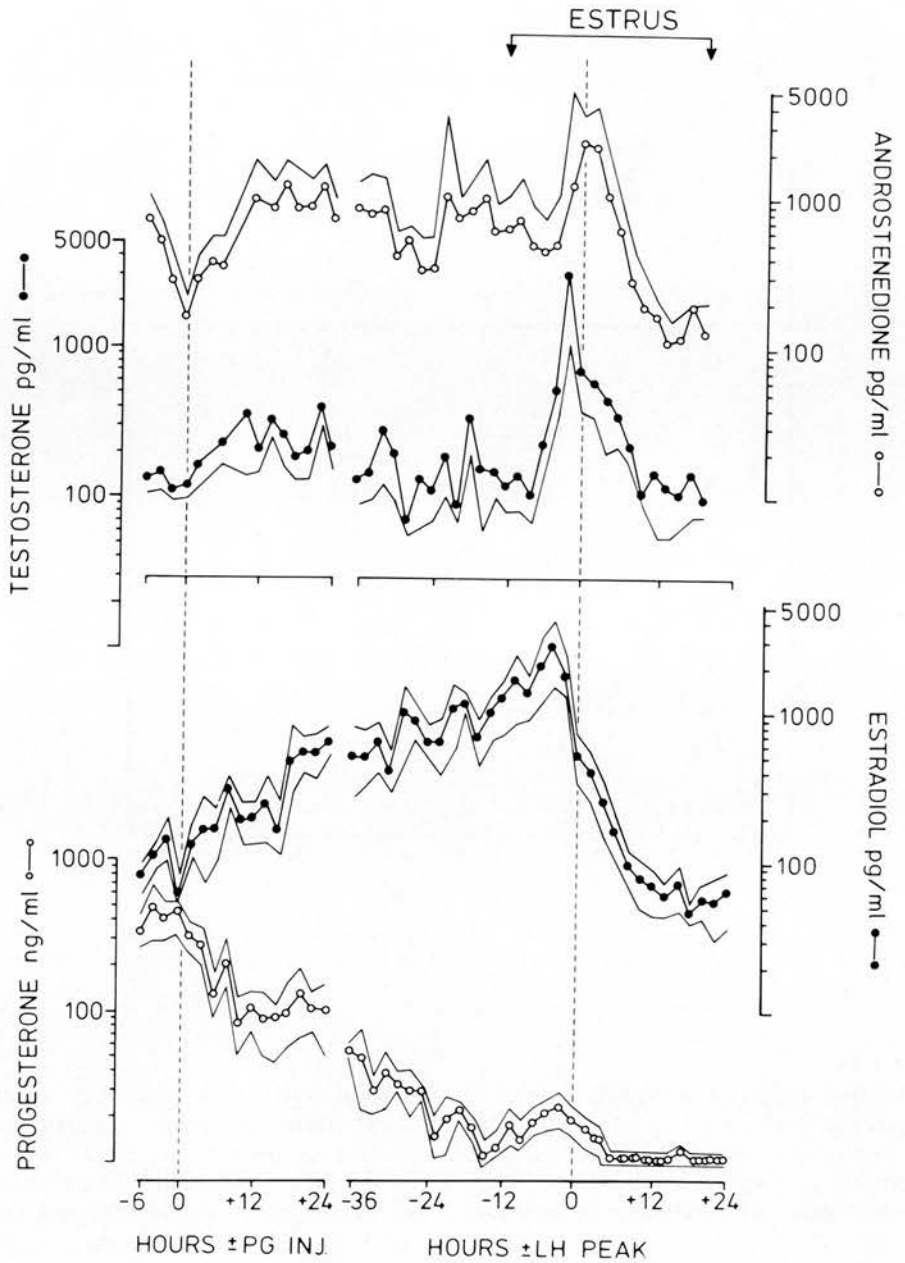



FIG. 2. Mean concentration (± SEM) of progesterone, estradiol, androstenedione, and testosterone in ovarian venous plasma of four ewes with ovarian autotransplants. Results have been grouped around the injection of 100 μg cloprostenol (PG Inj.) and the peak LH value.

ficient of variation of the assay of the hormone concerned (Table 2).

Although the secretion of all hormones showed marked fluctuations, the most obvious episodic pulses were recorded for LH and estradiol (Fig. 6). Each LH pluse was followed within a few minutes by a rise in the secretion of estradiol. The number of LH and estradiol pulses in the 20 h of observation increased from 8 and 6 pulses, respectively, during the luteal phase to 18 and 20 pulses, respectively, during the follicular phase of the cycle ($\chi^2$ = 3.87 and

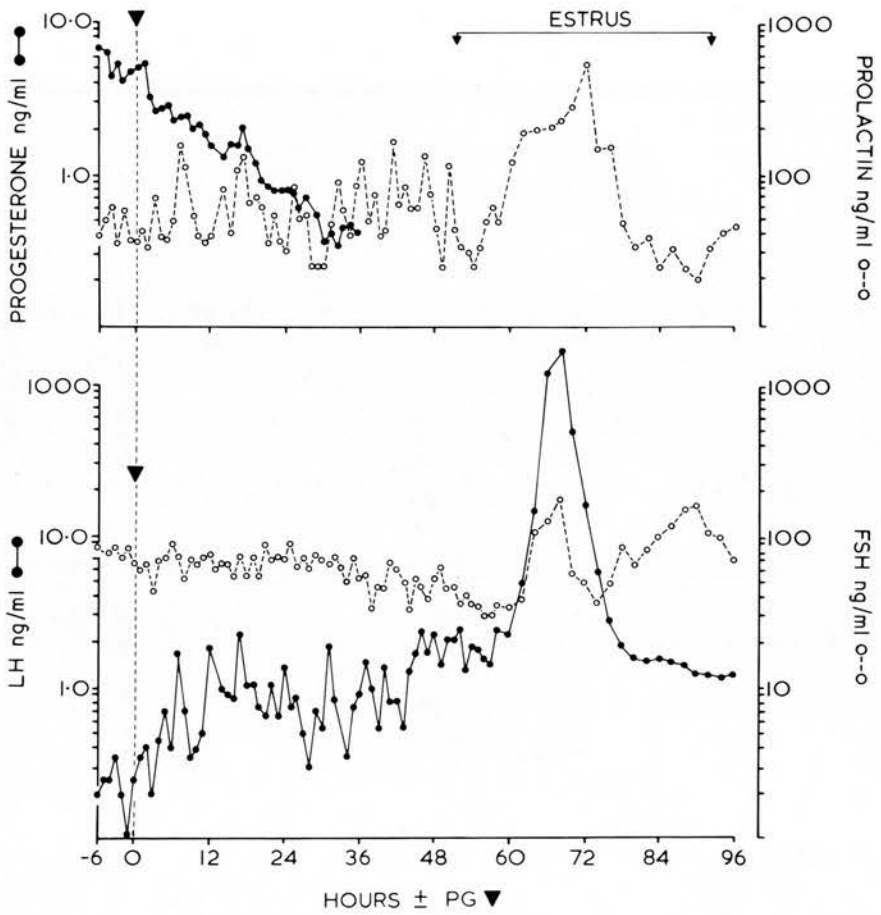



FIG. 3. Concentration of progesterone, FSH, LH, and prolactin in jugular venous plasma in ewe 9U6 with ovarian autotransplant. Values have been grouped around the injection of 100 μg cloprostenol (▼) given on Day 10 of the cycle.

5.76, $P<0.05$). There was a marked increase in the basal and peak secretion of estradiol although there was no significant rise in basal LH concentration or the pulse amplitude (Table 2). The overall mean LH concentration increased significantly due mainly to the increased number of episodic pulses. The pulses of prolactin and FSH did not always coincide with those of LH (Figs. 6, 7), and there was no significant change in the frequency of the pulses of these two hormones in the two phases of the cycle. Compared with the luteal phase, the concentration of FSH during the follicular phase was significantly lower (64 ± 9 vs 93 ± 11 ng/ml).

## DISCUSSION

The changes in LH, progesterone, and estradiol secretion in this study confirm and extend those previously reported following spontaneous (Pant et al., 1977; Hauger et al., 1977) and prostaglandin-induced luteal regression (Chamley et al., 1972; Baird and Scaramuzzi, 1976). We have reported (Baird et al., 1968; Baird et al., 1976) that secretion of androstenedione (and testosterone) correlated with that of estradiol and was stimulated by endogenous (Baird et al., 1976) and exogenous LH (McCracken et al., 1969). In the present study the rise in LH secretion occurring during luteal regression was associated initially with an increased secretion of androgens as well as estrogens (Figs. 2, 4). However, in the 36 h before the preovulatory LH surge there was no further rise in the secretion of androstenedione and testosterone in spite of a tenfold increase in

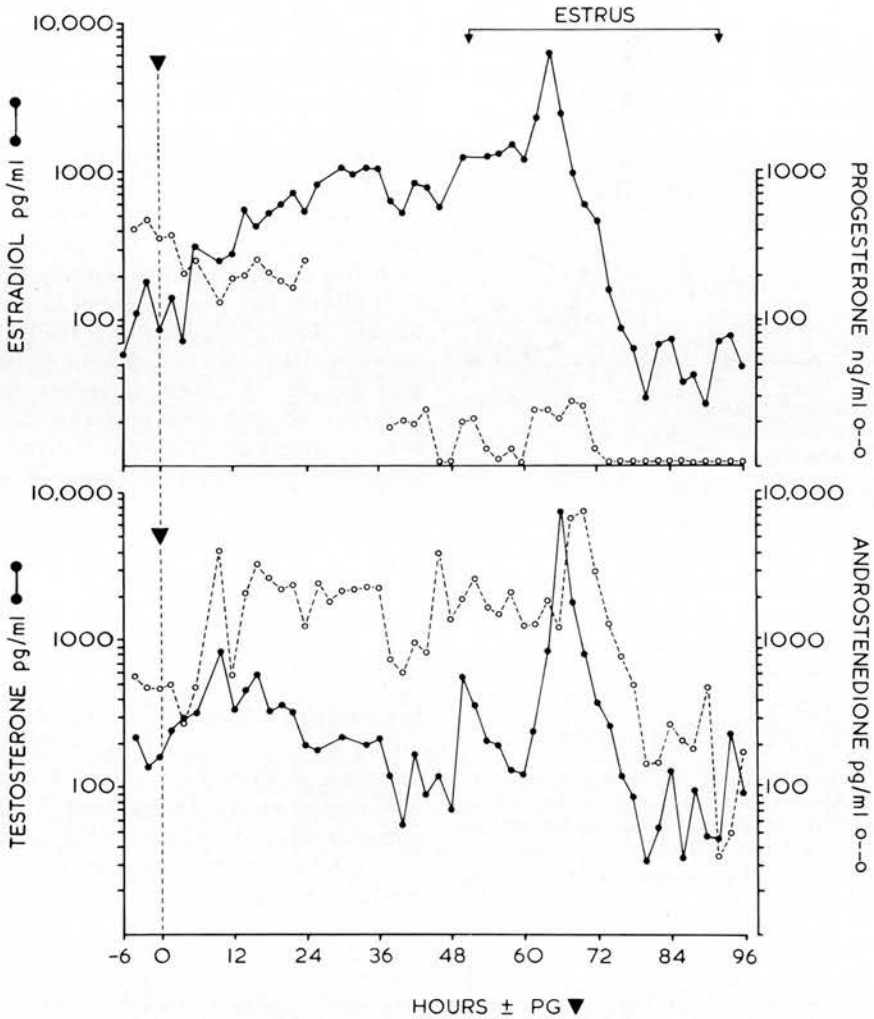



FIG. 4. Concentration of estradiol, progesterone, testosterone, and androstenedione in ovarian venous plasma of ewe 9U6 with an ovarian autotransplant. Values have been grouped as in Fig. 3.

the secretion of estradiol. As an increasing proportion of androgen produced by the preovulatory follicle is converted to estradiol, it seems likely that the quantity of estradiol secreted is limited not solely by the aromatase activity but also by the amount of androgen available as precursor. In the sheep virtually all the estradiol secreted into the ovarian vein as derived from the dominant follicle(s) (Bjersing et al., 1972; Moor, 1973; Baird and Scaramuzzi, 1976b). Although atretic follicles and stroma can synthesize androstenedione and testosterone (Moor et al., 1975), the dominant follicle is the source of over 90% of androgen secreted into the ovarian vein (Baird and Scaramuzzi, 1976b; Scaramuzzi et al., 1980; Scaramuzzi et al., 1981; McNatty et al., 1981). Thus, although in the present study the source of steroids was not studied directly, it can be assumed that the ovarian secretion of estradiol, androstenedione, and testosterone reflects predominantly the activity of the preovulatory follicle.

Although the cellular origin of estrogens in the follicle remains a controversial issue, it is generally accepted that androgens arise from the theca (Moor, 1977). As receptors for LH are present on the cells of the theca (Carson et al., 1979), it is hardly surprising that androgen secretion is so respon-

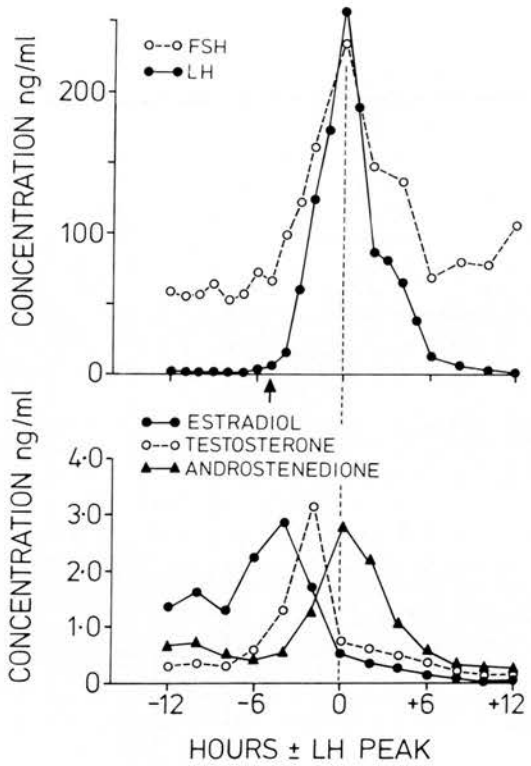



FIG. 5. Mean concentration of estradiol, testosterone, and androstenedione in ovarian venous plasma and LH and FSH in jugular venous plasma of four ewes with ovarian autotransplants. Samples have been grouped around the LH peak. Start of the LH surge, defined as a concentration >5 ng/ml, is indicated by ↑.

sive to each episodic pulse of LH (Baird et al., 1976). The rise in frequency of LH pulses occurring during luteal regression is probably related to the fall in progesterone concentration although a direct stimulatory effect of estradiol cannot be excluded (Legan and Karsch, 1979; Baird and McNeilly, 1981). In seasonal anestrus the final stages of follicular development and ovulation can be induced by infusion or repeated pulses of LH alone without any addition of FSH (Ryan and Foster, 1980; Goodman and Karsch, 1980; McNeilly et al., 1980). Thus the increased frequency of episodic pulses of LH that occurs at luteal regression stimulates secretion of androgen precursor, and is the most important factor in determining the preovulatory increase in estradiol secretion (Baird, 1978b).

In contrast to LH, there was no rise in the frequency of episodic pulses of FSH at the time of luteal regression. In fact the overall concentration of FSH was significantly suppressed due to a decrease in the amplitude of the pulses (Table 2). As LHRH is thought to be the sole hypothalamic hormone responsible for stimulating the release of both gonadotropins, the divergent secretion of FSH and LH during the follicular phase is probably due to a differential effect of estradiol or some other ovarian factor (e.g., inhibin) on the sensitivity of the anterior pituitary to LHRH. Estradiol in physiological

TABLE 2. Concentration of LH, FSH, and prolactin in jugular venous plasma and the ovarian secretion of estradiol in the follicular and luteal phase of the estrous cycle of five ewes (mean ± SEM).

| | No. of pulses in 20 h | Concentration | | | |
|---|---|---|---|---|---|
| | | Overall (n = 5) | Basal | Peak | Amplitude |
| LH (ng/ml) | | | | | |
| Follicular | 18* | 1.2 ± 0.4** | 0.8 ± 0.2 | 1.6 ± 0.3 | 0.7 ± 0.1 |
| Luteal | 8 | 0.9 ± 0.4 | 0.5 ± 0.2 | 1.4 ± 0.4 | 0.9 ± 0.3 |
| FSH (ng/ml) | | | | | |
| Follicular | 12 | 64 ± 9* | 58 ± 5 | 86 ± 8*** | 30 ± 5* |
| Luteal | 11 | 93 ± 11 | 66 ± 11 | 125 ± 9 | 51 ± 7 |
| Prolactin (ng/ml) | | | | | |
| Follicular | 7 | 90 ± 14 | 68 ± 9* | 104 ± 17* | 35 ± 8* |
| Luteal | 6 | 55 ± 11 | 39 ± 5 | 55 ± 6 | 16 ± 1 |
| Estradiol secretion (ng/min) | | | | | |
| Follicular | 20* | 8.19 ± 2.31* | 5.69 ± 0.72*** | 12.39 ± 2.05*** | 6.60 ± 1.53*** |
| Luteal | 6 | 2.21 ± 0.32 | 1.44 ± 0.28 | 4.26 ± 0.98 | 2.81 ± 0.76 |

*P<0.05; **P<0.01; ***P<0.001; significantly different from luteal phase (paired Student's t test or $\chi^2$ test).

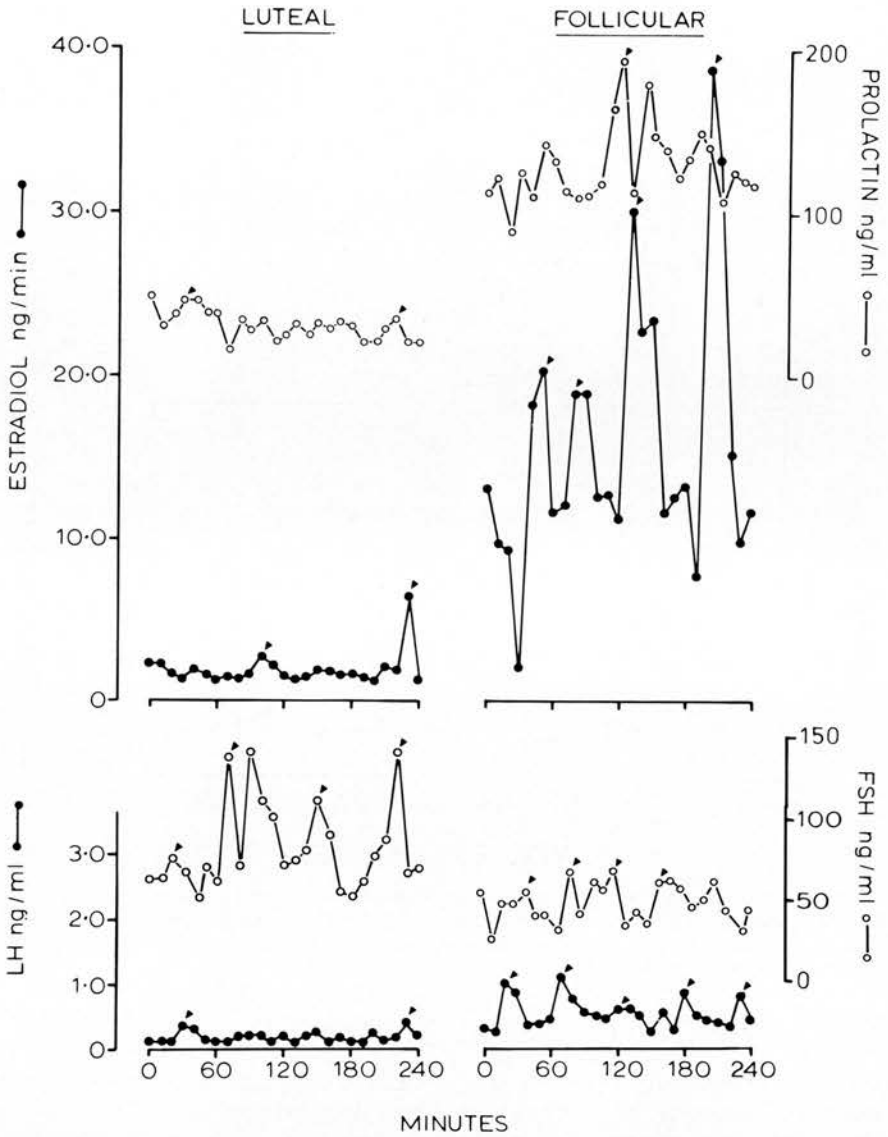



FIG. 6. Secretion of estradiol and concentration of LH, FSH, and prolactin in ewe OU57. Samples were collected between 1000 and 1400 h on Day 10 (luteal) and 24 h after injection of 100 μg cloprostenol (follicular). An episodic pulse is indicated by the arrow ▼ (see text for definition).

concentrations ($10^{-11}$ to $10^{-9}$ M) suppresses the output of FSH from isolated ovine anterior pituitary cells cultured in vitro (Miller et al., 1977), and release of FSH from rat pituitary glands in response to LHRH is inhibited at a lower concentration of estradiol than of LH (Schally et al., 1973).

Although FSH is essential for folliculogenesis and is thought to play a role in estrogen synthesis by stimulating aromatization of androgens to estrogens by granulosa cells (Dorrington et al., 1975), the final rise in estradiol secretion by the dominant follicle in women (Ross et al., 1970) occurs in the face of a falling secretion of FSH (Fig. 1). It is possible that the relatively high concentration of FSH in follicular fluid protects the dominant follicle (McNatty et al., 1981) from the deleterious effect of low levels of FSH in blood.

As in previous studies there was a very rapid decline in estradiol secretion within hours of the preovulatory LH surge (Chamley et al.,

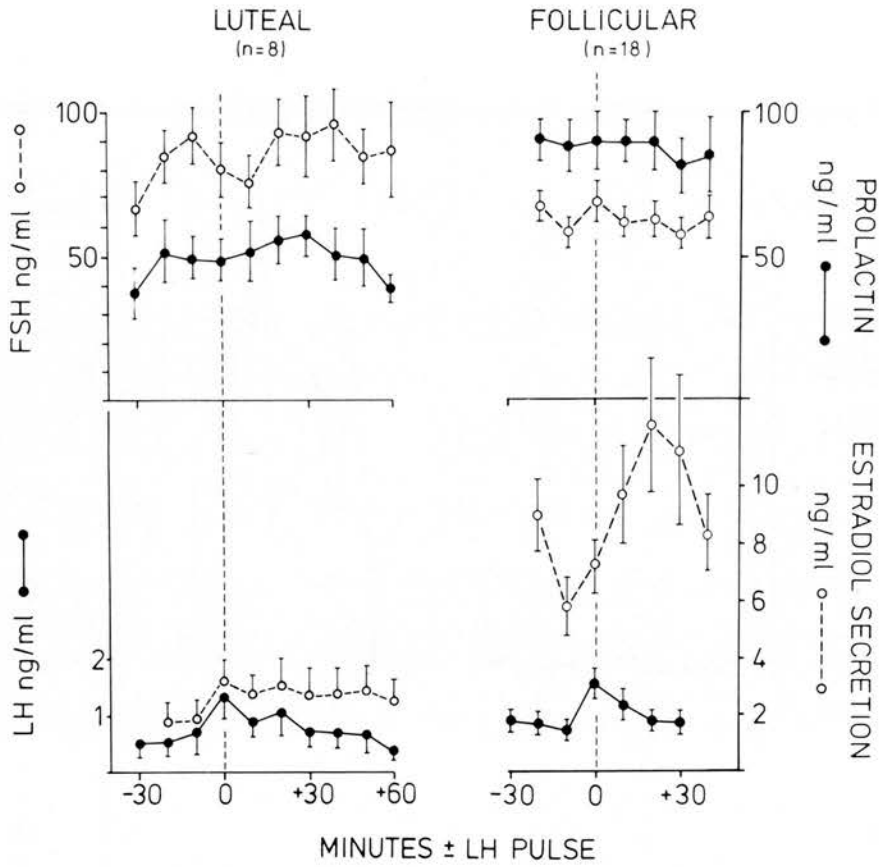



FIG. 7. Concentration of LH, FSH, and prolactin in jugular venous plasma and ovarian secretion of estradiol in five ewes with ovarian or utero-ovarian autotransplants. Results have been grouped around the peak concentration of LH. Each point represents the mean ± SEM of 8 or 18 observations in the luteal (Day 10) or follicular (24 h after cloprostenol) phase, respectively.

1972; Baird and Scaramuzzi, 1976). In the present study the sampling was sufficiently frequent to define the precise relationship between the changes in the secretion of ovarian steroids and pituitary gonadotropins. It was apparent that following a short-lived stimulation of estradiol, androstenedione, and testosterone, there was a very rapid decline of all ovarian steroids (Chamley et al., 1972; Baird and Scaramuzzi, 1976a). That the secretion of estradiol was inhibited before that of testosterone and androstenedione is compatible with the suggestion that LH in large amounts inhibits aromatase activity (Rado et al., 1970; Moor, 1974). Certainly there is no evidence of lack of androgen precursor, and the concentration of FSH, which is known to stimulate aromatase activity in granulosa cells, increases markedly at this time. Testosterone and its 5α-reduced metabolites, e.g., dihydrotestosterone, have been demonstrated to inhibit the conversion of androgens to estrogens by granulosa cells in vitro (Hillier et al., 1980). It is possible, therefore, that the preovulatory surge of LH stimulates a large increase in the secretion of thecal androgens which then accumulate within the follicle and inhibit aromatase. The total inhibition of androgens and estrogens by the follicle after the LH surge is probably due to desensitization and loss of LH receptors on the theca cells (Harwood et al., 1978).

The cause or function of the second peak of FSH which occurs about 22 h after the preovulatory LH peak is unknown (Salamonsen et al., 1973; Pant et al., 1977; Bindon et al., 1979). In other species, e.g., rat, the second peak of FSH determines the number of follicles available for ovulation at the next estrus

(Sheela-Rani and Moudgal, 1977) although in the sheep the ovulation rate is unaltered by unilateral ovariectomy as late as Day 14 (Land, 1973; Findlay and Cumming, 1977). There is no associated release of LH or prolactin, and the secretion of ovarian steroids is lower than at any other phase of the estrous cycle. It would seem likely that a change in the pituitary sensitivity of LHRH is responsible for the selective release of FSH, as antiserum to LHRH will inhibit the first but not the second FSH peak (Narayana and Dobson, 1979). The second FSH peak occurs at a time when the secretion of ovarian steroids and hence negative feedback is minimal. However, the factors regulating the secretion of FSH are complex and in both the rat (Schwartz and Channing, 1977) and the sheep (Goodman et al., 1980) steroids and/or factors in addition to estradiol are involved.

## ACKNOWLEDGMENTS

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# ACTIVE IMMUNIZATION OF EWES AGAINST LUTEINIZING HORMONE RELEASING HORMONE, AND ITS EFFECTS ON OVULATION AND GONADOTROPHIN, PROLACTIN AND OVARIAN STEROID SECRETION

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SUMMARY

Three Scottish Blackface ewes were immunized against luteinizing hormone releasing hormone (LH-RH) conjugated to bovine serum albumin (BSA) and three control ewes were immunized against BSA alone. When the antibody titre to LH-RH became raised the treated animals failed to show oestrus or ovulate; they had significantly lower levels of plasma luteinizing hormone (LH) and higher levels of prolactin than the controls, whereas the levels of follicle-stimulating hormone (FSH) were unaltered.

The integrity of the hypothalamus-pituitary-gonadal system of these animals was then challenged by the injection of a highly active analogue of LH-RH and by ovariectomy. An i.v. injection of 5 μg D-serine-t-butyl$^{6}$ *des*-glycine-$NH_2{}^{10}$ LH-RH ethylamide raised plasma LH and FSH. Ovariectomy caused an eight- and ninefold rise in plasma levels of LH and FSH respectively in controls, but failed to increase plasma levels of LH and FSH in the LH-RH-immunized ewes. Plasma prolactin concentrations in the LH-RH-immunized ewes were significantly reduced by ovariectomy.

## INTRODUCTION

Inhibition of luteinizing hormone releasing hormone (LH-RH) activity by antibodies to LH-RH has been studied in the rat (Fraser & Gunn, 1973; Koch, Chobsieng, Zor, Fridkin & Lindner, 1973; Arimura, Debeljuk & Schally, 1974; Fraser, Gunn, Jeffcoate & Holland, 1974*a*; Kerdelhue, Catin, Kordon & Jutisz, 1976; Fraser & Baker, 1978), hamster (de la Cruz, Arimura, de la Cruz & Schally, 1976), rabbit (Arimura, Sato, Kumasaka, Worobec, Debeljuk, Dunn & Schally, 1973; Fraser & Gunn, 1973) and monkey (Hodges & Hearn, 1977; McCormack, Plant, Hess & Knobil, 1977). Results have shown that in these species inhibition of LH-RH leads to a reduction in secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and to an impairment of reproductive function.

In the present study, ewes were immunized against LH-RH and the effects on the levels of LH, FSH and prolactin in the circulation and on ovarian steroid secretion were measured. The integrity of the hypothalamus-pituitary-gonadal system of these animals was then challenged by the injection of a highly active analogue of LH-RH and by ovariectomy.

## MATERIALS AND METHODS

### *Immunization*

Six mature Scottish Blackface ewes (used to being handled and having blood samples taken) were studied. The LH-RH was conjugated to bovine serum albumin (BSA) by carbodiimide

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as previously described (Fraser, Gunn, Jeffcoate & Holland, 1974*b*). Each of three ewes received 2·5 mg of the conjugate in Freund's complete adjuvant, 1 ml of the emulsion being injected at four s.c. sites on the inside of each leg. Three control ewes were immunized with 2·5 mg BSA. The primary immunizations were carried out on 13 August 1976 and booster injections were given 13 and 17 weeks later. The booster immunizations consisted of 1 mg conjugate in Marcol 52 (ESSO Petroleum Ltd) containing 10% Arlacel A (Sigma Chemicals Ltd, London) given at three i.m. sites in the shoulder at 13 weeks and two i.m. sites in the hind legs at 17 weeks. Controls were treated similarly, being immunized with 1 mg BSA.

Antibody titre to LH-RH was measured in doubling dilutions of serum from 1 : 100 to 1 : 12 800 incubated with $^{125}$I-labelled LH-RH (10 000 counts/min) in a final volume of 300 μl. Free and bound labelled LH-RH were separated using 5 vol. ice-cold ethanol and the titre was expressed as the highest dilution to bind 33% of the label. A radioimmunoassay was established using each antiserum and specificity was assessed by addition of fragments and analogues of LH-RH. The LH-RH was iodinated in the presence of chloramine T and purified with Vycor glass (Mortimer, McNeilly, Rees, Lowry, Gilmore & Dobbie, 1976).

*Oestrus and ovarian activity*

The control and immunized ewes were run with a vasectomized raddled ram from the beginning of September and were checked daily for raddle markings indicating oestrus had occurred. Jugular venous blood was sampled at 2 week intervals for measurement of LH-RH antibody titre and prolactin. When all the immunized ewes had failed to show overt oestrus, blood was collected twice weekly to measure plasma progesterone concentrations.

The ovaries of all the ewes were inspected by laparoscopy under sodium pentobarbitone (Nembutal) anaesthesia on the day that they received their second booster injection (13 December). Four days later the immunized ewes were given LH-RH analogue (see below). Four days after receiving the analogue, the controls and the immunized ewes were laparotomized under Nembutal anaesthesia and ovarian venous blood was collected for the assay of the plasma concentration of oestrone, oestradiol-17β and androstenedione. All the ewes were then ovariectomized and the ovaries taken for histology (see below).

*Blood sampling and LH-RH treatment*

Three days after the second booster immunization (7 days later for two controls) blood samples were collected from the control and LH-RH-immunized ewes at 20 min intervals for 6 h, 2 h after insertion of indwelling jugular venous catheters. The plasma was separated for the measurement of LH, FSH and prolactin concentrations. On the following day the LH-RH-immunized ewes were given a single i.v. injection of 5 μg D-serine-t-butyl$^{6}$ *des*-glycine-$NH_2^{10}$ LH-RH ethylamide (LH-RH analogue) and jugular samples were again taken at 20 min intervals for 6 h. Daily jugular venous blood samples were then taken until ovariectomy 4 days later for determination of plasma progesterone concentrations.

*Histology*

The ovaries were fixed in Bouin's fluid and histological sections stained with haematoxylin and eosin.

*Effects of ovariectomy*

Three weeks after ovariectomy, blood samples were again taken from jugular venous catheters at 20 min intervals for 6 h and the plasma concentrations of LH, FSH and prolactin were measured.

*Radioimmunoassays*

Luteinizing hormone, FSH, prolactin, progesterone, oestrone, oestradiol-17β and androstenedione were measured in duplicate by radioimmunoassays previously described

(McNeilly & Andrews, 1974; Baird, Burger, Heavon-Jones & Scaramuzzi, 1975; McNeilly, McNeilly, Walton & Cunningham, 1976; Martensz, Baird, Scaramuzzi & Van Look, 1976; Clarke, Scaramuzzi & Short, 1977). Sensitivities of the assays were 0·1 ng NIH LH-S-14/ml, 16 ng NIH FSH-S-10/ml and 0·1 ng prolactin NIH P-S-6/ml.

### *Analysis of results*

To analyse differences in the peripheral plasma hormone profiles between immunized and control intact ewes during the 6 h sampling period, the overall mean for each animal was calculated and then analysed by Student's *t*-test.

For the comparison of plasma hormone concentrations before and after castration, the overall mean for each animal was compared using a paired *t*-test.

## RESULTS

### *Antibody titre*

Low levels of LH-RH antibody were detected in all three ewes after the primary immunization (Fig. 1). The titres rose significantly in each ewe after the first series of booster injections and remained raised for the remainder of the experiment (Fig. 1). When the ewes were bled 3 weeks after ovariectomy, antibody titres for ewes nos 23, 27 and 39 had risen to 1 : 14 000, 1 : 1800 and 1 : 11 000 respectively.

Specificity of the antibodies was directed towards the $CO_2H$-terminal end of LH-RH. Antisera from ewes nos 23 and 39 cross-reacted 100% with 3–10 LH-RH, <0·001% with 1–6 LH-RH and 10% with the LH-RH analogue. Antiserum from ewe no. 27 cross-reacted 3%, <0·001% and 0·8% with these peptides.

### *Oestrus and ovulation*

The control ewes had regular oestrous cycles (see Fig. 1). One LH-RH-immunized ewe never showed oestrus, one had a single oestrus and the remaining ewe showed four oestrous periods but became acyclic after the first booster injection (Fig. 1). This ewe (no. 23) continued to show luteal phase levels of plasma progesterone (1·7–5·2 ng/ml) after her last heat but corpora lutea were not observed at laparoscopy 8 days later. The source of this progesterone was possibly the luteinized follicles which were found in the ovaries of this sheep. The plasma concentrations of progesterone in the other LH-RH-immunized ewes were always below 0·4 ng/ml and the ovaries of these two ewes did not possess corpora lutea at laparoscopy.

In the control ewes, plasma progesterone concentrations indicated normal luteal function during mid-cycle with levels rising to 5·1, 5·8 and 4·9 ng/ml respectively. The ovaries of all the control ewes possessed corpora lutea at laparoscopy.

### *Plasma prolactin levels throughout immunization*

The plasma prolactin levels from immunization until ovariectomy are shown in Fig. 1. In the controls, prolactin levels declined as the ewes began to cycle. In the LH-RH-immunized ewes the levels of prolactin also declined but the fall was not as great as in the controls and levels rose again in December.

### *Plasma concentrations of LH, FSH and prolactin before ovariectomy*

The peripheral plasma concentrations of LH, FSH and prolactin measured in control and LH-RH-immunized ewes during a 6 h period are shown in Fig. 2. The mean concentration of LH was significantly ($P<0·01$) lower in the immunized ewes compared with the mean concentration in the controls (Table 1). Variation in the plasma LH levels of the control ewes (Fig. 2) was due to pulsatile LH secretion. This fluctuation was absent in the LH-RH-immunized ewes and their plasma LH concentrations consistently remained below 1 ng/ml.

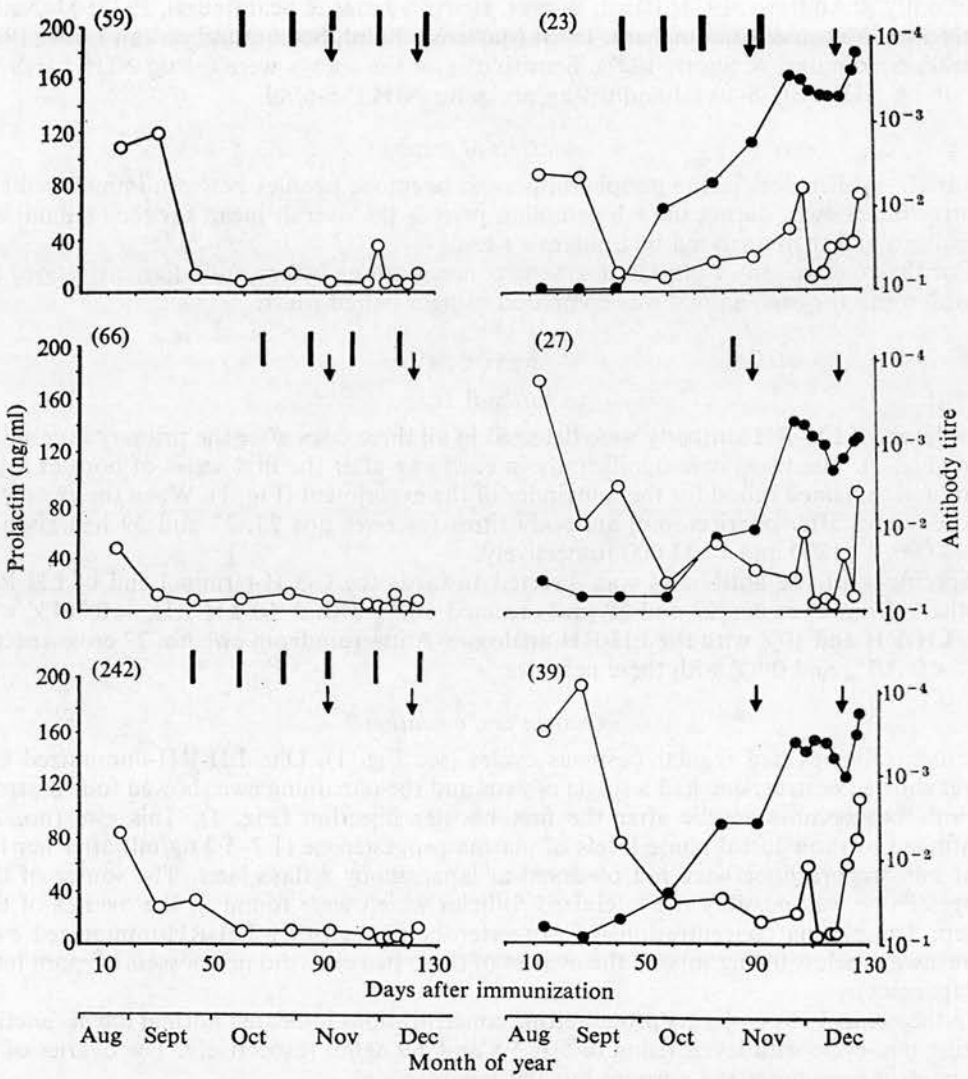



Fig. 1. Peripheral plasma prolactin concentrations (○) and antibody titres to luteinizing hormone releasing hormone (LH-RH) (●) during the experimental period. The arrows show the times at which boosters were given. Vertical lines show occurrence of oestrus. Controls immunized against bovine serum albumin are on the left and ewes immunized against LH-RH on the right. The reference number for each ewe is given in parentheses.

The mean plasma prolactin concentration was significantly ($P < 0{\cdot}02$) higher in the LH-RH-immunized ewes compared with that of the controls. Plasma FSH concentrations did not differ between the two groups.

*Effect of LH-RH analogue*

Injection of 5 μg LH-RH analogue produced significant and sustained increases in plasma LH and FSH concentrations in the three immunized ewes (Fig. 3). Plasma progesterone concentrations after treatment with the LH-RH analogue remained below 1 ng/ml, thus indicating that the analogue did not cause ovulation in any of the immunized ewes. This

Table 1. *Mean plasma concentrations* (±S.D.) *of LH, FSH and prolactin in ewes immunized against LH releasing hormone (LH-RH) and in control ewes immunized against bovine serum albumin, while intact and 3 weeks after ovariectomy*

| | Plasma hormone concentration (ng/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Intact | | | Ovariectomized | | |
| Group | LH | FSH | Prolactin | LH | FSH | Prolactin |
| Control | 1·3±0·3 | 96±63 | 4·7±2·0 | 10·8±4·3 | 871±279 | 4·8±4·7 |
| LH-RH-immunized | 0·6±0·1* | 81±8 NS | 27·6±11·8** | 0·6±0·1* | 108±29* | 10·8±4·3 NS |

Significance of difference from control values (Student's *t*-test): $*P<0·01$; $**P<0·02$; NS, not significant.
A paired *t*-test was carried out to compare values before and after ovariectomy: in control ewes levels of LH and FSH rose ($P<0·002$) while prolactin levels were not significantly different and in LH-RH-immunized ewes levels of LH and FSH were not significantly different and prolactin levels declined ($P<0·05$).

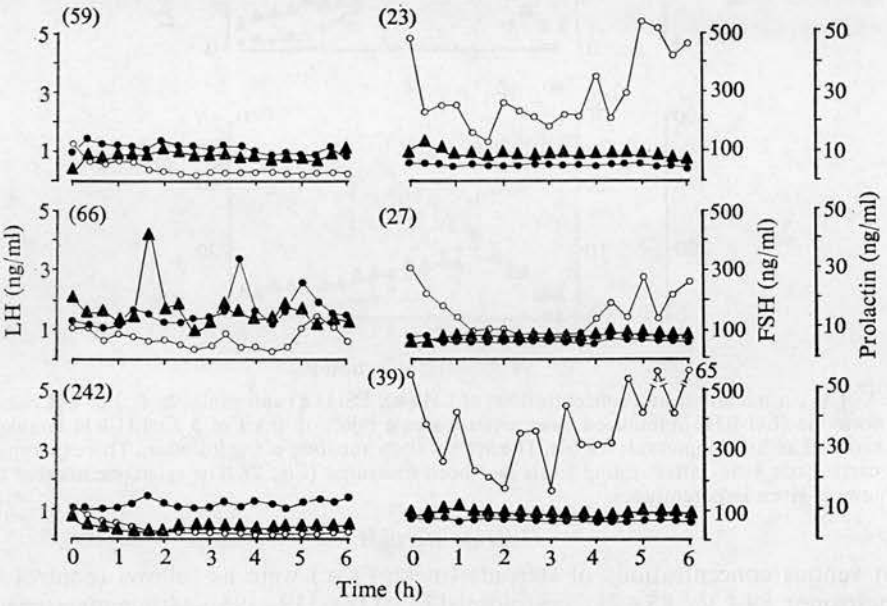



Fig. 2. The peripheral plasma concentrations of LH (●), FSH (▲) and prolactin (○) in control and LH releasing hormone (LH-RH)-immunized ewes sampled at 20 min intervals for 6 h. Control ewes immunized against bovine serum albumin are on the left and ewes immunized against LH-RH are on the right. Sheep nos 59 and 242 were sampled on day 4 of the oestrous cycle and sheep no. 66 on day 10. The immunized ewes were not cyclic.

was confirmed by inspection of the ovaries at laparotomy 4 days later, and also by histological examination of ovarian sections.

### *Ovarian histology*

Normal luteal tissue was present in ovaries from all the control ewes. Apart from two corpora albicantia in ewe no. 23, no luteal tissue was seen in the ovaries of the LH-RH-immunized ewes. Numerous primordial follicles were found in all sheep. Several antral follicles were present in ovaries of control sheep. At least one antral follicle was present in the ovaries of the LH-RH-immunized ewes and two cystic luteinized follicles in one ovary from ewe no. 23.

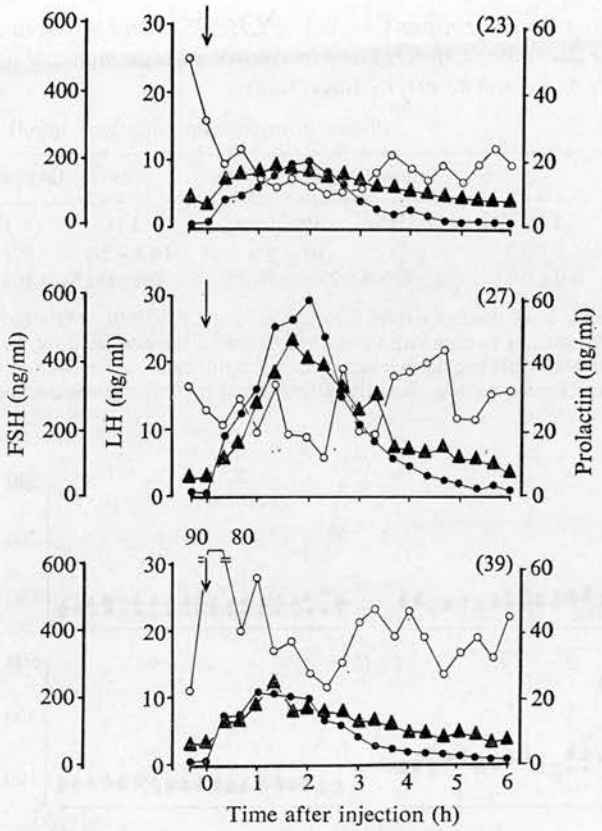



Fig. 3. The peripheral plasma concentrations of LH (●), FSH (▲) and prolactin (○) in LH releasing hormone (LH-RH)-immunized ewes given a single injection (i.v.) of 5 μg LH-RH analogue and sampled at 20 min intervals for 6 h. The arrows show the time of the injection. This experiment was carried out 1 day after resting levels had been measured (Fig. 2). The reference number for each ewe is given in parentheses.

### *Ovarian steroids*

Ovarian venous concentrations of steroids (mean ± S.D.) were as follows (control values first), oestrone: 89 ± 36, 85 ± 31; oestradiol-17β: 122 ± 119, 196 ± 140; androstenedione: 363 ± 198, 484 ± 230. No significant differences were found between the two groups.

### *Effect of ovariectomy on plasma concentrations of LH, FSH and prolactin*

The plasma concentrations of LH, FSH and prolactin in the control and LH-RH-immunized ewes, 3 weeks after ovariectomy, are shown in Fig. 4. Ovariectomy caused a significant ($P < 0{\cdot}02$) increase in the plasma LH levels of the control ewes but not in the LH-RH-immunized ewes (Table 1).

The plasma FSH levels of the control ewes rose significantly ($P < 0{\cdot}02$) after castration but there was no significant increase in FSH levels in the ovariectomized LH-RH-immunized ewes. Plasma prolactin levels were unaltered after ovariectomy in control ewes but in the immunized ewes, levels were significantly reduced ($P < 0{\cdot}01$) after ovariectomy and they were no longer significantly higher than in the control ewes.

## DISCUSSION

Active immunization against LH-RH before the onset of the breeding season prevented ovulation in each of the cases studied. Two of the ewes began to cycle during the early stage

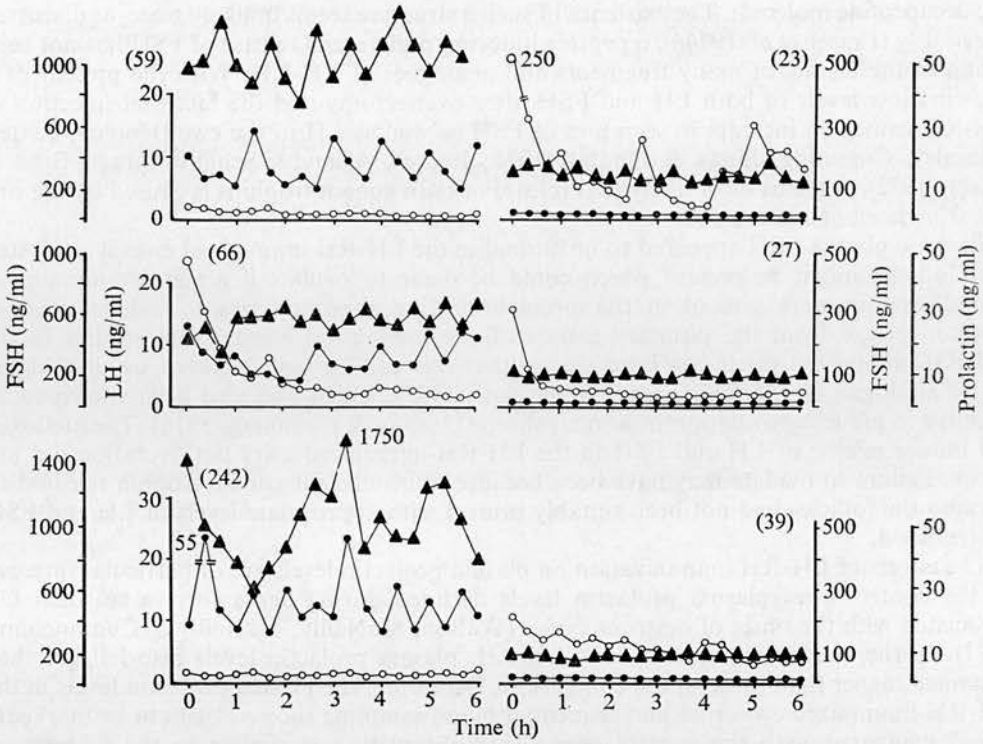



Fig. 4. The peripheral plasma concentrations of LH (●), FSH (▲) and prolactin (○) in control and LH releasing hormone (LH-RH)-immunized ewes 3 weeks after ovariectomy. Controls immunized against bovine serum albumin are on the left and ewes immunized against LH-RH are on the right. The reference number for each ewe is given in parentheses.

of immunization showing that cyclicity was possible when antibody titre was low but when a higher degree of inhibition of LH-RH was attained cycles stopped. One immunized ewe failed to show oestrus at any time during immunization and this may have been due to factors other than the presence of antibody to LH-RH. This is unlikely since all the ewes studied had had normal cycles throughout the whole of the previous breeding season.

Normal luteal tissue was not present in the ovaries of LH-RH-immunized ewes but some follicular growth was continuing as shown by histology and by the normal levels of oestradiol-17β found in the ovarian venous blood of these ewes.

In the ewes immunized against LH-RH, plasma levels of LH were significantly lower than in the controls but the levels of FSH were unaltered. After ovariectomy, it was clear that both plasma LH and FSH levels in the immunized ewes were lower than in the controls. It is probable that small amounts of unbound LH-RH reach the pituitary gland of animals immunized against LH-RH so that secretion of LH and FSH continues albeit at a very reduced rate. It is possible that this condition favours release of FSH in preference to LH. Small amounts of LH-RH stimulate FSH secretion in man (Mortimer, Besser & McNeilly, 1975). In hypophysectomized rats with pituitary transplants, chronic treatment with LH-RH stimulates secretion of FSH but not of LH (Arimura, Debeljuk, Shiino, Rennels & Schally, 1973). The levels of LH and FSH in plasma after ovariectomy in ewes immunized against LH-RH are in agreement with a preliminary report by Foster, Webb & Crighton (1977) who found reductions in both LH and FSH in plasma from ovariectomized ewes producing antibodies to LH-RH. It is possible that antibodies to LH-RH could cross-react with and inhibit a separate FSH-RH if it consisted in part of an amino acid sequence in common with

the decapeptide molecule. The existence of such a structure seems unlikely since, as discussed previously (Fraser *et al.* 1974*a*), a peptide inducing preferential release of FSH has not been found in the testing of many fragments and analogues of LH-RH. With the present data showing low levels of both LH and FSH after ovariectomy and the fact that injection of LH-RH causes an increase in secretion of FSH as well as LH in the ewe (Hooley, Baxter, Chamley, Cumming, Jonas & Findlay, 1974; Reeves, Arimura, Schally, Kragt, Beck & Casey, 1972), it seems most likely that release of both gonadotrophins is caused by the one releasing hormone in the ewe.

Because plasma FSH appeared to be normal in the LH-RH-immunized ewes it suggested that follicles might be present which could be made to ovulate if a suitable amount of gonadotrophin were present in the circulation. One approach was to induce gonadotrophin release from the pituitary glands of the immunized ewes. From studies in the LH-RH-immunized rat it has been shown that this can be best achieved using a highly active analogue of LH-RH (Fraser & Sandow, 1977), which had also been shown to be effective in inducing ovulation in normal sheep (Findlay & Cumming, 1976). The analogue did induce release of LH and FSH in the LH-RH-immunized ewes but ovulation did not occur. Failure to ovulate may have been because of insufficient gonadotrophin released or because the follicles had not been suitably primed with appropriate levels of LH and FSH in the blood.

The effects of LH-RH immunization on plasma prolactin levels are of particular interest. In the control ewes plasma prolactin levels declined during September, a seasonal fall associated with the onset of oestrous cycles (Walton, McNeilly, McNeilly & Cunningham, 1977). In the ewes immunized against LH-RH, plasma prolactin levels also fell but they remained higher than those of the controls. In December, the plasma prolactin levels in the LH-RH-immunized ewes rose and sequential blood sampling showed them to be markedly raised compared with the control ewes. This observation is similar to the findings of Kerdelhue *et al.* (1976) who showed that when female rats stopped cycling after the injection of antibodies to LH-RH, prolactin levels increased. The reasons for the high levels of prolactin are unknown but it is possible that they result from the low levels of progesterone and normal levels of oestrogen in the blood which occur when cycles have been stopped by inhibition of LH-RH.

We wish to thank Professor R. V. Short for helpful discussion and Dr T. G. Baker for advice on histology. We are grateful to Miss Diane M. Blakeley, Mr D. W. Davidson and Mr D. G. Patterson for technical assistance and to the NIAMDD for hormone preparations of pituitary hormones. Dr E. Vogl (Hoechst U.K.) and Dr J. Sandow kindly supplied LH-RH and the analogue.

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# Induction of Ovulation and Normal Luteal Function by Pulsed Injections of Luteinizing Hormone in Anestrous Ewes

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**ABSTRACT.** To determine whether failure of the final stages of follicular maturation during anestrus in sheep was due to inadequate LH stimulation, two experiments were carried out in midanestrus, 1 yr apart, in six Finn-Merino ewes with an ovary or an ovary and uterus autotransplanted to the neck. In the first experiment, iv injections of ovine LH were given at a rate (one injection per 3 h for 24 h, one per 2 h for 24 h, and one per hour for 24 h) equivalent to the natural increase in frequency of LH pulses which occurs in the preovulatory period of the estrous cycle during the breeding season. In the second experiment, the frequency of LH pulses was kept constant at one per 3 h for 72 h. Each injection resulted in a pulse of LH which was qualitatively and quantitatively similar to an endogenous LH pulse. Jugular and ovarian venous samples were collected and assayed for LH, FSH, PRL, 17$\beta$-estradiol, and progesterone. An increase in the secretion of 17$\beta$-estradiol, a preovulatory LH peak, and normal corpus luteum function were induced in all four ewes in the first experiment and in two of the four ewes in the second experiment. In these animals, the pattern of secretion of LH, FSH, 17$\beta$-estradiol, and progesterone was similar to that occurring spontaneously in the estrous cycle. In both experiments, the concentration of PRL remained elevated at a level consistent with midanestrus. It is concluded that 1) FSH levels are adequate for, and raised PRL levels do not directly interfere with, follicular development in anestrus; 2) an increase in the number of pulses of small physiological amounts of LH alone is sufficient to induce follicular maturation in anestrus, resulting in ovulation and formation of a corpus luteum with normal function; and 3) the failure of normal preovulatory follicular development in the anestrous ewe is due mainly to an inadequate frequency of the pulsatile release of LH. (*Endocrinology* **110:** 1292, 1982)

SEASONAL anestrus in the ewe is associated with an absence of ovulation and subsequent luteal function, and an apparent increased sensitivity to the negative feedback and a decreased sensitivity to the positive feedback effects of estrogen (1, 2). The frequency of pulses of LH is reduced compared to that found during the breeding season (3, 4), although each pulse of LH can elicit an increase in the release of 17$\beta$-estradiol from the ovarian follicle(s) (3). The serum levels of FSH appear normal (5), and while serum levels of PRL are elevated (5, 6), suppression of these levels does not result in a resumption of ovarian activity, ovulation, or normal corpus luteum function (7, 8). Thus, it appears possible that the reason for the failure of normal follicular development during seasonal anestrus in the ewe may be the low frequency of LH pulses. To test this hypothesis, we injected ovine LH in amounts sufficient to simulate endogenous LH pulses either at a frequency changing in a manner equivalent to the normal pattern of pulsatile LH release in the periovular period in the ewe or at a constant frequency, and measured the changes in 17$\beta$-estradiol, FSH, PRL, and progesterone.

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## Materials and Methods

### *Experimental animals*

Four Finnish Landrace × Merino ewes with ovarian transplants (9) and two with uteroovarian transplants (10) were studied during seasonal anestrus in early August 1979 (Exp 1) and again in July 1980 (Exp 2). Seasonal anestrus in these ewes extends from the middle of June to the end of September. For the duration of the experiments, the ewes were housed under natural lighting conditions at Dryden Field Station, Roslin, Midlothian, Scotland.

### *Experimental design*

Two experiments were then carried out using four animals in each experiment. In Exp 1 (August 1979), pulses were injected for 72 h with increasing frequency, *i.e.* once every 3 h for 24 h, once every 2 h for 24 h, and once every hour for a further 24 h. In Exp 2 (July 1980), LH was injected once every 3 h for 72 h.

For LH injections, a stock of ovine LH (NIH-LH-S13; 50 $\mu$g/ml 0.9% sodium chloride soln) was prepared and diluted in 0.9%

saline containing 10% plasma previously obtained from each ewe. Based on preliminary data, a bolus injection of 10 μg LH in 2 ml given over a 10-sec period via a jugular vein cannula resulted in a pulse of LH similar in both magnitude and duration to that seen in the preovulatory period (Fig. 1).

*Collection of blood samples*

On the first morning of the experiment, both jugular veins were cannulated. On the side of the ovarian or uteroovarian anastomosis, a Silastic cannula (id, 2.0 mm; od, 3.2 mm; Dow-Corning Corp., Midland, MI) was advanced so that the tip lay near the site of the anastomosis of the ovarian or uteroovarian and jugular vein (11). Two iv catheters were then placed about 15 cm apart in the opposite jugular vein (Braunula; 18-gauge, size 2; Armour Pharmaceutical Co., Ltd., Eastbourne, United Kingdom). The cannulae were filled with sterile heparinized saline (20 IU/ml), and the animals were returned to the metabolism crates.

After a rest period of 4 h, timed samples of ovarian venous blood (10 ml) were collected as described previously (11), either hourly (Exp 1) or every 2 h (Exp 2) for 2 h before and 72 h after the first LH injection. Jugular venous blood samples (3 ml) were collected hourly in both experiments; all blood samples were collected immediately before any injections. In addition in Exp 1, ovarian and jugular samples were collected every 10 min for 20 min before until 60 min after the initial (0 h), 9th (+23 h), 10th (+26 h), and 19th (+45 h) injections of LH.

All blood samples were centrifuged, and the resulting plasma was stored at −20 C until assayed for 17β-estradiol (ovarian samples) or LH, FSH, PRL, and progesterone (jugular samples).

*RIAs*

LH, FSH, and PRL were measured in duplicate by specific double antibody RIAs, as described previously (12–14). The sensitivities of the assays were 0.1 ng LH (NIH-LH-S14)/ml, 16 ng FSH (NIH-FSH-S10)/ml, and 0.05 ng PRL (NIH-P-S6)/ml. All samples from a single animal were included in one assay. The mean intra- and interassay coefficients of variation of three quality control sera (at approximately 30%, 55%, and 80% bound to free ratio) were 7% and 10%, 8% and 12%, and 5% and 8% for LH, FSH, and PRL, respectively.

17β-Estradiol and progesterone were measured by specific RIAs, as described previously (15). Intra- and interassay coefficients of variation were 6% and 10%, and 9% and 13% for 17β-estradiol and progesterone, respectively.

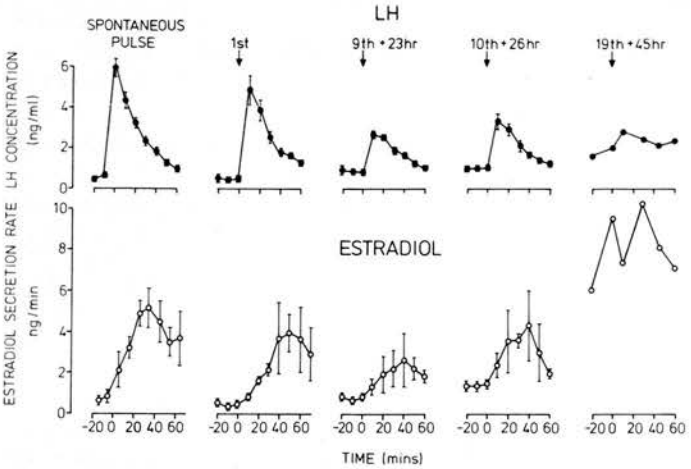



FIG. 1. Plasma levels (mean ± SEM) of LH in jugular venous blood and ovarian secretion of 17β-estradiol in ovarian venous blood samples around a spontaneous pulse of LH (n = 3) and after the first (n = 4), 9th (n = 4), 10th (n = 3), and 19th (n = 1) iv injections of ovine LH (10 μg; NIH LH S-13). The frequency of LH injections was increased over a 72-h period as follows: one per 3 h for 24 h, one per 2 h for 24 h, and one per hour for 24 h.

*Analysis of results*

Because the values were not all normally distributed means, SDs, SEs, and normal ranges for the changes in plasma levels of LH, FSH, and PRL were calculated after log transformation. Results have been compared to the changes in plasma levels of LH, FSH, and PRL in the same ewes during the preovulatory period after prostaglandin-induced luteal regression during the breeding season, as previously described (11, 16). For the present study, the 95% confidence limits of these changes have been calculated after log transformation. Results were analyzed using Student's *t* test or analysis of variance.

## Results

*Exp 1*

*LH and 17β-estradiol.* In two ewes, ovarian venous blood samples were collected throughout the experiment, while in the other two ewes, collections were terminated 23 and 45 h after the first injection of LH, because the cannulae became blocked. The acute changes in plasma levels of LH and 17β-estradiol after the injection of LH shown in Fig. 1 are comparable to the spontaneous pulse of endogenous LH and 17β-estradiol observed in the control sampling period before the start of Exp 1.

Each injection of LH was followed by an increase in the secretion of 17β-estradiol (Fig. 1) and resulted in a sustained elevation in the secretion of 17β-estradiol from 0.55 ± 0.07 ng/min [±SEM; range, 0.10–1.26 ng/min (basal secretion rate); n = 4] before the first injection of LH to 1.62 ± 0.19 ng/min (range, 0.71–2.96 ng/min; n = 3) 24–30 h later. Over this time, the concentration of LH increased from 0.59 ± 0.05 (range, 0.3–1.0 ng/ml) before the first injection to 1.13 ± 0.08 (range, 0.7–1.9 ng/ml; Figs. 2a and 3b). Between 44 and 51 h after the first injection of LH, the secretion of 17β-estradiol increased further to reach a peak of 4.66–11.49 ng/min. This coincided with a surge of LH 52–57 h after the first LH injection, which reached peak levels of 41–98 ng/ml and lasted 8–14 h (Figs. 2a and 3b). Within 6 h after the concentration of LH increased above 5 ng/ml, the secretion of 17β-estradiol decreased rapidly over 6–9 h to levels between 0.05–0.2 ng/min (n = 2; Fig. 3b). The changes in plasma concentrations of LH and secretion

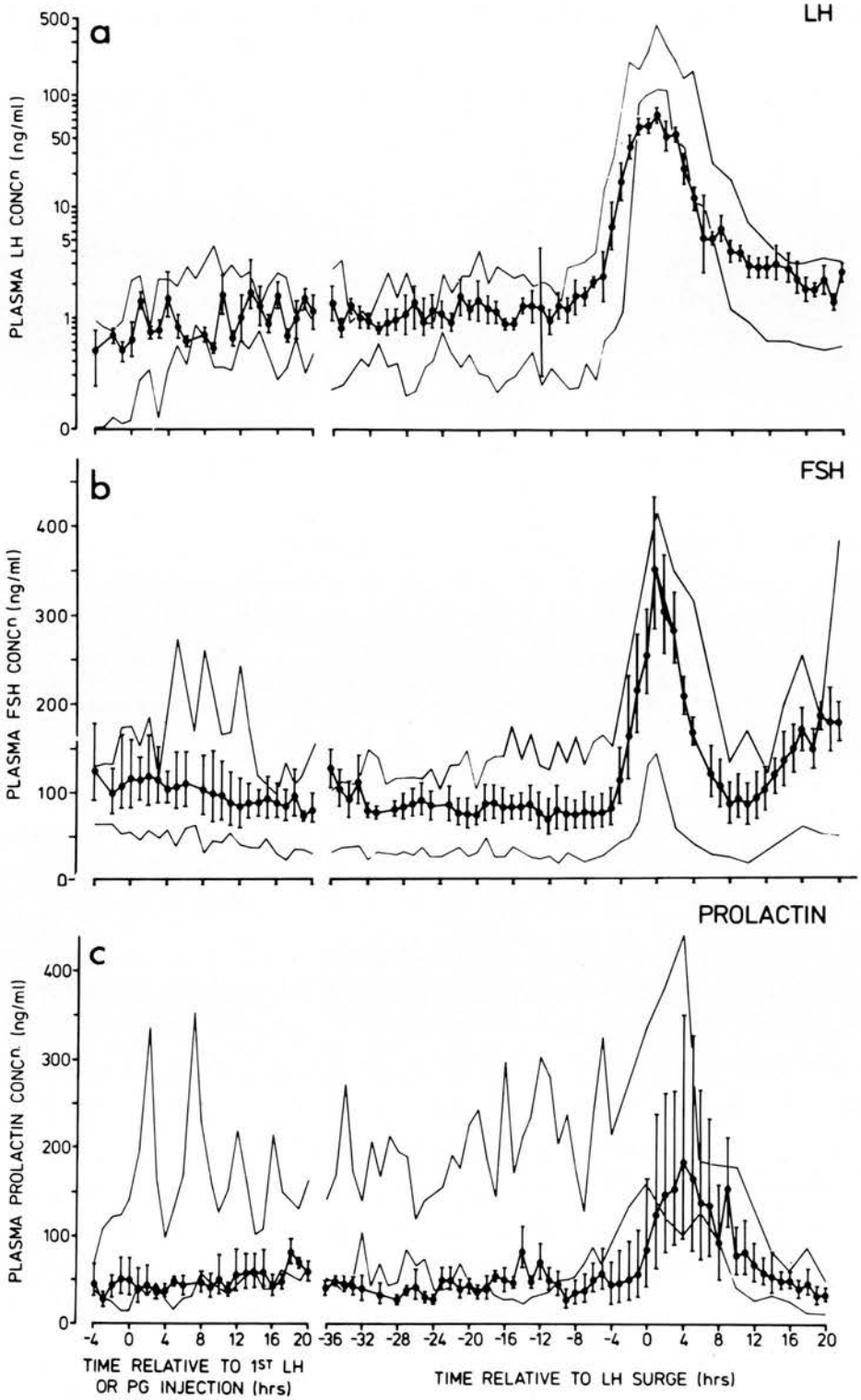



FIG. 2. Plasma levels (mean ± SEM after log transformation) of LH, FSH, and PRL centered around the peak value of LH in the four ewes injected with LH pulses at an increasing frequency (see Fig. 1). The 95% confidence limits (——) of the changes in the same hormones during the preovulatory period in the normal estrous cycle during the breeding season in the same ewes are shown for comparison.

rates of 17β-estradiol from 36 h before to 14 h after the surge were within the range seen during the normal spontaneous preovulatory period during the estrous cycle in these ewes (19) (Fig. 2a).

*FSH.* During the period of increased secretion of 17β-estradiol after the first injection of LH, plasma levels of FSH declined from 120 ± 22 ng/ml (range, 62–400 ng/ml) to 84 ± 7 (range; 63–156 ng/ml; Figs. 2b and 3b). Levels then increased to a peak of 246–718 ng/ml coincident with the surge of LH. After this, the concentration

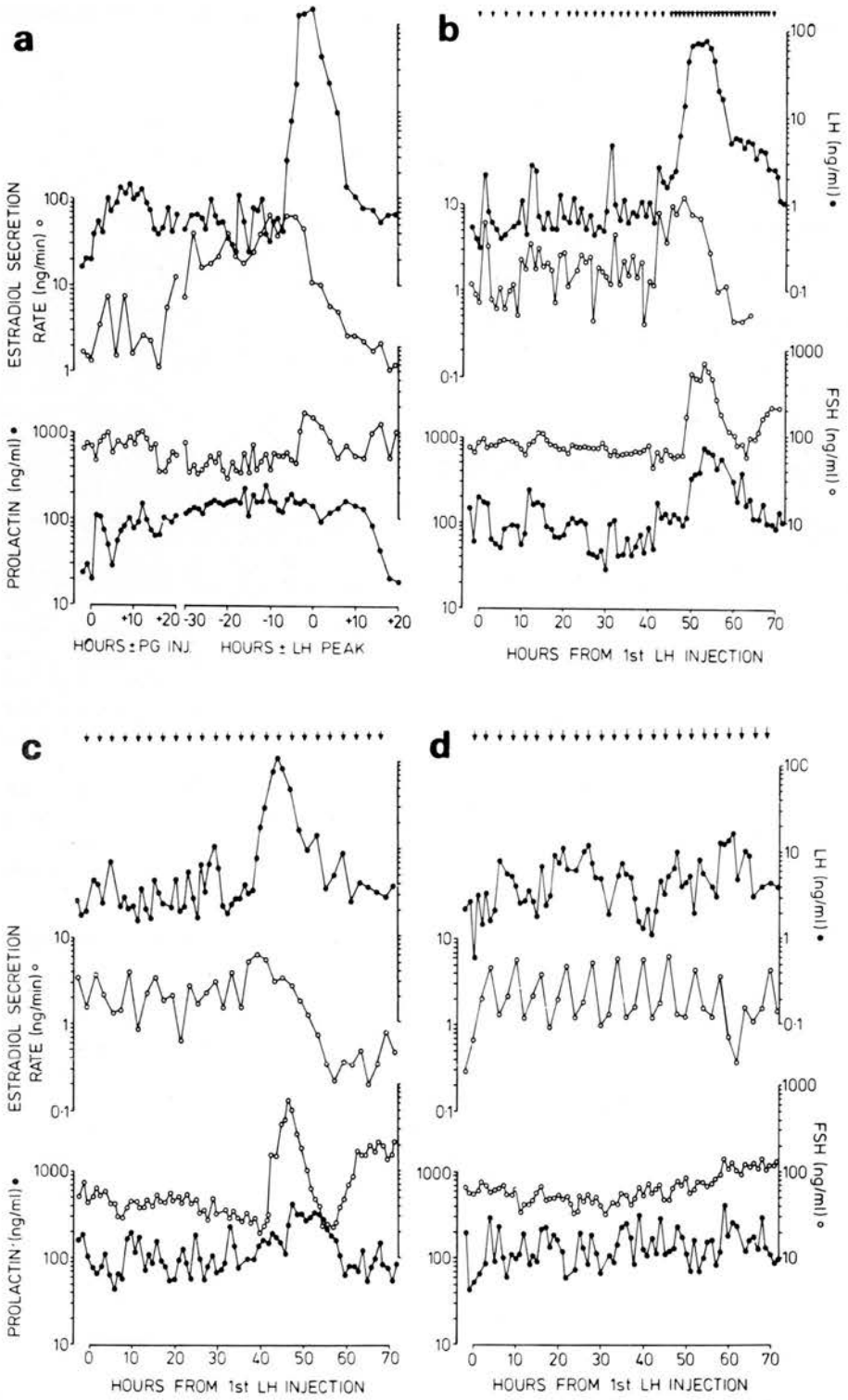



FIG. 3. Plasma levels of LH, FSH, and PRL in jugular venous blood and ovarian secretion of 17$\beta$-estradiol in ovarian venous blood in individual ewes during the preovulatory period of the normal estrous cycle (a) and when receiving LH injections iv at a frequency increasing from one per 3 h (b) to one per hour (c) and a constant frequency of one per 3 h (d). The *arrows* indicate when LH was injected. Results in a, b, and c are from the same animal.

of FSH declined before rising again to reach maximum values (104–248 ng/ml) 13–18 h after the first peak (Figs. 2b and 3b) and were within the range of levels found during the normal estrous cycle (Fig. 2b).

*PRL.* In the 7 days before the start of the experiment, plasma concentrations of PRL were significantly greater ($P < 0.001$) than those during the normal estrous cycle (106 ± 15 *vs.* 48 ± 2 ng/ml, respectively; n = 4). Up to 15 h before the preovulatory LH peak, PRL concentrations were at or below the lower limit of those in the normal estrous cycle (Fig. 2c). In one animal, levels remained

low throughout, whereas in the other three animals, there was a rise in the concentration of PRL associated with the LH peak. Levels then returned to normal within 20 h of the LH peak.

*Progesterone.* Before the LH surge, plasma levels of progesterone were all less than 0.1 ng/ml. Within 2 days after the LH surge, they increased to 0.31 ± 0.10 ng/ml and reached peak values of 4.05 ± 0.51 ng/ml by day 14 after the LH surge in all four animals (Fig. 4).

*Exp 2*

*LH and 17β-estradiol.* Examples of the two patterns of response seen after LH was injected at 3-h intervals are illustrated in Fig. 3, c and d. In two animals, the changes in plasma levels of LH and secretion rates of 17β-estradiol were similar to those in Exp 1. An increase in the secretion rate of 17β-estradiol to maxima of 4 and 6.4 ng/min 39 and 43 h after the first injection of LH was followed by a surge of LH, with peak values of 85 and 140 ng/ml occurring 6 and 7 h later, respectively (Fig. 3c).

In the other two animals, levels of LH (0.5–3.5 ng/ml) were within the normal range until 48 and 56 h after the first LH injection, when levels increased to between 11–17 ng/ml for 4 and 5 h (Fig. 3d). In the one animal in which complete data are available, the secretion of 17β-estradiol responded to each injection of LH without any evidence of a sustained increase in basal secretion (Fig. 3d). The basal secretion of 17β-estradiol before the first injection of LH was lower in these two ewes than in those which ovulated in response to the injections (0.28–0.95 *vs.* 1.7–3.5 ng/min).

*FSH.* In all four animals, FSH levels declined over the first 30 h after the first LH injection from 142 ± 33 ng/ml (n = 12) before the experiment to 81 ± 21 ng/ml (n = 12) 29–31 h later. This was not statistically significant, since FSH levels in one animal were 3–10 times higher than those in the other three animals at the start of the experiment. In percentage terms, FSH levels declined between 21–47% (mean, 34%). FSH levels increased to peak values of 322 and 619 ng/ml coincidentally with the LH surge in the two animals which ovulated (Fig. 3c), then declined and rose again to reach maximum levels of 160 and 190 ng/ml 23 and 21 h later, respectively. These changes were within the normal range for the estrous cycle. In the two animals which failed to ovulate, FSH levels increased gradually to values similar to or slightly above those at the start of the experiment, but did not show any surges (Fig. 3d).

*PRL.* PRL levels in all four animals at the start of the experiment were significantly higher ($P < 0.01$; n = 4) than those seen in the normal cycle (95 ± 18 *vs.* 48 ± 2). Levels ranged between 41–340 ng/ml in all animals throughout the experiment. In the two animals which ovulated, an increase in PRL levels occurred (lasting 8–9 h) around the time of the LH surge (Fig. 3c); levels then returned to normal.

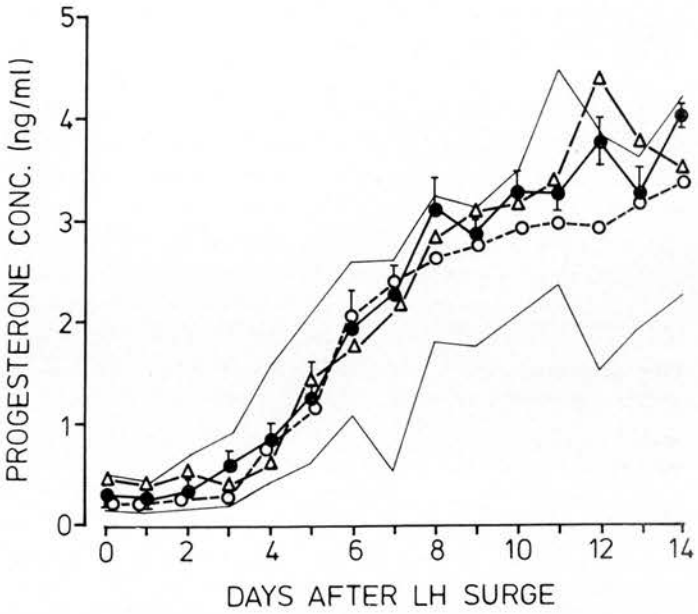



FIG. 4. Plasma levels (mean ± SEM; ○) of progesterone in the four ewes which ovulated after receiving LH injections at an increasing frequency and in the two ewes (● and △) which ovulated after LH was injected at a constant frequency. For comparison, the 95% confidence limits (——) of the changes in progesterone in the luteal phase during the normal breeding season in the same ewes are shown.

*Progesterone.* Progesterone levels increased normally in both animals, with a preovulatory LH surge, while in the other two ewes, levels remained below 1 ng/ml (Fig. 4).

## Discussion

The results of the present study demonstrate that repeated injections of LH in amounts which simulate endogenous pulses of LH in the follicular phase of the normal estrous cycle of the ewe will induce and sustain 17β-estradiol secretion, resulting in the induction of an endogenous surge of LH, ovulation, and a normally functioning corpus luteum. Previous studies have demonstrated that during anestrus, the ovary contains follicles which can respond with an increase in 17β-estradiol and androstenedione secretion when stimulated by an endogenous pulse of LH (3). A similar situation occurs during the luteal phase of the normal estrous cycle (15). During the preovulatory period, the basal level of LH increases (17–19), associated with an increase in the frequency of LH pulses (17, 20). This results in an increase in the secretion of 17β-estradiol from the preovulatory follicle, which in turn induces the preovulatory surge of LH, ovulation, and the formation of a corpus luteum (16, 20–22). In the first experiment, the frequency of LH injec-

tions was increased to mimic in part this natural increase in frequency during the follicular phase of the cycle. It is of note that the endogenous surge of LH occurred around 55 h (range, 52–57 h) after the first injection of LH, a time very similar to the 60 h (range, 52–68 h) between the induction of luteal regression by prostaglandin injection and the LH surge in the estrous cycle (16). Clearly, the stimulation of the follicle by the LH injections over the first 52–57 h was sufficient to maintain follicular 17$\beta$-estradiol secretion at a level equivalent to that seen in the preovulatory phase of the normal estrous cycle in the breeding season (19), and the last 14–20 injections of LH at the end of the experiment were irrelevant. Indeed, as in the normal cycle (16, 22), when the concentration of LH in the preovulatory surge exceeded and was maintained above 5 ng/ml, the secretion of 17$\beta$-estradiol from the follicle was inhibited and failed to respond to further pulses of LH even 20 h after the start of the LH surge when levels had returned to baseline.

In all four animals in Exp 2, the ovary responded to each injection of LH with increased secretions of 17$\beta$-estradiol of comparable magnitude. It is not clear why in only two of the four animals this signal was sufficient to induce a preovulatory surge of LH. It may be that these animals are displaying the differential sensitivity to the positive feedback effects of 17$\beta$-estradiol, as has been described previously for other breeds of sheep in anestrus (1, 8, 23). The lower basal secretion of 17$\beta$-estradiol in the two ewes which failed to ovulate suggests that follicular development was less advanced at the start of the experiment. It may be that if the secretion of 17$\beta$-estradiol had been further stimulated by increasing the frequency of LH injections, as in Exp 1, a LH surge would have been induced. It is also possible that the ewes in Exp 2 were in a deeper state of anestrus than those in Exp 1. This seems unlikely, since the experiments were carried out within 21 days of the same relative stage of anestrus in the 2 yr. On the other hand, it may be that in breeds of sheep with a longer and deeper anestrus than the Finn-merino ewes used in the present studies, *e.g.* Scottish Blackface ewes (1, 7), the present regimen of pulse injections of LH may need to be extended before ovulation can be induced.

Similar results have been obtained in the prepubertal lamb, where ovulation was induced in two of three lambs when LH injections were given hourly over 48 h, whereas not one of three lambs ovulated when LH was injected every 3 h (24). In rhesus monkeys, either prepubertally or in adults with hypothalamic lesions, ovulation was induced with pulses of LH induced by the hourly injection of LHRH (25, 26). Similarly, in hypogonadotrophic or anorexic women, follicular development and ovulation have been induced by LHRH injections, at intervals of 90 min (27) or 2 h (28, 29). However, there have been no reports on the effects of changing these pulse frequencies, although in women, there is an increase in the pulse frequency of LH as ovulation nears (30).

The physiological importance of this change in LH pulse frequency is uncertain. Pulsatile stimulation of the ovary by LH is not a prerequisite for follicular growth and development, since this can be achieved by single daily injections of large amounts of gonadotrophins (31). Similarly, in anestrous sheep, ovulation can be induced by LH infusion or injection of hCG or PMS gonadotropin (23, 32–34). However, if ovulation is induced by the injection of LHRH or a LHRH agonist such as to induce the release of LH and FSH equivalent to the preovulatory surge in the normal cycle, the subsequent corpus luteum fails to secrete normal amounts of progesterone both *in vivo* (7, 35–37) and *in vitro* (37) unless the ewes are pretreated with PMS gonadotropin (38). In the normal estrous cycle, LH is released in a pulsatile fashion, and an increase in the frequency of LH pulses in the preovulatory period appears to be the physiological stimulus for the final phase of follicular development (22). The present data support this suggestion, and since LH alone was injected, it seems unlikely that an alteration in the secretion of FSH is important in the etiology of seasonal anestrus.

The plasma levels of FSH in anestrus in the present study are within the same range as those seen during the estrous cycle, as reported previously (5, 7, 8, 39). However, it has been suggested that in the ewe, as in the hamster (40) and rat (41), the second FSH peak occurring at ovulation is necessary in some way to prime follicles for growth (42). In the present study, follicular growth and development, at least in terms of estradiol secretion, appeared normal when induced by LH pulses alone without a prior surge of FSH, and subsequent corpus luteum function was normal, suggesting that preovulatory intrafollicular development had proceeded normally (43). It is interesting to note that the change in FSH levels seen before ovulation in the animals which ovulated in this study was the same as that found in the normal estrous cycle, with a peak of FSH occurring coincidentally with the LH surge, and a second peak of FSH occurring some 10–20 h later. It appears, therefore, that the follicle destined to ovulate can in some way protect itself from the declining levels of FSH in the preovulatory period, possibly by maintaining intrafollicular levels of FSH (44) as has been suggested in the human follicle (45). Thus, it is unclear in the sheep what role the increases in FSH which occur at the time of ovulation may play, but it seems probable that the lack of FSH stimulation is not of prime importance in the failure of the final stages of follicular development of anestrus.

In both of the experiments reported here, the plasma levels of PRL at the time of each experiment and for 20–

26 weeks beforehand were some 2- to 10-fold higher than those found during the breeding season (5–7). While it has been suggested that these raised levels of PRL may be implicated in some way in the reduction of ovarian follicular development (43, 46, 47), it is clear from the present study that any inhibitory effect of PRL on the ovary can be overcome, in the sheep at least, by adequate LH stimulation. Nevertheless, high levels of PRL may reduce steroidogenesis (48, 49). It is possible, therefore, that high levels of PRL might, if LH pulsatility was reduced, result in a reduction in ovarian activity. This may not have been seen in Exp 1, since at the time of the experiment, PRL levels were within the range seen during the preovulatory period in the normal estrous cycle. It is of considerable interest, however, that a surge of PRL occurred at the time of the LH surge in all six animals which ovulated in Exp 1 and 2, but not in the two which failed to ovulate. A similar increase in PRL levels occurs during the preovulatory period in the normal estrous cycle (50). Thus, even though PRL levels are elevated during anestrus, this mechanism remains intact.

It is clear from the present study that the corpus luteum formed after ovulation secretes normal amounts of progesterone. However, it remains to be determined whether ewes in which ovulation is induced by LH pulses alone would be fertile.

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# Evidence for direct inhibition of ovarian function by prolactin

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## Introduction

There is no doubt that in breast-feeding women, suckling with its associated hyperprolactinaemia prevents the resumption of ovarian activity for prolonged periods (see McNeilly, 1979). The extent of this suppression varies greatly among species but in all for which there are adequate data it appears to depend critically upon the intensity of the suckling stimulus (Lamming, 1978). Our recent data from women show that in the pattern of suckling, frequency and duration, throughout the day, are both key factors in maintaining the elevation of basal levels of prolactin associated with lactation (McNeilly, Howie & Houston, 1980a; Howie & McNeilly, 1982). Suckling also releases large quantities of prolactin, maintaining a physiological hyperprolactinaemic state which is directly associated with the duration of lactational amenorrhoea (Delvoye, Badawi, Demaegd & Robyn, 1978; Duchen & McNeilly, 1980). The question remains, how does suckling suppress ovarian activity?

The levels of prolactin in blood during peak lactation appear to be many times the requirement for production of milk. It therefore becomes pertinent to ask whether this prolactin is released solely to stimulate milk production or whether it is involved directly in the suppression of ovarian activity. If there is a direct involvement then there are two loci for this action; an effect at the hypothalamic–pituitary level or a direct effect on the ovary. While the former has received some attention (see McNeilly, 1979, 1980a), relatively little information is available (for species other than the rat) on whether or how prolactin might act on the ovary and, in particular, how high levels might interfere or block follicular development during lactation (McNeilly, 1980b). The purpose of this paper is to review the evidence for a direct action of prolactin on the ovary in the context of the effects of prolactin in the control of normal ovarian cyclicity.

## Prolactin and the normal ovarian cycle

While this is not the place to present an exhaustive review of the literature pertaining to the role of prolactin in the normal ovarian cycle it is necessary to discuss briefly how prolactin is involved so that this may be set against any directly inhibitory role associated with hyperprolactinaemic states.

### *Changes in blood concentrations of prolactin in ovarian cycles*

In the majority of species studied adequately so far prolactin levels change little during the luteal phase of the cycle but increase around the time of ovulation in association with the

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increasing levels of oestrogens at this time (see McNeilly, 1980a; Cowie, Forsyth & Hart, 1980). In the rat, however, the corpus luteum of pseudopregnancy or pregnancy will not function unless prolactin levels are sustained after ovulation (see Richards, 1980). In primates no clear-cut rise in blood levels of prolactin can be seen although there is some evidence of a small increase around the time of the ovulatory surge of LH in women (see McNeilly, 1980a).

*Prolactin and follicular growth and development*

While there is evidence for a requirement for prolactin in maintaining growth and/or development of follicles, the mechanisms are poorly understood. Studies in which blood levels of prolactin have been suppressed pharmacologically with bromocriptine (sheep: Niswender, 1974; cow: Hoffmann *et al.*, 1974; women: Besser, Parke, Edwards, Forsyth & McNeilly, 1972), ergocornine (ewes: Louw, Lishman, Botha & Baumgartner, 1974) or with antiserum to prolactin (cows: Hoffmann *et al.*, 1974) suggest that only minimal amounts of prolactin are needed for normal follicular growth. It does appear that some prolactin is necessary for steroidogenesis, in women at least, because human granulosa cells grown *in vitro* in the absence of prolactin fail to secrete progesterone even in the presence of adequate amounts of gonadotrophin (McNatty, Sawers & McNeilly, 1974; McNatty, Bennie, Hunter & McNeilly, 1975).

Whether changes in the blood level of prolactin, provided they are within the normal range, are of relevance to gonadal function may be in doubt. In follicular fluid of developing human follicles prolactin concentrations change in a discrete pattern during the menstrual cycle independently of the blood level (McNatty *et al.*, 1974). High levels (4–6 times those in serum) are found in follicular fluid during the early follicular phase of the cycle and decrease progressively towards midcycle when an inverse relationship between prolactin and progesterone levels within the follicular fluid is observed. Similar observations have been made for both the sheep (McNatty, 1977) and cow (Henderson, McNeilly & Swanston, 1982). These results suggest that changes of hormones within the follicle and not necessarily in the blood may be of prime importance for follicular development at least in terms of prolactin. Indeed, more recent studies on granulosa cells suggest that this may well be the case. In the pig the number of prolactin receptors on granulosa cells decrease with increasing size of follicle (Rolland & Hammond, 1975) a change which appears to be steroid mediated (Hammond & Krall, 1979) and dependent on the stage of cytodifferentiation (Veldhuis, Klase & Hammond, 1980). Exposure of granulosa cells from mature follicles to oestradiol *in vitro* increases the number of prolactin receptors and then prolactin stimulates progesterone secretion, apparently interacting synergistically with oestradiol (Veldhuis & Hammond, 1980; Veldhuis *et al.*, 1981).

A similar sequence of events appears to occur in the rat. Before ovulation prolactin binding to theca and granulosa cells is low but increases in granulosa cells in response to the preovulatory LH surge (Richards & Midgley, 1976; Richards *et al.*, 1976; Rao, Richards, Midgley & Reichert, 1977). In immature female rats that have been hypophysectomized and treated with diethylstilboestrol FSH will increase the number of prolactin receptors on granulosa cells *in vivo* and *in vitro* (Wang, Hsueh & Erickson, 1979). Prolactin alone added to such cells will increase progesterone secretion, decrease 20α-hydroxysteroid dehydrogenase activity and secretion of 20α-hydroxypregn-4-en-3-one without affecting oestrogen secretion (Eckstein & Nimrod, 1979; Wang *et al.*, 1979, 1980).

*Prolactin and corpus luteum function*

Over the years confusion has arisen as to the role of prolactin in corpus luteum function. The so-called luteotrophic effect of prolactin has been interpreted by many authors to indicate that prolactin *per se* will promote steroidogenesis. Failure to demonstrate such an effect after

prolactin administration during the luteal phase of the cycle in the sheep (Karsch *et al.*, 1971), cow (Smith, McShaw & Casida, 1957) and pig (Anderson, 1966) and the absence of an effect of lowering prolactin levels with bromocriptine in sheep (Niswender, 1974) and cows (Hoffmann *et al.*, 1974) suggested that prolactin played little or no role in luteal maintenance in these species. On the other hand, a decrease in progesterone secretion from the corpus luteum has been shown for women (Schulz *et al.*, 1976) when prolactin levels are suppressed below normal by bromocriptine treatment. It is also clear that in the sheep (Denamur, Martinet & Short, 1973), goat (H. L. Buttle, personal communication) and ferret (Murphy, 1979) both LH and prolactin are necessary for maintenance of normal luteal progesterone production after hypophysectomy.

In rats and mice postovulatory increases in plasma prolactin concentrations are essential to induce the functional corpora lutea of pregnancy or pseudopregnancy (Everett, 1961; Dominic, 1966). Mating in the rat is associated with an increased diurnal secretion of prolactin (Smith, Freeman & Neill, 1975), blockade of which by ergocornine or bromocriptine treatment leads to failure of progesterone secretion and corpus luteum function (Wuttke & Dohler, 1973).

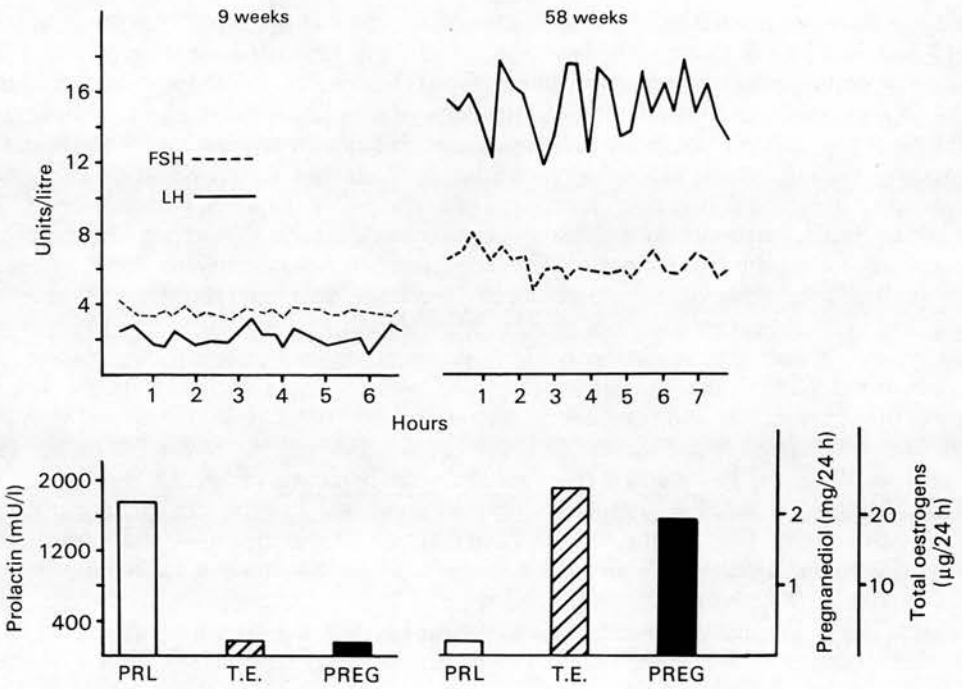
Prolactin alone will stimulate the secretion of progesterone from the rat corpus luteum (Richards & Williams, 1976) and is responsible for the induction of luteal LH receptors (Holt, Richards, Midgley & Reichert, 1976). Within the corpus luteum prolactin enhances the uptake of cholesterol esters (Armstrong, Miller & Knudsen, 1969), increases cholesterol esterase activity (Behrman, Orczyk, Macdonald & Greep, 1970; Klemcke & Brinkley, 1980) and decreases 20α-HSD activity, limiting the secretion of 20α-dihydroprogesterone (Wiest, Kidwell & Balogh, 1968). Prolactin also maintains the luteal LH receptor after prostaglandin treatment (Grinwich, Hichens & Behrman, 1976) or hypophysectomy (Behrman, Grinwich, Hichens & Macdonald, 1978; Gibori & Richards, 1978) and stimulates and/or maintains the content of cytosolic oestradiol receptors in luteal cells synergizing with oestradiol to maintain progesterone secretion (Gibori, Richards & Keyes, 1979). Prolactin-associated accumulation of cholesterol esters has also been demonstrated in rabbit ovarian interstitium (Hilliard, Spies, Lucas & Sawyer, 1968) and cow ovary (Bartosik, Romanoff, Watson & Scricco, 1967).

In contrast to its luteotrophic role prolactin may also act as a structural luteolysin in the rat (Malven, 1969) and mouse (Grandison & Meites, 1972) and is responsible for the final structural demise of the corpora lutea in the normal non-pregnant, non-pseudopregnant cycle (Billeter & Fluckiger, 1971; Bohnet, Shiu, Grinwich & Friesen, 1978). No comparable function has been observed in any other species so far studied.

It is clear that prolactin is involved directly at the ovarian level for the growth and development of the follicle and for the maintenance of luteal function in those species investigated. However, the precise mechanisms by which prolactin acts remain to be resolved.

## Prolactin and the suppression of ovarian activity *post partum*

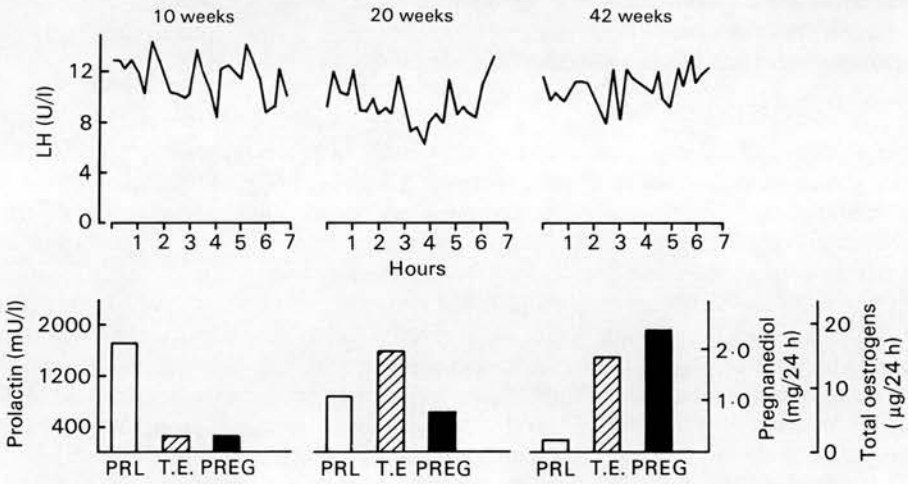
In a large number of species lactation is associated with a period of ovarian inactivity of varied length (see Lamming, 1978; McNeilly, 1979). This period is related to enhanced prolactin secretion and in women an association between hyperprolactinaemia and lactational amenorrhoea has been demonstrated (Delvoye *et al.*, 1978; Duchen & McNeilly, 1980; McNeilly *et al.*, 1980a; Howie & McNeilly, 1982). The sequence of hormonal events associated with the suppression and gradual resumption of ovarian activity, in women in particular, has been reviewed previously (McNeilly, 1979; McNeilly *et al.*, 1980a; Howie & McNeilly, 1982) and will only be outlined here. During the phase of complete ovarian inactivity prolactin values are elevated, FSH levels are within the normal range while LH concentrations are low. In the early post-partum period the low levels of LH may be associated with a reduced frequency and amplitude of LH pulses (Text-fig. 1; A. Glasier, A. S. McNeilly & P. W. Howie, unpublished observations) and an absence of positive feedback and enhanced negative feedback effects of




**Text-fig. 1.** Changes in LH and FSH at 15-min intervals over a 6·5-h period (10:00–14:30 h) in a breast-feeding woman at 9 and 58 weeks *post partum*. At 9 weeks, ovarian activity, as measured by urinary excretion per 24 h of total oestrogens (T.E.) and pregnanediol (PREG), was suppressed (T.E. < 10 µg/24 h; PREG < 0·5 mg/24 h) while prolactin levels (PRL) over the 6·5-h period and basally were between 2 and 6 times normal. Results are compared to the pattern of LH and FSH secretion in the same woman 8 weeks after weaning at 58 weeks *post partum* on Day 10 of a normal menstrual cycle. (A. Glasier, A. S. McNeilly & P. W. Howie, unpublished observations.)

oestrogen on gonadotrophin secretion (Baird, McNeilly, Sawers & Sharpe, 1979; Glass, Rudd, Lynch & Butt, 1981). The resumption in ovarian activity occurs as a consequence of a decrease in the suckling stimulus which results in a fall of basal prolactin concentrations and an increase in basal levels and pulsatile release of LH (Text-fig. 1; McNeilly *et al.*, 1980a; Howie & McNeilly, 1982; A. Glasier, A. S. McNeilly & P. W. Howie, unpublished observations). However, while lactation continues ovulation is invariably associated with prolonged follicular phases and deficient corpora lutea secreting inadequate amounts of progesterone. Only when lactation ceases completely or when prolactin levels return to normal are ovulatory cycles associated routinely with normal corpus luteum function. A similar situation and sequence of events occurs during the re-establishment of ovarian cyclicity during lactation in the cow (e.g. Schams *et al.*, 1978; Stevenson & Britt, 1979; Carruthers & Hafs, 1980; Webb, Lamming, Haynes & Foxcroft, 1980), sheep (Fitzgerald & Cunningham, 1981; Wright, Geytenbeck, Clarke & Findlay, 1981) and pig (Stevenson, Cox & Britt, 1981). Whether the high levels of prolactin or the suckling stimulus alone or together are responsible for the suppression of LH secretion during lactation has been discussed previously (McNeilly, 1980a). What is of interest is to determine whether the high levels of prolactin *per se* might interfere with follicular development, ovulation or corpus luteum function by a direct action on the ovary. In 40% of the women investigated during lactation when ovarian activity was fully suppressed, basal concentrations and pulsatile secretion of LH had increased to levels indistinguishable from those

seen when follicular development and ovulation had returned (Text-fig. 2). The most significant difference between these two situations, i.e. similar LH levels in the absence and then the presence of follicular development was the much greater plasma concentration of prolactin in the former compared to the latter. Nevertheless even though follicular growth and development and ovulation did occur, the resulting corpus luteum remained inadequate until prolactin levels returned to normal. These results suggest that during lactation in women elevated levels of prolactin can interfere directly at the ovarian level to prevent the normal action of LH thereby inhibiting follicular growth. Even when this does occur the resulting corpus luteum did not appear to have gained its normal ability to secrete progesterone. Deficient corpora lutea could arise either from a direct inhibitory action of high concentration of prolactin on the corpus luteum itself or because the corpus luteum was formed from a follicle inadequately primed before ovulation.




**Text-fig. 2.** Changes in LH over a 6·5-h period (10:00–14:30 h) in a breast-feeding woman when fully breast feeding with no ovarian activity at 10 weeks, on Day 10 of a menstrual cycle with a deficient corpus luteum at 20 weeks *post partum* while partly breast feeding and at Day 10 of a normal menstrual cycle at 42 weeks *post partum* when the baby was fully weaned. PRL = prolactin; T.E. = total oestrogens; PREG = pregnanediol. (A. Glasier, A. S. McNeilly & P. W. Howie, unpublished observations.)

*Hyperprolactinaemia and the corpus luteum*

Several lines of evidence suggest that high levels of prolactin do not interfere with steroidogenesis in the corpus luteum. In the rat the corpus luteum of pregnancy and pseudopregnancy is dependent on prolactin until Day 8 and will not function unless prolactin levels remain elevated during this time (e.g. Garris & Rothchild, 1980). In hysterectomized sheep there is no change in progesterone secretion from the persistent corpus luteum, even in anoestrus (Baird, Land, Scaramuzzi & Wheeler, 1976) when prolactin levels are elevated (Walton, McNeilly, McNeilly & Cunningham, 1977). Similarly, corpora lutea formed after ovulation had been induced by pulsed injections of LH in anoestrous ewes secreted normal levels of progesterone (McNeilly, O'Connell & Baird, 1980b). In dairy cows, normal luteal function occurs after ovulation in spite of elevated levels of prolactin (Peters, Vyvoda & Lamming, 1979; Carruthers & Hafs, 1980), while in monkeys with lesions of the arcuate nucleus and associated hyperprolactinaemia, corpus luteum function was normal after ovulation induction with pulsed injections of LH-RH (Knobil, Plant, Wildt, Belchetz & Marshall, 1980). While pathological

hyperprolactinaemia in women with ovulatory menstrual cycles may be associated with short or deficient luteal phases (Seppala, Hirvonen & Ranta, 1976; Muhlenstedt, Wuttke & Schneider, 1977), this is not necessarily the case (Sarris *et al.*, 1978). Indeed, when hyperprolactinaemia is induced by pharmacological agents in the luteal phase of the menstrual cycle (TRH: Jewelewicz, Dyrenfurth, Warren, Frantz & Vande Wiele, 1974; sulpiride: Robyn *et al.*, 1976) or when bromocriptine therapy in hyperprolactinaemic patients is given only during the follicular phase of the cycle thus allowing hyperprolactinaemia during the luteal phase of the cycle (Polatti, Bolis, Ravagni-Probizer, Baruffini & Cavalleri, 1978; Bennink, 1979), luteal function was within the normal range. The only species in which prolactin has been shown to inhibit progesterone secretion from the corpus luteum is the tammar wallaby. In the tammar, embryonic diapause occurs with development being arrested at the 80-cell stage, and pregnancy will only continue when the suckling stimulus of the young is removed or diminished (Tyndale-Biscoe & Hawkins, 1977). This can be prevented by administration of exogenous prolactin (Tyndale-Biscoe, 1979).

It seems probable from these observations that raised levels of prolactin in the luteal phase do not inhibit steroidogenesis and suggest that deficient corpus luteum function in hyperprolactinaemic states arise from ovulation of inadequately primed follicles.

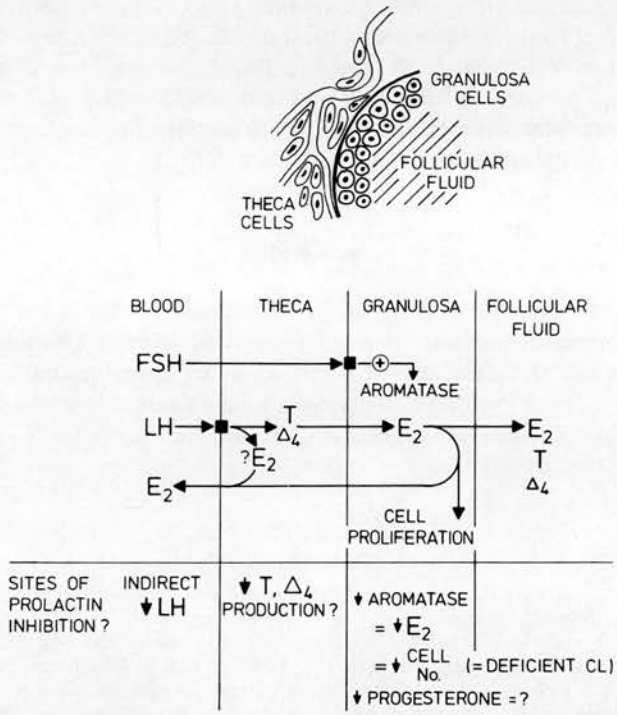
*Hyperprolactinaemia and the follicle*

Direct evidence of an inhibitory effect of high levels of prolactin was obtained from in-vitro experiments with human granulosa cells. Increasing amounts of prolactin added to the culture medium inhibited the secretion of progesterone in a dose dependent manner (McNatty *et al.*, 1974). While this mechanism is still not understood a similar inhibition of progesterone secretion, without effect on oestradiol, has been demonstrated *in vitro* with granulosa cells from small but not large pig follicles (Veldhuis *et al.*, 1980, 1981) and from whole mouse follicles (McNatty, Neal & Baker, 1976). Increasing levels of prolactin stimulate progesterone secretion in rat granulosa cells *in vitro* by a dose-related manner, and this is associated with dose-related decrease in oestradiol secretion (Wang *et al.*, 1980). Prolactin can suppress both FSH- and LH-induced increases in oestradiol production without affecting progesterone production, suggesting that prolactin inhibits at a site distal to the gonadotrophin-induced formation of cAMP, i.e. the effect is specific for aromatase or the oestrogen synthetase system (Wang *et al.*, 1980). Indeed, it has now been shown that high levels of prolactin will block FSH induction of aromatase in this system (Dorrington & Gore-Langton, 1981).

What might be the consequences of these observations? It is clear that oestradiol is a key factor in the development of the follicle and in particular in the growth and differentiation of granulosa cells (Richards, 1980). Thus any suppression of oestrogen secretion would lead to decreased numbers of granulosa cells and since there is little or no mitosis of these cells once transformed into granulosa lutein cells (Diekman, O'Callaghan, Nett & Niswender, 1978) any decrease in numbers within the follicle would lead to decreased steroidogenic potential in the corpus luteum, i.e. the deficient corpus luteum. McNatty (1979) has shown that raised concentrations of prolactin in plasma and human ovarian follicular fluid were associated with a reduced number of granulosa cells with an inadequate ability to produce steroids and a marked reduction in the values of circulating oestrogens.

While these studies show that raised values of prolactin may inhibit granulosa cell oestrogen secretion by interfering with aromatization of androgens, it is also quite feasible that prolactin might interfere with the production of androgens by the theca interna (Text-fig. 3). The secretion rate of oestradiol from the preovulatory follicle is reduced when sheep are made hyperprolactinaemic by repeated administration of TRH (McNeilly & Baird, 1977). More recently, with experiments in which prolactin was infused locally into the ovarian arterial circulation, we have demonstrated that this reduction in steroid secretion is not associated with a reduction in the pulsatile secretion of LH (A. S. McNeilly, M. O'Connell & D. T. Baird, unpublished results). In these experiments the secretion of androstenedione and oestradiol was also inhibited.

Since androstenedione is thecal in origin and its release is stimulated by LH, it seems probable that high levels of prolactin may also inhibit ovarian follicular steroidogenesis, not only by interfering with aromatase but also by reducing the production by the theca of the androgen precursor necessary for oestrogen production (Text-fig. 3). The results suggest that high levels of prolactin can inhibit follicular development by interfering with steroidogenesis and this could explain, in part at least, the failure of normal luteal function in lactation since it may be presumed that intrafollicular cellular development may be inadequate when ovulation occurs.




**Text-fig. 3.** Diagrammatic representation of the control of steroidogenesis within the follicle, indicating the possible sites of inhibition by high levels of prolactin. T = testosterone; $E_2$ = oestradiol; $\Delta_4$ = androstenedione; ↓ = decrease or inhibition of synthesis or release; ⊕ = stimulation; ■ = hormone receptor.

### Does prolactin really work like this?

It seems clear that prolactin may have two divergent actions on the ovary, both stimulatory (permissive) and inhibitory. While there is little evidence to refute the former as a general role in many species, the direct inhibitory role may vary considerably amongst species. In spite of maintained hyperprolactinaemia, normal ovarian activity can and does occur *post partum* in dairy cows (see Carruthers & Hafs, 1980), dairy goats (Hart, 1975) and in marmosets (McNeilly, Abbott, Lunn, Chambers & Hearn, 1981). Ovulation with normal luteal function can be induced in hyperprolactinaemic states by appropriate gonadotrophin treatment in sheep (LH: McNeilly *et al.*, 1980b; PMSG: e.g. Rhind, Robinson, Chesworth & Crofts, 1980), rhesus monkeys (LH-RH: Knobil *et al.*, 1980) and women (e.g. Kemmann, Gemzell, Beinert, Belling & Jones, 1977). In women with pathological hyperprolactinaemia, LH-RH-induced release of gonadotrophins will induce normal oestradiol secretion from the ovary (e.g. Lachelin, Abufadil

& Yen, 1978; Caro & Woolf, 1980) and no differences in the amounts of exogenous gonadotrophin required to induce ovulation have been shown (Fraser, Markham & Shearman, 1978; McGarrigle *et al.*, 1978). However, it may well be that any inhibitory effect of prolactin depends upon the physiological balance within an individual between the concentrations of gonadotrophin and prolactin such that the inhibitory role for prolactin can be overcome by large quantities of gonadotrophin either exogenously administered or endogenously induced by LH-RH treatment.

There is some evidence that, even though cycles may be endocrinologically normal in hyperprolactinaemic states, fertility may be reduced, e.g. in ewes induced to ovulate during lactation (Rhind *et al.*, 1980) and in women with transient hyperprolactinaemia (Fleming, Craig, England, Macnaughton & Coutts, 1978; Lenton, Brook, Sobowale & Cooke, 1979). Perhaps some direct interaction between high levels of prolactin within the follicle and oocyte maturation may be implied, in particular since prolactin has been localized within the oocyte (Nolin, 1980) and implicated in oocyte maturation (Baker & Hunter, 1978).

## Conclusions

While there is abundant evidence that prolactin is involved directly in the normal regulation of ovarian function the precise mechanisms remain to be discovered. There is also clear evidence that in some species abnormally elevated prolactin levels in physiological situations are associated with the inhibition of normal ovarian activity by an action directly on the ovary. Nevertheless, there are exceptions to this and these differences will only be resolved when we understand more fully how prolactin acts normally.

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# Inhibition of ovarian function in subordinate female marmoset monkeys (*Callithrix jacchus jacchus*)*

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**Summary.** Plasma concentrations of progesterone, cortisol, LH and prolactin were measured in dominant and subordinate female marmosets in 10 well-established peer groups. Subordinate females never ovulated, had a reduced LH response to LH-RH and showed no positive feedback LH surge after oestrogen administration. There was no evidence of elevated plasma cortisol levels or hyperprolactinaemia in subordinates and all showed a similar prolactin response to TRH in comparison with dominants. However, subordinates showed a reduced prolactin response to metoclopramide. These results clearly indicate that high circulating levels of cortisol or prolactin are not responsible for the inhibition of ovulation in female marmosets.

## Introduction

Prolactin release in response to specific or non-specific stress has been reported for many mammalian species (Raud, Kiddy & Odell, 1971; Horrobin, 1973; Lamming, Moseley & McNeilly, 1975; McNeilly, 1980). Elevated prolactin levels (hyperprolactinaemia) have also been linked with the inhibition of reproductive function (high-prolactinamenorrhoea) in women (Besser, Parke, Edwards, Forsyth & McNeilly, 1972; Tyson, Friesen & Anderson, 1972; Van Look, McNeilly, Hunter & Baird, 1977; Baird, McNeilly, Sawers & Sharpe, 1979) and in captive subordinate female talapoin monkeys, *Miopithecus talapoin* (Bowman, Dilley & Keverne, 1978; Keverne, 1979). In both cases, high prolactin concentrations were clearly associated with the inhibition of the ovulatory surge of pituitary LH in response to oestrogen. It was proposed by Bowman *et al.* (1978) and Keverne (1979) that in talapoin monkeys, increased prolactin values were due to the stress of attacks from dominant females. However, in these studies only two captive groups of talapoin monkeys were used, "only the most dominant and subordinate individuals" were compared, and it was not established how frequently high prolactin levels were associated with behavioural subordination and infertility in females. As acknowledged by Bowman *et al.* (1978), not all subordinate female talapoin monkeys are normally infertile because these animals are polygamous (Rowell & Dixson, 1975). Furthermore, all the females were ovariectomized and blood oestrogen levels were maintained by implants of oestradiol-17β.

In the present study, 10 groups of captive marmoset monkeys (*Callithrix jacchus jacchus*) were used and the females were left intact. Marmosets are monogamous (Epple, 1967; Rothe, 1975; Stevenson, 1978) and only the single dominant female in any group reproduces because the subordinates do not ovulate (Abbott & Hearn, 1978). We investigated whether (1) the

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ovarian inhibition of subordinate female marmosets was associated with increased peripheral prolactin concentrations, and (2) a pituitary LH surge in these infertile animals could be elicited similar in magnitude and duration to that occurring at ovulation in normal cyclic females (Hodges & Hearn, 1977).

## Materials and Methods

The animals and their management have been described elsewhere (Hearn, Lunn, Burden & Pilcher, 1975).

### *Peer groups and behavioural status*

Ten peer groups were formed, each consisting of 3 male and 3 female post-pubertal marmosets (Abbott & Hearn, 1978). Dominant or subordinate status was assigned on the basis of an animal's aggressive and submissive interactions with others in the group (Abbott & Hearn, 1978; Abbott, 1979). In each group, the dominant male (Male 1) and the dominant female (Female 1) rapidly established a pair bond (Abbott & Hearn, 1978; Abbott, 1979). The subordinate or second and third ranking males (Males 2 and 3, respectively) and subordinate females (Females 2 and 3) formed no such social or sexual relationship with any animal in the group (Abbott, 1978, 1979). In 7 out of the 10 groups, one of the subordinate females had to be removed after the first 2–3 days because of persistent attacks from an animal of higher rank, as previously found (Abbott, 1978, 1979).

### *Blood sampling and hormone assays*

Blood samples of 0·3 ml were withdrawn from the femoral vein into a heparinized syringe through a 0·41 mm diameter needle (27 gauge). Sampling was carried out between 09:30 and 12:30 h. The females were held in a restraining device and were not anaesthetized (Hearn, 1977). All samples were taken 2–4 min after removal of the animals from their cages and then immediately placed on ice. The blood was centrifuged at 500 ***g*** for 20 min at 4°C and the plasma stored at −20°C until assayed. Females were bled at 2–3-day intervals for 6 months, starting 5–12 months after the peer groups had been established. The frequency of sampling was sufficient to cover the approximately 10–12-day period of plasma progesterone elevation during the luteal phase of the 16–17-day ovarian cycle (Hearn & Lunn, 1975). Plasma prolactin and cortisol concentrations were estimated from samples 2–20 days apart.

### *Hormone assays*

Plasma concentrations of progesterone (Chambers & Hearn, 1979), LH (Hodges, 1978), chorionic gonadotrophin (Chambers & Hearn, 1979) and prolactin (McNeilly, Abbott, Lunn, Chambers & Hearn, 1981) were determined by specific radioimmunoassays validated for use in the marmoset monkey. All samples were assayed in duplicate. Plasma cortisol was measured using a competitive protein-binding kit (Cortipac; The Radiochemical Centre, Amersham, U.K.) and cross-reactions with other steroids and corticosteroids have been described (Technical Bulletin 75/10). No significant departure from parallelism between the human standards (2–47·8 μg/100 ml) and serial dilutions of marmoset plasma (1:8–1:128) was demonstrated ($P < 0·10$) by a two-factor analysis of variance with replication. All samples were diluted to read within this range. Two pools of female marmoset plasma (diluted to 1:20 (v/v) with distilled water) were used to determine the inter- and intra-assay coefficients of variation (CV). One pool was repeatedly measured in duplicate in 5 assays and gave a mean value (±s.e.m.) of 100 ± 3·5

μg/100 ml with a CV between assays of 10·4%. The other pool was repeatedly measured in one assay and gave a value of 99·6 ± 3·5 μg/100 ml with a CV of 7·8% ($n = 8$).

*Ovarian cycle and detection of pregnancy*

The luteal phase of each ovarian cycle was defined as lasting from the rise of plasma progesterone concentrations above 20 ng/ml until levels decreased again below that value. When plasma progesterone rises above 20 ng/ml in cyclic female marmosets, progesterone values quickly reach 50 ng/ml and more for at least 10–12 days, typical of the previously described luteal phase of the cycle (Hearn & Lunn, 1975).

Since females were excluded from the study while pregnant, and so as not to miss any early spontaneous abortion (Abbott, 1979), pregnancies were determined by radioimmunoassay of serial plasma samples for progesterone and chorionic gonadotrophin and by monthly transabdominal uterine palpation (Hearn & Lunn, 1975; Chambers & Hearn, 1979).

*LH stimulation with LH-RH and oestradiol benzoate*

At the end of the 6-month period of serial blood sampling, dominant and subordinate females were each given 2 μg LH-RH (Hoechst U.K. Ltd, Hounslow, Middlesex, U.K.) intravenously (Hodges, 1979), following an initial blood sample (0·4 ml). The animals were bled at 30, 45, 60 and 90 min thereafter. As controls, 6 dominant and 4 subordinate females received a similar volume of saline (9 g NaCl/l) 3 weeks later, and were bled after 0, 30, 45, 60 and 90 min.

One month after receiving LH-RH, each dominant and subordinate female was given 35 μg oestradiol benzoate (Organon Laboratories Ltd, Morden, Surrey, U.K.) in a single subcutaneous injection of arachis oil (0·2 ml), and then bled after 0, 8, 24, 28, 36, 48 and 60 h (Hodges & Hearn, 1978): 6 dominant and 4 subordinate females received a single injection of arachis oil 2 weeks later, as controls, and were bled similarly. A positive feedback response to an oestrogen challenge was considered to be an increase in circulating LH to values exceeding two standard deviations of the mean control values (Hodges & Hearn, 1978).

*Prolactin stimulation with TRH and metoclopramide*

TRH (25 μg; Roche Products Ltd, London, U.K.) was given intravenously in 0·2 ml saline to 7 dominant and 9 subordinate female monkeys at the time of LH-RH infusion, and blood samples were taken −30, 0, 15, 30, 45, 60 and 90 min. The same females were injected i.m. with 2 mg metoclopramide in 0·2 ml saline (Maxolon: Beecham Research Labs, Brentford, Middlesex, U.K.) 1 week later and blood samples were taken at −30, 0, 30, 60 and 90 min. Both TRH and metoclopramide are known to raise prolactin levels in female marmoset monkeys (McNeilly *et al.*, 1981). TRH was injected simultaneously with LH-RH since LH-RH has no significant effect on prolactin concentrations (McNeilly *et al.*, 1981) and TRH produces no marked change in LH concentration (Hodges, 1978). As controls, 5 dominant and 5 subordinate females were given saline 4 weeks later.

*Analysis*

Six females were excluded from the LH-RH experiment and 1 from the oestradiol benzoate experiment because they had high progesterone levels (i.e. luteal phase or pregnant). As in other species (Fink, 1979), high progesterone concentrations inhibit LH release (J. K. Hodges, personal communication). Animals in poor condition at the time of an experiment were also excluded (see Text-figure legends for numbers included in each experiment).

The plasma cortisol and prolactin concentrations in subordinate females obtained during the

6-month sampling period were each compared against those of the dominant female in their group by Student's *t* test. The plasma LH concentrations after LH-RH administration, and the plasma prolactin concentrations after TRH and metoclopramide injection were all analysed by a two-way analysis of variance for repeated measures. Comparisons of individual means were made using an *a posteriori* Newman–Keuls test (Winer, 1962). A one-way analysis of variance for repeated measures was performed separately on values from dominant and subordinate females if a significant behavioural status-by-time sampled interaction was achieved ($P < 0{\cdot}05$).

## Results

### *Progesterone and pregnancy*

The dominant female in each of the 10 groups exhibited circulating concentrations of progesterone characteristic of the follicular and luteal phases of a normal ovarian cycle (Table 1). In contrast, subordinate females had only follicular-phase concentrations of progesterone, indicating that none had ovulated. All of the dominant females subsequently became pregnant, but pregnancy was not detected in any subordinate female.

**Table 1.** Mean ± s.e.m. plasma progesterone concentrations (ng/ml) in dominant and subordinate female marmosets in 10 peer groups sampled every 2–3 days for 6 months

| | Dominant females | | | Subordinate females |
|---|---|---|---|---|
| Group | Follicular phase | Luteal phase | Peak luteal values | All values |
| 1 | 4·9 ± 1·6 | 78·3 ± 11·0 | 109·1 ± 20·5 | 3·2 ± 0·4 |
| | | | | 4·4 ± 0·7 |
| 2 | 8·1 ± 2·0 | 91·8 ± 14·1 | 141·2 ± 11·3 | 7·8 ± 1·6 |
| 3 | 2·7 ± 0·6 | 50·7 ± 9·8 | 136·3 ± 30·8 | 2·7 ± 0·3 |
| 4 | 6·0 ± 0·9 | 65·8 ± 3·5 | 101·5 ± 13·9 | 3·0 ± 0·2 |
| 5 | 9·2 ± 2·9 | 93·2 ± 29·4 | 146·8 ± 22·2 | 6·2 ± 2·0 |
| | | | | 6·1 ± 2·0 |
| 6 | 3·6 ± 0·4 | 43·9 ± 4·2 | 75·3 ± 9·8 | 3·5 ± 0·4 |
| | | | | 4·9 ± 0·8 |
| 7 | 3·2 ± 0·4 | 59·2 ± 4·9 | 97·7 ± 16·6 | 2·9 ± 0·3 |
| 8 | 4·0 ± 0·5 | 47·6 ± 4·7 | 56·3 ± 5·8 | 3·0 ± 0·6 |
| 9 | 7·5 ± 1·5 | 82·2 ± 7·4 | 119·8 ± 23·4 | 3·9 ± 0·4 |
| 10 | 2·6 ± 0·3 | 72·6 ± 3·7 | 109·9 ± 9·1 | 3·0 ± 0·2 |

### *Cortisol*

In 2 of the 5 groups sampled, there were similar plasma cortisol concentrations in the dominant and subordinate females (Groups 7 and 9; Table 2). In Group 6, the dominant female had cortisol levels similar to those of the second-ranking female, but higher than those of the third. In the remaining 2 groups, both dominant females had cortisol levels higher than those of their respective subordinates.

### *LH stimulation*

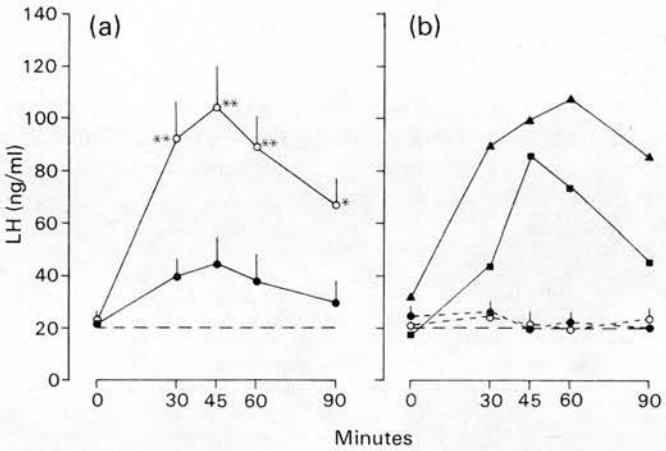
*LH-RH.* There was a significant difference between dominant and subordinate females in their LH responses to LH-RH ($P < 0{\cdot}01$) which, because of their identical basal LH concentrations, led to a significant behavioural status-by-time sampled interaction ($P < 0{\cdot}001$; Text-fig. 1a). Following the LH-RH injection, plasma LH concentrations in dominant females were elevated by 30 min and rose to a peak after 45 min ($P < 0{\cdot}001$; Text-fig. 1a). By 90 min, LH levels were still elevated above baseline. In contrast, there was no significant elevation of plasma

**Table 2.** Plasma cortisol concentrations (mean ± s.e.m. for no. of observations in parentheses) in female marmosets from 5 peer groups

| | Plasma cortisol (μg/100 ml) | | |
|---|---|---|---|
| Group | Female 1 | Female 2 | Female 3 |
| 3 | 414 ± 76 (6) | 285 ± 42* (8) | |
| 6 | 184 ± 12 (16) | 238 ± 50 (7) | 88 ± 12** (8) |
| 7 | 189 ± 8 (14) | 182 ± 17 (12) | |
| 8 | 523 ± 69 (6) | 320 ± 36* (8) | |
| 9 | 190 ± 6 (10) | 280 ± 55 (9) | |

Values significantly different from those of Female 1, $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ (Student's $t$ test).

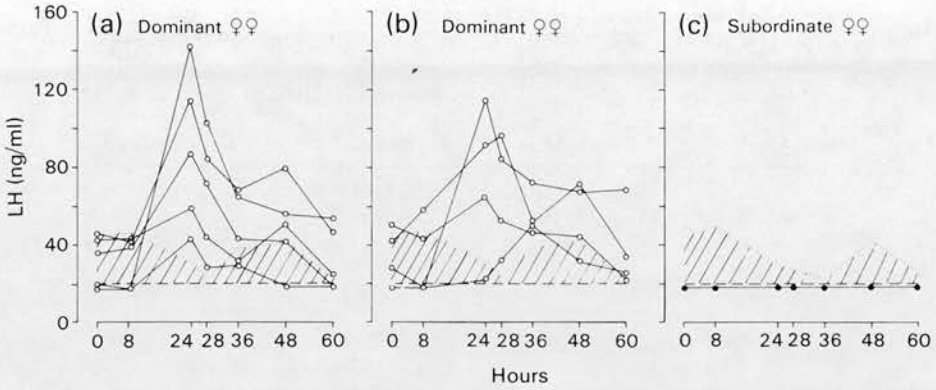
LH concentrations in subordinate females. Nevertheless, 2 out of the 9 subordinates tested showed LH responses equal in magnitude and duration to those of the dominant females (Text-fig. 1b).




**Text-fig. 1.** Plasma LH concentrations (mean ± s.e.m.) in (a) 4 dominant (○——○) and 9 subordinate (●——●) female marmosets given 2 μg LH-RH i.v. at 0 min and (b) 2 of the 9 subordinates responding to 2 μg LH-RH (▲;■) and 6 dominant (○- - - -○) and 4 subordinate (●- - - -●) females given saline (at 0 min). Values significantly different from those at 0 min: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. The sensitivity limit of the assay (20 ng/ml) is shown by the broken horizontal line.

*Oestradiol benzoate.* The LH responses of dominant and subordinate females to a single injection of oestradiol benzoate are shown in Text-fig. 2. Plasma LH concentrations increased abruptly in 8 of the 9 dominant females between 8 and 24 h after the oestrogen injection and, in all 8 females, the criterion set for positive feedback was reached at the 24 h sample. The remaining animal showed a positive feedback response between 24 and 36 h.

None of the 13 subordinate females showed any positive feedback response of LH to the oestrogen challenge: plasma LH levels remained below the sensitivity limit of the assay throughout the 60 h (Text-fig. 2c). These sustained low LH concentrations were not observed when subordinates were given the oil vehicle (range of individual values: <20–43 ng/ml). Some females in both dominant and subordinate categories also showed an inexplicable drop in plasma LH concentrations 24–36 h after oil administration.




**Text-fig. 2.** Plasma LH concentrations in (a) 5 dominant, (b) 4 dominant, and (c) 13 subordinate female marmosets after the s.c. administration of 35 μg oestradiol benzoate at 0 h. The shaded area represents the area covered by two standard deviations from mean control values of 6 dominant females in (a) and (b) and 4 subordinate females in (c) given oil. The sensitivity limit of the assay (20 ng/ml) is shown by the broken horizontal line.

*Prolactin*

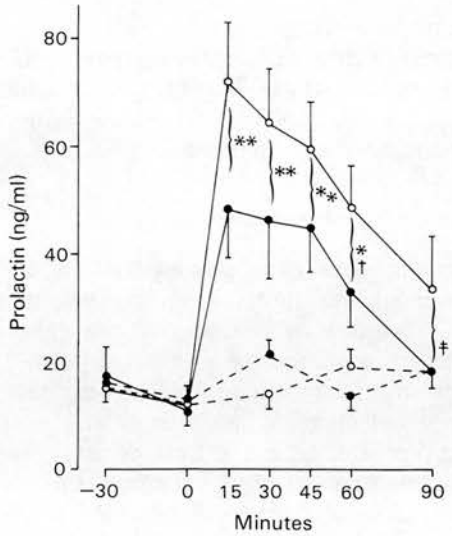
There were no differences in plasma prolactin levels between dominant and subordinate females in 5 out of the 10 groups (Table 3). In another 4 groups, a subordinate had circulating prolactin concentrations lower than those of the dominant animal. In the last group, the subordinate had higher prolactin levels than the dominant.

**Table 3.** Plasma prolactin concentrations (mean ± s.e.m. for no. of observations in parentheses) in female marmosets from 10 peer groups

| | Plasma prolactin (ng/ml) | | |
|---|---|---|---|
| Group | Female 1 | Female 2 | Female 3 |
| 1 | 19·6 ± 3·4 (10) | 20·8 ± 3·4 (10) | 8·3 ± 2·0** (9) |
| 2 | 17·0 ± 4·6 (15) | 7·8 ± 1·1** (13) | |
| 3 | 22·0 ± 7·5 (9) | 11·5 ± 1·1* (17) | |
| 4 | 17·2 ± 1·6 (26) | 7·5 ± 0·7** (25) | |
| 5 | 13·7 ± 1·2 (18) | 17·2 ± 2·1 (17) | 15·2 ± 1·8 (18) |
| 6 | 13·9 ± 1·4 (19) | 11·8 ± 1·7 (18) | 10·7 ± 2·0 (8) |
| 7 | 15·6 ± 2·6 (15) | 14·3 ± 0·6 (15) | |
| 8 | 13·4 ± 1·7 (15) | 11·5 ± 2·2 (8) | |
| 9 | 13·9 ± 1·2 (16) | 14·7 ± 1·1 (16) | |
| 10 | 11·1 ± 1·1 (26) | 15·5 ± 1·5* (28) | |

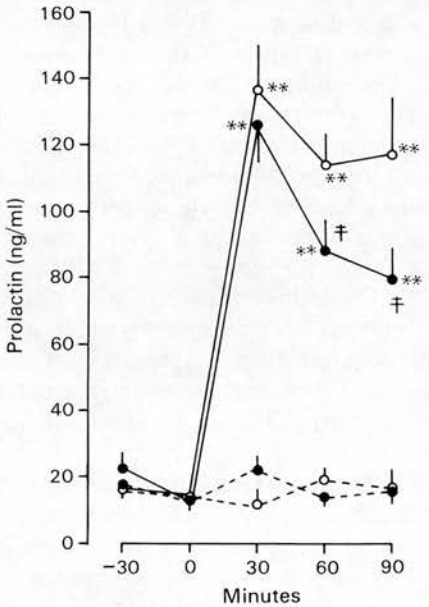
Values significantly different from those of Female 1, $*P < 0.05$, $**P < 0.01$ (Student's $t$ test).

*TRH.* Circulating concentrations of prolactin in dominant and subordinate females were similar before and after TRH treatment, and both types of female showed a significant prolactin response to TRH ($P < 0.001$; Text-fig. 3). Prolactin concentrations were elevated after 15 min, but had significantly declined by 60 min and returned to baseline by 90 min. While the dominant females consistently showed a greater response than did the subordinates, this was not statistically significant, perhaps because of the large variability in the prolactin response of both types of female.




**Text-fig. 3.** Plasma prolactin concentrations (mean ± s.e.m.) in 7 dominant (○——○) and 9 subordinate (●——●) females given 25 μg TRH i.v. at 0 min. As controls, 5 dominant (○- - - - -○) and 5 subordinate (●- - - - -●) females were given saline (at 0 min). Values significantly different from those at −30 or 0 min: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$. Values at 30–90 min significantly different from those of the peak response at 15 min: $\dagger P < 0{\cdot}05$, $\ddagger P < 0{\cdot}01$.

*Metoclopramide.* The pre-treatment prolactin levels of dominant and subordinate females were indistinguishable (−30 and 0 min; Text-fig. 4). Prolactin levels rose sharply in all animals between 0 and 30 min after administration of metoclopramide ($P < 0{\cdot}001$) and mean (±s.e.m.)




**Text-fig. 4.** Plasma prolactin concentrations (mean ± s.e.m.) in 7 dominant (○——○) and 9 subordinate (●——●) female marmosets given 2 mg metoclopramide i.v. at 0 min. As controls, 5 dominant (○- - - - -○) and 5 subordinate (●- - - - -●) females were given saline (at 0 min). Values significantly different from those at −30 and 0 min: $**P < 0{\cdot}01$. Values significantly different at 60–90 min from peak response at 30 min: $\ddagger P < 0{\cdot}01$.

peaks of 136·7 ± 13·1 ng/ml and 126·6 ± 11·1 ng/ml were reached in the dominant and subordinate females, respectively. However, by 60 and 90 min, prolactin levels had significantly declined only in the subordinate females (Text-fig. 4), producing a significant behaviour status-by-time sampled interaction ($P < 0{\cdot}05$). Nevertheless, prolactin levels in the subordinate females were still well elevated above the pre-treatment values ($P < 0{\cdot}001$).

## Discussion

This is the first report describing changes in plasma levels of LH, cortisol and prolactin in subordinate female marmoset monkeys. The results clearly show that subordinate females suffer from complete ovarian failure apparently due to gonadotrophin insufficiency, although the circulating concentrations of cortisol and prolactin are not elevated.

Inhibition of ovarian function in subordinate female marmosets was maintained during the complete 6-month sampling period (animals had been in their peer groups between 5 and 18 months; Table 1) and plasma progesterone concentrations never reached the luteal-phase values of the dominant females. The low progesterone values from subordinate females are consistent with previous findings of low total urinary oestrogen excretion in subordinate female marmosets. Urinary oestrogen concentrations were suppressed 10-fold in comparison with those of dominant females, and resembled the excretion pattern from bilaterally ovariectomized animals (Lunn, 1978). Furthermore, histological examination of the ovaries from 9 subordinate females revealed many primary and atretic follicles, but no preovulatory follicles, corpora lutea or corpora albicantia (D. H. Abbott, T. G. Baker & S. F. Lunn, unpublished). Failure to observe appropriate elevations in circulating LH concentrations (Text-figs 1b and 2c; D. H. Abbott, unpublished) in the face of this evidence of chronically low ovarian hormone secretion, suggests that a deficiency in hypothalamic LH-RH and/or pituitary LH had accompanied the behavioural subordination of female marmosets, and that the impairment of ovarian function was probably due to insufficient gonadotrophic stimulation.

When challenged with a standard dose of LH-RH (Hodges, 1979), 7 out of 9 subordinate females showed a negligible increase in plasma LH values, unlike the large LH response from dominant females (Text-fig. 1). The pituitary of subordinate female marmosets therefore appears to be less responsive to LH-RH. Nevertheless, the poor pituitary LH response of subordinate animals to exogenous LH-RH might just as well reflect insufficient endogenous LH-RH stimulation from the hypothalamus, leading to a reduced pituitary LH content and a reduced pituitary capacity to synthesize LH. The LH responses of subordinate females to different doses of LH-RH and to multiple injections of a given dose of LH-RH require evaluation before these possibilities can be resolved. However, as 2 of the 9 subordinate females showed a normal LH response to LH-RH administration, the lack of a pituitary response to LH-RH may only be present in some subordinates or may only occur intermittently in each individual. Unfortunately, there is no reliable information concerning basal plasma LH levels in dominant and subordinate animals, because LH values for intact marmosets frequently fall below the sensitivity limit of the available assay (Hodges & Hearn, 1977) and there is at present no suitable assay for marmoset FSH.

Each subordinate female marmoset was also insensitive to a standard oestrogen challenge, unlike the response found in dominant females, and hence the positive feedback surge of LH required for ovulation did not occur (Text-fig. 2). In fact, plasma LH levels in each of the 13 subordinates given oestrogen were suppressed below the sensitivity limit of the assay, and below the LH values of subordinates given oil, as if an intense negative feedback system was in operation. This would imply that if there was a preovulatory follicle present in the ovary of a subordinate female marmoset, the increased circulating oestrogen might fail to induce an ovulatory surge of LH from the pituitary by one of two ways. If the female marmoset is similar to the female rhesus monkey (Knobil, Plant, Wildt, Belchetz & Marshall, 1980) and the pituitary

can respond to the increased circulating oestrogen with an LH surge provided that a regular LH-RH release is maintained, then the lack of a pituitary LH response to oestrogen in subordinate females might arise from either a lack of normal hypothalamic LH-RH release or pituitary insensitivity to LH-RH (see above) which would produce the anovulatory condition. On the other hand, if the female marmoset is similar to many other mammalian species (see Fink, 1979, for references) and an increase in the release of hypothalamic LH-RH is apparently required to release an LH surge from the pituitary, then hypothalamic insensitivity to increased circulating oestrogens would lead to sterility, regardless of whether or not the pituitary was responsive to LH-RH. The evidence from this study favours the latter possibility because exogenous LH-RH elicited a normal LH response from two of the subordinate female marmosets although they were acyclic (Text-fig. 1b). However, an oestrogen challenge would have to be administered to LH-RH-treated subordinates before pituitary insensitivity could be ruled out.

It is possible that an insensitive positive feedback mechanism in subordinate female marmosets might reflect an extremely sensitive negative feedback response of the hypothalamus to the administered oestrogen. In other words, the negative feedback system has been 'set' at too sensitive a level in subordinates so that even very low circulating concentrations of oestrogen inhibit the secretion of LH-RH and hence LH. This could explain why the injected oestradiol benzoate completely suppressed plasma LH levels in subordinates below the sensitivity limit of the assay for 60 h (Text-fig. 2) while LH-RH administration elicited at least some plasma LH response (Text-fig. 1). Consequently, the pituitary of subordinate females would also be starved of hypothalamic LH-RH stimulation. In these respects, subordinate adult females may well resemble pre-pubertal female marmosets in which the LH response to LH-RH is similarly poor (J. K. Hodges & D. H. Abbott, unpublished). The subordinate female marmoset may be useful in examining the factors controlling ovarian inactivity, since 6 out of the 13 subordinates had had cycles before inclusion in a peer group (Abbott, 1979) and all the subordinates exhibited ovarian cyclicity 10–30 days after removal from the peer group (Abbott & Hearn, 1978).

The poor LH responses of subordinate female marmosets cannot be attributed to increased levels of cortisol (Table 2) or prolactin (Table 3). The adrenal glands from subordinates were the same size and weight as those from dominant females (S. F. Lunn & D. H. Abbott, unpublished). The stress of behavioural subordination in this female monkey therefore does not induce hyperprolactinaemia or elevated cortisol levels, a result in marked contrast to that reported by Bowman *et al.* (1978) and Keverne (1979), who found that behavioural subordination in female talapoin monkeys was associated with elevated prolactin and cortisol concentrations, suggesting that the suppression of oestrogen-induced LH surges in subordinate females was causally related to the elevated prolactin. The different prolactin and cortisol findings between talapoin and marmoset monkeys may partly arise because subordinate female talapoins continue to receive aggression long after captive groups are established (Bowman *et al.*, 1978; Keverne, 1979), whereas aggressive interactions virtually cease 2–3 days after captive marmoset groups are set up (Abbott & Hearn, 1978; Abbott, 1979), and this difference in aggression may, in turn, be related to the oestrogen treatment of the former. Nevertheless, since subordinate females in both species fail to show oestrogen-induced LH surges, a common mechanism(s) inhibiting positive feedback may be operating independently of circulating prolactin concentrations. In subordinate female marmosets, prolactin responses to TRH were consistently, but not significantly, lower than those of dominant females (Text-fig. 3). Metoclopramide, a pituitary cell-receptor antagonist of dopamine (a neurotransmitter which acts to inhibit prolactin release from the pituitary; Hökfelt & Fuxe, 1972; Olson, Fuxe & Hökfelt, 1972) produced a more striking difference (Text-fig. 4): at 30 min after injection of metoclopramide, prolactin levels of subordinates were similar to those of dominant females, but had fallen by 60 and 90 min. It is therefore possible that, because of increased dopamine release or turn-over, pituitary prolactin secretion is inhibited in subordinates, hence the reduced

response to pituitary stimulation. This explanation would be in accordance with the lower prolactin levels found in subordinates in 4 out of the 10 groups when compared with the dominant female (Table 3). Increased dopamine turn-over is also known to suppress LH secretion (LeBlanc, Lachelin, Abu-Fadil & Yen, 1976; Lachelin, LeBlanc & Yen, 1977; Fuxe *et al.*, 1976; Judd, Rakoff & Yen, 1978; McNeilly, 1980), presumably by reducing the hypothalamic output of LH-RH (de Cotte, De Menzes, Bennett & Edwardson, 1980). This information would fit with the finding of reduced LH secretion in subordinate female marmoset and talapoin monkeys. Changes in plasma prolactin levels under such conditions would therefore be only a secondary effect, as indeed acknowledged by Bowman *et al.* (1978). Since oestrogen blocks dopamine at its receptor (Labrie, Baulieu, Caron & Raymond, 1978), ovariectomy followed by oestrogen treatment of the subordinate talapoins and reduced oestrogen levels of the subordinate marmosets might contribute directly to the dissimilar prolactin findings in these two species of monkey.

The mechanisms by which the dominant female marmoset inhibits ovulation in her subordinates are unknown but they probably involve a combination of pheromones from her frequent scent marking (Epple, 1973; Epple, Golob & Smith, 1979) and physical and visual contact (Abbott, 1979). Such a system would help to enforce the marmoset's monogamous mating system and encourage movement of subordinate females away from a group to pair with a male or become dominant in another group where the suppression of fertility would be removed.

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# INHIBITION OF GONADOTROPHIN SECRETION BY INDUCED HYPERPROLACTINAEMIA IN THE MALE RAT

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SUMMARY

Pituitary glands were transplanted under the kidney capsule to induce hyperprolactinaemia in both immature (41 days old) and adult (150 days old) intact male rats. Blood samples were taken for hormone analysis at regular intervals after transplantation and the animals were killed 42–45 days after the operation.

Serum levels of prolactin rose in all groups of rats with transplants except in those animals bearing only one pituitary gland. Increased adrenal weight in all transplanted groups suggested that effective hyperprolactinaemia was always achieved. Serum levels of LH and FSH were significantly suppressed in all animals with transplants; the pituitary LH content was as reduced in all animals except those bearing one pituitary transplant but reductions in the pituitary FSH content were inconsistent. Although the amount of luteinizing hormone releasing hormone (LH-RH) in the hypothalamus did not differ significantly between groups, the effects of hyperprolactinaemia on serum and pituitary levels of LH and FSH suggested a functional lack of LH-RH (possibly due to an increase in the sensitivity of the hypothalamus to the negative feedback effects of testicular steroids).

Serum levels of testosterone remained normal in spite of reductions in the serum concentrations of LH and FSH and, in immature animals, a reduction in testicular binding of LH/human chorionic gonadotrophin.

These results are discussed in the light of previous conflicting reports on the effects of induced hyperprolactinaemia on the regulation of secretion of LH and FSH.

## INTRODUCTION

An increasing amount of evidence suggests that prolactin plays an important part in the functioning of the mammalian testis (Bartke & Lloyd, 1970; Hafiez, Lloyd & Bartke, 1972; Negro-Vilar, Krulich & McCann, 1973). Prolactin appears to act synergistically with luteinizing hormone (LH) to maintain testicular production of androgens in the mouse (Bartke, 1971), rat (Hafiez *et al.* 1972; Johnson, 1974), hamster (Bartke, Croft & Dalterio, 1975) and man (Rubin, Govin, Lubin, Poland & Pirke, 1975; Ambrosi, Travaglini, Beck-Peccoz, Bara, Elli, Paracchi & Faglia, 1976). High levels of prolactin are associated with hypogonadism in men (Boyar, Kapen, Finkelstein, Perlow, Sassin, Fukushima, Weitzman & Hellman, 1974; Thorner, McNeilly, Hagen & Besser, 1974). Although transplantation of a prolactin releasing tumour into adult male rats leads to testicular atrophy (Fang, Refetoff & Rosenfield, 1974), transplantation of normal pituitary glands, which also results in raised serum levels of prolactin, fails to induce any significant alteration in testicular activity (Bartke, Smith, Michael, Peron & Dalterio, 1977).

To resolve these differences, the changes in the concentrations of gonadotrophins in the pituitary gland and serum, testosterone in the serum and luteinizing hormone releasing

hormone (LH-RH) in the hypothalamus have been studied in groups of prepubertal and adult male rats bearing pituitary grafts under the kidney capsule. The testicular binding of gonadotrophins and prolactin was also studied to determine whether the observed reduction in gonadotrophin secretion had influenced gonadal function.

## MATERIALS AND METHODS

### *Animals*

Male rats of the inbred PVG strain were used throughout this study both as donors and recipients of the pituitary glands. After pituitary transplantation, rats were allocated randomly to cages, allowed food and water *ad libitum* and maintained on a lighting schedule of 14 h light : 10 h darkness.

### *Experimental procedures*

Two experiments were performed. In the first experiment two or four pituitary glands from adult male rats were transplanted under the kidney capsule of prepubertal (41 days old; body weights 118–146 g) male rats (six animals/group) under ether anaesthesia. Rats in the control group were sham-operated. In the second experiment, one, two or three pituitary glands were transplanted into adult (150 days old; body weights 220–267 g) male rats (five animals/group) as described for the immature animals; control rats were again sham-operated. Blood samples (1 ml) were withdrawn from a tail vein of immature rats under light ether anaesthesia 8, 21 and 35 days after the operation; samples (2 ml) from adult animals were collected similarly 4, 11, 18, 25 and 32 days after the operation.

Immature and adult rats were killed in random order by decapitation 45 and 42 days, respectively, after transplantation and trunk blood was collected into polystyrene tubes. After storage at 4 °C for 4 h all tubes were centrifuged at 600 *g* for 30 min at 4 °C and the resultant serum was stored at −20 °C until assayed for prolactin, LH, follicle-stimulating hormone (FSH) and testosterone. Immediately after decapitation the pituitary gland, hypothalamus and an area of cortex were dissected out, weighed, placed in tubes, snap-frozen in a bath containing methanol and solid $CO_2$ and stored at −20 °C. The testes, ventral prostate gland, seminal vesicles and adrenal glands were dissected out and weighed. One of each pair of testes was gently homogenized in 2 ml Krebs–Ringer bicarbonate solution (KRB) in a glass homogenizer. The homogenate was diluted wtih KRB to a concentration of 250 mg testis/ml and stored at −20 °C.

### *Testicular binding of hormones*

The binding of human chorionic gonadotrophin (HCG) to testicular LH/HCG receptors was assessed (Sharpe, 1976) with HCG CR119 (11 600 i.u./mg) labelled with $^{125}I$ by the lactoperoxidase method (Miyachi, Vaitukaitis, Nieschlag & Lipsett, 1972). The binding of FSH and prolactin was assessed with $^{125}I$-labelled bovine FSH (Cheng, 1976) and $^{125}I$-labelled ovine prolactin (Shiu, Kelly & Friesen, 1973), again using the lactoperoxidase method. To reduce procedural losses to a minimum and allow measurement of the binding of all three hormones, homogenates of whole testis were used. These were thawed, re-suspended and 0·2 ml suspension was added to 7 ml polystyrene tubes which were then incubated in duplicate with either $^{125}I$-labelled HCG or $^{125}I$-labelled bovine FSH (2 ng hormone; sp. act. 20–30 Ci/g HCG and 40–90 Ci/g FSH) in 0·1 ml KRB containing 0·2% bovine serum albumin (BSA) for 3 h at 37 °C with shaking. Non-specific binding of $^{125}I$-labelled HCG or FSH to each homogenate was assessed by the addition of either 100 i.u. HCG (Pregnyl, Organon) or 5 μg ovine FSH (ovine FSH-NIH-S10). Incubation was terminated by the addition of 5 ml cold 0·9% saline and the tubes were centrifuged at 1500 *g* for 30 min at 4 °C. The supernatant fluid was decanted and the precipitate counted in a well-type gamma counter (LKB Wallac, Sweden).

Testicular binding of $^{125}$I-labelled ovine prolactin was assessed in a similar manner except that 0·01 M-Tris-HCl buffer, pH 7·4, containing 0·1% BSA and 10 mM-$Mg^{2+}$ was used to dilute the tracer and to stop the reaction (Shiu *et al.* 1973). Incubation was carried out for 16 h at 4 °C. Non-specific binding was assessed after the addition of 2 μg ovine prolactin (NIH-P-S10, 25 i.u./mg) in Tris-HCl buffer.

The specific binding of LH, FSH and prolactin was taken as the total minus the non-specific binding and is given as pg hormone bound/50 mg homogenate.

### *Measurement of hypothalamic and pituitary hormone content*

Immediately after thawing, each hypothalamus or sample of cortex was extracted by homogenization in 1·0 ml 0·01 M-HCl. After neutralization with 1 M-NaOH, the homogenate was centrifuged for 30 min at 4 °C and 600 ***g*** and the supernatant fluid was then removed and assayed for LH-RH.

Individual pituitary glands were homogenized in 4 ml 0·9% NaCl solution containing 1% BSA. Each homogenate was then centrifuged for 5 min at 4 °C and 1500 ***g***. The supernatant fraction was removed and stored at −20 °C until assayed for LH, FSH and prolactin.

### *Radioimmunoassays*

Serum and pituitary levels of prolactin and LH were measured using reagents kindly provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), NIH, Bethesda, Maryland, U.S.A. The radioimmunoassay method of Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong (1975), as described by de Jong & Sharpe (1977), was used for the determination of FSH. Results are expressed in terms of the standard NIAMDD reference preparations (NIAMDD RP-1) for each hormone. Serum testosterone was measured by a specific radioimmunoassay (Corker & Davidson, 1978); the antiserum against testosterone was raised in a rabbit and was kindly provided by D. & S. Tillson, Alza Corporation, U.S.A.

The radioimmunoassay described by Mortimer, McNeilly, Rees, Lowry, Gilmore & Dobbie (1976) was used for the determination of LH-RH and used a rabbit antiserum raised against LH-RH conjugated to BSA by carbodiimide (Fraser, Gunn, Jeffcoate & Holland, 1974).

Statistical analysis was by analysis of variance or Student's *t*-test.

## RESULTS

### *Serum levels of LH, FSH, prolactin and testosterone*

Variations in the serum concentrations of LH, FSH, prolactin and testosterone are shown in Figs 1 and 2. In immature rats the serum level of prolactin was significantly ($P<0·001$) higher in rats bearing transplants than in control animals, except on day 35 in rats with two transplanted glands (Fig. 1*a*). Serum levels of LH were lower in transplanted than in control rats on days 21, 35 and 45 (Fig. 1*b*). Because of the large variations between concentrations within groups, the differences were statistically significant only on days 35 and 45 in the animals with two pituitary transplants. Serum levels of FSH were significantly lower than in the controls on days 35 and 45 in both groups of transplanted rats and also on day 21 in the group with two transplanted pituitary glands (Fig. 1*c*). Serum testosterone could only be assessed in the last sample and levels were not significantly different between control animals and those bearing two or four pituitary transplants (serum levels of testosterone $5·6 \pm 5·0$ (S.D.), $3·6 \pm 1·2$ and $5·0 \pm 2·0$ ng/ml respectively).

In adult rats a significant increase in the serum concentration of prolactin was seen consistently only in the group with three pituitary transplants (Fig. 2*a*). In animals with two pituitary transplants the level of prolactin was significantly raised only on days 11 and 25

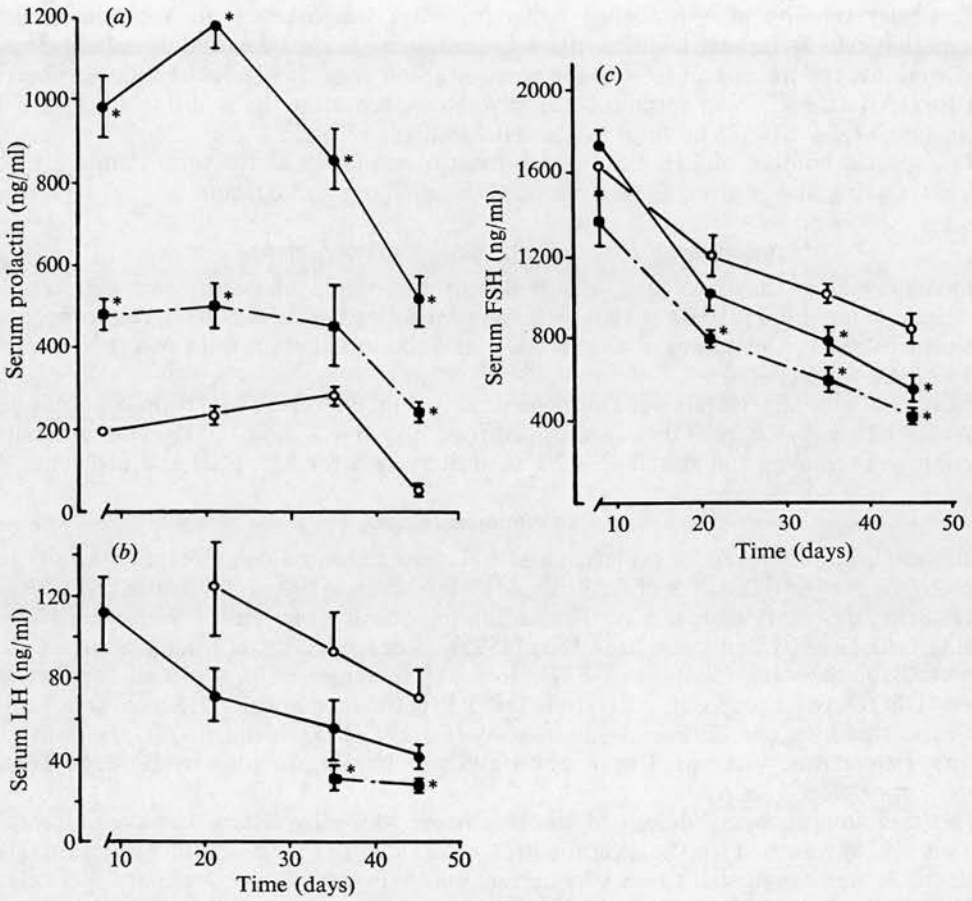



Fig. 1. Effect of hyperprolactinaemia induced by pituitary transplants on serum levels (means ± S.E.M., $n = 6$) of (*a*) prolactin, (*b*) LH and (*c*) FSH in immature male rats. Operations were performed on day 41. Rats were sham-operated (controls, ○) or implanted with two (■) or four (●) pituitary glands underneath the kidney capsule. * Indicates significantly different ($P < 0{\cdot}05$) from control values. Statistical analysis was by Student's *t*-test.

after the operation, whereas in rats bearing one pituitary transplant, levels of prolactin did not differ significantly from control values. In all groups serum concentrations of prolactin were lower in terminal samples. In spite of this failure to demonstrate consistently raised levels of prolactin, serum levels of LH were significantly suppressed in rats with two pituitary transplants 11 days after the operation and in all three groups on and after day 25 (Fig. 2*b*). Similarly, serum levels of FSH were significantly lower in all groups 25 days after transplantation (Fig. 2*c*). Serum concentrations of testosterone were not significantly different (Fig. 2*d*) and were similar to those in the immature group.

### *Pituitary and hypothalamic hormone content*

A reduction in the pituitary content of prolactin was observed in immature rats with two or four transplanted pituitary glands, but the reduction was significant ($P < 0{\cdot}05$) only in the animals with two transplants (Table 1). Transplantation had no effect on the amount of prolactin in the pituitary gland of adult rats.

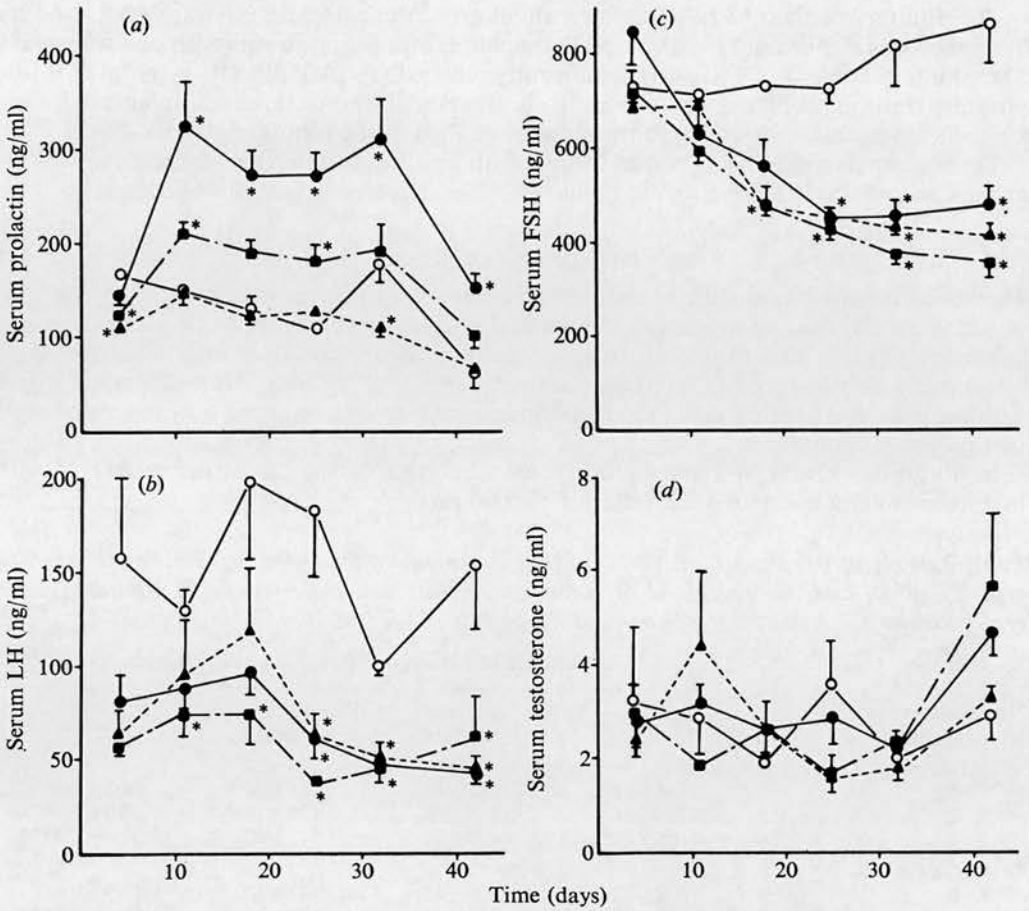



Fig. 2. Effect of hyperprolactinaemia induced by pituitary transplants on serum levels (means ± S.E.M., $n = 5$) of (*a*) prolactin, (*b*) LH, (*c*) FSH and (*d*) testosterone in adult male rats. Operations were performed on day 150. Rats were sham-operated (controls, ○) or implanted with one (▲) two (●) or three (●) pituitary glands under the kidney capsule. * Indicates significantly different ($P < 0.05$) from control values. Statistical analysis was by Student's *t*-test.

Table 1. *Pituitary content of LH, FSH and prolactin, and hypothalamic content of luteinizing hormone releasing hormone (LH-RH) (means ± 1 S.D.) in control male rats and male rats with pituitary glands grafted under the kidney capsule on days 41 or 150 of life*

| No. of implanted pituitary glands | *n* | Pituitary wt (mg) | Pituitary content (μg/mg) | | | Hypothalamic LH-RH content (ng) |
|---|---|---|---|---|---|---|
| | | | Prolactin | LH | FSH | |
| 41 days | | | | | | |
| 0 | 6 | 5·6 ± 0·6 | 1·8 ± 0·5 | 90·6 ± 18·4 | 38·2 ± 6·6 | 8·0 ± 1·8 |
| 2 | 5 | 4·8 ± 0·2 | 0·9 ± 0·3* | 51·0 ± 8·0** | 30·7 ± 2·6* | 8·6 ± 1·9 |
| 4 | 6 | 5·3 ± 0·6 | 1·3 ± 0·7 | 63·1 ± 11·6* | 30·1 ± 1·6* | 6·8 ± 2·3 |
| 150 days | | | | | | |
| 0 | 5 | 5·2 ± 0·2 | 1·0 ± 0·6 | 26·1 ± 10·7 | 88·5 ± 34·0 | 5·4 ± 1·3 |
| 1 | 4 | 5·2 ± 0·2 | 0·9 ± 0·3 | 17·6 ± 6·6 | 48·3 ± 13·8 | 5·1 ± 1·9 |
| 2 | 4 | 5·1 ± 0·1 | 1·1 ± 0·3 | 14·8 ± 2·6* | 44·2 ± 11·6* | 6·4 ± 1·4 |
| 3 | 3 | 5·2 ± 0·2 | 1·0 ± 0·4 | 16·2 ± 5·7* | 42·6 ± 13·9* | 4·6 ± 0·4 |

* $P < 0.05$; ** $P < 0.01$: versus control (by Student's *t*-test).

The pituitary content of LH was lower in all groups of rats with pituitary grafts and the difference was significant ($P<0.05$) in all animals except for adult rats with one transplant. The pituitary content of FSH was significantly reduced ($P<0.05$) in rats given two or four pituitary transplants on day 41 whereas in adult rats with two or three transplants there was a significant decrease ($P<0.05$) in the amount of FSH in the pituitary gland.

The hypothalamic LH-RH content showed no significant differences between any of the groups and no LH-RH (< 5 pg/mg tissue) could be detected in samples of cortex.

### *Body and organ weights*

There was no significant difference between control and pituitary-grafted groups in body weight or in the relative weights of the seminal vesicles or ventral prostate glands in either immature or adult rats (Table 2). In rats transplanted on day 41 of life, testicular weight was significantly reduced ($P<0.05$) in animals with four pituitary transplants compared with controls, but in adult rats a reduction in testicular weight occurred only in animals with two pituitary transplants.

In all groups bearing pituitary grafts there was a highly significant increase ($P<0.001$) in adrenal weight compared with that of control rats.

Table 2. *Weights (means ± 1 S.D.) relative to body weight of the testes, seminal vesicles, ventral prostate gland and adrenal gland of control male rats and male rats with pituitary glands grafted under the kidney capsule on days 41 or 150 of life*

| No. of implanted pituitary glands | *n* | Relative organ weights (mg/100 g body weight) | | | |
|---|---|---|---|---|---|
| | | Testes | Seminal vesicles | Ventral prostate gland | Adrenal gland |
| 41 days | | | | | |
| 0 | 6 | 1254 ± 70 | 434 ± 85 | 133 ± 20 | 16·3 ± 0·6 |
| 2 | 5 | 1206 ± 84 | 438 ± 56 | 125 ± 19 | 28·0 ± 3·0*** |
| 4 | 6 | 1165 ± 60* | 473 ± 46 | 143 ± 12 | 28·0 ± 0·8*** |
| 150 days | | | | | |
| 0 | 5 | 1078 ± 37 | 509 ± 67 | 147 ± 14 | 12·9 ± 0·6 |
| 1 | 4 | 1059 ± 107 | 431 ± 44 | 140 ± 13 | 18·2 ± 1·0*** |
| 2 | 4 | 992 ± 35** | 507 ± 59 | 146 ± 15 | 17·6 ± 1·5*** |
| 3 | 3 | 1058 ± 79 | 585 ± 121 | 144 ± 31 | 19·2 ± 1·1*** |

* $P<0.05$; ** $P<0.01$; *** $P<0.001$: versus control (by Student's *t*-test).

Table 3. *Testicular binding of LH/HCG and bovine FSH to testicular homogenates of control male rats and male rats implanted with pituitary glands under the kidney capsule on days 41 or 150 of life (means ± 1 S.D.)*

| No. of implanted pituitary glands | *n* | Testicular binding (pg/50 mg homogenate) | |
|---|---|---|---|
| | | LH/HCG | FSH |
| 41 days | | | |
| 0 | 6 | 300 ± 20 | 26 ± 8 |
| 2 | 6 | 244 ± 46* | 34 ± 4 |
| 4 | 6 | 228 ± 32* | 36 ± 9 |
| 150 days | | | |
| 0 | 5 | 244 ± 10 | 55 ± 8 |
| 1 | 5 | 254 ± 26 | 57 ± 13 |
| 2 | 5 | 250 ± 18 | 58 ± 11 |
| 3 | 5 | 222 ± 28 | 50 ± 13 |

* $P<0.05$: versus control (by Student's *t*-test).

*Testicular binding of LH/HCG, FSH and prolactin*

As shown in Table 3, although a small but significant ($P < 0{\cdot}05$) reduction in the binding of LH/HCG to the testes was observed in the immature rats bearing pituitary grafts, there was no significant change in this parameter in rats transplanted on day 150 (Table 3). No significant change was seen in the binding of FSH. Prolactin was bound by the testes of both immature (range, 3·1–10·2 pg/50 mg tissue) and adult (range 2·4–20·0 pg/50 mg tissue) control rats but binding was not seen in any rat transplanted on day 41 and only in six out of the 15 animals with transplants on day 150 (range 1–17 pg/50 mg tissue).

## DISCUSSION

In the present study an increase in the concentration of prolactin in the circulation of immature or adult rats, induced by pituitary gland transplants under the kidney capsule, was associated with decreases in the serum levels of LH and FSH. In spite of this, no change in the serum levels of testosterone was observed, which is similar to the findings of Bartke *et al.* (1977). This suppression of serum concentrations of LH and FSH occurred even in rats given one pituitary transplant on day 150, which seemingly failed to increase the serum level of prolactin above the control value. This may be a false assumption since all blood samples, except the final one, were collected under light ether anaesthesia, a method known to induce hypersecretion of prolactin (Neill, 1972). The increased adrenal weight in all groups with pituitary transplants which may be attributed to prolactin (Schlein, Zarrow & Denenberg, 1974), suggests that basal levels of prolactin were raised in all animals with transplants.

Long-term maintenance of reduced serum levels of LH and FSH in the present study contrasts with results in ovariectomized rats bearing pituitary transplants (Beck & Wüttke, 1977). These authors suggested that the initial decline in the concentration of LH seen in their ovariectomized hyperprolactinaemic rats was the result of an increase in the hypothalamic turnover of dopamine (Olson, Fuxe & Hökfelt, 1972; Fuxe, Hökfelt, Agnati, Löfström, Everitt, Johansson, Wüttke & Goldstein, 1976), resulting in a suppression of the serum level of LH and abolition of the pulsatile release of LH, presumably by reducing the hypothalamic output of LH-RH. After 5–9 days, the hypothalamus compensated for this increase in dopamine turnover,and secretion of LH and therefore presumably also of LH-RH returned to normal (Beck, Engelbart, Gelato & Wüttke, 1977; Beck & Wüttke, 1977). In the present study, however, the gonads were present and the concentration of testosterone in the blood remained unaltered. If the initial effect of the rising serum levels of prolactin was to increase the hypothalamic dopamine turnover, with a consequent reduction in hypothalamic secretion of LH-RH and LH, then perhaps normal levels of testosterone induce a reduction in the level of output of LH-RH and a somewhat lower rate of synthesis and secretion of both LH and FSH by a change in sensitivity to negative feedback.

In the present study, serum levels of LH were reduced even 4 days after transplantation of pituitary tissue (Figs 1 and 2), a finding similar to that of Beck & Wüttke (1977). On the other hand, FSH levels did not decrease significantly until day 17. This pattern of change in the concentrations of LH and FSH is similar to that observed after immunization against LH-RH (Fraser & Sandow, 1977), when a gradual reduction in the amount of LH-RH reaching the pituitary gland was observed to affect the synthesis and secretion of LH more than that of FSH. Small amounts of LH-RH also maintain normal serum levels of FSH in hypophysectomized rats bearing a pituitary transplant under the kidney capsule whereas secretion of LH is negligible (Arimura, Debeljuk, Shiino, Rennels & Schally, 1973).

The data on pituitary and serum levels of LH and FSH and the pattern of their consequent reduction in the presence of raised concentrations of prolactin are consistent with a reduction in the hypothalamic output of LH-RH. Our failure to demonstrate a significant reduction in the hypothalamic LH-RH content in rats with a raised serum level of prolactin

may not be surprising as the amount of LH-RH in the whole hypothalamus was measured and only a small proportion of this pool is secreted into the hypophysial portal vessels.

In spite of the maintenance of reduced concentrations of LH and FSH in the circulation, no significant change was seen in the serum level of testosterone or the weights of the accessory sex organs. This is at variance with previous results (Bartke *et al.* 1977; Negro-Vilar, Saad & McCann, 1977) which suggested that prolactin stimulated the growth of the male accessory organs in the rat. This difference may be related to age (Negro-Vilar *et al.* 1977, used 30-day-old animals) or to the length of time the rats were exposed to a high concentration of prolactin (approximately 6 weeks in the present study compared with 24–48 weeks in the study reported by Bartke *et al.* 1977).

Testicular weight was significantly reduced only in rats given four pituitary transplants on day 41 or two pituitary transplants on day 150; it may be significant that this reduction occurred in the rats with the greatest reduction in the serum levels of FSH. Bartke *et al.* (1977) also failed to observe any reduction in testicular weight even after exposure for 42 weeks to high concentrations of prolactin secreted by pituitary grafts. These findings are at variance with the results of Fang *et al.* (1974), who demonstrated complete testicular atrophy in male rats bearing a pituitary tumour secreting prolactin and growth hormone which affected gonadotrophin secretion.

The binding of FSH to the testis was not significantly affected by the presence of pituitary grafts. While no change in the binding of LH/HCG to the testis was seen in adult rats with pituitary transplants, a significant reduction in the binding was seen in immature transplanted rats with raised serum levels of prolactin when they were mature at the end of the experiment (Table 3). Recent evidence has strongly suggested that FSH may be responsible for the maintenance of testicular LH receptors in the prepubertal rat (Chen, Payne & Kelch, 1976), and the reduction in the number of LH/HCG receptors seen in this study may reflect the chronic reduction in the concentration of FSH in the circulation. Despite the reduction in the binding of LH/HCG, serum levels of testosterone did not differ in pituitary transplanted and control rats. Although only a small percentage of LH/HCG receptors need to be occupied to elicit maximum steroidogenesis (Catt & Dufau, 1973; Mendelson, Dufau & Catt, 1975), it is perhaps surprising that the reduction in the number of LH/HCG receptors, together with the lower concentrations of LH in the circulation of implanted rats, is not reflected in lower serum levels of testosterone. It may be that the associated hyperprolactinaemia is able to compensate for these changes by sensitizing the testis to stimulation by LH (Bartke *et al.* 1977).

Specific binding of prolactin was demonstrable in the testes of control rats but was not detectable in the testes of animals implanted with pituitary glands at 41 days of age and was only detectable in 40% of the pituitary-implanted adult animals. This may represent occupancy of the receptors or a loss of receptors related to maintenance of raised endogenous levels of hormone (Raff, 1976).

A significant increase in adrenal weight was seen in all groups of pituitary-transplanted rats which is in agreement with previous reports (Witorsch & Kitay, 1972; Schlein *et al.* 1974; Winters, Colson, MacDonald & Porter, 1975; Bartke *et al.* 1977; Negro-Vilar *et al.* 1977). This increase in weight is related to an absolute increase in the weight of the adrenal cortex with concomitant increases in cytochrome P450 and protein content and the apparent output of corticosterone (A. S. McNeilly, I. Mason, J. Dobbie, E. A. Cowden & R. M. Sharpe, unpublished observations). It is possible that the increase in adrenal weight is also related to a direct action of prolactin via its adrenal receptors (Posner, Kelly, Shiu & Friesen, 1974). Whether an alteration in adrenal steroid output is related to the reduction in gonadotrophin secretion is unknown. An increase in the concentration of dehydroepiandrosterone and dehydroepiandrosterone sulphate of adrenal origin has been reported in hyperprolactinaemic men and women (Carter, Tyson, Warne, McNeilly, Faiman & Friesen, 1977; Giusti, Bassi, Borsi, Cattanco, Giannotti, Lanza, Pazzagli, Vigiani & Serio,

1977). The role of the adrenal gland in the response of the hypothalamic-pituitary-gonadal system to hyperprolactinaemia requires further investigation.

The present study demonstrates that, in the presence of the testes, hyperprolactinaemia induced by pituitary transplants results in a long-term decrease in serum concentrations of both LH and FSH although the decrease in LH in 41-day-old rats was inconsistent. This decrease probably results from a suppression of hypothalamic secretion and release of LH-RH, although the total hypothalamic content of LH-RH is not reduced. It is of considerable interest that despite low levels of both LH and FSH in the circulation and, in immature rats, reduced testicular binding of LH/HCG, serum levels of testosterone remained normal. These results suggest that prolactin may sensitize the hypothalamus and/or pituitary gland to the negative feedback of gonadal steroids. The increase in adrenal weight suggests a possible role of adrenal steroids in modulating the secretion of LH and FSH and requires further investigation.

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# EFFECT OF ADRENALECTOMY OR CASTRATION ON THE INHIBITION OF GONADOTROPHIN SECRETION INDUCED BY HYPERPROLACTINAEMIA IN THE ADULT MALE RAT

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SUMMARY

To investigate the role of adrenal and gonadal steroids in the long-term suppression of gonadotrophin secretion induced by prolactin the effects of adrenalectomy or castration on the serum and pituitary levels of LH, FSH and prolactin and the hypothalamic content of LH releasing hormone (LH-RH) have been studied in adult male rats with hyperprolactinaemia produced by the transplantation of pituitary glands under the kidney capsule.

Levels of LH and FSH in serum were significantly suppressed in all intact pituitary-grafted rats. Adrenalectomy on the day of pituitary implantation or 20 days later did not affect this suppression. However, castration on days 0, 28 or 49 after pituitary grafting resulted in a rise in levels of FSH in serum indistinguishable from that in control rats. While the rise in levels of LH after castration on day 0 was the same as the controls, this increase was significantly reduced 2 days after castration on days 28 and 49 after pituitary grafting.

Castration resulted in an increase in the pituitary content of LH and a reduction in the hypothalamic content of LH-RH but no change in the pituitary content of FSH. Hyperprolactinaemia did not appear to affect these responses.

The present results showed clearly that the gonad but not the adrenal must be present for prolactin to exert an inhibitory effect on gonadotrophin secretion.

## INTRODUCTION

Previous studies have shown that hyperprolactinaemia produced by pituitary grafts in adult male rats results in a reduction in the serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Bartke, Smith, Michael, Peron & Daltero, 1977; McNeilly, Sharpe, Davidson & Fraser, 1978).

Associated with hyperprolactinaemia is a significant increase in adrenal weight (Schlein, Zarrow & Denenberg, 1974; McNeilly *et al.* 1978; Vasquez & Kitay, 1978) and an increase in secretion of adrenal progesterone (Piva, Gagliano, Motta & Martini, 1973). Greeley & Kizer (1979) have suggested that the adrenal modulates some of the inhibitory actions of prolactin on the secretion of LH in the adult male rat although these experiments were only short term. On the other hand, prolactin, either injected or endogenously secreted from pituitary grafts, can delay but not prevent the increase in levels of LH in serum after castration in adult male rats (Grandison, Hodson, Chen, Advis, Simpkins & Meites, 1977; Winters & Lóriaux, 1978). The present study was undertaken to determine the role of adrenal and gonadal steroids in the long-term inhibition of gonadotrophin secretion in hyperprolactinaemia.

## MATERIALS AND METHODS

### *Animals*

Adult male rats (130–150 days old; 240–280 g body wt) of the inbred PVG strain were used both as donors and recipients of anterior pituitary glands. Rats were allocated randomly to the treatment groups, allowed access to food and water *ad libitum* and maintained on a lighting schedule of 14 h light : 10 h darkness. Adrenalectomized rats were given 1% NaCl (w/v) instead of water.

### *Experimental procedures*

*Experiment 1*

On day 0 of the experiment two anterior pituitary glands were transplanted under the kidney capsule of 14 adult male rats under ether anaesthesia. Four of these rats were bilaterally adrenalectomized at the same time (day 0), and five 20 days later (day 20). Five rats with a pituitary transplant remained intact. Five adult male rats without a pituitary transplant were bilaterally adrenalectomized on day 0; five remained intact as untreated controls.

*Experiment 2*

Two pituitary glands were grafted under the kidney capsule under light ether anaesthesia in 15 rats on day 0. Four of these rats were castrated at the time of pituitary transplantation on day 0, five were castrated on day 28 and six on day 49 after the pituitary transplant. Four rats were sham operated and acted as a control group and a further four were castrated on day 0 to act as controls for the effects of castration.

### *Blood sampling and collection of tissues*

Blood samples (2 ml) were withdrawn from a tail vein under light ether anaesthesia before the operation and then at intervals of 3–7 days after the operation until the end of the experiment (experiment 1, day 43; experiment 2, day 70). In experiment 2 additional blood samples were taken on days 1, 2, 4, 5 and 6 after castration. Serum was separated by centrifugation at 600 ***g*** for 30 min at 4 °C and the samples were stored at −20 °C until assayed for LH, FSH, prolactin and testosterone.

At the end of each experiment, animals were killed in random order by cervical dislocation, decapitated and trunk blood was collected. Immediately after decapitation the pituitary gland was dissected out, weighed, placed in a tube and snap frozen in a bath containing methanol and solid $CO_2$, and stored at −20 °C until assayed for LH, FSH and prolactin. The hypothalamus was dissected out and immediately extracted as described by Sharpe, Fraser & Sandow (1979), and the extract stored at −20 °C until assayed for LH releasing hormone (LH-RH).

The ventral prostate gland, seminal vesicles, adrenal glands and testes were dissected out and weighed. In experiment 1, adrenalectomized animals were examined macroscopically to confirm that no adrenal tissue was present. In experiment 2 testes were weighed at the time of castration.

### *Radioimmunoassays*

Before assay, each pituitary gland was thawed and the hormones were extracted as described previously (McNeilly *et al.* 1978). The levels of prolactin and LH in serum and pituitary glands were assayed using reagents provided by the NIAMDD, NIH, Bethesda, Maryland, U.S.A. as described previously (McNeilly *et al.* 1978; McNeilly, de Kretser & Sharpe, 1979). Serum and pituitary levels of FSH were measured by the method of Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong (1975) as described by de Jong & Sharpe (1977). All results are expressed in terms of the appropriate NIAMDD reference preparations (NIAMDD RP-1). Testosterone was measured by a specific radioimmunoassay (Corker & Davidson, 1978).

The hypothalamic content of LH-RH was measured by the method of Nett, Akbar, Niswender, Hedlund & White (1973) using $^{125}$I-labelled LH-RH prepared as described by Mortimer, McNeilly, Rees, Lowry, Gilmore & Dobbie (1976).

Statistical analysis was by analysis of variance and Student's *t*-test.

## RESULTS

### *Organ weights*

In both experiments the weight of the adrenal gland was significantly ($P<0{\cdot}001$) increased in rats with a pituitary transplant compared to control rats (expt 1: $16{\cdot}9\pm0{\cdot}4$ (S.E.M, $n=5$) *v.* $13{\cdot}5\pm0{\cdot}4$ ($n=5$); expt 2: $16{\cdot}9\pm0{\cdot}5$ ($n=15$) *v.* $13{\cdot}7\pm0{\cdot}3$ ($n=8$) mg/100 g body wt). Castration did not affect adrenal weight.

In experiment 1, there was no significant difference between any groups in body weight or in the weights relative to body weight of the testes, seminal vesicles or ventral prostate glands.

In experiment 2, testes were weighed at the time of castration on days 28 and 49 in pituitary-grafted rats and compared with the weight of the testes in intact control rats at the end of the experiment. The presence of pituitary transplants for either 28 or 49 days did not affect testis weight (range: 810–860 mg/100 g body wt). Seminal vesicles were weighed at the end of the experiment and castration resulted in the expected highly significant decrease ($P<0{\cdot}001$) in their weight in all groups compared with intact control rats ($77\pm4$ ($n=19$) *v.* $419\pm81$ ($n=4$) mg/100 g body wt respectively). In rats with a pituitary transplant a significant progressive decline in the weight of the seminal vesicles occurred with increasing time after castration [21 days after ($103\pm4$; $n=6$)>($P<0{\cdot}001$) 42 days after ($70\pm3$, $n=5$)>($P<0{\cdot}05$) 70 days after ($59\pm3$; $n=4$)]. The weight of seminal vesicles in rats with pituitary transplants were not significantly different after 70 or 42 days of castration from those of control rats 70 days after castration ($59\pm3$ and $70\pm3$ *v.* $66\pm2$ mg/100 g body wt respectively).

### *Levels of prolactin, LH, FSH and testosterone in serum*

#### *Prolactin*

The levels of prolactin in serum showed wide fluctuations within and between groups during the course of both experiments (range 18–218 ng/ml) presumably because all samples were taken under ether anaesthesia. Levels in blood collected after the animals had been killed by

Table 1. *Effect of adrenalectomy (ADX) on the levels (mean* ± S.E.M.) *of prolactin, LH, FSH and testosterone in the serum of terminal samples from adult male rats with or without two pituitary glands implanted under the kidney capsule 43 days previously. Adrenalectomy was performed at the same time as pituitary implantation (ADX day 0) or 20 days later (ADX day 20)*

| | | Level in serum (ng/ml) | | | |
|---|---|---|---|---|---|
| Treatment | *n* | Prolactin | LH | FSH | Testosterone |
| No transplantation | | | | | |
| Intact | 5 | $70\pm8$ | $44\pm11$ | $647\pm87$ | $2{\cdot}35\pm0{\cdot}46$ |
| ADX day 0 | 5 | $38\pm4$** | $36\pm6$ | $511\pm67$ | $2{\cdot}79\pm1{\cdot}89$ |
| Pituitary transplantation | | | | | |
| Intact | 5 | $127\pm13$** | $5\pm1$* | $287\pm30$** | $1{\cdot}95\pm0{\cdot}45$ |
| ADX day 0 | 4 | $96\pm11$ | $8\pm2$* | $193\pm45$** | $3{\cdot}89\pm0{\cdot}87$ |
| ADX day 20 | 5 | $101\pm12$ | $6\pm1$* | $204\pm46$** | $3{\cdot}14\pm0{\cdot}79$ |

*$P<0{\cdot}05$; **$P<0{\cdot}01$: *v.* intact rats without a pituitary transplant (Student's *t*-test).

Table 2. *Levels (mean ± S.E.M.) of LH, FSH and prolactin in serum samples taken at the time of death (day 70) from control or castrated adult rats with or without two pituitary glands transplanted under the kidney capsule on day 0*

| Treatment | *n* | Level in serum (ng/ml) Prolactin | LH | FSH |
|---|---|---|---|---|
| No transplantation | | | | |
| Intact | 4 | 34 ± 8 | 32 ± 3 | 577 ± 34 |
| Castrated on day 0 | 4 | 34 ± 4 | 375 ± 47** | 1541 ± 237** |
| Pituitary transplantation | | | | |
| Castrated on day 0 | 4 | 77 ± 10* | 481 ± 71** | 1278 ± 94*** |
| Castrated on day 28 | 5 | 59 ± 7* | 311 ± 46** | 1328 ± 73*** |
| Castrated on day 49 | 6 | 58 ± 6* | 283 ± 59** | 1063 ± 61*** |

*$P<0.05$; **$P<0.01$; ***$P<0.001$: *v.* intact rats without a pituitary transplant (Student's *t*-test).

cervical dislocation showed less intragroup variation. Compared with control animals all rats with a pituitary transplant had significantly raised serum levels of prolactin at the end of each experiment ($P<0.05$; Tables 1 and 2). Adrenalectomy in rats without a pituitary transplant resulted in a significant decrease ($P<0.01$) in levels of prolactin but was without effect in rats with a pituitary transplant. Castration did not affect prolactin levels in serum in either control rats or those implanted with pituitary glands.

*Luteinizing hormone*

In experiment 1 (adrenalectomy) levels of LH in serum were significantly suppressed ($P<0.05$) for the duration of the experiment (43 days) in rats with, compared to rats without, a pituitary transplant, a significant decrease ($P<0.001$) occurring within 2 days of implantation of the pituitary glands (Fig. 1, Table 1). Adrenalectomy did not affect levels of LH in rats either with or without a pituitary transplant.

In experiment 2, the presence of a pituitary transplant significantly suppressed levels of LH ($P<0.05$–$P<0.01$) from day 6 until the last day before castration on days 28 or 49 (Fig. 2). Castration in the control group and in all groups with pituitary transplants resulted in a

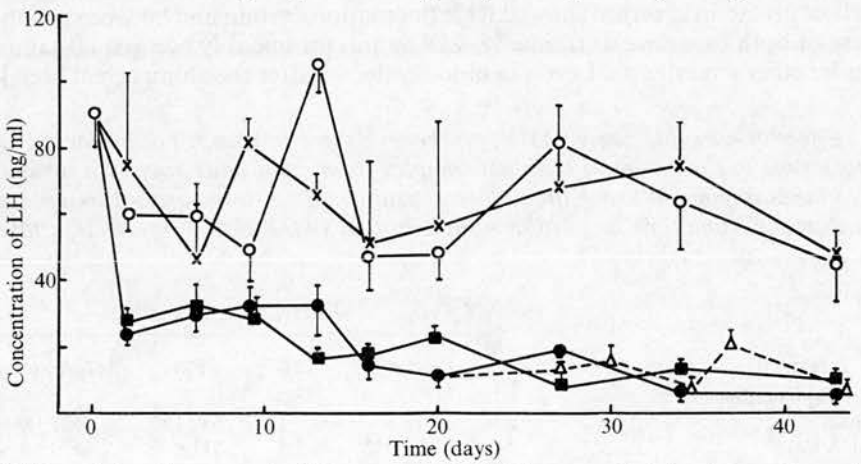



Fig. 1. Effect of adrenalectomy on levels (mean ± S.E.M.) of LH in serum of adult male rats. Rats acting as controls were either sham-operated (○, $n = 5$) or adrenalectomized on day 0 (×, $n = 4$). Fourteen rats implanted with a pituitary gland under the kidney capsule on day 0 were adrenalectomized on day 0 (■, $n = 4$) or day 20 (△, $n = 5$) after the implantation of the pituitary glands. Until day 20 all intact rats with pituitary transplants were treated as a single group (●, $n = 10$ up to day 20, $n = 5$ up to day 43).

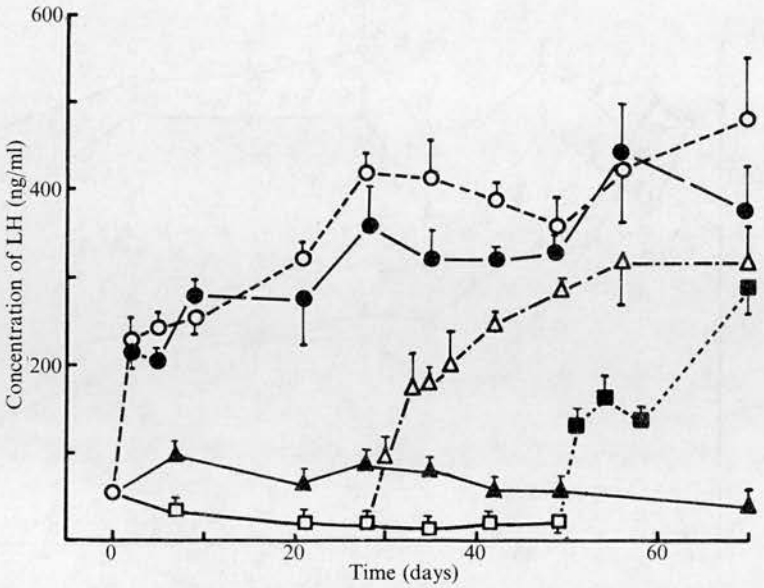



Fig. 2. Effect of castration on levels (mean ±S.E.M.) of LH in serum of adult male rats. Rats acting as controls were either sham operated (▲, $n = 4$) or castrated on day 0 (●, $n = 4$). Fifteen rats implanted with two pituitary glands under the kidney capsule on day 0 were castrated on day 0 (○, $n = 4$), on day 28 (△, $n = 5$) or on day 49 (■, $n = 6$) after the transplantation of the pituitary glands. Until day 49 all intact rats with pituitary transplants were treated as a single group (□).

significant increase in levels of LH in serum within 48 h. The increase in the levels of LH in serum after castration of day 0 in both control rats and rats with a pituitary transplant was not significantly different and levels of LH continued to increase throughout the course of the experiment (Fig. 2). When rats with a pituitary transplant were castrated on either day 28 or day 49 the increase in levels of LH on day 2 after castration was significantly less ($P < 0{\cdot}01$) than in control rats. However, by 20 days after castration serum levels of LH in all groups had increased to the same level and were not significantly different at this time or at the end of the experiment (Table 2).

*Follicle-stimulating hormone*

In experiment 1 the presence of a pituitary transplant in intact (non-adrenalectomized) rats caused a decrease in levels of FSH in serum by day 6 after pituitary implantation, this decrease becoming and remaining significant ($P < 0{\cdot}05$ to $P < 0{\cdot}001$) from day 9 until the end of the experiment on day 43 (Fig. 3). Adrenalectomy did not affect the levels of FSH in rats without a pituitary transplant (Fig. 3). However, in rats with a pituitary transplant, adrenalectomy on day 0 resulted in a larger decrease in levels of FSH on days 9, 13 and 16 ($P < 0{\cdot}05$, $< 0{\cdot}01$ and $< 0{\cdot}05$ respectively) compared with non-adrenalectomized pituitary-transplanted rats. Thereafter no significant difference in levels of FSH between intact and adrenalectomized rats with pituitary transplants were observed, even after adrenalectomy on day 20 (Fig. 3; Table 1). In experiment 2, the presence of a pituitary transplant significantly suppressed levels of FSH ($P < 0{\cdot}05$ to $P < 0{\cdot}001$) (Fig. 4). However, the increase in levels of FSH in serum during the first 9 days after castration was the same in all groups, and levels were not significantly different in the terminal samples between all the groups of castrated rats (Table 2).

*Testosterone*

In experiment 1 levels of testosterone in serum (Table 1) were unaffected by adrenalectomy in rats without a pituitary transplant and were similar to levels in adrenalectomized rats with a

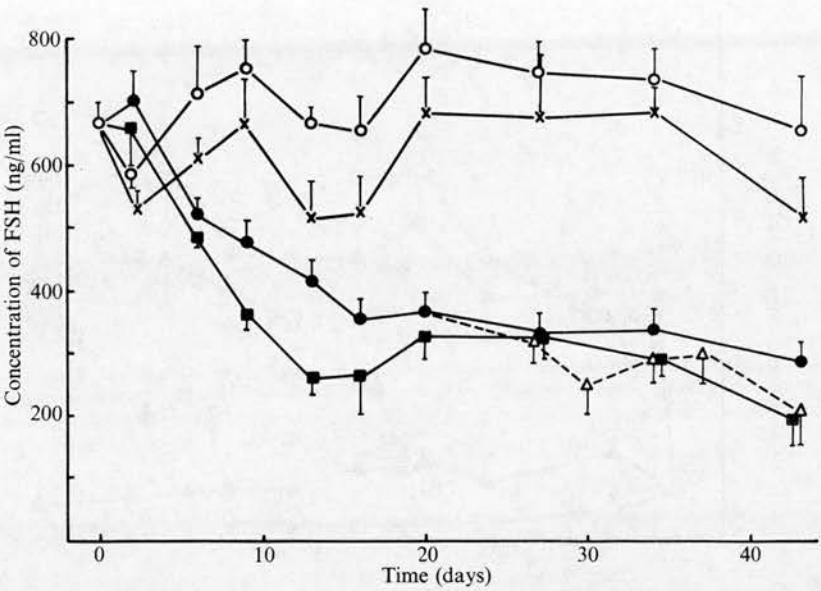



Fig. 3. Effect of adrenalectomy on levels (mean ± S.E.M.) of FSH in serum of adult male rats. Rats acting as controls were either sham-operated (○, $n = 5$) or adrenalectomized on day 0 (×, $n = 4$). Fourteen rats implanted with a pituitary gland under the kidney capsule on day 0 were adrenalectomized on day 0 (■, $n = 4$) or day 20 (△, $n = 5$) after the implantation of the pituitary glands. Until day 20 all intact rats with pituitary transplants were treated as a single group (●, $n = 10$ up to day 20, $n = 5$ up today 43).

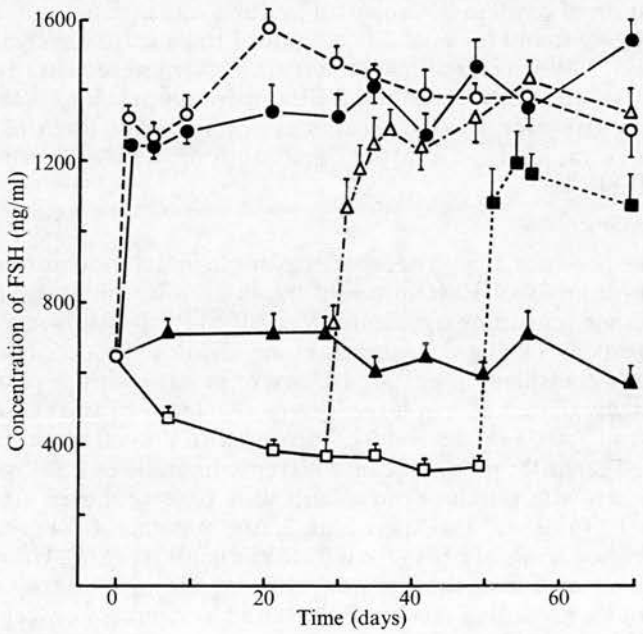



Fig. 4. Effect of castration on levels (mean ± S.E.M.) of FSH in serum of adult male rats. Rats acting as controls were either sham-operated (▲, $n = 4$) or castrated on day 0 (●, $n = 4$). Fifteen rats implanted with two pituitary glands under the kidney capsule on day 0 were castrated on day 0 (○, $n = 4$), on day 28 (△, $n = 5$) or on day 49 (■, $n = 6$) after the transplantation of the pituitary glands. Until day 49 all intact rats with pituitary transplants were treated as a single group (□).

pituitary transplant. Serum levels of testosterone were significantly higher ($P<0.05$) after adrenalectomy on day 0 in rats with a pituitary graft.

*Hormone content of the hypothalamus and pituitary gland*

Adrenalectomy or castration did not affect the weight of the pituitary gland.

*Experiment 1*

Adrenalectomy did not affect the pituitary content of LH, FSH or prolactin either within the control groups or within the pituitary-grafted groups (Table 3). The presence of a pituitary graft significantly reduced ($P<0.05$ to $P<0.001$) the pituitary content of both LH and prolactin compared to controls. This decrease in prolactin content was even greater after adrenalectomy on either day 0 ($P<0.01$) or day 20 ($P<0.05$). While the pituitary content of FSH was also decreased this achieved significance ($P<0.05$) only in rats with a pituitary transplant adrenalectomized at the time of pituitary implantation (day 0, Table 3).

Table 3. *Effect of adrenalectomy (ADX) on the pituitary concentration (mean ± S.E.M.) of prolactin, LH and FSH in adult male rats with or without two pituitary glands implanted under the kidney capsule 43 days previously. Adrenalectomy was performed at the same time as the pituitary implantation (ADX day 0) or 20 days later (ADX day 20)*

| | | Pituitary concentration (μg/mg) | | |
|---|---|---|---|---|
| Treatment | *n* | Prolactin | LH | FSH |
| No transplantation | | | | |
| Intact | 5 | 1·69 ± 0·22 | 20·8 ± 2·1 | 4·4 ± 0·7 |
| ADX day 0 | 4 | 1·83 ± 0·33 | 16·0 ± 0·5 | 4·6 ± 0·6 |
| Pituitary transplantation | | | | |
| Intact | 5 | 1·00 ± 0·13* | 11·5 ± 0·9** | 3·2 ± 0·2 |
| ADX day 0 | 4 | 0·51 ± 0·13*** | 9·0 ± 0·4** | 2·1 ± 0·1* |
| ADX day 20 | 5 | 0·74 ± 0·19* | 10·6 ± 0·6** | 3·0 ± 0·5 |

*$P<0.05$; **$P<0.01$; ***$P<0.001$: *v.* intact rats without a pituitary transplant (Student's *t*-test).

Table 4. *Effect of castration on the pituitary concentration of LH, FSH and prolactin and on hypothalamic content of LH releasing hormone (LH-RH) (mean ± S.E.M.) in adult male rats with or without two pituitary glands transplanted under the kidney capsule on day 0*

| | Time after castration | | Pituitary concentration (μg/mg) | | | Hypothalamic content (ng) |
|---|---|---|---|---|---|---|
| Treatment | (days) | *n* | Prolactin | LH | FSH | LH-RH |
| No transplantation | | | | | | |
| Intact | 0 | 4 | 0·80 ± 0·18 | 20·6 ± 2·5 | 5·6 ± 0·3 | 4·7 ± 0·8 |
| Castrated on day 0 | 70 | 4 | 0·51 ± 0·05 | 31·9 ± 1·9** | 6·8 ± 0·2 | 1·1 ± 0·3** |
| Pituitary transplantation | | | | | | |
| Castrated on day 0 | 70 | 4 | 0·13 ± 0·01** | 36·4 ± 2·1** | 5·7 ± 0·4 | 0·7 ± 0·3** |
| Castrated on day 28 | 42 | 5 | 0·08 ± 0·02*** | 34·8 ± 1·3** | 6·0 ± 0·7 | 0·9 ± 0·2** |
| Castrated on day 49 | 21 | 6 | 0·10 ± 0·02*** | 29·5 ± 1·8* | 5·3 ± 0·3 | 1·1 ± 0·2** |

*$P<0.05$; **$P<0.01$; ***$P<0.001$: *v.* intact rats without a pituitary transplant (Student's *t*-test).

*Experiment 2*

In control animals, castration resulted in a non-significant decrease in the pituitary content of prolactin. In contrast, the presence of a pituitary transplant resulted in significant reductions ($P<0{\cdot}01$–$P<0{\cdot}001$; Table 4) in the pituitary content of prolactin whenever castration was performed.

The pituitary content of LH was significantly increased ($P<0{\cdot}05$–$P<0{\cdot}01$; Table 4) after castration, but neither the presence of a pituitary transplant nor the time after castration affected this significantly.

Neither castration nor the presence of a pituitary transplant affected the pituitary content of FSH (Table 4).

The hypothalamic LH-RH content was significantly reduced ($P<0{\cdot}001$) in all the groups of castrated rats compared with that in intact controls (Table 4). There was no significant difference in content in relation to time after castration or to the presence or absence of a pituitary transplant.

## DISCUSSION

The results of the present studies confirm previous reports that the secretions of pituitary transplants (presumably prolactin) in adult male rats lead to a prolonged suppression of the serum levels of both LH and FSH in serum and of the content of LH and prolactin in the pituitary gland (Bartke *et al.* 1977; McNeilly *et al.* 1978), and show clearly that this long-term suppression requires the presence of the gonads but not the adrenal glands.

Adrenalectomy 20 days after pituitary implantation did not affect the suppression of LH or FSH secretion. However, while adrenalectomy at the time of pituitary implantation did not affect the suppression of LH secretion the initial suppression of FSH secretion was enhanced. These studies have only examined the effects of adrenalectomy on the basal secretion of LH and FSH. It has been shown that hyperprolactinaemia inhibits the LH response to LH-RH (Winters & Lóriaux, 1978; Greeley & Kizer, 1979), an effect apparently dependent on the presence of the adrenal (Greeley & Kizer, 1979). Our results show that in the long-term, the contribution of the adrenal to the hyperprolactinaemia-induced inhibition of gonadotrophin secretion is minimal.

In contrast, castration resulted in a normal, although delayed, rise in LH and a normal rise in FSH despite the high levels of prolactin in serum that were maintained. This delayed increase in LH is similar to that reported previously in adult male rats bearing pituitary transplants (Grandison *et al.* 1977; Winters & Lóriaux, 1978), injected with prolactin (Grandison *et al.* 1977; Celotti, Massa & Martini, 1978) or implanted with prolactin in the median eminence (Grandison *et al.* 1977). It has also been shown that while the rise in levels of LH in serum after castration may be delayed by the presence of a pituitary transplant, levels of FSH in serum are not affected. In the present study castration resulted in a significant increase in the pituitary content of LH without affecting the pituitary content of FSH. A similar increase in LH content and no change in FSH content occurred in rats with pituitary transplants indicating that hyperprolactinaemia does not affect the pituitary response to castration. Castration results in the expected decrease in hypothalamic LH-RH content (Badger, Wilcox, Meyer, Bell & Cicero, 1978). Since hyperprolactinaemia alone does not affect hypothalamic LH-RH content (McNeilly *et al.* 1978) it seems probable that the decrease in hypothalamic LH-RH content seen in the present study in the castrated rats with pituitary transplants is attributable to castration alone and not to an effect of the maintained levels of prolactin in serum.

In contrast the increase in the weight of the adrenal gland occurs only in rats with a pituitary transplant (McNeilly *et al.* 1978) and castration does not appear to affect this response.

Herbert, Cisneros & Rennels (1977) have shown that castration results in a significant decrease in the prolactin content of the rat pituitary gland. While a similar though non-significant reduction in content was seen after castration in the present study, castration in the presence of a pituitary transplant resulted in a highly significant reduction. In the present, as in the previous studies (McNeilly *et al.* 1978), hyperprolactinaemia alone was associated with a small but consistent decrease in the prolactin content of the pituitary gland. It appears, therefore, that the reduction in prolactin content of the pituitary gland in castrated rats with a pituitary transplant is due to an interaction of hyperprolactinaemia and withdrawal of gonadal steroids.

The progressive decline in the weight of the seminal vesicles after castration of the rats with pituitary transplants clearly shows that prolactin alone cannot maintain the weight of the seminal vesicles. While prolactin as such may increase their weight in the immature rat (Negro-Vilar, Saad & McCann, 1977) this is not the case in adult mice or rats (Bartke & Lloyd, 1970; Sivelle, McNeilly & Collins, 1978).

The present results clearly show that hyperprolactinaemia alone cannot prevent the increased secretion of either LH or FSH in response to castration. This suggests that the negative feedback effects of gonadal steroids are an essential component of the mechanism whereby hyperprolactinaemia suppresses gonadotrophin secretion. In contrast, steroids of adrenal origin appear to play a negligible part.

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# CHANGES IN PITUITARY LHRH RECEPTOR LEVELS IN SITUATIONS OF INCREASED OR DECREASED GONADOTROPHIN SECRETION IN THE MALE RAT

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Anterior pituitary LHRH receptor numbers were studied by measuring binding of $^{125}$I-labelled (D-Ser-Bu$^t$)$^6$-des-Gly$^{10}$ LHRH ethylamide in male rats in which gonadotrophin output is markedly altered. Rats made hyperprolactinaemic by transplantation of 2 pituitary glands under the kidney capsule 98 days previously had significantly lower pituitary contents and serum levels of LH and FSH than controls, although serum concentrations of testosterone remained within the normal range. Pituitary LHRH receptor numbers in these animals were significantly reduced. A condition of further suppression of pituitary contents and serum levels of LH and FSH was achieved by active immunization against LHRH for one year, which also reduced serum concentrations of testosterone to non-detectable levels. This was associated with a marked reduction in LHRH receptor numbers. In contrast, rats castrated 49 days previously demonstrated the expected marked rise in pituitary content and serum levels of LH and FSH and had a marked rise in the number of pituitary LHRH receptors. Castration of animals with pituitary transplants resulted in a similar response. Rats in which testosterone was neutralized by active immunization also showed an increase in pituitary contents and serum levels of LH and FSH, but these changes were lower than in castrated rats, probably owing to the presence of small amounts of non-antibody-bound testosterone. These animals had only a marginal rise in pituitary LHRH receptor numbers.

Our results, showing a lowering in LHRH receptors when hypothalamic LHRH stimulation of the pituitary is thought to be reduced (hyperprolactinaemia and LHRH immunization) and an elevation in receptors when LHRH output is thought to be increased (castration and testosterone immunization), add further support to the view that LHRH regulates its own receptors.

*Keywords*: hyperprolactinaemia; LHRH immunization; castration; testosterone immunization.

Differences in serum concentrations and pituitary content of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) under different physiological conditions are mediated through the feedback influence of gonadal steroids and other factors on the synthesis and release of

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hypothalamic luteinizing hormone-releasing hormone (LHRH) and changes in pituitary responsiveness to LHRH (see Lincoln and Short, 1980, for review). An understanding of the exact role of LHRH in these interrelationships has been hampered by the limitations in measuring changes in output of LHRH because of technical problems in collecting hypophysial portal blood (Eskay et al., 1977; Rotsztejn et al., 1977). At the gonadotroph, changes in the number of LHRH receptors may play a part in the mechanism by which pituitary responsiveness is altered (Debeljuk et al., 1974). It has also been suggested that LHRH receptor numbers are controlled by autoregulation (see Clayton and Catt, 1981a, for review) so that they reflect changes in LHRH output.

In the present study we have examined anterior pituitary LHRH receptor numbers under conditions in which gonadotrophin output is markedly altered but little is known about amounts of LHRH being released, namely after castration, hyperprolactinaemia and active immunization against LHRH or testosterone in the adult male rat.

## MATERIALS AND METHODS

*Animals and experimental procedures*

For the immunization experiments, Sprague–Dawley rats aged 90 days were obtained from Charles River (U.K.) Ltd. and housed under conventional conditions. 5 rats were immunized with LHRH conjugated to bovine serum albumin (BSA) as described previously (Fraser et al., 1974a). 2.5 mg LHRH (Hoechst, U.K.) and 2.5 mg BSA (Sigma) were dissolved in 1 ml of distilled water (pH 5.5) and conjugated by the addition of 100 mg carbodiimide in 200 $\mu$l water. The mixture was dialysed against distilled water followed by 0.9% saline and after emulsification in Freund's complete adjuvant a total of 1 ml emulsion was injected between 8 intradermal sites in each rat.

Testosterone was conjugated to BSA through position 15 (Rao and Moore, 1976) and 10 rats immunized with 100 $\mu$g conjugate as for LHRH. 10 control animals were injected similarly using BSA in Freund's complete adjuvant. At 6 months and 11 months the animals were given booster immunizations as above except that Freund's incomplete adjuvant was used. One month after the final booster, the rats were rendered unconscious by exposing them to an atmosphere of $CO_2$ generated by dry ice. After 1 min, blood was collected by decapitation and the anterior pituitary glands rapidly dissected out and used immediately for the binding experiments. Serum was separated by centrifugation and stored at $-20°C$ prior to measurement of hormone concentrations and antibody titres.

For the studies on hyperprolactinaemic rats, adult males (200–250 g body weight) of the inbred PVG strain were used both as donors and recipients of anterior pituitary glands. They were allocated at random to treatment groups. Two pituitary glands were transplanted under the kidney capsule of 2 groups of 5 rats, while 2 groups of 5 control animals were sham-operated. One group of control rats and one group of rats with pituitary transplants were castrated 49 days later. All animals were killed as described above, 98 days after the pituitary transplant. The animal's own anterior pituitary gland and the transplanted glands were rapidly dissected out and used immediately for binding experiments, and an aliquot stored at −20°C for measurement of protein concentration and content of LH and FSH. Serum was separated and stored as above.

*Measurement of antibody titre and hormone levels*

The titre of anti-LHRH or anti-testosterone antibodies was determined by incubation of serial dilutions of serum together with 10000 cpm $^{125}$I-labelled LHRH or [$^{3}$H]testosterone using the conditions described previously for the radioimmunoassay of LHRH (Jeffcoate et al., 1974) and testosterone (Corker and Davidson, 1978). Sera from the LHRH-immunized rats were also tested for their ability to bind $^{125}$I-labelled LHRH agonist. The dilution of serum required to bind 33% of the labelled hormone was taken as the antibody titre.

The serum levels and pituitary content of LH and FSH were measured by radioimmunoassay as described previously (Fraser and Sandow, 1977; de Jong and Sharpe, 1977) and results expressed in terms of the appropriate NIAMDD reference preparation (RP-1). Serum concentrations of testosterone were measured by radioimmunoassay after extraction with 10 vols hexane:diethyl ether (4:1, v/v) (Corker and Davidson, 1978). Serum from testosterone-immunized animals was acidified by addition of an equal volume of 1 M HCl to release antibody-bound steroid prior to this procedure.

*LHRH receptor assay*

Anterior pituitary glands were homogenized individually in 400 μl 10 mM Tris–HCl (pH 7.6) in 1 ml glass homogenizers and the suspension filtered through nylon gauze. LHRH receptors were determined in duplicate 50 μl aliquots of individual pituitary homogenates by the method of Clayton et al. (1980) as used by us previously (Clayton et al., 1982a). Briefly, the homogenates were equilibrated with $^{125}$I-labelled (D-Ser-Bu$^{t}$)$^{6}$-des-Gly$^{10}$ LHRH ethylamide (Hoechst AG), prepared as described previously (Sharpe and Fraser, 1980), using a near-saturating concentration of 0.1 nM tracer plus 1 nM unlabelled analogue in a total

volume of 250 μl assay buffer (10 mM Tris–HCl, pH 7.6, containing 0.2% BSA). After 80 min in ice, a 16-fold dilution of ice-cold 0.01 M phosphate buffer containing 0.9% NaCl, pH 7.4, was added to stop the reaction and the incubate was immediately filtered under vacuum through glass fibre filters (Whatman GF/C) presoaked in 2% BSA. After washing with 2 ml phosphate buffer, the filters were counted for bound radioactivity in a gamma-counter. Protein concentration in the pituitary homogenates was determined by the method of Lowry et al. (1951) using BSA as a standard. Results for binding of LHRH agonist have been expressed as fmoles bound per pituitary. When the results were corrected for protein concentration, none of the values was significantly altered. All statistical comparisons were made using Student's *t*-test.

## RESULTS

*Effect of immunization against testosterone or LHRH*

In testosterone-immunized rats the titre of antibody varied between animals from as little as 1:50 to a maximum of 1:4000, with the majority of animals exhibiting a titre in the range 1:800 to 1:4000. Despite this variation, the antibodies were clearly effective in all the testosterone-immunized animals, as evidenced by the raised ($P < 0.001$) serum levels and pituitary contents of LH and FSH (Fig. 1). Serum concentrations of testosterone were markedly increased in the testosterone-immunized rats (controls, $1.2 \pm 0.2$ ng/ml; testosterone-immunized, $61.7 \pm 9.6$ ng/ml, means ± SEM). There was a small but significant increase ($P < 0.05$) in the number of LHRH receptors on the anterior pituitary gland (Fig. 1).

In the LHRH-immunized rats the titre of antibody was between 1:4000 and 1:25000, i.e. well in excess of that required to show the characteristic reduction in serum levels and pituitary content of LH and FSH (Fraser, 1980, Fig. 1). Testosterone concentrations were non-detectable (< 0.1 ng/ml) in the LHRH-immunized rats. The pituitaries of these animals showed a marked reduction ($P < 0.001$) in their ability to bind $^{125}$I-labelled LHRH agonist. The possibility that antibodies in the serum of these animals was capable of binding the labelled agonist and thus perhaps interfering in the binding assay was checked and revealed complete absence of any detectable binding in 4 of the rats and a very low binding ability (1:100) in the remaining animal.

*Effect of pituitary transplant*

The presence of transplanted pituitaries produced a significant increase ($P < 0.01$) in serum levels of prolactin (controls, $39 \pm 4$ ng/ml;

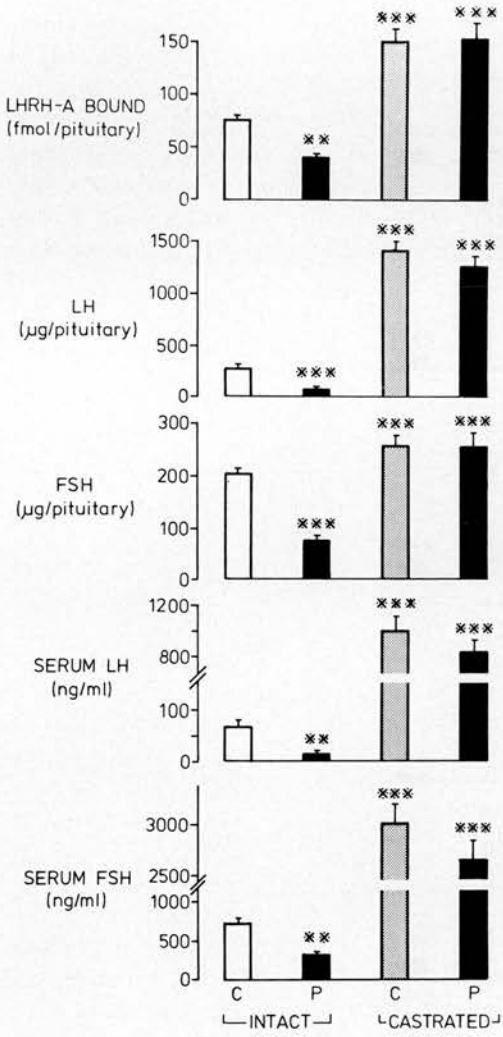



Fig. 1. Effect of immunization against testosterone (T) or LHRH on pituitary LHRH receptors, pituitary content and serum concentrations of LH and FSH in adult male rats. 10 rats per group, controls and T-immunized, and 5 per group LHRH-immunized. Values are means ± SEM. ** $P < 0.05$, *** $P < 0.001$.

transplants, 107 ± 13 ng/ml, means ± SEM). Rats with pituitary transplants demonstrated significant ($P < 0.05$) reductions in serum levels of LH and FSH compared to control rats, and had significantly ($P < 0.001$) lower pituitary contents of LH and FSH (Fig. 2). Despite this, serum

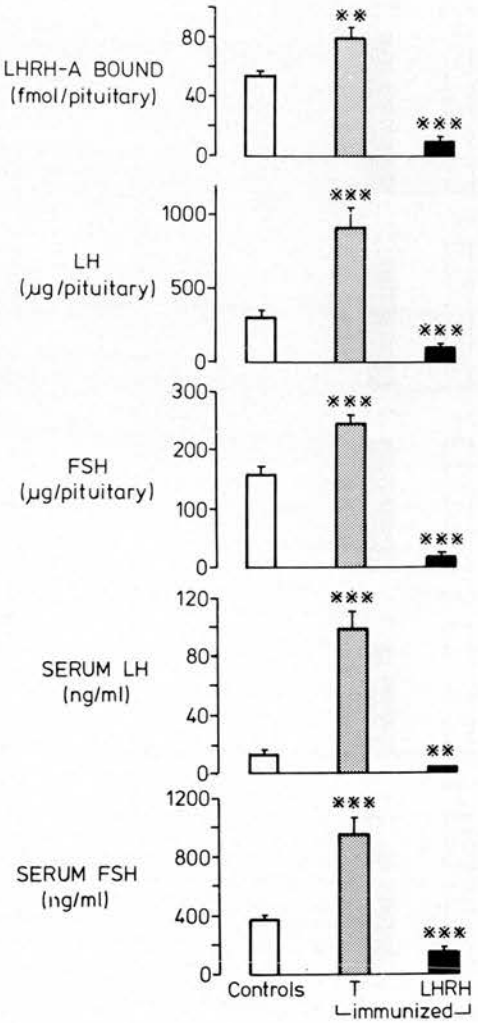



Fig. 2. Effect of hyperprolactinaemia induced by pituitary transplants (P) on pituitary LHRH receptors, pituitary content and serum concentrations of LH and FSH in intact adult male control rats (C), in rats given a transplant of 2 pituitary glands 98 days previously (P) and left intact or castrated 49 days after receiving a pituitary transplant. 5 rats per group, values are means ± SEM. ** $P < 0.01$, *** $P < 0.001$ by Student's $t$-test.

concentrations of testosterone were unaltered (controls, $3.5 \pm 0.2$ ng/ml; transplants, $3.1 \pm 0.3$ ng/ml, means ± SEM). LHRH receptor numbers on the pituitaries of the transplant animals were significantly ($P < 0.01$) reduced (Fig. 2).

*Effect of castration*

Rats castrated 49 days previously demonstrated the expected highly significant rises ($P < 0.001$) in serum levels and pituitary content of LH and FSH (Fig. 2) while serum testosterone concentrations were $< 0.1$ ng/ml. These changes were accompanied by a marked rise ($P < 0.001$) in the numbers of pituitary LHRH receptors when compared to intact rats (Fig. 2). The presence of transplanted pituitaries significantly raised ($P < 0.01$) serum levels of prolactin (controls, $32 \pm 4$ ng/ml; transplants, $121 \pm 12$ ng/ml, means $\pm$ SEM), but did not significantly alter any of these changes (Fig. 2).

*Transplanted pituitaries*

The tissue consisting of the original 2 anterior pituitary glands recovered from within the kidney capsule had an atrophied appearance. The LH and FSH contents in the total tissue were much lower than the animal's own pituitary gland, values for intact animals being $7 \pm 1$ $\mu$g for LH and $13 \pm 1$ $\mu$g for FSH, and in castrated rats $30 \pm 14$ $\mu$g for LH and $7 \pm 2$ $\mu$g for FSH (means of total tissue $\pm$ SEM). Binding of LHRH agonist was about one-third of that from the control rats, being $25 \pm 3.9$ fmoles/pituitary for the intact rats and $24 \pm 4.2$ fmoles/pituitary for the castrated rats, and about half of that observed in the anterior pituitaries of the intact rats bearing the transplants (see Fig. 2).

There are difficulties in obtaining a valid comparison in binding between the transplanted glands and the normal pituitary. Since the transplanted tissue consisted of 2 glands, expression as binding of LHRH per gland increases the differences between the normal and transplanted tissue. The protein content of the homogenates of the 2 transplanted glands was only 70% of that of one normal pituitary, so expression of results per mg protein virtually abolishes the difference. However, because of low protein hormone content and regression of cells other than prolactin cells following transplantation (Nikitovitch-Winer and Everett, 1959), expression of receptor numbers on this basis is probably misleading.

## DISCUSSION

Castration, hyperprolactinaemia and immunization against LHRH or testosterone produce marked changes at different points in the pituitary–testicular axis and the present study describes changes in the numbers of pituitary LHRH receptors under these conditions, which may be of central importance in understanding the mechanisms involved. As

shown previously, transplantation of pituitaries under the kidney capsule in the intact male rat caused reductions in serum levels and pituitary content of LH and FSH, although serum concentrations of testosterone were unaffected (Bartke et al., 1977; McNeilly et al., 1978). It is also known that in these animals pituitary responsiveness to LHRH is reduced (Winters and Loriaux, 1978; Tresguerres et al., 1981). We have now demonstrated that pituitary LHRH receptor numbers are reduced in this situation, which helps to explain the decreased activity of the gonadotroph cells. Since testosterone levels in the blood are unchanged, the feedback effects of testosterone presumably do not play an important part in this change, suggesting a decrease in hypothalamic LHRH release as the likely stimulus. Decreased output of LHRH during hyperprolactinaemia has been suggested previously (McNeilly et al., 1980) but has not yet been demonstrated. Our observations in the male agree with the decrease in pituitary LHRH receptors observed in lactating female rats in which serum prolactin concentrations are also elevated (Clayton et al., 1980).

In the animals actively immunized against LHRH the serum concentrations and pituitary contents of LH and FSH were also reduced, but more markedly than in the hyperprolactinaemic rat so that serum concentrations of testosterone were non-detectable (Fraser et al., 1974b; Fraser, 1980). These animals had drastically reduced numbers of pituitary LHRH receptors. The results confirm and extend other reports which showed a slight reduction in pituitary LHRH receptors after short-term inhibition of LHRH by a chemical antagonist or by neutralization after passive immunization with an LHRH antiserum (Clayton et al., 1982a, b). It can be assumed that in these animals virtually complete inactivation of endogenous LHRH is achieved, indicating this as the likely cause of the reduced number of receptors. Since the animals are also deprived of testicular steroids a role for these cannot be excluded, particularly as experiments in vitro have revealed that androgens can have a direct action on LHRH receptors (Giguere et al., 1981), although it appears that this effect is inhibitory in nature.

Primary control of pituitary LHRH receptors via changes in LHRH levels rather than gonadal steroids is suggested from experiments involving the response to castration. The marked rise in LHRH receptor numbers in castrated animals observed in the present study agrees with previous reports (Clayton and Catt, 1980; Frager et al., 1981; Naess et al., 1981). This rise can be overcome by testosterone replacement (Clayton and Catt, 1981b; Frager et al., 1981; Naess et al., 1981), but it is important to note that the castration-induced rise in LHRH receptors can be prevented by concomitant administration of LHRH antibodies

(Frager et al., 1981; Clayton et al., 1982a) or an LHRH antagonist (Clayton et al., 1982b) or by hypothalamic lesions (Clayton et al., 1982b; Pieper et al., 1982), and that LHRH antibodies can also reduce LHRH receptor numbers in chronically castrated rats (Clayton et al., 1982a). Thus, depriving an animal of gonadal steroids either by castration or immunization against LHRH has opposite effects on pituitary LHRH receptor numbers, suggesting that it is the differing exposure to endogenous LHRH which primarily mediates these changes.

Castration of rats with pituitary transplants resulted in a similar increase in pituitary LHRH receptor numbers as after castration of normal rats, when examined 7 weeks later. This is not surprising in view of the fact that in this and previous studies (McNeilly et al., 1980) the presence of transplanted pituitaries did not affect the long-term gonadotrophin response to castration.

In the testosterone-immunized rat the reduction in available testosterone induced by the circulating antibodies led to a rise in pituitary content and serum concentrations of LH and FSH, but this effect was less pronounced than in the castrated animal, probably because of the presence of small amounts of non-antibody-bound testosterone. The fact that pituitary LHRH receptors were only marginally elevated in these animals is perhaps a reflection of this response which was intermediate between the intact and castrated animal.

Finally, it was of interest that the pituitary tissue transplanted under the kidney capsule, and thus deprived of LHRH support, still possessed LHRH receptors. While the number of receptors appeared less than in normal pituitaries, it was surprising that they were higher than in LHRH-immunized rats. Perhaps this may be accounted for by the longer period of LHRH deprivation in the LHRH-immunized group (transplants 3 months; LHRH-immunized 12 months). While we have shown in this study and from previous tests in vitro (Clayton et al., 1982a) that the presence of LHRH antibodies does not significantly interfere with the LHRH receptor assay in vitro, the higher binding in the transplanted pituitaries raises the possibility that the long-term presence of LHRH antibodies in vivo may have some masking or deleterious effect on pituitary LHRH receptors.

One factor which could have contributed to the changes in LHRH agonist binding, but which we have not measured, is an alteration in receptor affinity. This seems unlikely, however, since receptor affinity has been shown to be unaltered in several other situations which induce changes in LHRH receptor numbers (Clayton et al., 1980; Clayton and Catt, 1981b; Frager et al., 1981).

In conclusion, our results showing a lowering in LHRH receptors

when hypothalamic LHRH stimulation of the pituitary is thought to be reduced (hyperprolactinaemia and LHRH immunization) and an elevation in receptors when LHRH output is thought to be increased (castration and testosterone immunization) add further support to the view that LHRH regulates its own receptors (Clayton and Catt, 1981a; Clayton et al., 1982a, b; Frager et al., 1981; Pieper et al., 1982; Marian et al., 1981). The most convincing support for this theory is supplied by the comparison of the castrated with the LHRH-immunized rat. They are similar in that they are both deprived of gonadal steroids, but the LHRH-immunized animal is also deprived of LHRH and shows a drastic reduction in pituitary LHRH receptors, while the castrated rat demonstrates a marked increase in receptor numbers.

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# Increased Sensitivity to the Negative Feedback Effects of Testosterone Induced by Hyperprolactinemia in the Adult Male Rat

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**ABSTRACT.** High plasma levels of PRL induced by transplants of two donor pituitaries under the kidney capsule of adult male rats resulted in a prolonged suppression of plasma levels of LH and FSH although testosterone levels were maintained within normal limits. Castration of rats with pituitary transplants resulted in a normal though delayed rise in serum levels of both LH and FSH to levels equivalent to those in normal castrated controls. This increase in gonadotropin levels occurred in spite of maintenance of elevated PRL levels. Two experiments were carried out in which testosterone was restored after castration by Silastic testosterone-containing implants of various lengths (Exp 1: 60, 30, and 10 mm; Exp 2: 30, 20, 10, 5, and 2 mm). In both experiments 60- and 30-mm testosterone implants prevented the postcastration rise in LH and FSH in both control and hyperprolactinemic rats. However, although the shorter testosterone implants delayed this rise in control rats, levels of LH and FSH increased by 4 days and were not significantly different from castrated rats without testosterone implants by 15 days after castration. In contrast, this rise in gonadotropins was abolished or considerably delayed by the shorter implants in hyperprolactinemic rats, demonstrating an increase in sensitivity of the hypothalamic pituitary axis to the negative feedback effects of testosterone in these animals.

These results suggest that 1) to maintain suppression of gonadotropin secretion in hyperprolactinemia high levels of PRL alone are insufficient and gonadal steroids are required, and 2) high levels of PRL appear to sensitize the hypothalamic-pituitary axis to the negative feedback effects of gonadal steroids. (*Endocrinology* **112:** 22, 1983)

PREVIOUS studies have shown that hyperprolactinemia produced by pituitary grafts in adult male rats results in a reduction in plasma levels of LH and FSH (1, 2). Furthermore, we have shown that this reduction is dependent on the presence of the gonads but not the adrenal glands, suggesting that the negative feedback effects of gonadal steroids are an essential component of the mechanism whereby hyperprolactinemia suppresses gonadotropin secretion (3).

Since plasma levels of testosterone in hyperprolactinemic rats were not significantly different from control rats, it seemed possible that hyperprolactinemia acted by increasing the sensitivity of the hypothalamic-pituitary axis to the negative feedback effects of gonadal steroids, thereby reducing the secretion of the gonadotropins (4). The present study was undertaken to test this hypothesis by examining the effects of replacing different levels of testosterone on the gonadotropin response to castration. A small part of this study has been reported previously in abstract (4).

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## Materials and Methods

### *Animals*

Adult male rats (130–160 days old; 230–260 g BW) of the inbred PVG strain were obtained from Bantin and Kingman Ltd. (Hull, United Kingdom) and used both as donors and recipients of anterior pituitary glands. Rats were allocated randomly to the treatment groups, allowed access to water and food *ad libitum*, and maintained on a lighting schedule of 14 h light, 10 h darkness.

### *Experimental procedures*

Silastic elastoma implants containing testosterone were prepared at lengths varying from 2–60 mm, using Silastic tubing of 1.57 mm id, 3.18 mm od (Dow Corning Corp., Midland, MI) and testosterone (Sigma, Poole, Dorset, United Kingdom). After preparation all implants were stirred in 1 liter of a sterile solution of 0.9% NaCl (wt/vol) for 24 h at room temperature with three exchanges of saline at 2, 6, and 12 h and then stored dry in sealed plastic vials. Immediately before use implants were again stirred with 0.9% NaCl (wt/vol) for 4 h at room temperature.

*Exp 1.* On day 0 of the experiment, two anterior pituitary glands were transplanted under the kidney capsule of 32 adult male rats under ether anesthesia. Donor pituitary glands were ob-

tained from adult male rats of the same inbred strain. Thirty-two adult male rats without a pituitary transplant acted as controls. Twenty-eight rats in each group were castrated 22 days later (day 22); 4 rats in each group remained intact. Immediately before castration the rats were given a sc Silastic implant of testosterone of 60 mm (n = 8), 30 mm (n = 8), or 10 mm (n = 8), while 4 rats were castrated and left without an implant to act as controls. Animals were killed 12 days after castration (day 34).

*Exp 2.* The experimental design was identical to Exp 1 except that a total of 36 adult male rats were used in each group. Immediately before castration on day 22, rats were given a sc Silastic implant of testosterone of 30 mm (n = 4), 20 mm (n = 6), 10 mm (n = 6), 5 mm (n = 6), or 2 mm (n = 6), while 4 rats received no implant to act as controls. Four rats remained intact. Animals were killed 21 days after castration (day 43).

### *Blood sampling and collection of tissues*

Blood samples (1–2 ml) were withdrawn from a tail vein under light ether anesthesia before each operation and at 3- to 7-day intervals until the end of each experiment. Serum was separated by centrifugation at 600 × *g* for 30 min at 4 C, and the samples were stored at −20 C until assayed for LH, FSH, PRL, and testosterone.

At the end of each experiment, animals were killed in random order by decapitation under $CO_2$ anesthesia, and trunk blood was collected. Immediately after decapitation the hypothalamus was dissected out and immediately extracted as described previously (5), and the extract was stored at −20 C until assayed for LHRH and TRH.

The pituitary gland, ventral prostate gland, seminal vesicles, and adrenal glands were dissected out and weighed. The kidneys were examined macroscopically to confirm the presence of pituitary gland implants.

### *RIAs*

The levels of PRL and LH in serum were assayed using reagents provided by the NIAMDD, NIH (Bethesda, MD) as described previously (2, 6). Serum levels of FSH were measured by the method of Welchen *et al.* (7) as described by de Jong and Sharpe (8). All samples were measured in a single assay with a within assay variation (as percent coefficient of variation) of 7%, 9%, and 11% and sensitivities of 2.5 ng RP-1/ml, 6 ng RP-1/ml, and 100 ng RP-1/ml for PRL, LH, and FSH, respectively.

Testosterone was measured by a specific RIA (9) with an intra- and interassay percent coefficient of variation of 6% and 11%.

The hypothalamic content of LHRH was measured by the method of Nett *et al.* (10), using [$^{125}$I]LHRH prepared as described by Mortimer *et al.* (11). The hypothalamic content of TRH was measured by the method of Fraser and McNeilly (12) with an intrassay variation of 8%.

### *Statistical analysis*

Statistical analysis was by analysis of variance and Student's *t* test.

## Results

### *Organ weights*

As found previously (2, 3), in both experiments the weight of the adrenal glands was significantly ($P < 0.001$) increased in rats with a pituitary transplant compared to control rats. Castration with or without testosterone implants did not affect adrenal weight.

Pituitary weights were not significantly different in any group in either experiment (data not shown).

Castration resulted in the expected highly significant decrease ($P < 0.001$) in weights of the seminal vesicles and the ventral prostate gland, which was prevented by the presence of testosterone implants in a dose-dependent manner (data not shown). These changes were not influenced by the presence of a pituitary transplant in either experiment.

### *Hypothalamic content of LHRH and TRH*

The hypothalamic content of LHRH in control rats or rats with a pituitary transplant did not differ significantly but was reduced ($P < 0.001$) in all castrated rats without testosterone implants.

In castrated rats with or without a pituitary transplant replacement of testosterone with 60-, 30-, 20-, or 10-mm implants resulted in hypothalamic contents of LHRH that were not significantly different from intact control rats (Table 1). Similarly, in pituitary-transplanted rats with 5- and 2-mm testosterone implants (Exp 2), hypothalamic LHRH content was not different from intact controls; however, in castrated rats without a pituitary transplant, replacement of testosterone with 5- and 2-mm implants resulted in significantly higher ($P < 0.001$) contents of LHRH than in castrated control rats, although levels were still significantly lower ($P < 0.05$) than in intact rats.

Neither the presence of a pituitary transplant nor castration with or without testosterone replacement had any effect on the hypothalamic content of TRH.

### *Serum hormone levels*

*PRL.* The levels of PRL in serum showed wide fluctuations within and between groups during the course of both experiments (range, 4–265 ng/ml) because samples were routinely taken under light ether anesthesia. Levels in the terminal serum samples showed less intragroup variation. Compared with control animals, all rats with a pituitary transplant had significantly ($P < 0.05$) raised serum levels of PRL at the end of both experiments (Exp 1: 34 ± 8 *vs.* 96 ± 11 ng/ml, n = 32; Exp 2: 47 ± 9 *vs.* 79 ± 6, n = 36, for control and hyperprolactinemic rats, respectively). Castration with or without testosterone replacement did not affect PRL levels in either control

TABLE 1. Plasma levels of testosterone and hypothalamic content of LHRH (mean ± SEM) in control male rats (C) and male rats with pituitary glands grafted under the kidney capsule (P) 43 days earlier

| Group | n | Testosterone implant (mm) | Plasma testosterone (pg/ml) | Hypothalamic LHRH content (ng) |
|---|---|---|---|---|
| Exp 1 | | | | |
| Intact | | | | |
| C | 4 | 0 | 3837 ± 358 | 4.3 ± 0.4 |
| P | 4 | 0 | 3908 ± 467 | 3.1 ± 0.2 |
| Castrate | | | | |
| C | 4 | 0 | 341 ± 67 | 0.9 ± 0.2 |
| P | 4 | 0 | 243 ± 74 | 1.2 ± 0.1 |
| C | 8 | 60 | 9290 ± 277 | 4.0 ± 0.1 |
| P | 8 | 60 | 9126 ± 470 | 3.1 ± 0.2 |
| C | 8 | 30 | 3955 ± 184 | 2.7 ± 0.3 |
| P | 8 | 30 | 4956 ± 326 | 3.3 ± 0.2 |
| C | 8 | 10 | 2135 ± 196 | 3.1 ± 0.3 |
| P | 8 | 10 | 1821 ± 110 | 3.1 ± 0.3 |
| Exp 2 | | | | |
| Intact | | | | |
| C | 4 | 0 | 2145 ± 705 | 2.6 ± 0.2 |
| P | 4 | 0 | 4061 ± 1250 | 2.8 ± 0.2 |
| Castrate | | | | |
| C | 4 | 0 | 246 ± 23 | 0.9 ± 0.2 |
| P | 4 | 0 | 239 ± 43 | 0.8 ± 0.2 |
| C | 4 | 30 | 3090 ± 227 | 3.0 ± 0.5 |
| P | 4 | 30 | 2962 ± 150 | 2.5 ± 0.4 |
| C | 6 | 20 | 2418 ± 166 | 2.5 ± 0.3 |
| P | 6 | 20 | 2497 ± 182 | 3.2 ± 0.1 |
| C | 6 | 10 | 1220 ± 51 | 1.9 ± 0.3 |
| P | 6 | 10 | 1264 ± 84 | 2.8 ± 0.2 |
| C | 5 | 5 | 697 ± 6 | 1.5 ± 0.2 |
| P | 5 | 5 | 694 ± 32 | 2.4 ± 0.1 |
| C | 6 | 2 | 656 ± 85 | 1.2 ± 0.3 |
| P | 6 | 2 | 665 ± 87 | 1.8 ± 0.3 |

Rats were either intact or had been castrated 21 days previously and given Silastic elastoma implants containing testosterone at lengths of 30, 20, 10, 5, or 2 mm at the time of castration.

rats or those implanted with pituitary glands.

*Testosterone.* The presence of a pituitary transplant *per se* had no significant effect on the levels of testosterone in serum of intact rats or in castrated rats with Silastic implants of testosterone. Serum levels of testosterone in castrated rats were directly related to the length of the Silastic implant except in Exp 2, in which there was no significant difference between the 2- and 5-mm groups (Table 1). Levels in rats with testosterone implants were within the normal range of values seen in intact rats, although the overall mean level in rats with a 60-mm implant (Exp 1) was significantly higher ($P < 0.01$; Table 1). In intact rats in both experiments there was a wide range of testosterone levels (0.78–13.45 ng/ml), and although there was no significant difference in overall mean levels in control rats and those implanted with pituitary glands (Table 1), maximum values tended to be lower in the latter group.

*LH.* In both experiments levels of LH in serum were significantly suppressed ($P < 0.01$) for the duration of the experiment in rats with a pituitary transplant compared to those without (Fig. 1). In Exp 1 castration resulted in a significant increase in levels of LH in serum within 24 h in the control group. However, in the group with a pituitary transplant, this increase was delayed until 96 h. By 14 days after castration, levels of LH were not significantly different in the two groups (Fig. 2).

In control rats this postcastration rise in LH was inhibited by both the 60- and 30-mm testosterone implants and indeed caused a significant ($P < 0.001$) suppression in serum levels of LH compared to precastration levels (Fig. 2).

However, in castrated control rats with a 10-mm testosterone implant, after initial suppression, a significant

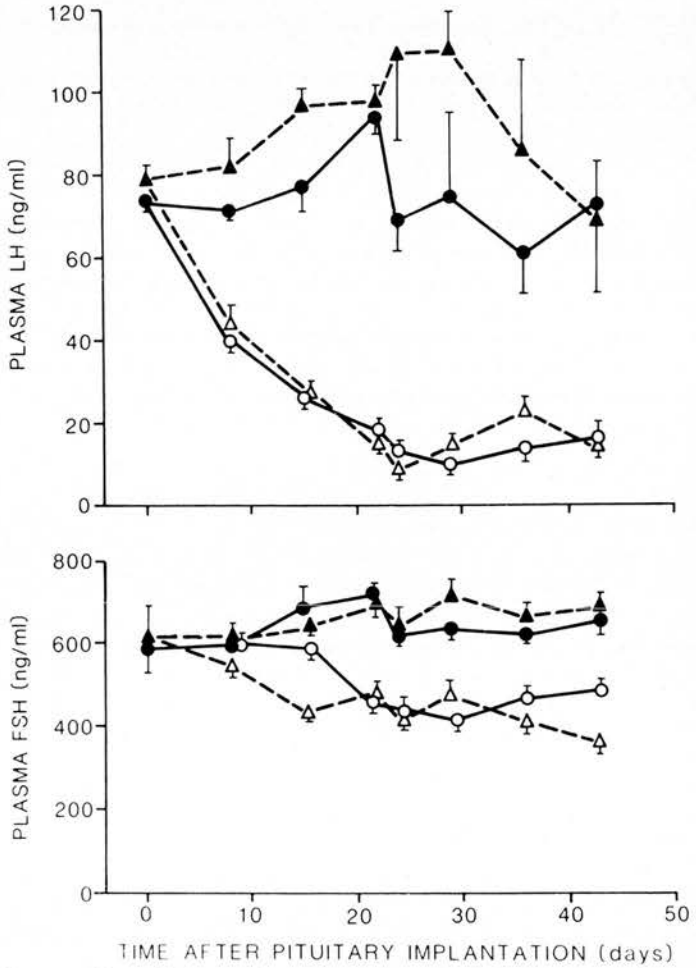



FIG. 1. Effect of hyperprolactinemia induced by pituitary transplants on serum levels (mean ± SEM, n = 32) of LH (*upper panel*) and FSH (*lower panel*) in adult male rats. Rats were sham-operated controls (●, ▲) or implanted with two pituitary glands underneath the kidney capsule (○, △). Results are shown for two separate experiments performed 12 months apart (——, Exp 1; – – –, Exp 2).

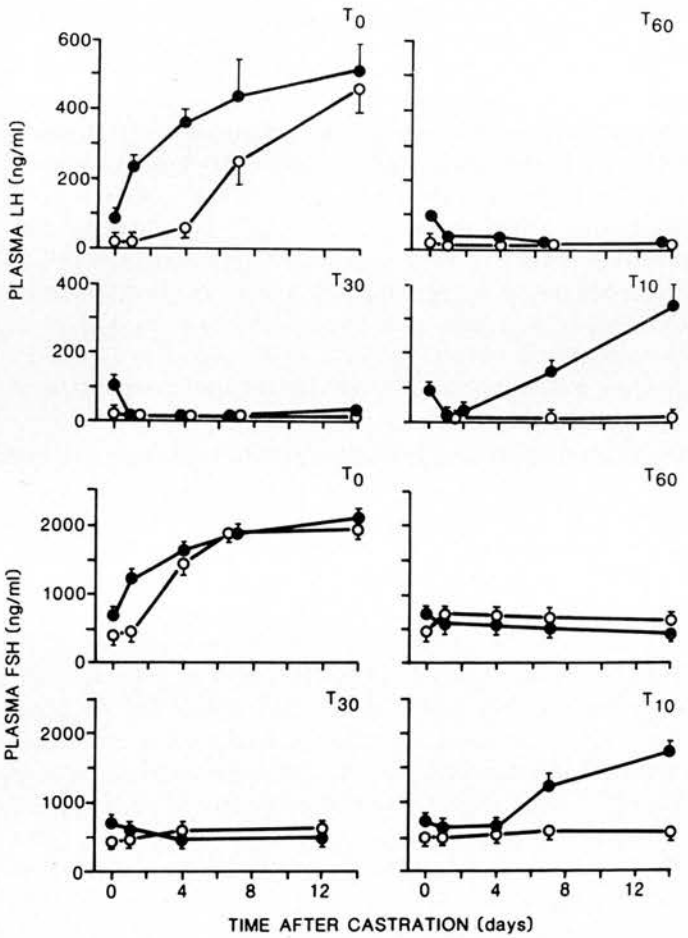



FIG. 2. Effect of castration and testosterone replacement on levels (mean ± SEM) of LH (*upper four panels*) and FSH (*lower four panels*) in serum of control male rats (●, n = 4) or male rats with two pituitary glands implanted underneath the kidney capsule 28 days before castration (○; n = 6; Exp 1). At the time of castration rats were given testosterone-containing Silastic elastoma implants of 0 ($T_0$; n = 4), 60 ($T_{60}$; n = 8), 30 ($T_{30}$; n = 8), or 10 ($T_{10}$; n = 8) mm sc.

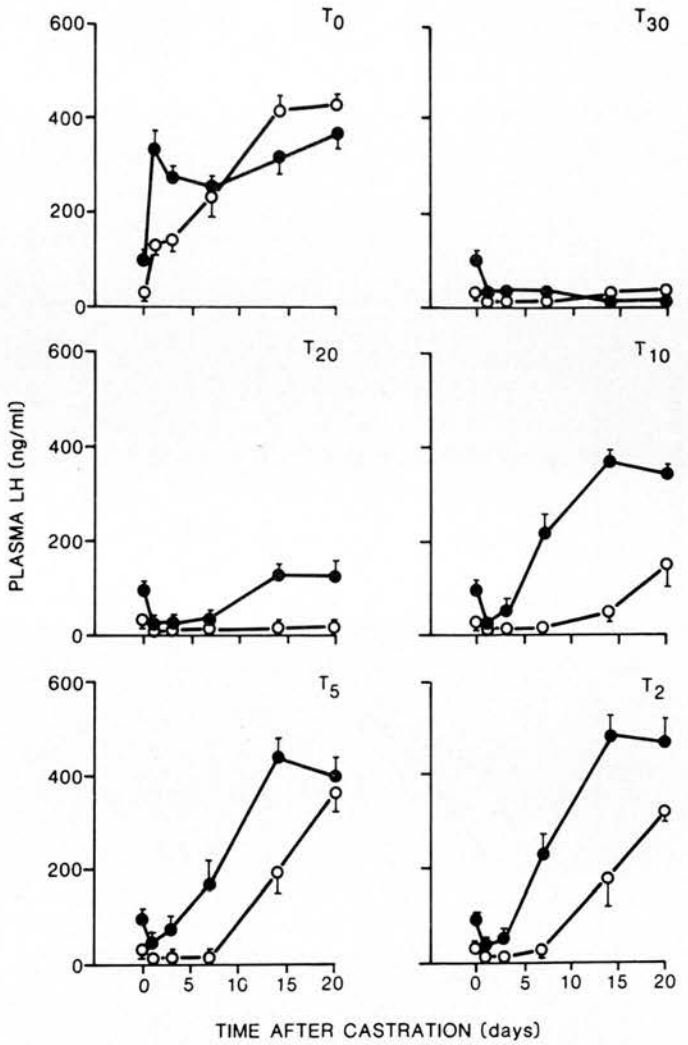



FIG. 3. Effect of castration and testosterone replacement on levels (mean ± SEM) of LH in the serum of control male rats (●) or male rats with two pituitary glands implanted underneath the kidney capsule 28 days before castration (○; Exp 2). At the time of castration rats were given testosterone-containing Silastic elastoma implants of 0 ($T_0$; n = 4), 30 ($T_{30}$; n = 4), 20 ($T_{20}$; n = 6), 10 ($T_{10}$; n = 6), 5 ($T_5$; n = 6), or 2 ($T_2$; n = 6) mm sc.

($P < 0.01$) and sustained increase in levels of LH in serum occurred by day 7 after castration and by day 14 had reached levels similar to those in controls without a testosterone implant.

In contrast, the postcastration rise in LH in rats with pituitary transplants was inhibited completely by the 60-, 30-, and 10-mm testosterone implants (Fig. 2). A similar contrast was observed in Exp 2. In control rats the postcastration rise was completely prevented by the 30-mm testosterone implant, but a significant rise in LH levels in serum occurred with all of the shorter implants such that by day 20 post castration, the serum levels of LH in the groups with 10-, 5-, and 2-mm implants were not significantly different from castrated rats without an implant (Fig. 3). However, in rats with a pituitary transplant, the postcastration increase in serum levels of LH was either prevented completely (30- and 20-mm implant groups) or was delayed (10-, 5-, and 2-mm implant groups) compared to control rats (Fig. 2).

*FSH.* In both Exps 1 and 2 the presence of pituitary transplants in intact rats caused a decrease in levels of FSH in serum, which remained significant ($P < 0.01$) from day 15 after pituitary implantation until the end of the experiment on day 43 (Fig. 1).

In both Exps 1 and 2, castration resulted in a significant increase ($P < 0.01$) in serum levels of FSH within 24 h in the control group, levels reaching maximum values between 2 and 7 days later (Figs. 2 and 4). A similar increase occurred in rats with a pituitary transplant, although serum levels of FSH were significantly less ($P < 0.05$) at 24 h postcastration (Figs. 2 and 4). This postcastration

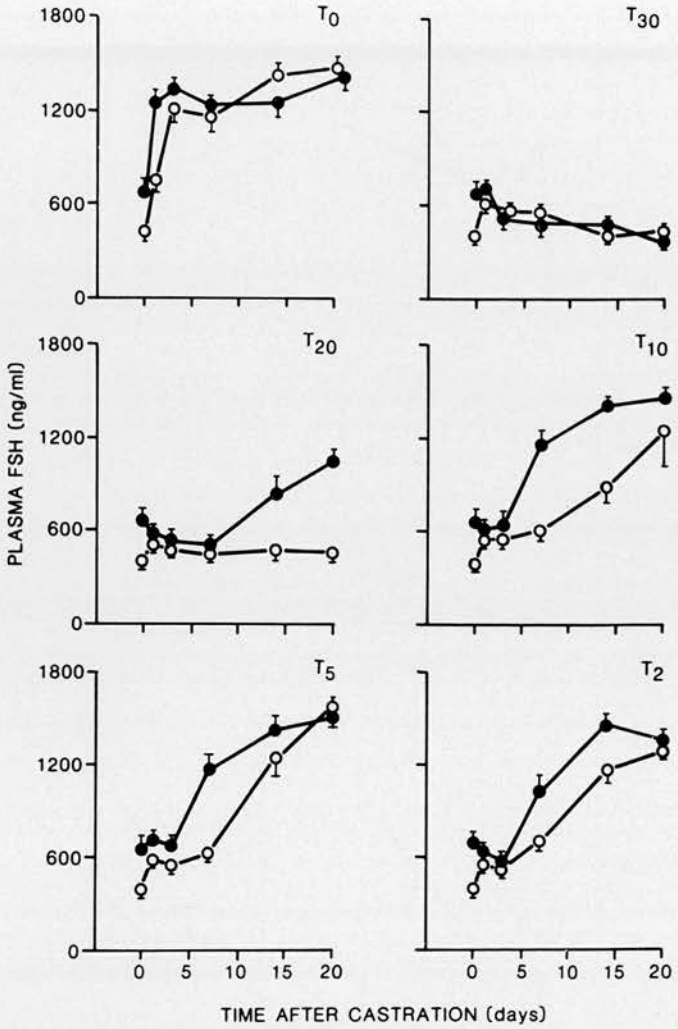



FIG. 4. Effect of castration and testosterone replacement on levels (mean ± SEM) of FSH in the serum of control male rats (●) or male rats with two pituitary glands implanted under the kidney capsule 28 days before castration (○, Exp 2). At the time of castration rats were given testosterone-containing Silastic elastoma implants of 0 ($T_0$; n = 4), 30 ($T_{30}$; n = 4), 20 ($T_{20}$; n = 6), 10 ($T_{10}$; n = 6), 5 ($T_5$; n = 6), or 2 ($T_2$; n = 6) mm sc.

increase in FSH was prevented by 60-mm (Exp 1) and 30-mm (Exps 1 and 2) testosterone implants both in control rats and rats with a pituitary transplant (Figs. 2 and 4) but was only prevented by the 20-mm testosterone implant in rats with a pituitary transplant (Fig. 4). However, in control rats with 20-, 10-, 5-, and 2-mm testosterone implants, there was a delayed increase in levels of FSH at day 7 after castration, and by day 20 levels were not significantly different from castrated control rats (Fig. 4). In contrast, in rats with a pituitary transplant, an increase in FSH levels occurred in the groups with 10-, 5-, and 2-mm testosterone implants, although there was a significant delay in this response compared with that of controls (Fig. 4).

## Discussion

The present study was carried out to determine whether the prolonged suppression of gonadotropin secretion induced by secretions (presumably PRL) from pituitary glands transplanted under the kidney capsule was due to an increased sensitivity to the negative feedback effects of gonadal steroid. The results clearly support this concept since the postcastration rise in both LH and FSH could be inhibited by lower circulating testosterone levels in rats with a pituitary transplant than in control rats.

After induction of hyperprolactinemia the pattern of change of serum levels of LH and FSH with a decline in LH preceding that of FSH confirms our previous studies and clearly suggests a decrease in the release of hypothalamic LHRH (2, 3). A similar pattern of change occurs after active or passive immunization against LHRH (13). In addition, pituitary stores of both LH and FSH are reduced (3) and there is a significant reduction in LHRH receptors on the pituitary glands of these animals (14). Since the maintenance of LHRH receptors requires continued exposure to LHRH (15), all of these results suggest that the major reason for the decreased secretion of LH and FSH is a decrease in the amount of LHRH released from the hypothalamus and reaching the pituitary gland. A decrease in gonadotropin response to an LHRH challenge occurs in similar male rat models (16, 17) and in hyperprolactinemic female rats (18) and has been attributed to a decrease in pituitary sensitivity to LHRH. However, in view of the reduced pituitary content of both LH and FSH and the reduced number of LHRH receptors, it is more probable that this change in pituitary sensitivity is secondary to a decrease in hypothalamic secretion of LHRH.

The present study confirms previous reports that serum levels of testosterone remain within the range seen in normal rats, in spite of the prolonged suppression of gonadotropins induced by the pituitary grafts (1, 3). Exactly how the testis of the hyperprolactinemic rat is able to compensate for the reduced gonadotropin stimulation and maintain normal levels of testosterone is unresolved. It is probably a consequence of the increase in the number of LH receptors and in the sensitivity of the Leydig cells to LH stimulation that has been observed in these animals (19, 20).

Castration alone resulted in a normal but delayed rise in LH and a normal rise in FSH despite the high levels of PRL that were maintained throughout in both experiments. Thus, long term suppression of gonadotropin secretion in adult male rats with pituitary transplants requires the presence of the testis (3), showing clearly that high levels of PRL only suppress gonadotropin secretion by amplifying the negative effects of gonadal

steroids. This increase in sensitivity to negative feedback was shown clearly by the fact that the gonadotropin response to castration could be prevented or reduced by lower levels of testosterone in hyperprolactinemic as opposed to control rats. This would explain how gonadotropin secretion is suppressed by normal levels of testosterone in these intact hyperprolactinemic rats. A similar delay but no long term inhibition of the response of serum levels of LH to castration has also been reported in adult rats bearing pituitary transplants (3, 17, 21) injected with PRL (21, 22) or implanted with PRL in the median eminence (21), whereas serum levels of FSH were not affected.

Measurement of the hypothalamic content of LHRH has not been helpful in elucidating the mechanism by which this occurs, since no differences were found between intact control rats or rats with a pituitary transplant (2). As in previous studies, castration resulted in a decrease in hypothalamic content of LHRH (23) and hyperprolactinemia did not affect this response (3). Conversely, replacement of testosterone by implants in these castrated animals, resulting in serum levels of testosterone within the normal range for intact rats, restored the hypothalamic content of LHRH, as has been shown previously (24).

The degree to which hypothalamic LHRH returned to precastration levels appeared to depend not on the level of testosterone but on the gonadotropin response to testosterone. Hypothalamic LHRH content was not significantly different from intact controls when the postcastration rise in LH was completely inhibited by testosterone implants. However, as the amounts of LH released after castration increased in each group, so the hypothalamic content of LHRH declined toward levels seen in castrated animals without a testosterone implant. Although it appears that high levels of PRL in the group of rats with a pituitary transplant may have prevented the postcastration drop in hypothalamic LHRH, this probably reflects the increase in sensitivity to the negative feedback of testosterone in this group, which leads to a reduction in the amount of LH released after castration. Thus, PRL *per se* may not alter hypothalamic LHRH production directly but may act in concert with gonadal steroids to affect the release of LHRH from the hypothalamus.

The mechanism whereby hyperprolactinemia induces an increase in the negative feedback effects of testosterone in the present study are unclear. A similar increase in sensitivity to the negative feedback of gonadal steroids occurs in seasonal breeders such as sheep (ewe, Ref. 26; rams, Ref. 27) and hamsters (28), and in women during lactation (29). In both women and sheep this altered sensitivity is associated with elevated serum levels of PRL (30), and an increase in sensitivity to negative feedback has been observed after pharmacologically induced hyperprolactinemia in women (31). Thus, in some circumstances at least, increased levels of PRL are associated with a change in steroid sensitivity.

Increased levels of PRL in the rat cause an increase in the hypothalamic turnover of dopamine (32, 33) and its release from the hypothalamus (34, 35). Although the proximity of dopamine and LHRH neurones in the median eminence has led to speculation that dopamine may be an inhibitory modulator of LHRH release (33, 34, 36, 37), the possible site at which gonadal steroids may be involved is not clear. Indeed, in terms of the suppression of LH secretion in hyperprolactinemia, the relevance of the increase in dopamine turnover in the tuberoinfundibular neurones of the hypothalamus to the suppression of LH secretion in hyperprolactinaemia has been questioned, since this increase continues even after castration, when LH levels increase normally (37). Neurones have been identified in the rostral preoptic regions of the hypothalamus, that contain "PRL-like" material (38, 39) and are testosterone responsive (40). However, there is little evidence that neurones in these areas are directly involved in the central control of LH and FSH secretion (37, 41). It is clear that the mechanisms by which hyperprolactinemia interacts with gonadal steroids to cause long term suppression of gonadotropin secretion are as yet unknown and clearly require further investigation.

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# Influence of pinealectomy on the suppression of gonadotrophin secretion induced by hyperprolactinaemia in the adult male rat

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SUMMARY

To investigate the role of the pineal gland in the long-term suppression of gonadotrophin secretion induced by prolactin, the effects of pinealectomy were studied in adult male rats with hyperprolactinaemia produced by the transplantation of two pituitary glands under the kidney capsule.

Pinealectomy had no effect on basal levels of LH, FSH or prolactin. The presence of pituitary transplants induced a significant twofold increase in prolactin levels and a prolonged suppression in both LH and FSH. These changes were not affected by pinealectomy. Castration resulted in a similar rise in plasma levels of LH and FSH in rats with and without pituitary transplants. In control rats this rise in LH and FSH was reduced by testosterone-containing silicone elastomer implants (s.c) of 10 mm in length and delayed by implants of 30 mm. These rises in LH and FSH were significantly delayed (10-mm implant) or abolished (30-mm implant) in rats with pituitary transplants indicating an increase in sensitivity of the hypothalamic-pituitary axis to the negative feedback effects of testosterone in these animals compared to controls. These responses were not affected by pinealectomy.

These results suggest that the pineal gland is not involved in the mechanism whereby pituitary grafts, possibly through their secretion of prolactin, cause long-term suppression of gonadotrophin secretion.

## INTRODUCTION

High plasma levels of prolactin induced by pituitary grafts under the kidney capsule in the adult male rat result in a prolonged suppression of plasma levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Bartke, Smith, Michael, Peron & Dalterio, 1977; McNeilly, Sharpe, Davidson & Fraser, 1978). This suppression is dependent on the presence of the testes but not the adrenal glands (McNeilly, Sharpe & Fraser, 1980). Since circulating levels of testosterone in rats with pituitary transplants are the same as those of control rats, we have suggested that high levels of prolactin suppress gonadotrophin secretion by increasing the sensitivity of the hypothalamo-pituitary axis to the negative feedback effects of gonadal steroids (McNeilly, Sharpe, Davidson & Fraser, 1980). Subsequent studies have confirmed this by showing that the increase in circulating levels of LH and FSH after castration, which is not affected by hyperprolactinaemia alone (McNeilly, Sharpe & Fraser, 1980), could be abolished or delayed by smaller implants of testosterone in rats with pituitary grafts (McNeilly, Sharpe & Fraser, 1980, 1983).

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However the mechanism by which hyperprolactinaemia increases hypothalamic sensitivity to negative feedback remains unclear. A similar change in sensitivity to negative feedback occurs during the non-breeding season in the ewe (Karsch, Goodman & Legan, 1980) and hamster (Turek, 1979). Such changes are directly related to alteration in daylength and are mediated through the pineal gland (see Lincoln & Short, 1980). This increase in sensitivity to negative feedback in sheep coincides with an increase in prolactin secretion (Lincoln & Short, 1980; McNeilly, 1980). Recent evidence suggests that in the male (Wallen & Turek, 1981) and female (Hoffman & Cullin, 1977) rat, daylength may also influence gonadotrophin secretion by means of the pineal gland by altering sensitivity to the negative feedback effects of gonadal steroids.

To investigate whether the pineal gland is involved in the mechanism whereby increased levels of prolactin suppress gonadotrophin secretion in the rat we have investigated the effects of pinealectomy on basal gonadotrophin secretion, the response to pituitary grafts under the kidney capsule and the changes in response to castration with or without testosterone replacement.

## MATERIALS AND METHODS

### *Animals*

Adult male rats (130–160 days old; 225–250 g body wt) of the inbred PVG strain were obtained from Bantin & Kingman Ltd, Hull, North Humberside, and used both as donors and recipients of anterior pituitary glands. Rats were allocated randomly to the treatment groups, water and food were available *ad libitum* and they were maintained on a lighting schedule of 14 h light : 10 h darkness.

### *Experimental procedures*

Silastic elastomer implants (Dow Corning, Midland, Michigan, U.S.A.; 10 mm and 30 mm in length) containing testosterone (Sigma (London), Poole, Dorset) were prepared exactly as described previously (McNeilly, Sharpe & Fraser, 1980, 1983). Immediately before use implants were stirred with 0·9% (w/v) NaCl for 1–2 h at room temperature.

On day 0 of the experiment, 24 adult male rats were pinealectomized under anaesthesia with pentobarbitone sodium (Sagatal; May & Baker, Dagenham, Essex; 35 mg/kg) and xylazine (Rompum; Bayer UK Division, Bury St Edmunds, Suffolk; 6 mg/kg); six rats were sham-operated as controls. Forty-nine days later (day 49) two anterior pituitary glands were transplanted under the kidney capsule of 12 pinealectomized and 12 control rats (three sham-operated, nine unoperated) under ether anaesthesia. After a further 28 days (day 77) all these rats together with an equal number of pinealectomized ($n = 12$) or intact ($n = 12$) rats without pituitary transplants were castrated under ether anaesthesia. At the time of castration, rats were given a subcutaneous Silastic implant of 10 mm ($n = 4$) or 30 mm ($n = 4$) in length. Four rats were castrated and left without an implant to act as controls for the effects of castration. All animals were killed 21 days (day 98) after castration.

### *Blood sampling and collection of tissues*

Blood samples (1·5 ml) were withdrawn from a tail vein under light ether anaesthesia before the start of the experiment (day 0) and then at 20 and 40 days after pinealectomy, 7 (day 56) and 14 days (days 63) after the pituitary transplant and immediately before (day 77) and 7 days after (day 84) castration. At the end of the experiment (day 98), between 10.00 and 11.00 h, animals were killed in random order by decapitation under ether anaesthesia and trunk blood was collected. Plasma was separated by centrifugation at 600 ***g*** for 30 min at 4 °C and samples were stored at −20 °C until assayed for LH, FSH and prolactin.

The seminal vesicles and adrenal glands were dissected out and weighed.

*Radioimmunoassays*

Plasma levels of LH, FSH and prolactin were assayed using reagents provided by the NIAMDD, NIH, Maryland, U.S.A. as described previously (McNeilly *et al.* 1978; McNeilly, de Kretser & Sharpe, 1979). All results are expressed in terms of the appropriate NIAMDD reference preparations (NIAMDD RP-1). All samples were measured in the same assay with intra-assay variations (as % coefficient of variations for three quality control sera at 25–80% $B/B_0$ in each assay) of 7, 11 and 10% for LH, FSH and prolactin respectively.

Statistical analysis was by analysis of variance and Student's *t*-test.

## RESULTS

### *Levels of LH, FSH and prolactin in plasma*

*Effect of pinealectomy*

Plasma levels of LH, FSH and prolactin 20 and 40 days after pinealectomy were not significantly different from those in intact rats (Table 1).

Table 1. *Effect of pinealectomy (PNX) on plasma levels of LH, FSH and prolactin in adult male rats. Values are means* ± S.E.M., *24 rats per group*

| Hormone | Time after PNX (days) | Hormone levels (μg/l) Intact | PNX |
|---|---|---|---|
| LH | 20 | 74±10 | 84±7 |
| | 40 | 67±12 | 72±7 |
| FSH | 20 | 425±20 | 364±19 |
| | 40 | 410±28 | 398±18 |
| Prolactin | 20 | 18±2 | 16±3 |
| | 40 | 20±2 | 23±2 |

*Effect of pituitary transplants*

In intact rats, the presence of a pituitary transplant resulted in a significant ($P<0.01$) increase in plasma levels of prolactin compared with control rats without a pituitary transplant (Table 2). Plasma levels of LH were significantly ($P<0.01$) lower in rats with a pituitary transplant than controls at 7, 14 (Table 2) and 28 (Fig. 1*a*) days after the pituitary transplant. Plasma levels of FSH were only significantly ($P<0.05$ to $P<0.01$) lower at 14 (Table 1) and 28 (Fig. 1*b*) days after the pituitary transplant.

Pinealectomy did not affect these changes in plasma levels of LH, FSH and prolactin (Table 2).

Table 2. *Effect of pituitary transplants (P) under the kidney capsule on plasma levels (means* ± S.E.M.) *of LH, FSH and prolactin in intact or pinealectomized (PNX) adult male rats. C are control rats; 12 rats per group*

| Hormone | Time after pituitary transplant (days) | Hormone levels (μg/l) Intact C | Intact P | PNX C | PNX P |
|---|---|---|---|---|---|
| LH | 7 | 63±8 | 26±4* | 74±8 | 27±3* |
| | 14 | 72±10 | 25±6* | 81±7 | 21±7* |
| FSH | 7 | 416±26 | 399±22 | 398±64 | 364±19 |
| | 14 | 504±40 | 320±21* | 485±20 | 306±24* |
| Prolactin | 7 | 21±2 | 44±3* | 23±2 | 40±2* |
| | 14 | 17±3 | 50±6* | 24±2 | 54±2* |

* $P<0.05$–$P<0.01$ compared with respective control (analysis of variance and Student's *t*-test).

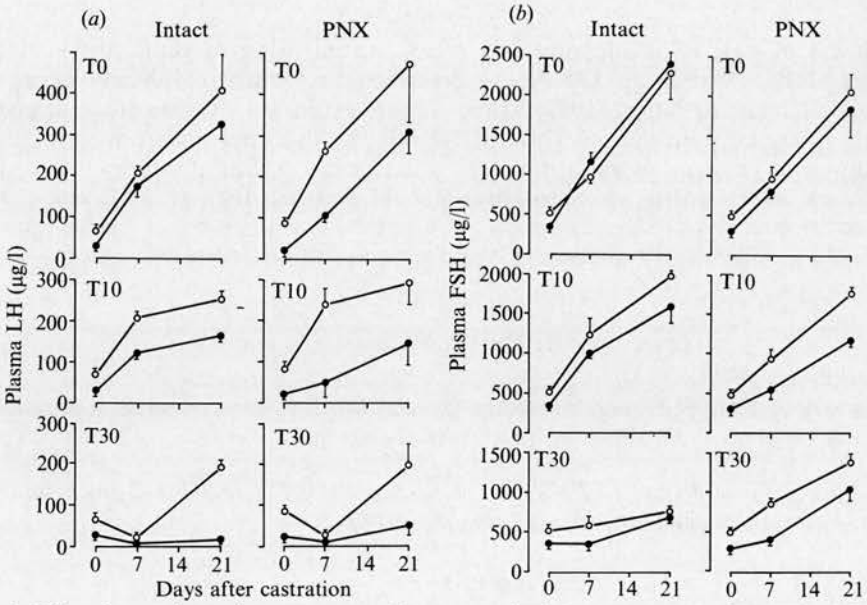



Fig. 1. Effect of castration and testosterone replacement on levels (means ± S.E.M.) of (*a*) LH and (*b*) FSH in the plasma of control male rats (○) or male rats with two pituitary glands implanted underneath the kidney capsule 28 days before castration (●). Rats were either intact or had been pinealectomized (PNX) 49 days before pituitary implantation. At the time of castration rats were given testosterone-containing Silastic implants of 10 (T10) or 30 (T30) mm in length s.c. or left untreated (T0).

*Effect of castration and testosterone replacement*

*Prolactin.* Castration with or without testosterone replacement did not affect the plasma levels of prolactin in either pineal-intact or pinealectomized rats. Levels remained significantly ($P<0{\cdot}01$) higher in rats with (pineal-intact $44\pm4$ (S.E.M.) μg/l, pinealectomized $48\pm5$ μg/l; $n=36$) a pituitary transplant than those without (pineal-intact $18\pm1$ μg/l, pinealectomized $22\pm2$ μg/l; $n=36$).

*Luteinizing hormone* (Fig. 1*a*). In pineal-intact rats with or without a pituitary transplant, castration resulted in a significant ($P<0{\cdot}001$) and continued increase in plasma levels of LH. This increase in the levels of LH after castration in both control rats and rats with a pituitary transplant was not significantly different. In pinealectomized rats a similar response was seen although the increase in LH 7 days after castration was significantly ($P<0{\cdot}05$) less in pinealectomized rats with a pituitary transplant.

The presence of a 10-mm testosterone implant did not significantly ($P>0{\cdot}5$) affect the LH response to castration observed in control pineal-intact rats although the maximum levels seen 21 days after castration were reduced. A 30-mm testosterone implant delayed the LH response to castration in control pineal-intact rats such that a significant ($P<0{\cdot}001$) increase over precastration levels was not seen until 21 days after castration. Furthermore, in control intact rats with a pituitary transplant the 10-mm testosterone implant significantly ($P<0{\cdot}05$) reduced and the 30-mm implant completely prevented the post-castration rise in LH.

Pinealectomy of rats with and without pituitary transplants did not affect the changes in plasma LH after castration with testosterone replacement in rats with or without a pituitary transplant.

*Follicle-stimulating hormone* (Fig. 1*b*). In pineal-intact rats, castration resulted in a significant ($P<0{\cdot}001$) and sustained increase in plasma levels of FSH. There was no significant difference in this response between rats with or without a pituitary transplant. A

similar increase in plasma levels of FSH in rats with or without transplants was seen in animals with a 10-mm testosterone implant although the maximum level of FSH was significantly ($P<0{\cdot}05$) less 21 days after castration in rats with than rats without a pituitary transplant.

The FSH response to castration was more markedly reduced ($P<0{\cdot}001$) in rats with a 30-mm testosterone implant. There was no significant difference in this response between rats with or without a pituitary transplant.

In pinealectomized rats a similar response was seen to castration without or with a 10-mm testosterone implant compared to intact rats although the increase in FSH 7 days after castration was significantly ($P<0{\cdot}05$) less in pinealectomized rats with a pituitary transplant. However, the post-castration rise in FSH was significantly ($P<0{\cdot}05$) greater in the presence of a 30-mm testosterone implant in pinealectomized control than in pineal-intact control rats. In this same group, the rise in FSH after castration in rats with pituitary transplants was greater in pinealectomized rats than pineal-intact controls, but the difference was not significant.

### *Weights of adrenal gland and ventral prostate gland*

The weight of the adrenal gland was significantly ($P<0{\cdot}001$) increased in rats with a pituitary transplant compared with control rats (Table 3). There was no significant effect of pinealectomy on this increase.

Table 3. *Effect of pinealectomy on the weights of the adrenal gland and seminal vesicles (means* ± S.E.M.*) in castrated male rats (C) and castrated male rats with donor pituitary grafts under the kidney capsule (P). Rats were either pinealectomized (PNX) or sham-operated (intact) 98 days previously (day 0) and received pituitary grafts on day 49. All rats (n = 4 per group) were castrated 28 days later (day 77) and given Silastic implants (length 0, 10 or 30 mm) containing testosterone. All rats were killed 21 days later (day 98)*

| Group | Testosterone implant (mm) | Adrenal gland (mg) | | Seminal vesicles (mg) | |
|---|---|---|---|---|---|
| | | Intact | PNX | Intact | PNX |
| C | 0 | 31 ± 3 | 32 ± 3 | 133 ± 12 | 204 ± 24 |
| P | 0 | 44 ± 3 | 41 ± 2 | 140 ± 4 | 136 ± 6 |
| C | 10 | 34 ± 2 | 35 ± 3 | 523 ± 69 | 540 ± 73 |
| P | 10 | 48 ± 3 | 43 ± 3 | 410 ± 41 | 543 ± 100 |
| C | 30 | 35 ± 1 | 34 ± 1 | 1232 ± 27 | 1251 ± 56 |
| P | 30 | 51 ± 4 | 42 ± 2 | 1054 ± 137 | 1129 ± 97 |

Testosterone replacement after castration resulted in a dose-dependent significant ($P<0{\cdot}001$) increase in the weight of the seminal vesicles (Table 3). This increase was not affected by the presence of a pituitary transplant or by pinealectomy.

## DISCUSSION

The results of the present paper confirm previous reports that secretions from pituitary transplants (presumably prolactin) in adult male rats leads to a prolonged suppression of the plasma levels of LH and FSH but only in the presence of the gonads (McNeilly, Sharpe & Fraser, 1980). They also confirm the observation (McNeilly, Sharpe, Davidson & Fraser, 1980; McNeilly *et al.* 1983) that high levels of prolactin appear to act by increasing the sensitivity of the hypothalamo-pituitary axis to gonadal steroids as shown by the prevention or suppression of the post-castration rise in gonadotrophins in rats with pituitary transplants by shorter testosterone-containing Silastic implants than in control rats without a pituitary transplant.

The failure of pinealectomy to alter this response substantially clearly suggests that increased levels of prolactin do not act either directly by means of the pineal gland or indirectly by interacting with any humoral pineal secretion to alter hypothalamo-pituitary sensitivity. Indeed pinealectomy was without effect on the basal secretion of both LH and FSH (Table 1) confirming a previous report (Wallen & Turek, 1981). However, an increase in sensitivity to the negative feedback effects of testosterone has been reported in the adult male rat exposed to a 6 h light : 18 h darkness cycle and was abolished by pinealectomy (Wallen & Turek, 1981). These authors observed no significant pineal-dependent alteration in sensitivity to negative feedback when rats were exposed to a 16 h light : 8 h darkness cycle, the same light : darkness cycle as that employed throughout the present study. Thus it is possible that an even greater alteration in sensitivity to negative feedback induced by pituitary grafts might be seen if rats were also maintained on a short-day photoperiod.

Indeed some indication that this might be the case was seen since there was a significant delay in the post-castration rise in both LH and FSH in pinealectomized compared to control rats (Fig. 1). However, by 21 days after castration this difference was no longer apparent.

The mechanism whereby secretions from the pituitary transplant (presumably prolactin) suppresses gonadotrophin secretion remains obscure. Although it has been suggested that the adrenal gland may play a role (Greeley & Kizer, 1979) we failed to confirm this observation (McNeilly, Sharpe & Fraser, 1980). In the present study, the increase in the weight of the adrenal gland, which occurs only in rats with a pituitary transplant (McNeilly *et al.* 1979), was not affected by pinealectomy. However, the pattern of suppression of gonadotrophins, with a decline in LH before FSH, is similar to that seen after immunoneutralization of luteinizing hormone releasing hormone (LH-RH) (Fraser & Sandow, 1977). The reduction in pituitary content of both LH and FSH (McNeilly *et al.* 1978; Fraser, Popkin, McNeilly & Sharpe, 1982) and the reduction in LH-RH receptors on the pituitary gland (Fraser *et al.* 1982) in male rats with pituitary transplants clearly suggest a decrease in the release of LH-RH from the hypothalamus (McNeilly *et al.* 1983). Because of the close proximity of LH-RH and dopamine neurones in the median eminence it has been suggested that this decrease in LH-RH release may be due to the inhibitory effects of dopamine directly on the LH-RH neurones (Hokfelt & Fuxe, 1972; Weiner & Ganong, 1978; Selmanoff, 1981).

It is clear that increased levels of prolactin in the rat cause an increase in the hypothalamic turnover of dopamine (e.g. Selmanoff, 1981). We have also shown increased dopamine and 3,4-dihydroxyphenylacetic acid levels in hypophysial portal blood in rats with pituitary transplants identical to those used in the present study (A. Brar, A. S. McNeilly & G. Fink, unpublished observations). However, it has also been shown that the increased hypothalamic turnover of dopamine in pituitary-grafted rats continues even after castration when LH levels increase normally (Kalra, Simpkins & Kalra, 1981). It is also unclear how steroids interact with the effects of prolactin at the level of the hypothalamus. Neurones containing 'prolactin-like' material (Fuxe, Hokfelt, Eneroth, Gustafsson & Skett, 1977; Tabeau, Desclin, Parmentier & Pasteels, 1978) and that are testosterone responsive (Simpkins, Kalra & Kalra, 1980) have been identified in the rostral preoptic regions of the hypothalamus. There is little evidence, however, that these neurones are involved in the central control of LH and FSH secretion (Kalra & Kalra, 1980). Thus the link between the sites within the hypothalamo-pituitary axis which are involved in steroid modulation of gonadotrophin secretion and prolactin-induced increase in dopamine turnover remains obscure. The present results clearly suggest that the pineal gland is not involved.

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# Modulation of Prolactin, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) Secretion by LHRH and Bromocriptine (CB154) in the Hypophysectomized Pituitary-Grafted Male Rat and Its Effect on Testicular LH Receptors and Testosterone Output

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## ABSTRACT

Hypophysectomized adult male rats with a pituitary transplant under the kidney capsule were used to study the effects of chronic treatment with LHRH, with or without CB154, on the secretion of LH, FSH and prolactin and the action of these hormones on testicular LH receptors and testosterone secretion.

The presence of a pituitary transplant resulted in the expected increase in circulating levels of prolactin. Treatment with LHRH (5 or 50 μg) caused a significant increase in basal serum levels of FSH, but not LH, after 7 or 14 days of treatment. Treatment with CB154 reduced serum prolactin levels but did not affect the secretion of LH or FSH.

The presence of a pituitary transplant alone increased testicular binding of hCG without increasing testis weight. Administration of LHRH was associated with an increase in testis weight and with the capacity of the whole testis to bind hCG. Administration of CB154 to rats having transplants and receiving 5 μg LHRH reduced testicular binding of hCG without affecting testis weight.

Basal testicular testosterone secretion *in vitro* was increased in all groups receiving pituitary transplants. However, hCG-stimulated testosterone secretion *in vitro* was increased significantly over that in hypophysectomized controls only when gonadotropin secretion was maintained by LHRH treatment with or without CB154.

These results suggest that: 1) small amounts of LHRH stimulated a greater and more prolonged increase in the levels of FSH than of LH; 2) something from the pituitary transplant, presumably prolactin, induces or maintains LH receptors in the testis without affecting testis weight or hCG-stimulated testosterone secretion *in vitro*; and 3) LHRH treatment and the increased gonadotropin secretion caused by it, increase testis weight with an associated increase in LH binding and ability to secrete testosterone after hCG stimulation *in vitro*.

## INTRODUCTION

Luteinizing hormone releasing hormone (LHRH) releases both LH and FSH in all mammalian species including the rat. While steroids modify the response, the differential regulation of LH and FSH is not understood. In the pituitary gland, LHRH appears to stimulate LH release preferentially. In the prepubertal state and in men with hypogonadotrophic hypogonadism of hypothalamic origin, LHRH releases more FSH than LH, suggesting that FSH synthesis and secretion is maintained to a greater extent than LH when the pituitary is exposed to only low amounts of LHRH (Reiter et al., 1976; Bremner et al., 1977). A similar situation occurs when LHRH levels are reduced after active immunization against LHRH; whereas LH levels are reduced, FSH levels remained unaffected in the ewe (Clarke et al., 1978) or are reduced to a much lesser extent in the rat (Fraser and Sandow, 1977). Debeljuk et al. (1973) showed that chronic treatment with twice daily injections of LHRH in hypophysectomized male rats bearing a pituitary graft under the kidney capsule induced the secretion of FSH from the transplanted pituitary gland although LH remained unstimulated. These authors used only a single dose (5 μg) of LHRH. Thus, to determine whether LH secretion could be induced with a larger dose of LHRH, we have re-examined the effect of chronic treatment with LHRH on hypophysectomized adult male rats with a pituitary transplant under the kidney capsule.

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The effects of both high and low doses of LHRH were examined together with the effect of CB154-induced suppression of prolactin secretion on pituitary responsiveness to LHRH.

To date all studies on the hormonal regulation of the testicular LH receptor and testosterone secretion have involved the use of exogenous ovine, bovine or human hormones. In the present study, it was possible to modulate the secretion of endogenous prolactin and gonadotropins independently. Thus we have re-examined the effects of gonadotropins and prolactin on the testis in terms of changes in LH receptors and testosterone response to hCG stimulation *in vitro*.

## MATERIALS AND METHODS

### *Animals and Treatment*

Hypophysectomized, adult male Wistar rats (178–190 g BW) were obtained from Charles River, Co. Adult male rats of the same inbred strain were used to provide donor pituitary glands. Animals were allowed food and 5% dextrose solution *ad libitum* and maintained on a lighting schedule of 14L:10D.

Nine days after hypophysectomy, a single anterior pituitary gland was transplanted under the kidney capsule under ether anesthesia. Six hypophysectomized rats were sham operated and served as controls. Twenty-three days after the grafting operation, when it was assumed that LH and FSH cells in the pituitary transplant were regressed, animals were allocated at random to 1 of the following treatment groups: Group 1, controls injected with the vehicle; group 2, low dose LHRH (5 μg twice daily); group 3, high dose LHRH (50 μg twice daily); group 4, low dose LHRH (5 μg twice daily) plus CB154 (100 μg twice daily). All injections were given s.c. at 0900 h and 1800 h daily for 14 days. The LHRH (Hoechst) was diluted in normal saline containing 0.25% gelatin and was injected in a final volume of 0.25 ml. CB154 was diluted in ethanol:normal saline (4:6, v/v) and injected in a volume of 0.1 ml.

### *Blood Sampling and Collection of Tissues*

Blood samples (0.5 ml) were collected by tail vein puncture under light ether anesthesia on Days 7 and 14 of treatment before injection of either LHRH or CB154. Blood samples were allowed to clot at 4°C for 6 h and serum was removed after centrifugation and stored at −20°C until assayed.

All animals were killed on Day 14 of LHRH treatment and the testes and adrenal glands were dissected out and weighed. One of each pair of testes was decapsulated, weighed and stored at −20°C until used to assess the binding of [$^{125}$I]-labeled hCG. The remaining testis was used immediately to assess *in vitro* testosterone production. Tissue from any animal showing pituitary remnants upon macroscopic inspection of the pituitary fossa was discarded.

### *Testicular Binding of Human Chorionic Gonadotropin (hCG)*

The binding of hCG to testicular LH/hCG receptors was assessed as described previously (Sharpe, 1976, 1977; Sharpe and McNeilly, 1978) using hCG (CR119; 11600 IU/mg) labeled with $^{125}$I by the lactoperoxidase method (Miyachi et al., 1972). Each testis was thawed and gently homogenized in 2 ml Krebs Ringer bicarbonate solution (KRB) in a glass homogenizer. The homogenate was diluted with KRB to a concentration of 200 mg testis/ml and 200 μl aliquots were added to 7 ml polystyrene tubes. The tubes in duplicate were then incubated for 3 h at 34°C with shaking after the addition of 6 nG [$^{125}$I]-hCG in 0.1 ml KRB containing 0.2% bovine serum albumin (BSA). Nonspecific binding of [$^{125}$I]-hCG was assessed by parallel incubation of homogenate in the presence of 100 IU hCG (Pregnyl, Organon). At the end of the incubation, 2 ml cold 0.9% saline was added to all tubes which were then centrifuged at 1500 × g for 15 min at 4°C. The supernatant fluid was decanted and the precipitate counted in a well-type gamma counter (LKB Wallac, Sweden).

### *In vitro Testosterone Production*

*In vitro* testosterone production was assessed as described previously by Sharpe (1977) and Sharpe and McNeilly (1978). Immediately after each animal was killed, 1 testis was decapsulated, halved and each half placed separately into preweighed 83 × 11 mm polystyrene tubes and weighed. One half was incubated with and the other without 33 IU hCG (Pregnyl, Organon)/ml in 0.3 ml KRB containing 1.5 mg glucose/ml. After 4 h incubation at 34°C in a shaking water bath, the medium was aspirated and centrifuged at 1500 × g for 5 min at 4°C; 0.1 ml of the supernatant was then removed and stored at −20°C until assayed for testosterone after appropriate dilution.

### *Radioimmunoassays*

Serum levels of prolactin and LH were measured using reagents provided by the NIAMDD, Bethesda, MD. We measured FSH by the radioimmunoassay method of Welschen et al. (1975) as described by de Jong and Sharpe (1977). Results are expressed in terms of standard NIAMDD reference preparations (NIAMDD RP-1) for each hormone. All samples were assayed in 1 assay with an intra-assay coefficient of variation of 6, 8 and 7% for prolactin, LH and FSH, respectively and sensitivities of 4 ng prolactin/ml, 0.8 ng LH/ml and 1.2 ng FSH/ml.

In a group of hypophysectomized control rats, blood samples were withdrawn before and 40 min after the s.c. injection of LHRH (5 μg, n = 6) or LHRH (5 μg) plus CB154 (100 μg, n = 6). No significant difference ($P>0.1$ was found in the mean "apparent" LH, FSH or prolactin levels before and after injection, respectively: LH, 4.9 ± 1.2 (± SEM) ng/ml and 4.2 ± 0.9 ng/ml; FSH 150 ± 7 ng/ml and 153 ± 9 ng/ml; prolactin, 19.3 ± 1.9 ng/ml and 20.4 ± 2.0 ng/ml. These values were considered to represent the apparent hormone values attributable to non-specific serum interference in the radioimmunoassays. Despite this interference LH, FSH and prolactin were

recovered quantitatively after addition of standards to rat plasma samples (recovery of 95 ± 4, 103 ± 2 and 97 ± 3% for LH, FSH and prolactin, respectively). In addition, serum from hypophysectomized sheep (n = 3) supplied by Dr. H. Buttle, NIRD, Shinfield, caused the same apparent serum interference.

Testosterone was measured by a specific radioimmunoassay without column chromatography (Corker and Davidson, 1978); the antiserum against testosterone was raised in a rabbit and was provided by Dr. S. Tillson, Alza Corporation, USA.

Statistical analysis was by Student's t test or analysis of variance.

## RESULTS

### *Basal Serum Levels of LH, FSH and Prolactin*

The basal serum levels of LH, FSH and prolactin are given in Table 1. Serum LH was apparently detectable in all serum samples, even from hypophysectomized control rats having no pituitary transplant. While mean levels of LH were higher after treatment with LHRH, this increase was not significant because of the wide variation in results. A small but nonsignificant increase in "apparent" basal serum levels of FSH was seen in all control hypophysectomized rats with a pituitary transplant compared with sham operated hypophysectomized control rats (Table 1). Treatment with LHRH significantly increased ($P<0.05$ to $P<0.001$) basal serum FSH levels on both Days 7 and 14 after treatment. Basal levels of prolactin were detectable in all sera collected from control hypophysectomized rats without a pituitary transplant. The presence of a pituitary transplant significantly increased ($P<0.05$ to $P<0.01$) the basal prolactin levels. Treatment with CB154 reduced this increase and basal values of prolactin were not significantly different from hypophysectomized rats without a transplant.

### *Body and Organ Weights*

Whereas the body weights of hypophysectomized control rats were significantly lower ($P<0.05$) than those with a pituitary transplant, this reflected the body weights of animals at the start of the experiment (Table 2). During the 44 day period from hypophysectomy, all animals lost between 3–6 g BW. The weight of the adrenal gland did not vary significantly among treatment groups. Testis weight was significantly greater in all groups treated with LHRH and/or CB154 when compared with hypophysectomized rats without a pituitary

TABLE 1. Basal serum levels (mean ± SEM) of prolactin (PRL), LH and FSH on Days 7 and 14 of treatment with injections twice daily of saline, LHRH or LHRH plus CB154 in hypophysectomized adult male rats with or without a pituitary transplant under the kidney capsule.

| Group | Treatment | Expected hormone profile | Serum levels (ng/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | LH | | FSH[a,b] | | PRL[b] | |
| | | | Day 7 | Day 14 | Day 7 | Day 14 | Day 7 | Day 14 |
| Hypox | Saline | None | 3.7 ± 1.3 (4) | 5.6 ± 0.6 (9) | 153 ± 8 (4) | 155 ± 7 (9) | 20.2 ± 1.5 (4) | 18.8 ± 1.1 (9) |
| Hypox + transplant | Saline | PRL | 4.9 ± 0.7 (6) | 5.5 ± 1.3 (3) | 160 ± 4 (6) | 164 ± 10 (3) | 35.2 ± 4.3 (6) | 39.3 ± 3.3 (3)** |
| | LHRH (5 μg) | PRL, FSH (LH) | 3.5 ± 0.6 (7) | 6.5 ± 1.7 (7) | 215 ± 10 (7)*** | 234 ± 14 (7)*** | 43.1 ± 7.5 (7)** | 40.2 ± 10.6 (7)** |
| | LHRH (50 μg) | PRL, FSH (LH) | 6.2 ± 1.1 (7) | 6.8 ± 1.5 (3) | 201 ± 16 (7)* | 194 ± 11 (3)** | 81.0 ± 17.3 (7)** | 42.7 ± 11.7 (3)* |
| | LHRH (5 μg) CB154 (100 μg) | FSH (LH) | 6.0 ± 0.9 (4) | 7.6 ± 1.9 (4) | 190 ± 10 (4)** | 206 ± 20 (4)*** | 21.8 ± 4.0 (4) | 19.3 ± 0.5 (4) |

*$P<0.05$; **$P<0.01$; ***$P<0.001$.

[a]Significance values refer to difference from hypophysectomized pituitary-transplanted saline treated rats.

[b]Significance values refer to difference from saline-treated hypophysectomized rats.

TABLE 2. Weights of body, adrenal gland and testis and testicular binding of hCG in hypophysectomized adult male rats with or without a pituitary transplant under the kidney capsule after 14 days treatment with LHRH ± CB154. Results are mean ± SEM.

| Group | Treatment | n | BW (g) | Adrenal weight (mg/100 g BW) | Testis weight (mg/100 g BW) | Testicular binding of hCG | |
|---|---|---|---|---|---|---|---|
| | | | | | | pg/20 mg | pg/testis |
| Hypox | Saline | 9 | 177 ± 4[a] | 5.0 ± 0.2 | 118 ± 9 | 72 ± 6 | 742 ± 60 |
| Hypox + transplant | Saline | 3 | 190 ± 11 | 4.6 ± 0.5 | 131 ± 16 | 136 ± 22*** | 1700 ± 340*** |
| | LHRH (5 μg) | 7 | 195 ± 5 | 5.5 ± 0.4 | 205 ± 11** | 147 ± 20*** | 2959 ± 411*** |
| | LHRH (50 μg) | 4 | 194 ± 9 | 6.7 ± 1.2 | 426 ± 125* | 92 ± 13 | 3793 ± 1493** |
| | LHRH (5 μg) + CB154 (100 μg) | 4 | 198 ± 5 | 5.6 ± 0.3 | 206 ± 24* | 81 ± 19 | 1538 ± 227*** |

[a]Significance indicates hypophysectomized rats without a pituitary transplant vs hypophysectomized rats with transplant.

*P<0.05; **P<0.05; ***P<0.001.

transplant while the presence of a pituitary transplant alone was without significant effect (Table 2).

*Testicular Binding of hCG*

Binding of hCG/testis was significantly greater (P<0.05 to P<0.001) in all groups with a pituitary transplant compared to hypophysectomized controls (Table 2). Whereas treatment with LHRH (5 μg) significantly increased (P<0.05) this binding compared with the effect of the pituitary transplant alone, concomitant treatment with CB154 significantly reduced (P<0.01) this increase. In contrast, a significant increase (P<0.001) in the binding of hCG/20 mg testis compared with hypophysectomized controls was seen only in rats bearing pituitary transplants and treated with the vehicle or 5 μg LHRH. Binding (pg hCG bound/20 mg testis) was not significantly different from that in hypophysectomized controls in the other groups (Table 2).

*In vitro Testosterone Production*

The basal output of testosterone (ng/testis) after 4 h incubation at 34°C was significantly greater in all groups which had a pituitary transplant when compared with hypophysectomized controls (Fig. 1). Pretreatment with LHRH or CB154 did not significantly influence this increase in basal output. Testosterone production increased in all groups after incubation with hCG (Fig. 1). The presence of a pituitary transplant alone did not affect the hCG-stimulated testosterone output in hypophysectomized rats. However, pretreatment of hypophysectomized, rats with pituitary transplants with LHRH ± CB154 resulted in a significantly greater (P<0.05 to P<0.001; Fig. 1) increase in testosterone after hCG treatment than in either of the 2 previous groups. There was no effect due to the amount of LHRH given or whether CB154 was given or not.

## DISCUSSION

The increase in the basal serum levels of FSH but not LH after 7 and 14 days treatment with twice daily injections of 5 μg LHRH in hypophysectomized adult male rats with a pituitary transplant under the kidney capsule confirms the previous report of Debeljuk et al. (1973) using the same test system. In addition, in the present study we have shown that treatment over the same period with a

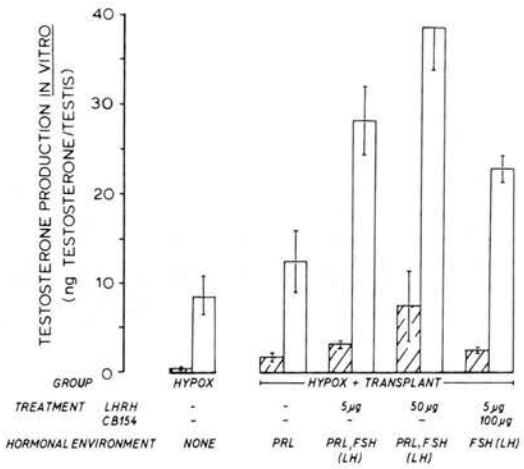



FIG. 1. Testosterone production *in vitro* in the absence (■) or presence (□) of hCG (33 IU/ml) by testicular tissue taken from control hypophysectomized rats and hypophysectomized rats with a pituitary transplant under the kidney capsule after 14 days treatment with LHRH ± CB154. Results are mean ± SEM (n = 4–7/group). Hormone environment refers to the circulating hormones present in the animals during the 14 days prior to sacrifice. LH is in parentheses because it was only present for a short time after LHRH injection.

10-fold greater amount of LHRH (50 μg) also failed to increase basal levels of serum LH significantly. Since basal FSH levels were similar under both treatments, this would suggest that only small amounts of LHRH are required to stimulate maximally the basal output of FSH from the pituitary. For normal LH secretion more LHRH is required, because treatment of LHRH-deficient patients with LHRH resulted first in an increase in FSH secretion followed by an increase in LH secretion (Mortimer et al., 1975). Similarly, withdrawal of LHRH after active immunization results first in a drop in LH secretion followed later by a decline in FSH secretion (Fraser and Sandow, 1977; Clarke et al., 1978). The failure of a 10-fold increase in the amount of LHRH to raise the basal serum levels of LH suggests either that the 14-day treatment period is insufficient or that no increase in basal LH output can be achieved by a twice daily injection schedule of LHRH, i.e. the appropriate stimulus for release is missing in the model presently used. Luteinizing hormone is normally released in pulses in women (Midgley and Jaffe, 1971), men (McNeilly, et al., 1974), ewe (Baird, 1978), ram (Lincoln, 1977), bull (Katongole et al., 1971) and in the castrated male rat (Gay and Sheth, 1971) presumably in response to release of LHRH from the hypothalamus. While the pulse frequency of LH is not known in the intact male rat, the frequency in the castrated male rat is of the order of 24–72 pulses/24 h (Gay and Sheth, 1971). Hence the present injection schedule of 2 injections/24 h, even of large amounts of LHRH, presumably does not provide the correct stimulus to maintain basal LH release. Short term (60 min) increases in LH do occur at the time of LHRH injection (McNeilly, de Kretser and Sharpe; unpublished observations).

In the present study the presence of elevated prolactin levels from the pituitary transplant or suppression of prolactin levels with CB154 did not affect the LHRH-induced secretion of FSH or LH. The 'basal' level of prolactin in hypophysectomized rats of the order of 15–20 ng/ml is similar to that found by other authors (Lu et al., 1977) and presumably represents nonspecific binding of serum proteins to the antiserum against rat prolactin. Nevertheless, the presence of a pituitary graft under the kidney capsule resulted in a significant increase in circulating prolactin levels and treatment with CB154 suppressed the output of prolactin as described previously (Lu et al., 1971).

The presence of a pituitary transplant did not affect adrenal weight. This contrasts with results in intact adult male rats where the presence of a single pituitary transplant induces a significant increase in adrenal weight associated with an increase in the weight of the zona fasciculata (McNeilly et al., 1978; Dobbie, McNeilly, Cowden and Sharpe, unpublished observations). This suggests that the increase in weight is not an action of prolactin alone but requires an additional pituitary agent, presumably ACTH, to cause the increase in adrenal weight seen in intact male rats.

An increase in testis weight occurred in all groups treated with LHRH. No increase occurred in the group with the pituitary transplant alone suggesting that prolactin alone has no influence on testicular growth. This was further emphasized by the fact that suppression of prolactin release from the pituitary transplants by CB154 treatment did not affect the gonadotropin-induced increase in testis weight. Since we have demonstrated in the present study that basal levels of FSH are elevated and both FSH and LH are released in response to each injection of LHRH, the increase in testicular weight is

almost certainly attributable to this induced gonadotropin secretion. Similar results were obtained by Debeljuk et al. (1973) using the same experimental model.

As indicated by the binding of hCG, the results of the present study show that prolactin alone may increase the number of LH (hCG) receptors in the testis. This increase was observed both in terms of binding/20 mg testis and binding/whole testis. These results support observations in which administration of exogenous prolactin induced an increase in LH receptors in the testis of the prolactin-deficient dwarf mouse (Bohnet and Friesen, 1976), the light-deprived hamster (Bex and Bartke, 1977) and the hypophysectomized male rat (Zipf et al., 1978a).

An increase in basal FSH (LH) levels in the absence of PRL (LHRH + CB154 treated group) resulted in an increase in LH receptors/testis but did not increase the concentration of LH receptors/20 mg testis. This confirms previous results in the hypophysectomized rat treated with exogenous FSH (Chen et al., 1976; Dufau et al., 1978). When the testis was exposed to increased plasma levels of FSH (LH) and prolactin, there was an additive effect of these hormones on the number of LH receptors/testis. The increase in concentration of LH receptors/20 mg testis in this situation was presumably due to the prolactin because no increase in concentration was seen following treatment with LHRH + CB154.

The failure to demonstrate an increase in hCG binding/20 mg testis when rats were treated with 50 $\mu$g LHRH may be due to LH-induced loss of receptors (Sharpe, 1977; Sharpe and McNeilly, 1978) since the LH response to 50 $\mu$g LHRH is probably greater than that to 5 $\mu$g LHRH.

The presence of a pituitary transplant significantly increased the basal output of testosterone *in vitro*. While this may be due to prolactin, it appears unlikely since the increased output occurred even when prolactin levels were suppressed *in vivo* by CB154 treatment. It is possible that the small amounts of both LH and FSH, which were apparently released by the unstimulated pituitary transplant, were sufficient to influence basal testosterone output, a situation similar to the partial maintenance of spermatogenesis and prostate weight in the same experimental model (Lu et al., 1977).

Exposure of the testis over a 14 day period to the increased levels of LH and FSH during LHRH treatment significantly increased the subsequent ability of the testis to release testosterone *in vitro* in response to hCG stimulation. This is similar to the well established effects of FSH administration to hypophysectomized rats (Chen et al., 1976; Dufau et al., 1978). Even though there was a significant reduction in the binding of hCG to the testis in the CB154 treated group, the testosterone production *in vitro* while also being reduced was not changed significantly. This further emphasizes the absence of direct correlation between the number of testicular LH binding sites and the ability of the testis to release testosterone after LH stimulation (Sharpe and McNeilly, 1978; Zipf et al., 1978b).

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# DIFFERENCES BETWEEN DISPERSED LEYDIG CELLS AND INTACT TESTES IN THEIR SENSITIVITY TO GONADOTROPHIN-STIMULATION IN VITRO AFTER ALTERATION OF LH-RECEPTOR NUMBERS

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The sensitivity to hCG-stimulation in vitro of intact hemi-testes and collagenase-dispersed Leydig cells has been compared directly following either an hCG-induced loss of LH-receptors or after a hyperprolactinaemia-induced increase in LH-receptors.

Injection of hCG 65 h previously, reduced hCG-binding to dispersed Leydig cells by over 84%. The sensitivity of the steroidogenic response of these cells to hCG-stimulation in vitro was reduced 22-fold whereas intact testes from the same animals showed only a 3-fold reduction in sensitivity to hCG. Dispersed Leydig cells from control rats were 8 times more sensitive to hCG-stimulation than intact testes from the same rats, a difference not evident with hCG-injected rats. In contrast, there was no difference between intact testes and dispersed Leydig cells from control and hCG-injected rats in their sensitivity to stimulation with dibutyryl cyclic AMP in vitro. Induction of hyperprolactinaemia increased hCG-binding to dispersed Leydig cells by 55%. The sensitivity of these cells to hCG-stimulation in vitro was increased by a factor of 4.5, a difference not found with intact testes from the same animals.

These results show that experimental manipulation of LH-receptor numbers alters the sensitivity of dispersed Leydig cells, but not of the intact testis, to hCG-stimulation in vitro, a difference which appears to reside at the receptor level. Possible explanations for these findings are discussed together with their implications with respect to the distribution of LH-receptors over the Leydig cell surface.

*Keywords*: testosterone; prolactin; dibutyryl cyclic AMP; hCG.

Many target tissues for hormones contain an excess of receptors for the trophic hormone, and it has been suggested that the role of the 'spare' receptors is to control the sensitivity of the target tissue to hormonal stimulation, particularly in situations where the hormone negatively regulates the numbers of its own receptor (Raff, 1976). Thus, as serum-hormone levels increase so the number of available receptors for the hormone decrease, thereby reducing the probability of hormone–receptor interaction and hence the sensitivity of the target tissue to the hormone. The Leydig cell possesses 'spare' LH-receptors (Catt and Dufau, 1973; Mendelson et al., 1975), which are negatively regulated by LH and hCG (Hsueh et al., 1976;

Sharpe, 1976; Purvis et al., 1977), but contrary to the above prediction, studies using intact testes have reported no major change in sensitivity to hCG-stimulation in vitro following the LH(hCG)-induced loss of up to 80% of the available LH(hCG)-receptors (Sharpe and McNeilly, 1978; Sharpe et al., 1979). However, in similar studies where dispersed Leydig cells rather than intact testes were used, a clear reduction in sensitivity to hCG-stimulation in vitro was observed (Dufau et al., 1978; Purvis et al., 1978). These completely opposite results might be due to differences in laboratory procedure or may indicate an important functional difference between the intact testis and dispersed Leydig cells. To clarify the situation, we have compared directly the sensitivity to hCG-stimulation in vitro of intact testes and dispersed Leydig cells from the same animals following either an hCG-induced loss of LH-receptors or after an increase in LH-receptor numbers induced by hyperprolactinaemia (Sharpe and McNeilly, 1979).

## MATERIALS AND METHODS

### *Animals and treatments*

To induce loss of Leydig cell LH-receptors, Liverpool-Hooded (L/H) rats aged 55 days were injected subcutaneously with either 50 IU hCG (pregnyl) in 0.2 ml 0.9% saline or with the vehicle, and were killed with ether 65 h later when the paired testes were removed and decapsulated. One testis from each rat was used to prepare dispersed Leydig cells whilst the contralateral testis was halved and weighed but was otherwise kept intact for use in the procedures detailed below.

To increase the number of Leydig cell LH-receptors, inbred PVG male rats aged 100 days were made hyperprolactinaemic by transplanting 2 anterior pituitary glands beneath the kidney capsule; control rats were sham-operated and pituitary donors were male rats of the same age and inbred strain. 3 weeks later the paired testes were removed under ether anaesthesia, decapsulated, and used either to prepare dispersed Leydig cells or were halved and weighed but otherwise kept intact.

### *Preparation of dispersed Leydig cells*

A pool of 8 decapsulated testes were incubated in Krebs–Ringer bicarbonate solution (KRB; 1 ml/testis) containing 0.25 mg/ml collagenase (Type 1; Sigma) for 12 min at 34°C in a shaking water bath. After dilution, tubular material was allowed to settle and the supernatant was aspirated and centrifuged at 4°C for 5 min at 250 *g*. The precipitated cells were then washed with 10 ml KRB, recentrifuged and resuspended in KRB containing glucose (2 mg/ml) and bovine serum albumin (BSA; fraction V, Sigma; 0.25%) (= KRBG). Nucleated cells were then counted in a haemocytometer and the cell suspensions diluted to give $5 \times 10^6$ cells/ml.

The percentage of nucleated cells exhibiting 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, was determined by staining using a modification of the method

of Levy et al. (1959). To a 0.4-ml aliquot of cells the following were added in sequence: 100 μl β-nicotinamide adenine dinucleotide (6 mg/ml; Sigma), 80 μl pregnenolone (1 mg/ml) and 200 μl nitro-blue tetrazolium (1 mg/ml, Sigma), and the cell suspension incubated for 30 min at 34°C. The cells were then precipitated by centrifugation, resuspended in KRB and stained cells counted in a haemocytometer.

*In vitro testosterone production*

This was determined using either intact hemi-testes or dispersed Leydig cells. For the former, each hemi-testis was placed in a glass scintillation vial to which was added 1 ml KRBG followed by various concentrations of either hCG (pregnyl) or dibutyryl cyclic AMP (Sigma) in 50 μl KRBG. For dispersed Leydig cells, aliquots of 0.2 ml (containing $10^6$ nucleated cells) were dispensed into 63 × 11 mm polystyrene tubes followed by various concentrations of hCG or dibutyryl cyclic AMP in 50 μl KRBG. Vials and tubes were then incubated for 3 h at 34°C in a shaking water bath under an atmosphere of 95% $O_2$ : 5% $CO_2$. The medium was then aspirated, centrifuged for 5 min at 1500 *g* and the supernatant stored at −20°C prior to the measurement of testosterone by radioimmunoassay (Corker and Davidson, 1978) as described previously (Sharpe, 1977; Sharpe et al., 1979).

Testosterone production was plotted against the logarithm of the dose of hCG or dibutyryl cyclic AMP, and using this dose–response curve, 'sensitivity' to stimulation was computed as the concentration of the stimulator required to achieve a half-maximal ($ED_{50}$) response. Measurement of sensitivity in other terms (e.g. $ED_{10}$ or the minimum effective dose) produced essentially the same pattern of results as those obtained using $ED_{50}$.

To confirm that observed differences in $ED_{50}$ were statistically significant, dose–response curves for control and treated groups were compared using 2-factor analysis of variance (with replication). If the relationship to each other of the 2 dose–response curves varied significantly ($P < 0.05$ for the interaction of dose × treatment), the actual testosterone response at doses of the stimulator which were closest to the $ED_{50}$ doses for both dose–response curves were compared directly using Student's *t* test. If these were both significantly different ($P < 0.05$), a significant difference in sensitivity was inferred.

*In vitro binding of $^{125}$I-hCG*

Aliquots of dispersed Leydig cells were incubated for 2 h at 34°C in the presence of 20 ng/ml $^{125}$I-hCG (hCG CR115; 13 200 IU/mg). The $^{125}$I-labelled hCG was prepared using lactoperoxidase (Miyachi et al., 1973) and had properties similar to those previously described (Sharpe et al., 1979). In studies on Leydig cells from hCG-treated rats, the $^{125}$I-hCG used had a specific activity of 77 μCi/μg whilst that used with cells from pituitary-transplanted rats had a specific activity of 72 μCi/μg. Non-specific or non-displaceable binding of $^{125}$I-hCG was determined by parallel incubation in the presence of 100 IU unlabelled hCG (pregnyl). All incubations

were performed in triplicate. At the end of incubation, tubes were diluted with 2 ml 0.9% saline, centrifuged at 1500 *g* for 10 min at 4°C, the supernatant decanted and radioactivity in the precipitated cells assessed by counting in a gamma spectrometer.

## RESULTS

*Effect of treatments on staining for 3β-HSD and the binding of $^{125}$I-hCG*

There were no major differences between any of the groups in the percentage of nucleated cells exhibiting 3β-HSD activity. Using L/H rats, 32.4 and 30.6% of cells from control and hCG-injected rats resp., exhibited 3β-HSD activity, whilst 38.5 and 33.7%, resp., of cells from control and hyperprolactinaemic PVG rats stained positively for this enzyme. This suggests that the treatments did not affect the proportion of Leydig cells obtained by collagenase digestion, although it should be noted that the total yield of nucleated cells from the testes of hCG-injected L/H rats in 2 separate experiments was less than 40% of that obtained with testes from control rats.

Using collagenase-dispersed Leydig cells from L/H rats, the specific binding of $^{125}$I-hCG per $10^6$ nucleated cells in controls was 165.3 ± 10.7 pg (mean ± S.D.) whilst in hCG-injected rats, binding was reduced to less than 16% of this value (26.4 ± 4.6 pg). In control PVG rats the binding of $^{125}$I-hCG per $10^6$ cells was 132.0 ± 10.8 pg whilst induction of hyperprolactinaemia increased the binding by 55% (204.5 ± 12.4 pg).

*Effect of hCG-induced loss of LH-receptors on sensitivity to stimulation with hCG in vitro*

The basal production of testosterone by dispersed Leydig cells from control L/H rats was 0.5 ng/$10^6$ nucleated cells and, after incubation in the presence of hCG concentrations of 2.3 pM or more, testosterone production was increased to a maximum of approx. 12 ng/$10^6$ cells (Fig. 1). Compared to this, dispersed Leydig cells from hCG-injected rats showed an increased ($P < 0.001$) basal production of testosterone and a reduced ($P < 0.001$) maximum steroidogenic capacity (Fig. 1).

The mean basal production of testosterone by intact testes from control L/H rats was 120 ng/testis and this increased to a maximum of 960 ng/testis following incubation in the presence of hCG concentrations of 45 pM or more (Fig. 1). In comparison, intact testes from hCG-injected rats showed an increased ($P < 0.001$) basal production of testosterone, with some evidence ($P = 0.05$) of an increased capacity to secrete testosterone (Fig. 1).

Using the dose–response curves illustrated in Fig. 1, sensitivity to hCG-stimulation in vitro was assessed in terms of the concentration of hCG required to elicit a half-maximal ($ED_{50}$) response (Table 1). It was found that intact testes from hCG-injected rats showed a slight, but statistically insignificant ($P > 0.05$), 3-fold reduc-

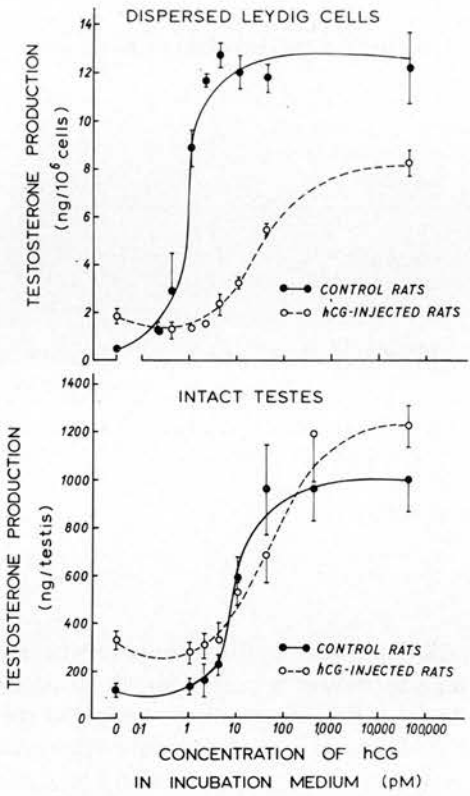



Fig. 1. Relationship between the concentration of hCG in the incubation medium and testosterone production in vitro by dispersed Leydig cells and intact testes from control and hCG-injected rats. Each point represents the mean ± S.D. of triplicate incubations.

tion in sensitivity to stimulation with hCG in vitro when compared with controls, but when a similar comparison was made using dispersed Leydig cells, the reduction in the sensitivity of cells from hCG-injected rats was 22-fold (Table 1). This difference in results between intact testes and dispersed Leydig cells may have been due partly to the fact that dispersed cells from control rats were apparently 8 times more sensitive to hCG than intact testes from the same animals (Table 1). In contrast, dispersed Leydig cells and intact testes from hCG-injected rats showed almost identical sensitivities to hCG-stimulation in vitro (Table 1).

*Effect of hCG-induced loss of LH-receptors on sensitivity to stimulation with dibutyryl cyclic AMP in vitro*

The preceding results demonstrated that intact testes from hCG-injected rats

Table 1
The sensitivity ($ED_{50}$) of testicular tissue from control and hCG-injected rats to stimulation in vitro with either hCG or dibutyryl cyclic AMP

| Tissue preparation | Treatment group | |
|---|---|---|
| | Controls | hCG-injected |
| *Sensitivity to hCG* | | |
| Dispersed Leydig cells | 1.0 pM | 22.4 pM * |
| Intact testes | 8.0 pM | 25.2 pM NS |
| *Sensitivity to dibutyryl cyclic AMP* | | |
| Dispersed Leydig cells | 500 μM | 600 μM NS |
| Intact testes | 270 μM | 350 μM NS |

The $ED_{50}$ are derived from the dose–response curves illustrated in Figs. 1 and 2. Statistical significance of results was determined using 2-factor analysis of variance (with replication) and Student's *t* test.
NS not significant
* $P < 0.001$
} Compared with control treatment group.

showed only a small reduction in sensitivity to hCG-stimulation in vitro whereas dispersed Leydig cells from the same animals showed a very large reduction in sensitivity. To test if this disparity was due to differences at the receptor level the experiment was repeated and sensitivity to dibutyryl cyclic AMP-stimulation was assessed. For both dispersed Leydig cells and intact testes from control rats, the shape of the testosterone dose–response curves (Fig. 2) was essentially the same as that observed in the previous experiment (Fig. 1). However, although Leydig cells and intact testes from hCG-injected rats showed a similar basal secretion of testosterone to that observed previously, the maximum steroidogenic response to dibutyryl cyclic AMP stimulation was much greater than in controls (Fig. 2) and was considerably larger that the corresponding response elicited by maximal hCG-stimulation in vitro (Fig. 1). Despite these changes, tissue from control and hCG-injected rats showed similar sensitivities ($P > 0.2$) to stimulation with dibutyryl cyclic AMP (Table 1). Moreover, intact testes from both control and hCG-injected rats showed similar sensitivities to stimulation with dibutryl cyclic AMP as dispersed Leydig cells from the same animals (Table 1).

*Effect of hyperprolactinaemia-induced increases in LH-receptors on sensitivity to stimulation with hCG in vitro*

As an hCG-induced decrease in LH-receptors severely reduced the sensitivity of dispersed Leydig cells to stimulation with hCG in vitro, we tested whether an increase in LH-receptors would have the opposite effect and increase sensitivity to hCG. The rats used for this study were of the PVG strain and, with the exception of

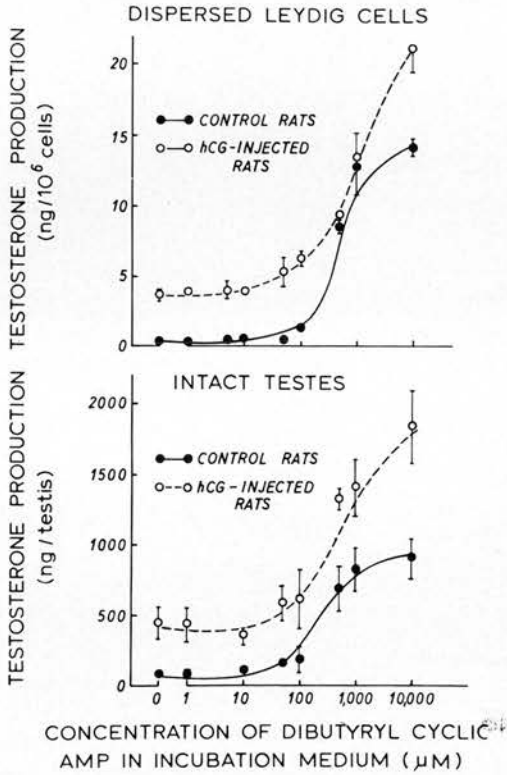



Fig. 2. Relationship between the concentration of dibutyryl cyclic AMP in the incubation medium and testosterone production in vitro by dispersed Leydig cells and intact testes from control and hCG-injected rats. Each point represents the mean ± S.D. of triplicate incubations.

the characteristically lower steroidogenic response to maximal hCG-stimulation in vitro (Fig. 3), the shape and sensitivity to hCG of the testosterone dose–response curves for dispersed Leydig cells and intact testes from control rats of this strain were very similar to those obtained using rats of the L/H strain (compare Fig. 3 with Fig. 1 and Table 2 with Table 1). In terms of the basal secretion of testosterone and the steroidogenic response to maximal stimulation with hCG in vitro, dispersed Leydig cells and intact testes from hyperprolactinaemic rats showed a similar picture: thus, basal testosterone production was reduced ($P < 0.001$) by over 60% and the steroidogenic maximum was reduced ($P < 0.001$) by 40–50% in relation to control values (Fig. 3). Despite these changes, dispersed Leydig cells from hyperprolactinaemic rats were 4 times more sensitive than cells from controls to stimulation with hCG (Table 2). In contrast, intact testes from hyperprolactinaemic rats

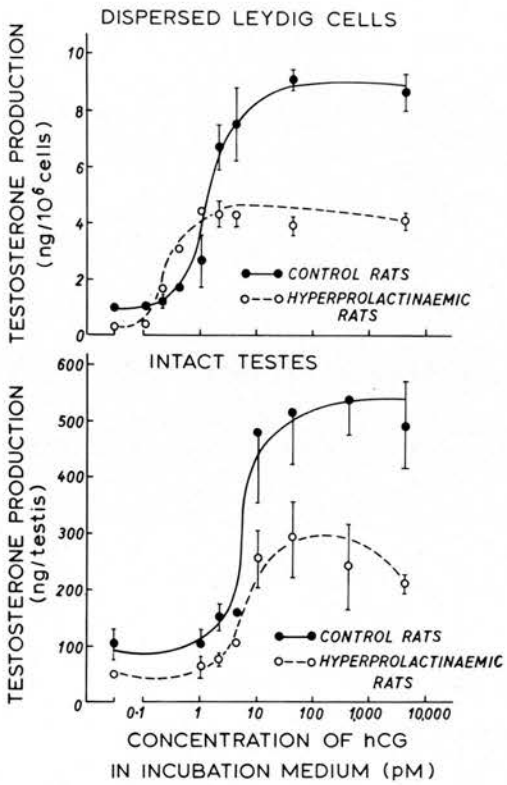



Fig. 3. Relationship between the concentration of hCG in the incubation medium and testosterone production in vitro by dispersed Leydig cells and intact testes from control and hyperprolactinaemic rats. Each point represents the mean ± S.D. of triplicate incubations.

Table 2
The sensitivity ($ED_{50}$) to hCG-stimulation in vitro of testicular tissue from control and hyperprolactinaemic rats

| Tissue preparation | Treatment group | |
|---|---|---|
| | Controls | Hyperprolactinaemic |
| Dispersed Leydig cells | 1.3 pM | 0.3 pM * |
| Intact testes | 5.6 pM | 5.6 pM $^{NS}$ |

The $ED_{50}$ are derived from the dose–response curves illustrated in Fig. 3. Statistical significance of results was determined using 2-factor analysis of variance (with replication) and Student's $t$ test.
$^{NS}$ not significant
$^{*}$ $P < 0.05$
} Compared with control treatment group.

showed no change in sensitivity to hCG (Table 2). This difference was further emphasised by the fact that dispersed Leydig cells from hyperprolactinaemic rats were 18 times more sensitive to hCG than intact testes from the same animals, whereas a similar comparison with controls showed only a 4-fold difference in sensitivity (Table 2).

## DISCUSSION

The sensitivity of Leydig cells to stimulation with hCG is presumably determined initially by the probability of hCG-receptor interaction. The latter is itself presumably determined directly by the number of available LH(hCG)-receptors, sensitivity increasing with increase in the number of receptors (assuming that the receptors are evenly distributed). It follows that a reduction in the number of LH-receptors, as occurs following injection of hCG (Hsueh et al., 1976; Sharpe, 1976), should result in a decrease in sensitivity to stimulation with hCG in vitro. This prediction was matched by the present findings which showed a 22-fold reduction in the sensitivity to hCG of dispersed Leydig cells from hCG-injected rats, and confirms the earlier findings of two other groups. Thus, Dufau et al. (1978) reported a 20- to 40-fold reduction in the sensitivity of dispersed Leydig cells to hCG-stimulation in vitro following an hCG-induced loss of 90% of LH-receptor numbers, whilst Purvis et al. (1978) found a 5- to 10-fold reduction in sensitivity following induction of 77% receptor loss. In contrast to these findings, the present results show that the sensitivity to hCG-stimulation in vitro of intact testicular tissue (i.e. hemi-testes) from hCG-injected rats was reduced to only a small extent, if at all. This again confirms previous detailed findings which showed that the induced loss of 50–80% of the LH-receptor population did not result in any major reduction in sensitivity to hCG in vitro of intact testes from rats treated with LH (Sharpe and McNeilly, 1978), or with an agonist of LH-RH (Sharpe et al., 1979). However, the present report is the only one in which the sensitivity to hCG of intact testes and dispersed Leydig cells from the same animals has been compared directly.

A similar, although smaller, discrepancy to that detailed above was also evident when LH-receptor numbers were increased by the induction of hyperprolactinaemia. Thus, dispersed Leydig cells from hyperprolactinaemic rats were 4 times more sensitive to hCG-stimulation in vitro than cells from control rats, but this difference was not evident with intact testes from the same animals. Although these changes were perhaps complicated by the pronounced impairment of steroidogenesis in hyperprolactinaemic rats, the latter appears to reflect an overall reduction in steroidogenesis rather than an inhibition at a specific point(s) in the steroidogenic pathway (I.A. Swanston, A.S. McNeilly and R.M. Sharpe, unpublished data), and would not explain the present observations.

A complicating factor in the evaluation of these findings was the fact that dispersed Leydig cells from control rats were more sensitive to hCG-stimulation in

vitro than intact testes from the same animals. In L/H rats the discrepancy was by a factor of 8, whereas cells from PVG rats were over 4 times more sensitive than intact testes from the same animals. This initially heightened sensitivity to hCG-stimulation in vitro may, in part, explain why hCG-injection caused a much greater reduction in sensitivity of dispersed Leydig cells than of intact testes, although it is difficult to assess the contribution of this factor.

It seems likely that the observed discrepancies in sensitivity to hCG of dispersed Leydig cells and intact testes are due to differences at the receptor level, as incubation of tissue with dibutyryl cyclic AMP revealed no difference in sensitivity to stimulation, even after the injection of hCG. This also makes it unlikely that the increased initial sensitivity to hCG of dispersed Leydig cells simply reflects the easier diffusion of hCG between the dispersed cells rather than into a compact mass of testicular tissue, a view which is reinforced by the observation that, following hCG-induced loss of receptors, dispersed Leydig cells and intact testes from the same animals showed no difference whatsoever in their sensitivity to hCG.

Another possibility is that differences in the degradation of hCG in vitro by intact testes and dispersed Leydig cells might contribute to the observed discrepancies in sensitivity to hCG-stimulation in vitro. This might also explain the apparently shallower slope of the testosterone dose–response curves using tissue from hCG-injected rats (Fig. 1), an observation also reported by both Dufau et al. (1978), and Purvis et al. (1978). Although this possibility remains, it should be noted that degradation of hCG in vitro has only been reported for broken cell preparations (Ketelslegers et al., 1975), and an explanation along these lines would have to be complicated to account for the disappearance of the difference in sensitivity to hCG between dispersed Leydig cells and intact testes following injection of hCG.

The significantly greater reduction in the sensitivity of dispersed Leydig cells, compared to the intact testis, following hCG-induced receptor loss, might be explained on the assumption that the injected hCG did not have a uniform effect on Leydig cells throughout the testis. Thus, those cells adjacent to capillaries may have been 'desensitized' whilst those furthest away from the blood vessels were least affected. During subsequent incubation of intact hemi-testes in vitro, hCG would penetrate to the Leydig cells via the interstitial spaces (rather than via the vasculature) and might thus reach those Leydig cells which were relatively unaffected by the injected hCG. Whilst factors such as this may be important, this explanation would not account for the greater initial sensitivity to hCG in vitro of dispersed Leydig cells from control rats and would also not explain the present observations in hyperprolactinaemic rats. More importantly, recent work indicates that the intratesticular transport of hCG to the Leydig cells is accomplished to a major extent by the interstitial lymph (Sharpe, 1980), which would tend to undermine the basic assumption on which the above explanation is based.

As argued earlier, the present observations on differences or changes in sensitivity to hCG-stimulation in vitro appear to reflect differences at the level of the

LH(hCG)-receptor. On this basis the greater sensitivity to hCG of dispersed Leydig cells, rather than intact testes, from control rats suggests that somehow more LH-receptors become exposed on the Leydig cell surface following collagenase digestion. As collagenase is relatively impure and may contain other enzymes (e.g. trypsin-like activity) it is possible that exposure of Leydig cells to it, may uncover previously masked or cryptic receptors. Along somewhat similar lines, as Leydig cells occur in close-knit clusters in the rat testis (Fawcett, 1972; Fawcett et al., 1973), it is possible that LH-receptors may be located on adjacent Leydig cell surfaces which have gap junctions (Connell and Connell, 1977), and are therefore less accessible to hCG than receptors on exposed areas of the cell surface. Obviously, these 'hidden' receptors would become more accessible when fully exposed following isolation of the cells with collagenase. However, to account for the differential effect of injected hCG on the subsequent sensitivity to stimulation of dispersed Leydig cells on the one hand, and of the intact testis on the other, initial differences in the number of 'accessible' and 'less accessible' LH-receptors in the intact testis would have to be large and their proportions would have to change during hCG-induced loss of LH-receptors. Such speculation is beyond the scope of the present paper, but it is worth mentioning that in ovarian luteal cells there is marked variation in the surface distribution of LH-receptors, with many receptors located on relatively 'inaccessible' areas of the cell surface where adjacent luteal cells abut (Bramley and Ryan, 1978; Anderson et al., 1979).

In addition to the findings on the sensitivity to stimulation of testicular tissue, there are several other interesting aspects to the present data. It had been shown previously that hyperprolactinaemia caused an increase in hCG-binding to testicular homogenates but severely impaired the steroidogenic responsiveness of intact testes to hCG-stimulation in vitro (Sharpe and McNeilly, 1979). The present results confirm these changes and show that they are due to alterations in hCG-binding and steroidogenic responsiveness per Leydig cell rather than to changes in the number of Leydig cells. Another interesting finding was the clear distinction between the ability of testicular tissue from hCG-injected rats to respond in vitro to dibutyryl cyclic AMP on the one hand, and to hCG on the other. The magnitude of the maximum steroidogenic response to the former was far in excess of that obtained with testicular tissue from controls and was also much greater than the response elicited by maximal hCG-stimulation in vitro of testicular tissue from identically treated rats. This may be explained by the fact that although less than 1% of LH-receptors need to be occupied to stimulate steroidogenesis maximally in untreated rats, there is a parallel increase in cyclic AMP production with increasing receptor-occupancy (Mendelson et al., 1975). It follows that because of their greatly reduced LH-receptor numbers, Leydig cells from hCG-treated rats may be unable to generate sufficient cyclic AMP to drive the increased steroidogenic capacity of these cells.

To conclude, these findings show considerable differences in the sensitivity to hCG-stimulation in vitro of isolated Leydig cells and organized testicular tissue

from the same animals. Whilst the physiological significance of these differences is uncertain, the data may indicate that functional changes in Leydig cells occur when they become separated from each other and/or from the seminiferous tubules. With respect to the latter, the results would lend further support to the growing body of data showing that normal Leydig cell function is influenced by the presence and functional status of the seminiferous tubules (e.g. Aoki and Fawcett, 1978; Rich et al., 1979).

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# Gonadotrophin-Induced Reduction in LH-Receptors and Steroidogenic Responsiveness of the Immature Rat Testis

By

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The autoregulation of testicular LH-receptors and steroidogenic responsiveness was assessed in rats following treatment with various doses of hCG or oLH. Irrespective of dose, both oLH and hCG caused greater LH-receptor loss at 48 h than at 20 h after injection and the effectiveness of oLH was dramatically increased if treatment was spread over several injections. Maximal testosterone production *in vitro* was reduced by 50 % at 20 h but not at 65 h after injection of 10 IU hCG, an effect which was not attributable to LH-receptor loss. Sequential treatment with oLH failed to reduce testicular sensitivity or responsiveness to hCG despite causing 50 % LH-receptor loss. Cycloheximide significantly inhibited the hCG-induced receptor loss at 20 h after injection but its effectiveness had largely diminished by 48 h.

*Key words:* LH-receptors – Leydig cell – testosterone production.

Recent evidence has shown that treatment of rats with human chorionic gonadotrophin (hCG) or luteinizing hormone (LH) results in a marked decrease in available testicular LH-receptors (Hsueh et al. 1976; Sharpe 1976; Haour & Saez 1977; Purvis et al. 1977); this autoregulation of LH-receptors may also be accompanied by a decrease in the testicular capacity to secrete testosterone (Hsueh et al. 1977; Sharpe 1977b). Although these findings demonstrate that the testis is capable of regulating its sensitivity and responsiveness to LH-stimulation, it remains to be shown precisely how these changes are induced and whether they are of physiological importance. In this paper we attempt

to evaluate these problems by examining LH-receptor loss and steroidogenic responsiveness after injection of various doses of hCG and in particular after sequential administration of small doses of ovine LH. This work, together with further findings on the ability of cycloheximide to inhibit hCG-induced receptor loss (Sharpe 1977a) are discussed in relation to the normal physiology of the male.

## Materials and Methods

*Animals and treatments*

The animals used were from our in-bred colonies of PVG and L/H rats; housing and lighting conditions were as described elsewhere (de Jong & Sharpe 1977). Although immature rats of both strains respond similarly to hCG treatment (Sharpe 1976; 1977a), rats from only one strain were used in individual experiments. Administration of hCG (Chorulon) or ovine LH (NIH-LH-S19) was by subcutaneous injection in 0.5 ml 0.9 % NaCl, control rats receiving the vehicle alone. Where repeated injections of ovine LH were given, 0.2 % bovine serum albumin (BSA; Sigma, fraction V) was added to the 0.9 % NaCl. Injection of $^{125}$I-hCG (10 ng) by itself or together with 10 IU unlabelled hCG and subsequent assessment of *in vivo* binding of the hormone was as described previously (Sharpe 1976). Intra-testicular injection of saline or cycloheximide (Sigma) was as described elsewhere (Sharpe 1977a).

*Iodination of hCG*

Labelling of hCG (hCG CR119; 11 600 IU/mg) with $^{125}$I was either by a gentle Chloramine-T method or by the enzymatic method of Miyachi et al. (1972). The specific activities obtained were in the range 22–50 $\mu$Ci/$\mu$g and 54–65 $\mu$Ci/$\mu$g for each method respectively. The labelled hormone showed high retention of biological activity (Sharpe 1976) and there was no deterioration during storage at 4°C for periods in excess of two months.

*Binding of $^{125}$I-hCG*

Homogenates of testis were prepared as described previously (Sharpe 1976) and after filtration and centrifugation the sedimented pellet was resuspended at 200 mg/ml in Krebs-Ringer bicarbonate solution containing 0.2 % BSA. Then 0.1 ml of this suspension was incubated (in duplicate) in 85 × 13 mm polystyrene tubes together with 15 ng/ml $^{125}$I-hCG for 3 h at 37°C in a shaking water bath (100 cycles/min). Non-specific or non-displaceable binding of $^{125}$I-hCG was assessed by incubation in the presence of 100 IU hCG. Other details were as given previously (Sharpe 1976).

*In vitro testosterone production*

The steroidogenic responsiveness of hemi-testes from control or LH/hCG-treated rats was assessed as described previously (Sharpe 1977b) during incubation for 4 h at 34°C in the presence of various concentrations of hCG (45 fM to 45 mM). Statistical analysis of results was by analysis of variance or Student's *t*-test.

## Results

*In vivo binding of $^{125}$I-hCG*

The amount of radioactivity in the testes following injection of 10 ng $^{125}$I-hCG (0.12 IU) by itself or together with 10 IU unlabelled hCG is shown in Fig. 1. Injection of the mixture of labelled and unlabelled hCG resulted in much higher levels of radioactivity in the testes compared to injection of $^{125}$I-hCG alone, as reported previously (Sharpe 1976). However, the pattern of $^{125}$I-hCG uptake by the testes was similar with either treatment, increasing between 4 and 8 h after injection and then falling steeply between 12 and 48 h, such that at 48 h, levels were less than 6 % of those at 8 h. Blood levels of $^{125}$I-hCG were highest at 8 h and then declined with a half-life similar to that of unlabelled hCG (Fig. 1).

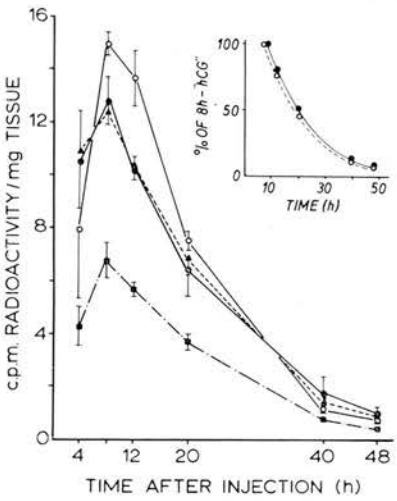



*Fig. 1.*

Binding of $^{125}$I-hCG *in vivo* to immature rat testes following injection of 0.12 IU $^{125}$I-hCG by itself (■ – . – ■) or together with 10 IU unlabelled hCG (o—o). Blood levels of $^{125}$I-hCG after injection of 0.12 IU $^{125}$I-hCG by itself (●—●) or together with 10 IU unlabelled hCG (▲ – – ▲) are also shown. Results are expressed as cpm radioactivity/mg and are the mean ± SD of 3 animals at each point. Inset shows the disappearance curves in blood for unlabelled (o – – o) and $^{125}$I-labelled hCG (●—●).

*Table 1.*
Relationship between dose of hCG injected and subsequent *in vitro* testicular binding of $^{125}$I-hCG.

| Dose of hCG injected (IU) | Time when killed (h) | $^{125}$I-hCG bound | |
|---|---|---|---|
| | | pg | % of control |
| 0 | 20 | 197.1 ± 24.9 | (100) |
| 10 | | 94.0 ± 40.3 | 47.7** |
| 1 | | 183.1 ± 74.2 | 92.9 |
| 0.1 | | 206.6 ± 34.1 | 104.8 |
| 10 | 48 | 36.6 ± 12.5 | 18.6*** |
| 1 | | 107.7 ± 39.4 | 54.6** |
| 0.1 | | 134.7 ± 14.6 | 68.3** |

Values shown are the mean ± SD for 4 rats (28 days old) per group.
** $P < 0.01$
*** $P < 0.001$
} compared with control.

*Table 2.*
Testicular binding of $^{125}$I-hCG *in vitro* following treatment with ovine LH.

| LH-treatment | | Time when killed (h) | $^{125}$I-hCG bound | |
|---|---|---|---|---|
| dose ($\mu$g) | No. of injections | | pg | % of control |
| 0 | 1 | 20 | 260.3 ± 34.4 | (100) |
| 100 | 1 | | 122.9 ± 18.0 | 47.2*** |
| 25 | 4° | | 93.3 ± 17.8 | 35.8*** |
| 0 | 1 | 48 | 266.6 ± 13.5 | (100) |
| 100 | 1 | | 68.3 ± 14.1 | 25.6*** |
| 25 | 4° | | 39.3 ± 14.2 | 14.7*** |

Values shown are the mean ± SD for 3 or 4 rats (36 days old) per group.
° injections given at 4 h intervals.
*** $P < 0.001$ compared with control.

Assuming that the *in vivo* binding of $^{125}$I-hCG to the testis proportionately reflects the total binding of both labelled and unlabelled hormone, the data suggests that at 8 h after injection of 10.12 IU hCG, 5.5 ng hCG is bound per testis, decreasing to 0.2 ng at 48 h. The comparable figures after injection of 0.12 IU hCG are 46 pg at 8 h, decreasing to 1.8 pg at 48 h.

*In vitro binding of $^{125}$I-hCG*

Following injection of 10 IU hCG, testicular binding of $^{125}$I-hCG was reduced to 48 % of control levels at 20 h and to 19 % at 48 h after injection (Table 1). Injection of 1 or 0.1 IU hCG had no significant effect on binding of $^{125}$I-hCG at 20 h after injection but both doses significantly reduced binding at 48 h (Table 1). A single injection of 100 $\mu$g ovine LH also reduced binding of $^{125}$I-hCG more effectively at 48 h than at 20 h after injection, and this reduction in binding was enhanced at both times by giving the LH as four equally spaced injections (Table 2). In a more detailed experiment shown in Fig. 2, a single injection of 20 $\mu$g ovine LH caused a 23 % reduction in binding

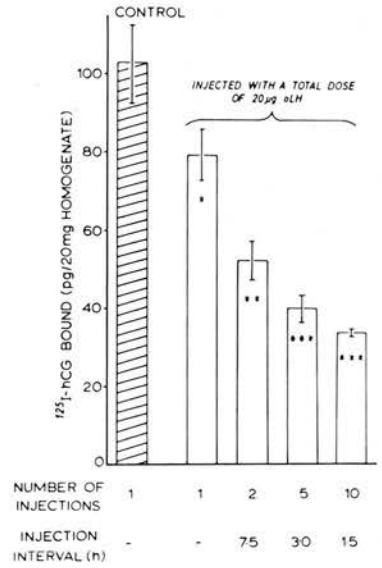



*Fig. 2.*
The effect of sequential injections of ovine LH on testicular binding of $^{125}$I-hCG *in vitro*. Values shown are the mean ± SD for 3 or 4 animals (23 days old) per group killed 48 h after the first injection.

$^{*}P < 0.05$
$^{**}P < 0.01$
$^{***}P < 0.001$
} compared with control

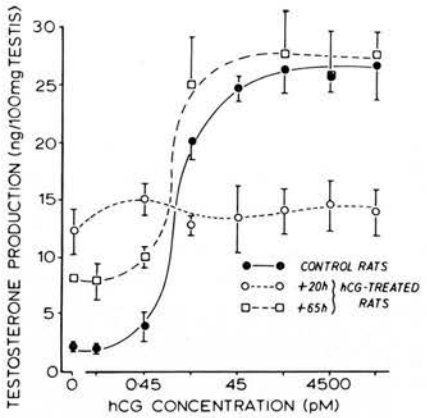



*Fig. 3.*

The effect of hCG treatment on testicular testosterone production *in vitro*. Each point represents the mean ± SD of either 9 (controls) or 3 (10 IU hCG-treated) determinations. Animals were 25 days old.

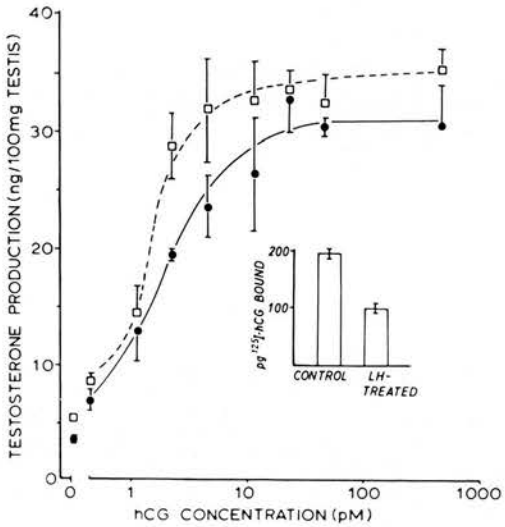



*Fig. 4.*

The effect of sequential injections of ovine LH on testicular testosterone production *in vitro*. Each point represents the mean ± SD of 3 determinations. Animals were 25 days old and were injected five times with 4 μg oLH at 3 h intervals and killed 48 h after the first injection. Inset shows *in vitro* testicular binding of $^{125}$I-hCG in the same rats.

●——● control rats.
□ - - □ oLH-treated rats.

of $^{125}$I-hCG at 48 h after injection, whereas administration of the LH as 2, 5 or 10 equally spaced injections resulted (respectively) in 50, 60 and 67 % reductions.

*In vitro testosterone production*

Control rat testes showed a sigmoidal steroidogenic response to hCG *in vitro* with maximal testosterone secretion occurring when hCG was present in concentrations of between 4.5 and 45 pM (Fig. 3). In contrast, testes from rats injected 20 h previously with 10 IU hCG showed elevated baseline testosterone levels with no additional response to hCG even with concentrations up to 45 nM, so that maximum testosterone output was only 50 % of that in controls (Fig. 3). At 65 h after injection of 10 IU hCG, maximal steroid output had returned to control levels although basal testosterone secretion was still elevated (Fig. 3).

Testes from rats injected with small sequential doses of ovine LH showed only marginally elevated basal testosterone output at 48 h after injection with no reduction in the maximum steroidogenic capacity (Fig. 4). There was an

*Table 3.*
The effect of cycloheximide on hCG-induced changes in testicular binding of $^{125}$I-hCG *in vitro*.

| Treatment* | Time when killed (h) | $^{125}$I-hCG bound | |
|---|---|---|---|
| Peripheral/ Testicular | | pg | % of control |
| Saline/Saline | 20 | 216.2 ± 30.6 | (100) |
| Saline/CHX | | 190.0 ± 18.3 | 87.9 |
| hCG/Saline | | 67.9 ± 29.5 | 31.4 |
| hCG/CHX | | 146.7 ± 19.2 | 67.9 |
| Saline/Saline | 48 | 204.4 ± 12.4 | (100) |
| Saline/CHX | | 181.5 ± 16.5 | 88.8 |
| hCG/Saline | | 37.9 ± 7.8 | 18.5 |
| hCG/CHX | | 76.2 ± 22.6 | 37.3 |

Values shown are the mean ± SD for 3 animals (30 days old) per group. Significance values are given in the text.

* Rats were injected subcutaneously with saline or 10 IU hCG and intra-testicularly (both testes) with either 25 μg cycloheximide (CHX) in 25 μl saline or with the vehicle only.

overall difference ($P < 0.01$) in the responsiveness of testes from control and LH-treated rats suggestive of heightened sensitivity to hCG in the latter, despite the concomitant reduction in LH-receptors (Fig. 4).

*Effect of cycloheximide*

Intra-testicular injection of cycloheximide slightly, but non-significantly ($P < 0.1 > 0.05$) reduced binding of $^{125}$I-hCG to testicular tissue from rats injected peripherally with saline (Table 3). Peripheral injection of hCG significantly ($P < 0.001$) reduced binding of $^{125}$I-hCG at 20 h and 48 h after injection; intra-testicular injection of cycloheximide significantly ($P < 0.01$) inhibited this effect of hCG but was more effective at 20 h than at 48 h in this respect ($P < 0.05$).

## Discussion

In agreement with previous findings (Hsueh et al. 1976; 1977), the present results demonstrate that the reduction in the *in vitro* binding of $^{125}$I-hCG following injection of various doses of hCG or ovine LH, is greater at 48 h than at 20 h after injection. This reduction in binding could be due either to receptor loss or receptor-occupancy by the injected hormone. The latter was assessed by measuring the *in vivo* binding of $^{125}$I-hCG as described previously (Sharpe 1976); the results show that at 20 h after injection of a high dose of hCG (10.12 IU total), there is still a considerable amount of bound hormone, but at 48 h the *in vivo* bound hCG has fallen to less than 6 % of levels found at 8 h, when receptor-occupancy was highest. This temporal change in bound hCG shows good agreement with the findings of Hsueh et al. (1977) who eluted the bound hormone and measured it by radioimmunoassay. Therefore, the hCG-induced reduction in the *in vitro* binding of $^{125}$I-hCG at 20 h may, at least in part, be due to receptor-occupancy, but this explanation can not account for the even greater loss of binding observed at 48 h. This fact is perhaps even more evident when a low dose (0.1 IU) of hCG is injected, as *in vivo* binding of $^{125}$I-hCG is less than 50 pg per testis even at its peak (8 h), yet by 48 h the *in vitro* binding of $^{125}$I-hCG has been reduced by over 30 %.

The use of hCG in rats, even in low doses, is of questionable physiological significance in view of its long half-life (16–20 h; see Fig. 1) compared to rat LH (12 min; Bogdanove & Gay 1969). However, as shown here and by other workers (Hsueh et al. 1976) injection of ovine LH, which has a similar half-life to rat LH (Gay & Bogdanove 1968), has comparable effects to hCG in inducing LH-receptor loss. Indeed, experimental elevation of endogenous LH levels in the male rat also results in receptor loss (Auclair et al. 1977). In all

experiments reported to date, high (and presumably) unphysiological doses of hormone have been used to induce LH-receptor loss, and as LH levels in the male rat never normally show drastic elevations at any age (de Jong & Sharpe 1977), autoregulation of testicular LH-receptors would seem unlikely to be of physiological importance. However, the present findings show that receptor loss can be enhanced by sequential ("episodic") treatment with low doses of ovine LH as ten, 90-min spaced injections of 2 $\mu$g LH nearly trebled receptor loss compared with 20 $\mu$g LH given as a single injection. Exposure to relatively small amounts of LH continuously over a long period, which approximates the physiological state, would therefore seem to be the most effective means of causing receptor loss; this may also explain why hCG with its protracted half-life is so effective in this respect.

An hCG-induced reduction in available LH-receptors might be expected to increase the concentration of hCG required to stimulate a given output of testosterone *in vitro* and reported reductions in testosterone output (Hsueh et al. 1977; Sharpe 1977b) might be taken as evidence for this decreased 'sensitivity'. Against this interpretation is the fact that steroidogenic output was only reduced in animals given high doses of hCG and was not overcome by addition of dibutryl cyclic AMP (Sharpe 1977b). The present findings confirm that at 20 h after injection of 10 IU hCG there is a 50 % reduction in the steroidogenic capacity of the testis and as this can not be overcome by hCG in concentrations up to 45 nM, it rules out the possibility that the effect simply results from the fewer LH-receptors available. Furthermore, although sequential treatment with oLH caused a marked decrease in LH-receptors, *in vitro* sensitivity to hCG was not decreased but apparently slightly increased. This is perhaps surprising and requires further investigation as it may represent an acceleration of the normal maturational increase in testicular sensitivity to LH (Odell & Swerdloff 1976).

Exactly how LH and hCG exert their negative effects on LH-receptors and steroidogenesis is unknown. It was previously suggested (Sharpe 1977a) that autoregulation of LH-receptors might be dependent on protein synthesis in view of the ability of cycloheximide to partially inhibit the hCG-induced reduction in binding of $^{125}$I-hCG at 20 h after injection. The present data, while confirming the previous findings show that by 48 h the effectiveness of cycloheximide in preventing 'receptor-loss' has considerably diminished. Although this drug does not interfere with binding of LH/hCG to its receptor *in vitro* (Mendelson et al. 1975), it may indirectly reduce binding *in vivo* by preventing or delaying the hCG-induced increase in blood flow to the testis (Sharpe 1976, 1977a). Alternatively, as recent evidence in both the male and female rat suggests, LH-receptor complexes may be translocated within the cell (Ascoli & Puett 1977; Chen et al. 1977), and cycloheximide may interfere with this process. This would mean that at 20 h, but not 48 h, after injection of

hCG and cycloheximide, occupied receptors would still be located on the plasma membrane rather than internally (as a result of translocation), allowing free exchange between the bound hCG and the excess $^{125}$I-hCG added during *in vitro* incubation.

It appears certain that LH/hCG exerts its effect on LH-receptors on the one hand and steroidogenesis on the other in different ways. Steroidogenic responsiveness is only reduced by large unphysiological doses of hCG, and is a more transient effect than LH-receptor loss (Hsueh et al 1977; Sharpe 1977b). The fact that exposure to very small amounts of LH still caused marked LH-receptor loss without affecting overall steroidogenic output suggests that it is an everyday process which perhaps provides circumstantial support for the concept of translocation. More importantly it means that receptor-loss due to exposure to the normal endogenous levels of LH, being a continous low-level process, might go undetected and demonstration of the physiological significance of receptor autoregulation has to be achieved by using unphysiological doses of hormone to magnify the effect.

## Acknowledgment

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## DISCUSSION

*Payne:* What age are the rats which were used in your studies? I think we have to be very careful in regard to the age of the animals when making statements on changes in sensitivity of Leydig cells or testes after *in vitro* treatment with LH or hCG. Very different results may be seen during pubertal development when LH receptors and Leydig cell responsiveness to LH is rapidly changing compared to Leydig cells obtained from mature testes when LH receptors and Leydig cell responsiveness to LH are relatively constant.

*Sharpe:* The rats were of slightly varying ages but were all prepubertal.

I do think we need to be cautious, although I have found that even when one causes LH-receptor loss over 6–7 days when large maturational increases in testicular LH-receptors are occurring in control rats, it is still possible to meaningfully assess testicular responsiveness and sensitivity.

*Catt:* We have heard several times this morning about the marked differences that may be observed in receptor regulation and responses in rats according to the age of the animals employed for study. It would seem prudent to take the effects of age, hormone preparation, route of administration and other factors into account when comparing the results obtained in different laboratories, and to take care to repeat others conditions before concluding that a given result is or is not a reproducible finding. I was also interested by your comment on the possibility of continuous turnover of receptors and its magnification by procedures that increase plasma LH levels. We have felt for some time that the turnover of LH receptors is a process that may particularly involve occupied receptors, so that much of the turnover is of complexes formed by hormone-receptor interaction. It seems possible that the occupancy of receptors with

very high affinity by hormone might form a relatively irreversible complex that is processed and degraded, rather than vacated and reutilized. Do you have any views on the existence of such a mechanism?

*Sharpe:* I am becoming gradually more convinced that translocation of LH-receptor complexes may occur, perhaps routinely. I think translocation is consistent with practically all of the available data. Our findings with cycloheximide might be explained on the basis of this drug interfering with such a process as I have mentioned in our paper.

*Swerdloff:* Dr. Sharpe, how much testicular tissue obtained from biopsy was required for your receptor studies? What type of subjects were studied?

*Sharpe:* As much as we could get our hands on! Generally in the order of 4–8 mg replicates and therefore 20–30 mg per biopsy. The biopsies are from infertile men who are being sampled for testicular pathology.

*Cooke:* We have shown *in vitro* with Leydig cell suspension incubated with cycloheximide that it is possible to completely wash out the cycloheximide with 2 washes with buffer followed by centrifugation. This indicates that cycloheximide is not retained by the Leydig cells. The effects you have found with cycloheximide injected *in vivo* may indicate that you get a very rapid effect as I would not expect the cycloheximide to be retained for very long by the testis *in vivo*.

*Sharpe:* I have no information on how long cycloheximide is working for or how long it is retained intratesticularly in active form. I think it is more consistent with my findings if one assumes a longer action than you describe *in vitro*.

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# THE EFFECT OF INDUCED HYPERPROLACTINAEMIA ON LEYDIG CELL FUNCTION AND LH-INDUCED LOSS OF LH-RECEPTORS IN THE RAT TESTIS

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Anterior pituitary glands were transplanted beneath the kidney capsule of intact, adult male rats to induce hyperprolactinaemia. This resulted in reduced serum levels of LH and FSH and increased adrenal weight. In pituitary-transplanted rats, testicular hCG-receptor binding was increased by 55 to 175%, whilst the capacity of the testis to secrete testosterone in vitro was greatly reduced. Injection of ovine LH into control and pituitary-transplanted rats resulted in similar percentage reductions in hCG-receptor binding in the two groups. This treatment impaired the in vitro steroidogenic responsiveness of testes from control rats at 24 h after injection, but had no major effect on the already-impaired, steroidogenic responsiveness of testes from pituitary-transplanted rats.

Although induction of hyperprolactinaemia resulted in marked changes in Leydig cell function, these alterations were possibly due to the chronically reduced serum gonadotrophin levels in hyperprolactinaemic rats as well as a direct effect of prolactin on the Leydig cell.

The role of prolactin in the male is still unknown although the demonstration of specific prolactin receptors on the Leydig cell (Aragona and Friesen, 1975; Charreau et al., 1977) suggests that it exerts some effect at this level. There is evidence from a variety of situations to suggest that prolactin may augment the steroidogenic response to LH (for review see Bartke et al., 1977) perhaps by its reported effects on the activity of key steroidogenic enzymes (Hafiez et al., 1971; Musto et al., 1972). More recently, findings in the prolactin-deficient dwarf mouse (Bohnet and Friesen, 1976), in the seasonally-regressed hamster (Bex and Bartke, 1977) and in the hypophysectomized immature (Zipf et al., 1978) or adult (McNeilly et al., 1979) rat, all suggest that prolactin can increase the number of Leydig cell LH-receptors. The latter are negatively regulated by LH and hCG (Hsueh et al., 1976; Sharpe, 1976), and there is preliminary evidence to suggest that prolactin may also interfere with this process (Payne and Zipf, 1978). Therefore, in an attempt to clarify the role of prolactin in the intact rat we have examined the effect of experimentally-induced hyperprolactinaemia on Leydig cell function and on the LH-induced loss of LH-receptors.

## MATERIALS AND METHODS

### *Animals and treatments*

Adult male rats (120–150 days of age) of the inbred PVG strain were used both as donors and recipients of pituitary transplants. Two separate experiments were performed 6 months apart. In these, either one (Expt. 1) or two (Expt. 2) anterior pituitary glands were transplanted beneath the kidney capsule under ether anaesthesia to induce hyperprolactinaemia; control rats were sham-operated. In Expt. 1, animals were killed by decapitation 49 days after operation. In Expt. 2, rats received one of the following 3 treatments 28 days after operation. (1) A single subcutaneous injection of 0.5 ml 0.9% saline containing 0.2% bovine serum albumin (BSA, fraction V; Sigma) = vehicle. (2) A single subcutaneous injection of 250 μg NIH-LH-S18 in 0.5 ml vehicle. (3) 5 subcutaneous injections each of 50 μg LH-S18 in 0.5 ml vehicle, injections being given at 3-h intervals.

There were 8 sham-operated and 8 pituitary-transplanted rats in each of these 3 treatment groups. Rats were killed by cervical dislocation at either 24 or 48 h after the first injection. In both Expts. 1 and 2, trunk blood was collected for the subsequent determination of serum-hormone levels and the paired testes and adrenals were removed, cleaned of fat and weighed. Testes were decapsulated and halved and each half weighed. One hemi-testis from each rat was then frozen at −20°C for the subsequent determination of the in vitro binding of $^{125}$I-hCG whilst in Expt. 2 the remaining testicular tissue was used immediately to assess in vitro testosterone production.

### *In vitro binding of $^{125}$I-hCG*

Hemi-testes were thawed and individually homogenized in Krebs–Ringer bicarbonate solution (KRB, 1 ml/100 mg tissue) as described previously (Sharpe, 1976). Aliquots of 0.2 ml of this suspension (containing 20 mg tissue) were incubated in 63 × 11 mm polystyrene tubes together with 20 ng $^{125}$I-labelled hCG/ml for 3 h at 34°C in a shaking water bath. The $^{125}$I-labelled hCG (NIAMDD hCG CR119; 11 600 IU/mg) was prepared using lactoperoxidase (Miyachi et al., 1972); the labelled hormone used to measure hCG–receptor binding in Expt. 1 had a specific activity of 65 μCi/μg whilst that used in Expt. 2 had a specific activity of 68 μCi/μg. Assessment of the non-specific or non-displaceable binding of $^{125}$I-hCG and the separation procedure were as described previously (Sharpe, 1976). Receptor-occupancy was not assessed, but in other situations where large doses of LH or hCG have been injected into rats the degree of receptor-occupancy was minimal (Tsuruhara et al., 1977; Sharpe and McNeilly, 1978).

### *In vitro testosterone production*

Hemi-testes were placed individually into glass scintillation vials containing 1 ml KRB to which was added glucose (2 mg/ml), BSA (0.2%) and one of 4 concentrations of hCG (Pregnyl; Intervet). Vials were incubated for 4 h at 34°C in a shaking

water bath and the medium then aspirated, centrifuged for 5 min at 1500 *g* and the supernatant stored for 4 weeks at −20°C. Testosterone in the incubation medium was measured by radioimmunoassay (Corker and Davidson, 1978) with modifications as described by Sharpe et al. (1979). In previous studies, storage of testicular incubates for periods of up to 2 months did not affect the measurable content of testosterone in the medium.

*Serum-hormone measurements*

Serum levels of LH and prolactin were determined by radioimmunoassay using materials supplied by the NIAMDD (U.S.A.). The method used for the assay of LH was as described by Fraser and Sandow (1977) whilst prolactin was assayed according to the method of McNeilly and Friesen (1978). Serum levels of FSH were measured by radioimmunoassay as described previously (de Jong and Sharpe, 1977) using the method of Welschen et al. (1975).

Results were analysed by two-factor analysis of variance (with replication) and Student's *t* test.

## RESULTS

*Serum-hormone levels and organ weights*

In rats bearing one (Expt. 1) or two (Expt. 2) pituitary transplants, the serum levels of prolactin were increased relative to controls but only in the second of these groups was the increase statistically significant (Table 1). However, both groups of pituitary-transplanted rats had decreased serum levels of FSH and LH and showed increased adrenal weight (Table 1); testicular weight was similar in control and pituitary-transplanted rats.

*In vitro binding of $^{125}$I-hCG*

In rats bearing one pituitary transplant for 49 days (Expt. 1), the testicular binding of $^{125}$I-hCG was increased ($P < 0.001$) by 175% (278 ± 30 pg bound/20 mg testis; mean ± SD) when compared to control animals (102 ± 36 pg bound/20 mg testis). In rats bearing 2 pituitary transplants for 29–30 days (Expt. 2), testicular binding of $^{125}$I-hCG was increased ($P < 0.001$) by 55–88% when compared to controls (compare saline-treated groups in Table 2).

In both control and pituitary-transplanted rats in Expt. 2 a single injection of 250 μg ovine LH significantly reduced ($P < 0.001$) the in vitro testicular binding of $^{125}$I-hCG, this reduction being considerably greater ($P < 0.001$) at 48 h than at 24 h after injection (Table 2). Administration of the ovine LH as 5 sequential injections of 50 μg significantly increased ($P < 0.001$) its effectiveness at reducing the in vitro binding of $^{125}$I-hCG at 48 h, but not at 24 h, after injection (Table 2). Despite the fact that testicular tissue from pituitary-transplanted rats always bound significantly more ($P < 0.001$) $^{125}$I-hCG than tissue from control rats, injection of ovine

Table 1
Organ weights and serum-hormone levels in control and pituitary-transplanted rats (mean ± S.D.)

| Treatment group | Number of animals | Organ weights (mg) | | Serum-hormone levels (ng RP-1/ml) | | |
|---|---|---|---|---|---|---|
| | | Testes | Adrenals | Prolactin | FSH | LH |
| *Expt. 1* | | | | | | |
| Controls | 4 | 2587 ± 294 | 39.8 ± 3.3 | 34 ± 16 | 581 ± 50 | 55 ± 19 |
| Pituitary-transplanted | 6 | 2426 ± 162 | 48.3 ± 3.4 ** | 46 ± 28 | 344 ± 47 *** | 22 ± 10 ** |
| *Expt. 2* | | | | | | |
| Controls | 8 a) | 2745 ± 124 | 27.3 ± 2.7 | 22 ± 18 | 661 ± 131 | 46 ± 31 |
| Pituitary-transplanted | 8 a) | 2715 ± 149 | 36.8 ± 2.4 *** | 105 ± 46 *** | 489 ± 83 ** | 20 ± 10 * |

a) Data is for saline-treated rats only.

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$
} compared with respective control.

Table 2
The in vitro binding of $^{125}$I-hCG by testicular homogenates from control and pituitary-transplanted rats at 24 and 48 h after injection of saline or ovine LH

| Treatment group | Time when killed | Control rats | | Pituitary-transplanted rats | |
|---|---|---|---|---|---|
| | | pg bound/ 20 mg testis | % of saline-treated group | pg bound/ 20 mg testis | % of saline-treated group |
| Saline | 24 h | 144.5 ± 4.6 | (100) | 272.3 ± 24.1 | (100) |
| 1 × 250 μgLH | 24 h | 92.7 ± 16.2 | 64.2 | 171.0 ± 11.4 | 62.8 |
| 5 × 50 μgLH | 24 h | 86.6 ± 14.9 | 59.9 | 180.3 ± 12.4 | 66.2 |
| Saline | 48 h | 133.3 ± 13.1 | (100) | 206.9 ± 6.6 | (100) |
| 1 × 250 μgLH | 48 h | 52.5 ± 8.0 | 39.4 | 89.5 ± 8.2 | 43.3 |
| 5 × 50 μgLH | 48 h | 34.0 ± 11.9 | 25.5 | 59.1 ± 8.1 | 28.6 |

Each value is the mean ± S.D. for 4 rats.
Results were analysed by 2-factor analysis of variance (with replication) and significance values are given in the text.

LH caused similar percentage reductions in the binding of $^{125}$I-hCG in both groups (Table 2).

*In vitro testosterone production*

The influence of hyperprolactinaemia on the steroidogenic function of the testis was assessed by comparing testosterone production in vitro by testes from control (sham-operated) and pituitary-transplanted rats which had been injected with saline 24 or 48 h earlier. In controls, the mean basal production of testosterone (i.e. in the absence of in vitro hCG-stimulation) was 136 (range 108–184) ng/testis, rising to a maximum of 301 (273–564) ng/testis following incubation in the presence of either 45 or 4500 pM hCG (Fig. 1); incubation with 4.5 pM hCG had a small but insignificant effect on testosterone production. In pituitary-transplanted rats the basal production of testosterone in vitro was 71 (62–119) ng/testis, which was significantly lower ($P < 0.05$) than in controls, and incubation in the presence of hCG concentrations of up to 4500 pM failed to increase testosterone secretion significantly above the basal level (Fig. 1). Therefore, compared with controls, testes from pituitary-transplanted rats showed a much reduced ($P < 0.001$) capacity to secrete testosterone in vitro, i.e. reduced testicular responsiveness.

Following a single injection of 250 μg LH into control rats the capacity of the testis to secrete testosterone after incubation with hCG was reduced ($P < 0.05$) at 24 but not at 48 h after injection, when compared to controls injected with saline (Fig. 1); administration of the LH in 5 sequential injections had a similar but more pronounced ($P < 0.001$) effect (Fig. 1). Therefore, prior treatment of control rats

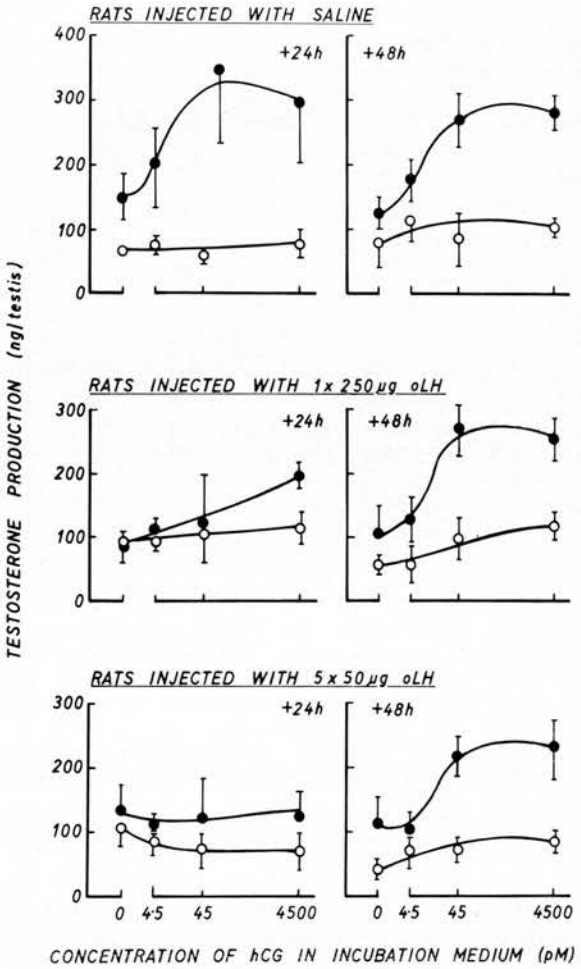



Fig. 1. Testosterone production in vitro by testes from control (•) and pituitary-transplanted (○) rats at 24 and 48 h after injection of saline or ovine LH. Each point is the mean ± S.D. of triplicate incubations.

with LH led to a reduction in testicular responsiveness in vitro 24 h later.

Injection of pituitary-transplanted rats with LH significantly increased ($P < 0.05$) the basal production of testosterone at 24 h when compared with saline-injected, pituitary-transplanted rats (Fig. 1). Otherwise, this treatment had no discernible effect, and maximal testosterone production in vitro (i.e. hCG-stimulated) by testes from pituitary-transplanted rats injected with saline or LH was similar and barely differed from the basal steroid production (Fig. 1).

## DISCUSSION

The present results show that elevation of blood levels of prolactin by pituitary transplants under the kidney capsule in adult male rats leads to marked changes in Leydig cell function. Although the serum levels of prolactin in rats bearing a single pituitary transplant (Expt. 1) in the present study were not significantly elevated above those found in controls, the clear increase in adrenal weight in these animals, which can be attributed to prolactin (Schlein et al., 1974), suggests that effective hyperprolactinaemia was achieved (see also McNeilly et al., 1978).

Confirming previous longitudinal studies (Bartke et al., 1977; McNeilly et al., 1978), the present data show that hyperprolactinaemia is associated with a chronic reduction in the serum levels of LH and FSH although, despite this change, there was no reduction in testicular weight. Induction of hyperprolactinaemia resulted in a clear increase in testicular hCG–receptor binding, a finding which is consistent with previous reports on the effects of prolactin in hypophysectomized or hormone-deficient animals (Bohnet and Friesen, 1976; Bex and Bartke, 1977; Zipf et al., 1978; McNeilly et al., 1979). Our most recent findings show that this is the result of an increase in the number of LH(hCG)–receptors per Leydig cell (unpublished data).

In a previous investigation using intact, pituitary-transplanted rats, we failed to demonstrate any increase in hCG–receptor binding (McNeilly et al., 1978). We now have evidence that this was due to a methodological problem associated with the earlier study in which testicular tissue was homogenized prior to freezing (rather than after thawing as in the present and most other studies) and was inadequately resuspended prior to incubation with $^{125}$I-hCG.

It is well established that LH(hCG) negatively regulates its Leydig cell receptor (Hsueh et al., 1976; Sharpe, 1976; Sharpe et al., 1979) and this 'autoregulation' is believed to occur continuously in vivo (Sharpe and McNeilly, 1978). Therefore, the increase in LH–receptor numbers in hyperprolactinaemic rats may be a consequence of a lower rate of LH–receptor loss, because of the chronic reduction in circulating LH levels, rather than the result of a direct effect of prolactin on the Leydig cell. It follows that, if the rate of LH–receptor synthesis is constant, lowering the LH levels by hyperprolactinaemia would be expected to increase LH–receptor numbers progressively with time, and this appears to be the case. Moreover, as injection of control and hyperprolactinaemic rats with ovine LH resulted in similar percentage reductions in LH–receptor numbers in both groups, this suggests that prolactin does not affect the autoregulation of Leydig cell LH–receptors in the intact rat. Payne and Zipf (1978) reached a different conclusion when they showed that injection of intact, adult rats with LH + prolactin for 3 days failed to reduce LH–receptor numbers to the levels found following treatment with LH alone. However, Payne and Zipf (1978) failed to control for the effect of prolactin alone, which presumably would have lowered endogenous LH levels and hence increased the LH–receptor concentration.

The ability of prolactin treatment to increase hCG–receptor binding in the hypophysectomized rat (Zipf et al., 1978; McNeilly et al., 1979) can only be explained by a direct effect of prolactin on the Leydig cell. Therefore, although the observed increase in hCG–receptor binding in intact, hyperprolactinaemic rats may be explained by the chronic reduction in serum levels of LH, a direct stimulatory effect of prolactin on the synthesis of Leydig cell LH–receptors cannot be excluded. It is also possible that, in physiological amounts, prolactin may have completely different effects to those observed during hyperprolactinaemia.

Lowering of endogenous gonadotrophin levels by hypophysectomy or immunization against LH-releasing hormone results in a severe reduction in the in vitro steroidogenic responsiveness of the rat testis (Hauger et al., 1977; Sharpe and Fraser, 1978). It is probable therefore that the marked reduction in in vitro testicular responsiveness in the hyperprolactinaemic rats is directly related to the chronic lowering of the blood levels of gonadotrophins. Other authors have found no effect of short-term prolactin treatment on testicular responsiveness in vivo (Zipf et al., 1978), although Bartke and his co-workers have long held that prolactin treatment can augment the in vivo steroidogenic response to LH in a number of experimental situations (reviewed by Bartke et al., 1978). Certainly, in the present study administration of LH to hyperprolactinaemic rats failed to have any stimulatory effect on subsequent steroidogenic function of the testis in vitro.

Treatment of control rats with ovine LH severely reduced testicular responsiveness at 24 h after injection, and this almost certainly reflects the characteristic impairment of steroidogenesis that results from over-stimulation of the testis with LH/hCG (Sharpe, 1977; Tsuruhara et al., 1977; Sharpe et al., 1979). No inhibitory effect of LH was observed in hyperprolactinaemic rats, but this may have been because steroidogenic responsiveness was already greatly reduced in these animals.

Finally, it should be pointed out that the maximum steroidogenic response of control rat testes in the present investigation (performed in October) was only 273–564 ng/testis which is less than 50% of that observed in rats of this and other strains at other times of the year (Sharpe and Fraser, 1978; Sharpe, unpublished data), and may be a 'seasonal' effect similar to that reported by other workers (de la Torre et al., 1977).

To conclude, although the present results show that induction of hyperprolactinaemia leads to marked changes in Leydig cell function in the intact rat, these alterations may result from the chronic lowering of serum gonadotrophin levels as well as a direct effect of prolactin on the Leydig cell.

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# A Comparison of the Effectiveness of FSH, LH and Prolactin in the Reinitiation of Testicular Function of Hypophysectomized and Estrogen-Treated Rats

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## ABSTRACT

The effects of FSH, LH and prolactin on the testes and accessory sex organs of hypophysectomized and estrogen-treated rats were compared. In both types of animals there was a similar reduction in the weights of the reproductive organs and in the numbers of resting and pachytene spermatocytes, although greater numbers of step 7 spermatids were lost in estrogen-treated rats. Levels of FSH, but not LH or prolactin, were reduced in estrogenized animals.

In rats hypophysectomized for 28 days, treatment with FSH increased the numbers of all germ cells examined while accessory sex organ weights were unchanged. Conversely, treatment with LH stimulated accessory sex organ weights, but not germ cell numbers. In estrogen-treated rats, FSH increased the numbers of step 7 spermatids and pachytene spermatocytes, but to a lesser extent than was found in hypophysectomized animals. In both hypophysectomized and estrogen-treated rats, prolactin inhibited the stimulatory effects of FSH. Injections of LH produced no change in accesory sex organ weights or germ cell numbers in estrogen-treated rats.

It is concluded that estradiol directly inhibits the action of gonadotrophins on the testis.

## INTRODUCTION

The administration of estrogens to male rats has been shown to induce a regression of the reproductive system similar to that found after hypophysectomy (Lacy, 1963; Lacy et al., 1973). This effect has been shown to be associated with a reduction in gonadotrophic hormone secretion (Greep and Jones, 1950; Swerdloff and Walsh, 1973; Verjans et al., 1974). Accordingly, estrogen treatment has been used as an alternative to hypophysectomy in the investigation of the hormonal control of spermatogenesis in the rat (Lacy and Lofts, 1965; Steinberger and Duckett, 1965) and mouse (Davies et al., 1974). Furthermore, estrogen preparations have been used as components of an oral contraceptive for males (Lacy et al., 1973; Briggs and Briggs, 1974).

There is some evidence, however, which suggests that the effects of estrogens on the reproductive system are not exclusively mediated through the suppression of pituitary function. Various workers have shown that estrogens directly inhibit the activity of various enzymes involved in androgen synthesis (Samuels et al., 1964; Oshima et al., 1967; Yanaihara and Troen, 1972) and may produce a decrease in circulating testosterone levels while LH levels remain within normal limits (Chowdhury et al., 1974; Danutra et al., 1973). The function of the accessory sex organs may also be directly affected by estrogens since treatment with these hormones has been shown to alter the uptake (Giorgi et al., 1972) and subsequent metabolism (Leav et al., 1971) of androgens by the prostate.

The proper interpretation of data relating to the use of estrogens in applied and experimental studies depends on an accurate assessment of the mode of estrogen action. To provide information of this nature we 1) determined serum FSH, LH and prolactin levels in animals treated orally with ethinyl estradiol

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and 2) compared the effects of FSH, LH and prolactin when injected into hypophysectomized or estrogen-treated rats.

## MATERIALS AND METHODS

Mature male Wistar rats weighing 220–240 g were kept in controlled environmental conditions and were allowed free access to commercial diet and tap water. Thirty animals hypophysectomized by the parapharyngeal route were obtained from Anglia Laboratories Ltd. and were given rock salt in addition to their normal diet.

### *Experimental Design*

The day of hypophysectomy or the first day of estrogen treatment was designated as Day 0. Twenty-eight days after hypophysectomy, animals were divided into 5 groups of 6 animals each and were injected i.m. with LH (NIH-LH-S18), FSH (NIH-FSH-S10) and/or prolactin (NIH-P-S9) daily until Day 42 (Table 1). The hormones were given at a dose of 100 μg/100 g body weight in 0.1 ml of 0.9% (w/v) NaCl. The animals were weighed every 7 days throughout the experimental period and when the growth curves indicated incomplete hypophysectomy, the animal was eliminated from the study.

Thirty intact rats were treated orally with ethinyl estradiol (17α-ethinyl-1,3,5(10-estrariene-3, 17βdiol, Schering, A. G., Berlin) at a dose of 100 μg/100 g body weight daily for 42 days. The estrogen was administered in 1% sodium carboxymethyl cellulose by using a hypodermic syringe fitted with a short length of plastic tubing which was gently inserted into the esophagus. After 28 days of estrogen treatment, the animals were divided into 5 groups of 6 animals and were treated with gonadotrophins and/or prolactin as described above for hypophysectomized animals.

### *Treatment of Material*

On day 42, all animals were killed by decapitation. The testes, epididymides, seminal vesicles (full), coagulating glands, ventral prostrate and adrenal glands were dissected out and weighed. The sella turcica of the hypophysectomized rats was examined and animals with pituitary fragments were discarded.

Small pieces of testes and epididymis were fixed in Bouin's fixative, embedded in parafin wax, sectioned at 4 μm and stained in Heidenhain's iron-haematoxylin and orange-G for general histological examination. Spermatogenesis was evaluated by the method of Clermont and Harvey (1967). Only round cross sections of tubules at stage VII of the cycle of the seminiferous epithelium were selected (Leblond and Clermont, 1952). The nuclei of Sertoli cells, resting (preleptotene) spermatocytes, pachytene spermatocytes and step 7 spermatids were counted. These crude counts were converted into true counts by the formula of Abercrombie (1946) and were corrected by the 'Sertoli cell factor' to eliminate the bias introduced by linear shrinkage or expansion of the tubules caused by the histological procedures or hormonal treatments (Clermont and Morgentaler, 1955; Clermont and Harvey 1967).

TABLE 1. Mean (± SEM) body and organ weights of hypophysectomized (hypox) and estrogen-treated rats given gonadotrophins and/or prolactin.

| Treatment | No. of animals | Body weight (g) | | Testes (mg) | | Epididymides (mg) | | Seminal vesicles (mg) | | Coagulating glands (mg) | | Prostate (mg) | | Adrenal glands (mg) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Intact control | 6 | 395 | 8 | 3088 | 136 | 1000 | 19 | 1105 | 88 | 186 | 19 | 414 | 23 | 51 | 3 |
| Hypophysectomized | 6 | 196 | 4 | 565 | 120 | 154 | 15 | 34 | 1 | 13 | 1 | 14 | 1 | 19 | 3 |
| Ethinyl estradiol E | 6 | 241 | 9 | 573 | 44 | 172 | 10 | 49 | 3 | 10 | 1 | 16 | 2 | 85 | 3 |
| Hypox + FSH | 4 | 212 | 1 | 1370 | 196[b] | 209 | 27 | 36 | 4 | 11 | 2 | 9 | 1 | 12 | 1 |
| E + FSH | 6 | 241 | 9 | 914 | 82[b] | 173 | 10 | 53 | 21 | 10 | 8 | 19 | 2 | 89 | 4 |
| Hypox + LH | 6 | 217 | 3 | 994 | 42[a] | 297 | 10[b] | 184 | 21[b] | 42 | 9[b] | 65 | 15[b] | 11 | 1 |
| E + LH | 5 | 233 | 6 | 648 | 28 | 148 | 18 | 409 | 1 | 12 | 7 | 21 | 9 | 75 | 4 |
| Hypox + prolactin | 5 | 199 | 3 | 426 | 36 | 127 | 5 | 25 | 1[a] | 7 | 1[a] | 9 | 1[a] | 11 | 2 |
| E + prolactin | 6 | 251 | 10 | 591 | 32 | 163 | 7 | 51 | 7 | 13 | 1 | 21 | 9 | 75 | 4 |
| Hypox + FSH + prolactin | 4 | 203 | 2 | 1188 | 81[b] | 187 | 18 | 24 | 3[a] | 8 | 2[a] | 13 | 1 | 11 | 1 |
| E + FSH + prolactin | 6 | 241 | 4 | 772 | 69[a] | 179 | 15 | 56 | 6 | 14 | 1 | 21 | 6 | 77 | 5 |

Values significantly different from those of hypophysectomized or estrogen treated controls; [a]$P<0.05$, [b]$P<0.01$—body weights excluded.

*Hormone Assays*

Trunk blood was collected from hypophysectomized and estrogen-treated control animals and was allowed to clot at 4°C for 2 h before centrifugation at 700g for 15 min. The serum was removed and stored at −20°C until assay. Serum FSH, LH and prolactin were measured by double antibody radioimmunoassays using reagents supplied by NIAMDD, Bethesda, MD. The reference preparations, antiserum and hormones for iodination were of the first series (RP-1, anti rat S1 and I-1, respectively) for each assay. The iodination and assay techniques were identical to those previously described for human FSH, LH and prolactin (McNeilly and Hagen 1974; McNeilly and Andrews 1974). All samples were assayed in a single assay to avoid interassay variation. The intra-assay coefficients of variation estimated by assaying quality control sera at 3 different concentrations were 11% for FSH, 8% for LH and 7% for prolactin. The assay sensitivities were 65 ng/ml for FSH, 7 ng/ml for LH and 4ng/ml for prolactin.

## RESULTS

*Body and Organ Weights*

The effects of the hormonal replacement therapy on the weights of the testes and accessory sex organs are shown in Table 1.

After hypophysectomy or estrogen-treatment, there was a significant reduction in the weights of the testes, epididymides and accessory sex organs. Although the final body weights differed slightly between groups, none of the treatments promoted an increase in body weights over the experimental period.

Testicular weight of hypophysectomized and estrogen-treated rats increased significantly after treatment with FSH or FSH plus prolactin. A small increase in testis weight was recorded following administration of LH to hypophysectomized but not estrogen-treated animals.

The weights of the epididymides, seminal vesicles, coagulating glands and prostrate were significantly increased in hypophysectomized rats receiving LH, but no increase in the weights of these organs occured in estrogen-treated rats given LH. Treatment with prolactin alone failed to affect testicular weight in either hypophysectomized or estrogen-treated animals. In hypophysectomized rats, the accessory sex organ weights were significantly reduced after administration of prolactin alone or prolactin plus FSH.

The weights of the adrenal glands were significantly reduced after hypophysectomy and were unchanged by any of the treatments. The increased adrenal weights found in all rats treated with estrogen was not affected by treatment with gonadotrophins and/or prolactin.

*Histology of the Seminiferous Tubule*

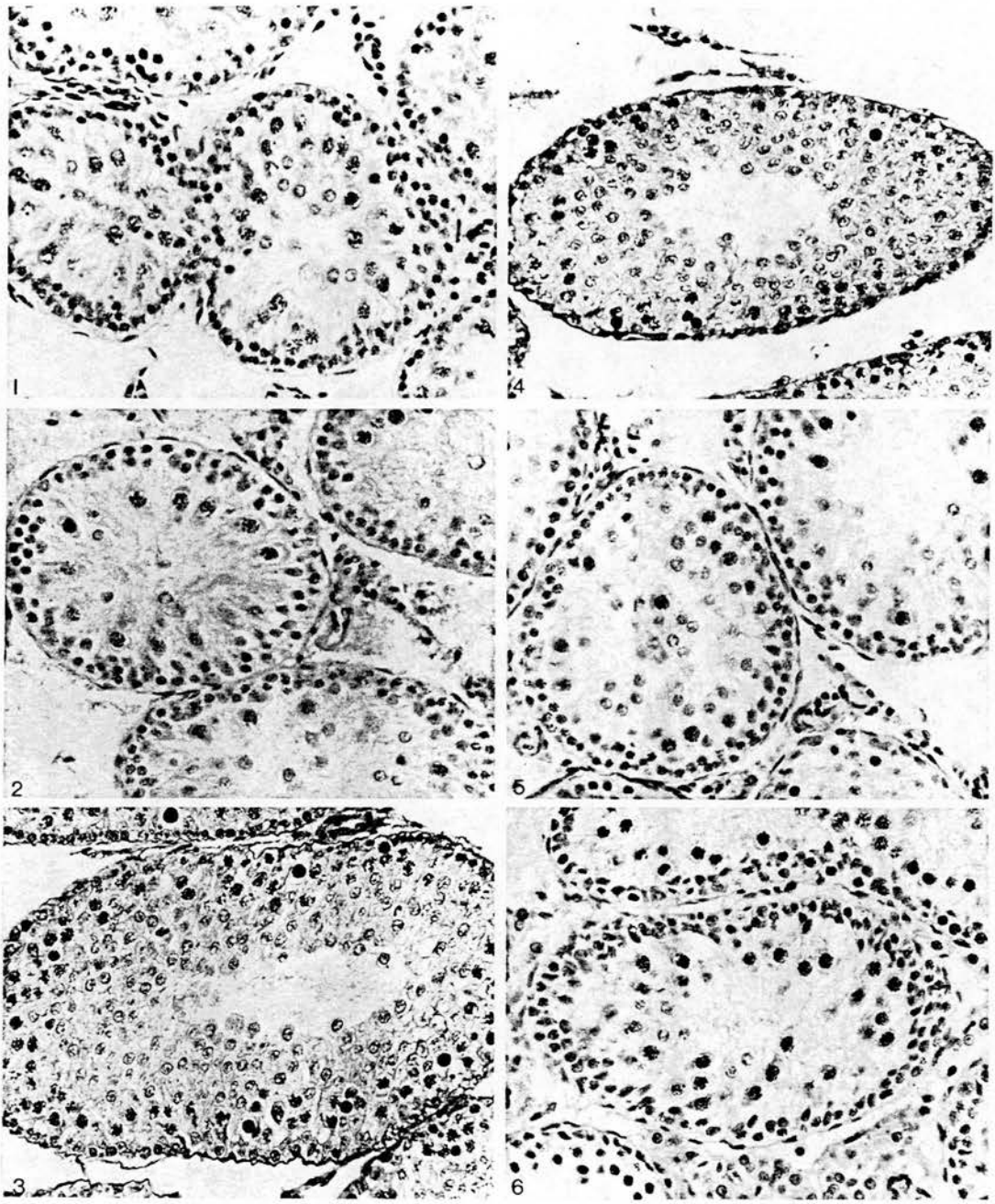
Examples of the histology of the seminiferous tubules from rats receiving various hormone combinations are shown in Plate 1 (Figs. 1–6) and the corrected cell counts are shown in Figs. 7–9.

*No therapy.* In hypophysectomized animals, there was some variation in the degree of damage seen in different tubules, but germ cell development had not proceeded beyond step 7 of spermiogenesis. In severly damaged tubules, the germinal epithelium was represented by Sertoli cells, spermatogonia and resting spermatocytes. In other tubules, a small number of older spermatocytes and Golgi or cap phase spermatids were also present although the cells were often desquamated into the lumen (Fig. 1). The quantitative aspects of spermatogenesis was severely affected in all tubules. The numbers of resting and pachytene spermatocytes and step 7 spermatids were significantly reduced from control values of 19.252 ± 0.288, 17.373 ± 0.755 and 61.411 ± 2.617, respectively ($P < 0.001$ in all cases).

The general appearance of the tubules from animals treated only with estrogen was similar to that found in hypophysectomized rats (Fig. 2), as was the numbers of resting and pachytene spermatocytes. However, the numbers of step 7 spermatids were significantly lower than that found in hypophysectomized animals ($P < 0.001$).

*FSH treatment.* Hypophysectomized animals receiving FSH showed an obvious increase in tubular diameter compared with hypophysectomized controls and the germ cells were well organized into the cellular associations characteristic of intact animals (Fig. 3). There had been a significant increase in the numbers of resting ($P < 0.001$, Fig. 9) and pachytene spermatocytes ($P < 0.01$, Fig. 8) and step 7 spermatids ($P < 0.001$, Fig. 7) compared with hypophysectomized animals receiving no therapy.

There was also an obvious stimulation of spermatogenesis in estrogen-treated animals given FSH, but the germinal epithelium was poorly organized and the lumen of the tubules was often completely occluded with displaced spermatids. In spite of a significant increase



Representative sections of the testes of hypophysectomized and estrogen-treated rats given gonadotrophin replacement therapy.

FIG. 1. Hypophysectomized (Hypox) control. The most mature germ cell present was the step 7 spermatid. The numbers of spermatids, spermatogonia and spermatocytes were considerably reduced. X440.

FIG. 2. Estrogen (E) treated control. Essentially similar in appearance to hypophysectomized control animals except that fewer spermatids and spermatocytes were present. X450.

FIG. 3. Hypox + FSH. Increased numbers of step 7 spermatids and pachytene spermatocytes. X440.

FIG. 4. E + FSH. The increase in numbers of step 7 spermatids and pachytene spermatocytes not as great as that found in Hypox + FSH. X420.

FIG. 5. Hypox + LH. Tubules similar in appearance to Hypox control. X430.

FIG. 6. E + LH. Tubules similar in appearance to estrogen treated control. X450.

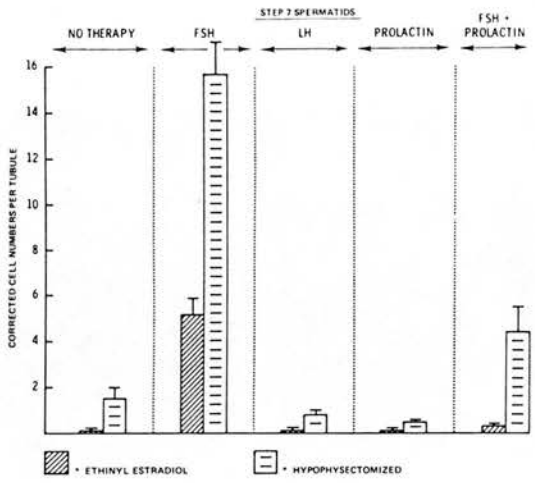



FIG. 7. Number of step 7 spermatids.

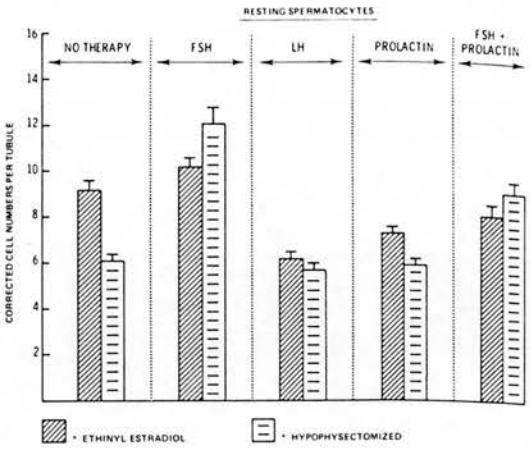



FIG. 9. Number of resting spermatocytes.

in the numbers of pachytene spermatocytes and step 7 spermatids, the numbers of these germ cells was significantly lower than that found in hypophysectomized rats receiving FSH ($P<0.001$ Hypox. + FSH vs. estrogen + FSH, Figs. 7, 8).

*LH treatment.* In hypophysectomized rats given LH, the general appearance of the seminiferous epithelium resembled that of the hypophysectomized controls and there was no improvement in the numbers of germ cells examined. In estrogen-treated animals, the qualitative and quantitative aspects of spermatogenesis were generally similar to those seen in animals receiving ethinyl estradiol alone.

*Prolactin treatment.* The tubules of all the animals receiving prolactin resembled those of the appropriate control animals receiving no therapy.

*FSH plus prolactin.* While the general development and organization of the tubules in hypophysectomized animals given FSH plus prolactin was similar to that found in hypophysectomized rats receiving FSH alone; the increase in germ cell numbers was significantly less ($P<0.001$ FSH + prolactin vs. FSH alone, Figs. 7–9). There was no increase in germ cell numbers in estrogen-treated rats and the tubules resembled those from animals receiving only estrogens.

*Serum Hormone Levels.* These are shown in Table 2. Treatment with ethinyl estradiol significantly reduced the levels of serum FSH whilst those of LH and prolactin did not change.

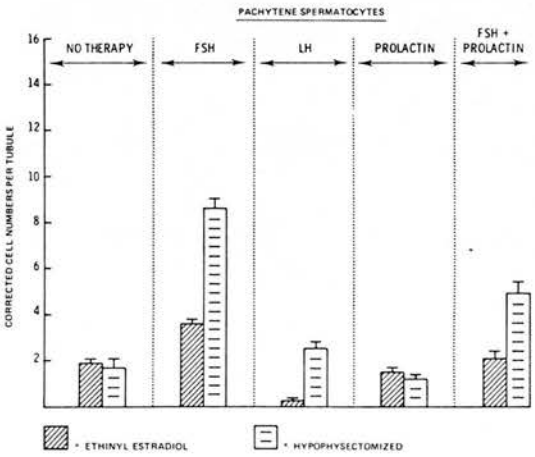



FIG. 8. Number of pachytene spermatocytes.

## DISCUSSION

In the present study, ethinyl estradiol induced a regression of the reproductive system similar to that which followed hypophysectomy. This had been anticipated since the dose used was in excess of that previously found to inhibit spermatogenesis when given orally (Lacy et al., 1973). This dose was selected so that the testes would regress maximally within the experimental period. It is still lower than that used in previous studies in the male (Grayhack, 1965; Lacy and Lofts, 1965).

It is clear from the levels of circulating gonadotrophins shown in Table 2 that the method by which ethinyl estradiol produced testicular and accessory sex organ regression

TABLE 2. Serum FSH, LH and prolactin levels in estrogen-treated and hypophysectomized rats (mean ± SEM).

| Treatment | FSH (ng/ml) | LH (ng/ml) | Prolactin (ng/ml) |
|---|---|---|---|
| Intact control | 447.4 49.4[a] | 25.4 3.7 | 120.20 14.9 |
| Ethinyl estradiol | 165.1 13.3 | 25.4 1.5 | 142.8 7.7 |
| Hypophysectomized | ND | ND | ND |

ND = Not detectable.

[a] = Significantly different, $P<0.001$.

differed from that found in hypophysectomized animals. In the latter, regression of the reproductive system occurred in the absence of gonadotrophins whereas in estrogen-treated rats, this event was associated with a reduction of FSH levels only, those of LH and prolactin remaining unchanged. This finding is in agreement with the observation of Mallampati and Johnson (1973) who found that serum FSH levels were reduced, serum LH levels slightly raised and accessory sex organ weights reduced in rats treated with 50 $\mu$g of estradiol benzoate. Similarly, Chowdhury et al. (1974), using 50 $\mu$g of estradiol-17$\beta$, also found that accessory sex organ regression occurred in the presence of normal LH levels. Like Danutra et al. (1973), Chowdhury et al. (1974) found that although serum LH levels were normal in estrogen-treated rats, serum testosterone levels were significantly reduced. It appears, therefore, that in the present study, ethinyl estradiol may have induced accessory sex organ atrophy by directly blocking the action of endogenous LH on the Leydig cells. The fact that LH levels were not reduced with a dose of estrogen higher than that used in the studies quoted above may be due to the oral route of administration. This could have resulted in a lower amount of estrogen reaching the pituitary than would have occurred if a similar dose had been given i.m.

Estrogen treatment also inhibited the action of exogenous LH because the accessory sex organ weights were not increased by such treatment as they were in hypophysectomized rats. From the present study, it is not possible to determine the exact mechanism of this inhibition. It may have resulted from a block of the stimulatory effect of LH on steroid production by the interstitial tissue. Moger (1976) has found that acute treatment with LH resulted in a smaller increase in serum testosterone levels in estradiol-treated rats than in intact controls. Alternatively, in the present study, LH may have stimulated androgen production by the interstitial tissue while estradiol inhibited the action of testosterone on the accessory sex organs. An effect of this type has been reported by Leav et al. (1971) who demonstrated decreased metabolism of testosterone to dihydro-testosterone in estrogen-treated dogs.

In hypophysectomized and estrogen-treated rats, the considerable reduction in the numbers of step 7 spermatids and pachytene spermatocytes relative to resting spermatocytes suggests a great loss of cells during meiotic prophase and subsequent maturation divisions. These stages of spermatogenesis appear to be more sensitive to estrogens than to hypophysectomy because fewer spermatids were lost in hypophysectomized rats, although comparable numbers of pachytene and resting spermatocytes were lost in both groups of animals. Therefore, although the two treatments were identical qualitatively, estrogen-treatment appeared to produce slightly more testicular damage. Impairment of spermatogenesis in estrogen-treated rats may have resulted from a 65% reduction in circulating FSH levels. However, the fact that spermatogenesis was quantitatively inferior in estrogenized rats when compared with hypophysectomized animals, in spite of the presence of some circulating FSH, suggests that estrogens were directly interfering with tubular function.

Further evidence for this direct effect is apparent when the effects of exogenous FSH on hypophysectomized and estrogen-treated rats are compared. In hypophysectomized

animals, FSH appeared to stimulate all stages of spermatogenesis as judged by the increased numbers of each cell type examined. In estrogen-treated rats, however, FSH produced no increase in the numbers of step 7 spermatids and pachytene spermatocytes. While a similar proportion of pachytene spermatocytes completed the reduction division and subsequent development into step 7 spermatids, in both cases, the relative numbers of resting spermatocytes maturing to pachytene spermatocytes was considerably lower in estrogen-treated rats than hypophysectomized animals. Ethinyl estradiol appeared to have produced a partial inhibition in the response of the germinal epithelium to FSH, mainly by restricting the development of resting spermatocytes. This direct effect of estradiol on the siminiferous tubules has not been reported before.

The gametogenic effect of FSH was not accompanied by any stimulation of the accessory sex organs and the increased accessory sex organ weights found in hypophysectomized rats receiving LH was not associated with any stimulation of the germinal epithelium. The selective action of these gonadotrophic hormones is in general agreement with the original observations of Greep and Fevold (1937) and suggests that FSH has a specific action on the early stages of the reinitiation of spermatogenesis in hypophysectomized rats in which the testes have regressed.

In both hypophysectomized and estrogen-treated animals, prolactin appeared to have an antigonadal effect. Thus, in hypophysectomized rats treated with prolactin alone or prolactin plus FSH, there was a decrease in accessory sex organ weights. This may represent a direct inhibitory effect of prolactin on accessory sex organ growth or may result from prolactin blocking the action of some residual circulating androgens. When given with FSH, prolactin had an antispermatogenic effect. In hypophysectomized rats treated with FSH plus prolactin, there was a smaller increase in germ cell numbers than in animals receiving FSH alone and, in estrogen-treated rats given FSH plus prolactin, there was no increase in germ cell numbers at all. This specific anti-FSH effect of prolactin has not previously been observed in the rat, although it has been demonstrated in the pigeon (Bates et al., 1937). Fang et al. (1974) have reported testicular involution, arrest of spermatogenesis and regression of the accessory sex organs in rats with high circulating prolacting levels resulting from prolactin secreting tumours. Since serum testosterone levels were reduced but FSH levels normal and LH levels only slightly raised, Fang et al. (1974) suggested that prolactin may have blocked the action of gonadotrophins on the testis. Similarly men with galactorrhoea due to hyperprolactinaemia have been found to be hypogonadal although serum FSH and LH levels were normal (Thorner et al., 1974). These reports raise the question as to whether the testicular regression found in estrogenized animals may have been due, in part, to a rise in circulating prolactin levels since estrogens have been shown to have this effect in the male (Danutra et al., 1973; Mallampati and Johnson 1973). However, in the present study, prolactin levels in estrogen-treated rats did not differ significantly from untreated controls, although prolactin levels in the latter were higher than is usually found in intact male rats. This may have been due to stress induced by moving the animals on the day of killing in spite of using decapitation to eliminate stress immediately prior to killing. It is possible that these high values may have masked an increase in prolactin levels following estrogen administration, although it is doubtful if any raised prolactin levels for the short duration of the experiment could account for such severe damage seen in the seminiferous tubules of estrogen-treated rats since, in the study of Fang et al. (1974), some 20 weeks of hyperprolactinaemia were required to produce a 45% reduction in testis weight.

The current investigation has shown that the deleterious effects of estrogens can occur in the presence of normal levels of LH. Estrogen treatment completely negated the stimulatory effects of LH on the Leydig cells and partly counteracted the trophic effects of FSH on the germinal epithelium. The effects of estrogens cannot, therefore, be solely attributed to their inhibitory effect on the pituitary, but are also due to a direct action on the testis and accessory sex organs. This effect should be taken into account when interpreting data from studies of testis function involving oestrogen administration.

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**Leydig cell function in hyperprolactinaemic adult rats.** By R. M. Sharpe, A. S. McNeilly, D. W. Davidson and I. A. Swanston. *MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh, EH3 9EW*

We have examined Leydig cell function in intact adult rats in which hyperprolactinaemia was induced 21 days previously by the transplantation of two anterior pituitary glands beneath the kidney capsule. As reported previously (McNeilly, Sharpe, Davidson & Fraser, 1978), this treatment chronically reduced ($P<0{\cdot}001$) the serum concentrations of both luteinizing hormone (LH) and follicle-stimulating hormone, but did not significantly alter the serum levels of testosterone (controls, 2·5; range 0·6–14·0 ng/ml; $n=34$; hyperprolactinaemic, 3·1; range 0·6–12·2 ng/ml; $n=35$). Mean paired testicular weight was slightly but significantly ($P<0{\cdot}01$) reduced from $2592\pm125$ (S.D.) mg in controls to $2471\pm141$ mg in hyperprolactinaemic rats, whilst seminal vesicle weight was similar in either group. Although these data suggest that normal Leydig cell function was maintained in hyperprolactinaemic rats, studies *in vitro* revealed a different picture.

As determined by the binding of $^{125}$I-labelled human chorionic gonadotrophin (HCG) *in vitro* to isolated Leydig cells, the number of LH(HCG) receptors per Leydig cell was 61% higher in hyperprolactinaemic than in control rats, although binding affinity remained unaltered. Leydig cell responsiveness was determined by incubating decapsulated testes *in vitro* as described previously (Sharpe & McNeilly, 1979). The basal production of both pregnenolone and testosterone by testes from hyperprolactinaemic rats was reduced ($P<0{\cdot}01$) to less than half of control values, and a similar reduction was observed in the maximal steroidogenic response to either HCG or dibutyryl cyclic AMP *in vitro*. In contrast, when pregnenolone (100 μg/ml) was added as substrate to the incubation medium, the basal production of testosterone by decapsulated testes from hyperprolactinaemic and control rats was similar (controls, $183\pm12$ ng/testis, $n=3$: hyperprolactinaemic, $213\pm18$ ng/testis), although the maximal response to HCG stimulation *in vitro* was still reduced by 40% in the former group.

As the conversion of cholesterol to pregnenolone is rate-limiting and LH-dependent, it is concluded that the altered in-vitro steroidogenic responsiveness of Leydig cells from hyperprolactinaemic rats stems primarily from the prolactin-induced reduction in levels of LH in the circulation. This may also partly explain the increase in LH-receptor numbers, although in this respect, it is known that prolactin can have direct effects on the Leydig cell (McNeilly, de Kretser & Sharpe, 1979). However, these results offer no explanation for the maintenance of apparently normal serum concentrations of testosterone in hyperprolactinaemic rats despite the chronic lowering of the serum levels of LH.

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BRITISH MEDICAL JOURNAL 29 JULY 1972



# Hormonal Responses to Synthetic Luteinizing Hormone and Follicle Stimulating Hormone-Releasing Hormone in Man

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## Summary

The effects of the gonadotrophin-releasing hormone, synthetic decapeptide luteinizing hormone/follicle stimulating hormone-releasing hormone (LH/FSH-RH), have been studied in 18 normal men and five women in the follicular phase of their menstrual cycle. Rapid and dose-dependent (25 to 100 μg) increases in serum immunoreactive LH were seen, which reached a peak 20 to 30 minutes after a rapid intravenous injection. Similar but much smaller increases in serum immunoreactive FSH were seen. These conclusions have been validated by using two different immunoassay systems for each hormone. The LH/FSH-RH therefore causes both LH and FSH release in man as in animals but does not affect growth hormone, thyrotrophin, or ACTH. The gonadotrophin responses were the same in the women as in the men but were insufficient in the men to cause statistically significant changes in the serum levels of the gonadal steroid hormones, testosterone or oestradiol, or in their precursors 17 α-hydroxyprogesterone or progesterone. In the women, however, there was a rise in oestradiol after the 100-μg doses. The use of LH/FSH-RH will provide an important test to define the level of the lesion in hypogonadal patients and also should be valuable in the treatment of some types of male and female in-

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TABLE II—*Basal Serum LH and FSH Values in Intensively Studied Subjects*

| Dose | LH (mU/ml) | | FSH (mU/ml) | |
|---|---|---|---|---|
| | Assay 1 | Assay 2 | Assay 1 | Assay 2 |
| Six males* | | | | |
| 25 μg .. .. | 2·3 ± 0·5 | 2·2 ± 0·3 | 1·4 ± 0·2 | 5·0 ± 1·3 |
| 50 μg .. .. | 2·2 ± 1·1 | 2·5 ± 0·4 | 1·7 ± 0·2 | 5·7 ± 0·8 |
| 100 μg .. .. | 1·2 ± 0·6 | 2·8 ± 0·7 | 1·1 ± 0·4 | 3·4 ± 1·6 |
| Two females† | | | | |
| 50 μg .. .. | 0·7, 0·9 | 3·1, 2·8 | 3·1, 2·9 | 5·2, 5·7 |
| 100 μg .. .. | 1·5, 2·0 | 1·9, 1·9 | 1·9, 2·7 | 4·6, 3·1 |

*Mean ± 1 S.E. given.
†Individual values given.

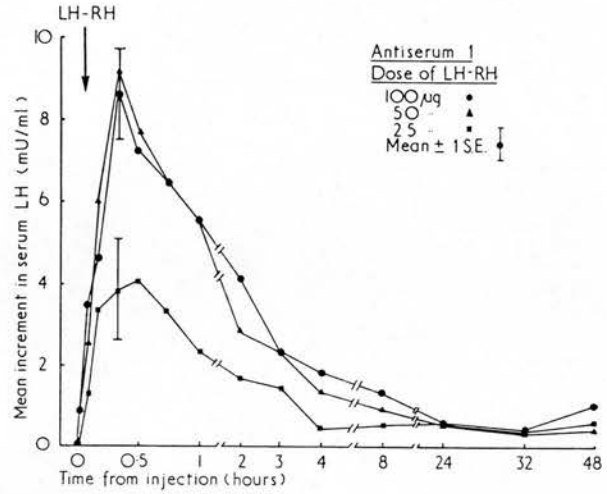
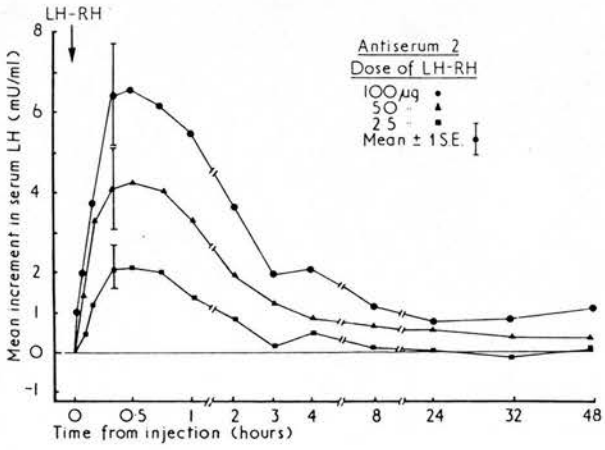



FIG. 1—LH assay 1. Mean increments in serum LH after 25, 50, and 100 μg LH/FSH-RH in six normal males. Four subjects were studied at each dose level.




IG. 2—LH assay 2. Legend as for Fig. 1.

The *FSH* levels showed similar but much smaller initial hanges compared with the LH results. With assay 1 (Fig. 3) a ignificant rise was seen with each dose and there was a definite ose-response relationship. However, in every subject the ıcrease in serum FSH was small and in no case did the re-onse exceed the upper limit of the normal male range (4·5 ıU/ml). With assay 2 (Fig. 4) a definite FSH response was en with only 50 and 100 μg doses of LH/FSH-RH and the ffects appeared to be sustained for several hours longer.

The LH and FSH responses in the two women given 50 and 00 μg LH/FSH-RH in their follicular phases did not differ om the results obtained in the males and reached their peak 20 to 30 minutes after the injection.

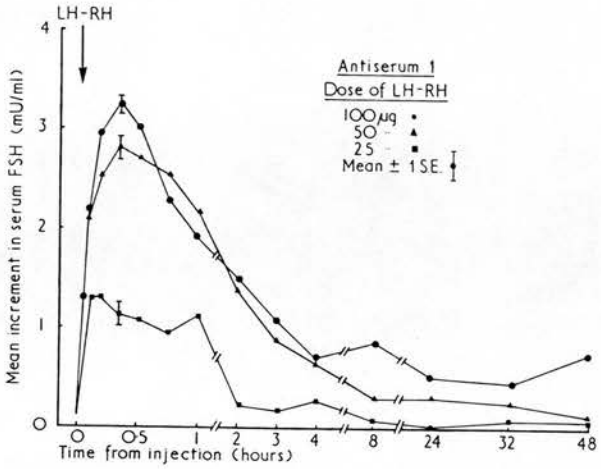



FIG. 3—FSH assay 1. Mean increments in serum FSH after 25, 50, and 100 μg LH/FSH-RH in six normal males. Four subjects were studied at each dose level.

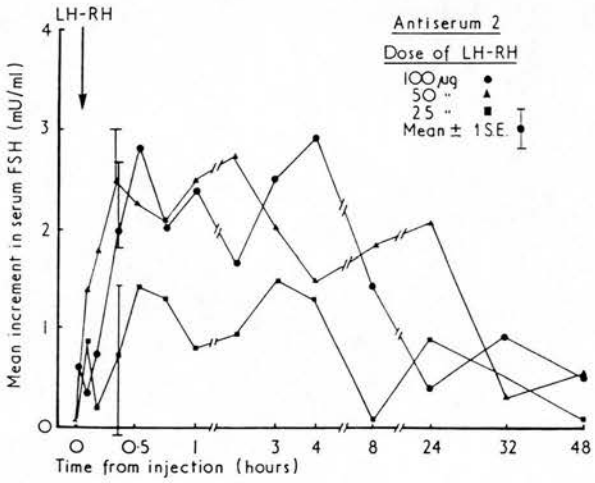



FIG. 4—FSH assay 2. Legend as for Fig. 3.

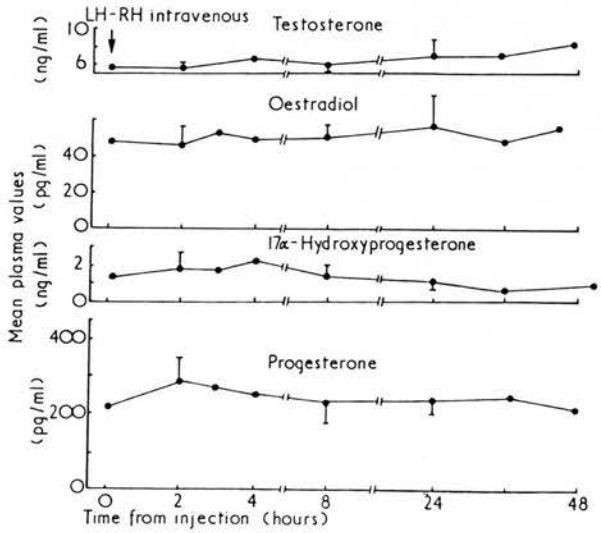



FIG. 5—Effects on gonadal steroid hormones of an intravenous injection of 100 μg LH/FSH-RH into four male subjects. Mean and representative S.E.s are shown.

*Gonadal Steroids.*—There were no statistically significant changes in any of the four specific gonadal steroid hormones assayed in the male subjects, and this was also true for plasma 17β-hydroxyandrogens on any dose. The results after 100 μg LH/FSH-RH are shown in Fig. 5, and there is a trend suggesting that the plasma testosterone is rising 24 to 48 hours after the dose. In the women plasma oestradiol rose from basal values of 84 and 68 pg/ml in the two subjects to reach a peak value of 105 and 115 pg/ml four and eight hours after injection of 100 μg LH/FSH-RH. This was in response to increases of 4·5 and 6·9 in LH, 2·2 and 3·7 FSH mU/ml in the first subject (assays 1 and 2 respectively); and of 7·0 and 2·0 LH, 0·7 and 0·7 FSH mU/ml in subject 2; there were no significant changes in the other gonadal steroids in the women at this dose and none at all after 50 μg. Serum *GH*, *TSH*, and plasma *cortisol* did not change significantly in any subject after any dose.

LIMITED STUDY

After the initial study a standard LH/FSH-RH test procedure was adopted: 100 μg was injected intravenously between 9 and 10 a.m. and blood was sampled before and 20 and 60 minutes afterwards for LH and FSH. Our provisional normal ranges for this test are given in Tables III and IV.

*Side Effects.*—None were encountered in any subject and the menstrual cycles after administration of LH/FSH-RH were unaltered.

TABLE III—*Responses in Serum LH and FSH to 100 μg LH/FSH-RH Given Intravenously at 9-10 a.m. in 18 Normal Male Subjects*

| | LH (mU/ml) | | FSH (mU/ml) | |
|---|---|---|---|---|
| | Assay 1 | Assay 2 | Assay 1 | Assay 2 |
| Mean value: | | | | |
| 0 Min | 3·1 | 2·4 | 2·0 | 3·6 |
| 20 Min | 12·0 | 7·7 | 4·5 | 5·4 |
| 60 Min | 8·6 | 6·3 | 3·2 | 5·6 |
| Range: | | | | |
| 0 Min | 0·4—6·9 | 1·7—4·6 | 0·4—5·8 | 1·0—7·4 |
| 20 Min | 7·1—26·8 | 2·0—17·0 | 2·8—8·3 | 1·5—10·5 |
| 60 Min | 4·6—14·5 | 2·7—17·0 | 2·0—5·2 | 1·2—11·0 |
| Range of increments: | | | | |
| 20 Min | 1·8—24·9 | 0·2—12·1 | 0·6—6·5 | —0·3—4·5 |
| 60 Min | 1·7—10·0 | 0·1—12·1 | —0·9—2·8 | —0·2—5·6 |

TABLE IV—*Responses in Serum LH and FSH to 100 μg LH/FSH-RH given Intravenously at 9-10 a.m. in five Normal Women During Follicular Phases of their Menstrual Cycles*

| | LH (mU/ml) | | FSH (mU/ml) | |
|---|---|---|---|---|
| | Assay 1 | Assay 2 | Assay 1 | Assay 2 |
| Mean value: | | | | |
| 0 Min | 3·0 | 2·3 | 2·4 | 5·4 |
| 20 Min | 8·6 | 6·7 | 6·2 | 6·3 |
| 60 Min | 7·6 | 6·1 | 5·4 | 7·5 |
| Range: | | | | |
| 0 Min | 1·3—5·1 | 1·9—2·8 | 1·3—3·2 | 3·0—8·3 |
| 20 Min | 2·4—13·2 | 2·9—11·0 | 3·3—8·9 | 3·0—10·0 |
| 60 Min | 3·4—10·2 | 2·7—9·2 | 2·9—7·3 | 3·8—12·0 |
| Range of increments: | | | | |
| 20 Min | 0·6—9·2 | 1·0—8·3 | 0·4—6·9 | —0·2—2·0 |
| 60 Min | 1·8—5·3 | 0·8—6·5 | 0·0—6·0 | 0·8—3·7 |

## Discussion

We have shown that in man, as in animals, the synthetic decapeptide LH/FSH-RH is a potent releaser of immunoreactive LH, that the effect is seen within two minutes of an intravenous injection, and that it is dose-dependent over the range of 25 to 100 μg. In view of the intrinsic lack of specificity and sensitivity of gonadotrophin radioimmunoassays we have validated these conclusions by using two completely different assay systems for each hormone. In all, 25 male and female subjects were given the compound on 32 occasions, and only twice did the serum LH fail to rise outside the normal range.

There is no doubt that serum immunoreactive FSH also rose in response to LH/FSH-RH since the increments seen could not be accounted for by any cross-reactivity of the antisera used in either assay. We have therefore confirmed that this hormone releases FSH as well as LH but that it does not affect any of the other non-gonadotrophin pituitary hormones studied, GH, TSH, or ACTH. However, the rise in FSH is much smaller than that of LH, and of the 32 occasions that the substance was administered serum FSH failed to rise outside the normal range 24 times. It remains to be determined whether there is another more specific FSH-releasing hormone or whether the release of LH or FSH depends on the interaction of circulating gonadal hormone levels and the known LH/FSH-RH.

There appear to be no differences between the gonadotrophin responses to LH/FSH-RH in the men or women studied during the follicular phases of their cycles. Preliminary results, however, suggest that much bigger responses in both LH and FSH can be obtained in women during the luteal phases compared with the follicular phases.

In the male subjects there were no statistically significant changes in the circulating gonadal steroid hormones, testosterone and oestradiol, or in their precursors, 17α-hydroxyprogesterone or progesterone, in response to the induced LH and FSH release. The upward trend in plasma testosterone at 48 hours after 100 μg LH/FSH-RH suggests that more prolonged gonadal stimulation is required to get a definite response, and either larger doses or different methods of administration of LH/FSH-RH will be necessary. In the women, however, after 100 μg of the releasing hormone definite increases in oestradiol were seen, indicating significant follicular stimulation. Further study will indicate the doses required to induce ovulation.

The availability of LH/FSH-RH should provide a powerful therapeutic agent for the treatment of infertility in many women and men. In addition it will provide a useful test of pituitary function in a manner analagous to the use of thyrotrophin-releasing hormone (Hall *et al.*, 1972; Ormston, *et al.*, 1972) in the study of diseases of the thyroid-pituitary axis. The use of LH/FSH-RH in hypogonadal subjects should allow precise localization of the defect at either the hypothalamic, pituitary, or gonadal level. A patient who shows no gonadotrophin response to clomiphene but an adequate response to LH/FSH-RH would be expected to have a lesion above the level of the pituitary, but an absent response to the releasing hormone would indicate pituitary disease. If the clomiphene and LH/FSH-RH responses are present the defect would be in the gonads. Furthermore, elucidation of whether so-called "isolated gonadotrophin deficiencies" are indeed deficiencies of hypothalamic-releasing hormones rather than of pituitary gonadotrophin production will be possible. Such studies are currently in progress. We have defined a simple clinical test procedure involving administration of 100 μg LH/FSH-RH intravenously with sampling for LH and FSH before and 20 and 60 minutes afterwards for use when investigating these problems, and we have defined our provisional normal range of responses. However, in view of the inherent problems of gonadotrophin radioimmunoassay it will be necessary for each laboratory to establish its own control data.

We are grateful to Dr. R. Roussel and Dr. W. Bogie, of Hoechst U.K. Ltd., for help and provision of the LH/FSH-RH, to Dr. A. Stockell-Hartree and Dr. W. Butt for the iodination materials, to D. Borthwick, S. Williams, M. McKendrick, A. Hewison, J. Young, P. Hamer, Y. Emment, M. D. Mansfield, E. Youssefnajadian, and J. Woodham for help, and to Professor Russell Fraser and Professor E. F. Scowen for advice.

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# Effect of Synthetic Luteinizing Hormone Releasing Hormone (LH/FSH-RH) in Women with Menstrual Disorders

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## Summary

**Synthetic luteinizing hormone/follicle stimulating hormone-releasing hormone (LH/FSH-RH) (50 μg) was given intravenously to six women with oligomenorrhoea and to four women with secondary amenorrhoea. Peripheral venous blood was withdrawn at regular intervals over a 24-hour period. The concentrations of LH, FSH, and oestradiol-17β were determined by radioimmunoassay. In all subjects there was a variable rise in LH (3-16 times the mean basal level): in six a small rise in FSH (two to three times the mean basal level) and in seven a twofold to threefold rise in oestradiol three to eight hours after the rise in gonadotrophins.**


## Introduction

There is good evidence to believe that the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) is under a neuroendocrine control emanating from the hypothalamus (Guillemin and Schally, 1963). Evidence for a specific LH-releasing hormone (LH-RH) in rat hypothalamic extracts was first presented by McCann *et al.* (1960) and in sheep by Courrier *et al.* (1961).

Recently Schally *et al.* (1971) showed that a purified extract of pig hypothalami (LH-RH) has both LH and FSH releasing properties in rats. Human subjects given this purified porcine LH-RH also release LH and FSH (Kastin *et al.*, 1971), as do monkeys, sheep, and rabbits (Reeves *et al.*, 1970; Schally *et al.*, 1970). Kastin *et al.* (1970) also isolated two fractions from human hypothalami with LH-RH and FSH-RH activity, and when given separately both produced a significant increase in FSH and LH after administration to volunteers.

Schally *et al.* (1971) proposed a provisional structure for porcine LH-RH, which was revised and found to be a decapeptide (Matsuo *et al.*, 1971). A product with this amino-acid sequence has now been manufactured by Hoechst Pharmaceuticals and was used in the present study.

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## Patients and Methods

Patients selected for this study were all volunteers. They were admitted to hospital on the day before the test and were kept in bed for the duration of the study but were not fasting. A No. 16-gauge angiocath was inserted into an antecubital fossa vein and connected to an extension tube and three-way tap. The tube and angiocath were flushed with heparinized sterile distilled water between each sample. Blood was withdrawn to determine basal hormone levels at 8, 8.30, and 8.50 a.m. A 50-μg dose of synthetic LH-RH (HOE 471) was dissolved in 0·9% sterile saline solution and diluted to 10 ml in the same material. This was injected via the intravenous line at 9 a.m. Blood samples were then withdrawn at the following times after the injection: 5, 10, 15, 20, and 30 minutes; and 1, 1½, 2, 3, 6, 9, and 24 hours. The blood samples were centrifuged immediately, the plasma being separated and deep frozen at −25°C until assayed. FSH and LH were determined by radioimmunoassay using a pituitary standard LER 907 (Wide and Porath, 1966) and oestradiol-17 β by radioimmunoassay using an anti-serum to oestradiol—6-carboxymethyl oxime-bovine serum albumin (Emment *et al.*, 1972).

The first six patients had oligomenorrhoea with cycles of six to eight weeks. These patients do not have the premenstrual rise in LH and FSH (Newton, 1972c; Ross *et al.*, 1970) which occurs in the normal menstrual cycle (Newton *et al.*, 1971a); the injection of LH-RH was given on the first day of bleeding. The last four patients had secondary amenorrhoea for not less than six months and had previously been investigated to assess their basal endocrine state (Newton 1972a, 1972b).

## Results

The data in Tables I and II show the first significant rise of LH and FSH after the injection of synthetic LH-RH, the time at which the peak level was reached, the duration of raised gonadotrophin levels, and the factorial increase of LH and FSH over the mean basal sample (mean of three). As can be seen from Table I there was a rise in LH in all 10 patients. Three showed the first significant rise 5 minutes after the injection, five after 10 minutes, and two after 15 minutes. The peak value for LH was reached after 30 minutes in four patients, 45 in two patients, and 90 minutes in two patients. The duration of response varied from 45 minutes to 8 hours 50 minutes, with five patients between 2 hours 50 minutes and 2 hours 55 minutes. The

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# Luteinizing Hormone and Follicle Stimulating Hormone-Releasing Hormone Test in Patients with Hypothalamic-Pituitary-Gonadal Dysfunction

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## Summary

**A standard intravenous 100 μg luteinizing hormone/follicle stimulating hormone-releasing hormone (LH/FSH-RH) test was used to assess the pituitary gonadotrophin responses in 155 patients with a variety of diseases of the hypothalamic-pituitary-gonadal axis. In all but nine patients there was an increase in circulating levels of either LH or FSH in response to the releasing hormone though 137 (88%) were clinically hypogonadal. It was not possible with this test to distinguish between hypothalamic and pituitary causes of hypogonadotrophic hypogonadism, since a variety of LH and FSH responses emerged within the disease groups. However, primary gonadal failure characteristically resulted in exaggerated gonadotrophin response. The potential therapeutic use of the gonadotrophin releasing decapeptide is suggested in certain patients with hypogonadotrophic hypogonadism.**

## Introduction

In 1971, Schally and his co-workers isolated a single polypeptide with gonadotrophin releasing activity from porcine hypothalamic extracts (Schally *et al.*, 1971 a, 1971 b). Early work with this synthetic decapeptide showed that in animals it released both LH (luteinizing hormone) and FSH (follicle stimulating hormone) in a manner that was indistinguishable from that of the natural material isolated from porcine hypothalami (Matsuo *et al.*, 1971, Schally *et al.*, 1971 c). In man there were similar dose-related LH and FSH responses when doses between 25 and 100 μg of the decapeptide were given intravenously with a peak occurring between 20 and 30 minutes after the dose (Besser *et al.*, 1972 a). The FSH responses were smaller than the LH responses. The actions of the releasing hormone (LH/FSH-RH) were otherwise specific with no resulting changes in growth hormone (GH), thyroid stimulating hormone (TSH), or adrenocorticotrophic hormone (ACTH), and there were no side effects after administration of the synthetic material. It is also now established that there is no interaction between LH and FSH secretion in response to LH/FSH-RH, and either the TSH or prolactin responses to thyrotrophin releasing hormone (TRH) or the ACTH, GH, or prolactin responses to hypoglycaemia when these stimuli are given together (Mortimer *et al.*, 1973). We have therefore suggested that this material can be used in a simple test procedure to assess the reserve capacity of the hypothalamic-pituitary-gonadal axis for LH and FSH secretion. The results of this test are now reported in 155 patients with a variety of disorders of this system.

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## Patients and Methods

The details of the diagnosis in the patients studied, together with the incidence of clinical and biochemical deficiencies of GH, ACTH, TSH, and the gonadotrophins, are shown in table I. The criteria of Hall *et al.* (1972) were used to define the presence of endocrine deficiency of GH, ACTH, and TSH, and in females hypogonadism was considered to be present if puberty was delayed beyond 19 years of age or if amenorrhoea (in the absence of pregnancy) persisted for longer than three months. This was often accompanied by loss of libido, body hair, and involutional changes of the breasts and genitalia. In males a loss of potency, reduction in beard growth or body hair, and oligospermia or azoospermia were taken as an indication of hypogonadism. In 21 out of 63 male patients regarded as hypogonadal on clinical grounds basal 17β-hydroxyandrogens (17-OHA) were measured and in 17 cases the levels were notably below the normal range, while in the remainder levels were at the lower limit.

"Isolated gonadotrophin deficiency" was accepted as a diagnosis in patients with partial or absent puberty and low or undetectable basal serum gonadotrophin levels which failed to respond to clomiphene administration; there was no evidence of deficiency of other pituitary hormones in these patients except for occasionally impaired GH responses to hypoglycaemia (Marshall *et al.*, 1972). "Delayed puberty" was accepted as the diagnosis in patients with a similar clinical picture, but in whom basal serum gonadotrophin levels or clomiphene responsiveness were normal.

### LH/FSH-RH TEST

In order to study the hypothalamic-pituitary-gonadal axis a standard test was devised (Besser *et al.*, 1972 a) similar to the TRH test in assessing hypothalamic-pituitary-thyroidal function (Ormston *et al.*, 1971). A 100 μg dose of the synthetic LH and FSH releasing hormone (LH/FSH-RH, Hoechst) was given intravenously as a bolus between 8 and 10 a.m. and blood samples were withdrawn before and 20 and 60 minutes after the injection for measurement of LH and FSH. Specimens were rapidly separated and stored at −20°C until assayed. The responses in a group of 39 *normal male volunteers* and seven *normal females* in the follicular phase of their menstrual cycles were determined to establish a provisional normal range of responses at each sampling time (table II). An *absent* response was defined as one in which the administration of LH/FSH-RH failed to produce a rise greater than three times the within-assay coefficient of variation in the basal levels of the gonadotrophins. This was a change of 15% for LH and 18% for FSH. If there was a change in gonadotrophin level but not into the normal

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TABLE III—*Serum LH and FSH Responses to LH/FSH-RH in 155 Patients with Hypothalamic-Pituitary-Gonadal Dysfunction*

| | Total | LH | | | | FSH | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Normal | Absent | Impaired | Exaggerated | Normal | Absent | Impaired | Exaggerated |
| Hypothalamic disease: | | | | | | | | | |
| Isolated gonadotrophin deficiency | 15 | 4 | 3 | 8 | | 9 | 3 | 3 | |
| Craniopharyngioma | 10 | 4 | 5 | 1 | | 2 | 5 | 3 | |
| Isolated TRH deficiency | 1 | | | 1 | | | | 1 | |
| Tumours | 2 | | 1 | 1 | | 1 | | 1 | |
| Pituitary disease: | | | | | | | | | |
| Functionless pituitary tumours | 31 | 11 | 1 | 19 | | 28 | | 3 | |
| Acromegaly | 27 | 6 | | 21 | | 11 | | 16 | |
| Cushing's disease | 3 | 1 | | 1 | 1 | 2 | | 1 | |
| Sheehan's syndrome | 2 | | | 2 | | 2 | | | |
| Idiopathic hypopituitarism | 6 | 1 | 4 | 1 | | 4 | 2 | | |
| Amenorrhoea syndromes: | | | | | | | | | |
| Delayed puberty—primary amenorrhoea | 4 | 1 | | 2 | 1 | 2 | | 2 | |
| Anorexia nervosa | 13 | 6 | | 2 | 5 | 8 | | 2 | 3 |
| Secondary amenorrhoea and galactorrhoea | 15 | 7 | | | 8 | 14 | | | 1 |
| Polycystic ovary syndrome | 3 | 2 | | | 1 | 2 | | 1 | |
| Turner's syndrome | 2 | | | | 2 | | | | 2 |
| Male syndromes: | | | | | | | | | |
| Delayed puberty | 3 | 3 | | | | 2 | | 1 | |
| Precocious puberty | 1 | 1 | | | | 1 | | | |
| Testicular feminization | 1 | | | | 1 | | | | 1 |
| Primary gonadal failure | 7 | 2 | | | 5 | 1 | | | 6 |
| Anorexia nervosa | 1 | | | 1 | | | | 1 | |
| Galactorrhoea | 3 | 2 | 1 | | | 2 | | 1 | |
| Gynaecomastia | 2 | 2 | | | | 2 | | | |
| Miscellaneous: | | | | | | | | | |
| Internal hydrocephaly | 2 | 1 | 1 | | | 2 | | | |
| Werner's syndrome | 1 | | | | 1 | | | | 1 |
| Total | 155 | 54 (35%) | 16 (10%) | 60 (39%) | 25 (16%) | 95 (61%) | 10 (7%) | 36 (23%) | 14 (9%) |

*Tumours.*—Two patients with other space-occupying lesions involving the hypothalamus were tested. One female with histiocytosis X had an impaired LH but normal FSH response. The other, a male with a tumour of unknown nature, had an absent LH but impaired FSH response. These responses were not improved by clomiphene administration.

## PITUITARY DISEASE

*Functionless Pituitary Tumours.*—There were 31 patients with apparently non-secreting tumours of the pituitary and 25 were clinically hypogonadal. Eighteen patients had been treated with surgery or irradiation or both, and of these 17 were hypogonadal. Despite this there was some LH and FSH secretion after LH/FSH-RH in all patients except one male with absent LH but normal FSH response, and he had had a hypophysectomy, was hypogonadal, hypothyroid, and GH deficient but had a normal ACTH reserve. In the other eight male patients, all of whom had either reduced or absent potency, an impaired LH response was seen in seven; only in two did the FSH fail to rise normally. Of 22 females investigated 16 had had secondary amenorrhoea for between six months and 22 years, but all showed secretion of gonadotrophins, though there was an impaired LH response in 11 and one showed impaired FSH release. Clomiphene was administered to seven patients and there was a rise above the upper limit of the normal basal range for serum LH in five women but in neither man; menstruation followed in three of the five responsive patients after clomiphene. In five patients with secondary (pituitary) hypothyroidism four had an impaired LH response to LH/FSH-RH though in only one was FSH release impaired. Impaired LH but normal FSH release was, however, seen in four patients with a normal protein bound iodine who were clinically euthyroid; of these three had a delayed TSH rise after TRH. Despite normal LH responses a high basal FSH level with an exaggerated response to LH/FSH-RH was seen in one postmenopausal woman with a pituitary tumour and primary hypothyroidism due to thyroiditis. The TSH response to TRH was characteristically high.

*Acromegaly.*—In all there were 27 patients, and some LH and FSH secretion was seen in each. Twenty-one had been treated with surgery or irradiation or both. There were 12 males 10 of whom were hypogonadal at the time of testing. Ten had an impaired LH response at 20 minutes but only five had an impaired 60-minute value. The serum FSH responses were normal in 11 of the 12 patients. All patients had active acromegaly in that the GH failed to suppress to less than 5 ng/ml during a glucose tolerance test. Eleven patients had GH levels above 30 ng/ml. After partial hypophysectomy in one male it was still possible to induce the release of LH and FSH, though the levels were impaired. GH levels were still raised in this patient. In the 15 female patients tested amenorrhoea had been present in each for between one and 10 years, and 11 had active disease. Two were aged 62 and 64 years at the time of the study but their basal gonadotrophin levels were low, and they have been considered with the hypogonadal group. Reduced basal LH levels were seen in 12 of the 15, but in seven a normal value was achieved by 60 minutes. The basal FSH levels were reduced in seven but each achieved a normal 60-minute value.

*Cushing's Disease—Untreated.*—Two women were investigated. One aged 40 had secondary amenorrhoea of five years' duration with an impaired and delayed LH rise but a normal FSH level by 60 minutes. The other, aged 38, had irregular periods but with a normal LH and FSH response. The only male patient had a rise in LH and FSH outside the normal range despite normal basal levels and was not clinically hypogonadal.

*Sheehan's Syndrome.*—Two patients with secondary amenorrhoea after postpartum haemorrhages showed impaired release of LH, while FSH was within the normal range.

*Idiopathic Hypopituitarism.*—Six patients are included in whom there was no obvious disease to account for their hypopituitarism. Of five males three had an absent LH response; in one it was impaired but normal in the fifth. The FSH response was normal in three and absent in two. A normal FSH response was seen in one male patient in whom LH levels had remained undetectable, and the same differential response was seen in the only female patient in this group.

## AMENORRHOEA SYNDROMES

*Delayed Puberty and Primary Amenorrhoea.*—There were four patients in this group, aged between 19 and 26 years, without evidence of ACTH, GH, or TSH deficiency. In two both LH and FSH responses were impaired, one had a normal response of both gonadotrophins, and the fourth had an exaggerated LH but a normal FSH rise. Menstruation followed clomiphene administration in this patient.

*Anorexia Nervosa in Females.*—Secondary amenorrhoea was associated with anorexia nervosa in 13 females aged 12-27 years.

sexes with delayed puberty had normal LH and FSH release when tested, suggesting that hypothalamic or higher centres are involved in the initiation of puberty.

In patients with "functionless" pituitary tumours or those with active acromegaly, impaired LH release was often seen, though FSH reserve under the test conditions appeared intact. The ability of the pituitary to release gonadotrophins occurred in many patients independently of the functional pituitary reserve for other hormones. The three patients with pituitary-dependent Cushing's disease each showed a different type of response—normal, impaired, or exaggerated. Impaired or absent TSH responses to TRH were seen in these three patients and this is characteristic of this condition (Hall *et al.*, 1972).

When the 15 patients with amenorrhoea and galactorrhoea were investigated it was clear that basal gonadotrophin levels were normal or high in nine, and that normal or even exaggerated responses could occur after administration of the decapeptide. The response did not correlate with basal prolactin levels nor the levels achieved after administration of TRH. The cause of amenorrhoea in these women was clearly not due to inability to synthesize the gonadotrophins but was probably the result of failure of their cyclical release. Hyperprolactinaemia may be responsible, at least in part, for this functional abnormality, since when the prolactin levels were reduced with bromergocryptine galactorrhoea ceased and normal menstruation resumed; potency returned in male patients similarly treated (Besser *et al.*, 1972 b). Whether prolactin interferes at the hypothalamic-pituitary level or has a direct action on the gonads remains to be elucidated.

Gonadotrophin releasing hormone responsiveness was present in every patient with anorexia nervosa, and here again it is probably the failure of cyclical release rather than inability to synthesize gonadotrophins which is the major factor in the initiation and perpetuation of the amenorrhoea. Patients with primary gonadal failure showed the expected exaggerated response to the decapeptide.

In view of the fact that in most patients with hypogonadotrophic hypogonadism, of whatever cause, LH and FSH secretion can be induced with the synthetic decapeptide LH/FSH-RH, it is evident that the pituitaries of these patients contain LH and FSH. It would therefore appear that it is the impairment of release which is the primary cause of the hypogonadism rather than pituitary gonadotroph destruction in these patients. It is disappointing that the LH/FSH-RH test will not differentiate between hypothalamic and pituitary causes of hypogonadotrophic hypogonadism. However, since such a high proportion of these patients can be made to release LH and FSH in response to the decapeptide, it is possible that repeated administration of LH/FSH-RH might restore their fertility, and the results of therapeutic trials with this material are awaited.

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# INTRAVENOUS, INTRAMUSCULAR, SUBCUTANEOUS AND INTRANASAL ADMINISTRATION OF LH/FSH-RH: THE DURATION OF EFFECT AND OCCURRENCE OF ASYNCHRONOUS PULSATILE RELEASE OF LH AND FSH

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SUMMARY

The duration of action of 100 $\mu$g of the synthetic gonadotrophin releasing hormone, the decapeptide LH/FSH-RH, was compared in three subjects after intravenous, intramuscular and subcutaneous administration, given in random order at 7–10 day intervals. The levels of serum LH and FSH were followed over 9 hr. Each route of administration was equally effective in promoting LH and FSH release, and the magnitude of the responses and their time course were the same in each case. Two of these subjects then received 2 mg of the pure material dissolved in 0·5 or 1 ml of 0·9% NaCl by intranasal application. Although definite LH and FSH responses were seen, this route was less effective in promoting gonadotrophin release despite the higher dose used. In three further subjects blood sampling was carried out at 10 min intervals for 4 hr, following i.v., i.m. or s.c. injection of LH/FSH-RH; pulsatile release of LH and FSH was observed in each subject but the fluctuations in LH and FSH were asynchronous. The therapeutic implications of subcutaneous and intranasal administration of the material are discussed.

## INTRODUCTION

The synthetic gonadotrophin releasing hormone (LH/FSH-RH) has been shown to stimulate the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) in humans (Besser *et al.*, 1972). It has also been demonstrated that secretion of both hormones can be induced with this decapeptide in the majority of patients who are hypogonadal due to pituitary or hypothalamic disease and in those with isolated gonadotrophin deficiency (Marshall *et al.*, 1972; Mortimer *et al*, 1973a). The potential therapeutic implications for restoration of fertility in these patients with long-term treatment are clear and we have therefore studied the time courses of the LH and FSH responses to LH/FSH-RH after different routes of administration. In addition, frequent serial sampling was carried out

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over 4 hr in order to study the time relationships between the circulating levels of LH and FSH more closely.

## MATERIALS AND METHODS

100 μg of LH/FSH-RH (Hoechst) was administered intravenously, intramuscularly and subcutaneously dissolved in 1 ml of sterile water to three healthy male subjects (aged 26–36 years) at 7–10 day intervals to allow a within-subject comparison of the routes of administration of the decapeptide. The three treatments were given in random order. The levels of serum LH and FSH were measured at the following intervals: −30, 0, 20, 30 and 60 min then hourly for 9 hr. Eight weeks after receiving the last dose of the decapeptide, two of these subjects then received 2 mg of pure LH/FSH-RH, i.e. free of stabilizing substances, dissolved in 0·5 or 1 ml of 0·9% NaCl. The solution was administered by repeated intranasal application via a Pasteur pipette until the whole volume had been delivered over 5–15 min. Three further males (aged 22–24 years) were injected with the 100 μg LH/FSH-RH either intravenously (i.v.), intramuscularly (i.m.), or subcutaneously (s.c.) and blood was sampled at 10 min intervals for 4 hr. All experiments began between 08.30 and 09.30 hours after at least 24 hr of sexual continence. Samples were withdrawn from the antecubital vein through an indwelling cannula and serum was stored at −20°C until assayed.

Serum LH and FSH were determined using a double antibody technique and samples were assayed in duplicate. The preparations used as standards were MRC 68/40 (each ampoule assumed to contain 39·8 units) for LH and MRC 68/39 (32·8 units per ampoule) for FSH, and results were expressed in mU/ml. The minimum detectable level of LH was 0·4 mU/ml and 0·2 mU/ml of FSH. There was no interassay cross-reactivity at many times the levels measured and the details are given by Mortimer *et al.* (1973b). The within assay coefficient of variation was 5% for LH and 6% for FSH; all samples for each subject were measured in the same assay.

## RESULTS

All subjects had basal serum LH levels within the normal male range for this laboratory (from less than 0·4 to 6·0 mU/ml). The peak response in each of the three subjects given LH/FSH-RH by different routes occurred between 20 and 60 min, following injection of 100 μg of LH/FSH-RH, by all three routes of administration (Fig. 1). In one subject a high LH peak of 14 mU/ml was achieved 20 min after intravenous administration; this was considerably greater than the response to either s.c. or i.m. injection in this subject at the times measured but in the other subjects there was little difference between the timing or magnitude of the peaks after the different routes. The duration of effect on LH was similar in all subjects whatever the route of administration, and levels returned to the basal values within 5–7 hr. The basal levels of serum FSH were also within the normal male range (less than 0·2 to 5·9 mU/ml) and peak responses occurred 20–60 min after injection of the decapeptide (Fig. 2). The FSH peaks did not necessarily occur simultaneously with the LH peaks in the same subject. Levels of FSH remained elevated above the basal values for 3–5 hr, although, unlike LH, they did not exceed the upper limit of the basal male range. As with LH, the magnitude of the elevation of FSH and the time course of the response did not vary with the route of administration.

Intranasal application of LH/FSH-RH was less effective in promoting gonadotrophin release. In one subject the maximum levels of both LH (4·9 mU/ml) and FSH (1·5 mU/ml) were less than by other routes of administration and the values returned to basal levels within 4 hr. The other subject also had an impaired LH response, the maximum level reaching 5·9 mU/ml, while there was no clear FSH rise.

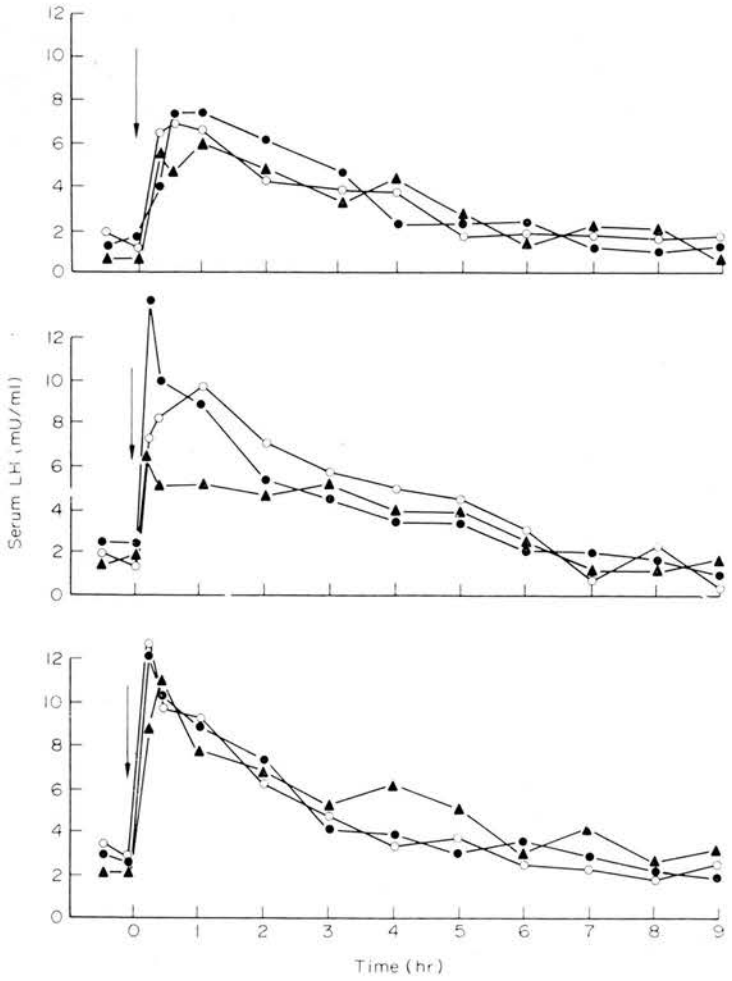



FIG. 1. The serum LH responses in three subjects following i.v (●), i.m. (○) and s.c. (▲) injection of LH/FSH-RH 100 μg.

When 10 min sampling was carried out for 4 hr (Fig. 3) in the other three subjects it was apparent that peak levels of either LH or FSH could occur as soon as 10 min after the injection.

Throughout the 4 hr period, marked pulsatile fluctuations in both LH and FSH were seen. These fluctuations were significant since they were far greater than could be accounted

for by assay variability. The peaks in the serum levels of the two hormones clearly did not occur synchronously; and neither did the times at which the maximal values were seen. There was a gradual diminution in the levels until sampling ceased.

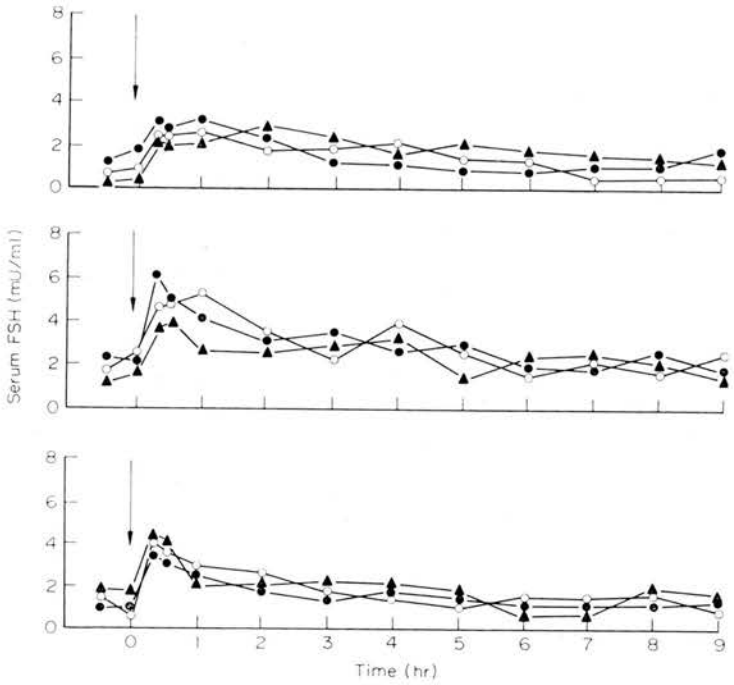



FIG. 2. The serum FSH responses in three subjects following i.v. (●), i.m. (○) and s.c. (▲) injection of LH/FSH-RH 100 μg.

## DISCUSSION

This study shows that 100 μg of synthetic LH/FSH-RH given by i.v., i.m. or s.c. injection released virtually the same amounts of LH and FSH with identical time courses irrespective of the route of administration. The decapeptide was capable of increasing the circulating levels of LH and FSH above the basal levels for between 5–7 and 3–5 hr respectively after injection. A time interval of 7–10 days between testing did not appear to modify succeeding responses to the releasing hormone since the curves for either serum LH or FSH were virtually superimposable. The time course reported here suggests that in order to maintain raised circulating levels of LH and FSH with this releasing hormone in normal males, injections of 100 μg should be given at least 6-hourly. Since any of the routes of administration provided the same response, patients could be instructed to inject the material subcutaneously. We have not so far recorded any adverse effects resulting from subcutaneous injections apart from occasional initial local discomfort lasting a few seconds, with erythema around the site of the injection. The erythema may persist for several hours before fading but is not accompanied by any systemic or other local effects. When intranasal application was considered it was clear that the gonadotrophin responses were small even though as

much as 2 mg of the material had been used. In one subject there was no FSH response at all. This suggests that intranasal administration is a less reliable route than the others. The method of introducing the material as nose drops was also more time consuming than subcutaneous injection. Before this route can be used routinely an improved method of dispensing the active material must be found if reliable stimulation of the pituitary is to occur.

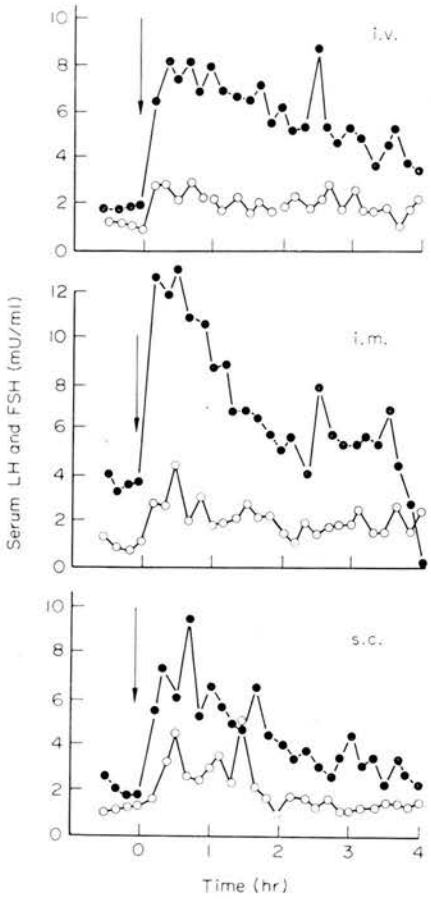



FIG. 3. The serum LH (●) and FSH (○) responses in three different subjects following either i.v., i.m. or s.c. injection of LH/FSH-RH 100 μg.

Asynchronous pulsatile fluctuations of basal serum LH and FSH levels in males have been reported by Naftolin *et al.* (1972) and we have also observed similar fluctuations throughout the day in normal men and women. The lack of synchrony between these pulses of LH and FSH has been taken as an indication that two separate LH and FSH releasing hormones exist rather than the single one suggested by Schally *et al.* (1971). However, the present study shows that administration of the single synthetic decapeptide is capable of producing exaggerated asynchronous, pulsatile fluctuations in both serum LH and FSH whatever the route of administration. This effect would appear to be achieved by the differen-

tial effect of the decapeptide on the mechanisms responsible for release of LH and FSH by the pituitary gonadotroph cells.

Arimura *et al.* (1973) have reported that after the initial FSH rise produced by the subcutaneous injection of LH/FSH-RH there is a secondary rise in FSH seen at 3 or 4 hr. We have not observed this, but it is clear from our studies that marked fluctuations in FSH levels may be seen after any route of administration of LH/FSH-RH. The secondary rise in FSH observed by Arimura and co-workers might therefore be related more to these random fluctuations of FSH after the decapeptide than to any distinctive feature of subcutaneous injections. It is clear that infrequent sampling of blood can easily lead to an imprecise description of the time course of releasing hormones.

The occurrence of pulsatile release of both gonadotrophins indicates that half life estimates for LH and FSH following the use of LH/FSH-RH are difficult to interpret. An exponential disappearance of LH and FSH after a single dose of the decapeptide does not occur and this may account for some of the variability of disappearance rates for these hormones found by previous authors (Arimura *et al.*, 1973). The measurement of circulating levels of LH and FSH at arbitrary times after LH/FSH-RH administration probably reflects a combination of the biological decay of the processes involved in synthesis and release of the pituitary hormones as well as the simple disappearance times of the hormones in the circulation. The disappearance rates for gonadotrophins in humans are therefore probably more accurately determined following intravenous administration of the pure hormone (Marshall *et al.*, 1973).

The occurrence of episodic release of LH and FSH after i.v., s.c. or i.m. administration, as well as during continuous infusion of the decapeptide (Mortimer *et al.*, 1973c), suggests that there are short periods of time when the pituitary gonadotroph cell is refractory to the actions of the releasing hormone, although it is also possible that the actions of the exogenous decapeptide may be augmented by those of endogenous hypothalamic regulatory hormone(s) for the gonadotrophins, secreted intermittently. The time intervals between the episodes are unlikely to be related to the period required for re-synthesis since recent work suggests that large amounts of gonadotrophins in the form of storage granules are available in the pituitary cells ready for release (Phifer *et al.*, 1973).

Although the exact nature of endogenous gonadotrophin releasing hormone(s) in the human remains controversial (Schally *et al.*, 1971; Bowers *et al.*, 1973), the single synthetic decapeptide is capable of producing differential circulating levels of both LH and FSH. In view of the efficacy of 100 $\mu$g of the material in maintaining levels of both gonadotrophins following subcutaneous injection when compared with intranasal administration by currently available methods, it is suggested that at least 6-hourly administration of this dose or possibly less frequent administration of a higher dose of the decapeptide may be required for the treatment of infertility in patients with hypogonadotrophic hypogonadism.

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# COMPARISON OF SINGLE AND REPEATED APPLICATIONS OF A LONG-ACTING SYNTHETIC ANALOGUE OF LHRH [D-SER (TBU)$^6$ EA$^{10}$ LHRH] IN THE ASSESSMENT OF PITUITARY GONADOTROPHIN SECRETORY CAPACITY

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SUMMARY

A long-acting synthetic analogue of LHRH [D-Ser (TBU)$^6$ EA$^{10}$ LHRH] was administered intramuscularly to thirteen normal women in the early-to-mid follicular phase of the menstrual cycle. Six women received a single injection of 5 μg of analogue and seven received 10 μg of the analogue, followed by 20 and 10 μg at 10 and 24 h after the first injection, respectively. The concentrations of LH, FSH and oestradiol in plasma were measured before, and at intervals up to 72 h after treatment. In the group receiving 5 μg of analogue the concentrations of both LH and FSH rose rapidly to a peak value five times the pre-injection value, 5 h after injection. The concentrations of both gonadotrophins remained elevated for 10 h, but 24 h after injection had returned to control values. The concentration of oestradiol rose progressively to reach a peak value 7 h after injection. In the second group of women, receiving three injections of analogue, there was a rapid twelvefold increase in the concentration of LH in plasma within 4 h of the initial injection, and plasma concentrations remained elevated for 10 h. The concentration of FSH rose progressively to a peak value, five times the pretreatment value, 5 h after the initial injection and was still elevated after 10 h. The concentrations of both gonadotrophins remained elevated above pretreatment values for at least 34 h after the start of treatment. The concentration of oestradiol rose gradually to a peak value 7 h after the initiation of treatment and remained elevated for 34 h. These results indicate that a single injection of the LHRH analogue is effective in demonstrating pituitary secretory activity, but repeated applications are required to induce and maintain a rise in plasma LH similar in magnitude and duration to the pre-ovulatory, mid-cycle surge. This analogue of LHRH provides a simple means of evaluation of the secretory capacity of the anterior pituitary.

The hope, following the synthesis of LHRH by Schally and his co-workers (Matsuo *et al.*,

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1971), that a single-dose LHRH test would permit distinction between pituitary and hypothalamic causes of hypogonadotrophic hypogonadism has not been fulfilled (Marshall *et al.*, 1972). Repeated injections or continuous infusions of LHRH however, have been shown to demonstrate the synthetic capacity of the pituitary gonadotroph (Bremner & Paulsen, 1974; Kley *et al.*, 1974; Serra *et al.*, 1975; Hoff *et al.*, 1977) and have been used to investigate amenorrhoea due to hypogonadotrophism (Serra *et al.*, 1975; Jewelewicz *et al.*, 1974). However, these tests are cumbersome and difficult to apply in routine clinical practice. Recently, a long-acting analogue of LHRH, D-Ser (TBU)$^{6}$ EA$^{10}$ LHRH (Hoechst 766), has been shown to induce a prolonged release of LH and FSH similar to that produced by continuous infusion of LHRH (Sandow *et al.*, 1977; Kuhl *et al.*, 1976; Dericks-Tan *et al.*, 1977; Friedrich *et al.*, 1978; Nillius & Wide, 1977). The biphasic pattern of gonadotrophin release in response to the analogue (Nillius & Wide, 1977; Dericks-Tan *et al.*, 1977) resembles that seen in response to an LHRH infusion (Bremner & Paulsen, 1974; Hoff *et al.*, 1977; Jewelewicz *et al.*, 1974, 1977), and, in demonstrating pituitary secretory capacity, could be useful as a clinical test of pituitary function.

Many anovulatory patients have a normal response to a single injection of LHRH, but are unable to release LH in response to oestrogen. This failure of positive feedback may be due to a defect in hypothalamic feedback or in the capacity of the pituitary to release adequate quantities of LH. This study was undertaken to compare LH and FSH release in response to single and repeated applications of the LHRH analogue, in an attempt to develop a clinically useful test of the secretory capacity of the anterior pituitary.

## MATERIAL AND METHODS

Thirteen healthy women of proven fertility volunteered for study between days 1 and 10 of a normal menstrual cycle. All had ovulated in the previous cycle as determined by measurement of plasma progesterone concentrations greater than 6 ng/ml. The LHRH analogue, D-Ser (TBU)$^{6}$ EA$^{10}$ LHRH (Hoechst 766), was administered intra-muscularly in two dose regimens. Six women received a single dose of 5 $\mu$g. The remaining seven women received 10 $\mu$g initially, 20 $\mu$g after 10 h and a further 10 $\mu$g 24 h after the first injection. Ten ml of venous blood were collected 24 h and immediately before treatment, at hourly intervals for 10 h after initiation of treatment and thereafter twice daily, until 72 h after the start of the treatment. Plasma was separated and deep frozen, until all samples could be assayed together for LH, FSH and oestradiol.

### *Hormone Assay Methods*

Plasma levels of LH and FSH were measured in duplicate at two dilutions in the specific double-antibody radioimmunoassays described in detail by Hunter & Bennie (1979). Results are expressed in terms of iu/l of the appropriate standards obtained from the National Institute of Biological Standards and Control, Holly Hill, London (LH: 68/40, 77 iu/ampoule, FSH: 69/104, 10 iu/ampoule). At the end of the assay incubation period, tubes were centrifuged for 30 min, at 4°C, at 600 g, the supernatants poured off and the precipitates containing antibody-bound hormone counted in an LKB-Wallac gamma-counter. Three quality control plasmas were included in each assay (B/Bo approximately 80, 55 and 30%) and gave intra-assay coefficients of variation of 8% and 7%, and inter-assay variations of 12% and 11% for LH and FSH respectively. Plasma levels of

oestradiol were measured by a specific radioimmunoassay described in detail previously (Baird *et al.*, 1974). Intra-assay and inter-assay variation of two quality control plasmas (B/Bo 75 and 30%) were 8% and 12% respectively.

*Statistical analysis*

Mean values and standard deviations were derived from log means and standard deviations. Mean values are given in the text with the 95% confidence limits in parentheses. Student's single-tailed *t* test was used to determine significance of differences between logarithmic data.

## RESULTS

The mean concentrations of LH, FSH and oestradiol in plasma after administration of the LHRH analogue are shown in Fig. 1. In the first group of women the concentration of LH increased rapidly to a peak value of 54·4 (20·7–98·6) u/l 5 h after the injection. The concentration then declined rapidly but was still elevated above pretreatment values 10 h ($P<0·05$), but not 24 h after injection of the analogue. The concentration of FSH rose progressively to a peak value of 26·6 (15·0–40·2) u/l 5 h after injection, and was still elevated above pretreatment values 10 h ($P<0·05$) but not at 24 h after injection of the analogue. The concentration of oestradiol increased from a mean control value of 46·6 (27·9–69·4) pg/ml to a peak value of 201·8 (104·4–325·0) pg/ml after 7 h and remained elevated 34 h ($P<0·05$) but not at 48 h after injection.

The pretreatment concentrations of LH, FSH and oestradiol in the second group of women did not differ from those in the first group of subjects. After the initial injection of 10 μg of the LHRH analogue, there was a rapid rise in the concentration of LH, to a peak value of 120·4 (65·0–188·5) u/l, 4 h after the injection. This was greater than the peak value in the first group of subjects ($P<0·001$). The plasma concentration decreased rapidly, but 10 h after the initial injection was still elevated above pretreatment levels ($P<0·001$), and at this time did not differ from the respective value in the first group of women. Fourteen h after the second injection, and 10 h after the third injection of the analogue, the concentration of LH was greater than both the pretreatment levels ($P<0·001$) and the corresponding values in the first group of subjects ($P<0·05$: $P<0·01$ respectively).

The concentration of FSH in plasma rose progressively to a peak value of 19·9 (10·6–31·7) u/l 5 h after the initial injection of the analogue. This value did not differ from the peak FSH value in the first group of women. The concentration of FSH remained elevated above pretreatment levels ($P<0·01$) 10 h after the injection. Fourteen hours after the second and 10 h after the third injection, the plasma concentrations of FSH were greater than the pretreatment levels ($P<0·01$: $P<0·05$ respectively), but did not differ significantly from the corresponding values in the first group of women.

A later rise in the concentration of oestradiol, from a mean control value of 59·6 (28·9–100·0) pg/ml, culminated in a peak value of 137·0 (74·5–214·2) pg/ml 7 h after the initial injection of the analogue. This value did not differ significantly from basal values. A rapid decline to pretreatment values during the next 2 h was followed by a rise to a second peak of 161·9 (102·8–231·0) pg/ml 14 h after the second injection that was significantly greater than basal values ($P<0·05$). Concentrations of oestradiol had returned to pretreatment values 10 h later. The concentration of oestradiol in plasma did not differ from respective values in the first group except between 8 and 9 h after the start of treatment,

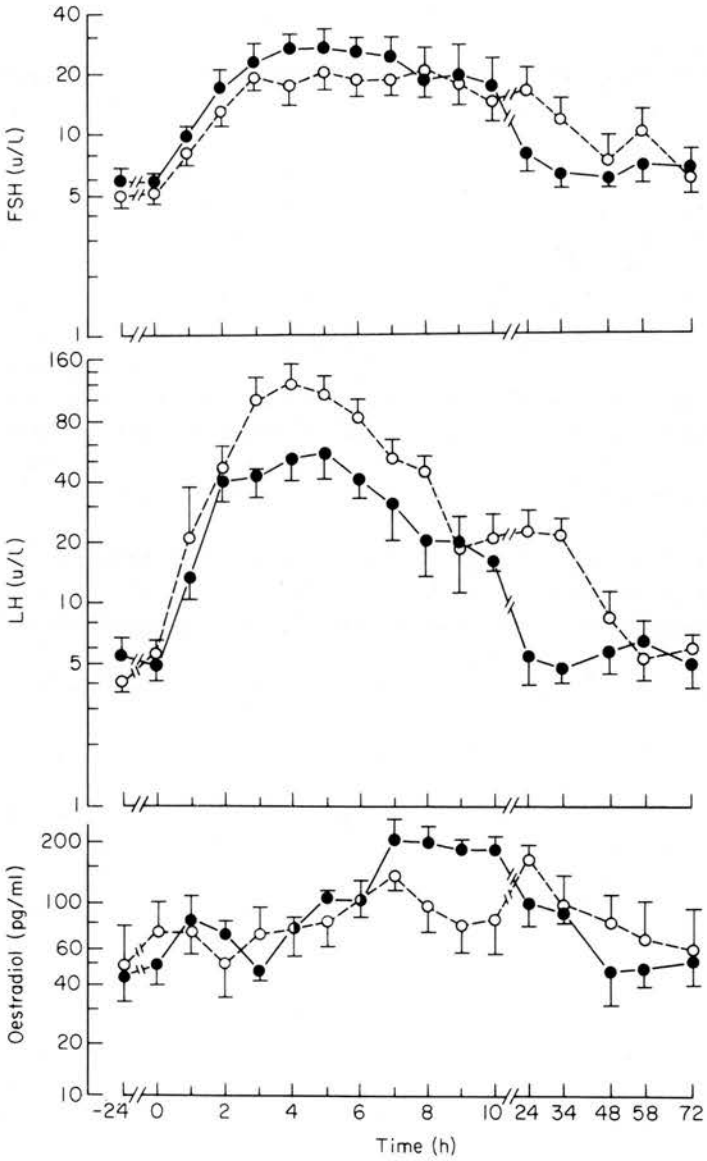



Fig. 1. Concentration of LH, FSH and oestradiol in plasma in normal women in the follicular phase of the cycle after injection of D-Ser (TBU)$^{6}$ EA$^{10}$ LHRL (HOE 766). Mean ± SEM. 5 μg HOE 766 ●——●; 10–20–10 μg HOE 766 ○——○.

when values in the first group were greater than those in the second group of women ($P < 0{\cdot}05$).

## DISCUSSION

The results of this study confirm previous reports (Sandow *et al.*, 1976; Wiegelmann *et al.*,

1976; Dericks-Tan *et al.*, 1977; Friedrich *et al.*, 1978) that D-Ser (TBU)$^{6}$ EA$^{10}$ LHRH is a potent analogue of LHRH with prolonged stimulatory effects upon the release and synthesis of pituitary gonadotrophins. The concentration of LH in plasma remained significantly elevated for at least 10 h after a single intramuscular injection of either 5 $\mu$g or 10 $\mu$g of the analogue, whereas the concentration of FSH in plasma remained elevated for at least 24 h. The peak concentration of LH after injection of 10 $\mu$g of analogue was significantly greater than that occurring after injection of 5 $\mu$g, thus confirming the dose–dependency of the LH response reported by Dericks-Tan *et al.*, (1977). No dose–dependency was apparent in the FSH response to the analogue. The concentration of LH in plasma 10 h after injection of 5 $\mu$g and of 10 $\mu$g did not differ. Thus, although inducing a greater elevation in the concentration of LH, the larger dose of the analogue did not have a more prolonged effect on LH release than did the lower dose. The magnitude of the LH response to a single injection either of 5 $\mu$g or of 10 $\mu$g of the analogue was similar to that of the pre-ovulatory LH surge, but the duration of both responses was markedly shorter. A single injection of either 5 $\mu$g or 10 $\mu$g therefore, will demonstrate pituitary synthetic function but does not confirm a synthetic capacity to maintain a release of LH sufficient to effect ovulation.

Repeated injections of the analogue (three doses over 24 h) resulted in an elevation of LH concentrations similar in magnitude and duration to the mid-cycle pre-ovulatory LH surge, plasma concentrations remaining significantly elevated for at least 34 h. Repeated injections of the LHRH analogue, have been shown to result in refractoriness of the pituitary response. Wiegelmann *et al.*, (1977) reported a reduction in the LH response after the seventh daily injection of 2·5 $\mu$g, but the pituitary remained responsive to daily injections of 1·25 $\mu$g for 12 days. Dericks-Tan *et al.*, (1977) injected 5 $\mu$g of the analogue at 8 hourly intervals and showed a reduction in the LH response after the third injection. Although we did not measure the peak concentrations of LH or FSH after the second and third injections of the analogue in this study, the levels at 14 and 10 hours respectively after these injections, did not differ. However, as the response to the first injection of 5 $\mu$g resulted in a peak concentration of LH comparable to that of the pre-ovulatory LH surge, repeated injections of this dose would be sufficient to assess pituitary synthetic capacity.

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# Assessment of pituitary secretory capacity in women with hypogonadotrophic hypogonadism by using a long-acting synthetic analogue of luteinizing hormone releasing factor

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**Summary.** The gonadotrophin responses to single and repeated injections of a long-acting synthetic analogue of luteinizing hormone releasing factor (LRF), D-Ser(TBU)$^{6}$EA$^{10}$LHRH, were investigated in 10 women with normoprolactinaemic hypogonadotrophic hypogonadism. Abnormal luteinizing hormone (LH) responses were observed in two of the five patients treated with 5μg of the analogue and in all five patients treated with three injections of 10, 20 and 10 μg administered at intervals of 10–14 h. However, the LH response to repeated injections of the analogue was of similar magnitude and duration to that observed in normal women in response to an oestrogen provocation test in the early-to-mid follicular phase of the cycle. Thus failure of the LH response to oestrogen provocation in women with hypogonadotrophism results from hypothalamic rather than primary pituitary dysfunction. This study confirms the usefulness of this analogue of LRF in the assessment of pituitary secretory function in women with abnormal responses to oestrogen positive feedback.

The prolonged and potent effect of the luteinizing hormone releasing factor (LRF) analogue, D-Ser(TBU)$^{6}$EA$^{10}$LHRH, on gonadotrophin secretion in normal women is well established (Dericks-Tan *et al.* 1977; Sandow *et al.* 1977; Friedrich *et al.* 1978) and has been exploited in the development of a simple clinical test of pituitary secretory capacity (Vaughan Williams *et al.* 1980). Furthermore the release of luteinizing hormone (LH) in response to a single injection of 5μg of the analogue, administered in the early-to-mid follicular phase of the cycle, is similar in magnitude and duration to that induced by a similarly timed oestrogen provocation test (C. A. Vaughan Williams, A. S. McNeilly & D. T. Baird, unpublished work).

Women with hypogonadotrophic hypogonadism invariably exhibit absence of the LH response to oestrogen positive feedback (Shaw *et al.* 1975*b*) as a result of either a primary disorder of pituitary secretory function or from abnormal hypothalamic function. The LRF analogue D-Ser(TBU)$^{6}$EA$^{10}$LHRH has therefore been used to investigate pituitary secretory capacity in women with hypogonadism to differentiate between hypothalamic and pituitary causes of abnormal gonadotrophin secretion and of failure of the LH response to oestrogen positive feedback.

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## Materials and methods

Ten women with secondary amenorrhoea resulting from significant weight loss and with concentrations of LH in plasma (mean of at least two random measurements) below the normal early follicular-phase range (2·5–15·3 units/l) were recruited for study. Informed consent was obtained from all subjects and the study was approved by the Ethical Committee of the Edinburgh Royal Infirmary. Concentrations of follicle stimulating hormone (FSH) were below the normal follicular-phase range (2·9–12·6 units/l) in three of these subjects. Concentrations of prolactin and thyroxine in plasma were within normal limits and pituitary radiology was normal. None of the subjects had shown any increase in total oestrogen excretion in response to treatment with clomiphene (100 mg daily) for 5 days. Clinical data are summarized in Table 1.

Five patients received a single intramuscular injection of 5μg of the LRF analogue and five received an initial injection of 10μg, followed by further injections of 10 and 20 μg after 10 and 24 h respectively. Venous blood (10 ml) was collected 24 h and immediately before treatment, at hourly intervals for 10 h after the initial or single injection, and thereafter twice daily until 72 h after the start of treatment. Additional blood samples were collected at hourly intervals for 6 h after the second and third injections. Plasma was stored at −20°C until assayed for LH, FSH and oestradiol.

The gonadotrophin and oestrogen responses were compared with those in normal women in the early follicular phase of the cycle and which have been reported in detail previously (Vaughan Williams *et al.* 1980).

The LH response to three injections of the analogue was compared with that observed in normal women in response to an oestrogen provocation test. Seven women with regular ovulatory cycles received two injections of oestradiol benzoate (1 mg intramuscularly) at an interval of 24 h, the first injection being administered between days 3 and 6 of the cycle. Blood samples were collected 24 h and immediately before the first injection, and at 12–14 h intervals until 96 h thereafter, for the measurement of concentrations of LH, FSH and oestradiol in plasma (C. A. Vaughan Williams, A. S. McNeilly & D. T. Baird, unpublished work).

## Hormone assay methods

Concentrations of LH and FSH in plasma were measured in duplicate in the specific double-antibody radioimmunoassay (RIA) of Hunter & Bennie (1979). The intra-assay coefficients of variation were 8 and 7% and interassay coefficients of variation 12 and 11% for LH and FSH respectively and the sensitivities of the systems were 0·3 units/l for LH and 1·0 units/l for FSH. Results are expressed in units per litre of the appropriate standards obtained from the National Institute of Biological Standards and Control, Holly Hill, London (LH: MRC 68/40, 77 i.u./ampoule; FSH: MRC 69/104, 10 i.u./ampoule).

**Table 1.** Clinical data on 10 patients treated with DSer(TBU)$^{6}$EA$^{10}$LHRH

| Patient no. | Duration of amenorrhoea (months) | IBW[a] (%) | Plasma concentrations[b] LH (units/l) (2·5–15·3) | FSH (units/l) (2·9–12·6) | Oestradiol (pg/ml) (100–150) |
|---|---|---|---|---|---|
| 1 | 37 | 86 | 1·2 | 4·4 | 13 |
| 2 | 23 | 86 | 2·3 | 6·6 | 48 |
| 3 | 90 | 86 | 2·4 | 3·8 | 5 |
| 4 | 13 | 72 | 2·0 | 2·3 | 7 |
| 5 | 17 | 81 | 2·4 | 2·1 | 70 |
| 6 | 24 | 82 | 2·1 | 4·1 | 112 |
| 7 | 14 | 84 | 1·8 | 2·2 | 115 |
| 8 | 19 | 78 | 2·2 | 3·2 | 57 |
| 9 | 39 | 73 | 1·0 | 4·3 | 47 |
| 10 | 24 | 89 | 0·3 | 3·4 | 60 |

[a] IBW, Ideal Body Weight; Geigy Scientific Tables.
[b] Values in parentheses give normal ranges.

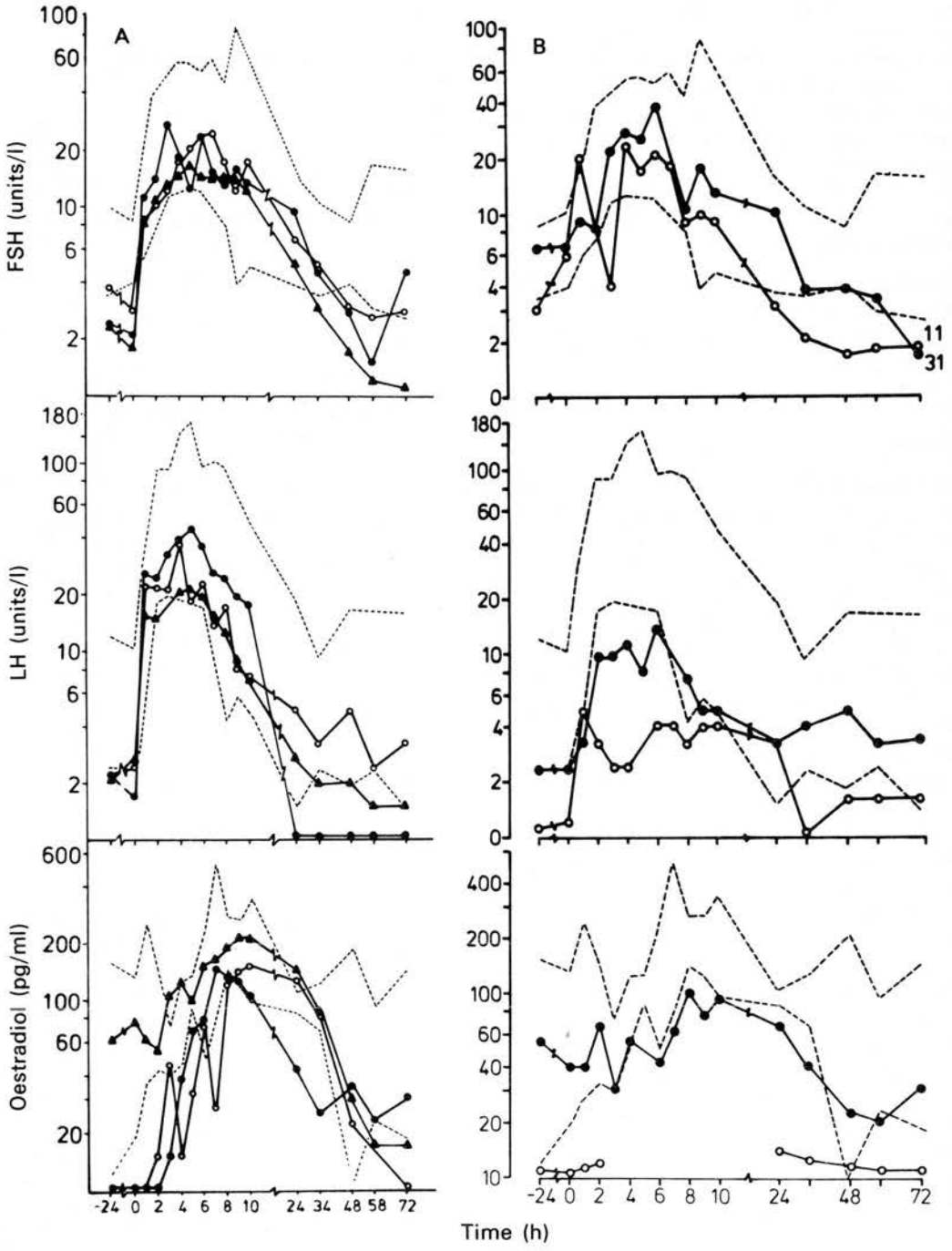



**Fig. 1.** Concentrations of LH, FSH and oestradiol in plasma in five hypogonadotrophic patients in response to a single injection of 5 μg of D-Ser(TBU)$^{6}$EA$^{10}$LHRH(99% confidence limits of normal mean values are shown by the broken lines). A, Normal responses (three patients ○, ●, ▲); B, abnormal responses (two patients, ○, ●).

The specific RIA method of van Look *et al.* (1977) was used for the measurement of concentrations of oestradiol in plasma. The sensitivity of the method was 5±1·0 (SD) pg, the interassay coefficient of variation for concentrations from 28 to 205 pg/ml was 6·1–13·3% and the intra-assay coefficient of variation 9·3%. Results are expressed in pg/ml.

### Statistical analysis

Values expressed in this paper were derived from the respective log values, which were used in Student's single-tail *t*-test. Mean values (±SD) are given in the text.

### Results

The gonadotrophin and oestradiol responses to a single injection of 5 μg of the analogue are shown in Fig. 1. Three subjects exhibited normal responses (Fig. 1A) but the LH and oestradiol responses in the remaining two subjects were below the normal ranges, although the FSH responses were within normal limits during the first 10 h after treatment (Fig. 1B). Gonadotrophin responses in five women treated with three injections of the analogue were very similar and mean concentrations of LH, FSH and oestradiol in plasma are shown in Fig. 2. The initial increase in the concentration of LH in plasma was within normal limits during the first 2 h after injection but concentrations thereafter were below the normal limits. Maximal concentrations of LH after the second and third injections of 42·9 (+23·9; −15·4) and 30·1 (+19·3; −11·3) units/l respectively did not differ from that of 47·1 (+24·2; −16·0) units/l in response to the first injection, but occurred progressively earlier, at 3 and 2 h after injection respectively.

The integral LH response to each application of the analogue was determined from the sum of the increments in the concentration of LH in plasma during the first 6 h after injection. As expected the initial response, of 175·2 (+86·6; −58·0) units $6\ h^{-1}\ l^{-1}$ was below the normal range (203–974 units $6\ h^{-1}\ l^{-1}$). The integral LH response to the second injection [163·0 (+89·8; −57·9) units $6\ h^{-1}\ l^{-1}$] did not differ significantly from the first but the response to the third injection was markedly reduced to 58·8 (+48·4; −26·6) units $6\ h^{-1}\ l^{-1}$ ($P<0·001$).

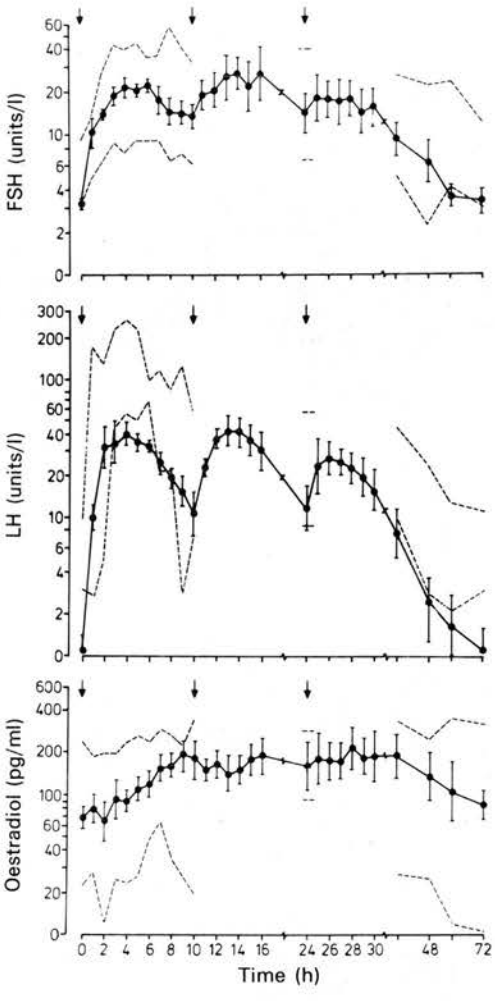



**Fig. 2.** Concentrations of LH, FSH and oestradiol in plasma (mean±SEM) in five hypogonadal patients in response to three injections (shown by the arrows) of 10, 20 and 10 μg of D-Ser(TBU)$^{6}$EA$^{10}$LHRH (99% confidence limits of normal mean values are shown by the broken lines).

Concentrations of LH reached pre-ovulatory levels, but were not sustained above the normal follicular range for more than 6–8 h. However, the concentrations of LH in response to three doses of the analogue did not differ significantly from those measured at comparable intervals in normal women during the positive-feedback phase of the LH response to oestrogen provocation in the early-to-mid follicular phase of the cycle (Fig. 3).

The FSH response to the initial injection of the analogue followed the normal pattern and peak concentrations of FSH after the second and third

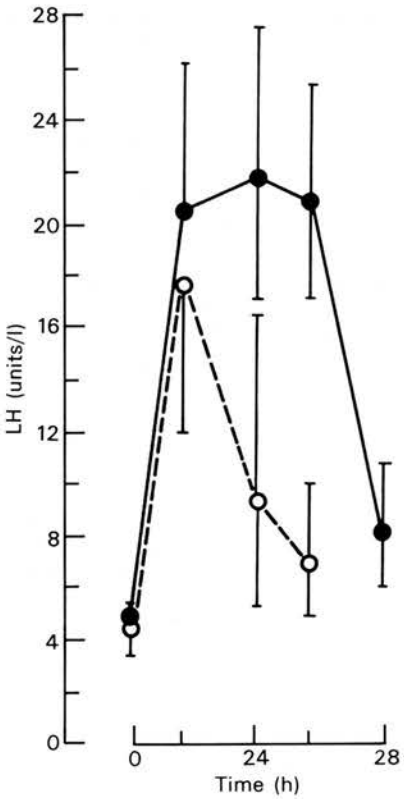



**Fig. 3.** Concentrations of LH in plasma (mean±SEM) in five hypogonadotrophic patients in response to repeated injections of D-Ser(TBU)$^{6}$EA$^{10}$LHRH (●——●) and in seven normal women in response to an oestrogen provocation test in the early-to-mid follicular phase of the cycle (○– – –○) (C. A. Vaughan Williams, A. S. McNeilly & D. T. Baird, unpublished work).

injections did not differ from those observed after the initial injection. The integral FSH responses were determined in a similar manner to those of LH. The initial response of 90·0 (+21·8; −17·6) units 6 $h^{-1}$ $l^{-1}$ did not differ significantly from the control value (mean 62·2 units 6 $h^{-1}$ $l^{-1}$). There was a progressive reduction in the magnitude of the integral FSH responses to subsequent injections of the analogue. The response to the second injection of 43·2 (+101·3; −30·3) units 6 $h^{-1}$ $l^{-1}$ did not differ from the initial response, but that to the third injection was markedly reduced to 10·3 (+23·7; −7·2) units 6 $h^{-1}$ $l^{-1}$ ($P<0·005$).

The oestradiol response followed the normal pattern, concentrations in plasma remaining elevated for 48 h after the first injection of the analogue.

## Discussion

Hypogonadotrophic hypogonadism is invariably associated with absence of the LH response to oestrogen positive feedback (Shaw *et al.* 1975*b*) and failure of the gonadotrophin response to clomiphene (Marshall & Fraser 1971; Beumont *et al.* 1973; Wakeling *et al.* 1976) and results either from a primary pituitary disorder of gonadotrophin secretion or from abnormal hypothalamic function.

The normal LH response to oestrogen positive feedback has not been fully elucidated. However, evidence from studies in rhesus monkeys indicates that LH is released in response to the direct effect of oestrogen on the pituitary, while continued priming of the gonadotrophs is essential to the maintenance of pituitary secretory capacity and thus the integrity of the response (Knobil *et al.* 1979, 1980).

The long-acting LRF agonist D-Ser(TBU)$^{6}$ EA$^{10}$LHRH provides an effective means of demonstrating pituitary secretory capacity in normal women (Vaughan Williams *et al.* 1980) and a single injection of 5 μg of the analogue in the follicular phase of the cycle induces an LH response of equal magnitude and duration to that induced by a similarly timed oestrogen provocation test, whereas the LH response to three injections of the analogue exceeded that induced by oestrogen provocation. Thus an LH response of normal magnitude to 5 μg of the LRF analogue observed in three of the five hypogonadotrophic women in the present study indicates that failure of the LH response to oestrogen positive feedback does not result from a primary pituitary defect. Similarly an LH response to three injections of the analogue of a magnitude and duration comparable with that provoked by oestrogen stimulation in normal subjects confirms that the secretory capacity of the pituitary in these hypogonadotrophic women is adequate for the manifestation of a normal LH response to oestrogen provocation.

However, LH responses to the LRF analogue were impaired in seven of the 10 patients in this study, whereas FSH responses were within normal limits. These are similar to the gonadotrophin responses to the natural decapeptide exhibited by women with hypogonadotrophic hypogonadism resulting from moderate degrees of weight loss, to which the FSH response appears to be less sensitive than does that of LH (Warren *et al.* 1975; Beumont *et al.* 1976). The

abnormal LH responses probably reflect the inappropriate stimulation of the pituitary afforded by the LRF analogue, as administration of synthetic LRF in a pulsatile manner, which mimics the presumed physiological pattern of LRF release, not only restores a normal pattern of gonadotrophin secretion and normal LH responses to LRF in similar subjects (Marshall & Kelch 1979) but also restores ovulatory cycles (Crowley & McArthur 1980; Leyendecker *et al.* 1980; Shoemaker *et al.* 1980).

Thus it would appear that hypogonadotrophic hypogonadism is the end result of abnormal secretion of LRF which is inappropriate for the maintenance of the normal secretory function of the pituitary. Hence the pituitary becomes incompetent to respond to the direct effect of oestrogen positive feedback with an increase in the secretion of LH.

Normal FSH responses in association with impairment of the LH responses suggest that maintenance of the synthesis and release of FSH is less dependent on priming of the pituitary gonadotrophs by LRF than is that of LH secretion.

Refractoriness of the pituitary response to repeated stimulation with the analogue has been demonstrated in normal women (Dericks-Tan *et al.* 1977; Nillius *et al.* 1978; Baumann *et al.* 1980) probably resulting from 'down-regulation' of receptors (Barr & Roth 1977; Khan *et al.* 1977) but appears to develop less readily in hypogonadal subjects (Hanker *et al.* 1978; C. A. Vaughan Williams, A. S. McNeilly & D. T. Baird, unpublished work). The LH response to the third injection of the LRF analogue was markedly reduced in the group of hypogonadal women. However, concentrations of oestradiol in plasma had been elevated for at least 17 h at the time this injection was administered and would thus be expected to result in reduced pituitary responsiveness to LRF (Shaw *et al.* 1975*a*).

The increase in oestradiol secretion observed in these women after injection of the analogue can be confidently attributed to increased gonadotrophin secretion in response to treatment, as a spontaneous return to ovulatory cycles in such patients rarely occurs before the reattainment of their ideal body weight. The magnitude of LH response to the third injection of the analogue in normal women was not measured and the relative reduction in the response in hypogonadotrophic subjects cannot therefore be assessed.

The progressive reduction in the magnitude of the FSH response to the second and third injections of the analogue was far greater than that of the LH response and may reflect the greater sensitivity of FSH secretion to the negative-feedback effects of oestrogen (Yen *et al.* 1970).

However, chronic treatment of hypogonadotrophic women with natural LRF induces a marked reduction in the magnitude of the FSH response independent of oestrogen negative feedback, while the LH response is augmented (C. A. Vaughan Williams, A. S. McNeilly & D. T. Baird, unpublished work). Thus the FSH response to LRF appears to be more readily desensitized than does that of LH.

The present study confirms that pituitary secretory function is not primarily impaired in women with hypogonadotrophic hypogonadism and that failure of the LH response to oestrogen positive feedback in these women results from hypothalamic dysfunction. Furthermore our data suggest that FSH secretion is less dependent on priming of the pituitary by LRF than is that of LH, but that the FSH response to LRF is more readily desensitized.

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# Asynchronous Changes in Circulating LH and FSH after the Gonadotrophin Releasing Hormone

HYPOTHALAMIC extracts have been reported to contain two gonadotrophin releasing factors, one for luteinising hormone (LH) and one for follicle stimulating hormone (FSH)[1,2] but only a single peptide has been isolated from these preparations[3]. This hypothalamic hormone has been shown to be a decapeptide which is capable of releasing both LH and FSH in humans[4]. We report here the relationship between the changes in circulating concentrations of both LH and FSH during continuous infusion of this material.

There were four male volunteers aged between twenty-four and twenty-seven yr old. Each subject received a continuous infusion of 0.9% NaCl solution during a control period of 1 h, followed immediately by a 1 h infusion of either a solution containing 190 $\mu$g of the gonadotrophin releasing hormone LH/FSH-RH (Hoechst) in 0.9% NaCl, or 0.9% NaCl alone. Experiments were carried out at weekly intervals after at least 24 h of sexual continence. Samples for serum LH and FSH were withdrawn at 5 min intervals for the first 55 min then at 1 min intervals until the end of the control infusion. Sampling was continued at intervals of 1 min during the initial 10 min of the LH/FSH-RH infusion and at 5 min intervals thereafter. Serum

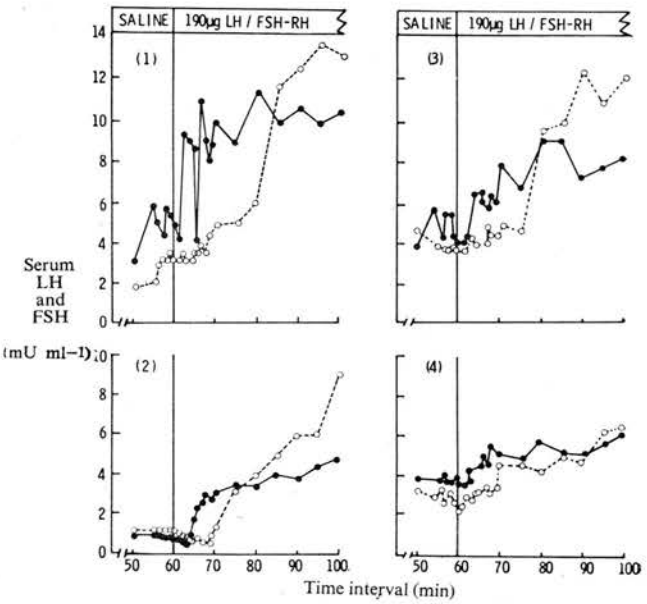



**Fig. 1** Asynchronous changes in circulating levels of LH (○) and FSH (●) in four subjects continuously infused with gonadotrophin releasing hormone.

was stored at −20°C until assayed. Radioimmunoassay of LH and FSH was performed as previously described[5], and results expressed in mU ml$^{-1}$ of LH (MRC 68/40) and FSH (MRC 68/39) taking the contents of each ampoule as 39.8 and 32.8 U respectively. There was no crossreactivity between the hormones at the levels measured. The coefficient of variation for LH within assay was 5% (in the range 0.4 to 15 mU ml$^{-1}$) and 6% for FSH (in the range 0.2 to 10 mU ml$^{-1}$). Because of this assay variance, and the known fluctuations of basal gonadotrophin concentrations[6], a response resulting from the infused material material was considered significant only when a change five times the coefficient of variation was observed above the maximum gonadotrophin level seen in the control infusion. For LH this was a change of 25% and for FSH 30%.

Basal levels of LH and FSH were observed to fluctuate in a random fashion. Following infusion of LH/FSH-RH, marked fluctuations in FSH were observed as the concentration increased in subjects 1 and 3. The fluctuations were less marked in subject 4 and a smoother response was seen in subject 2 (Fig. 1). A significant FSH response occurred within 3 to 10 min of starting the infusion of the decapeptide. Concentrations continued to increase for 20 to 30 min before levelling off although often varying in a pulsatile fashion.

Compared with the FSH response, the LH response was delayed 9 to 20 min after the start of the releasing hormone infusion. A smooth increase was seen initially although later it also began to fluctuate. LH levels continued to increase although the FSH response had begun to level off. Clearly the circulating concentrations of the gonadotrophins were asynchronous both in the time of release and in their fluctuations in spite of a continuous infusion of the synthetic decapeptide.

Although it has been suggested that there are separate releasing hormones for the gonadotrophins[6,7] our work indicates that the occurrence of asynchronous concentrations of the gonadotrophins does not necessarily mean that there must be separate releasing hormones. It would seem that the threshold for the initiation of FSH release is less than that for LH, although greater concentrations of LH are subsequently reached. These differential time-related responses cannot be accounted for by the known differences in disappearance rates for LH and FSH[8,9]. After bolus injections of LH/FSH-RH, however, the time course of release of the hormones from the pituitary seems to be similar although not necessarily synchronous in individual subjects[10].

It would also seem that if a constant concentration of releasing hormone is maintained then the critical factor resulting in pulsatile release of LH and FSH is pituitary cell function. Sudden decrease in circulating hormone concentrations may reflect a refractory phase of the pituitary gonadotrophic cell to stimulation. It is possible that the effects of endogenously produced gonadotrophin releasing hormone(s) may be superimposed on the effects of the exogenous decapeptide in our experiments but this would not invalidate the conclusion that continuous administration of the single decapeptide releasing hormone produces differential release of LH and FSH asynchronously.

We suggest that this infusion technique may be used to explore hormonal feedback mechanisms in relation to gonadotrophin secretion and the possible intermittent refractoriness of the gonadotroph. The further possibility that the frequency and amplitude of the induced pulsatile gonadotrophin secretion in response to the hypothalamic releasing hormone may be involved in the coding of the trophic message to the glands remains to be explored.

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# PAPERS AND ORIGINALS

# Gonadotrophin-releasing Hormone Therapy in Hypogonadal Males with Hypothalamic or Pituitary Dysfunction

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ritish Medical Journal, 1974, 4, 617-621


## ummary

**ubcutaneous self-administration of synthetic onadotrophin-releasing hormone 500 μg eight-hourly or up to one year by 12 male patients (five prepubertal) ith clinical hypogonadism due to hypothalamic or ituitary disease resulted in the synthesis and continued elease of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). There was a rise in circulating ndrogen levels in all patients. Improvements in pubertal atings were seen in some prepubertal patients. Potency eturned in the adults and spermatogenesis was induced nd maintained in the four patients who had received reatment for more than four months, total counts eaching between 7·8 and 432 × $10^6$ spermatozoa. A fall n the FSH response to the releasing hormone occurred uring spermatogenesis though LH was little affected. uring the initial weeks of therapy FSH secretion usually ccurred before that of LH though LH secretion was reater as treatment continued. FSH secretion also ersisted for longer when treatment was stopped.**


## ntroduction

We have recently reviewed the results of a standard 100-μg test ose of luteinizing hormone/follicle stimulating hormone-leasing hormone (LH/FSH-RH) in 155 patients with hypo-alamic-pituitary-gonadal dysfunction (Mortimer *et al.*, 1973 a). Though 88% of these patients were clinically hypogonadal at the time of testing and had low endogenous gonadotrophin secretion an LH response or FSH response or both occurred in 90%. It was therefore hoped that repeated administration of the synthetic decapeptide would result in the induction of gonadotrophin synthesis, its release, and gonadal stimulation. We here report the results of such treatment.

## Dose Regimen

Studies of the time course of action of the releasing hormone given by intramuscular, intravenous, and subcutaneous routes showed it to be equally effective in promoting increased circulating levels of LH and FSH by each route with a duration of action of between five and seven and three and five hours respectively (Mortimer *et al.*, 1973 b). It was therefore decided that when sufficient supplies of the material became available long-term therapy in patients with hypogonadotrophic hypogonadism would be initiated by repeated subcutaneous injections. A dose of 500 μg was used since experience showed that patients with hypothalamic or pituitary disease would require a higher dose than normal subjects to produce an adequate response. Therapy was continued with this dose administered by the patients themselves subcutaneously into the anterior abdominal wall or thigh. Injections were repeated at eight-hour intervals in view of the known time course of gonadotrophin secretion. One patient, treated when the supply of the drug was unavoidably curtailed, received a more varied regimen and is considered separately. All patients were tested before treatment and at intervals during treatment with 100 and 500 μg LH/FSH-RH, measurement of serum LH and FSH levels being carried out over a four-hour period. The duration of treatment varied from four weeks in the prepubertal patients to between 26 weeks and one year in the adults.

## Patients

Twelve male patients with clinical hypogonadism and pituitary or hypothalamic disease were studied.

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PREPUBERTAL PATIENTS

*Craniopharyngioma.*—Four patients aged 14 to 22 years had craniopharyngiomas. Three had had excision of the tumour followed by irradiation, while one had had irradiation only. These procedures had been performed between six months and 10 years, previously. All were receiving intramuscular growth hormone therapy. Three were also receiving thyroxine and three hydrocortisone replacement.

*Isolated Growth Hormone Deficiency.*—One patient, aged 20 years, had isolated growth hormone deficiency. He was also receiving intramuscular growth hormone.

None of these patients except one (craniopharyngioma) showed an LH or FSH response to clomiphene given in a dose of 3 mg/kg/daily for 10 days.

ADULT PATIENTS

*Isolated Gonadotrophin Deficiency.*—Four patients aged 22 to 24 years had so-called isolated gonadotrophin deficiency. Previously we suggested that this syndrome probably represents a deficiency of LH/FSH-RH rather than a primary gonadotrophin deficiency (Marshall *et al.*, 1972). All had low or undetectable basal serum gonadotrophin levels which failed to respond to clomiphene. There was no evidence of a deficiency of other pituitary hormones. These patients had been treated previously with either intramuscular testosterone proprionate or human chorionic gonadotrophin (HCG) or both. One patient, previously impotent and azoospermic, had received intramuscular Pergonal (human menopausal urinary gonadotrophins; HMG) and had successfully fathered a child.

*Craniopharyngioma.*—One 33-year-old man had had a craniopharyngioma decompressed and irradiated 19 years previously and had also fathered a child after HMG therapy. He was unresponsive to clomiphene.

*Acromegaly.*—A 32-year-old man had received external pituitary irradiation for acromegaly one year before treatment and he was hypogonadal. Basal growth hormone levels were reduced from 17 to 4 ng/ml and the paradoxical rise during a glucose tolerance test was reduced from 55 to 19 ng/ml. His acromegaly clinically improved. He had low 17β-hydroxyandrogen (17-OHA) levels and greatly impaired potency.

*Hypothalamic Tumour.*—One patient, aged 37 years, had a diffuse hypothalamic tumour of unknown origin which went into remission after high-dose dexamethasone therapy and hypothalamic irradiation. At the time of the study he was maintained on hydrocortisone, thyroxine, and DDAVP (Edwards *et al.*, 1973) as replacement therapy. He was unresponsive to clomiphene.

All these patients had been off any form of gonadal steroids or gonadotrophin therapy for at least four months before the study began and were clinically hypogonadal, with loss of potency and body hair and small genitalia. All the patients had azoospermia in the first ejaculate produced during treatment except for the man with the craniopharyngioma, who had a total count of 600,000 dead spermatozoa, presumably lying in situ from HCG therapy terminated four months earlier.

## Assays

*Serum LH and FSH* levels were measured by specific radioimmunoassays as described previously (Mortimer *et al.*, 1973 c) and the results expressed in mU/ml of M.R.C. standard 68/40 for LH and 68/39 for FSH, the contents of each ampoule being taken as 39·8 and 32 units respectively. Normal adult male basal levels of LH and FSH range from less than 0·4 to 6 mU/ml and from less than 0·2 to 5·9 mU/ml respectively.

*Plasma 17-OHA* levels were estimated by the method of Anderson (1970). This measures predominantly testosterone and dihydrotestosterone. (Normal male range at 9 a.m. for plasma 17-OHA 5·0-22·5 ng/ml.)

*Plasma sex-hormone-binding globulin (SHBG)* was measure by the method of Rosner (1972) and the results were expresse in $10^{-8}$ mol of dihydrotestosterone bound per litre. The norm adult male range is 2·10 to 5·1 $\times$ $10^{-8}$ mol/l. Higher levels a characteristically seen in prepubertal subjects (August *et a* 1969; Forest and Bertrand, 1972).

*Non-protein-bound ("free") androgen levels* were estimated b calculating the ratio of total 17-OHA to SHBG. When expresse as a percentage the normal adult male range is 1·42% to 2·0%

*Plasma oestradiol* was measured by radioimmunoassa (Hotchkiss *et al.*, 1971). The normal adult male range is fro less than 10 to 50 pg/ml. All blood samples were separate immediately and stored at −20°C until assayed.

## Gonadotrophin Responses

PREPUBERTAL PATIENTS

*Craniopharyngioma.*—Of the four patients with craniopharyn giomas two had undetectable serum LH levels and two had leve of 0·7 mU/ml before treatment; however, all had detectab circulating FSH (between 0·3 and 1·0 mU/ml). The respons during the initiation of therapy in one of these patients is show in fig. 1. Three patients failed to show a response in LH to a intravenous test dose of 100 μg of the releasing hormone befo treatment though a small but significant rise in FSH was seen i each case. When the first subcutaneous dose of 500 μg was give all three patients showed a rise in both LH and FSH. In th fourth patient, with detectable LH and FSH basally, there wa no further response to 100 μg of the releasing hormone but simultaneous increase in both gonadotrophins occurred whe 500 μg was injected. This patient was the only one who had show a response to clomiphene. As treatment with 500 μg was starte and continued there were progressive increases in both gonado trophins in all four patients, with LH secretion becoming equa to or exceeding that of the FSH response. Thus though initiall there was a larger FSH than LH response before treatment wa started, by the end of four weeks the response of LH was greate than that of FSH. Therapy was discontinued after four weeks i three patients as it was felt unwise to induce puberty while the were still receiving growth hormone therapy.

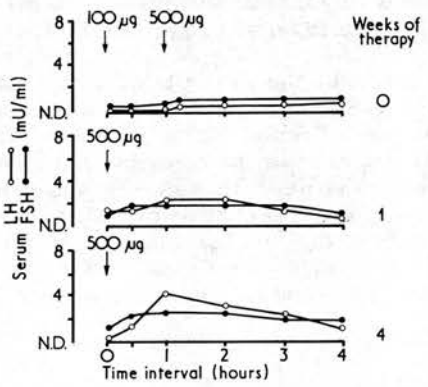


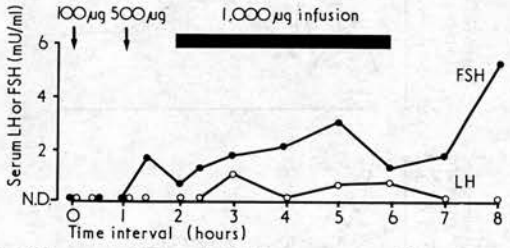

FIG. 1—Serum LH and FSH responses during four weeks of LH/FSH-R therapy in prepubertal male patient with craniopharyngioma. N.D. = N detectable.

*Isolated Growth Hormone Deficiency.*—The patient wit isolated growth hormone deficiency had detectable LH and FS levels. He had an impaired LH response but a normal FSH response to the initial 100 μg of releasing hormone but norm LH and FSH secretion was induced by LH/FSH-RH therap maintained for 20 weeks.

ADULT PATIENTS

All four patients with the so-called *isolated gonadotroph*

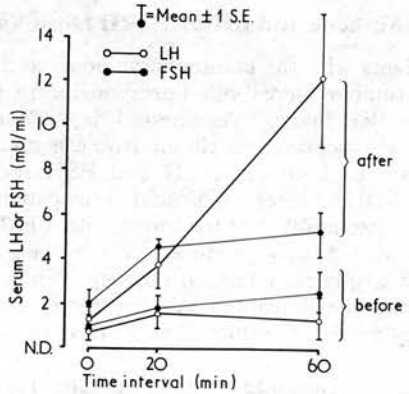
*leficiency* had undetectable LH levels and in only one was FSH letectable; LH and FSH secretion was induced by long-term LH/FSH-RH therapy in each case. Two of these patients howed initial responses similar to that seen in the prepubertal patients with craniopharyngiomas, in that detectable secretion of FSH returned before that of LH. The responses in one such adult patient are shown in fig. 2. The other two patients showed a small LH response but an absent FSH response to an intravenous dose of 100 μg of the releasing hormone before treatment. LH secretion was greater initially than FSH secretion on treatment. The man with the *craniopharyngioma* had undetectable LH but measurable FSH levels basally and impaired LH but normal FSH responses to an intravenous dose of 100 μg of the releasing hormone. After the first week of therapy the levels of FSH were higher than those of LH though these relative responses were reversed by the second week. The patient with the *hypothalamic tumour* of unknown origin had detectable gonadotrophins basally and these showed normal responses to the 100 μg test. FSH secretion was greater during the first five weeks of therapy but thereafter LH was the predominant hormone secreted. The man with *acromegaly* had a basal LH level in the range seen in adult males with normal testosterone levels and he showed a normal response to 100 μg of the releasing hormone. His basal FSH values in contrast were raised and showed an excessive response to the same dose of the decapeptide.




G. 2—Initial serum LH and FSH responses to intravenous bolus of 00 μg and 500 μg LH/FSH-RH followed by infusion of 1,000 μg over four ours in man with isolated gonadotrophin deficiency.

FFECTS OF THERAPY ON LH/FSH-RH TEST

n five patients (two prepubertal boys with craniopharyngiomas, he man with the craniopharyngioma, the man with the diffuse ypothalamic tumour, and one of the men with isolated gonadoophin deficiency) the standard 100-μg LH/FSH-RH test was epeated after four to six weeks of therapy after being off eatment for one to seven days. Before treatment in these atients the response of FSH was much greater than that of LH, hereas after treatment the secretion of LH was greater than at of FSH. The levels of both LH and FSH were higher after eatment than before (fig. 3).




. 3—Serum LH and FSH responses to standard test dose of 100 μg avenous LH/FSH-RH in five patients before and after four to six weeks herapy with releasing hormone.

## Induction of Androgen Secretion and Puberty in Prepubertal Patients

Of the five prepubertal patients three were still growing on intramuscular growth hormone therapy. Gondadotrophin-releasing hormone therapy was discontinued after four weeks in these subjects since it was considered undesirable to induce puberty while they still had growth potential. All had shown a rise in gonadotrophin and 17-OHA levels during that time. The remaining two patients, aged 19 and 22 years, were also on growth hormone therapy but since their growth was slowing down it was decided to attempt to induce puberty in the hope that there would be potentiation of growth as in normal puberty. Before treatment one patient had pre-adolescent genitalia with vellus over the pubes indistinguishable from that of the abdominal wall—that is, stage 1 (Tanner, 1958). After eight weeks of treatment this patient showed an increase in spontaneous erections and stage 2 pubic hair development. The other patient before treatment had slight pubertal enlargement of the testes and scrotum with sparse growth of pigmented downy hair at the base of the penis (stage 3). After nine weeks of treatment there was an increase in testicular volume of from 6 to 12 ml on the right and from 4 to 8 ml on the left. Spontaneous erections became frequent and there was an increase in coarse pubic hair.

Gonadotrophin secretion continued within the normal basal adult male range in these subjects while treatment was maintained though the 17-OHA levels increased only slightly, from pretreatment levels of 0·4 to 1·7 ng/ml to between 1·4 and 2·4 ng/ml—that is, below the normal adult range. Plasma oestradiol levels did not change and were within the normal adult male range. Plasma SHBG was above the adult range before treatment in four patients (between 7·7 and 22·6 $\times 10^{-8}$ mol/l.), as is usually seen before puberty (August *et al.*, 1969; Forest and Bertrand, 1972); in the other patient it was normal (4·6 $\times 10^{-8}$ mol/l.). In the three patients treated for four weeks only there was no consistent change in SHBG. In the two treated for longer the SHBG levels fell. In these patients changes in circulating levels of free androgens inferred from the ratio of total 17-OHA to SHBG and expressed as a percentage rose from between 0·02% and 0·18% basally to between 0·03% and 0·32% after treatment but were still below the normal adult male range.

## Induction of Androgen Secretion and Potency in Adult Patients

All seven adult patients studied had an increase in potency within seven to 14 days of beginning treatment. Before treatment 17-OHA levels reached between 1·0 and 3·1 ng/ml. In every case there was an increase in 17-OHA levels though only two patients showed a rise to within the normal range on more than two occasions. These two subjects achieved maximum levels of 15·5 and 7·5 ng/ml after two and four weeks of treatment respectively but values then fell and were maintained between 3·1 and 4·8 ng/ml. In the remaining five patients, though levels were subnormal (between 0·4 and 4·8 ng/ml) improved potency was well maintained. Three patients had normal potency. Plasma 17β-oestradiol levels remained within the normal range throughout treatment except in three patients on three isolated occasions, when raised levels of 55, 98, and 171 pg/ml were recorded.

SHBG levels were above normal before treatment in one patient (5·8 $\times 10^{-8}$ mol/l.), low in two patients (1·4 and 1·5 $\times 10^{-8}$ mol/l.), and normal in the other four (2·7 to 5·0 $\times 10^{-8}$ mol/l.). During treatment SHBG levels in all seven patients rose to a maximum of between 2·6 and 9·6 $\times 10^{-8}$ mol/l. Peak values occurred at three weeks in one patient, between six and 10 weeks in four, and at 22 and 23 weeks in the other two. Levels then fell but remained above the normal range in four of these patients (between 5·4 and 8·0 $\times 10^{-8}$ mol/l. at 21 to 30 weeks of therapy), and though the free androgen levels rose (17-OHA:

SHBG ratio 0·04% to 0·13% basally, rising to a maximum of between 0·19% and 0·57% during therapy) they were still well below the adult male range. Of the other three patients one had low SHBG levels throughout, with free androgen levels rising above the normal range at nine to 18 weeks, being between 4·5% and 7·4%. They were still raised at 33 weeks. One patient had normal SHBG levels throughout 10 weeks of therapy, and his free androgen levels rose from 0·3% to a maximum of 0·49%. The other patient had a normal SHBG level of $4{\cdot}4 \times 10^{-8}$ mol/l. before treatment, rising to $6{\cdot}0 \times 10^{-8}$ mol/l. at three weeks and then falling to $4{\cdot}4 \times 10^{-8}$ mol/l. at 28 weeks. Free androgens rose from 0·13% to a maximum of 0·30% on therapy.

SPERMATOGENESIS

Four of the adults were on treatment for between 26 weeks and one year and spermatogenesis occurred in each of them. Initially one patient with *isolated gonadotrophin deficiency* was impotent but after nine days of treatment he was able to produce a specimen which showed azoospermia. By 17 weeks, however, he produced $1{\cdot}6 \times 10^6$ spermatozoa, and his count subsequently rose to $2{\cdot}7 \times 10^6$ and then to $7{\cdot}8 \times 10^6$ with a motility of 30% at 41 weeks (fig. 4). Another patient with *isolated gonadotrophin deficiency* had azoospermia intially but after six months produced three spermatozoa, two of which were motile. Four weeks later his count has risen to 660,000, with a motility of 60%, and after a further four weeks to $1{\cdot}6 \times 10^6$, with a motility of 20%; at 50 weeks his sperm count had reached $36{\cdot}7 \times 10^6$ with a motility of 50%. In both these patients the maximum FSH levels achieved after a 500-μg dose of the releasing hormone fell progressively as the sperm count rose but the LH levels were not consistently affected.

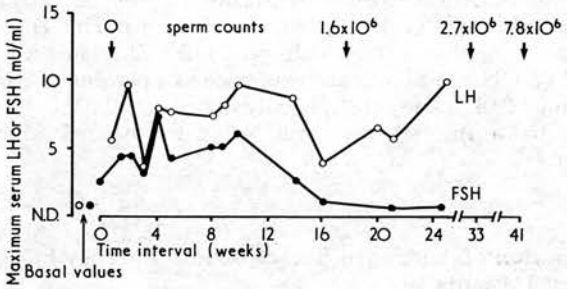



FIG. 4—Maximum serum LH and FSH responses to therapetuic 500-μg dose of LH/FSH-RH in man with isolated gonadotrophin deficiency. Increasing total sperm counts are shown at top.

The results in the patient with the *craniopharyngioma* who had previously fathered a child after HMG therapy are shown in fig. 5. Initially he was completely impotent but this had improved by the end of the second week of treatment. At four weeks an ejaculate contained 600,000 dead spermatozoa, presumably

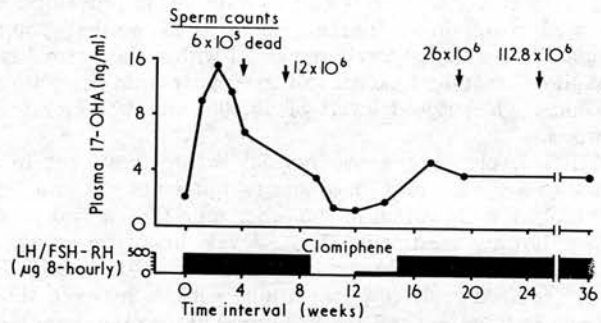



FIG. 5—Plasma 17-OHA levels during therapy with releasing hormone in man with craniopharyngioma. Clomiphene started at week 9, therapy reintroduced with 100 μg 8-hourly from week 12 to week 15, then increased to 500 μg 8-hourly. Increasing total sperm counts are shown at top.

lying there since gonadotrophin therapy some four month previously. By six weeks a total count of $12 \times 10^6$ spermatozo had been recorded, and this subsequently rose to $26 \times 10^6$ a 19 weeks, $112{\cdot}8 \times 10^6$ at 33 weeks, with a motility of 40%, an $432 \times 10^6$, with 70% motility, after one year of treatment. Th pattern of the FSH response in the previous two patients wa repeated, LH again showing no clear changes.

The fourth patient in whom spermatogenesis was induce was the man with *acromegaly* (fig. 6). Initial investigation showed that he had a basal 17-OHA level of only 1·7 ng/ml.O treatment this rose to a maximum of 5·2 ng/ml at six weeks an was then maintained at between 3·8 and 4·5 ng/ml. When see initially he had a much reduced potency but was able to produc a specimen for seminal analysis, which contained no spermatozoa During treatment, however, he showed a progressive rise i total sperm counts from 50,000 to $13{\cdot}6 \times 10^6$ at 25 weeks an $60{\cdot}8 \times 10^6$ at 48 weeks with a motility of 40%. Before treatmen he had a basal LH level of 3·8 mU/ml but raised FSH level o 17·3 mU/ml. Initially the FSH values after a 500-μg dose o LH/FSH-RH were greater than 50 mU/ml, but these graduall fell to 1·6 mU/ml during therapy as the sperm count rose. Th LH values in contrast fluctuated between 6 and 15 mU/m without showing a well-defined pattern of response.

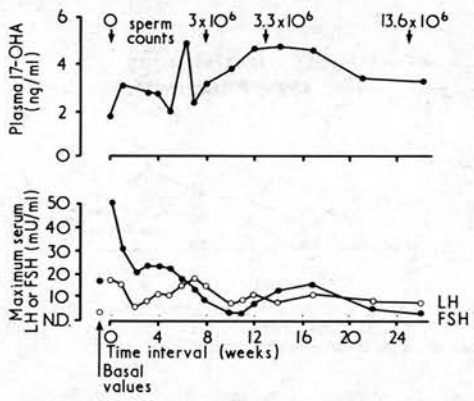



FIG. 6—Man with treated acromegaly. Top: Levels of 17-OHA throughou LH/FSH-RH therapy. Increasing sperm counts are shown at top. Botton Maximum serum LH and FSH responses to therapeutic 500-μg dose LH/FSH-RH.

TESTICULAR VOLUME

In all seven adult patients there was a clear increase in testicul volume—from between 1 and 3 ml before treatment to betwee 6 and 12 ml after 12 to 35 weeks—together with an increase i the size of the penis. Beard growth and body hair improved i three patients.

## Effect of Clomiphene and LH/FSH-RH Dose Variation

The adult patients with the craniopharyngioma and the diffu hypothalamic tumour were both unresponsive to clomipher before LH/FSH-RH therapy. At nine and six weeks respective LH/FSH-RH was stopped and clomiphene was given in a do of 3 mg/kg/day. In both cases LH and FSH levels becan undectable, 17-OHA levels fell, and the patients becan impotent again. Resumption of treatment with LH/FSH-RH a dose of 100 μg 8-hourly produced only a small increase 17-OHA and a negligible effect on potency. When full dosa was given again gonadotrophin and androgen secretion a normal potency returned within 10 to 21 days in both patier (fig. 5).

One patient had a more variable dosage regimen. Th patient, with isolated gondotrophin deficiency, whose init results are shown in fig. 2, had basal 17-OHA levels of 1·4 ng/r

e was treated initially with 500 μg of the releasing hormone ocutaneously 12-hourly. There was an increase in potency in e next two weeks though the levels of 17-OHA never rose ove 2·1 ng/ml. During the first three weeks of treatment culating levels of basal gonadotrophins increased with more H being secreted than FSH. At that time, however, the dose of H/FSH-RH was reduced to 200 μg 12-hourly because of short pplies and there was a marked decrease in gonadotrophin els. By 19 weeks FSH secretion continued in response to 0 μg of the releasing hormone though LH had become detectable—that is, the initial response pattern had again en restored. An increase in dose to 500 μg eight-hourly, wever, produced a rise in both LH and FSH.

## scussion

l the patients in this study had evidence of hypothalamic or uitary disease resulting in clinical hypogonadism with low culating levels of 17-OHA. Two patients failed to show any nadotrophin responses to the standard intravenous 100-μg t dose of LH/FSH-RH before treatment was started, whereas all but two of the other 10 there was impairment of either H secretion or FSH secretion or both. In every case, however, peated administration of the synthetic decapeptide resulted in e initiation and maintenance of secretion of both gonadophins. It appears from our studies, therefore, that long-term H/FSH-RH administration not only causes the release of the nadotrophins but also promotes synthesis of new hormone, d these effects may persist for up to a year of continuous erapy.

In four of the five clinically prepubertal subjects FSH cretion either occurred first or rose to the normal male range response before LH. The exception was the patient with a niopharyngioma who was clomiphene responsive before atment. A rise in FSH before LH was also seen in two adults th so-called isolated gonadotrophin deficiency and in the man th the craniopharyngioma. It appears that in patientswith pogonadotrophic hypogonadism secretion of FSH most mmonly occurs before that of LH during continued treatment th the releasing hormone. Interestingly a similar pattern of fferential gonadotrophin responses during LH/FSH-RH ting was reported to occur at the time of normal puberty by anchimont *et al.* (1974). Prepubertal patients showed an ult type of FSH responses to LH/FSH-RH though reduced H responses; after puberty the adult pattern with more LH n FSH secretion was reported, but these effects were better en in female than male patients.

From our studies it is evident that prepubertal boys may nieve puberty if treatment with the releasing hormone is ntinued. There is an increase in testicular size with a rise in culating androgens, greater hair growth, and enhanced tency. Also within four to six weeks the prepubertal type of nadotrophin responses to the releasing hormone reverses to e normal adult pattern.

It is also evident that if therapy is maintained potency will urn in the adult patients. Surprisingly, however, in six of the ven adult patients there was an early increase in potency, seven 14 days after starting therapy, which was maintained despite culating 17-OHA levels well below the lower limit of the rmal male range. At these levels most other patients seen are potent. The unusual degree of potency recorded by these bjects is not explained on the basis of normal free androgen els in the presence of low total androgens, since levels of the ding protein SHBG were either normal or high in five of em and their free testosterone levels were well below the rmal adult male range. In only one patient did low basal e androgen levels rise to the normal range during therapy and nain there. He also had low SHBG levels. Plasma oestradiol els were also within the normal male range except on rare occasions. The rapid return of potency in these patients may therefore have been due to factors other than those which are simply androgen-mediated. Of relevance to these observations may be the findings of Moss and McCann (1973) and Pfaff (1973), who noted a marked increase in the number of lordotic responses in ovariectomized and hypophysectomized rats after subcutaneous treatment with the synthetic decapeptide. These workers suggested that LH/FSH-RH may have a direct promoting action on sexual behaviour in rats indepedent of its hormone effects, and this may also be the case in man.

Apart from the return of potency, spermatogenesis was induced in four patients. Total sperm counts increased from zero (or 600,000 dead spermatozoa in one patient) to maximum values of 7·8, 36·7, 60·8, and 432 $\times$ $10^6$ in these patients. The patient with acromegaly was particularly interesting. Before treatment he had low 17-OHA levels with LH levels in the normal range together with azoospermia despite raised FSH levels. The LH response to a standard 100-μg test dose of LH/FSH-RH was normal but the FSH response was excessive. A similar pattern of response in patients with oligospermia or azoospermia has been noted previously (Mortimer *et al.*, 1973 a). As treatment continued, however, spermatogenesis was induced, presumably as a result of the production of intratesticular androgens in the presence of FSH, and the maximum FSH values after administration of the releasing hormone began to fall as the sperm count began to rise. This pattern of response though less marked also occurred in the other patients in whom spermatogenesis was induced. This suggests that the factor produced during spermatogenesis, often called "inhibin," was being released into the circulation and was exerting a negative feedback effect on FSH secretion. It seems, therefore, that the site of action of inhibin was primarily at the pituitary level since circulating LH/FSH-RH levels were being maintained by repeated subcutaneous injections. It is also evident that pituitary responsiveness in male subjects may be modified by circulating substances other than testosterone or oestrogen.

In conclusion, therefore, we suggest that long-term treatment with LH/FSH-RH 500 μg eight-hourly self-administered by subcutaneous injection may provide an efficient means of treating patients with hypogonadotrophic hypogonadism due to diseases of the hypothalamus or pituitary and may result in the return of potency and fertility. This treatment may replace the more expensive conventional therapy with natural human gonadotrophins. The promotion of potency out of proportion to the increase in circulating androgens suggests that its place in the treatment of psychogenic impotence should be explored, and these studies are under way. Lower doses have been found to be ineffective in these patients.

We should like to thank Miss Janet Hook, Mrs. Jean Woodham, and Mrs. Susan Walsby for technical work; Dr. W. Bogie (Hoechst U.K.) for supplying the LH/FSH-RH; and the staff of the metabolic ward, St. Bartholomew's Hospital, for their invaluable help. These studies have been supported by the Joint Research Board of St. Bartholomew's Hospital, the Peel Medical Research Trust, and the Medical Research Council.

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# Long-term Infusion of Growth Hormone Release Inhibiting Hormone in Acromegaly: Effects on Pituitary and Pancreatic Hormones

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## Summary

**Growth hormone release inhibiting hormone (GH-RIH) was infused at a rate of 1·3 μg/min for 28 hours into four patients with acromegaly, two of whom also had clinical diabetes mellitus. Growth hormone and glucagon were suppressed throughout the infusion though delayed secretion of insulin occurred in association with both meals and an oral glucose load. Glucose tolerance was improved in one diabetic patient who was taking chlorpropamide while the other required much less insulin than usual. Secretion of endogenous thyroid-stimulating hormone was lowered in one euthyroid patient on carbimazole. Luteinizing hormone, follicle-stimulating hormone, ACTH, and prolactin were not affected. Serum somatomedin levels were reduced in one patient. There was a rapid rebound of all the suppressed hormones when the infusions stopped. Longer-acting analogues of GH-RIH will be needed before long-term therapy of acromegaly or diabetes mellitus becomes possible, but such preparations should be available soon for clinical trial.**

## Introduction

We have previously shown that short-term infusions of growth hormone release inhibiting hormone (GH-RIH) are effective in man in suppressing growth hormone (GH) (Hall *et al.*, 1973; Besser *et al.*, 1974; Mortimer *et al.*, 1974), glucagon and insulin (Mortimer *et al.*, 1974), and the thyrotrophin and follicle-stimulating hormone (TSH and FSH) responses after intravenous-injection of the thyrotrophin-releasing hormone (TRH) (H *et al.*, 1973): during an infusion of GH-RIH a diabetic patie with a glucagon-secreting tumour was rendered hypoglycaem (Mortimer *et al.*, 1974). Some of these effects have been confirm by others (Siler *et al.*, 1973; Hansen *et al.*, 1973; Alberti *et a* 1973; Siler *et al.*, 1974).

The effect of GH-RIH given as bolus injections is short liv For this reason infusions have been used and the longest stud so far described have been over four hours (Besser *et al.*, 197 Mortimer *et al.*, 1974). This preliminary work led to the stu of the effects of long-term infusions of GH-RIH in patients w acromegaly with or without diabetes mellitus to determi whether this hormone was effective over a prolonged period time and to observe any beneficial or adverse effects in t control of GH secretion or glucose tolerance. The circulati levels of the other pituitary hormones and somatomedin w also measured. In addition GH-RIH was combined w protamine zinc in an attempt to produce a depot preparation a its actions were studied.

## Patients

Two male and two female patients aged 50 to 65 years w studied. All had evidence of active acromegaly. Two h previously had external pituitary irradiation, one a transfron partial hypophysectomy, and the fourth patient was untreat Two patients had clinical diabetes mellitus, a woman bei treated with oral chlorpropamide 500 mg/day (case 1) and a m receiving 102 U/day of soluble insulin in divided doses (case One woman also had thyrotoxicosis but was euthyroid at t time of study on treatment with carbimazole 10 mg/day (case Both females were post-menopausal with high levels of circul ing gonadotrophins. All the participants were inform volunteers who gave their written consent.

## Methods

### GH-RIH INFUSIONS

The normal dietary intake of each patient was assessed bef the study began and was then kept constant throughout t period of investigation. Each patient was studied as follow day 1 (starting at 08.30)—a glucose tolerance test (G.T.T.) w performed during a control infusion of 0·9% NaCl; blo samples were withdrawn half an hour before, at zero time, a then half hourly for three hours after an oral load of 50 g glucos day 2 (12.00)—during a control infusion of 0·9% NaCl blo was sampled one hour before and hourly for four hours duri and after a normal lunch, and the "lunch curve" was estimate day 3 (08.00)—GH-RIH was infused for 28 hours at a rate 1·3 μg/min since this had been found to be the minimu effective dose for lowering GH levels in acromegaly in earli studies (Besser *et al.*, 1974). Patients were connected to a consta infusion pump via a 200-cm catheter so that the patients could out of bed during the day. Blood was sampled throughout t 28 hours. A lunch curve was obtained during the first day of t GH-RIH infusion and the oral G.T.T. was repeated at 08.

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the second morning of the infusion so that comparisons could made with the control infusions.

Blood samples were withdrawn via an indwelling venous nnula introduced at least 30 minutes before sampling. Samples ere also taken hourly for five hours and at 24 hours after the H-RIH infusions had stopped. These were assayed for plasma H, glucose, glucagon, insulin, serum TSH, corticosteroids, teinizing hormone (LH), follicle-stimulating hormone (FSH), olactin, and somatomedin. Urine was collected during the ntrol and GH-RIH infusions in four-hour periods until idnight and as an eight-hour overnight collection for measure-ent of GH excretion.

The two women also had blood samples withdrawn through indwelling venous cannula throughout a 28-hour control riod when not receiving GH-RIH to compare the levels of rum TSH, prolactin, LH, FSH, and cortisol with an infusion y. In one patient this was performed the day before GH-RIH fusion and in the other three weeks after the completion of the itial studies.

*GH-RIH.*—The cyclic tetradecapeptide was synthesized and rified as previously described by Coy *et al.* (1973).

### OTAMINE ZINC GH-RIH

otamine zinc GH-RIH (PZ/GH-RIH) was prepared in an morphous suspension in sodium phosphate buffer at pH 9-7·3 with phenol as a preservative according to the method scribed for the preparation of protamine zinc insulin (*British armacopoea*, 1973). Each 0·5-ml ampoule of the suspension ntained 0·125 mg of zinc together with 500 μg GH-RIH. Z/GH-RIH 500 μg was injected subcutaneously into the same romegalic subject who had received intravenous, subcutaneous, d intramuscular injections of the same dose of GH-RIH and no has been reported previously (Besser *et al.*, 1974). Two of e acromegalic patients from this study received PZ/GH-RIH 0 μg subcutaneously at four-hour intervals. GH levels were llowed for 12 hours in these patients.

### SAYS

ne following were measured by specific radioimmunoassay: sma GH using M.R.C. 69/46 as standard (Forsyth *et al.*, 71), urinary GH by the method of Hanssen (1972), plasma ncreatic glucagon (C-terminal antiserum) with M.R.C. ndard 69/104 (Bloom, 1971), serum insulin with M.R.C. ndard 66/304 (Sönksen *et al.*, 1973), serum TSH with M.R.C. ndard 69/38 (Hall *et al.*, 1973), serum prolactin with Friesen ndard (McNeilly, 1973), serum LH with M.R.C. standard /40, and FSH with M.R.C. standard 68/39 (Mortimer *et al.*, 73). Serum corticosteroids were measured by a competitive otein-binding method (Baum *et al.*, 1974) and plasma glucose a neocuprine method (Technicon). Serum somatomedin was easured using a modified chick bioassay (Hall, 1971); normal els are equivalent to the activity of 1 ml of reference serum—t is, 1 unit—and differences of 0·15 units or more are signifi-nt.

## sults

### SMA GROWTH HORMONE

e mean basal GH levels in these four patients were 54, 74, 117, l 158 ng/ml. During the control studies the striking feature the great surges of GH seen during the lunch and G.T.T. mpling period. The plasma GH after lunch on the control day e to 135, 125, 256, and 300 ng/ml respectively, while during G.T.T. GH levels reached 66, 146, 290, and 300 ng/ml. By trast, during the infusion of GH-RIH, GH fell to minimum ls of 18, 7, 28, and 38 ng/ml and the maximum values seen after food or glucose administration were 41, 26, 60, and 100 ng/ml respectively. The levels rose rapidly, however, when the infusions were discontinued and maximum values during the five hours after the infusion had stopped were considerably higher than the basal levels (120-376 ng/ml). Twenty-four hours after the end of the infusion they were still raised with levels between 75 ng and 460 ng/ml. The basal values and the surges in GH in response to food and glucose were clearly suppressed during the infusion of GH-RIH (fig. 1).

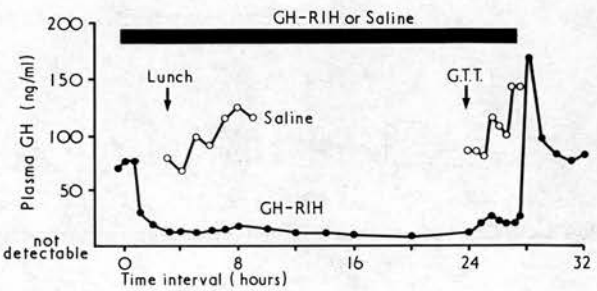



FIG. 1—Case 4. Effect of infusion of GH-RIH on plasma GH levels compared with saline control.

### URINARY GROWTH HORMONE

Urinary GH excretion was measured as an indication of GH production. The total 24-hour urinary GH output in the patient illustrated in fig. 2 (case 4) was 1,050 ng (normal range 37-84 ng/24 hr), about half of that being excreted during the night. The urinary GH output during the 28 hours of the GH-RIH infusion was greatly reduced (268 ng/28 hr), with a clear reduction in the overnight excretion. There was a partial recovery in urinary GH excretion in the 24 hours after the infusion of GH-RIH (467 ng/24 hr). The other three patients had urinary GH measured only between 08.00 and 24.00 hours on the day before, the day of, and the day after the GH-RIH infusion (table I). The results confirm that the infusion of GH-RIH reduced urinary GH levels throughout the day time though an increase in excretion occurred after its cessation. Urinary GH excretion therefore followed closely the changes in plasma GH levels.

TABLE I—*Urinary GH Secretion (ng) before, during, and after GH-RIH Infusions in Three Acromegalic Patients*

| Case No. | Day before GH-RIH | Day of GH-RIH | Day after GH-RIH |
|---|---|---|---|
| 1 | 177 | 138 | 184 |
| 2 | 285 | 112 | 156 |
| 3 | 163 | 87 | 261 |
| Mean | 208·3 | 112·3 | 200·3 |

### SERUM SOMATOMEDIN

Serum somatomedin levels were significantly raised in two patients and normal in the others (table II). The patient in case 1 had a normal basal level of 1·02 units which fell significantly to a minimum value of 0·83 units at 26 hours and was still depressed at 28 hours (0·91 units). The level returned to 1·01 units 24 hours after the end of the infusion. The other patients did not show significant suppression of serum somatomedin levels.

TABLE II—*Serum Somatomedin Levels (Somatomedin U/ml) before, during, and after 28-hour Infusion of GH-RIH in Four Acromegalic Patients*

| Time (hr) | 0 | 6 | 12 | 24½ | 26 | 28 | 32 | 48 |
|---|---|---|---|---|---|---|---|---|
| Case 1 | 1·02 | 1·06 | 0·97 | 1·0 | 0·83† | 0·91† | 0·94 | 1·01 |
| Case 2 | 1·28 | 1·44 | 1·33 | 1·36 | 1·3 | 1·46 | 1·21 | 1·57 |
| Case 3 | 0·99 | 1·08 | 1·19 | 1·00 | 0·99 | 1·01 | 0·93 | 1·03 |
| Case 4 | 1·10 | 1·17* | 1·22 | 1·18 | 1·28 | | 1·36 | 1·15 |

*Value at four hours.
†Significantly less than basal value ($P<0·05$).

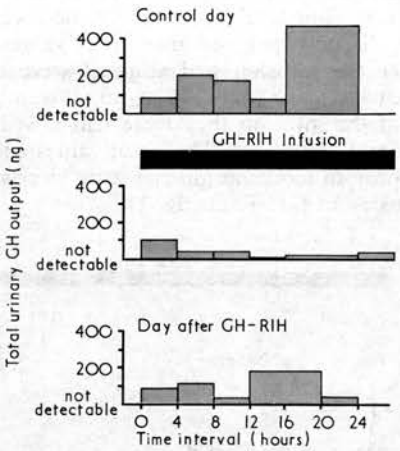



FIG. 2—Case 4. Effect of infusion of GH-RIH on total urinary GH excretion.

PLASMA GLUCAGON

Basal plasma glucagon levels ranged from 27 to 72 pg/ml. These were suppressed throughout the period of the infusion of GH-RIH to 6-30 pg/ml, and when the infusion ceased levels rebounded to 49-206 pg/ml in the next five hours. Twenty-four hours after the infusion the levels were from 51 to 72 pg/ml. Levels were much higher on the control day during the lunch curve in all four subjects, maximal levels reaching 63 to 93 pg/ml, but when GH-RIH was infused the levels were from 3 to 47 pg/ml. Glucagon levels seemed to be reduced during the first hour of the G.T.T. in three of the four patients and thereafter there was evidence of breakthrough secretion in two of them. Clearly, therefore, GH-RIH suppressed glucagon secretion basally and postprandially, but not consistently after a glucose load (fig. 3, tables III and IV).

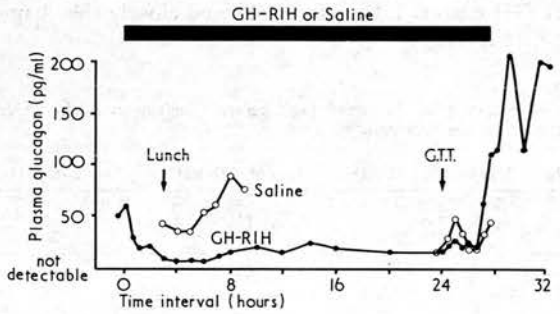



FIG. 3—Case 4. Effect of infusion of GH-RIH on plasma glucagon levels compared with saline control.

PLASMA INSULIN

Basal plasma insulin levels ranged from 20·5 to 45 μU/ml in three subjects. Insulin was not assayed in the patient on soluble insulin. In three patients levels fell during the initial infusion of GH-RIH to 6·0-11·3 μU/ml. During the lunch period on the control infusion day the levels rose to maximum values of 115 and > 500 μU/ml in two subjects and 73·7 μU/ml in the patient on chlorpropamide compared to values of 73, 121, and 24·5 μU/ml on the day of GH-RIH. Similarly, basal levels before the G.T.T. was performed during the GH-RIH infusion were 9, 49, and 16·7 μU/ml but these rose to a maximum level of > 250, > 150, and 27·3 μU/ml respectively after the ingestion of 50 g of glucose. Corresponding maximum values on the control day were 149, 176, and 99 μU/ml. It was evident, therefore, that there was a delayed breakthrough secretion of insulin in response to a normal lunch or oral glucose load though GH and glucago secretion were suppressed. In one subject the breakthroug resulted in higher levels of insulin being achieved during th G.T.T. despite the infusion of GH-RIH (fig. 4).

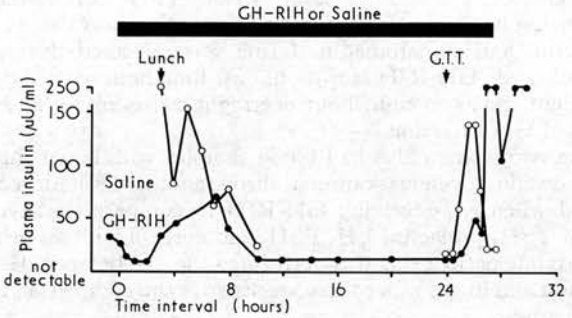



FIG. 4—Case 4. Effect of infusion of GH-RIH on plasma insulin leve compared with saline control.

TABLE III—*Plasma Glucagon Levels (pg/ml) in Four Acromegalic Patien during Lunch on Control Saline and GH-RIH Infusions*

| Case No. | | Time (hr) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | −1 | 0 | 1 | 2 | 3 | 4 | 6 |
| 1 | Control | 58 | 56 | 60 | 64 | 76 | 93 | 56 |
| | GH-RIH | 30 | 32 | 47 | 38 | 37 | 30 | 31 |
| 2 | Control | 32 | 34 | 64 | 43 | 44 | 35 | 33 |
| | GH-RIH | 19 | 16 | 25 | 18 | 15 | 17 | 22 |
| 3 | Control | 53 | 52 | 63 | 45 | 47 | 43 | 36 |
| | GH-RIH | 19 | 12 | 23 | 17 | 15 | 16 | 26 |
| 4 | Control | 41 | 36 | 34 | 52 | 60 | 90 | 75 |
| | GH-RIH | 6 | 3 | 6 | 5 | 9 | 14 | 18 |

TABLE IV—*Plasma Glucagon Levels (pg/ml) in Four Acromegalic Patien during Oral Glucose Tolerance Tests on Control Saline and GH-RIH Infusio*

| Case No. | | Time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 0 | 30 | 60 | 90 | 120 | 150 | 180 |
| 1 | Control | 48 | 42 | 38 | 55 | 41 | 30 | 37 | 28 |
| | GH-RIH | 49 | 58 | 83 | 55 | 43 | 65 | 41 | 38 |
| 2 | Control | 32 | 26 | 36 | 35 | 16 | 27 | 29 | 23 |
| | GH-RIH | 26 | 42 | 32 | 28 | 21 | 30 | 28 | 28 |
| 3 | Control | 44 | 62 | 60 | 48 | 35 | 36 | 26 | 50 |
| | GH-RIH | 63 | 65 | 38 | 35 | 35 | 44 | 51 | 107 |
| 4 | Control | 16 | 28 | 49 | 32 | 17 | 14 | 33 | 42 |
| | GH-RIH | 16 | 20 | 20 | 19 | 24 | 16 | 62 | 111 |

PLASMA GLUCOSE

*During the Lunch Curve*

Basal levels of plasma glucose were measured at 11.00 and 12.0 hours on both the control and infusion days, then lunch w eaten. On the infusion day GH-RIH had been infused contin uously from 08.00 hours. In cases 1, 3, and 4 basal levels we higher on the GH-RIH day—by 46, 30, and 69 mg/100 ml- compared with the control, whereas in case 2 the levels we lower by 42 mg/100 ml (table V). The peaks reached aft GH-RIH were higher in cases 1, 3, and 4 by 41, 58, and mg/100 ml than on control saline but were the same in case

*Oral G.T.T.*

The result of the glucose tolerance test in case 1 was improv by GH-RIH. This patient in this case was the fema diabetic who was being treated with chlorpropamide and w continued on this treatment throughout the study. The diabe man on a 150-g carbohydrate diet, who was normally controll with 102 units/day of soluble insulin (case 2), received 30 un of soluble insulin subcutaneously at 08.00, 12.00, and 18. hours on the two control days. On the GH-RIH day he had o

TABLE V—*Plasma Glucose Levels in Four Acromegalic Patients*

| Case No. | Lunch Curve | | Glucose Tolerance | | |
|---|---|---|---|---|---|
| | Mean Basal Glucose (mg/100 ml) | Peak after Lunch (mg/100 ml) | Fasting (mg/100 ml) | Peak (mg/100 ml) | At 3 Hours (mg/100 ml) |
| Control | 168 | 163 | 209 | 340 | 249 |
| GH-RIH | 214 | 204 | 162 | 287 | 207 |
| Control | 213 | 187 | 161 | 282 | 196 |
| GH-RIH | 171 | 187 | 234* | 280 | 226 |
| Control | 180 | 196 | 111 | 237 | 108 |
| GH-RIH | 210 | 254 | 119 | 246 | 101 |
| Control | 124 | 135 | 97 | 205 | 82 |
| GH-RIH | 193 | 164 | 107 | 163 | 107 |

* Value after 24 hours off insulin.

one injection of 30 units subcutaneously at 08.00 hours at the start of the infusion. Though his basal plasma glucose levels were increased during GH-RIH infusion the peak response after an oral glucose load was little changed despite his receiving only one-third of his normal insulin requirements during that 24 hours. This patient did not in fact receive further insulin until 24 hours after the end of the infusion when the plasma GH level was 228 ng/ml, plasma glucagon 22 pg/ml, and plasma glucose 332 mg/100 ml with glycosuria and ketonuria. In one non-diabetic patient (case 3) the glucose tolerance was little altered during the GH-RIH infusion except for a higher two-hour value, but this had fallen by two and a half hours. In the other non-diabetic patient (case 4) the peak plasma glucose was delayed during the GH-RIH infusion until 120 minutes at which time a level of 163 mg/100 ml was recorded. This was less than during the control study (fig. 5).

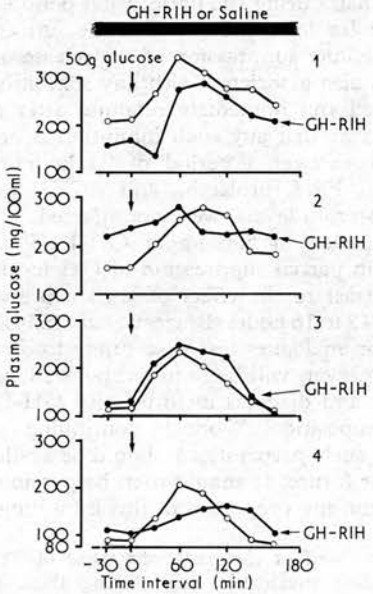



FIG. 5—Effect of infusion of GH-RIH (●) on plasma glucose levels compared with saline control (o) in patients with acromegaly and diabetes mellitus (cases 1 and 2) and acromegaly only (cases 3 and 4).

In cases 2, 3, and 4 the mean plasma glucose levels measured 4 and 24½ hours after the start of the GH-RIH infusion were higher by 73, 8, and 10 mg/100 ml respectively but lower in the diabetic patient in case 1 by 47 mg/100 ml. The peak plasma glucose values achieved were greater by 9 mg/100 ml in case 3 but lower by 53, 2, and 42 mg/100 ml in cases 1, 2, and 4 during GH-RIH compared with the control studies. Three hours after the glucose load the levels were higher in cases 2 and 4 (by 30 and 25 mg/100 ml) but lower in cases 1 and 3 (by 42 and 7 mg/100 ml).

When the 28-hour infusions of GH-RIH were stopped plasma glucose levels fell from 186 to 89 mg/100 ml and from 97 to 89 mg/100 ml in cases 2 and 3 respectively in the five hours after the infusions whereas in case 4 they rose from 57 to a maximum of 89 mg/100 ml.

## SERUM TSH

Serum TSH levels were followed before, during, and after the infusion of GH-RIH in all four subjects. In three of them TSH levels ranged between undetectable ( <0·4) and 4 μU/ml but since they were undetectable at various times throughout the study it was impossible to show any significant changes induced by the inhibiting hormone. One of the acromegalic female patients who had previously been thyrotoxic but was euthyroid on carbimazole at the time of study with normal serum thyroxine and triiodothyronine levels, however, had TSH levels above the normal range of <0·4-4 μU/ml. Her levels were between 5·0 and 7·2 μU/ml on the control day and during the GH-RIH infusion they fell progressively (fig. 6). There was a clear rebound, however, when the infusion was discontinued. Since changes in serum TSH of 1 μU/ml or more are clearly significant the extent of the fall and the immediate rebound on stopping the GH-RIH infusion indicated that the TSH release was inhibited by GH-RIH.

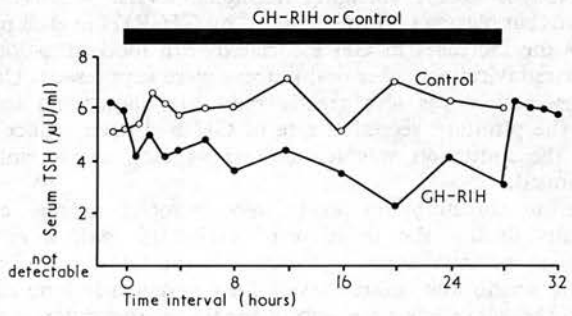



FIG. 6—Case 4. Effect of infusion of GH-RIH on serum TSH levels compared with control day in euthyroid acromegalic patient being treated with carbimazole for thyrotoxicosis.

TABLE VI—*Plasma GH Levels (ng/ml) after Single Subcutaneous Injection of 500 μg PZ/GH-RIH given at Time 0 to Acromegalic Patient who had Previously received Same Dose Intravenously, Intramuscularly, and Subcutaneously combined with Arachis Oil or 16% Gelatin (Besser et al., 1974) and in Cases 1 and 3 of this paper who received 500 μg PZ/GH-RIH Subcutaneously every Four Hours*

| Time (hr): | -¼ | -½ | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acromegalic patient | 49 | | 49 | 18 | 18 | 28 | 26 | 78 | 82 | 71 | 83 | 75 | 29 | 40 | 60 |
| Case 1 | | 80 | 95 | 45 | 50 | 60 | 45 | 105 | 55 | 40 | 55 | 95 | 105 | 85 | 95 |
| Case 3 | | 100 | 125 | 70 | 90 | 85 | 90 | 104 | 102 | 95 | 90 | 85 | 80 | 95 | 95 |

SERUM LH, FSH, PROLACTIN, AND CORTICOSTEROIDS

Serum LH and FSH levels were within the normal range (LH, 0·4-6·0 mU/ml; FSH 0·2-5·9 mU/ml) in the male acromegalics, whereas both females were postmenopausal with LH levels fluctuating from 5·9 to 14·4 mU/ml in one patient and from 5·6 to 28·3 in the other. Their FSH levels varied from 21·6 to 32·1 mU/ml and from 1·6 to 5·3 mU/ml respectively. The infusion of GH-RIH did not significantly alter the gonadotrophin levels in any of the patients. Similarly prolactin levels were within the normal range (up to 20 ng/ml) and these remained uninfluenced by the infusion of GH-RIH. The female acromegalic patient on carbimazole had prolactin levels within the normal range. Levels of serum corticosteroids measured in one patient were not influenced by GH-RIH when compared to a control day, the normal circadian rhythm being maintained.

PZ/GH-RIH

Plasma growth hormone levels after a single subcutaneous injection in one patient of 500 μg of PZ/GH-RIH are shown in table VI. Two other acromegalics (cases 1 and 3) received also the same dose subcutaneously at four-hourly intervals, and GH levels were followed for 12 hours in each patient. The preparation extended the action of GH-RIH to between four and five hours compared to between two and four hours when it was combined with arachis oil or 16% gelatin (Besser *et al.*, 1974). Increasing the dose to 2 mg PZ/GH-RIH did not prolong its action any further.

SIDE EFFECTS

None of the patients suffered any adverse side effects during these studies. One acromegalic man spontaneously reported that his sweating was decreased during the infusion of GH-RIH but returned within a few hours after its cessation.

## Discussion

We have previously reported that GH-RIH will suppress plasma GH, glucagon, and insulin levels in acromegalic patients during infusions lasting up to 150 minutes (Mortimer *et al.*, 1974). It was important, therefore, to look at the effects of prolonged infusions of GH-RIH in acromegalic and diabetic patients over a time period which was representative of their normal daily activities in order to observe the effects and interactions of the changing hormonal levels. We have now shown that plasma GH was reduced by GH-RIH in each patient while the increases in GH associated with food ingestion and the paradoxical rise after oral glucose were suppressed. Urinary GH secretion was also greatly reduced. These data indicate that the pituitary secretion rate of GH had been reduced and that the reduction was maintained as long as the infusion continued.

Serum somatomedin levels were reduced in one of the patients during the infusion of GH-RIH with a rebound after the infusion stopped. Presumably the GH levels were not suppressed to low enough levels for a sufficiently long time to exert the same effect on somatomedin in the other subjects. Plasma glucagon levels were also suppressed throughout the infusion and in response to a normal lunch in all the patients. In the three subjects in whom plasma insulin levels could be measured, however, clear breakthrough secretion of the hormone, though delayed, occurred both in response to lunch and after an oral glucose load. This was encouraging since our initial studies showing impaired insulin secretion after acute GH-RIH administration suggested that the precipitation or exacerbation of diabetes mellitus might become a serious problem during long term GH-RIH therapy. Though basal glucose levels tended to be higher in three subjects when GH-RIH was being infused the plasma glucose peaks after oral glucose were the same or lowe in three patients, and only higher by 9 mg/100 ml in the other. The patient with diabetes mellitus on chlorpropamide had much improved glucose tolerance. Though the patient with diabete mellitus on soluble insulin had increased basal plasma glucos levels when receiving GH-RIH the peak level was no significantly increased during his G.T.T. or food when compare to the control studies. This occurred despite receiving onl one-third of his normal insulin dose. Improvement in circulating glucose levels, therefore, probably depends on the degree o suppression of GH and glucagon since breakthrough secretio of insulin occurs. Further studies in patients with clinica diabetes mellitus without acromegaly are being performed to se if the suppression of plasma GH and glucagon levels results i better control of carbohydrate metabolism.

In the patient treated with carbimazole slightly raised basa TSH levels were clearly suppressed during the infusion o GH-RIH. Since the TSH response to exogenous syntheti TRH is also inhibited it seems likely that in this patient GH-RI was preventing the action of endogenously secreted TRH on th pituitary thyrotrophs. This suggests that tonic TRH secretio by the hypothalamus plays an important role in maintainin TSH secretion and thyroidal homoeostasis and that control i not solely mediated at the pituitary level. The serum prolacti levels in this patient were within the normal range despite raise TSH levels and were unaffected by GH-RIH. It will b important, therefore, to monitor thyroid function test result carefully in patients being treated with long-term GH-RIH i order to detect and prevent the occurrence of hypothyroidism All the hormones which were suppressed during the infusio showed a rapid rebound after the infusion had stopped, often t levels higher than during the preinfusion period. Sampling wa not continued for long enough after the infusions to estimat whether the definite suppression of the release of hormones b GH-RIH was also associated with any inhibition of synthesi but the marked and immediate rebound after the end of th infusions suggests that any such inhibition is not necessarily dominant feature over a period of 28 hours. The pituitar hormones LH, FSH, prolactin, and ACTH (as reflected b serum corticosteroid levels) were not affected.

The combination of 500 μg of GH-RIH with protamin zinc resulted in partial suppression of GH levels for up to fi hours, in contrast to the effect of such a preparation in rat which acts for 12 to 16 hours (Brazeau *et al.*, 1974). Longer-actir preparations or analogues less susceptible to destruction in th circulation, however, will be required before long-term therap of acromegaly and diabetes mellitus with GH-RIH becomes practicable proposition. Work is continuing rapidly in th direction and such preparations should be available for clinic use in the near future. It should then be possible to exploit th extremely promising properties of this inhibiting hormone.

We thank the staff of the metabolic ward of St. Bartholomew Hospital for their invaluable help during these studies; Mr. Sanders and the staff of the department of chemical pathology f the measurement of plasma glucose levels and routine biochemic investigations; and Mr. C. Baum for the measurement of seru corticosteroids. Dr. P. Sönksen very kindly provided the reage for the insulin assays and Dr. H. Friesen the human prolactin us as standard. These studies were supported by the Joint Resear Board of St. Bartholomew's Hospital, the Peel Medical Resear Trust, and the Medical Research Council. Dr. A. S. McNeilly is receipt of a grant from the Wellcome Foundation. Dr. S. R. Bloc has an M.R.C. Clinical Fellowship and is also supported by t British Diabetic Association.

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# Azathioprine in Ulcerative Colitis: Final Report on Controlled Therapeutic Trial

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*British Medical Journal*, 1974, **4**, 627-630

## Summary

**Eighty patients, all of whom were suffering from a frank clinical attack of ulcerative colitis, were admitted to the trial. The attack was treated with a standard course of corticosteroids and the patients were immediately placed on treatment with either azathioprine in a dose of 2·5 mg/kg body weight or dummy tablets. The trial tablets were continued for one year while the patients were maintained under regular clinical, sigmoidoscopic, histological, haematological, and biochemical surveillance. If a patient relapsed during such maintenance treatment he or she was treated with a further course of corticosteroids without interrupting maintenance treatment.**

**In the treatment of an actual attack of ulcerative colitis the results in the attacks which brought the 80 patients into the trial show that no benefit came from the addition of azathioprine to a standard course of corticosteroid therapy.**

**Patients admitted in their first attack of ulcerative colitis showed no benefit from the one-year maintenance treatment with azathioprine, the benefits of which were confined to patients admitted in a relapse of established disease. Even in these the difference between the treated group and the control group failed to reach statistical significance, but the difference was big enough to suggest that there is a prima facie case for regarding azathioprine as of some benefit in this group of patients.**

## Introduction

In an interim report we dealt with the results obtained in 40 patients with ulcerative colitis treated in a controlled therapeutic trial of azathioprine for one year (Jewell and Truelove, 1972). We admitted a further 40 patients to the trial and report here the clinical results for the whole group of 80 patients.

## Patients

All the patients had classical ulcerative colitis. The diagnosis was made on the basis of a history of bloody diarrhoea coupled with sigmoidoscopic evidence of diffuse inflammation, biopsy evidence of an inflammatory reaction compatible with ulcerative colitis, and changes of ulcerative colitis on the barium enema film.

All patients had a frank attack of the disease on admission to the trial. Some were in their first attack of the disease, and others were suffering from a relapse of established disease. It was explained to the patients that a new treatment was being tested and they were free to volunteer to be included. The necessity for repeated attendances and serial sigmoidoscopic examinations with biopsy was included in the explanation. Married women of fertile age were advised to avoid conception during the trial.

## Experimental Design

A two-by-three stratified design was used. Patients were treated initially as either inpatients or outpatients depending on an overall clinical assessment of the severity of their attack. They were also subdivided according to the length of history into three categories: first attack; short history (less than five years); and long history (more than five years). As a result of this double specification patients fell into six groups.

Within each of these groups patients were allotted at random to real or dummy azathioprine treatment, a system of restricted randomization being used so that each block of six patients contained three on azathioprine and three on dummy tablets. The trial treatment was prescribed as "azathioprine special," and the hospital pharmacists worked from a master sheet indicating whether a particular patient was to be given real or dummy azathioprine.

The attack which brought the patient into the trial was treated with a standard course of corticosteroids together with general medical measures in the case of the inpatients. The regimen of corticosteroid treatment for outpatients was oral prednisolone 5 mg four times a day and prednisolone disodium retention enemata nightly. If the therapeutic response was good this regimen was maintained for one month and then tailed-off over the next two weeks.

As the attack was severe in all inpatients treatment began with a five-day intensive course of intravenous therapy as follows: nothing by mouth except water, intravenous control of water and electrolyte balance, prednisolone 21-phosphate 40 mg daily in the intravenous fluid, tetracycline 1 g daily in divided doses in the intravenous fluid, and rectal drip of hydrocortisone hemisuccinate sodium 100 mg twice daily. If the clinical response was good this was followed by resumption of feeding and oral prednisolone 40 mg daily in divided doses. When discharged the patients were on the outpatient regimen and treatment was tailed-off after one month from the start of treatment.

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The dose of azathioprine was about 2·5 mg/kg body weight. With the first 40 patients the dose was reduced after three months to 1·5-2·0 mg/kg body weight whereas with the second 40 patients the dose was maintained at 2·5 mg/kg body weight throughout the entire trial period. The dummy tablets were prescribed in an equivalent manner.

The patients were seen at a special clinic at least once a month. At each attendance the symptoms were recorded, a full blood examination was made, and sigmoidoscopy with biopsy was performed. Apart from the first few patients to be admitted all the patients had biochemical observations on the blood at each monthly attendance, using a Technicon 12.

The attack of ulcerative colitis which brought the patient into the trial was classified as mild, moderate, or severe by the criteria of Truelove and Witts (1955). The sigmoidoscopic appearances at each examination were graded from 0 to 3 (0=normal appearances, 1=mild inflammation, 2=moderate inflammation, and 3=severe inflammation). All the biopsy specimens were examined by one pathologist, who classified them according to the criteria of Truelove and Richards (1956) into the following categories: (*a*) no significant inflammation, (*b*) mild to moderate inflammation, and (*c*) severe inflammation.

During the one-year trial period some patients suffered from one or more relapses of the ulcerative colitis. A relapse was defined as the occurrence of diarrhoea with blood in the motions and with sigmoidoscopic evidence of inflammation. Each relapse was treated according to the regimens already outlined. If a patient suffered three relapses during the trial period he was taken out of the trial and treated openly.

A few patients were defined as failures. These were patients who failed to go into clinical remission within six weeks of corticosteroid treatment, either during the attack which brought them into the trial or in a subsequent relapse during the trial period.

## Results

### VALUE OF AZATHIOPRINE IN ACUTE ATTACK

As the patients admitted to the trial were all in a frank attack of ulcerative colitis and as they were immediately placed on treatment with azathioprine or dummy tablets as well as a standard regimen of corticosteroid treatment the value of azathioprine in the treatment of an actual attack of the disease could be assessed.

On entry to the trial the azathioprine group and the control group were closely similar with respect to the severity of the attack which brought them into the trial (table I). At the end of one month there was no obvious difference between the two groups. Though the azathioprine group had 31 patients in remission as against 27 in the control group, this difference was so small that it could be expected to occur often by chance. In each treatment group there were two patients who had already failed by the end of one month.

The sigmoidoscopic gradings were roughly similar in the treated and control group at entry to the trial, with the azathioprine group showing the more severe picture. After one month, however, the azathioprine group showed on average a more favourable sigmoidoscopic response though the difference between the two treatment groups was not statistically significant (table II). Likewise, the rectal biopsy findings were closely similar in the two treatment groups on entry to the trial (table III), but after one month the azathioprine group showed a more favourable response to treatment than the control group though the difference was not statistically significant (excluding the two patients in each group who had already failed and had been withdrawn from the trial before the end of the first month), P=0·11.

Hence azathioprine in a dose of 2·5 mg/kg body weight is of negligible value in the treatment of an attack of ulcerative colitis when added to a standard course of corticosteroid therapy.

TABLE I—*Clinical Severity of Attacks on Entry to Trial and at End of One Month in Two Treatment Groups*

| Severity of Attack | On Entry to Trial | | After One Month | |
|---|---|---|---|---|
| | Azathioprine Group | Control Group | Azathioprine Group | Control Group |
| Remission .. | | | 31 | 27 |
| Mild .. .. | 16 | 17 | 4 | 6 |
| Moderate .. | 21 | 19 | 2 | 3 |
| Severe .. | 3 | 4 | 1 | 2 |
| Failed .. | | | 2 | 2 |
| Total .. | 40 | 40 | 40 | 40 |

TABLE II—*Sigmoidoscopic Grades on Entry to Trial and at End of One Month in Two Treatment Groups*

| Sigmoidoscopic Grade | On Entry to Trial | | After One Month | |
|---|---|---|---|---|
| | Azathioprine Group | Control Group | Azathioprine Group | Control Group |
| 0 | | | 15 | 9 |
| 1 | 7 | 15 | 20 | 18 |
| 2 | 29 | 21 | 3 | 9 |
| 3 | 4 | 4 | | 2 |
| Failed | | | 2 | 2 |
| Total | 40 | 40 | 40 | 40 |

TABLE III—*Histological Grades of Rectal Biopsy Specimens on Entry to Trial and at End of One Month in Two Treatment Groups*

| Histological Grade | On Entry to Trial | | After One Month | |
|---|---|---|---|---|
| | Azathioprine Group | Control Group | Azathioprine Group | Control Group |
| 1 | 1 | 1 | 24 | 15 |
| 2 | 20 | 21 | 7 | 9 |
| 3 | 19 | 18 | 7 | 14 |
| Failed | | | 2 | 2 |
| Total | 40 | 40 | 40 | 40 |

### VALUE OF AZATHIOPRINE AS MAINTENANCE TREATMENT

The clinical responses of the treated and control groups are shown in table IV. The patients on azathioprine had a more favourable course than those on dummy tablets. Thus, there were 16 of the azathioprine-treated patients who were symptom free throughout the trial (after recovering from the original attack) compared with nine of the patients on dummy treatment. At the other extreme only three azathioprine-treated patients were classified as "failures" compared with seven in the dummy-treated group. For statistical analysis the patients in both treatment groups were classified according to two regroupings of the numbers of relapses as follows: (*a*) 0, 1-3, or failure; and (*b*) 0, 1 or 2, or 3 or failure. On Fisher's exact test the significance of the differences between the azathioprine and control groups on both (*a*) and (*b*) regroupings was P=0·18. Thus, the differences between the two treatment groups were not significant at the conventional level of 0·05 probability.

Treatment with azathioprine had no discernible effect on the course of the disease in patients who were admitted to the trial in their first attack of ulcerative colitis (table V). Such differences as existed between the two treatment groups were confined to those patients who were admitted to the trial during a relapse of established disease. Even though the results were still not statistically significant at the conventional level of 0·05 probability, they were close to this value and there is therefore a prima facie case for regarding azathioprine as of some value in the maintenance treatment of ulcerative colitis which has become chronic. Further analysis of the data has shown that this beneficial effect of azathioprine on established ulcerative colitis was manifest during the first six months of the trial period and continued to show itself during the second six months.

The effect of azathioprine as a maintenance treatment was closely similar in the first part of the trial when the dose was reduced to 1·5-2·0 mg/kg body weight after the first three months to that observed in the second part of the trial when the dose was

80

In "Hypothalamic Hormones" pp 325-335. Eds M. Motta, P.G. Crosignani & L. Martini. London, New York & San Francisco: Academic Press. 1975.

# GONADOTROPHIN RELEASING HORMONE THERAPY IN THE INDUCTION OF PUBERTY, POTENCY, SPERMATOGENESIS AND OVULATION IN PATIENTS WITH HYPOTHALAMIC-PITUITARY-GONADAL DYSFUNCTION

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## INTRODUCTION

We have recently reviewed the results of a standard 100 μg luteinizing hormone/follicle stimulating hormone releasing hormone (LH/FSH-RH) test in 155 patients with hypothalamic-pituitary-gonadal dysfunction (Mortimer *et al.*, 1973a). Although 88% were clinically hypogonadal when initially tested, with low endogenous gonadotrophin secretion, only 10% of the patients failed to show any response to the releasing hormone. It was hoped therefore that repeated administration of the releasing hormone would result in the induction of gonadotrophin synthesis and release with gonadal stimulation.

Previous studies of the time course of action of 100 μg LH/FSH-RH given by intravenous, intramuscular and subcutaneous routes showed that these were equally effective in promoting increased circulating levels of LH for 5-7 h and FSH for 3-5 h (Mortimer *et al.*, 1973b). The similarity in time course of gonadotrophin release after subcutaneous and intravenous injection of LH/FSH-RH has also been confirmed by other workers (Arimura *et al.*, 1973a), although it was suggested that the subcutaneous route was associated with a secondary rise in FSH. This however may have been the result of inadequate sampling since asynchronous pulsatile release of the gonadotrophins is seen following intravenous, subcutaneous or intramuscular administration of the releasing hormone (Mortimer *et al.*, 1973b). Intranasal application has been considered for the administration of LH/FSH-RH in view of the ease in which other oligopeptides are absorbed via this route and have been shown to have therapeutic value, especially DDAVP in the treatment of diabetes insipidus (Edwards *et al.*, 1973). The releasing hormone is undoubtedly absorbed via the nasal mucosa although the amount required is greater than that by other routes in order to produce the equivalent gonadotrophin response (Mortimer *et al.*, 1973b). London *et al.* (1973) deducted that a dose of 2.5 mg

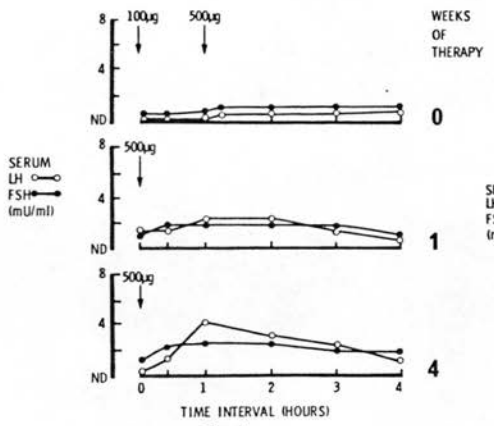



*Fig. 1*

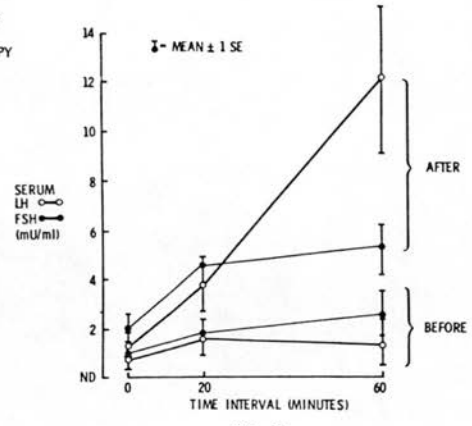



*Fig. 2*

*Fig. 1* Pre-pubertal male with a craniopharyngioma: serum LH (○) and FSH (●) responses during 4 weeks of LH/FSH-RH therapy.

*Fig. 2* The serum LH (○) and FSH (●) responses to a standard test dose of 100 μg intravenous LH/FSH-RH in five patients before and after 4 to 6 weeks of LH/FSH-RH therapy.

intranasally would produce the same effect as 100 μg intravenously on LH secretion, whereas 1.25 mg intranasally would be required for the same FSH releasing ability.

In view of these findings we decided that subcutaneous injection of LH/FSH-RH would provide the most reliable means of administering the hormone. Earlier work had shown that patients with so-called isolated gonadotrophin deficiency might fail to respond adequately to 100 μg, but this was improved by using a dose of 500 μg of the decapeptide (Marshall *et al.*, 1972). It was decided therefore to use a dose of 500 μg given by self-administered subcutaneous injection 8 hourly into the thigh or anterior abdominal wall.

All patients had been off any form of gonadal steroids or gonadotrophins at least four months prior to the start of gonadotrophin releasing hormone therapy.

## INDUCTION OF PUBERTY AND POTENCY

It has been noted previously that acute intravenous injection of the releasing hormone resulted in increased circulating gonadotrophin levels, together with a rise in plasma testosterone concentrations in adult and pubertal males although a testosterone response was not seen in pre-pubertal subjects (Roth *et al.*, 1973). Continued therapy in pre-pubertal males has been reported by Zarate *et al.* (1973a). They treated two brothers with hypogonadotrophic hypogonadism and anosmia, with intramuscular injections of the releasing hormone 500 μg daily for 5 days. Although there was a rise in both gonadotrophins following intramuscular injection of LH/FSH-RH, basal levels of FSH only were increased after five weeks treatment. A 50 μg LH/FSH-RH test was carried out the day before and two days after therapy, but the response, which was previously impaired compared to normal patients of a similar age, was not improved. These authors suggested that more frequent injections, or larger doses would have been more effective. This has been our experience also and we have previously reported the effects of 4-6 weeks subcutaneous therapy with 500 μg 8 hourly in five pre-pubertal patients; four with

craniopharyngiomas which had been treated either by excision and irradiation, or irradiation only, and one with isolated growth hormone deficiency (Mortimer *et al.*, 1974a; Besser and Mortimer, 1974; Mortimer *et al.*, 1974b). The responses during the initiation of therapy in one patient with a craniopharyngioma are shown in Fig.1. It was noted in four of these patients who were clomiphene responsive that a rise in FSH occurred before that of LH, and was initially greater. However as therapy continued there was a progressive increase in both gonadotrophins but with LH secretion becoming the predominant hormone secreted. Two of the pre-pubertal patients (and three adults with isolated gonadotrophin deficiency, craniopharyngioma or a diffuse hypothalamic tumour of unknown origin) were retested after this preliminary therapy with 100 μg LH/FSH-RH after being off treatment for 1-7 days. Before therapy in these patients the FSH response was considerably greater than that of LH, whereas following treatment LH secretion was now greater than that of FSH (Fig.2). The initial pre-pubertal type of response had therefore reverted to the adult pattern (Franchimont *et al.*, 1974). One pre-pubertal patient with a craniopharyngioma had no LH or FSH response to 100 μg LH/FSH-RH but had a simultaneous increase in both gonadotrophins when 500 μg was given. This patient therefore did not show the clear disparity between the initiation of FSH secretion, followed by a rise in LH. It was of interest that he was the only patient to respond to clomiphene on initial testing which indicated perhaps that a certain degree of hypothalamic maturation had taken place despite being clinically pre-pubertal. As treatment continued however LH secretion became predominant in this patient also. In three patients therapy was discontinued at 4-6 weeks since it was felt unwise to induce puberty while they were still receiving growth hormone therapy. However in two (a pre-pubertal patient with a craniopharyngioma aged 22 yrs, and one with isolated growth hormone deficiency, aged 20 yrs) therapy was continued with 500 μg 8 hourly for 20 weeks since their growth rate was already slowing. It was hoped that the initiation of puberty would potentiate further growth. Before therapy one had pre-adolescent genitalia with the vellus over the pubis indistinguishable from that of the abdominal wall, i.e. stage 1 of Tanner (1958). After 8 weeks of treatment he developed stage 3 pubic hairs and later there was hair growth over the legs. Testicular volumes before therapy were 4 ml and 6 ml, which increased to 6 ml and 8 ml respectively at 8 weeks. The other patient before therapy had slight pubertal enlargement of the testes and scrotum with sparse growth of pigmented downy hair at the base of the penis (stage 3). After 9 weeks there was an increase in testicular volume from 6 to 12 ml on the right and 4 to 8 ml on the left. Spontaneous erections in both these patients became frequent although 17-hydroxy-androgens (a measure of testosterone and dihydrotestosterone, Anderson, 1970) increased only slightly during treatment, from 0.4-1.7 initially to 1.4-2.4 ng/ml during therapy. These levels were well below the normal adult range (5.0-22.5 ng/ml). It remains to be seen whether continued therapy will result in full testicular development and testosterone production in these two patients.

Seven adult males were also treated. Four had so-called isolated gonadotrophin deficiency, although the primary defect is probably a deficiency of the releasing hormone (Marshall *et al.*, 1972, Mortimer *et al.*, 1973a); one a craniopharyngioma which had been decompressed and irradiated 19 yrs previously; one with acromegaly treated by irradiation, and one with a diffuse hypothalamic tumour of unknown origin. During therapy with the releasing hormone all had an increase in potency after 7-14 days of treatment although only one patient (with the craniopharyn-

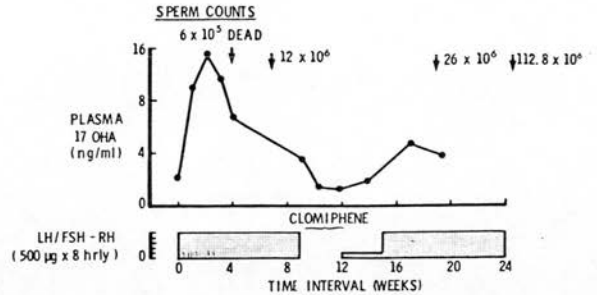



*Fig.3* Adult male with a craniopharyngioma: plasma 17 OHA levels during therapy with the releasing hormone. Clomiphene started at week 9, therapy reintroduced with 100 µg × 8 hourly from weeks 12-15 then increased to 500 µg 8 hourly. Increasing sperm counts shown at the top.

gioma) had a rise of 17 OHA into the normal adult male range. As treatment continued 17 OHA levels increased but in only two patients were the levels within the normal range on more than two occasions. Despite this, potency has been well maintained and has been 'normal' in 3 patients. The rapid return of potency in these patients with a very consistent time course of 7-14 days, with levels of 17 OHA at which most other patients we see are impotent, suggests that there may be factors at work other than those which are androgen mediated. Moss and McCann (1973) and Pfaff (1973) have previously noted a marked increase in the number of lordotic responses in ovariectomised and hypophysectomised rats following subcutaneous treatment with the releasing hormone. This work indicated that LH/FSH-RH was exerting a direct central stimulating effect on sexual behaviour in rats which was independent of gonadotrophins or gonadal steroids. In view of these findings we have initiated a series of experiments to investigate the possible therapeutic value in psychogenic impotence. In preliminary uncontrolled studies carried out so far in two patients with a history of 1½ to 2 years of psychogenic impotence, treatment with LH+FSH-RH resulted in a remarkable return of potency after 7-10 days of a 3 weeks course of therapy. The treatment was not repeated and both patients continued to have normal potency during the 3 months of follow up so far achieved.

## INDUCTION OF SPERMATOGENESIS
(Figs 3 and 4)

Since the gonadotrophin releasing hormone has been shown to produce a consistent increase in circulating LH and FSH levels it was hoped that it would be of value in patients with oligo- or azoospermia. A therapeutic trail involving its use in ten males with normal external genitalia and considered to be clinically endocrinologically intact with normal urinary gonadotrophins measured by the mouse bioassay, was carried out by Zarate *et al.* (1973b). Treatment with 500 µg intramuscularly, twice daily for 6 months resulted in an increase in sperm counts in three of the four patients with azoospermia to a maximum of less than 6 million. In six patients with oligospermia three showed an increase from a total count of 1-5 million basally to greater than 10 million at 3 months. The count then fell despite continued therapy. Three other oligospermic patients showed no significant improvement although all had an increase in motility. In our initial studies we have sought to treat patients with well-documented hypothalamic or pituitary dis-

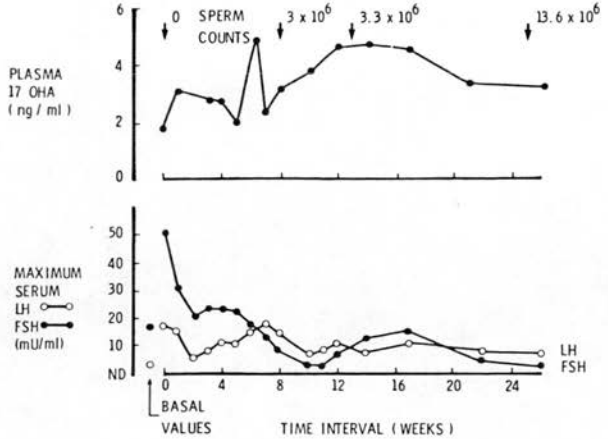



*Fig.4* Adult male with treated acromegaly. Top: levels of 17 OHA throughout LH/FSH-RH therapy. Increasing sperm counts are shown at the top. Bottom: maximum serum LH (○) and FSH (●) responses to the therapeutic 500 μg dose of LH/FSH-RH. Levels were measured over 4 h on each test day.

ease in order to assess the role of the gonadotrophin releasing hormone as possible therapy for infertility. Spermatogenesis was induced in four patients who were treated for up to 41 weeks (Mortimer *et al.*, 1974b). The results in an adult male with a craniopharyngioma are shown in Fig.3. This patient had previously fathered a child during a long and expensive course of intramuscular Pergonal (human menopausal urinary gonadotrophins). Later, he received intramuscular HCG (human chorionic gonadotrophin) to maintain testicular androgen production, but this was discontinued 4 months prior to LH/FSH-RH therapy. Initially he was impotent and clomiphene unresponsive; however at the end of the second week of treatment potency improved, and at 4 weeks he produced an ejaculate containing 60,000 dead sperms, which evidently had been lying *in situ* as a result of previous gonadotrophin therapy. During LH/FSH-RH treatment the total sperm count rose to 112.8 millions at 33 weeks with a motility of 40%. On treatment 17 OHA levels rose initially then started to level off presumably as a result of the introduction of feedback mechanisms acting primarily at the pituitary level. At 9 weeks therapy was stopped and substituted by clomiphene (3 mg/kg/day for 10 days). As a result LH and FSH levels became undetectable, 170 OHA levels fell and again he became impotent. Releasing hormone therapy using 100 μg 8 hourly was reinstituted, but this produced only a small rise in 17 OHA with a negligible effect on potency. When the 500 μg dose 8 hourly was reinstituted gonadotrophin and androgen secretion was enhanced and potency returned. A similar pattern of response was also seen in the patient with a diffuse hypothalamic tumour although the doses were reversed as a control. It would appear, therefore, in these patients that although pituitary stores of LH and FSH had been increased using the synthetic decapeptide as therapy, the administration of clomiphene failed to release sufficient, if any, endogenous releasing hormone to stimulate the gland.

Spermatogenesis was also induced in the acromegalic patient (Fig.4). He initially had markedly reduced potency but was able to produce a specimen of seminal fluid which contained no sperms. During therapy, however, the total

sperm count rose to reach a maximum of 13.6 millions at 25 weeks together with a modest increase in 17 OHA from 1.7 ng/ml, basally to a peak of 5.2 ng/ml at 6 weeks and was then maintained between 3.8 and 4.5 ng/ml. His potency was much improved. Before therapy he had a normal basal LH level of 3.8 mU/ml (normal range less than 0.4 to 6.0 mU/ml, of MRC standard 68/40), but an elevated FSH of 17.3 mU/ml (normal range less than 0.2 to 5.9 mU/ml of MRC standard 68/39), using radioimmunoassays which have been previously described (Mortimer *et al.*, 1973c). When tested with 100 μg LH/FSH-RH there was a normal LH response but an exaggerated FSH rise, similar to the pattern of response we have reported previously in a patient with azoospermia (Mortimer *et al.*, 1974a). The maximum FSH response at the beginning of treatment showed levels greater than 50 mU/ml but these decreased as therapy continued falling to 1.6 mU/ml at 26 weeks. This occurred as the sperm count continued to rise. The LH pattern of response however was little changed and levels fluctuated between 6 and 15 mU/ml. A decrease in the maximum FSH level during therapy with an almost unchanged LH response was common to all four patients in whom spermatogenesis was induced. This included two adults with 'isolated gonadotrophin deficiency' in whom sperm counts rose from zero to 5.4 million and 7.8 million after 24 and 41 weeks therapy respectively. Since in all these patients plasma oestradiol levels have been within the normal adult male range except on rare occasions, and 17 OHA levels have been below the normal range almost continuously, it would appear that another substance was produced during spermatogenesis resulting in the differential suppression of FSH secretion while LH remained uninfluenced. This has been called inhibin, but as yet remains unidentified. However, it would seem that the primary site of action of this factor is at the pituitary level since circulating LH/FSH-RH levels were maintained by repeated injections of the synthetic decapeptide. It is to be hoped that this elusive substance will soon be isolated so that feedback mechanisms in the male may be more fully explored.

## INDUCTION OF OVULATION (Fig.5)

During a normal menstrual cycle secretion of LH and FSH from the pituitary results in steroidogenesis and gametogenesis in the ovaries. This causes a rise in circulating oestrogens which appears to induce a mid cycle burst of LH and FSH secretion presumably by a positive feedback mechanism at the hypothalamus and mediated by the discharge of endogenous gonadotrophin releasing hormone. Support for this concept comes from work by Nillius and Wide (1971), who showed that the injection of 1 mg of 17β-oestradiol caused a rise in serum LH levels (but not FSH however) 48 to 72 hours after intramuscular administration. The effect of progesterone on hypothalamic-pituitary feedback mechanisms has also been implicated since 5 or 10 mg of progesterone given after the 17β-oestradiol produced a second rise in LH (Odell and Swerdloff, 1968). However a significant increase in the secretion of progesterone before the LH peak in normal females has never been detected and so this action of progesterone appears to have little physiological significance. More recently it has been shown that there is a rise in immunoreactive LH/FSH-RH at the time of the mid cycle peak (Arimura *et al.*, 1974); we have subsequently confirmed this and shown that the secretion occurs in a pulsatile pattern. It was to be hoped therefore that when the releasing hormone was given to non-cycling women that consistent ovulation could

be produced. However the ability of exogenous LH/FSH-RH to produce a sufficient rise in circulating gonadotrophin levels to cause ovulation at all, has been in doubt. In 1972, Akande *et al.* reported a series of studies in eight patients with secondary amenorrhoea. In four the diagnosis was not recorded, one had anorexia nervosa, one thyrotoxicosis, one diabetes mellitus and one has post oral contraceptive amenorrhoea. The releasing hormone in doses ranging from 25 to 250 μg were given by intravenous, subcutaneous or intramuscular injections to these patients. One woman received a 24 hr infusion of 300 μg intravenously. All patients showed a marked increase in circulating gonadotrophins although in only one case (diagnosis not recorded) was ovulation produced. This patient, however, had ovulated spontaneously while basal blood samples were being collected over the preceding 28-35 days. These authors concluded that the presence of a suitably primed follicle would be necessary before ovulation could be produced regularly. The therapeutic possibilities of the decapeptide were further explored by Keller (1973). Ovulation and pregnancy resulted, although since clomiphene therapy was combined with releasing hormone therapy it was difficult to distinguish whether it was the clomiphene or LH/FSH-RH which produced the desired effects. Brechwald *et al.* (1974) found during their studies that the administration of 100 to 200 μg of the releasing hormone for 18 to 21 days did not result in any ovarian stimulation resulting in a rise in plasma oestradiol. They also investigated its use in ovulation induction following HMG (human menopausal gonadotrophin), and also with HCG after LH/FSH-RH therapy. The decapeptide was given by subcutaneous injection and also as intravenous infusions. Although they reported ovulation with these mixed regimes it was unclear if the dose of LH/FSH-RH used had provided a significant contribution. They concluded that the synthetic decapeptide was not suitable for the induction of ovulation in HMG stimulated ovaries and that its value was limited by pituitary responsiveness. These latter two studies also were unsatisfactory in that the aetiology of the primary or secondary amenorrhoea in their patients was not reported and the possibility of spontaneous ovulation was not adequately excluded. Recently Zarate *et al.* (1974) have also achieved ovulation in two out of 16 women with secondary amenorrhoea following depot medroxyprogesterone acetate, or chlormadinone for contraceptive purposes. These authors used doses of 50 to 100 μg of LH/FSH-RH once or twice daily over 7 to 20 days. In addition intravenous infusions of 500 μg lasting 5 hours or longer were used. These larger doses were effective in causing oestrogen changes in vaginal cytology and cervical gland secretion. However, the occurrence of spontaneous ovulation was not entirely excluded. From these studies therefore it was evident that the dose and timing of the administration of the releasing hormone to produce ovulation was not known. However, it was reasonable to assume that larger doses would have to be used if satisfactory pituitary stimulation was to be obtained in view of the failure of lower dose regimes. We decided therefore to use 500 μg subcutaneously 8 hourly as in the males. It was also necessary to select patients who had been fully investigated with well-documented amenorrhoea excluding cases of irregular periods so that the results of ovulation induction would be meaningful. We decided to induce pituitary stimulation with the releasing hormone in four females aged between 19 and 29 years who had had secondary amenorrhoea for 5-7 years following anorexia nervosa. Of these patients two were at their ideal body weight and two were between 3.8 and 5.8 kg below this at the start of therapy. They were all clomiphene unresponsive (3 mg/kg for 10 days) on at least two occasions at 3-5 months prior to the

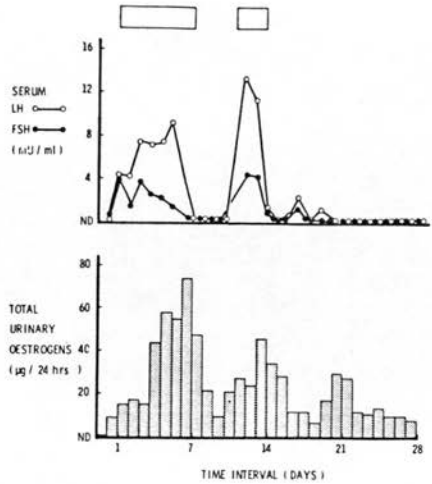



*Fig.5* Female with anorexia nervosa with secondary amenorrhoea of 5 years duration. Top: serum LH (○) and FSH (●) levels 12 hrs after last dose of 500 μg LH/FSH-RH × 8 hourly. Bottom: total 24 hr urinary oestrogens during therapy.

study. Basal urinary oestrogens were between 4 and 17 μg/24 hr measured fluorimetrically (Brown *et al.*, 1968). All four subjects received LH/FSH-RH 500 μg subcutaneously 8 hourly, for 7 days. Treatment was then stopped and restarted on days 12, 13 and 14. Serum gonadotrophins were measured 12 hr after the last dose of the releasing hormone so that the levels represented the minimum circulating concentrations throughout the 24 hours. The results in one such patient are shown in Fig.5. Basal serum gonadotrophins were low or absent in all four subjects (normal female range in the follicular phase of the cycle LH, between 1.3 and 5.1 mU/ml and FSH between 1.3 and 5.2 mU/ml). During therapy there was a rise in the daily 'basal' gonadotrophins with more LH secreted than FSH. During the first seven days urinary oestrogens rose to a maximum of between 62 and 135 μg/24 hr (normal mid cycle values range between 30 and 120 μg/24 hr). When therapy was discontinued serum gonadotrophins again became low or undetectable and there was a fall in urinary oestrogens. The reinstitution of therapy however resulted in an increase in serum gonadotrophins with the 'basal' daily levels rising to within the normal mid cycle range (LH 10 to 30 mU/ml, FSH 4-9 mU/ml). At this time a further rise occurred in urinary oestrogens, to between 15 and 55 μg/24 hr. Therapy was then discontinued. However three of the four patients showed a rise in 24 hr gonadotrophins, and all had a rise in urinary oestrogens between days 18 and 28 which reached a maximum of 30 to 115 μg/24 hr (gonadotrophin levels are awaited in the fourth patient). Twenty-four hour urinary oestrogen levels were paralleled by increases in plasma 17β oestradiol levels in the three subjects in whom they were measured. Levels before therapy were between 14 and 45 pg/ml, rising to between 111 and 220 pg/ml during the first seven days of treatment (the normal mid cycle peak being between 80 and 240 pg/ml). The levels then fell to undetectable levels in one subject and to 14 to 22 pg/ml in the others at days 8 to 10. Further therapy on days 12, 13 and 14 resulted in a rise to between 33 and 94 pg/ml. Levels then fell but rose again on days

18 to 28 to between 63 and 161 pg/ml despite being off therapy. These data therefore indicated that the patients had adequate pituitary LH and FSH reserve although release of the gonadotrophins was impaired while off therapy. Presumably this was due to a persistent deficiency or unavailability of the endogenous releasing hormone. Synthetic LH/FSH-RH therapy resulted in rises in total 24 hour urinary oestrogens and plasma oestradiol which were comparable with those seen in the normal cycle. Since there was evidence of spontaneous release of LH and FSH resulting in oestrogen secretion despite being off therapy on days 18 to 28, it would appear that there had been positive feedback of the rise in circulating oestrogens at the hypothalamic-pituitary level. It would seem therefore that the hypothalamus, pituitary and gonads were no longer functioning in isolation but the normal cycle of events had been completed. Following these initial studies one patient went on to menstruate, for the first time in 6 years. However there was no biochemical evidence in her or the others of ovulation. Two patients were retested with clomiphene 12-14 weeks after this therapy. Both were now clomiphene responsive with a rise in urinary oestrogens followed by menstruation. Evidence of ovulation has not yet been confirmed. One of the other two patients instead of receiving clomiphene, had a further course of 14 days of LH/FSH-RH 500 μg 8 hourly subcutaneously. During the initial releasing hormone therapy total urinary oestrogens rose from 8 μg/24 hours to a maximum of 62 μg/24 hours on day 3, falling to a minimum of 13 μg/24 hours on day 9, but rising to 36 μg/24 hours on day 13. Until day 7 plasma progesterone levels were undetectable (less than 1 mg/ml) but a progressive rise to 9.9 mg/ml was recorded on day 13. Urinary pregnanediol also increased from 1.6 mg and 1.1 mg/24 hours on days 0 and 7, to 4.6 mg/24 hours on day 13. This was clear evidence that ovulation had been induced. HCG 4,500 units was given on day 14 by intramuscular injection (the evidence for ovulation was not known at this time). Total urinary oestrogens then rose from 36 μg/24 hours on day 13 to 137 μg/24 hours on day 18. Levels then fell to 12 μg/24 hours on day 21. Following HCG there was a further rise also in plasma progesterone from 9.9 to 22 ng/ml on day 16, and urinary pregnanediol to 11.4 mg/24 hours. Concentrations then fall to undetectable on day 22, when the patient menstruated for 4 days for the first time in seven years. The second patient will be treated in the same way and it is hoped that the preliminary stimulation of the hypothalamic-pituitary-gonadal axis, together with a further course of the releasing hormone (with and without HCG) will result in ovulation and menstruation.

From our studies it appears that doses of LH/FSH-RH in the region of 500 μg 8 hourly for 7-14 days will induce adequate steroidogenesis by the ovary and ovulation may also occur. The addition of HCG on day 14 of such treatment may therefore be unnecessary. It is hoped that this regimen will result in reliable ovulation and since gonadotrophin and oestrogen levels produced were within the physiological ranges, that the hyperstimulation syndrome will be avoided. It remains to be seen if feedback mechanisms induced by this therapy will continue intact whatever the dose of LH/FSH-RH used. If this is so then it will provide an additional safety factor and considerably simplify the treatment of female infertility.

## CONCLUSION

From the data presented above it is clear that repeated subcutaneous therapy with LH/FSH-RH results not only in release of gonadotrophins but also continued synthesis at least up to 41 weeks. As a consequence puberty, potency and spermatogenesis may be induced and maintained in patients with hypothalamic or pituitary disease with secondary gonadal hypofunction. This situation with respect to reliable ovulation induction is less clear although recent observations are extremely encouraging. Long term therapy with LH/FSH-RH therefore provides a potentially inexpensive means of treating infertility although the dose required needs to be large in patients with pre-existing disease. It is to be hoped that when longer acting preparations are available either in different repository media or of a structure more resistent to enzymatic destruction, that 8 hourly subcutaneous injections (although well tolerated) will become unnecessary. The use in 'non-organic' causes of impotence still has to be defined although this is potentially a field which could open up many exciting new horizons for neuro-endocrinologists ascending from the pituitary and the hypothalamus to higher levels.

## ACKNOWLEDGEMENTS

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# GONADOTROPHIN RELEASING HORMONE THERAPY

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## SUMMARY

In view of the high proportion of patients with demonstrable hypothalamic or pituitary disease who responded to a single intravenous bolus injection of Gn-RH it was hoped that repeated administration (500 µg 8 hourly) of the decapeptide would prove to be of therapeutic value.

Treated prepubertal males (craniopharyngioma, isolated growth hormone deficiency) showed an improvement in pubic hair, a marked increase in the frequency of erections and testicular size increased.

Nine adult males with hypothalamic or pituitary disease have been treated ; in all these patients, a rapid return of potency has been observed between 2 and 17 days after starting therapy. In four of them treated for 12-18 months potency has been well maintained. In addition, spermatogenesis was induced within 7 to 26 weeks of starting therapy. FSH levels have fallen as the sperm count has risen (« inhibin ») although LH levels have been little altered. One patient's wife is now pregnant.

Female patients with anorexia nervosa (clomiphene unresponsive) and documented amenorrhea for 5-7 years, have been treated with Gn-RH resulting in ovulation and mensturation. The addition of HCG 4 500 units on day 14 in one patient also resulted in pregnancy.

Gn-RH may be of value in the treatment of male and female infertility.

## INTRODUCTION

Following the work of McCann 1962, McCann and Dhariwal 1966, it was suggested that hypothalamic regulation of pituitary gonadotrophin secretion was by means of two separate releasing hormones for luteinising hormone (LH) and follicle stimulating hormone (FSH). However, Schally *et al.* (1971) were able to isolate only a single gonadotrophin releasing hormone (Gn-RH) from porcine hypothalami. Administration of this decapeptide results in both LH and FSH release and therefore has left unresolved the identity of FSH-RH, if indeed it exists at all in man.

When the synthetic material became available many centres including our own investigated its properties. It was shown that the decapeptide released both LH and

FSH in a dose related manner when 25 to 500 μg was given as an intravenous bolus (BESSER *et al.*, 1972). Subsequently a standard 100 μg test was devised sampling for LH and FSH before and 20 and 60 minutes after the injection of Gn-RH. This test was then applied to 155 patients with hypothalamic-pituitary-gonadal disease. The results showed that although 88 p. 100 were clinically hypogonadal when tested initially, only 10 p. 100 of the patients had an absent response to the releasing hormone. It was also evident that this test could not distinguish between hypothalamic and pituitary disease since identical responses could be obtained in either group, although primary gonadal failure resulted in an exaggerated response (MORTIMER *et al.*, 1973). In view of the large number of patients with demonstrable hypothalamic or pituitary disease who did respond it was hoped that repeated administration of the decapeptide would prove to be of therapeutic value.

Studies of the time course of action of 100 μg Gn-RH given by intravenous, intramuscular or subcutaneous routes showed that they were equally effective in elevating LH for 5-7 hours and FSH for 3-5 hours (MORTIMER *et al.*, 1974 *a*). Intranasal administration unfortunately was not particularly effective since at least 2 mg had to be given by this route to produce the same effect as 100 μg by the other routes (LONDON *et al*, 1973). In our studies, therefore, self administration of Gn-RH by the subcutaneous route was chosen and in view of the time course of gonadotrophin release injections were given 8 hourly. A dose of 500 μg was used since earlier studies had shown that patients with isolated gonadotrophin deficiency' might fail to respond to 100 μg, but this could be improved by increasing the dose (MARSHALL *et al.*, 1972). Patients who had previously received gonadal steroids or intramuscular gonadotrophin treatment were taken off all therapy for at least 4 months prior to Gn-RH administration.

## PREPUBERTAL MALES : PUBERTY AND POTENCY

Initially five prepubertal patients were treated aged 14 to 22 years. Of these, four had craniopharyngiomas and one isolated growth hormone deficiency. All were clomiphene negative except one (craniopharyngioma). Treatment resulted in progressive increases in both gonadotrophins with the FSH response being greater initially than that of LH. This prepubertal pattern of response (FRANCHIMONT *et al.*, 1974) was later reversed becoming adult in type. (fig. 1). Of the five patients, two were still growing with intramuscular growth hormone therapy and so Gn-RH treatment was discontinued after 4 weeks since it was considered undesirable to induce puberty while they still had growth potential. However, therapy was continued in two of them (craniopharyngioma, isolated growth hormone deficiency). Before treatment one had pre-adolescent genitalia at Stage 1 puberty (TANNER, 1958). After 8 weeks of treatment this patient showed an increase in spontaneous erections with Stage 3 pubic hair. The other was Stage 3 before therapy but after nine weeks showed an improvement in pubic hair with a marked increase in the frequency of erections. Testicular size increased from 4-6 ml before treatment to 6-12 ml after 20 weeks in these patients.

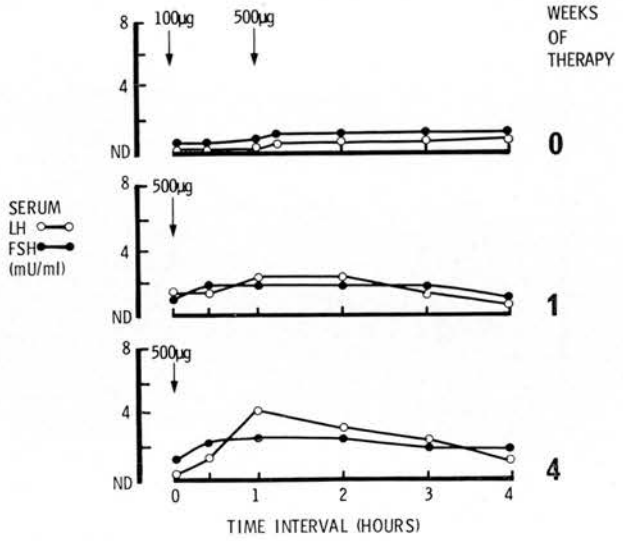



FIG. 1. — *Serum LH ○ and FSH ● responses to Gn-RH in a prepubertal male aged 14 years with a craniopharyngioma*

## ADULT MALES : POTENCY AND SPERMATOGENESIS

Nine adult males with hypothalamic or pituitary disease have been treated. Of these, six had isolated gonadotrophin deficiency ; one craniopharyngioma ; one acromegaly treated with external pituitary irradiation and one a diffuse hypothalamic tumour of unknown origin. In all patients there was an increase in potency between 2 and 17 days after starting therapy, despite testosterone levels below the normal adult male range in all but two. The rapid return of potency in these patients despite levels of testosterone normally associated with impotence suggests that these behavioural effects may not be entirely androgen mediated. It is of interest that MOSS and MCCANN (1973), PFAFF (1973) have reported an increase in the number of lordotic responses in ovariectomised and hypophysectomised rats following subcutaneous administration of the releasing hormone. The role of Gn-RH in psychogenic impotence remains to be defined and we await the results of a double blind crossover trial being carried out in conjunction with Professor R. HALL and co-workers in Newcastle.

Four adult males have been treated for 12-18 months and potency has been well maintained. In addition, spermatogenesis was induced within 7 to 26 weeks of starting therapy. Two with « isolated » gonadotrophin deficiency and the craniopharyngioma patient had low or undetectable basal gonadotrophins, and all were clomiphene unresponsive. During initial therapy the prepubertal type of gonadotrophin response to Gn-RH was seen although later as in the prepubertal boys this pattern became adult in type. During therapy the maximum LH levels following a therapeutic 500 µg dose of Gn-RH showed no definite pattern. However, in all three patients the maximum FSH response began to decline before spermatazoa were seen

on seminal analysis and showed a progressive fall into the normal basal male range throughout therapy (fig. 2).

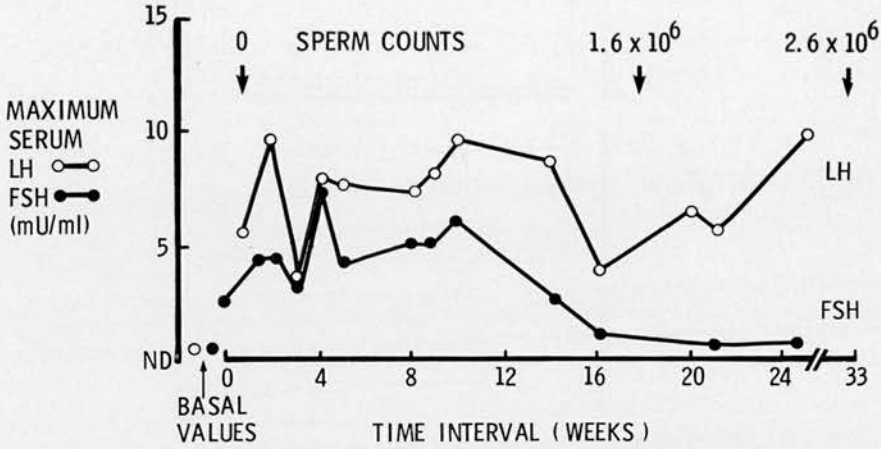



FIG. 2. — *Maximum serum LH ○ and FSH ● responses to 500 μg Gn-RH subcutaneously in a man with isolated gonadotrophin deficiency*

The gonadotrophin results in the patient with acromegaly were different since he had an elevated basal FSH level of 17.3 mU/ml (normal range less than 0.2 to 5.9 mU/ml, MRC standard 68/39) although the basal LH was normal at 3.8 mU/ml (normal range less than 0.4 to 6 mU/ml, MRC standard 68/40). He had much reduced potency, low plasma testosterone and was azoospermic. When tested with 100 μg Gn-RH he had a normal LH response but an exggerated FSH rise. The maximum FSH response at the beginning of treatment showed levels greater than 50 mU/ml but fell progressively before and following the appearance of spermatozoa (fig. 3). During treatment in these four patients total sperm counts have risen from zero (or 600.000 dead spermatozoa in one patient who had received human chorionic gonadotrophin 4 months prior to the study) to maximum levels of 7.8 and 60.8 million (isolated gonadotrophin deficient patients), 146 million (acromegalic) and 432 million (craniopharyngioma) with a motility of 40-70 p. 100. This was accompanied by an increase in testicular volume from 1-3 mls before to between 8-15 mls after 52 weeks of treatment. In all patients in whom spermatogenesis has occurred FSH levels have fallen as the sperm count has risen although LH levels have been little altered. This suggests that a factor (inhibin) has been released which selectively impairs FSH secretion. Also, since circulating Gn-RH levels were maintained by repeated subcutaneous injection it would seem that the site of action of inhibin is primarily at the pituitary level. It is also clear that pituitary responsiveness in male subjects may be modified by circulating substances other than testosterone or oestrogen (plasma 17β-oestradiol levels were within the normal male range throughout therapy except on three isolated occasions).

Following one year of therapy the acromegalic patient was treated with bromocriptine in order to suppress growth hormone secretion (THORNER *et al.*, 1974). Gn-RH therapy was continued and the total sperm maintained around 130 million. This patient's wife is now 3 months pregnant.

Gn-RH therapy has been used also to treat patients with oligo-or azoospermia. (ZARATE *et al.*, 1973). Treatment with 500 μg subcutaneously, twice daily for 6 months resulted in an increase from a total count of 1-5 million basally to 10 million. The count then fell despite continued therapy. Three of their patients showed no significant improvment although all had an increase in motility. It remains to be seen if Gn-RH therapy is of benefit in patients with primary gonadal dysfunction resulting in infertility.

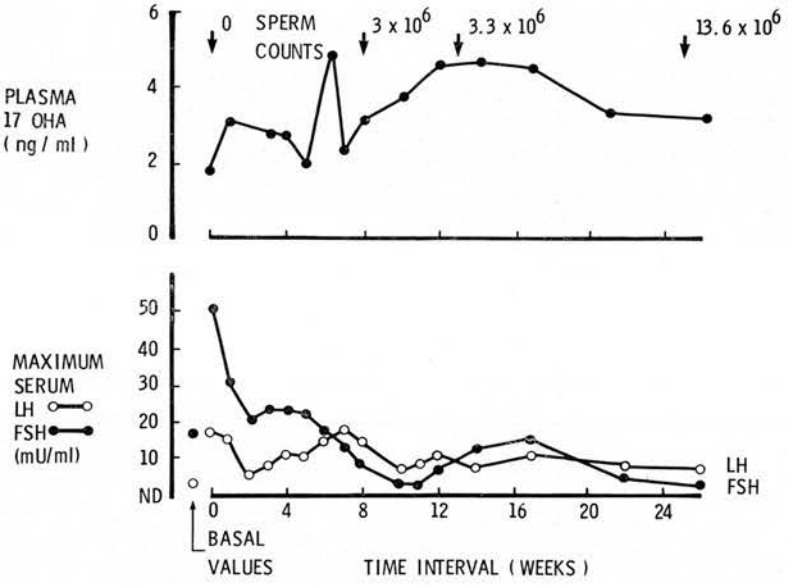



FIG. 3

Top : *Plasma 17 hydroxyandrogen levels* (normal range 5-22.5 ng/ml) *and increasing total sperm counts in a man with acromegaly.*

Bottom : *Maximum serum LH* ○ *and FSH* ● *responses to 500* μg *Gn-RH subcutaneously.*

## FEMALES : OVARIAN STEROIDOGENESIS, OVULATION AND MENSTRUATION

In 1972, AKANDE *et al.*, reported a series of studies in 8 patients with secondary amenorrhoea although the diagnosis was not recorded in all of them. However, although a rise in gonadotrophins was observed ovulation occurred in only one who had ovulated spontaneously while basal sampling was being carried out prior to the study. Other studies were subsequently reported (KELLER 1973 ; BRECKWOLDT *et al.*, 1974) but the value of Gn-RH therapy for ovulation induction remained undecided. More recent studies have produced ovulation in 2 of 16 women with secondary amenorrhoea following depot-medroxyprogesterone acetate or chlormadinone therapy for contraceptive purposes (ZANARTU *et al.*, 1974). However, the low success rate achieved suggests that the optimal regimen for reliable ovulation induction remains to be found.

In our own study, we have treated four patients aged between 19 and 29 years with anorexia nervosa and documented amenorrhoea for 5-7 years. These patients were either at their ideal body weight or between 3.5 and 5.8 kgs below this, and each was clomiphene unresponsive. Basal total urinary oestrogens were between 4 and 17 μg/24 hours measured fluorimetrically (BROWN *et al.*, 1968). Initial therapy with 500 μg Gn-RH was given by subcutaneous injection 8 hourly for 7 days, then stopped and restarted on days 12-14. During this study samples for serum gonadotrophins were collected 10 hours after the last dose of releasing hormone given the previous night. Basal serum gonadotrophins were low or absent in all these patients although during therapy these was a rise with more LH than FSH secreted. During the first seven days urinary oestrogens rose to a maximum between 62 and 135 μg/24 hrs (normal mid-cycle range between 30 and 120 μg/24 hrs). When therapy was discontinued after 7 days gonadotrophins became low or undetectable with a fall in urinary oestrogens. There was then a further rise when treatment was restarted between days 12-14. In all subjects there was a rise in 24 hours total urinary oestrogens between days 18 and 28 despite being off therapy preceeded by a rise in basal serum gonadotrophins. These results therefore indicate that the patients had adequate pituitary LH

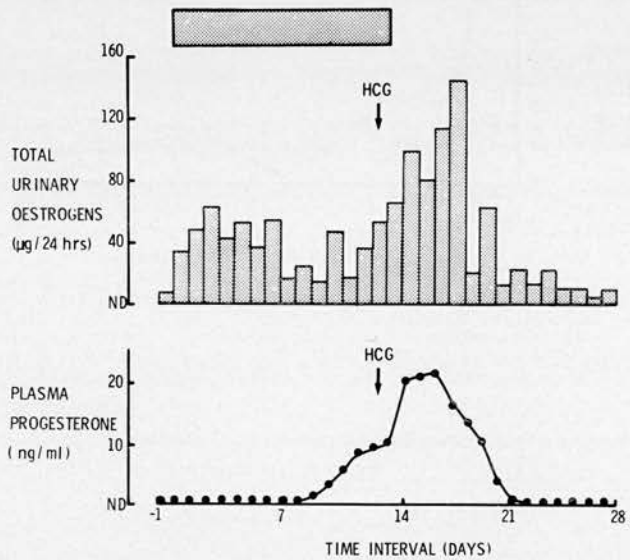



FIG. 4. — *Total 24 hrs urinary oestrogen and plasma progesterone responses to Gn-RH 500 μg 8 hourly subcutaneously in a woman with anorexia nervosa*

and FSH reserve although the release of the gonadotrophins was impaired while off therapy due to a persistent deficiency of endogenous Gn-RH. Therapy resulted in rises in total 24 hour urinary oestrogens (and plasma oestradiol) to levels comparable to those seen in normal cycles. The occurrence of spontaneous release of gonadotrophins between days 18-28 despite being off treatment indicates that there had been positive feedback of the rise in circulating oestrogens at the hypothalamic-pituitary level. Following this treatment one patient menstruated for the first time in 6 years, although there was no biochemical evidence of ovulation in any of the 4

patients. Two patients were retested after 12-14 weeks off therapy with clomiphene and both now showed a rise in urinary oestrogens followed by a rise in urinary pregnanediol indicative of ovulation. Both patients also menstruated. The other two instead of clomiphene received a further course of Gn-RH 500 μg 8 hourly except this was continued for 14 days, at which time an injection of 4.500 units of HCG was given intramuscularly (fig. 4). In both patients there was a rise in urinary oestrogens together with a rise in plasma progesterone from less than 1 ng/ml to 6.4 and 9.9 ng/ml on day 14 *before* HCG was given. Both patients later menstruated.

Further studies involving the administration of Gn-RH 500 μg 8 hourly for 28 days has resulted in ovulation (as determined by a rise in urinary pregnanediol from undetectable to 6.5 mg/24 hrs) and menstruation. The addition of HCG 4.500 units on day 14 in one patient resulted in ovulation and pregnancy. Continuous therapy initially for 1 month and then for a further 4 months in a patient with isolated gonadotrophin deficiency has resulted in a cyclical increase in 24 hour total urinary oestrogens from 1 to 30 μg/24 hrs and increase in breast size although ovulation and menstruation have not yet occurred.

It would appear therefore that Gn-RH therapy will result in ovarian steroidogenesis and ovulation. This may be followed by menstruation although no female has reported the increase in libido noted by the men. Since Gn-RH therapy for ovulation induction results in the production of oestrogen and progesterone levels normally seen during the menstrual cycle, it is hoped that hyperstimulation of the ovary and multiple births will be avoided.

## CONCLUSION

Studies with Gn-RH indicate that the single decapeptide is capable of promoting synthesis as well as the differential release of LH and FSH by the modulation of pituitary responsiveness by gonadal steroids and inhibin. It may be of value in the treatment of male and female infertility and suggests that although a distinct FSH-RH may yet exist in man, it is not essential for these events to occur. Future studies involving depot preparations and analogues more resistant to enzymatic degradation may further widen the scope of Gn-RH therapy.

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## ACKNOWLEDGEMENTS

We are grateful to the Editor of the British Medical Journal for permission to reproduce figures 1, 2, 3 (from MORTIMER *et al.*, 1974 *b*) ; Hoechst (U. K.) for suppling the synthetic Gn-RH and the Medical Research Council, Peel Medical Research Trust and the Joint Research Board, Saint Bartholomew's Hospital for their financial support.

## RÉSUMÉ

### TRAITEMENT PAR L'HORMONE DE LIBÉRATION DES GONADOTROPINES ((Gn-RH)

Puisqu'une proportion élevée (88 p. 100) de patients avec des troubles hypothalamiques ou hypophysaires répondent au Gn-RH, il était possible d'espérer que des administrations répétées (500 µg toutes les 8 heures) du décapeptide auraient une certaine valeur thérapeutique.

Quelques adolescents mâles prépubères traités avec le Gn-RH ont effectivement montré une augmentation importante de la fréquence d'érection et de la taille testiculaire.

De même, 9 patients mâles adultes souffrant de troubles hypothalamiques ou hypophysaires ont tous montré un retour rapide de la puissance entre 12 et 17 jours après le début du traitement au Gn-RH. Celle-ci s'est maintenue chez quatre d'entre eux traités pendant 12 à 18 mois. En outre, la spermatogenèse a été induite 7 à 26 semaines après le début du traitement. Les niveaux plasmatiques de FSH ont diminué lorsque le nombre de spermatozoïdes s'est élevé dans l'éjaculat (« inhibine ») bien que les niveaux de LH n'aient pas été modifiés. La femme de l'un des patients est actuellement enceinte.

Des femmes avec anorexie nerveuse et aménorrhée depuis 5-7 ans et ne répondant pas à un traitement au clomiphène ont reçu des injections de Gn-RH pendant des durées variables jusqu'à 28 jours. L'ovulation et la menstruation ont été obtenues. L'injection supplémentaire de 4 500 unités de HCG le 14e jour chez une patiente a permis l'installation d'une grossesse.

Ainsi, le Gn-RH peut être utile dans le traitement de la stérilité mâle ou femelle.


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# THE EFFECTS OF CHRONIC TREATMENT WITH LHRH ON GONADOTROPHIN SECRETION AND PITUITARY RESPONSIVENESS TO LHRH IN WOMEN WITH SECONDARY HYPOGONADISM

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SUMMARY

Twenty women with secondary hypogonadism and four normal women in the early follicular phase of the cycle were treated for 7 days with 10, 50 or 100 μg synthetic LHRH administered intramuscularly at 4 h intervals. Concentrations of pituitary and ovarian hormones in plasma were measured at intervals during the treatment period. The episodic pattern of LH secretion and the gonadotrophin responses to acute stimulation with LHRH were evaluated before and after treatment.

In normal women the concentrations of gonadotrophins and the LH response to LHRH remained unchanged, whilst the FSH response to LHRH was reduced after treatment. Concentrations of oestradiol rose progressively in response to treatment, indicating follicular development.

In hypogonadal subjects with unimpaired pituitary function, treatment with LHRH induced a marked but transient increase in the concentrations of LH and FSH in plasma and a progressive rise in that of oestradiol. The concentration of progesterone was increased in four of the 11 subjects. However, the amplitude of LH pulses and the responses of FSH and LH to LHRH after treatment were suppressed below pretreatment values.

Women with hypogonadotrophic hypogonadism and diminished pituitary responses to LHRH exhibited a sustained increase in the concentrations of LH, FSH and oestradiol in plasma to normal follicular phase levels. The amplitude of LH pulses and the LH response to LHRH were increased after treatment but did not reach normal values. The FSH response to LHRH was further reduced

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after treatment. Treatment of similar subjects with 10 μg LHRH induced a small increase in the concentration of gonadotrophins but not of ovarian steroids.

These data demonstrate that the effect of chronic treatment with LHRH on women with secondary hypogonadism depends on the level of endogenous gonadotrophins. The dose of LHRH used in hypogonadal women receiving treatment by intermittent injection may require adjusting according to the level of gonadotrophin secretion if an optimal response is to be obtained.

Repeated administration of luteinizing hormone releasing hormone (LHRH) increases the basal secretion of LH and FSH and the LH response to LHRH in the majority of women with hypogonadotrophic hypogonadism, suggesting that the primary lesion is a deficiency of endogenous LHRH (Marshall *et al.*, 1972; Jewelewicz *et al.*, 1974). However, attempts to restore ovarian activity in women with secondary hypogonadism have been disappointing until recently (Nillius & Wide, 1975; Mortimer *et al.*, 1976; Henderson *et al.*, 1976). Although follicular maturation could be induced in some subjects by the injection of 500 μg LHRH at 8 hourly intervals, ovulation was infrequent or followed by a defective luteal phase (Henderson *et al.*, 1976; Nillius, 1979).

More recently several authors have reported successful induction of ovulation and subsequent pregnancies in hypogonadotrophic women treated with small 'physiological' doses of LHRH at more frequent intervals (Leyendecker *et al.*, 1980; Crowley & McArthur, 1980; Valk *et al.*, 1980; Schoemaker *et al.*, 1982). These clinical studies have stemmed from the knowledge that LH, and presumably therefore LHRH, is secreted in a pulsatile fashion in many species including man (Yen *et al.*, 1972; Knobil, 1980; Baird & McNeilly, 1981). In the rhesus monkey in which endogenous LHRH secretion has been abolished by destruction of the arcuate nucleus, cyclical ovarian function could be restored by administration of small doses of LHRH at intervals of 90 min (Knobil, 1980). In the clinical studies referred to above, neither the optimal dose nor the time interval of injection have been investigated although there is a wide variation in the level of LH and FSH in women presenting with 'hypothalamic' amenorrhoea (Kletsky *et al.*, 1975; Bohnet *et al.*, 1976a; Van Look *et al.*, 1977a).

The present study was therefore undertaken to investigate whether chronic treatment with three different doses of LHRH could restore ovarian activity in amenorrhoeic women in whom the activity of the hypothalamic–pituitary axis was suppressed to a varying degree.

## MATERIALS AND METHODS

### *Patients*

Twenty women with secondary amenorrhoea of at least 6 months duration were recruited for the study after full endocrine investigation had excluded primary ovarian failure or general endocrine disease (Table 1). Written informed consent was obtained in each subject.

The subjects with secondary amenorrhoea were divided into three groups based on whether the level of gonadotrophins and prolactin were within the normal range for the early follicular phase of the cycle (LH 2·5–15·3 U/l; FSH 2·9–12·6 U/l; Prolactin 60–500 mU/l):

(1) HGA: Hypogonadotrophic ($n=9$);

Table 1. Clinical data on amenorrhoeic subjects

| Group | Subject | Plasma concentration | | | | | | Dose of LHRH (μg) |
|---|---|---|---|---|---|---|---|---|
| | | LH (U/l) | FSH (U/l) | PRL (mU/l) | $E_2$ (pg/ml) | Androstenedione (pg/ml) | Progesterone (ng/ml) | |
| HGA | 1 | 0·6 | 2·3 | 270 | 44 | 1193 | 0·46 | 10 |
| | 2 | 0·8 | 0·8 | 102 | 27 | 5111 | 0·76 | |
| | 3 | 1·1 | 0·6 | 238 | 60 | 1323 | 0·70 | |
| | 15 | 2·0 | 4·0 | 143 | 25 | 2138 | 0·99 | 50 |
| | 18 | 2·1 | 0·8 | 300 | 58 | 1957 | 0·35 | |
| | 19 | 1·5 | 2·9 | 460 | 39 | 1983 | 0·74 | |
| | 11 | 0·6 | 2·7 | 414 | 32 | 2062 | 1·06 | 100 |
| | 12 | 1·0 | 5·4 | 280 | 126 | 2085 | 0·46 | |
| | 13 | 0·7 | 2·2 | 114 | 61 | 2025 | 0·63 | |
| NGA | 16 | 4·5 | 3·8 | 271 | 74 | 2824 | 1·02 | 50 |
| | 17 | 5·8 | 7·2 | 440 | 79 | 866 | 0·53 | |
| | 4 | 11·3 | 3·0 | 140 | 199 | 2865 | 1·00 | 100 |
| | 6 | 6·4 | 9·7 | 230 | 68 | 3285 | 0·72 | |
| | 9 | 5·5 | 4·6 | 300 | 17 | 1151 | 1·12 | |
| | 7 | 5·2 | 5·5 | 120 | 36 | 2561 | 0·90 | |
| HPA | 5 | 12·1 | 2·6 | 870 | 272 | 2363 | 0·99 | 100 |
| | 8 | 5·2 | 5·6 | 990 | 101 | 3495 | 0·74 | |
| | 20 | 3·0 | 3·0 | 1985 | 251 | 2265 | N/A | |
| | 10* | 1·9 | 5·1 | 4850 | 40 | 3562 | 0·51 | |
| | 14* | 2·2 | 2·3 | 5213 | 20 | 2261 | 0·73 | |

* Enlarged pituitary fossa.
N/A = not available.
$E_2$ = oestradiol.

(2) NGA: Normogonadotrophic ($n = 6$);
(3) HPA: Hyperprolactinaemic ($n = 5$).

The HGA corresponded to Group I and NGA and HPA to Group 3 of Kletsky *et al.* (1975).

There was radiological evidence of the presence of a pituitary tumour in two of the five subjects in Group HPA although TSH response to 200 μg TRH and GH response to insulin-induced hypoglycaemia (0·15 U/kg) were normal in all five subjects.

None of the patients had ovulated in response to a single treatment with clomiphene 100 mg daily for 5 days. All but one of the NGA subjects and all five HPA subjects had exhibited a normal biphasic LH response to an oestrogen provocation test (oestradiol benzoate 1 mg intramuscularly on two consecutive days) 2 weeks before this study, but the positive feedback LH response was absent in all nine HGA subjects and in one NGA subject (No. 7).

*Treatment regimen*

Synthetic LHRH (Hoechst) in 0·5 ml was self-administered by intramuscular injection at a dose of 10, 50 or 100 μg (Table 1), with the exceptions described below, at 4 hourly

intervals for 7 days. Treatment of normal subjects started on Day 1 or 2 of the cycle. The injection due at 02.00 h was omitted to allow undisturbed sleep.

Twenty millilitres of venous blood were collected 24 h and immediately before treatment and at 10–14 h intervals thereafter for 5 days, morning samples being taken 3 h after the previous injection of LHRH and evening samples 1 h after injection. A further 20 ml of blood was collected after 167 h (7 days) of treatment, 3 h after the last injection of LHRH. Concentrations of LH, FSH, prolactin, oestradiol, and androstenedione in plasma were measured in all samples and the concentration of progesterone in plasma was also measured in the last sample.

To assess the pattern of LH secretion, 5 ml venous blood were collected at 15 min intervals over a period of 6 h on the days before and after treatment. Sampling on the last day started 3 h after the last injection of LHRH.

To determine the pituitary responsiveness, the first injection of LHRH (10.00 h, Day 1) and that 100 h after the start of treatment (14.00 h, Day 5) were administered intravenously. Ten millilitres of venous blood were collected immediately before and 15, 30, 45, 60, and 90 min after injection for the measurement of concentrations of LH and FSH in plasma.

*Control subjects*

Four healthy women aged 20–30 years, with regular cycles of 26–32 days and a mid-luteal phase concentration of progesterone in plasma greater than 10 ng/ml, were treated with 50 $\mu$g LHRH at similar intervals starting on Day 1 or 2 of the cycle, and blood samples were collected at the same intervals.

*Hormone assay methods*

Plasma was separated immediately after collection and stored at $-20^{\circ}$C until all samples from each subject could be assayed together. Concentrations of LH and FSH in plasma were measured in duplicate using the specific double-antibody RIA method of Hunter & Bennie (1979). The intra-assay coefficients of variation were 8% and 7% and the interassay coefficients of variation, 12% and 11% for LH and FSH, respectively. The sensitivity of the systems were 0·3 U/l for LH and 1·0 U/l for FSH. Results were expressed in U/l of the appropriate standards obtained from the National Institute of Biological Standards and Control, Holly Hill, London (LH: MRC 68/40, 77 IU/A; FSH: MRC 69/104, 10 IU/A).

Prolactin was measured by the double-antibody RIA described by McNeilly & Hagen (1974) using MRC standard 75/504 (10 mU/a). Results are expressed in mU/l (1 mU/l MRC 75/504 = 0·045 ng Friesen or Lewis prolactin). The sensitivity of the assay was 18·5 mU/l and the inter- and intra-assay coefficients of variation, 9% and 6% respectively.

Oestradiol, androstenedione, and progesterone were measured by radioimmunoassays, the details of which have been previously published from this laboratory (Van Look *et al.*, 1977b; Baird *et al.*, 1974; Scaramuzzi *et al.*, 1975). The sensitivity, inter- and intra-assay coefficient of variation for the three methods was as follows:

| | | |
|---|---|---|
| Oestradiol: | 5 pg/ml; | 13·3% and 9·3%; |
| Androstenedione: | 100 pg/ml; | 14·1% and 8·3%; |
| Progesterone: | 140 pg/ml; | 14·8% and 8·0% |

*Episodic secretion of LH*

Since progesterone is known to alter the frequency of LH pulses (Yen *et al.*, 1972), those subjects with increased level of progesterone following therapy were excluded from the comparison between the pre- and post-treatment characteristics of LH pulses.

Concentrations of LH in plasma tended to decline during the sampling periods. The regression equation for each individual series of LH values was therefore determined and values above the 99% confidence limit of the regression line were identified. These values were then excluded from a calculation of the regression in the remaining (basal) values. All values were then corrected for the regression in the basal concentration and pulses identified as two consecutive (peak) values in excess of the mean of the preceding two (basal) values, the increment of at least one peak value above the mean basal value being greater than expected on the basis of the intra-assay coefficient of variation (i.e. greater than three times the CV of 8% (Bäckström *et al.*, 1982).

*Statistical methods*

Values expressed in this paper were derived from the respective log values, which were used in Student's single-tail *t*-test. Mean values are given in the text with SD in parentheses.

## RESULTS

*Basal concentrations of LH, FSH, oestradiol, and progesterone during treatment*

Concentrations of LH and FSH in plasma remained unchanged in the control group whereas the concentration of oestradiol increased more than two-fold within 23 h of the start of treatment, and remained elevated throughout the study period ($P<0{\cdot}05$ to $P<0{\cdot}005$) (Fig. 1a). Concentrations of progesterone in plasma were raised above the baseline level on the seventh day in two subjects, reaching to 1·9 and 1·6 ng/ml, respectively.

Basal concentrations of gonadotrophins and oestradiol increased in all three groups of amenorrhoeic women within 23 h of the start of treatment with 50 or 100 μg LHRH (Fig. 1b, c & d). In the HGA group, concentrations of LH increased to, and were maintained within, the normal follicular phase range while the concentration of oestradiol increased progressively to a peak value of 112 (+136; −61) pg/ml after 167 h, three times the basal value (Fig. 1b). There was no increase in progesterone secretion in this group.

In contrast, in the NGA and HPA groups the levels of LH and FSH declined after the initial temporary stimulation (Fig. 1c & d). Concentrations of oestradiol increased progressively to reach peak values of 245 (+136; −88) and 271 (+110; −78) pg/ml, respectively, after 57 h of treatment and then declined.

Concentrations of progesterone in plasma were elevated on the seventh day in three NGA subjects (Nos. 4, 6, and 9) to 1·3, 3·0, and 2·6 ng/ml, respectively, and in one HPA subject (No. 8) to 3·8 ng/ml.

In the HGA group treated with 10 μg LHRH, concentrations of LH and FSH increased within 23 h and remained elevated throughout the treatment period (Fig. 2) but did not reach normal follicular phase levels. There was no increase in the concentration of oestradiol nor of progesterone. Concentrations of prolactin and androstenedione in plasma remained unaltered during treatment with LHRH in all four groups (Table 2).

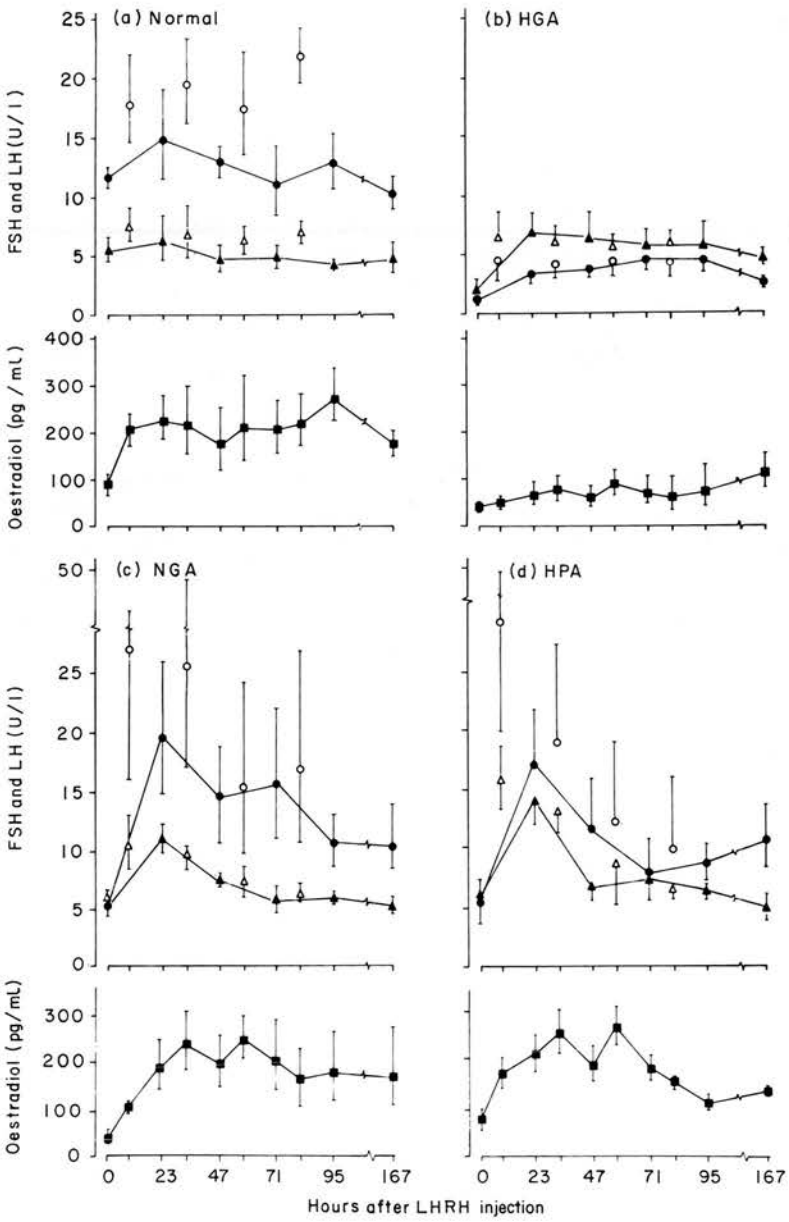



Fig. 1. Concentration of LH, FSH and oestradiol (mean ± SEM) in plasma of women during treatment with LHRH (50 or 100 μg) every 4 h for 167 h. (A) Control women ($n=4$); (B) hypogonadotrophic (HGA) ($n=6$); (C) normogonadotrophic (NGA) ($n=6$); (D) hyperprolactinaemic (HPA) ($n=5$). (●) Basal LH at 09.00 h; (○) LH 60 min after LHRH at 23.00 h; (▲) Basal FSH at 09.00 h; (△) FSH 60 min after LHRH at 23.00 h.

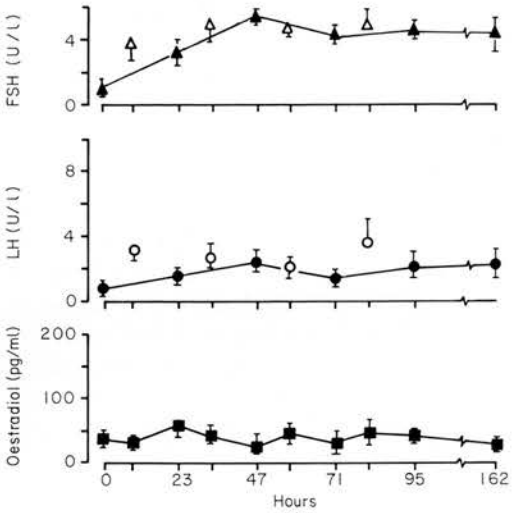



Fig. 2. Concentrations of LH, FSH and oestradiol (mean ± SEM) in plasma of three women with gonadotrophic hypogonadism (HGA) during treatment with 4 hourly injections of 10 μg LHRH. (●) Basal LH at 09.00 h; (○) LH 60 min after LHRH at 23.00 h; (▲) basal FSH at 09.00 h; (△) FSH 60 min after LHRH at 23.00 h.

Table 2. Integral LH and FSH responses to LHRH and basal concentrations of prolactin, oestradiol and androstenedione in plasma before and after 164 hours treatment with LHRH 50 or 100 μg every 4 h

| Group | | LH (U/l) | | FSH (U/l) | | Basal prolactin (mU/l) | | Basal concentration of steroids (pg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Oestradiol | | Andro-stenedione | |
| | | Before | After | Before | After | Before | After | Before | After | Before | After |
| Normal | Mean | 46·0 | 44·5 | 10·8 | 1·8* | 281 | 470 | 89 | 257* | 1902 | 2769 |
| ($n=4$) | SD + | 20·4 | 11·6 | 4·1 | 11·9 | 156 | 176 | 33 | 104 | 460 | 1823 |
| | − | 14·1 | 9·2 | 3·0 | 1·6 | 100 | 128 | 24 | 73 | 370 | 1099 |
| HGA | Mean | 12·4 | 21·3* | 10·8 | −1·9* | 271 | 209 | 37 | 78 | 1811 | 1840 |
| ($n=6$) | SD + | 103·6 | 27·0 | 26·9 | 14·3 | 154 | 23 | 19 | 192 | 312 | 608 |
| | − | 11·1 | 11·9 | 7·7 | 3·2 | 98 | 21 | 13 | 56 | 266 | 457 |
| NGA | Mean | 160·5 | 44·2 | 10·5 | 0·8* | 253 | 276 | 43 | 204** | 1904 | 2355 |
| ($n=6$) | SD + | 276·8 | 74·5 | 31·5 | 9·7 | 95 | 94 | 60 | 307 | 931 | 1079 |
| | − | 101·6 | 27·8 | 12·4 | 7·7 | 69 | 70 | 25 | 123 | 625 | 740 |
| HPA | Mean | 133·4 | 55·5 | 27·2 | 6·2* | 1513 | 2168 | 78 | 112 | 2306 | 2267 |
| ($n=5$) | SD + | 124·4 | 16·2 | 60·1 | 5·9 | 3304 | 5694 | 69 | 61 | 626 | 422 |
| | − | 64·4 | 12·5 | 18·9 | 3·0 | 1038 | 1570 | 37 | 40 | 492 | 1355 |

Significance of difference from value before treatment: * $P<0·05$; ** $<P<0·02$.

*Gonadotrophin response to LHRH*

The integral LH and FSH responses to acute stimulation with LHRH were determined from the sums of the increments in the concentrations in plasma during the 90 min following intravenous injection of LHRH. The responses before and after treatment with LHRH for 100 h are summarized in Table 2.

Before treatment both the LH and FSH responses were normal or exaggerated in the NGA and HPA groups, but as expected the LH response in the HGA group was impaired. After treatment the LH response to LHRH remained unchanged in normal women, whereas the FSH response was reduced by 85% (Table 2). In the NGA and HPA groups the LH and FSH responses to LHRH were reduced in comparison with pretreatment values ($P < 0{\cdot}05$). In contrast, the LH response to LHRH in the HGA group treated with 50 or 100 $\mu$g LHRH was double that before treatment ($P < 0{\cdot}05$). As in the other groups the FSH response to LHRH was markedly reduced after treatment. In the three HGA subjects treated with 10 $\mu$g LHRH the pituitary response remained unchanged.

*Pulsatile secretion of LH*

Before treatment, pulses of LH were identified in all four control subjects occurring at a frequency of $2{\cdot}2 \pm 0{\cdot}4$ per 4 h. The frequency of LH pulses was similar to normal in the NGA group but was markedly suppressed in both HGA and HPA groups ($P < 0{\cdot}005$ and $< 0{\cdot}01$, respectively) (Fischer's exact probability test). In keeping with the basal levels, the amplitude of LH pulses was similar to normal in NGA and HPA groups but markedly suppressed in the HGA group (Table 3) ($P < 0{\cdot}001$).

Observations of the effect of chronic LHRH treatment of the pattern of LH secretion were not available in all subjects (Table 3). In the two available control subjects there was a decrease in the frequency and amplitude of pulses after therapy (Table 3). In all three groups of patients the frequency of LH pulses was unaffected by treatment. However, the amplitude of LH pulses after treatment with LHRH was reduced in both the NGA and HPA groups from 2·7 and 4·9 U/l to 1·3 and 3·1 U/l, respectively ($P < 0{\cdot}005$). In contrast,

Table 3. Mean LH pulse amplitude and frequency and concentration of oestradiol in normal and amenorrhoeic women before and after treatment with LHRH 50 or 100 $\mu$g for 7 days

| | | Frequency (pulses/4 h) | | Amplitude | | Oestradiol | |
|---|---|---|---|---|---|---|---|
| Group | | Before | After | Before | After | Before | After |
| Normal | Mean ($n$) | 2·2 (5) | 1·0 (2) | 3·8 | 1·8 | 76 | 133 |
| | ± SD | +0·4; −0·4 | | +3·2; −1·7 | | +43; −27 | |
| HGA | Mean ($n$) | 1·3 (6) | 1·4 (6) | 0·6 | 1·5** | 42 | 97* |
| | ± SD | +1·4; −0·9 | +1·7; −1·0 | +0·4; −0·2 | +1·4; −0·7 | +36; −20 | +112; −52 |
| NGA | Mean ($n$) | 2·0 (3) | 1·6 (3) | 2·7 | 1·3** | 59 | 136* |
| | ± SD | +0; −0 | +0·8; −0·5 | +2·0; −1·1 | +0·8; −0·8 | +33; −21 | +420; −103 |
| HPA | Mean ($n$) | 0·9 (4) | 0·4 (2) | 4·9 | 3·1 | 86 | 128 |
| | ± SD | +2·0; −1·0 | +0·7; −0·5 | +12·2; −3·5 | | +234; −63 | +22; −19 |

Significance of difference of value after treatment: * $P < 0{\cdot}05$; ** $P < 0{\cdot}005$.

in the HGA group the amplitude of LH pulses increased in response to treatment from 0·6 to 1·5 U/l ($P < 0{\cdot}005$) although it was still well below the values for the control group.

## DISCUSSION

The present study has confirmed numerous reports that the amount of LH and FSH released in response to LHRH is related to the basal level of gonadotrophins (e.g. Akande *et al.*, 1972). Thus in the HGA group, who were selected on the basis of the fact that the level of LH was below normal, the response to LHRH was markedly impaired. The low basal levels of LH and FSH in this group are probably due to a decreased secretion of endogenous LHRH because the amplitude and frequency of LH pulses were markedly reduced (Table 3). In contrast, the amplitude of LH pulses in the HPA group was slightly greater than normal, although there was a marked reduction in frequency. Hence it can be concluded that hyperprolactinaemic hypogonadism is associated mainly with a reduction in the frequency of episodic secretion of LHRH from the hypothalamus (Bohnet *et al.*, 1976b). As the amplitude of endogenous pulses of LH and the responsiveness of the pituitary to LHRH is maintained in hyperprolactinaemia, it seems likely that the quantity of LHRH released during each episodic discharge from the hypothalamus is near normal.

Most studies which have successfully stimulated ovarian activity in hypogonadal subjects with LHRH have employed large doses at infrequent intervals (500 μg every 8 h, e.g. Nillius, 1979) or small doses (1·5–20 μg every $1\frac{1}{2}$–2 h; Leyendecker *et al.*, 1980; Crowley & McArthur, 1980; Valk *et al.*, 1980). The small increase in LH and FSH secretion observed in the HGA group treated with 10 μg LHRH every 4 h was insufficient to stimulate ovarian function as indicated by the persistently low concentrations of oestradiol. This dose in normal women produces a nearly physiological response and when injected more frequently can usually restore cyclical ovarian activity in women with hypogonadotrophic hypogonadism (Crowley & McArthur, 1980).

The experiments involving injection of larger doses of LHRH (50–100 μg) at intervals of 4 h demonstrated a variable response depending upon the degree of suppression of gonadotrophin secretion.

In the hypogonadotrophic group (HGA) the basal level of both FSH and LH rose in response to the injections and there was a gradual increase in the concentration of oestradiol indicating some follicular activity. After 7 days' treatment there was no evidence of desensitization; rather there was a significant increase in the amount of LH released in response to LHRH indicating that even this relatively large dose of LHRH was capable of priming the pituitary in women in whom endogenous hypothalamic activity was markedly suppressed. It is not possible from the present study to determine whether ovulation and cyclical ovarian activity could be restored using this regime of infrequent LHRH injections. Perhaps if the injections had been continued for longer, follicular growth would have been stimulated. Using a dose of 100 μg every 2 h, Schoemaker *et al.* (1982) induced ovulation with evidence of hyperstimulation of the ovaries in some subjects.

Those amenorrhoeic subjects in whom the levels of FSH and LH were within normal range (NGA and HPA) rapidly became less responsive to subsequent injections so that by 7 days the FSH and LH response to LHRH was significantly depressed. The desensitization to LHRH was reflected by the transient rise in oestradiol concentration which rose to maximal levels by 30 h and declined thereafter. We have recently observed

similar findings when much lower doses of LHRH (2–3 μg) are administered to normogonadotrophic amenorrhoeic subjects at 90 min intervals (Baird, McNeilly & Glasier, unpublished data). The optimum dose and frequency of LHRH which will induce ovulation in such subjects is unknown although it is possible that the regime may require adjusting individually (Schoemaker *et al.*, 1982).

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# EFFECT OF SEROTONIN AND PROGESTERONE ON INDUCED OVULATION IN IMMATURE RATS

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SUMMARY

Serotonin (50 and 100 mg/kg), given subcutaneously, inhibited induced ovulation in immature rats treated with pregnant mare serum gonadotrophin (PMSG). A single injection was effective if given 52–55 h after the PMSG, i.e. in the 3 h period after the critical period before ovulation. The injection of serotonin inhibited the release of luteinizing hormone (LH) from the pituitary since the pituitary levels were higher than in the control animals and there was complete inhibition of the plasma LH surge. The anti-ovulatory effect was reversed by administration of progesterone and endogenous plasma progesterone levels were reduced in the late evening after serotonin treatment. The site of action of serotonin appeared to be peripheral since it inhibited induced ovulation in hypophysectomized rats but was without effect in intact rats treated intraventricularly. It is suggested that progesterone is essential for the occurrence of induced ovulation and serotonin inhibits either its secretion at the ovarian level or its passage away from the ovary.

## INTRODUCTION

Ovulation may be inhibited in the immature rat treated with pregnant mare serum gonadotrophin (PMSG) by either single or multiple injections of serotonin (5-hydroxytryptamine, 5HT) (O'Steen, 1964, 1965). The most effective time for administration was found to be during the 'critical period' on the day before expected ovulation. In addition increasing hypothalamic levels of 5HT, by the administration of monoamine oxidase inhibitors, reduces the ova number in both immature rats (Kordon, Javoy, Vassent & Glowinski, 1968; Kordon, 1969) and adult hamsters (Lippmann, 1968) treated with PMSG.

Systemic administration of 5HT to the adult rat at certain times of the cycle will also inhibit ovulation (Labhsetwar, 1971; Wilson & McDonald, 1974). Based on the results of a previous study it was suggested that 5HT exerted its inhibitory action at a peripheral site (Wilson & McDonald, 1974). Furthermore, it has been shown that progesterone can reverse the inhibitory effect of 5HT when the latter is given at 17.00 h on pro-oestrus. This suggested that the pre-ovulatory release of progesterone

(Barraclough, Collu, Massa & Martini, 1971) may be important for successful ovulation. Similar proposals have also been made by Kobayashi, Hara & Miyake (1969). However, antisera to progesterone did not inhibit spontaneous ovulation in the normal cyclic rat or induced ovulation in the immature rat treated with PMSG (Ferin, Tempone, Zimmering & Vande Weile, 1969; Ferin, Zimmering & Vande Weile, 1969). In order to investigate this further we have examined the effect of 5HT on ovulation in PMSG-treated immature rats in which LH and progesterone levels were also determined.

## MATERIALS AND METHODS

### *Animals*

Female Wistar rats (Tuck & Sons, Rayleigh; Biorex Ltd, London) were housed under 14 h light and 10 h darkness (lights on from 06.00 to 20.00 h). On day 30 of life rats weighing over 60 g were treated with 20 i.u. PMSG at 13.00 h. Animals were autopsied on day 33, ovarian and uterine weights were recorded and the number of ova shed was counted.

In a second experiment female Sprague–Dawley rats (Tuck & Sons, Rayleigh) were housed under 14 h light and 10 h darkness (lights on from 05.00 to 19.00 h). Those animals weighing over 60 g on day 27 were treated with 5 i.u. PMSG and autopsied on day 30.

### *Administration of compounds*

Serotonin (May & Baker; Koch-Light Laboratories) was given as the creatinine sulphate salt and all doses are expressed in these terms. The compound was dissolved in distilled water at a concentration of 10 mg/ml for s.c. injection or 2 mg/ml for intraventricular administration. Human chorionic gonadotrophin (HCG; Pregnyl) and PMSG (Gestyl; Organon Laboratories Ltd) were dissolved in 0·9 % sodium chloride solution and administered subcutaneously in a volume of 0·1 ml/rat. Progesterone and oestradiol (Organon Laboratories Ltd) were made up in arachis oil and administered subcutaneously in a volume of 0·2 ml/rat. Methysergide and BOL 148 (bromo-lysergic acid; Sandoz, Basle) were dissolved in 0·9 % sodium chloride solution and injected subcutaneously in a volume of 0·2 ml.

### *Intraventricular injection*

Animals were anaesthetized with a 2·5 % solution of Avertin (tribromoethanol plus amylene hydrate; Sterling Winthrop Laboratories Ltd). Intraventricular injection was carried out by the method of Noble, Wurtman & Axelrod (1967) using a volume of 0·02 ml/rat. At autopsy the brains were perfused with formol–saline. After further fixation, frozen sections were examined directly and only those brains showing a needle track entering the lateral ventricle were included in the results.

### *Hypophysectomy*

Animals were anaesthetized with 30 mg sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd)/kg. On day 27 hypophysectomy was carried out by the intra-aural approach (Falconi & Rossi, 1964). At autopsy the sella turcica was examined under a dissecting microscope for pituitary remnants. Only animals completely hypophysectomized have been included in the results.

*Collection of pituitaries and blood*

Pituitaries were collected from Wistar rats at different times after an injection of 20 i.u. PMSG on day 30, as described in the Results. There were between four and nine pituitaries collected at each time interval. They were homogenized in 0·9% NaCl solution (one pituitary/2 ml) and stored at −15 °C until assayed for luteinizing hormone (LH) at the one dilution. Plasma LH was assayed in blood taken from Sprague–Dawley rats treated on day 27 with 5 i.u. PMSG. Collections were made, at 1 or 2 h intervals between 16.00 and 22.00 h on day 29, into heparinized syringes after heart puncture in animals anaesthetized with Avertin. Samples were collected from one to six rats at each time (most groups consisted of three rats). Plasma used for assaying progesterone levels was taken from Wistar rats treated with 20 i.u. PMSG on day 30. Blood was collected into heparinized syringes from the posterior vena cava from animals anaesthetized with ether. Collections were made at 2 h intervals between 14.00 and 24.00 h on day 32, from six to twelve rats at each interval. In both sets of collections only one blood sample was taken from each rat. The blood was centrifuged at 400*g* at 4 °C and the plasma stored at −15 °C until assayed.

*Assay of LH and progesterone*

Pituitary LH was assayed by the ovarian ascorbic acid depletion method of Parlow (1961) as modified by Zarrow, Yochim & McCarthy (1964). The mean precision for 13 assays was 0·25.

Plasma LH was determined by a double antibody radioimmunoassay using reagents from the National Institutes of Health, Bethesda, U.S.A. Results are expressed in terms of NIH-LH-S1.

Plasma progesterone was estimated by a rapid competitive protein-binding assay. Details of the method have been reported previously (Horth & Palmer, 1972; Wilson, Horth, Endersby & McDonald, 1974).

## RESULTS

*Effect of serotonin on induced ovulation*

Daily injections of 100 mg 5HT/kg were given. This dose, even given over 3 days, had no toxic effect on the immature rats nor was there any appearance of ill health or discomfort after the injection. Daily injections of 5HT at 0, 24 and 48 h after PMSG-treatment significantly reduced the number of rats ovulating, although the average number of ova in the rats that did ovulate was not reduced (Table 1). Injections of 5HT at 0 and 24 h, or 48 h only, were ineffective in inhibiting ovulation (Table 1). Since these results do not agree with the findings of O'Steen (1965), groups of rats treated with PMSG were given a single injection of 100 or 50 mg 5HT/kg at different times on the afternoon of day 32. Table 2 shows that 5HT inhibited ovulation when given between 52 and 55 h after the PMSG, i.e. between 16.00 and 19.00 h on day 32.

The effect of different doses of 5HT given 52 h after PMSG treatment (Table 3) shows that all levels inhibited ovulation in a graded dose-response manner, without affecting the average number of ova in the rats which ovulated. Ovarian weights were reduced with the high dose of 5HT but lower doses had no effect. The increase

in uterine weight, seen after treatment with 100 mg 5HT/kg was due to the larger percentage of non-ovulating rats with fluid-filled uteri. Sprague–Dawley rats seemed to be less sensitive to the inhibitory effects of 5HT as only a high dose was effective (Table 3).

Table 1. *Effect of serotonin (5HT, 100 mg/kg) on ovulation in Wistar rats treated with 20 i.u. pregnant mare serum gonadotrophin (PMSG) on day 30 of life (means* ± S.E.M.)

| Treatment with 5HT (h after PMSG injection) | | | Body wt (g) | No. of rats ovulating 72 h after PMSG | No. of ova/ ovulating rat 72 h after PMSG |
|---|---|---|---|---|---|
| 0 | 24 | 48 | | | |
| — | — | — | 62·3 ± 1·7 | 38/44 | 18·3 ± 1·6 |
| 100 | 100 | 100 | 63·0 ± 2·6 | 4/12* | 23·0 ± 5·7 |
| 100 | 100 | — | 60·0 ± 3·5 | 7/8 | 14·8 ± 2·4 |
| — | — | 100 | 65·6 ± 2·1 | 13/18 | 15·1 ± 1·5 |

Significance of difference from PMSG-treated control rats: * $P < 0.01$.

Table 2. *Effect of serotonin (5HT) administered at different times on day 32 on ovulation in Wistar rats treated with 20 i.u. pregnant mare serum gonadotrophin (PMSG) on day 30 of life (means* ± S.E.M.)

| Dose (mg/kg) | Time of 5HT treatment: After PMSG (h) | On day 32 (h) | Body wt (g) | No. of rats ovulating on day 33 | No. of ova/ ovulating rat on day 33 |
|---|---|---|---|---|---|
| — | — | — | 62·3 ± 1·7 | 38/44 | 18·3 ± 1·6 |
| 100 | 48 | 13.00 | 65·6 ± 2·1 | 13/18 | 15·1 ± 1·5 |
| 50 | 50 | 14.00 | 62·4 ± 2·6 | 4/5 | 33·5 ± 3·3 |
| 50 | 51 | 15.00 | 61·0 ± 2·8 | 3/5 | 27·7 ± 7·8 |
| 50 | 52 | 16.00 | 62·6 ± 2·0 | 2/10** | 31·5 ± 9·5 |
| 50 | 53 | 17.00 | 67·2 ± 1·3 | 3/10* | 26·0 ± 6·6 |
| 50 | 55 | 19.00 | 69·0 ± 2·0 | 1/13*** | 65·0 |
| 50 | 57 | 21.00 | 66·7 ± 2·6 | 4/6 | 35·5 ± 11·1 |

Significance of difference from PMSG-treated control rats; * $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$.

### *Reversal of the inhibitory effects of serotonin*

Attempts were made to reverse the inhibitory effects of 5HT with compounds known to antagonize the action of 5HT. Table 4 shows that 0·5 mg BOL 148/kg and 2 mg methysergide/kg by themselves have no effect on the number of rats ovulating, although both significantly increased the mean number of ova/rat. They did not reverse the inhibitory effect of 5HT at these dose levels. Both BOL 148 and methysergide are known to enter the central nervous system (CNS) in significant concentrations at 1 mg/kg and 4 mg/kg respectively (Parrett & West, 1958; Corne, Pickering & Warner, 1963). However, both compounds at the higher dose level inhibited ovulation (Table 4). There was no mutual antagonism between 5HT and the anti-5HT compounds. Table 4 also shows the effects of 0·5 $\mu$g oestradiol/rat and 2 mg progesterone/rat given 48 h after the PMSG treatment. Neither hormone affected the number of rats ovulating when given by itself, and oestradiol did not reverse the inhibitory

effects of 5HT. However, progesterone significantly increased the average number of ova when given alone and reversed the action of 5HT, when given 4 h before the amine.

Table 3. *Effect on ovulation of different doses of serotonin (5HT) given 52 h after pregnant mare serum gonadotrophin (PMSG) in immature female rats (means* ± S.E.M.)

| Dose (mg/kg) | Body wt (g) | Ovarian wt (mg) | Uterine wt (mg) | No. of rats ovulating | No. of ova/ ovulating rat |
|---|---|---|---|---|---|
| | | Wistar | | | |
| 0 | 68·8 ± 3·3 | 51·2 ± 2·7 | 125·6 ± 4·4 | 17/20 | 20·0 ± 2·5 |
| 25 | 61·7 ± 2·7 | 59·3 ± 3·1 | 136·6 ± 4·2 | 7/19*** | 27·1 ± 3·4 |
| 50 | 62·7 ± 1·3 | 57·6 ± 4·6 | 142·4 ± 10·9 | 3/10** | 26·0 ± 6·6 |
| 100 | 63·3 ± 2·7 | 40·1 ± 3·0*** | 155·8 ± 7·0**** | 3/20**** | 15·3 ± 9·0 |
| | | Sprague–Dawley | | | |
| 0 | 67·4 ± 1·3 | 30·9 ± 2·1 | 117·0 ± 5·0 | 12/17 | 13·3 ± 1·5 |
| 50 | 61·1 ± 0·8 | 40·4 ± 7·7 | 140·6 ± 11·1 | 5/7 | 12·2 ± 0·9 |
| 100 | 64·8 ± 1·7 | 28·5 ± 1·7 | 157·1 ± 8·8**** | 2/8* | 11·5 |

Wistar rats received 20 i.u. PMSG at 30 days of age; autopsy at day 33. Sprague–Dawley rats received 5 i.u. PMSG at 27 days of age; autopsy at day 30.

Significance of difference from PMSG-treated control rats: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$; **** $P < 0.001$.

Table 4. *Effects of serotonin (5HT), methysergide and BOL 148 and ovarian steroids on ovulation in Wistar rats treated with 20 i.u. pregnant mare serum gonadotrophin (PMSG) on day 30 of life (means* ± S.E.M.)

| Treatment on day 32 | Dose (mg/kg) | Body wt (g) | No. of rats ovulating on day 33 | No. of ova/ ovulating rat on day 33 |
|---|---|---|---|---|
| — | — | 62·3 ± 1·7 | 38/44 | 18·3 ± 1·6 |
| 5HT | 50 | 61·7 ± 2·7 | 3/10 | 26·0 ± 6·6 |
| Methysergide | 2 | 60·0 ± 1·8 | 9/12 | 34·5 ± 6·7* |
| 5HT + methysergide | 50 + 2 | 64·0 ± 2·1 | 4/13 | 27·2 ± 6·4 |
| Methysergide | 4 | 62·8 ± 3·9 | 1/6 | 39·0 |
| 5HT + methysergide | 50 + 4 | 67·7 ± 5·6 | 2/6 | 34·0 |
| BOL 148 | 0·5 | 70·2 ± 2·3 | 4/6 | 42·0 ± 5·2**** |
| 5HT + BOL 148 | 50 + 0·5 | 69·3 ± 3·3 | 0/6 | — |
| BOL 148 | 1 | 76·7 ± 2·2 | 1/4 | 23·0 |
| 5HT + BOL 148 | 50 + 1 | 67·6 ± 3·3 | 2/5 | 29·0 |
| Oestradiol | 0·075 | 66·2 ± 4·4 | 6/9 | 29·5 ± 6·0 |
| 5HT + oestradiol | 50 + 0·075 | 62·0 ± 2·8 | 2/6 | 27·0 ± 9·0 |
| Progesterone | 30 | 62·3 ± 3·2 | 11/11 | 31·7 ± 5·3** |
| 5HT + progesterone | 50 + 30 | 65·0 ± 2·4 | 7/7*** | 29·4 ± 6·6 |

Methysergide and BOL 148 given 51·5 h after PMSG. Oestradiol and progesterone given 48 h after PMSG. 5HT given 52 h after PMSG.

Significance of difference from PMSG-treated control rats: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$.

*Investigation of the site of action of serotonin*

Treatment with 30 i.u. PMSG followed 72 h later by 10 i.u. HCG induced 100% ovulation in rats hypophysectomized on day 27 (Table 5). When 5HT (100 mg/kg)

was given at the same time as the HCG there was a significant reduction in both the number of rats ovulating and in the number of ova shed.

Administration of 5HT (1 mg/kg) into the lateral ventricle of intact rats had no inhibitory effect on induced ovulation either 49 or 53 h after the PMSG treatment i.e. at 13.00 and 17.00 h on day 32. However, 5HT administered at 13.00 h significantly increased the average number of ova (Table 6). The condition of the rats after intraventricular injection was normal and no deaths occurred.

Table 5. *Effect of serotonin (100 mg/kg) on ovulation in hypophysectomized Wistar rats treated with 30 i.u. pregnant mare serum gonadotrophin (PMSG) and 10 i.u. human chorionic gonadotrophin (HCG) (means* ± S.E.M.)

| Treatment | Body wt (g) | Ovarian wt (mg) | Uterine wt (mg) | No. of rats ovulating | No. of ova/ ovulating rat |
|---|---|---|---|---|---|
| PMSG + HCG | 52·4 ± 2·8 | 58·0 ± 5·2 | 83·1 ± 4·8 | 18/18 | 18·8 ± 2·6 |
| PMSG + HCG + 5HT | 68·6 ± 2·6 | 33·3 ± 4·1 | 76·7 ± 11·7 | 5/9* | 7·6 ± 1·8** |

HCG and 5HT were given 72 h after PMSG.
Significant difference from PMSG + HCG-treated control rats: * $P < 0·02$; ** $P < 0·01$.

Table 6. *Effect of intraventricular administration of serotonin (5HT) on ovulation in Wistar rats treated with 20 i.u. pregnant mare serum gonadotrophin (PMSG) on day 30 of life (means* ± S.E.M.)

| Treatment on day 32 | Time of 5HT administration on day 32 (h) | Body wt (g) | No. of rats ovulating on day 33 | No. of ova/ ovulating rat on day 33 |
|---|---|---|---|---|
| 0·9 % NaCl solution (0·02 ml) | 13.00 | 62·2 ± 1·3 | 6/6 | 14·5 ± 5·4 |
| 5HT (1 mg/kg) | 13.00 | 69·1 ± 3·6 | 6/6 | 30·3 ± 4·3* |
| 5HT (1 mg/kg) | 17.00 | 84·5 ± 2·0 | 9/11 | 28·4 ± 6·9 |

Significant difference from saline-treated control rats: * $P < 0·05$.

*Effect of serotonin on pituitary and plasma levels of LH*

In immature Wistar rats treated with 20 i.u. PMSG on day 30, a significant reduction in pituitary LH content occurs between 14.00 and 16.00 h on the afternoon of day 32 (Wilson *et al.* 1974). When 5HT was given at 17.00 h on day 32 pituitary LH levels rose and were not significantly different from the control values at 14.00 h and were significantly higher than control values at 12.00 h on day 33 (Table 7). After PMSG treatment plasma LH levels increase significantly between 16.00 and 20.00 h on the day before ovulation (Wilson *et al.* 1974). Table 7 shows the results obtained from control and 5HT-treated Sprague–Dawley rats after 5 i.u. PMSG on day 27. While PMSG alone induced a normal pre-ovulatory surge of LH, injection of 5HT at 17.00 h completely abolished the response (see Fig. 1).

*Effect of serotonin on plasma progesterone levels*

Table 7 shows the results obtained after treatment of Wistar rats with 20 i.u. PMSG on day 30. Levels of plasma progesterone rose after 18.00 h and reached a

maximum at 22.00 h. When 5HT was given at 17.00 h progesterone levels increased until 20.00 h but were significantly lower than those of the control rats at 22.00 h. By 24.00 h the effects of the 5HT had declined and there was no significant difference between the control and 5HT-treated animals.

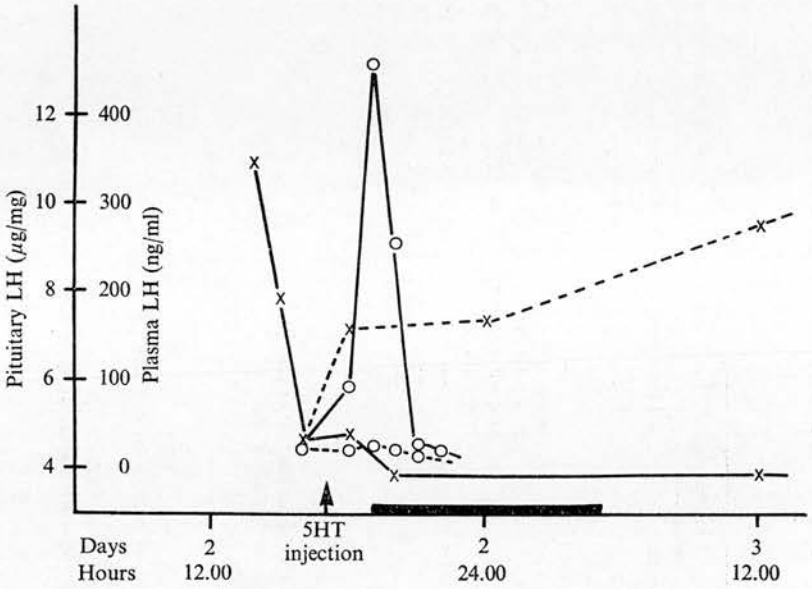



Fig. 1. Effect of 100 mg serotonin (5HT)/kg, given s.c., on pituitary and plasma LH levels in immature rats on the 2nd and 3rd day after PMSG treatment (days after PMSG indicated on the abscissa); 5HT was given at 17.00 h on the 2nd day. ×—×, Control pituitary LH; ×— —×, 5 HT-treated pituitary LH; ○——○, control plasma LH; ○----○, 5HT-treated plasma LH.

## DISCUSSION

Although previously the high dose of 100 mg 5HT/kg was found to be toxic in adult rats, in these experiments the same dose did not cause any deaths or appearances of ill-health in the immature rats, even when given daily for 3 days. The results, therefore, are unlikely to be due to non-specific toxicity, but rather due to a particular action of 5HT.

The present experiments show that 5HT inhibits ovulation when given daily for 3 days to immature rats treated with PMSG, confirming the results of O'Steen (1964, 1965). Strauss & Meyer (1962) have shown that the 'critical period' for ovulating hormone release in immature rats kept under our lighting system occurs between 14.00 and 16.00 h on the day before expected ovulation. Single injections of 5HT at different times on the afternoon of this day (i.e. day 32) showed that ovulation was inhibited only when the drug was given between 16.00 and 19.00 h; that is in the 3 h period after the 'critical period'. This is in agreement with our findings in adult cyclic rats (Wilson & McDonald, 1974). It seems likely that after systemic administration the site of the anti-ovulatory action of 5HT is a peripheral one since it significantly reduced the incidence of ovulation in hypophysectomized rats treated with PMSG and HCG. In addition no anti-ovulatory effect was seen when 5HT was

Table 7. *Effect of serotonin (5HT, 100 mg/kg) on pituitary and plasma LH and plasma progesterone in immature rats treated with pregnant mare serum gonadotrophin (PMSG) (means ± S.E.M.)*

| Time of sampling | | Luteinizing hormone | | | | | |
|---|---|---|---|---|---|---|---|
| | | Pituitary (μg/mg)[a]‡ | | Plasma (ng/ml)[b]§ | | Plasma progesterone (ng/ml)[a] | |
| Time after PMSG treatment (h) | Time of day, 2 days after PMSG treatment | Control | 5HT treated | Control | 5HT treated | Control | 5HT treated |
| 50 | 14.00 | 10·9 ± 3·3 (5) | — | — | — | 9·1 (6) | — |
| 51 | 15.00 | 7·9 ± 1·1 (5) | — | — | — | — | — |
| 52 | 16.00 | 4·6 ± 0·4 (7)** | — | 23·0 ± 6·4 (4) | — | 12·6 (6) | — |
| 54 | 18.00 | 4·7 ± 0·6 (4)* | 7·2 ± 1·6 (6) | 93·2 ± 37·2 (5) | 23·8 ± 11·4 (3) | 14·7 (6) | 12·5 (6) |
| 55 | 19.00 | — | — | 462·0 (1) | 27·8 ± 5·2 (3) | — | — |
| 56 | 20.00 | 3·9 ± 0·5 (4)* | — | 255·0 ± 66·0 (6) | 24·6 ± 3·5 (3)*** | 31·4 (12) | 29·6 (6) |
| 57 | 21.00 | — | — | 31·0 (2) | 17·9 ± 4·6 (3) | — | — |
| 58 | 22.00 | — | — | 23·7 ± 11·6 (3) | — | 36·3 ± 8·9 (12) | 16·9 ± 3·4 (12)**** |
| 60 | 24.00 | — | 7·4 ± 1·3 (6)† | — | — | 27·3 ± 4·4 (12) | 25·9 ± 5·7 (12) |
| 72 | 12.00 (3 days after PMSG) | 3·9 ± 0·9 (9)* | 9·5 ± 2·7 (13)† | — | — | — | — |

Number of samples shown in parentheses.
[a] Pituitary and plasma samples taken from Wistar rats treated with 20 i.u. PMSG on day 30 of life.
[b] Plasma samples taken from Sprague–Dawley rats treated with 5 i.u. PMSG on day 27 of life.
5HT treatment at 53 h after PMSG, i.e. 17.00 h 2 days after PMSG treatment.
‡ LH expressed in terms of NIH-LH B3.
§ LH expressed in terms of NIH-LH S1.
Significant difference from pituitary LH in control rats at 14.00 h: * $P < 0.05$, ** $P < 0.02$.
Significant difference from pituitary LH in control rats at 20.00 h on day 32 and 12.00 h on day 33: † $P < 0.05$.
Significant difference from plasma LH in control rats at 20.00 h: *** $P < 0.01$.
Significant difference from plasma progesterone in control rats at 22.00 h: **** $P < 0.05$.
Pooled plasma LH values from treated and control rats between 18.00 and 20.00 h are significantly different $P < 0.01$.

administered centrally via the lateral ventricle. Similar results have also been found after intraventricular injection in adult pro-oestrous rats (Wilson & McDonald, 1974). In the immature rat, administration of 5HT into the lateral ventricle just before the 'critical period' resulted in a significant increase in the mean number of ova shed. This suggests that 5HT could act on the brain to facilitate gonadotrophin release.

The results obtained after the administration of antagonists to 5HT are difficult to interpret. Neither compound was successful in blocking the action of 5HT. At the lower dose, both compounds significantly increased the mean number of ova shed. Both antagonists are known to enter the brain at the higher doses used (Parrett & West, 1958; Corne *et al.* 1963), and at this dose level ovulation was blocked. This may be a further indication that at the level of the central nervous system 5HT in fact has a facilitatory action. Similar results with methysergide have been obtained in immature mice treated with PMSG (Brown, 1967).

In both adult cyclic rats and immature rats treated with PMSG, there is a rise in plasma progesterone levels coincident with the pre-ovulatory surge of LH on the afternoon preceding ovulation (Barraclough *et al.* 1971; Wilson *et al.* 1974). At this time the inhibitory effects of 5HT may be reversed by the administration of progesterone, but not oestradiol, in both adult rats (Wilson & McDonald, 1974) and immature rats treated with PMSG. Estimation of plasma progesterone levels after 5HT treatment at 17.00 h on the day before ovulation showed a transitory but significant depression in levels at 22.00 h compared with control animals. In addition, 5HT treatment prevented the fall in pituitary LH content which occurs after 16.00 h and inhibited the rise in plasma LH normally seen between 18.00–20.00 h.

Progesterone is known to exert a facilitatory effect on pituitary gonadotrophin release in immature rats treated with PMSG (McCormack & Meyer, 1963; Zarrow & Hurlbut, 1967). It also advances the timing of the LH surge on the day before ovulation (Ying & Meyer, 1973). Similar facilitatory actions of progesterone have also been found in adult rats (Everett, 1948; Zeilmaker, 1966; McDonald & Gilmore, 1969). Brown-Grant (1969) has suggested that the pre-ovulatory release of progesterone may act as a 'fail-safe' mechanism in stimulating LH release via a positive feedback mechanism. Recently Kobayashi, Hara & Miyake (1973) have shown that in pro-oestrous rats treated with sodium pentobarbitone, progesterone administered after the critical period significantly increases the incidence of ovulation.

There is, therefore, much evidence indicating that progesterone may be involved in stimulating the LH surge necessary for ovulation at the hypothalamic level. It is possible that the anti-ovulatory action of 5HT late on the afternoon of pro-oestrus is due to an inhibition of the endogenous progesterone surge that normally occurs on the late afternoon of pro-oestrus as (1) the effect of 5HT can be reversed by administration of progesterone and (2) the levels are transitorily reduced by 5HT treatment. Thus when the progesterone action is prevented there is no release of pituitary LH into the plasma. Since we have shown that the antiovulatory action of 5HT is probably a peripheral one, we suggest that the site of action is at the ovarian level (and possibly the adrenal level too) inhibiting either the secretion of progesterone or its passage from the ovary to its target site in the central nervous system. This may well be due to a vasoconstrictor action of 5HT on the ovarian (and adrenal) blood vessels in a similar manner to that already suggested in the adult rat (Wilson & McDonald, 1974).

The findings of both this report and the previous one on adult rats show that the anti-ovulatory action of systemic 5HT is a pharmacological one and is probably due to peripheral vasoconstriction; thus any action 5HT may have at the hypothalamic level is masked by this peripheral effect. In addition, using 5HT as a pharmacological tool these experiments have revealed the physiological importance of progesterone in stimulating the LH surge before induced ovulation.

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# STEROIDAL CONTROL OF THE RELEASE OF THE PREOVULATORY SURGE OF LUTEINIZING HORMONE IN THE RAT

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SUMMARY

Experiments were carried out on 4 day cyclic rats or immature rats induced to ovulate by administration of pregnant mare serum gonadotrophin. Removal of the ovaries and adrenal glands at 17.00 h of pro-oestrus, i.e. after the critical period, prevented the appearance of the surge of LH. Sham-operation or removal of only one of the sets of glands had no effect. This indicates that the preovulatory increase in the concentration of oestradiol is not solely responsible for the surge of LH; the presence of a steroid, secreted by the ovaries and adrenal glands in the late afternoon of pro-oestrus, is also required.

Attempts were made to reinstate the surge of LH in ovariectomized, adrenalectomized rats by administration of one of the steroids normally secreted in late pro-oestrus. Corticosterone, 20α- and 20β-hydroxy-4-pregnen-3-one and 17α-hydroxyprogesterone all had no effect. Progesterone injected at the time of the operation stimulated the release of LH but only after the plasma concentration had reached its maximum 3–5 h after injection. Testosterone also stimulated the release of LH some hours after administration.

## INTRODUCTION

During the rat oestrous cycle, the increase in the concentration of oestradiol seen at di-oestrus and pro-oestrus is essential for the occurrence of the preovulatory surge of luteinizing hormone (LH) observed in the later afternoon of pro-oestrus (Neill, Freeman & Tillson, 1971; Tapper, Naftolin & Brown-Grant, 1972). At the same time as the surge of LH there are increases in plasma concentrations of several steroids, including progesterone, testosterone, androst-4-ene-3,17-dione (androstenedione), 17α-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one and corticosterone. Such increases have been observed in both adult cyclic rats and immature rats induced to ovulate with pregnant mare serum gonadotrophin (PMSG; Barraclough, Collu, Massa & Martini, 1971; Raps, Barthe & Desaulles, 1971; Dupon & Kim, 1973; Horikoshi & Suzuki, 1974; Shaikh & Shaikh, 1975; Parker, Costoff, Muldoon & Mahesh, 1976).

The physiological importance of these steroids is not clear, although it has been shown that progesterone, testosterone and 20α-hydroxy-4-pregnen-3-one all stimulate the release of LH in either oestrogen-primed ovariectomized rats or PMSG-treated immature rats (McCormack & Meyer, 1963; Caligaris, Astrada & Taleisnik, 1971; Swerdloff, Jacobs &

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Odell, 1972; Brown-Grant, 1974). The preovulatory surge of progesterone has been investigated most fully and it is suggested that it synergizes with the earlier preovulatory increase in the concentration of oestrogen and thus enhances the surge of LH (Kalra, Fawcett, Krulich & McCann, 1973; Kobayashi, Hara & Miyake, 1973; Krey, Tyrey & Everett, 1973; Mann & Barraclough, 1973; Tapper, Greig & Brown-Grant, 1974). However, progesterone is not considered to be essential for ovulation because Ferin, Tempone, Zimmering & van de Weile (1969) have reported that antiserum to progesterone does not inhibit ovulation (although in their report, the antiserum in fact prevented ovulation in eight out of 20 rats).

It has been reported previously that serotonin inhibits ovulation when given subcutaneously after the critical period at 17.00 h of pro-oestrus in both cyclic adults and immature rats treated with PMSG (Wilson & McDonald, 1974; Wilson, Horth, McNeilly & McDonald, 1975). This effect was due to peripheral vasoconstriction, and since the anti-ovulatory effect could be reversed by concomitant administration of progesterone but not oestrogen, it was suggested that serotonin prevented the passage of progesterone from the ovaries and adrenal glands to the brain so that further secretion of LH was not stimulated. Perhaps the oestrogen secreted earlier in the day was not sufficient to stimulate the release of enough LH for ovulation so that the synergistic action of progesterone was essential. Any of the other steroids that are secreted in the later afternoon of pro-oestrus may also be involved in this positive feedback effect.

The experiments reported here investigate this possibility using progesterone as the representative steroid. All sources of endogenous progesterone were removed on the evening of pro-oestrus, after the critical period (so that the oestradiol could exert its full effect) but before the appearance of the surge of LH in the plasma. The effect of this procedure on the release of LH was noted.

## METHODS

For the experiments with immature animals, Sprague–Dawley rats (Tuck & Son, Essex) were obtained at 22 days of age and exposed to a lighting schedule of 14 h light : 10 h darkness (lights on 5.00–19.00 h). On day 27, all rats which weighed over 60 g were injected subcutaneously with 5 i.u. PMSG (Folligon; Intervet Ltd) which induces ovulation in 85% of animals. On day 29, the day of the expected surge of LH, the rats were sham-operated, ovariectomized, adrenalectomized or both ovariectomized and adrenalectomized between 16.30 and 17.00 h. The operations were carried out under ether anaesthesia and when both adrenalectomy and ovariectomy were performed, the adrenal glands were removed first. Groups of five to eight rats were then killed by decapitation at 1 h intervals after the operation until 22.00 or 23.00 h on day 29 or 01.00 h on day 30. In one experiment blood was collected only at 18.00 h. Blood was collected from a cervical cut into polycarbonate tubes and centrifuged for 10 min at 400 *g* and 4 °C. The plasma was stored at −20 °C until assayed for LH and progesterone.

The concentration of LH was determined by double-antibody radioimmunoassay using reagents from the National Institutes of Health, Bethesda, U.S.A. The LH standard was LH-RP-1 and the antiserum was S1. The sensitivity of the assay was 4 ng/ml. The intra- and interassay coefficients of variance were 8 and 15% respectively. Some samples were assayed for LH according to the method of Naftolin & Corker (1971) using LH standard LER-1056-$C_2$ (LH potency 1·73 NIH-S1 units/mg) provided by Dr L. Reichert and No.15 anti-ovine LH serum provided by Dr G. Niswender. The sensitivity of the assay was 20 ng LER-1056-$C_2$ and the intra- and interassay coefficients of variance were 11 and 16% respectively. Progesterone was assayed by the method of Brenner, Guerrero, Cekan & Diczfalusy (1973). The sensitivity of the assay was 0·1 ng and the intra- and interassay coefficients of variance were 5 and 8% respectively.

For the experiment with adult animals, Sprague–Dawley rats weighing 200–250 g were kept in the same lighting system as described for the immature animals and vaginal smears were taken daily. Only those rats having at least two regular 4 day oestrous cycles were used in the experiments. Groups of four to six rats were either sham-operated or adrenalectomized and ovariectomized while under ether anaesthesia between 16.30 and 17.00 h on the day of pro-oestrus. Groups were then killed at 1 h intervals from the time of the operation until 21.00 h and the blood was collected and treated as above and the plasma assayed for LH and progesterone.

When steroids were injected into immature PMSG-treated rats, they were given subcutaneously dissolved in 0·2 ml corn oil (except corticosterone which was suspended in corn oil) at 13.00 or 17.00 h on day 29 or, for oestradiol benzoate at 17.00 h on day 28. All of the steroids were obtained from Sigma Chemical Co. Ltd.

In the experiments investigating the plasma concentration of progesterone after a subcutaneous injection, 3 μg [1,2,6,7-$^3$H]progesterone (250 Ci/g; Radiochemical Centre, Amersham) in 0·3 ml benzene were added to 15 mg unlabelled progesterone in 1·5 ml corn oil. The final concentration was, therefore, 750 μCi and 15 mg progesterone in 1·8 ml oil + benzene. Six Sprague–Dawley rats weighing 120–125 g were ovariectomized and adrenalectomized and three animals were left intact. Each rat received 1·7 mg (83 μCi) progesterone s.c. in 0·2 ml vehicle at 09.30 h, and then at 1 h intervals, for 7 h approximately, 0·25 ml blood was collected from the tail vein into 50 μl capillary tubes. The tubes were blocked at one end and centrifuged for 3 min at 400 *g*. The tube was then cut at the plasma/cell interface and the plasma blown into a 15 ml bottle and stored at −20 °C until extracted.

Progesterone was extracted from plasma by whirling for 45 s with 5 ml petroleum ether (boiling point 40–60 °C; A. R. BDH). The aqueous phase was then frozen at −20 °C and the petroleum ether layer was poured into a scintillation vial. The procedure was carried out twice so that the final volume of petroleum ether was 10 ml, which was evaporated under a stream of nitrogen at 45–50 °C. Scintillating fluid [2,5 diphenyl oxale (0·5%) and 1,4-bis-(4 methyl-5-phenyl oxazyl-2-yl) benzene (0·025%) dissolved in toluene] (12 ml) and ethanol (6 ml) were added to the vial and the radioactivity was counted in a Packard (Tricarb Model 3003) liquid scintillation spectrometer. Quenching was corrected for by the external standard method and all radioactivity was corrected for efficiency by a computer program (Felts & Mayes, 1967).

The effects of the operation on hormone concentration were analysed statistically by one-way analysis of variance and if the $F$ value was significant, comparisons between groups were made with Duncan's multiple range test. If the variances were too large, the Mann–Whitney U test was used. The effects of injected steroids on the concentrations of LH were assessed by Student's $t$-test and Fisher's contingency tables.

## RESULTS

### *Effects of ovariectomy and/or adrenalectomy on immature rats treated with PMSG*

Figure 1*a* shows that intact immature rats treated with 5 i.u. PMSG on day 27 of life had surges of LH and progesterone on day 29 (equivalent to the day of pro-oestrus) with peak concentrations of both hormones at 19.00 h. When similar rats were sham-operated under ether anaesthesia the surge of LH occurred at the same time as in the unoperated rats and the rise in progesterone was advanced by at least 2 h (Fig. 1*b*). In rats that were ovariectomized between 16.30 and 17.00 h, the surge of LH occurred as usual and the rise in progesterone was advanced by 2 h as seen in the sham-operated rats (Fig. 1*c*). In adrenalectomized rats, both surges occurred at 20.00 h. The surge of progesterone was much reduced compared with the other groups, but the concentration at 20.00 h was significantly higher ($P < 0·05$) than at 18.00 h (Fig. 1*d*). When animals were both ovariectomized and

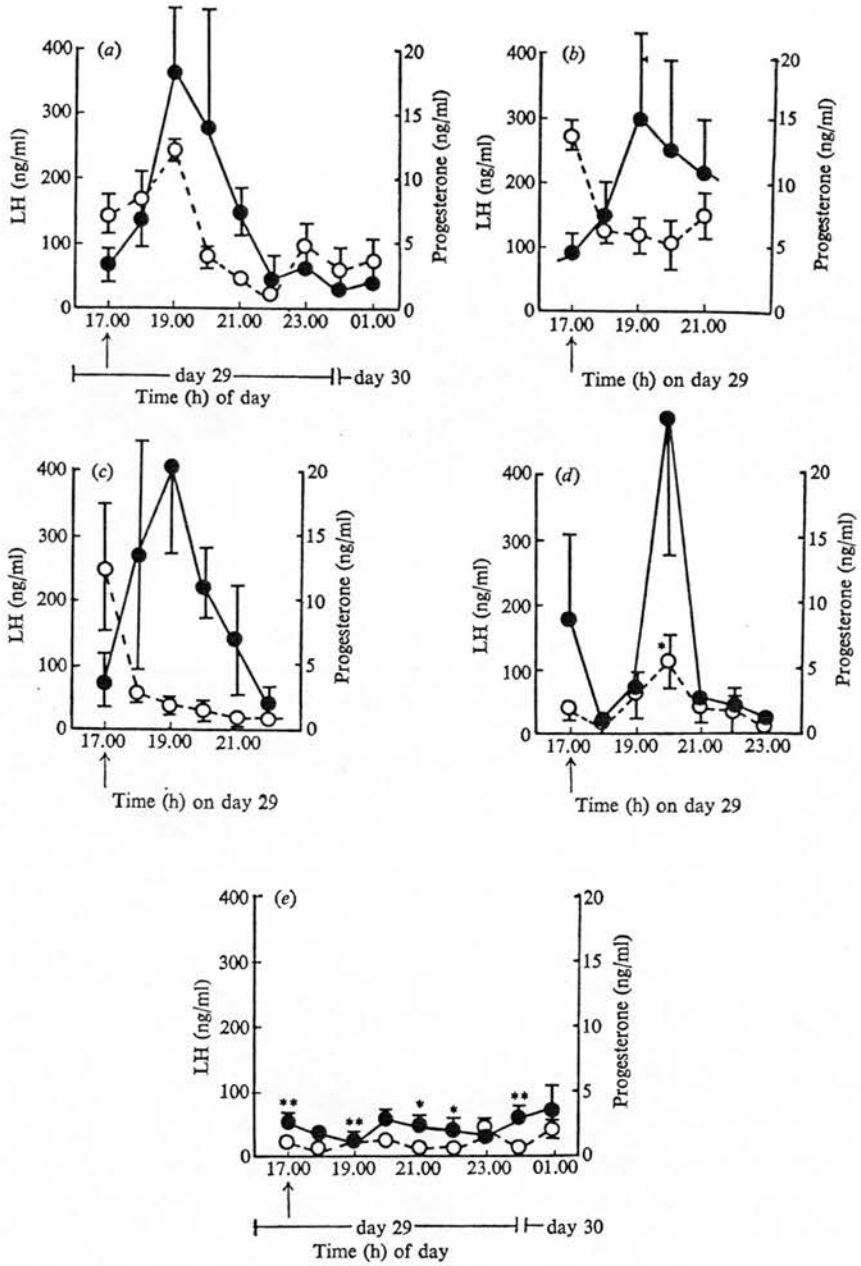



Fig. 1. Variations in the plasma concentrations of LH (●) and progesterone (○) in (*a*) intact controls, (*b*) sham-operated, (*c*) ovariectomized, (*d*) adrenalectomized, (*e*) ovariectomized and adrenalectomized rats on day 29 of life after treatment with 5 i.u. pregnant mare serum gonadotrophin on day 27. Operations were carried out at 16.30–17.00 h on day 29 (arrows). Each point is the mean of five to eight samples taken from individual rats. The vertical lines indicate ± S.E.M. Where standard errors are not shown, they are smaller than the height of the symbols. The LH concentration is expressed in terms of NIH-LH-S1. $*P<0{\cdot}05$; $**P<0{\cdot}01$: in figure (*d*) difference from progesterone concentration at 18.00 h is calculated by Student's *t*-test. In figure (*e*) LH concentrations in the ovariectomized and adrenalectomized rats are compared with the maximum concentration in the sham-operated group using Duncan's multiple range test. Progesterone concentrations were similarly compared and were all significantly different at $P<0{\cdot}01$.

adrenalectomized, the concentrations of both LH and progesterone remained at low basal concentrations throughout the period between the operation and 01.00 h on the day of oestrus (Fig. 1*e*). Analysis of variance showed that these low values were significantly different from the concentrations seen in sham-operated rats (LH: $P<0{\cdot}05$, $F=2{\cdot}48$ (9, 50); progesterone: $P<0{\cdot}01$, $F=46{\cdot}7$ (5, 21)).

*Effect of ovariectomy and adrenalectomy on adult cyclic rats*

The effect of removal of the ovaries and adrenal glands from adult rats showing regular 4 day cycles was observed (see Fig. 2). Operations were performed between 16.00 and 17.00 h on the day of pro-oestrus. After sham-operation the surges of LH and progesterone reached their peaks at 18.00 h. After ovariectomy and adrenalectomy, the plasma concentrations of both hormones remained low throughout the period of sampling. The difference between these patterns of plasma concentrations was significant (LH: $P<0{\cdot}01$, $F=7{\cdot}2$ (5, 27)).

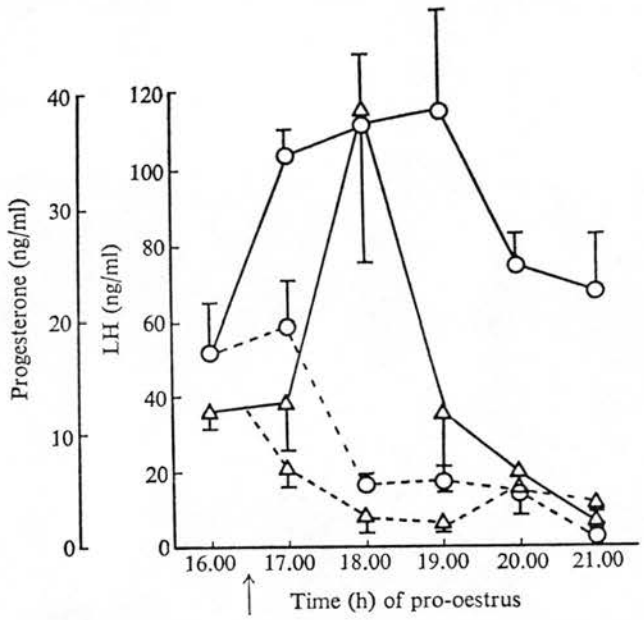



Fig. 2. Variations in the concentrations of LH (△) and progesterone (○) in the plasma of adult cyclic rats on the day of pro-oestrus after sham-operation (solid lines) or ovariectomy and adrenalectomy (broken lines) at 16.30–17.00 h (arrow). Each point is the mean of four to six samples from individual rats. Vertical lines represent ±S.E.M. The concentration of LH is expressed in terms of LER 1056-$C_2$ (LH potency of 1·73 NIH-S1 units/mg). All the LH and progesterone concentrations in the ovariectomized and adrenalectomized rats were significantly different ($P<0{\cdot}01$) from the maximum concentration in the sham-operated group as calculated by Duncan's multiple range test.

*Effect of administration of progesterone to rats ovariectomized and adrenalectomized just before the expected surge of LH*

These experiments were carried out in immature rats treated with 5 i.u. PMSG on day 27 and ovariectomized and adrenalectomized between 16.30 and 17.00 h on day 29. Control groups of sham-operated and ovariectomized/adrenalectomized animals injected with oil showed that the surge of LH occurred normally in the former group and was significantly

($P<0{\cdot}05$) reduced in the latter group. This is similar to the changes shown in Figs 1*b* and *e* and, therefore, the detailed results are not given. When the rats received 2 mg progesterone subcutaneously at the time of the operation, the plasma concentration of progesterone was higher than the concentration normally noted during the surge in intact rats (i.e. 30 ng/ml). From the time of the injection, the values rose until they reached a peak 5 h later (at 22.00 h). The concentrations of LH started to rise approximately 4 h after the injection and reached a peak 1 h after the peak concentration of progesterone at 23.00 h, so that a surge of LH occurred 6 h after injection of progesterone (see Fig. 3). Another

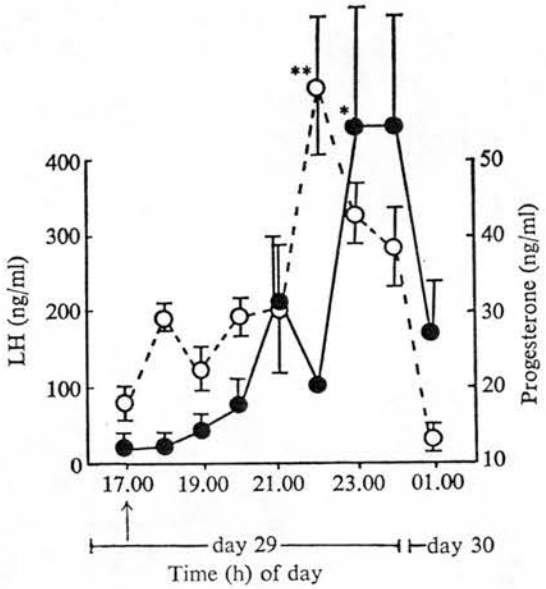



Fig. 3. Variations in the concentrations of LH (●) and progesterone (○) in the plasma of immature rats on day 29 after treatment with 5 i.u. pregnant mare serum gonadotrophin on day 27 and ovariectomy and adrenalectomy and s.c. injection of 2 mg progesterone at 16.30–17.00 h on day 29 (arrow). Each point represents the mean of six or eight samples taken from individual rats and the vertical lines represent ±S.E.M. LH concentration is expressed in terms of NIH-LH-S1. $*P<0{\cdot}02$; $**P<0{\cdot}01$: peak concentrations of LH and progesterone compared (by Student's *t*-test) with concentrations at 17.00 h.

group of ovariectomized and adrenalectomized rats was given 0·5 mg progesterone at 13.00 h, i.e. 3·5–4·5 h before the operation and 6 h before the normal time of the LH surge. In these rats, the concentration of progesterone rose to a high value in 3 h and at this time a surge of LH occurred with a peak between 16.00 and 17.00 h, i.e. 3–4 h after injection of progesterone (see Fig. 4).

### *Effect of various steroids on release of LH in rats ovariectomized and adrenalectomized just before the expected surge of LH*

The experiments were carried out on immature PMSG-treated rats and blood was only collected at 18.00 h, i.e. near the expected time of the LH surge. Table 1 shows that in sham-operated rats there were high concentrations of LH in ten out of 21 animals, with a mean plasma concentration of about 26 ng/ml. This was significantly ($P<0{\cdot}05$) higher than the values found at 18.00 h in the ovariectomized and adrenalectomized animals where only one out of 26 rats had a raised plasma concentration of LH. Adrenalectomy

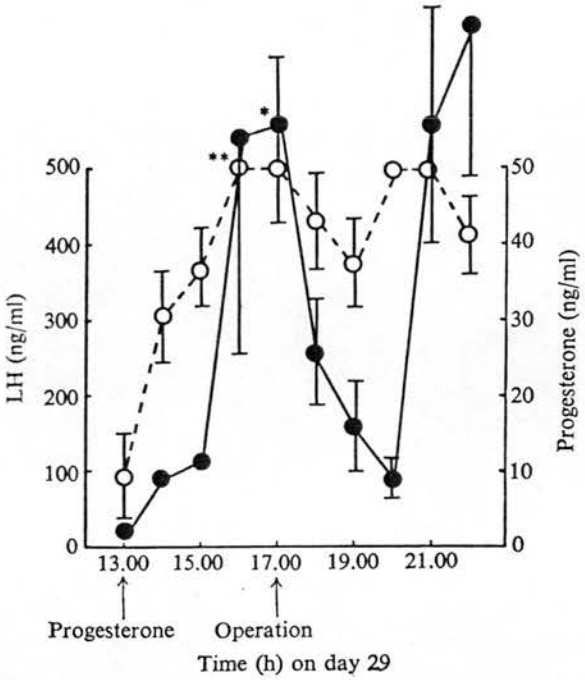



Fig. 4. Variations in the concentrations of LH (●) and progesterone (○) in the plasma of immature rats on day 29 after treatment with 5 i.u. pregnant mare serum gonadotrophin on day 27 and s.c. injection of 0·5 mg progesterone at 13.00 h and ovariectomy and adrenalectomy at 16.30–17.00 h on day 29. Each point is the mean of four or five samples taken from individual rats, the vertical lines represent ±S.E.M. LH concentration is expressed in terms of NIH-LH-S1. $*P<0{\cdot}02$; $**P<0{\cdot}001$: peak LH and progesterone concentrations compared with concentrations at 13.00 h by the Mann–Whitney test and Student's *t*-test respectively.

and ovariectomy carried out on the morning of day 29, at 10.30 h, also prevented the surge of LH.

When the various steroids (0·5 mg/rat; see Table 1) were injected subcutaneously at the time of the operation (17.00 h on day 29), none of them induced a surge of LH at 18.00 h and although in some animals the mean plasma concentration of LH was higher than in the oil-treated rats, the differences were never significant. However, when the steroids were given 4 h before the operation, at 13.00 h, then progesterone as noted previously induced a rise in the concentration of LH at 18.00 h and testosterone produced a similar response. The other steroids, i.e. 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, 17α-hydroxyprogesterone and corticosterone had no significant effect. Oestradiol (1 μg/rat) induced a surge of LH when given on the afternoon of day 28.

*Plasma progesterone concentrations after a subcutaneous injection of labelled progesterone*

Labelled progesterone (1·7 mg) was injected subcutaneously into intact or ovariectomized adrenalectomized rats and the radioactivity present in the plasma was noted at 1 h intervals. The changes seen in the two groups of rats were very similar, so that the results have been combined. The means are shown in Fig. 5, from which it can be seen that the amount of radioactivity in the plasma reached a peak 3 h after injection, before decreasing slowly. The occurrence of the peak concentration varied in individual rats between 3 and 5 h

## DISCUSSION

In the present paper the effect of removal of the ovary and/or adrenal glands in the late afternoon of pro-oestrus on the preovulatory surge of LH has been described. The study was carried out in immature rats induced to ovulate with PMSG and in adult cyclic rats. Removal of both sets of glands affected the immature and adult rats similarly and so most of the experiments were carried out on the more convenient immature model which was assumed to be representative of the adult cyclic rat.

When the ovaries alone were removed, the steroids secreted by the adrenal glands would still be present and in ovariectomized animals, the surges of both progesterone and LH still occurred. It is known that ovariectomy at any time after 03.00 h on the day of pro-oestrus has no effect on the surge of LH (Kalra, 1975). The stress of the operation probably stimulates adrenal secretion of progesterone (Nequin & Schwartz, 1971; Lawton, 1972; Campbell, Schwartz & Firlit, 1977) as the plasma concentration of progesterone was raised immediately after the operation, 2 h earlier than usual. This effect was also seen in the sham-operated rats.

When the rats were adrenalectomized, the concentration of progesterone was much reduced, although a significant increase did occur. Perhaps most of the progesterone normally secreted in intact rats is of adrenal origin, but it is also possible that the operation reduced ovarian secretion. The concentrations of both progesterone and LH rose to a peak 1 h later than usual. This delaying effect of adrenalectomy on the surge of LH has been noted previously (Kobayashi, 1974; Mann, Korowitz & Barraclough, 1975) and suggests that the adrenal gland synchronizes the surge of LH to a particular lighting schedule. If the adrenal glands are absent or the adrenal corticosterone rhythm irregular, then the oestrous cycles are also irregular (Feder, Brown-Grant & Corker, 1971; Ramaley, 1975).

The surge of LH occurred when either the adrenal glands or the ovaries were present on the late afternoon of pro-oestrus, but when both were removed, it did not. The stress of the double operation may have inhibited the surge, but this is unlikely as it still occurred after the stress of the sham-operation or removal of one of the pairs of glands.

Ovariectomy and adrenalectomy prevented the surge of LH despite the presence of endogenous preovulatory oestradiol. This suggests that in the oestrous cycle, the normal level of oestradiol secretion is not sufficient to stimulate the release of LH by itself and that a second hormone is required to synergize with it. Removal of both the ovaries and the adrenal glands presumably reduced the concentrations of all steroids in the plasma to negligible values. When some of the possible contenders for a synergistic action with oestradiol were injected into ovariectomized and adrenalectomized rats, only progesterone and testosterone induced a surge of LH. 20α-Hydroxy-4-pregnen-3-one was not effective although previous work carried out on oestrogen-primed ovariectomized rats suggested that it would be (Swerdloff *et al.* 1972; Brown-Grant, 1974).

Similar results have been obtained in rats ovariectomized and adrenalectomized on the day before pro-oestrus and given a small replacement dose of oestrogen (1 μg oestradiol benzoate/rat) at the time of the operation. In these rats the surge of LH did not occur unless either progesterone was injected as well or, alternatively, a higher dose of oestrogen (5–20 μg/rat) was given (Kalra *et al.* 1973; Mann & Barraclough, 1973; Tapper *et al.* 1974). Thus when oestrogen is present in high concentrations it can act alone to stimulate the release of LH in both acutely and chronically ovariectomized and adrenalectomized rats (Mann, Korowitz, Macfarland & Cost, 1976). This may explain why ovariectomy and adrenalectomy of pro-oestrous 5 day cyclic rats does not affect the surge of LH (Campbell *et al.* 1977). The concentrations of oestradiol on the last day of dioestrus in 5 day cyclic rats are significantly higher than in animals with 4 day cycles (Naftolin, Brown-Grant & Corker, 1972) and so perhaps enough oestradiol is secreted in the 5 day cycle to act alone in stimulating the surge of LH. When 1 μg oestradiol benzoate was injected on the day

(mean ± S.E.M.; 3·3 ± 0·42 h). The radioactivity remaining in the plasma after extraction rose slowly over the 7 h after injection, indicating a slow increase in the concentration of water-soluble metabolites.

Table 1. *Effect of various steroids on the plasma concentration of LH (means ± S.E.M.) at 18.00 h on day 29 in immature rats treated with 5 i.u. pregnant mare serum gonadotrophin on day 27 and ovariectomized and adrenalectomized at 17.00 h on day 29*

| Treatment† | Time of injection on day 29 (h) | No. of rats with >20 ng LH/ml | Plasma LH (ng/ml) |
|---|---|---|---|
| Oil | 17.00 | 1/26 | 7·2 ± 1·2 |
| Oil, sham-operation | 17.00 | 10/21**** | 25·9 ± 5·9* |
| Oil, operation at 10.30 h | 17.00 | 1/7 | 12·8 ± 5·2 |
| Progesterone | 17.00 | 1/6 | 15·5 ± 8·0 |
| Progesterone + oestradiol benzoate (0·05 μg) | 17.00 | 0/7 | 5·0 ± 1·0 |
| 20α-Hydroxy-4-pregnen-3-one | 17.00 | 1/8 | 10·8 ± 7·0 |
| 17α-Hydroxyprogesterone | 17.00 | 0/3 | 3·3 ± 2·7 |
| Corticosterone | 17.00 | 1/6 | 12·3 ± 6·6 |
| Testosterone | 17.00 | 0/6 | 4·8 ± 1·2 |
| Progesterone | 13.00 | 4/6** | 30·7 ± 10·4* |
| 20α-Hydroxy-4-pregnen-3-one | 13.00 | 0/6 | 5·44 ± 1·2 |
| 20β-Hydroxy-4-pregnen-3-one | 13.00 | 0/6 | 3·3 ± 1·0 |
| 17α-Hydroxyprogesterone | 13.00 | 0/6 | 4·95 ± 1·7 |
| Corticosterone | 13.00 | 2/8 | 15·9 ± 5·7 |
| Testosterone | 13.00 | 4/6** | 37·8 ± 13·3* |
| Oestradiol benzoate (1 μg) | 17.00 on day 28 | 5/8*** | 40·5 ± 11·3* |

LH is expressed in terms of LER-1056-$C_2$ (LH potency of 1·73 NIH-S1 units/mg).
† All steroids were given as 0·5 mg/rat (except oestradiol benzoate).
* $P < 0·05$; ** $P < 0·025$; *** $P < 0·01$; **** $P < 0·001$: compared with oil-treated rats ovariectomized/adrenalectomized at 17.00 h (by Fisher's contingency tables).

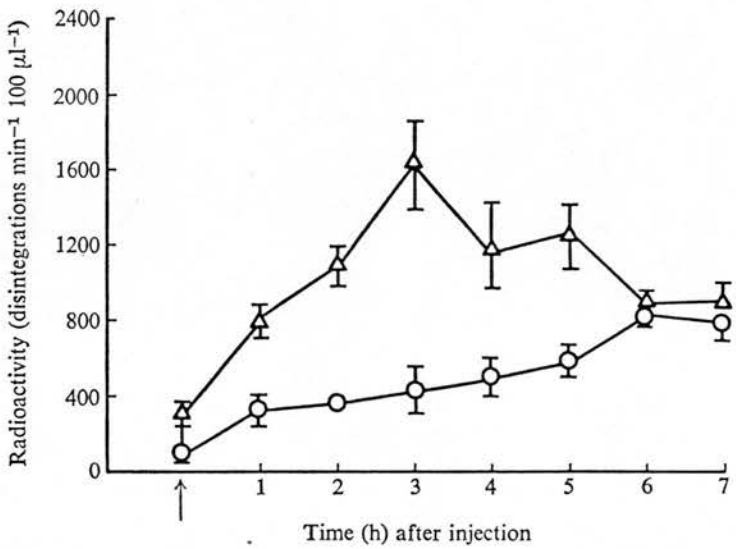



Fig. 5. Variations in the radioactivity (disintegrations/min) in the petroleum ether (b. p. 40–60 °C; nine rats, △) and the aqueous (four rats, ○) fractions after extraction of plasma obtained from rats injected (arrow) s.c. with 1·7 mg (83 μCi) [$^3$H]progesterone/rat. Vertical lines represent ± S.E.M.

before pro-oestrus (17.00 h on day 28), the combination of the exogenous and endogenous oestrogen was capable of stimulating a surge of LH in our ovariectomized/adrenalectomized rats.

When progesterone or testosterone was administered to the ovariectomized/adrenalectomized rats at pro-oestrus (17.00 h on day 29), the increase in the concentration of LH occurred several hours later than usual. Perhaps the unphysiological concentration and pattern of plasma concentrations of steroids delayed gonadotrophin release. Another possibility is that although progesterone and testosterone can stimulate the release of gonadotrophin, neither is the physiological synergist in the oestrous cycle. This would explain why antiserum to progesterone did not inhibit ovulation (Ferin *et al.* 1969); other steroids and their antisera should be tested to elucidate this. Alternatively, a mixture of steroids may be the synergistic agent. We have tried, unsuccessfully, to give progesterone with a basal dose of oestradiol; perhaps other combinations should be tried. It is unlikely that oestradiol alone is required at this time because plasma concentrations of oestradiol are low in the late afternoon of pro-oestrus in the intact rat; in addition, administration of oestradiol in the afternoon of pro-oestrus did not reverse the antiovulatory effect of serotonin (see Introduction; Wilson & McDonald, 1974). It is, therefore, also unlikely that testosterone is active only after being aromatized to oestradiol.

The peak concentration of progesterone in the plasma occurs 3–5 h after a subcutaneous injection (Figs 3, 4 and 5). It is interesting to note that the effects of progesterone on the central nervous system of oestrogen-primed animals, such as induction of lordosis, the release of LH or increased hypothalamic multiple unit activity, also occur 3–5 h after the injection (Boling & Blandau, 1939; Terasawa & Sawyer, 1970; Caligaris *et al.* 1971; Brown-Grant, 1974). A delay of 3–5 h after the time of injection in the peak plasma concentration would be expected of a substance such as progesterone which is lipid soluble, since it would presumably dissolve in lipid membranes throughout tissues before being released into the blood. The slow decrease in the concentration after the peak agrees with this explanation.

It seemed that the surge of LH only appeared when the progesterone peak occurred. When progesterone was given at 17.00 h, the highest plasma concentration was seen at 22.00 h and the surge of LH at 23.00 h. When progesterone was given at 13.00 h, high levels were seen at 16.00 h and the surge of LH occurred at this time.

In conclusion, we have shown that removal of the steroid surges which occur on the late afternoon of pro-oestrus prevents the preovulatory surge of LH in both adult cyclic rats and rats induced to ovulate with PMSG. It is suggested that in the normal 4 day oestrous cycle of the rat, oestrogen alone cannot stimulate the release of the surge of LH and the synergistic action of another steroid(s) is essential.

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# Effect of oestrogen and an LH-RH agonist on the release of gonadotrophins in ovariectomized ewes deprived of LH-RH

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**Summary.** Endogenous LH-RH in ewes was inhibited by active immunization or by injection of LH-RH antiserum. Plasma levels of LH and FSH were elevated in 3 ovariectomized control ewes but low in 3 LH-RH immunized ovariectomized ewes. Oestradiol benzoate (50 μg i.m.) caused a marked rise in LH concentrations in control ewes but not in the immunized ewes. In the immunized ewes the low plasma levels of FSH decreased even further 8–36 h after injection of oestrogen, indicating a direct inhibitory action of the steroid on the pituitary. Both groups responded to the oestrogen injection by a rise in plasma levels of prolactin and by exhibiting normal oestrous behaviour.

When the control ewes were again challenged with oestradiol benzoate and, after 10 h, given an i.v. injection of 75 ml antiserum to LH-RH, the LH surge was abolished in one animal and reduced in another. These experiments indicate that the continued presence of LH-RH is necessary for the occurrence of the oestrogen-induced LH surge in the ewe.

Administration of a stimulatory analogue of LH-RH released LH and FSH in control and immunized ewes but the responsiveness to further injections at intervals of 3 h decreased, particularly for FSH.

## Introduction

The way in which the negative feedback effect of oestrogen on the release of luteinizing hormone (LH) can change to positive feedback and bring about the preovulatory LH surge is still not clear. It has not been established whether the LH surge is preceded by a surge of luteinizing hormone-releasing hormone (LH-RH), composed of an increase in the number and/or amplitude of LH-RH pulses, or whether the rising oestrogen concentrations in the blood are the primary cause of the LH surge by a direct action on the pituitary, with LH-RH secretion remaining constant. There is also no reason why these mechanisms might not act together, with some differences in their relative importance amongst species. Increased levels of LH-RH in the hypophysial portal blood are associated with the LH surge in the rat (Sarkar, Chiappa, Fink & Sherwood, 1976), but the changes in LH-RH secretion in other species during the induced LH surge before ovulation in other species are unknown because of the problem of obtaining portal blood.

One experimental approach to this problem is to inhibit endogenous LH-RH immunologically, by active immunization or by injection of LH-RH antiserum. Injection of LH-RH antiserum to rats and hamsters at 12:00 h on the day of pro-oestrus prevents the peovulatory

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LH surge (Koch, Chobsieng, Zor, Fridkin & Lindner, 1973; de la Cruz, Arimura, de la Cruz & Schally, 1976), and to chickens blocks the progesterone-induced LH surge (Fraser & Sharp, 1978). In marked contrast, LH-RH antiserum was without effect on the oestrogen-induced LH surge in the rhesus monkey (McCormack, Plant, Hess & Knobil, 1977). Active immunization against LH-RH prevents ovulation in the rat (Fraser & Baker, 1978), sheep (Clarke, Fraser & McNeilly, 1978; Jeffcoate, Foster & Crighton, 1978), marmoset monkey (Hodges & Hearn, 1977) and stump-tailed macaque (H. M. Fraser, unpublished), but the mechanisms causing this have not been studied.

We have therefore investigated the effect of immunological inhibition of endogenous LH-RH on the ability of oestrogen to induce an LH surge in ovariectomized ewes. We have also attempted to restore pituitary function in actively immunized animals by administering a stimulatory analogue of LH-RH which is immunologically different from LH-RH, and which releases gonadotrophins, after a single injection, from the pituitaries of rats (Fraser & Sandow, 1977) and sheep (Clarke *et al.*, 1978) immunized against LH-RH.

## Materials and Methods

### *Animals*

Three Scottish Blackface ewes were immunized in August 1976 with LH-RH conjugated to bovine serum albumin (BSA) by carbodiimide, and 3 control ewes were immunized against BSA alone (Clarke *et al.*, 1978). Booster immunizations in Freund's incomplete adjuvant were given 3, 4, 5 and 7 months later. Four months after immunization, all the ewes were ovariectomized and when blood samples were taken 3 weeks later the levels of LH and FSH were elevated in the controls but not in the ewes immunized against LH-RH (Clarke *et al.*, 1978).

### *Effect of oestradiol benzoate on gonadotrophin secretion and oestrous behaviour*

In January 1977, 3 weeks after ovariectomy, all ewes were treated with progestagen pessaries (Cronolone: Searle) for 12 days. An i.m. injection of 50 μg oestradiol benzoate (Intervet, Organon Laboratories Ltd) in 1 ml arachis oil was given 2 days after removal of the pessary and the ewes were run in a pen with a raddled vasectomized ram. The ewes were observed every 2 h to detect the onset of heat. Blood samples were taken by jugular venepuncture every 4 h until the oestrogen injection then every 2 h for a further 36 h.

In February 1978 the effect of injecting antiserum to LH-RH on the oestrogen-induced LH surge in the 3 control ovariectomized ewes was investigated. Blood samples were taken from an indwelling jugular catheter inserted 24 h before starting the experiment and the animals were kept in individual crates. Progestagen pessaries were not given and the animals were not tested for oestrous behaviour. In a control test an injection of 50 μg oestradiol benzoate induced an LH surge in all 3 ewes. The experiment was repeated 2 weeks later, except that the ewes were given an i.v. injection of 75 ml LH-RH antiserum (taken from Ewe 39) 10 h after administration of the oestradiol benzoate, i.e. 2–8 h before the expected time of the LH surge. The specificity of the antiserum was similar to that described previously (Lincoln & Fraser, 1979) but the antibody titre was three times higher, being 1 : 105 000.

### *Effect of an LH-RH agonist on gonadotrophin release*

Different dose regimens of (D-Serine-t-butyl$^{6}$,*des*-Glycine-$NH_2{}^{10}$) LH-RH ethylamide (LH-RH agonist: Hoechst A.G.) were given to assess the ability of the pituitaries of the control and LH-RH immunized ewes to respond to exogenous LH-RH. An indwelling jugular venous catheter was inserted and the animals caged individually, 24 h before starting the experiment.

Blood samples were taken at 20-min intervals for 2 h before treatment. A series of LH-RH agonist injections were given between February and May 1977 as follows: (1) a single injection of 5 μg (February), (2) 8 injections of 1 μg at intervals of 3 h (beginning of March), (3) 2 injections of 100 ng 3 h apart (end of March) and (4) 8 injections of 20 ng every 3 h (May). All injections were given i.v. in 1 ml saline (9 g NaCl/l).

*Collection of pituitaries*

In April 1978, 11 months after the agonist experiments the immunized ewes were given a final booster immunization, and 2 weeks later all the animals were killed. After pentobarbitone sodium anaesthesia, the jugular vein and carotid artery were cut, the animals bled out and the pituitary gland rapidly removed. The anterior pituitary gland was dissected free of other tissue, weighed, and bisected. One half was placed in Bouin's fixative, and after sectioning, stained with Alcian blue-PAS-orange G. The remainder of the anterior pituitary was weighed before homogenization in 5 ml 0·1 M-phosphate buffer, pH 7·4, containing 0·2% BSA. After centrifugation the supernatant was stored at −20°C until required for assay of LH, FSH and prolactin.

*Radioimmunoassays*

The concentration of LH was determined by radioimmunoassay (Martensz, Baird, Scaramuzzi & Van Look, 1976) of duplicate 200 μl (LH-RH immunized ewes) or 50 μl (control ewes) quantities of plasma and results were expressed in terms of ng NIH-LH-S14/ml. The sensitivity of the assay was 0·3 ng/ml and the intra- and inter-assay coefficients of variation were 8 and 10% respectively. FSH was measured in duplicate quantities of 150 μl (all ewes) and 50 μl (some control samples) using the radioimmunoassay described by McNeilly, McNeilly, Walton & Cunningham (1976) and results were expressed as ng NIH-FSH-S10/ml. Assay sensitivity was 20 ng/ml with intra- and inter-assay coefficients of variation being 9 and 12% respectively. Prolactin was measured in duplicate quantities of 30 μl plasma by radio-immunoassay (McNeilly & Andrews, 1974) and results were expressed in terms of ng NIH-PRL-S6/ml. This assay had a sensitivity of 0·05 ng/ml and intra- and inter-assay coefficients of variation of 8 and 11% respectively. LH-RH antibody titre was assessed as before (Clarke *et al.*, 1978) and expressed as the initial dilution binding 33% of $^{125}$I-labelled LH-RH.

*Statistical analysis*

Differences in endogenous levels of LH, FSH and prolactin were compared using a 2-factor analysis of variance with replication. Changes in concentrations of FSH after treatment with oestradiol benzoate and LH-RH agonist were analysed by analysis of variance without replication.

## Results

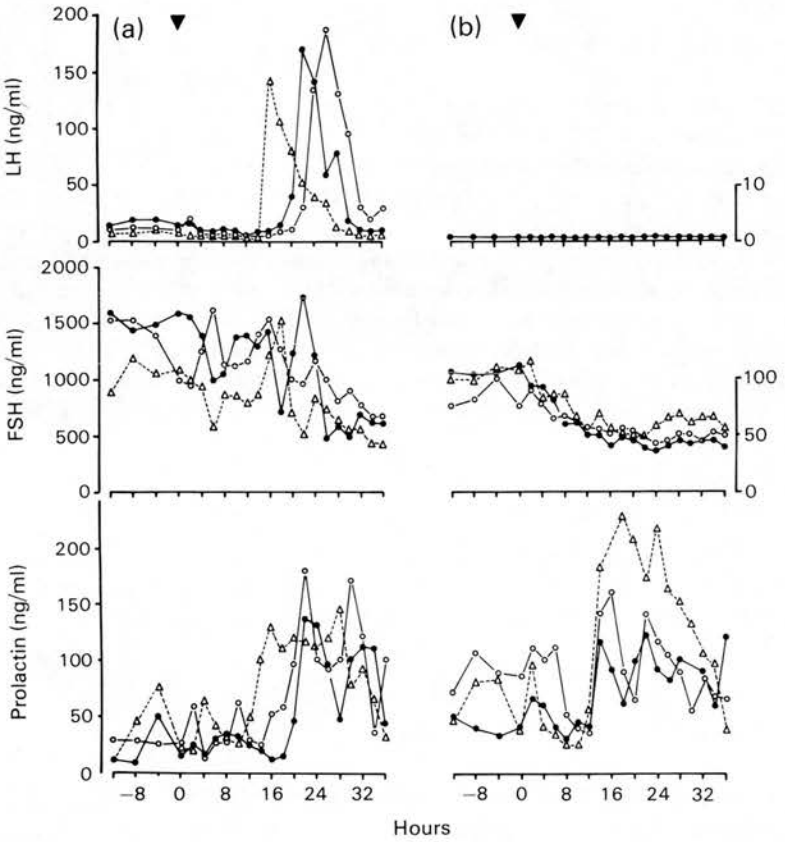
Throughout the period of study the immunization against LH-RH was successful in inhibiting the action of the endogenous hormone since plasma gonadotrophin levels were consistently low (<0·8 ng LH/ml, 30–100 ng FSH/ml) in all 3 treated animals, while values in control animals were markedly elevated (e.g. see Text-figs 1 and 3). LH-RH antibody levels during the experiments were high in 2 ewes and relatively low in the remaining animal (Table 1).

**Table 1.** Hormone content of anterior pituitary glands and range of LH-RH antibody titre during the experimental period in ovariectomized ewes immunized against LH-RH

| Group | Ewe No. | Pituitary hormone content (μg/gland) | | | LH-RH antibody titre |
|---|---|---|---|---|---|
| | | LH | FSH | Prolactin | |
| Control | 59 | 4064 | 691 | 5934 | — |
| | 66 | 4606 | 500 | 2808 | — |
| | 242 | 1525 | 386 | 8286 | — |
| LH-RH immunized | 23 | 32 | 42 | 3824 | 1:5000–1:12 800 |
| | 27 | 380 | 120 | 1452 | 1:800–1:1600 |
| | 39 | 35 | 27 | 3987 | 1:17 000–1:105 000 |

*Effect of exogenous oestrogen on gonadotrophin secretion and oestrous behaviour*

In the control ewes the administration of oestrogen was followed by a decline in LH lasting 12 h but after 16–24 h a marked rise in LH concentrations occurred. In contrast, in all the LH-RH immunized ewes LH levels remained at <0·8 ng/ml throughout (Text-fig. 1).



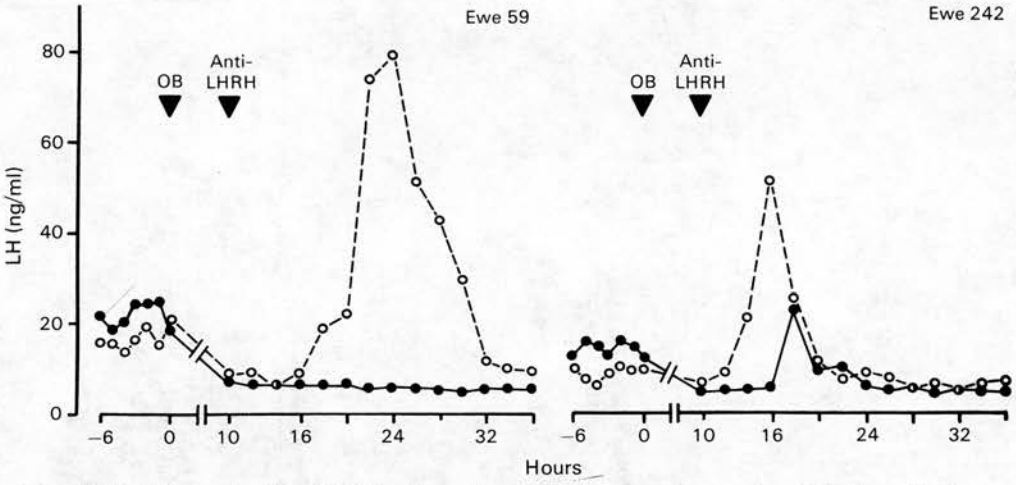

**Text-fig. 1.** Plasma levels of LH, FSH and prolactin in (a) 3 control ovariectomized ewes and (b) 3 ovariectomized ewes immunized against LH-RH after the i.m. injection of 50 μg oestradiol benzoate (arrow). Note the difference (× 10) in the scale for (a) and (b).

There was a tendency for plasma FSH concentrations to decline during the last 8 h of the sampling period in the control ewes but no clear-cut response was evident. In all 3 LH-RH immunized ewes there was gradual decline in FSH levels, beginning 4 h after the oestrogen injection, and plasma FSH values at 8–36 h were significantly ($P < 0{\cdot}001$) lower than preinjection levels (Text-fig. 1).

Preinjection concentrations of prolactin were significantly ($P < 0{\cdot}01$) higher in the immunized ewes, than in the control but oestrogen injection resulted in a prolonged rise, beginning 12–20 h after the injection, in both groups (Text-fig. 1).

Oestrous behaviour appeared normal in all ewes and occurred 19 ± 2 h (controls) and 15 ± 10 h (immunized) (mean ± s.d.) after the oestrogen injection.

Injection of antiserum to LH-RH completely blocked the oestrogen-induced LH rise in one ewe and severely reduced it in another (Text-fig. 2). In the remaining animal the LH rise had not occurred by 17 h after the oestrogen injection when sampling was stopped; during the control test in this animal the plasma LH levels had been markedly elevated (39 ng/ml) by 16 h. The FSH response was unaltered in all 3 ewes.



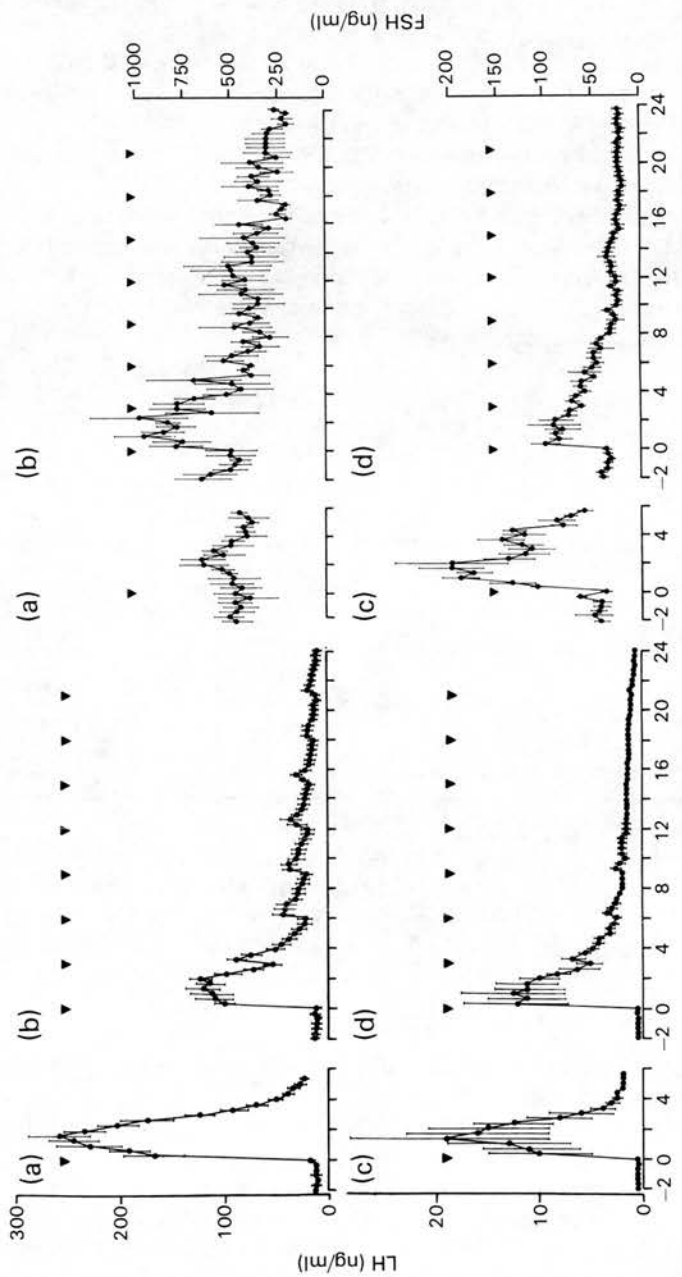

**Text-fig. 2.** Plasma levels of LH in 2 ovariectomized ewes after an i.m. injection of 50 μg oestradiol benzoate (OB) during a control test (○) and after injecting 75 ml antiserum to LH-RH (●).

*Effect of LH-RH agonist on gonadotrophin release*

Before the agonist injections, LH and FSH concentrations were elevated in the control ewes but very low in the immunized animals.

*Schedule 1.* The injection of 5 μg LH-RH agonist induced a rapid release of LH with peak values being obtained 1–2 h later in both groups (Text-fig. 3). Concentrations in the LH-RH immunized ewes were much lower although the pattern of release appeared similar. The magnitude of the response in the immunized ewes was related to antibody titre, being highest (39 ng/ml) in the animal with the lowest titre and 10 ng/ml in the other 2 ewes. FSH levels also rose in both groups, with the increase in the LH-RH immunized ewes being less than that in the controls (Text-fig. 3).

Plasma levels of prolactin were not affected by the agonist. During the 6-h study period they were significantly higher ($P < 0{\cdot}001$) in the immunized animals, the mean ± s.e.m. values being 38 ± 9·3, 42 ± 13 and 49 ± 9 ng/ml in the LH-RH immunized ewes and 27 ± 8, 32 ± 9 and 32 ± 16 ng/ml in the controls.




**Text-fig. 3.** Plasma levels of LH and FSH (mean ± s.e.m.) in 3 control ovariectomized ewes (a, b) and 3 ovariectomized ewes immunized against LH-RH (c, d) following a single injection of 5 μg LH-RH agonist (Schedule 1) (a, c) or 8 injections of 1 μg LH-RH agonist at 3 h intervals (Schedule 2) (b, d). Note the difference for LH (× 10) and FSH (× 5) in the scale for (a, b) and (c, d).

*Schedule 2.* When 1 μg LH-RH agonist was injected on 8 occasions 3 h apart the control ewes showed a marked rise in LH levels in response to the first injection but there was a progressive diminution of response to each subsequent injection (Text-fig. 3). The LH-RH immunized ewes also responded most to the first injection, LH levels in the animal with a low titre reaching 23 ng/ml with highest levels in the other animals being 7 and 9 ng/ml. After subsequent injections plasma LH values were considerably lower and after the fifth injection there was very little response (Text-fig. 3).

There was a rise in plasma FSH levels in both groups, but the response was less sustained than that for LH, and after treatment for 15 h (i.e. after the 5th injection) the FSH levels were significantly lower ($P < 0{\cdot}001$) in both groups than the preinjection levels (Text-fig. 3).

*Schedule 3.* LH concentrations in the immunized ewes rose from $0{\cdot}6 \pm 0{\cdot}1$ ng/ml to a maximum of $3{\cdot}1 \pm 0{\cdot}3$ ng/ml (mean ± s.e.m.) after the first injection of 100 ng LH-RH agonist, but the response to the second injection was reduced, levels rising to only $1{\cdot}8 \pm 0{\cdot}2$ ng/ml. Concentrations of FSH rose from $56 \pm 9$ to a maximum of $94 \pm 17$ ng/ml after the first injection and there was no clearly defined rise after the second injection. Control ewes were not studied.

*Schedule 4.* It was impossible to distinguish any response of the control ewes to the 8 injections of 20 ng LH-RH agonist. In Ewes 27 and 39 (immunized) this treatment caused LH levels to rise from 0·5 to 0·8–1·6 ng/ml after each injection but there was no enhancement of responsiveness. In the remaining LH-RH immunized ewe this dose of agonist was without effect on LH levels. In all 3 LH-RH immunized ewes plasma FSH values seemed unaffected by the treatment and remained below 70 ng/ml. At this time of year (May) the normal seasonal rise in prolactin levels had occurred in both groups of ewes and concentrations (mean ± s.e.m.) during this study period were $91 \pm 28$, $109 \pm 26$, $121 \pm 26$ ng/ml in the controls and $95 \pm 30$, $93 \pm 33$ and $106 \pm 36$ ng/ml in the LH-RH immunized ewes. These values were not significantly different.

*Pituitary hormone contents and histology*

Pituitary contents of LH and FSH were considerably reduced in the LH-RH immunized ewes, appearing inversely proportional to antibody titre, while pituitary contents of prolactin were similar in both groups (Table 1). Histological examination of the pituitaries revealed that acidophils were the predominant cell type in all animals. Identifiable gonadotroph cells were clearly fewer in number in pituitaries from the LH-RH immunized ewes and appeared small and shrunken compared to those of controls.

## Discussion

The presence of circulating antibodies to LH-RH in ovariectomized ewes inhibits the action of the endogenous hormone as judged by the low levels of LH and FSH in the blood and by their reduced content in the anterior pituitary gland. A very small amount of LH-RH probably reaches the gonadotroph cells, particularly if antibody production is low, as illustrated by the fact that pituitary content of LH and FSH was highest in the ewe with the lowest antibody titre. Studies of rats with different LH-RH antibody titres have given similar results (Fraser & Baker, 1978). The presence of small amounts of biologically active LH-RH may also explain why some FSH is detectable in the plasma of LH-RH immunized ewes. Nevertheless, in these ewes the action of LH-RH is effectively blocked and as the source of the ovarian steroid hormones is also absent they provide an in-vivo system with which to study the ability of oestrogen and LH-RH agonists to stimulate the secretion of LH and FSH independently of endogenous LH-RH secretion. This study has established that the ability of oestrogen to induce an LH surge is

completely abolished in ewes immunized against LH-RH, showing that LH-RH is necessary for the LH surge to occur.

It was also important to establish how much, if any, gonadotrophin the pituitaries of these LH-RH immunized animals could release following exogenous LH-RH stimulation. This was done by injecting an agonist of LH-RH which is effective in small amounts and is not inhibited by the circulating LH-RH antibodies (Fraser & Sandow, 1977; Clarke *et al.*, 1978; Jeffcoate *et al.*, 1978). A comparison of Text-figs 1 and 3 shows that in control ewes a dose of 1 μg LH-RH agonist can produce an LH rise equivalent to that stimulated by an oestrogen test. Although the response of the LH-RH immunized ewes was much less it nevertheless showed that the pituitaries of these animals could release LH and FSH but that oestrogen alone could not exert any stimulatory gonadotrophin-releasing action on the pituitary.

When interpreting these findings to assess the role of LH-RH it must be remembered that the pituitaries of these animals had been deprived of the normal priming effect of endogenous LH-RH, which is important in contributing to the increased responsiveness to oestrogen, and their capacity to synthesize new hormone may therefore be impaired. These differences were avoided by carrying out the experiment in the control ewes and inhibiting LH-RH by injecting antiserum, but not until 10 h after the oestrogen injection. When oestrogen was allowed to act on a normal pituitary receiving LH-RH priming, the inhibition of LH-RH just before the expected LH surge completely abolished positive feedback in one animal and severely reduced it in another. Subsequent studies have confirmed the abolition of the LH surge in 5 intact ewes treated with oestrogen and an LH-RH antiserum (H. M. Fraser & A. S. McNeilly, unpublished observations). These results are similar to the inhibitory effects of injecting antibodies to LH-RH on the LH surge in the rat (Koch *et al.*, 1973), hamster (de la Cruz *et al.*, 1976) and fowl (Fraser & Sharp, 1978), but are in marked contrast to the lack of effect in the rhesus monkey, in which administration of antibodies both before and after oestrogen injection failed to abolish the LH surge (McCormack *et al.*, 1977).

The usefulness of animals actively immunized against LH-RH would be enhanced if it were possible to induce normal synthesis and release of LH and FSH by exogenous releasing hormone. This might be attempted by injecting massive doses of LH-RH, but most of this would be inhibited by the circulating antibody (see Lincoln & Fraser, 1979). The use of an LH-RH agonist has the advantage that it is highly potent and can by-pass the circulating antibody because of immunochemical differences from LH-RH (Fraser & Sandow, 1977). However, the present attempts to stimulate pituitary activity by repeated injections of 20 ng–1 μg LH-RH agonist resulted in a decreased pituitary responsiveness in the control and immunized ewes, as has been found for higher doses of agonist in rams (Fraser & Lincoln, 1980), monkeys (Fraser, Laird & Blakeley, 1980) and women (Dericks-Tan, Hammer & Taubert, 1977). In the present study, using low doses, it was clear that a 3-h interval is not long enough for the gonadotroph cells to recover from the exposure to agonist.

In the LH-RH immunized ewes the administration of oestrogen caused the already low levels of FSH in the blood to decrease even further within a few hours. Studies of sheep pituitaries *in vitro* have shown that oestradiol-17β can have a direct inhibitory action on the secretion of FSH (Miller, Knight, Grimek & Gorski, 1977). Since, in LH-RH immunized animals, the pituitary is constantly deprived of LH-RH stimulation, the present results *in vivo* provide further evidence of a negative feedback action of oestrogen directly on the pituitary gonadotroph cells.

In the LH-RH immunized ewes a single injection of 1 or 5 μg LH-RH agonist gave a particularly rapid release of FSH which was slightly more pronounced than that of LH when compared with controls. Both gonadotrophins were readily available for release despite the lack of previous exposure to endogenous LH-RH. However, the FSH response soon became negligible in all the animals, and levels fell below preinjection values in both groups after the fifth agonist injection. This indicates that the mechanisms involved in release of additional stores of FSH are rapidly affected by repeated exposure to LH-RH agonist.

Prolactin concentrations were higher in the LH-RH immunized ewes than in controls during the months of January and February but this difference disappeared with the seasonal influences which normally induce high levels of prolactin (Walton, McNeilly, McNeilly & Cunningham, 1977). An elevation in prolactin levels in these ewes before ovariectomy has been described previously (Clarke *et al.*, 1978), but the reason is unclear. It may be connected with the fact that in these animals all aspects of negative feedback have been removed by the absence of the ovaries and by the absence of elevated gonadotrophin levels. If this results in an increased output of LH-RH this might decrease hypothalamic turnover and output of dopamine, thus increasing prolactin secretion (McNeilly, 1980). Administration of LH-RH agonist was without effect on prolactin secretion while the injection of oestrogen resulted in a clear rise in prolactin levels in both groups. This effect of oestrogen is probably brought about by its ability to block dopamine receptors on the prolactin-secreting cells (Labrie *et al.*, 1978).

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# Effect of Immunoneutralization of Luteinizing Hormone Releasing Hormone on the Estrogen-Induced Luteinizing Hormone and Follicle-Stimulating Hormone Surges in the Ewe

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## ABSTRACT

The importance of luteinizing hormone releasing hormone (LHRH) in inducing the preovulatory luteinizing hormone (LH) surge in the ewe was investigated by administration of LHRH antiserum approximately 10 h before the expected onset of the rise in LH in the blood. Ten ewes with synchronized estrous cycles were studied. Luteolysis was induced by prostaglandin and 26 h later the ewes were injected with 50 μg estradiol benzoate in arachis oil, and after a further 10 h, 5 control ewes received 100 ml ovine antiserum to bovine serum albumin (BSA) i.v., and 5 remaining animals 100 ml ovine antiserum to LHRH (LHRH antiserum). All 5 control ewes demonstrated the expected sharp rise in plasma LH concentrations, peak values occurring 26 ± 2 h (mean ± SEM) after estradiol benzoate administration. This rise was absent in the LHRH antiserum treated animals, who also failed to ovulate as judged from the absence of a rise in plasma progesterone concentrations during the following 16 days. Plasma LH concentrations remained low during the 16-day period in the treated animals.

In the control ewes there was a clear surge of follicle-stimulating hormone (FSH) coincidently with the LH surge. This did not occur in the ewes treated with antibodies to LHRH. However, plasma FSH concentrations rose despite the presence of antibodies to LHRH and although a clear second FSH peak which occurred in the controls was not evident in the LHRH antibody treated animals, there was no significant difference in the amount of FSH released during this time period between the two groups. Also, plasma FSH concentrations during the remaining 16 days were unaltered by the LHRH antibodies.

Thus in the ewe, the estrogen-primed hypothalamo-pituitary system cannot produce an LH surge if LHRH is neutralized by giving antiserum before the onset of the surge and basal LH output is also reduced. The major preovulatory FSH surge is also inhibited when LHRH is neutralized but basal secretion of the hormone remains unaltered.

## INTRODUCTION

The relative importance of the hypothalamus and anterior pituitary gland as sites of the positive feedback action of estradiol on the gonadotropin surge has still to be established. In the ewe, the fall in progesterone concentrations in the blood after regression of the corpus luteum is followed by an increase in the pulsatile release of LH from the pituitary which stimulates an increase in production of estradiol from the ovaries (Baird and McNeilly, 1981). As in other species, this increase in estradiol plays a crucial role in triggering the preovulatory LH surge, acting at either the hypothalamus to increase LHRH output or at the anterior pituitary gland to increase pituitary responsiveness.

Because of technical difficulties in collecting hypophysial portal blood, there have been no reports on the concentration of LHRH during the time around the LH surge in the ewe. Alternative means of studying the role of LHRH are required, such as blocking the action of the releasing hormone. This is best achieved by injecting antibodies to LHRH. In the cyclic rat and hamster, administration of antibodies to LHRH during the early afternoon of proestrus blocks the surge of LH and FSH and ovulation (Fraser and Gunn, 1973; Koch et al., 1973; Arimura et al., 1974; de la Cruz et al., 1975; Kerdelhue et al., 1976; Fraser, 1977; Blake and Kelch, 1981; Hasegawa et al., 1981) and the progesterone-induced LH surge in ovariec-

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tomized estrogen-primed rats (Lu and Yen, 1980) and chickens (Fraser and Sharp, 1978). Similar experiments in the rhesus monkey failed to abolish the LH surge (McCormack et al., 1977) and these results, along with others (Knobil, 1980; Wildt et al., 1981) emphasized the possibility that the anterior pituitary may be the site of positive feedback in this species.

In ovariectomized ewes, we have demonstrated that the estrogen-induced LH surge was abolished in ovariectomized ewes actively immunized against LHRH (Fraser et al., 1981). However, pituitaries of these animals have low LH content (Fraser et al., 1981) and have been deprived of previous exposure to LHRH. To overcome this problem we have studied the effect of neutralizing LHRH by passive immunization in normally cycling ewes pretreated with estradiol benzoate to allow maximal stimulation of the hypothalamic-pituitary system before neutralizing LHRH.

## MATERIALS AND METHODS

### *Experimental Animals*

The estrous cycles of 10 adult Dam-line ewes kept at the Animal Breeding Research Organization, Roslin, Midlothian, Scotland were synchronized during the breeding season (November) with progestogen pessaries (Cronolone; Searle). The progestogen was removed after 12 days and on Day 10 of the following cycle, luteal regression was induced by intramuscular injection of 100 μg of a potent analogue of prostaglandin $F_{2\alpha}$ ICI 80996 (cloprostenol).

Twenty-six hours later the ewes were injected intramuscularly with 50 μg estradiol benzoate (Intervet, Organon Laboratories Ltd.) in 1 ml arachis oil. After insertion of a cannula ending in a 3-way tap into the jugular vein, all ewes were placed in holding pens. Ten hours after estradiol benzoate administration, 5 ewes were given an intravenous infusion of 100 ml of ovine antiserum to bovine serum albumin over a 30-min period and 5 ewes were infused with 100 ml ovine antiserum to LHRH (LHRH antiserum). The properties of this antiserum have been described previously (Lincoln and Fraser, 1979).

Blood samples were obtained at the time of prostaglandin administration (Day 1), at the time of estradiol benzoate injection (time 0) and at 8, 9 and 10 h (pre-antibody). After antibody administration, blood samples were collected at hourly intervals for 45 h. In order to determine the occurrence of ovulation, blood samples were also collected by jugular venipuncture at Days 3, 4, 7, 9, 11, 14 and 16 after estradiol benzoate administration.

### *Radioimmunoassays*

Concentrations of LH, FSH and progesterone were measured in duplicate quantities of plasma by methods which have been described in detail by Martenz et al., 1976, McNeilly et al., 1976 and Scaramuzzi et al., 1975. The sensitivities of the assays were 0.3 ng LH (NIH-LH-S14)/ml, 12 ng FSH (NIH-FSH-S10)/ml and 0.2 ng progesterone/ml. Intraassay coefficients of variation were 8, 9 and 10% and interassay variations 10, 12 and 12% for LH, FSH and progesterone, respectively.

### *Statistical Methods*

To determine the effects of LH antiserum on the amount of LH and FSH released after estradiol benzoate, cumulative totals of hormone released were calculated by adding the values after subtraction of the baseline level at the time of injection of estradiol benzoate. Statistical analysis of results was by Student's *t* test.

## RESULTS

### *Luteinizing Hormone*

The expected marked rise in plasma LH levels occurred in all 5 control ewes receiving antiserum to BSA (Table 1 and Fig. 1). None of the ewes treated with antiserum to LHRH exhibited a LH surge although there were indications of a small rise in LH in 3 of the 5 animals (Table 1 and Fig. 1). Plasma LH concentrations remained significantly depressed ($P<0.02$ to $<0.01$, by Student's *t* test) in the LHRH antiserum treated animals when examined between Days 3–16 after estradiol benzoate administration; mean values of single daily blood samples ranged from 0.8–1.3 ng/ml in controls and 0.2–0.4 ng/ml in the treated ewes.

### *Follicle-Stimulating Hormone*

In the control ewes a clear surge of FSH occurred coincidently with the LH surge in all ewes, although in one animal the surge was considerably less (about 30%) than those of the other 4 (Table 2 and Fig. 1). In the ewes receiving LHRH antiserum, this clear surge of FSH did not occur and the maximal plasma levels during thie time of the LH/FSH surge in control ewes was significantly less ($P<0.05$) than that of the controls. The cumulative FSH levels during 0–30 h after antibody administration (i.e. during the period of the gonadotropin surge in the controls) was lower in the LHRH antiserum treated ewes than in 4 of the 5 controls, although this value only reached significance ($P<0.01$) when the value for the poor-responding control was excluded (Table 2, Fig. 1). However, it was also evident that FSH concentrations increased during this period despite the presence of LHRH antibody.

TABLE 1. Effect of LHRH antiserum on the peak height and total amount of LH released after estradiol benzoate administration.

| Group | Animal no. | Basal LH (ng/ml) | | | LH surge | | | |
|---|---|---|---|---|---|---|---|---|
| | | Luteal phase (n=1) | Follicular phase | | Time to | | Peak height (ng/ml) | Area under curve (arbitrary unit) |
| | | | Pre E (n=1) | Pre-antibody (n=3) | Onset (h) | Peak (h) | | |
| Control | 27 | 0.2 | 0.7 | 0.3 | 20 | 25 | 80 | 451 |
| | 40 | 0.2 | 0.3 | 0.3 | 24 | 35 | 115 | 624 |
| | 101 | 0.3 | 2.4 | 1.3 | 17 | 22 | 200 | 837 |
| | 248 | 0.3 | 0.6 | 0.3 | 11 | 25 | 200 | 692 |
| | 383 | 1.0 | 0.6 | 0.4 | 17 | 21 | 400 | 989 |
| | Mean ± SEM | 0.4 ± 0.1 | 0.9 ± 0.4 | 0.5 ± 0.2 | 18 ± 2 | 26 ± 2 | 199 ± 55 | 719 ± 92 |
| Treated | 35 | 0.3 | 0.3 | 0.4 | 39 | 39 | 0.4 | 1 |
| | 52 | 0.5 | 0.5 | 0.4 | 20 | 20 | 1.0 | 3 |
| | 215 | 0.2 | 0.7 | 0.6 | 20 | 24 | 12.0 | 45 |
| | 231 | 0.6 | 1.1 | 0.7 | 39 | 39 | 2.8 | 4 |
| | 288 | 0.4 | 1.5 | 0.4 | 26 | 27 | 3.2 | 7 |
| | Mean ± SEM | 0.4 ± 0.1 | 0.8 ± 0.2 | 0.5 ± 0.1 | 29 ± 4 | 30 ± 4 | 4 ± 2 | 12 ± 8 |
| P value | | NS | NS | NS | NS | NS | <0.001 | <0.001 |

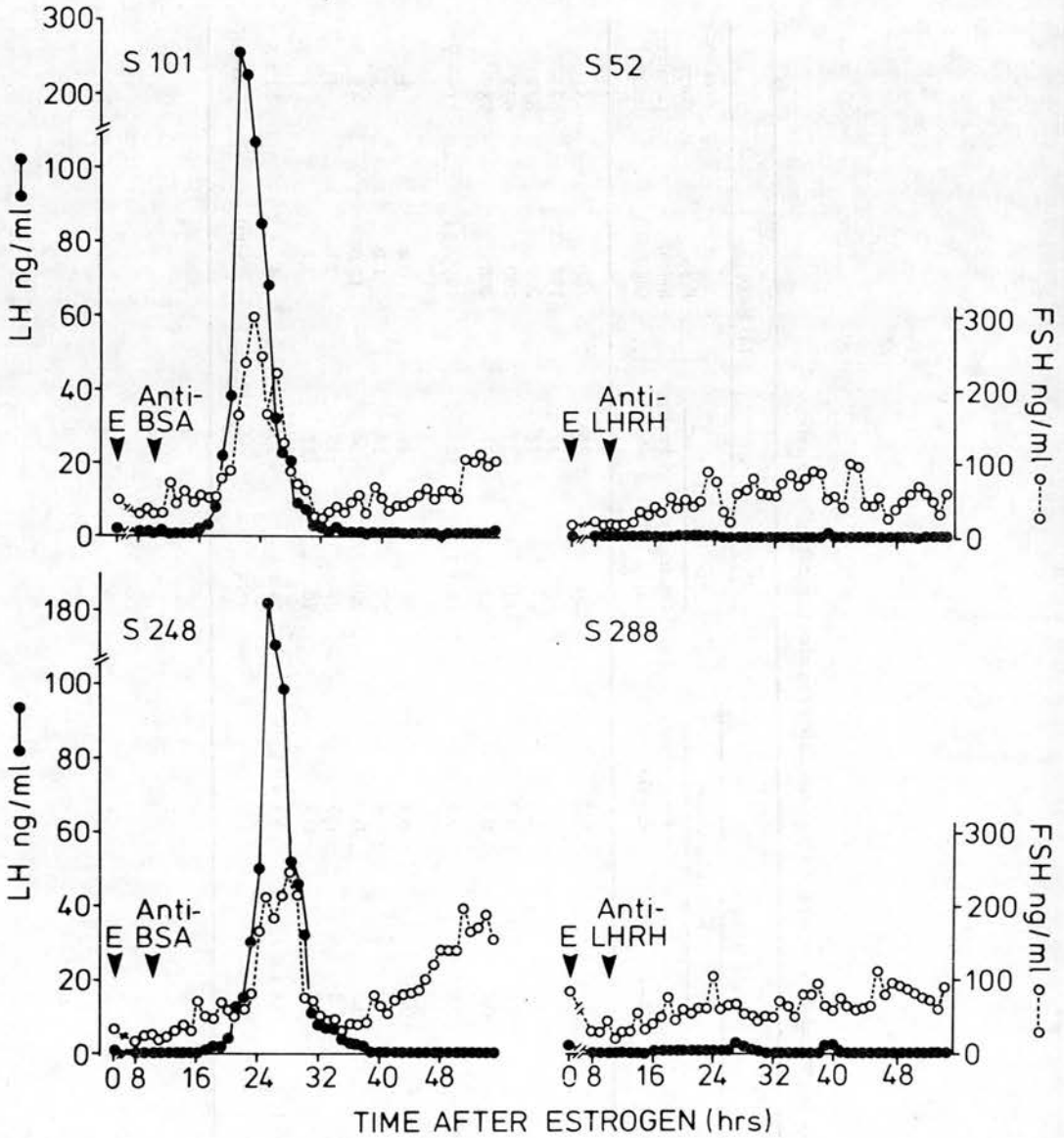



FIG. 1. Effect of i.v. infusion of LHRH antiserum on the estradiol benzoate *(E)* induced gonadotropin surges in 2 representative ewes and in 2 control ewes injected with antiserum to bovine serum albumin *(Anti-BSA)*.

In the control ewes there was an obvious second FSH surge between 31–45 h after antibody administration (Table 2, Fig. 1). Although a clear rise in FSH did not occur in the LHRH antibody treated ewes, the cumulative plasma FSH concentrations were not different from those in controls. Plasma FSH concentrations were similar between the 2 groups during the remaining 3- to 16-day period after estradiol benzoate, mean values ranging from 39–90 ng/ml in controls and 65–88 ng/ml in the LHRH antiserum treated ewes.

*Effect on Ovulation*

As assessed by measuring plasma concentrations of progesterone, all the ewes had active corpora lutea at the time of prostaglandin administration and levels in all ewes had declined by the time of estradiol benzoate injection. Four of the 5 control ewes demonstrated a clear elevation of plasma progesterone beginning 3 days after estradiol benzoate administration but this rise was absent in all 5 ewes who received LHRH antiserum (Fig. 2).

TABLE 2. Effect of LHRH antiserum on the height of the first and second FSH peaks and the total amount of FSH released during and after the LH surge induced by estradiol benzoate administration.

| Group | Animal no. | Basal FSH (ng/ml) | | | First peak height (ng/ml) | Cumulative FSH levels (arbitrary unit) Time after antibody injection | | Second peak height (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| | | | Follicular phase | | | | | |
| | | Luteal phase (n=1) | Pre E (n=1) | Pre-antibody (n=3) | | 0–30 h | 31–45 h | |
| Control | 27 | 37 | 23 | 19 | 152 | 1254 | 325 | 52 |
| | 40 | 36 | 21 | 15 | 59 | 433 | 238 | 51 |
| | 101 | 37 | 49 | 30 | 246 | 1769 | 546 | 82 |
| | 248 | 88 | 33 | 24 | 180 | 1519 | 1520 | 170 |
| | 383 | 115 | 66 | 49 | 356 | 2446 | 1319 | 151 |
| | Mean ± SEM | 63 ± 16 | 38 ± 8 | 27 ± 6 | 199 ± 49 | 1484 ± 329 | 804 ± 271 | 101 ± 25 |
| Treated | 35 | 29 | 20 | 15 | 74 | 938 | 196 | 31 |
| | 52 | 84 | 15 | 17 | 80 | 1119 | 577 | 82 |
| | 215 | 52 | 23 | 18 | 28 | 293 | 558 | 54 |
| | 231 | 63 | 22 | 21 | 56 | 462 | 780 | 93 |
| | 288 | 107 | 85 | 34 | 20 | 688 | 659 | 75 |
| | Mean ± SEM | 67 ± 13 | 33 ± 13 | 21 ± 3 | 52 ± 12[a] | 700 ± 151 | 554 ± 98 | 67 ± 11[b] |
| P value | | NS | NS | NS | <0.05 | NS | NS | NS |

[a]These values are the maximal plasma levels of FSH observed during the time period over which the LH/FSH surge occurred in the control animals.

[b]These values are the maximal plasma levels of FSH observed during the time period of the second FSH surge in the control animals.

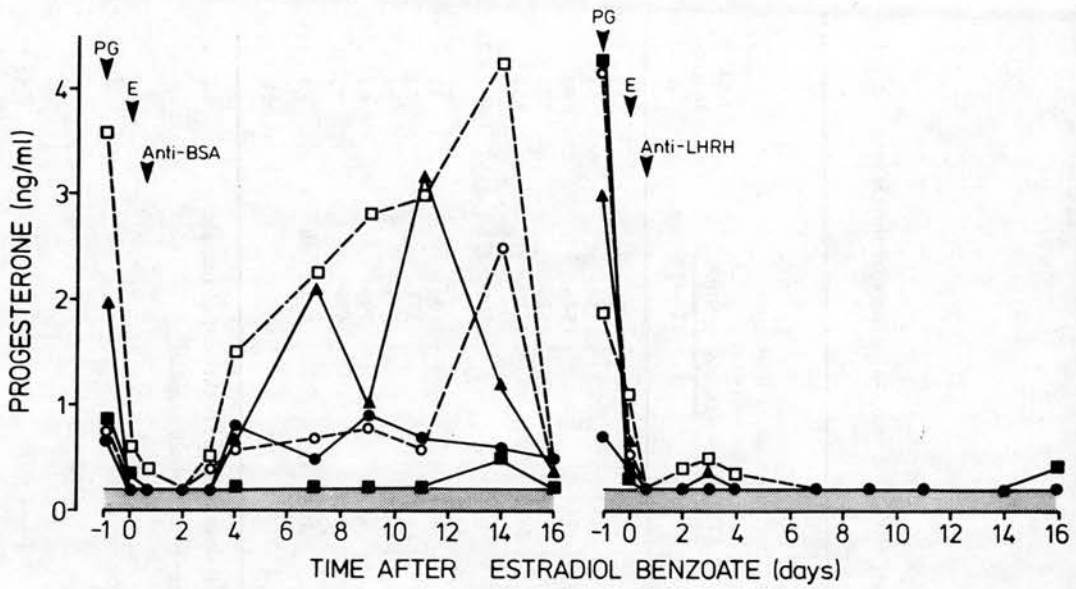



FIG. 2. Plasma progesterone concentrations in individual ewes with synchronized estrous cycles after luteolysis has been induced by prostaglandin (*PG*) administration and followed by administration of estradiol benzoate (*E*) and i.v. infusion of antiserum to bovine serum albumin (*Anti-BSA*) (*left panel*) or antiserum to LHRH (*Anti-LHRH*) (*right panel*).

## DISCUSSION

In the present study the timing of LHRH antiserum administration in the ewe (36 h after prostaglandin injection to induce luteolysis and 10 h after estrogen administration) was chosen to allow LHRH priming of the anterior pituitary gland up to approximately 10 h before the expected onset of the LH surge. The clear inhibition of the LH surge after inhibition of LHRH by giving antiserum demonstrates that the presence of LHRH is necessary, either as tonic secretion or in increased output for the LH surge to occur. This agrees with the results from similar studies in the rat, hamster and bird (Koch et al., 1973; Arimura et al., 1974; de la Cruz et al., 1975; Kerdelhue et al., 1976; Fraser, 1977; Fraser and Sharp, 1978; Lu and Yen, 1980; Blake and Kelch, 1981; Hasegawa et al., 1981). Since the frequency of LH pulses in the ewe increases prior to the onset of the LH surge (Baird and Scaramuzzi, 1976; Baird and McNeilly, 1981), and since one pulse of LH is likely to be due to one release episode of LHRH from the hypothalamus (Lincoln and Fraser, 1979), then the LH surge in this species is likely to be the result of increased frequency of LHRH release from the hypothalamus. This does not exclude the possibility that estrogen may also increase the pituitary response to LHRH. However, this mechanism on its own is insufficient to cause the LH surge in the ewe; the small rise in LH observed after LHRH antiserum administration which was observed in some of the animals may have represented a direct stimulatory effect of estrogen on the pituitary or incomplete neutralization of LHRH. Whatever the reason, the LH released represents less than 5% of that released in control animals.

Narayana and Dobson (1979) reported that injection of LHRH antiserum at the time of onset of behavioral estrus abolished the LH surge in 1 animal and delayed it by 16 h in another 3 ewes. These authors also concluded that LHRH controls the LH surge in the ewe. The more convincing demonstration of this in the present report is probably due to more effective neutralization of endogenous LHRH by our antiserum. The absence of the LH surge in our treated ewes was confirmed by the low levels of plasma progesterone during the treatment cycle. Our study employing specific neutralization of LHRH by antibodies, agrees with the inhibition of the LH surge by depres-

sants of the central nervous system in ewes (Radford and Wallace, 1974; Webb et al., 1981).

These findings contrast with those in the rhesus monkey which indicate that while the pituitary gland must be primed by LHRH before estrogen administration, the releasing hormone is not required after estrogen injection to cause the LH surge (McCormack et al., 1977; Knobil, 1980; Wildt et al., 1981). This raises the question of whether there is a species difference in relation to the importance of the hypothalamus and pituitary as sites of positive feedback.

The FSH surge which occurs coincidentally with the LH surge was also inhibited by the LHRH antiserum although the effect was less dramatic. The FSH concentrations in plasma of the LHRH antibody treated ewes did not demonstrate a clear surge as in the controls, and "peak" height was reduced. A suppression of the FSH surge occurring coincidentally with the LH surge after neutralizing LHRH prior to the onset of the LH surge, agrees with other results in the ewe (Narayana and Dobson, 1979) and rat (Koch et al., 1973; Arimura et al., 1974; Kerdelhue et al., 1976; Fraser, 1977; Blake and Kelch, 1981; Hasegawa et al., 1981) and with the effects of central nervous system depressants in the ewe (Webb et al., 1981). This clearly suggests that the surge of FSH which is coincident with the preovulatory LH surge occurs as a result of maintained secretion of LHRH during this time.

Administration of LHRH antibodies prior to the LH/FSH surges inhibits the second rise of FSH in the morning of estrus in the rat (Blake and Kelch, 1981; Hasegawa et al., 1981; H. M. Fraser, unpublished observations) and in the ewe (Narayana and Dobson, 1979). However, the suppression in FSH observed in the present work is less than in these reports. In fact, the results clearly showed an elevation in FSH output after neutralization of LHRH even though the two clear peaks observed in the controls did not occur. There may be a number of possible explanations for this phenomenon but we consider the most important to center around the influence of the negative feedback effect of ovarian steroids on the pituitary gland in this species. During the preovulatory phase of the estrus cycle in the sheep, plasma FSH levels decline as the concentration of the gonadal steroids increase in response to increased pulsatile secretion of LH (Baird and McNeilly, 1981). Administration of LHRH antiserum, by inhibiting this increase in LH, should result in a failure to sustain the increased gonadal inhibition on FSH secretion, leading to an increase in FSH output from the pituitary, probably in response to minimal LHRH stimulation remaining after immunoneutralization. This concept is strengthened when the mechanisms for the naturally occurring second FSH surge are considered. This rise in FSH occurs at a time of apparent minimal LHRH secretion since pulsatile secretion of LH is dramatically reduced (Baird and McNeilly, 1981) and LHRH antiserum administered after the LH/FSH surge but before the second FSH rise does not abolish the second rise in the rat and ewe (Narayana and Dobson, 1979; Blake and Kelch, 1981; Hasegawa et al., 1981), suggesting it is not controlled by LHRH. Further, as gonadal steroid secretion is at a minimum at this time (Baird et al., 1981), this again suggests that the increase in FSH is related to a removal of a negative influence of gonadal steroids or other factors such as "inhibin" (Baird and McNeilly, 1981).

During the 3–16 days after administration of LHRH antiserum no effects on plasma FSH levels were observed, while LH was reduced. This was also the case after active immunization against LHRH in the ewe (Clarke et al., 1978). Again it may be possible to explain the presence of basal amounts of FSH on the basis of removal of the negative feedback effects of ovarian steroids and small amounts of LHRH escaping neutralization. However, LHRH must still be the primary controller of FSH release in the ewe when large amounts of hormone are required to be released, as in the FSH surge coincident with the LH surge, and in response to ovariectomy as plasma FSH levels are not elevated when ewes actively immunized against LHRH are ovariectomized (Clarke et al., 1978).

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# Differential effects of LH-RH immunoneutralization on LH and FSH secretion in the ewe

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**Summary.** Neutralization of LH-RH by injection of an ovine antiserum to LH-RH in ewes during the late follicular phase of the oestrous cycle resulted in an immediate blockade of pulsatile secretion of LH. Plasma concentrations of FSH gradually rose in the antiserum-treated ewes during the 36-h study period but levels declined in control ewes. These results show that, in the ewe, pulsatile LH secretion is dependent on LH-RH from the hypothalamus, while FSH is largely unresponsive to short-term reduction of LH-RH stimulation. Since reduction in LH secretion is likely to reduce ovarian function, the changes in FSH secretion may be attributed to the removal of a negative feedback influence of an ovarian factor, perhaps oestradiol, on FSH secretion.

## Introduction

Passive immunoneutralization of luteinizing hormone-releasing hormone (LH-RH) prevents the preovulatory surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the rat (Koch, Chobsieng, Zor, Fridkin & Lindner, 1973; Arimura, Debeljuk & Schally, 1974; Kerdelhue, Catin, Kordon & Jutisz, 1976; Kawakami & Higuchi, 1979), hamster (de la Cruz, Arimura, de la Cruz & Schally, 1976) and ewe (Narayana & Dobson, 1979; Fraser & McNeilly, 1982). However, little is known about the changes associated with neutralization of LH-RH on tonic gonadotrophin secretion in the intact female. In the present report, we investigated the effects of LH-RH antibodies on gonadotrophin secretion during the late follicular phase of the oestrous cycle in the ewe, with particular reference to the role of LH-RH in causing pusatile release of LH, and the differences in the control of LH and FSH. In addition, since administration of antibodies to LH-RH in the rat has been reported to cause prolonged cyclic failure associated with hyperprolactinaemia (Kerdelhue *et al.*, 1976), we examined the long-term effects of the antibodies on oestrous cycles and plasma prolactin concentrations.

## Materials and Methods

Eleven Scottish Blackface ewes (4–5 years old, 52–57 kg body weight) with regular oestrous cycles were treated with progestagen-impregnated vaginal pessaries (Chronogest, Intervet Laboratories, Cambridge, U.K.) to synchronize cycles during the mid breeding season in January. After 12 days the pessaries were withdrawn and a catheter ending in a three-way tap was inserted into the jugular vein and the ewes were transferred to individual crates indoors. At 24 h after pessary withdrawal blood samples were taken at 20-min intervals for 4 h. At this time 5 ewes were given an intravenous infusion of 100 ml ovine antiserum to bovine serum albumin over a 20-min period and 6 ewes were

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infused with 100 ml ovine antiserum to LH-RH. Blood samples were collected at 10-min intervals for a further 4 h and at hourly intervals until 36 h after antiserum injection.

The catheters were removed and the animals returned to the pasture. Blood samples were taken by jugular venepuncture on Days 3, 4 and 7 after withdrawal of progestagen and at intervals of 2–3 days for 87 days thereafter. Oestrus was detected by running the ewes with a raddled vasectomized ram until the end of the breeding season and to determine the onset of the following breeding season.

Plasma was stored at −20°C until required for radioimmunoassay of LH and FSH in the sequential blood samples and LH, FSH, prolactin, progesterone and LH-RH antibody titre in the daily samples.

*Antiserum*

The LH-RH antiserum used to treat intact ewes was a pool obtained from various blood samples from Ewe 39 immunized against LH-RH conjugated to BSA (Fraser, Clarke & McNeilly, 1981). The properties of this antiserum have been described previously (Lincoln & Fraser, 1979; Fraser & McNeilly, 1982). Control antiserum was obtained from a ewe immunized against BSA.

*Radioimmunoassays*

The concentration of LH was determined by radioimmunoassay (Martensz, Baird, Scaramuzzi & Van Look, 1976) of duplicate quantities of plasma and results were expressed in terms of ng NIH-LH-S14/ml. The sensitivity of the assay was 0·3 ng/ml and the intra- and inter-assay coefficients of variation were 8 and 10% respectively. FSH was measured in duplicate using the radioimmunoassay described by McNeilly, McNeilly, Walton & Cunningham (1976) and results were expressed as ng NIH-FSH-S10/ml. Assay sensitivity was 20 ng/ml with intra- and inter-assay coefficients of variation being 9 and 12% respectively. Prolactin was measured in duplicate quantities of plasma by radioimmunoassay (McNeilly & Andrews, 1974) and results were expressed in terms of ng NIH-PRL-S6/ml. This assay had a sensitivity of 0·05 ng/ml and intra- and inter-assay coefficients of variation of 8 and 11% respectively. Progesterone was measured by radioimmunoassay as described previously (Scaramuzzi, Corker, Young & Baird, 1975) with a detection limit of 0·2 ng/ml and intra- and inter-assay coefficients of variation of 10 and 12% respectively. LH-RH antibody titre of the plasma from the ewes after passive immunization was assessed as before (Clarke, Fraser & McNeilly, 1978) and expressed as the initial dilution of plasma binding 33% of a constant amount of $^{125}$I-labelled LH-RH.

*Statistical analysis*

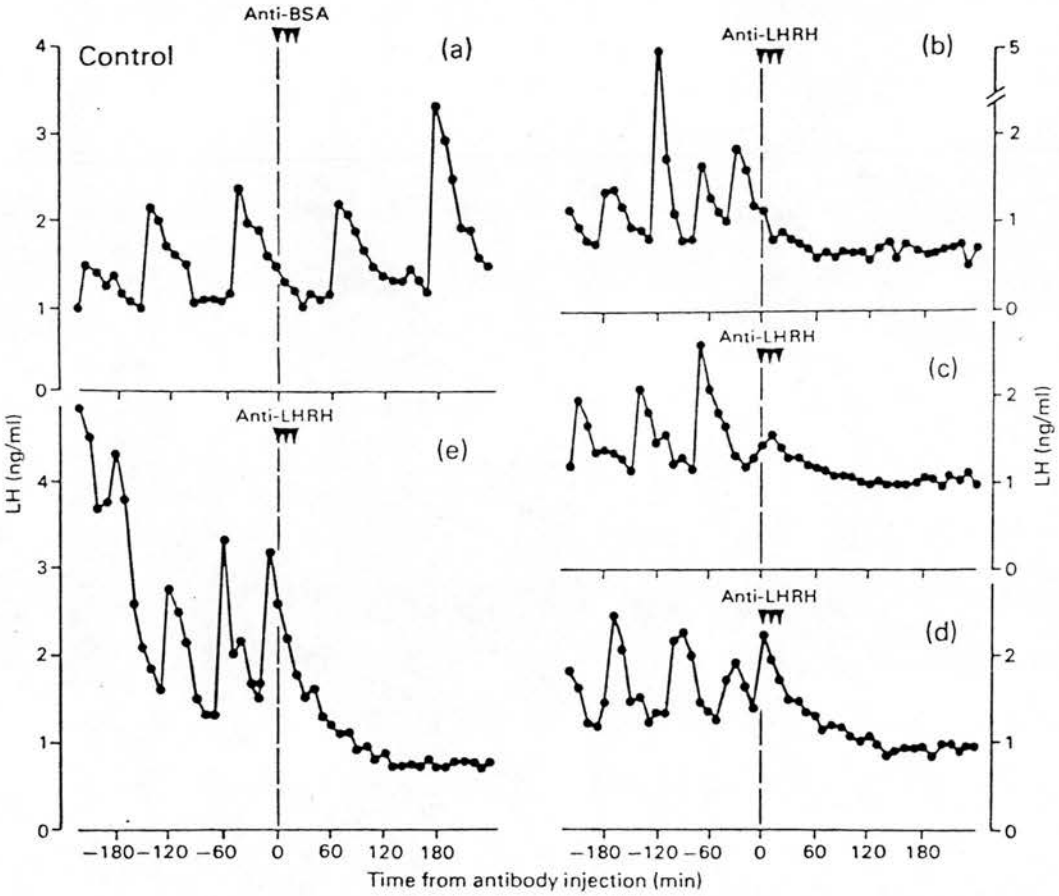
A rise in LH was considered to be a pulse if the value of two consecutive samples was greater than the mean of the two previous samples (basal value) and the value of at least one of the peak samples exceeded the mean basal value by more than twice the co-efficient of variation of the assay (Bäckström, McNeilly, Leask & Baird, 1982).

Because of considerable individual variation between animals, plasma concentrations of FSH were assessed as the percentage change from the mean of the 4-h preinjection value for each ewe. Differences in FSH between control and antibody-treated animals during the period 5–30 h after antibody injection were analysed by Student's *t* test using the mean value over this time period for each individual ewe. Student's *t* test was also used for analysis of the long-term effects on LH and FSH.

## Results

*Short-term effects on LH and FSH secretion*

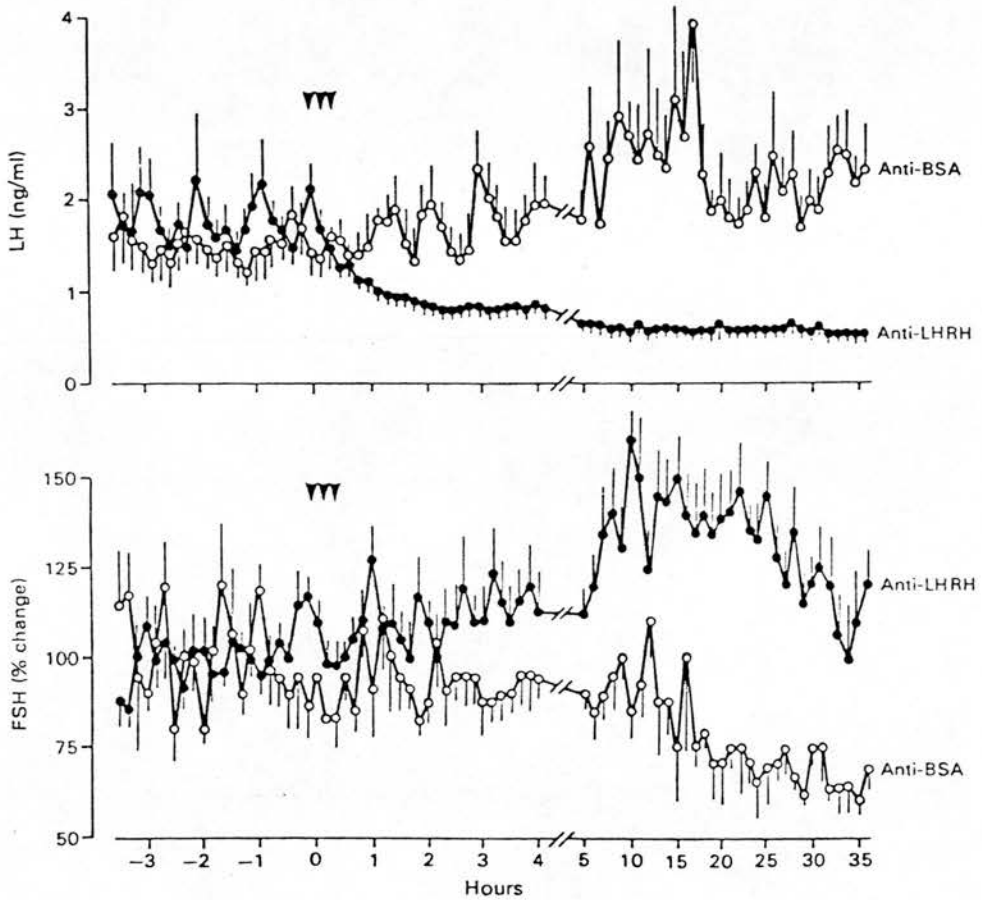
The effects of antiserum injection on pulsatile LH release in the intact ewes are shown in Text-fig. 1. In all ewes clear LH pulses occurred at a frequency of one per 60–80 min. In the 5 control ewes, injection of antiserum to BSA had no effect on LH pulses while in all 6 ewes treated with the LH-RH antiserum LH pulses were abolished. Because of the high frequency of withdrawal of blood samples we observed the effects of the antibody on different stages of an LH pulse (Text-fig. 1). In Text-fig. 1(b), for example, the LH pulse appeared to be due just at the time of antibody administration but the expected pulse failed to occur, indicating that the antibody had intercepted the LH-RH from the hypothalamus, causing an immediate neutralization of its action.




**Text-fig. 1.** Effect of an i.v. injection of (a) antiserum to BSA (control) or (b, c, d, e) antiserum to LH-RH on plasma LH concentrations during the follicular phase of the oestrous cycle in the ewe. The LH-RH antiserum injection in the 4 treated ewes is at different times in relation to the LH pulse.

Mean values for plasma LH and FSH concentrations during the 36-h period after antibody injection are shown in Text-fig. 2. In the control animals LH pulses continued while in the LH-RH antibody-treated ewes LH pulses were abolished. In contrast, the antibodies to LH-RH did not cause a decline in pituitary output of FSH during the 36-h sampling period. The mean ± s.e.m. plasma concentrations of FSH representing the 100% preinjection values were 89 ± 15 ng/ml for

controls and 70 ± 8 ng/ml for the treated ewes. In the controls, FSH concentrations gradually declined during this period (1–2 days after progestagen withdrawal) while in the LH-RH antibody-treated animals there was a small increase in FSH concentrations during the period 5–30 h after antibody administration (Text-fig. 2). During this period, plasma FSH levels in LH-RH antibody treated ewes were significantly higher ($P < 0{\cdot}01$) than in controls ($t = 3{\cdot}7$; d.f. = 9).

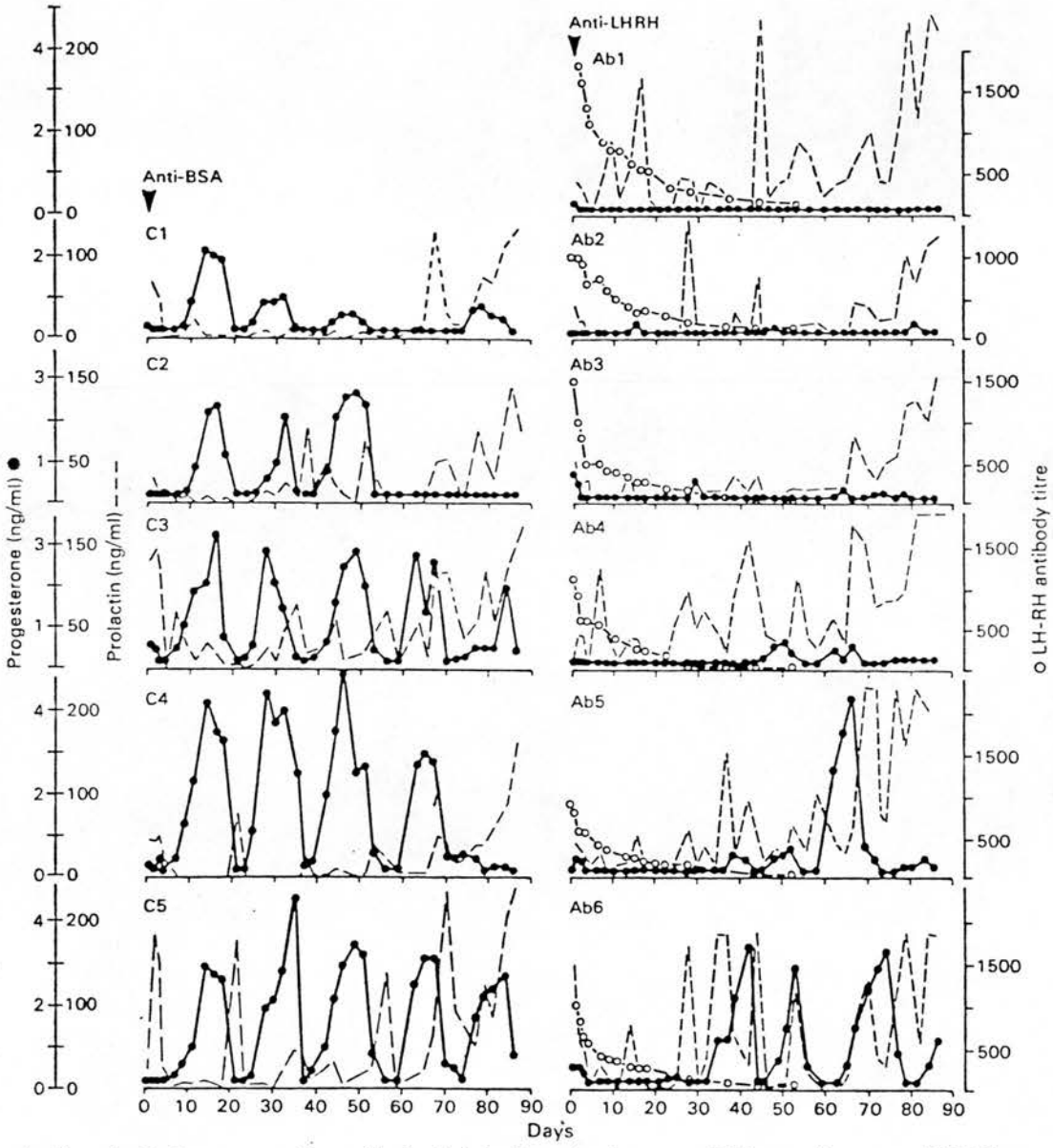



**Text-fig. 2.** Effects of an i.v. injection (arrows) of antiserum to BSA (control) or antiserum to LH-RH on the mean plasma concentrations of LH and FSH during the follicular phase of the oestrous cycle in the ewe. The oestrous cycles had been synchronized using progestagen-impregnated pessaries which were removed 28 h before administration of antiserum. Values are mean ± s.e.m. for 5 control ewes and 6 LH-RH antibody-treated ewes.

*Long-term effects of LH-RH immunoneutralization*

All control animals demonstrated a clear elevation of plasma progesterone beginning 7–9 days after progestagen withdrawal, indicating that a normal ovulation had occurred (Text-fig. 3). In contrast, none of the treated ewes showed a rise in progesterone. Ovulation was presumably preceded by an LH surge in control animals but this was only detected in one ewe on Day 4, probably occurring in other control ewes on Days 5 and 6 when blood samples were not taken. An earlier onset of the LH surge which would have been detected during our study period had been anticipated but this may have been delayed by use of progestagen. However, we have already established that the LH surge is prevented in the ewe by this antiserum (Fraser & McNeilly, 1982).

Behavioural oestrus was not studied before the ovulation immediately after treatment, but all control ewes demonstrated oestrus 20–22 days after withdrawal of the progestagen pessary, while none of the LH-RH antiserum-treated ewes came into heat during this period.




**Text-fig. 3.** Long-term effects of a single injection of antiserum to BSA or antiserum to LH-RH on plasma levels of progesterone, prolactin and LH-RH antibody titre. Results for the individual animals in each group have been arranged according to descending order of cyclic activity.

The control ewes continued to cycle for various times until the end of the breeding season, 4 of the 5 ewes having at least 2 further normal cycles (Text-fig. 3). In the treated ewes, LH-RH antibody titre declined rapidly during the first 7 days after administration, this period representing the approximate half-disappearance time in the circulation. Thereafter levels fell more slowly,

reaching titres of $<1:100$ by 30–40 days (Text-fig. 3). In 3 of the 6 ewes there was no evidence of return of ovulation during the remainder of the breeding season. Ewe 4 had progesterone levels indicating 2 ovulations with an inadequate luteal phase, ewe 5 showed evidence of an inadequate CL followed by a normal cycle, while the remaining animal began normal cycles by Day 32 (Text-fig. 3). LH levels remained suppressed ($P < 0{\cdot}05$) during the first 3 weeks after antibody treatment, but then returned to within the normal range. Plasma concentrations of FSH were similar in both groups during this period (data not shown).

Control ewes demonstrated elevations of plasma prolactin on a number of occasions, most often before ovulation (Text-fig. 3). Ewes treated with LH-RH antiserum also showed variable elevations in prolactin concentrations associated with anovulation. These elevations were not sustained nor did they bear any obvious relation to whether or not cycles were re-established. The onset of the following breeding season was similar in both groups, all ewes having demonstrated behavioural oestrus by October.

## Discussion

By taking sequential blood samples at 10-min intervals in ewes producing LH pulses at 60–80-min intervals we have been able to demonstrate conclusively that i.v. injection of antibodies to LH-RH leads to the immediate neutralization of LH-RH. In one ewe, the antibodies were administered when a pulse of LH was imminent. The fact that the LH pulse was not observed strongly indicates that the antibodies intercepted and neutralized a pulse of LH-RH which would have preceded it. These results confirm and extend previous studies on the effects of immunoneutralization of LH-RH on LH pulses in the ram (Lincoln & Fraser, 1979), ovariectomized rats (Snabes & Kelch, 1979) and castrated rat (Ellis, Desjardins & Fraser, 1983). This conclusion is supported by the demonstration that in ovariectomized ewes LH pulses are preceded or accompanied by LH-RH pulses measured by radioimmunoassay of hypophysial portal blood (Clarke & Cummins, 1982; Levine, Pau, Ramirez & Jackson, 1982).

The fact that the LH-RH antibodies failed to reduce secretion of FSH during the 36-h study period shows that, unlike LH, secretion of FSH is not dependent on short-term changes in LH-RH release. Similar effects have been observed in other situations. Although injection of LH-RH antibodies before the preovulatory gonadotrophin surges in the rat and ewe will prevent the surge of FSH as well as LH (Koch *et al.*, 1973; Arimura *et al.*, 1974; Narayana & Dobson, 1979; Blake & Kelch, 1981; Hasegawa, Miyamoto, Yazaki & Igarashi, 1981; Fraser & McNeilly, 1982), the second rise in FSH occurring in the rat on the morning of oestrus and in the ewe cannot be prevented by LH-RH antibody administration after the first FSH surge (Narayana & Dobson, 1979; Blake & Kelch, 1981; Hasegawa *et al.*, 1981). Also, plasma FSH levels began to rise after prevention of the preovulatory LH and FSH surge in the ewe (Fraser & McNeilly, 1982). After injection of LH-RH antibodies in the ram, no decline in plasma FSH concentrations was detected during the 24-h study period, despite an immediate fall in LH (Lincoln & Fraser, 1979). In the male rat, serum FSH values did decline 24 h after LH-RH antibody administration, but the fall was slower and less pronounced than for LH (Fraser, Sharpe, Lincoln & Harmer, 1982). Also, in the ovariectomized rat, rhesus monkey and ferret treated with LH-RH antibodies, FSH concentrations decline more slowly than do those of LH (Koch *et al.*, 1973; McCormack, Plant, Hess & Knobil, 1977; Gledhill, Fraser & Donovan, 1982).

Maintenance of plasma FSH levels during this time may be due to a number of causes, including, (1) low values of LH-RH as a result of incomplete neutralization, (2) autonomous release of FSH, (3), a separate hypothalamic FSH-RH, or (4) changes in secretion of ovarian factors acting directly on the pituitary gonadotrophs during this period. It seems reasonable to assume that small

amounts of LH-RH will remain biologically active after injection of LH-RH antibodies. Although LH pulses cease, LH is still detectable in the blood and this may reflect low LH-RH stimulation, which also contributes to maintaining FSH release. While evidence for a separate hypothalamic FSH-RH is poor, the pituitary does appear to possess a degree of autonomous FSH release; for example, stimulation of gonadotrophin release by exogenous LH-RH in the ram leads to a rapid decline in plasma LH concentrations when the exogenous stimulus is withdrawn, while FSH values remain elevated for several hours (Lincoln, 1979). In the present study we consider the most important influence on the pattern of FSH observed to be the changes in the levels of ovarian factors which feedback on the pituitary gonadotrophs. This conclusion is supported by studies in the rat which showed that immunoneutralization of LH-RH failed to prevent the rise in serum FSH concentrations which occurs during the first few hours after ovariectomy (Kawakami & Higuchi, 1979).

The identity of the ovarian factor which suppresses FSH secretion remains to be established. Androgens do not seem to be of primary importance (Dobson & Ward, 1977) and oestradiol has been implicated as the most likely candidate by Radford, Nancarrow & Findlay (1978), who also observed maintenance of plasma FSH concentrations with suppressed LH levels after administration of an anaesthetic to ewes during the late follicular phase. In ovariectomized ewes administration of oestradiol suppressed FSH concentrations (Goodman, Pickover & Karsch, 1981), although these authors proposed that another factor, possibly "inhibin" (Cummins, O'Shea, Bindon, Lee & Findlay, 1983), also plays a role in the selective suppression of FSH during the normal cycle in the ewe. Our own studies show that oestradiol is clearly involved since results from similar experiments on ewes with ovarian transplants have shown that LH-RH immunoneutralization suppresses oestradiol-17β secretion (A. S. McNeilly, H. M. Fraser & D. T. Baird, unpublished observations). Studies of sheep pituitaries *in vitro* have shown that oestradiol-17β can have a direct inhibitory action on the secretion of FSH (Miller, Knight, Grimek & Gorski, 1977). Also, in ovariectomized ewes actively immunized against LH-RH and with suppressed plasma levels of FSH, these values were reduced even further by administration of oestradiol benzoate (Fraser *et al.*, 1981). Therefore, removal of this negative feedback by reduction in ovarian secretion of oestradiol by LH-RH immunoneutralization probably plays an important part in causing FSH concentrations to rise after LH-RH neutralization, while in control ewes the rising concentrations of oestradiol cause the decline in FSH.

The long-term suppression of ovulatory cycles that we have observed after a single administration of LH-RH antiserum in the present study would agree with similar observations in the rat (Kerdelhue *et al.*, 1976). In contrast, it has been found that, in similar experiments, ovulatory cycles occurred 1–2 weeks after LH-RH antiserum administration in the hamster (de la Cruz *et al.*, 1976), mouse (S. L. Laing, R. E. Gosden & H. M. Fraser, unpublished observations) and stumptailed macaque monkey (H. M. Fraser, unpublished observations). Such a rapid return of pituitary function would be expected from the time course of decline in LH-RH antibody titres observed in this and other studies and in the return of normal circulating gonadotrophin concentrations after 1–3 weeks in intact and ovariectomized animals (McCormack *et al.*, 1977; Lincoln & Fraser, 1979; Gledhill *et al.*, 1982; Fraser *et al.*, 1982). We are therefore unable to explain the present results on the basis of continued direct neutralization of LH-RH. As in the study of Kerdelhue *et al.* (1976), we also observed a tendency for hyperprolactinaemia in the LH-RH antibody-treated ewes, but this did not appear to be a sustained effect and may reflect some disturbance in neutronsmitter activity in response to LH-RH neutralization (McNeilly, 1980). Since the animals were approaching the period of natural anoestrus at this time, perhaps the hypothalamo–pituitary–ovarian axis was particularly sensitive to such disruption.

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# Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe

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**Summary.** In the adult non-pregnant ewe the secretion of FSH is sufficient to ensure a continuous growth and development of antral follicles to 3–5 mm size at all times. Further development and increased secretion of oestradiol through the final 72 h to ovulation depends on adequate stimulation by LH. During anoestrus and the luteal phase of the cycle LH pulses occur too infrequently to stimulate sufficient oestradiol to evoke an LH surge. Moreover, during the luteal phase progesterone secreted by the corpus luteum not only reduces the frequency of LH pulses but also inhibits the ability of oestrogen to evoke an LH surge. At the time of luteal regression the frequency of LH pulses increases to at least one per hour due to the fall in progesterone secretion. This change in pulse frequency of LH is associated with a decrease in the secretion of FSH, probably because of a direct inhibitory action of oestrogen on the anterior pituitary gland. The dominant follicle is probably relatively independent of circulating levels of FSH due to the high concentration of oestradiol and FSH within the microenvironment of the follicular cavity.

Once the oestrogen secretion achieves a certain level a preovulatory surge of LH (and FSH) occurs. Increased sensitivity of the anterior pituitary to LH-RH and increased secretion of LH-RH from the hypothalamus both play a part in producing the LH surge. The rise in prolactin at this time probably reflects a decrease in hypothalamic dopamine turnover which is necessary for maximum release of LH-RH.

The preovulatory LH surge initially stimulates and then totally inhibits further secretion of oestrogen and androgen from the ovulatory follicle. This suppression of steroid secretion is accompanied by a second peak of FSH at about the time of ovulation. The function of this second peak of FSH remains unknown although it may be responsible for the development of the large antral follicles which occur on Days 3 and 4. It is probably more important in those mammals like the rat and hamster which only form a functional corpus luteum if pregnancy occurs and in which oestrogen is necessary for implantation.

## Introduction

hough endocrinology is involved in the study of control mechanisms and the way ysiological systems adapt to change, most of our knowledge has been pieced together from periments involving cross-sectional measurements. For example, a study of the secretion of uitary gonadotrophins throughout the ovarian cycle was only possible by measuring the uitary content in groups of animals killed at various intervals (Greep, 1961). These oss-sectional studies established much of our basic knowledge but did not allow a study *in vivo* the dynamics of the endocrine system.

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In the past decade several advances in techniques have facilitated more detailed studies endocrine control mechanisms. In particular, the development of relatively simple, sensitive a specific assays for the measurement of the major reproductive hormones in samples of blood a urine has been particularly important in expanding the scope of experimental design. It is only years since the publication of the first chemical method for the measurement of oestroge (Brown, 1955); and the first method able to measure oestrogens in the blood of non-pregna women required 50 ml blood and had a productivity of 4 samples per week (Baird, 1968)!

The sheep has proved an ideal model with which to exploit the potential of these n experimental techniques. Of comparable size to man, it is large enough to withstand ser collections of blood from easily accessible veins for the measurement of hormones. It has ev been possible to relocate structures such as the ovary, uterus and adrenal to more accessil subcutaneous sites where their venous effluent can be collected more easily (Goding, Bai Cumming & McCracken, 1971). The sheep is tolerant of experimental procedures, includi surgery, and is free from many of the health hazards of other species, e.g. rhesus monkey. It h been possible to exploit genetic differences between breeds, e.g. in ovulation rate, to test c trolling physiological mechanisms. Of particular importance to the study of the reproduct system specific radioimmunoassays for all three pituitary gonadotrophins (FSH, LH a prolactin) have been available for at least 5 years (Cole & Cupps, 1977). These assays a sufficiently sensitive to detect small short-term changes in the concentrations of hormones and allow study of the dynamic relationship between the anterior pituitary gland and the gonad.

This paper reviews our knowledge of the control of follicular development and function (i hormone secretion) in cyclic sheep. Our knowledge of follicle growth and development is s based on cross-sectional studies (e.g. Brand & de Jong, 1973) although recent advances biophysics, e.g. use of ultrasound scanning, make serial longitudinal measurements of at lea large antral follicles a possibility (Hackeloer, 1977).

## Folliculogenesis

Follicular development or folliculogenesis is thought to start before birth and in most mamma to continue throughout life. Once recruited from the pool of primordial follicles developme continues until either atresia or ovulation occurs (Peters, Byskov, Himelstein-Braw & Fabe 1975). However, the rate of division of cells (mitotic index) within a follicle varies depending its stage of development and hence the rate of growth of a follicle is not constant. I incorporating labelled thymidine, it has been found that it takes approximately 19–20 days f the mouse follicle to proceed through to ovulation once it enters the growth phase (Pete & Levy, 1966). Using less direct methods involving a study of the mitotic index of granulo cells, Turnbull, Braden & Mattner (1977) concluded that the smallest antral follic (approximately 0·2–0·3 mm diameter) in the sheep takes 25–35 days to become a matu preovulatory Graafian follicle (about 8 mm). The number of follicles entering the growth phase inversely related to the total number of oocytes in both ovaries (Mauléon & Mariana, 197 Although the number of small pre-antral follicles entering the growth phase in the remaini ovary is increased after unilateral ovariectomy, the total number when compared to t combined total of both ovaries is reduced (Dufour, Cahill & Mauléon, 1979). However, t percentage of antral follicles that become atretic is lower and so the number of large antr follicles, and hence ovulation rate, remains the same.

### *Gonadotrophin requirements for folliculogenesis*

Although initiation of follicle growth appears to continue following hypophysectomy, t number of non-atretic pre-antral and antral follicles is markedly reduced (Dufour *et al.*, 197

ius at some point in their early development (probably 0·06–0·07 mm diameter) follicles quire pituitary gonadotrophins without which further development is severely impaired or ases altogether. Once an antrum is formed (in the sheep 0·2–0·3 mm diameter) withdrawal of nadotrophins leads to immediate atresia.

Even in the presence of gonadotrophins most antral follicles become atretic and only a small inority survive to ovulate. Whether an individual follicle becomes atretic or not depends on its nsitivity to gonadotrophins as well as on the amount of gonadotrophins secreted by the tuitary. Administration of exogenous FSH or PMSG increases the ovulation rate by creasing the proportion of large antral follicles which become atretic (Peters *et al.*, 1975; auléon & Mariana, 1977). It is a reasonable assumption, therefore, that follicles become retic because they receive inadequate amounts of FSH or lack the ability to respond to FSH.

The sensitivity of individual follicles to gonadotrophins is probably determined by the mber of receptors for LH, FSH and possibly prolactin (Richards & Midgley, 1976). In turn, e development of gonadotrophin receptors is influenced by the local concentration of steroid rmones within the follicle. For example, oestradiol enhances the sensitivity of the granulosa lls to FSH and together with FSH generates LH receptors. On the other hand, it is known, at ist for rats, that androgens (particularly dihydrotestosterone) hasten atresia (Louvet, Harman, hrieber & Ross, 1975). The limited data which are available on the concentrations of drogens and oestrogens in follicular fluid of sheep suggest that, as in women, there is great riation in the concentration of steroids in follicular fluid (Moor, Hay, Dott & Cran, 1978). In althy, non-atretic follicles the concentration of oestradiol is much higher than that of drogens while the reverse applies to atretic follicles. Thus, whether a follicle becomes atretic or t may depend on the oestrogen/androgen ratio in its microenvironment.

## Action of gonadotrophins

### *SH*

Classically, FSH stimulates the growth and development of egg-bearing follicles (Greep, 61). Undoubtedly FSH plays a crucial role in stimulating cell division and the mitotic index of anulosa cells grown in monolayer is increased when FSH is added to the culture medium. In ts, receptors for FSH are present on the granulosa cells of the smallest antral follicles while LH ceptors occur on theca cells and granulosa cells of the mature Graafian follicle (Richards & idgley, 1976). Both oestradiol and FSH are important in generating LH receptors on anulosa cells. In the absence of LH receptors granulosa cells do not luteinize and secrete ogesterone in response to an LH surge; instead those follicles without LH receptors on the anulosa cells become atretic.

Recent data confirm that a similar situation exists in the sheep (Carson, Findlay, Burger & ounson, 1979). LH receptors are present on the theca cells of follicles at all stages of velopment (even in early atresia) but are confined to the granulosa cells of large (4–6 mm) n-atretic follicles. Granulosa cells from non-atretic follicles bind FSH irrespective of size. The creased binding of both FSH and LH to large non-atretic follicles is due to the increased mber of receptors and increased number of cells rather than a change in the affinity of binding.

### *H*

The steroidogenic action of LH on the ovary is well established. Its main action is to increase roid secretion by stimulating the conversion of cholesterol to pregnenolone (Marsh, 1976). ie initial steps involve interaction with the receptor on the cell membrane, the activation of the enyl cyclase enzyme and the production of cyclic AMP. Because this early step in steroid nthesis is stimulated by LH, it is hardly surprising that all steroids on the biosynthetic pathway

from pregnenolone to androgens and oestrogens are increased following administration of L
(McCracken, Uno, Goding, Ichikawa & Baird, 1969).

A certain basal level of LH is necessary to maintain steroid secretion by all cell types in th
ovary. Infusion of LH antiserum causes swift cessation of progesterone secretion from th
corpus luteum (Fuller & Hansel, 1970; McCracken, Baird & Goding, 1971). LH, like mar
other pituitary hormones, is released in pulses so that the concentration in blood is not consta
(Yuthasastrakosol, Palmer & Howland, 1977). Each pulse of LH is followed within 10 min by
rapid increase in the secretion of oestradiol and androstenedione from the follicle (Bair
Swanston & Scaramuzzi, 1976a). The rapid increase in androgen in response to LH is to b
expected for there are numerous LH receptors in the theca cells. However, the increase
oestradiol secretion implies that either the theca cell in the sheep can synthesize oestradiol or th
there is a very rapid transfer of androgen precursor from theca to granulosa cell. If the latter
correct it seems likely that the oestradiol secreted into the ovarian vein is produced by tho
granulosa cells lining the basement membrane which are nearest the capillaries supplying th
theca layer. In the rat these parabasal cells have in fact been shown histochemically to have
higher concentration of cytochrome P450 than those granulosa cells nearer the follicular antru
(Zoller & Weiss, 1978).

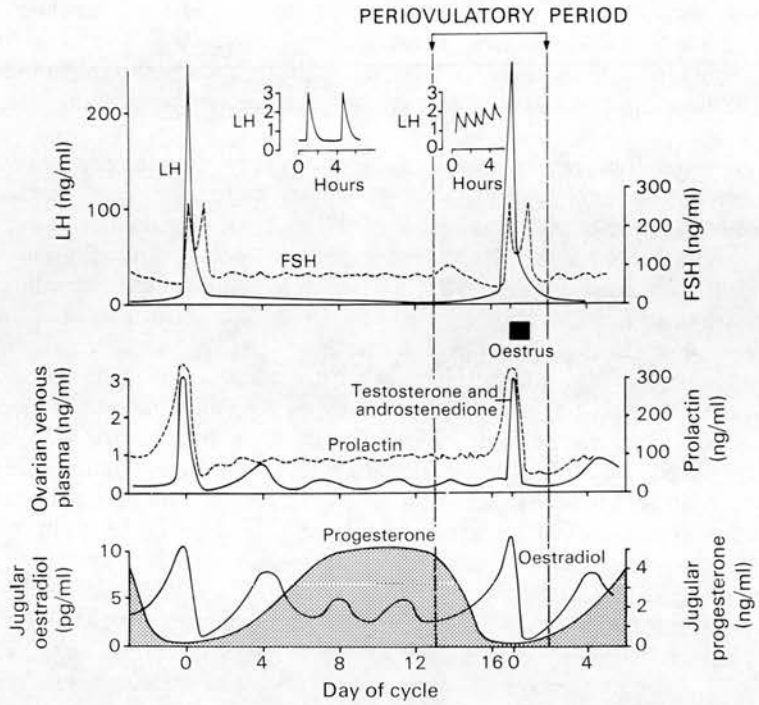
*Prolactin*

Serum levels of prolactin increase after luteal regression in the ewe and remain elevated un
the end of the ovulatory surge of LH (Reeves, Arimura & Schally, 1970; Cumming, Brow
Goding, Bryant & Greenwood, 1972; Kann & Denamur, 1974; Text-fig. 1). This increa
appears to be related both to the rise in the concentration of oestradiol and to a decrease
hypothalamic dopamine turnover necessary to allow the increase in pulse frequency of LH-R
and LH release occurring at this time (McNeilly, 1980). During the luteal phase prolactin leve
remain low without significant variation.

The action, if any, of prolactin in development of the follicle in the sheep is not clea
Suppression of prolactin with ergocornine hydrogen maleinate or bromocriptine throughout t
oestrous cycle does not affect the occurrence of ovulation, ovulation rate, or corpus luteu
function (Louw, Lishman, Botha & Baumgartner, 1974; Niswender, 1974). It may therefore b
presumed that if prolactin is necessary for normal follicular development in the ewe, the circ
lating levels required are considerably lower than occur spontaneously and at present no cle
role for prolactin in follicular development in the ewe has been demonstrated. On the other han
prolactin is required together with LH for the maintenance of the corpus luteum (Denamu
Martinet & Short, 1973).

High circulating levels of prolactin are associated with the cessation of ovulation duri
lactational and seasonal anoestrus (Kann & Martinet, 1975; Walton, McNeilly, McNeilly
Cunningham, 1977) and with low levels of progesterone during early pregnancy induced b
PMSG treatment at the end of the breeding season (Rhind, Chesworth & Robinson, 197
Whether the increased levels of prolactin alone are directly implicated in the reduction of ovari
activity is unclear. Suppression of prolactin levels with bromocriptine during anoestrus does n
result in (1) a return of oestrous cycles (B. P. Fitzgerald & F. J. Cunningham, person
communication), (2) an increase in the number of intact ewes showing positive feedback
response to oestrogen (Land, Carr, McNeilly & Preece, 1980), or (3) the number of ew
ovulating in response to LH-RH treatment or their subsequent luteal function (McNeilly
Land, 1979).

In contrast, ovulation and normal luteal function can be induced during anoestrus, wh
prolactin levels are elevated, by the injection of LH in amounts sufficient to mimic a normal L
pulse and given at a frequency similar to that seen during the preovulatory period of a norm
oestrous cycle (A. S. McNeilly, M. A. O'Connell & D. T. Baird, unpublished). This suggests th
high levels of prolactin do not significantly affect folliculogenesis provided the follicles recei




**Text-fig. 1.** Hormone changes throughout the oestrous cycle of the ewe. The gonadotrophins are the mean of several published series (Goding *et al.*, 1973; Salamonsen *et al.*, 1973; Pant *et al.*, 1977). The concentrations of oestradiol and progesterone in jugular venous plasma are those published by Hauger *et al.* (1977). The concentration of androstenedione and testosterone in ovarian venous plasma are from Baird *et al.* (1976b, 1981). The inset is to represent the rise in basal level and increased frequency of LH pulses in the follicular phase of the cycle.

quate gonadotrophic support. Nevertheless, TRH-induced hyperprolactinaemia during the ovulatory period may result in a reduced secretion of oestradiol in some ewes without ecting LH pulsatility (McNeilly & Baird, 1977).

At present the evidence suggests that prolactin is probably required for normal follicular elopment and is certainly necessary for normal corpus luteum function.

## Control of gonadotrophin secretion

synthesis and secretion of gonadotrophins from the anterior pituitary gland is stimulated by -RH released from the hypothalamus into the hypothalamic–hypophysial portal vessels nk, 1979).

There is thought to be only one releasing hormone (LH-RH) for gonadotrophins. The ount of gonadotrophin secreted is a function of the amount of LH-RH reaching the pituitary nd and the responsiveness of the anterior pituitary to LH-RH. Ovarian steroids influence the othalamus and pituitary to modulate the secretion of gonadotrophins. Because it is not sible to monitor the electrical activity of LH-RH-secreting neurones or to measure LH-RH in tal blood, changes in hypothalamic activity can only be inferred from changes in the uency of LH pulses. LH is released in pulses at intervals ranging from one per 24 h in estrus (Yuthasastrakosol *et al.*, 1977) to more than one every hour during the late follicular se of the cycle (Baird, 1978a). Each pulse of LH is presumed to represent the response of the

anterior pituitary to a pulse of LH-RH. Neutralization of LH-RH with specific antiseru immediately blocks LH pulses (Clarke, Fraser & McNeilly, 1978). Thus steroid hormor influence the secretion of gonadotrophins by changing the frequency and magnitude of release LH-RH from the hypothalamus as well as altering the responsiveness of the pituitary gland LH-RH.

Oestradiol is probably the most important hormone in regulating the release of LH and FS After ovariectomy or immunization against oestradiol there is a very marked rise in t concentration of both gonadotrophins. Because of the inability of immunized ewes to respond oestrogen and produce a surge of LH, ovulation does not occur although luteinized cysts a common (Pant, Dobson & Ward, 1978). However, during the breeding season t post-castration rise of LH cannot be prevented fully by the administration of oestradiol alone physiological amounts (Legan, Karsch & Foster, 1977). The addition of progesterone amounts normally secreted by the corpus luteum are necessary if luteal phase levels of LH are be achieved (Legan & Karsch, 1979; Karsch, Legan, Ryan & Foster, 1980). Progesterc causes a marked reduction in the frequency of LH pulses although their amplitude increas Thus the secretory products of the follicles (oestradiol) and the corpus luteum (progesterone) a necessary for the control of tonic LH secretion during the luteal phase of the oestrous cycle.

The effects of steroids on FSH secretion have been less well studied. In the ovariectomiz ewe FSH is inhibited by oestradiol but not progesterone although a 2–3-week treatment perioc required for physiological amounts of oestradiol to reduce FSH values to levels similar to those intact animals (Cumming *et al.*, 1974). Oestradiol and large amounts of LH-RH will also indu the release of FSH under the same conditions as LH is released (Jonas *et al.*, 1973). Howev smaller pulses of LH are not always accompanied by release of FSH, suggesting that, as in t ram, the relative proportion of LH and FSH may be determined by both the frequency a amplitude of LH-RH pulses (Text-fig. 2).

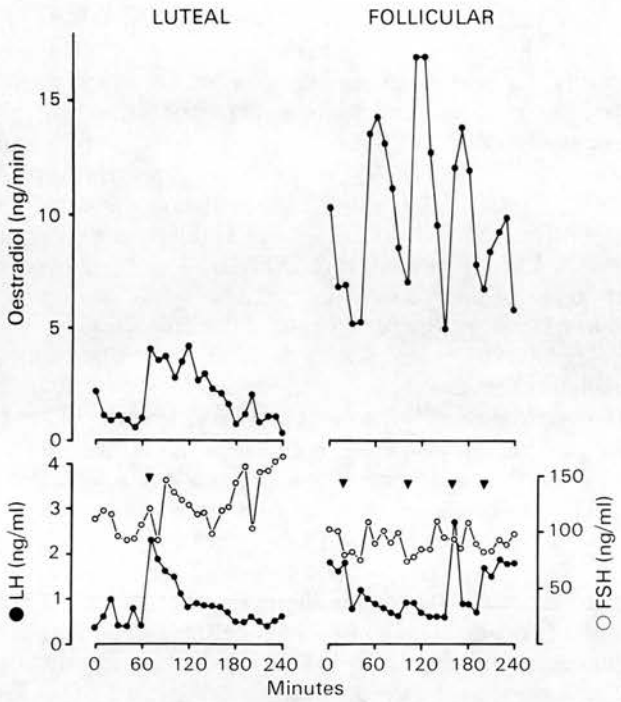
Experiments with ewes suggest that androgens may play some role in regulating the secreti of gonadotrophins. Immunization against androstenedione results in multiple follicu development and an increase in ovulation rate (Scaramuzzi, Davidson & Van Look, 197 Although the secretion of LH is elevated the concentration of FSH is suppressed (Martensz Scaramuzzi, 1979). The reason for the suppression of FSH is probably the increased ovari secretion of oestradiol due to multiple development of non-atretic large follicles (Scaramuz Baird, Clarke, Martensz & Van Look, 1980). These immunized ewes exhibit many of t endocrine characteristics of the high fecundity breeds, e.g. decreased sensitivity to the feedba effects of oestrogen, implying that androstenedione may normally modulate the sensitivity of t hypothalamic–pituitary unit to the feedback effect of ovarian steroids.

## Hormones and follicular function throughout the oestrous cycle

It is convenient to divide the 17-day cycle (Day 0 = oestrus) into the luteal phase lasting fr Day 2 to 13 and the peri-ovulatory period from Day 14 (oestrus −3) to Day 1 (Text-fig. 1).

### *Luteal phase*

During the luteal phase the corpus luteum secretes increasing quantitities of progesterone that its concentration in peripheral plasma reaches a plateau between Days 6 and Consistently one or two large follicles develop on Day 2 or 3 as indicated by a rise in t secretion of oestradiol (Holst, Braden & Mattner, 1972). Following this peak the secretion oestradiol and androgens continues to fluctuate as follicles develop up to 4 mm diameter befc becoming atretic. Although the concentration of FSH shows no consistent trend at this time, secretion of LH gradually declines. Closer inspection of the pattern of LH concentration reve




**Text-fig. 2.** The concentration of LH and FSH in jugular venous plasma and the ovarian secretion rate of oestradiol in a ewe with an ovarian autotransplant to the neck. Samples of blood were collected every 10 min for 4 h from 10:00 to 14:00 on Day 10 of the oestrous cycle (luteal) and 24–28 h after luteal regression had been induced by the injection of 100 μg of a prostaglandin analogue (cloprostenol; ICI) (follicular). Note the greater secretion of oestradiol in the follicular phase associated with an increased frequency of LH pulses (▼). (Data from Baird *et al.*, 1981.)

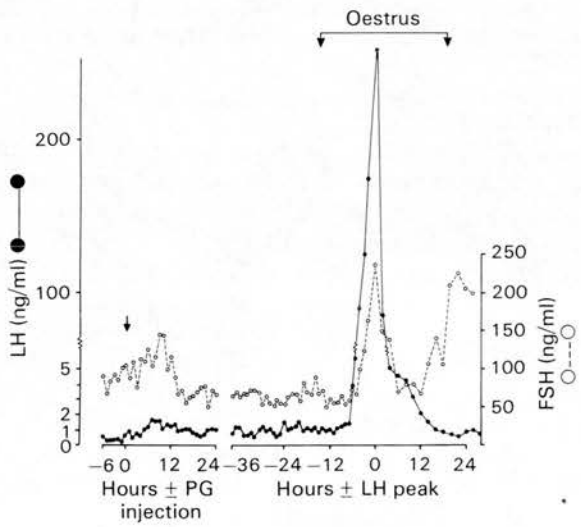
at LH is secreted in pulses which occur at intervals of approximately every 3 h during the teal phase. These pulses of LH stimulate secretion of oestradiol and androgens from the follicle aird *et al.*, 1976a). The suppression of the basal level and pulse frequency of LH by ogesterone during the luteal phase reduces the effective stimulation of oestrogen secretion from e follicle. Progesterone has an additional important effect on the uterus which requires a period priming of 7–10 days before it will synthesize adequate quantities of prostaglandin (PG) F-2α aird, 1978b). It is this feedback loop between the ovary and the uterus which regulates the ngth of the luteal phase. In the presence of the low basal levels of LH which exist by Day –13, the corpus luteum becomes increasingly sensitive to the luteolytic effect of PGF-2α nich is released in small amounts into the uterine vein. By Day 13 sufficient PGF-2α reaches e ovary via a counter-current transfer between the utero-ovarian vein and the ovarian artery to use a decline in secretion of progesterone. Further release of PGF-2α from the uterus is hanced by this decline in progesterone levels so that the whole cascade of events proceeds until nctional and eventually structural luteal regression is complete.

### *ri-ovulatory period*

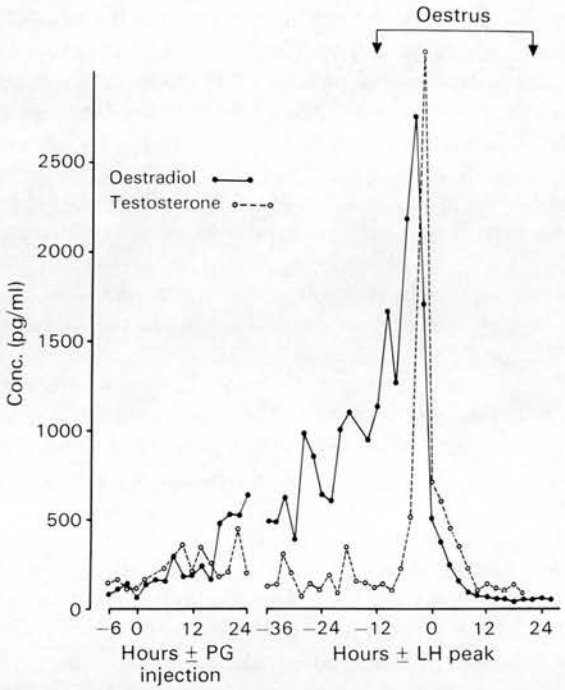
Because antral follicles (up to 5 mm diameter) are usually present in the ovary at all times ring the luteal phase and even during anoestrus, the factors regulating the final 2 or 3 days of llicular maturation of the ovulatory follicle are of particular interest. During the interval

between luteal regression and the end of oestrus, the dominant follicle(s) undergo a series structural and functional changes which culminate in ovulation (Hay & Moor, 1975). TH sequence of events during this peri-ovulatory period is similar whether luteal regression occu spontaneously or whether it is induced by surgical enucleation of the corpus luteum administration of exogenous PGF-2α (Baird & Scaramuzzi, 1976; Legan & Karsch, 197 Karsch, Foster Legan, Ryan & Peter, 1979). For convenience we have studied the hormo changes following luteal regression induced by the injection of the potent synthetic analogue PGF-2α, cloprostenol, on Day 10 of the cycle, as well as during the spontaneous cycle (Baird Scaramuzzi, 1975; Baird, Land, Scaramuzzi & Wheeler, 1976b). The endocrine events duri the peri-ovulatory period can be divided into three phases.

*Luteal regression to LH surge.* The period between luteal regression and the onset of the L surge is characterized by a striking increase in the secretion of oestradiol from the follicle( which is going to ovulate (Text-figs 1 and 2). The rise in secretion of androstenedione a testosterone is much less so that there is a progressive increase in the oestrogen to androgen rat (Text-fig. 4). This change in ratio is also found in follicular fluid (Moor *et al.*, 1978) and probab reflects the increasing utilization of androgen precursor as it is aromatized to oestrogen by t preovulatory follicle. The increasing secretion of oestradiol from the preovulatory follicle h three important effects—it stimulates further PGF-2α release from the uterus and hastens t onset of irreversible structural regression of the corpus luteum; it induces oestrous behaviou and it suppresses the secretion of FSH so that during the final 48 h before the LH surge the le of FSH falls significantly (Text-fig. 3). The follicle(s) destined to ovulate are probably protect from the deleterious effect of declining levels of FSH by the high intrafollicular concentration FSH and oestradiol. It is possible, however, that this decline in FSH before ovulation haste atresia in those large antral follicles which will not ovulate but which are very dependent gonadotrophic support.




**Text-fig. 3.** Mean concentration of LH and FSH in jugular venous plasma in 4 ewes with ovarian autotransplants injected with 100 μg cloprostenol (arrow) on Day 10 of the oestrous cycle. The samples have been grouped around the injection of cloprostenol or the LH peak. The mean interval from the injection to the LH peak was 60 h (range 54–69 h). Oestrous behaviour started at 48 h and extended to 79 h. Note the 5-fold rise in basal LH following luteal regression induced by injection of cloprostenol. LH expressed in ng NIH-LH-S14 and FSH in ng NIH-FSH-S10. (Data from Baird *et al.*, 1981.)




**Text-fig. 4.** Mean concentration of oestradiol and testosterone in ovarian venous plasma of 4 ewes with ovarian autotransplants. Note the marked rise in concentration of testosterone which occurs as the LH surge commences. See legend Text-fig. 3 for explanation of grouping of samples. (Data from Baird *et al.*, 1981.)

The question now arises—what is the stimulus for this final rise in secretion of oestradiol? here is strong evidence that the factor responsible is the rise in basal secretion of LH which curs in response to the decline in the concentration of progesterone associated with luteal gression (Baird & Scaramuzzi, 1976; Hauger, Karsch & Foster, 1977; Karsch *et al.*, 1979). If e fall in progesterone concentration is prevented by the insertion of a progesterone-releasing plant, the rises in basal LH and preovulatory oestradiol secretion are suppressed. LH mulates a rapid increase in the secretion of androgens and oestrogens when infused into the ary *in vivo* (McCracken *et al.*, 1969). Each spontaneous LH pulse is followed within 10 min an increased secretion of oestradiol from the ovary. Taken together with the known roidogenic effect of LH these results support the concept that LH stimulates preovulatory strogen secretion. Associated with the rise in basal LH concentration is a marked increase in e frequency of LH pulses (Baird, 1978a). By 24 h after the decline of progesterone secretion e pulse frequency has doubled to one pulse every 75 min. This increase in pulse frequency mulates the largest non-atretic antral follicle so that the secretion of oestradiol rises steeply. though the pulse frequency increases, the amplitude decreases, probably due to a direct effect the increasing levels of oestradiol on the anterior pituitary (R. L. Goodman & F. J. Karsch, published). In spite of the decrease in pulse amplitude, the ovary responds to each pulse of LH th a bigger increase in oestradiol secretion. This increase in ovarian sensitivity to LH is obably related to the increased number of LH receptors present on the theca cells of the large tral follicle (Carson *et al.*, 1979).

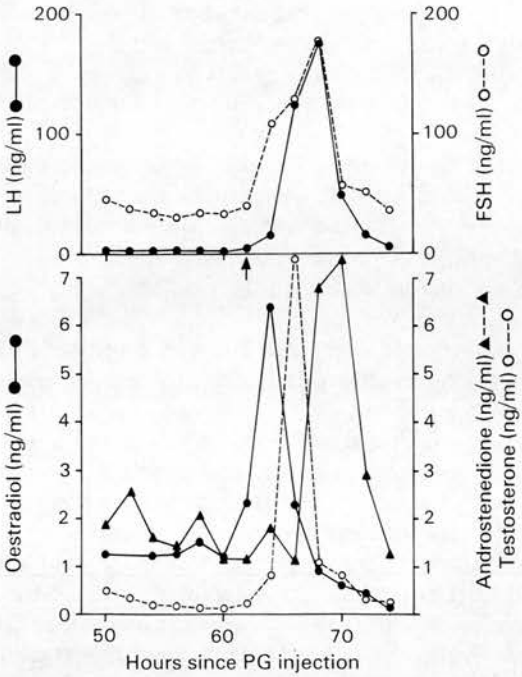
Further evidence of the importance of LH in stimulating the final maturation of the Graafian llicles comes from experiments in which LH or hCG was given to ewes during anoestrus egan & Karsch, 1979; Goodman & Karsch, 1980). In 4/7 ewes a rise in the secretion of

oestradiol, an LH surge and ovulation occurred following the infusion of hCG alone. When L was injected into anoestrous ewes repeatedly for 72 h in amounts to mimic the changes in L pulses which occur during the follicular phase of the cycle in the breeding season, hormo changes characteristic of the peri-ovulatory period occurred, i.e. rise in oestradiol secretion a fall in FSH concentration followed by a surge of LH and FSH (A. S. McNeilly, M. A. O'Conn & D. T. Baird, unpublished). These results indicate that during seasonal anoestrus ovulati does not occur in spite of the presence of large antral follicles because LH pulses occur t infrequently to stimulate oestrogen secretion to a level at which it will induce a preovulato surge of LH.

*Preovulatory LH surge.* Approximately 60 h after the initiation of luteal regression, at abo the time of the onset of behavioural oestrus, there is a marked rise in the concentration in plasn of both FSH and LH. We have defined the onset of this preovulatory LH surge as commenci when the concentration of LH reaches at least 5 ng/ml. There is strong experimental evidence f most species studied, including the sheep, that the preovulatory surge of LH and FSH stimulated by the rise in oestrogen secretion from the preovulatory follicle (Goding *et al.*, 197 It is likely that the positive feedback effect of oestradiol is exerted at the levels of both t hypothalamus and pituitary. By oestrus (Day 0) the pulsatile discharges of LH are occurring frequently (every 45 min or less) that it becomes difficult to distinguish individual pulses from t rising basal level of LH. At the same time the sensitivity of the anterior pituitary gland LH-RH is increased by oestrogen so that by Day 0 small amounts of exogenous LH-RH w release large quantities of LH (Reeves *et al.*, 1971). In the rhesus monkey positive feedback c be obtained by oestrogen acting on the pituitary gland alone (Knobil, Plant, Wildt, Belchetz Marshall, 1980). These monkeys in which endogenous LH-RH had been abolished b radiofrequency lesions in the arcuate region of the hypothalamus were maintained on a pul frequency of LH-RH (one per h) which closely approximates that observed during the fin stages of development of the preovulatory follicle in the sheep and women (Santen & Bardi 1973; Baird, 1978a). The preliminary evidence for sheep suggests that the increase in pul frequency of LH is essential if adequate follicular development and oestrogen secretion is to occ (A. S. McNeilly, M. A. O'Connell & D. T. Baird, unpublished). In sheep and women progestero decreases and oestradiol increases the frequency of LH pulses, indicating that und physiological situations these steroids act in part at the level of the hypothalamus (Santen Bardin, 1973; Legan & Karsch, 1979). Unfortunately, it is not yet possible to address the questions directly in the rhesus monkey because pulsatile release of gonadotrophins cannot b demonstrated in the intact animal (Knobil, 1980). Whether this is a true species difference (more likely) a limitation of the existing radioimmunoassays available for measurin gonadotrophins in the monkey is not yet known. However the fact that, in contrast to inta animals, progesterone is unable to inhibit the positive feedback effect of oestradiol hypothalamic-lesioned monkeys maintained on hourly injections of LH-RH indicates that least this steroid has an effect on hypothalamic activity as in other species (Wildt, Hutchiso Marshall & Knobil, 1980). It would be of interest to know whether normal cyclic activity of t ovary and positive feedback effect of oestradiol could be observed in lesioned anima maintained at an exogenous pulse frequency of $<1/h$.

The preovulatory surge of LH initiates a sequence of events in the ovary which lead t ovulation about 24 h later. There is an initial stimulation of the secretion of oestradiol a androgens by the dominant follicle(s) followed by a marked inhibition of steroid secretio (Text-fig. 5). The secretion of testosterone and androstenedione is increased to a greater exte than that of oestradiol so that the oestrogen/androgen ratio, which rises progressive throughout the follicular phase, very rapidly declines.

These paradoxical effects of LH, i.e. stimulation and inhibition of steroid secretion, appear be dependent on dose and duration. Infusion of LH into ewes on Day 10 of the oestrous cyc first stimulates and then inhibits oestrogen secretion from the dominant follicle (Baird, McNeill




**Text-fig. 5.** Concentration of oestradiol, testosterone and androstenedione in ovarian venous plasma and LH and FSH in jugular venous plasma of a ewe with ovarian autotransplant. The samples were collected between 50 and 76 h after the induction of luteal regression by the injection of 100 μg cloprostenol on Day 10 of the cycle. The start of the LH surge, defined as a concentration >5 ng/ml, is indicated by the arrow. (Data from Baird *et al.*, 1981.)

Connell & Swanston, 1980). The fact that the secretion of oestradiol is inhibited before that of drogen suggests that aromatase may be inhibited directly (Moor, 1974). However, the ntual total suppression of steroid secretion would be compatible with desensitization and loss LH receptors such as occurs in the rat (Webb & England, 1979). Probably both mechanisms involved.

The LH surge induces important changes in the morphology and structure of the granulosa s. In contrast to that for women and rats, there is very little cytological evidence of einization of granulosa cells before ovulation in the sheep (Bjersing *et al.*, 1972) although re is a slight increase in the concentration of progesterone in ovarian venous blood (Wheeler, rd, Land & Scaramuzzi, 1975) and follicular fluid in the 12 h prior to ovulation. The oplasm of the theca cells shrinks shortly after the onset of oestrus so that by ovulation the ca interna is insignificant (Bjersing *et al.*, 1972).

While the LH surge prepares the dominant follicle for ovulation, it probably has disastrous sequences for the remaining large follicles (>2 mm) which are already suffering from the tive decline in FSH. At ovulation all but one or two large follicles show prominent signs of esia. Administration of hCG together with PMSG increases the proportion of atretic follicles rnbull *et al.*, 1977). From experiments with rats it has been concluded that the increase in icular atresia produced by hCG is mediated by androgens (Louvet *et al.*, 1975). Probably the ca cells of those follicles which have not developed to the stage at which the granulosa cells tain adequate amounts of LH receptors are unable to luteinize in response to LH (hCG) and stimulated to produce androgens in large quantities.

*LH surge to ovulation.* Ovulation occurs approximately 24 h after the preovulatory LH

surge (Cumming *et al.*, 1971). During this time the secretion of oestradiol, androstenedione a testosterone decline rapidly so that at ovulation steroid secretion by the ovary is lower than any other time in the oestrous cycle. Although the concentrations of LH and prolactin al decline during this period, the concentration of FSH rises again to reach a peak comparable size to that occurring 24 h earlier.

The cause and function of this second peak of FSH remains unknown although it occurs many other species, e.g. rat and hamster. Primates including man are an exception and it may relevant that the decline in ovarian steroid secretion at ovulation in primates is not nearly complete as in other species. The primate corpus luteum secretes oestradiol as well progesterone early in its formation and helps to maintain a high concentration of ovari steroids (Baird, Baker, McNatty & Neal, 1975).

Whatever the mechanism it seems likely that this second peak of FSH is in some way relat to the events which follow the preovulatory LH surge. Infusion of exogenous LH in the ewe Day 10 of the oestrous cycle is followed within 24 h by a second peak of FSH (Baird *et a* 1980). As oestrogen secretion is inhibited following the infusion of LH, it is tempting to relate t rise in FSH secretion to the reduction in negative feedback effect of this steroid. However, similar experiments with rats, Chappel & Barraclough (1977) were unable to prevent the seco peak of FSH when steroid levels were maintained with oestradiol implants and concluded th the second peak of FSH was due to lack of ovarian 'inhibin' acting at the pituitary. In the e the first but not the second FSH peak can be inhibited by the administration of pentobarbitone antiserum to LH-RH (Dobson & Ward, 1977; Narayana & Dobson, 1979). During the seco FSH peak there is no coincidental rise in LH secretion and the frequency of LH pulses remai unaltered. All these experiments would point to a change in the sensitivity of the pituita gonadotroph to LH-RH as being responsible for the selective release of FSH at this time. Dire experiments with exogenous LH-RH would help to confirm this hypothesis.

It is hard to believe that the second FSH peak has no function in the ewe. In rats if its effect neutralized by the administration of antiserum to FSH, the number of follicles available f ovulation at the next oestrus is reduced (Sheela Rani & Moudgal, 1977). In sheep, because t second FSH peak is larger in breeds with high rates of ovulation, it has been suggested that determines the number of small antral follicles which are recruited for development and whi will eventually ovulate 17 days later (Cahill, 1979). However, it is difficult to reconcile th hypothesis with the fact that the rate of ovulation is apparently unaffected when the corp luteum is regressed prematurely at any stage after Day 4 (Bindon, Blanc, Pelletier, Terqui Thimonier, 1979). It is perhaps more likely that the two or three large antral follicles present Days 3 or 4 of the cycle are a result of the second FSH peak.

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*Extrait du* **Colloque de la Société nationale pour l'étude de la Stérilité et de la Fécondité - 1982**
**PROLACTINE, NEUROTRANSMISSION ET FERTILITÉ**

# PROLACTIN AND THE CONTROL OF OVARIAN FUNCTION

by

A.S. McNEILLY *

**RÉSUMÉ**

**Prolactine et régulation de la fonction ovarienne.** — Les études sur l'influence de la PRL sur la fonction ovarienne ont été principalement centrées sur la relation hyperprolactinémie/ovulation. Au cours du cycle ovarien normal, la PRL semble accroître le nombre des récepteurs LH des cellules de la granulosa des follicules en développement. Les taux de PRL diminuent dans le liquide folliculaire quand ceux de progestérone et d'œstradiol augmentent. La raison de ces modifications n'est pas élucidée. Après l'ovulation, la PRL participe avec LH à un *complexe lutéotrophique* bien qu'isolément la PRL n'ait pas d'effet stéroïdogénique.

En cas d'hyperprolactinémie, lorsque l'activité ovarienne est supprimée, la sécrétion des gonadotrophines et particulièrement de LH est déficiente. Des études *in vitro*, réalisées surtout sur les cellules de la granulosa du rat, ont montré que des niveaux élevés de PRL empêchaient l'induction de l'aromatase et donc la sécrétion d'œstrogène. Cependant, des études *in vivo* en cas d'hyperprolactinémie n'ont pas mis en évidence de baisse sévère de l'activité aromatase des cellules de la granulosa, ce qui suggère que les précédentes doivent être interprétées avec précaution. L'hyperprolactinémie pourrait entraîner une réduction de la sécrétion thécale d'androgènes en inhibant les effets de LH.

Cependant, les données disponibles tendent à indiquer que la suppression d'une activité ovarienne normale dans de nombreux cas d'hyperprolactinémie, tout particulièrement l'aménorrhée post-partum en cas d'allaitement, est due à une carence de stimulation de l'ovaire par LH, plutôt qu'à une action d'inhibition primordiale et directe de la prolactine sur l'ovaire.

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# INTRODUCTION

The role of prolactin in the control of ovarian function in the human has received increased attention since the purification of human prolactin in the early seventies. Most attention has been paid to the possible inhibitory effects of high circulating levels since it is those situations — both pathological and physiological hyperprolactinaemia — which are relevant to clinical management. As yet, no major clinical problems appear to be directly caused by either « normal » or abnormally low levels of prolactin. However, in order to understand the mechanisms whereby prolactin might disrupt normal ovarian activity there is a need to increase our knowledge on the normal mechanism of action of prolactin within the ovary. In addition it is clear that in many situations in which prolactin levels are increased there is also a decrease in gonadotrophin stimulation, usually Luteinizing hormone (LH) of the ovaries. Thus it is possible that many of the *in vivo* effects of prolactin are more correctly effects of reduced gonadotrophic stimulation to the ovary.

A more complicated problem is the relationship between responses *in vitro* to true physiological changes. Recently several papers have dealt with the actions of prolactin on ovarian follicular granulosa cells which have been maintained in short or long term culture (up to 10 days). The responses of these cells to various stimulators and the influence of prolactin on these responses have yielded data which appear to increase our understanding of how hyperprolactinaemia may work. However critical appraisal of these results in comparison to events *in vivo* cast some doubt on this suggestion.

Some of these problems will be discussed here.

## *Prolactin and normal ovarian function*

During the normal ovarian cycle in many of the species so far examined, an increase in levels of prolactin occurs around the time of the preovulatory LH surge with little change occurring at other times [1, 2]. This surge appears to occur not only as a result of the increase in œstrogen secretion from the preovulatory follicle but also because of a decrease in hypothalamic inhibition (presumably decrease in dopamine secretion) of prolactin secretion associated with the release of LHRH driving the LH surge [2, 3]. While the increase in prolactin is relatively large in some species e.g. rat and sheep, the rise in primates, especially man, is modest and its role, if any, in the ovary is unclear [2, 4]. However, during the development of the preovulatory follicle the levels of prolactin within the follicular fluid decline while those of both œstradiol and progesterone increase [5]. This observation, first made in the human has now been confirmed in the cow [6] and sheep [7]. The decrease in prolactin levels may be due to increased utilization since the number of prolactin receptors on the granulosa cells increase with follicle maturation [8], although precisely what role prolactin plays in the final stages of follicular development remains

unclear. Indeed a decrease in granulosa cell prolactin receptors has been reported in the pig [9].

Certainly in the human, prolactin appears to be essential for the maintenance of progesterone secretion by granulosa cells *in vitro* [10]. At the same time there are indications that prolactin may be involved in oocyte maturation, possibly in an inhibitory manner since not only does prolactin-like immunoreactivity disappears from the rat oocyte as it matures [11], oocyte maturation in the pig appears to be stimulated by incubation with antiprolactin antiserum [12].

There appears to be little data on effects of prolactin on the theca of developing follicles, although autoradiographical studies suggest that prolactin binding sites are present [13]. In the rat prolactin does not appear to affect androgen production by the theca [14] but there is some indication that in the cow, prolactin may initially enhance *in vitro* adrostenedione secretion. Clearly this area requires further investigation since the production of œstrogen from the follicle depends almost entirely on supply of androgen precursor from the thecal layer [15]. These alterations in androgen production by the theca are of critical importance to normal follicle growth and a proper understanding of the role, of prolactin, if any, is essential.

The amount of prolactin required for normal ovarian activity appears to be minimal since follicle development and ovulation continue normally in most cases when prolactin levels are suppressed pharmacologically (e.g. sheep [16]; cows [17]; women [18]) or in post-hypophysectomy states in both sheep [19] and women [20] where ovulation has been induced by gonadotrophin therapy. Nevertheless, there are reports that excessively low prolactin levels, resulting from bromocriptine therapy (e.g. [21]) may reduce luteal function in women. However, in view of the possible inhibitory effects of dopamine agonist on gonadotrophin secretion [3, 4] this may be due to a reduction in LH drive rather than lack of prolactin.

It is clear that, in almost all species studied, prolactin is essential for the normal functioning of the corpus luteum (see [22] for review). The situation may be similar in man since the human corpus luteum does contain prolactin receptors, at least in the early luteal phase [23]. However, as with the granulosa cells the exact mechanism of action remains to be elucidated. In the rat, prolactin acts to inhibit the reduction of progesterone to 20 $\alpha$-dihydroprogesterone and may prevent or delay prostaglandin-induced luteolysis [22]. Whether such actions occur in other species remains to be determined. One action which may be common among species is to increase or maintain the number of LH receptors on the luteal cells [8, 24] but in no case does prolactin appear to have any intrinsic capacity to stimulate steroidogenesis.

### *Hyperprolactinaemia and ovarian function*

Hyperprolactinaemic states are usually associated with anovulation and amenorrhoea. In the majority of cases pharmacological suppression of the elevated prolactin levels results in a resumption of normal cyclicity [25].

While in many cases the LH and FSH response to an LHRH challenge is within normal limits, this response may not be maintained to repeated challenges [26] suggesting that there is also an impairment of gonadotrophin secretion. This is further suggested by the observation that pulsatile secretion of LH is often reduced and is restored to normal by normalization of the prolactin levels [27]. Thus absence of ovarian activity may be due merely to an inadequacy of gonadotrophin drive at the level of the ovary and the elevated levels of prolactin may play no direct inhibitory role at ovarian level.

In support of this are the observations that even in hyperprolactinaemic states follicle growth, ovulation and normal corpus luteum can be induced by gonadotrophin therapy as exogenous hormone of LHRH treatment in women [e.g. 28], sheep [29] and monkeys [30], at levels similar to those in normoprolactinaemic states.

Similarly, in some species e.g. cow [31] and marmoset monkey [32], normal ovarian activity resumes in the early postpartum period even though hyperprolactinaemia is maintained.

Nevertheless, there are some indications that high levels of prolactin may be inhibitory directly at the ovarian level. During the resumption of ovarian activity in women postpartum follicular growth in terms of œstrogen secretion remains suppressed in spite of a return of an apparently normal pulsatile pattern of LH secretion an normal levels of FSH [4]. Resumption of follicle development is associated with a significant decline in prolactin levels[33, 34] in the absence of any significant change in gonadotrophin levels. This suggests the possibility that prolactin may act directly on the ovary to inhibit follicle growth. If this is the case how might it act ?

Studies with granulosa cells *in vitro* have shown that, in the rat, high levels of prolactin can inhibit the FSH-induction of aromatase in granulosa cells from immature ovaries [35] and reduce œstradiol secretion [36]. A similar suppression of œstradiol secretion with stimulation of progesterone production occurs *in vitro* for rat granulosa cells from Graafian follicles [37]. However, the majority of these effects in the rat occur around day 3 of culture, a time at which they will have undergone luteinization, both *in vitro* and *in vivo* if they had remained within their follicle. Thus the relevance of these studies to normal physiological situations remains unclear. A reduction of the aromatase system of granulosa cells in immature follicles would clearly lead to a reduction in cell division and a cessation of normal follicular development. However, in order to maintain pseudopregnancy or pregnancy in the rat [22] there has to be an increase in the secretion of prolactin to levels which should, according to the *in vitro* evidence, be inhibitory. In spite of this there is no deficiency in the aromatase system of follicles during pregnancy in the rat and stimulation of thecal androgen production by HCG will result in a continuation of normal follicular development and ovulation in spite of maintained hyperprolactinaemia [38, 39].

In recent studies in which prolonged hyperprolactinaemia was maintained by donor pituitary transplants under the kidney capsule, adult female rats continued to ovulate and exhibit pseudopregnant cycles event though there was some indication of a reduction in the follicular aromatase system [40]. In this study as in pregnancy hyperprolactinaemia was associated with a reduction in

gonadotrophin secretion. Thus the reduction in aromatase may be due to inadequate LH/FSH stimulation rather than to a direct effect of high levels of prolactin.

In the human, high levels of prolactin were shown to inhibit progesterone secretion by granulosa cells which luteinized *in vitro* [5]. However, such a suppression of progesterone was not seen when granulosa cells recovered from preovulatory follicles were used [41]. These cells were taken from follicles just before the time of ovulation and had been exposed to the endogenous preovulatory LH surge. Clearly this discrepancy in results required clarification in particular since it has been shown that in the human, follicles which have greater than normal follicular fluid levels of prolactin contain fewer than normal numbers of granulosa cells with reduced steroid capacity [42].

It is clear from the above discussion that most research has concentrated on prolactin effects on granulosa cells. Howerver, as pointed out earlier, the function of the granulosa cells is dependent on a supply of androgen precursor from the thecal layer, stimulated by LH. Thus it is possible that high levels of prolactin might prevent follicle maturation by interfering with LH stimulated androgen production. Indeed, *in vitro* studies with dispersed rat ovarian cells have shown that high levels of prolactin can prevent LH-stimulated androstenedione production although not until the 3rd-4th day in culture [14]. However, a more immediate reduction in androstenedione secretion was induced by infusion of high levels of prolactin in the follicular phase of the sheep œstrous cycle and resulted in a reduction in œstradiol secretion [4]. In spite of this, ovulation with normal luteal function occurred suggesting that a 10 to 20 fold increase in prolactin levels in the 40-70 hours prior to ovulation in the sheep at least, did not significantly alter follicle development of subsequent luteal function. It was clear, however, that LH secretion was unaffected by the infusion of prolactin suggesting that, if an adequate LH signal is maintained then the inhibitory effects of hyperprolactinaemia directly on the ovary (if any) can be overcome.

Such a suggestion may explain the failure of pulsatile secretion of LH to drive follicle growth in breast feeding women postpartum. It has been shown previously that breast feeding women are more sensitive to the negative feedback effects of œstrogen [43]. In addition it has been shown that, in spite of very low levels of œstrogen, LH levels remain at the lower limit of normal in breast feeding mothers who maintain adequate suckling stimulation and have absent follicle development. If gonadotrophin secretion was really normal it would be expected that these women, with low œstrogen levels, should have LH levels in the postmenopausal range. The fact that this is not the case suggests that LH secretion, even though it appears to be of a normal pulsatile nature [4], is in fact suppressed. Thus as a follicle develops in response to the pulsatile secretion of LH, the increase in œstrogen feeds back on the hypothalamo-pituitary axis and, because of the increase in sensitivity to negative feedback, switches off further LH secretion resulting in failure to maintain follicle growth. This continues while suckling occurs at a frequency sufficient to maintain hyperprolactinaemia. As suckling decreases, so prolactin levels decline suggesting a change in hypothalamic catecholamine status which appears to occur around the time that the increased sensitivity to negative

feedback of œstrogen is lost and positive feedback resumes [43]. It might be presumed therefore, that this alteration in the hypothalamus allows an increase in LHRH secretion sufficient to maintain pulsatile secretion of LH in spite of rising œstrogen levels as follicle development continues. Thus follicles will now receive continued LH stimulation sufficient to allow ovulation.

Nevertheless, first ovulatory cycles in women postpartum usually have inadequate of deficient luteal function [33, 44]. Since prolactin levels at this time are still elevated, it is possible that prolactin may play a role in the suppression of normal follicular development thus leading to inadequate luteal function.

## CONCLUSIONS

*1)* It is clear that in the majority of species examined, prolactin is an important part of the luteotrophic complex needed to maintain follicle growth and corpus luteum function. The precise mechanism by which it acts remains to be elucidated.

*2)* While hyperprolactinaemia is associated with reduced or absent follicle development, the involvement of prolactin by a direct inhibitory action on the ovary remains to be confirmed. Evidence suggests that in many instances apparent effects of hyperprolactinaemia may be more correctly assigned to a lack fo gonadotrophic, especially LH, stimulation.

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# THE GONADOTROPHINS AND THEIR SUBUNITS IN FOETAL PITUITARY GLANDS AND CIRCULATION.

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## SUMMARY

Specific radioimmunoassays and column chromatography on Sephadex G-100 have been used to assess the changes in foetal pituitary content and serum concentration of LH, FSH and LH β-subunit and the common α-subunit during gestation. All pituitary glands contained free α-subunit and intact LH. The free α-subunit: LH ratio reached a peak between 10 and 14 weeks of gestation and decreased to adult ratios by term. No FSH was detected in pituitary ($n = 7$) extracts from 9.5–12.5 weeks while FSH was detectable in all foetal pituitaries at term. No significant sex difference in intact hormone or subunit concentrations in the pituitary was seen. In contrast to these results the pituitary of an anencephalic foetus (36 week of gestation) contained mainly free α-subunit.

During early gestation foetal blood levels of LH-HCG, FSH and free α-subunit were significantly higher than at term. The immunoreactive LH-HCG consisted of both LH presumably from foetal pituitary origin and HCG.

These results suggest that during foetal development the first gonadotrophin substance synthetized in the foetal pituitary gland is the common α-subunit. Under hypothalamic influence the synthesis of the β-subunit then takes place leading to the production of the intact gonadotrophins.

## INTRODUCTION

The pituitary glycoprotein hormones, luteinizing hormone (LH), follicle stimulating hormone (FSH), thyrotrophin (TSH) and human chorionic gonadotrophin (HCG) produced by the placenta during pregnancy all consist of two non-identical smaller components, designated α- and β-subunit [1, 2]. The α-subunit has almost identical protein structure in all the hormones within any species; in contrast, the β-subunit is characteristic of a particular hormone and varies in structure from hormone to hormone [3–5].

The adenohypophysis develops early in embryonic life from an evagination of the ectoderm, Rathske's pouch, which extends towards the base of the brain where it undergoes proliferation. The presence of basophil cells with the capacity to synthetize and store protein hormones soon after the anatomical formation of the human anterior pituitary gland at 8 weeks of gestation has been demonstrated by various methods [6–8].

Immunoreactive LH and FSH have been detected in foetal pituitary extracts as early as 9.5–10 weeks of gestation—the youngest foetuses studied [9, 10]. Furthermore, from the 16th to 40th week of gestation foetal pituitary tissue cultured *in vitro*, secretes, in molar terms, about 200 times as much of α-subunit as intact LH and FSH, and only small amounts of the LHβ subunit [11]. That a considerable amount of gonadotrophic substance in the foetal pituitary gland is the common α-subunit has been confirmed by Hagen and McNeilly[10].

In this presentation we will review our findings and the evidence for the existence of free subunits in the human foetal pituitary gland and circulation. In addition, the physiological implications of these findings on the development of the hypothalamic-pituitary control mechanism will be discussed.

Radioimmunological determination of various proteins implies the use of different heterogenous standards, therefore, comparison of concentrations of different hormones will always be arbitrary. In addition, the limitation in measuring the hormone content of an endocrine gland and not the synthesis and secretion and the accuracy of the assessment of foetal age will affect the results.

## MATERIALS AND METHODS

*Pituitary glands*: Twenty foetal pituitary glands were obtained from patients undergoing hysterotomy during pregnancy at the age of gestation of 9.5–32 weeks, and pituitaries were removed within 30 min of incision of the uterus. Extraction of pituitary glands were performed as described [10].

*Serum measurements*: Blood samples were collected at the time of delivery from 26 foetal cord arteries and veins (period of gestation 37–41 weeks; 16

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Table 1. The specificity of the radioimmunoassays for LH, FSH, LHα and LHβ.

| Hormone or subunit preparation | Assay* | | | |
|---|---|---|---|---|
| | LH | FSH | LHα | LHβ |
| LH IRC2/69 (Dr. A. S. Hartree) | 100 | <1 | 8 | 25 |
| HCG CR115 (Dr. R. E. Canfield) | 100 | <0.1 | 4 | 0.2 |
| FSH CPDS6 (Dr. W. R. Butt) | 2 | 100 | 1 | <1 |
| LHα 4.1.72 (Dr. A. S. Hartree) | 25 | <1 | 100 | 1 |
| HCGα CR115 (Dr. R. E. Canfield) | 12 | <1 | 100 | <1 |
| LHβ 18.10.72 (Dr. A. S. Hartree) | 60 | <1 | <1 | 100 |

* Percentage inhibition.

females and 10 male foetuses) and from 17 early foetuses (period of gestation 10–16 weeks; 5 female and 11 male).

*Chromatography.* Pituitary extracts and serum samples were chromatographed on a Sephadex G-100 column (1.5 × 90 cm). The column was standardized with LH IRC2/69 (Dr. A. Stockell-Hartree), LHα (Dr. A. Stockell-Hartree) and HCG CR 115 (Dr. R. E. Canfield) as described [10, 12].

*Radioimmunoassays.* Serum LH, FSH, LHβ and the common α-subunit were measured by double antibody radioimmunoassays [10, 12, 13]. The specificity of the radioimmunoassays are shown in Table 1. In the LH assay equipotency on a weight basis of LH IRC2/69 (Dr. A. Stockell-Hartree) and HCG CR 115 (Dr. R. E. Canfield) was found. All results are expressed in ng of LH IRC2/69 (1 ng LH IRC2/69 = 2 mU MRC 68/40). In the FSH assay, FSH MRC 69/104 (assuming 10 I.U./ampoule) was used as standard but all results are expressed in ng FSH CPDS6/ml (1 ng FSH CPDS6 (immunopotency 5.000 I.U./mg) = 5 mU FSH 69/104). In the α-subunit assay LHα 1.4.72 (Dr. A. Stockell-Hartree), FSHα N611C (Dr. A. F. Parlow), TSHα N785B (Dr. A. F. Parlow) and HCGα CR 115 (Dr. R. E. Canfield) were equipotent on a weight basis. In the LHβ assay the LH preparation IRC2/69 caused significant displacement of $I^{125}$ labelled LHβ subunit when it was present at concentrations > 4 ng/ml serum.

## RESULTS

*Pituitary extracts.* Immunoreactive LH and α-subunit were detected in pituitary extracts as early as 9.5 weeks of gestation—the earliest extracts studied. However, the amount of LHβ subunit measured in the 7 extracts from 9.5–12.5 week old foetuses could, in 5 of the extracts, be due to crossreaction of LH in the LHβ assay. No FSH was detected in the 7 extracts from 9.5–12.5 week old foetuses.

In Fig. 1 the ratio between LH and the subunits in 19 foetal pituitary extracts and the values of 4 normal adult extracts are shown.

The α-subunit to LH ratio seems to reach peak values between the 10th to 14th week of gestation and then to decrease towards term. In contrast to the adult pituitary extracts in which the amount of LH on a weight basis was double that of the α-subunit, the foetal extracts contained significantly ($p < 0.01$) more α-subunit than LH. No significant change in the ratio of LH:LHβ subunit could be detected from the 9.5–32 weeks of gestation. The adult pituitaries contained 100 to 1000 times more LH and α-subunit than pituitaries obtained from foetuses of 9.5–11.5 weeks. Pituitary extracts from three older foetuses (19.5–32 weeks) showed hormone and subunit ratios similar to those of the adult extracts.

Unlike normal pituitary extracts, the extract from an anencephalic foetus (age of gestation 36 weeks) contained predominantly α-subunit, but also significant amount of LH with an α-subunit:LH ratio of

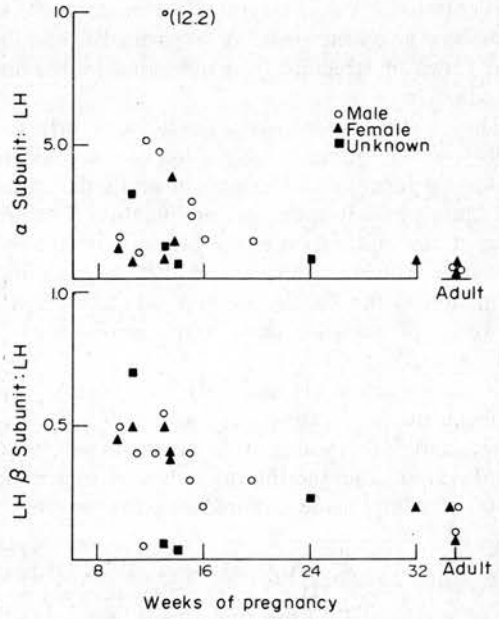



Fig. 1. The ratios of α-subunit: LH and LHβ subunit:LH in foetal and adult pituitary extracts.

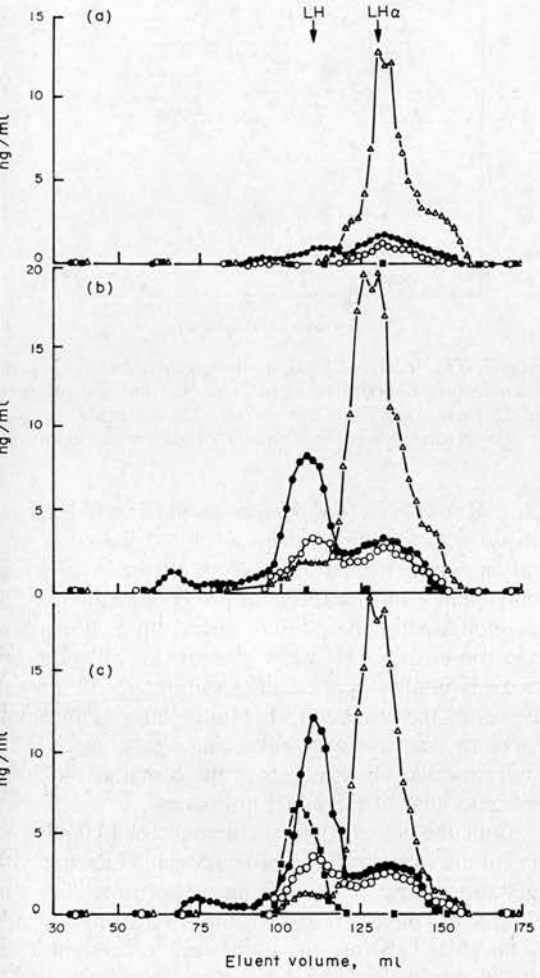



Fig. 2. Elution pattern after column chromatography on Sephadex G-100 of pituitary extracts from a 9.5-week old male (a), a 13.5-week old female (b) and a 32-week old female (c) foetus. All fractions were assayed by specific radioimmunoassays for LH (●), FSH (■), LHβ- (○) and α-subunits (△). The peaks for standard hormone preparations LH (68/40) and LHα subunit (A. Stockell-Hartree) are indicated (↓) at the top. (From *J. Endocr.* page 54, 1975).

7.8. No FSH could be detected. The ratio of LHβ:LH was 0.20, which is within the normal foetal range (0.02–0.7).

It has been confirmed by gel filtration that a considerable amount of gonadotrophic substance in the foetal pituitary gland is a molecular species recognized both immunologically and physically as the common α-subunit (Fig. 2). After column chromatography the ratio between LH and the α-subunit in the pituitaries in molar terms seems to decrease from about 1:30 at 9.5 weeks of gestation to 1:2 at 32 weeks.

All foetal pituitary extracts of more than 12 weeks of gestation contained not only a form of LH which behaved immunologically and physically like standard LH, but also a molecular species which eluted before LH, but reacted in the LH assay [10]. Whether

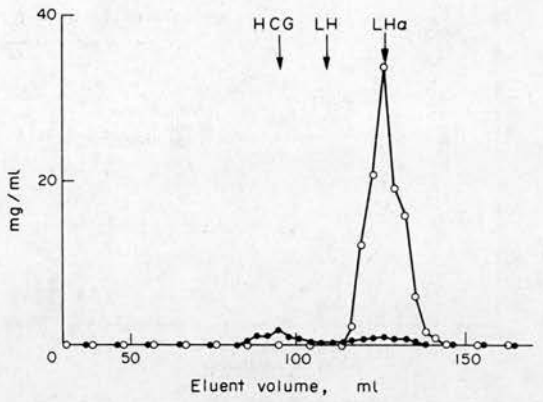



Fig. 3. Elution pattern after column chromatography on Sephadex G-100 of foetal cord serum at term. All fractions were assayed by specific radioimmunoassays for LH-HCG (●) and α-subunit (○). The peaks for standard HCG (CR 115), LH (68/40) and LHα subunit (A. Stockell-Hartree) are indicated (↓) at the top.

this 'big LH' is aggregated LH, LH bound to proteins or quite another form of LH remains to be seen. Only one form of α- and LHβ-subunit could be recognized in these extracts.

*Foetal serum levels.* As in the pituitary gland a molecular species, which behaves both immunologically and physically as the common α-subunit, is found in the foetal circulation (Fig. 3). Although a wide range of values is found, higher blood levels of α-subunit occur at the age of 10–16 weeks than at term (Fig. 4). The α-subunit concentrations in the foetal circulation is 50–500 times higher than those seen in adult premenopausal women and men. Low to undetectable levels of the LHβ subunit was found (Fig. 5). This might be due to crossreaction of LH-HCG and α-subunit in the LHβ-assay.

The concentrations of LH-HCG and FSH in the foetal circulation varies, but higher levels of these hormones were measured between the 11.5 to 16 weeks of gestation than at term (Figs. 6 and 7). The levels of FSH at term are within the range of FSH measured in premenopausal women.

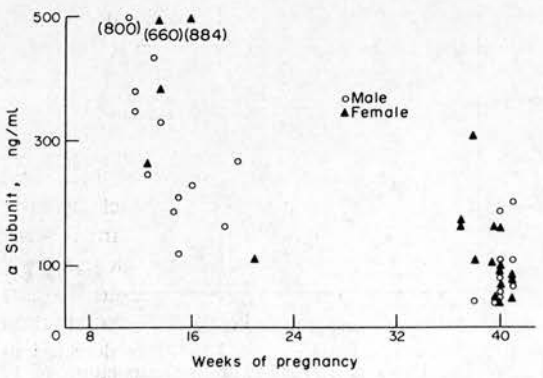



Fig. 4. The levels of α-subunit in the circulation of 17 early foetuses (age of gestation 11 to 21 weeks) and in cord veins of 26 foetuses at term are shown. The concentrations of the subunit were measured by radioimmunoassay.

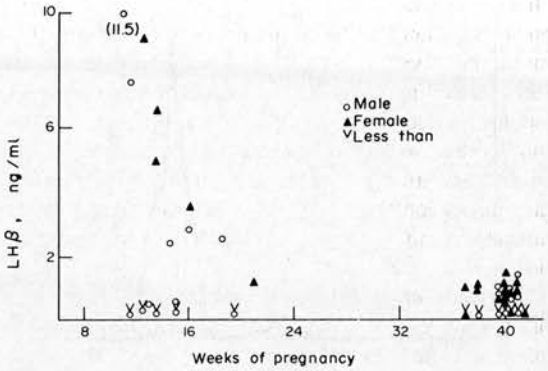



Fig. 5. The levels of LH$\beta$ subunit in the circulation of 17 early foetuses (age of gestation 11 to 21 weeks) and in cord veins of 26 foetuses at term are shown. The concentrations of the subunit were measured by radioiommunoassay.

Immunoreactive LH-HCG in foetal serum seems to consist of both LH of foetal origin and HCG from the placenta. Firstly, the levels of LH$\beta$ subunits obtained in the foetal circulation represented only 2% of the LH levels recorded, and with the known 25% crossreaction of LH in the LH$\beta$ assay, the foetal LH must consist of molecular species other than normal LH. Secondly, foetal serum at term contains a molecular species similar to HCG when examined by gel filtration and immunoreactivity, while only a small fraction is similar to standard LH (Fig. 3).

## DISCUSSION

*Pituitary content.* The proposition that free $\alpha$-and $\beta$-subunits in pituitary extracts are not due to dissociation of the intact hormones has been discussed in detail [10, 14, 15].

Immunoreactive LH and FSH have been detected in foetal pituitary extracts as early as 9.5 to 10 weeks of gestation—the youngest foetuses studied [10].

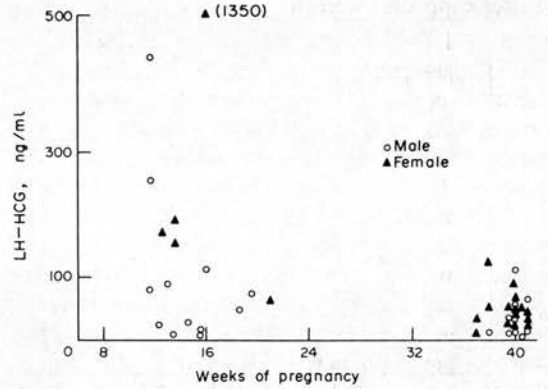



Fig. 6. The levels of LH-HCG in the circulation of 17 early foetuses (age of gestation 11 to 21 weeks) and in cord veins of 26 foetuses at term are shown. The concentrations of the hormone were measured by radioimmunoassay.

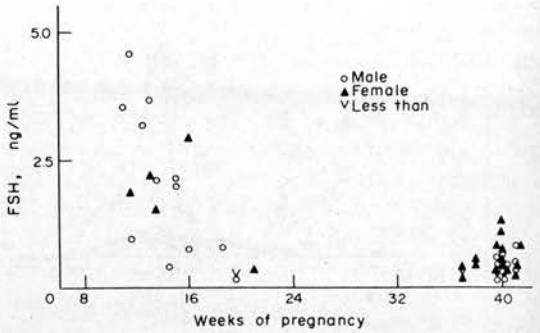



Fig. 7. The levels of FSH in the circulation of 17 early foetuses (age of gestation 11 to 21 weeks) and in cord veins of 26 foetuses at term are shown. The concentrations of the hormone were measured by radioimmunoassay.

Siler-Khodr *et al.* [16] demonstrated LH and FSH activity in foetal pituitary tissue of 5–7 weeks of gestation cultured *in vitro*; therefore, the onset of gonadotrophin synthesis seems to precede the anatomical development of the pituitary gland. Immunoreactive $\alpha$-subunit and LH were present in all pituitary extracts studied (period of gestation 9.5–32 weeks); however, the $\alpha$-subunit: LH ratio after gelfiltration seem to decrease with advancing age (Fig. 2). This indicates that the synthesis of the common $\alpha$-subunit precedes that of the intact hormones.

Both the content and the amount of LH released in culture from the foetal pituitary gland increase with gestational age to a maximum between the 20th and 30th weeks of gestation, thereafter decreasing toward term [9, 16]. Before the 20th week of gestation no sex difference could be detected in the amount of the gonadotrophins released from the pituitary gland or in their content. However, from the 20th to 30th week of gestation female pituitaries contained and released more of the gonadotrophins than male [9, 16]. No sex difference in the ratio of $\alpha$-subunit to LH was recorded, indicating that female pituitaries also contained more of the $\alpha$-subunit than male. Unlike normal pituitary extracts the extract from an anencephalic foetus at 36 weeks of gestation contained predominantly $\alpha$-subunit and hardly any of the intact hormones [10]. This agrees with the approximately 2% of LH and FSH found in the pituitary gland of an anencephalic foetus compared with the glands of normal newborn infants [9].

*Serum levels.* Circulating subunit levels are not due to the dissociation of intact hormones. This has been verified in adults by the infusion of purified LH in normal subjects and the observation that this was not followed by a rise in $\alpha$- and LH$\beta$-subunit [17]. Furthermore, it has been shown that the handling of the sample has no influence on the result. This has been demonstrated on serum samples obtained from postmenopausal women in which the levels of LH, FSH and $\alpha$-subunits were compared both immediately and after having been left at 4°C or 37°C for one week (Hagen, unpublished observation). It has also been

demonstrated in tests carried out for the purpose of comparing the levels of TSH, TSH$\beta$- and $\alpha$-subunits after multiple freezing and thawing of a single serum sample [18].

The highest levels of LH-HCG and FSH in the circulation during foetal life occur when the content of gonadotrophins in the pituitary gland is increasing i.e. between 12 and 20 weeks of gestation [9, 19]. The present results confirm these reports since higher blood levels of both intact hormones (LH and FSH) and $\alpha$-subunit occurred in early foetal life (age of gestation 10–16 weeks) than at term (Figs. 4, 6 and 7). However, due to the relatively small number of samples, it was not possible to confirm [9, 19, 20] the higher levels of FSH in the circulation of female than male foetuses. Foetal LH-HCG is not influenced by the sex of the foetus.

The blood concentration of the glycoproteins is dependent on the rate of secretion, their distribution within the body and their metabolism. There is no information on these parameters in the foetus. In adult males the initial half life of LH is from 21–60 min [21, 22] and that of the LH$\alpha$ and LH$\beta$ subunit from 14–17 min [22]. If the values for the half lives of subunits and intact hormones measured in adults apply to the foetus as well, the high circulating levels of $\alpha$-subunit compared to the intact hormones found in the foetus indicate that the secretion of $\alpha$-subunit in molar terms is 5–10 times that of LH.

*Transfer of glycoproteins across the placental barrier.* The observation that the respective concentrations of LH-HCG and $\alpha$-subunit in maternal blood at term are 200 and 20 times higher than those of foetal blood [12, 23] indicate that the intact hormones and their subunits are transferred between maternal and foetal circulation in only small amounts if any. Secondly, the $\alpha$-subunit levels in the mother increase steadily during pregnancy [23], whereas the levels in the foetus are lower at term than at the 10th to 16th week of gestation. Thirdly, no difference in the levels of the intact glycoprotein hormones or their subunits are seen between foetal cord artery and vein (Table 2) and no correlation between hormone or subunit levels in the maternal and the foetal circulation have been demonstrated [12]. On the other hand, most LH-HCG found in the foetal circulation at term is of a molecular species similar to HCG when examined by gel filtration and immunoreactivity while a small fraction is similar to LH [12, 24]. However, whether this is true HCG or LH of a bigger molecular species than normal LH remains to be seen. In addition, no physical or immunological difference between the $\alpha$-subunit found in the foetus and the mother at term has been demonstrated [12]. From these studies it seems likely that the foetal pituitary gland contributes, at least in part, to the intact hormones and $\alpha$-subunit found in the foetal circulation.

Table 2. The concentration of LH-HCG, $\alpha$-subunit and LH$\beta$-subunit in foetal cord artery and vein at term

| Hormone or subunit | Artery* (ng/ml) | Vein* (ng/ml) |
|---|---|---|
| LH-HCG' | 39.6 (20.7–97) | 38.5 (18.8–124) |
| $\alpha$-subunit | 99 (40–204) | 97 (43–316) |
| LH$\beta$ subunit | 1.0 (0.5–1.4) | 0.9 (0.8–1.6) |

* mean and range are shown; $n = 22$.

*Ontogeny of gonadotrophin control.* In adults the production of hormones from the anterior pituitary gland is to a large extent regulated by signals from the midbrain and the cerebral cortex; this control is mediated by the monoaminergic neurons and their neurohumeral chemotransmitters, which terminate in the median eminence and the pituitary gland [25], and by neurohormones secreted by the hypothalamus carried through the portal vessels to the anterior pituitary gland [26]. The secretion of both LH and FSH is apparently controlled by only one releasing hormone, the decapeptide gonadotrophin-releasing hormone (LHRH) [27]. The sex hormones oestrogen, progesteron and testosteron secreted by the gonads exert a predominantly negative feedback on the hypothalamus and pituitary [28]; however, oestrogens have a stimulatory effect as well [29, 30].

Hypothalamic control of the foetal adenohypophysis depends at any rate on the presence of an intact portal circulation, the development of nerve endings of tuberal neurons containing monoamines in the median eminence and pituitary gland, and the hypothalamic content of the hypophysiotrophic hormones [25, 31]. In humans, portal vessels appear about the 4th month of gestation, but the portal circulation is not completed before the second half of intrauterine life [32, 33]. Neurosecretory nerve fibres and structures containing monoamines are seen in the median eminence at the 15th to 18th week of gestation [33, 34]; however, Hyyppä[35] found an accumulation of nerve fibres containing monoamines in the median eminence at the 11th week of gestation. At the same time he found that the portal vessels penetrated the median eminence. Furthermore, the age of gestation at which LHRH is present in the human hypothalamus is controversial. Winters *et al.* [36] demonstrated immunoreactive LHRH in foetal hypothalami as early as 4.5 weeks of gestation. Gilmore and Dobbie[37] on the other hand were unable to demonstrate biologically active LHRH in hypothalamic extracts before the 16th week of gestation.

These studies indicate that releasing hormones and portal vessels exist in foetal life, but that the hypothalamic-pituitary control system is not developed until mid- to late-pregnancy. However, it is still possible that the pituitary gland is under influence of the hypothalamic hormones at a very early stage, because the small hypothalamic peptides could reach the pituitary gland by diffusion, or *via* the cerebro-spinal fluid circulation during this period [38].

The stage of foetal development at which the inhibitory feedback mechanisms of the sex hormones mature is not known. Steriodogenic activity in the foetal testis can be demonstrated from the 6th week of gestation, leading to testosterone production at the age of 12 weeks [19, 39, 40]. The steroid synthesis of the human foetal ovary is practically negligible [41]; however, in the foetal circulation oestrone, oestradiol-17$\beta$ and oestriol are present around the 12th to 15th week of gestation, with increasing levels at the age of 20 weeks [19, 42]. The increase in foetal sex hormones correlates with the fall in pituitary content and the serum concentrations of LH and FSH; this might indicate the development of a sex hormone-hypothalamic-pituitary feedback mechanism at this time [9].

The pattern of change in the ratio of pituitary LH, FSH, LH$\beta$ subunits and $\alpha$-subunits during foetal life suggests that the first gonadotrophin substance synthetized in the foetal pituitary gland is the common $\alpha$-subunit, after which, under hypothalamic influence, the $\beta$-subunits synthetize, and this leads to the production of the intact hormones. This is supported by the fact that, in normal pituitaries, the hypothalamic gonadotrophin-releasing hormone not only releases LH and FSH but also stimulates their synthesis [27]. Furthermore, the pituitary extract from an anencephalic foetus (period of gestation 36 weeks) contained large amounts of $\alpha$-subunit but practically no intact hormones [10].

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## DISCUSSION

*Posner.* Do you know of measurements of the kind made in amniotic fluid?

*Hagen.* I suppose you mean of the subunits. Yes, we have measured the α-and HCGβ-subunit concentration in a few samples of the amniotic fluid around term. The α-subunit concentration was very high (more than 500 ng/ml) and no HCGβ-subunit was found.

*Thorburn.* I just wondered what evidence you have that the fetal pituitary actually secretes the α-subunit. You measure high levels in the blood but this doesn't say it's secreted, have you any studies on perfusion of pituitaries or other experimental evidence to support your contention.

*Hagen.* No, we have not performed such studies, but Franchimont and coworkers have shown that the foetal pituitary *in vitro* is able to secrete free α-subunit. What we have shown is, that both the α-subunit and the intact gonadotrophins are present in the pituitary gland, and that we can pick up FSH, presumably LH and free α-subunit in the circulation. We can not distinguish between α-subunit of foetal and maternal origin, but I think it is like the LH-HCG story, that the α-subunit that we find in the foetal circulation is mainly of placental origin.

*Jaffe.* I can corroborate one of your latter comments. Some years ago Dr. Midgley and I performed gel filtration and radioimmunoassay of umbilical cord blood at term and did indeed find that the bulk of the gonadotrophin was HCG rather LH. I think also it might be helpful for people not working in this field if we labeled α-subunit just as α-subunit rather than as LH α-subunit. The term LH α-subunit might be misleading.

*Hagen.* I agree, when we are talking about circulating levels of α-subunit. But I still think it is nice to know, whether standards used in the assays or for standardizing columns, are HCGα, TSHα or FSHα.

*Friesen.* Do you know anything about the recovery in your extractions of pituitary α and β subunits? Is there a differential extraction of the two?

*Hagen.* I agree this is an important question. Because of the mild extraction procedures used, it is not likely that a dissociation of intact hormones into subunits has occurred. No, we have not performed any recovery studies. However, we have extracted pieces of the same pituitary gland on different days, and shown that this had no influence on the elution pattern of the hormones and their subunits. This finding does not exclude the possibility, that the extraction procedure and gel filtration influence our results, but I do not think it is very likely.

*Friesen.* In view of the possibility that α-subunit is secreted independently do you know of any suggestion that the α-subunit might itself have an independent action apart from something to do with the gonadal function?

*Hagen.* In adults, you can see a rise in circulating levels of α-subunit after the administration of LHRH and after large doses of TRH. We have shown that the rise in α-subunit precedes that of the intact hormones. Therefore, I think that the α-subunit secreted is of pituitary origin. In collaboration with Dr. Ken McNatty from Edinbourgh we have looked at the subunit concentration during the normal menstrual cycle. We found that in the circulation the α- and perhaps the LHβ-subunit showed a midcycle peak, similar to those seen for LH and FSH, but no changes during the follicular- and luteal phase. Secondly, in the follicular fluid we found the same concentration of α-subunit as in the corresponding blood samples, and in a few follicles even higher. Because of the non-specificity of our LHβ-assay, we have not been able to demonstrate free LHβ-subunit neither in the circulation nor in the follicular fluid. *In vitro* studies performed by Ken McNatty and others have not been able to show any biological function of the subunits themselves. However, we have speculated that in the normal menstrual cycle the subunits could penetrate the follicle and inside the follicle recombine and act as the intact hormones. I do not think it is an important function, but only a minor thing. Whether the α-subunit in the foetus and the mother has a biological function I do not know.

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# IMMUNOREACTIVE α- AND β-SUBUNITS OF LUTEINIZING HORMONE IN HUMAN PERIPHERAL BLOOD AND FOLLICULAR FLUID THROUGHOUT THE MENSTRUAL CYCLE, AND THEIR EFFECT ON THE SECRETION RATE OF PROGESTERONE BY HUMAN GRANULOSA CELLS IN TISSUE CULTURE

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SUMMARY

The concentration of the common α-subunit of the glycoprotein hormones and of the β-subunit of luteinizing hormone (LHβ) in peripheral blood and follicular fluid was measured throughout the menstrual cycle, and the effects of these subunits, either alone or in combination, on the production of progesterone by human granulosa cells in tissue culture were investigated.

Changes in the serum concentration of α-subunit and immunoreactive 'LHβ-like' material throughout the menstrual cycle were similar to those of LH. The concentrations of the subunits before the mid-cycle gonadotrophin peak were not significantly different from those during the luteal phase of the menstrual cycle. Gel filtration of a pooled serum sample obtained at mid-cycle confirmed the presence of immunoreactive α-subunit together with intact LH; however, because of the cross-reactivity of LH in the LHβ assay a distinct peak of LHβ-subunit could not be demonstrated.

In follicular fluid, α-subunit was detectable in large follicles (≥ 8 mm) throughout the menstrual cycle at concentrations similar to those found in serum. By contrast α-subunit in small follicles (< 8 mm) and 'LHβ-like' material in all follicles were only detectable during or just after peak concentrations in peripheral plasma.

The LH subunits did not increase the rate of progesterone secretion by human granulosa cells when each was added alone, even at concentrations five times higher than those in plasma. However, when both subunits were added simultaneously there was an increased rate of progesterone secretion comparable to that achieved with intact LH.

It is concluded that the common α-subunit circulates in blood independently of the intact hormones, and that it is present in a proportion of developing Graafian follicles without affecting either the viability or biosynthetic potential of their granulosa cells. During the late follicular phase however, when both the α- and LHβ-like subunits are present in follicular fluid, they may recombine and enhance steroid production by granulosa cells which are undergoing luteinization at this time.

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## INTRODUCTION

The pituitary glycoproteins, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), consist of two non-identical, non-covalently bound subunits, $\alpha$ and $\beta$ (Pierce, 1971; Saxena & Rathnam, 1971; Stockell Hartree, Thomas, Braikevitch, Bell, Christie, Spaull, Taylor & Pierce, 1971). The $\alpha$-subunit is both chemically and immunologically similar in all three, whereas differences in the $\beta$-subunit are responsible for the biological and immunological specificity of the hormones (Vaitukaitis & Ross, 1972).

Luteinizing hormone is present in peripheral plasma throughout the menstrual cycle, and, during the follicular phase, basal levels of this hormone are probably important for maintaining the secretion of oestrogen by the thecal cells of the developing Graafian follicle (McCracken, Uno, Goding, Ichikawa & Baird, 1969; Moor, 1973; Baird, Baker, McNatty & Neal, 1975). However, LH is not detectable in follicle fluid until the mid-cycle peak of this hormone in peripheral plasma (McNatty, Hunter, McNeilly & Sawers, 1975*b*). Indeed, if LH did enter the follicle prematurely it would probably interfere with both the mitotic activity and steroidogenetic potential of the granulosa cells (McNatty & Sawers, 1975). By contrast FSH is present in the follicular fluid of developing Graafian follicles at all stages of antral growth (McNatty *et al.* 1975*b*) and its presence appears to be essential for the survival and proliferation of these cells (McNatty, Bennie, Hunter & McNeilly, 1975*a*).

The $\alpha$- and LH$\beta$-subunits circulate independently of the intact hormones in plasma (Franchimont, Gaspard, Reuter & Heynen, 1972; Laburthe, Dolais & Rosselin, 1973; Hagen & McNeilly, 1975*a*), however no information is available on their concentrations in peripheral blood and follicular fluid throughout the normal menstrual cycle. The aim of the present study was to determine whether the levels of $\alpha$- and LH$\beta$-subunits showed specific changes in blood and follicle fluid throughout the menstrual cycle. Furthermore, it was hoped to correlate these findings with the effects of the subunits on the production of progesterone by human granulosa cells in tissue culture.

## MATERIALS AND METHODS

### *Subjects*

#### *Women not undergoing surgery*

For the measurements of FSH, LH, $\alpha$- and LH$\beta$-subunits in peripheral serum, blood samples were obtained daily from four women (aged 25 to 35 years) between 08.00 and 10.30 h during one menstrual cycle. Before the study all four women had had at least four regular consecutive menstrual cycles (28–33 days). Ovulation and normal corpus luteum function were confirmed in each cycle by measurement of serum progesterone levels. Serum was separated and stored at $-20$ °C until assayed. All samples from an individual subject were measured in the same assay.

#### *Women undergoing surgery*

Samples of peripheral blood and of follicular fluid from ovaries or wedge biopsies for the measurement of $\alpha$- and LH$\beta$-subunits and LH were obtained from 47 patients (aged 27–48 years) at various stages of the menstrual cycle. These patients were undergoing hysterectomy for a number of gynaecological disorders. The blood samples were obtained during surgery and immediately before oophorectomy. The indications for surgery were either stage 0 carcinoma of the cervix (7 patients), menorrhagia due to endometriosis (3),

fibroids (8), dysmenorrhoea or chronic pelvic pain (29). The previous menstrual cycles of these women varied in length from 22 to 33 days and 17 had ovulated during the cycle under study, the remaining 30 women were in the follicular phase.

### *The menstrual cycle in women undergoing surgery*

The stage of the menstrual cycle was assessed in all 47 patients from the endometrial histology (Noyes, Hertig & Rock, 1950), the date of the last menstrual period, the concentrations of LH, FSH, oestradiol and progesterone in plasma and the presence or absence of a corpus luteum. The menstrual cycle was divided into six phases: early follicular, (patient still menstruating) (EF); mid-follicular (MF); late follicular (LF); early luteal (EL); mid-luteal (ML) and late luteal (LL).

### *Collection of follicular fluid and the culture of granulosa cells*

Eighty Graafian follicles of varying sizes (4–20 mm diameter) were dissected from the ovaries. The follicular fluid was aspirated from each follicle into a 1 ml syringe and stored at −20 °C until assayed for $\alpha$- and LH$\beta$ subunits and LH.

Granulosa cells were scraped from the collapsed follicle into Medium 199 containing Hanks' salts, HEPES buffer (20 mmol/l), gentamicin (50 $\mu$g/ml), amphotericin-B (2·5 $\mu$g/ml) and 1 % glutamine (Flow Laboratories, Irvine, Scotland).

The technique of culturing granulosa cells was identical to that previously described by McNatty & Sawers (1975). The cells were grown for 10 days in 1 ml culture medium containing 20 % foetal calf serum (v/v). The endogenous gonadotrophic activity of the culture medium was 1·7 i.u. LH/l and 1·8 i.u. FSH/l (McNatty & Sawers, 1975). The culture medium was replaced each day and stored at −20 °C until assayed for progesterone by the radioimmunoassay technique described previously (Neal, Baker, McNatty & Scaramuzzi, 1975). All cultures were carried out in duplicate and the precision obtained in relation to the production of progesterone for all experiments was 8·1 ± 0·78 (S.E.M.) %, $n = 26$ which was similar to that reported by McNatty & Sawers (1975). The number of 'live' cells at the beginning of culture was determined by the techniques of McNatty & Sawers (1975).

### *Follicle-stimulating hormone and the subunits of luteinizing hormone*

The gonadotrophins added to the cultures were: human LH (Stockell Hartree, IRC-2, 24.6.69) containing 7550 units LH/mg and < 25 units FSH/mg; human FSH (MRC 73/519) containing 2200 units FSH/mg and 8·8 units LH/mg; human LH$\alpha$ (MRC 72/20) containing 7·4 % LH (IRC 2/69) biological activity; human LH$\beta$ (MRC 71/342) containing 5·8 % LH (IRC 2/69) biological activity (Dr A. Stockell Hartree, personal communication). The immunological potencies of LH and FSH were assessed by Dr W. M. Hunter, Edinburgh using the following standards: LH MRC 68/40 assuming 77 units/ampoule; FSH MRC 68/39 assuming 32·8 units/ampoule. The concentrations of LH and FSH are expressed as i.u./l, and both LH$\alpha$ and LH$\beta$ as ng/ml. The gonadotrophins were diluted and stored in culture media in ampoules at −20 °C until added to cell cultures. All cultures received FSH so that the final concentration of this hormone in the culture medium was 30 i.u./l.

*Radioimmunoassays for LH, FSH, LHα and LHβ*

Serum LH, FSH, LHβ and the common α-subunit were measured by double antibody radioimmunoassays (McNeilly & Hagen, 1974; Hagen & McNeilly, 1975*a*, *b*). To overcome non-specific serum interference in the subunit assays, standards were diluted in horse serum (No. 3 Wellcome Reagents Ltd). Under these conditions the detection limits for the α- and LHβ-subunit assays were 0·4 and 0·5 ng/ml serum respectively. In the α-subunit assay, LHα, FSHα, (N611C, Dr A. F. Parlow) and TSHα (N785B, Dr A. F. Parlow) were equipotent on a weight basis. The LH standard (MRC 68/40; assuming 77 units per ampoule) did not cause significant displacement of $^{125}$I-labelled α-subunit in the α-subunit assay unless it was present at concentrations > 20 i.u./l serum (i.e. 8 % cross-reaction). In the LHβ-subunit assay the LH standard (MRC 68/40) caused significant displacement of $^{125}$I-labelled β-subunit when it was present at concentrations > 8 i.u./l serum (i.e. 25 % cross-reaction). Both the α and LHβ assay were highly specific in terms of FSH (i.e. < 1 % cross-reaction).

Identical procedures were used to measure the LH subunits in follicular fluid and plasma; however, because of the smaller volumes of follicular fluid, the sensitivity of the assays for both subunits was 0·6 ng/ml follicular fluid. The assay of LH in follicular fluid was identical to that described by McNatty *et al.* (1975*b*). The limit of detection for measuring LH in the smallest follicle examined was 2·8 i.u./l, and the concentration of LH was less than 20 i.u./l in all fluids. The concentrations of the subunits in pooled sera obtained from women at mid-cycle after fractionation by column chromatography (see below) were measured by more sensitive assays. By increasing the sample volume to 200 μl in a total incubation volume of 400 μl and using the same reagents, incubation time and separation procedure as described by Hagen & McNeilly (1975*a*, *b*), the sensitivities of the α- and LHβ-subunit assays were 0·12 and 0·04 ng/ml respectively. The percentage of cross-reactions between LH (MRC 68/40) and the respective subunits in the two assays remained unaltered.

*Column chromatography*

Pooled sera from women at mid-cycle were analysed after gel filtration on Sephadex G-100 (1·5 × 90 cm). Markers of purified LH IRC 2/69, $LH_2$ (Hagen & McNeilly, 1975*a*), LHα (Dr A. Stockell Hartree) and LHβ (Dr A. Stockell Hartree) were used. The reagents and elution procedure have been described in detail elsewhere (Hagen & McNeilly, 1975*b*). All fractions were assayed for LH, LHβ- and α-subunits by radioimmunoassay. Statistical analyses were carried out using Student's *t*-test.

## RESULTS

All four women not undergoing surgery showed a significant ($P < 0·01$) increase in the concentration of both immunoreactive α- and LHβ-subunits in serum, associated with the mid-cycle peak of LH and FSH (Fig. 1). In two subjects a significant rise ($P < 0·01$) in immunoreactive α-subunit levels was not coincident with any rise in FSH (Fig. 1*b* and *c*). The serum from one woman which had a double LH peak did not show any rise in LHβ-subunit associated with the first LH peak, but a significant rise in LHβ was associated with the second LH peak (Fig. 1*c*). There were no significant differences in the levels of α- and LHβ-subunits in the follicular phase before the mid-cycle peak compared with those in the luteal phase of the menstrual cycle.

Gel filtration of pooled sera from women at the mid-cycle peak showed distinct peaks

of immunoreactive material corresponding to LH and LHα-subunit (Fig. 2). The LHβ assay recognized a large peak corresponding to LH and a small retarded peak eluted as LHβ-subunit.

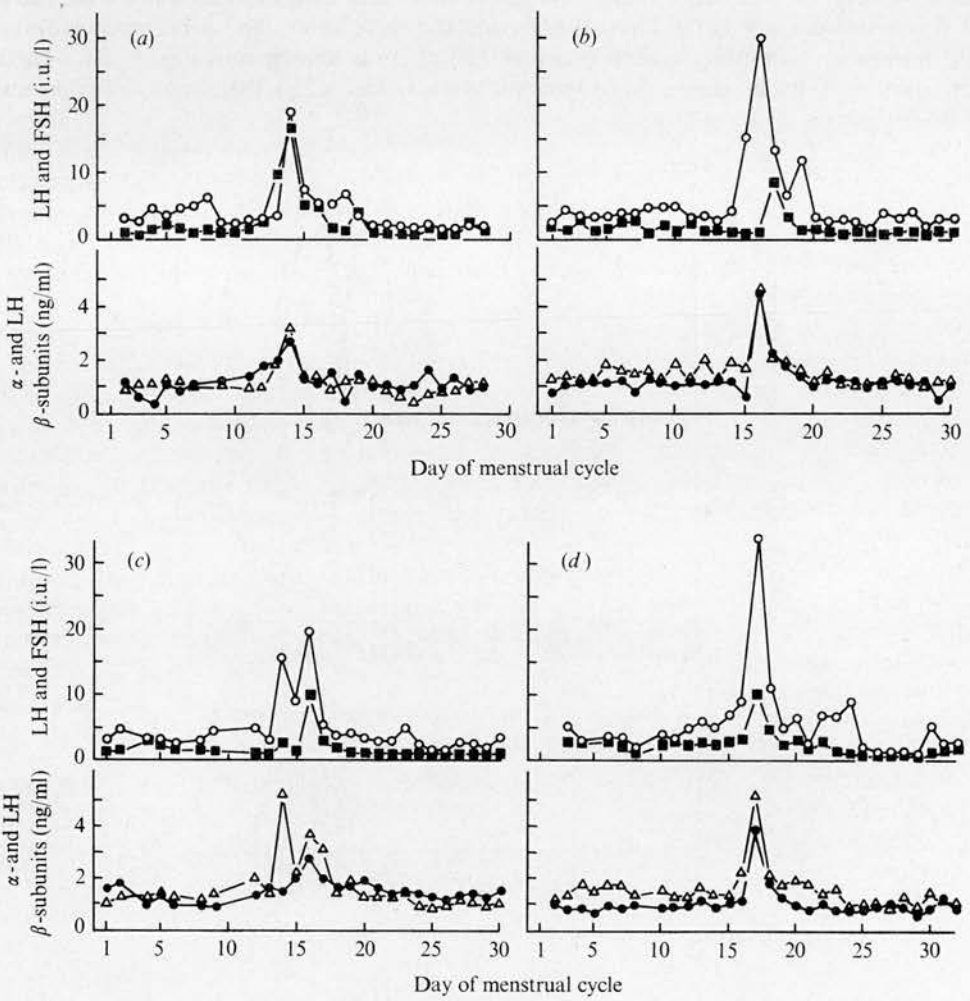



Fig. 1. Levels of LH (○), FSH (■), LHβ-subunit (●) and α-subunit (△) during the menstrual cycle in four subjects, *a*, *b*, *c* and *d*. Note the double LH and FSH peaks, and the lack of rise in LHβ subunit related to the first peak in subject *c*.

### *Concentration of α- and LHβ-subunits in plasma of women undergoing surgery*

Figure 3 shows the concentration of α- and LHβ-subunits and LH in plasma in relation to the stage of the menstrual cycle. In these patients, the rise in the plasma concentrations of immunoreactive α- and LHβ-subunits in the late follicular phase was similar to that observed in normal women not undergoing surgery.

*Concentration of α-subunit in follicular fluid*

The mean concentration of immunoreactive α-subunit in antral fluids in relation to follicle size and the stage of the menstrual cycle is shown in Fig. 4(*a*). The minimum detectable level of α-subunit in the smallest follicle examined (4 mm) was 0·6 ng/ml, and this figure was chosen as the limit of detection for all follicles. The percentage of follicles with detectable α-subunit at each phase of the cycle is shown in Table 1. The greatest proportion of follicles containing α-subunit were found when there were peak levels in

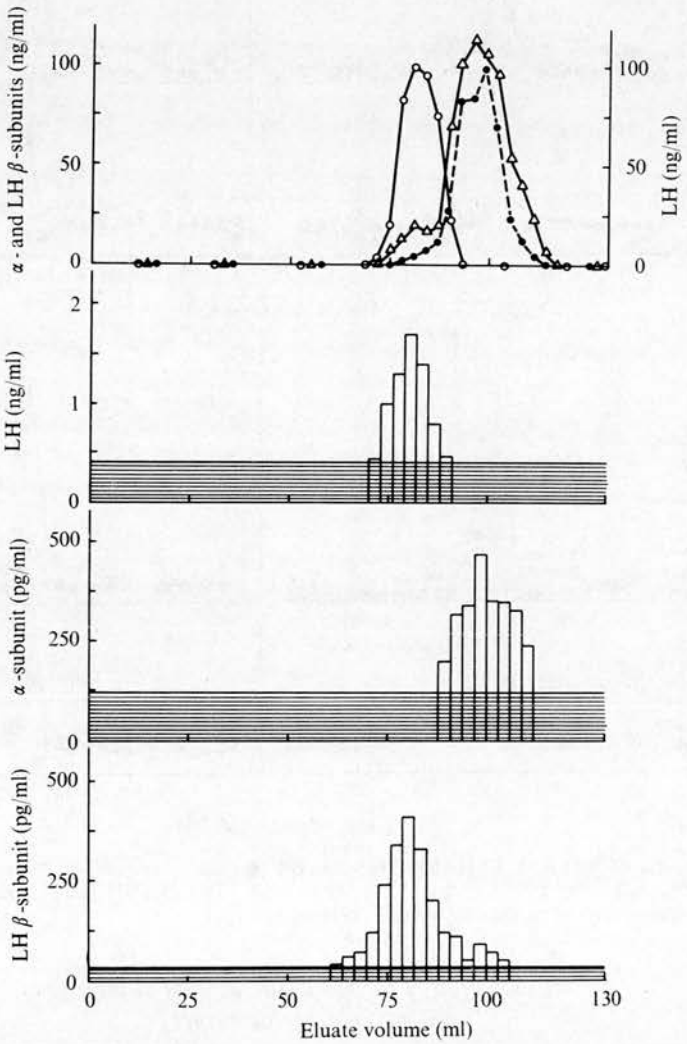



Fig. 2. Column chromatography on Sephadex G-100 of standards and pooled sera taken at the mid-cycle LH peak. All fractions were assayed for LH (○), LHβ-subunit (●) and α-subunit (△) and the elution patterns were assessed relative to that for $LH_2$ (○) (repurified LH IRC2/69), LHα (△) (Dr A. Stockell Hartree) and LHβ (●) (Dr A. Stockell Hartree), upper panel. The hatched area indicates the lower limit of sensitivity of the assays. LH is expressed in ng LH IRC2/69/ml; 1 ng LH IRC2/69 equals 2 mu. LH MRC 68/40.

peripheral plasma (Figs 3 and 4*a*). The α-subunit was detectable in a proportion of large follicles (≥ 8 mm) at every phase of the menstrual cycle, at concentrations similar to those found in plasma. By contrast, α-subunit was only detectable in small follicles (< 8 mm) during the mid- to late proliferative phase and the early secretory phase of the cycle.

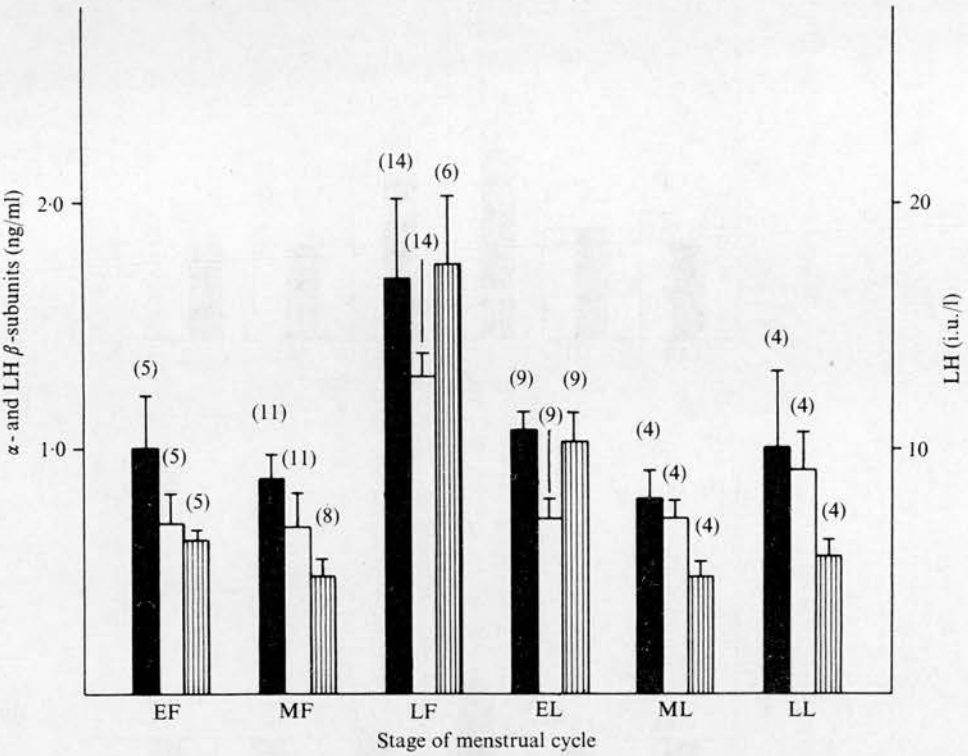



Fig. 3. Concentration of α-subunit (black bars), LHβ-subunit (white bars) and LH (hatched bars) in peripheral plasma in relation to the stage of the menstrual cycle. Results shown represent means+1 S.E.M. For abbreviations see Table 1. Numbers in parentheses represent number of observations.

### *Concentration of LHβ in follicular fluid*

The mean concentration of LHβ-like material in antral fluids in relation to follicle size and the stage of the menstrual cycle is shown in Fig. 4*b*. The percentage of follicles with detectable LHβ at each phase of the cycle is shown in Table 1. The minimum detectable level of LHβ in the smallest follicle examined (4 mm) was 0·6 ng/ml and this figure was chosen as the limit of detection for all follicles. The β-subunit was only detectable in small or large follicles during the late follicular and early luteal phase of the cycle related to the peak concentration in peripheral plasma. The concentration of immunoreactive β-subunit in follicular fluid at mid-cycle was similar to that in plasma.

### *Concentration of LH in follicular fluid*

Figure 5 shows the mean concentration of LH in antral fluids in relation to follicle size and the stage of the menstrual cycle. The minimum detectable level of LH in the smallest

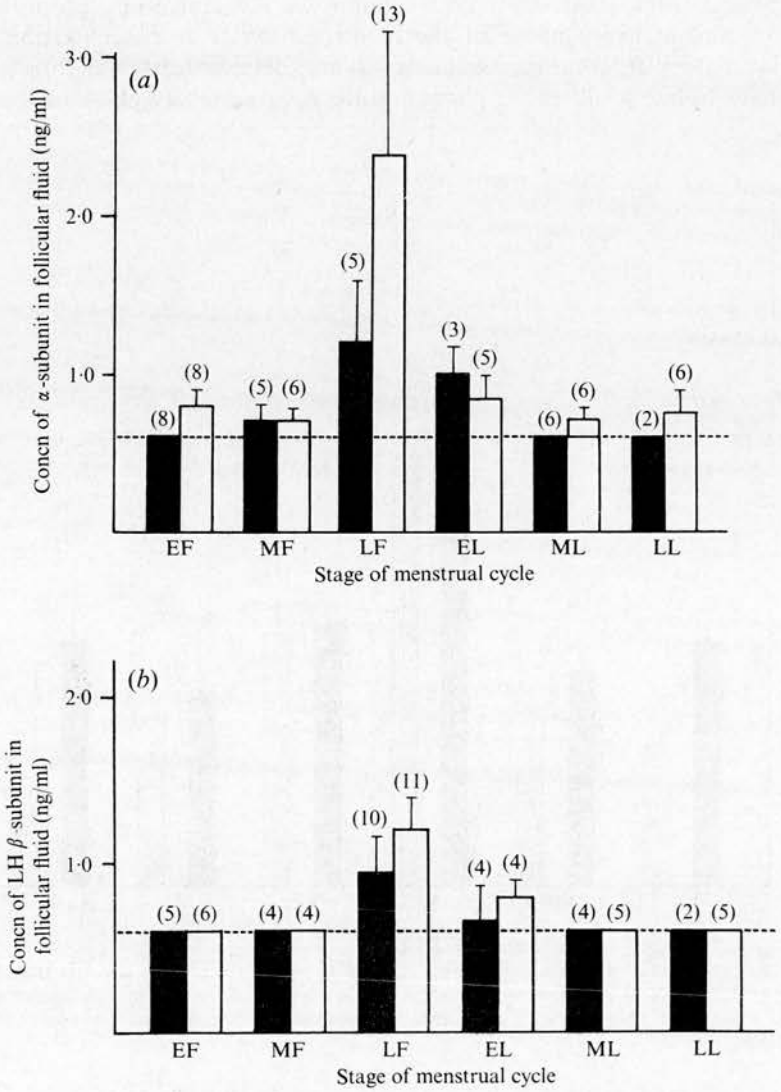



Fig. 4. Concentration of (*a*) α-subunit and (*b*) LHβ-subunit in follicular fluid. Results shown represent means+1 S.E.M. For abbreviations see Table 1. Numbers in parentheses represent number of observations. Black bars, follicles < 8 mm diameter; white bars, follicles ⩾ 8 mm diameter. The minimum detectable level of the subunits in the smallest follicle examined is indicated by the broken line.

follicle examined (4 mm) was 2·8 i.u./l, and this figure was chosen as the limit of detection for all follicles. Because of the limited amount of fluid available it was not possible to measure LH in all cases. Luteinizing hormone was only detectable in small follicles (< 8 mm) during the late follicular phase and in large follicles (⩾ 8 mm) during the mid- and late follicular phase.

Table 1. *Percentage of follicles with detectable levels of α- and LHβ subunits in relation to size at each phase of the human menstrual cycle*

| | | Stage of menstrual cycle | | | | | |
|---|---|---|---|---|---|---|---|
| Size of follicle | Subunit | EF | MF | LF | EL | ML | LL |
| < 8 mm | α | 0 | 25 | 45 | 25 | 0 | 0 |
| | LHβ | 0 | 0 | 30 | 25 | 0 | 0 |
| ⩾ 8 mm | α | 33 | 25 | 50 | 25 | 16 | 16 |
| | LHβ | 0 | 0 | 45 | 25 | 0 | 0 |

EF, MF and LF refer to early, mid- and late follicular phases, while EL, ML and LL refer to early, mid- and late luteal phases.

*Effect of subunits of LH on the viability and mitotic activity of granulosa cells in culture*

When cells were exposed to LHα and LHβ alone or in combination there was no significant increase or decrease in cell numbers during the 10 days of culture when compared with untreated controls.

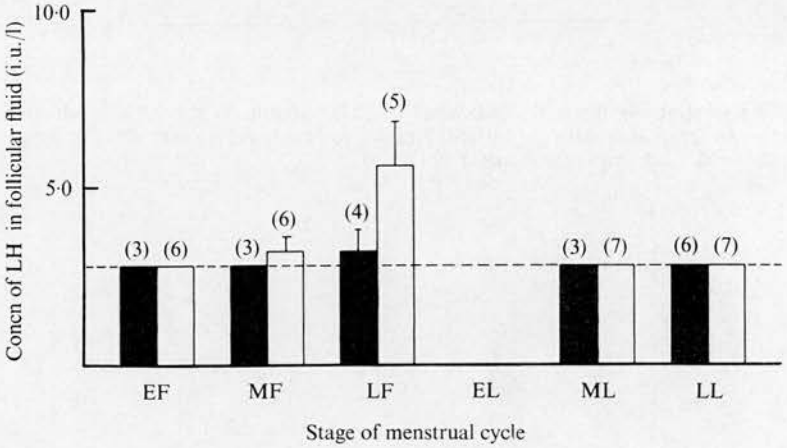



Fig. 5. Concentration of LH in follicular fluid. Results shown represents means+1 S.E.M. For abbreviations see Table 1. Numbers in parentheses represent number of observations. Black bars, follicles < 8 mm diameter, white bars ⩾ 8 mm diameter. The minimum detectable level of LH in the smallest follicle examined is indicated by the broken line.

*Effect of differing doses of LHα- or LHβ-subunits on the daily production rate of progesterone by granulosa cells in culture*

Figures 6 and 7 show the daily production of progesterone by granulosa cells after daily exposure to LHα and LHβ at concentrations between 1 and 100 ng/ml. The daily production was expressed as a fraction of that achieved by the untreated control; if the subunit had no effect on steroidogenesis then the production rate/day was 1, but if stimulation occurred then the production rate was > 1. There was no increase in the daily production of progesterone when the concentration of α- or β-subunits was between 1 and 20 ng/ml; however, there was a progressive increase in production if the concentration of either subunit was increased from 40 to 100 ng/ml.

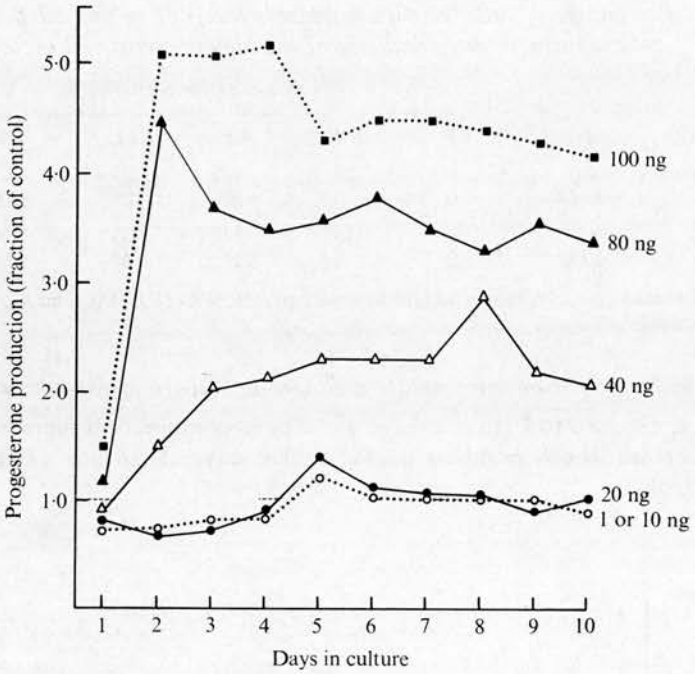



Fig. 6. Effect of differing doses (as indicated) of LHα-subunit on the daily production rate of progesterone by granulosa cells in culture. Results for each experiment are the mean of four duplicate cultures. Each culture contained 30 i.u.FSH/l.

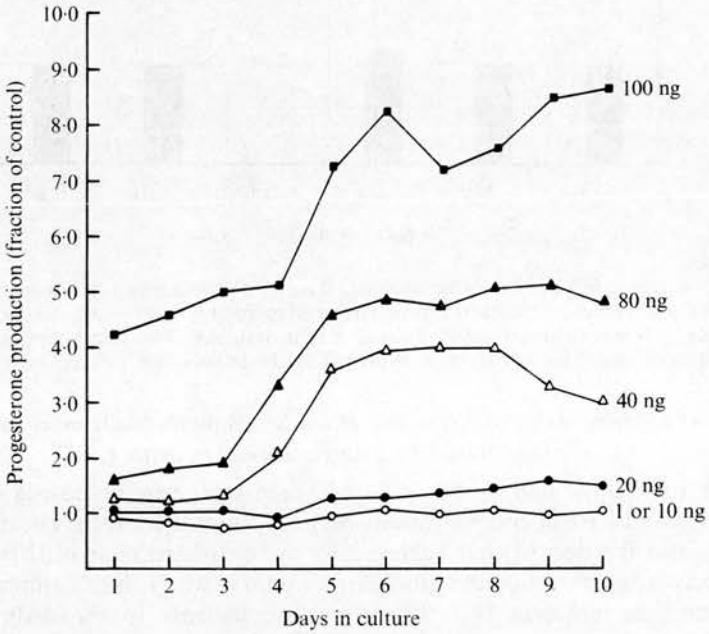



Fig. 7. Effect of differing doses (as indicated) of LHβ-subunit on the daily production rate of progesterone by granulosa cells in culture. Results for each experiment are the mean of four duplicate cultures. Each culture contained 30 i.u.FSH/l.

*Effect of addition of LHα- and LHβ-subunits on the daily production rate of progesterone by granulosa cells in culture*

The effect of daily addition of 10 or 40 ng of both LHα and LHβ is shown in Fig. 8. By day 10 of culture, 10 ng LHα + 10 ng LHβ increased the daily production rate sevenfold, while 40 ng of both subunits induced a 15-fold increase. By contrast 30 mu. LH (equivalent to approximately 4·3 ng LH: Dr W. M. Hunter, personal communication) induced a ten-fold increase in the production rate on day 10 of culture. Thus α- and β-subunits added together were only 30 % as active as an equivalent amount of intact LH.

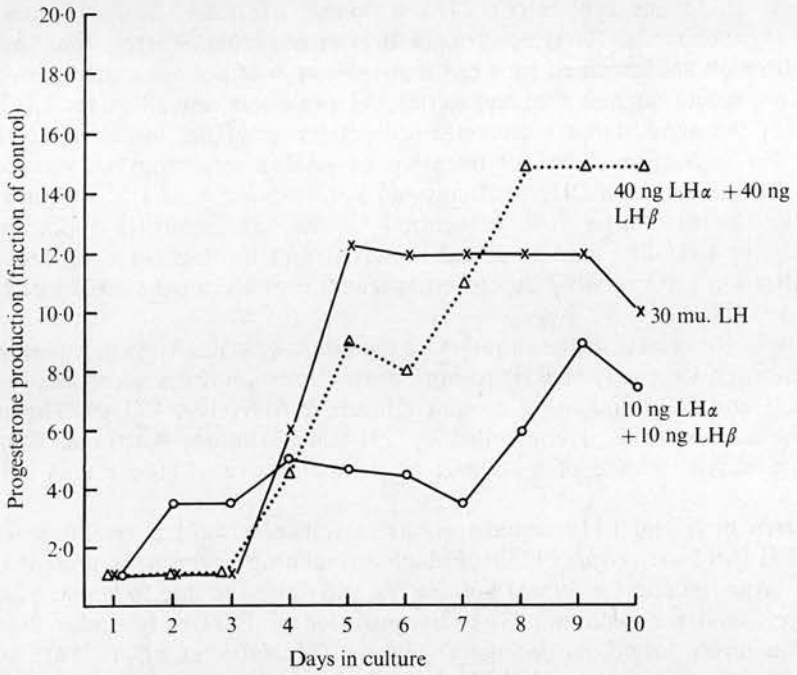



Fig. 8. Effect of LHα- and LHβ-subunits on the production rate of progesterone by granulosa cells in culture. Results of each experiment are the mean of three duplicate cultures. Each culture contained 30 i.u.FSH/l.

## DISCUSSION

The radioimmunoassay for measuring α-subunit is sufficiently sensitive and specific to determine basal levels of this material in unfractionated serum, plasma and follicular fluid even in the presence of increased levels of TSH, FSH and concentrations of LH < 20 i.u./l (Hagen & McNeilly, 1975*a*). In serum and follicular fluid the highest concentration of LH recorded was 34·3 i.u./l. The maximum contribution of the raised LH level to α-subunit will be 0·4 ng/ml (Hagen & McNeilly, 1975*a*). Thus if α-subunit level is measured at 5·3 ng/ml and LH at 34·3 i.u./l (Fig. 1*d*), at least 4·5 ng/ml will be α-subunit. Although the LHβ radioimmunoassay has the sensitivity for measuring low concentrations of LHβ-like material in serum, plasma or follicular fluid, LHβ could not be isolated from serum without contamination from intact LH. Furthermore, since concentrations of LH in plasma or serum in excess of 8 i.u./l interfere in the LHβ assay it is not possible to state unequivocally that the immunoreactive LHβ-like material is indeed LHβ.

In the normal menstrual cycle it is well established that the levels of FSH and LH are higher during the follicular phase than during the luteal phase and there is a mid-cycle peak in concentrations of both hormones the day before ovulation (Faiman & Ryan, 1967; Burger, Catt & Brown, 1968; Midgley & Jaffe, 1968; Franchimont, 1969; Abraham, Odell, Swerdloff & Hopper, 1972). The results of the present study are in agreement with this recognized pattern of secretion. This study also shows that the $\alpha$-subunit may circulate in free form with highest concentrations at the time of the mid-cycle peak of LH and FSH. However, no difference in the levels of either $\alpha$- or 'LH$\beta$-like' subunits during the follicular phase before the mid-cycle peak and the luteal phase could be shown. This was shown in the four normal subjects (Fig. 1), and also in the group of 47 patients undergoing surgery (Fig. 3). In one subject (Fig. 1*c*), a double LH peak, similar to that described previously (Franchimont, 1969) was found. It is of particular interest that the initial rise of 'LH', although accompanied by a rise in $\alpha$-subunit, was not associated with any change in LH$\beta$. This would suggest that the initial LH peak was not all intact LH and it also suggests that the subunits may circulate independently of the intact hormone. For the $\alpha$-subunit this is confirmed by gel filtration of pooled sera from women at mid-cycle (Fig. 2), when the values of LH, $\alpha$-subunit and LH$\beta$-subunit were 1·7, 0·47 and 0·09 ng/ml respectively. The first large peak recognized by the LH$\beta$ assay (Fig. 2), the levels of immunoreactive LH$\beta$-like activity found in serum and the lack of a distinct LH$\beta$ peak after gel filtration are probably due to cross-reaction of LH in the LH$\beta$ assay (Hagen & McNeilly, 1975*b*).

The stimulus for release of the subunits by the pituitary is not known, but administration of LH releasing hormone (LH-RH) to both normal men and women results in the release of intact LH and FSH and the $\alpha$-subunit (Hagen & McNeilly, 1975*a*). Therefore, if the release of gonadotrophins is controlled by LH-RH (Arimura, Kastin & Schally, 1974), then a concomitant release of $\alpha$-subunit and possibly even LH$\beta$-subunit may occur at mid-cycle.

The pattern of $\alpha$ and LH$\beta$ concentrations in follicular fluid is reminiscent of that of FSH and LH (McNatty *et al.* 1975*b*). Follicle-stimulating hormone is present in a greater number of large follicles ($\geqslant$ 8 mm) both in the early and the late follicular phase. Similar results were found for $\alpha$-subunit. The concentration of FSH in follicular fluid was only 60 % of the levels found in peripheral plasma (McNatty *et al.* 1975*b*), however the concentration of the $\alpha$-subunit in follicular fluid is similar to, and in some patients even higher than, that found in plasma. This difference may either be due to lack of uptake/utilization of the subunit or dissociation of intact gonadotrophin in follicular fluid, or merely due to differences in the rate of transfer across the blood–follicle barrier. Intact LH was only found in small and large follicles during the late follicular phase of the menstrual cycle (Fig. 5; McNatty *et al.* 1975*b*). Similar results were found for the LH$\beta$ subunit, however these results are unlikely to be entirely due to cross-reaction by LH in the LH$\beta$ assay, because the least detectable amount of LH which can interfere in the LH$\beta$ assay is 8 i.u./l and the mean level of LH found in large follicles during the late follicular phase was only 5·6 i.u./l (Fig. 5).

The two- to ninefold increase in production of progesterone by granulosa cells *in vitro* after daily exposure to either LH$\alpha$ or LH$\beta$ subunits at doses from 40 to 100 ng (Figs. 6 and 7) can easily be explained by their known contamination with LH (Hagen & McNeilly, 1975*a*; C. Hagen, unpublished observations).

When added together the subunits induced a seven to fifteenfold increase in progesterone production, which indicates around 33 % recombination of the subunits to biologically active LH.

It is unlikely in view of the findings *in vitro* that the subunits, either alone or in combi-

ıation, influence the rate of granulosa cell mitosis or their viability unless both subunits re present in high concentrations in the follicular fluid before the midcycle gonadotrophin ›eak; if this occurred, then the recombined subunits might block mitotic activity (McNatty ‹ Sawers, 1975). Furthermore, when $\alpha$-subunit is present in follicular fluid, even together vith FSH, it is unlikely to alter or disrupt the developing steroidogenic potential of the ranulosa cells (Figure 4*a*; McNatty & Sawers, 1975). However, at mid-cycle when both :- and presumably LH$\beta$-subunits are present in the follicular fluid, it is not unreasonable o expect recombination of the subunits to occur and this would enhance steroidogenesis ›y the granulosa cells undergoing luteinization in the large preovulatory follicle (McNatty *t al.* 1975*b*; McNatty & Sawers, 1975).

The biological activities of the glycoproteins and their subunits have been studied both *n vivo* and *in vitro*. It is probable that the small amount of biological activity present in nost current subunit preparations is due to contamination of these preparations with ntact hormone (Cornell & Pierce, 1973; Morgan, Canfield, Vaitukaitis & Ross, 1974; Parlow & Shome, 1974); an alternative suggestion (Yen, Llerena, Little & Pearson, 1968; Braunstein, Vaitukaitis & Ross, 1972) that the subunits have biological activity but are ess potent *in vivo* because of the much faster clearance rate from the circulation compared vith the intact hormones is unlikely to be correct as indicated by the generally close greement between subunit potency when measured *in vivo* and *in vitro* (Kammerman & Canfield, 1972; Reichert, 1972; Catt, Dufau & Tsuruhara, 1973; Cole, Davis, Huseby & Rice, 1973). However, subunit preparations have only been purified after dissociation of he intact hormone and not directly from the gland of origin. Therefore, whether the ubunit preparations used have the same biological activity as the subunits found in ree form in the gland of origin or circulation is not known. The present studies show that he $\alpha$-subunit circulates independently of the intact hormones in the blood and enters ollicular fluid. Furthermore they support the conclusions of previous studies suggesting hat the subunits have little or no effect on steroidogenesis and that the role of the subunits n follicular development is, at most, of only minor significance.

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# THE GONADOTROPIC HORMONES AND THEIR SUBUNITS IN HUMAN MATERNAL AND FETAL CIRCULATION AT DELIVERY

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# The gonadotropic hormones and their subunits in human maternal and fetal circulation at delivery

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*The blood levels of HCG, LH, their α and β subunits, and FSH were measured by double-antibody radioimmunoassays in 20 normal pregnant women and in matched fetal cord arterial and venous samples at term. High levels of HCG, α subunit, and HCGβ subunit, with low levels of LHβ and FSH, were detected in maternal sera. In the fetal circulation the major detectable components were α subunits and presumably HCG. There was no significant arteriovenous difference in any of the hormones in the fetal circulation and no correlation between levels of hormones in maternal and fetal circulation. Column chromatography of sera confirmed that α subunits were present independent of intact hormones in both maternal and fetal sera.*

THE GLYCOPROTEIN hormones—luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (HCG)—are composed of two nonidentical subunits, α and β, which in the intact molecule are joined by noncovalent bonds.[6, 15, 17, 18] Recent findings suggest that, under some circumstances, the individual subunit can be synthesized and released independently of intact hormone. Thus, the fetal pituitary gland and the placenta may secrete a relative preponderance of the α and β subunits of LH and HCG, respectively.[2, 3] In the present study the nature of circulating LH and HCG has been examined in the human maternal and fetal circulation at the time of delivery. The hormones and their subunits have been identified by means of specific radioimmunoa and by their characteristics on column chroma raphy.

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## Subjects and methods

**Subjects.** Blood samples were collected at time of delivery from 20 normal pregnant wo In 10 of these cases samples of fetal umbilical a and vein blood were also taken. Blood sa were allowed to clot and the serum was sepa by centrifugation and stored at –20° C. unti sayed. All samples were measured in the same as

**Assay methods.** All hormones were measure double-antibody radioimmunoassays. The mat used and the specificity of the assays are show Tables I and II. With the exception of the subunit assay, all the procedures shown in the t have been described previously.[5, 10, 11] Serum L subunit was measured with a rabbit anti-LHβ serum in a final concentration of 1:150,000 146, kindly supplied by Dr. H. S. Jacobs[7]). iodination procedure, buffer, incubation vol incubation time, and separation technique we described for the LHα assay.[4] The sensitivity o assay was 0.2 ng. per milliliter. The specificit the assay is shown in Fig. 1.

**Column chromatography.** Samples of mat and fetal serum, 3 ml. each, were applied to a

e I. Materials used in the radioimmunoassays for LH, HCG, their subunits, and FSH

| ay | *Preparation used for iodination* | *Preparation used as standard* | *Antiserum* |
|---|---|---|---|
| | LH (IRC 2/29),* Hartree | LH (MRC 68/40) assuming 77 I.U./amp†, § | Rabbit anti-LH (F87), Butt |
| | FSH (CPDS 6),* Butt | FSH (MRC 69/104) assuming 10 I.U./amp‡, § | Rabbitt anti-FSH (M93), Butt |
| | | LH$\alpha$ (1/4/72), Hartree | Rabbit anti-FSH, Niederer and McNeilly |
| | | LH$\beta$ (10/18/72), Hartree | Rabbit anti-LH$\beta$, Jacobs |
| | | HCG (CR 115), Canfield | Rabbit anti-LH, Wellcome |
| | | HCG$\alpha$ (CR 115), Canfield | Rabbit anti-HCG$\alpha$ (M110), Lynch |
| | | HCG$\beta$ (CR 115), Canfield | Rabbit anti-HCG$\beta$ (SB6), NIAMD |

RC2/69 and CPDS 6 are code numbers for the LH and FSH preparations obtained from Drs. Hartree and Butt, ctively.

mU. LH MRC 68/40 = 1 ng. IRC2/69 = 100 ng. LER 907.

mU. FSH MRC 69/104 = 1 ng. CPDS 6 = 200 ng. LER 907.

ll LH and FSH results are reported in ng./ml. IRC 2/69 and CPDS 6, respectively. MRC = Medical Research cil.

e II. The specificity of the radioimmunoassays for LH, HCG, their subunits, and FSH. The figure shown is otency of the hormone or subunit expressed as a percentage of the material to which the assay is directed

| *Hormone or subunit* | *LH* | *FSH* | *LH$\alpha$* | *LH$\beta$* | *HCG* | *HCG$\alpha$* | *HCG$\beta$* |
|---|---|---|---|---|---|---|---|
| LH (IRC 2/69) | — | 0.8 | 8 | 25 | 50 | 12 | 3 |
| FSH (CPDS 6) | 2 | — | < 0.1 | < 0.1 | < 0.1 | 0.1 | < 0.1 |
| LH$\alpha$ (Hartree) | 25 | < 0.1 | — | 1 | 7 | 100 | 3 |
| LH$\beta$ (Hartree) | 60 | < 0.1 | < 0.1 | — | < 0.1 | < 0.1 | < 0.1 |
| HCG (CR 115) | 100 | < 0.1 | 4 | 0.2 | — | 0.5 | 10 |
| HCG$\alpha$ (CR 115) | 12 | < 0.1 | 100 | < 0.1 | < 0.1 | — | < 0.1 |
| HCG$\beta$ (CR 115) | < 0.1 | < 0.1 | < 0.1 | < 0.1 | 3 | < 0.1 | — |

) cm. column of Sephadex G-100 (Pharmacia) eluted with 0.05M phosphate buffer, pH 7.4, aining 10 mg. per milliliter of bovine serum min. The flow rate of the column was adjusted ml. per hour and 3 ml. fractions were collected ° C. The column was calibrated with the same , LH$\beta$, HCG$\alpha$, and HCG$\beta$ preparations as for standards in the radioimmunoassays, and rified LH (IRC 2/69), $LH_2$.[4] All results were pared with the Wilcoxon or the Mann-Whitney

## esults

he levels of HCG were significantly higher ($p$ .01) in the maternal (range, 1,200 to > 20,000 per milliliter) than in the fetal circulation ge, 5.8 to 30.0 ng. per milliliter) (Fig. 2). lar values were obtained when LH was mea-l, presumably due to complete cross-reaction of G in the LH assay (range, maternal—1,258 to 3 ng. per milliliter; fetal—18.8 to 70 ng. per liter) (Fig. 3). No cross-reaction of HCG or

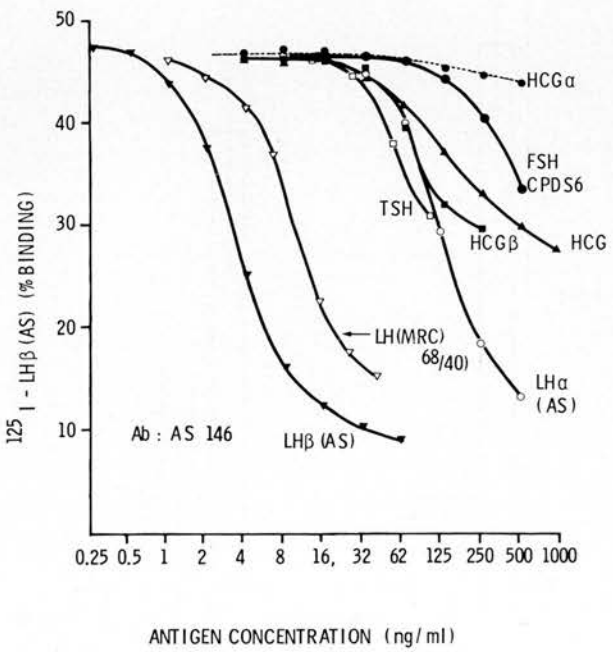



**Fig. 1.** Cross-reaction of the intact glycoprotein hormones and their subunits in the LH$\beta$-subunit assay.

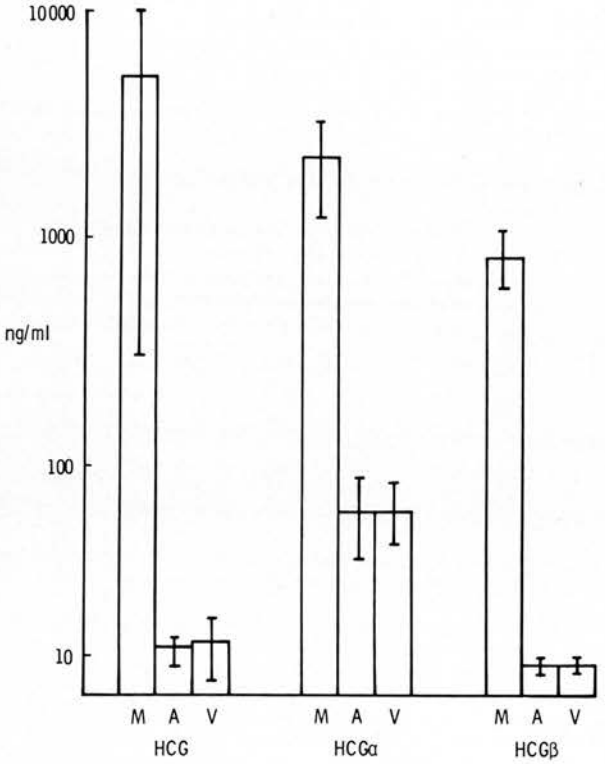



**Fig. 2.** Concentrations (mean ± S.D.) of HCG, HCG$\alpha$, and HCG$\beta$ subunit in maternal *(M)* and fetal cord artery *(A)* and vein *(V)* at term.

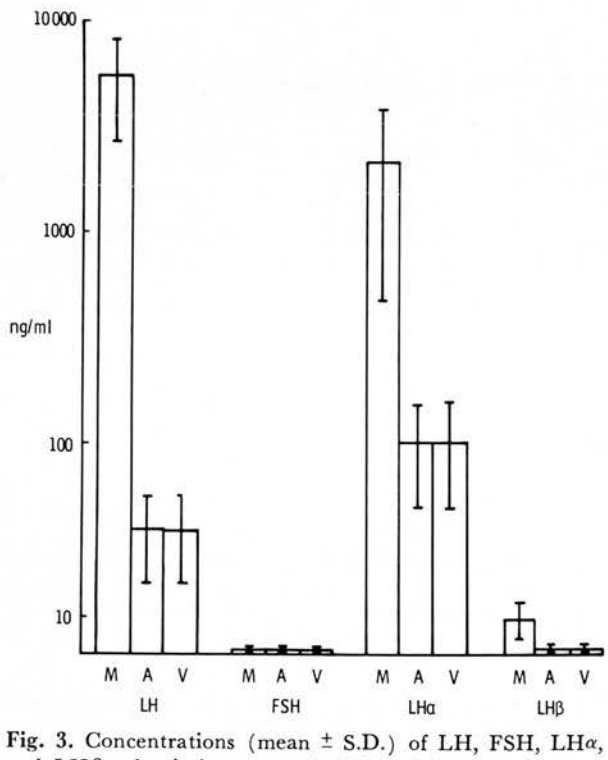



**Fig. 3.** Concentrations (mean ± S.D.) of LH, FSH, LH$\alpha$, and LH$\beta$ subunit in maternal *(M)* and fetal cord artery *(A)* and vein *(V)* at term.

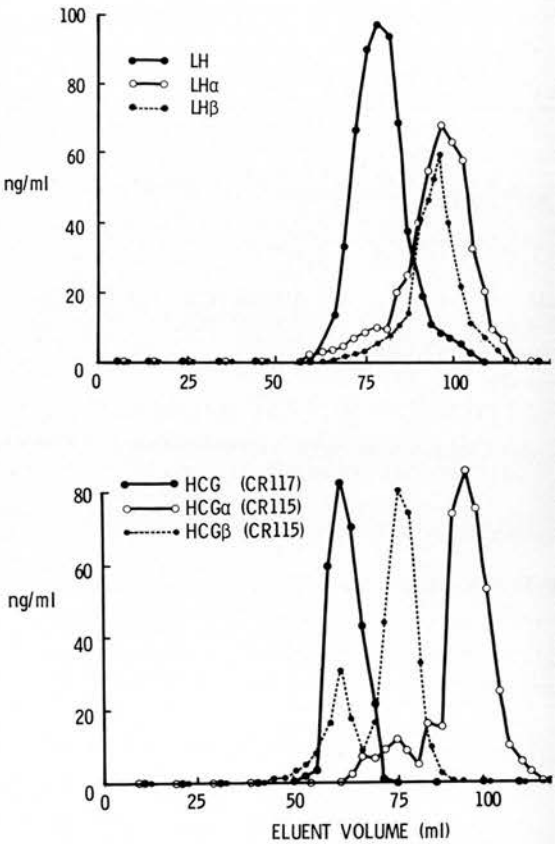
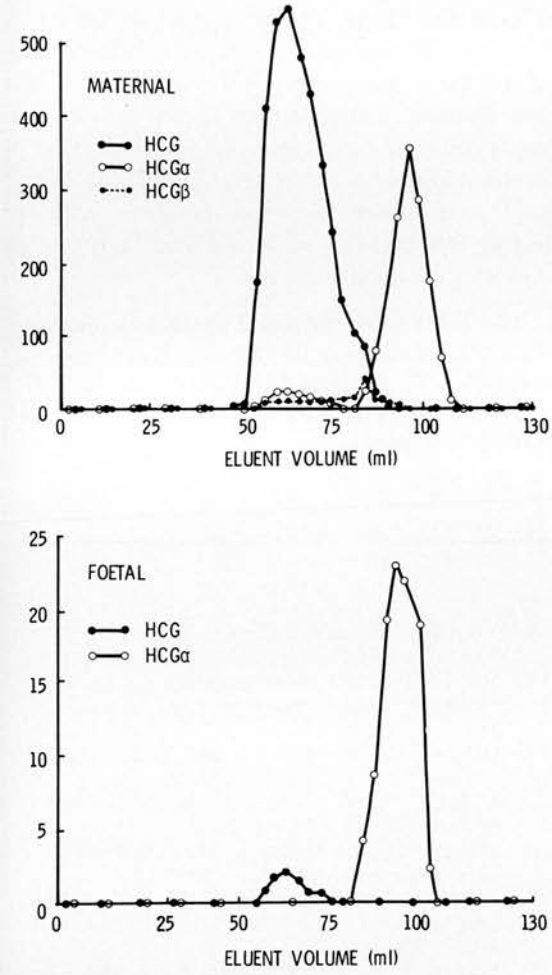



**Fig. 4.** Column chromatography on Sephadex G-1( standard hormone preparations LH ($LH_2$), LH$\alpha$ ( tree), LH$\beta$ (Hartree), HCG (CR 115), HCG$\alpha$ 115), and HCG$\beta$ (CR 115). The elution patterns determined by radioimmunoassay.

subunits ($<$ 0.1 per cent w/w) was found in FSH assay and the levels of FSH in the mate and the fetal circulation were similar, 2.1 ± 0.4 1.7 ± 0.2 ng. per milliliter (mean ± 1 S.D.), res tively (Fig. 3). In both maternal and fetal b levels of HCG$\alpha$ (range, maternal—850 to 4,70( per milliliter; fetal—43.0 to 114.0 ng. per millili and LH$\alpha$-like material (range, maternal—68( 7,940 ng. per milliliter; fetal—40 to 222 ng. milliliter) were significantly greater ($p < 0$ than those of the HCG$\beta$ (range, maternal—63 840 ng. per milliliter; fetal—4.3 to 8.1 ng. per m liter) or LH$\beta$ (range, maternal—4.4 to 29.5 per milliliter; fetal—0.5 to 1.5 ng. per millili In the fetal circulation the $\alpha$ subunit level was nificantly ($p < 0.01$) higher than that of the ir hormones, but no significant differences ($p > 0$ in the levels of any of the hormones or their units were seen between fetal cord artery and (Figs. 2 and 3).




5. Column chromatography of maternal and fetal serum at term. The elution patterns of hormones determined using the HCG, HCG$\alpha$, and HCG$\beta$ immunoassays. Note that in both sera the first peak sponds to HCG and the large second peak to the $\alpha$ nit. No HCG$\beta$ could be detected in the fetal cord n.

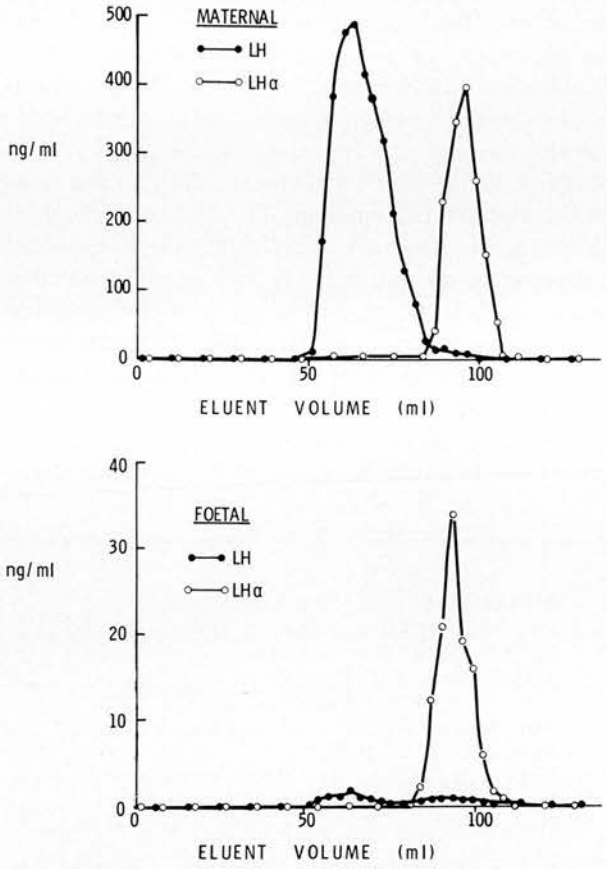



**Fig. 6.** Column chromatography of maternal and fetal cord serum at term. The samples were the same as in Fig. 5, but elution patterns of hormones were determined using the LH, LH$\alpha$, LH$\beta$, and FSH radioimmunoassays. Again the first peak corresponds to HCG not LH, and no LH, LH$\beta$, or FSH were detectable in either sera.

he elution patterns of LH, HCG, and their sub- s are shown in Fig. 4. Gel filtration of maternal m revealed a large peak corresponding to intact G and another more retarded peak correspond- o the $\alpha$ subunit whether measured by the HCG, G$\alpha$, LH, or LH$\alpha$ radioimmunoassays (Figs. 5 6). A small peak of a molecular species which physically and immunologically behaved as G$\beta$ was also observed (Fig. 5). In fetal serum e was virtually no intact hormone but a large corresponding to the $\alpha$ subunit (Figs. 5 and 6).

### omment

s in previous studies it has been shown that the s of HCG are considerably higher in the mater- nal than the fetal circulation.[4, 13, 14] Whether or not any of the material measured as HCG in the maternal circulation could be due to pituitary LH or its subunits remains uncertain, but there seems little doubt that FSH occurs as a separate entity in the blood of both mother and child. Furthermore, the levels of FSH are comparable to those seen in premenopausal woman (range, 0.2 to 3.4 ng. per milliliter) and are not, in contrast to previous findings, lower.[8, 12, 16] That the hormone measured in the LH assay in the fetus is mainly HCG and not LH is shown both by column chromatography (Figs. 2 and 3) and, because of the 25 per cent cross-reaction of LH in the LH$\beta$ assay (Table II), by the very low to undetectable levels of LH$\beta$. It is known that the placenta in vitro can secrete both intact HCG and its subunits.[2] In the present studies it has been shown that these subunits can be distinguished as a separate entity in both the ma-

ternal and fetal circulation and that the relative concentrations of $\alpha$ subunits is greater than both $\beta$ subunits and the intact hormone in the blood of the child. Three explanations can pe put forward for this finding. (1) It is possible that the half-life of the hormone and its subunits differs in the maternal and fetal circulation. (2) It is possible that the barrier to the entry of HCG into the fetus is less complete for the subunit. (3) It is possible that the fetal pituitary gland as shown in vitro[3] secretes considerable amounts of $\alpha$ subunit. At the present time there are no data with which to distinguish between these possibilities.

According to previous studies[1, 9] a higher concentration of HCG is found in the umbilical vein than in the artery. In the present studies we c not find a significant arteriovenous difference in of the hormones in agreement with Geiger, Ka and Franchimont[4]; neither could we demonst any significant correlation between hormone l in the maternal and the fetal circulation. This gests that if the hormones or their subunits transferred between maternal and fetal circula it is only in small amounts.

The assays were performed by Miss D. Bradford. thank Dr. T. Chard for helpful discussions. We thank Drs. W. R. Butt, C. R. Canfield, A. S. Har H. S. Jacobs, and S. S. Lynch, the MRC and NIRMD for reagents used in the radioimmunoassay

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# IDENTIFICATION OF HUMAN LUTEINIZING HORMONE, FOLLICLE-STIMULATING HORMONE, LUTEINIZING HORMONE β-SUBUNIT AND GONADOTROPHIN α-SUBUNIT IN FOETAL AND ADULT PITUITARY GLANDS

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(*Received* 12 *December* 1974)

SUMMARY

Specific radioimmunoassays were used to assess the content of LH, FSH, the gonadotrophin α-subunit and the LH β-subunit in four adult, 19 normal foetal pituitary glands (9·5–32 weeks of gestation) and a pituitary extract from an anencephalic foetus (36 weeks). The hormones and subunits were further identified by column chromatography on Sephadex G-100. All pituitary glands contained free α-subunit and intact LH but the α-subunit:LH ratio was significantly higher in the early foetal pituitaries (9·5–16 weeks) than in the four adult pituitaries. Only small or undetectable amounts of LH β-subunit and 'undetectable' FSH were found in these early foetal pituitaries (9·5–11·5 weeks). The concentration of intact hormones or subunits in the pituitaries showed no significant sex difference in any of the groups. In contrast to these results, only α-subunit was detectable in the pituitary of the anencephalic foetus.

For 14 early foetuses (age of gestation 10–16 weeks) the serum levels of LH–HCG, FSH, and α-subunit in the circulation were significantly higher than in 26 foetuses at term (37–41 weeks). On the basis of these results a theory for the development of the gonadotrophin secretion from the foetal pituitary gland is outlined.

## INTRODUCTION

Pituitary luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyrotrophin (TSH) each consist of two subunits, a hormone-non-specific α-subunit and a hormone-specific β-subunit (Pierce, 1971; Saxena & Rathnam, 1971; Stockell Hartree, Thomas, Braikevitch, Bell, Christie, Spaull, Taylor & Pierce, 1971). No known biological activity has been associated with the gonadotrophin subunits (Rathnam & Saxena, 1971; Reichert, 1972; Catt, Dufau & Tsuruhara, 1973), even though in man they may circulate independently of the intact hormones (Franchimont, Gaspard, Reuter & Heynen, 1972; Laburthe, Dolais & Rosselin, 1973; Hagen & McNeilly, 1975*a*) and very high levels of α-subunit have been demonstrated in the foetal circulation at term (Hagen & McNeilly, 1975*b*). One question that arises is whether these subunits can be found as separate entities in the pituitary gland. In the present studies the contents of LH, FSH, LH β-subunit and gonadotrophin α-subunit have been examined in extracts of normal adult and foetal pituitary glands, and a pituitary gland from an anencephalic foetus. Hormone and subunit levels in foetal blood have also been studied.

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## MATERIALS AND METHODS

### *Extraction of pituitary glands*

Four adult pituitary glands were obtained at *post-mortem* from patients with no known endocrine abnormalities (two women, aged 52 and 74 years; two men, aged 23 and 72 years). Twenty foetal pituitary glands were obtained from patients undergoing hysterotomy during pregnancy, and pituitaries were removed within 30 min of incision of the uterus (Table 1). All pituitaries were stored at −20 °C until extraction. In order to minimize dissociation and aggregation of the hormones and their subunits, a mild extraction procedure was used, and all procedures were performed at 4 °C. The frozen pituitary glands were thawed and homogenized for 30 s in 4 ml 0·05 M-phosphate buffer (pH 7·4) containing bovine serum albumin (Armour), 20 mg/ml; the homogenate was mixed for 2 h, centrifuged at 1500 *g* for 20 min and the supernatant used for further assessment.

Table 1. *Number, sex, and age of the pituitaries extracted*

| Age (weeks of gestation) | Number of pituitaries | | |
|---|---|---|---|
| | Female | Male | Unknown |
| 9·5 | 1* | 1* | — |
| 10·5 | 1 | — | 1* |
| 11 | — | 1 | — |
| 11·5 | — | 1 | — |
| 12·5 | — | 1 | — |
| 13 | 1 | 1 | 1 |
| 13·5 | 2* | — | — |
| 14 | — | — | 1 |
| 15 | — | 2 | — |
| 16 | — | 1 | — |
| 19·5 | — | 1 | — |
| 24 | — | — | 1* |
| 32 | 1* | — | — |
| 36† | 1* | — | — |
| Adult | 2* | 2* | — |

* Hormones identified by column chromatography.
† Anencephalic foetus.

### *Serum measurements*

Blood samples were collected at the time of delivery (period of gestation 37–41 weeks) from 26 foetal umbilical cord veins (16 female and 10 male foetuses), and from 14 early foetuses (period of gestation 10–16 weeks; 4 female and 10 male). Blood samples were allowed to clot, the serum was separated by centrifugation and stored at −20 °C until assayed.

### *Chromatography*

The pituitary extracts were diluted in 2 ml 0·05 M-phosphate buffer (pH 7·4) containing bovine serum albumin (10 mg/ml), applied to a 1·5 × 90 cm column of Sephadex G-100 and eluted with protein buffer. The flow rate of the column was adjusted to 4 ml/h; 2 ml fractions were collected at 4 °C.

The column was calibrated with LH 68/40 (provided by the Medical Research Council), FSH CPDS 6 (kindly provided by Dr W. R. Butt), LH $\alpha$- (AS) (kindly provided by Dr A. Stockell Hartree) and LH $\beta$- (Q) (kindly provided by Dr R. M. Lequin), diluted in 2 ml of the protein buffer. All fractions were assayed for LH, FSH, LH $\beta$- and $\alpha$-subunits.

### *Radioimmunoassays*

The concentrations of LH, FSH, LH $\beta$- and $\alpha$-subunits were measured by double antibody radioimmunoassays (McNeilly & Hagen, 1974; Hagen & McNeilly, 1975*a*, *b*). In the LH assay, LH MRC 68/40 (assuming 77 i.u./ampoule) was used as standard, but all results are expressed in ng LH IRC 2/69/ml (kindly provided by Dr A. Stockell Hartree; 1 ng LH IRC 2/69 = 2 mu. LH MRC 68/40). This assay measures LH IRC 2/69 and human chorionic gonadotrophin (HCG CR 115, biopotency 13·000 i.u./mg, kindly provided by Dr R. E. Canfield) equally on a weight basis. The LH $\beta$- (Q) and LH $\alpha$- (AS) preparations showed 60 % and 25 % cross-reaction, while FSH CPDS 6 showed less than 2 % cross-reaction.

In the FSH assay, FSH MRC 69/104 (assuming 10 i.u./ampoule) was used as standard but all results are expressed in ng FSH CPDS 6/ml (1 ng FSH CPDS 6 (immunopotency 5·000 i.u./mg) = 5 mu. FSH 69/104). LH IRC 2/69, LH $\alpha$- (AS) and LH $\beta$- (Q) all showed less than 1 % and HCG CR 115 less than 0·1 % cross-reaction.

In both the LH $\alpha$- and LH $\beta$-subunit assays, LH $\alpha$- (AS) and LH $\beta$- (AS) preparations kindly supplied by Dr A. Stockell Hartree were used as standards. LH $\alpha$- (AS), HCG$\alpha$ CR 115, TSH$\alpha$ and FSH$\alpha$ subunits (N-785-B and N-611-G respectively; both kindly provided by Dr A. F. Parlow) were equipotent in the LH $\alpha$- assay on a weight basis. LH MRC 68/40 showed 8 % and FSH CPDS 6 and LH $\beta$- (Q) less than 0·1 % cross-reaction respectively in the LH $\alpha$-subunit assay.

In the LH $\beta$-subunit assay the LH MRC 68/40, LH $\alpha$- (AS), FSH CPDS 6, HCG CR 115 preparations showed 25, 1, 0·1 and 0·2 % cross-reaction respectively.

### *Statistics*

All results were compared using the Mann–Whitney U test.

## RESULTS

### *Column standardization*

The void volume of the column was 51 ml estimated by the elution of blue dextran 2000 (Pharmacia Ltd). Fig. 1 shows the relative elution pattern for FSH, LH and its subunits. The LH $\alpha$- and LH $\beta$-subunits were most retarded on the Sephadex G-100 column and were eluted at 130 and 134 ml respectively. These two subunits could be distinguished on the basis of their behaviour in radioimmunoassays. We have shown (Hagen & McNeilly, 1975*b*) that highly purified $\alpha$-subunits of LH and HCG have identical elution patterns. Highly purified FSH and LH were eluted as distinct peaks of immunoreactivity at 106 and 108 ml respectively and these two glycoproteins could also be distinguished on the basis of their behaviour in radioimmunoassays. Less than 5 % and 2 % of the added intact LH and FSH preparations could be detected by any of the radioimmunoassays in the region where subunits were eluted.

### *Pituitary extracts*

Since it was not possible to weigh accurately the foetal pituitaries or to determine how much of the early foetal pituitaries had been removed, the content of LH, FSH and the subunits in the pituitary extracts are reported in terms of the ratio between LH, FSH and the subunits (Fig. 2). No significant difference ($P > 0{\cdot}05$) in any of the hormone ratios was found between pituitaries obtained from female and male subjects, either foetal or adult. In contrast to the adult pituitary extracts in which the amount of LH on a weight basis was double that of the $\alpha$-subunit, the foetal extracts contained significantly ($P < 0{\cdot}01$) more $\alpha$-subunit than LH or FSH. In addition the content of LH greatly exceeded that of FSH, and the ratios of LH:FSH and $\alpha$-subunit:LH were significantly ($P < 0{\cdot}01$) higher in

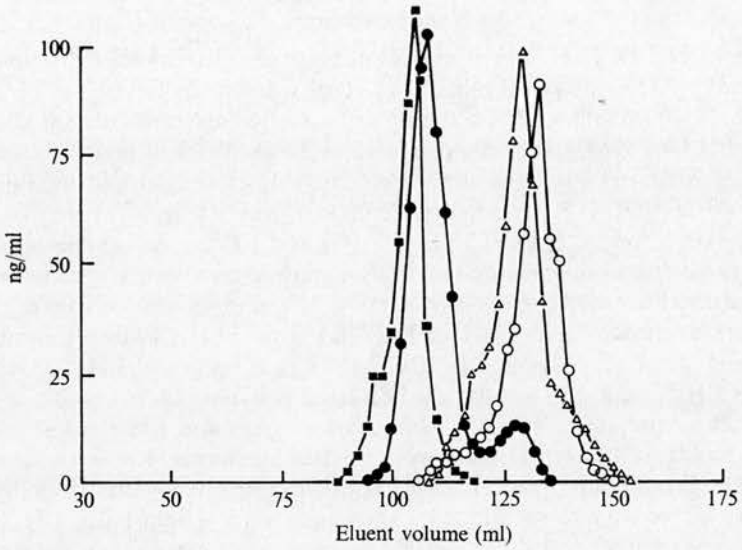



Fig. 1. Elution pattern after column chromatography on Sephadex G-100 of standard hormone preparations, LH 68/40 (●), FSH CPDS 6 (■), LH α-subunit (△) (Hartree) and LH β-subunit (○) (Lequin). Elution patterns were determined by specific radioimmunoassays.

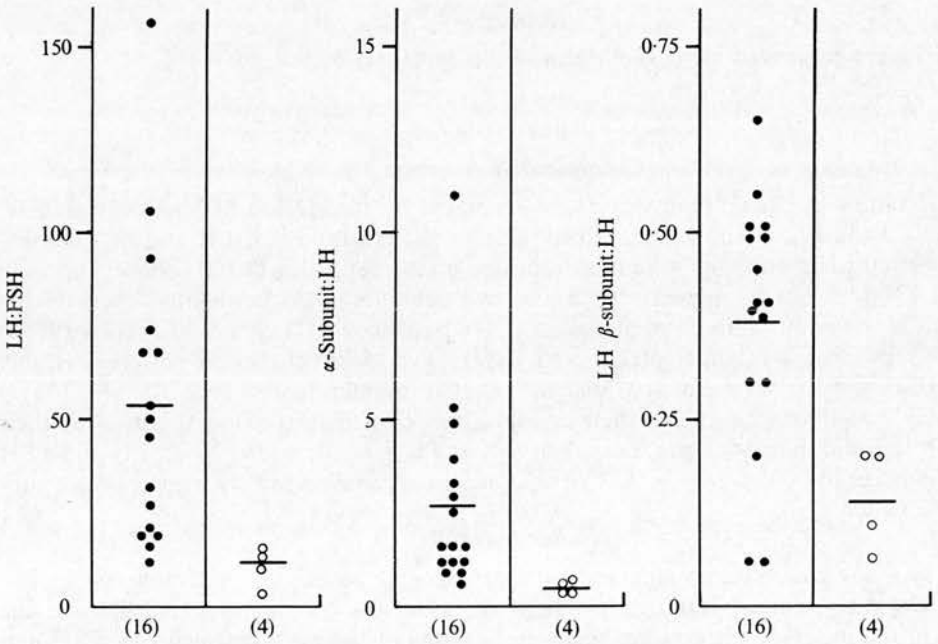



Fig. 2. The ratios of LH:FSH, α-subunit:LH, LH β-subunit:LH in foetal pituitary extracts (closed circles, 9·5–16 weeks of gestation) and adult pituitary extracts (open circles). Horizontal lines indicate mean values. The hormone or subunit concentrations were measured by specific radioimmunoassays. The number of pituitary extracts in each group is shown in parentheses.

the foetal than in the adult pituitary extracts (Fig. 2). In contrast, no significant difference ($P > 0{\cdot}05$) was seen in the ratio of LH:LH $\beta$-subunit in the adult and foetal pituitary extracts. The adult pituitaries contained 100 to 1000 times more LH and $\alpha$-subunit than pituitaries obtained from foetuses of 9·5–11·5 weeks. Pituitary extracts from the three older foetuses (19·5–32 weeks) showed hormone and subunit ratios similar to those of the adult extracts. In contrast the pituitary extract from the anencephalic foetus contained predominantly $\alpha$-subunit, but also significant amounts of LH with an $\alpha$-subunit:LH ratio of 7·8. Only small amounts of FSH could be detected which is shown in the very high LH:FSH ratio, 126. The ratio of LH $\beta$-subunit:LH was 0·20, which is within the normal foetal range (0·02–1·6).

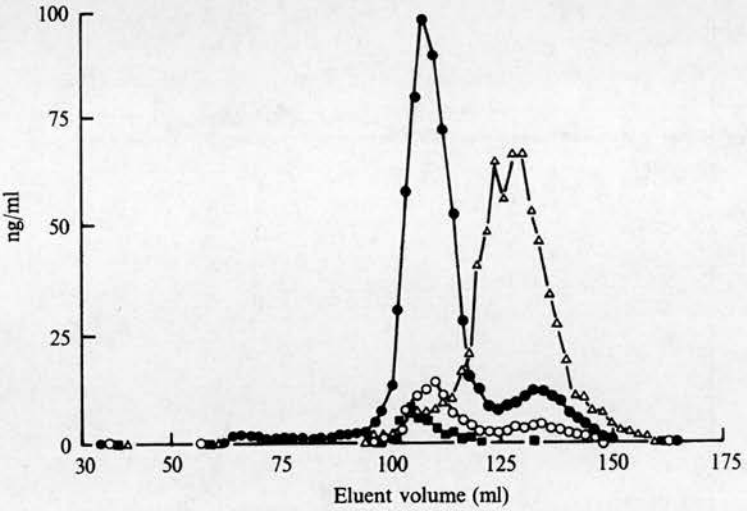



Fig. 3. Elution pattern after column chromatography on Sephadex G-100 of a pituitary extract from a 72-year-old man. All fractions were assayed by specific radioimmunoassays for LH (●), FSH (■), LH $\beta$- (○) and $\alpha$-subunits (△).

### *Chromatography*

All pituitary extracts contained considerable amounts of free $\alpha$-subunit which eluted around 130 ml, i.e. equivalent to standard LH $\alpha$-subunit (Figs 3, 4 and 5). In contrast all extracts contained only small or undetectable amounts of material identifiable as LH $\beta$-subunit. Chromatography of a single pituitary extract obtained from a 72-year-old man (Fig. 3) showed almost equal amounts on a weight basis of LH and $\alpha$-subunit. The amount of FSH compared with that of LH eluted varied in the four adult pituitaries from 5 to 30 % in the two women and from 5 to 20 % in the two men. The LH $\beta$-subunit peak could not be accounted for on the basis of the known 1 % immunological cross-reactivity of LH $\alpha$- in the LH $\beta$-subunit radioimmunoassay.

No FSH was found in the extracts from 9·5- to 12·5-week-old foetuses (Fig. 4). However, significant amounts of FSH were seen in the extracts from 13- to 32-week-old foetuses but this amount was insufficient to be detected after column chromatography. In the pituitary from a 36-week-old anencephalic foetus significant amounts of FSH were also found (Fig. 5).

### *Serum levels*

The levels of LH, FSH and $\alpha$-subunit in the circulation were significantly ($P < 0{\cdot}01$) higher in the early foetuses (period of gestation 10–16 weeks) than in the circulation of the

foetuses at term (period of gestation 37–41 weeks) (Fig. 6). However, although the mean level of LH $\beta$-subunit in the early foetuses was higher than at term, because of the wide scatter in levels this difference was not significant ($P > 0{\cdot}05$). No significant ($P > 0{\cdot}05$) sex

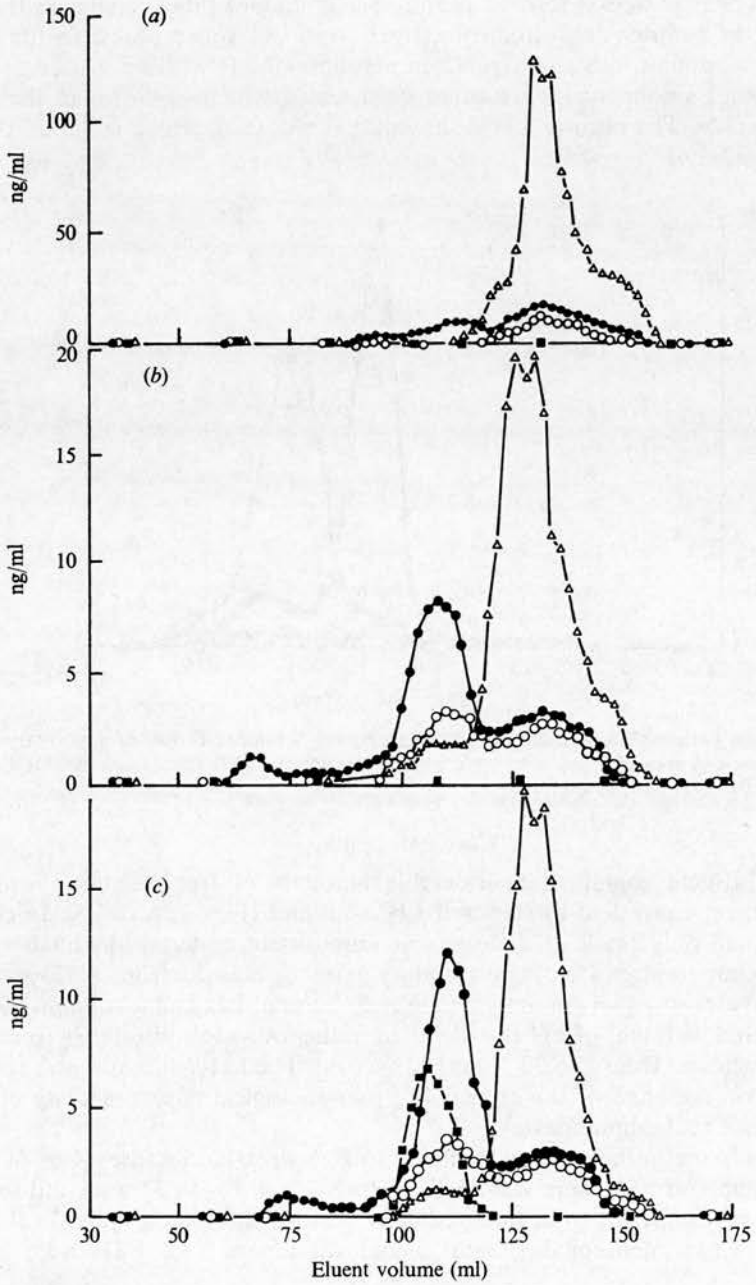



Fig. 4. Elution pattern after column chromatography on Sephadex G-100 of pituitary extracts from a 9·5-week-old male (*a*), a 13·5-week-old female (*b*) and a 32-week-old female (*c*) foetus. All fractions were assayed by specific radioimmunoassays for LH (●), FSH (■), LH $\beta$- (○) and $\alpha$-subunits (△).

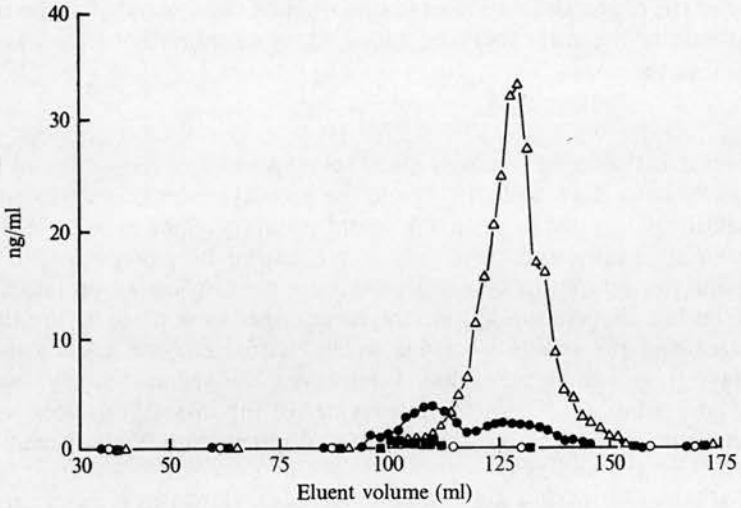



Fig. 5. Elution pattern after column chromatography on Sephadex G-100 of pituitary extract from a 36-week-old anencephalic foetus. All fractions were assayed by specific radioimmunoassays for LH (●), FSH (■), LH $\beta$- (○) and $\alpha$-subunits (△).

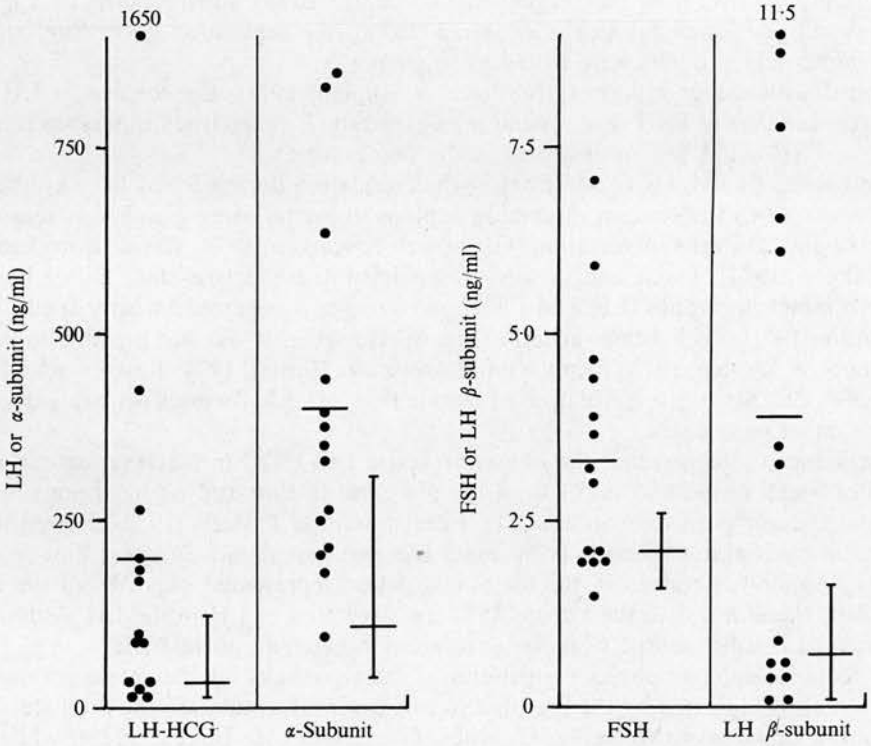



Fig. 6. The levels of LH–HCG, $\alpha$-subunit, FSH and LH $\beta$-subunit in the circulation of 14 foetuses aged from 10 to 16 weeks (closed circles) and the range (vertical bars) in cord veins of 26 foetuses at term (period of gestation 37–41 weeks) are shown. Horizontal lines indicate mean values. The concentrations of the hormones and subunits were measured by specific radioimmunoassays.

difference in any of the hormone or subunit levels could be demonstrated in the two groups. It was apparent that in the early foetuses, blood levels of intact hormones and subunits showed considerable variation.

## DISCUSSION

It has been shown that the foetal pituitary gland secretes more of the $\alpha$-subunit than intact LH *in vitro* (Franchimont & Pasteels, 1972) and the present report confirms that the major content of gonadotrophic substances in the foetal pituitary gland is a molecular species which both immunologically and physically is recognized as gonadotrophin $\alpha$-subunit. The mild extraction procedure was chosen to minimize the dissociation of intact hormones into subunits. Whether dissociation by proteolytic enzymes took place before the pituitary glands were frozen and the results were due to differential enzyme activity in the foetal and adult pituitary gland cannot be excluded. However, this seems unlikely since it would be expected that degradation by proteolytic enzymes of the intact hormones would result in smaller components of varying molecular size and not immunologically and physically distinct subunits (Figs 3, 4 and 5).

Dissociation of intact hormones into subunits has been shown to occur at acid or basic pH (Papkoff & Samy, 1967; Ingham, Alof & Edelhoch, 1973), or at high osmolality, for example, exposure to 8 M-urea (De la Llosa & Jutisz, 1969). The mild extraction procedure used in this study is unlikely to have produced breakdown of intact hormones into subunits (Ingham *et al.* 1973; J. G. Pierce, personal communication). Furthermore, no aggregation of subunits or intact hormones occurred during the separation procedure, since purified hormones and subunits were eluted as single peaks.

In agreement with earlier reports (Grumbach & Kaplan, 1973), the content of LH in all glands exceeded that of FSH (Fig. 2) and no significant ($P > 0{\cdot}05$) sex difference in the ratio of LH to FSH could be demonstrated in the two groups.

The highest levels of LH–HCG and FSH in the circulation during foetal life have been reported to occur when the content of gonadotrophins in the pituitary gland is increasing, i.e. between 12 and 20 weeks of gestation (Grumbach & Kaplan, 1973; Reyes, Boroditsky, Winter & Faiman, 1974). The results of the study confirm these reports since higher blood levels of both intact hormones (LH and FSH) and $\alpha$-subunit occurred in early foetal life (age of gestation 10–16 weeks) than at term (Fig. 6). However, it was not possible to confirm (Grumbach & Kaplan, 1973; Penny, Olambiwonnu & Frasier, 1974; Reyes *et al.* 1974) the higher levels of FSH in the circulation of female than of male foetuses probably due to the smaller number of samples.

There is evidence to suggest that the immunoreactive LH–HCG in foetal serum consists of both LH of foetal origin and HCG from the placenta. In this study it has been shown that the foetal adenohypophysis contains LH. Franchimont & Pasteels (1972) showed that the foetal pituitary is able to secrete both intact LH and its subunits *in vitro*. Finally the levels of LH $\beta$-subunits obtained in the foetal circulation represented only 2% of the LH levels recorded; therefore, with the known 25% cross-reaction of LH in the LH $\beta$-subunit assay, the foetal LH must consist of molecular species other than normal LH.

That the foetal adenohypophysis contributes at least partially to the serum immunoreactive $\alpha$-subunit is indicated by the fact that in the maternal circulation the level steadily increases during pregnancy (McNeilly, Gardner, Gau, Jeffrey & Hagen, 1975), while in the foetal circulation the levels are lower at term than in early foetal life (Fig. 6). However, the exact contribution of foetus and placenta to hormones and subunits in the foetal circulation remains to be determined.

Thus, the results from the present study indicate that both intact gonadotrophins and the subunits are present as separate entities in the pituitary gland. The pattern of change in

the ratio of pituitary LH, FSH, LH $\beta$-subunits and $\alpha$-subunits during foetal life suggests that the first gonadotrophin substance synthesized in the foetal pituitary gland is the common $\alpha$-subunit, and then under hypothalamic influence the synthesis of the $\beta$-subunit takes place which leads to the production of the intact hormones. This is underlined by the fact that the pituitary from the anencephalic foetus (period of gestation 36 weeks) only contained small amounts of intact hormones compared with the amount of $\alpha$-subunit (Fig. 5), and that hypothalamic gonadotrophin releasing hormone stimulates not only release but also synthesis of LH and FSH in the pituitary gland (see Schally, Arimura & Kastin, 1973).

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**Secretion of human chorionic gonadotrophin and its subunits during pregnancy.** By A. S. McNeilly, J. Gardner, G. Gau, D. Jeffrey and C. Hagen. *Department of Reproductive Physiology, St Bartholomew's Hospital Medical College, London, EC1A 7BE*

The placental glycoprotein hormone, human chorionic gonadotrophin (HCG) consists of a hormone specific $\beta$-subunit (HCG$\beta$) and an $\alpha$-subunit common to the pituitary glycoprotein hormones luteinizing hormone, follicle-stimulating hormone and thyrotrophin. Previous studies (Franchimont, Gaspard, Reuter & Heynen, 1972; Vaitukaitis, 1974) indicated that the placenta contained and secreted both intact HCG and free $\alpha$-subunits. Using specific radioimmunoassays for HCG and HCG$\beta$ and $\alpha$-subunits, the changes in blood levels of these components during pregnancy have been investigated. The secretion patterns from placentae during continuous flow culture, and their distribution throughout the placenta have also been studied.

During pregnancy, serum levels of HCG (800–9300 ng/ml) did not show any significant change from week 16 of pregnancy to term, while levels of $\alpha$-subunit showed a significant mean increase from $518 \pm 50$ (S.E.M.) ng/ml to a maximum value of $1062 \pm 80$ ng/ml at term. The changes in serum levels of HCG and $\alpha$-subunit were unrelated throughout pregnancy.

In six experiments slices of placenta at term, collected at normal vaginal delivery were maintained in continuous flow culture for 8–36 h. Initial production of free $\alpha$-subunit was 10–200 times that of intact HCG. Secretion of both HCG and $\alpha$-subunit, plateaued after 8–10 h of culture when the $\alpha$-subunit secretion rate varied between three and five times that of HCG. The amount of $\alpha$-subunit and HCG released was directly related to the flow rate of medium through the culture system.

Analysis of the distribution of HCG and its subunits throughout the placenta showed that both HCG and $\alpha$-subunit were distributed evenly throughout the placenta, and were not directly related to the amount of trophoblast present. The placental content of HCG varied between 1 and 4 mg, while that of the $\alpha$-subunit varied between 15 and 80 mg.

The results indicate that free $\alpha$-subunit, probably of placental origin, circulates in increasing concentration throughout pregnancy. The placenta at term produces $\alpha$-subunit *in vitro* at a greater rate than intact HCG and the predominant glycoprotein hormone-related material in the placenta at term is free $\alpha$-subunit.

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# Presence and activity of LH-RH in the mid-term human fetus

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**Summary.** Immunoreactive LH-RH was present in all the hypothalamic and cortical extracts of mid-term human fetuses studied and in the cortical tissue removed from the two youngest fetuses. Gonadotrophin-releasing activity of hypothalamic and cortical extracts was demonstrated by the significant rises of circulating LH after infusion into oestrogen and progesterone-primed ovariectomized rats.

## Introduction

Although immunoreactive LH-RH has recently been identified in human fetal brain tissue from as early as 4·5 weeks after conception (Winters, Eskay & Porter, 1974) there is little information existing as to the exact time at which pituitary gonadotrophin synthesis and release commences. Siler-Khodr, Morgenstern & Greenwood (1974) have claimed that both pituitary LH and FSH are present within 5 weeks of conception and Tamura, Minaguchi & Sakamonto (1973) have shown that when second trimester human pituitaries are grown in organ culture LH may be released spontaneously or by the addition of synthetic LH-RH to the medium. However, an investigation *in vivo* by Gennser, Liedholm & Thorell (1976) failed to note any change in plasma gonadotrophins when LH-RH was administered to fetuses at 15–22 weeks of gestation. The purpose of the present study was (1) to investigate further the presence of LH-RH in human fetal hypothalamic and cortical tissue by direct radioimmunoassay and (2) to examine the gonadotrophin-releasing activity of these same extracts when infused into the carotid artery of suitably primed ovariectomized rats.

## Materials and Methods

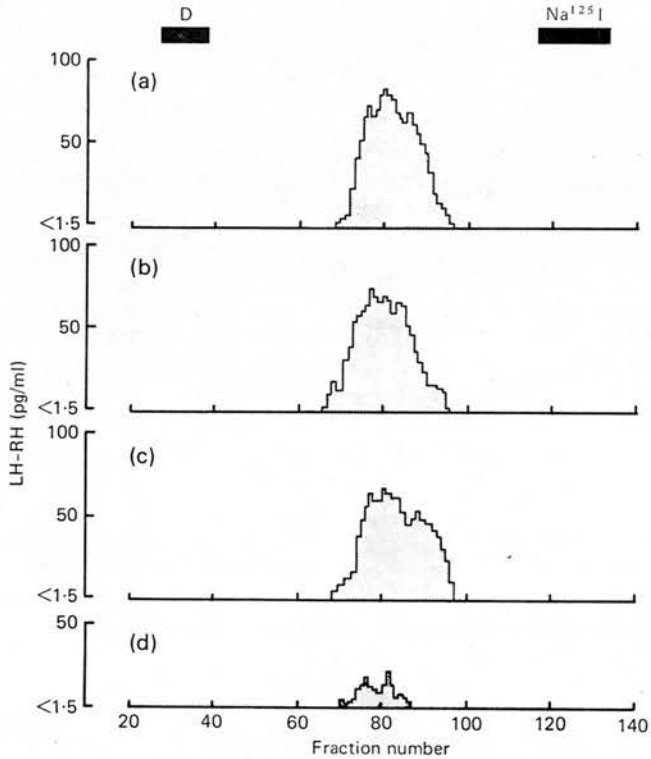
Sixteen mid-term human fetuses, ranging in size from 46 to 179 mm crown–rump length, were collected within 5–10 min of their removal from the uterus by hysterotomy (performed for gynaecological reasons on otherwise healthy pregnant women). Fetal age was assessed by comparing weight and crown–rump length with those in the tables published by Iffy *et al.* (1975). Sex was determined by examination of the external and internal genitalia and confirmed by subsequent gonadal histology. The hypothalamus and a sample of cortical tissue from the frontal lobe were removed and immediately frozen on solid $CO_2$ before storage at −20°C. Extracts were later prepared by thawing the samples and adding 1·0 ml 0·5% HCl for every 100 mg tissue present. The mixture was homogenized and centrifuged at 0°C and the supernatant was neutralized with solid $NaHCO_3$ before being frozen and stored. Each preparation yielded beftween 1·0 and 2·0 ml extract.

### *Experiment 1. Detection of fetal LH-RH*

Samples (0·5 ml) of hypothalamic and cortical extracts from 8 fetuses were individually thawed and then chromatographed on a Biogel P2 column (32·5 × 1 cm) with 2·5% human serum albumin and 0·05 M-$Na_2PO_4$ buffer (pH 7·5) at a flow rate of 3 ml/h. Fractions of 0·5 ml were collected and LH-RH

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was measured with the highly sensitive radioimmunoassay developed by Mortimer *et al.* (1976), in which the minimal detectable LH-RH titre is 0·2 pg/ml. The assays were performed in duplicate; the intra- and inter-assay coefficients of variation were 12 and 15·5% respectively. Extracts of rat stalk–median eminence (SME) were prepared in the same manner as the human tissue and the elution patterns for synthetic LH-RH in buffer, rat SME and human fetal hypothalami were similar and are shown in Text-fig. 1.




**Text-fig. 1.** Elution patterns of (a) synthetic LH-RH + buffer, (b) synthetic LH-RH + plasma, (c) rat stalk–median eminence and (d) human fetal hypothalami chromatographed on a Biogel P2 column with 2·5% HSA, 0·05 M-$Na_2PO_4$ buffer, pH 7·5. The elution positions of dextran (D) and $Na^{125}I$ are shown.

*Experiment 2. Assessment of fetal LH-RH activity*

Other samples (1·0 ml) of hypothalamic and cortical extracts from all 16 fetuses were thawed and each was then infused under ether anaesthesia into the carotid artery of a mature heparinized Sprague–Dawley rat. Infusions of 1·0 ml of carrier solution (neutralized HCl) were carried out as controls and carrier solution containing synthetic LH-RH in concentrations ranging from 0·2 pg to 200 ng/ml was administered to investigate dose-related responses. All 66 rats used were sexually mature and had been ovariectomized for at least 4 weeks. Priming doses of 50 μg oestradiol benzoate and 25 mg progesterone in 1·0 ml corn oil were injected subcutaneously 72 h before the experiment because pretreatment with these steroids has been shown to sensitize ovariectomized rats to the action of exogenous LH-RH (Ramirez & McCann, 1963). Blood was collected from the iliac vein immediately before and at 15 min after infusions. Concentrations of LH and FSH were measured in the separated plasma by a double-antibody radioimmunoassay using material supplied by the NIAMDD, Bethesda, Maryland. The intra-assay coefficient of variation was 8% for LH and 10% for FSH. The inter-assay coefficient of variation was 12% for LH and 15% for FSH. The results are expressed as ng equivalents of NIAMDD-

LH-RP-1 or NIAMDD-FSH-RP-1/ml. The limits of detection were established as 4 ng/ml for LH and 40 ng/ml for FSH.

## Results

### *Experiment 1*

LH-RH was detected by radioimmunoassay in all the fetal hypothalamic samples (Table 1). The concentration was highest in the preparations from the two youngest fetuses, and it was only from these that the cortical samples contained any detectable amounts of LH-RH.

**Table 1.** LH-RH content, measured by specific radioimmunoassay, in human fetal hypothalamic and cortical tissue

| Sex | Crown–rump length (mm) | Age (weeks) | LH-RH content (pg/mg wet wt of tissue) | |
|---|---|---|---|---|
| | | | Hypothalamus | Cortex |
| ♂ | 46 | 11 | 11·84 | 0·25 |
| ♂ | 73 | 13–14 | 3·51 | 0·15 |
| ♀ | 90 | 15 | 1·48 | ND |
| ♂ | 103 | 15–16 | 2·32 | ND |
| ♀ | 110 | 16–17 | 0·71 | ND |
| ♂ | 115 | 16–17 | 1·67 | ND |
| ♀ | 122 | 17 | 0·84 | ND |
| ♀ | 154 | 19 | 0·59 | ND |

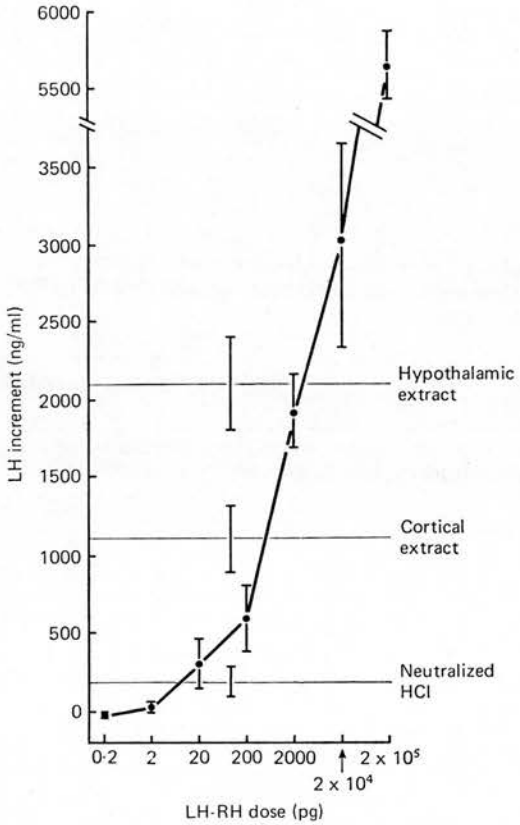
ND, not detectable.

**Table 2.** Plasma gonadotrophin concentrations (mean ± s.e.m.) in progesterone and oestrogen-primed ovariectomized rats before and at 15 min after carotid infusion of various substances

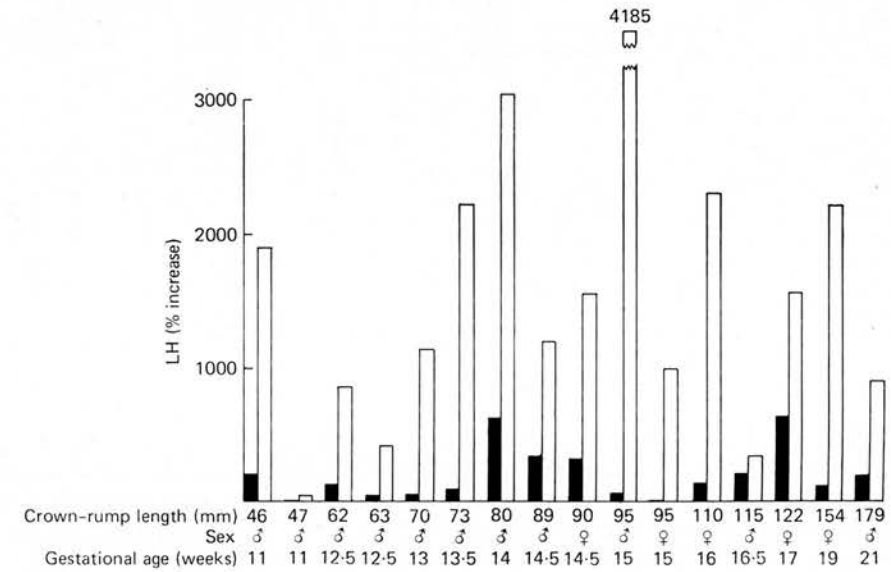
| Substance infused | No. of rats | LH (ng/ml) | | FSH (ng/ml) | |
|---|---|---|---|---|---|
| | | Pre-infusion | Post-infusion | Pre-infusion | Post-infusion |
| Cortical extract | 16 | 240·6 ± 29·4 | 1352·2 ± 207·8 | 501·3 ± 28·8 | 751·1 ± 64·2 |
| Hypothalamic extract | 16 | 340·2 ± 49·5 | 2446·7 ± 300·3 | 554·0 ± 35·1 | 964·4 ± 139·0 |
| 200 ng LH-RH | 5 | 404·4 ± 19·7 | 6053·4 ± 892·7 | 562·6 ± 63·2 | 1289·8 ± 284·3 |
| 20 ng LH-RH | 5 | 333·8 ± 119·9 | 3354·8 ± 799·1 | 691·2 ± 72·0 | 1080·4 ± 134·9 |
| 2 ng LH-RH | 3 | 407·0 ± 140·8 | 2339·3 ± 191·9 | 761·0 ± 152·6 | 1209·7 ± 124·9 |
| 200 pg LH-RH | 4 | 191·0 ± 30·6 | 786·3 ± 244·7 | 950·0 ± 117·3 | 1048·0 ± 106·5 |
| 20 pg LH-RH | 4 | 205·5 ± 29·0 | 509·0 ± 160·0 | 750·5 ± 99·1 | 713·8 ± 101·5 |
| 2 pg LH-RH | 4 | 202·3 ± 37·0 | 222·3 ± 37·2 | 748·8 ± 21·4 | 736·0 ± 43·7 |
| 0·2 pg LH-RH | 4 | 239·0 ± 36·8 | 215·8 ± 40·8 | 1054·3 ± 161·9 | 959·5 ± 165·3 |
| Neutralized HCl | 5 | 390·0 ± 73·9 | 570·4 ± 68·4 | 561·2 ± 72·1 | 861·2 ± 113·6 |

### *Experiment 2*

The effects on rat plasma gonadotrophin levels of the carotid infusions are shown in Table 2 and in Text-figs 2 and 3. The results were analysed statistically by a number of two-sample *t* tests. Doses of LH-RH ranging from 2 to 200 ng and the infusions of hypothalamic extract all brought about a significant elevation of plasma LH ($P < 0·001$) in comparison to the rise induced in the controls treated with neutralized HCl. The increase in circulating LH caused by infusion of cortical extract was also significant ($P < 0·05$), but to a lesser degree. However, only the highest dose of the synthetic decapeptide significantly raised plasma FSH concentrations ($P < 0·05$).




**Text-fig. 2.** Increments (mean ± s.e.m.) in plasma LH of oestrogen and progesterone-primed ovariectomized rats infused with various doses of LH-RH, neutralized HCl and fetal hypothalamic and cortical extracts (see Table 2 for numbers of animals).




**Text-fig. 3.** Percentage increase in circulating plasma LH 15 min after infusion of individual extracts of fetal hypothalamic (open bars) and cortical (solid bars) tissue into the carotid artery of oestrogen and progesterone-primed ovariectomized rats.

## Discussion

The differential effect of LH-RH on plasma LH and FSH levels is in broad agreement with the results of Libertun, Cooper, Fawcett & McCann (1974) who found no significant effects on plasma FSH when rats were treated with various doses of LH-RH.

Few pregnancies in women are now terminated by hysterotomy and investigations into human prenatal endocrinology are therefore limited by the availability of tissue. Although there are insufficient data from the present study to permit any firm comments about the pattern of LH-RH content and activity in relation to fetal age and sex, it would appear that the concentrations of the hormone measured in the hypothalamus and cortex (Table 1) are not necessarily correlated with the hormonal activity demonstrated by bioassay (Text-fig. 2). Fetal hypothalamic and cortical tissues appear to contain much higher amounts of LH-RH than are measured by radioimmunoassay in any of the samples collected. However, estimates of LH-RH activity in the human brain by using a bioassay must be made with some caution because the decapeptide has not been established as the sole biologically active form of the gonadotrophin-releasing hormone. Certain fragments of the decapeptide have been shown by Debeljuk, Arimura & Schally (1973) to have biological activity and it is possible that these fragments may be present in proportionally greater concentrations in the fetal than in the adult brain. Moreover, as pointed out by Seyler & Reichlin (1974), the bioassay dose–response curves for LH-RH extracted from rat, porcine and human hypothalami differ from those with the synthetic hormone.

Although the presence of LH-RH has been identified in the early and mid-term human fetal brain, the experiments of Gennser *et al.* (1976) have demonstrated that the human fetal pituitary *in vivo* is unable to respond to exogenous LH-RH; endogenous LH-RH may also therefore be incapable of stimulating gonadotrophin release. However, the experiments reported here have shown that extracts of fetal hypothalamus and cortex containing this releasing hormone do at least exhibit biological activity when injected into suitably primed rats.

We thank Professor M. C. Macnaughton, Dr P. W. Howie and Dr Helen McEwan and their staff at the Glasgow Royal Infirmary; Mrs D. Jeffries, Mrs H. McLean and Mr W. Barrie for their excellent technical assistance; Dr V. A. Moss, Glasgow University, and Professor J. Landon, Department of Chemical Pathology, St. Bartholomew's Hospital; the Medical Research Council for financial assistance; and the NIAMDD Pituitary Hormone Distribution Programme and Dr A. F. Parlow for the rat FSH and LH radioimmunoassay kits.

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# Radioimmunoassay and Chromatographic Similarity of Circulating Endogenous Gonadotropin Releasing Hormone and Hypothalamic Extracts in Man

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# Radioimmunoassay and Chromatographic Similarity of Circulating Endogenous Gonadotropin Releasing Hormone and Hypothalamic Extracts in Man

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**ABSTRACT.** A highly sensitive radioimmunoassay for the gonadotropin releasing hormone has been developed in order to study its physiological importance in man. In view of the expected low concentrations in peripheral blood, large volumes of human plasma were extracted by two different methods and the characteristics of the radioimmunoassayable material compared with those of synthetic decapeptide and extracts of human hypothalami. The results indicate that radioimmunoassayable gonadotropin releasing hormone is present in some human plasmas but the plasma concentrations are less than 2.5 pg/ml. Peripheral levels were more consistently measurable in women at midcycle and after the menopause. The hormone was undetectable in the plasma of normal men, human cerebrospinal fluid, and fetal cerebral tissue, but was present in fetal hypothalami. (*J Clin Endocrinol Metab* 43 882, 1976)

FOLLOWING the isolation and subsequent synthesis (1) of the gonadotropin releasing hormone (LH-RH), assays have been developed which are capable of determining the high concentrations found in hypothalamic extracts of various species. They have not been sufficiently sensitive, however, to determine peripheral circulating levels with confidence. Thus, while there is both indirect and direct evidence for the existence of this hypothalamic hormone in the peripheral circulation (2–7), the levels reported by different groups vary considerably (Table 1) and it has not proved possible to extract sufficient hormone to permit further investigation of its nature. Consequently, the levels of LH-RH reported by these authors have been questioned.

It was our intention to develop a highly sensitive radioimmunoassay for LH-RH and compare the chromatographic characteristics of the material extracted from large volumes of plasma with those of the synthetic decapeptide and naturally occurring releasing hormone in human fetal hypothalami. The assay was then applied to the measurement of LH-RH levels in plasma samples from women with normal menstrual cycles, adult men, and in human cerebrospinal fluid and fetal cerebral extracts.

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## Materials and Methods

### A. *Collection of samples*

Pooled plasma samples for chromatography were obtained from 10 normal men and 25 post-menopausal women. Blood (9 ml) was collected in lithium heparin tubes containing 1 ml of Trasylol (10,000 KIU) and centrifuged immediately. The supernatant was then decanted into 60 ml polystyrene tubes containing $10^{-3}$ molar phenylmethylsulphonylfluoride (PMSF), since this collection procedure had been shown previously to minimize hormone degradation by proteolytic enzymes present in blood. Hemolyzed samples were rejected. Samples were frozen immediately and kept at −20 C until assayed.

Individual plasma samples (10 ml) were taken at hourly intervals for eight h from three women on days 7, 13, or 14 (the day of a rise in basal body temperature), 21, and 28 of the menstrual cycle. Two had hyperprolactinemia and secondary amenorrhea. However, a normal menstrual cycle resumed after bromocriptine therapy (8).

TABLE 1. Estimates of plasma LH-RH levels in man

| | | | pg/ml |
|---|---|---|---|
| Unextracted (Keye *et al.* 1973 (5)) | Pre-pubertal children (mean) | | 30.7 |
| | Men (mean) | | 67.9 |
| | Women (mean) | | 69.6 |
| Methanol extraction (Jeffcoate and Holland 1974 (6)) | Normal subjects | | <0.25–3.5 |
| Ethanol extraction (Arimura *et al.* 1974 (7)) | Women | follicular | Undetectable–1.8 |
| | | *midcycle | 2–17 |
| | | luteal | Detectable in 2/5 women |
| Vycor extraction Present study | Men | | <0.2 |
| | Women | follicular | <0.2 |
| | | midcycle | <0.2–1.5 |
| | | luteal | <0.2 |
| | | post-menopausal | <0.2–2.5 |

* Lower level estimated from published illustration.

Plasma samples were also obtained at random from six post-menopausal women and 10 normal men. Human hypothalamic and cerebral tissue was obtained from eight aborted fetuses between 5 and 18 weeks old, extracted with N HCl and neutralized with sodium bicarbonate before assay. Cerebrospinal fluid (CSF) was obtained from eight patients prior to air encephalography. Six had radiological evidence of pituitary tumors with sloping or double floors to the sella turcica and hyperprolactinemia and two had acromegaly. Samples were assayed individually from 5 patients and then pooled with a further three samples (one acromegalic and two with abnormal pituitary fossae). They were treated with bromocriptine (Parlodel, CB154) and external pituitary irradiation.

### *B. Extraction*

Pooled human plasma, CSF and hypothalamic, and cerebral extracts were concentrated either with leached silica glass (Vycor, Code 7930, Mesh size 140. Corning Glass Int., Corning, New York) or with methanol. The Vycor was activated by heating to 600 C for 1 h before use and 100mg was added for each 10 ml of sample since this was shown to result in the maximum recovery of synthetic hormone. The sample was vortexed, centrifuged, and the supernatant discarded. The residue was washed with 2 ml of de-ionised water followed by 1ml of N HCl and the hormone then eluted from the glass by rotation for 30 min with 1 ml 60% acetone/water. The extracts were dried at 50 C in a sand bath under a stream of nitrogen or in a vortex evaporator apparatus and then reconstituted in 0.5 ml 2.5% human serum albumin (HSA) sodium phosphate buffer, pH 7.5 and assayed immediately (although they have been shown to remain stable in this form for up to 7 days at 4 C). With this method, 50% of added hormone was recovered from plasma samples. The methanol extracts were prepared by the addition of 1.5 volumes of methanol and the supernatant dried as described above for volumes less than 10 ml. When volumes of 200–500 ml of plasma were extracted, the supernatant was dried initially at 50 C in a rotary evaporator and the residual material then re-extracted with 80% methanol water and the supernatant dried at 50 C under a stream of nitrogen or in a vortex evaporator. Samples were then reconstituted in buffer as above.

### *C. Gel filtration*

Samples, 0.5 ml, were chromatographed on a Biogel P2 column (32.5 × 1 cm) with 2.5% human serum albumin (HSA) 0.05M sodium phosphate buffer pH 7.5 at a flow rate of 3 ml per h. Fractions of 0.5 ml were collected and stored at 4 C until assayed.

### *D. Preparation of iodinated hormone*

Synthetic LH-RH (1 $\mu$g) was iodinated with 1mCi $^{125}$I in the presence of chloramine T (5 $\mu$g) for 25 sec. The reaction mixture was diluted in 10 ml of buffer and the labelled hormone ex-

tracted with 100 mg of Vycor glass as described for plasma samples. The iodinated hormone was flash-frozen in 0.5 ml aliquots by dropwise addition to polystyrene tubes standing in a mixture of dry ice and ethanol and was stored at −80 C until required. The tracer was stable for up to 3 weeks without loss of immunoreactivity.

*E. Antiserum and specificity*

The antiserum was raised in white New Zealand rabbits by Dr. S. Jeffcoate (9). There was 100% cross-reaction, on a molar basis, with both the complete hormone and the 3–10 octapeptide. Loss of the C-terminal glycine resulted in a fall in cross-reactivity to 0.33%. There was no cross-reactivity with thyrotropin releasing hormone, growth hormone release inhibiting hormone, oxytocin, lysine vasopressin, ACTH, βMSH, LH, FSH, TSH, GH, and prolactin at many times their physiological levels. The addition of Trasylol® or PMSF in the amounts used did not interfere in the assay.

*F. Assay protocol*

Standards were prepared using synthetic LH-RH in 2.5% HSA phosphate buffer pH 7.5 and 100 μl of standard or sample in duplicate was incubated with 50 μl antiserum (final dilution 1:60,000) for 24 h at 4 C. Labelled hormone (approximately 3 pg) in 0.05 ml buffer was then added and incubated for a further 24 h. Separation of the bound and free fractions was achieved by a second antibody technique and the radioactivity of the precipitate (bound fraction) was counted for 100 sec in an Ultrogamma 1280, LKB. The coefficient of variation within assay after Vycor extraction was 12% at a mean plasma level of 10 pg/ml (N = 20). Inter-assay variation at a mean plasma level of 10 pg/ml was 15.5% (N = 8).

## Results

*Gel filtration*

*a. Untreated samples (Fig. 1a).* Synthetic decapeptide added to 0.5 ml protein buffer or 0.5 ml plasma eluted with the same mobility. The elution patterns of the synthetic hormone and human hypothalamic extracts were similar. There was no detectable immunoassayable LH-RH in 0.5 ml of fresh plasma from a post-menopausal

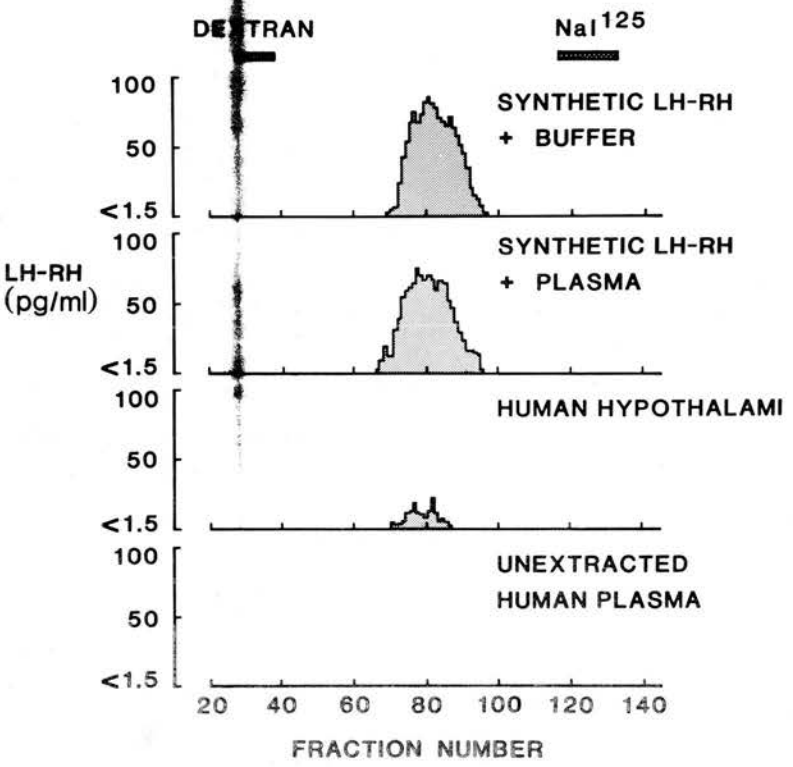



FIG. 1a. Gel filtration by upward flow through BioGel P2 of samples not extracted with Vycor or methanol (LH-RH measured by radioimmunoassay).

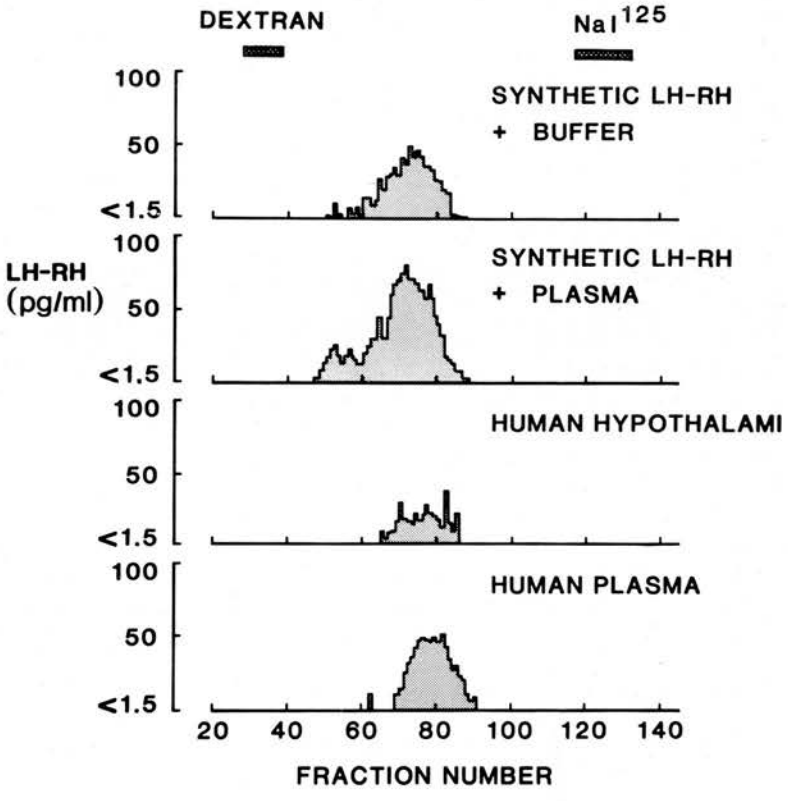



FIG. 1b. Gel filtration of Vycor extracted samples.

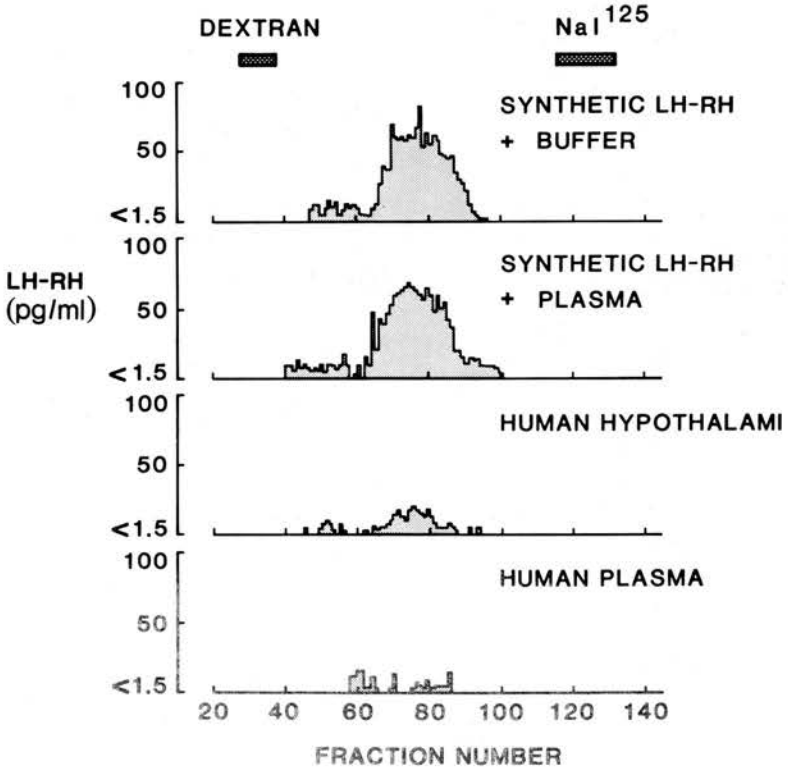



FIG. 1c. Gel filtration of methanol extracted samples.

woman. The mean LH-RH content of the human fetal hypothalami was 170 pg.

*b. Vycor extracts (Fig. 1b).* The extraction of synthetic LH-RH from buffer or plasma with Vycor resulted in the elution of one major and some minor peaks. Similar peaks were found with extracts of human hypothalami and 355 pg of immunoreactive releasing hormone was present in an extract of 2.5 l of plasma from postmenopausal women with a mobility similar to that of the other samples. Recovery of synthetic hormone from the same volume of blank plasma was 50%; thus the mean endogenous releasing hormone content of the plasma was approximately 0.28 pg/ml. A Vycor extract of 750 ml of plasma from normal males contained no detectable hormone.

*c. Methanol extracts (Fig. 1c).* Methanol extracts of synthetic LH-RH and human hypothalami were similar to those above, with a proportion eluting before the main peak. A methanol extract of 1.5 l plasma from post-menopausal women contained 54 pg of material. In view of the variable losses incurred during methanol extraction of large volumes of plasma, estimations of the concentration of the hormone in plasma were not made.

*d. LH-RH levels (Fig. 2).* In order to improve the sensitivity, 10 ml of plasma were treated with Vycor with a final recovery of 50% of added hormone. This resulted in an improvement in assay sensitivity to 0.2 pg/ml from 1.5–3 pg/ml (A) depending on individual assays. A parallel curve was obtained when synthetic hormone was added to 10 ml volumes of buffer and extracted, although recovery was greater by 10–15%. Curve B represents dilutions of untreated human hypothalamic extracts. Parallelism was also seen following Vycor extraction.

Curve C shows serial dilutions of 10 ml plasma Vycor extracts obtained from the normal woman on day 13 of the menstrual cycle. No immunoreactive material was detected at any other time in the cycle. Both

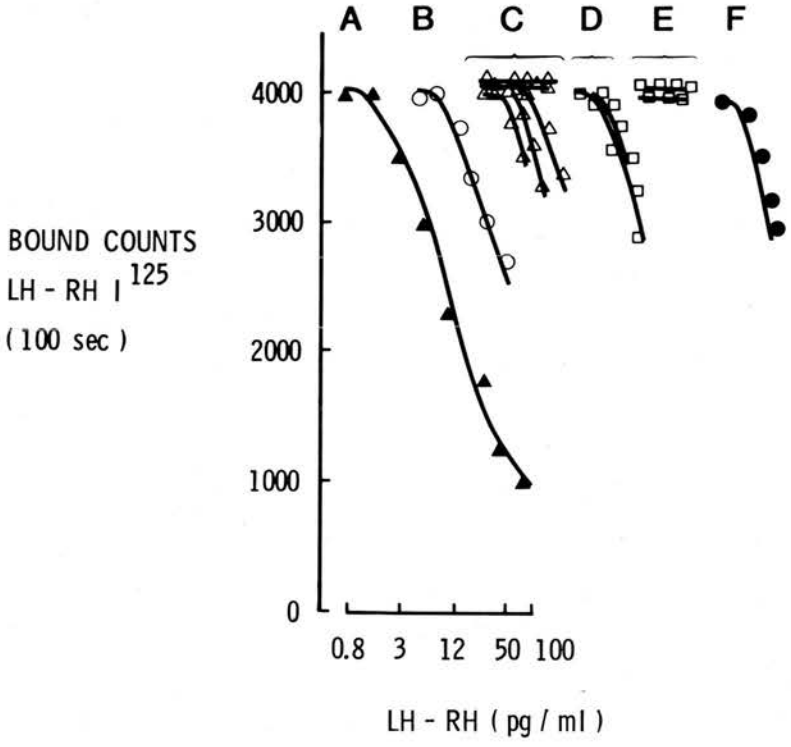



FIG. 2. Dilution curves of immunoreactive LH-RH. A = standard curve in buffer. B = human hypothalamic extracts. C = Vycor extracts of plasma at mid-cycle. D = Vycor extracts of plasma from post-menopausal women. E = Vycor extracts of pooled normal male plasma. F = methanol extract of post-menopausal plasma. (B–F represent concentrated extracts and the abscissa scale, in terms of concentrations, does not apply. See text for details of LH-RH levels).

patients who received bromocriptine showed no acute change in LH-RH or gonadotropin levels. LH-RH levels were detectable only at mid-cycle, as in the normal women. Even then, endogenous releasing hormone was detectable only on 9 of 24 occasions when plasma was sampled. Levels at mid-cycle ranged between <0.2 and 1.5 pg/ml. All subjects had a rise in basal body temperature and serum gonadotropin levels at this time.

Plasma samples (D), 10 ml from 6 post-menopausal female patients taken at random showed detectable LH-RH in seven out of ten samples. Levels ranged between <0.2 and 2.5 pg/ml.

Individual 10 ml plasma samples from 10 normal males (E) did not show any detectable immunoreactive material. On two occasions there was inhibition of binding but non-parallelism with standard LH-RH.

F is a dilution curve of a methanol extract of the pooled fractions 59–86 from the post-menopausal plasma shown in Fig. 1c. These fractions were therefore free from plasma proteins which eluted in the void volume. Dilution curves of methanol extracts containing residual plasma protein sometimes showed non-parallelism. This effect was probably due to protein interference since a non-parallel effect was seen also when the albumin content of buffer was greater than 12.5 mg/100 ml and serially diluted.

CSF from 5 individual subjects contained no detectable LH-RH. A pooled sample of 15 ml from eight patients was therefore extracted with Vycor, but no immunoreactive material was found.

Cerebral extracts from 8 human fetuses, corresponding to the hypothalamic extracts shown in (B), were assayed singly and also after pooling and extraction with Vycor. However, no immunoreactive material was detected.

## Discussion

Endogenous immunoreactive LH-RH in the peripheral circulation appeared indistinguishable from the synthetic decapeptide or human hypothalamic extracts. The production of 'big' LH-RH which eluted before the major peaks were artifacts produced during extraction, but since this occurred with extracts and control samples it suggests at least partial similarity between them. In untreated samples there was no evidence of a naturally occurring 'big' or protein-bound fraction. The nature of this apparent change in mol wt, therefore, may simply reflect a change in its hydrophobic nature during extraction or be due, in part, to aggregation of the hormone. Both synthetic and endogenous releasing hormone had an affinity for Biogel leading to elution later than that expected from its mol wt.

Extraction using Vycor glass has not been reported previously for a hypothalamic hormone although it has been used in the radioimmunoassay of ACTH and $\beta$MSH (10,11). Although the net recovery (50%) is less than methanol extraction (75–80%), large volumes of plasma or other fluids can be extracted and the residual material reconstituted in standard buffer solutions. In contrast, methanol extraction does not precipitate all protein, especially with large volumes of plasma, and the residual protein may lead to errors in the radioimmunoassay.

With any radioimmunoassay, specificity is crucial. Although immunoreactivity, parallelism, and chromatographic similarity are essential, they must be viewed only as evidence against non-identity. Similarity of the material is assumed by inference.

The antiserum used showed no cross-reactivity with known anterior pituitary hormones or synthetic hypothalamic peptides. However, since it was directed towards the C-terminus, differences in the N-terminus would not be detected. It is possible that C-terminal peptides might occur endogenously which would be measured and therefore we have not assumed that the immunoreactive hormone levels found in this study necessarily represent concentrations of the intact biologically active hormone. Further immunization programs

are being undertaken to produce antisera specific for the N-terminus, and hopefully for the intact hormone, to investigate the possible occurrence of C- and N-terminus dissociation.

Immunoreactive LH-RH may be measured in the circulation, although levels are frequently undetectable. This may indicate that LH-RH is released in a pulsatile manner. It is important, therefore, that the timing of specimens should be chosen so that pulses may be measured. This may require continuous sampling from an indwelling catheter.

Our preliminary results indicate that in three women the releasing hormone was detectable only at midcycle, and then intermittently (34% of samples) with a range of $<0.2$ to 1.5 pg/ml. There was no detectable change in the plasma LH-RH levels following the acute administration of the dopaminergic drug, bromocriptine. The range in post-menopausal women was greater (up to 2.5 pg/ml) and levels were more consistently detectable (70%). LH-RH was never detected in normal males or in CSF.

Further studies using this assay system are in progress to determine the clinical usefulness of circulating LH-RH levels in differentiating between hypothalamic and pituitary disease.

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# Changes in Circulating Levels of LH, FSH, LHβ- and α-Subunit After Gonadotropin-Releasing Hormone, and of TSH, LHβ- and α-Subunit After Thyrotropin-Releasing Hormone

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**ABSTRACT.** The effect of the gonadotropin-releasing hormone (LRH) and thyrotropin-releasing hormone (TRH) on the blood levels of LH, FSH and TSH, and LHβ- and α-subunit have been studied in 4 normal subjects during the first 20 min after administration of these releasing hormones. Increases in serum immunoreactive LH, LHβ and α-subunit were seen in all subjects after LRH (100 μg iv) but in all subjects the rise in LH was preceded by a rise in α-subunit. All subjects showed an increase in TSH and 3 of the 4 subjects a rise in α-subunit after TRH (200 μg) but the α-subunit responses were smaller and less consistent than after LRH. Levels of LHβ remained unchanged after TRH.

The results demonstrate that the immunoreactive α-subunit of the pituitary glycoprotein hormones can be released independently of the intact hormones and that release occurs in response to the same releasing hormones, LRH and TRH, that release the intact hormones. (*J Clin Endocrinol Metab* **41**: 466, 1975)

THE pituitary glycoprotein hormones, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) consist of two nonidentical, noncovalently bound subunits, α and β (1). The α-subunit appears to be similar in all three hormones while the β-subunits are dissimilar and hormone specific (1–4), and it has recently been demonstrated that the human pituitary contains both intact hormone and subunits in free form (5,6). Edmonds *et al.* (1974, 7) have shown that release of free α-subunit in four normal men after the injection of either LRH (100 μg or 3000 μg iv) or TRH. In hypothyroid patients release of free α-subunit after TRH (500 μg iv) has also been demonstrated (8). In contrast we have been able to confirm the release of free α-subunit only after the administration of LRH (100 μg iv) but not TRH (200 μg iv) in 6 normal men.

Our previous results (5) were obtained when samples were taken at 20 and 60 min after injection of releasing hormones; the failure to demonstrate an α-subunit rise after TRH could be explained if there was differential secretion of α-subunit immediately after TRH when compared to LRH. To investigate this possibility we have determined the changes in LH, FSH, TSH, LHβ- and α-subunit by serial sampling during the first 20 min after iv injection of both LRH (100 μg) and TRH (200 μg) in normal male volunteers.

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## Materials and Methods

*Subjects.* Four male volunteers aged 27–32 yr participated in two experiments, 1 to 5 days apart. A forearm venous cannula was inserted and blood samples were withdrawn at 1-min intervals for 20 min after a single intravenous injection of LRH (100 μg) or TRH (200 μg). Serum was separated and stored at −20 C until assayed. All samples from an individual subject were measured in the same assay.

*Methods.* LH, FSH, TSH, LHα and LHβ-subunit were measured by double antibody radioimmunoassays, which have been described in detail (9,10,12). The materials used, the specificity of the assays and the intra-assay variation are shown in Tables 1 and 2. There was complete cross-reaction between all the α-subunits in the LHα assay. The LH, FSH, TSH and α-subunit assays were specific at the hormone and subunit

TABLE 1. Materials used and intra-assay variation of the radioimmunoassays for LH, FSH, TSH, LHα- and LHβ- subunit

| Assay | Preparation used as standard | Intra-assay variation[1] |
|---|---|---|
| LH | LH (MRC 68/40, assuming 77 IU/amp) | 4% |
| FSH | FSH (MRC 69/104, assuming 10 IU/amp) | 6% |
| TSH | TSH (MRC 68/38, assuming 6.8 IU/amp) | 5% |
| LHα | LHα (4.1.72) Dr. A. S. Hartree | 7% |
| LHβ | LHβ (18.10.72) Dr. A. S. Hartree | 6% |

[1] Percent coefficient of variation of ten replicates each at three dose levels.

levels obtained. In the LHβ-subunit assay 38 mIU of LH (MRC 68/40) was required to cause 50% displacement of LHβ from its antibody; this displacement was equivalent to that caused by 3 ng of LHβ. In terms of the other glycoprotein hormones and their subunits, this assay was specific.

Because of the intra-assay variance and the known fluctuations of basal gonadotropin concentrations in the human (13) a response resulting from the infused material was considered significant only when a change was observed above basal levels, which was at least five times the intra-assay coefficient of variation.

*Statistics.* The relation between changes in intact hormones and subunits were analyzed by estimation of the linear correlation coefficient without correcting for cross reactivity.

## Results

*LRH.* LH increased in all 4 subjects after LRH although FSH was released in only 2 subjects (Fig. 1, no. 2 and 3). An increase in α-subunit levels was seen in all 4 subjects and occurred 1 to 7 min before any significant change in LH, and the α-subunit reached peak levels (percentage increase, range 176–1760%) before or at the same time as the peak of LH (range 340–6400%). The changes in α-subunit levels were directly related to changes in LH ($r = 0.8079$, $n = 80$, $P < 0.001$) but not to changes in FSH ($r = 0.3050$, $n = 80$, $P > 0.001$).

The changes in LHβ-subunit levels could not, except for subject no. 2 (Fig. 1) be accounted for in terms of the known cross reaction with LH. The LHβ-subunit rose significantly before, at the same time or after the rise in LH, but the peak levels of LHβ-subunit (range 113–2200%) in all subjects were reached 1 to 4 min after the peak of LH. These changes in LHβ-subunit showed a significant correlation to the changes in α-subunit ($r = 0.9405$, $n = 80$, $P < 0.001$), LH ($r = 0.8082$, $n = 80$, $P < 0.001$) and FSH ($r = 0.7008$, $n = 80$, $P < 0.001$).

*TRH.* All subjects showed a significant TSH response within 3 to 7 min after the injection of TRH (Fig. 2). A rise in α-subunit occurred

TABLE 2. The specificity of the radioimmunoassays for LH, its subunits, FSH and TSH

| Hormone or subunit preparation | Assay | | | | |
|---|---|---|---|---|---|
| | LH[2] | FSH[2] | TSH[2] | LHα | LHβ |
| LH IRC2/69[1] (Dr. A. S. Hartree) | 100 | <1 | <1* | 8 | 25 |
| FSH CPDS 6[1] (Dr. W. R. Butt) | 2* | 100 | <1 | 1 | <1 |
| TSH[1] (NIH) | 10* | 2 | 100 | 10 | 3 |
| LHα 4.1.72[1] (Dr. A. S. Hartree) | 25 | <1 | <1* | 100 | 1 |
| HCGα CR115 (Dr. R. E. Canfield) | 12 | <1 | <1* | 100 | <1 |
| FSHα N 611 C (Dr. A. F. Parlow) | — | — | <1* | 100 | 2 |
| TSHα N 785 B (Dr. A. F. Parlow) | — | <1 | — | 100 | 10 |
| LHβ 18.10.72[1] (Dr. A. S. Hartree) | 60 | <1 | <1* | <1* | 100 |
| FSHβ N 596 C (Dr. A. F. Parlow) | <1 | <1 | <1* | 5 | 2 |
| TSHβ N 745 B (Dr. A. F. Parlow) | <1 | <1 | — | 2 | <1 |

The potency if the hormone or subunit is expressed as a percentage (wt/wt) of the material to which the assay is directed.

* Nonparallel inhibition.

[1] Preparation used for iodination in the respective assay.

[2] For the specificity studies LH, FSH and TSH are estimated and expressed in ng/ml of IRC 2/69, FSH CPDS 6 and NIH respectively.

2 mIU LH MRC 68/40 = 1 ng IRC2/69 = 100 ng LER 907.

5 mIU FSH MRC 69/104 = 1 ng CPDS 6 = 200 ng LER 907.

7 μU TSH MRC 68/38 = 1 ng NIH.

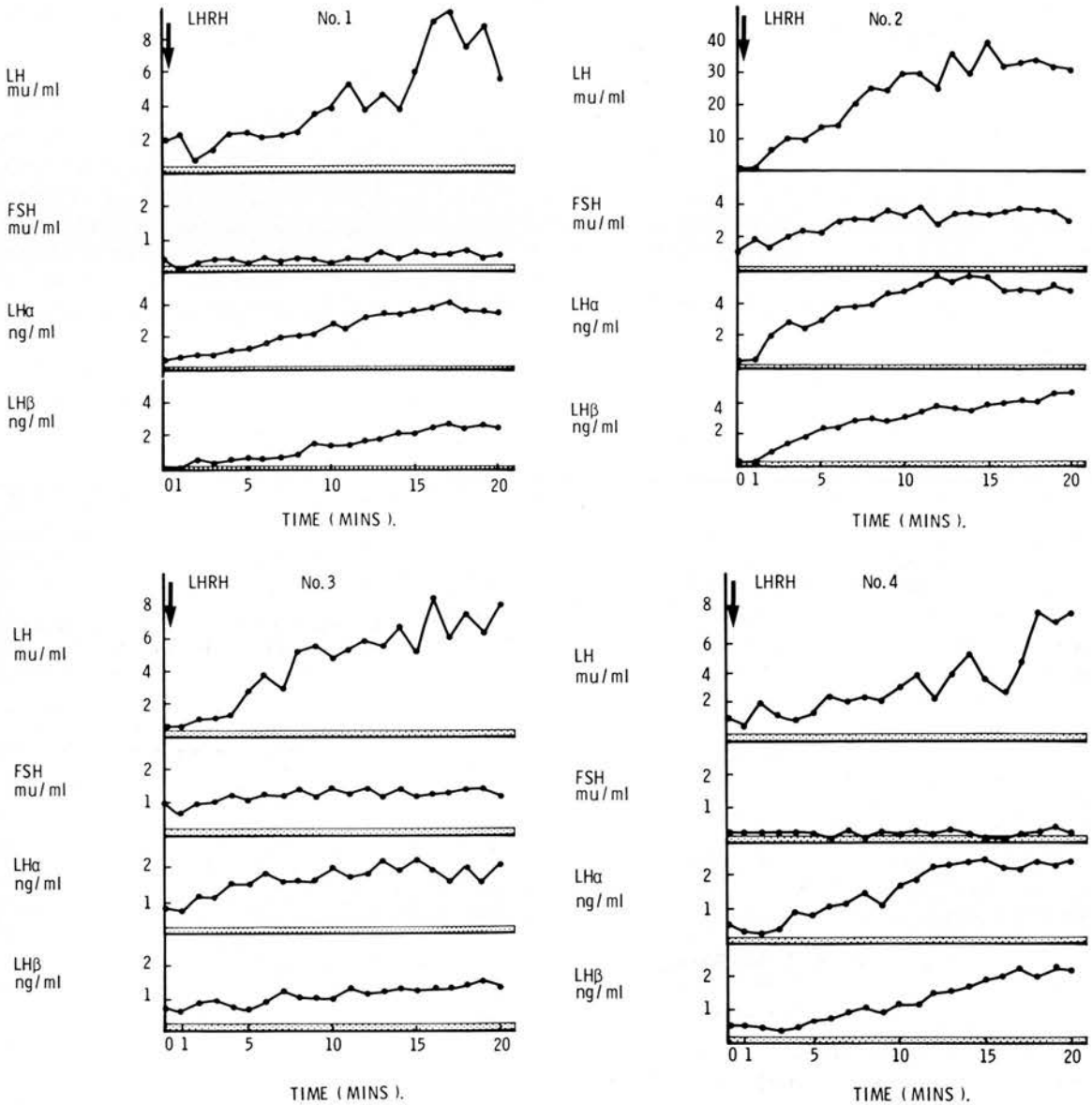



FIG. 1. Serum concentrations of LH, FSH, LHβ- and α-subunit, measured in an LHα assay, in 4 subjects following administration of LRH (100 μg). Shaded area indicates the sensitivity of each assay.

in 3 of the 4 subjects (Fig. 2, no. 1, 3 and 4). These changes occurred 3 min before, at the same time as and 7 min after the rise in TSH in each of these subjects respectively, and the α-subunit reached peak levels (range 96–270%) 4 to 8 min before the peak of TSH (range 301–960%). A significant correlation ($r = 0.5456$, $n = 80$, $P < 0.001$) between the levels of TSH and the α-subunit was seen. In all subjects the peak responses of α-subunit after TRH (200 μg) were less than after LRH (100 μg). The LHβ levels showed no significant changes throughout the test.

## Discussion

The decapeptide LRH causes release of LH and FSH without affecting other pituitary hormones (12,13). In normal subjects the release of LHβ- and α-subunit independ-

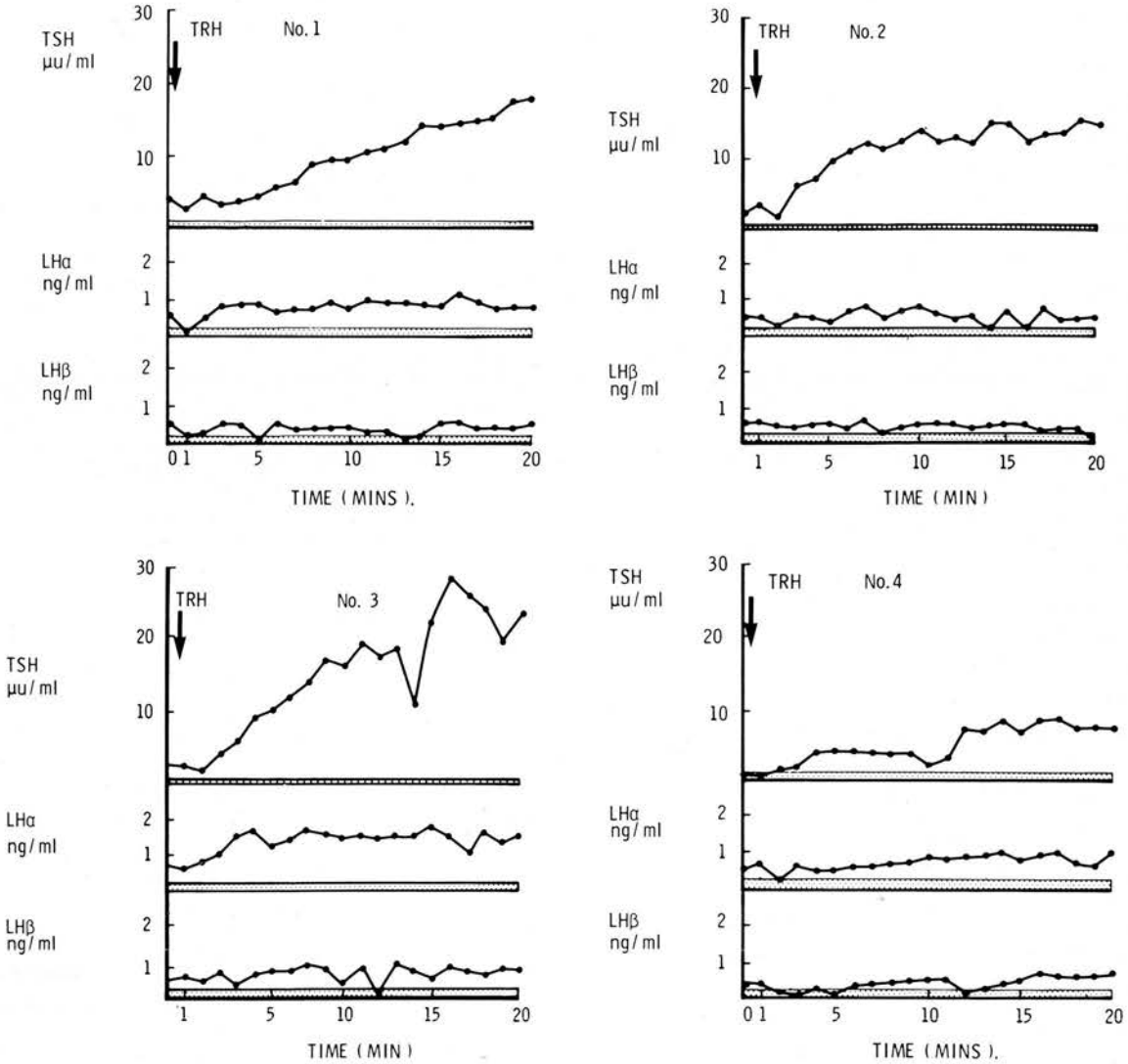



FIG. 2. Serum concentrations of TSH, LH$\beta$- and $\alpha$-subunit, measured in an LH$\alpha$ assay, in the same 4 subjects as shown in Fig. 1, following administration of TRH (200 $\mu$g). Shaded area indicates the sensitivity of each assay.

ent of release of the intact hormones, has also been found (7,9). It seems likely that this rise in circulating levels of subunits is due to secretion from the pituitary gland rather than from peripheral dissociation of intact hormones. First, the pituitary contains free subunits (5,6,16) and therefore has the potential to secrete these substances. Second, the present study demonstrates that after administration of LRH the circulating levels of $\alpha$-subunit rose before that of the intact hormones (Fig. 1). Third, it has recently been shown that infusion of highly purified LH was not followed by an increase in serum $\alpha$-subunit concentrations (7).

The lack of FSH response to LRH in 2 subjects (Fig. 1, no. 1 and 4) was not reflected in the other hormones or subunits and emphasize the previous reports of independent changes in circulating levels of LH and FSH after LRH (15).

The administration of TRH to normal male subjects is followed by an increase in circulating levels of TSH, a smaller rise in FSH, and no change in LH (13,16). The difference in $\alpha$-subunit rise after LRH (100 $\mu$g) and

H (200 μg) could be due to a difference ween the two α-subunits which is not ealed by the cross reaction studies per- ned. All current subunit preparations e been prepared from intact hormones l not directly from the pituitary gland, and refore may not exactly represent the ubunit present in free form in the circula- ı.

n 5 hypothyroid patients a greater percent rease in the TSHβ subunit, than intact H was seen 5 minutes after the injection 200 μg of TRH, and 4 of the 5 patients owed earlier peak responses of TSHβ as npared to TSH (17). However, in this dy a later peak response of LHβ than t of LH was seen, though we have no ex- nation for this finding. The earlier peak els of α-subunit as compared to intact rmones both after LRH and TRH could be e to the different half-lives of these stances (18,19).

We have shown previously a concomitant ease of α- and LHβ-subunits after LRH not after TRH (9). In the present study it mphasized that the differential release of ubunit after administration of the two easing hormones is not due to an early ease and disappearance of the TSHα- unit. It is apparent, however, that injec- ı of LRH to male subjects results not only he release of LH and FSH, but also in the ease of free α- and LHβ-subunit.

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# EFFECTS OF LACTATION ON FERTILITY

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1 Epidemiological studies
  *a* Lactation and the birth interval
  *b* Ovulation and menstruation post partum
2 Endocrinology of lactational amenorrhoea
  *a* Endocrinology of lactation
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3 Pathological hyperprolactinaemia
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ıas long been recognized that the return of menstruation and ility is delayed in women who suckle their babies, and it has n suggested that breast feeding acts as a natural birth cer—Nature's own contraceptive (Short, 1976). In societies ere breast milk is the sole nutrition for the baby lactational enorrhoea may last for two to three years, giving a birth inter- of three to four years. Our understanding of the mechanisms lerlying lactational amenorrhoea has advanced rapidly in the few years, owing mainly to the recognition and purification ıuman prolactin and the establishment of specific and sensi- radioimmunoassay methods for its detection in blood.

This paper reviews our present knowledge of the mechanisms lerlying the maintenance of infertility associated with lacta-ı.

## 1 Epidemiological Studies

### a *Lactation and the Birth Interval*

n many societies the newborn infant is totally reliant on ast milk for its survival. Thus breast feeding is of funda-ıtal importance for the growth and development of the off-ng. It is now clear that, associated with the production of ĸ, lactation also inhibits reproductive activity. It should be phasized that the relationship between lactation and lacta-al amenorrhoea is not through the process of milk secretion is related to the endocrine consequences of the continual lication of the suckling stimulus.

That breast feeding plays a profound role in the control of ility is illustrated by a comparison between the length of the rtile period and alterations in the mode of breast feeding, ticularly in societies moving from a rural to an urban way of

In the nomadic !Kung hunter–gatherers of the Kalahari, ies are breast fed with minimal supplementation for three to years. This is related to a prolonged period of infertility ociated with the period of lactation and results, in the total ence of contraception, in a birth interval of approximately years, i.e., an average period of lactational infertility of over three years. When the !Kung forsake the nomadic way of life and settle in agricultural communities where breast feeding is supplemented with grain meal and cows' milk the birth interval is reduced to under three years, a decrease associated primarily with the reduced intensity of breast feeding (Kolata, 1974). An obvious consequence of the decreased birth interval is an increase in the number of children born in the reproductive life-span of the women and a consequent increase in population growth.

A similar situation has been documented in Rwandese women (Bonte *et al.* 1974), where in the rural areas babies are breast fed on demand and 50% of women had conceived within 23 months post partum. In the urban areas where breast feeding was confined to a more rigid timetable, with a consequent reduction in the number of suckling episodes, the interval to conception post partum for 50% of women had dropped to nine months, only five months longer than if the women had not breast fed at all.

It is important to recognize that one consequence of the move from a rural to an urban way of life may be an improvement of the nutritional status of the mother. Thus while the decrease in the intensity of breast feeding may well be directly related to the decrease in the infertile period associated with lactation, it may also be related to the better nutritional status of the mother. However, in North American Hutterite communities where mothers breast feed their babies on demand, have a high standard of health and nutrition and use no form of contraception, the average birth interval was still 21.7 months (Eaton & Mayer, 1953). This gave an average period of lactational infertility of 12–13 months even when nutrition was presumably optimal. Similar studies have been carried out in Eskimos and South American communities with the same conclusions. Lactation by maintaining a prolonged period of infertility reduces the possible number of children born during the reproductive life-span of a woman and as a consequence acts as a natural contraceptive and a limiter of population growth. Thus Short (1976) has concluded that, throughout the world as a whole, more births are prevented by lactation than by all other forms of contraception put together.

### b *Ovulation and Menstruation post Partum*

While in many cases the duration of amenorrhoea parallels the period of full lactation this is certainly not always the case. Several studies have shown that there may be no intervening menstruation between two successive conceptions, clearly showing that menstruation is an unreliable indicator of fertility. Many of the difficulties in giving a reliable estimate of the period of lactational infertility lie in the definition of full lactation. In many western societies babies are fed on a rigid time regimen and it is often the practice to eliminate night-time feeds early in the course of lactation. While the amount of milk taken by the baby fed according to a time regimen may well be comparable to that taken by a baby who is fed on demand, even where the baby sleeps with the mother, the consequence in terms of the influence of breast feeding on fertility may be very different.

Few studies have related the period of lactational infertility to the number of suckling episodes each day, although there are several studies of the duration of amenorrhoea itself, and of the time at which ovulation recommences. During breast feeding the first ovulation post partum has been detected as early as day 33 and sporadic ovulations between day 34 and day 42 have been demonstrated by either measurement of basal body temperature or endometrial biopsy (Chartier & Gillain, 1964; Sharman, 1967; Kava *et al.* 1968; Perez *et al.* 1972). The

number of women ovulating at this time is very small. Perez *et al.* (1972) showed that of 170 subjects, 24 ovulated while fully nursing, 49 while partially nursing and 97 only after the baby had been weaned. In agreement with this El-Minawi & Foda (1971) showed by endometrial biopsy that 96% of the breast-feeding women investigated were anovulatory, an observation supported by Berman *et al.* (1972) in Alaskan Eskimos. In contrast with these findings, Kamal *et al.* (1969) reported an incidence of pregnancy during lactation in Egyptian women as high as 57%, of whom 44% were still amenorrhoeic.

It is clear that there is considerable disagreement concerning the time of recurrence of ovarian function and obviously lactational amenorrhoea is not, in a proportion of women, a time when infertility can be guaranteed. As indicated however, full lactation does not adequately define the nature of the lactation and more attention should be paid to relating the onset of ovarian activity with the number of suckling episodes each day. Nevertheless, in a considerable number of women breast feeding does suppress ovarian activity and they remain infertile if not amenorrhoeic until breast feeding ceases.

## 2 Endocrinology of Lactational Amenorrhoea

Lactational amenorrhoea is a result of failure of ovarian activity associated with lactation. To understand the mechanisms that relate to this situation it is necessary to review the hormonal control of lactation, and the status of the hypothalamo-pituitary gonadal axis during the period of lactational amenorrhoea.

### a *Endocrinology of Lactation*

It is not the purpose of this review to detail the endocrine events that lead to the onset and maintenance of milk secretion in women, since this has been reviewed recently (see McNeilly, 1977). However, a brief summary of these events is necessary to our understanding of the possible mechanisms involved in lactational amenorrhoea. It should first be pointed out that the secretion of prolactin from the pituitary is primarily under inhibitory control from the hypothalamus and the inhibitory substance is currently thought to be dopamine. During pregnancy the secretory alveolar tissue in the breast proliferates under the influence of placental steroids, placental lactogen and possibly prolactin. Blood concentrations of prolactin increase progressively throughout pregnancy under the influence of the increasing concentrations of placental steroids. During pregnancy, milk secretion is inhibited by a direct action of steroids on the breast. At delivery, blood concentrations of prolactin are eight to 20 times the normal and remain high in the immediate seven days post partum even in the absence of suckling. Milk secretion begins two to three days post partum in response to the suckling of the baby and the time of onset of lactation is directly related to the withdrawal of the inhibitory influence of the placental steroids immediately post partum. In non-breast-feeding mothers prolactin concentrations decline and reach the normal concentrations of non-pregnant women 15–20 days post partum. In breast-feeding women blood concentrations of prolactin remain high for the duration of lactation, and prolactin is released at the majority of suckling episodes in response to tactile stimulation of the nipple. As lactation progresses the amount of prolactin released at each episode may decline but basal concentrations of prolactin remain high. The mechanism behind this is unknown. High basal concentrations of prolactin have been demonstrated in long-term lactation (eighteen months to two years) in women studied in Zaire and the degree of elevation was directly related to the number of suckling episodes. Prolactin concentrations remained at least three times the normal basal values for more than one year, when breast feeding occurred more than six times each day. If breast feeding took place only one to three times each day, prolactin concentrations returned to normal basal values within six months post partum (Delvoye *et al.* 1977). Since high concentrations of prolactin may be implicated in the maintenance of lactational amenorrhoea it is clearly necessary to relate all observations on the onset of ovarian activity to the number of daily suckling episodes during lactation.

### b *Gonadotrophins during Lactation*

i *Follicle-stimulating hormone (FSH).* As a result of the high concentrations of placental oestrogens and progesterone, blood concentrations of FSH are suppressed during pregnancy and at term are low normal or undetectable. In all women, whether breast feeding or not, blood concentrations increase post partum until by 15–20 days post partum they are within the normal range of the follicular phase. Thereafter in lactating women FSH remains in the upper normal range during the first year of lactation (Reyes *et al.* 1972; Bonnar *et al.* 1975; Rolland *et al.* 1975; Keye & Jaffe, 1976). During the second year post partum FSH concentrations decline to normal concentrations of the early follicular phase (Delvoye *et al.* 1978).

ii *Luteinizing hormone (LH).* Blood concentrations of LH are not measurable immediately post partum because of the cross-reaction of human chorionic gonadotrophin in the radioimmunoassays used. It is probable, however, that concentrations are extremely low, as the pituitary content of LH during late pregnancy is less than 1% of that of normally cyclic women (de la Lastra & Llados, 1977). By 15–20 days post partum LH concentrations have increased and are low normal (Reyes *et al.* 1972; Bonnar *et al.* 1975; Rolland *et al.* 1975; Keye & Jaffe, 1976). Despite the return to low normal basal levels of LH, no pulsatility of LH secretion (a feature of normally cyclic women) occurs during lactation (Bohnet & Schneider, 1977); this indicates a reduced hypothalamic secretion of gonadotrophin-releasing hormone (GnRH). LH concentrations increase when prolactin concentrations fall to normal at the time of weaning (Rolland *et al.* 1975).

In non-lactating women LH concentrations remain low during the early puerperium, with a return to normal cyclic concentrations during the third to fifth week when prolactin concentrations return to normal (Bonnar *et al.* 1975).

iii *Gonadotrophin response to GnRH and oestrogen.* The secretion of both LH and FSH is under the control of GnRH released from the hypothalamus (see Fink, 1979). To determine whether the low concentrations of LH seen during lactational amenorrhoea were due to a decreased ability of the pituitary to release LH and FSH, the sensitivity of the pituitary to GnRH stimulation has been assessed by several groups of workers. During the first seven days post partum the FSH and LH response is absent or low both in breast-feeding and non-breast-feeding mothers. In fully breast-feeding mothers a diminished LH response but normal FSH response to GnRH was seen at six to eight weeks of lactation (Jeppsson *et al.* 1974; LeMaire *et al.* 1974; Andreassen & Tyson, 1976; Keye & Jaffe, 1976). In long-term lactation of one to two years' duration, the FSH increase in response to GnRH remains exaggerated, while the LH increase is similar to that seen in the normal luteal phase (Delvoye *et al.* 1978). This situation is similar to that seen in

ertal girls where the FSH response to GnRH is greater he LH response, although in lactating women the LH se is much greater than it is before puberty.

non-breast-feeding women the LH and FSH responses to are exaggerated from three to six weeks post partum and how responses equivalent to those seen in the normal follii phase of the cycle (Keye & Jaffe, 1976). It is apparent re that while the LH and FSH responses to GnRH are shed in the immediate puerperium, during lactation the ry contains adequate stores of both LH and FSH, as by the responses to GnRH.

ring the normal menstrual cycle the ovulatory discharge of s induced by the increasing concentrations of oestradiol ed during the follicular phase. Injection of oestrogen will this response, initially suppressing LH and FSH concenns (negative feedback) and then inducing a release of both nd FSH (positive feedback). Administration of oestrogen tating and non-lactating women at 7, 30 and 100 days of ion suppressed LH and FSH concentrations to a greater in lactating than in non-lactating women, and failed to e positive feedback in the lactating women (Baird *et al.* . These results suggest that, while the pituitary can nd to GnRH, the hypothalamic response to oestrogen ation is altered and is implicated in the maintenance of orrhoea.

### c *Ovarian Activity in Lactation*

llowing delivery, the placental steroids, including oestranid progesterone, are cleared rapidly from the blood and basal values within seven days. Progesterone concenns do not increase until ovarian cyclicity resumes and a s luteum is formed. Oestradiol is secreted predominantly the developing ovarian follicle and thus monitors follicular opment. In non-lactating women a rise in basal oestradiol ntrations occurs at about 17 days post partum, indicating mption of follicular development at a time when prolactin ntrations have returned to normal (Bonnar *et al.* 1975; nd *et al.* 1975); and ovarian function together with ovulaan return as soon as 19–24 days post partum (Rolland *et* 75).

lactating women oestradiol concentrations are signifi- lower than in non-lactating women from day 17 onwards ar *et al.* 1975; Rolland *et al.* 1975; Baird *et al.* 1979) and n so until the resumption of ovarian activity. In one study, y oestrogen output remained basal while prolactin concenns were high in breast-feeding women for up to 250 days partum, indicating absence of ovarian follicular develop- (Baird *et al.* 1979). Thus, despite circulating concenns of FSH and LH within the normal early follicular phase , no ovarian follicular development occurs during the of lactation so long as the prolactin concentrations are (Reyes *et al.* 1972; Bonnar *et al.* 1975; Rolland *et al.* .

ese observations suggest that the ovary may be refractory nadotrophin stimulation when under the influence of pro- However, while gonadotrophin stimulation immediately partum (7–15 days) failed to stimulate oestrogen secretion te *et al.* 1974; del Pozo *et al.* 1975), thereafter either exos gonadotrophin (Nakano *et al.* 1975; Andreassen & , 1976) or GnRH-induced increases in endogenous otrophin (Zanartu *et al.* 1974; Andreassen & Tyson, did stimulate ovarian follicular development and oestrogen secretion. Thus, as far as oestrogen secretion is concerned, the ovary remains responsive to gonadotrophin stimulation.

These observations lead to the conclusion that high concentrations of prolactin, while being closely associated with ovarian inactivity, may not act directly on the ovary. It must be recognized, however, that maintenance of ovarian function by exogenous gonadotrophins may overcome any direct inhibitory effect of prolactin on the gonad. In vitro, high concentrations of prolactin, equivalent to those seen in lactation, inhibit progesterone production by human granulosa cells even in the presence of high concentrations of both LH and FSH (McNatty *et al.* 1974). Thus prolactin may alter the intrafollicular mechanisms whereby granulosa cells are programmed for their subsequent secretory activity as luteal cells following ovulation.

To summarize the reproductive endocrine status in lactational amenorrhoea, prolactin concentrations are increased in response to the repeated suckling stimulus of breast feeding. Blood concentrations of FSH are high normal and of LH low normal without pulsatile secretion, and yet there is an absence of ovarian activity as judged by an absence of oestrogen secretion. Despite adequate releasable LH and FSH in the pituitary, oestrogen fails to induce a positive feedback release of LH and the hypothalamo-pituitary axis appears more sensitive to the negative feedback effects of oestrogen.

Upon weaning there is an immediate drop in blood concentrations of prolactin and an increase in blood concentrations of LH and oestradiol, indicating prompt resumption of ovarian activity, and ovulation occurs within 14–30 days (Rolland *et al.* 1975).

## 3 Pathological Hyperprolactinaemia

With the advent of radioimmunoassays for human prolactin it has become abundantly apparent that, in 15–20% of cases of secondary amenorrhoea, prolactin concentrations are high. In many cases hyperprolactinaemia results from increased secretion of prolactin from a tumour either within the pituitary or interfering with the median eminence. Galactorrhoea is not frequently associated with hyperprolactinaemia. As in lactation, pathologically high concentrations of prolactin are associated with normal or low blood concentrations of LH and FSH and adequate release in response to GnRH (see e.g., Thorner *et al.* 1974). Pulsatility of LH secretion is also absent and there is failure of positive feedback of LH secretion following administration of oestrogen (Glass *et al.* 1975). Suppression of prolactin concentrations to normal, either by surgical removal of a prolactin-secreting microadenoma (Hardy, 1973) or by treatment with Parlodel (bromocriptine mesylate; CB154, Sandoz) (e.g. Besser *et al.* 1972), leads to a resumption of normal menstrual cyclicity. These results clearly support the contention that high concentrations of prolactin are related to the lack of ovarian cyclicity.

As in lactational amenorrhoea, in pathological hyperprolactinaemia the ovaries remain responsive to either gonadotrophin stimulation (Kemmann *et al.* 1977) or GnRH-stimulated LH and FSH secretion (Lachelin *et al.* 1977), and normal ovarian cyclicity can be maintained in the face of high prolactin concentrations provided an adequate dose of gonadotrophin is given (Kemmann *et al.* 1977). This short résumé of pathological hyperprolactinaemia serves to illustrate the relationship between high prolactin concentrations and the absence of ovarian cyclicity and suggests that there may be a causal relationship between the two, as seems to be the case in lactational amenorrhoea.

### 4 Mechanism of Lactational Amenorrhoea

It is clear from the review of the literature that high blood concentrations of prolactin are closely associated with both lactational and some pathological forms of amenorrhoea. In both situations the question arises whether prolactin per se is implicated as the causal agent in hyperprolactinaemic anovulation or whether either the suckling stimulus in lactation or an undefined disturbance of hypothalamic activity (resulting in reduced secretion of prolactin-inhibiting factor and resultant increase of prolactin concentrations) is the primary cause of the amenorrhoea. Or the high concentrations of prolactin may be secondary, associated with but not causally related to the amenorrhoea. Several studies suggest that prolactin per se is indeed a major factor.

As we have seen, specific suppression of prolactin, either by removal of a prolactin-secreting microadenoma or by specifically blocking prolactin secretion by Parlodel in both pathological hyperprolactinaemia and in lactation, results in a resumption of normal ovarian and menstrual cyclicity. While Parlodel treatment may act by repletion of depleted hypothalamic dopamine reserves, the resumption of cyclicity following removal of an adenoma secreting prolactin alone clearly suggests that prolactin per se is a principal causative agent in hyperprolactinaemic anovulation.

Similarly, a loss in the pulsatile secretion of LH and of the positive feedback effect of oestrogen on LH and FSH release, and an increase in sensitivity to the negative feedback effects of oestrogen, can all be caused by selective increase of prolactin concentrations in women either by treatment with thyrotrophin-releasing hormone or by specific dopamine receptor-blocking agents (e.g. sulpiride) (Robyn *et al.* 1976; Bohnet & Schneider, 1977). In fact, amenorrhoea has been induced in women by hyperprolactinaemia caused by the long-term administration of sulpiride (Robyn *et al.* 1976).

The maintenance of the anovular state could be achieved by an action of the high concentrations of prolactin on the secretion of GnRH. If this is reduced so that LH pulsatility is abolished, the developing follicles will not receive the correct signal to ensure adequate oestrogen secretion. Even if this is achieved the block of the positive feedback action of oestrogen by the high concentrations of prolactin will prevent or drastically reduce the release of the mid-cycle ovulatory discharge of LH and lead to either anovulation or an inadequate luteal phase.

It is clear that, while the available evidence strongly suggests that it is the high concentrations of prolactin per se that are responsible for the maintenance of hyperprolactinaemic amenorrhoea, the mechanisms behind this action remain to be elucidated. It is also apparent that a clearer understanding of the relationship between suckling frequency, high prolactin concentrations and resumption of ovarian activity during lactation, and a simple reliable method of detecting when ovulation has recommenced, must be sought before the full potential of breast feeding as a reliable and safe method of contraception can be exploited.


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**Fertility regulation during human lactation**

# PHYSIOLOGY OF LACTATION

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**Summary.** In adult life the human breast, unlike that of other species, is sufficiently developed to allow milk secretion to occur after only a brief period of hormonal stimulation. During pregnancy the ducts and secretory alveoli develop under the influence of both oestrogen and progesterone. Growth hormone and placental lactogen are not required; the role of prolactin in mammogenesis remains questionable. Lactogenesis, the onset of milk secretion, is inhibited during pregnancy despite high levels of prolactin, by a direct inhibitory action of steroids on the breast. The clearance of placental steroids after delivery removes this inhibition and milk secretion is initiated.

Inhibition of prolactin secretion prevents milk secretion, suggesting that prolactin is the essential hormone for lactation in man. Prolactin release occurs in response to suckling and the amount released depends on the strength and duration of the suckling process. No release of prolactin occurs in response to stimuli other than stimulation of the nipple.

Removal of milk from the mammary gland is effected by the milk-ejection reflex (MER) involving the release of oxytocin in response to suckling. Unlike prolactin, oxytocin may be released in response to stimuli associated with breast-feeding, e.g. the cry of the infant. The MER may be inhibited by psychological and physical stress, either by inhibiting oxytocin release or by preventing its action upon the breast contractile elements. The susceptibility of the MER to disturbance requires consideration when encouraging the establishment of breast-feeding.

Breast-feeding is geared directly to the needs of the infant, since not only does the suckling infant obtain its present meal by inducing oxytocin release, but also by stimulating prolactin release it orders the next (Tindal, 1972).

## Introduction

Failure to lactate in almost all mammalian species will result in death of the newborn and a halt to the continuation of the species. The key role of lactation in human reproduction has been ignored to a great extent in the modern western world where the advent of artificial feeding has resulted in a dramatic decline in the number of women breast-feeding. A recent resurgence of interest has seen a

rise in the number of women wishing to breast-feed. While many of these lactate successfully, a large number find difficulty either in establishing or maintaining lactation. The purpose of this paper is to review our current knowledge of the physiology of human lactation. Recent major advances have taken place in our understanding of the hormonal control of lactation, advances made possible by the purification of human prolactin and the development of highly sensitive radioimmunoassay methods for its measurement (Hwang, Guyda & Friesen, 1971). Recent investigations show that prolactin, together with oxytocin, holds the key to lactation in the human.

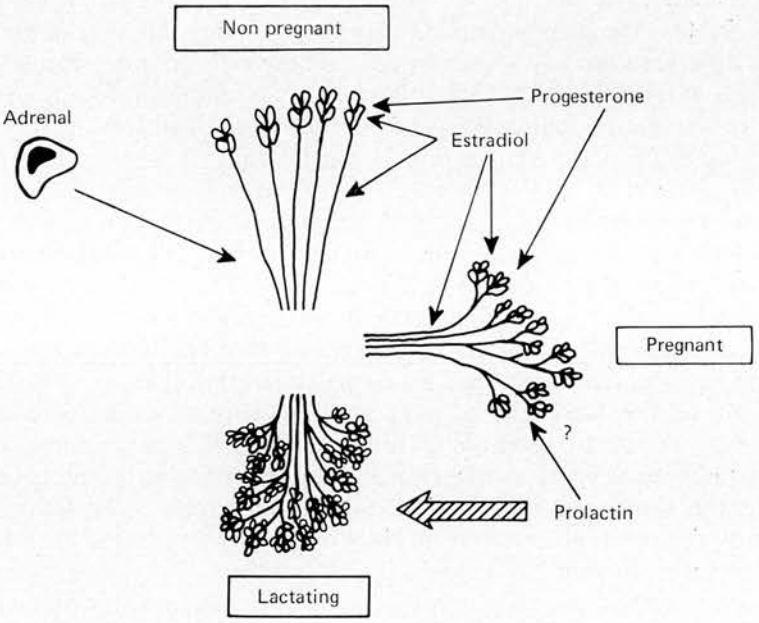
## Mammary growth and development during pregnancy and lactation

### *Puberty and the menstrual cycle*

In the non-pregnant state the adult human breast has achieved sufficient differentiation to require only minimal exposure to the appropriate hormonal stimuli in order to begin secreting milk. Hence as little as 14 days' exposure to conjugated oestrogens followed by stimulation of prolactin secretion results in the establishment of 'normal' lactation (Tyson *et al.*, 1976). This is in contrast to the situation in animals where the mammary tissue requires exposure to the hormonal changes of pregnancy to achieve sufficient development to allow milk secretion.

During puberty and presumably under oestrogen stimulation, the milk ducts leading from the nipple to the secretory alveoli within the breast, sprout, branch and form terminal glandular tissue buds from which develop the future secretory alveoli and lobules. Under the further influence of progesterone secreted in the luteal phase of the menstrual cycle limited ductal–lobular–alveolar growth occurs together with growth of the mammary connective tissue (stroma) and deposition of fat. The growth of the breast during puberty requires ovarian steroid hormones since ovariectomy before puberty (Baron, 1958) or pathological ovarian agenesis (Newton, 1961) results in failure of pubertal breast development. The adult non-pregnant human breast is composed of a mixture of fatty and glandular tissue interspersed with fibrous tissue septa which divide the breast tissue into 15–20 lobes, each of which is divided into a number of lobules. There are groups of alveoli scattered among these lobes with small ducts leading to lactiferous ducts, one from each lobe. Under the areola each duct expands to form a lactiferous sinus which opens directly upon the surface of the nipple. Apart from fat and connective tissue (stroma) the major part of the breast parenchyma is duct (Dabelow, 1957; Mayer & Klein, 1961; Newton, 1961). At this stage the term 'resting breast' is often applied to the human mammary gland that is not secreting milk. However, changes in breast volume during the normal menstrual cycle have been reported with an increase in volume related to increased oestradiol and progesterone secretion in the luteal phase (Milligan, Drife & Short, 1975). Small amounts of secretion can be aspirated from at least 75% of non-lactating breasts (Petrakis *et al.*, 1975) and synthesis of milk protein (immunoglobulin A) in plasma cells associated with the lobules has been demonstrated during culture of human

breast tissue (Drife *et al.*, 1976). The hormonal control, if any, of these changes during the normal menstrual cycle is not clear. Unlike mammary tissue from other species (Forsyth, 1971), human breast tissue does not require hormones to retain functional morphology while in short-term cultures (Archer, 1968; Ceriani, Contesso & Nataf, 1972) and ductal epithelium shows spontaneous DNA synthesis (Flaxman & Lasfargues, 1973). This may relate to the greater development of the human breast compared to animal mammary tissue that occurs in the non-




**Text-fig. 1.** Hormonal requirements for mammogenesis and lactogenesis in the human. Lobulo-alveolar and ductal development in the breast before and during pregnancy appear to be steroid dependent with an undetermined role for prolactin or placental lactogen during pregnancy. Lactation requires prolactin for lactogenesis and maintenance of milk secretion.

pregnant, non-lactating state. Stimulation of mitosis in epithelial cells occurs when insulin is added to human breast cultures (Barker, Fanger & Farnes, 1964; Archer, 1968; Ceriani *et al.*, 1972). A further increase in this mitotic activity occurs if prolactin is added together with insulin (Ceriani *et al.*, 1972; Dilley & Kister, 1975) and this increase is greater if human rather than ovine or bovine prolactin is used (Dilley & Kister, 1975). The basal activity of the epithelial cells in the normal human breast during the menstrual cycle may therefore depend to some extent on both insulin and prolactin, although both oestrogen and progesterone are intimately involved (Text-fig. 1).

*Pregnancy and lactation*

During early pregnancy ductular–lobular–alveolar mammary development is further stimulated resulting in hypertrophy of the pre-existing lobular–alveolar structures and the formation of new lobules (Dabelow, 1957). Development continues with the formation of prominent mammary breast lobules and dilation of the alveolar lumina. By mid-pregnancy the proliferation of mammary lobules and ducts ceases and is replaced by differentiation of the alveolar tissue preparatory for secretion (Mayer & Klein, 1961). The epithelial lining of the mammary ducts and alveoli before the 3rd month of gestation and in non-pregnant women is of two-cell thickness. At the end of the 3rd month of pregnancy the mammary alveoli begin to lose their superficial epithelial layer and the single cell layer remaining differentiates into secretory cells. These cells contain secretory material from the end of the 4th month onwards and are no longer proliferative (Dabelow, 1957). At this stage the mammary gland is fully developed for lactation and milk secretion may begin under the appropriate endocrine stimulation (Text-fig. 1).

The endocrine control of mammogenesis in rodents and a few other species has been extensively investigated and the results suggest that a complex of hormones including oestrogens, progesterone, adrenal steroids, growth hormone and prolactin are involved (see Cowie & Tindal, 1972). In man the situation is somewhat different in that a certain degree of mammogenesis has occurred before pregnancy. Several clinical observations suggest that the human breast does not require the quantitative hormonal changes occurring during pregnancy to allow development of the breast to a stage where adequate milk secretion for full lactation may occur. In relation to pregnancy, milk secretion may occur after premature delivery as early as the 16th week (Newton, 1961) and after abortion as early as the 12th week of gestation (Smith, Shearman & Korda, 1972). Thus the necessity for the high levels of prolactin (Hwang *et al.*, 1971) and of human placental lactogen throughout pregnancy is doubtful.

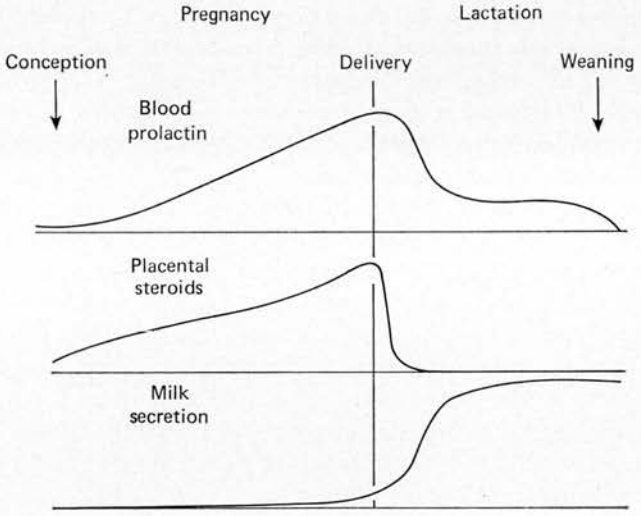
It is also apparent that galactorrhoea occurs both in men and women in response to many different circumstances (see Frantz *et al.*, 1973). Breast development sufficient to allow full lactation also occurs in women with congenital absence of growth hormone (Rimoin *et al.*, 1968) suggesting that, unlike the other species so far investigated (Cowie & Tindal, 1972), growth hormone is not involved in human breast development. Thus ductal development has been noted in men (Frantz, 1972), women (Fujii *et al.*, 1959) and in cultures of human breast tissue (Ceriani *et al.*, 1972) after treatment with oestrogens. Addition of progesterone to human breast tissue in culture stimulated ductal and lobular–alveolar development (Text-fig. 1) with the formation of small closely packed alveoli (Ceriani *et al.*, 1972).

## Milk secretion

*Initiation of milk secretion* (*lactogenesis*)

From mid-pregnancy alveolar mammary cells actively synthesize milk fat and proteins, although only small amounts are released into the lumen of the ducts (Dabelow, 1957; Mayer & Klein, 1961). The copious milk secretion necessary for

the initiation of full lactation does not occur until delivery or soon after. In the traditional view prolactin release was thought to occur just before delivery or in the early puerperium and be responsible for the initiation of milk secretion (lactogenesis) in the breast previously prepared for lactation by the steroids of pregnancy. However, direct measurements in the human show that prolactin levels in blood increase progressively during pregnancy and reach a maximum at the time of delivery (Hwang *et al.*, 1971; Tyson & Friesen, 1973; Jaffe *et al.*, 1973; Robyn *et al.*, 1977). After delivery the prolactin levels fall but basal concentrations are not reached until at least 2 weeks post-partum, depending on the duration and suckling frequency of lactation (Text-fig. 2).




**Text-fig. 2.** The temporal relationships between blood levels of prolactin and placental steroids and the initiation of milk secretion. Although levels of prolactin are high during pregnancy milk secretion occurs only as the levels of placental steroids decline, suggesting a direct inhibitory action of these steroids on the breast. Copious milk secretion is induced by the suckling-induced increase in prolactin levels which remain above non-pregnant levels as long as an adequate suckling stimulus is maintained.

Thus, despite high levels of the lactogenic hormones prolactin and placental lactogen during pregnancy, the breast remains unresponsive in terms of milk secretion until the immediate post-partum period. During pregnancy the placenta maintains high levels of both oestrogens and progesterone (Hytten & Leitch, 1971). These steroid levels decline rapidly after delivery of the placenta while the levels of prolactin remain elevated; thus the initiation of milk secretion is correlated with the disappearance of steroids (Text-fig. 2). While it was originally suggested that steroids acted by suppressing prolactin secretion (Newton, 1961) this is clearly not the case. Thus oestradiol valerate or testosterone enanthate (Deladumone) given for the suppression of lactation failed to alter prolactin levels (Del Pozo *et al.*, 1972; Jaffe *et al.*, 1973; Tyson & Friesen, 1973), although successfully blocking lactation in the majority of cases. It seems probable therefore that the

steroids act peripherally on the breast tissue itself and inhibit milk secretion directly or indirectly at the level of the alveolar cell. Thus, milk secretion during pregnancy is prevented by the high levels of oestrogens and progesterone circulating and only when these steroid levels have declined after delivery does milk secretion begin (Text-fig. 2).

Milk secretion occurs post-partum whether suckling begins or not, and some milk secretion is apparent for up to 3–4 weeks post-partum (Cooke *et al.*, 1976). This period of secretion coincides with the time post-partum during which blood prolactin levels remain above normal even in the non-breast-feeding state (Bonnar *et al.*, 1975; Cooke *et al.*, 1976). However, the stimulus of breast-feeding is required to initiate the copious milk secretion of full lactation.

The role of prolactin in lactogenesis appears crucial. Complete suppression of lactogenesis occurs in women treated with bromocriptine (2$\alpha$ bromergocriptine, CB154 Sandoz) which selectively suppresses prolactin secretion (Rolland & Schellekens, 1973; Del Pozo & Flückiger, 1973). A similar failure of lactation occurs in Sheehans syndrome (Newton, 1961) which results in very low levels of prolactin post-partum (Tyson *et al.*, 1976).

Since prolactin is essential for the initiation and continuation of milk secretion, and prolactin release during lactation occurs in response to suckling (see below), it is of considerable interest that at delivery there is a dramatic increase in nipple sensitivity (Robinson & Short, 1977). This may be a physiological mechanism to ensure adequate prolactin secretion in the vital immediate post-partum period. The reason for the dramatic increase is unclear, but it is tempting to suggest that it is in part related to the withdrawal of oestrogen. Thus Tyson *et al.* (1976) reported the failure of nipple stimulation to evoke prolactin release during oestrogen treatment in two women, although prolactin release did occur in response to the same stimulus when oestrogen treatment was terminated.

*Maintenance of milk secretion* (*galactopoiesis*)

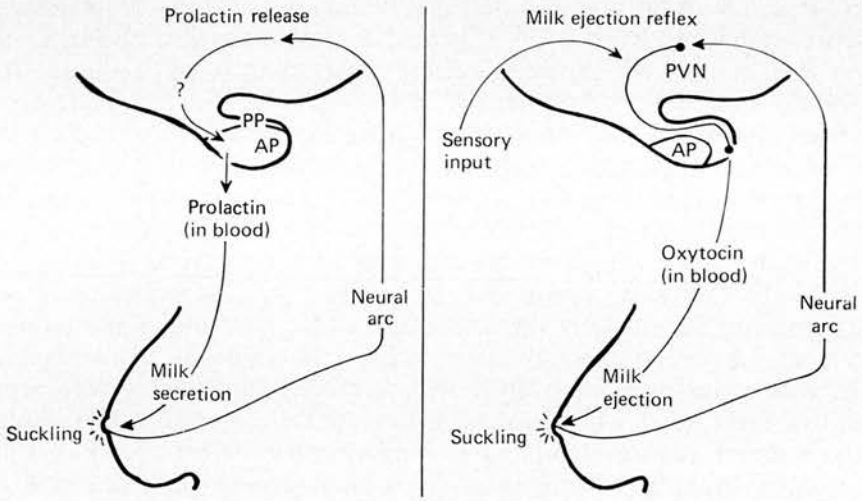
Effective and sustained milk production is achieved 10–14 days post-partum and reaches a plateau around 3 weeks post-partum.

The amount of milk secreted does not depend on the initial breast size, but does relate to the increase in breast volume during pregnancy (Hytten, 1954) an increase which relates mainly to the development of the secretory alveolar cells. There is some evidence that 'small' breasts may be related to a lower incidence of successful breast-feeding (Lussky, 1951) but whether this relates to a reduced amount of secretory tissue or to a reduced storage capacity for secreted milk within the breast is unknown.

The hormonal requirements for the maintenance of established lactation vary among species. In species other than man, only in the rabbit can lactation be maintained by prolactin alone. Man may represent a similar situation to the rabbit since the selective inhibition of prolactin secretion by bromocriptine (CB154; 2$\alpha$ bromergocriptine) will result in the total cessation of milk secretion (Del Pozo *et al.*, 1972; Rolland & Schellekens, 1973; Rolland *et al.*, 1975a; Cooke *et al.*, 1976). Growth hormone is not essential since normal lactation occurs in total absence of human growth hormone (Rimoin *et al.*, 1968).

The ovarian steroids similarly play little or no role in the maintenance of milk secretion since ovariectomy does not affect lactation (Jacobsohn, 1961). Similarly, lactation induced by the repeated application of the suckling stimulus alone in a post-menopausal woman (Newton, 1961) and the secretion of milk in men with pituitary tumours secreting high levels of prolactin (Besser *et al.*, 1972) further emphasize that ovarian hormones are not required.

During puerperal lactation in women the blood levels of prolactin vary widely (Reyes, Winter & Faiman, 1972; Jaffe *et al.*, 1973; Rolland *et al.*, 1975b; Bonnar *et al.*, 1975), this variation being related to the release of prolactin in response to the suckling of the infant.




**Text-fig. 3.** Diagrammatic representation of the pathways involved in suckling induced prolactin and oxytocin release. Both hormones are released in response to nipple stimulation during suckling. The release of these hormones is otherwise independent. Prolactin release does not occur in response to other stimuli associated with nursing while oxytocin release resulting in milk ejection may occur spontaneously or be induced by, e.g., the cry of the infant.

Prolactin release occurs in response to direct mechanical stimulation of the nipple (Text-fig. 3), a reflex arc which appears to be complete although less accentuated even in the non-pregnant state (Noel, Suh & Frantz, 1972; Kolodny, Jacobs & Daughaday, 1972). The amount of prolactin released during suckling depends upon the strength and duration of the stimulation. Thus prolactin secretion increases as soon as the suckling stimulus begins and ceases when the stimulus ends (Frantz, Kleinberg & Noel, 1972). If both breasts are suckled at the same time, thus doubling the stimulus, the amount of prolactin released is also increased (Tyson, 1977).

It would appear that the stimulus for the prolactin release during suckling is solely neural in origin since anaesthesia of the nipple or total denervation of the nipple as a result of reduction mammoplasty prevented any prolactin increase in

response to suckling (Tyson, 1977). Psychological factors associated with breast-feeding, in particular the anticipation of nursing sufficient to induce the milk-ejection reflex from oxytocin release, do not play an important role in prolactin release since these stimuli do not release prolactin in nursing mothers (Noel *et al.*, 1972).

As lactation advances and milk yield decreases, prolactin declines to reach normal basal levels. This is coupled with a decline in the prolactin release in response to suckling (Tyson, Friesen & Anderson, 1972). The reason for this decline in response is not clear, since the amount of prolactin which can be released from the pituitary by other stimuli (e.g. thyrotrophin releasing hormone, TRH) does not alter (Tyson *et al.*, 1972).

The decline in basal levels of prolactin may reflect the decrease in the number of suckling periods as lactation advances (Rolland *et al.*, 1975b). Thus when suckling occurs on demand basal levels of prolactin remain elevated during advanced lactation even though the prolactin release to suckling is still reduced (Robyn *et al.*, 1977). A decline in the intensity of the suckling stimulus, which in turn would decrease the amount of prolactin released, as well as a decline in the number of periods of suckling, may be an additional important factor during the declining phase of lactation. Thus in women who had lactated for some months and were expressing all their milk manually, an increased amount of nipple stimulation by manual expression produced a marked increase in milk production over a 7-day period (Macy *et al.*, 1930). This may suggest that the amount of prolactin circulating during lactation is the important factor controlling the amount of milk secreted. Support for this view is gained from the studies of Tyson *et al.* (1972, 1976) in which treatment of women with declining milk yields with repeated injections of TRH which will release prolactin (see Bowers *et al.*, 1971) resulted in subsequent breast engorgement and a marked enhancement of milk secretion. Zarate *et al.* (1976) failed to demonstrate a similar response in a group of hypogalactic women. However, in the latter case, blood levels of prolactin appeared to be high before treatment with TRH, suggesting that the reason for the hypogalactia in this group of patients was not reduced prolactin levels.

Suckling leading to the release of prolactin is a powerful stimulus in lactation and this alone can induce milk secretion. Thus full lactation can be induced in post-menopausal women (Newton, 1961), in women who have never borne children (Foss & Short, 1951) and even in men (Greenway, 1937) as a result of frequent suckling by an infant or by nipple stimulation.

### Action of prolactin on milk secretion

Prolactin acts directly on the secretory alveolar cell of the breast to initiate and stimulate the synthesis and secretion of the milk proteins casein, lactalbumin and lactoglobulin, lactose, and lipids. From mid-pregnancy onwards alveolar cells are secreting small amounts of these components. The lumina of the alveoli before delivery become filled with this colostral secretion which is rich in epithelial cells and differs in chemical composition from milk (see Vorherr, 1974). After delivery, milk secretion proper begins under the influence of prolactin. At the level of the secretory alveolar cell prolactin binds to specific receptor sites on the cell plasma

membrane (Falconer, 1972; Shiu, Kelly & Friesen, 1973) and stimulates formation of RNA and subsequent protein synthesis. Prolactin mediates its action via the membrane receptor of the alveolar cell since prolactin coupled to sepharose beads which would prevent entry into the cell can stimulate isolated mammary epithelial cells (Turkington, 1970). In a more elegant study, Shiu & Friesen (1976), using a specific antiserum raised against the prolactin receptor, thus blocking the binding of prolactin to its own receptor, prevented prolactin stimulated casein synthesis in rabbit mammary tissue. In this study insulin action was unaffected. These results clearly suggest that prolactin does not act within the alveolar cell.

Prolactin is also involved in the synthesis of lactose by stimulating the synthesis of $\alpha$ lactalbumin, the whey protein (B protein). The rate-limiting enzyme in the biosynthetic pathway of lactose is lactose synthetase, consisting of galactosyltransferase (A protein) and $\alpha$ lactalbumin; $\alpha$ lactalbumin specifies glucose as a substrate for transfer to lactose and is stimulated by prolactin. Progesterone appears to inhibit this stimulation of lactose synthetase directly (Kuhn, 1969; Assairi *et al.*, 1974) and suggests one mechanism by which steroid inhibition of lactation may occur (see above).

Milk secretion is inhibited if milk is not removed regularly from the gland. Mammary tissue elasticity allows milk storage for up to 48 hr before the rate of milk synthesis and milk secretion declines (Vorherr, 1974). The accumulation of milk in the alveoli and smaller milk ducts causes distension and mechanical atrophy of the epithelial structures. Rupture of the dilated alveolar walls leading to the formation of large hollow spaces may also occur (Mayer & Klein, 1961). The alveolar cells eventually disintegrate and involution continues with deposition of mammary fat and an increase in the density of connective tissue (Newton, 1961). The process of involution is governed predominantly by local mechanical factors including alveolar distensions, tissue hypoxia and phagocytosis, and hormonal changes appear to play little part. The histological changes occurring during involution of the human breast have been reviewed by Mayer & Klein (1961).

## Milk ejection

So far the mechanisms involved in the initiation and maintenance of milk secretion have been discussed. Of vital importance to lactation is that the milk within the breast is available to the suckling infant. This is effected by the milk-ejection reflex (MER) which is a neuroendocrine reflex involving the passage of afferent nerve impulses from the nipple via the spinal cord to the hypothalamic paraventricular nucleus and down to the posterior pituitary (Text-fig. 3). This results in the release of the octapeptide oxytocin which is carried in the blood-stream to the mammary gland where it causes contraction of the myoepithelial cells surrounding each alveolus and the ducts, thus effecting the expulsion of milk (see Cowie & Tindal, 1972; Folley, 1969; Tindal, 1972).

The release of oxytocin and the subsequent milk ejection process is recognized as milk let down or 'draught'. While oxytocin release occurs in response to nipple stimulation (Tindal, 1972) it may also be released spontaneously in response to stimuli such as the crying or sight of the infant, a situation readily demonstrable

in animals (McNeilly, 1972). Although oxytocin and prolactin are released in response to the suckling stimulus, their release appears to be independent of one another and oxytocin will not evoke a release of prolactin (Schams, 1972; McNeilly & Hart, 1973; McNeilly & Friesen, 1976; McNeilly, unpublished observations). The amount of oxytocin required to elicit full milk ejection in women is of the order of 1–2 m$\mu$ (2–4 ng) iv resulting in a peripheral blood concentration of between 5 and 20 pg/ml during suckling (Fisch, Sala & Schwarcz, 1964). At other times oxytocin release is highly irregular and basal levels are normally undetectable (see Chard, 1973). Very few studies have been made on the measurement of oxytocin during lactation, but these results support the data of Fisch *et al.* (1964) that blood levels increase around the time of suckling (Cobo *et al.*, 1967; Coch *et al.*, 1968; Vorherr, 1972) and are very low (5–20 pg/ml).

The milk ejection reflex (MER) is susceptible to inhibition by psychological and physical stress. Inhibition of oxytocin release may occur (Cross, 1961) but often release of catecholamines in response to stress causes constriction of the mammary blood vessels preventing oxytocin access to the breast myoepithelial cells (Vorherr, 1971). The degree of psychological stress which will cause inhibition of the MER is greater at the beginning of lactation than in established lactation (Newton & Newton, 1948), a point which requires important consideration in encouraging breast-feeding. Difficulty in overcoming inhibition of the MER may also relate to the success of lactation since successful breast-feeders showed a higher incidence of 'draught' (MER) than unsuccessful breast feeders (Newton & Newton, 1950).

More recently Bruce, Cofre & Ramirez (1973) have demonstrated a significant decrease in oxytocin-induced milk ejection in the rat by oestrogen. This may be relevant when considering the use of contraceptives containing oestrogens during puerperal lactation.

It is apparent that the MER in women has received scant attention from researchers. This is related to the extreme difficulty of measuring oxytocin (see Chard, 1973) even with highly sensitive radioimmunoassay techniques. It is hoped that in the future further efforts to resolve this dilemma will be successful since the failure of milk ejection alone will cause a total cessation of successful lactation and may be an important factor in the failure of breast-feeding.

I thank gratefully Professor R. V. Short and Dr J. R. McNeilly for their helpful advice, and Mrs D. M. Jackson for typing this review.

## Discussion

*Thomson:* Obstetricians and midwives often teach mothers to stretch the nipples and to undertake other training exercises for lactation. Is there any physiological basis for these manoeuvres?

*McNeilly:* It certainly cannot have any physiological role, but massaging the nipple may perhaps make the woman realize that the breast is for producing milk and it may make it easier for the baby to suck. It is well-known that animals lick their own nipples—this is of importance in the rat, in particular—and it is related to

maternal bonding. This is a new topic in human physiology, and we do not know whether maternal bonding is required for lactation, or whether it has any other role.

*Thomson:* Does massaging have any hardening or desensitizing effect on the nipple? Feet get blistered unless some walking practice has been done. Is there an analogue for the nipple?

*McNeilly:* Certainly cracking of the nipple can cause problems.

*Tyson:* Would you expand on the induction of lactation in males and in ovariectomized individuals?

*McNeilly:* Recently, because placental lactogens have been found in a number of species other than man, some workers have suggested that placental lactogens during pregnancy are vital for lactation. I do not believe this suggestion for several reasons; there are species (e.g. rabbit and pig) which do not have a placental lactogen and yet lactate successfully. Some animals (e.g. dog and cat) do not require pregnancy to lactate, and there are well-documented cases in the literature of men lactating. Spontaneous galactorrhoea occurs when blood prolactin levels are high, and while the secretion may not be milk in the true sense, it accumulates in the mammary gland which is not being emptied. The secretion may be more like that from an involuting mammary gland in a normal lactation and could not be expected to have the same composition as normal milk in full female lactation.

There is documented evidence that some medicine men in developing countries can induce lactation by either of two methods. First, by nipple stimulation—which causes release of both oxytocin and prolactin, but especially the latter and high prolactin levels will induce lactogenesis in the mammary gland. Secondly, they use a root which contains a phenothiazine-type compound which also stimulates prolactin release. Historically, there is a well-documented case of a couple in the 15th or 16th century who shared the suckling of their infants. The first child was suckled by the mother, the second by the father, the third by the mother and so on (Foss & Short, 1951).

*Gupta:* What are the effects of progesterone? Is the blocking of lactalbumin synthesis dose-related? Clinically, progestogens can be given in certain doses which do not affect the quantity of milk secretion: do progestogens in low doses block lactalbumin synthesis?

*McNeilly:* It is not yet possible to say whether lactalbumin synthesis is blocked in man—we can only presume that it is because it happens in the rat, mouse and rabbit. I would guess that if oestrogen was also given there would be a synergism reducing the amount of progestogen that would block, so it probably is dose dependent.

*Gupta:* There may be a species difference.

*McNeilly:* Yes, possibly.

*Senanayake:* Does prolactin affect the biochemistry of the milk? The first drops of milk that come out after the child starts suckling may have less lipid and fat than milk expressed later. Does prolactin affect this, or is there some other mechanism involved?

*McNeilly:* It does affect it. The first drops of the milk expressed have been in the ducts since the last feed; they are the residues of the milk secreted at the end of the

previous lactation. The milk expressed at the end of the suckling will be the most recently synthesized milk, which will be of a different composition because it is produced at a different time. If prolactin is given, it is possible to alter the milk composition. Dr Tyson's data clearly show that with stimulation of prolactin release towards the end of a feed, when blood levels of prolactin are low, the composition of the milk alters.

*Senanayake:* I have recently read reports stating that the fat content of human milk varies so greatly that it might be better to give milk with a uniform fat content. Some of the milk manufacturers use this as an argument against the usefulness of breast milk. Is this true, or is it useful to have a varying fat content as the child suckles through each period of feeding? I am sorry to be the devil's advocate.

*McNeilly:* I become angry when I see that sort of advertisement, because lactation is geared for the infant, and has always been. We sometimes forget this. Many women to whom I talk in Edinburgh do not realize that lactation is actually designed for their infant. As far as they are concerned, milk comes out of a bottle, originally from a cow.

If the milk fat content varies in human milk, it is varying for a purpose. This is true for all animals, and I do not see why man should be an exception.

Lactation is perfectly designed for feeding a child. When it is suckling, oxytocin is released and thence prolactin, so that while feeding, a child is also ordering its next meal. There is a continuous chain of events. If there is a change in composition, it is a physiological response.

*Gray:* First, could the progesterone effect be a species difference? Most of the work in humans has been done with synthetic progestogens. Second, Dr McNeilly said that post-partum fall in oestrogen is apparently associated with the onset of lactogenesis. Rolland *et al.* (1975a) and Bonnar *et al.* (1975) stressed this, but how strong is the evidence that the post-partum fall in oestrogen is crucial to lactogenesis? Last, there have been suggestions by Hall (1975) that changes in fat content of milk during a feed may be important in the development of the child's appetite control mechanism.

*McNeilly:* In answer to the last comment, the physiology appears to be right and the milk manufacturers wrong.

On your first question, progestational agents, if they work, have to do so through the same receptors as progesterone. This means that all we are doing is decreasing the amount of progesterone which has to be given to obtain the same effect.

*Gray:* But they presumably act as blockers?

*McNeilly:* Some do, but the ones that are active in this case are not blockers of progesterone but agonists.

In answer to the second question, in the study with Bonnar and Nott our data showed clearly that the post-partum fall in oestrogen and the onset of lactogenesis are related. The paper by Rolland *et al.* showed the correlation between oestrogen, progesterone and lactogenesis. Oestrogen and progesterone are cleared at the same rate after the placenta has gone. I do not know which of the two is important in man, but from animal experiments, especially those on the rat, it is probably

progesterone. Progesterone blocks lactogenesis. Progesterone cover after parturition in rats and rabbits prevents lactogenesis, but that effect does not occur if oestrogens are given.

*Gray:* Would not human experience suggest the exact opposite? Stilboestrol is a well-known means of suppressing lactation, and all the work on progestational agents in lactating women suggests either no effect, at least on output, or some marginal increase in volume. So it might be another species difference.

*McNeilly:* I agree entirely.

*Thomson:* The literature is not entirely unanimous that stilboestrol has a suppressing effect. There has been much dispute over the years whether, having 'suppressed' lactation by stilboestrol, removal of the stilboestrol then causes lactation to restart.

*McNeilly:* Giving stilboestrol raises prolactin levels so its withdrawal leaves prolactin levels high and women will start to lactate again. This is well-known, and has been utilized as a means of inducing lactation. Giving oestrogens to menstruating women pushes up the prolactin level and induces lactation.

*Robyn:* I should like to comment on the possible interplay of human placental lactogen (HPL) and prolactin during pregnancy. Much emphasis was placed on HPL as a lactogenic hormone when prolactin was not accepted in man as a pituitary hormone distinct from growth hormone. But now that prolactin is recognized as a lactogenic hormone in the human, the role of HPL may be merely to antagonize prolactin effects during pregnancy. HPL binds well to prolactin receptors but actually does not have much biological activity. Much more HPL is available in the circulation during pregnancy than prolactin. Perhaps, therefore, HPL may block the effect of prolactin, preventing milk secretion during pregnancy. After delivery, when HPL decreases rapidly, while prolactin remains at high levels, lactation occurs. Thus HPL may be one of several factors involved in the control of the onset of lactation after delivery.

*McNeilly:* That is possible. For instance, in the rat the placental lactogen (RPL) binds to rat mammary tissue, and there is no binding of rat prolactin to the mammary gland until the time of parturition when placental lactogen is cleared. The problem in the human is that at present we cannot even show prolactin binding to the mammary gland, let alone binding of placental lactogen. On a weight-to-weight basis, HPL is equipotent with prolactin, and we are talking in terms of 1000 times more HPL present than prolactin.

Placental lactogens in organ cultures or injected *in vivo* will induce lactogenesis, so they are biologically active in lower species, although perhaps not in man. If they block the action of prolactin by acting as antagonists one would expect them not to possess inherent lactogenic activity. I can accept that they could block the receptor, but should they actually promote the response itself? It is an interesting point.

Placental lactogen is certainly not necessary for lactation. It is called placental lactogen because it has lactogenic activity in the rat and the rabbit, but at present we do not know whether it is lactogenic in the human.

*Thomson:* Are we completely sure, or only 99% sure, that prolactin is important in man for lactation?

*McNeilly:* We are absolutely certain, because if it is suppressed with bromocriptine there is no lactation.

*Harfouche:* I am interested in this because galactorrhoea can occur in a woman who is not pregnant, and also in the human male. Probably a strong element of motivation has to be present. In galactorrhoea in females there is at least a kind of pseudocyesis, probably partly psychological. Has this been reported in male animals where there is no such desire, as a result of mechanical stimulation alone?

*McNeilly:* Professor Short has drunk the milk of a lactating billy goat. He said it was dreadful! Males of some species can be induced to lactate by nipple stimulation, by repeated attempts to obtain milk. I am afraid that I do not know anything about the psychological background of the billy goat!

*Robyn:* Little is known about nipple stimulation reflex inducing prolactin release in women. It has been suggested in animals that this reflex can be conditioned, so that, for example, cows can start prolactin release some time before milking takes place. Are there any data which indicate that this might be possible in women too?

*McNeilly:* Schams (1972) suggested that prolactin is released by cows in response to washing the udder, but we have to be careful when we discuss animals. For instance, cows and goats have a big 'cistern' of stored milk and can release that milk without any oxytocin or prolactin, merely by a local reflex, the tap reflex, which is mechanical stimulation of the mammary gland causing myoepithelial cell contraction. That is why cows walking across a field for milking may have milk squirting out of their udders.

I know of no evidence for prolactin release in animals before some sort of mammary stimulation occurs. The innervation of a cow's teat is the same as that of the human, but the cow has a longer one to be washed and thus stimulated.

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# olactin during Pregnancy and Lactation in the Rabbit*

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STRACT. By using a specific heterologous double-body RIA, changes in the blood levels of PRL during gnancy, pseudopregnancy, and lactation have been stigated for the first time in the rabbit. Blood levels tuate during early and midpregnancy in a manner lar to that in pseudopregnancy. Levels decline in third trimester of pregnancy and increase dramati-y (3- to 25-fold) at or 1–2 days before delivery. itary levels of PRL showed no significant alteration, fetal serum and amniotic fluid levels of PRL remain (<10 ng/ml) throughout pregnancy.
No significant PRL-like, GH-like, or placental lacto-like activity could be demonstrated either in serum extracts of placenta (n = 262) taken between days 10 and 31 of pregnancy.

Postpartum blood levels of PRL were similar in lactating and postpartum nonlactating females. In lactating females, suckling evoked an immediate increase (15- to 25-fold) in circulating PRL levels.

Handling the female or the iv injection of oxytocin during lactation did not cause PRL release. In contrast, manual teat stimulation caused an immediate increase in blood levels of PRL and a response pattern very similar to that of natural suckling. These results suggest that PRL release during suckling occurs solely in response to the tactile stimulation of the teats. (*Endocrinology* **102**: 1548, 1978)

OR MANY years the rabbit has been used in the study of reproduction and ation. In this species, PRL is essential for naintenance of lactation (1) and also plays le at the ovarian level during pregnancy Until now no data have been available on measurement of pituitary PRL levels in d during these reproductive phases in the bit. Recently, we developed a specific het-ogous RIA for rabbit PRL (3). The present er reports the measurement of PRL during gnancy in maternal and fetal serum and niotic fluid, and in serum during pseudo-gnancy and lactation. The stimuli causing release of PRL during lactation have been stigated. In addition, serum and placental acts have been extensively evaluated by eral different lactogenic, GH, and placental ogen (PL) radioreceptor assays (RRA) in effort to determine whether a placental ogen is present in the rabbit.

## Materials and Methods

### *nals*

ew Zealand White female rabbits, weighing 2.8–4.0 kg, were mated with a fertile buck (day 1 of pregnancy) and housed individually in metal cages under a 12-h light:12-h dark lighting schedule. All animals received water and Purina lab chow *ad libitum*. Three days before term, females were transferred to nesting boxes.

The experiments were divided into three sections: a) collection of serial blood samples during pregnancy and lactation; b) collection of maternal serum, amniotic fluid, fetal serum, and placentae at different stages of pregnancy; and c) investigation of the release of PRL during suckling and the stimuli responsible for the release.

### *Serial blood sampling during pregnancy and lactation*

Daily blood samples (3 ml) were collected between 1100–1500 h by venepuncture from the marginal ear vein in seven rabbits from day 3 postmating until day 28 of lactation (n = 2) or day 18 postpartum (n = 4). One rabbit aborted on day 24 of pregnancy and died on day 27 postmating. Two further rabbits became pseudopregnant after mating, as confirmed by raised serum progesterone levels on day 10, and were sampled daily until day 28 postmating.

Because venepuncture may cause a release of PRL in the rabbit (3), samples collected from animals showing obvious signs of stress, or which required repeated venepunctures were not analyzed for PRL. Normally, sampling was completed within 1 min of the first handling of the animal, a time insufficient to detect any increase in PRL levels

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even if stressed (McNeilly, unpublished observations).

*Collection of samples during pregnancy*

Four rabbits were killed by an overdose of sodium pentobarbital iv on each of days 10, 15, 20, 25, 28, 29, 30, and 31 postmating. Three-milliliter blood samples were withdrawn as described above before the animals were killed. Immediately after death a midline abdominal incision was made and the uterus was exposed, removed, and placed on ice. The position of each fetal attachment site was noted on each uterine horn. At each site a sample of amniotic fluid was withdrawn by syringe, the amniotic was sac split, and the fetus was removed and weighed. Where possible, a sample of fetal blood was collected after section of the umbilical cord. The whole placenta was then dissected free of the uterine wall and weighed. From day 15 onwards the placenta was separated into the decidua and placental labryinth (4) and weighed separately. The maternal pituitary was then removed and weighed, and this together with the placental tissues and amniotic fluid samples was frozen immediately on dry ice and stored at −20 C.

All blood samples were allowed to clot and were stored overnight at 4C; the serum was removed by centrifugation and stored at −20 C until assayed.

*PRL release during lactation*

After delivery the pups were left with the mothers until day 5 postpartum. During this time two mothers killed their pups on day 1. In one case the pups appeared too weak to feed despite the mother's repeated attempts to induce suckling, and all of the pups died by day 5 postpartum. Two females successfully raised their litters, one of seven pups, the other of two pups. The pups were taken from the mothers on day 5 postpartum and returned to the mothers once each day to suckle. The suckling period lasted between 3–5 min and was terminated when the young were satiated. Pups were weighed before and after suckling to assess the milk yield. Daily blood samples were taken before, during and after suckling by an indwelling ear arterial cannula, as described previously (3), on days 11 and 22 of lactation in one rabbit and days 11, 22, and 24 of lactation in the other rabbit. To determine the stimuli responsible for the PRL release during suckling, serial blood samples were also obtained a) during handling of the mother in a manner normally adopted during the suckling period but without teat stimulation, and b) after the iv injection of 1 IU oxytocin (Syntocinon, Sandoz) on each of three occasions in the lactating females between day 14–16 of lactation.

The effect of teat stimulation was assesse each of three nonlactating postpartum female days 5, 7, and 9 postpartum. Teat stimulation effected by manual stimulation of all teats over min period and serial blood samples were colle as described above.

*Extraction of pituitaries and placentae*

Each maternal pituitary was homogenized ml 0.15 M $NaHCO_3$, pH 9.5 mixed, and shaker 4 h at 4 C. After centrifugation at 15,000 × *g* fc min at 4 C, the supernatant was diluted 1/1 0.025 M Tris HCl, pH 7.4, containing 10 mM M and 0.1% bovine serum albumin (BSA) and st in aliquots at −20 C until assayed. This met results in the retention of over 90% of the biolo activity of the rabbit pituitary PRL (McN unpublished observations).

Each placenta was homogenized in 0.1 $NH_4HCO_3$, pH 8.5, (1/5, wt/vol) at 4 C for 3C with a Polytron. Extracts were shaken for 1 h C and centrifuged at 15,000 × *g* for 30 min at Analysis of these extracts (n = 262) by rece assays (see later) failed to demonstrate any like or lactogenic activity not explicable by pituitary PRL content measured by RIA and nonspecific interference of protein, as seen with extracts of lung and spleen. In view of these ap ently negative data, experiments were performe determine whether the extraction proce adopted was suitable or whether different co tions were required to demonstrate GH-like acti or lactogenic activity. One-gram amounts ta from a pool of placental labyrinth tissue were tracted for the times shown and under each of conditions listed in Table 1. Equal amount decidual placental tissue were similarly extrac Control tissue was taken from the lungs and spl of rabbits killed on day 28 of pregnancy. All extr were prepared by centrifugation and the supe tant was aliquoted and assayed immediatel reduce the chance of loss in biological activit any active component.

*Assay methods*

PRL was measured by a specific heterolo double-antibody RIA described in detail in preceding article (3). Briefly, this method us guinea pig antiserum to human PRL, ovine [1 iodo-PRL and a rabbit pituitary PRL standard

Lactogenic activity was measured by RRA using the rabbit mammary receptor with o [$^{125}$I]iodo-PRL as tracer (5) and the adult fe

BLE 1. PRL-like activity (PRL-LA), GH-like activity (GH-LA), and placental lactogen-like activity (PL-LA) as estimated by PRAs and PRL level estimated by RIA in rabbit placental extracts

| ıterial extracted | Extraction medium | pH | Time (h) | PRL RIA (ng/g) | | PRL-LA (μg/g) | | | | PL-LA (μg/g) | | GH-LA (μg/g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mammary RRA | | Liver RRA | | | | | |
| | | | | F[a] | M[b] | F | M | F | M | F | M | F | M |
| entas | 0.05 M $NH_4HCO_3$ | 9.5 | 3 | <12 | 22 | <0.01 | 0.24 | 0.05 | 0.21 | 0.40 | 0.35 | 0.02 | 0.04 |
| | 0.05 M $NH_4HCO_3$ | 8.7 | 3 | 20 | 56 | 0.32 | 0.36 | 0.22 | 0.36 | 0.41 | 0.55 | 0.04 | 0.05 |
| | 0.1 M $NH_4HCO_3$ | 9.2 | 3 | 15 | 24 | 0.06 | 0.19 | 0.03 | 0.13 | 0.28 | 0.18 | 0.01 | 0.04 |
| | 0.1 M $NH_4HCO_3$ | 8.0 | 3 | 12 | 16 | 0.03 | 0.32 | 0.10 | 0.14 | 0.30 | 0.22 | 0.02 | 0.03 |
| | 0.1 M $NH_4HCO_3$ | 8.0 | 0.5 | 16 | 16 | 0.36 | 0.33 | 0.14 | 0.10 | 0.28 | 0.34 | 0.04 | 0.03 |
| | 0.1 M PBS[c] | 7.6 | 3 | 12 | 8 | 0.23 | 0.20 | 0.23 | 0.17 | 0.27 | 0.31 | 0.01 | 0.02 |
| | 0.1 M NaAc | 5.6 | 3 | 4 | 4 | 0.20 | 0.21 | 0.06 | 0.08 | 0.11 | 0.07 | 0.03 | 0.02 |
| lium alone | 0.05 M $NH_4HCO_3$ | 8.7 | | UD[d] | UD | 0.02 | | 0.06 | | 0.02 | | 0.01 | |
| g | 0.05 M $NH_4HCO_3$ | 8.7 | 3 | 28 | | 0.28 | | 0.37 | | 0.28 | | 0.03 | |
| :en | 0.05 M $NH_4HCO_3$ | 8.7 | 3 | 6 | | 0.12 | | 0.09 | | 0.28 | | 0.02 | |
| ım,[e] 1:3 | | | | UD | | 0.14 | | 0.10 | | 0.60 | | 0.06 | |

F, placental labyrinth.
M, decidual plate.
BS, phosphate-buffered saline.
UD, undetectable.
Serum obtained after treatment of rabbit with CB154.

liver receptor with ovine [$^{125}$I]iodo-PRL as er (6). In both assays, ovine PRL (oPRL; NIH--10; 26 IU/mag) was used as the standard. GH- activity was measured by using the pregnant ›it liver receptor with human [$^{125}$I]iodo-GH as tracer and human GH (hGH; NIH-HS-1146; 2.6 mg) as the standard as described in detail elsere (7). PL-like activity was measured by using ovine liver receptor with ovine [$^{125}$I]iodo-PRL racer and oPL (Chan-Friesen 20) as the stan- l (8). As a control in all these receptor assay ems, rabbit serum was added after dilution (1:3) ı 0.025 M Tris-HCl, pH 7.4, containing 0.1% A, as a sample with an approximately equal ein content to that of the placental extracts.

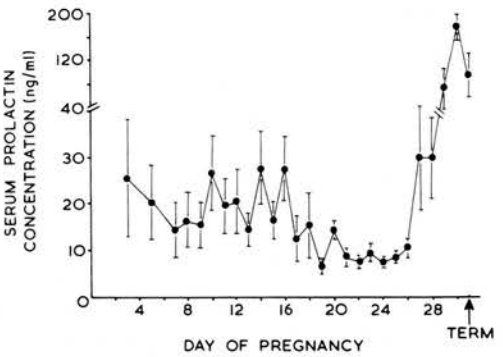



FIG. 1. Changes in the serum PRL concentration during pregnancy in the rabbit. Each point represents the mean (±1 SD) of at least four animals.

## Results

### *L levels during pregnancy and pseudo-gnancy*

Changes in the maternal blood level of PRL ing pregnancy showed a similar pattern in animals sampled either serially or when ed in groups at different stages of preg- ıcy for the collection of placentae. There- e, the serum PRL levels in each of these studies were pooled (Fig. 1). Levels varied ing the first 16 days of pregnancy (range 00 ng/ml; Figs. 1,2) and then declined to a re steady baseline (range 2–19 ng/ml; Figs. 1 and 2) until 2–4 days before parturition (day 31). Levels then increased 3- to 25-fold in all animals at, or 1–2 days before, parturition (range, 65–280 ng/ml; Figs. 1 and 2).

A similar pattern was seen in pseudopregnancy up to day 28 (Fig. 3). In the one animal which aborted, no increase in PRL levels occurred at the time of abortion (Fig. 3). PRL levels in fetal blood (n = 125) and amniotic fluid (n = 187) collected at days 10, 15, 20, 25, 28, 29, 30, and 31 (term) ranged between 3–10 ng/ml and showed no variation throughout pregnancy. Measurement by RRA of the lac-

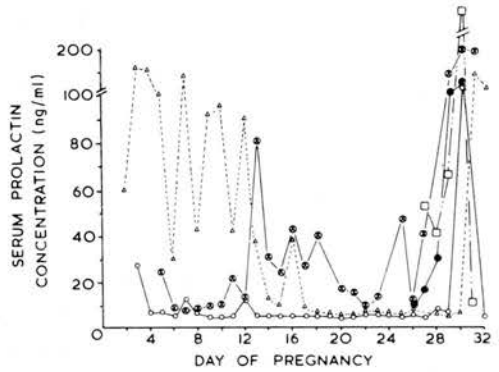



FIG. 2. Changes in serum PRL concentration in individual female rabbits during pregnancy. Parturition occurred on day 31 in all animals.

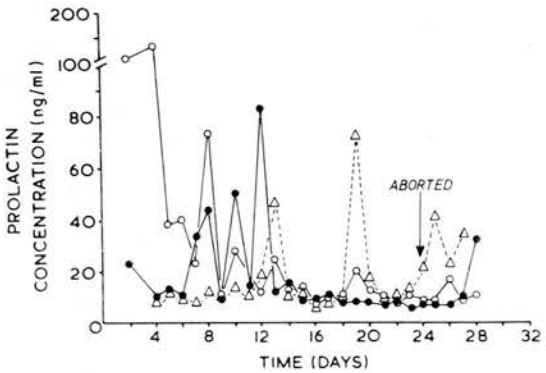



FIG. 3. Changes in serum PRL concentration during pseudopregnancy in two female rabbits (○——○; ●——●) and in one female (△——△) who aborted on day 24 of pregnancy.

togenic activity in the maternal sera during pregnancy showed an identical pattern to the PRL levels measured by RIA.

### *Pituitary and placentae*

Pituitary levels of PRL (range, 1.5–2.6 μg/mg wet wt) showed no significant change during pregnancy, although there was a significant ($P < 0.01$) increase in pituitary weight, comparing weights on days 29 and 30 with weights in nonpregnant and day 10 pregnant rabbits (Fig. 4).

Initial analysis of placental extracts (n = 262) collected from day 10 of pregnancy onwards by RRA (5) failed to demonstrate any lactogenic activity. Subsequent experiments using different extraction procedures, two PRL RRAs, and RRAs for PL and GH also failed to show any significant activity wh was not explicable, in the main, by the r specific effect of protein in the assay syst and by the amount of pituitary PRL pre in the extracts (Table 1).

### *PRL levels during lactation*

Growth rate of the pups and milk yiel the two females who successfully lacta were similar to those reported elsewhere Daily blood levels throughout lacta showed a wide variation (range, 10–195 ng/ Fig. 5a). In the two females who killed t pups at birth (Fig. 5b), PRL levels were vated only on the day of parturition. The female who unsuccessfully attempted to h her pups (all of which died on day 5 postp

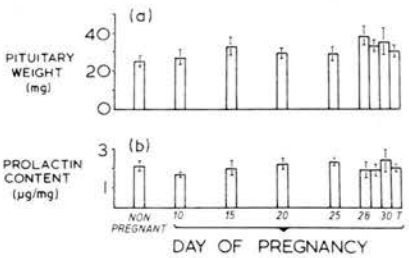



FIG. 4. Pituitary weights (a) and PRL contents (b) du pregnancy in the rabbit. (Mean ± 1 SD.)

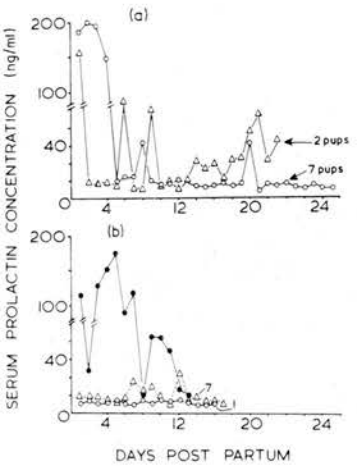



FIG. 5. Serum levels of PRL postpartum in two fem rabbits which successfully lactated (a), two females wh cannibalized their young (b; △——△; ○——○) and female whose litter died on day 5 postpartum ●——●).

um) showed elevated PRL levels over the irst 12 days postpartum.

Suckling caused an immediate increase range 15- to 25-fold) in blood levels of PRL -Fig. 6) on both days 11 and 22 of lactation. 'he pattern of release was different with PRL evels remaining elevated during the whole )bservation period after suckling on day 11, vhereas on day 22, levels began to decline oon after suckling finished and returned to )asal values within 60 min.

Handling of the lactating females in the ame manner as during the suckling experinents, but in the absence of the pups, or the v injection of 1 IU oxytocin did not alter PRL evels (Fig. 7). Manual teat stimulation for a 4-min period resulted in an immediate in-:rease in PRL levels in all three of the rabbits )n each of days 5, 7, and 9 postpartum (Fig. 3).

## Discussion

The pattern of maternal serum PRL levels ;een during pregnancy in the rabbits is similar

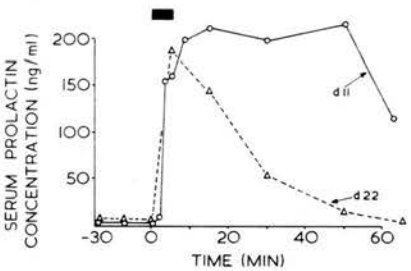



FIG. 6. Change in serum PRL level in one lactating female rabbit in response to suckling on days 11 and 22 of lactation. The period of suckling (4 min) is indicated by the black bar.

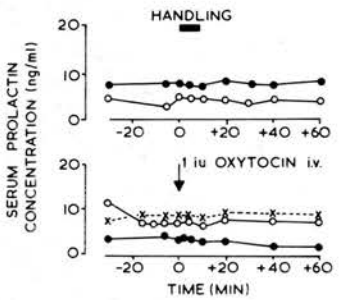



FIG. 7. The effect of handling in sight and sound of their young or of the iv injection of 1 IU oxytocin on serum PRL concentrations in lactating rabbits. The period of handling (4 min) is indicated by the black bar and each line represents a different animal.

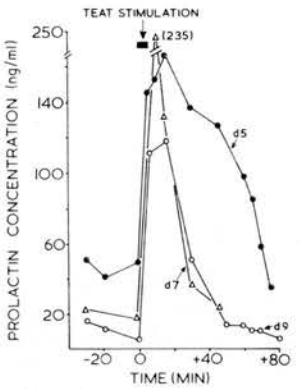



FIG. 8. The effect of manual teat stimulation for 4 min (indicated by the black bar) on serum PRL levels in a postpartum female rabbit on days 5, 7, and 9 postpartum.

to that reported in the mouse (9), rat (10), sheep (11, 12), goat (13), and cow (14), with fluctuating basal levels early in pregnancy, a decline, and then a dramatic increase at parturition. The drop in serum PRL levels between days 20–26 postmating occurred both in pregnant and nonpregnant animals. The reason for this is not apparent. No dramatic change in steroid secretion occurs around this period of pregnancy (15–18), suggesting that the change is independent of steroid levels. It is probable that the increase of PRL at or just before parturition is related to an increase in estrogen levels which occurs at this time (17, 18) because estrogen stimulates PRL secretion in the rabbit (McNeilly, unpublished observations).

The absence of any change in pituitary PRL content confirmed the earlier observations of Meites and Turner (19). Together with the maternal serum levels of PRL, this would suggest that if PRL were to play an important role in mammary development or ovarian function during pregnancy the amounts of PRL required would be small. An increase in serum levels of PRL cannot be a requirement for the onset of lactogenesis in the rabbit, which occurs around day 20 of pregnancy (20). Previous studies (21) have demonstrated high binding of PRL to the rabbit mammary gland at all stages of pregnancy, suggesting that the absence of PRL binding to the mammary gland is not the limiting step. It may be, therefore, that the onset of lactogenesis, albeit to a limited degree, is related not to an in-

crease in serum PRL or in the PRL-receptor population in the mammary gland, but to a decrease in the blood level of progesterone (17), a hormone which is known to inhibit lactogenesis in the rabbit mammary gland (22).

The low levels of PRL found at all stages of pregnancy in the fetus and amniotic fluid are in contrast to the results obtained in primates (23). In the guinea pig it has been suggested that PRL plays a role in the salt and water balance within the amniotic fluid (24). It is surprising, therefore, that low levels of PRL should be found in the rabbit, a species in which PRL alters the salt and water balance (25, 26).

The failure to demonstrate significant amounts of a lactogenic or GH-like activity in the rabbit placenta or in maternal serum confirms previous findings (27, 28), although an increase in GH secretion of pituitary origin occurs during the second trimester of pregnancy (McNeilly, unpublished observations).

The pattern of PRL secretion postpartum and its relationship to maternal behavior may provide preliminary evidence for the role of PRL in behavior (29). PRL levels remained high for more than 2 days in those females who successfully reared or who tried to nurse their young. By contrast, PRL levels in those females who killed their young were only elevated on the day of delivery. This data would support the earlier studies of Zarrow and coworkers (29), *i.e.* implicating PRL as one of the major components governing maternal behavior in the postpartum rabbit.

Suckling resulted in an immediate and prolonged release of PRL in established lactation (day 11). Later in lactation the release of PRL was not so prolonged and PRL levels began to decline as soon as the suckling of the young was terminated. If the release of PRL had ceased immediately after suckling in this latter case, then the decline in serum levels of PRL would be related to the metabolic clearance rate of PRL. On the three occasions in which PRL levels were measured during suckling at this stage of lactation (days 22 and/or 24), the half-life of PRL was estimated as 22, 19, and 26 min, very similar to that of other species (*e.g.* 30). The reason for the decline in output of PRL in response to suckling is unknown but a similar decline in PRL output after suckling or milking has been observed in other species (12, 31–34). During lactation, basal levels of PRL were similar to those in nonlactating postpartum females. This would suggest that the release of PRL in response to suckling is of major significance to the onset and maintenance of lactation, and confirms the indirect evidence (1, 35) that PRL is the essential hormone for lactation in the rabbit.

The nature of the stimulus for PRL release during suckling has been clarified in the present experiments. Handling of the female within sight and sound of her pups did not cause any release of PRL. Similarly, the injection of oxytocin caused no change in PRL levels, confirming data in other species (32, 36). In contrast, manual stimulation of the teats caused an immediate increase in blood PRL levels. As with the PRL release occurring in response to natural suckling, the release in response to manual teat stimulation decreased as lactation advanced. The reasons behind this decreased release of PRL are unknown and are under investigation at the present time. The present results strongly suggest that PRL release during suckling occurs in response to tactile stimuli arising from the teat and that other factors related to suckling are not involved.

The present paper describes for the first time the changes in serum levels of PRL during pregnancy, pseudopregnancy, and lactation in the rabbit, and although some of the data are limited, they suggest that the pattern of PRL secretion is similar to that described in other species.

It is apparent that much further work is required, in particular in respect to PRL levels during pseudopregnancy and lactation in the rabbit, but with the availability of a suitable RIA (3) this task may now be pursued.

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# BINDING OF PROLACTIN TO THE RABBIT MAMMARY GLAND DURING PREGNANCY

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ıding of prolactin to the rat mammary gland is low during pregnancy and only increases at rturition (Holcomb, Costlow, Bushchow & McGuire, 1976). Holcomb *et al.* (1976) have ggested that failure to demonstrate binding of prolactin may be related to occupancy of the olactin receptor by placental lactogen. However, the rabbit apparently does not produce a ıcental lactogen (Kelly, Tsushima, Shiu & Friesen, 1976; McNeilly & Friesen, 1977), and it s therefore of considerable interest to determine whether the results of binding of prolactin the rabbit mammary gland during pregnancy showed significant differences from those tained in the rat.

Mammary glands were taken from pregnant New Zealand White rabbits killed by an i.v. erdose of sodium pentobarbitone on each of days 10, 15, 20, 25, 28, 29, 30 and 31 (term) ter mating. Mammary glands from four non-pregnant and three lactating females (on day of lactation) were taken as controls. Mammary glands were weighed and stored at −20 °C ıtil used. The binding of ovine prolactin to cell membrane fractions of each mammary gland ıs assessed as follows. Mammary tissue (10 g) taken from different areas of the gland was ›mogenized in 0·3 M-sucrose solution (1 : 5, w/v) and cell membranes were prepared by quential centrifugation at 15000 and 100000 *g* (Shiu, Kelly & Friesen, 1973). The 100000 *g* llet was resuspended in 0·025 M-Tris-HCl (pH 7·4) containing 10 mM-$Mg^{2+}$, and the protein ncentration was estimated according to the method of Lowry, Rosebrough, Farr & Randall 951). All subsequent dilutions were made in 0·025 M-Tris-HCl (pH 7·4) containing mM-$Mg^{2+}$ and 0·1% bovine serum albumin. The specific binding of $^{125}$I-labelled ovine olactin (NIH P-S10; 28 i.u./mg) prepared by the lactoperoxidase method (Thorrell & hansson, 1971) was determined as follows. To 200 µl membrane preparation containing 00 µg protein were added 100 µl unlabelled ovine prolactin (10 µg/ml solution) or 100 µl ıffer and 200 µl $^{125}$I-labelled ovine prolactin (80 000 c.p.m.; approximately 1 ng). After ixing, the tubes were incubated overnight at room temperature. Three ml cold buffer were ıded to each tube and the membranes were precipitated by centrifugation at 2000 *g* for 20 in at 4 °C. The precipitates were counted in a well-type gamma counter (LKB-Wallac, veden) and the specific binding of prolactin was assessed as the amount of $^{125}$I-labelled ovine olactin bound which could be displaced by excess unlabelled ovine prolactin (Shiu, Kelly & iesen, 1973). Scatchard analyses were then carried out. Tubes contained 0·1 ml membrane eparation diluted to give specific binding of between 5 and 7% $^{125}$I-labelled ovine prolactin, ıd different amounts of unlabelled ovine prolactin with a constant amount of $^{125}$I-labelled ine prolactin tracer. Incubation and separation were as described above. For Scatchard ıalysis, the molecular weight of ovine prolactin was taken as 23000.

The results are shown in Fig. 1. Whereas prolactin binding was low or undetectable in non-egnant animals (<0·1–0·8%), significant binding of prolactin was observed throughout preg-ıncy in all mammary preparations examined. Even at day 10 of pregnancy, a substantial (40- to 00-fold) increase in binding had occurred, even though there was little increase in the weight of e mammary glands. Specific binding of prolactin showed a further increase during lactation.

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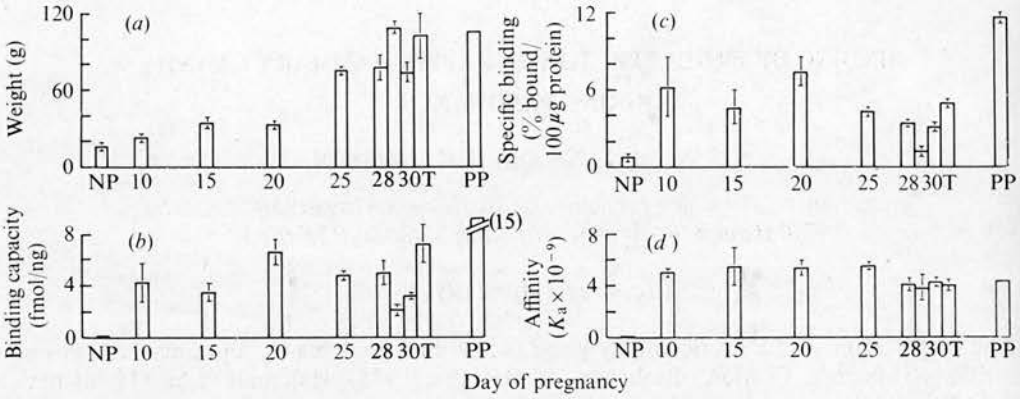



Fig. 1. Changes in the weight (*a*), the specific binding of $^{125}$I-labelled ovine prolactin (*b*), the prolactin binding capacity (*c*) and the binding affinity (*d*) of the rabbit mammary gland during pregnancy. The vertical lines represent ± S.E.M. The binding capacity and affinity were determined by Scatchard analysis. NP, non-pregnant; T, term; PP, *post-partum*.

The binding capacity determined by Scatchard analysis showed an identical pattern to t specific binding. In both cases, a significant drop in binding ($P<0{\cdot}001$) occurred on day 29 pregnancy coincident with the time when blood levels of prolactin in the rabbit increase befc parturition (McNeilly & Friesen, 1977) suggesting that occupancy of receptors may occur. I significant change in binding affinity was seen throughout pregnancy and the affinity consta ($K_a = 5 \times 10^{-9}$ mol/l) was similar to that previously reported in the lactating rabbit mamma gland (Shiu & Friesen, 1974).

The results show a clear difference from the pattern of binding reported in the rat (Holcor *et al.* 1976). It is apparent that in the rabbit, the inhibition of lactogenesis until around day of pregnancy (Denamur & Delouis, 1972) and inhibition of full lactation until after parturiti is not related to an absence of prolactin receptors in the mammary gland.

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# olactin Receptor Content of Rabbit Milk*

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**ABSTRACT.** Rabbit milk fat globule membranes have been shown to contain PRL receptors by a variety of criteria, including hormonal specificity and inhibition by specific anti-PRL receptor antisera. Collection of milk samples throughout the period of lactation facilitated a temporal study of the receptor content of the membrane fraction of these milk samples by Scatchard analysis after treatment with 5 M $MgCl_2$ to dissociate endogenously bound PRL. Total receptor content was low after parturition (3.3 ± 1.3 fmol/mg membrane protein) but increased subsequently, reaching maximal levels (43.7 ± 3.4 fmol/mg) by day 21 of lactation. No significant difference in the $K_a$ ($4.9 \times 10^9$ ± 0.35 $M^{-1}$) of the milk receptor was detected over a 4-week suckling period. No apparent relation seemed to exist between rabbit serum PRL values measured by RIA in serum samples taken just before milking and milk PRL receptor content. Milk receptor content, however, was significantly ($P < 0.02$) correlated with the PRL receptor content of the gland when animals were sacrificed immediately after milking. (*Endocrinology* **107:** 816, 1980)

CONSIDERABLE body of morphological and bio- chemical evidence has accumulated in support of Bargmann-Knoop model of milk secretion (Ref. 1 references therein); central to this model is the elopment of the milk fat globule by the plasma mem- ıe as it reaches the apex of the mammary epithelial before its expulsion in the alveolar lumen. This ma membrane lining the milk fat globules can be ıted with ease by differential centrifugation after ure of the granules (2) and provides a purified sample lasma membrane-derived material which is wholly helial in origin and, in most cases, only hours old (1). number of workers have reported finding radioim- ıoassayable PRL in milk samples (3, 4), and Nolin Witorsch (5) have demonstrated the presence of PRL and its receptor in the apices of mammary and within the alveolar lumen by immunohisto- nical techniques. Likewise, Bullough and Wallis (6) reported finding $^{125}$I-labeled PRL in association alveolar lumen contents after *in vivo* administration regnant mice. Considering these findings in terms of Bargmann-Knoop hypothesis, it seemed likely that ma membrane PRL receptors could be demonstrated ilk samples. A logical source for these samples was the rabbit, since PRL receptors are both abundant and well characterized in rabbit mammary tissue (7, 8), and the daily production rate of milk in the rabbit (up to 270 ml/day) provides sufficient material for a temporal study of receptor levels by Scatchard analysis. Finally, a recently developed heterologous RIA for rabbit PRL (9) has allowed us to examine hormone levels as well as receptor content, measures which would seem particularly relevant in view of the unique ability of PRL to induce its own receptor (Refs. 8 and 10 and references therein) and its mandatory role in lactogenesis in the rabbit (11).

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## Materials and Methods

### *Hormones*

Hormones used were NIH preparations (oPRL-S12, bGH-1003A, rat PRL RP2, and rat GH B2), a Connaught insulin preparation (26 IU/mg), or were prepared in our laboratory to greater than 90% purity (ovine placental lactogen) or 33% purity (human PRL and human placental lactogen).

### *Animals*

New Zealand White rabbits were obtained during the middle portion of their first pregnancy from North American Laboratories (Winnipeg, Canada) or from Fauna Breeding Laboratories (Montreal, Canada). Animals were housed individually in hooded nesting cages and maintained at 20 C or a 12-h light, 12-h dark cycle, with access to food and water *ad libitum*. Litters were not disturbed until the third day after delivery in order to avoid pup rejection. The day of delivery is referred to as day 0.

*ection of samples*

ıckling mothers were removed from their rooms between
ı–1700 h every second day for the first 8 days and then at
6, 21, and 28 days post partum. Blood samples were taken
kly by ear venupuncture, and then 100 mU oxytocin (Syn-
ıon, Sandoz, Ltd., Hanover, NJ) plus a mild anesthetic dose
ɛmbutal were administered by the same route. Milk samples
ml) were then expressed manually over a 10- to 30-min
ɔd, and the animals were returned to their litters while still
thetized. Serum and milk samples were stored at −20 C
l assay. After collection of the final milk sample, mothers
ɛ killed by nembutal overdose, and the mammary glands
ɛ rapidly removed and transferred to −20 C storage. Most
ıals were killed between 28–35 days after parturition, since
ıing generally occurs at this time (12).

*, content of serum samples*

he specific heterologous double antibody RIA developed by
Neilly and Friesen (9) was used to determine the PRL
ɛntration of the serum samples referred to above.

*ɔaration of receptor-containing fractions*

fter thawing, milk samples (5 ml) or tissue samples (5 g
ıelial tissue) were homogenized for 45 sec in 3 vol of ice-
0.3 M sucrose-25 mM Tris-HCl at maximum setting on the
'tron PT-10 (Brinkmann Instruments, Westbury, NY). Ho-
enates were centrifuged for 20 min at 15,000 × *g* to remove
in particles and debris, and then at 150,000 × *g* for 45 min
ɛllet the clear vitreous membrane layer. This procedure has
ı shown to yield an enriched fraction of milk plasma mem-
ıe-like material (13). The 150,000 × *g* pellet was resus-
ɛd in 2.0 ml 5 M $MgCl_2$-0.1 M Tris-HCl (pH 7.5) buffer at
, and after 5 min this was diluted with 15 ml chilled 25 mM
-HCl (pH 7.5) to facilitate subsequent sedimentation of the
ıbrane. To remove excess magnesium chloride, this suspen-
was again centrifuged at 150,000 × *g* for 45 min, and the
ɛt was finally resuspended in 4.4 ml 25 mM Tris-HCl, (pH
and stored at −20 C until assay. Preliminary experiments
ved that treatment with 5 M $MgCl_2$, designed to dissociate
ɔgenous bound PRL (14, 15), resulted in a 11.2 ± 0.48%
an ± SEM; n = 4) loss of membrane protein. This treatment
lted in an increase of up to 55% in specific binding compared
ɔntrol non-$MgCl_2$-treated membranes. Kelly *et al.* (14) has
ɔrted a greater loss of membrane protein (27 ± 1.8%) after
$Cl_2$ treatment of rabbit mammary and rat liver membranes.
rt from the possibility that the PRL receptor may be more
ɛtant than milk membrane proteins generally to solubiliza-
, we believe that this discrepancy relates to our use of
ıcentrifugation rather than a low speed spin to harvest the
ıbranes after $MgCl_2$ treatment. Since our protein determi-
ɔns were carried out on the $MgCl_2$-treated pellet, we have
found it necessary to include a correction factor to account
ɔolubilization of membrane proteins, although we, too, as-
e that the receptor is solubilized to the same extent as the
r membrane proteins. Our polyethylene glycol separation
ɛm ensures that all bound PRL is recovered in the bound/
separation step.

*Receptor assay*

All assays were carried out with ovine [$^{125}$I]PRL ([$^{125}$I]oPRL) labeled by the lactoperoxidase method routinely used in our laboratory (7). The standard procedure (7) was used for all assays, except that the polyethylene glycol bound/free separation method (16) was used rather than low speed centrifugation of membranes because not all [$^{125}$I]oPRL specifically bound was precipitable by the latter procedure. Using the polyethylene glycol procedure, nonspecific binding averaged approximately 10% of the total counts (range, 5–14%). A 15- to 18-h incubation at 22 C was used for assays, since this was found to be necessary for the attainment of maximum specific binding. Specific binding was markedly reduced at 4 C. Preliminary assay of the resuspended milk membranes at various dilutions was undertaken to establish the optimum concentration for Scatchard analysis. In all cases this optimum ranged from 100–200 μl resuspended membranes, with a final protein content of around 1 mg (range, 0.35–2.8 mg)/assay tube. Specific binding was linearly related to membrane concentration up to 25% specific binding. Scatchard analysis of every sample was performed with nine-point duplicate determinations over the range of 0–100 ng added oPRL, and the initial linear portion (0–30 ng oPRL) was used to calculate the capacity and $K_a$ by least squares analysis.

Antisera to PRL and GH-receptors were used to characterize the specificity of hormone binding using the methods described previously (17, 18).

*Protein determinations*

The protein content of membrane samples after $MgCl_2$ treatment was estimated by the method of Lowry *et al.* (19) using bovine serum albumin as standard.

*Statistics*

Statistical significance was determined using the one-way analysis of variance, followed by Dunnet's multiple comparison procedure (20). Correlation coefficients were obtained by regression analysis using the least squares method (21).

## Results

*Characterization of the PRL receptor in milk*

Analysis of the binding of [$^{125}$I]oPRL to the 15,000 × *g* pellet, the 150,000 × *g* pellet, and the 150,000 × *g* supernatant showed that approximately 80% of the specific binding was located in the 150,000 × *g* pellet. Displacement analysis of these fractions (Fig. 1A) showed high affinity ($K_a$ = 3–6 × $10^9$ $M^{-1}$) binding localized in the 150,000 × *g* pellet, and no detectable specific binding in the 150,000 × *g* supernatant. Further characterization of this binding was undertaken entirely with the 150,000 × *g* pellet.

The specificity of hormone binding was examined with a variety of cold hormones as competitive inhibitors for [$^{125}$I]oPRL and showed the pattern characteristic of the

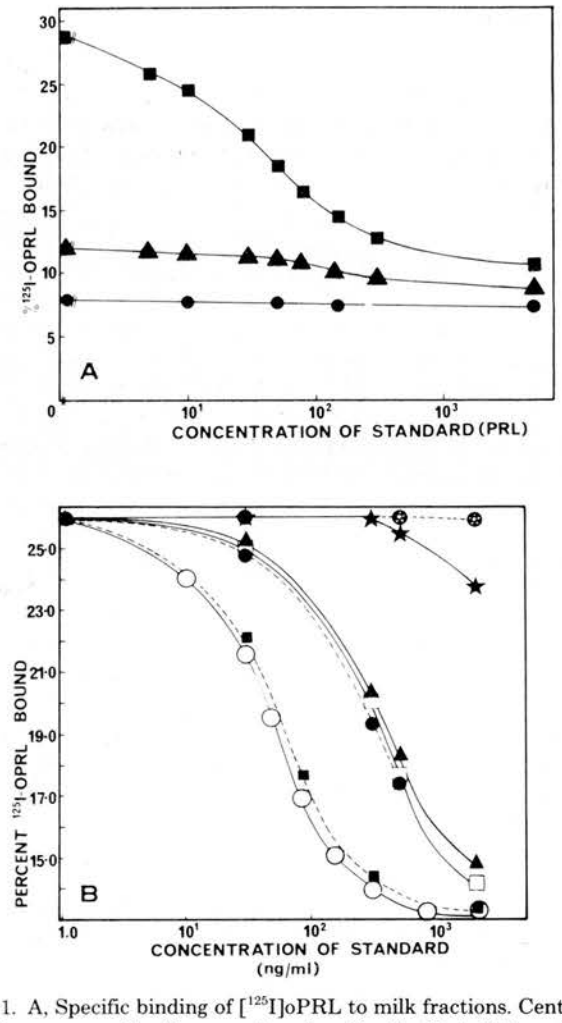



FIG. 1. A, Specific binding of [$^{125}$I]oPRL to milk fractions. Centrifugal fractions, prepared by the procedure described in *Materials and Methods*, were used to set up displacement curves using [$^{125}$I]oPRL and 1 mg protein/tube. Incubation conditions are outlined in *Materials and Methods*. ■, 150,000 × *g* pellet; ▲, 150,000 × *g* supernatant; ●, 15,000 × *g* pellet. B, Specificity of binding of PRL receptor in milk. Protein (800 μg) from the 150,000 × *g* pellet was added to varying concentrations of the hormones shown and the reaction was initiated by the addition of [$^{125}$I]oPRL followed by incubation and bound/free separation, as previously described. ○, oPRL; ■, ovine placental lactogen; ●, human PRL; □, rabbit PRL; ▲, human placental lactogen; ★, rabbit and bovine GH; ✪, insulin and FSH.

mammary gland receptor (Fig. 1B) (22). The most effective inhibitors were oPRL and placental lactogen, which displaced bound [$^{125}$I]oPRL with equivalent potency, followed by human placental lactogen, rat PRL, and a less potent (30%) preparation of human PRL. Minimal displacement was seen with high concentrations of rat GH and bovine GH, and no displacement was produced by insulin and FSH. Additional specificity studies were undertaken with the specific anti-rabbit mammary prolactin receptor and the specific antirabbit liver GH receptor antisera, described by Shiu and Friesen (17, 22) and Waters and Friesen (18), respectively. Pretreatment of milk PRL receptors with PRL receptor antisera inhibit the binding of [$^{125}$I]oPRL to these membranes, wh anti-GH receptor and normal guinea pig sera were wi out effect (Fig. 2). This anti-GH receptor antisera tota inhibited the binding of ovine [$^{125}$I]GH to liver me branes at a 1:100 dilution in a parallel experiment (d not shown).

*PRL receptor content of milk over the postpartum per*

We consider that the studies discussed above dem strate the presence of PRL receptors in the membra fraction of rabbit milk. Accordingly, milk samples w collected at various times after parturition, and af fractionation, the 150,000 × *g* pellets were analyzed PRL receptor content by Scatchard analysis, as descrit in *Materials and Methods*.

Figure 3 presents the results obtained from five rabb allowed to suckle for up to 35 days post partum. Fig 3A expresses receptor content per ml milk, and Fig. expresses receptor content per mg membrane prote Data for the following time periods was pooled: days 8–9, 12–13, 16–17, 21, and 28–31. In both cases an incre ($P < 0.05$) in receptor content over that observed on d 2 had occurred by days 12–13 and beyond. Mean recep content was maximal by day 21 [296 ± 64 fmol/ml or 4 ± 3.4 fmol/mg (mean ± SEM)] and was maintained o days 28–31 (309 ± 33 fmol/ml or 45.1 ± 5.7 fmol/r respectively). Receptor content was very low just af parturition (day 2 values were 24.4 ± 12.6 fmol/ml or ± 1.3 fmol/mg, respectively). This pattern resembles

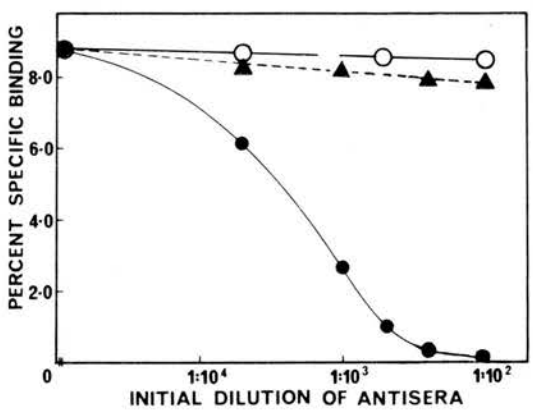
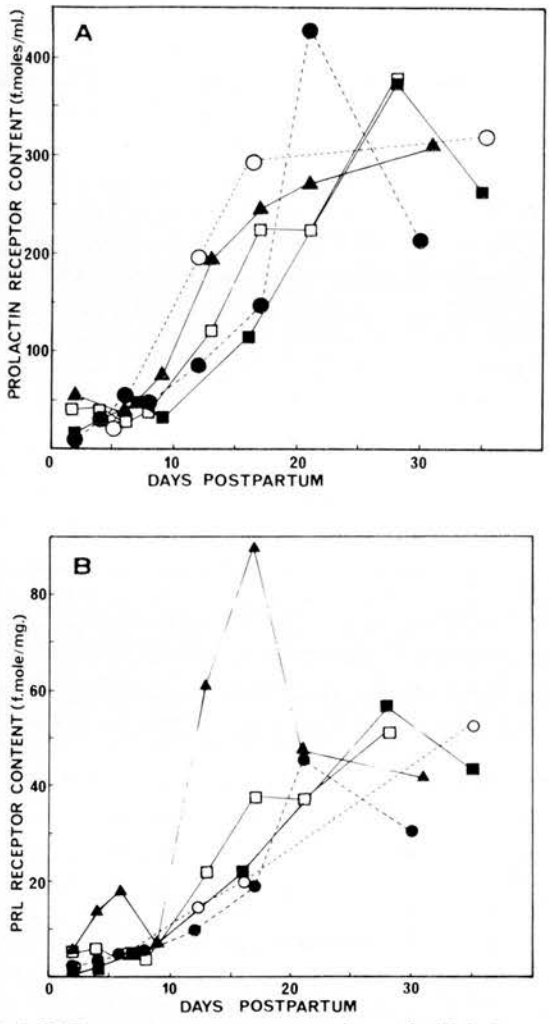



FIG. 2. Effect of antireceptor antisera on [$^{125}$I]oPRL binding to r membrane receptors. Membrane fraction protein (600 μg) was pr cubated with 100 μl of the stated dilutions of antisera for 24 h at 4 300 μl radioreceptor assay buffer. Membrane fractions were t washed by centrifugation in 3 ml ice-cold assay buffer and resuspen in 400 μl assay buffer. This was followed by the addition of [$^{125}$I]oP (85,000 cpm) in 100 μl buffer and a subsequent 18-h 22 C incuba and polyethylene glycol separation. Tubes were included with ex cold oPRL (400 ng) and no antisera to allow for a determinatio total specific binding. ●, Anti-PRL receptor; ▲, anti-GH receptor normal G.P. serum.




. 3. A, PRL receptor content, expressed per ml milk before weaning. nples of milk (5 ml), collected by the procedure described in *Mate-ls and Methods*, were homogenized and fractionated to give a milk mbrane fraction which was used to determine PRL receptor content Scatchard analysis, as described previously. Results for five individ- rabbits are shown, and the same symbols are used throughout for h animal. B, PRL receptor content, expressed per mg protein in the k membrane fraction. Protein analysis, by the method of Lowry *et* (13) was used on the membrane fractions in conjunction with the a from the previous figure.

ttern reported by Djiane *et al.* (8) for the glands of n-CB154-treated animals, and this prompted us to mpare the content of receptors in the milk samples tained just before killing with the receptor content of embranes derived from the glands themselves. Figure hows that even with a small sample number, a signif-ınt correlation exists between gland and milk receptor ntents, whether expressed on the basis of milligrams of embrane protein or weight of gland and volume of milk. Despite considerable individual variation, there was no nificant change in the association constant throughout e postpartum period [$K_a = 4.9 \times 10^9 \pm 0.35$ M$^{-1}$ (mean

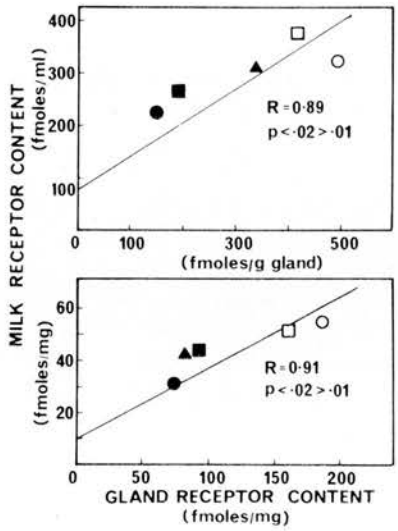



FIG. 4. Correlation between milk and gland PRL receptor content. Representative 5-g samples of epithelial tissue were taken just after the last milking, and 150,000 × *g* particulate fractions were obtained as described previously. Scatchard analysis of these pellets gave the values for PRL receptor shown on the abscissa when expressed either per g gland or per mg protein. Using these data and those shown in Fig. 3, analysis was performed on these values, and the significance of values was stated. Each point represents one rabbit.

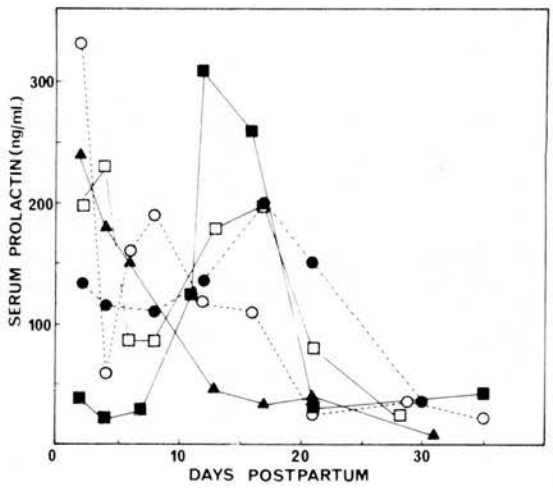



FIG. 5. Serum levels of rabbit PRL in samples taken just before milking (1500–1700 h) for the five rabbits used in this study. A specific heterologous RIA, referred to in the text, was used for the determination of these values.

± SEM)], in agreement with the finding of Djiane *et al.* (8) using the glands themselves.

### *PRL content of serum*

As can be seen from Fig. 5, no clear relationship exists between milk receptor content and serum PRL levels. In four of five animals, initial serum levels were high compared to those during the first 27 days of pregnancy, presumably because of a continuation of the enhanced

PRL secretion seen after parturition (9). PRL levels declined in all animals toward the end of the lactating period, as has been reported for a number of other species (Ref. 9 and references therein).

The significance of the peaks of serum PRL in midlactation is uncertain but may be the result of an intensified suckling stimulus before removal of the animal for sampling (9).

## Discussion

There seems little doubt that PRL receptors do, indeed, occur in rabbit milk, in view of the complete agreement between the immunological and hormonal specificities as well as the kinetic properties of the milk and gland receptors. The inhibition of [$^{125}$I]oPRL binding by anti-PRL receptor antiserum is particularly relevant here, since this antiserum has been shown to block a number of the biological responses of mammary tissue to PRL (23). The finding that high affinity PRL binding is restricted to the 150,000 × *g* fraction of milk, containing the milk fat globule membranes, provides further support for the presence of membrane-derived PRL receptors in milk.

If we assume that there is a good correlation between gland and milk receptor contents throughout lactation, then the pattern shown in Fig. 3 is not in complete agreement with the only report on the PRL receptor content of rabbit mammary gland throughout lactation. Djiane *et al.* (8) found that PRL receptor content (whether expressed per mg membrane protein or per total mammary gland) was maximal by 6 days of lactation and had declined by 14 days. However, in these experiments, CB154 (bromoergocryptine) was administered for 36 h before sacrifice to reduce circulating PRL levels and, hence, abolish receptor occupancy. Measurement of PRL receptor in nonergocryptine-treated animals showed a gradual increase in receptor content, plateauing after 14 days, which more closely resembles the pattern reported here. We have used $MgCl_2$ (5 M) treatment of membranes to abolish occupancy, according to the report of Kelly *et al.* (14) and our own results (15), and find that this procedure results in the complete release of bound hormone and the recovery of free receptor. We believe that total blockade of the hormone is responsible for the positive autoregulation of its own receptor (*i.e.* PRL) is not an entirely satisfactory way to avoid the problem of occupancy, especially in view of the relatively fast turnover of the PRL receptor ($t_{\frac{1}{2}} < 3$ h) (24). Indeed, Djiane *et al.* (8) found that ergocryptine treatment just before the PRL surge at parturition abolished the difference between treated and untreated animals, implying that the treatment prevented the normal rapid induction of receptor at this time.

It is perhaps noteworthy that the period of maxim milk production in the rabbit (days 15–25) (12) al correlates with the period of maximal milk recept content, although the limited number of values after tl time precludes a decision on whether this correlati holds true during the subsequent decline in milk produ tion. The question of the degree of occupancy of the PI receptor required to produce a certain biological respon has not yet been resolved, although it is worth noti that in some situations (25, 26) a correlation has be found between receptor levels and milk production.

There appears to be no correlation between the seru content of PRL and the milk receptor content (an indeed, the milk yield) (12). It seems likely that tl magnitude of the increase in PRL levels after sucklii (9) is more closely related to these parameters than random daily values (27). This is particularly evide after day 21 of lactation, when PRL levels are quite lo in the presence of high receptor levels. An estimate the extent of occupancy obtained by comparing recept contents before and after $MgCl_2$ treatment would hav perhaps, been a more valid parameter to relate to seru PRL levels, but the technical problems involved in o taining a sufficient quantity of milk for two Scatcha plots precluded this approach.

The constancy of the affinity of PRL for its recept throughout lactation is in agreement with the findings Djiane *et al.* (8) using the gland but does not agree wit the report of Perry and Jacobs (28), who found a 2.5-fol increase in affinity between the early postpartum perio and days 25–30 of lactation. The latter authors, in fac found no difference in the mammary PRL receptor co tents of rabbits during the early and late postpartu periods.

We wish to emphasize that much of the precedin discussion is based on a close correlation between glan and milk receptor contents; while this appears to be tru after day 28 in our limited samples, the correlation ha not been confirmed before this time. Nevertheless, w hope that these studies will be extended to the clinic situation, where the unavailability of lactating huma mammary tissues has impeded the study of human PR receptors. It may well be that the high circulating estro gen levels suppress PRL receptors in preparturien women, and that these receptors are induced post partu by the PRL surges associated with suckling. A study o milk fat globule membrane PRL receptors may we provide a simpler and more physiological approach t this problem than mammary cell tissue culture tech niques.

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# THE ORIGIN OF PROLACTIN IN AMNIOTIC FLUID: FETAL OR MATERNAL?

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During pregnancy prolactin in the maternal serum increases progressively towards term (Hwang *et al.*, 1971; L'Hermite *et al.*, 1972; Tyson *et al.*, 1972). High levels of prolactin are also found in amniotic fluid throughout gestation (Hwang *et al.*, 1971; Tyson *et al.*, 1972) although the source of this prolactin and its function remains obscure.

This paper summarizes our studies on the changes in prolactin concentration in the maternal and fetal serum, fetal pituitary and amniotic fluid during the first two trimesters of pregnancy. In view of the possible contribution of prolactin of fetal origin to the accumulation of prolactin in the amniotic fluid the hypothalamic control of prolactin secretion in early fetal life has been examined.

## MATERIALS AND METHODS

### *Maternal Serum and Amniotic Fluid Samples*

Blood samples (n = 220) were collected during the first 30 weeks of pregnancy from 138 women attending the antenatal clinic at St. Bartholomew's Hospital. All pregnancies were normal. In 75 patients undergoing therapeutic terminations between 9 and 23 weeks of pregnancy, samples of maternal blood and amniotic fluid were collected.

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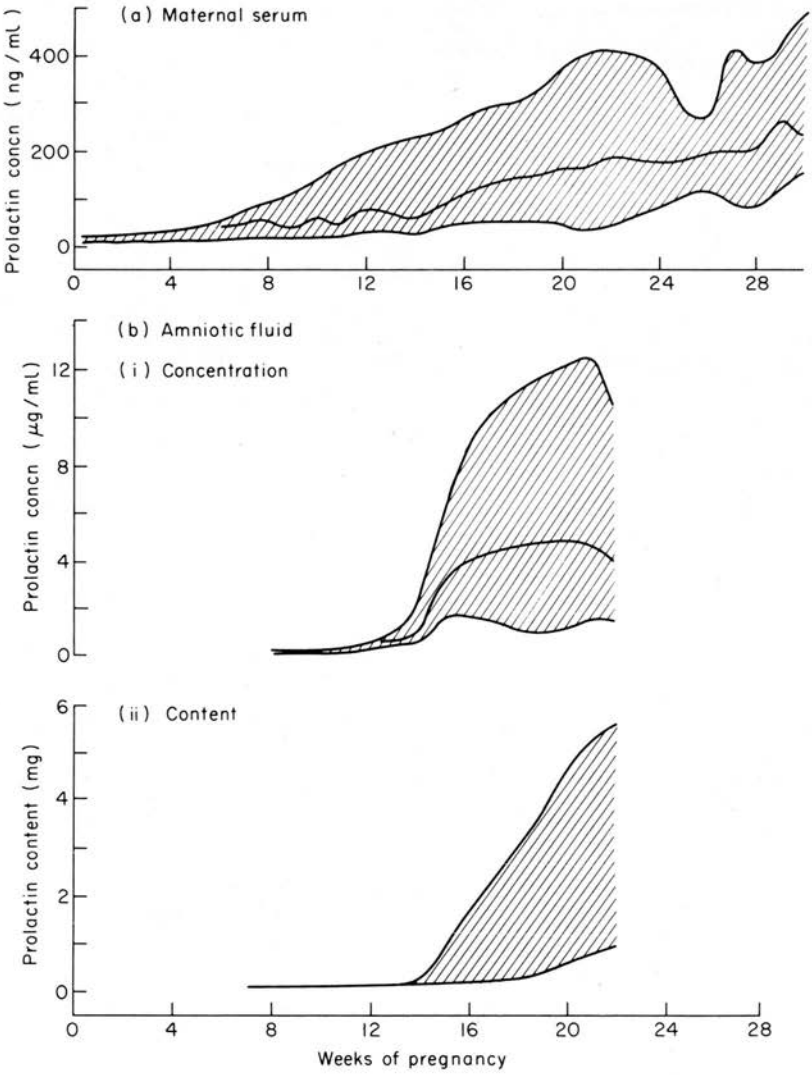



*Fig. 1* Changes in maternal serum (a) and amniotic fluid (b) concentration of prolactin during the first half of pregnancy. Results show mean and range. The prolactin content of amniotic fluid (ii) was calculated from the estimated mean amniotic fluid volume reported by Fuchs (1966) and the upper and lower concentrations of prolactin at each week of pregnancy.

*Fetal Samples*

Twenty-two fetal blood samples were collected at hysterotomy between 10 and 22 weeks of pregnancy. Fetal pituitaries, hypothalami and cortices were collected in 11 of these cases, immediately frozen and stored at −20°C until extracted. Pituitaries were extracted at 4°C into 0.01 N HCl as described by Scott and Lowry (1974). Each fetal hypothalamus and cortex was extracted in a similar

manner to the pituitaries (Campbell *et al.*, 1964) and pituitary, hypothalamic and cortical extracts were stored at −20°C until assay.

*Assay of Prolactin Releasing-Inhibiting Activity and TRH*

The prolactin releasing or inhibiting activity of the fetal hypothalamic and cortical extracts *in vivo* was assayed in ovariectomized estradiol and progesterone primed female rats (McDonald and Gilmore, 1971). Extracts, TRH (from 1 ng to 200 ng), dopamine (50 μg) or dopamine (50 μg) plus TRH (100 ng) were injected into the right carotid artery of rats under light ether anaesthesia. Blood samples were withdrawn from the iliac vein before and 15 and 20 min after the injection of the test substances, the serum separated and stored at −20°C until assayed for prolactin.

TRH in the hypothalamic and cortical extracts was estimated by specific radioimmunoassay described in detail elsewhere (Jeffcoate *et al.*, 1973). The assays were kindly performed by Dr. S. Jeffcoate, St. Thomas' Hospital, London.

*Radioimmunoassay for Prolactin*

Human prolactin was measured by a specific double-antibody radioimmunoassay described in detail previously (McNeilly, 1973; McNeilly and Hagen, 1974). Rat prolactin was measured by a double-antibody radioimmunoassay using reagents supplied by the NIAMDD (Bethesda, Maryland, U.S.A.).

## RESULTS

The overall pattern of results is shown in Figs 1 and 2. Maternal serum levels increase progressively during the period of gestation studied and levels were consistently above normal (20 ng/ml) by week 15 of pregnancy (Fig.1a). Amniotic fluid levels between 4 and 10 weeks of pregnancy were low and comparable to those in the maternal circulation (Fig.1b). The levels progressively increased between 10 and 15 weeks to between 4 and 100 times those in maternal serum.

*Fetal Prolactin Levels*

Prolactin was detectable in all samples examined ranging from 8 to 220 ng/ml (Fig.2a). There was no relation between prolactin level and stage of gestation and no significant difference between levels in male and female fetuses.

Total fetal pituitary prolactin content increased with gestational age from between 8 and 18 ng before week 12 to 70 to 80 ng by week 18 (Fig.2b). Prolactin content was greater in male than female fetuses from week 15 of pregnancy onwards.

*Fetal Hypothalamic TRH and Prolactin Release Activity*

Immunoreactive TRH levels within the fetal hypothalamus were 1.3 to 12.8 times greater than that of the cortex and increased after week 15 of pregnancy. TRH content was not correlated to the prolactin releasing activity *in vivo* of the hypothalamic or cortical extracts. Up to week 15 hypothalamic extracts either had no effect or caused release of prolactin equivalent to between 0 and 1 ng (n=3) or 1 and 10 ng (n=2) of TRH. All hypothalamic extracts from fetuses of 15½ weeks or older inhibited prolactin release (n=10). Cortical extracts caused either release (n=4), no change (n=4), or inhibition (n=4).

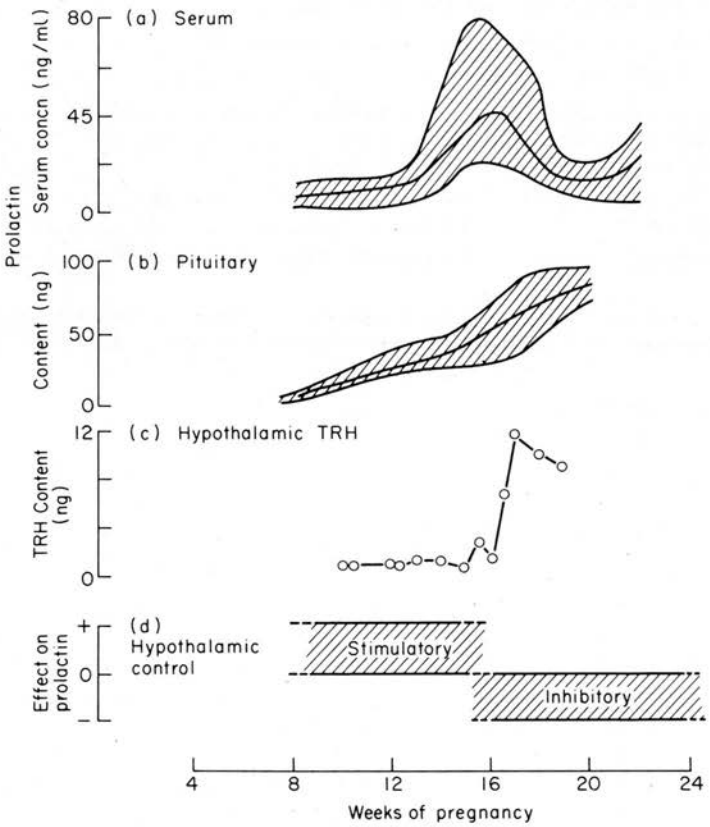



*Fig. 2* Changes in fetal serum concentration (a) and pituitary content (b) of prolactin (mean and range), hypothalamic TRH content (c) estimated by radioimmunoassay and hypothalamic control (d) of prolactin secretion in the fetus. The latter shows the effect of fetal hypothalamic extracts on prolactin secretion in the *in vivo* rat bioassy.

*Correlations Between Prolactin Levels*

No significant correlation ($p > 0.1$) could be demonstrated between prolactin levels in any of the compartments examined. In addition, no correlation could be demonstrated between prolactin levels and osmolarity in any compartment.

## DISCUSSION

The present report confirms previous observations on prolactin levels during pregnancy (Hwang *et al.*, 1971; L'Hermite *et al.*, 1972; Tyson *et al.*, 1972) and shed some light on the development of hypothalamic control mechanisms of prolactin release in the fetus. However, the source of prolactin in amniotic fluid remains obscure. It is apparent that there is a gradual increase in prolactin concentration in amniotic fluid up to the 14th week of gestation after which the concentration reaches a plateau. However, since amniotic fluid volume continues

to increase beyond this stage (Fuchs, 1966) the total amount of prolactin in the amniotic fluid continues to increase (Fig.1c). Studies in the monkey (Freisen *et al.*, 1972; Josimovich *et al.*, 1974) suggest that transfer of prolactin into and out of the amniotic fluid is slow. Since the maximum levels of prolactin become considerably higher than those elsewhere, amniotic fluid may be acting as a 'sump' with variable contributions from other compartments.

An obvious source of this prolactin would be the maternal circulation since the increase in prolactin concentration in both maternal circulation and amniotic fluid occur at a similar stage of gestation (Fig.1). However, it is apparent that a proportion of this prolactin could derive from the fetus. Prolactin was present in the fetal circulation from the earliest stage (6 weeks, 10 ng/ml) at which samples could be collected and prolactin was also detectable (total content 18 ng) in the pituitary at the same stage of gestation. Fetal pituitary prolactin levels increased as gestation progressed confirming previous reports (Aubert *et al.*, 1975; Levina, 1968). Whether this prolactin is transferred from the fetus to the amniotic fluid remains unknown but the fetus remains a potential source.

It has been suggested that TRH may be a physiological prolactin releasing factor in man. It is of interest, that in the fetus, while the levels of TRH in the cortex remain relatively unchanged, those in the hypothalamus increase markedly after the 16th week of gestation. However, the *in vivo* activity of these extracts revealed that up to the 16th week of gestation the hypothalamus is either devoid of activity or possesses only releasing activity for prolactin. After the 16th week, however, the fetal hypothalamic extracts inhibited prolactin secretion thus exhibiting an influence similar to that in the adult. Whether at this stage of fetal development pituitary hormone secretion is indeed under hypothalamic control is uncertain. It has been suggested by Siler-Khodr and colleagues (1974) that some stimulatory action in the fetus during early gestation may be necessary for normal pituitary prolactin output. The present results lend support to this concept. In addition blood levels and pituitary concentrations of prolactin in anencephalic fetuses are not significantly different from normal fetuses (Aubert *et al.*, 1975; Hayek *et al.*, 1973) despite the absence of any hypothalamic factor(s). This would suggest that the pituitary lactotrophs in the anencephalic pituitary are not secreting maximally and supports the concept that at an early stage of fetal development prolactin secretion from the pituitary may be under a stimulatory influence from the hypothalamus.

A peak in circulating levels of prolactin in the fetus appeared to occur between 14 and 16 weeks of pregnancy. This was coincident with an increase in total pituitary content. The decline in fetal serum levels of prolactin, between 16 and 20 weeks of pregnancy, occurred at a time when the fetal hypothalamus inhibited prolactin output in the rat bioassay preparation. It may be tentatively suggested that the decline in serum levels is a direct result of the development of the inhibitory hypothalamic control occurring between the 15th and 17th week of fetal life.

It is apparent from the present study that during the whole of fetal development during pregnancy the fetus can secrete pituitary prolactin. The lack of correlation between levels of prolactin in the maternal and fetal serum suggest an independence of control. Both compartments therefore may contribute to the store of prolactin in amniotic fluid.

## ACKNOWLEDGEMENTS

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# The Relationship between Suckling-Induced Prolactin Response and Lactogenesis

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**ABSTRACT.** The PRL response to suckling was studied during the first week of the puerperium. Mean basal levels of PRL showed no significant change during the first week of the puerperium, but there were progressive rises in both the maximum suckling-induced response and the total area under the response curve, which reached peak values on the fourth day after delivery.

Despite large variations between individuals in basal PRL levels (range, 0.3–7.0 U/liter), peak suckling-induced response (range, 0.1–9.9 U/liter), and total response (range, 0.6–63.0 arbitrary units), there was much less variability within individuals between consecutive feeds.

Using an electronic balance, 20 patients on days 5 and 6 were classified either as good feeders (>60 g milk/feed) or poor feeders (≤60 g milk/feed) on the basis of 2 consecutive test weights. The mean PRL response to suckling in 11 good feeders was no different from that in 9 poor feeders, and there was no significant correlation between milk yield and PRL response. Six patients whose infants were in the special care nursery had lactation initiated and maintained by breast pump for an average of 5.6 days. Although the PRL response to the breast pump was very small, these patients also had satisfactory milk yields (mean, 86 g).

Although the presence of PRL is essential for lactation, the data in this paper suggest that there is no close temporal correlation between PRL concentrations and milk yield. (*J Clin Endocrinol Metab* **50:** 670, 1980)

THE ADVANTAGES of breast feeding are fully recognized (1) and there has been an encouraging increase in the prevalence of breast feeding (2). The major problem which now prevents successful lactation is the high discontinuation rate within the first 3 months after delivery (2–4). Many factors may influence the decision to discontinue breast feeding, but the most common reason advanced by mothers is inadequacy of their milk supply (1).

The eventual success of lactation is improved greatly if a satisfactory flow of milk is established during the first week of the puerperium (5–7). Although PRL must be present to allow lactation to take place (8), the exact quantitative relationship between PRL and milk yield has not been established. In a recent report, Aono *et al.* (9) reported a close correlation between milk yield in the first 5 days after delivery and the PRL response to suckling. This observation suggests that the use of drugs to stimulate PRL release might improve lactational performance (10, 11). This paper reports on the individual variations in PRL-induced suckling responses and their relationship to the success of lactogenesis.

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## Materials and Methods

The PRL response to suckling was studied during the first 6 days of the puerperium in 54 volunteers who were breast feeding after delivering mature infants of normal birth weight in the Simpson Memorial Maternity Pavilion, Edinburgh. In Table 1, clinical details are given of the number of patients, their ages parities, and the birth weight and sex distribution of their infants. Since all patients were studied only once, the data are cross-sectional for each day of the puerperium. Full informed consent was obtained from all of the women before their inclusion in the study. All subjects were feeding on an approximate 4-h regimen, feeding 3 min on each breast on each occasion on day 1, 5 min on each breast on day 2, 7 min on each breast on day 3, and 10 min on each breast from day 4 on.

In addition to the normal subjects, six women whose infants were in the special care nursery were also studied at a mean 5.6 days after delivery. In all cases, these women were having lactation initiated by regular milk expression with a humalactor breast pump and none of them had experienced normal suckling before the time of the study.

### *Assessment of PRL response*

A catheter was placed in a forearm vein 30 min before suckling, and 2-ml blood samples were taken for serum PRL estimation immediately before suckling and 10, 20, 30, 45, 60, 90, and 120 min after the onset of feeding. As shown in Fig. 1, the PRL results were expressed in 3 ways, namely as the basal PRL value, the maximum suckling-induced PRL increase, and

the total suckling-induced PRL response (area under the response curve). In 15 subjects, intrapatient variation was studied by measuring the PRL response to 2 consecutive suckling episodes.

*Hormone assay*

Sera were separated and stored at −20 C until assayed. PRL was measured in duplicate by RIA using reagents supplied by Dr. H. G. Friesen, Winnipeg, Canada (12, 13). The inter- and intraassay precisions were 13% and 6%, respectively. Results were expressed in terms of M.R.C. pituitary PRL standard 75/506 (14). Results are quoted in units per liter (22 $\mu$U = 1 ng PRL).

*Test weighing*

Test weighing was assessed by the difference in baby's weight before and after nursing using an electronic integrating balance (model PS15, A, Galenkamp and Co. Ltd., London, England). This balance gave reproducible readings to within 2 g.

TABLE 1. Clinical details of mothers and their infants who were studied once each during the first week of the puerperium

| Day | No. of subjects | Mean age (yr) | Parity primigravid: parous | Infant | |
|---|---|---|---|---|---|
| | | | | Birth wt | Male:female |
| 1 | 5 | 24.4 | 3:2 | 3.37 | 2:3 |
| 2 | 7 | 22.2 | 5:2 | 3.71 | 3:4 |
| 3 | 9 | 29.1 | 4:5 | 3.66 | 5:4 |
| 4 | 9 | 27.5 | 4:5 | 3.60 | 4:5 |
| 5 | 11 | 27.4 | 7:4 | 3.61 | 6:5 |
| 6 | 13 | 26.1 | 8:5 | 3.24 | 5:8 |

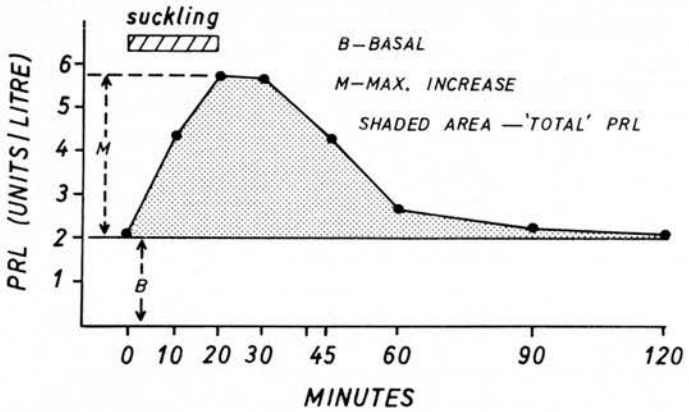



FIG. 1. Methods of measuring PRL response.

*Clinical success of lactation*

In 20 of the patients who were studied on the fifth or sixth day of the puerperium, the clinical success of lactation was assessed as the mean of two consecutive test weights. There was a strong correlation between the first and second test weights ($r = 0.855$; $P < 0.001$). An arbitrary division was made into two groups on the basis of the mean test weight results; there were 11 good feeders (5 primigravid and 6 parous; mean age, 26.8 yr; mean birth wt, 3.48 kg) who all produced more than 60 g milk/feed, and 9 poor feeders (6 primigravid and 3 parous; mean age, 26.2 yr; mean birth wt, 3.48 kg) who all produced 60 g or less per feed.

*Statistics*

Comparisons between groups were made using Student's unpaired $t$ test.

## Results

*PRL values on days 1–6 of the puerperium (Table 2)*

Considering all of the suckling patients together, there were no significant changes in the mean basal PRL concentrations during the first week of the puerperium. Between days 1 and 4, the maximum suckling-induced increase of PRL rose progressively from a mean of 1.7 to 5.4 U/liter ($P < 0.05$), and the total PRL release rose from a mean of 7.5 to 25.3 U ($P < 0.05$). Thereafter, the maximum increase and the total PRL fell, but the mean values on day 6 were not significantly different from those on day 4.

*Inter- and intrapatient variations in PRL*

Wide interpatient variations were recorded on each day of the puerperium for the basal PRL level (range, 0.3–7.0 U/liter), the maximum increase of PRL (range, 0.1–9.9 U/liter), and the total PRL level (range, 0.6–63.0 U). In contrast, intrapatient variation was assessed in 15 subjects by comparing the PRL responses in 2 consecutive feeds, and a positive correlation was found between the maximum PRL increases during the 2 feeds ($r = 0.731$; $P < 0.001$; Fig. 2). Similarly, positive correlations were found between the consecutive basal PRL values ($r = 0.931$; $P < 0.001$) and the total PRL responses ($r = 0.715$; $P < 0.01$).

TABLE 2. Basal PRL, maximum PRL increase, and total PRL release in response to suckling (mean ± SD), during the first 6 days of the puerperium

| | Day 1 (n = 5) | Day 2 (n = 7) | Day 3 (n = 9) | Day 4 (n = 9) | Day 5 (n = 9) | Day 6 (n = 13) |
|---|---|---|---|---|---|---|
| Basal PRL (U/liter) | 2.6 ± 1.7 | 3.9 ± 1.3 | 3.3 ± 2.4 | 2.8 ± 1.5 | 2.8 ± 2.3 | 2.7 ± 1.8 |
| Maximum PRL increase (U/liter) | 1.7 ± 0.9[a] | 3.4 ± 2.1 | 4.3 ± 2.4 | 5.4 ± 3.6[b] | 3.8 ± 3.0 | 3.3 ± 2.5 |
| Total PRL (arbitrary units) | 7.5 ± 2.9[a] | 12.7 ± 11.7 | 19.9 ± 11.7 | 25.3 ± 20.1[b] | 19.4 ± 12.3 | 15.1 ± 15.5 |

[a] Significantly different from [b] ($P < 0.05$).

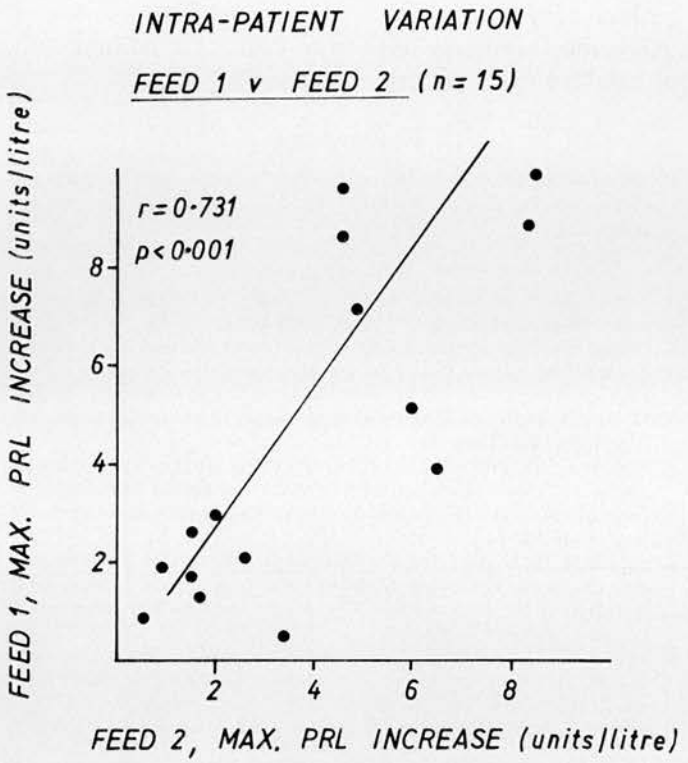



FIG. 2. Maximum PRL release in response to suckling in 2 consecutive feeds in 15 patients on days 5 or 6 of the puerperium.

*PRL responses in good and poor feeders*

Comparison of the mean PRL responses on days 5 or 6 in 11 good feeders [mean test weight, 80 ± 3 g (SEM)] and 9 poor feeders [mean test weight, 46 ± 4 g (SEM)] is shown in Fig. 3.

The mean PRL responses were similar at all points for the good and poor feeders. Considered as a single group, no significant correlations were found between the test weight values and basal PRL levels (r = 0.115), maximum increment of PRL (r = 0.040), or total PRL release (r = 0.079; $P > 0.1$ in all cases).

As also shown in Fig. 3, six humalactor patients who had a mean milk yield of 86 g/feed had a mean basal PRL level of 1.06 U/liter (±0.25 SEM), with no significant response during milk expression. The PRL response in the humalactor patients was significantly less than that in both the poor and the good feeders at all points of the response curve ($P < 0.01$ at all points).

*Comparison of test weights in large and small PRL responders*

The 10 patients with the highest PRL responses on days 5 and 6 of the puerperium were compared with the 10 who had the lowest responses (Fig. 4). Despite the large differences in PRL responses to suckling, the mean test weights did not differ significantly between the two groups.

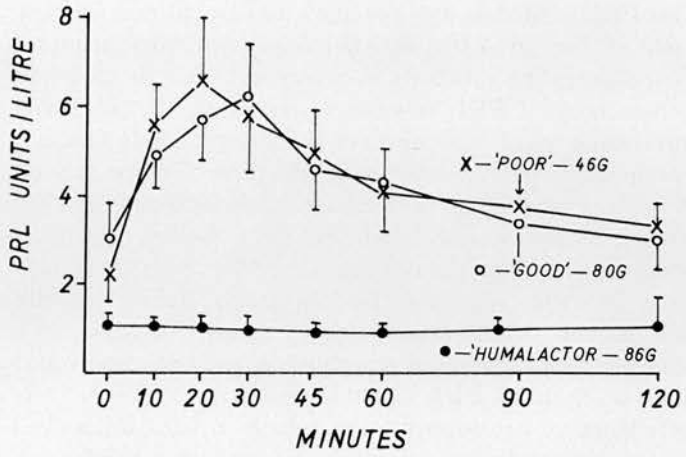



FIG. 3. PRL responses in 11 good feeders, 9 poor feeders, and 6 patients on humalactor breast pump on days 5 and 6 of the puerperium.

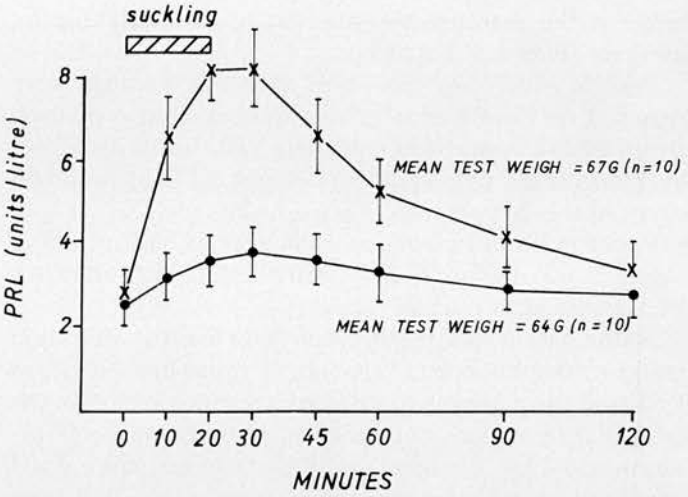



FIG. 4. Comparison of mean test weights in 10 high (X) and 10 low (●) PRL responders on day 5 or 6 of the puerperium.

## Discussion

It is well known that PRL is essential for the initiation and maintenance of lactation because when bromocriptine is used to reduce the basal PRL level and the suckling-induced PRL response, milk production ceases within 48 h (8, 15). If there was an exact quantitative relationship between PRL levels and milk yield, this would suggest that drugs which increase PRL would increase milk output, but we could find no relationship between PRL response to suckling and milk yield. Furthermore, entirely satisfactory milk production was found in those women using a breast pump which failed to provide a PRL discharge.

Our findings differ from those of Aono *et al.* (9), who reported a correlation between the total milk yield during the first 5 days of the puerperium and the suckling-induced PRL response. However, Aono *et al.* (9) assessed the PRL response as the difference between the concentrations before and 30 min after suckling. We found that

the PRL response was not maximal by 30 min in over half of our subjects. Furthermore, standardization of blood-sampling methods is essential if one is to avoid stress-induced PRL release. Aono *et al.* (9) measured total milk yield by emptying the breasts with a breast pump after each feed during the first 5 days. Breast emptying may itself have an effect on lactogenesis (16), and it is noteworthy that the poor feeding group in Aono's study was mainly primigravidae with small nipples. Even in their poor feeding group, there was milk left in the breast after feeding, suggesting that milk ejection was as much of a problem as milk production. It may be that the PRL levels in their study reflected the efficiency of breast emptying, which in turn influenced the milk yield. In our own study, we preferred to influence the natural course of events as little as possible by using test weighing as the clinical end point and to overcome some of the inaccuracies inherent in test weighing by using an electronic balance.

Results which are consistent with our findings were reported by Zarate *et al.* (7), who compared a placebo group with a group who had their PRL levels increased by TRH for the first 4 postpartum weeks. They found no effect of this hyperprolactinemia on the yield or composition of milk. Furthermore, Franks *et al.* (18) reported a patient who lactated successfully despite unusually low PRL levels after pituitary surgery.

A distinction has to be made between the initiation and the maintenance of lactation, because basal levels of PRL and the response to suckling are much higher in the immediate puerperium than after several months of lactation (19–21). Tyson *et al.* (22) reported that TRH increased breast engorgement in women who had been feeding for more than 80 days, hence, this treatment may be of value in long established lactation. From a practical point of view, however, most problems of breast feeding arise early in lactation (2–4).

The very low PRL responses in the patients who had only used a breast pump after delivery are of interest. The humalactor acts by negative pressure, and the machine used in this study applied very little stimulation to the nipple. Despite the absence of a PRL response to breast emptying, milk yield was equivalent to or greater than that in the group of normal breast-feeding mothers, suggesting that adequate breast emptying is of paramount importance in establishing lactation. This is further supported by one patient who was producing 1.6 liters milk/day at 6 weeks, having initiated and maintained lactation solely by humalactor.

Illingworth and Stone (23) showed that breast engorgement during the first week of the puerperium was associated with the increased success of lactation. We have recently established that there is no relationship between engorgement and postpartum levels of PRL (24). The combined evidence suggests that artificial stimulation of PRL levels is unlikely to have an important influence on the establishment of successful breast feeding.

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# HORMONAL PROFILES IN LACTATING AND NON-LACTATING WOMEN IMMEDIATELY AFTER DELIVERY AND THEIR RELATIONSHIP TO BREAST ENGORGEMENT

BY

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**Summary**

Prolactin, human placental lactogen (HPL), oestrone, oestradiol and progesterone levels in plasma were measured before and during the first seven days after delivery in women who did not breast feed. The results confirmed the rapid clearance of placental steroids from the circulation after delivery. Plasma prolactin levels remained elevated during the early puerperium and the range of values were the same in non breast-feeding women and a group of breast feeding women. Of the 25 women studied, six developed breast engorgement. No difference in hormonal profiles were found leading to the conclusion that there is no endocrine basis for breast engorgement in non-breast feeding women.

DURING pregnancy, the glandular component of the breast proliferates under the action of oestrogens and progesterone, mainly of placental origin. Blood levels of both prolactin and human placental lactogen (HPL) rise progressively throughout pregnancy but their respective roles in mammary development remain obscure (McNeilly, 1977). It is clear, however, that prolactin is the principal hormone involved in milk secretion during lactation (Tyson and Friesen, 1973).

Following delivery, the onset of milk secretion coincides with the clearance of placental steroids from the circulation (Kulski *et al*, 1977), thus allowing the high levels of pituitary prolactin to act on the steroid-primed breast tissue and initiate lactogenesis. Milk secretion begins within 48 to 72 hours of delivery and, in the absence of suckling, ceases within a few days. This spontaneous milk secretion may give rise to breast engorgement.

The degree of breast engorgement which occurs in puerperal mothers who do not breast feed is very variable. Some have marked discomfort and overdistension, while in others the changes are minimal. It has been shown that there is a good correlation between clinical estimation of the degree of breast engorgement and the volume of milk obtained from the breast (Newton and Newton, 1951). The object of our study was to determine whether there was any endocrine basis for the differences in degrees of breast engorgement observed in a group of non-lactating puerperal women.

TABLE I

*Clinical assessment of breast engorgement*

| Score | Milk production | Breast congestion | Breast discomfort |
|---|---|---|---|
| 0 | No milk leakage | No change | No subjective change |
| 1 | Few drops only | Slight hardness | Mild discomfort |
| 2 | Moderate leakage | Moderate hardness and redness | Moderate discomfort |
| 3 | Marked leakage* | Very hard reddened breasts | Marked discomfort, requiring analgesics |

* Requiring frequent changes of breast pad.

## PATIENTS AND METHODS

The 25 women who volunteered for the study were all enrolled during the last month of their pregnancies. All had previously decided not to breast feed and none had attempted breast feeding in a previous pregnancy. They all had uncomplicated pregnancies and received no medication other than routine analgesics or antibiotics at any stage during the study. All delivered vaginally, with the exception of two women who had Caesarean sections under epidural anaesthesia.

It is the hospital policy to use no agents to suppress lactation and management is conservative. The women were instructed to avoid handling their breasts and to wear a firm supporting brassière. If uncomfortable symptoms of engorgement developed a firm binder was applied and analgesics offered if necessary.

The degree of breast engorgement was assessed daily during the first week after delivery by the patient herself and by a single observer, using a simple numerical rating system similar to that described in previous studies (Cooke *et al*, 1976; Rolland and Schellekens, 1973). Details of the system are given in Table I. No attempt was made to evaluate milk production by expressing the breast as it was felt that this might interfere with the study. Note was made of the degree of spontaneous outflow. The patient rated the degree of discomfort herself by comparing it with the pre-delivery state and an assessment was made of the degree of congestion by gentle palpation and observation of distension and overlying skin changes. The final assessment of degree of engorgement in each individual was obtained by adding together the three highest daily scores obtained during the six days of the study. For comparison, blood samples were taken at least two hours after a suckling episode from 17 women who were breast feeding their babies.

Ten ml venous blood samples were taken at the time of enrolment to the study, within an hour of completion of the third stage of labour and daily during the first six days of the puerperium. In some subjects, blood samples were taken twice daily during the first 72 hours of the puerperium. The blood was immediately centrifuged and stored at $-20°C$ until the assays were performed.

### *Hormone assays*

Plasma prolactin was measured by a specific double antibody radioimmunoassay using reagents supplied by Professor H. G. Friesen, Winnipeg, Canada (Hwang *et al*, 1975; McNeilly and Hagen, 1974). The inter- and intra-assay precision was 13 per cent and 6 per cent

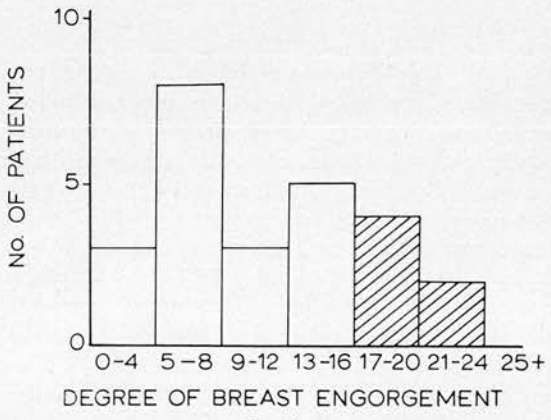



FIG. 1

Distribution of patients in relation to the degree of breast engorgement. Patients showing scores greater than 17 (hatched columns) showed severe clinical signs of breast engorgement.

respectively. Plasma human placental lactogen (HPL) was measured using the Phabedas kit (Radiochemical Centre, Amersham). Progesterone, oestrone and oestradiol in plasma were measured by previously described radioimmunoassays (Baird *et al*, 1974; Scaramuzzi *et al*, 1976; Van Look *et al*, 1977).

## Results

The mean age of women entering the study was 26 years (range 19 to 34) and 17 of them were parous. The degree of breast engorgement is shown in Figure 1. The six women with the greatest degree of engorgement (score of more than 17) will be considered separately later.

The mean and range of plasma levels of progesterone, oestradiol, oestrone and HPL in the six women with severe breast engorgement are shown in Figure 2. At no time did the levels for any of these hormones fall outside the range of levels found in the non-lactating group as a whole, even when the breast-engorged subjects

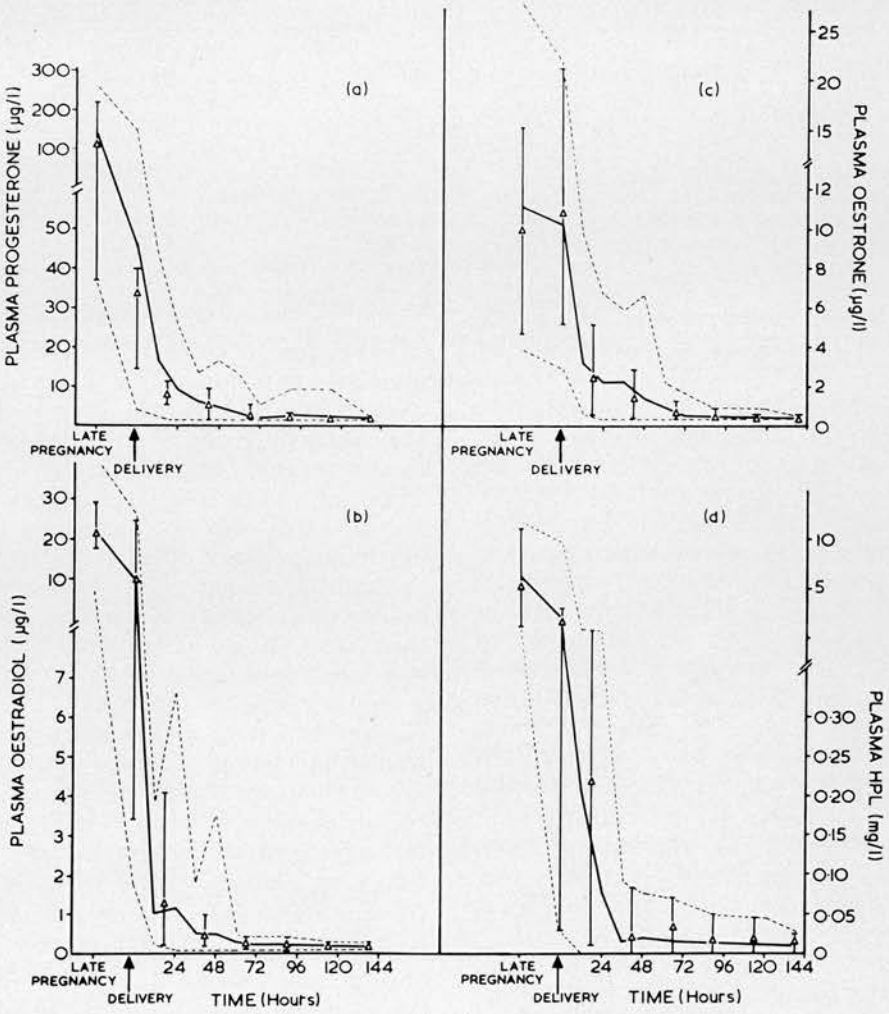



FIG. 2

Plasma levels of (a) progesterone, (b) oestradiol, (c) oestrone and (d) HPL immediately before and for six days after delivery in non breast-feeding women (n = 25). The mean value (solid line) and range (dotted lines) are shown for each hormone. The mean (△) and range of hormone levels for the six patients showing severe breast engorgement are also shown.

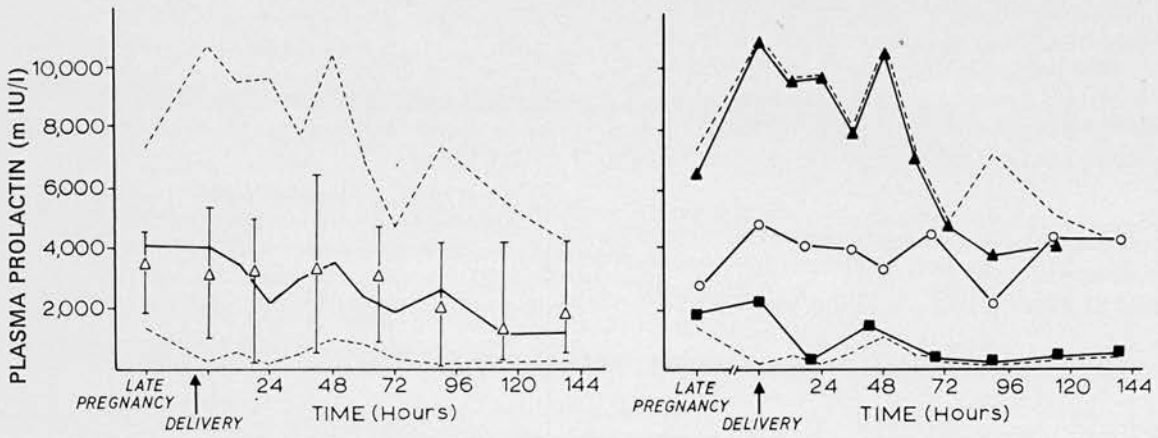



FIG 3.

Plasma levels of prolactin immediately before and for six days after delivery in non breast-feeding women (mean = solid line; range = dotted lines. (a) The mean (△) and range of plasma prolactin levels for all six patients showing severe engorgement. (b) Serial prolactin levels in each of three patients with breast engorgement—scores of 15 (▲), 17 (○) and 19 (■).

were excluded. No difference was seen in the levels of these hormones in late pregnancy (Fig. 2).

Plasma prolactin levels in those subjects with breast engorgement are shown in Figure 3 (a). The mean and range of values lie within the range of plasma levels of prolactin in the non-breast feeding group as a whole. The individual sequential values for plasma prolactin in three of the breast-engorged women are shown in Figure 3 (b). It can be seen that the level of prolactin was not related to the degree of breast engorgement, the highest basal levels of prolactin being associated with less severe breast engorgement (score = 15) than the lowest overall prolactin levels (score = 19). This is further emphasized when comparison is made of the individual degree of breast engorgement as estimated clinically and the mean prolactin level obtained during the first six days after delivery. No significant correlation was found (Fig. 4).

The mean and range of prolactin levels obtained during the study of the 25 non-lactating mothers was comparable to that of the basal levels of prolactin in a group of 17 breast feeding mothers (Fig. 5). Blood samples were taken at least two hours after a period of breast feeding in the latter group.

## DISCUSSION

The endocrine events of the early puerperium in relation to the onset of milk production are of considerable interest, not only in relation to chemical suppression of unwanted lactation and in the understanding of the potential adverse effects of contraceptive steroids on milk production, but also to those concerned with developing methods of enhancing poor lactation in mothers attempting to breast feed. There are no other studies in the literature in which these changes are considered in a group of non-lactating women.

Our object was to study the endocrine changes and degree of breast engorgement in women undergoing no form of suckling stimulus. We felt that our results might throw some light on the marked differences in lactational performance seen in puerperal women who breast feed their infants. While it is known that frequent suckling is important for the success of lactation (Egli *et al*, 1961), the fact that breast engorgement occurs in some women who do not suckle suggests that the ability to produce milk is not related simply to suckling. It has also been shown (Illingworth and Stone, 1952) that lactating women who experience breast engorgement during the initial stages of lactation

subsequently breast feed more successfully than those who have no engorgement.

The results of our study clearly show that there is no obvious endocrine basis for the differences in degree of breast engorgement experienced by non-lactating puerperal women.

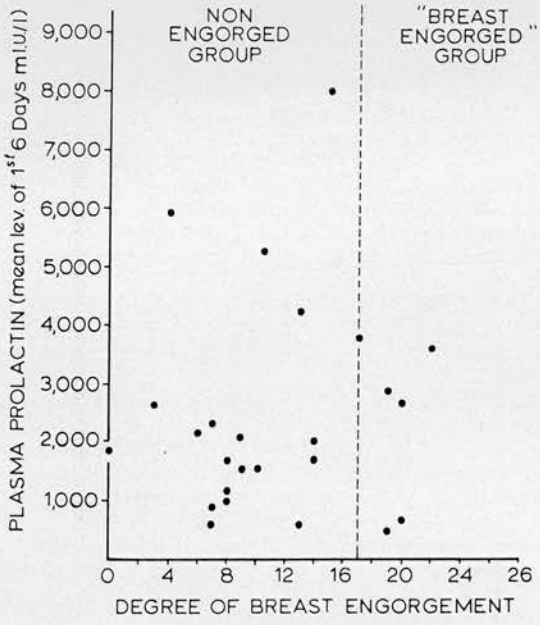



FIG. 4

Correlation between plasma levels of prolactin (mean plasma level over the first six days after delivery) and the degree of breast engorgement.

They confirm the results of previous studies showing that levels of oestrogen and progesterone fall sharply following delivery, while levels of prolactin fall more gradually during this period (Reyes *et al*, 1972; Bonnar *et al*, 1975). Since lactation can be completely inhibited with bromocriptine which suppresses prolactin levels into the non-pregnancy range (Cooke *et al*, 1976), it is clear that prolactin is essential for lactation. It was therefore considered possible that the degree of breast engorgement in non breast-feeding women might relate to the levels of prolactin in the early puerperium.

A marked individual variation in prolactin levels was seen during the period of the study, levels remaining elevated in some women throughout the first six days of the puerperium, while in others levels fell rapidly to the non-pregnant range within three days. It is interesting that, at this early stage of the puerperium, ranges of prolactin levels in non-lactating women were comparable to basal levels seen in a group of mothers who were breast feeding. Our results clearly suggest that there is no direct relationship between the degree of breast engorgement, presumably related to the ability of the breast to secrete milk, and the actual level of circulating prolactin.

The onset of lactogenesis and milk secretion in human lactation appears to closely parallel the disappearance of steroid hormones from the circulation after delivery (Kulski *et al*, 1977).

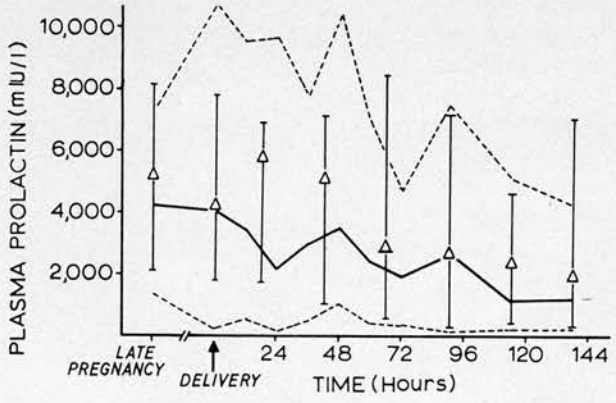



FIG. 5

Plasma levels of prolactin before and for the first six days after delivery in non breast-feeding women (mean = solid line, range = dotted line, n = 25) and in a group of breast-feeding women (mean (△) and range, n = 17).

It is uncertain which of the placental steroids acts as the inhibitor, or how this inhibition is brought about but recent evidence suggests that the active steroid may be progesterone (Hartmann *et al*, 1973; Kulski *et al*, 1977) as in animals. Nevertheless lactation may be inhibited by treatment with oestrogens (Daniel *et al*, 1967).

Overall increase in breast volume during pregnancy appears to determine subsequent milk output (Hytten, 1954). This suggests that local factors in the breast are likely to be relevant in determining its potential for milk production. In our study, the six women with the highest scores, and thus with the most marked degree of breast engorgement, were selected for comparison with the group as a whole. Only 4 out of the 25 women, all in the 'engorged' category, actually required analgesics to relieve breast discomfort and the majority were aware of mild discomfort only. One clinical study of postpartum breast engorgement concluded that placebo was effective in relieving symptoms of engorgement in 90 per cent of subjects and hence the universal use of agents to suppress lactation was unjustified (MacDonald and O'Driscoll, 1965). Our findings seem to support the view that such agents are of benefit only to a minority of women and thus suppressive therapy should be selective.

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# HOW LONG SHOULD A BREAST FEED LAST?

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## SUMMARY

Patterns of milk flow were studied in 50 mothers who were breast-feeding normal birth-weight babies on days 5—7 of the puerperium by progressive test weighing at 5-min intervals during two consecutive feeds.

Compared with a regime in which mothers attempted to feed for 10 × 10 min on alternate breasts, a regime of 5 × 5 × 5 × 5 min increased the amount of milk taken in the first 10 min but did not influence the final milk intake or the suckling-induced prolactin release.

The wide variation of breast-feeding patterns between mothers was demonstrated in respect of the duration of the feed (mean 17.3 min; S.D. ± 3.1; range 7—30 min), the initial rate of milk flow (mean 6 g/min; S.D. ± 4.2, range 1—14 g/min) and the final milk intake (mean 70.9 g; S.D. ± 20.5; range 42—125 g).

The advice to breast-feed for 20 min was inappropriate for the majority of mothers because the nutritive feeding time was 15 min or less in 75% of the feeding episodes. The milk intake correlated with the initial rate of milk flow but not with the duration of the feed, the infant's birth weight, or the time since the last feed.

It is suggested that the duration of a breast feed should be determined by the infant's response and not by an arbitrary time schedule.

breast feed duration; milk intake; prolactin

## INTRODUCTION

Although there has been a rise in the prevalence of breast feeding, a large number of mothers only lactate for a short time after delivery [1,9]. The clinical management during the early puerperium is important because

mothers who establish a good milk flow quickly are more likely to breast-feed for a longer period of time [4].

A common complaint among breast-feeding mothers is that they are given conflicting advice on practical aspects such as the recommended frequency and duration of feeding. Such conflicting advice may cause anxiety and it has been shown that emotional stress can prejudice the success of lactation by inhibiting the oxytocin-induced let-down reflex [7].

Recent work has indicated that early and frequent suckling in the immediate puerperium encourages good milk flow [8]. The objective of the present investigation was to determine the best advice to be given to mothers regarding the optimum duration of a breast feed. This was done by studying the duration of nutritive suckling as shown by progressive test weighing at 5-min intervals during a 20 min breast feed. The traditional pattern of 10 min on each breast was compared with a regime in which the baby was changed from one breast to the other at 5-min intervals. This was done to see if the more frequent change of breast encouraged lactation by promoting a greater prolactin discharge and encouraging the let-down reflex.

## SUBJECTS AND METHODS

Fifty breast-feeding mothers who had delivered healthy babies weighing more than 2800 g in the Simpson Memorial Maternity Pavilion, Edinburgh, agreed to participate in the study of progressive test weighing. Twenty-four of the mothers were primigravid and 26 were parous; the infants' sex ratio was 29 male to 21 female. The mothers were feeding on demand in response to the baby's hungry cry; this policy achieved a mean interval between feeds of 3 h 25 min ± S.D. 28 min.

### *Test weighing*

During two consecutive breast feeds between day 5 and day 7 of the puerperium, babies were weighed before the feed and at 5-min intervals during the feed using a Mettler Electronic Balance (Model PS15, A, Galenkamp & Co. Ltd., Christopher Street, London) which gave reproducible results to within 2 G. During one feed, the mother was advised to feed the baby for 10 min on alternate breasts (10 × 10 regime) and during the next feed, to change the breast side at 5-min intervals during the 20 min period (5 × 5 × 5 × 5 regime). In half of the subjects, the 10 × 10 regime was performed first, and the 5 × 5 × 5 × 5 regime second; the order was reversed in the remainder. Although all mothers were advised to feed for 20 min a number of babies either fell asleep or came off the breast before the 20 min of feeding was complete; on the other hand, a small number of babies were slow to settle and fed for longer than 20 min.

*Definitions*

The duration of feeding was taken as the total time the baby remained on the breast; nutritive feeding was considered to have stopped when the infant took less than 5 g in a 5 min suckling period; the initial rate of milk flow was defined as the intake of milk during the first 5 min expressed as g per min.

*Prolactin studies*

The prolactin response to suckling was studied in ten separate mothers who were managed during two consecutive feeds according to the 10 X 10 and 5 X 5 X 5 X 5 regimes as described above. These mothers did not have test weighing. A cannula was placed in the forearm vein and blood samples were taken for prolactin estimation at 0, 10, 20, 30, 45, 60, 90 and 120 min after the onset of suckling. Prolactin was estimated by radioimmunoassay as previously described [6].

*Statistics*

Statistical analysis was performed using the paired Student's *t*-test.

RESULTS

*Milk intakes*

All the test weighing was performed between days 5 and 7 of the puerperium, by which time lactation was established and the mean test weigh per feed had reached a plateau as follows; day 5, mean test weigh 68.4 g per feed, day 7, 69.8 g per feed.

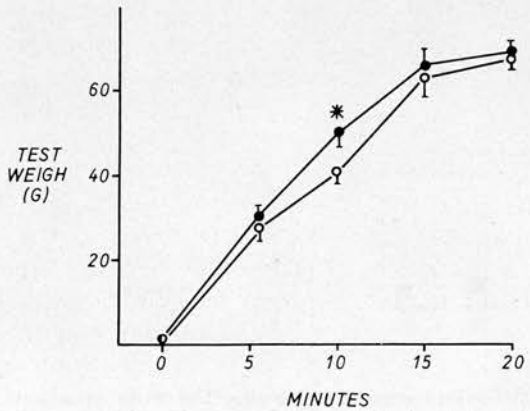



Fig. 1. Feed volumes taken at 5-min intervals during a breast feed by the 10 x 10 (○———○) or 5 x 5 x 5 x 5 (●———●) regimes (n = 50).

TABLE I

Mean prolactin response (±S.D.; U/l) to suckling during the 10 × 10 and 5 × 5 × 5 × 5 suckling regimes (n = 10)

| Regime | Minutes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 20 | 30 | 45 | 60 | 90 | 120 |
| 10 × 10 | 3.19 ± 2.05 | 4.62 ± 2.77 | 5.88 ± 3.40 | 7.04 ± 4.76 | 5.02 ± 3.10 | 5.14 ± 3.18 | 4.16 ± 2.49 | 3.78 ± 2.21 |
| 5 × 5 × 5 × 5 | 2.88 ± 2.06 | 4.33 ± 2.22 | 5.08 ± 2.74 | 6.25 ± 4.45 | 4.72 ± 2.82 | 4.40 ± 3.03 | 3.81 ± 2.02 | 3.47 ± 2.02 |

*Comparison of 10 × 10 and 5 × 5 × 5 × 5 regimes (Fig. 1)*

The mean final milk intake of 71.1 ± 21.1 g (S.D.) for the 10 × 10 regime did not differ significantly from 70.7 ± 20.1 g (S.D.) for the 5 × 5 × 5 × 5 regime. During the first 10 min of the 5 × 5 × 5 × 5 regime, a mean of 51.7 ± 21.4 g (S.D.) (73% of the feed) was taken which was significantly more than 42.8 ± 17.9 g (66% of the feed) of the 10 × 10 regime; $t = 4.46$, $P < 0.01$. During the final 5 min of the 20 min period, a mean of 4.9 g and 6.1 g were taken by the 5 × 5 × 5 × 5 and 10 × 10 regimes, respectively.

In both regimes, 70% of the milk from the first breast and 80% of the milk from the second breast was taken during the first 5 min of suckling.

The suckling induced prolactin responses (Table I) showed no significant differences between the two regimes.

*Inter- and intra-patient variation*

Wide differences were observed between mothers in respect of the final milk intake (mean 70.9 ± 20.5 g (S.D.), range 35—125), the initial rate of

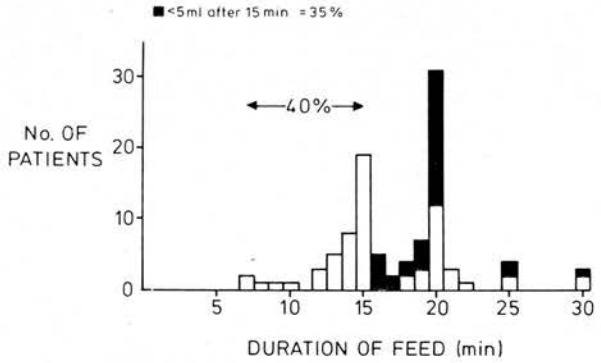



Fig. 2. Duration of suckling in 100 breast-feeding episodes showing the wide variation between individuals and the high incidence of non-nutritive suckling towards the end of a feed.

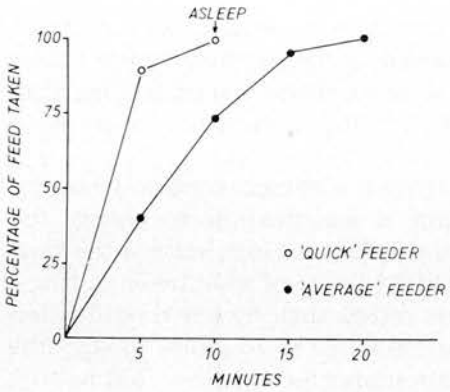



Fig. 3. Comparison of a 'fast' feeding infant who showed early signs of satiation, with an average feeder on the 5 × 5 × 5 × 5 regime who may feed for 20 min.

milk flow (mean 6 g/min ± 4.2 S.D., range 1—14) and the duration of feeds (mean 17.3 min ± 3.1 S.D., range 7—30 min). By contrast, the intra-patient variation was much less and significant correlations were found between the first and second feeds of individual mothers for the final milk intake ($r$ = 0.56; $P < 0.001$), the initial rate of milk flow ($r$ = 0.554, $P < 0.001$) and the duration of feeding ($r$ = 0.684; $P < 0.001$).

*Duration of feeds*

The duration of the 100 feeding episodes are shown in Fig. 2. In 40%, the duration of feeding was 15 min or less and in a further 35%, less than 5 g were taken by the baby after 15 min. In 5%, nutritive feeding lasted for more than 20 min. One mother, whose two feeds lasted 8 and 12 min, gave 95% of her total feed of 70 g during the first 5 min of feeding as illustrated in Fig. 3. Because her infant fell asleep before the recommended time of 20 min, she wished to discontinue breast feeding since she thought that the baby could not get enough milk during such a short feeding time.

*Final feed volume*

The final milk intake showed a significant correlation with the initial rate of milk flow ($r$ = 0.587; $P < 0.001$) but not with the duration of feeding ($r$ = 0.128), the infant's birth weight ($r$ = 0.067) or the time since the last feed ($P > 0.1$ in all cases).

## DISCUSSION

The central point to emerge from this study is that it is impractical to recommend a policy regarding the length of a breast feed which would be appropriate for all mothers. The data point clearly to the wide variability

between mothers in respect of the length of nutritive feeding, the initial rate of milk flow and the final milk intake. Indeed, some mothers whose babies fell asleep after a very short feed wished to discontinue breast feeding altogether, because they were assuming erroneously that a short feed must mean an inadequate feed.

It was of interest to note that by changing the clinical routine from the 10 × 10 regime to the 5 × 5 × 5 × 5 regime, it was possible to change the amount of milk taken by the baby during the first 10 min but not the final milk intake. This would suggest that the total volume of milk taken during a feed is determined by physiological factors rather than by any modification of the clinical management. The prolactin discharge in response to suckling was similar during the two regimes and this supported the view that neither of the suckling patterns offered any distinct advantage.

In a previous study of patterns of milk flow during breast feeding, it was shown that babies take about 80% of the milk during the first 4 min on each breast [5]. Our findings confirm this pattern of rapid flow in the first minutes followed by a marked slowing down at the end of the feed. In 75% of the mothers in this study, nutritive suckling stopped within 15 min and, in 17%, the nutritive feeding time was 10 min or less although the baby was often kept on the breast for longer. The importance of non-nutritive suckling is not known. The calorific content of hind milk is greater because of its higher fat content [3] so that the small volumes taken at the end of the feed may be important to the baby. Similarly, the non-nutritive suckling time may play a psychological role in establishing and maintaining mother–infant attachment. On the other hand, it seems unnecessary to waken babies who have shown signs of satiation by falling asleep simply to achieve a longer suckling time. In a previous study, Gunther [2] produced evidence which suggested that such extra stimulation might contribute to the problem of sore and cracked nipples.

The final milk intake correlated with the initial rate of milk flow, suggesting that the most alert and hungry babies were the ones who required and took the largest volumes of milk; alternatively it may indicate that the mothers with a well developed oxytocin ejection reflex or a well established milk supply were able to deliver the milk rapidly and achieve the most satisfactory feeds. Further studies will be required to determine which factors are of greatest importance in determining milk transfer from mother to baby. In the present study, the infants' birth weights and the intervals between feeds were confined to narrow ranges and this may explain why no correlations were found between these variables and the final milk intake.

The absence of any correlation between the test weigh and the duration of the feed indicates that it is illogical to offer advice regarding the length of a feed on the basis of an arbitrary time schedule. It is recommended that mothers should be told of the highly individual nature of breast feeding and should be encouraged to time their feeds according to the responses of their own infants.

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# Factors affecting the duration of breast feeding: 1. Measurement of breast milk intake in the first week of life

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**Summary**

Test-weigh measurements of 24 h milk intake by babies in the immediate puerperium (days 1–9) were carried out in a group of 18 mothers and babies using an accurate electronic balance. Estimated milk intakes were then calculated on the basis of one, and of two consecutive, breast feeds. These estimates were then compared with the actual total intake to give a measurement of reliability. Significant positive correlations were found between both the one-feed and the two-feed estimated intake, and the measured 24 h intake. Accuracy increased from day 3 onwards.

These results show that using an electronic balance an accurate estimate of 24 h milk intake during the first week of life can be obtained by using one or two test-weigh measurements. While this gives a minimally invasive and reliable method for estimating daily milk intake, it is not advocated as a routine procedure in postnatal wards.

milk intake; test weigh; breast feed; puerperium

## Introduction

Accurate measurement of breast milk intake by the infant in the first few days of life is an essential procedure when investigating the adequacy of breast milk supply,

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the nutritional intake of the infant, or the requirements for successful breast feeding.

The measurement of milk intake by the breast fed infant presents two particular problems, especially in the early postpartum days. Firstly, the procedure itself should intrude as little as possible into the process of breast feeding, and secondly the method of measurement should be accurate.

'Test weighing' of the infant before and after each feed is the traditional method of assessing feed volume, but the mother may feel this procedure to be threatening, especially if it is being used to assess her performance [4]. It is possible that test weighing itself may influence normal milk release by inhibiting the 'let down' reflex [5,6]. Furthermore, a restless baby is difficult to weigh on standard mechanical scales so that small milk intakes may not be recorded accurately.

Two other methods of measurement of breast milk intake have been suggested but they, too, have their drawbacks. Coward et al. [2] describe a technique involving intake of deuterium oxide by either the baby or the mother, followed by assay of salivary samples collected from the baby. This method has the advantage that it can give measurements over a prolonged time period. However, it requires specialised equipment, and it can intrude equally as much as test weighing on breast feeding, especially if the baby objects to the collection of salivary samples [1].

The other method involves emptying of the breast by a breast pump or other artificial means of breast expression [3]. This technique, however, does not measure actual milk intake by the infant, but gives a measure of the capacity of the breast to produce milk. Furthermore, the very act of emptying the breast may influence milk yield so that the results from this method may be difficult to interpret from a clinical viewpoint.

The objective of the study described in this paper was to see whether minimal intrusion by only one, or at the most two, test-weighs in 24 h would give assessments of 24 h feed volumes which would be sufficiently accurate for practical purposes. The problem of accuracy of recording was alleviated by the use of an electronic balance as described in the Methods below.

## Patients and Methods

*Patients*

Patients were breast-feeding mothers who had vaginal deliveries of babies of normal birthweight ( > 2500 g) and gestation ( > 38 weeks) in the Simpson Memorial Maternity Pavilion, Edinburgh. Eighteen mothers (mean age 27.4 yr (range 21–33 yr)) were recruited; nine were recruited antenatally so that test weighs could start immediately postpartum and a further nine women were recruited postnatally. Mothers who were recruited antenatally carried out test weigh measurements at every feed throughout their stay in hospital (range 4–9 days). Mothers who were recruited postnatally carried out test weigh measurements at every feed over either a 24 h or a 48 h period, ranging from day 1 to day 5. The local ethical committee gave its consent to the study, and the protocol was explained to each mother in detail.

Two babies were transferred to the Special Care Nursery for treatment of hyperbilirubinaemia; test weighs were continued in one of these babies. Ten of the mothers were primigravid and eight were parous; ten of the babies were boys and eight were girls.

*Test weighing*

In order to achieve the maximum accuracy possible, test-weigh measurements were carried out using a Mettler integrated electronic balance (Gallenkamp and Co. Ltd., Christopher Street, London) which gave readings which were reproducible to within 2 g. This balance achieves this accuracy by giving a reading integrated over 2 or 4 s periods and maintains accurate weighing even if the baby moves vigourously. A digital read-out facilitates the accuracy of the measurement. Minimal disturbance is caused, since the cot is placed on the scales with the baby fully clothed and undisturbed. The initial pre-feed weight is then measured, and the baby lifted and breast fed. The post-feed weight is recorded by returning the baby to the cot on the scales after feeding. Mothers carried out the test-weigh procedure themselves, following initial instruction. They recorded the test weigh measurements and the time of the feed so that there was minimal involvement of the nursing staff. Random checks were carried out by the researcher to ensure that the mothers were using the scales correctly.

*Information recorded*

Mothers measured the milk intake of the baby at each feed throughout the day for the duration of the study period. This information was recorded in 24 h periods, day 1 corresponding to the first 24 h postpartum.

A total of 63 completed days of measurement ranging from day 1 to day 9 were recorded. Intake at 366 breast feeds was measured.

*Calculation of estimated milk intake*

Two estimated milk intake totals were calculated and compared with the actual total intakes. One estimated total was based on intake at one feed, and the other was based on intake over two consecutive feeds.

These estimated totals were calculated as follows:

A. *One feed*: the measured intake of the feed nearest to the middle of the 24 h period was multiplied by the number of feeds given in the relevant 24 h period. Each 24 h period was calculated sequentially from the hour of birth.

B. *Two feeds*: the mean intake at two consecutive feeds nearest to the middle of the 24 h period was multiplied by the number of feeds given in the relevant 24 h period.

The two estimated totals were then compared with the measured total intakes. Methods of comparison included calculation of the correlation coefficient of the estimated against measured intakes, and calculation of percentage error of each estimated intake.

*Measured and estimated midnight milk intakes*

In a manner similar to the paragraph above, the measured milk intakes for the

24 h periods midnight to midnight were also calculated. Estimates of the midnight to midnight milk intake were based on (A) the intake of the first feed after 9 a.m. multiplied by the number of feeds in the midnight to midnight time period, and (B) the mean intake of the first two feeds after 9 a.m. multiplied by the number of feeds in the midnight to midnight time period.

## Results

*Number of feeds per day and mean milk intake* (Fig. 1)

The number of feeds increased from a mean (±S.E.) of 4.6 ± 0.47 on day 1 to 6.8 ± 0.32 on day 3, but there was no significant increase thereafter.

The mean (±S.E.) milk intake per feed was 7.4 ± 3.5 and 13.8 ± 4.3 g during the first two 24 h periods, respectively, but increased sharply to 38 ± 6 g on day 3 (48–72 h). After a further increase to 58 ± 7.8 g on day 4 (72–96 h), no further significant increase was found on days 5 or 6.

*Comparison of 1 feed and 2 feed estimated intakes with measured 24 h milk intake* (Figs. 2a, b)

The measured milk intake ranged from 0 to 1280 g in 24 h. The relationship of the measured milk intake to the one-feed estimated intake is shown in Figure 2a, and to the two-feed estimated intake in Figure 2b. Strong positive correlations were found both between the one-feed estimated intake and the measured 24 h intake ($r = 0.886$) and the two-feed estimated intake and the measured intake ($r = 0.938$).

*Percentage errors* (Fig. 3)

The mean percentage error for every feed on each postpartum day on the basis of

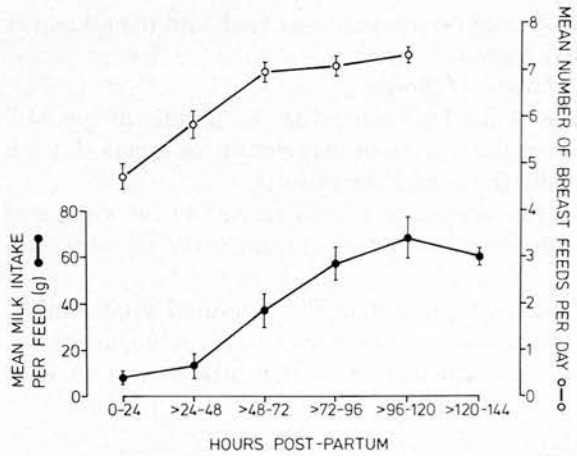



Fig. 1. Mean (±S.E.) milk intake per feed and number of breast feeds per day in the first six postpartum days.

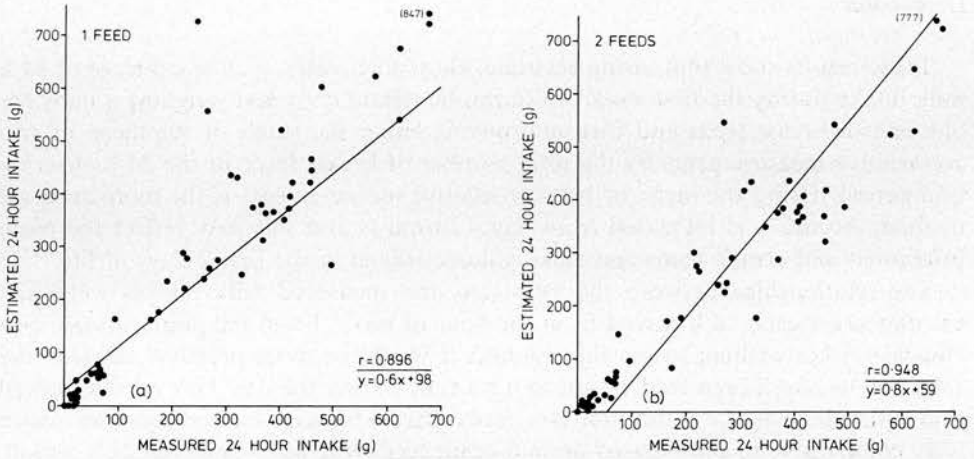



Fig. 2. Estimated 24 h milk intake calculated from (a) one feed, and (b) two consecutive feeds, correlated with the measured 24 h intake.

one and of two feeds is shown in Figure 3. From day 3 onwards the accuracy of estimated intakes was increased.

*Comparison of measured and estimated midnight to midnight milk intakes*

Strong positive correlations were also found between the measured midnight to midnight intake and the estimated intake based on the first feed after 9.00 a.m. ($r = 0.899$) and the measured midnight to midnight intake and the estimated intake based on the mean of the first two feeds after 9.00 a.m. ($r = 0.968$).

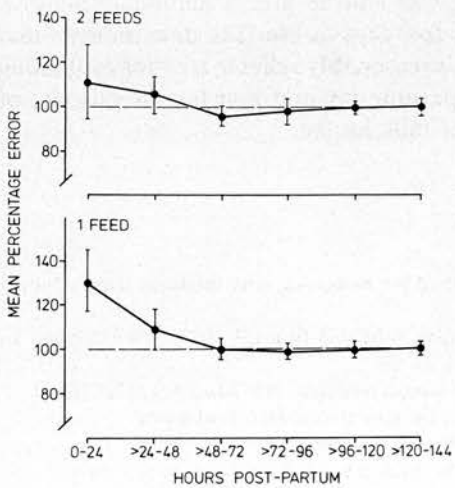



Fig. 3. Mean percentage error of estimated intake in the first six postpartum days calculated from one feed and from two feeds.

## Discussion

These results show that, using accurate, electronic scales, a close estimate of 24 h milk intake during the first week of life can be obtained by test weighing a baby for one or two breast feeds and then multiplying either the single or the mean of two consecutive measurements by the total number of breast feeds in the 24 h observation period. Using the mean of two consecutive measurements is the more accurate method. Accuracy is increased from day 3 onwards and this may reflect the more infrequent and erratic feeds and small volumes taken in the first 2 days of life.

The relationships between the estimated and measured milk intakes were first calculated for each 24 h period from the hour of birth. From the point of view of a clinical worker wishing to use this method, it would be more practical to make the calculations based on a feed closest to a set time during the day. Our results showed that estimates based on the first two feeds after 9.00 a.m. had correlations which were comparable to those based on mid-point feeds for each sequential 24 h period. From a practical point of view, estimates on the feeds after 9.00 a.m. would be more convenient but, on a routine basis, it should not be necessary to carry out test weighing.

The scales used in this study were accurate and easy to read, even when the baby was agitated. Normal ward scales cannot be read to an accuracy of less than 10 g and this accuracy is reduced even further if the baby is disturbed. This is particularly important in the early days, when measuring small amounts of colostrum or milk. The results of this study do not indicate test weighing should be advocated as a routine procedure on postnatal wards. In cases where staff may consider it to be necessary to determine an infant's milk intake, due thought should be given to the effect of maternal anxiety on milk release, and to the accuracy of the equipment used.

As described in this paper, test weighing was able to give a minimally invasive method of estimating milk intake in the first few days of life. The data indicate that this method of measurement may be used as a reasonably reliable tool for estimating milk intake from the third to the sixth postpartum day and that for the purpose of research studies may be used as a measure of milk intake.

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# Factors affecting the duration of breast feeding: 2. Early feeding practices and social class

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**Summary**

Breast milk intake by babies on the third and the sixth postpartum day was measured by a test-weigh procedure in a group of 47 mothers and babies. Women who gave the largest amount of breast milk to their babies on the third postpartum day continued to breast feed for longer than women who gave smaller amounts. These mothers also suckled most frequently, gave least additional fluid to their babies, and their infants regained their birth weight most rapidly. However, an increased early milk intake and duration of breast feeding were both associated with higher social class. These results suggest that, although practices in the initiation of breast feeding are relevant, factors in the background and environment of the mother are also of fundamental importance.

Thus recent emphasis on the importance of breast-feeding practices in the immediate puerperium should not divert attention from the equally important task of establishing the optimum conditions for breast feeding in the home environment of mothers.

breastfeeding; duration; social class; puerperium

## Introduction

It has been suggested in a number of studies [2,11] that early and frequent suckling in the immediate puerperium promotes the long-term continuation of breast

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feeding. Most hospitals have now adopted these practices in an effort to reduce the rate of premature discontinuation of breast feeding. There is as yet, however, little noticeable increase in the proportion of women continuing to breast feed successfully in spite of the fact that more women are choosing to start to breast feed [6]. All recent reports show that about 50% of women still discontinue breast feeding before 12 weeks postpartum [3,7,14]. This premature discontinuation of breast feeding is strongly associated with lower social class [7,14].

In a randomised study, Salariya et al. [11] provided some evidence that early and frequent suckling promoted good milk flow and a longer duration of breast feeding. They reported that the milk 'came in' more quickly in mothers who first suckled within 1 h of delivery on a regime of frequent feeding than in mothers who delayed first suckling for more than 6 h and fed by a rigid, 4 hourly regime. The assumption was made that the early establishment of milk flow led to a longer duration of breast feeding. There was, however, no objective measure of milk transfer in the early postpartum days, and no record was given of the social class of the mothers in the study.

The first objective of the study reported in this paper was to investigate whether or not the measured milk intake by the baby on the third postpartum day was reflected in the subsequent duration of breast feeding. A second objective was to study the clinical feeding practices to see if mothers who suckled most frequently were those who gave the largest amounts of milk on the third postpartum day and who breast fed for longest. It was recognised, however, that associations between frequent suckling, large feed volumes on day 3 and increased duration of breast feeding would not establish cause and effect relationships. It was equally possible that mothers from the highest social class group would be the most enthusiastic, suckle most frequently, establish milk flow most quickly and breast feed for longest. In this study, therefore, analysis was carried out to see whether the mother's environment, as measured by social class, was an important confounding factor in the subsequent duration of breast feeding.

## Patients and Methods

*Patients*

47 breast-feeding women and their babies were recruited from the postnatal wards of the Simpson Memorial Maternity Pavilion. All babies were normal birthweight ( > 2500 g) and gestation ( > 38 weeks) and were progressing normally on the third postnatal day; no baby who was jaundiced on that day was included in the study. To ease the problems of follow-up, all the mothers were resident in Edinburgh. All those mothers who fulfilled these criteria and were in hospital during the 4 week recruitment period were approached. No mothers refused to participate in the study.

*Test-weighing*

A test-weighing was carried out at one morning feed on the third postnatal day.

TABLE I

Clinical characteristics of mothers and babies in 'high', 'medium' and 'low' milk transfer groups

| Groups | Age (years) [a] | Parity | Mode of delivery | Maturity at delivery (weeks) [a] | Onset of labour | Infant birth weight (kg) [a] | No. of babies with bilirubin > 240 μmol |
|---|---|---|---|---|---|---|---|
| High milk transfer, 200 g/day ($n = 13$) | $27.6 \pm 3.2$ | 9 prim<br>4 para | 7 svd<br>3 forceps<br>3 section | $40 \pm 0.9$ | 5 spon<br>5 ind<br>3 el section | $3.5 \pm 0.28$ | 1 |
| Medium milk transfer, 50–200 g/day ($n = 22$) | $27 \pm 5.2$ | 14 prim<br>8 para | 15 svd<br>5 forceps<br>2 section | $39.9 \pm 1$ | 12 spon<br>9 ind<br>1 el. section | $3.4 \pm 0.46$ | 2 |
| Low milk transfer, 50 g/day ($n = 12$) | $26 \pm 3.6$ | 9 prim<br>3 para | 7 svd<br>3 forceps<br>2 section | $40 \pm 1.1$ | 6 spon<br>5 ind<br>1 el. section | $3.5 \pm 0.59$ | 2 |

[a] Mean ± S.D.

Test-weighing was performed as described by Houston et al. [5] and an estimated 24 h milk intake calculated.

On the basis of this estimated 24 h milk intake, the mothers were divided into three groups as follows: 'high' group (more than 200 g milk per 24 h, $n = 13$), 'medium' group (50–200 g per 24 h, $n = 22$) and a 'low' group (less than 50 g per 24 h, $n = 12$).

Mothers were not told of their allocation into these groups, although all mothers knew the intake of milk at test-weigh. The clinical characteristics of mothers and babies are shown in Table I.

*Clinical information*

The following information was recorded for each mother and baby during the first 144 h after delivery; the number of breast feeds given, the number and volume of complementary feeds, the time of the first breast feed, and the baby's weight on days 2, 4 and 6. The test-weigh was repeated on day 6 (120–144 h postpartum) and estimated intake calculated for that day.

All mothers were visited at home at 16 weeks postpartum, to determine the duration of breast feeding. Mothers were not told of this follow-up visit until they were contacted by letter or telephone before 16 weeks and permission requested for a follow-up visit. This was to forestall any improvement in duration of breast feeding due to extra interest and involvement which the mother might feel as a result of the project [1,12]. All mothers agreed to the follow-up visit.

*Social class*

Each mother was allocated to a social class group according to her husband's occupational grouping in the Registrar General's Classification of Occupation [9]. All mothers were married; it is at present unusual for unmarried women to choose to breast feed their babies. There were 17 women in social class I, 10 in social class II, 17 in social class III and 2 in social class IV. In view of the small numbers in social class IV, mothers from groups III and IV were combined and compared with mothers in groups I and II.

*Clinical practice*

At the time this study was performed, it was normal hospital practice to offer the baby a dextrose feed (5% dextrose in water) 3–4 h after delivery and to put the baby to the breast for the first time at the next feed. After each breast feed, the baby was offered an additional dextrose feed. Babies were fed on demand (that is, when the baby woke and cried) but a supplementary dextrose feed was offered overnight if the mother was tired.

*Statistics*

All statistics were performed using either Student's $t$-test, or chi-square.

## Results

*Duration of breast feeding* (Fig. 1)

Duration of breast feeding was related to the 'high', 'medium' and 'low' groupings of mothers on day 3. At 6 weeks postpartum, the continuation rate in the 'low' group was 42% which was significantly less than 77% in the 'medium' group ($P < 0.05$) and 92% in the 'high' group ($P < 0.01$). At 16 weeks the continuation rates were 69% in the 'high' group, 59% in the 'medium' group and 33% in the 'low' group; these differences were not significant.

*Sixth day test-weigh* (Fig. 2)

The mean ($\pm$S.E.) milk intake on day 6 was $558 \pm 44$ g in the 'high' group, $399 \pm 39$ g in the 'medium' group and $311 \pm 32$ g in the 'low' group ('high' vs. 'low' $P < 0.001$, 'low' vs. 'medium' $P < 0.05$).

*Baby weight* (Fig. 3)

Babies' weight loss from birth was similar in the three groups for the first 2 postpartum days. By the sixth day, differences between the groups were significant; babies in the 'high' group had a mean ($\pm$S.E.) loss of $17.6 \pm 30$ g, the 'medium' group was $95 \pm 24$ g and the 'low' group was $178.3 \pm 16$ g ('high' vs. 'low' $P < 0.001$, 'low' vs. 'medium' $P < 0.05$).

*The number of breast feeds* (Fig. 4)

The number of breast feeds increased during each day of the puerperium for all groups. Mothers in the 'high' group suckled more often than the other two groups throughout the first 6 days ('high' vs. 'low' 0.24 h, $P < 0.01$, 'high' vs. 'low', 120–144 h, $P < 0.05$).

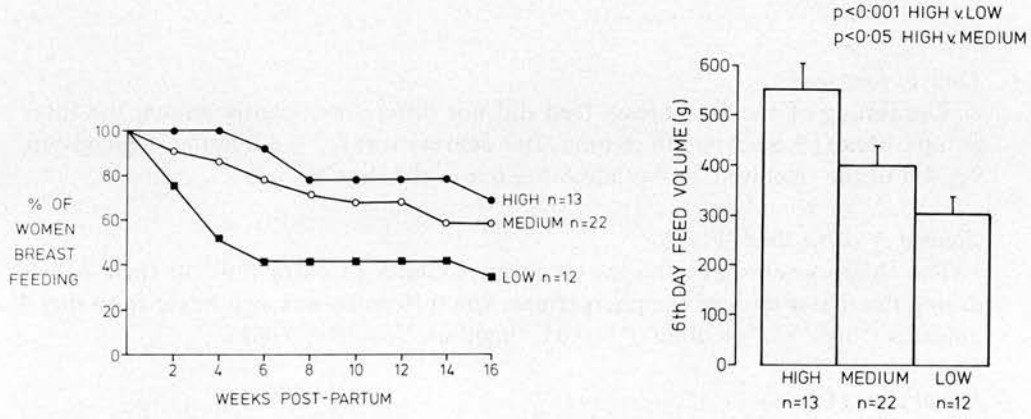



Fig. 1. Duration of breast feeding for women in 'high', 'medium' and 'low' intake groups.

Fig. 2. Sixth day feed volume (mean $\pm$ S.E.) for women in 'high', 'medium' and 'low' intake groups.

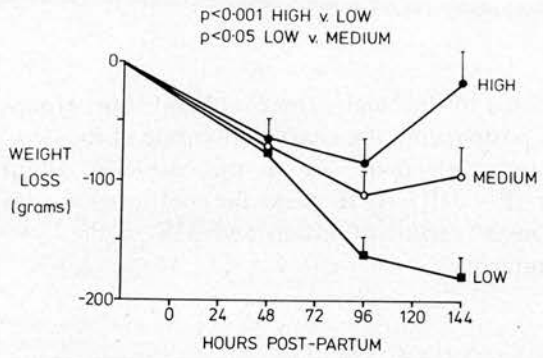



Fig. 3. Change in babies' weight (mean ± S.E.) from birth in 'high', 'medium' and 'low' intake groups.

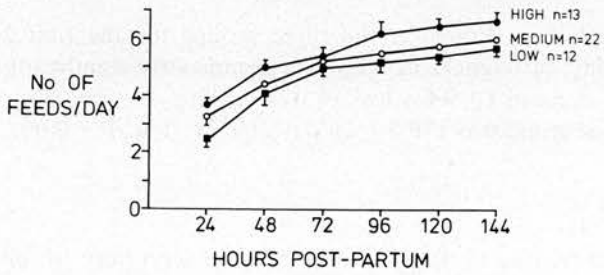



Fig. 4. Number of breast feeds per day (mean ± S.E.) in 'high', 'medium' and 'low' milk intake groups.

*Time of first feed*

The timing of the first breast feed did not differ significantly among the three groups. Mean (± S.E.) length of time after delivery was 6.7 ± 3 h in the 'high' group, 9 ± 4 h in the 'medium' group and 8.5 ± 6 h in the 'low' group.

*Volume of extra fluid* (Fig. 5)

The 'high' group gave the lowest mean volumes of extra fluid to their babies during the first 6 days of the puerperium. The difference was significant from day 4 onwards ('high' vs. 'medium' $P < 0.05$, 'high' vs. 'low' $P < 0.001$).

*Social class* (Fig. 6)

The continuation rate of women in social class groups I, II, III and IV are shown in Figure 6. The differences between the groups are significant both at 6 weeks and at 16 weeks ($P < 0.001$ at both points).

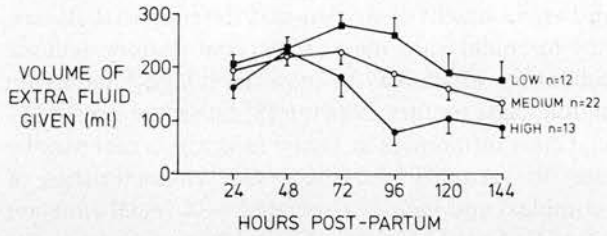



Fig. 5. Volume of extra fluid (mean ± S.E.) given in the first six days in 'high', 'medium' and 'low' intake groups.

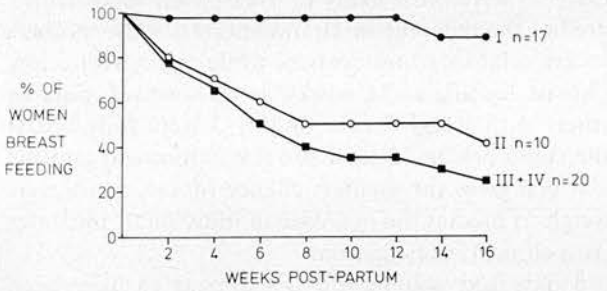



Fig. 6. Duration of breast feeding for women in social class groups I, II, III and IV.

## Discussion

As discussed in the Introduction, several studies have suggested that the early establishment of milk flow by frequent suckling in the immediate puerperium results in an increased duration of breast feeding. While the results of this study confirm the association between frequent suckling, the early establishment of milk flow and the success of breast feeding, they also introduce the possibility that social class is the common factor leading to these associations. The results reported here confirm the relationship of social class with duration of breast feeding and suggest that it is possible that social class I and II mothers may be more aware of the benefits of more frequent suckling and may establish a good milk flow at an earlier stage than other mothers. It is difficult to differentiate whether it is the frequent suckling per se or the advantages associated with higher social class which determine the outcome. The results of this study support the view that policies which encourage frequent suckling should be advocated. At the same time, it should be recognised that other equally important factors affect the duration of breast feeding. The relationship of social class to the success of breast feeding suggests that factors in the mothers' environment are of critical importance.

Although the association of successful breast feeding with social class is clear in these results, the underlying reasons for this association remain unclear. Many

factors vary in the background and environment of women in different social classes, education, nutrition and facilities for child care may all be contributory factors. Richards [10] describes some mechanisms which may be important, based mainly on the improved education of the middle class mother. Newton [8] raises the possibility that some of the cultural and social class differences in breast feeding success may be associated with different attitudes to sexuality and the sexual characteristics of breast feeding. The argument is complex, and factors associated with social class are described in more detail elsewhere [4]. For the purpose of this paper, and for the practical identification of women at risk of problems with breast feeding, it is sufficient to note the strong association with social class.

This study found a single test-weigh on the third postpartum day was able to discriminate the group of mothers who were most likely to breast feed successfully. The test-weigh was not able to predict the outcome in all individuals; some mothers with a high test-weigh on day 3 were relatively unsuccessful while some with a low intake on day 3 were still fully breast feeding at 16 weeks. It is, however, notable that all social class I and II mothers with a high intake on day 3 were fully breast feeding at 16 weeks. It may be the combination of good social environment and the early establishment of milk flow which gives the greatest chance of success. Nevertheless, the inability of the test-weigh to predict the outcome in individuals indicates that it has only a limited value as a clinical investigation.

In the present study, the third day feed volume did not appear to have been influenced to a major degree by the timing of the first feed. Starling et al. [13] have similarly been unable to confirm a relationship between breast feeding success and time of first suckling. More work is required to determine whether the time of first suckling is itself a major determinant of breast feeding success or whether its reported beneficial effect [11] is due to its association with some other more critical factor, such as the social environment of the mother.

It was of interest that mothers who gave the largest milk volumes to their babies were those who suckled most frequently and relied least upon additional fluids. It is not possible to determine whether the increased use of additional fluids reduced the baby's intake by inhibiting suckling or whether the additional fluids were most necessary in those with the lowest milk volumes. It is likely that both factors operate and that a 'vicious circle' is created whereby the use of additional fluids reduces milk volume which, in turn, increases the need for additional fluid. Further work is required to clarify this point.

The success of breast feeding depends on many interrelated factors. The recent emphasis on immediate mother–infant contact and early suckling should not divert attention away from the equally important task of establishing the optimum conditions for breast feeding in the mother's home environment.

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# DO BREAST FEEDING MOTHERS GET THE HOME SUPPORT THEY NEED?

*by* **M J Houston**
**P W Howie**
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**A S McNeilly**

General advice might also be given not to drink *any* unboiled water in other countries where the standard of hygiene and sanitation may be suspect. Giardia cysts can survive in cold water for one to three months but are killed by exposure to a temperature of 50°C (Wolfe, 1975). In practice, this may mean consuming only tea, coffee or alcoholic beverages and although it may not eliminate the risk of giardiasis completely (Vella, 1977), it should reduce it considerably. No ice should be taken in drinks as in most cases this is prepared from unboiled tap water.

**Acknowledgements**
The Director of Environmental Health, East Lothian District and his staff gave invaluable assistance in the investigation of this incident. Without this ready help, detailed follow-up of the individual cases would have been impossible.

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# Do Breast Feeding Mothers get the Home Support they Need?

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**Summary**
The outcome of breast feeding was studied in 28 mothers who received consistent additional home support in the form of fortnightly visits until weaning. Compared with a control group of 52 mothers who received routine support, the mothers in the study group had a longer duration of breast feeding and a later introduction of artificial milk or solid food.

None of the mothers in the study group stopped breast feeding because of 'insufficient milk' compared with 19% of the controls. The average number of home visits by health visitors as recalled by the mothers was 2·7 per patient in both groups and it is asked whether this level of support is sufficient for breast feeding mothers.

## Introduction

In the last few years, there has been an encouraging increase in the number of mothers choosing to breast feed (Martin, 1978; Howie and McNeilly, 1980). Despite this, many of these mothers either give up or introduce supplementary feeds within the first 12 weeks (Sloper *et al.*, 1975; Eastham *et al.*, 1976). The commonest reason given by the mothers for early discontinuation is 'insufficient milk' (Sjölin *et al.*, 1977; Martin, 1978). This report was prompted by the clinical impression that mothers participating in research projects during lactation breast fed for longer than would be anticipated from the success in the general population. It seemed probable that participation in a research project gave the mothers extra support and more opportunities to discuss their anxieties and problems.

Several studies have suggested that close personal support may encourage more successful breast feeding (Creery, 1973; Sloper *et al.*, 1977) but have not included control populations for comparison. The present study has compared the effect of one pattern of additional home support for breast feeding mothers with the routine care at present offered by health visitors.

### *Patients and Methods*

All patients were mothers who left hospital breast feeding, having delivered mature, normal birth weight babies in the Simpson Memorial Maternity Pavilion, Edinburgh. All the mothers were resident in Edinburgh and, since there is some evidence that

steroidal contraception may inhibit milk production (Gellen, 1977), none were intending to use hormonal contraception. The study group consisted of those mothers who met the entry criteria and were delivered within a period of 10 weeks. The control group were the comparable mothers who delivered in the 20 weeks subsequent to the period of recruitment to the study group. In this way, 28 mothers were recruited to the study group and 52 to the control group. Only three mothers declined to participate in the study.

*Study Group*
Patients in this group were visited in the post-natal ward by one of the two midwifery sisters involved in the study; all home visits were then carried out by the same sister and were additional to the routine visits by the health visitors. All mothers were visited once by the sister in the first week following discharge and thereafter visits were arranged fortnightly at times which were convenient to the mother. Each mother was given a telephone number through which she could contact the sisters during the day if any problems occurred. Routine records of the babies' feeding progress were kept by each mother; number of breast feeds, any extra bottle feeds and time of introduction of solids were noted. This information formed the basis for discussion at each visit.

The visits were conducted on a non-directive basis, merely giving the mothers the opportunity to raise problems. Decisions regarding feeding were made by the mother herself and no pressure was brought on her to continue breast feeding against her will. Visits were made fortnightly until the cessation of breast feeding.

*Control Group*
Mothers in this group received the normal care from the community services, but did not have the extra visits from the midwifery sisters. Twenty-four weeks after delivery, control mothers were interviewed at home about the progress of breast feeding.

*Babies*
Two babies in the control group were admitted to hospital, one because of respiratory infection and the other with failure to gain weight. The other babies were thriving.

*Statistics*
Comparisons between the groups were made using the chi square test.

**Results**
*Comparison of groups* (Table I)
No significant differences were found between the two groups in respect of age, parity or social class distribution. The number of routine home visits by health visitors as recalled by the mothers showed a wide range from one to ten visits per patient but the average number of 2·7 visits per patient was the same in the two groups. The study group received an additional $11{\cdot}5 \pm 0{\cdot}5$ visits per patient during the first 24 weeks from the sisters involved in the study.

*Duration of breast feeding*
In the study group, all mothers continued breast feeding until 12 weeks and 24 out of 28 (86%) were still feeding at 24 weeks; compared with the controls, the proportion of mothers breast feeding in the study group was higher throughout the 24 weeks of observation, the differences being significant at 12 weeks ($p<0{\cdot}01$) and 20 weeks ($p<0{\cdot}05$) respectively.

**Table I:** Comparison of age, parity, social class and number of home visits by health visitor or sister in control and study groups

| | Control n=52 | Study n=28 |
|---|---|---|
| *Age* (mean±SD) | 29±3·7 | 29±4·3 |
| *Parity* | | |
| Primigravid | 46% | 46% |
| Parous | 54% | 54% |
| *Social Class* | | |
| I | 30% | 39% |
| II | 35% | 36% |
| III and IV | 35% | 25% |
| *Visits by Health Visitor* (mean±SD) | 2·7±1·9 | 2·7±1·6 |
| *Extra visits* | 0 | 11·5 (±0·5) |

*Effect of Social Class* (Figure 1)
The effect of social class was shown by a progressive fall in continuation rates down the gradient of social class.
In social class I, no differences were observed between the study and control groups, the continuation rates at 24 weeks being 91% and 83% respectively. Differences were observed between the study and control groups in social class II ($p<0·05$ at 20 weeks) and social classes III and IV ($p<0·05$ at 12 weeks). There were no social class V mothers in either group.

*Introduction of supplements* (Figure 2)
Additional food in the form of formula milk or solids was introduced at an earlier stage in the control group as compared with the study group. This difference was most noticeable at 16 weeks when 83% of the control group were giving supplements compared with 28% of the study group ($p<0·001$); this difference was found in social classes I and II ($p<0·001$ in both groups) but not in social classes III and IV.

*Reasons for stopping breast feeding* (Table II)
The main reasons, stated by the mother herself, for stopping breast feeding within the first 24 weeks are shown in Table II. In the control group, 19% stated that either 'insufficient milk' or an unsettled baby was the major factor. None of the study group gave either of these reasons for stopping breast feeding. By contrast, the remaining reasons, not related to the adequacy of the milk supply, were given by 16% of the control and 14% of the study group.

## Discussion

The structure of modern society frequently isolates the young mother from the support of her friends and immediate family. This feeling of isolation may explain to a considerable measure why so many mothers stop breast feeding soon after leaving hospital. The present study has shown that in a group who were given consistent home support, it was possible to achieve a very high degree of successful breast feeding with 100% of the study

**Table II:** Reasons for stopping breast feeding before 24 weeks in control and study group mothers

| Reason Given | Control (n=52) | Study (n=28) |
|---|---|---|
| 'Not enough milk' | 8 } 19% | — |
| Unsettled baby | 2 } | — |
| Mother tired | 2 | 2 |
| Baby ill | 2 | — |
| Breast engorgement | 2 | — |
| Maternal choice | 1 | 1 |
| 'Baby refused breast' | 1 | — |
| Infected nipples | — | 1 |
| Total who stopped | 18 (35%) | 4 (14%) |

group still feeding at 12 weeks, and 86% at 24 weeks. The commonest reason for discontinuing breast feeding is 'insufficient milk' but this reason was never advanced by any of the mothers receiving regular visits. It is probable that a complaint of 'insufficient milk' usually indicates a lack of confidence on the mother's part rather than a true physiological incapacity to produce milk. The mother who loses confidence may introduce a bottle and this, in turn, will reduce the strength of the suckling stimulus and the adequacy of the subsequent milk supply. The increased confidence of the mothers receiving regular visits may explain why they were willing to postpone the introduction of supplementary or complementary feeds for longer than the mothers in the control group.

Social class has a major impact both on the incidence and the duration of breast feeding (West, 1980) and the beneficial effect of the additional visits on the duration of breast feeding was more obvious in the lower social class groups than in social class I. It would be wrong, however, to assume that mothers from social class I do not require additional support and the impact of the visits could be seen in the later introduction of supplementary feeds. The optimum timing for the introduction of supplementary food is still a matter of controversy (Waterlow, 1979; Jelliffe, 1979) but none of the babies in the study group appeared to suffer from the later introduction of supplements.

In this study, it was found that one visit every two weeks was sufficient to provide adequate support. Every visit, as far as possible, was made by the same sister and besides achieving close rapport, this ensured continuity of counselling. It is common practice for health visitors to visit at unscheduled times but, in this study, all visits were made by appointment. This had the advantage that no time was lost on wasted visits; furthermore, mothers found that they could cope with existing problems until the time of the next arranged visit.

Davies (1979) has drawn attention to the contented but undernourished breast-fed baby, and a pattern of regular visits would help in the early diagnosis and treatment of such cases.

Although the duration of breast feeding was used as the end-point of this study, the greater success in the mothers receiving additional support may reflect greater satisfaction

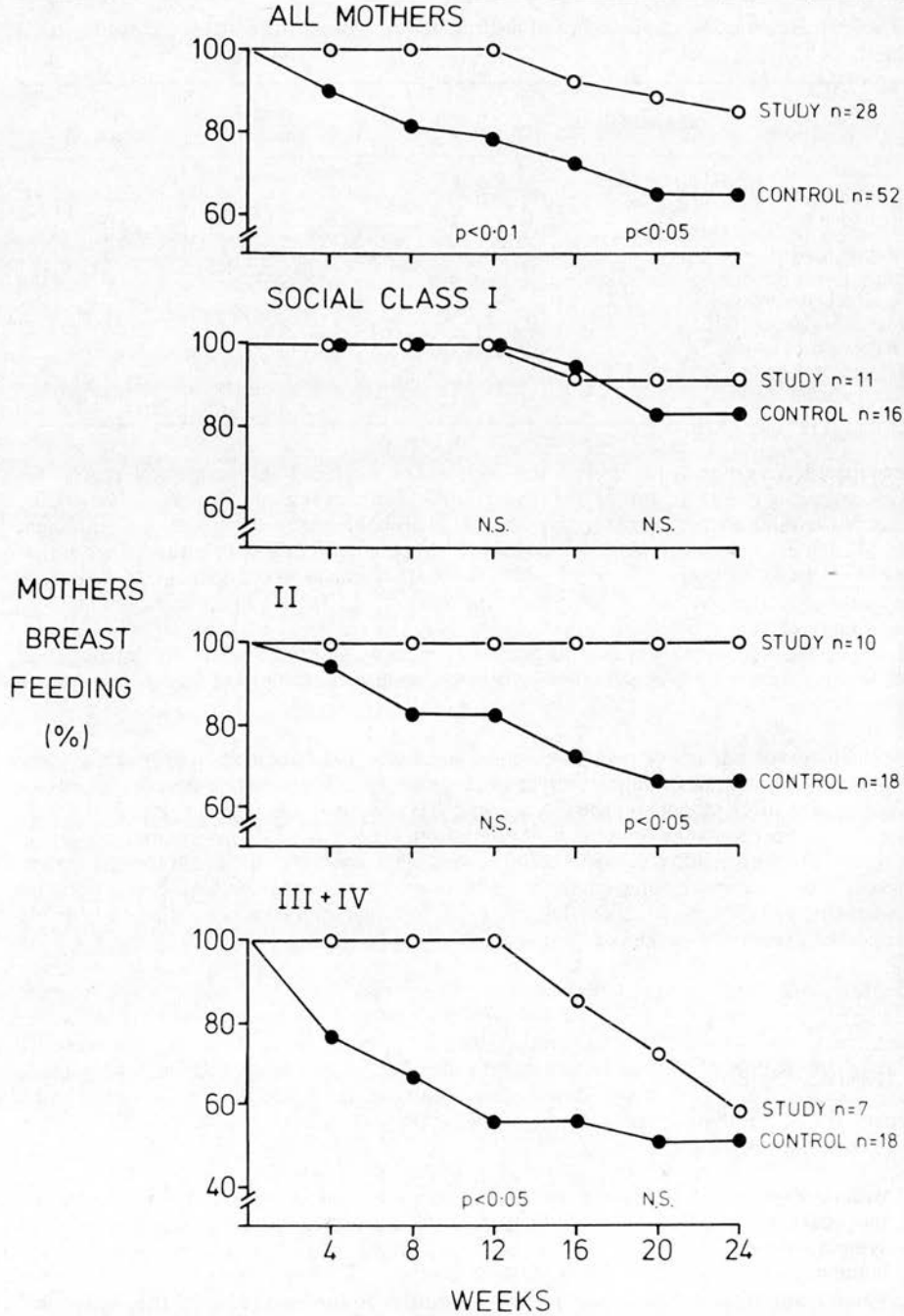



**Figure 1.** Comparison between study and control group mothers of the proportions still breast feeding 24 weeks after delivery.

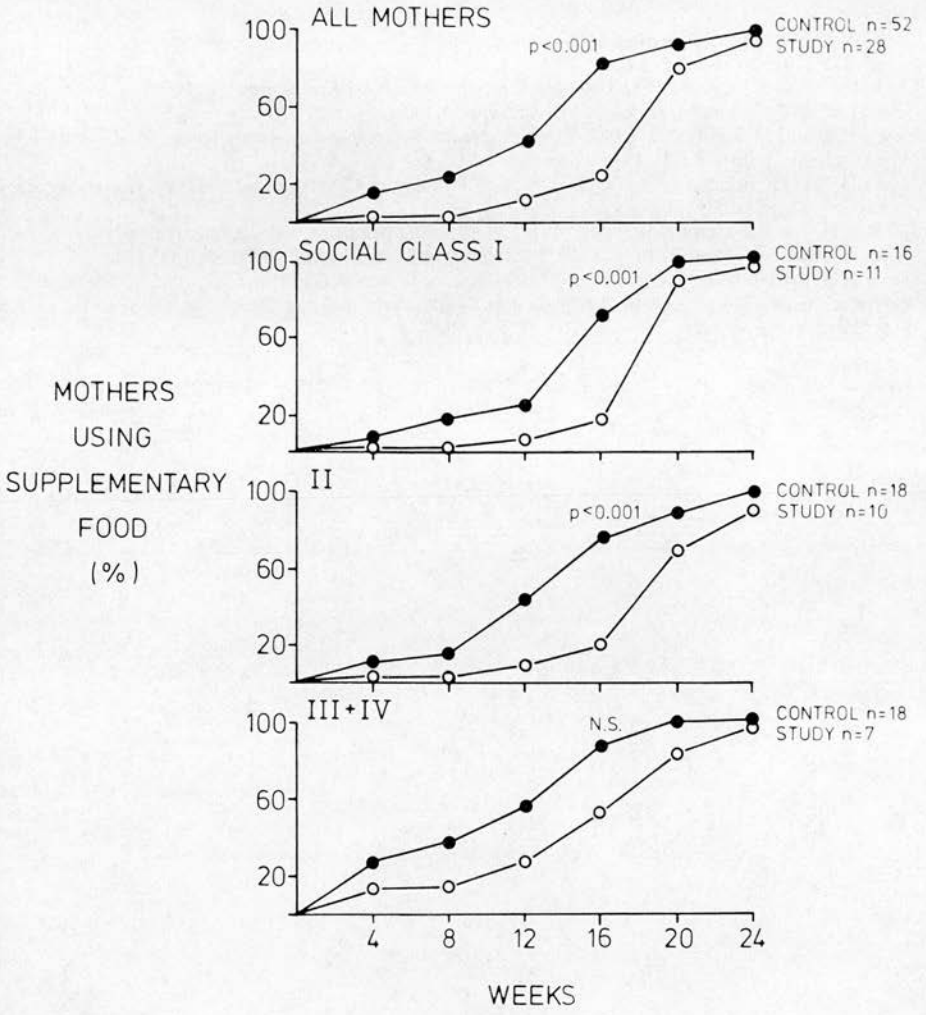



**Figure 2.** Comparison between study and control group mothers of the proportion of mothers who had introduced supplementary food (additional bottles or semi-solids).

with the experience of early motherhood. All the mothers said that they greatly appreciated the opportunity to share their feelings and their anxieties about the baby with a sympathetic listener and some had no other available confidant. The average number of home visits by the health visitors throughout breast feeding, as recalled by the mothers, was 2·7 in both the study and the control groups. It is questioned whether this level of support is sufficient to meet the needs of the breast feeding mother.

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# SIMULTANEOUS RELEASE OF OXYTOCIN AND NEUROPHYSIN DURING PARTURITION IN THE GOAT

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Neurophysin and the octapeptide hormones oxytocin and vasopressin are synthesized in the hypothalamus and stored in the posterior lobe of the pituitary gland. It has recently been shown that the release of both oxytocin and vasopressin or of vasopressin alone, in response to potent stimuli, is accompanied by a simultaneous release of neurophysin into the circulation (Burton, Forsling & Martin, 1971; McNeilly, Legros & Forsling, 1972). However, it has yet to be shown that neurophysin can be released at the same time as a specific release of oxytocin. This situation occurs in animals during both parturition (Folley & Knaggs, 1965) and lactation (Folley & Knaggs, 1966; McNeilly, 1972). The present report describes the simultaneous release of oxytocin and neurophysin during parturition in the goat.

Serial blood samples (approx. 10 ml each) were taken from an indwelling jugular cannula during the whole of labour in two pedigree British Saanen goats. Samples from a third pregnant goat at term but not in labour were used as controls. All plasma samples were stored at $-20$ °C until assay. Neurophysin was measured by radioimmunoassay of unextracted plasma (Martin, Chard & Landon, 1972), and oxytocin was measured by radioimmunoassay after fuller's earth extraction from acidified plasma (Chard, Boyd, Forsling, McNeilly & Landon, 1970). Arginine vasopressin (AVP) was determined by measurement of the antidiuretic activity of unextracted plasma in the hydrated ethanol-anaesthetized rat (Forsling, Jones & Lee, 1968).

The mean level of circulating neurophysin in the control animal was $4{\cdot}7 \pm 0{\cdot}6$ (S.D.) ng/ml while both oxytocin and AVP were undetectable ($< 2$ pg (1 $\mu$u.) oxytocin/ml and $< 10\mu$u. AVP/ml).

Oxytocin (9–115 $\mu$u./ml plasma) was released throughout second-stage labour in both goats and was accompanied by a simultaneous release of neurophysin (4–43 ng/ml plasma), with a rise in both peptides either just before or during the expulsion of each kid (Fig. 1). The correlation coefficient for plasma oxytocin and neurophysin concentrations was $+0{\cdot}70$ ($n = 54$, $P < 0{\cdot}001$). No significant change was observed in the concentration of AVP ($< 5\mu$u./ml plasma) in any of these experiments.

These results indicate that the specific release of oxytocin during parturition in the goat is accompanied by a release of neurophysin, in the absence of any rise in vasopressin levels. The significance of this simultaneous release remains to be determined;

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circulating neurophysin may simply represent a waste product of neurosecretion, or it may perhaps play some physiological role.

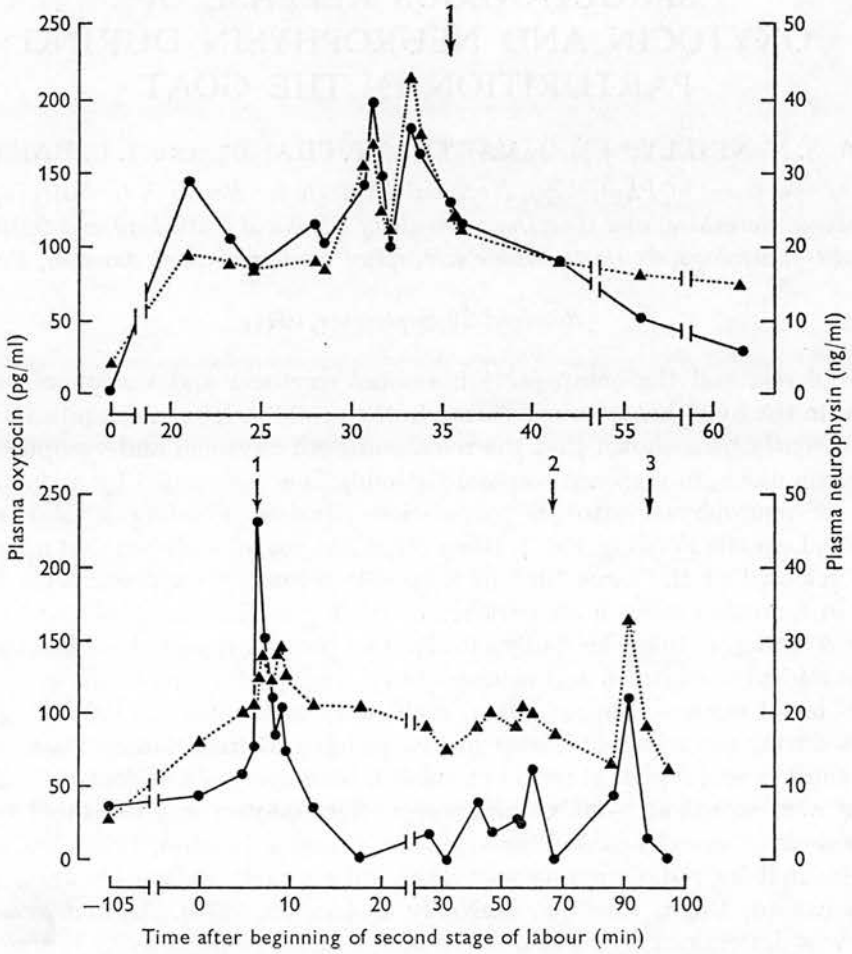



Fig. 1. Oxytocin (●—●) and neurophysin (▲.....▲) concentrations in jugular vein blood of two goats during second stage labour. Each arrow represents the birth of a kid.

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# RELEASE OF OXYTOCIN, VASOPRESSIN AND NEUROPHYSIN IN THE GOAT

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Neurophysin is considered to act as a carrier protein for oxytocin and arginine vasopressin (AVP) in the neurohypophysis, and recent evidence has suggested that neurophysin is released into the blood together with the neurohypophysial hormones in response to various stimuli (Cheng & Friesen, 1970). Neurophysin, extracted from bovine pituitary posterior lobes, consists of two major components designated neurophysin I and II (Hollenberg & Hope, 1968) both of which bind oxytocin and AVP *in vitro*, although neurophysin II appears to be located specifically in neurosecretory granules containing AVP (Dean, Hope & Kazie, 1968).

We now report results relating to the release of neurophysin, oxytocin and vasopressin into the blood in response to hand-milking, mating and haemorrhage in the goat. Consecutive serial blood samples (approximately 25 ml each) were taken from an indwelling jugular cannula during hand-milking in one goat and during mating in four oestrous female goats (McNeilly & Folley, 1970). In a further experiment, jugular blood samples were collected at intervals throughout progressive haemorrhage from the carotid artery in two castrated male goats under cyclopropane–oxygen anaesthesia. Basal plasma concentrations of neurophysin were determined in each of the following four groups (four animals per group): virgin, pregnant and lactating female, and castrated male goats. Estimates were made on single blood samples withdrawn from each animal by jugular venepuncture.

All plasma samples were stored at −20 °C until they were assayed for oxytocin, AVP and neurophysin. Oxytocin extracted from plasma with fuller's earth (Chard, Boyd, Forsling, McNeilly & Landon, 1970) was assayed by the intramammary pressure bioassay in the lactating guinea-pig (Tindal & Yokoyama, 1962) or by radioimmunoassay (Chard, Kitau & Landon, 1970; McNeilly, 1971). Arginine vasopressin was determined by measurement of the antidiuretic activity of unextracted plasma in the hydrated ethanol-anaesthetized rat (Forsling, Jones & Lee, 1968), and neurophysin was measured directly in plasma by a radioimmunoassay which, in the cow, is specific for neurophysin II (Legros, Franchimont & Hendrick, 1969). The neurophysin used as standard was bovine neurophysin II (K. W. Cheng & H. G. Friesen, personal communication) and complete cross-reaction was shown to occur between

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the bovine neurophysin II antiserum and a neurophysin complex extracted from goat posterior pituitary lobes (J. J. Legros, unpublished observations).

Although oxytocin (30 $\mu$u./ml plasma) was detected in one of the nine serial blood samples taken during hand-milking, and in nine of the 36 samples taken during mating (6 to 63 $\mu$u./ml plasma), no significant change was observed in the concentrations of AVP ($< 5$ $\mu$u./ml plasma) or neurophysin ($< 0{\cdot}5$ ng/ml plasma). However, there was a dramatic rise in the plasma concentration of both AVP and neurophysin but no detectable release of oxytocin during progressive haemorrhage (Table 1). The basal levels of neurophysin in virgin, pregnant and lactating female and castrated male goats were $< 0{\cdot}5$ to 7, $< 0{\cdot}5$, $< 0{\cdot}5$ and $< 0{\cdot}5$ ng/ml respectively.

Table 1. *Jugular blood levels of oxytocin, vasopressin (AVP) and neurophysin during progressive haemorrhage in the goat*

| Blood volume removed (ml) | Hormone concentrations per ml plasma in: Goat 156 (86·5 kg body wt) Oxytocin* ($\mu$u.) | AVP† ($\mu$u.) | Neurophysin* (ng) | Goat 296 (76·4 kg body wt) Oxytocin* ($\mu$u.) | AVP† ($\mu$u.) | Neurophysin* (ng) |
|---|---|---|---|---|---|---|
| 0 | < 5 | < 20 | 11 | < 5 | < 10 | 2 |
| 250 | < 5 | 25 | 18 | < 5 | < 10 | 2 |
| 500 | < 5 | 41 | 20 | < 5 | < 20 | 3 |
| 750 | < 5 | 200 | 19 | < 5 | 98 | 7 |
| 1000 | < 5 | 270 | 33 | < 5 | 2460 | 55 |
| 1250 | < 5 | 350 | 68 | < 5 | 2040 | 205 |
| 1500 | < 5 | 730 | 350 | < 5 | 1950 | 310 |
| 1750 | < 5 | 1140 | 500 | < 5 | 2250 | 500 |
| 2000 | < 5 | 850 | 1000 | < 5 | 1960 | 600 |

* Assayed by radioimmunoassay. † Assayed by bioassay.

Since the neurophysin radioimmunoassay used is thought to be specific for neurophysin II and since the component determined is found in association with AVP and not oxytocin, then the release of AVP appears to be accompanied by the release of a specific neurophysin, which may possibly be neurophysin II.

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# PLACENTAL TRANSFER OF OXYTOCIN IN THE GUINEA-PIG AND ITS RELEASE DURING PARTURITION

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SUMMARY

Oxytocin levels in pregnant and parturient guinea-pigs were studied by means of a sensitive and specific radioimmunoassay. Oxytocin was released from the maternal pituitary in substantial amount only during the expulsive phase of labour, when the mean concentration in carotid arterial blood in five animals was 503 pg/ml plasma (range 96–2900 pg/ml). Oxytocin was not found in the plasma of the first born at the moment of birth, but was usually detected in amounts ranging from 96 to 455 pg/ml in those born subsequently. The mean half-time of oxytocin in the maternal circulation during late pregnancy was $62 \pm 7{\cdot}5$ (S.E.M., $n = 5$) s. In-vivo experiments showed that the placenta was permeable to oxytocin in both directions.

## INTRODUCTION

The factors controlling parturition in the guinea-pig are poorly understood. In contrast to some other species, such as the sheep and goat, labour is neither preceded by a fall in circulating progesterone nor by a sharp rise in circulating oestrogens (Ash, Challis, Harrison, Heap, Illingworth, Perry & Poyser, 1973). In addition, both the concentration of total unconjugated oestrogens (Challis, Heap & Illingworth, 1971) and the capacity to convert labelled precursors to oestrogens (Challis & Illingworth, 1972) decreases during the last week of pregnancy.

Circulating levels of oxytocin during parturition have been measured in the cow, horse, sheep, goat and rabbit (Fitzpatrick, 1961; Knaggs, 1963; Fitzpatrick & Walmsley, 1965; Fuchs, 1966; Chard, Boyd, Forsling, McNeilly & Landon, 1970; Haldar, 1970; McNeilly, Martin, Chard & Hart, 1972; Allen, Chard & Forsling, 1973). These results show that there is little or no oxytocin in the maternal circulation during the first stage of labour, but high levels throughout the expulsive phase.

The uterus of the guinea-pig is sensitive to oxytocin at all stages of gestation (Bell, 1941; Porter, 1971), whereas, in most other species, the uterus responds to oxytocin only towards term. It has been demonstrated that the posterior pituitary of the foetal guinea-pig is highly active at the time of parturition (Burton & Forsling, 1972).

These observations prompted an investigation of the release of oxytocin in both mother and foetus at the time of parturition. During the course of these investigations it became apparent that information on the kinetics of oxytocin metabolism in the maternal circulation, and its placental transfer, would be necessary for the complete interpretation of the results. Therefore, the metabolism and placental transfer of oxytocin have also been studied.

## MATERIALS AND METHODS

### *Extraction and assay of oxytocin*

Oxytocin was extracted from plasma as described by Boyd, Jackson, Hollingsworth, Forsling & Chard (1972). The method was originally developed for 5-ml volumes; as the volume of plasma obtained from the guinea-pigs was generally less than 1 ml, it was necessary to determine whether a satisfactory recovery of oxytocin could be obtained under such conditions. Two sets of six different volumes of guinea-pig plasma, ranging from 0·5 ml to 5·0 ml, were extracted with Spherosil XOA 400. One set was extracted as blanks and the other set was extracted after the addition of 200 pg oxytocin to each volume.

Oxytocin levels were determined by a specific radioimmunoassay (Chard, Forsling, James, Kitau & Landon, 1970) after extraction. All results were corrected for recovery.

### *Animals*

Primigravid and multigravid animals were chosen from a colony of mixed and albino stock. Pregnancies were dated from the day on which a vaginal plug was found, or, in the case of post-partum mated animals, on the day of parturition (day 0).

### *Cannulation procedures and blood sampling*

The operation was performed 4–6 days before the expected day of parturition. The animals were anaesthetized with ether after sedation with tribromoethanol (Avertin, Winthrop Laboratories). One carotid artery was cannulated with vinyl catheter tubing (inner diameter 0·5 mm, outer diameter 0·8 mm; Dural Plastics Ltd, Australia) with the catheter being exteriorized through the back of the neck, and housed in a small plastic chamber taped to the animal (Hanwell, Fleet & Linzell, 1972). The cannulae were flushed daily with sterile heparin–saline (100 i.u./ml 0·9 % saline). Blood samples were withdrawn into cooled heparinized syringes and the blood transferred to centrifuge tubes. After centrifugation at 4 °C, the plasma was removed and stored at −20 °C until assayed.

When single samples were taken before parturition, the packed red cells (resulting after centrifugation) were resuspended in sterile 0·9 % saline and returned to the animal. During the expulsive phase of labour, resuspended red cells were returned to the animal only when the sampling was complete.

### *Parturition experiments*

Animals were observed closely throughout the day and night once they were within 24 h of delivery, as judged from pelvic relaxation and the position of the lowest foetal

head. The first stage of labour was sometimes extremely difficult to discern, and in some cases expulsive uterine contractions and the appearance of a foetal head were the first indications of uterine activity. Blood sampling was started as soon as there was any sign of parturient activity. Samples were withdrawn during strong expulsive contractions. The animals were disturbed as little as possible during the sampling and appeared unaffected by either the procedure or an audience. Blood samples were withdrawn from the foetuses by cardiac puncture as soon as possible after delivery and never later than 2–3 min after expulsion.

### *Time-course experiments*

These experiments were performed on five guinea-pigs (four conscious and one anaesthetized), through an indwelling carotid cannula, on day 62–63 of gestation. A control arterial blood sample was taken immediately before the experiment began. Oxytocin (8 or 20 ng) in 0·5 ml 0·9 % saline was injected through the cannula and washed through with sterile saline. Timed 1 and 2 ml blood samples were taken from the carotid cannula during the next 10 min.

### *Placental transfer experiments*

#### *Transfer of oxytocin from the maternal to the foetal circulation*

Four animals on days 61–65 of gestation were anaesthetized with sodium pentobarbitone (20 mg/kg, i.p.) supplemented with ether. The right carotid artery and left vein were cannulated; the abdomen was opened and the uterus exposed and kept moist with warm saline.

In three animals, oxytocin (8 or 20 ng) in 0·5 ml saline was injected into the jugular cannula. In the fourth animal, 0·9 % saline was substituted for oxytocin. Sequential 1 and 2 ml maternal blood samples were withdrawn through the carotid cannula during the next 5 or 10 min. The foetuses were exposed in turn and 1 ml cardiac and umbilical venous samples were taken.

#### *Transfer of oxytocin from the foetal to the maternal circulation*

Four animals on days 64–66 of gestation were anaesthetized as described above. The right carotid artery was cannulated and the uterus exposed. The umbilical vessels of one foetus were exposed and loose ligatures placed under the vitelline vein and artery. In three animals, oxytocin (4 to 8 ng) in 0·5 ml saline was injected into the vitelline vein. In the fourth animal, 0·9 % saline was substituted for oxytocin. The vein was immediately clamped above and below the injection site. Blood samples were collected from the maternal carotid cannula over the next 5 or 10 min. Foetal cardiac and umbilical venous blood samples were taken from the injected foetus, together with similar blood samples from the other foetuses in turn.

## RESULTS

### *Extraction and assay of oxytocin*

The average percentage recovery of oxytocin from plasma was $69{\cdot}5 \pm 1{\cdot}12$ (S.E.M., $n = 6$), and there was no significant variation with sample volume. The maximum

sensitivity of the assay, in terms of the lowest detectable oxytocin concentration/ml plasma, varied throughout these experiments as a result of varying volumes of plasma used in the initial extraction procedure. After extraction of oxytocin from 5 ml plasma, the maximum sensitivity of the assay was 4 pg/ml.

*Parturition*

Figure 1 shows the circulating levels of oxytocin in the maternal carotid arterial plasma of five guinea-pigs before, during and after parturition and the levels in foetal plasma at birth. No oxytocin was detected in the three animals studied before the

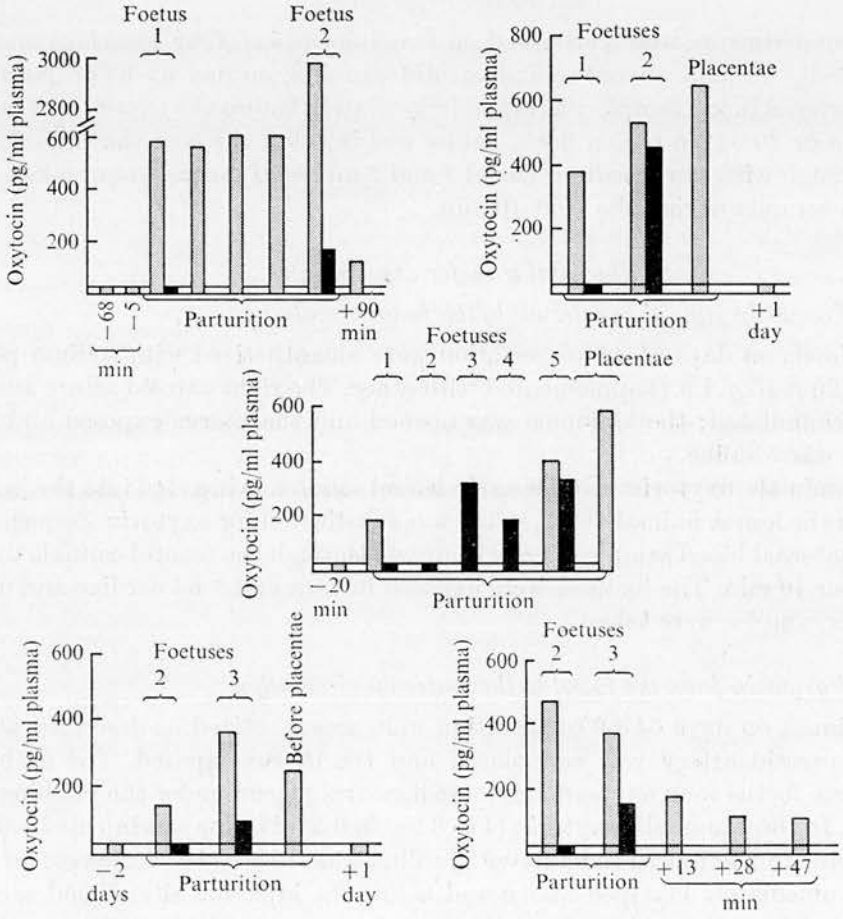



Fig. 1. Plasma levels of oxytocin in maternal guinea-pigs before, during and after parturition (stippled areas) and in foetal samples after birth (black areas). The line above the horizontal axis of each graph approximately represents the lowest detection limit of the assay. The terms 'before placentae' and 'placentae' indicate that the maternal blood samples were obtained before and during expulsion of the placentae, respectively.

expulsive phase of labour. High concentrations were found in all five animals once the expulsive phase began; the mean level was 503 pg/ml plasma (range 96–2900 pg/ml). These levels were maintained during the expulsion of the placentae. No oxytocin was detected 6 h after parturition.

Table 1 summarizes the results obtained in foetal plasma collected at delivery. No oxytocin was detected in the three animals in which samples were obtained from the first foetus. In all five animals, the incidence of positive levels of oxytocin increased with subsequent foetuses; the last foetus of a litter always showed positive levels. The levels of oxytocin in foetal blood at birth were invariably lower than the level found in the mother (Fig. 1).

Table 1. *Occurrence of oxytocin (pg/ml) in foetal plasma of the guinea-pig at the moment of birth*

| Guinea-pig no. | Foetus no. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | < 24 | 455 | — | — | — |
| 2 | N.S. | < 24 | 96 | — | — |
| 3 | < 16 | 156 | — | — | — |
| 4 | N.S. | < 48 | 146 | — | — |
| 5 | < 48 | < 48 | 312 | 174 | 327 |

N.S. No sample was obtained; —, no foetus.

Table 2. *Circulating levels of oxytocin in foetal guinea-pigs after injection of oxytocin into the maternal circulation*

| Guinea-pig no. | Oxytocin (ng) injected into mother | Sample | Time after injection (s) | Oxytocin (pg/ml plasma) |
|---|---|---|---|---|
| 1 | 8 | Foetus 1 (u.v.) | 60 | 183 |
| | | Foetus 1 (c.) | 155 | 198 |
| | | Foetus 2 (c.) | 226 | 218 |
| | | Foetus 3 (c.) | 354 | 171 |
| 2 | 20 | Foetus 1 (c.) | 98 | 45 |
| | | Foetus 1 (u.v.) | 155 | 45 |
| | | Foetus 2 (c.) | 230 | 45 |
| | | Foetus 2 (u.v.) | 268 | < 24 |
| | | Foetus 3 (c.) | 368 | 200 |
| | | Foetus 3 (u.v.) | 421 | 114 |
| | | Foetus 4 (c.) | 503 | < 24 |
| 3 | 20 | Foetus 1 (c.) | 44 | 41 |
| | | Foetus 1 (u.v.) | 85 | 35 |
| | | Foetus 2 (c.) | 164 | < 24 |
| | | Foetus 2 (u.v.) | 206 | 26 |
| | | Foetus 2 (u.v.) | 264 | < 17 |
| | | Foetus 1 (c.) | 542 | < 30 |

Samples were taken by cardiac puncture (c.) or from the umbilical vein (u.v.) as indicated.

### *Kinetics of oxytocin metabolism*

The disappearance of oxytocin from the maternal plasma, after an injection of oxytocin, was analysed as a double exponential function, with a half-time of $62{\cdot}5 \pm 7{\cdot}5$ (S.E.M., $n = 5$) s during the initial, rapid phase and of approximately 300 s during the second, slow phase. The latter could not be determined accurately due to the plasma levels of oxytocin 10 min after injection being close to the limits of sensitivity of the assay.

*Placental transfer experiments*

*Transfer of oxytocin from the maternal to the foetal circulation*

Table 2 shows that oxytocin was detected in 12 out of 17 foetal samples after injection into the mother. No oxytocin was detected after saline injection.

*Transfer of oxytocin from the foetal to the maternal circulation*

Table 3 shows that oxytocin was detected in maternal blood within 20 s of injection into a foetus: no oxytocin was detected in the maternal circulation 2 min after injection. No oxytocin was detected after saline injection.

Table 3. *Circulating levels of oxytocin in maternal guinea-pigs after injection of oxytocin into the circulation of one foetus from a litter*

| Guinea-pig no. | Oxytocin (ng) injected into foetus | Sample no. | Time after injection (s) | Oxytocin (pg/ml plasma) |
|---|---|---|---|---|
| 1 | 10 | 1 | 25 | 10 |
| | | 2 | 54 | 34 |
| | | 3 | 87 | 36 |
| | | 4 | 122 | < 11 |
| | | 5–8 | 182–316 | < 12 |
| 2 | 10 | 1 | 20 | 24 |
| | | 2 | 61 | 100 |
| | | 3 | 103 | 27 |
| | | 4 | 148 | < 8 |
| | | 5–7 | 200–430 | < 12 |
| 3 | 4 | 1 | 25 | 36 |
| | | 2 | 71 | 64 |
| | | 3 | 122 | < 9 |
| | | 4–8 | 171–552 | < 12 |

## DISCUSSION

The finding that oxytocin is released in large amounts from the pituitary of the guinea-pig during the expulsive phase of labour and not during earlier stages is in agreement with findings in other species, such as the goat (Knaggs, 1963; Folley & Knaggs, 1965; Chard, Boyd, Forsling, McNeilly & Landon, 1970; McNeilly, Martin, Chard & Hart, 1972), the sheep (Fitzpatrick, 1961; Fitzpatrick & Walmsley, 1965), the cow (Fitzpatrick & Walsmley, 1965), the rabbit (Fuchs, 1966; Haldar, 1970) and the horse (Allen, Chard & Forsling, 1973). However, in the present as in previous studies 'spurt' release of oxytocin (Folley & Knaggs, 1965; Fox & Knaggs, 1969; Gibbens, Boyd & Chard, 1972; McNeilly, 1972; McNeilly & Ducker, 1972) during earlier stages, or a small rise undetectable by the assay, cannot be excluded. The high levels of circulating oxytocin in maternal guinea-pigs during parturition agrees with the previous observation of a fall in maternal pituitary oxytocin content after parturition in this species (Burton & Forsling, 1972).

Oxytocin in the foetal circulation at birth might originate from either mother or foetus. The guinea-pig foetal pituitary at birth contains high levels of oxytocin and arginine vasopressin (Burton & Forsling, 1972), and there is evidence for foetal release

of oxytocin in man (Chard, Hudson, Edwards & Boyd, 1971), and the cow (Robinson, Zimmerman & Frantz, 1971) at birth. However, the present observations showing increasing levels of oxytocin in foetal blood with successive deliveries, suggest that, in the guinea-pig, this oxytocin originates from the maternal circulation. This is supported by the finding that oxytocin can cross the placenta in both directions. Thus, the first one or two foetuses of a litter would receive little oxytocin as their placental circulation is occluded by stretching of the umbilical cord at an early stage of parturition before the concentration of oxytocin in the maternal circulation has reached a high level.

The only previous observations on the placental transfer of oxytocin are those of Noddle (1964) showing that oxytocin can cross the placenta from mother to foetus in the ewe. The present results demonstrate that oxytocin can cross the placenta in both directions. The small number of samples, and the differences in clearance rate between mother and foetus, renders impossible an estimate of the percentage oxytocin transferred.

The half-time of $62 \pm 7{\cdot}5$ (S.E.M., $n = 5$) s for oxytocin in the guinea-pig is within the range obtained by other workers in different species such as the rat (Ginsburg & Smith, 1959; Fabian, Forsling, Jones & Lee, 1969), the rabbit (Chaudhury & Walker, 1959) the goat (Folley & Knaggs, 1965), the sheep (Fitzpatrick, 1961), the cow (Cleverley & Folley, 1969) and man (Ryden & Sjoholm, 1969; Forsling, Boyd & Chard, 1971; Chard *et al.* 1970).

The very high levels of oxytocin in the maternal plasma (2900 pg/ml, the maximum recorded here) would suggest an important rôle of oxytocin in the latter stages of parturition. However, hypophysectomized guinea-pigs can give birth to live young (Pencharz & Lyons, 1934; Illingworth, Ackland, Burton, Challis, Heap & Perry, 1973). No oxytocin can be detected in the maternal plasma of these animals during parturition, but it is present in foetal plasma. Parturition is prolonged and difficult compared with that of normal animals. Thus, although oxytocin is not essential for parturition, it is probably concerned with efficient expulsion of the young.

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# THE RELEASE OF OXYTOCIN AND PROLACTIN DURING PARTURITION IN THE GOAT

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It has been suggested that in certain circumstances oxytocin may stimulate the release of prolactin in the rat (Benson & Folley, 1956) and goat (Bryant, Greenwood & Linzell, 1968). In order to test this hypothesis in a physiological situation the changes in blood levels of oxytocin and prolactin were determined in a series of

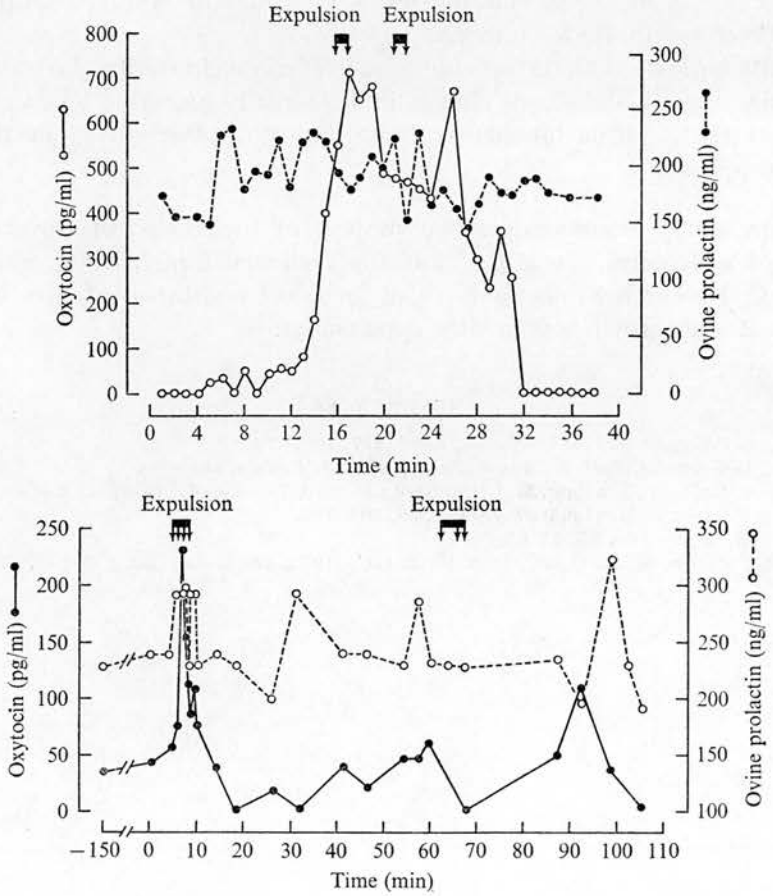



Fig. 1. Oxytocin and prolactin concentrations in jugular vein blood of two goats during second-stage labour. Expulsion indicates the birth of a kid.

samples taken during parturition in the goat, where oxytocin levels are known to be high (Folley & Knaggs, 1965; McNeilly, Martin, Chard & Hart, 1972).

The cannulation and blood sampling technique has been described previously (McNeilly *et al.* 1972). Jugular blood samples were taken continuously during the whole of labour in six pedigree British Saanen goats and all plasma samples were stored at −20 °C until assay. Oxytocin was measured by radioimmunoassay after fuller's earth extraction from acidified plasma (Chard, Boyd, Forsling, McNeilly & Landon, 1970). Prolactin was measured by a specific solid phase radioimmunoassay for ovine prolactin which showed complete cross-reaction with goat prolactin (Hart, 1972).

The basal circulating levels of prolactin in first-stage labour of all goats were between 102 and 506 ng/ml plasma while oxytocin remained undetectable ($< 2$ pg, 1 $\mu$u./ml).

During second-stage labour, oxytocin (6–195 $\mu$u./ml plasma) was released in all six goats, but these releases were not accompanied by any simultaneous rise in prolactin (Fig. 1) and the blood levels of prolactin remained within the range of levels during first-stage labour (102–506 ng/ml). This failure of oxytocin to evoke significant changes in the circulating levels of prolactin occurred despite a very substantial increase in oxytocin levels.

The results indicate that the specific release of oxytocin during parturition in the goat does not cause a significant change in the already increased levels of prolactin and suggests that, during labour, oxytocin does not play a role in the control of prolactin release.

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# THE AFFERENT PATHWAY OF THE MILK-EJECTION REFLEX IN THE MID-BRAIN OF THE GOAT

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SUMMARY

The position of the pathway for the release of oxytocin in the mid-brain was ascertained by exploration of a transverse stereotaxic plane (A4) in 23 anaesthetized goats. Electrical stimulation was applied between a monopolar electrode and an indifferent electrode in the scalp. Oxytocin release was monitored by simultaneous collection of blood samples during stimulation from a catheter in a jugular vein. The blood samples were extracted by the Sephadex G-25 or fuller's earth method and assayed for oxytocin content on the lactating guinea-pig preparation.

Oxytocin release occurred occasionally after stimulation of certain sites in the tectum, central grey and reticular formation. Regular releases of oxytocin, however, were only obtained after stimulation of a pathway which was compact and lay in the lateral tegmentum of the mid-brain in association with the spinothalamic tract. The position of this pathway corresponds to that described previously for the afferent pathway of the milk-ejection reflex in the mid-brain of the guinea-pig and rabbit. In these three species therefore, the impulses concerned in oxytocin release appear to ascend through the mid-brain in the spinothalamic tract.

## INTRODUCTION

The route taken by the afferent pathway of the milk-ejection reflex from the mammary gland to the neurohypophysis is still a subject of controversy. In a variety of species, milk-ejection responses have been obtained after electrical stimulation of several widely separated sites in the mid-brain, including the reticular formation, periaqueductal central grey and tectum, but no definite pathway was observed in any of these studies and it was therefore generally assumed that the fibres concerned in oxytocin release ascended in a diffuse manner through the mid-brain (see reviews by Denamur (1965) and Cross (1966)).

More recently, the afferent pathway of the milk-ejection reflex has been located in the mid-brain of the guinea-pig and rabbit (Tindal, Knaggs & Turvey, 1967, 1969) and its forward projection to the hypothalamus has been described in detail in the guinea-pig (Tindal & Knaggs, 1971). From these studies it was concluded that the pathway in the mid-brain was compact and lay bilaterally in the lateral tegmentum in association with the spinothalamic tract.

However, most of the available evidence (see Discussion) suggests that the milk-

ejection pathway in the ruminant differs considerably from that in the rodent and lagomorph and probably involves the dorsal column–medial lemniscal rather than the spinothalamic system. Therefore, to relate our studies in the guinea-pig and rabbit to the findings in the ruminant we investigated the milk-ejection pathway in the mid-brain of the goat.

## METHODS

Twenty-three adult female British Saanen goats from the Institute herd, ranging in weight from 54 to 89 kg, were used. A polyethylene cannula (internal diameter 2·0 mm) was inserted into an external jugular vein of each goat before the experiment began. Anaesthesia was induced and maintained by inhalation of a cyclopropane–oxygen mixture administered through a closed-circuit system. The anaesthetic was changed to a non-explosive fluothane–oxygen mixture for the surgical removal of a portion of the skull roof and incision of the dura mater when an electric drill and diathermy were used, but was then changed back to cyclopropane–oxygen for the remainder of the experiment.

The stereotaxic technique used for the goat has been described previously (Tindal, Knaggs & Turvey, 1968*a*). A monopolar 24 s.w.g. stainless steel electrode, insulated with epoxy resin except for the terminal 0·75 mm, was inserted in the mid-brain according to stereotaxic co-ordinates for the goat brain. The electrode was lowered in steps of 1·0 mm and at each site electrical stimulation, consisting of positive square-wave pulses, 1 ms pulse duration, 50 Hz, at 300 $\mu$A and 2–3 V, was applied for 25 s between the electrode and an indifferent electrode in the scalp. Only one electrode track was explored in each goat except for two cases where two tracks were investigated. At each site a 25-ml blood sample was collected from the jugular cannula during the electrical stimulation period and a further sample collected during the 25 s immediately after stimulation. A 2-min period was allowed between each stimulation and in the first three experiments a control blood sample was also taken in the 25 s immediately preceding each stimulation. The blood was collected into cooled, heparinized, polyethylene bottles, centrifuged, and the plasma extracted by dextran gel filtration on Sephadex G-25 (Folley & Knaggs, 1965) or by the fuller's earth method (Chard, Boyd, Forsling, McNeilly & Landon, 1970) and then assayed for milk-ejection activity by intra-arterial injection into the lactating guinea-pig preparation (Tindal & Yokoyama, 1962).

In each electrode track two or three sites were marked with a d.c. lesion (100 $\mu$A for 20 s) and at the end of the experiment the animal was killed with excess anaesthetic and the head perfused via the carotid arteries with 0·9 % NaCl solution followed by a mixture of acidified formalin and potassium ferro- and ferricyanides to mark the lesion sites with the Prussian blue reaction, and then with 10 % formalin to ensure fixation. Serial, transverse, frozen sections, 80 $\mu$m thick, were cut through the region of the brain containing the electrode track and stimulation sites were then determined microscopically and plotted on planes A 2, A 4 and A 9 of the goat brain atlas (Tindal *et al.* 1968*a*).

RESULTS

Twenty-five electrode tracks comprising a total of 217 stimulation sites were explored. While all the tracks were aimed at plane A 4, in fact 20 occurred at A 4, 4 at A 2 and 1 at A 9.

The control blood samples taken immediately before each stimulation period in the first three experiments were all negative ( $< 5$ $\mu$u. oxytocin/ml plasma) and in the remaining 22 experiments these were not taken. Oxytocin was found only in the sample taken during stimulation in 28 cases, only in the sample taken immediately

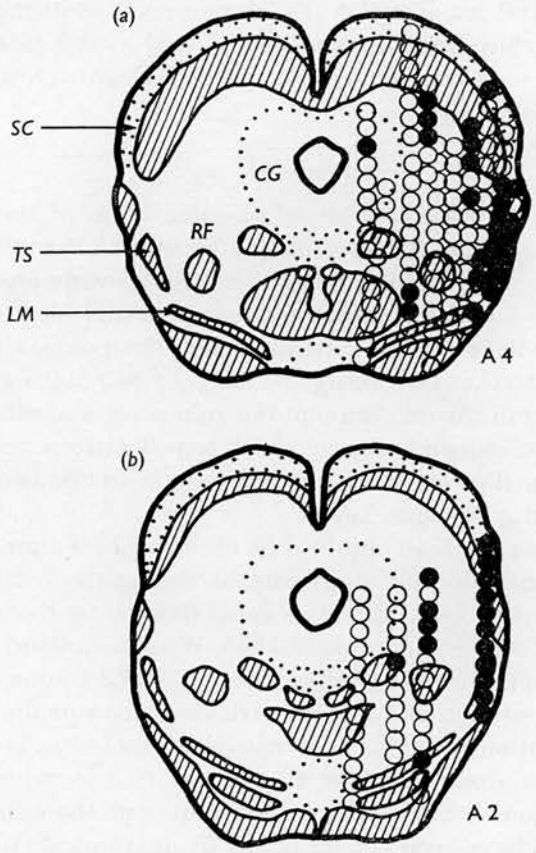



Fig. 1. Drawings of transverse sections of goat brain at stereotaxic planes A 4 (*a*) and A 2 (*b*). Solid circles (●) denote sites where electrical stimulation caused the release of oxytocin into the blood at a concentration greater than 20 $\mu$u./ml plasma. Open circles (○) denote sites where electrical stimulation caused no detectable oxytocin release ( $< 5$ $\mu$u./ml plasma) or in a few cases a small release of 5–20 $\mu$u./ml plasma. Note the concentration of responsive sites around the spinothalamic tract particularly at A 4. Abbreviations: *CG*, substantia grisea centralis; *LM*, lemniscus medialis; *RF*, formatio reticularis; *SC*, colliculus superior; *TS*, tractus spinothalamicus.

after stimulation in 22 cases and in both samples in 44 cases, the oxytocin levels ranging from 5 to 269 $\mu$u./ml plasma. At plane A 4 stimulation of 50 out of 156 sites resulted in the release of oxytocin. The majority of these positive sites was concen-

trated in the lateral tegmentum in and immediately dorsal to the spinothalamic tract. Although most of this plane was systematically investigated, other releases were found only at a few sites in the periaqueductal central grey, superior colliculus and reticular formation. Positive sites at A4 and A2 where amounts of oxytocin greater than 20 $\mu$u./ml plasma were released are shown in Fig. 1*a* and *b*.

Stimulation of 22 out of 49 sites at plane A2 resulted in the release of oxytocin (6–535 $\mu$u./ml plasma). One track contained a number of positive sites extending through the spinothalamic tract. Other positive sites occurred in the reticular formation, central tegmental fasciculus, and one site (535 $\mu$u./ml) was in the medial lemniscus, this being one of only two out of a total of nine sites within the medial lemniscus at planes A4 and A2 at which stimulation resulted in the release of oxytocin. The single track which was unintentionally sited at A9 passed through the spinothalamic tract where a release of 24 $\mu$u./ml was found with a few smaller releases immediately above and below this.

## DISCUSSION

The main finding in the present study of the mid-brain of the goat was that although oxytocin release occurred occasionally after stimulation of certain areas in the tectum, central grey and reticular formation, regular responses were obtained only after stimulation of a pathway which was compact and lay in the lateral tegmentum in association with the spinothalamic tract. The position of this pathway was exactly comparable to that previously described by us for the afferent pathway of the milk-ejection reflex in the mid-brain of the guinea-pig and rabbit (Tindal *et al.* 1967, 1969). The anatomical and physiological considerations for proposing the spinothalamic tract as the fibre system concerned have been discussed elsewhere (see Tindal *et al.* 1967; Tindal & Knaggs, 1970).

Milk-ejection responses have been obtained by other workers after electrical stimulation of sites in the mid-brain and hind-brain, including the central grey, medial lemniscus, tectum, reticular formation and subthalamus, in the rabbit (Cross & Silver, 1961; Holland, Woods & Aulsebrook, 1963; Woods, Holland & Powell, 1969) and the medial lemniscus in the goat (Andersson, 1951). The responses in the central grey have led to the suggestion that the periventricular–diencephalic system of fibres, which is the rostral extension of the dorsal longitudinal fasciculus, is concerned in the transmission of impulses from the mammary gland to the neurohypophysis. In support of this, connexions between the central grey and the hypothalamic paraventricular (PV) nucleus have been demonstrated by anatomical (Woods *et al.* 1969) and electrophysiological (Beyer, Tindal & Sawyer, 1962) techniques. In addition, stimulation of the mesencephalic central grey produces evoked potentials in the pituitary stalk of sheep (Richard, 1970) and evoked potentials are also elicited in the PV nucleus after stimulation of the mesencephalic central grey in the rabbit (Woods *et al.* 1969). Beyer & Mena (1965) also claimed that lesions of the mid-brain central grey region in the cat blocked lactation by abolishing the milk-ejection reflex, although in a similar previous study Beyer, Mena, Pacheco & Alcaraz (1962) had found that the block in lactation was not removed by oxytocin administration and had concluded that the cessation of lactation was due to interruption of prolactin secretion. In contrast to the above findings, however, Richard (1970) found that

lesions of the mid-brain central grey region of the sheep did not block the evoked potentials in the pituitary stalk produced in response to mammary nerve stimulation. Richard, Urban & Denamur (1970) also reported that lesions of the central grey alone did not block milk ejection and that it was necessary to lesion the whole of the mid-brain reticular formation in order to block the reflex in the conscious animal.

It would appear, therefore, that the mid-brain central grey is not on the direct pathway of the milk-ejection reflex and this is in accord with our description of the main pathway in the guinea-pig (Tindal *et al.* 1967; Tindal & Knaggs, 1971) which ascends from the lateral tegmentum of the mid-brain to the PV nucleus without involving the mid-brain central grey. The occasional effective stimulation sites found in the central grey may well represent collaterals of fibres from the main pathway since the mid-brain central grey receives a diffuse component from the spinothalamic system and this may constitute a part of the 'mid-brain–limbic system' described by Nauta (1960) and may thus be a possible route by which information concerned in the milk-ejection reflex reaches the various parts of the limbic system which have also been implicated in the release of oxytocin (Cross, 1966; Woods *et al.* 1969). Indeed, Powell & Rorie (1967) have shown that the septum is directly connected with the PV and supraoptic nuclei and the mesencephalic central grey.

Another suggestion (Rothballer, 1966), that the medial lemniscus was concerned with oxytocin release, was supported by finding oxytocin release after stimulation of the medial lemniscus in the mid-brain of the rabbit (Cross & Silver, 1961; Holland *et al.* 1963) and in the hind-brain of a single goat (Andersson, 1951). However, in a comparative anatomical study of the medial lemniscus in the hind-brain, Verhaart (1960) showed it to be in an entirely different position in the goat from that claimed by Andersson (1951). Also, destruction of the medial lemniscus in the mid-brain or destruction of the thalamic ventroposteriorlateral (VPL) nucleus, which is the main end-relay nucleus of the medial lemniscus, does not affect lactation in the cat (Beyer *et al.* 1962) nor milk ejection in the sheep (Richard *et al.* 1970) nor does it block evoked potentials in the pituitary stalk resulting from mammary nerve stimulation in the sheep (Richard, 1970) and, moreover, stimulation of the medial lemniscus does not produce evoked potentials in the PV nucleus (Woods *et al.* 1969). In addition, in the present work only two out of a total of nine stimulation sites in the medial lemniscus resulted in oxytocin release, whereas stimulation of the spinothalamic tract consistently resulted in oxytocin release which indicates that the milk-ejection reflex takes an extralemniscal route at least through the mid-brain in the goat.

We have assumed, with reasonable justification, that the milk-ejection activity of the goat blood is due to the release of oxytocin. The guinea-pig assay method used to measure the activity is highly specific for oxytocin (Folley & Knaggs, 1965; Knaggs, 1966); the only substance likely to interfere with the assay is vasopressin, which would be extracted along with the oxytocin by the Sephadex extraction procedure. This problem, however, was overcome in the last nine experiments since the blood was extracted by the fuller's earth method in which only 5% of any vasopressin present appears in the final extract (McNeilly, 1971). Therefore, it is reasonably certain that the milk-ejection responses were mainly, if not entirely, caused by the release of oxytocin. In further support of this, plasma from one of the series of blood samples, in which stimulation of the spinothalamic tract caused oxytocin release

(7–85 μu./ml plasma), was assayed for vasopressin content by the ethanol-treated rat antidiuretic assay. None of the samples contained any detectable vasopressin (< 10 μu./ml plasma). Moreover, we have shown previously (Tindal, Knaggs & Turvey, 1968*b*) that stimulation of the milk-ejection pathway in the guinea-pig results in the preferential release of oxytocin with no detectable release of vasopressin. The volume of blood (~ 500 ml) removed during these experiments did not cause vasopressin release and hence contribute to the milk-ejection activity found in the plasma extracts, since in a related experiment (McNeilly, 1971) it was shown that during progressive haemorrhage milk-ejection activity, presumably due to vasopressin release, only appears in the blood after removal of more than 500 ml of blood.

Much confusion exists at the spinal level as to which ascending sensory system carries the information concerned in the milk-ejection reflex. In the ruminant (sheep and goat), an intact dorsal funiculus ipsilateral to the stimulated side appears to be essential for the normal functioning of the reflex (Tsakhaev, 1953; Denamur & Martinet, 1959; Popovici, 1963; Richard *et al.* 1970), which suggests the involvement of the dorsal column–medial lemniscal system. In contrast, in the rat and rabbit, section of the lateral or ventrolateral funiculus respectively on one side blocks the reflex if suckling is confined to the side ipsilateral to the lesion (Eayrs & Baddeley, 1956; Mena & Beyer, 1968) suggesting that the spinothalamic, spinotectal or spinoreticular fibres are involved. One common factor which emerges from all these studies is that in all four species the pathway at the spinal level appears to be predominantly ipsilateral to the side suckled or milked. In this connexion the pathway has been shown to be ipsilateral from the mid-brain forwards to the PV nucleus in the guinea-pig (Tindal & Knaggs, 1971) and goat (Popovici, 1963).

It is at first sight somewhat difficult to reconcile these findings with our conclusion, particularly in the goat, that the spinothalamic system is involved since at the spinal level the spinothalamic fibres are mainly crossed and run in the lateral or ventral funiculus. However, there is some anatomical and electrophysiological evidence that a proportion of the spinothalamic fibres remain uncrossed and ascend ipsilaterally in the lateral or ventral funiculi (see Rose & Mountcastle, 1959). In this connexion Richard (1970) found that potentials evoked in the pituitary stalk of the sheep by stimulation of the mammary nerve, travelled in the ventrolateral funiculi and although responses were mainly contralateral, suggesting passage in the 'classical' spinothalamic system, a well-marked ipsilateral group of fibres was also present which preserved the response in animals with hemisection of the contralateral ventrolateral funiculus. Moreover, in the rat Eayrs & Baddeley (1956) found that the main pathway was ipsilateral, although there was a contralateral component which was adequate to maintain lactation after interruption of the main path but whose destruction had only a depressing effect. The possibility exists, therefore, that the fibres concerned in the transmission of the milk-ejection reflex occur mainly in the ipsilateral component of the spinothalamic system as suggested by Eayrs & Baddeley (1956) and the contralateral fibres are only involved to a lesser extent. This would explain the predominantly ipsilateral nature of the path in the lateral column of the spinal cord in the rat (Eayrs & Baddeley, 1956), rabbit (Mena & Beyer, 1968) and sheep (Richard, 1970). However, it does not explain the complete blockage of milk ejection by section of the ipsilateral dorsal funiculus in the thoracic region of the goat

(Popovici, 1963) and sheep (Denamur & Martinet, 1959; Richard *et al.* 1970). Richard *et al.* (1970) concluded from their results that the spino–cervico–thalamic system was involved and proposed that whereas the spinothalamic system carried the information to the hypothalamus, the spino–cervico–thalamic system nevertheless exerted an over-riding facilitatory control on transmission at some point along its course. However, the interpretation of their results in this way is somewhat confusing since, although they claimed that dorsal-column section blocked milk ejection, due to interruption of the spino–cervico–thalamic tract, this tract in fact passes in the dorsomedial portion of the lateral funiculus and its destruction would necessarily involve damage to the lateral funiculus and possibly simultaneous destruction of spinothalamic tract fibres which would abolish the reflex.

It may, moreover, be relevant that an interaction between ascending sensory systems has been proposed by Wall (1970) in a recent revision of the classical concept of dorsal column function where he states ' Impulses ascending in the dorsal column–medial lemniscal system are involved in controlling the analysis of messages arriving over the other somatosensory systems'. Wall reviews a number of clinical cases where destruction of dorsal column only or lateral column only does not affect sensory function whereas simultaneous loss of dorsal and lateral columns abolishes function. This situation might possibly have some bearing on the transmission of information concerned in the milk-ejection reflex, particularly in the ruminant.

On the basis of known anatomical projections Richard *et al.* (1970) suggested the subthalamus or mid-brain reticular formation to be likely sites for the interaction of the two sensory systems. The VPL nucleus is certainly not involved since, although the spinothalamic, spino–cervico–thalamic and dorsal column–medial lemniscal systems all project to the VPL nucleus, lesions of this nucleus do not block milk ejection or the appearance of evoked potentials in the pituitary stalk after mammary nerve stimulation (Richard, 1970; Richard *et al.* 1970). The spinal cord might represent another possible site of interaction since at this level the lemniscal system is known to have connexions with the extralemniscal system via collateral fibres (see Albe-Fessard, 1967). Other suitable sites for the interaction of these afferent systems might be the posterior thalamic complex, particularly the medial magnocellular division of the medial geniculate body (MMg). The spinothalamic system is well known to project bilaterally to these regions (Poggio & Mountcastle, 1960; Whitlock & Perl, 1961; Mehler, 1969) and the spino–cervico–thalamic system also passes through the MMg *en route* to the VPL nucleus (Hagg & Hongchien, 1970) and might well give off collaterals to this region. In addition, the dorsal column–medial lemniscal system also projects bilaterally to the posterior thalamic complex, particularly the MMg (Davidson, 1965; Mehler, 1965; Lund & Webster, 1967) and afferents from this system and the spinothalamic system were shown to converge on the cells of these two regions. The milk-ejection reflex pathway described by us previously (Tindal *et al.* 1967, 1969) was also shown to pass in close association with the posterior thalamic complex in the area occupied by the MMg, which might be expected if, as suggested, it was associated with the spinothalamic tract. The MMg also receives projections from the superior and inferior colliculi (Altman & Carpenter, 1961; Powell & Hatton, 1969) and these might be concerned in the influence of conditional stimuli on the milk-ejection reflex. The MMg therefore, is seen as a region of heterosensory con-

vergence which might well function as a polysensory integrative area for the milk-ejection reflex.

In conclusion, we have shown that the milk-ejection pathway in the mid-brain of the rodent, lagomorph and ruminant appears to be identical and occurs in the lateral tegmentum in association with the spinothalamic tract. It remains for future work to determine which of the sensory systems at the spinal level are involved in the transmission of the afferent impulses and whether any interaction between two or more ascending systems is involved. In this respect the ruminant may well represent a special case.

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# BROMOCRIPTINE AND α-ERGOCRYPTINE DO NOT INHIBIT OXYTOCIN SECRETION IN THE LACTATING RAT

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SUMMARY

Two experiments were performed to study the effects of bromocriptine and α-ergocryptine on oxytocin secretion in lactating rats. In both experiments, after overnight separation from their litters, rats were injected with either vehicle alone or ergot alkaloid plus vehicle; 4 h later the litters were returned.

In the first experiment the mothers were conscious. Treatment did not affect suckling behaviour, number of stretch reactions or litter weight gain in the first 30 min. Oxytocin injection before the second 30 min period of suckling caused no extra milk to be obtained.

In the second experiment the mothers were anaesthetized with ethyl carbamate (1·1 g/kg body weight) at the time of the ergot alkaloid or vehicle injection. Changes in intramammary pressure were recorded during suckling. Ergot alkaloids altered neither the number of milk ejections caused by suckling, nor the proportion of milk ejections equivalent to 0·2 mu. or more oxytocin.

In both experiments treatment with ergot alkaloids suppressed secretion of prolactin.

It is concluded that (a) in suppressing lactation, bromocriptine and α-ergocryptine do not inhibit oxytocin secretion as well as prolactin secretion, and that (b) prolactin secretion is not a necessary concomitant of oxytocin secretion.

## INTRODUCTION

The ergot alkaloids, including bromocriptine and α-ergocryptine, inhibit lactation by acting as dopamine agonists on type 2 dopamine receptors in the anterior pituitary gland thereby inhibiting prolactin secretion (Flückiger & Wagner, 1968; Shaar & Clemens, 1972; Flückiger & Del Pozo, 1978; Flint & Ensor, 1979; Kebabian & Calne, 1979). It is possible that they suppress lactation by also inhibiting secretion of oxytocin since dopamine has been proposed as an inhibitory transmitter in the pathway for the milk-ejection reflex (Prilusky & Deis, 1975; Seybold, Miller & Lewis, 1978). Other experiments strongly suggest that dopamine is an excitatory transmitter in this pathway (Clarke & Lincoln, 1975; Bridges, Hillhouse & Jones, 1976; Clarke & Merrick, 1978; Clarke, Lincoln & Merrick, 1979; Moos & Richard, 1979). Ergotamine inhibits litter weight gain during a test period of suckling in the rat, probably by an action on oxytocin secretion (Grosvenor & Turner, 1956), while it has been reported, without experimental details, that oxytocin secretion is not affected by bromocriptine but is inhibited by α-ergocryptine (Flückiger, 1978). Procedures have been established for assessing oxytocin secretion in response to the suckling stimulus in lactating rats, conscious or urethane-anaesthetized, after separation from their litters overnight (Grosvenor & Turner, 1956; Wakerley & Lincoln, 1971; Wakerley, O'Neill & ter Haar, 1978; Russell, 1980). In the present study these techniques were used to investigate further the

actions of α-ergocryptine and bromocriptine on oxytocin secretion. The results show that treatment with either ergot alkaloid did not inhibit oxytocin secretion.

## MATERIALS AND METHODS

### *Animals*

Three-month-old Sprague–Dawley rats were used. Litters were adjusted to eight pups on day 2 *post partum* (day of birth = day 1). Each litter was weighed daily to check lactational performance. Experiments were performed between days 9 and 11, most on day 10 *post partum*.

### *Drugs*

Bromocriptine (CB154; Sandoz, Feltham, Middlesex; Lot 780 01) and α-ergocryptine (Sigma, Poole, Dorset; Lot 77C–0308) were weighed out in 4 mg amounts. Each portion was dissolved in 0·2 ml absolute ethanol immediately before use and 0·2 ml 0·9% saline was added to each vial just before the appropriate dose was drawn up for injection. Each rat was injected with 4 mg/kg body weight of either drug or with an equivalent volume of vehicle.

Oxytocin (Syntocinon; Sandoz, Lot 202 L7) was used freshly diluted with 0·9% saline at concentrations of 1 unit/l and 2·5 units/l in the second series or 500 units/l in the first series (see below).

### *Experimental procedure*

There were two series of experiments; in both series animals were prepared by separating all but one of the pups in each litter from their mother overnight, from 18.00 h (Burnet & Wakerley, 1976). Overnight separation of pups from the mother increases the frequency of milk ejections in the subsequent test period, probably by effects on the vigour with which the pups suck and by facilitation of the milk-ejection reflex by distension of the mammary glands with milk (Lincoln, Hill & Wakerley, 1973; Friedman, 1975). Litters were kept at a nest temperature of 33–36 °C by a heating lamp.

#### *First series*

Each lactating female rat was injected subcutaneously with a solution of bromocriptine, α-ergocryptine or vehicle at 10.00 h (six or seven animals per treatment). At 14.00 h the urinary bladder of each pup was emptied by gentle suprapubic pressure and by stroking the perineum with cotton wool. Each litter was then weighed and returned to its mother. During the next 30 min the behaviour of each litter was continuously observed to measure the intensity of the suckling stimulus and the number of milk ejections (Russell, 1980). For each litter the following were recorded: (1) latency to attachment of first pup; (2) number of pups attached each minute; (3) duration of attachment; (4) number of 'stretch' reactions of litter, each one indicating a milk ejection (Wakerley & Lincoln, 1971). In using the suckling test to measure oxytocin secretion it is important to take into account variation in the intensity of the suckling stimulus since the number of pups and duration of suckling determine whether oxytocin is secreted at all and the frequency of milk ejections (Lincoln & Wakerley, 1975; Wakerley *et al.* 1978; Russell, 1980). Clearly, an inadequate suckling stimulus, rather than any treatment of the mother, may result in failure of oxytocin secretion.

After 30 min the litter was reweighed and each mother was injected subcutaneously either with 100 mu. oxytocin in 0·2 ml 0·9% saline (three rats from each previous treatment group) or with 0·2 ml 0·9% saline (three or four rats from each previous treatment group). The bladders of the pups were again emptied, the pups reweighed and at 14.40 h returned to their mothers and observed as before for a further 30 min (observations of stretch reactions were not made in the oxytocin-injected group). The litters were then weighed again, the mothers were anaesthetized with urethane (ethyl carbamate 1·5 g/kg body weight, in 25% (w/v)

solution, i.p.) and 1 ml blood was withdrawn by cardiac puncture for measurement of prolactin concentration by radioimmunoassay.

*Second series*

Each lactating female was anaesthetized at 10.00 h with urethane (1·1 g/kg body weight, in 25% (w/v) solution, i.p.) and injected subcutaneously at 10.10 h with a solution of bromocriptine (six rats), α-ergocryptine (seven rats) or vehicle (seven rats). The rats were prepared for continuous recording of intramammary pressure (right mid-inguinal gland) according to the method of Burnet & Wakerley (1976) through a plastic cannula (Portex 2FG, outside diameter 0·63 mm), using an Endevco transducer and Washington rectilinear pen recorder. Three hours after surgery was complete (14.00 h) doses of 0·2 and 0·5 mu. oxytocin in 0·2 ml 0·9% saline were injected intravenously to check sensitivity of the preparation, the litter was attached to the nipples and recordings made for 3 h (time of attachment of eight pups was taken as zero time). Pups aged 10 days are easier to work with because they shift nipples less readily than older pups (Hall, Cramer & Blass, 1975), and a litter of eight pups provides an optimally effective suckling stimulus, provided that the mother has previously nursed a litter of similar size (Wakerley *et al.* 1978).

The main variable recorded was the number of milk ejections per h, each characterized by an abrupt increase in intramammary pressure due to secretion of oxytocin (Lincoln *et al.* 1973). Injecting graded doses of oxytocin soon after or before each milk ejection to assay oxytocin secretion risks interfering with the measurement of frequency. To obtain an indication of the amount of oxytocin secreted per milk ejection, as well as the frequency, an approach modified from that of Wakerley *et al.* (1978) was used. Mid-way through the observation period 0·2 mu. oxytocin in 0·2 ml 0·9% saline was injected intravenously; each change in intramammary pressure due to endogenous oxytocin secretion was compared with the response to this dose and recorded as equivalent either to 0·2 mu. or more or to less than 0·2 mu. oxytocin. The proportion of each type of milk ejection was calculated for each animal. For most litters the occurrence or absence of a stretch or pushing reaction (Drewett, Statham & Wakerley, 1974) with each milk ejection was recorded. At the end of the experiment 1 ml maternal venous blood was withdrawn from the right atrium for measurement of prolactin concentration by radioimmunoassay. Increased concentration of prolactin in plasma of lactating rats anaesthetized with urethane depends on attachment of pups and is not an effect of urethane (Burnet & Wakerley, 1976).

*Prolactin radioimmunoassay*

Plasma was separated from heparinized blood samples immediately after withdrawal and stored at −18 °C. Concentration of prolactin was measured by a previously described method using reagents provided by the NIAMDD, Maryland, U.S.A. (McNeilly, Sharpe, Davidson & Fraser, 1978). The results are expressed in terms of the NIAMDD reference preparation RP-1.

Both experiments depended on treatment with the ergot alkaloids being timed so that the drugs would be absorbed and reach type 2 dopamine receptors before the suckling stimulus was applied, but would not affect the amount of milk stored in the mammary glands or lactogenesis (Flückiger & Del Pozo, 1978). Suppression of prolactin concentrations in maternal blood confirmed that the ergot alkaloids had reached type 2 dopamine receptors, at least in the anterior pituitary gland (Kebabian & Calne, 1979), while a milk yield similar to that in untreated controls during the suckling test confirmed that milk secretion had not yet been affected.

*Statistics*

Non-parametric methods were used for some comparisons (Kruskal–Wallis test, chi-squared test or calculation of Spearman's rank correlation coefficient, $r_s$); other data were compared by paired *t*-test or analysis of variance.

## RESULTS

### *First series: conscious lactating rats*

The results are shown in Table 1.

*First 30 min period of suckling*

Treatment with bromocriptine or α-ergocryptine did not affect significantly the latency to attachment of pups to the nipples, the duration of suckling or the mean number of pups suckled per min (analysis of variance).

Treatment with ergot alkaloids did not affect litter weight gain (analysis of variance) or the number of litter behavioural ('stretch') reactions (Kruskal–Wallis test).

Litter weight gain was not correlated with the number of stretch reactions for any group nor for all groups combined ($r_s = 0{\cdot}40$).

Two litters in the α-ergocryptine group showed no stretch reactions and gained 0·0 and 1·35 g respectively. The mothers of these litters took 23 and 27 min to adopt the nursing posture and to suckle six or more pups but they and their litters were unexceptional in the second 30 min period. For the other animals in the three groups the times to reach these criteria were $4{\cdot}7 \pm 2{\cdot}43$ (S.D.) min (controls), $2{\cdot}5 \pm 2{\cdot}07$ (bromocriptine) and $2{\cdot}8 \pm 1{\cdot}64$ (α-ergocryptine).

*Second 30 min period of suckling*

The latency to attachment of pups, the mean number of pups attached and the duration of suckling were the same in the first and second 30 min periods and were not affected by oxytocin injection (paired *t*-test). Litter stretch reactions continued without oxytocin injection.

Litter weight gain was less in the second 30 min than in the first 30 min period and was not affected significantly by oxytocin injection ($P < 0{\cdot}01$ without oxytocin, $P < 0{\cdot}05$ with oxytocin injection; paired *t*-test).

The mean percentage of the total weight increase which was gained in the second 30 min period was $31{\cdot}8 \pm 30{\cdot}76\%$ without oxytocin and $33{\cdot}0 \pm 20{\cdot}83\%$ with oxytocin injection. Because of the lack of effect of ergot alkaloid treatment on weight gain and number of milk ejections in the first 30 min period the series was not extended to include sufficient animals to analyse interaction of oxytocin injection and treatment with ergot alkaloids.

*Prolactin*

Both bromocriptine and α-ergocryptine suppressed prolactin concentration in maternal blood plasma, measured after suckling for 60 min followed by urethane anaesthesia ($P < 0{\cdot}005$; Kruskal–Wallis test).

### *Second series: urethane-anaesthetized lactating rats*

The results are shown in Table 2.

All of the rats responded to 0·2 mu. oxytocin injected intravenously, apart from two rats in the bromocriptine-treated group and one rat in the α-ergocryptine group which responded only to 0·5 mu. oxytocin; in these rats reduced sensitivity to oxytocin was not associated with fewer recorded milk ejections than the other rats.

*Milk ejections*

In only one animal (α-ergocryptine group) was there no secretion of oxytocin at all. There was no difference between the treatment groups for the total number of milk ejections in 3 h (Kruskal–Wallis test). There were no significant differences within or between groups for either of the first 2 h but the number of milk ejections in the bromocriptine-treated group in the third hour was less than in either the first or the second hours, and less than in the third

**Table 1.** *Conscious lactating rats whose litters were removed overnight, were injected with bromocriptine (4 mg/kg body wt, s.c.), α-ergocryptine (4 mg/kg body wt, s.c.) or vehicle alone. Litters were returned 4 h later and behaviour monitored for 30 min followed by a second period of 30 min after oxytocin (100 mu.) or vehicle had been administered to the mothers. Values are means ±S.D. except where indicated otherwise*

| | First 30 min | | | Second 30 min (Control, bromocriptine and α-ergocryptine groups combined) | |
|---|---|---|---|---|---|
| Parameter measured | Vehicle (control) ($n = 7$) | Bromocriptine ($n = 6$) | α-Ergocryptine ($n = 7$) | Oxytocin 10 min before suckling ($n = 9$)† | Vehicle only 10 min before suckling ($n = 11$)‡ |
| Litter weight gain (g) | 7·73 ± 3·07 | 8·82 ± 3·67 | 5·41 ± 3·36 | 3·88 ± 2·65* | 2·05 ± 1·14** |
| No. of litter stretch reactions, median (range) | 5 (2–7) | 6 (3–10) | 6 (0–9) | — | 4 (3–13) |
| Latency to pup attachment (min) | 2·7 ± 1·98 | 1·7 ± 1·21 | 4·4 ± 5·06 | 1·7 ± 0·87 | 4·8 ± 5·23 |
| Average no. of pups attached/min suckling | 7·1 ± 0·61 | 7·7 ± 0·24 | 6·3 ± 1·72 | 7·0 ± 1·06 | 7·4 ± 0·50 |
| Duration of suckling (min) | 24·6 ± 3·78 | 28·2 ± 1·47 | 24·4 ± 4·99 | 26·8 ± 2·99 | 25·2 ± 5·23 |
| Concn. (ng/ml) of prolactin in plasma, sampled at end of second 30 min under urethane anaesthesia, median (range) | 245 (144–322) | 16*** (15–35) | 23*** (11–57) | | |

*$P < 0·05$, **$P < 0·01$ compared with first 30 min (paired $t$-test); ***$P < 0·005$ compared with control group (Kruskal–Wallis).
‡, † Three or four and three rats respectively from each previous treatment group.

Table 2. *Lactating rats were anaesthetized with urethane and injected with bromocriptine (4 mg/kg body wt, s.c.), α-ergocryptine (4 mg/kg body wt, s.c.) or vehicle alone. Suckling was recommenced 4 h later, and intramammary pressure was recorded for 3 h. Oxytocin (0·2 mu. i.v.) was administered midway through the suckling period. Values are median and range is in parentheses*

| Parameter measured | Vehicle (control) ($n = 7$) | Bromocriptine ($n = 6$) | α-Ergocryptine ($n = 7$) |
|---|---|---|---|
| No. of milk ejections | | | |
| Total in 3 h | 10 (1–25) | 12 (3–15) | 16 (0–27) |
| Hour 1 | 1 (0–10) | 4·5 (2–7) | 5 (0–13) |
| Hour 2 | 4 (0–9) | 5·5 (1–7) | 6 (0–11) |
| Hour 3 | 4 (0–7) | 0·5* (0–4) | 4 (0–8) |
| Milk ejections equiv. to 0·2 mu. or more oxytocin (%) | 71 (0–100) | 70 (25–100) | 42 (26–100) ($n = 6$) |
| Proportion of rats | | | |
| With no milk ejections in first 2 h | 1/7 | 0/6 | 1/7 |
| With milk ejections in first 2 h only | 1/7 | 4/6 | 0/7 |
| Concn. (ng/ml) of prolactin in plasma at end of 3 h | 98 (18–439) ($n = 6$) | 29** (14–54) | 29** (12–97) |

*$P < 0·05$ compared with bromocriptine-treated group in hours 1 and 2 and $P < 0·025$ compared with control and α-ergocryptine-treated groups in hour 3; **$P < 0·025$ compared with control group (Kruskal–Wallis).

hour for the control and α-ergocryptine-treated groups ($P < 0·05$ and $P < 0·025$ respectively; Kruskal–Wallis test). Treatment with ergot alkaloids did not affect the proportion of milk ejections equivalent to 0·2 mu. or more oxytocin (Kruskal–Wallis test). Litter stretch reactions accompanied 71% of these milk ejections ($n = 112$) but only 46% of milk ejections equivalent to less than 0·2 mu. oxytocin ($n = 80$). These proportions were significantly different from each other ($P < 0·01$; chi-squared test); since behavioural reactions were recorded for only 12 litters (three to five litters per treatment group) these data have not been analysed further.

*Prolactin*

Concentration of prolactin in maternal plasma after suckling for 3 h was significantly lower in the groups treated with ergot alkaloids ($P < 0·025$; Kruskal–Wallis test).

## DISCUSSION

The relationship between the suckling stimulus and secretion of oxytocin in the lactating rat, conscious or anaesthetized with urethane, has been described fully previously (Lincoln *et al.* 1973; Drewett *et al.* 1974; Wakerley *et al.* 1978; Russell, 1980).

### *Conscious lactating rats*

Observation of behavioural ('stretch') reactions of a litter detects some milk ejections equivalent to 0·125 mu. oxytocin and all milk ejections equivalent to 0·3 mu. or more

oxytocin (Vorherr, Kleeman & Lehman, 1967). Neither bromocriptine nor α-ergocryptine affected the number of such milk ejections and the median number of milk ejections was similar to that in previous studies (Lincoln *et al.* 1973).

Weight gain of a litter during a period of suckling has been used frequently to study factors which affect oxytocin secretion, although the amount of milk obtained decreases with each milk ejection (Lincoln *et al.* 1973). Litter weight gain and number of milk ejections in the present experiment were not correlated. Since bromocriptine or α-ergocryptine affected neither litter weight gain nor number of observed milk ejections this is not important, but it is possible that in other experiments lack of effect of treatment on weight gain could conceal a change in the number of milk ejections. The insignificant effect of oxytocin treatment on litter weight gain in the second 30 min period supports the conclusion that milk let-down in the first 30 min period was not inhibited by prior treatment with ergot alkaloids.

*Urethane-anaesthetized lactating rats*

From a study of 425 rats, three patterns of milk ejection have been distinguished; first, in 65% of these rats a regular pattern of milk ejections continuing for several hours, secondly, in 20% no milk ejections, and thirdly, in 14% a regular pattern begins but then milk ejections stop (Tribollet, Clarke, Dreifuss & Lincoln, 1978). The control group used in the present study fitted this description, although others have found only the first pattern (Wakerley *et al.* 1978). Considering all three treatment groups, 65% of the rats in the present study began milk ejections in the first hour and continued for 3 h. The only effect of treatment on the number of milk ejections was a decrease in the third hour in the group treated with bromocriptine. However, four rats in this group could be classed as showing a type three pattern, and similarly one animal in the control group; these incidences were not significantly different from each other (chi-squared test), so the decreased number of milk ejections in the third hour may not have been caused by bromocriptine. The results of comparing each milk ejection with the effects of injecting 0·2 mu. oxytocin indicated that treatment with ergot alkaloids did not alter the amount of oxytocin secreted per milk ejection. The acute stimulatory effect of bromocriptine injected into a lateral cerebral ventricle increases frequency of milk ejections rather than their amplitude (Clarke *et al.* 1979). The relationship between the incidence of stretch reactions of the litters and the size of the mammary gland pressure responses is consistent with the findings of Vorherr *et al.* (1967), although Tribollet *et al.* (1978) reported that, in urethane-anaesthetized rats, usually all milk ejections are accompanied by stretch reactions.

The results from both conscious and urethane-anaesthetized rats show that neither bromocriptine nor α-ergocryptine inhibits oxytocin secretion stimulated by suckling. The inhibitory effect of L-DOPA on litter weight gain, and presumably oxytocin secretion, during the suckling test (Prilusky & Deis, 1975) is thus not mediated by type 2 dopamine receptors (Kebabian & Calne, 1979). Dopamine receptors in the neuro-intermediate lobe of the pituitary gland are thus either not type 2 receptors or are not involved in inhibitory control of oxytocin secretion (Holzbauer, Sharman & Godden, 1978).

It has been shown previously that urethane anaesthesia does not prevent the suckling stimulus from increasing prolactin secretion (Burnet & Wakerley, 1976; Wakerley *et al.* 1978). In the present study, treatment with ergot alkaloids inhibited prolactin secretion, as expected; it follows that prolactin itself does not affect oxytocin secretion, so the stimulatory effect of prolactin on hypothalamic dopamine secretion cannot be important for oxytocin secretion (Gudelsky & Porter, 1979). Conversely, although it is unlikely that oxytocin stimulates prolactin secretion (McNeilly & Friesen, 1978), oxytocin secreted in response to suckling did not reverse the inhibitory effects of the ergot alkaloids on prolactin secretion.

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# Release of oxytocin and prolactin in response to suckling

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**Abstract**

**The oxytocin and prolactin responses to suckling were measured in 10 women in early (n=5) and established lactation (n=5). Oxytocin was released in a pulsatile manner during suckling in all women, but the response was not related to milk volume, prolactin response, or parity of the mother. In all 10 women plasma oxytocin concentrations increased three to 10 minutes before suckling began. In five women this occurred in response to the baby crying, in three it coincided with the baby becoming restless in expectation of the feed, while in two it corresponded with the mother preparing for the feed. There was no prolactin response to stimuli other than stimulation of the nipple associated with suckling.**

**These results clearly indicate that the milk ejection reflex, with release of oxytocin, occurs in most women before the tactile stimulus of suckling. A second release of oxytocin follows in response to the suckling stimulus itself. Thus it is important that care is taken to protect breast feeding mothers from stress not only during suckling but also immediately before nursing, when conditioned releases of oxytocin will occur.**

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## Introduction

The milk ejection reflex, in which oxytocin is released in response to suckling, appears to be essential for the baby to take milk from the breast.[1] Despite this few studies on changes in blood oxytocin concentrations in response to suckling have been reported. Earlier studies in which intramammary pressure was recorded indicated that the rhythmic waves of intramammary pressure during suckling were best mimicked by discrete injections of oxytocin rather than constant infusions.[2] This indicated that, as in animals,[3] oxytocin is released in a pulsatile manner during suckling. This was substantiated by measurement of oxytocin in plasma in some studies[4 5] but not in others.[6 7]

Lucas *et al* suggested that release of oxytocin may not be essential for satisfactory flow of milk during breast feeding because no oxytocin could be detected during suckling in some women.[5] This conclusion must be viewed with extreme caution. In animals and women the milk ejection reflex may become conditioned to stimuli, such as the cry of the baby, as well as the physical action of suckling.[1 3] Indeed, spontaneous ejections of milk occurring at regular intervals independent of suckling have been reported.[8] No studies have investigated the patterns of release of oxytocin induced by stimuli occurring before suckling, and all previous investigations have been confined to mothers in the immediate postpartum period. Only a few reports are available correlating release of oxytocin to milk yield[5] or the prolactin response to suckling.[6 7]

We undertook the present study to measure the responses of oxytocin and prolactin before and during breast feeding in mothers in early (four to six days post partum) and established lactation (four and 10-11 weeks post partum).

## Patients and methods

We studied the responses of oxytocin and prolactin to suckling in 10 volunteers who were breast feeding after delivering mature infants of normal birth weight in the Simpson Maternity Pavilion, Edinburgh. The table gives clinical details. Patients were studied only once: five were studied four to six days post partum, three four to five weeks post partum, and two 10 to 11 weeks post partum. Full informed consent was obtained from all the women before their inclusion in the study. All patients were feeding on demand, giving about six feeds in 24 hours, with feeds lasting four to 10 minutes on each breast. None of the patients had introduced any supplementary or complementary food.

All the observations were made during a feed occurring around

1200, a catheter being placed in a forearm vein 40 minutes before the expected time of suckling. In all cases the baby remained with the mother, although before suckling it was held by one of the laboratory assistants helping with the blood collection. Blood samples (10 ml) were withdrawn over 25-35 s into an ice cooled syringe and the samples transferred to lithium heparin tubes in ice. Plasma was separated by centrifugation at 4°C and stored in two separate aliquots at −20°C until assayed. Blood samples were taken 15 minutes before

*Parity of mother, weight of baby at time of suckling, test weight of feed, and maximum plasma oxytocin concentration during sampling period and time when this occurred in relation to suckling*

| Case No | Parity | Age of baby (days) | Weight of baby (g) | Test weight (g) | Maximum plasma oxytocin (ng/l) | Oxytocin maximum before or during suckling |
|---|---|---|---|---|---|---|
| 1 | 2 | 4 | 3650 | 60 | 43 | Before |
| 2 | 1 | 4 | 4158 | 52 | 11 | Before |
| 3 | 2 | 4 | 4180 | 43 | 29 | Before |
| 4 | 1 | 5 | 4380 | 42 | 11 | Before |
| 5 | 1 | 6 | 3350 | 82 | 59 | During |
| 6 | 1 | 28 | 4448 | 48 | 20 | Before |
| 7 | 2 | 33 | 4400 | 73 | 29 | During |
| 8 | 2 | 37 | 5600 | 161 | 12 | Before |
| 9 | 1 | 75 | 7100 | 76 | 25 | During |
| 10 | 2 | 81 | 6473 | 165 | 50 | Before |

the start of suckling and then at one minute intervals from 10 minutes before until suckling ended 10-16 minutes after the start. A further sample was collected 10 minutes after the end of suckling. Because blood was being collected through a catheter that was led away from the mother the sampling procedure did not appear to intrude on the breast feed.

The volume of each feed was assessed by test weighing the baby before and after the feed with an electronic integrating balance as described previously.[9] Oxytocin was measured by radioimmunoassay[10] after extraction of the plasma using Sep-pak cartridges (Waters). Briefly, this entails absorption of acidified plasma samples on to C-18 Sep-pak cartridges, washing with 2 ml 0·1 mol formic acid, and eluting the oxytocin with 5 ml 60% acetonitrile in 0·1 mol formic acid. The eluates were dried under a stream of air and stored at −20°C until radioimmunoassay, when they were reconstituted in assay buffer. Recovery of oxytocin averaged 74 ± SEM 3% (n = 7), and the results were corrected accordingly. Plasma prolactin concentration was measured by a specific radioimmunoassay[11] using reagents supplied by Professor H G Friesen, Winnipeg, Canada. The interassay variation was 7·9%; results were expressed in terms of units of MRC pituitary prolactin standard 75/504/1.

## Results

*Pattern of oxytocin release*—Throughout the study oxytocin was detectable in most (226/240) samples (range 1·1-50·3 ng/l); in only

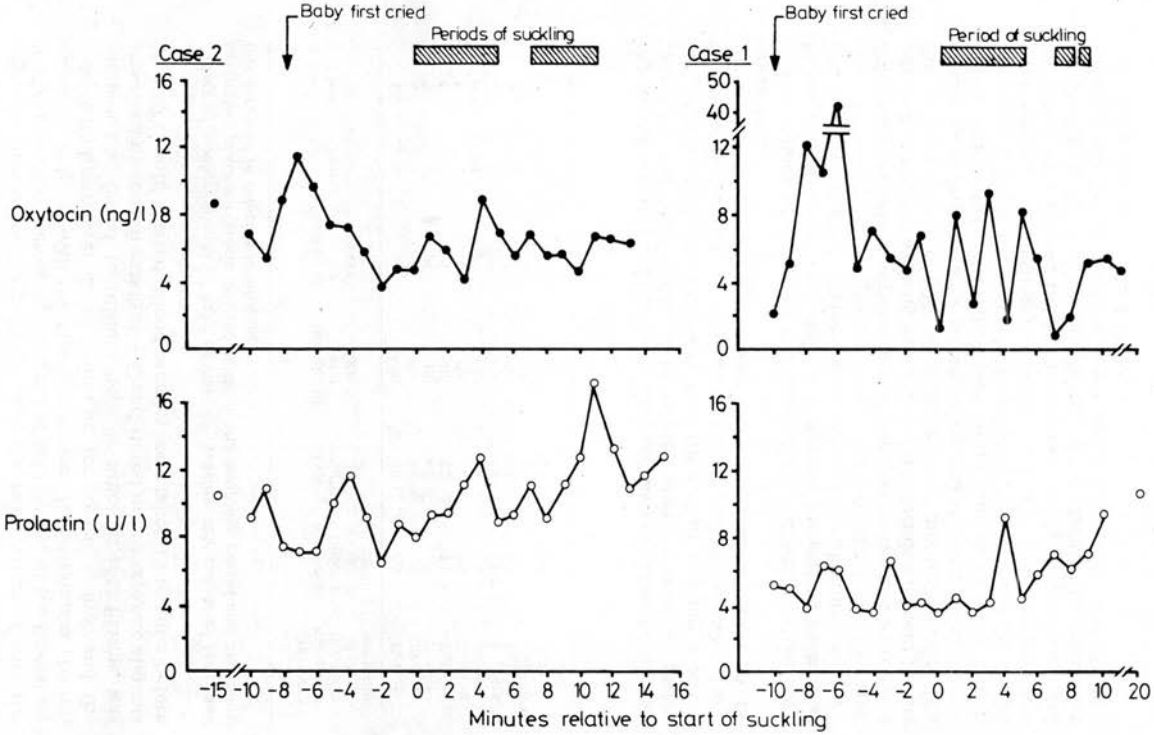



FIG 1—Changes in plasma oxytocin (●) and prolactin (○) concentrations before and during suckling in two women on day 4 post partum.

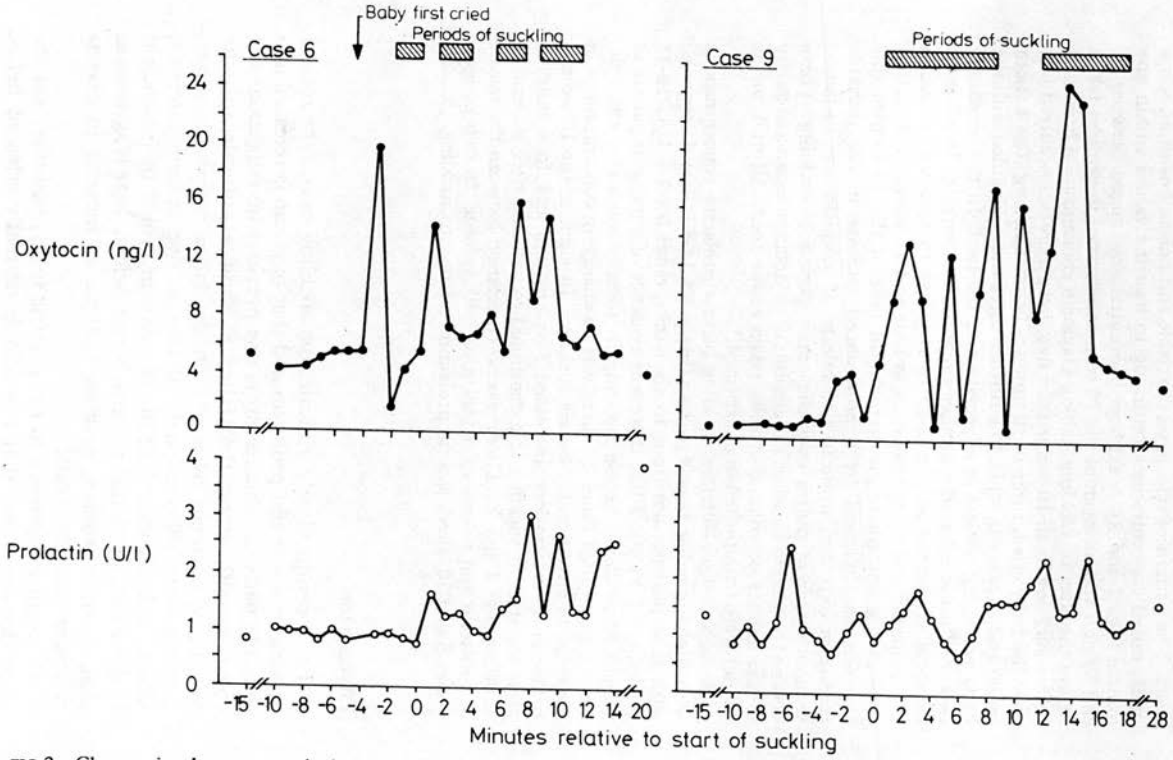



FIG 2—Changes in plasma oxytocin (●) and prolactin (○) concentrations before and during suckling 28 days (case 6) and 75 days (case 9) post partum.

14 were the concentrations below the sensitivity (< 1·0 ng/l) of the assay. The pattern of release was variable and pulsatile with, in some cases, raised concentrations returning to baseline values within one minute (figs 1 and 2). Maximum concentrations ranged between 11 and 59 ng/l and in seven of the 10 women occurred in samples taken before the start of suckling (table). Oxytocin concentration increased before suckling in all 10 women. In five these increases occurred just after the baby cried, three to 10 minutes before suckling (figs 1 (both cases) and 2 (case 6)), while in a further three they coincided with the baby becoming restless in expectation of the feed. In the remaining two they occurred as the mother prepared for nursing. In all cases oxytocin concentration increased in response to suckling, although this response was more consistent at four and 11 weeks post partum than in the immediate puerperium. In nine of the 10 women this increase was followed by a pronounced increase in the pulsatile release of oxytocin, while in one release of oxytocin was sustained without apparent pulses over the entire period of suckling. There was no correlation between the amount of oxytocin released and parity of the mother or volume of milk taken at the feed. All milk yields (table) were within the range expected.[9]

*Prolactin release*—Before suckling plasma prolactin concentrations were high immediately post partum (range 3·4-11·6 U/l) and decreased with time post partum (four to six weeks, range 0·7-1·4 U/l; 10-11 weeks, 0·6-1·3 U/l). While there was evidence of a pulsatile pattern of release of prolactin before suckling in some women (for example, cases 2 and 9 (figs 1 and 2)) there was no change in concentrations in response to any stimulus before suckling. In eight of the 10 women prolactin concentrations increased between one and four minutes after the start of suckling and continued to increase after the end of suckling (figs 1 and 2). There was no correlation between the release of prolactin and release of oxytocin or milk volume. In two patients (cases 5 and 10) there was no prolactin response to suckling.

## Discussion

These results clearly indicate that suckling causes the release of both oxytocin and prolactin and that oxytocin is released in a pulsatile pattern. This confirms one previous investigation[5] but contrasts with others that failed to show a pulsatile episodic pattern of oxytocin release.[6 7] In the latter reports, however, samples were collected only at two to five minute intervals, which, with the short half life of oxytocin, would be insufficient to show such a pulsatile pattern. Nevertheless, there is agreement that, in most women, suckling induces an increase in plasma oxytocin concentrations.

Of major importance in the present study, however, was the clear demonstration that oxytocin is normally released before the start of suckling. Lucas *et al*[5] suggested that, because they failed to detect any change in oxytocin concentrations in three

of their 10 patients during suckling and because the timing of the release of oxytocin did not always coincide with peak milk flow, release of oxytocin may not be essential for satisfactory transfer of milk from mother to child. In their study, however, insufficient samples were taken before the baby had fixed on the breast to determine whether release of oxytocin before suckling had occurred. From our results it appears that release of oxytocin occurs in most women before suckling begins. Possibly the primary function of sucking by the baby is removal of milk while release of oxytocin is a secondary effect. This has been reported in other species.[1 2] In women spontaneous ejection of milk may occur independently of suckling,[8] and this has been recognised by mothers for generations. Our failure to find a correlation between the amount of oxytocin released and the volume of milk may not be surprising. The amount of milk removed depends not necessarily on the volume of milk present in the breast but on the amount that the baby wishes to take. This, and the rate at which the milk is taken, varies widely between subjects,[12] and our failure to find a correlation between release of prolactin and milk volume confirms our previous report.[9] Indeed, the lack of a response of prolactin to suckling in two out of 10 women confirms other observations (A Glasier, A S McNeilly, and P W Howie, unpublished observations) that the response of prolactin to suckling in the morning is considerably reduced or even absent compared with that in the afternoon or evening. We also failed to see any response of prolactin to stimuli other than direct stimulation of the nipple, confirming previous reports.[13]

The present results support the concept that the milk ejection reflex, including release of oxytocin, is essential for the transfer of milk from mother to baby in breast feeding. An important part of this reflex is a conditioned release of oxytocin that occurs before the start of suckling. Ejection of milk associated with suckling may be inhibited by both physical and psychological stress, and women are more susceptible to these stresses in early lactation.[1 14] Clearly, care must be taken to protect breast feeding mothers from stress not only at the time of suckling but also immediately before nursing, when conditioned releases of oxytocin may occur.

Requests for reprints should be sent to Dr A S McNeilly.

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# Spontaneous milk ejection during lactation and its possible relevance to success of breast-feeding

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**Summary and conclusions**

**In a woman suckling twins it became apparent that both suckling-induced and precisely timed, spontaneous bursts of milk ejection were occurring. Observations on days 14, 28, 56, and 112 of lactation disclosed highly significant increases in intervals between episodes of spontaneous milk ejection. Furthermore, at all stages of lactation the interval between a feed and the next episode of spontaneous ejection was significantly longer than the interval between spontaneous ejections.**

**The decrease in frequency of episodes of spontaneous milk ejection during lactation may be related to the decreasing release of prolactin in response to suckling. Spontaneous milk-ejection episodes are felt only when the breast is full and may signal its readiness for a further suckling episode. Such bursts of milk ejection may stimulate the suckling response in babies, suggesting that rigid three- or four-hour feeding regimens may be unphysiological and pose a threat to the success of breast-feeding in the early postnatal period.**

## Introduction

In recent years there has been a resurgence of interest in breast-feeding. An increasing number of women start to breast-feed

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their babies, but while many continue to breast-feed for several months a large proportion stop within a few weeks of leaving hospital.[1 2] The reasons for stopping breast-feeding are many and varied but we do not know how often it is due to failure or inadequacy of normal physiological mechanisms.[2] Lactation comprises two separate physiological mechanisms—namely, milk secretion, which is controlled by prolactin released from the anterior pituitary in response to the suckling stimulus; and milk ejection, a neuroendocrine reflex in which oxytocin released from the posterior pituitary in response to suckling causes contraction of the alveoli of the breast and ejection of milk via the mammary ducts and nipple. Whereas the role of prolactin in lactation has received much attention,[3-5] that of oxytocin and the factors controlling milk ejection have been virtually ignored.[6-10]

Milk ejection during suckling may be inhibited by both physical and psychological stress, and women are more susceptible to these stresses during early lactation.[11] Newton and Newton's[11 12] classical study also showed that successful breast-feeders exhibit a greater milk-ejection response to suckling than unsuccessful breast-feeders, again emphasising the importance of oxytocin release in the success of breast-feeding.

Oxytocin release occurs in response not only to stimulation of the nipple during suckling[8 9] but also to other factors associated with breast-feeding—for example, the cry of the baby.[6 7 10] In addition, in anaesthetised rats, whose young are continually on the nipples, oxytocin release appears to occur spontaneously at precisely timed intervals independent of any suckling stimulus; the pups suckle only after spontaneous milk ejection has occurred.[13] This phenomenon has not been reported in other species. During normal lactation in a woman who was suckling twins it became apparent that both suckling-induced and precisely timed spontaneous milk ejections were occurring. We describe these findings and discuss their relevance to the management of mothers who wish to breast-feed.

## Observations

We made our observations during the third lactation of a normal, healthy woman after she had given birth to twins. She had successfully breast-fed her first and second children for nine and seven months. The twins were fed on demand, one at a time; each was allowed to suckle one breast only, the babies changing sides at each feed. Throughout the study period each baby suckled the breast for a mean of $13 \pm SD$ 3 minutes (20 observations) at a time, so that both babies were fed in 30 minutes. During every feed, for each twin milk let-down occurred 10-30 seconds after the start of suckling. In addition, however, episodes of spontaneous milk ejection occurred at predictable intervals between feeds, the sensation being identical with the mammary response experienced during feeding. This spontaneous milk ejection was never associated with any stimulus related to the babies. While some leakage of milk occurred from the unsuckled

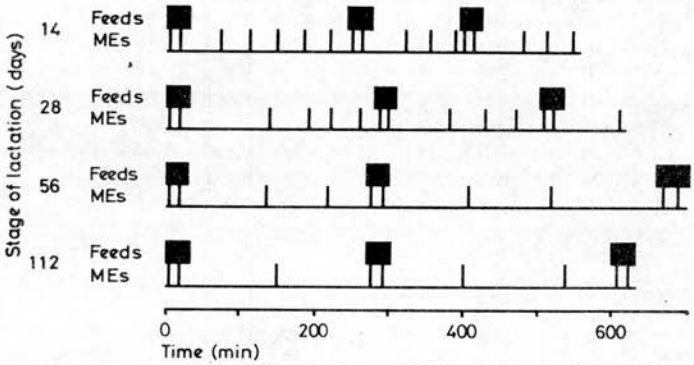
*Mean intervals (± SE of mean) between episodes of spontaneous milk ejection and between breast-feeding and next spontaneous milk ejection, and numbers of feeding periods and suckling-induced milk ejections in 24 hours. (Observations on woman breast-feeding twins)*

| | Stage of lactation (days) | | | |
|---|---|---|---|---|
| | 14 | 28 | 56 | 112 |
| Interval in minutes between spontaneous milk-ejection episodes (No of observations)* | 34·3 ± 0·9 (8) | 44·8 ± 6·3 (7) | 87·5 ± 8·5 (8) | 115·5 ± 6·6 (6) |
| Interval in minutes between breast-feeding and next spontaneous milk ejection (No of observations)† | 56·4 ± 5·4 (5) | 81·3 ± 10·1 (5) | 111·3 ± 9·4 (7) | 162·5 ± 24·6 (6) |
| No of feeding periods/24 hours | 6 | 6 | 5 | 5 |
| No of suckling-induced milk ejections/24 hours | 12 | 12 | 10 | 10 |

*Increase in time at each stage of lactation differs significantly ($0·05 > P > 0·001$) from previous interval.
†Interval to first milk ejection after breast-feeding is significantly greater ($P < 0·01$) than between spontaneous milk ejections and increases significantly ($P < 0·01$) as lactation advances.

breast in response to the first suckling episode of a feed, no such leakage occurred from the first breast when the second twin was being fed, nor during the majority of spontaneous milk ejections.

The timing of suckling-induced and spontaneous milk ejection was recorded over 48 hours on the 14th, 28th, 56th, and 112th days of lactation (table and figure). Spontaneous milk ejection occurred at




Intervals between feeds, milk ejections (MEs) associated with suckling, and spontaneous milk ejections at different stages of lactation in woman breast-feeding twins.

precise and regular intervals, which increased from 34·3±SE of mean 0·9 minute on day 14 to 115·5±6·6 minutes on day 112; this increase was highly significant at each stage of lactation ($P < 0.001$). Two suckling-induced milk ejections were observed at each feeding time, and an episode of breast-feeding delayed the onset of the next spontaneous milk ejection by intervals increasing from 22·1 minutes on day 14 to 47·0 minutes on day 112. Thus the time to first spontaneous milk ejection after a feed was significantly longer ($P < 0.01$) than the interval between spontaneous milk ejections at all stages of lactation. Possibly the double milk ejection occurring during the feeding period depletes the releasable pool of oxytocin or desensitises the hypothalamus or higher centres controlling milk release. On several occasions, however, suckling-induced milk ejection was induced only 10-15 minutes after spontaneous milk ejection (see figure). Some degree of breast distension was necessary for the mother to have the sensation of milk ejection, since she felt this only in the full breast when the second twin started suckling. (This accords with observations on rats, in which spontaneous milk ejection occurred only when there was distension of the mammary glands.[13]) Hence she may have had spontaneous milk ejection shortly after feeding but did not detect it owing to the lack of distension.

## Comment

The decrease in frequency of spontaneous milk ejection as lactation progresses, which has not been observed in rats (D W

Lincoln, personal communication), may be related to the decreasing release of prolactin in response to suckling.[3] Whether prolactin is released during these spontaneous milk-ejection episodes is not known. There is no evidence of prolactin release during lactation in response to any stimuli other than tactile nipple stimulation.[4]

The subjective observations reported here suggest that in addition to suckling-induced releases of oxytocin women have precisely timed spontaneous releases not unlike the presumptive episodic releases of luteinising hormone-releasing hormone during the menstrual cycle.[14] These spontaneous milk ejections are felt only when the breast is full, suggesting that there may be a neuroendocrine effect whereby the breast indicates its readiness for a further suckling episode. In so-called primitive societies women habitually sleep with the baby on the breast all night and carry it on the breast for much of the day.[15 16] Possibly these spontaneous bursts of milk ejection are the stimulus for the suckling response in the baby, who would therefore feed at intervals of 30-60 minutes during early lactation. This may mean that the rigid three- or four-hourly feeding regimens followed in many hospitals are unphysiological and as such may pose a threat to the initial success of breast-feeding. Milk secretion depends on the amount of prolactin released in response to suckling.[3 17 18] Thus an inadequate suckling stimulus will ultimately result in a reduced secretion of milk.[17] Since the mother's anxiety, often created by fear of being unable to lactate, will itself inhibit milk ejection, clearly she should have more psychological support. Moreover, advice on the optimal frequency of suckling should take account of physiological considerations. There is little value in having an adequate supply of milk in the breast if it is not made available to the hungry baby at the right moment.

We thank Professor R V Short and Drs D W and G A Lincoln for helpful discussions during this study.

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# Effect of breast-feeding on pituitary-ovarian function after childbirth

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## Summary

Pituitary and ovarian function at the end of pregnancy and during the first six weeks after delivery was investigated serially in women who fully breast-fed their infants and in women who did not. In the women who did not breast-feed the plasma prolactin level decreased rapidly and from the third day after delivery was significantly lower than in the breast-feeding mothers, reaching the normal range of the menstrual cycle by the thi week of the puerperium. In the breast-feeding mothe the plasma prolactin was still raised six weeks aft delivery. The levels of FSH in both groups were identi and increased over the third week of the puerperiu Plasma oestrogen fell steeply in both groups during t first two weeks after delivery. In the breast-feedi mothers plasma oestrogen remained depressed b increased in the non-lactating women, reflecting follicul development in the ovary in response to FSH; the plasr oestrogen levels were significantly higher in the no lactating women from the 17th day of the puerperiu onwards. These findings support the concept that breast-feeding women prolactin delays the return ovulation by inhibiting the ovarian response to FS stimulation.



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## Introduction

Breast-feeding postpones the return of ovulation and menstr tion after childbirth, and postpartum amenorrhoea is relate the duration of breast-feeding.[1-4] Studies on the influence lactation on ovulation have shown that breast-feeding wom

have little chance of becoming pregnant until the infant is over 10 weeks old, but in women who do not breast-feed ovulation can occur as early as the fourth week post partum.[5] To provide more information on the endocrine effects of breast-feeding we undertook a serial study of pituitary and ovarian hormones in women who fully breast-fed their infants and compared these results with the values in mothers who did not breast-feed their infants. Plasma follicle-stimulating hormone (FSH), luteinising hormone (LH), oestrogen, progesterone, prolactin, and human chorionic gonadotrophin (HCG) were studied in late pregnancy and for the first six weeks after delivery.

## Patients and methods

The study was explained to healthy women in late pregnancy and 60 women volunteered to take part. Serial studies proved feasible in 15 women who did not breast-feed, nine women who fully breast-fed up to or beyond the sixth-week after delivery, and three women who breast-fed for only three weeks. All had normal pregnancies and vaginal delivery of term infants and had no hormone or other drug treatment apart from analgesics in labour and in the first week after delivery. Three blood samples were taken between 36 and 39 weeks' gestation, and after delivery blood samples were taken on the first day, every second day for the first week, and thereafter every third day until the end of the sixth week. In the first week of the puerperium blood samples were taken between 9 and 11 am. After the patients left hospital blood samples were taken in the patient's home between 9 am and 12 noon. The blood was collected into heparinised tubes, and after separation the plasma was stored at −20°C until assayed. All the samples from each patient were assayed at the same time.

### HORMONE ASSAYS

All assays were performed in duplicate.

*Plasma LH and FSH* were measured by HCG and FSH double antibody radioimmunoassay.[6] The second international reference preparation of human menopausal gonadotrophin (2nd IRP-HMG) with a potency of 40 IU/ampoule was used as a standard. Dilutions of this standard made up in either phosphate buffer or plasma obtained from hypophysectomised subjects showed satisfactory parallelism when compared with dilutions of MRC LH 68/40 pituitary standard (potency, 40 U/ampoule LH) and postmenopausal plasma: 1 mU MRC LH 68/40 was equivalent to 5 mIU 2nd IRP-HMG. The antibody to FSH showed less than 1% cross-reaction with purified LH. The sensitivity for the LH assay was 1 mIU 2nd IRP-HMG/ml and 2 mIU 2nd IRP-HMG for the FSH assay.

*Plasma total oestrogens and progesterone* were estimated by a modification of the radioimmunoassay method of Hotchkiss.[7] The antibody used in the total oestrogen assay was raised against oestradiol-17β and had the following cross-reactions: 100% oestradiol-17β, 40% oestradiol-17α, 35% oestrone, and 8% oestriol. The only substance found to cross-react with the progesterone antibody was deoxycorticosterone (25%); all others tested were <0·5%.

*Plasma prolactin* was measured by a homologous double antibody radioimmunoassay using highly purified human prolactin for standards and iodination, as described elsewhere.[8] The sensitivity for the prolactin assay was 1·6 ng.

*Plasma HCG β-subunit* was estimated by a double antibody radioimmunoassay similar to that used for plasma prolactin.[9] The antibody used (National Pituitary Agency, NPA-SB6) was specific for HCG-β (100%) and cross-reacted with HCG-α (3%) only. All other hormones and subunits showed <0·1% cross-reaction.

*Analysis of results*—Logarithmic transformation of the plasma FSH, LH, HCG, and oestrogen results was used for statistical analysis with Student's *t* test

## Results

The mean plasma levels of prolactin, FSH, and oestrogen in the mothers who breast-fed for at least six weeks and the non-lactating mothers are shown in fig 1. At the 37th-38th week of pregnancy plasma prolactin, FSH, and oestrogen were similar in both groups. After delivery the plasma prolactin level in the non-lactating mothers decreased rapidly and was significantly lower than in the breast-feeding women from the third day of the puerperium. The plasma prolactin in the non-lactating women fell to the range of the normal menstrual cycle by the end of the third week of the puerperium: eight of the 15 women had prolactin levels below 25 μg/l by the end of the second week after delivery. In the lactating women the prolactin levels were still raised at the sixth week.

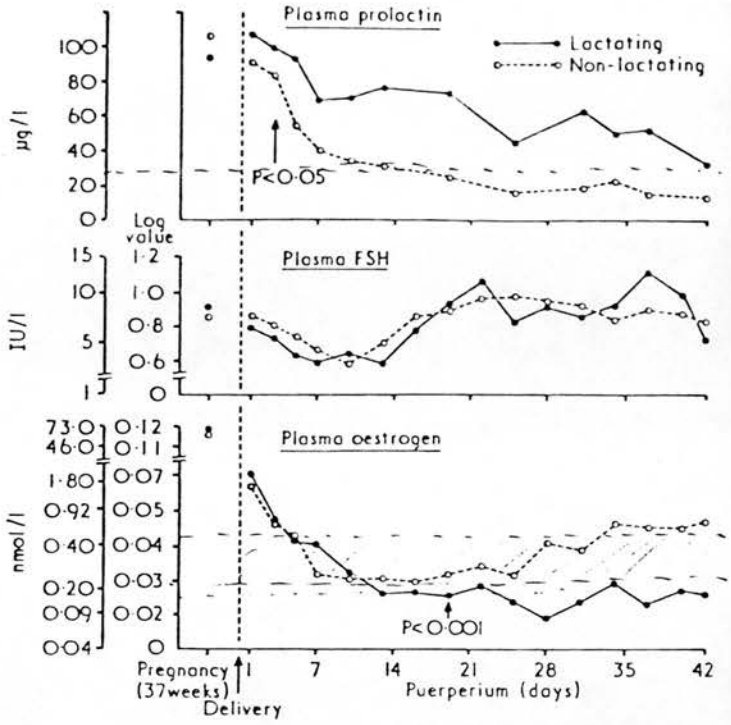



FIG 1—Comparison of serial levels of plasma prolactin, FSH, and oestrogen in last three weeks of pregnancy and first six weeks after delivery in nine mothers who fully breast-fed and 15 non-lactating mothers. Values for plasma FSH and plasma oestrogen were not a normal distribution and logarithmic transformation of data was used for statistical analysis.

*Conversion: SI to Traditional Units*—Oestrogen (oestradiol): 1 nmol/l ≈ 27·2 ng/100 ml.

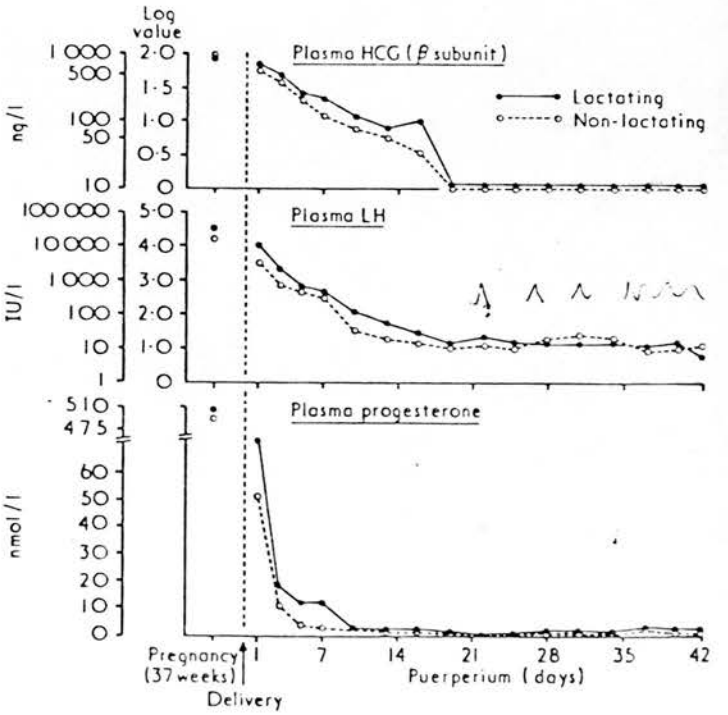



FIG 2—Comparison of plasma HCG (β-subunit), LH, and progesterone in nine lactating and 15 non-lactating mothers.

*Conversion: SI to Traditional Units*—Progesterone: 1 nmol/l ≈ 0·32 ng/ml.

The levels of plasma FSH in both groups were almost identical and fell during the first two weeks of the puerperium and increased from the 14th to the 21st day after delivery to levels in the upper limit of the range for the follicular phase of the menstrual cycle. Plasma oestrogen in both groups fell steeply during the first two weeks after delivery and remained depressed in the patients who were breast-feeding. In the non-lactating group oestrogen levels increased from the third week onwards and were significantly higher that in breast-feeding women from the 17th day of the puerperium.

The mean plasma levels of HCG (β-subunit), LH, and progesterone are shown in fig 2. The levels of HCG-β were virtually undetectable by the end of the third week after delivery. The plasma LH activity followed the same pattern as HCG during the first three weeks after delivery but during the fifth week an increase of LH was found in the non-lactating group, with a rise occurring in 10 out of the 15 women; the distribution of the LH results was skewed and no significant difference from the LH levels in the breast-feeding women was found using logarithmic values. The plasma progesterone levels were similar in both groups except during the fifth week after devliery, when a slight increase of progesterone was found in three out of 12 non-lactating women and one woman who was breast-feeding.

In the three women who breast-fed for three weeks the prolactin levels were below 25 μg/l before the end of the fourth week after delivery and the oestrogen levels increased between the fifth and sixth week.

## Discussion

During late pregnancy and the first two days of the puerperium levels of prolactin around 100 μg/l were found, which accords with other reports.[10 11] Thereafter a precipitate fall occurred in the non-lactating women, the prolactin falling to the normal non-pregnant range (12-25 μg/l) by the second or third week after delivery. In the women breast-feeding for six weeks or more plasma prolactin levels slowly decreased and at the sixth week the mean level was still above normal at 34·2 μg/l with a wide range (15-74 μg/l). The range of values reflects fluctuating levels in the mother as a result of the bursts of prolactin release that accompany breast-feeding. In the non-lactating women all the prolactin levels were within the normal non-pregnant range by the end of the third week of the puerperium. During the second and third weeks after delivery plasma FSH gradually increased to follicular phase levels, which accords with the findings of others.[2 12] By the third week after delivery the levels of plasma oestrogen in the non-lactating women began to increase, while those in the women who were breast-feeding remained low.

Our findings therefore indicate that plasma oestrogens do not increase in women who are fully breast-feeding despite the rise of FSH. This supports the view that the ovaries do not respond to FSH stimulation in the presence of increased prolactin levels.[6 7] Prolactin has an affinity for ovarian tissue,[13] and thus a local action may prevent follicular development in response to FSH stimulation. In concentrations above 30 μg/l, a level below that seen in the breast-feeding women, prolactin reduces steroid hormone production from human granulosa cells in vitro.[14] When exogenous gonadotrophins are given to women who are breast-feeding no change in ovarian steroid output can be detected.[15] In the early post-partum period the LH and FSH response to gonadotrophin-releasing hormone is depressed and this lack of response is more prolonged in women who lactate.[16] The periodic bursts of prolactin release that follow suckling may also therefore have an inhibitory effect on hypothalo-pituitary function. This, together with the inhibiting effect of prolactin on the ovary, would account for the suppression of ovulation and amenorrhoea in women who breast-feed. The return of ovarian activity in the non-lactating women was delayed for one to two weeks after the prolactin levels had declined. This was suggested by the absence of a rise in the plasma oestrogens until the end of the fourth week after delivery in the non-lactating women, although plasma FSH rose between the seventh and the 14th day.

The rise of plasma oestrogen in the non-lactating women indicates ovarian follicular response, and in two of the 15 women the level increased to that of the late follicular phase. In a significant proportion of women who do not breast-feed follicular development may therefore progress to a mature Graffian follicle and ovulation occur before the sixth week after delivery. Clinical studies on the effect of lactation without supplemental feeding have indicated that lactation provides effective birth control up to the 10th week after delivery.[3 5]

Although full breast-feeding will postpone the return of menstruation and ovulation after delivery, suppression of ovulation is limited in time as the plasma prolactin decreases even with continuing lactation. Possibly pharmacologically induced increases in plasma prolactin might be valuable as a method of prolonging the suppression of ovulation in women who are breast-feeding. Increasing the prolactin level might also be of value in establishing successful lactation in women who encounter problems with breast-feeding. Despite the fact that prolactin serves many diverse functions in animals, its only confirmed action in humans is the initiation and continuance of lactation.[16] Further research may show interesting possibilities for the use of prolactin, using its action on the ovary for the spacing of pregnancies.

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# Failure of Estrogen-Induced Discharge of Luteinizing Hormone in Lactating Women

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**ABSTRACT.** Pituitary-ovarian relationships were studied in seven lactating women by measuring the basal plasma concentrations of pituitary and ovarian hormones and their responses to an estrogen provocation test at 7, 30, and 100 days after delivery. The results were compared to a similar group of seven women who did not breast feed. The first ovulation occurred in five of the nonlactating women between 43–87 days after delivery, as judged by the urinary excretion of total estrogen and pregnanediol. In all lactating women, ovarian cyclicity was suppressed for at least 150 days after delivery or until weaning. The basal concentration of PRL in lactating women was significantly higher than in the nonlactating women at all three times measured. At 30 and 100 days, the concentration of estradiol was significantly lower in the lactating women, although the basal concentrations of FSH were similar in the two groups. After an injection of 1 mg estradiol benzoate, the concentrations of FSH and LH in plasma were suppressed to a greater extent in lactating than in nonlactating women. In addition, fewer of the lactating group (one of seven and none of seven at 30 and 100 days, respectively) than the nonlactating group (two of seven and five of seven) subsequently showed a rise in the concentration of LH 58–96 h after the estrogen injection (positive feedback). These results suggest that during lactation the hypothalamic-pituitary system is more sensitive to the negative feedback and relatively insensitive to the positive feedback effect of estrogen. (*J Clin Endocrinol Metab* **49**: 500, 1979)

LACTATION is associated with a period of reduced fertility in many mammalian species (1). In the rat (2), sheep (3), and rhesus monkey (4), suckling is associated with an afferent neural stimulus which in some way alters the central control of gonadotropin secretion. It has been suggested that the elevated secretion of PRL may in addition inhibit gonadal activity directly via an effect on the ovary (5) and/or indirectly via inhibition of the pulsatile release of LH (6). There is direct evidence in the tammar wallaby that elevated levels of PRL result in both increased sensitivity of the hypothalamus to steroid feedback and decreased sensitivity of the corpus luteum to stimulation by gonadotropins (7). After delivery in women, the period of postpartum amenorrhoea is rather variable even in those who do not breast feed their babies (8). Sharman (9), on the basis of endometrial biopsies, estimated that the first episode of menstrual bleeding was anovular, suggesting an impairment of the ability of estrogen to induce a discharge of LH (positive feedback). He concluded that the general effect of lactation is to retard the resumption of ovulation.

There is very little information as to the mechanism of the suppression of ovarian cyclicity during lactation in women. After delivery, the pituitary responsiveness to an acute injection of LRF is reduced until the fourth postpartum week but becomes exaggerated from the fifth to eighth weeks (10, 11). These results, domonstrating that the pituitary is responsive at least to exogenous LRF, suggest that the lack of ovarian cyclicity in nursing mothers may be due to a failure of the hypothalamic control of pituitary gonadotropin secretion. In the present study, the basal concentrations of pituitary and ovarian hormones and their responses to an injection of estradiol benzoate (estrogen provocation) were measured in a group of nursing mothers at 7, 30, and 100 days after delivery and the results were compared to a similar group of mothers who were not lactating.

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## Materials and Methods

### *Patients*

Fourteen women with a history of regular menstrual cycles before pregnancy were recruited from the postnatal wards of the Simpson Memorial Maternity Pavilion, Royal Infirmary, Edinburgh, Scotland. Informed consent was obtained from all patients submitted to the study, approval for which had been obtained by the local ethical committee. Breast feeding was established successfully in all seven patients selected for the lactating group. Lactation was not attempted in the remaining seven patients (nonlactating) and no measures other than local support to the breast were used to suppress lactation. There

was one primiparous patient in each group and there was no significant difference in the mean age [27.9 ± 1.3 *vs.* 27.4 ± 2.1 (SE) yr], height (161 ± 2.3 *vs.* 159 ± 2.8 cm), or weight (60.1 ± 2.6 *vs.* 59.1 ± 2.4 kg) in the first postpartum week of lactating and nonlactating groups, respectively. Each patient was instructed to keep a record of any vaginal bleeding from delivery until the study was terminated.

*Basal measurements*

In all subjects, ovarian function was monitored from the fourth postpartum week until the termination of the study by measuring the excretion in the urine of total estrogen and pregnanediol per 24 h three times per week (Sunday, Tuesday, and Thursday). A further measure of the spontaneous activity of the ovary and pituitary was made by the analysis of blood samples collected every morning between 0800–1100 h for the first three days of each estrogen provocation test (see below). Heparinized blood was centrifuged within 2 h of collection, and plasma was stored at −20C until analysis.

*Estrogen provocation test*

The response to estrogen was assessed by the measurement of LH, FSH, and PRL in plasma collected for 3 days before and 4 days after the administration of estradiol benzoate. Immediately after the collection of the third basal sample, 1 mg estradiol benzoate was injected im and further blood samples were collected at 24, 48, 58, 72, 82, and 96 h. In each subject, the estrogen provocation test was repeated at 7, 30, and approximately 100 days after delivery (day of delivery = day 1). In the nonlactating group, the third test was performed in the early proliferative phase of the menstrual cycle (days 1–5) when the estrogen excretion was less than 12 μg/24 h. For this reason, the timing of the third test was approximate, varying between 74–108 days in the nonlactating group and between 91–107 days in the lactating group.

*Hormone assays*

Urinary excretion of total estrogen was measured fluorimetrically (12) and that of pregnanediol was determined by gas-liquid chromatography (13). Plasma levels of LH and FSH were determined using double antibody RIAs utilising antisera to human LH (hLH; F87/4673) and human α-FSH (hFSH; M93) and $^{125}$I-labeled hLH (IRC2) and hFSH (CPDS/15), respectively (14). The results are expressed in terms of milliunits per ml reference preparation, *i.e.* hLH MRC 68/40 (77 U/ampoule; 1 mU LH = 11.6 ng LER 907) and hFSH/hLH MRC69/104 (LER 907). The cross-reaction of hCG in the LH assay was in excess of 65% but was negligible in the FSH assay. The interassay precisions were 9% and 11% for LH and FSH, respectively.

PRL was measured by a specific double antibody RIA using reagents supplied by Professor H. G. Friesen (Winnipeg, Canada) (15, 16). The inter- and intraassay precisions was 13% and 6%, respectively. Progesterone, androstenedione, and estradiol in plasma were measured by previously described RIAs (17–19). The interassay precision of these methods was 15% for progesterone, 15% for androstenedione, and 9.7% for estradiol.

*Statistical analysis*

Changes in peripheral hormone levels within each group were analyzed for statistical significance by means of a paired *t* test. Student's *t* test was used to compare the mean values between groups.

## Results

*Pattern of menstruation and ovarian activity*

In the nonlactating group, the first episode of vaginal bleeding occurred 25–70 days after delivery. Evidence of ovulation, as indicated by measurement of plasma progesterone or urinary pregnanediol excretion, was not found in any of the six women in whom steroid measurements were available before this episode of bleeding. In the remaining subject (NL 1), no steroid measurements were available before the first episode of vaginal bleeding on day 25. The second menstruation occurred 57–101 days after delivery and was preceded by an ovulatory cycle (indicated by a rise in the excretion of pregnanediol in the urine of 2.5 mg/24 h) in four out of seven subjects (Fig. 1). In these subjects, the length of the luteal phase (11–13 days), as judged by the interval between the rise in pregnanediol excretion and the onset of menses, total estrogen peak (23–69 μg/24 h), and maximum pregnane-

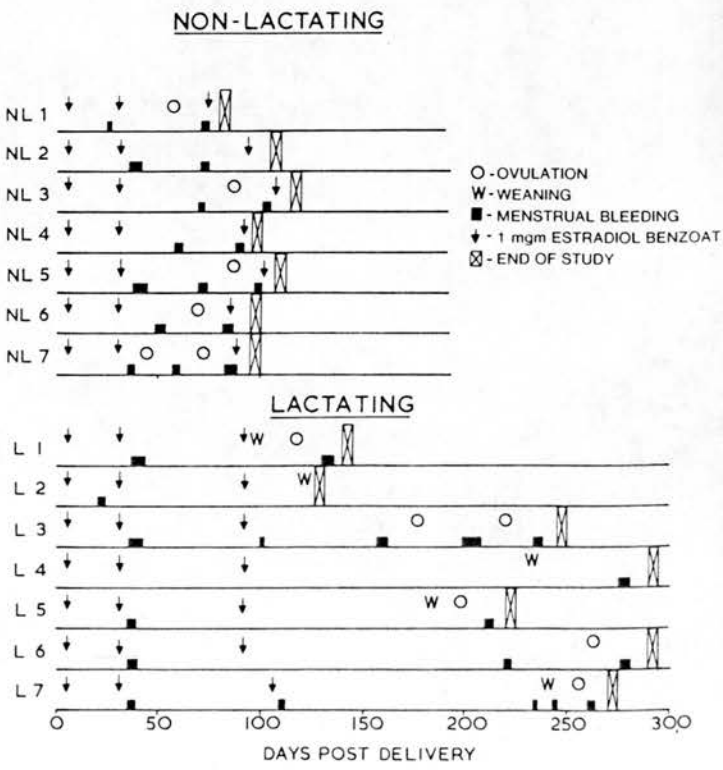



FIG. 1. Pattern of vaginal bleeding in nonlactating and lactating women after delivery. The pattern of ovarian activity was monitored from day 30 by the measurement of urinary excretion of total estrogen and pregnanediol. Estrogen provocation tests were performed as indicated by *arrows*.

diol excretion (4.2–6.5 mg/24 h) were within the range found in normal ovulatory cycles.

In one of the remaining three women (NL 5) evidence of ovulation was delayed until before the third episode of vaginal bleeding at 98 days, while the other two (NL 2 and NL 4) showed no evidence of ovulation when the study was terminated after the last estrogen provocation test (104 and 96 days, respectively). Based on menses minus 14, ovulation occurred between 43–87 days after delivery in five of the seven women in the nonlactating group.

In the lactating group, the first episode of vaginal bleeding occurred between 21–37 days after delivery. No patient in this group showed evidence of ovarian cyclicity until after weaning (*e.g.* L 1) or after at least 150 days of lactation (L 3). In two patients studied for over 250 days (L 4 and L 6), the excretion of total estrogen remained below 10 μg/24 h even though the frequency of breast feeding had dropped to two or three times per day and supplementary feeds were given.

### *Frequency of feeding*

The mean frequency (and range) of breast feeding was six (n = 5–7), five (n = 4–6), and four (n = 3–5) per day at 7, 30, and 100 days, respectively, in the lactating group.

### *Basal hormone values*

The plasma concentration of PRL was elevated in the first postpartum week (days 4–7) in both groups (Table I). In the lactating group, the basal concentration remained elevated from days 4–6 postpartum (Fig. 2). In the nonlactating group, there was a progressive fall in PRL concentration so that the basal value in the first

TABLE 1. The mean basal concentration of gonadotropins and steroids in the plasma of lactating (L) and nonlactating (N-L) women in the postpartum period

| Hormone | Days post partum | | | | | |
|---|---|---|---|---|---|---|
| | 7 | | 30 | | 100 | |
| | L | N-L | L | N-L | L | N-L |
| LH (mU/ml) | NM | NM | 3.45 ± 0.41 (21)[a] | 5.42 ± 0.82 (21) | 9.00 ± 0.97 (20)[a] | 6.67 ± 0.56 (20) |
| FSH (mU/ml) | 1.60 ± 0.05 (20) | 1.59 ± 0.04 (23) | 4.45 ± 0.33 (21) | 3.96 ± 0.38 (21) | 5.60 ± 0.41 (20) | 4.70 ± 0.39 (20) |
| PRL (ng/ml) | 172 ± 18 (21)[b] | 100 ± 11 (21) | 124 ± 19 (21)[b] | 19 ± 2 (21) | 49 ± 7 (20)[b] | 11 ± 1 (20) |
| Estradiol (pg/ml) | 61 ± 9 (21) | 58 ± 6 (21) | 37 ± 4 (20)[b] | 108 ± 16 (21) | 53 ± 6 (17)[b] | 125 ± 15 (18) |
| Progesterone (ng/ml) | 1.54 ± 0.16 (7) | 1.2–18.9[c] (7) | 0.53 ± 0.07 (6) | 0.70 ± 0.07 (7) | 0.76 ± 0.10 (7) | 0.96 ± 0.17 (5) |
| Androstenedione (ng/ml) | 2.20 ± 0.25 (21) | 2.02 ± 0.18 (20) | 1.27 ± 0.20 (20) | 1.53 ± 0.15 (21) | 1.24 ± 0.14 (19) | 1.70 ± 0.28 (17) |

There were seven subjects in each group; the number of observations is in parentheses. Values given are the mean ± SE. NM, Not measurable.
[a] $P < 0.05$ (L *vs.* N-L).
[b] $P < 0.001$ (L *vs.* N-L).
[c] Range.

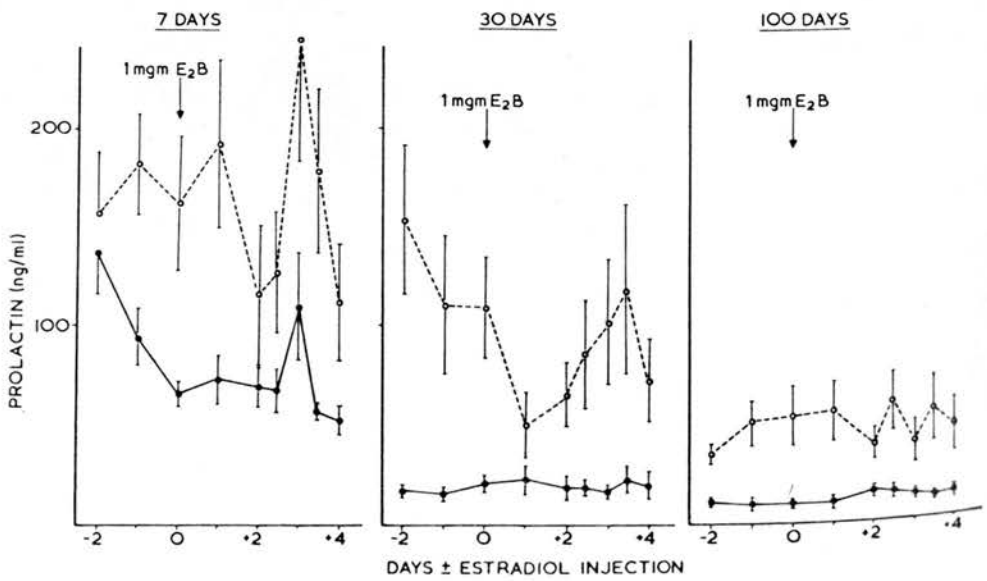



FIG. 2. Concentration of PRL in plasma of 14 women before and after the im injection of 1 mg estradiol benzoate ($E_2B$) at 7, 30, and 100 days post partum. Each point represents the mean ± SE of 7 women. ●—●, Nonlactating; ○- -○, lactating.

postpartum week was significantly lower than that in the lactating group (100 ± 11 *vs.* 172 ± 18 ng/ml; $P < 0.001$). By 30 days, the concentration of PRL in the lactating group was still elevated, while it had fallen to within the normal range for nonpregnant women in the nonlactating group.

In the first postpartum week, the plasma concentration of FSH in both groups was low and rose to within the normal range by 30–100 days; there was no statistically significant difference between the groups. In the first postpartum week, it was not possible to measure LH due to the presence of hCG which was recognized by the antibody used in the LH assay. At 30 days, the concentration of LH was higher in the nonlactating group, and by 100 days, the reverse applied (Table 1).

In both groups, in the first postpartum week the basal concentration of estradiol was within the range normally found in the early proliferative phase of the menstrual cycle (Table 1). By 30 and 100 days, the concentration rose significantly in the nonlactating group and was 3-fold higher than the corresponding values found in the lactating group ($P < 0.001$). The concentration of estradiol in the lactating group at 30 days (37 ± 4 pg/ml) was significantly lower ($P < 0.001$) than that found at any other time. There were no significant differences in the concentration of androstenedione or progesterone between the two groups at 7 and 30 days. The concentration of androstenedione fell significantly from 7 to 30 days in both groups ($P < 0.02$) and rose to 1.70 ± 0.28 ng/ml in the nonlactating group by 100 days.

*Estrogen provocation test*

In both groups, the concentration of estradiol rose to over 250 pg/ml 24 h after the injection and declined progressively to reach basal values by 96 h. There were no significant differences in the peak values observed at 7, 30, and 100 days in either group (overall peak values, 352 ± 35 and 323 ± 32 pg/ml in nonlactating and lactating groups, respectively).

There were significant differences in the patterns of FSH, LH, and PRL in response to the injection of estrogen both between the groups and in the same group at different times after delivery (Tables 2–5). To compare the results between groups and in the same groups at different times, the results have been expressed as a percentage of the mean basal values observed in the 3 samples before injection (Fig. 3). For each group, the coefficient of variation of the 21 basal samples was calculated and significant suppression or stimulation was considered to occur if the value fell outside the 95% confidence limits of the basal values ($P < 0.05$).

The concentration of FSH was suppressed after the injection in all groups at 30 and 100 days. There was no change in the concentration of FSH in response to estrogen at 7 days, although the detection of suppression would have been technically difficult due to the low basal

TABLE 2. Concentration of FSH (milliunits per ml) before and after estrogen provocation test at 30 days post partum

| Patient no. | Hours ± estrogen injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | −48 | −24 | 0 | 24 | 48 | 58 | 72 | 82 | 96 |
| Nonlactating | | | | | | | | | |
| NL 1 | 4.2 | 3.0 | 3.3 | 2.9 | 3.7 | 2.7 | 4.0 | 3.2 | 6.7[a] |
| NL 2 | 0.7 | 0.9 | 0.9 | 0.8 | 0.8 | 0.8 | 0.7 | 0.8 | 0.8 |
| NL 3 | 2.9 | 3.1 | 4.8 | 1.9 | 1.3 | 1.1 | 1.2 | 1.0 | 1.2 |
| NL 4 | 7.6 | 5.9 | 3.6 | 2.5 | 2.2 | 2.5 | 3.3 | 3.1 | 3.8 |
| NL 5 | 3.7 | 3.1 | 4.0 | 1.7 | 1.3 | 0.9 | 0.9 | 0.9 | 0.9 |
| NL 6 | 5.1 | 5.1 | 5.0 | 3.6 | 2.9 | 3.4 | 4.2 | 4.0 | 6.0 |
| NL 7 | 5.9 | 4.7 | 5.6 | 4.8 | 3.0 | 3.3 | 5.3 | 4.6 | 5.4 |
| Mean | 4.3 | 3.7 | 3.9 | 2.6 | 2.2 | 2.1 | 2.8 | 2.5 | 3.5 |
| SE | 0.8 | 0 6 | 0.6 | 0.5 | 0.4 | 0.4 | 0.7 | 0.6 | 0.9 |
| Lactating | | | | | | | | | |
| L 1 | 5.1 | 6.1 | 4.9 | 1.9 | 1.3 | 1.2 | 1.2 | 1.2 | 1.1 |
| L 2 | 2.3 | 4.3 | 2.8 | 1.4 | 0.9 | 0.9 | 1.0 | 0.8 | 0.9 |
| L 3 | 2.7 | 3.1 | 3.1 | 1.6 | 1.2 | 1.0 | 1.1 | 0.8 | 1.0 |
| L 4 | 6.2 | 4.7 | 7.3 | 2.8 | 2.1 | 1.8 | 1.7 | 2.1 | 8.3 |
| L 5 | 3.7 | 3.7 | 3.2 | 1.4 | 1.4 | 1.2 | 1.1 | 1.2 | 1.3 |
| L 6 | 7.0 | 4.9 | 3.5 | 1.5 | 1.0 | 1.0 | 0.8 | 0.9 | 0.9 |
| L 7 | 4.9 | 6.8 | 3.4 | 1.8 | 1.3 | 1.1 | 1.1 | 1.4 | 1.2 |
| Mean | 4.5 | 4.8 | 4.0 | 1.8 | 1.3 | 1.2 | 1.1 | 1.2 | 2.1 |
| SE | 0.7 | 0.5 | 0.6 | 0.2 | 0.1 | 0.1 | 0.1 | 0.2 | 1.0 |

[a] Peak values significantly above basal values ($P < 0.05$).

TABLE 3. Concentration of LH (milliunits per ml) before and after estrogen provocation test at 30 days post partum

| Patient no. | Hours ± estrogen injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | −48 | −24 | 0 | 24 | 48 | 58 | 72 | 82 | 96 |
| Nonlactating | | | | | | | | | |
| NL 1 | 4.4 | 3.9 | 3.4 | 4.3 | 5.8 | 3.2 | 6.0 | 3.9 | 15.9[a] |
| NL 2 | 1.4 | 1.5 | 1.6 | 1.2 | 1.9 | 1.2 | 1.2 | 1.6 | 1.2 |
| NL 3 | 3.0 | 6.4 | 5.0 | 2.5 | 1.5 | 2.0 | 1.9 | 2.1 | 2.1 |
| NL 4 | 4.8 | 4.0 | 3.5 | 2.8 | 4.0 | 4.3 | 6.2[a] | 4.2 | 5.2 |
| NL 5 | 2.8 | 2.0 | 2.7 | 1.4 | 1.0 | 1.8 | 0.8 | 1.2 | 1.7 |
| NL 6 | 9.8 | 11.4 | 10.2 | 7.2 | 6.7 | 7.9 | 10.5 | 7.7 | 10.1 |
| NL 7 | 6.4 | 12.1 | 13.6 | 10.5 | 8.4 | 9.0 | 13.3 | 7.6 | 14.1 |
| Mean | 4.7 | 5.9 | 5.7 | 4.3 | 4.2 | 4.2 | 5.7 | 4.0 | 7.2 |
| SE | 1.1 | 1.6 | 1.7 | 1.3 | 1.1 | 1.2 | 1.8 | 1.0 | 2.3 |
| Lactating | | | | | | | | | |
| L 1 | 4.4 | 6.0 | 4.2 | 2.0 | 2.2 | 2.1 | 2.7 | 1.8 | 3.2 |
| L 2 | 2.9 | 3.5 | 2.6 | 2.1 | 1.9 | 2.0 | 2.0 | 2.3 | 3.2 |
| L 3 | 3.3 | 3.3 | 2.8 | 2.0 | 2.3 | 2.1 | 2.3 | 2.1 | 3.0 |
| L 4 | 2.5 | 2.5 | 4.2 | 2.1 | 2.2 | 2.4 | 1.6 | 5.1 | 6.9[a] |
| L 5 | 1.6 | 1.2 | 1.2 | 1.4 | 0.9 | 1.2 | 0.7 | 0.9 | 1.0 |
| L 6 | 9.6 | 5.3 | 3.5 | 1.7 | 2.1 | 1.6 | 1.0 | 1.6 | 0.8 |
| L 7 | 3.5 | 2.4 | 2.0 | 1.8 | 2.0 | 2.4 | 1.9 | 1.7 | 1.6 |
| Mean | 4.0 | 3.5 | 2.9 | 1.9 | 1.9 | 2.0 | 1.7 | 2.2 | 2.8 |
| SE | 1.0 | 0.6 | 0.4 | 0.1 | 0.2 | 0.2 | 0.3 | 0.5 | 0.8 |

[a] Peak values significantly above basal values ($P < 0.05$).

TABLE 4. Concentration of FSH (milliunits per ml) before and after estrogen provocation test at 100 days post partum

| Patient no. | Hours ± estrogen injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | −48 | −24 | 0 | 24 | 48 | 58 | 72 | 82 | 96 |
| Nonlactating | | | | | | | | | |
| NL 1 | 5.6 | 5.4 | 5.5 | 2.0 | 3.2 | 6.9 | 4.7 | 6.8 | 8.3[a] |
| NL 2 | 3.5 | 3.3 | 3.4 | 2.1 | 2.3 | 3.3 | 3.5 | 2.5 | 2.3 |
| NL 3 | 1.6 | 1.7 | 4.6 | 2.4 | 2.4 | 2.6 | 3.8 | 4.7 | 6.5[a] |
| NL 4 | 7.3 | 7.4 | 8.4 | 3.7 | 3.7 | 4.2 | 6.6 | 9.0 | 12.0[a] |
| NL 5 | 3.8 | 5.0 | 4.1 | 1.8 | 1.2 | 1.4 | 2.1 | 1.6 | 3.0 |
| NL 6 | 5.0 | 6.2 | 4.5 | 3.5 | 3.5 | 5.3 | 3.8 | 4.7 | 6.7 |
| NL 7 | 3.9 | 4.1 | 3.6 | 1.8 | 1.3 | 1.7 | 1.9 | 2.9 | 4.3 |
| Mean | 4.5 | 4.7 | 4.9 | 2.4 | 2.5 | 3.6 | 3.8 | 4.6 | 6.2 |
| SE | 0.8 | 0.7 | 0.6 | 0.3 | 0.4 | 0.8 | 0.7 | 1.0 | 1.3 |
| Lactating | | | | | | | | | |
| L 1 | 4.7 | 4.9 | 6.3 | 2.3 | 2.5 | 2.4 | 4.0 | 3.7 | 4.8 |
| L 2 | 5.2 | 5.8 | 4.5 | 1.8 | 1.6 | 1.3 | 1.8 | 1.2 | 1.4 |
| L 3 | 6.0 | 6.3 | 7.2 | 3.6 | 2.1 | 2.2 | 2.1 | 1.8 | 1.9 |
| L 4 | 5.6 | 5.6 | 10.0 | 4.9 | 2.6 | 2.2 | 2.3 | 1.9 | 1.8 |
| L 5 | 7.8 | 7.5 | 5.0 | 2.7 | 1.7 | 2.2 | 3.6 | 3.4 | 6.3 |
| L 6 | 3.3 | 2.7 | 3.3 | 1.2 | 1.5 | 1.6 | 1.2 | 1.1 | 1.0 |
| L 7 | 4.4 | 7.6 | 3.4 | 1.7 | 1.5 | 1.1 | 1.4 | 1.5 | 1.0 |
| Mean | 5.3 | 5.8 | 5.7 | 2.6 | 1.9 | 1.9 | 2.3 | 2.1 | 2.7 |
| SE | 0.6 | 0.6 | 0.9 | 0.5 | 0.2 | 0.2 | 0.4 | 0.4 | 0.8 |

[a] Peak values significantly above basal values ($P < 0.05$).

TABLE 5. Concentration of LH (milliunits per ml) before and after estrogen provocation test at 100 days post partum

| Patient no. | Hours ± estrogen injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | −48 | −24 | 0 | 24 | 48 | 58 | 72 | 82 | 96 |
| Nonlactating | | | | | | | | | |
| NL 1 | 7.9 | 5.6 | 8.2 | 3.0 | 5.7 | 16.4[a] | 9.0 | 12.4 | 9.4 |
| NL 2 | 5.6 | 4.4 | 4.1 | 3.1 | 5.7 | 10.7[a] | 8.2 | 3.3 | 3.5 |
| NL 3 | 3.2 | 3.0 | 7.0 | | 3.8 | 4.6 | 9.2 | 7.8 | 11.0[a] |
| NL 4 | 11.6 | 5.5 | 9.0 | 2.9 | 5.0 | 4.7 | 8.8 | 10.6 | 9.4 |
| NL 5 | 3.2 | 7.7 | 4.6 | 2.0 | 2.1 | 1.0 | 3.2 | 1.4 | 8.4[a] |
| NL 6 | 10.1 | 10.6 | 7.5 | 7.5 | 9.5 | 13.1 | | 15.7 | 17.9[a] |
| NL 7 | | 7.3 | 6.6 | 3.7 | 2.5 | 3.8 | 5.0 | 9.2 | 9.8 |
| Mean | 6.9 | 6.3 | 6.8 | 3.7 | 4.9 | 7.8 | 7.2 | 8.6 | 9.9 |
| SE | 1.4 | 0.9 | 0.7 | 0.8 | 0.9 | 2.1 | 1.0 | 1.9 | 1.6 |
| Lactating | | | | | | | | | |
| L 1 | 12.3 | 15.1 | 18.1 | 5.4 | 9.4 | 9.9 | 17.1 | 15.6 | 16.5 |
| L 2 | | 11.2 | 11.9 | 2.4 | 3.2 | 3.7 | 3.4 | 3.1 | 2.7 |
| L 3 | 9.0 | 10.2 | 11.5 | 2.4 | 2.2 | 2.7 | 2.7 | 2.1 | 3.6 |
| L 4 | 2.8 | 6.0 | 15.6 | 2.1 | 2.3 | 1.3 | 2.0 | 1.0 | 0.8 |
| L 5 | 9.6 | 8.1 | 6.4 | 4.5 | 2.1 | 2.8 | 6.7 | 7.1 | 12.3 |
| L 6 | 8.9 | 7.0 | 2.8 | 3.1 | 2.4 | 1.6 | 2.0 | 1.6 | 1.3 |
| L 7 | 4.5 | 6.2 | 2.7 | 1.9 | 2.0 | 1.8 | 1.9 | 1.8 | 1.5 |
| Mean | 7.9 | 9.1 | 9.9 | 3.1 | 3.4 | 3.4 | 5.1 | 4.6 | 5.5 |
| SE | 1.4 | 12.0 | 2.3 | 0.5 | 1.0 | 1.1 | 2.1 | 2.0 | 2.4 |

[a] Peak values significantly above basal values ($P < 0.05$).

alues. In the lactating group, the concentration of FSH as suppressed (48–72 h after injection) to a minimum f about 30% of the basal value (Fig. 3). In the nonlac-ating group, the suppression was less marked (about 60%) and the mean value rose again by 96 h to reach 91 ± 21% and 133 ± 24% of the basal value in the 30- and 100-day tests, respectively. In the latter tests, the value at 96 h was significantly raised above the basal value in three of the seven subjects ($P < 0.05$).

The concentration of LH after in injection of estradiol benzoate was also suppressed more markedly in the lactating group at both 30 and 100 days (Fig. 3). In the lactating group, only one subject showed a positive feedback response to estrogen at 30 days and none showed it at 100 days. In the nonlactating group two of seven and five of seven showed positive feedback at 30 and 100 days, respectively. In the latter group, the peak value of LH in all seven subjects (mean, 187 ± 18%) occurred between 58–96 h after the injection of estrogen. The mean peak value of those five subjects showing positive feedback was 211 ± 14% above the basal values.

The pattern of PRL concentration after the estrogen injection varied depending on the basal concentration (Fig. 2). In the lactating group, there was an initial suppression followed by a short-lived increase in the concentration of PRL 72 h after the injection of estrogen when tested at 7 and 30 days. In the nonlactating group, a rise in PRL concentration at 72 h was only seen in the first postpartum week.

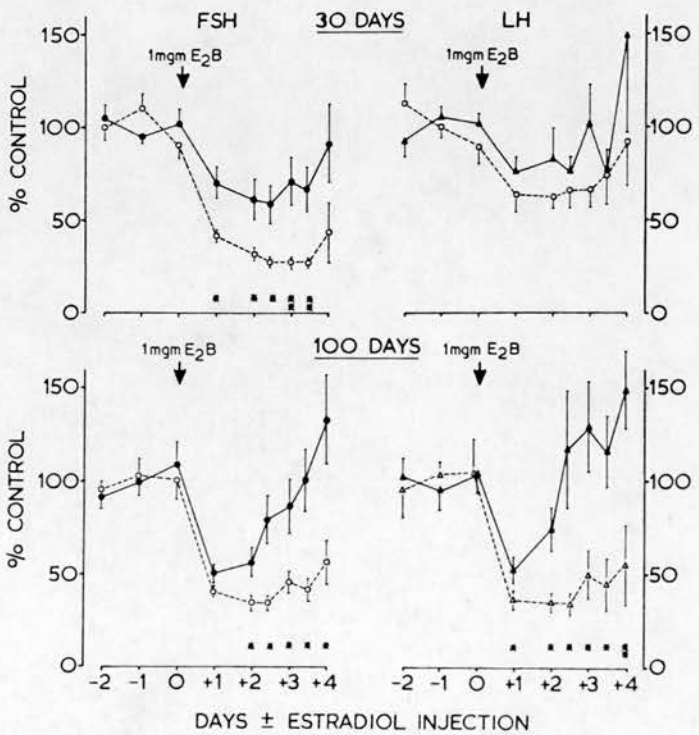



FIG. 3. The concentration of FSH and LH in plasma of 14 women before and after the im injection of 1 mg estradiol benzoate ($E_2B$) at 30 and 100 days post partum. The results have been expressed as a percentage of the mean basal values before the injection. Each point represents the mean ± SE of 7 women. ●—●, Nonlactating; ○– –○, lactating. Significance of difference between lactating and nonlactating groups was determined by Student's $t$ test (*, $P < 0.05$; **, $P < 0.01$).

## Discussion

The first episode of vaginal bleeding, which occurred between 21–70 days in all but one subject, was not preceded by an ovulatory ovarian cycle. In eight women, the bleeding followed the injection of estradiol benzoate and was probably related to withdrawal of exogenous estrogen. In others, *e.g.* NL 1 and L 2, bleeding occurred before the estrogen injection and was presumably due to the breakdown of involuting decidua. In six of the seven nonlactating women, the first ovarian cycle was anovulatory, although in one subject (NL 7), ovulation occurred as early as 43 days after delivery. These results, therefore, confirm the conclusion based on endometrial biopsies that the first ovarian cycle after delivery, even in nonlactating women, is frequently anovulatory (9).

Ovulation in the lactating women was delayed considerably longer than in the nonlactating group. Indeed, there was little evidence of ovarian activity, as judged by the quantity and pattern of steroids excreted in the urine until at least 150 days after delivery or until weaning. There is considerable evidence in experimental animals that this suppression of ovarian activity during lactation is dependent on a neural reflex originating from the breast during suckling (2–4). In sheep, denervation of the mammary gland results in more rapid restoration of ovarian cyclicity even if suckling continues (3). It has been suggested that, in women, the duration and completeness of lactational amenorrhoea is related to the frequency of breast feeding and, hence, nipple stimulation (20). In this study, weaning was usually followed by a rapid restoration of ovarian function and ovulation in most but not all subjects (*c.f.* L 4). In those subjects who ovulated before weaning (L 3 and L 6), the frequency of breast feeding had dropped to two or three times per day. Thus, although the effect of lactation is to delay the restoration of ovarian function, in Western society, the period of infertility is relatively short and some form of contraception must be used if pregnancy is to be avoided (8).

During the first week after delivery the concentration of PRL in both groups was grossly elevated above the value found in nonpregnant women, confirming previous reports (21–23). The progressive rise in the concentration of PRL during pregnancy is probably due to the elevated level of estrogen, which is known to stimulate the secretion of PRL (24). After an initial suppression, the concentration of PRL rose 72 h after the injection of estradiol benzoate. This pattern of response was only seen in the first postpartum week in both groups and at 30 days in the lactating women when the basal level of PRL was grossly elevated (over 100 ng/ml), indicating that hypertrophy of the pituitary lactotrophs induced pregnancy was still present.

In the first postpartum week, the concentration of estradiol fell gradually so that by day 7 in both groups it was within the range found in the early proliferative phase of the cycle. At this stage, the concentration of FSH was very low due to the prolonged negative feedback effects of steroids during pregnancy. In lactating women, although the concentration of FSH had risen to values normal for nonpregnant women, the concentration of estradiol at 30 and 100 days remained very low (23). One explanation of these anomalous findings is that during lactation the hypothalamic-pituitary system may become extremely sensitive to the negative feedback effects of estrogen, a suggestion compatible with the results of the estrogen provocation test. The mechanism responsible for this change in the sensitivity of the feedback mechanism is unknown, although PRL may inhibit hypothalamic activity directly and, hence, the secretion of pituitary gonadotropins (6, 25–27). The concentration of FSH rises more quickly postpartum in women given bromocriptine to suppress the secretion of PRL (28). However, in the present study, the basal concentration of LH in lactating women at 100 days was significantly higher than that in nonlactating women, although the estradiol concentration in plasma was lower. One possible explanation for this would be a loss of ovarian sensitivity to stimulation by LH. It may be that PRL in high concentration, such as is present during lactation, inhibits the synthesis of ovarian steroids as it is known to do with human granulosa cells in culture (5).

In the present study, lactation was characterized by a delay in the resumption of ovulatory ovarian cycles, and only one of the lactating women showed a rise of LH in response to estrogen (positive feedback). The magnitude of the LH rise in nonlactating women (mean, 211%; range, 162–239%) to this single estrogen injection in the early follicular phase was modest in comparison to that seen before ovulation in the normal menstrual cycle. A much larger response is seen in the mid and late follicular phase when the anterior pituitary has been primed by both estrogen and LRF for several days (29). Thus, although a preovulatory LH surge is not always elicited by this test, any significant rise in LH in response to estrogen provocation demonstrates the potential of the hypothalamic-pituitary unit to produce a surge of LH when suitably primed. The discriminating nature of the test is confirmed by the observation that five nonlactating women showing a positive response at 100 days had already ovulated, while a positive response was seen in none of the seven lactating women, none of whom had ovulated before the test.

The delay in the restoration of positive feedback in the postpartum period accounts for the frequent occurrence of anovulatory cycles. While at 30 days this failure to release adequate amounts of LH in response to estrogen may be due in part to the reduced pituitary sensitivity to LH/RH (11), it is more likely to be hypothalamic in

origin in lactating women, in whom the defect persists at 100 days when pituitary sensitivity is actually enhanced. Lactation, like the amenorrhoea-galactorrhoea syndrome (30), appears to be characterized by an increased sensitivity to the negative feedback effects of estrogen and a decreased sensitivity to positive feedback. The neuroendocrine mechanisms responsible for this change in the sensitivity of the hypothalamic-pituitary system remain unknown.

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# HYPERPROLACTINAEMIA AND LONG-TERM LACTATIONAL AMENORRHOEA

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SUMMARY

Clinical details of lactational and menstrual history post-partum have been correlated with measurements of serum prolactin, luteinizing hormone, follicle stimulating hormone, oestradiol and progesterone in Transkei women in order to gain further insight into the role of prolactin in the maintenance of post-partum lactational amenorrhoea. Of seventy-four breast-feeding mothers studied, sixty-six had elevated serum levels of prolactin. Forty-four women were amenorrhoeic with minimal or absent endocrine evidence of ovarian activity. Of the twenty-seven lactating women who reported menstruation, twelve had high levels of prolactin and no apparent ovarian activity, eleven had lower prolactin levels and increased oestradiol levels suggesting ovarian follicular development, while only four women had increased progesterone levels indicating definite luteal activity. Three lactating women were pregnant. The results support previous reports that lactational amenorrhoea is associated with hyperprolactinaemia and a possible sequence of endocrine events resulting in the return of post-partum fertility is proposed.

Breast feeding is known to delay the return of menstruation and fertility after childbirth (McNeilly, 1979). While several studies have reported the endocrine changes associated with short-term lactational amenorrhoea, only Delvoye *et al.* (1978) have investigated the endocrine changes associated with long-term lactational amenorrhoea of up to 2 years duration. They concluded that the duration of amenorrhoea was directly associated with the degree of hyperprolactinaemia which in turn was related to the suckling frequency. In rural Transkei it is customary for women to breast feed for up to 2 years, feeding on demand day and night. Sleeping with its mother, it is possible for a baby to suckle at any time of the night without waking her. Indeed, babies appear to have the nipple in or near their mouths for most of the night (Duchen, personal observations).

In such areas, there is minimal artificial contraception available and the birth interval is

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largely regulated by prolonged periods of post-partum lactational infertility (Vis *et al.* 1975). We have attempted to correlate clinical details of lactational and menstrual history post-partum with measurements of serum prolactin, luteinizing hormone (LH) follicle stimulating hormone (FSH), oestradiol and progesterone in Transkei women in order to gain further insight into the role of prolactin in the maintenance of post-partum lactational amenorrhoea.

## SUBJECTS AND METHODS

The aims of the study were explained by a nurse to healthy women with healthy small babies attending the well-baby clinics and paediatric wards of St Barnabas Hospital, Transkei. The women were asked to give information regarding their duration of breast feeding and associated menstrual history and to give a single blood sample.

Eighty-five women with an average post-partum interval of 11 months agreed to take part of whom seventy-four were breast feeding. Eleven women were not breast-feeding or pregnant and were menstruating normally. The results in this latter group were used to provide the normal range of hormonal levels for comparison with the breast-feeding group.

Blood samples (10 ml) were taken at random time in relation to suckling. Serum was separated and stored at $-15^{\circ}$C until assays could be undertaken at the MRC Unit in Edinburgh. The following questions were asked of each woman: (1) age of baby, (2) age of mother, (3) date of first post-partum menstrual bleed if any, (4) whether breast milk was supplemented by other feeds. Initially, the frequency of breast feeding was asked. However, since babies are fed on demand no mother could accurately tell how often her baby took the breast and only problems with feeding were recorded.

### *Radioimmunoassays*

Serum levels of LH, FSH, prolactin, oestradiol and progesterone were assayed by specific radioimmunoassays as described in detail previously (Baird *et al.*, 1974; McNeilly & Hagen, 1974; Van Look *et al.*, 1977). All samples were assayed in a single assay. Statistical analysis was by Students' *t* test.

## RESULTS

Results of hormone assays were correlated with clinical details obtained from the questionnaires. The hormone levels of the eleven normally cyclic women are shown in Fig. 1. Five women had high oestradiol, low progesterone and variable LH levels compatible with follicular development. The wide range of LH reflects the inclusion of one women whose LH level was more than 80 mu/ml, suggesting that blood was taken during the mid-cycle LH surge. In the remaining six women progesterone levels were high, suggesting established luteal activity. Mean prolactin levels were $209 \pm 38$ and $117 \pm 29$ uU/ml (mean $\pm$ SEM) respectively.

In the lactating group of women sixty-six of the seventy-four had serum levels of prolactin greater than the upper limit of normal for menstruating women (Fig. 2) and this hyperprolactinaemia was evident even at 14 months of lactation. Of the five lactating women with normal prolactin levels, two had reported difficulty with breast feeding, while no reason for the low levels of prolactin was apparent in the other three subjects.

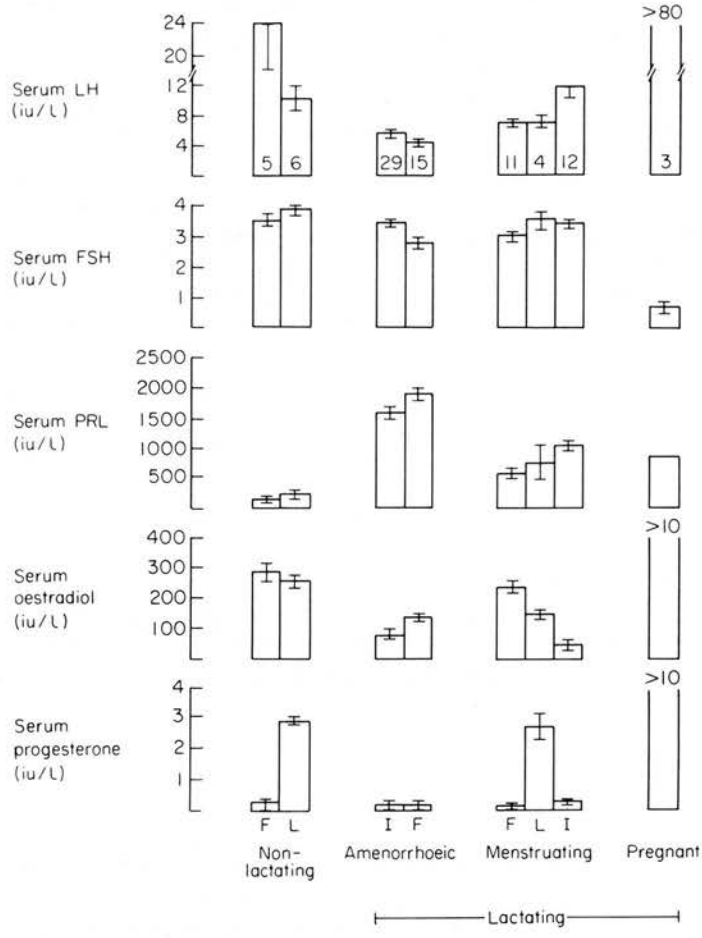



Fig. 1. Serum levels (mean ± SEM) of LH, FSH, prolactin (PRL) oestradiol and progesterone in non-lactating and lactating Transkei women. Results are grouped depending on clinical and endocrine status. Number of individual subjects per group is indicated within the histogram for serum LH. F = follicular phase; L = luteal phase; I = inactive.

Three lactating women had hormonal levels suggesting various stages of pregnancy with high LH (hCG), progesterone and oestradiol, low FSH and variable prolactin levels. The breast feeding women were classified as those who remained amenorrhoeic and those who reported menstrual bleeding. These were in turn sub-divided according to hormonal indices of ovarian function.

The hormonal profiles of women who remained amenorrhoeic are shown in Fig. 1. Of these forty-four women, twenty-nine showed no sign of ovarian activity, with very low oestradiol ( < 90 pg/ml) and progesterone ( < 0·2 ng/ml) levels. The remaining fifteen had raised oestradiol levels (120–150 pg/ml) suggesting a degree of early follicular activity, although the levels of oestradiol did not reach the levels found in non-suckling, menstruating women. The mean times post-partum of these groups were 4 and 3·5 months respectively. Prolactin levels in both these groups were markedly raised, with ranges of

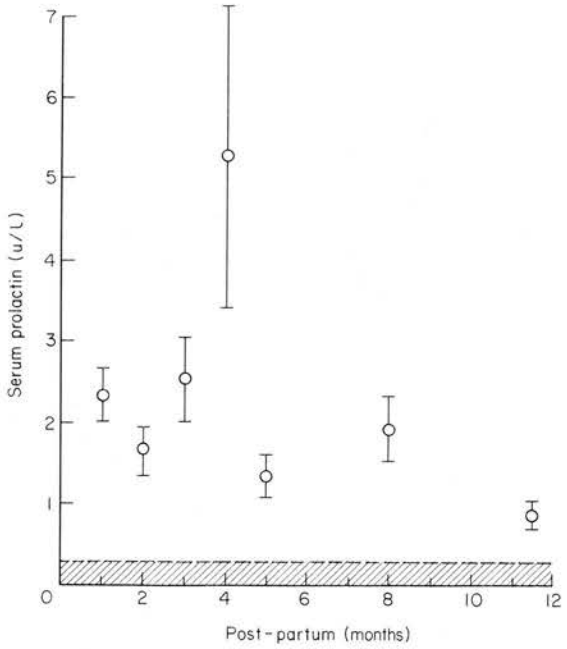



Fig. 2. Mean serum prolactin (±SEM) in lactating Transkei women during 12 post-partum months. The hatched area represents the normal range of prolactin values in a group of non-lactating women ($n=11$) in the same area.

1419–1809 and 1598–2179 μu/ml respectively. Mean levels of serum LH were significantly lower than normal ($P<0{\cdot}01$) in both groups, while FSH levels were in the normal range, being lower in those women with raised serum levels of oestradiol.

Of the twenty-seven breast-feeding women who reported menstruation, twelve (mean time post-partum = 7 months) had high serum levels of prolactin (range 809–1295 μu/ml) and no hormonal indications of ovarian activity. In a further eleven women (mean time post-partum = 8·5 months) oestradiol levels were in the follicular range (> 100 pg/ml) with low progesterone suggesting normal follicular activity. In six of these eleven women, prolactin levels were within the normal range and while levels were above normal in the remaining five these levels were less than those in the amenorrhoeic group ($P<0{\cdot}001$, Fig. 1). The remaining four women had evidence of definite luteal activity (mean progesterone 3·27 ng/ml) at 5, 7, 9 and 14 months post-partum; prolactin levels were variable, two within (129 and 319 μu/ml) and two above (610 and 2065 μu/ml) normal.

At monthly or 3-monthly intervals post-partum the number of women with either amenorrhoea or hyperprolactinaemia were calculated as a percentage of the total number at each time interval. Comparing these two percentages (Fig. 3) there appears to be a relationship between the incidence of amenorrhoea and that of hyperprolactinaemia. The majority of women who had menstruated and showed endocrine evidence of ovarian activity had normal prolactin levels. Nevertheless, in a few cases women were hyperprolactinaemic but had menstruated, and some women with normal prolactin levels remained amenorrhoeic.

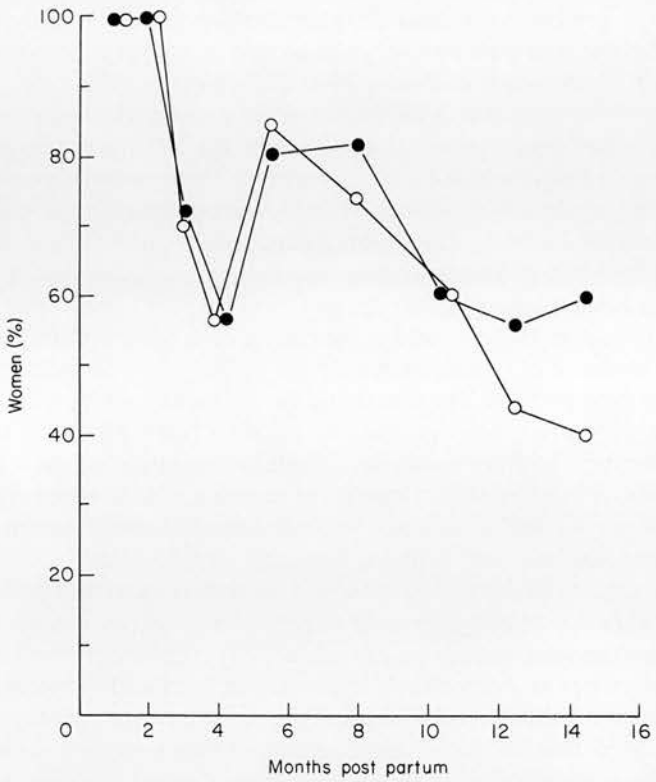



Fig. 3. Relationship between the percentage of Transkei women with hyperprolactinaemia (●——●) and amenorrhoea (○——○) at different times post-partum.

## DISCUSSION

Our results show that lactational amenorrhoea is clearly associated with pronounced hyperprolactinaemia which may last for 8 months or more post-partum, during which time many of the women (forty-four out of seventy-four) showed minimal ovarian endocrine activity. Indeed, our study included cases of women at 12 and 13 months post-partum who remained amenorrhoeic, anovulatory and hyperprolactinaemic whilst fully breast feeding their babies. Unfortunately, we were unable to obtain information about the frequency of feeds, as these women do not notice how often their babies suckle. Nevertheless, there is no doubt that the general custom is to feed many times a day on demand and freely throughout the night. This is very much more than the normal regimes adopted by European women, in whom prolactin levels may fall to normal with concomitant resumption of ovarian activity within 2–3 months post-partum even though breast-feeding continues (Rolland *et al.*, 1975).

Of the forty-four amenorrhoeic women twenty-nine showed no endocrine evidence of ovarian activity (oestradiol level $<90$ pg/ml). In the remaining fifteen, increased levels of oestradiol were found, comparable to those seen in the early follicular phase of the normal menstrual cycle. It is apparent from these results that some follicular development can

occur even in the presence of grossly elevated levels of prolactin, and this is compatible with reports that the post-partum ovary can secrete oestrogen in response to gonadotrophic stimulation (Andreasson & Tyson, 1976; Zaratu *et al.*, 1974).

The serum levels of prolactin in the twenty-seven women who had menstruated during lactation were significantly lower than those in the amenorrhoeic group, but were significantly higher than the non-lactating controls. Of these twenty-seven women, only fifteen had raised oestradiol levels suggestive of ovarian follicular development and only four had progesterone levels > 2ng/ml suggesting ovulation. As it is statistically improbable that only four out of fifteen women with normal menstrual cycles bled at random would be in the luteal phase of the cycle, our results would suggest that a proportion of these women were anovulatory, and experiencing oestrogen withdrawal bleeding only. We only have evidence of ovulation in four out of forty-four lactating women at 5, 7, 9 and 13 months post-partum. The remaining twelve menstruating women had no endocrine evidence of ovarian activity and are puzzling; their prolactin levels were high, consistent with their ovarian inactivity. Perhaps menstruation had been incorrectly diagnosed. It also transpired that at least three lactating women were pregnant. Two were 14 months post-partum and were breast feeding minimally, while the third was 12 months post-partum and had been menstruating regularly for 7 months.

While the results of our study do not explain the mechanisms by which lactation causes amenorrhoea, they do suggest a possible sequence of endocrine events. Whilst prolactin levels are grossly elevated, little or no ovarian activity is apparent despite normal levels of FSH but low levels of LH. As prolactin levels decline, levels of LH increase and oestradiol reaches normal mid-follicular phase levels. Although menstruation may occur during this period it is probable that it is due to oestrogen rather than progesterone withdrawal, since only 16% of the women in our study had evidence of luteal function. Finally, as serum levels of prolactin drop near or within the normal non-lactating range, ovarian cyclicity and ovulation resumes. Thus while prolactin is clearly implicated in the control of lactational infertility and amenorrhoea it is still not known precisely how it acts, either at the hypothalamus and/or the ovary.

## ACKNOWLEDGEMENTS

This study would not have been possible without the invaluable help of Dr Andrew Truscott and Sister Matyaba of St Barnabas Hospital. M.R. Duchen was supported in part by a grant from St Georges' Hospital Medical School. We gratefully acknowledge the skilled assistance of Miss K. Dosser and Messrs D.W. Davidson and I. Swanston; and Professors W.R. Butt and H.G. Friesen for supply of some of the reagents used for radioimmunoassay.

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# 12

# Relationship of Feeding Patterns, Prolactin, and Resumption of Ovulation Postpartum

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*PETER W. HOWIE*

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It has long been recognized that the return of menstruation and fertility are delayed in women who suckle their babies.[15] In societies where breast milk is the sole source of nutrition for the baby, lactational amenorrhea may last for 2 to 3 years, and it acts as a natural birth spacer.[22] While several epidemiologic studies have confirmed the antifertility effect of breast-feeding,[15] there are relatively few reports on the endocrine changes associated with the inhibition and subsequent reestablishment of fertility postpartum. Breast-feeding is associated with prolonged hyperprolactinemia, normal levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and low levels of estradiol and progesterone.[1,3,11,12,13,19,21] In long-term lactational amenorrhea of up to 2 years' duration, the actual duration of amenorrhea is associated with the degree of hyperprolactinemia,[6,7] which in turn is related to the suckling frequency.[6] Despite these reports, no longitudinal studies have been undertaken in which changes in suckling patterns have been related to the sequence of endocrine events leading to the resumption of ovulation, menstruation, and fertility in breast-feeding women. This chapter reports our preliminary findings in such an on-going study being carried out in Edinburgh. By collecting longitudinal data, a clearer understanding is possible regarding the precipitating factor(s) in the resumption of ovarian activity and the subsequent return of fertility. Therefore, the contraceptive requirement for the breast-feeding mother may be better defined.

We are extremely grateful to Sister A. Cook, Messers A. Hearle, H. Boyle, R. Stevenson, Miss S. Hall, and Miss G. Hall for assistance in the course of this study.

## SUBJECTS AND METHODS

### SUBJECTS

Fourteen women, aged 23 through 35 years, were recruited on the postnatal ward of the Simpson Memorial Maternity Pavilion, Edinburgh. Women were included in the study if they (1) were planning to breast-feed their infants, (2) were not intending to use steroidal contraceptives, and (3) lived within the Edinburgh area (allowing ease of visiting and collecting of specimens). Each subject gave informed consent and kept a daily record of the duration and number of suckling episodes, the introduction of supplementary food, and any periods of menstrual bleeding. Weekly 24-hour urine samples were collected for estimation of total estrogens and pregnanediol. Every 2 weeks, each subject was visited by the same research nursing sister, who collected a single blood sample (10 ml), at least 2 hours after the last feed, and the data cards. Blood samples were taken to the laboratory and centrifuged, and the plasma was stored at −20 C until assayed for prolactin, LH, and FSH. Urine specimens were assayed on the day of collection and aliquots kept at −20 C for future estimates of LH and FSH. Seven women who bottle-fed their babies acted as controls, and samples were collected as detailed for the breast-feeding mothers. Blood and urine collections were continued until the resumption of regular ovulation, as judged by levels of urinary pregnanediol > 1 mg/24 hr for 2 consecutive weeks.

### HORMONE ASSAYS

Urinary excretion of total estrogen was measured fluorometrically,[4] and that of pregnanediol was determined by gas-liquid chromatography.[5] Plasma levels of LH and FSH were determined using double antibody radioimmunoassays with interassay precision of 9% and 11%, respectively.[9,16] Results are expressed in terms of U (Unit) reference preparation /1, *i.e.,* LH MRC68/40 (77 U/ampule; 1mU = 11.6 ng LER 907) and FSH/ LH MRC69/104 (10 U/ampule = LER 907). Prolactin was measured by a specific double antibody radioimmunoassay, using reagents supplied by Dr. H.G. Friesen (Winnipeg, Canada) with an interassay variation of 9%.[8,16] Results are expressed in terms of U MRC prolactin reference preparation 75/504 (10 U/ampule, 22 $\mu$U = 1ng).

### STATISTICAL ANALYSIS

Results were analyzed using Analysis of Variance and Students t test.

## RESULTS

To illustrate the variation in the length of time required for each suckling episode—hereafter referred to as suckling duration—the variation in serum levels of prolactin (PRL), the changes in urinary steroid levels, and the presence or absence of menstruation are presented for four women who

breast-fed their babies for periods of from 12 to more than 44 weeks (Figs. 12-1 & 12-2).

A typical picture is shown of a bottle-feeding mother who resumes ovarian activity immediately after delivery and ovulates at 8 weeks. A mother who discontinued breast-feeding at only 12 weeks showed no ovarian activity while lactating, but resumed abruptly after cessation of lactation (Fig. 12-1B).

By contrast, a mother who breast-fed for more than 40 weeks and sustained night feeds during this time remained amenorrheic (Fig. 12-2A), maintained a high basal prolactin, and suppressed all ovarian activity, apart from a transient rise at 22 weeks. A fourth mother who breast-fed for 38 weeks maintained low basal PRL levels, had cyclic estrogen production from 12 weeks onward and inadequate pregnanediol production (Figs. 12-2B, 12-2C).

### SERUM PROLACTIN LEVELS AND SUCKLING PATTERN

The changes in serum levels of prolactin, suckling frequency, and duration, and their relationship to the introduction of supplementary food in the form of milk or solids, is shown in Fig. 12-3. No significant change in these parameters, except supplementary food, occurred before week 16 postpartum. Thereafter, mean prolactin levels, suckling frequency, and suckling duration declined in parallel with one another, and these changes were very significantly correlated ($n = 11$): prolactin levels in relation to (a) suckling frequency, $r = 0.918$; (b) suckling duration, $r = 0.935$; (c) suckling frequency to suckling duration, $r = 0.952$. They showed a significant inverse correlation to the introduction of supplementary food: against prolactin, $r = 0.930$; against suckling frequency, $r = -0.946$; against suckling duration, $r = -0.981$. Nevertheless, prolactin levels remained significantly above normal for at least 30 weeks postpartum while breast-feeding continued.

### EFFECT OF INTRODUCTION OF SUPPLEMENTARY FOOD (ISD) ON PROLACTIN, SUCKLING PATTERN, AND OVARIAN ACTIVITY

Supplementary food in the form of bottles of milk and/or solid food was introduced at $16 \pm 5$ (range 3-24) weeks postpartum. To eliminate this variation, data have been centered around the time of introduction of supplementation (Fig. 12-4). Before this time, no ovarian activity was seen in any breast-feeding woman. Supplementation was associated with a rapid decrease in the duration of suckling, a decline in the serum prolactin levels, and a resumption of follicular development (urinary estrogens 10 $\mu$g/24 hr). This resumption was slow, only 2 out of 14 showing estrogen secretion by 3 weeks. By 18 weeks, 12 out of 14 (85%) women had resumed ovarian activity and 10 out of 14 had ovulated (urinary pregnanediols > 1 mg/24 hr), the first ovulation occurring 5 weeks after the introduction of supplementary food.

Of the ten mothers who ovulated during the period of study, five ovulated for the first time after cessation of lactation and five ovulated during lactation. The five mothers who ovulated after lactation did so at 2, 2, 4, 5, and 7 weeks, respectively, after stopping breast-feeding. The five mothers who ovulated during lactation did so at 21, 22, 23, 27, and 32 weeks after delivery; by this

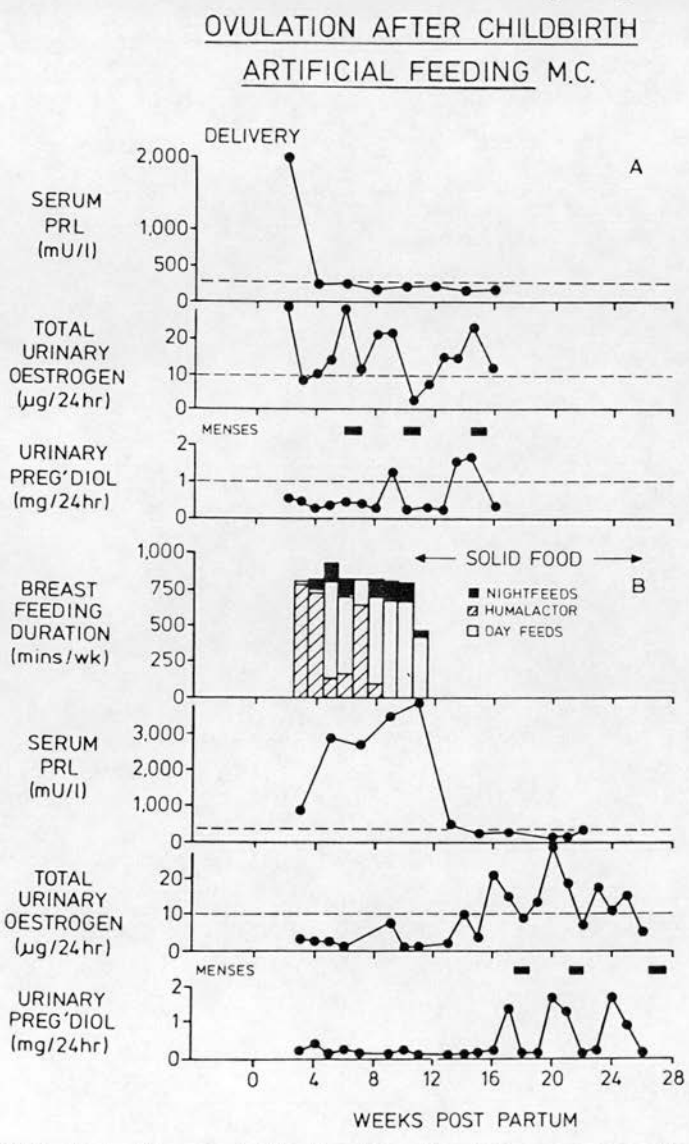



**FIG. 12-1.** Changes in the duration of suckling, serum prolactin, and urinary estrogens and pregnanediol postpartum in a breast-feeding woman (B). For comparison, the changes in prolactin and steroids are shown for a woman who bottle-fed her baby (A). Menstrual periods (■) and the duration of night-time (■) and day-time (□) feedings and periods when milk was expressed using a breast pump (▨) are indicated.

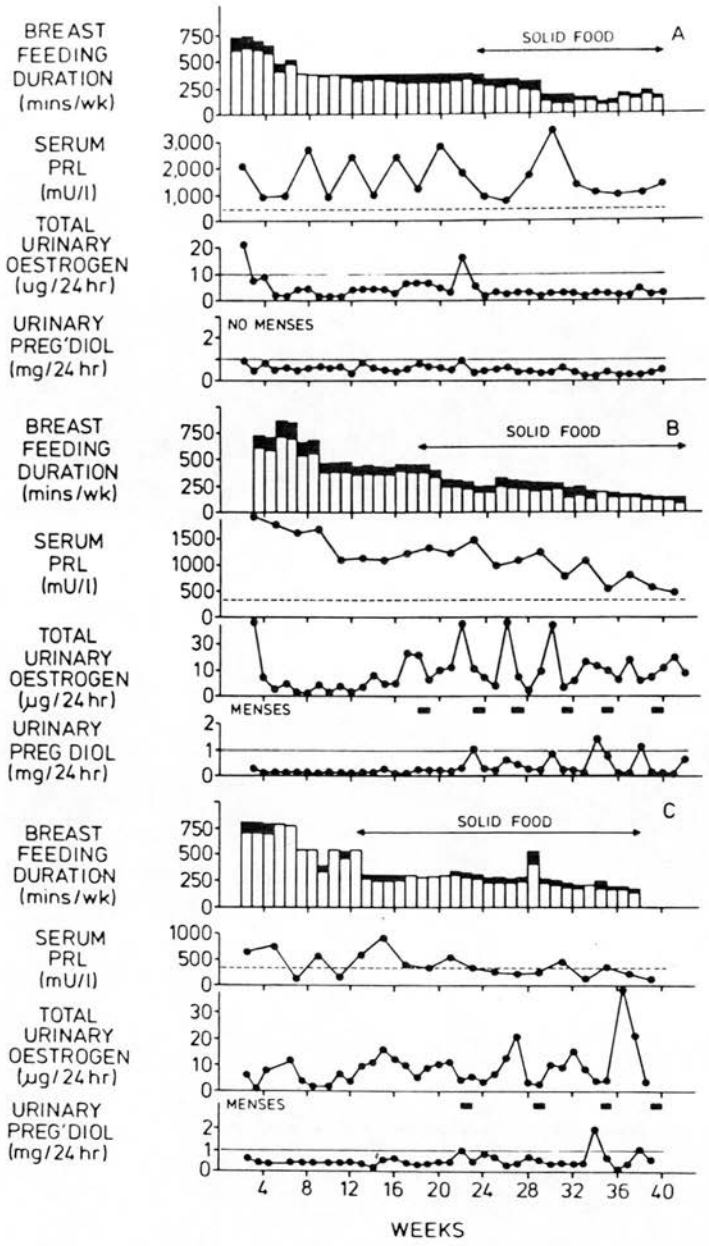



**FIG. 12-2.** Changes in the duration of suckling, serum prolactin, and urinary estrogens and pregnanediol in three breast-feeding women (A, B, & C). One (A) remained amenorrheic while the other two (B & C) menstruated (menses indicated by ■).

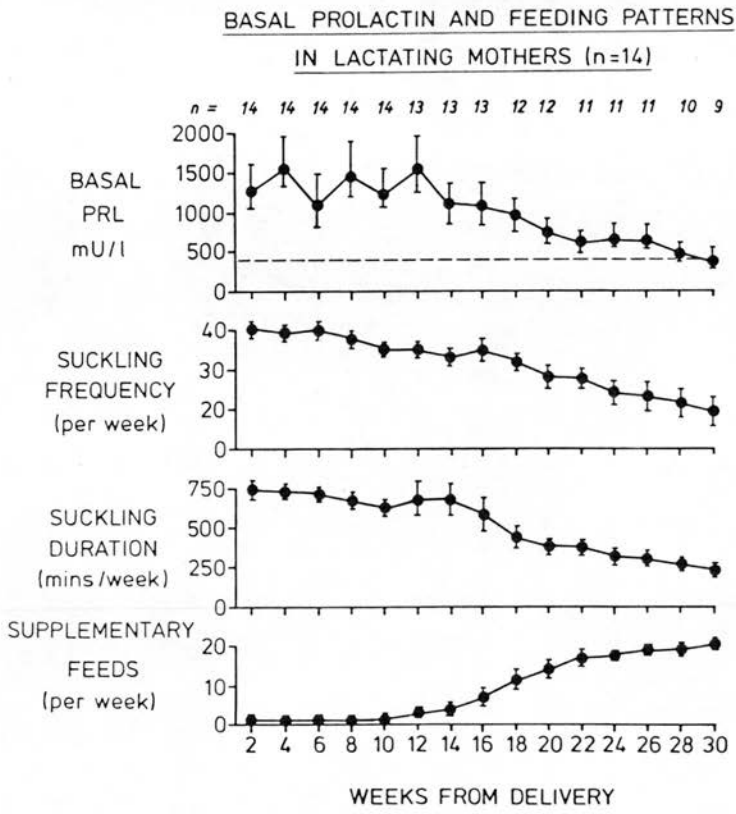



**FIG. 12-3.** Changes (mean ± S.E.M.) postpartum in serum levels of prolactin (log normal), suckling frequency and duration, and the number of supplementary feedings in 14 breast-feeding women.

time all these mothers had introduced three solid feeds per day and had substantially reduced their suckling frequency and duration (Table 12-1).

### ENDOCRINE AND SUCKLING EVENTS BEFORE AND AFTER RESUMPTION OF OVARIAN ACTIVITY

Before the resumption of ovarian activity, clearly shown by the increase in urinary estrogen levels (Fig. 12-5), prolactin levels remained significantly ($P < 0.05$) above normal but declined significantly between the week before and 5 weeks after resumed ovarian activity (Fig. 12-6). At that time prolactin levels were within the normal nonpregnant, nonlactating range (60 – 360 mu/l). Suckling frequency and duration declined steadily from 6 to 8 weeks before until 8 to 9 weeks after resumed ovarian activity. Serum levels of LH did not differ significantly during this period but levels of FSH declined significantly ($P < 0.05$) 1 week after the rise in estrogen secretion (Fig. 12-5).

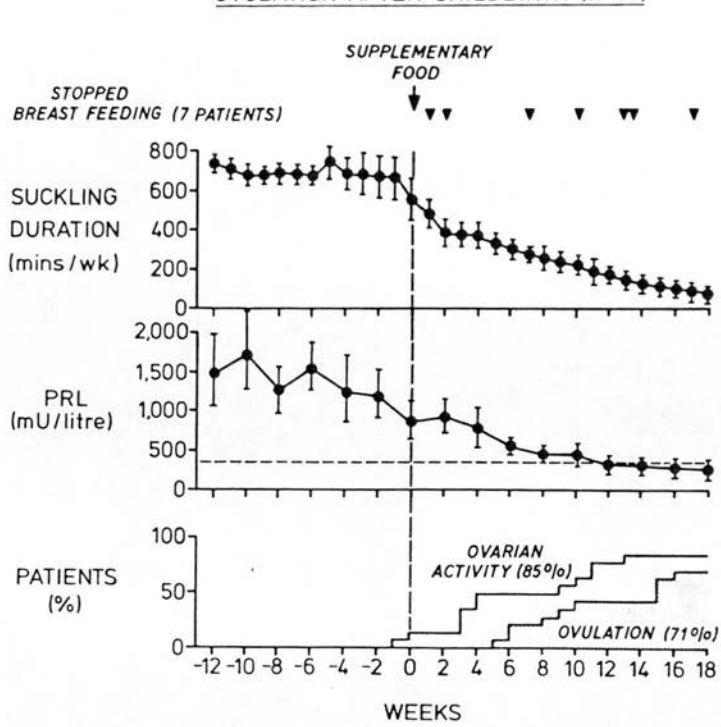



**FIG. 12-4.** Relationships between the introduction of supplementary food and mean ( ± S.E.M.) suckling duration, serum prolactin, and the percentage of women with ovarian activity of ovulation, in 14 women. The arrows (↓) indicate when individual women stopped breast-feeding.

TABLE 12-1. Suckling Duration, and Frequency and Number of Supplementary Feeds 3 Weeks Postpartum and at First Ovulation in Breast-feeding Women

| SUB-JECT NO. | SUCKLING FREQUENCY (EPISODES/DAY) | | SUCKLING DURATION (MIN/DAY) | | SUPPLEMENTARY FEEDS (NO/DAY) | |
|---|---|---|---|---|---|---|
| | *At Week 3* | *At Ovulation* | *At Week 3* | *At Ovulation* | *At Week 3* | *At Ovulation* |
| 1 | 6.0 | 5.7 | 120 | 57 | 0 | 3 |
| 2 | 6.3 | 4.5 | 109 | 41 | 0 | 3 |
| 3 | 6.4 | 1.3 | 160 | 51 | 0 | 3 |
| 4 | 5.0 | 2.6 | 50 | 31 | 0.2 | 3 |
| 5 | 6.3 | 2.0 | 108 | 31 | 0 | 3 |

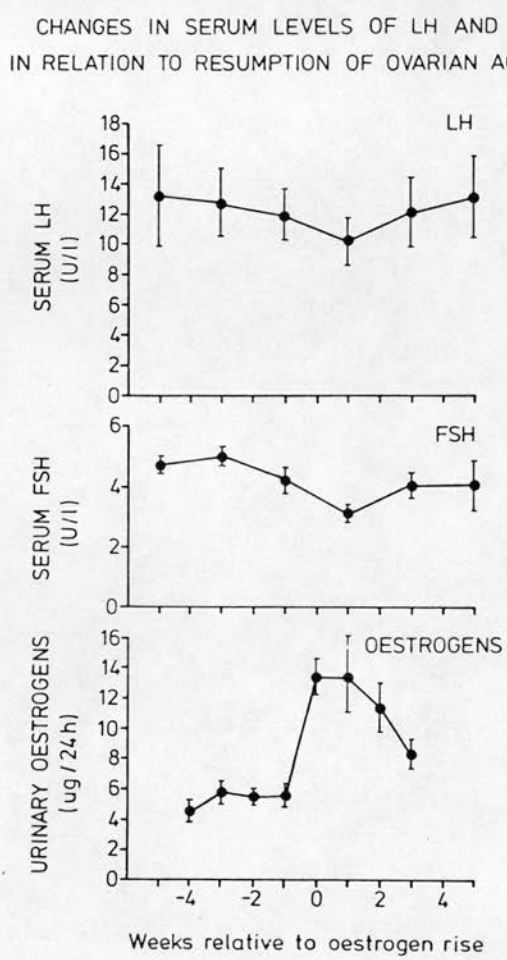



**FIG. 12-5.** Relationship between the resumption of ovarian activity (urinary estrogen levels) and mean (± S.E.M.) serum levels of LH and FSH.

## PROLACTIN AND SUCKLING PATTERN BEFORE AND AFTER RESUMPTION OF OVULATION

Three weeks before the resumption of ovulation, taken as an increase in urinary pregnanediol of 1 mg/24 hr, serum prolactin levels declined significantly ($P < 0.05$) and continued to decline until they were not significantly different from the normal at about 5 weeks after the resumption of ovulation. During this whole time period, both suckling frequency and duration declined steadily (Fig. 12-7).

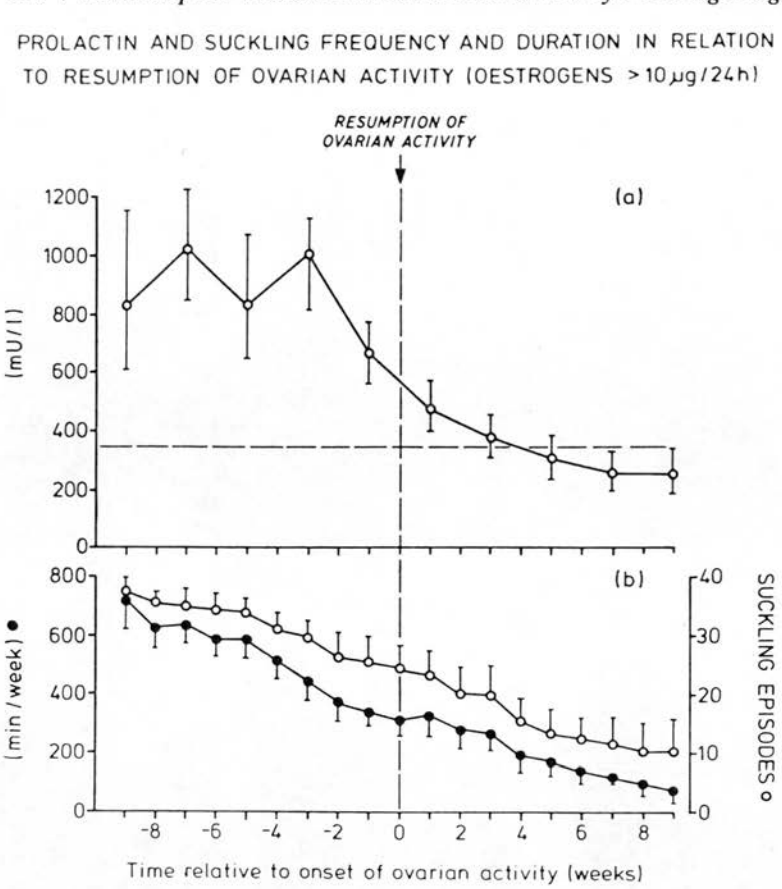



**FIG. 12-6.** Relationship between the resumption of ovarian activity and mean (± S.E.M.) serum levels of prolactin (log normal) and suckling frequency and duration (estrogens > 10 μg/24 hr).

## MENSTRUATION

In nonbreast-feeding women, first menstruation occurred 5 to 13 weeks postpartum. In contrast, 6 out of 14 women failed to menstruate while breast-feeding over a period of 11 to 53 weeks postpartum. Transient ovarian activity in the absence of ovulation and menstruation occurred in 4 out of 6 of these women. In the other 8 women, menstruation occurred between 18 and 33 weeks postpartum, never before the introduction of supplementary food.

The first menstrual period in women who were bottle-feeding was associated with an inadequate luteal phase both in duration (8/8) and peak urinary pregnanediol levels (7/8), only one achieving a level of 0.6 mg/24 hr (Fig. 12-8). The next menstrual period was preceded by normal peak levels of pregnanediol (all > 1.1 mg/24 hr) but in only 1 out of 8 was this of normal duration. By the third menstrual period, luteal function had returned to

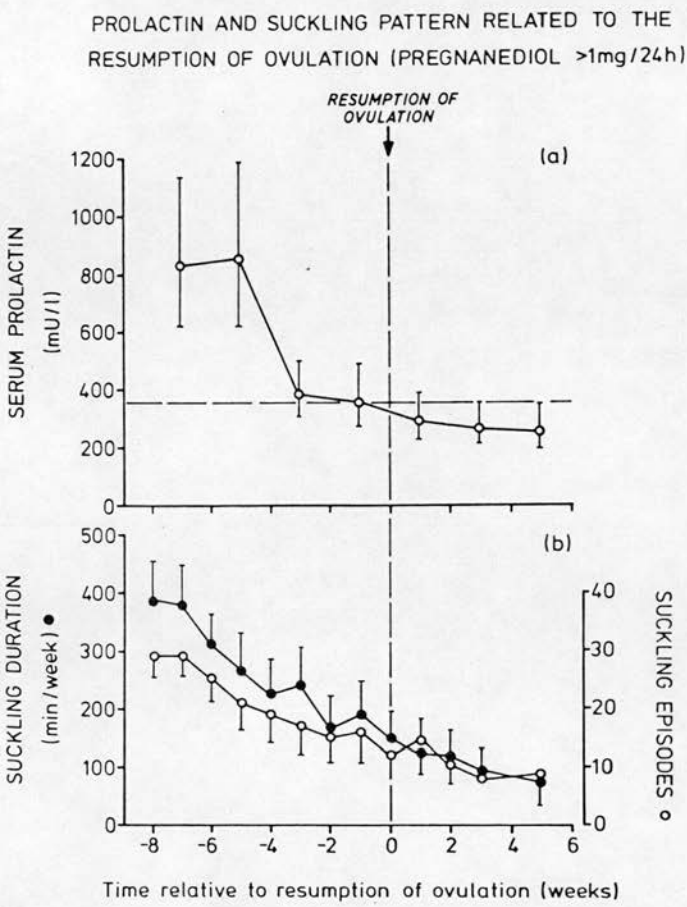



**FIG. 12-7.** Relationship between the resumption of ovulation and mean (± S.E.M.) serum levels of prolactin (log normal) and suckling frequency and duration (pregnanediol > 1 mg/24 hr).

normal. These changes were accompanied by a progressive increase in the estrogen levels prior to menstruation.

During breast-feeding, menstruation was always associated with reduced estrogen secretion and an inadequate luteal phase, in terms of both maximum levels and duration of excretion of pregnanediol (Fig. 12-8). A similar pattern was seen before the first menstrual period after breast-feeding had stopped. However, the second period was preceded by normal estrogen levels and a normal luteal phase.

## DISCUSSION

The present study documents clearly, for the first time, the relationship between the number and duration of suckling episodes and the serum levels of

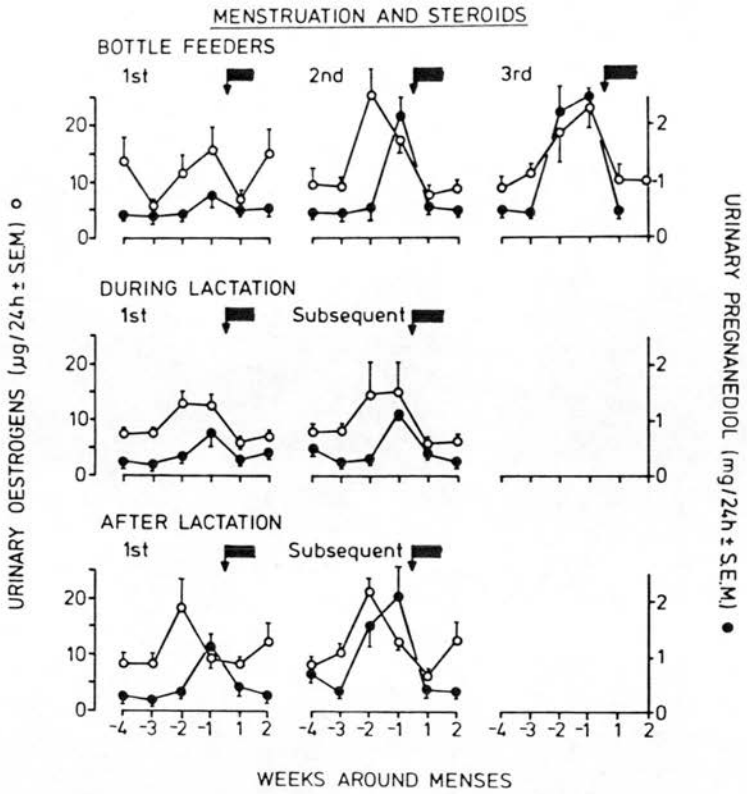



**FIG. 12-8.** Changes in mean (± S.E.M.) levels of estrogens and pregnanediol in relation to menstruation (▮) occurring in bottle-feeders, during lactation, and after lactation.

prolactin in relation to the resumption of ovarian follicular development, ovulation, and menstruation during breast-feeding. The results show clearly that normal cyclical ovarian activity resumes within 9 weeks postpartum in nonlactating women, whereas resumption of ovarian activity is delayed by breast-feeding, as demonstrated in previous epidemiologic or cross-sectional studies.[1,3,11,12,13,19,21] In contrast to the latter studies, the present longitudinal investigation allows a proper evaluation of the sequence of events and the precipitating factors which result in the return of fertility in breast-feeding women.

Basal levels of prolactin were related to both the number and duration of suckling episodes and remained above normal during breast-feeding. Previous results suggest that both these parameters are important in maintaining raised levels of prolactin during lactation.[6,18] Our present data do not suggest which, if either, of these two parameters is more important.

The most dramatic influence on the suckling pattern was the introduction of supplementary food, whether solid or in the form of bottles of milk. The initial change was a reduction in the duration of suckling, later followed by a

decline in the number of suckling episodes. Basal levels of prolactin declined in parallel with the decrease in suckling duration.

Of major significance was the effect of supplementary food on ovarian activity. Before supplementation, ovarian activity remained suppressed. Supplementation was associated with an increase in the number of women showing ovarian follicular development which was followed, after about a 6 week delay, by a parallel increase in the number of women ovulating. Since the resumption of ovarian activity was associated with both a reduction in the suckling stimulus and a parallel decline in the serum levels of prolactin, the present data do not allow any conclusions to be drawn concerning the relative importance of these parameters in maintaining infertility during non-supplemented breast-feeding.

Resumption of ovarian activity was associated with a decline in serum levels of prolactin, although these still remained above normal. On the other hand, resumption of ovulation was associated with a further drop in prolactin levels 3 to 5 weeks before ovulation. This drop was not associated with any accelerated decline in suckling frequency or duration, suggesting that there may be a threshold for both these parameters of suckling pattern which is required to maintain raised basal levels of prolactin and ovarian suppression.

Before and after the resumption of ovarian activity, the serum levels of both LH and FSH were within their normal early follicular phase ranges, confirming previous reports.[1,3,11,12,13,19,21] Preliminary studies have shown, however, that before the resumption of estrogen secretion, the pulsatile secretion of LH and estradiol is reduced or absent,[17] suggesting that breast-feeding may act initially by reducing the pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH). When follicular development did resume with cyclical estrogen secretion, these levels were low in women who continued to breast-feed, and in 50% of the women, ovulation, as judged by an increase in urinary pregnanediol, did not appear to occur. Recently, it has been shown that fully breast-feeding women have an impaired positive feedback response of LH and FSH to estrogen stimulation, suggesting that even though gonadotropin secretion is adequate for the growth and development of the follicle, the ovulatory surge of LH cannot occur and ovulation will not take place.[2]

In the present study, only 8 out of 14 women menstruated while breast-feeding. It is significant that in all these women, menstruation was preceded by a short-term rise in pregnanediol excretion. This suggests that ovulation may have taken place, but resulted in the formation of an inadequate corpus luteum rather than anovulation, as suggested previously on the basis of endometrial biopsies.[21] There was no instance in which a breast-feeding woman had a normal luteal phase; in fact, normal corpus luteum function was not observed in the majority of women (12 out of 14) until the second ovulatory cycle postweaning.

The reason why ovulation of an apparently normally developed follicle, at least in terms of estrogen secretion, results in an inadequate corpus luteum is unclear. It may be related to the direct intrafollicular antagonistic role of elevated levels of prolactin on normal follicular development[7,14] or to inadequate priming of the follicle because of an as yet undemonstrated reduction in gonadotropin stimulation. Nevertheless, these data clearly indicate that in the majority of cases, the first menstrual period in both breast-feeders and bottle-

feeders is associated with corpus luteum function, which is not compatible with fertility. The value of studies in which the return of "fertility" postpartum has been documented, either by menstrual history or endometrial biopsy, becomes questionable.

The evidence strongly indicates that breast-feeding, as practiced in the majority of Western societies, is associated with a cessation of ovarian activity for a variable period after delivery. The most important factor which appears to precipitate ovarian activity (often in the absence of ovulation) is the introduction of supplementary food. This results in a dramatic reduction in the length of time of each suckling episode and a more gradual decline in the number of suckling episodes, presumably because the introduction of supplementary food reduces the infant's nutritional need for breast milk. However, because the introduction of supplementary food to the infant results in a resumption of ovarian activity in the mother, with consequent earlier return of fertility, infant food supplementation must be carefully considered in many societies. This should be investigated not only on the grounds of the true nutritional impact on the infant, but also because of its potential fertility-promoting effect on the mother and the consequent, possibly drastic, effect on the welfare of the family.

## FUTURE DEVELOPMENTS

The present study shows that any decrease in suckling duration or frequency is associated with a decrease in serum levels of prolactin. As these levels fall, ovarian activity is resumed, but only when the serum prolactin levels are near or within the range found in a nonpregnant woman will ovulation, normal luteal function, and fertility return. Introduction of supplementary food can initiate these changes. While it is impractical to suggest that the suckling frequency or duration be maintained at presupplementation levels to sustain suppression of ovarian activity, maintenance of raised levels of prolactin by pharmacologic methods may offer an equally effective approach. Several agents are known to induce and maintain raised prolactin levels.

Thyrotropin-releasing hormone (TRH) acts directly on the pituitary to release both thyrotropin-stimulating hormone (TSH) and prolactin. It has been used to enhance lactation and in a preliminary trial, anovulation and amenorrhea appeared to be maintained.[23] However, because large doses of TRH may cause increased thyroid function, it seems unlikely that this would be suitable as a generally available contraceptive agent.

Phenothiazines, particularly chlorpromazine, are potent releasers of prolactin and appear to act by decreasing hypothalamic catecholamine turnover. The dosage required to maintain adequate hyperprolactinemia is such that symptoms related to decreased brain catecholamine turnover, *e.g.,* parkinsonian side-effects, may occur. However, a substantial delay in the return of fertility in lactating Bonnet monkeys has been achieved with very low doses of chlorpromazine delivered by intranasal spray.[18] Whether this would be applicable to women remains to be seen.

Of greatest promise is the use of the procaine amide derivatives sulpiride and metaclopramide. Both drugs act directly on the pituitary to cause increased

release of prolactin by blocking the action of prolactin inhibitory factor (PIF = dopamine). Neither drug appears to have side-effects, and both have been in use for many years as antiemetic agents. Sulpiride has been used to induce anovulation and amenorrhea in normally cyclic women but the high levels of prolactin resulted in galactorrhea in some women.[6] Since galactorrhea, the only observable side-effect of sulpiride and metaclopramide, is of no consequence in a breast-feeding woman, these drugs appear to offer the greatest potential as agents to maintain the infertility associated with breast-feeding in the presence of declining suckling frequency and duration.

The development of agents which maintain the natural contraceptive effect of lactation would allow breast-feeding mothers more control over the return of this fertility than is now possible and, it is hoped, would provide a more acceptable alternative to the use of steroidal contraceptives during lactation.

A proper understanding of the mechanisms whereby breast-feeding naturally suppresses ovarian activity should form the basis for future development in contraception for nonlactating women. It may be possible to develop methods in which the hypothalamic changes caused by breast-feeding, with its contraceptive effect, can be induced, without increasing serum prolactin levels. This would avoid the galactorrhea associated with presently available drugs such as sulpiride and would provide a more natural means of suppressing ovarian activity.

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# Effect of supplementary food on suckling patterns and ovarian activity during lactation

P W HOWIE, A S McNEILLY, M J HOUSTON, A COOK, H BOYLE

**Abstract**

**Patterns of infant feeding, basal prolactin concentrations, and ovarian activity were studied longitudinally in 27 breast-feeding mothers from delivery until first ovulation.**

**Suckling frequency (6·1 feeds/day) and suckling duration (122 mins/day) reached peak values four weeks post partum and remained relatively constant until the introduction of supplementary food at a mean of 16 weeks post partum. There were subsequently sharp declines in both the frequency and duration of suckling, both of which correlated closely with basal prolactin concentrations. None of the 27 mothers ovulated during unsupplemented breast-feeding, but within 16 weeks of introducing supplements ovarian follicular development had returned in 20 and ovulation in 14 mothers. The mothers who ovulated within 16 weeks of giving supplements had reduced frequency and duration of suckling more quickly and weaned more abruptly than those who continued to suppress ovulation.**

**These data suggest that the introduction of supplementary food may exert an important and hitherto unrecognised effect on the timing of first ovulation by reducing the frequency and duration of suckling episodes.**

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## Introduction

In western society the contraceptive effect of lactation has been largely dismissed on the grounds that it is ineffective and unreliable. By contrast, in developing countries the duration of lactational amenorrhoea may be prolonged and, by increasing inter-birth intervals, exert a profound effect on population growth.[1]

Since the suppression of ovarian activity may be related to the suckling stimulus, variations in infant feeding practices may account for the different contraceptive efficiencies in developed and developing countries. In this longitudinal study of breast-feeding mothers we report on the effect that the introduction of supplementary food may have on the frequency and duration of suckling and the resumption of ovarian activity after childbirth.

## Subjects and methods

Twenty-seven breast-feeding mothers aged 23-40 years were recruited to the study; all had delivered mature babies of normal birth weight and had decided not to use steroid contraception. Mothers were studied longitudinally from delivery throughout lactation and until ovulation was re-established.

Each mother kept a daily feeding data card on which she recorded the number of breast feeds and their duration; she also noted the number of supplementary feeds given either as bottles of formula milk or solid food from a spoon. The mother was visited at home every two weeks by a research sister to confirm that the feeding data card was being kept up to date.

A 24-hour urine sample was collected once weekly for assessment of total urinary oestrogen and pregnanediol concentrations. The resumption of ovarian follicular activity was recognised by a rise of total urinary oestrogens above 10 μg/24 hours and the resumption of ovulation by a urinary pregnanediol concentration above 1 mg/24 hours. A blood sample was taken for estimating basal prolactin concentrations at two hours or more after the last suckling episode every two weeks.

Urinary excretion of total oestrogen was measured fluorometrically[2] and pregnanediol was determined by gas-liquid chromatography.[3] Prolactin concentrations were measured by a specific double antibody radioimmunoassay using reagents supplied by Dr H G Friesen (Winnipeg, Canada)[4] with an interassay variation of 9%. Results are expressed in prolactin reference preparation MRC 75/504 (10 U/ampoule, 22 μU = 1 ng). To allow for skew distributions, mean prolactin concentrations were calculated by logarithmic transformation. Comparisons between groups were made using Student's *t* test.

## Results

The mean duration of breast-feeding among the 27 breast feeders was 40·5 weeks (range 12-82) and supplementary food was first introduced at a mean of 16·1 ± SE 0·9 weeks. First menses resumed at a mean of 32 ± 2·4 weeks and first ovulation at 36 ± 2·5 weeks.

Four weeks post partum peak values were reached for both the number of suckling episodes (6·1 ± SE 0·2/day), and suckling duration (122 mins ± 11/day); thereafter there were progressive falls in both these factors which were closely paralleled by changes in basal prolactin (fig 1). The mean basal prolactin concentrations correlated

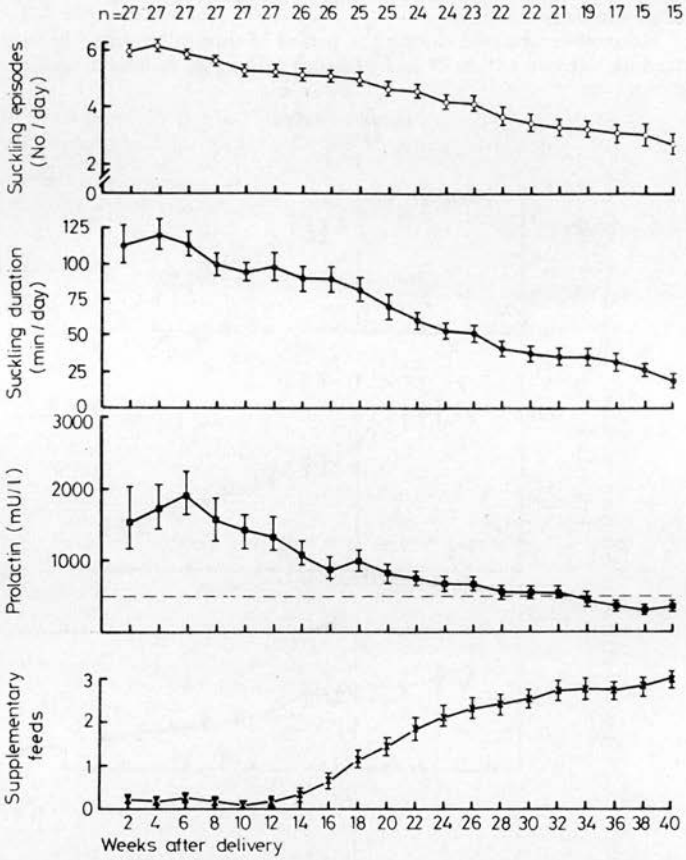



FIG 1—Mean (±SE) suckling episodes, suckling duration, basal prolactin, and supplementary feeds in 27 breast-feeding mothers after delivery. Numbers fall as mothers discontinue breast feeding.

closely with both the mean suckling frequency ($r = 0{\cdot}946$) and the mean suckling duration ($r = 0{\cdot}949$). The mean number of supplementary feeds were low for the first 12 weeks and progressively increased thereafter as suckling and basal prolactin fell; the mean number of supplementary feeds correlated negatively with mean basal prolactin concentration ($r = -0{\cdot}923$), mean suckling duration ($r = -0{\cdot}983$), and mean suckling frequency ($r = 0{\cdot}958$). Significant correlations were maintained for individual subjects as well as for the population means.

EFFECT OF SUPPLEMENTARY FOOD ON SUCKLING, BASAL PROLACTIN CONCENTRATION, AND OVARIAN ACTIVITY

Supplementary food was introduced between three and 24 weeks post partum. To eliminate this variation, data were centred on the introduction of supplementation (fig 2). Mean suckling frequency and duration were relatively constant during full (un-supplemented) breast-feeding, but both fell sharply at the time when supplements were introduced. Mean prolactin concentrations responded less acutely to the supplementary food but fell to the non-pregnant range 12 weeks after supplementation.

No mother ovulated during the period of unsupplemented breast-feeding but two out of 27 had evidence of ovarian follicular activity.

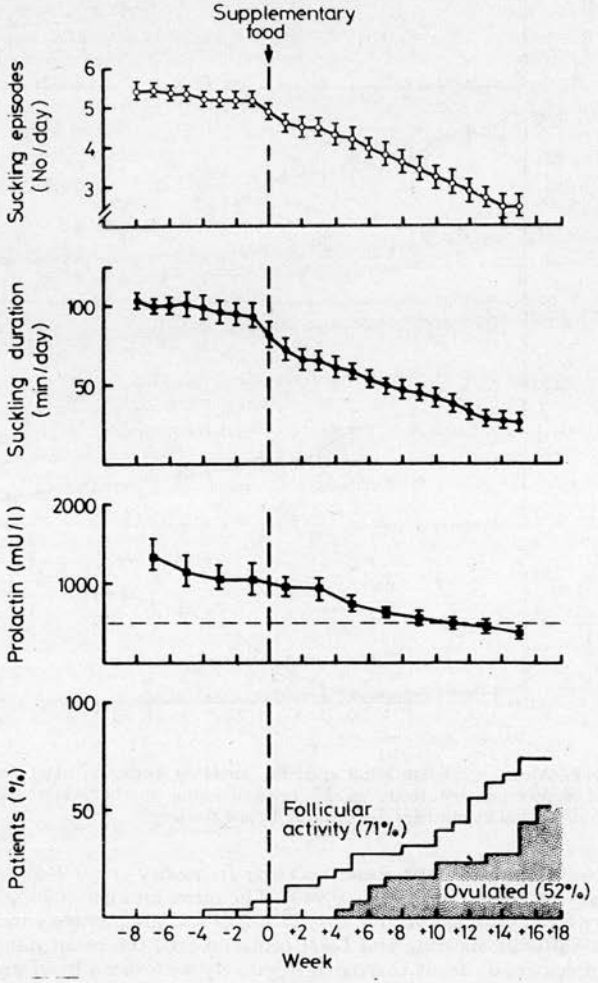



FIG 2—Mean (±SE) suckling episodes, suckling duration, basal prolactin, and gonadal activity in 27 breast-feeding mothers before and after introduction of supplementary food.

After the introduction of supplementary food the number of mothers showing evidence of ovarian activity progressively increased and, within 16 weeks, 20 out of 27 mothers had follicular activity and 14 out of 27 had ovulated.

INFANT FEEDING PATTERNS AND BASAL PROLACTIN IN OVULATING AND SUPPRESSED MOTHERS

Comparison was made between the 14 mothers who ovulated within 16 weeks of introducing supplementary food ("ovulating" group) and the 13 mothers who continued to suppress ovulation ("suppressed" group). The ovulating group had introduced supplements at 15·6 ± SE 0·9 weeks post partum compared with 16·6 ± 1·0 weeks in the suppressed group.

There were no differences between the two groups before the introduction of supplements in respect of suckling frequency, suckling duration, or basal prolactin concentrations (fig 3). After supplements, however, the suppressed group of mothers maintained suckling frequency and suckling duration at higher levels and introduced supplements more abruptly than the ovulating group ($p < 0.01$ in all cases). Mean basal prolactin concentrations remained above the non-pregnant range in the suppressed group for 16 weeks after supplements, but fell to the non-pregnant range within five weeks after supplements in the ovulating group.

## Discussion

The time at which breast-feeding mothers should be advised to introduce supplementary food is of great importance. It has been claimed that some mothers may be incapable of giving sufficient energy by breast milk alone from the third month of life onwards,[5] but this view has been disputed.[6] Alternatively, the early resort to supplementary food increases the risk of neonatal infection, which may have particularly important consequences on infant mortality in developing countries.[7]

Our data indicate that the introduction of supplementary food may have a profound impact on another factor—the restoration of fertility. The suckling stimulus is probably the primary factor responsible for the period of natural infertility during lactation. During pregnancy the nipple is relatively insensitive, but this is reversed immediately after delivery.[8] This increase in nipple sensitivity ensures a rapid input of afferent stimuli, which increase hypothalamic sensitivity to the negative feedback effect of ovarian steroids.[9] This in turn leads to a failure of adequate gonadotrophin production from the pituitary to induce ovulation.[10]

In a series of cross-sectional studies, Delvoye *et al*[11] showed that prolactin concentrations and the duration of postpartum amenorrhoea were related to the frequency of suckling episodes. Similarly El-Minawi and Foda[12] reported that partially nursing mothers had a shorter duration of amenorrhoea compared with those who were fully nursing. In a recent report Konner and Worthman[13] found that the prolonged birth spacing of up to four years in the !Kung hunter gatherers of the Kalahari desert was associated with frequent, short bursts of suckling during the

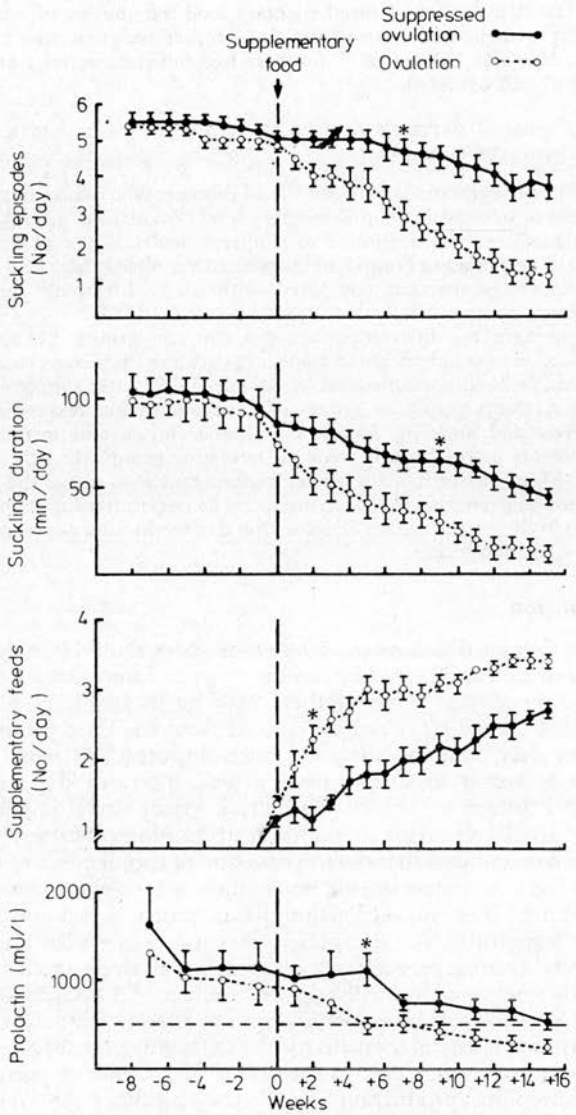



FIG 3—Comparison of suckling episodes, suckling duration, supplementary feeds, and basal prolactin (mean concentrations ± SE) in breast-feeding mothers who ovulated or continued to suppress ovulation after introduction of supplementary feeds. *$p < 0.01$.

**first three years of life. These considerations suggest that the duration and frequency of the suckling stimulus are the central control mechanisms in determining the resumption of ovulation after childbirth.**

In our longitudinal study the number of supplementary feeds was inversely correlated with suckling frequency, suckling duration, and the basal prolactin concentration, and the introduction of supplementary food was associated with abrupt falls in both the duration and the frequency of suckling. It could be argued that both these last will fall over time, irrespective of the introduction of supplementary food. We found, however, that the rate at which supplements were introduced influenced not only suckling frequency and duration, but also the resumption of ovulation. This suggested that supplementary food, by reducing the suckling stimulus, was an important determining factor in controlling ovarian activity.

In many countries contraception during lactation is either not available or not acceptable, so that breast-feeding continues to play an important part in determining the intervals between births. It has been estimated that in developing countries lactation prevents more pregnancies than all artificial methods of contraception.[14] It is important that policies concerning infant feeding and the introduction of supplementary food should not be formulated without full realisation of their impact on birth spacing and population growth.

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# FERTILITY AFTER CHILDBIRTH: POST-PARTUM OVULATION AND MENSTRUATION IN BOTTLE AND BREAST FEEDING MOTHERS

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SUMMARY

The resumption of post-partum menstruation and ovulation was studied in ten bottle feeding and twenty-seven breast feeding mothers. First menstruation occurred at 8·1 weeks (±1·0 SE) in bottle feeders and 32·5 weeks (±2·5 SE) in breast feeders ($P < 0{\cdot}001$); first ovulation occurred at 10·8 weeks (±1·0 SE) in bottle feeders and 36·4 weeks (±2·5 SE) in breast feeders ($P < 0{\cdot}001$).

In bottle feeders, ovulation preceded first menses in only 2/10 (20%) of mothers but was regularly established thereafter, occurring in 17/18 (94%) of second and subsequent cycles.

Breast feeding did not postpone ovulation indefinitely because 13/27 of the breast feeding mothers ovulated while still lactating; ovulation occurred in 9/27 (33%) of breast feeding mothers during the phase of lactational amenorrhoea but was followed by menstruation in every case. In breast feeding mothers, the frequency of ovular cycles progressively increased with time, ovulation being observed in 9/20 (45%) of first cycles during lactation, 20/30 (66%) of later cycles during lactation, 16/23 (70%) of first cycles after lactation and 26/31 (84%) of later cycles after lactation.

There was a disruption of menstrual rhythms during lactation, the mean interval between the first day of consecutive menstrual cycles being 37·0 days ± 3·3 SE during lactation compared with 29·8 days ± 1·0 SE after lactation and 29·5 days ± 1·0 SE in the bottle feeding mothers.

This study shows that bottle feeding is associated with an early resumption of post-partum menstruation and ovulation. In breast feeding mothers, there is complete suppression of ovulation during the greater part of lactational amenorrhoea but ovulation will return in a proportion of mothers just before

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first menses. After the return of menstruation during lactation, the frequency of ovular cycles progressively increases but does not return to normal until complete weaning has taken place.

There is epidemiological evidence that breast feeding prolongs the period of infertility after childbirth (for review see Buchanan, 1975; Badraoui & Hefnawi, 1979). This effect is of major demographic importance in developing countries where it has been estimated that lactational infertility prevents more pregnancies than all methods of artificial contraception put together (Rosa, 1975).

Studies which are based on the interbirth interval do not provide direct evidence of the mechanisms responsible for the reduced fertility during lactation because behavioural factors, such as sexual taboos or contraceptive usage, will influence the interval to next post-partum conception (Thomson *et al.*, 1975). The duration of lactational amenorrhoea gives a better indication of gonadal suppression (van Ginneken, 1977) but does not provide information on the frequency of anovular cycles before or after the return of menstruation. It is necessary, therefore, to determine the timing and frequency of ovulation in order to define fully the degree of gonadal suppression associated with lactation.

Studies, using cross-sectional data, have demonstrated that lactation is associated with hyperprolactinaemia, low levels of oestradiol and progesterone and levels of gonadotrophins which are either normal or just below the normal range (Delvoye *et al.*, 1978; Duchen & McNeilly, 1980). From such cross-sectional studies it is difficult to interpret the mechanisms responsible for the natural state of hypogonadism during lactation (McNeilly, 1979).

The present report is the first of a series drawn from a longitudinal study of bottle and breast feeding mothers which was designed to examine how suckling patterns, basal PRL levels and gonadotrophin secretion influenced the resumption of post-partum menstruation and ovulation. In this first report, the timing and frequency of post-partum ovulation was defined in a group of bottle and breast feeding mothers using urinary steroid excretion as an objective marker of ovarian function. Preliminary data from this study has already been published (McNeilly *et al.*, 1980) but the present report includes the results from the total cohort of mothers.

## SUBJECTS AND METHODS

### *Subjects*

Thirty-one breast feeding and ten bottle feeding mothers were recruited in the post-natal wards of the Simpson Memorial Maternity Pavilion, Edinburgh. Four of the breast feeding mothers were subsequently excluded; two because they left Edinburgh at 19 and 23 weeks and two because they started progestogen oral contraceptive at 7 and 10 weeks respectively. Ovulation had not occurred at the time of exclusion in any of these four patients.

The remaining twenty-seven breast feeders aged 23–40 years and the ten bottle feeders aged 24–34 years are the subjects of this report. Thirteen of the breast feeders were primigravidae and fourteen had previously breast fed one or more infants. Four of the bottle feeders were primigravidae and six were parous. None of the mothers used steroidal contraceptives during the period of study; eleven mothers used barrier contraception, six

were fitted with a coil and two used the rhythm method; seven mothers used no contraception. All lived within Edinburgh for ease of visiting and collecting specimens. Each mother gave informed consent and kept a daily record of the duration, number and timing of suckling episodes, the number of formula milk feeds, the introduction of solid feeds and periods of menstrual bleeding. Supplementary feeds were defined as either formula milk feeds or solid feeds. Weekly 24-h urine samples were collected for estimation of total oestrogens and pregnanediol. Every 2 weeks, each mother was visited in her own home by a research sister who collected the data card and a blood sample at least 2 h after the last breast feed.

*Methods*

Blood samples were centrifuged and plasma was stored at $-20°C$ until assayed for PRL. Urine samples were assayed on the day of collection when possible or stored in aliquots at $-20°C$ until time of assay.

Urinary excretion of total oestrogen was measured fluorimetrically (Brown *et al.*, 1968) and pregnanediol was determined by gas–liquid chromatography (Chamberlain & Contractor, 1968).

PRL was measured by specific double antibody radioimmunoassay (McNeilly & Hagen, 1974) using reagents supplied by Dr H. G. Friesen, Winnipeg, Canada, with an interassay variation of 9%. Results are expressed in U MRC PRL reference preparation 75/504 (10 U/ampoule; 32·5 $\mu$U = 1 ng).

*Definition of follicular development and ovulation*

Ovarian follicular development was defined as a total urinary oestrogen of $\geqslant 10$ $\mu$g/24 h. This definition was taken from a series of twenty-seven normal cycles in six women (McNeilly *et al.*, 1982). In these cycles, there was always at least one weekly value of total urinary oestrogen of greater than 10 $\mu$g/24 h. In the follicular phases of these twenty-seven normal cycles, urinary pregnanediol levels never rose above 0·8 mg/24 h so that a urinary pregnanediol of more than 1 mg/24 h was assumed to indicate corpus luteum activity and presumptive ovulation.

*Statistical methods*

Comparisons between groups were made using Student's *t* test.

## RESULTS

The endocrine patterns leading to the resumption of menstruation and ovulation are illustrated for a typical bottle feeding mother (Fig. 1) and a typical breast feeding mother (Fig. 2).

*Menstruation and ovulation in a bottle feeding mother (Fig. 1)*

In a typical bottle feeding mother, basal PRL levels had fallen to within the non-pregnant range by 4 weeks post-partum. Ovarian follicular development at 5 weeks post-partum was followed by first menstruation. Ovulation did not occur before first menses but did so in the second and third cycles post-partum.

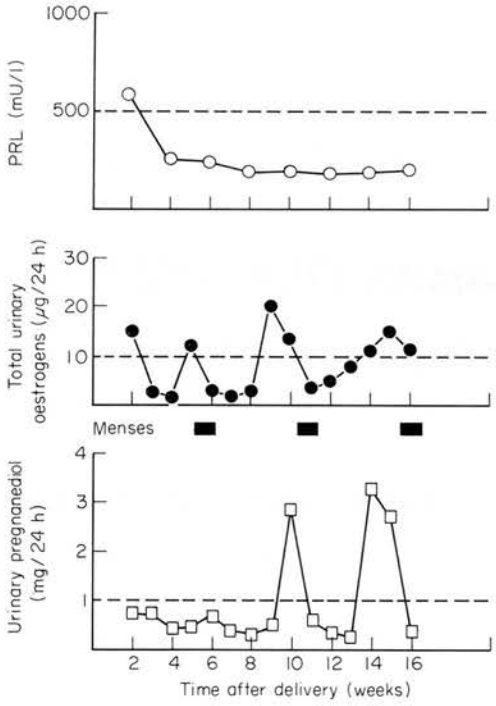



Fig. 1. Basal PRL levels, menstruation, and urinary steroid excretion in a bottle feeding mother (ovulation after first menses) during the post-partum period.

*Menstruation and ovulation in a breast feeding mother (Fig. 2)*

In the breast feeding mother, chosen as an example, suckling duration and frequency, calculated as daily mean values for individual weeks, remained relatively constant until solid food was introduced at 16 weeks post-partum. Thereafter there was an immediate fall in suckling duration despite the number of suckling episodes remaining constant for a further 4 weeks. Basal PRL levels fell after the introduction of solids but remained above the non-pregnant range until breast feeding stopped. Ovarian activity was suppressed throughout lactation but ovarian follicular development and ovulation restarted as soon as breast feeding stopped. The second cycle was anovular but ovulation was re-established the next cycle.

*First ovulation and first menses; comparison of bottle and breast feeding mothers*

In the twenty-seven breast feeding mothers, who breast fed for a mean of 40·3 weeks $\pm$ 3·1 SE, first menstruation occurred at a mean of 32·5 weeks $\pm$ 2·5 SE compared with 8·1 weeks $\pm$ 1·0 SE in the ten bottle feeders ($P < 0{\cdot}001$); two breast feeding mothers did not ovulate during the period of study (see below) and in the remaining twenty-five, first ovulation occurred at a mean of 36·4 weeks $\pm$ 2·5 SE compared with 10·8 weeks $\pm$ 1·0 SE in the bottle feeders (PRL $< 0{\cdot}001$).

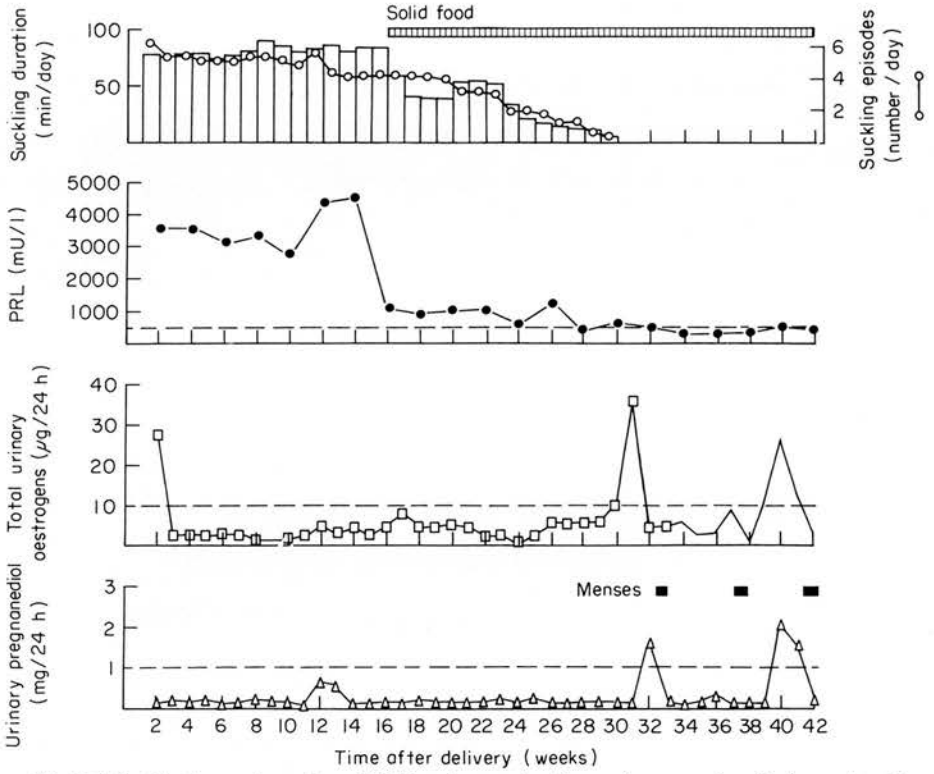



Fig. 2. Infant feeding patterns, basal PRL levels, menstruation and suppression of urinary steroid excretion in a breast feeding mother (amenorrhoea during lactation, ovulation before first menses) during the post-partum period.

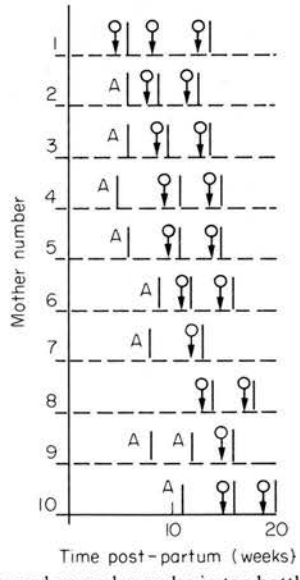



Fig. 3. Timing of menses, ovular and anovular cycles in ten bottle feeding mothers post-partum. | = menses; ♀ = ovulation; A = anovular.

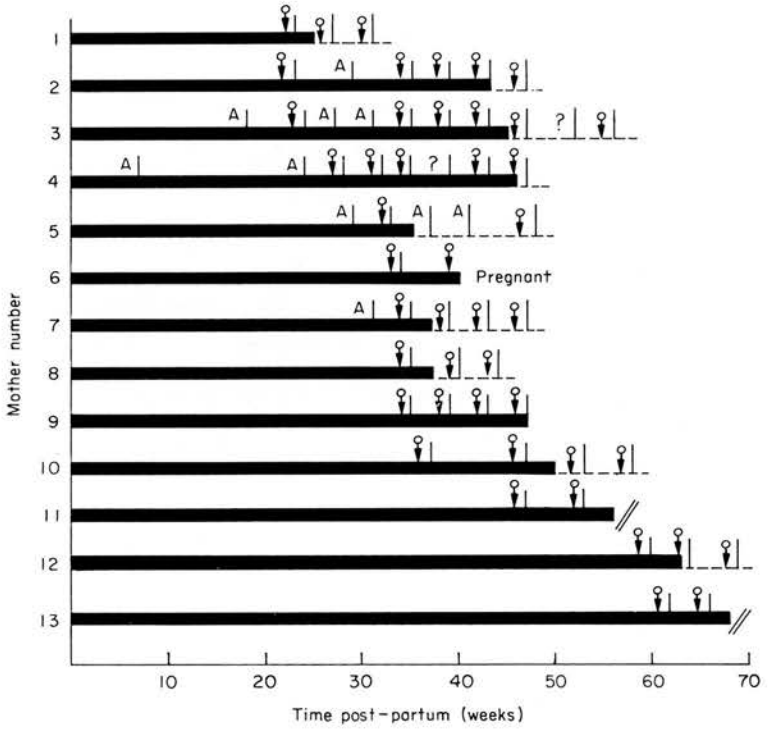



Fig. 4. Duration of breast feeding and timing of menses, ovulation and anovular cycles in thirteen nursing mothers who ovulated during lactation. ▬ = breast feeding; | = menses; ♀ = ovulation; A = anovular; ? = not documented.

*Timing and frequency of ovular cycles*

The timing and frequency of ovular cycles in the individual mothers are shown for the bottle feeders (Fig. 3) and for the breast feeders (Figs 4 and 5); the breast feeders are presented according to whether first ovulation occurred during (Fig. 4) or after lactation (Fig. 5).

*Ovular cycles in bottle feeding mothers (Fig. 3)*

Ovulation preceded first menses in only 2/10 (20%) of bottle feeders but, thereafter, ovulation was observed in 17/18 (94%) of second or subsequent cycles. Menstruation and ovulation had been re-established in all bottle feeding mothers by 15 weeks post-partum.

*Ovular cycles during lactation (Fig. 4)*

Thirteen of the twenty-seven breast feeding mothers (48%) ovulated during lactation between 22 and 61 weeks post-partum; nine mothers (33%) ovulated during the period of lactational amenorrhoea but ovulation was followed by menses in every case; one mother (number 6) conceived during her second ovular cycle at 39 weeks post-partum. Two mothers (numbers 11 and 13) in whom lactation was prolonged to 80 and 82 weeks

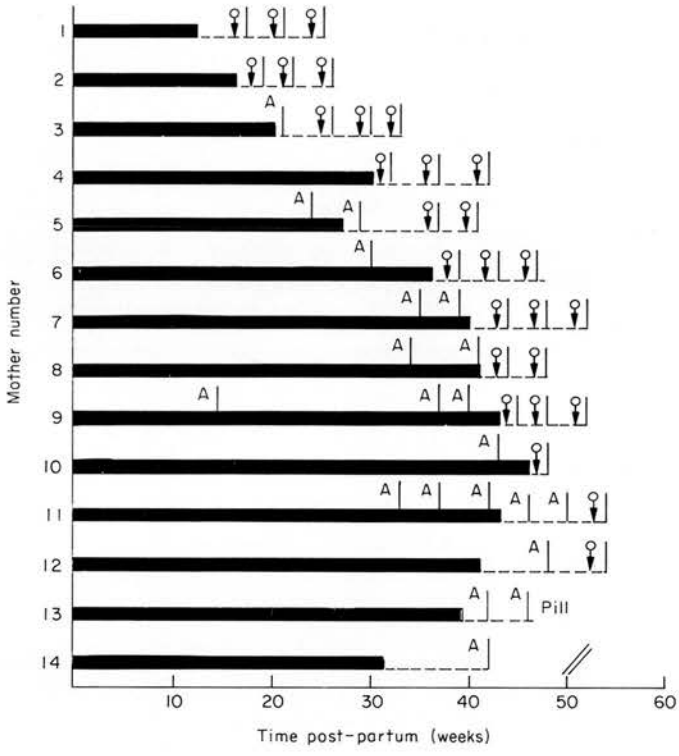



Fig. 5. Duration of breast feeding and timing of menses, ovulation and anovular cycles in fourteen nursing mothers who suppressed ovulation during lactation (for definition of symbols see legend to Fig. 4).

respectively, had observations discontinued after both had completed two consecutive ovular cycles.

*Ovulation after lactation (Fig. 5)*

Fourteen mothers suppressed ovulation throughout lactation, seven being amenorrhoeic and seven having one or more anovular cycles while breast feeding. In 12/14 of this group, ovulation resumed at a mean of 4·3 weeks, range 1–12 weeks, after complete weaning. Two mothers did not ovulate during the study period; one (number 13, Fig. 5) restarted oral contraceptives 7 weeks after lactation before re-establishing ovulation; the other (number 14, Fig. 5) had only one anovular cycle in the 20 weeks after stopping lactation, the reason for the failure to ovulate being unexplained.

*Timing of ovular and anovular cycles*

Cycles which occurred early in lactation tended to be anovular. When first menses was preceded by an anovular cycle, bleeding started at a mean of 27·2 weeks ($\pm$3·1 SE) but when first menses during lactation was preceded by ovulation, bleeding started at a mean of 39·6 weeks ($\pm$4·7 SE, $P < 0{\cdot}05$).

*Ovulation during lactational amenorrhoea*

Nine of the twenty-seven breast feeding mothers (33%) ovulated during lactational amenorrhoea but in every case, this was followed by menstruation. These nine ovulatory cycles occurred during a total of 200 months of lactational amenorrhoea in the twenty-seven breast feeding mothers.

*Frequency of ovular cycles after return of menses*

The proportion of ovular cycles increased progressively in lactating women, ovulation being observed in 9/20 (45%) of first cycles during lactation, in 20/30 (66%) of second or later cycles during lactation, in 16/23 (70%) of first cycles after lactation and 26/31 (84%) of second or later cycles after lactation.

*Intermenstrual intervals*

The mean interval between the first days of menstrual bleeding in cycles beginning during lactation was 37·0 days (±3·3 SE) compared with 29·8 days (±1·0 SE) in cycles beginning after lactation ($P < 0{\cdot}05$) and 29·5 days (±1·0 SE) in cycles of bottle feeding mothers ($P < 0{\cdot}05$).

## DISCUSSION

Most studies which have attempted to define the timing of ovulation after childbirth have used endometrial biopsy, basal body temperature, cervical mucus or vaginal cytology as markers of ovulation (Lass *et al.*, 1938; Tomkins, 1943; Sharman, 1951; Udesky, 1950; Cronin, 1968; Kamal *et al.*, 1969; El-Minawi & Foda, 1971; Perez *et al.*, 1972). All of these methods, however, present problems of interpretation. During lactation, the histological patterns in the endometrium may show inadequate coordination of the maturation signs characteristic of the normal menstrual cycle, making it difficult to determine whether ovulation has occurred or not (El-Minawi & Foda, 1971; Perez *et al.*, 1972). Similarly, temperature charts during lactation may show a much shorter plateau in the pre-menstrual phase (Cronin, 1968), making precise interpretation difficult. It is possible that the short plateau phase in the temperature chart is due to a shortening of the luteal phase in cycles during lactation. In the present study, we have used urinary steroid excretion as an objective marker of ovarian activity and regarded a urinary pregnanediol of greater than 1 mg/24 h as evidence of prior ovulation. Rolland *et al.* (1975) conducted a similar study to our own in ten mothers using plasma steroid levels as markers of ovulation, but did not continue observations beyond 100 days. A major advantage of urinary steroid excretion as an index of ovulation is that it provides a non-invasive method of monitoring which can be used longitudinally for many months after delivery.

Our study shows clearly that, compared with bottle feeding, breast feeding delays the return of ovulation after childbirth, thus confirming the findings of previous investigations (Sharman, 1951; Cronin, 1968; Perez *et al.*, 1972; El-Minawi & Foda, 1971). There was however, a quantitative change in the degree of ovarian suppression as lactation progressed. During the phase of lactational amenorrhoea, there was complete suppression of gonadal activity until just before the return of menstruation. This showed that recurrent ovulations did not occur during lactational amenorrhoea which was in agreement with the findings of Udesky (1950). It is unfortunate that ovulation can precede first menstruation because it means that first menses cannot be used as an infallible guide

when to restart contraception. On the other hand, the duration of lactational amenorrhoea varies widely, according to the breast feeding practices of different communities, so that a knowledge of lactational amenorrhoea can be useful in developing post-partum contraceptive strategies for a defined population (Potter *et al.*, 1973).

There is little information available on the frequency of ovulatory cycles in lactating women. Estimates of the frequency of ovulation in the cycle before first menses range from 14% (Udesky, 1950) to 23% (Cronin, 1968) and 78% (Perez *et al.*, 1972). These discrepant results may be related to the accuracy of the different methods used to assess ovulation but may also reflect different suckling patterns in the study populations. We have confirmed the finding of Perez *et al.* (1972) that ovulation is more likely to precede menstruation in cycles occurring late in lactation. By this time, suckling frequency and basal PRL levels have fallen and this may explain the greater chance of ovulation at that time.

Some reports have claimed that the reduction in fecundity associated with lactation is confined to the amenorrhoeic interval (van Ginneken, 1977). Chen *et al.* (1974), however, reported conception rates after the return of menses during lactation which were much lower than in non-nursing women using no contraception (Tietze, 1968). We have found that many cycles during lactation are anovular, which is in agreement with the report of Lass *et al.* (1938), and this may contribute to the lower fertility rates in breast feeding mothers who are menstruating. Further evidence of a disruption of normal ovarian activity during lactation comes from the longer intermenstrual intervals compared with those after lactation has been completed (Sharman, 1951; Berman *et al.*, 1972). The combined information suggests that lactational amenorrhoea is associated with complete gonadal inhibition which ends just before the return of menses; after first menses, there is a continuing suppression of gonadal activity which is less complete than during lactational amenorrhoea and is insufficient to prevent pregnancy in all cases.

The suppression of ovulation in breast feeding mothers is in sharp contrast to the early return of ovarian activity in bottle feeding mothers. Ovulation did not occur frequently in the first cycle of bottle feeders and our results correspond closely with those of Cronin (1968) who, using basal body temperature as a marker of ovulation, reported that 30% of first cycles were ovular compared with over 90% of later cycles. In the bottle feeders, ovulation occurred as early as 5 weeks post-partum in one mother and by 15 weeks in all mothers. This explains why bottle feeding mothers conceive readily within a few weeks post-partum in the absence of contraception and demonstrates that bottle feeding mothers who wish to avoid pregnancy must start taking contraceptive precautions from the immediate puerperium onwards.

Our study also shows that the timing of first ovulation in breast feeding mothers varies widely between individuals and further papers (Howie *et al.*, 1982) will examine how these differences relate to suckling patterns, basal PRL levels and gonadotrophin secretion.

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# FERTILITY AFTER CHILDBIRTH: INFANT FEEDING PATTERNS, BASAL PRL LEVELS AND POST-PARTUM OVULATION

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SUMMARY

The infant feeding patterns at the time of first ovulation after childbirth were determined in a longitudinal study of twenty-seven mothers who chose to breast feed their babies. Fourteen mothers suppressed ovulation throughout lactation and thirteen ovulated while still breast feeding. Those who ovulated while breast feeding had all introduced two or more supplementary feeds/day, reduced suckling frequency to less than six times/day and reduced suckling duration to less than 60 min/day at the time of first ovulation. Basal PRL levels had fallen to below 600 $\mu$U/l in all but one of the mothers at first ovulation.

Those mothers who suppressed ovulation for more than 40 weeks post-partum (late ovulation group) were compared with those who ovulated between 30 and 40 weeks post-partum (middle group) and with those who ovulated before 30 weeks post-partum (early group). The late ovulation group breast-fed for longest, suckled most intensively, maintained night feeds for longest and introduced supplementary feeds most gradually.

This study suggests suckling may be the most important factor inhibiting the return of ovulation during lactation and that policies which encourage increased suckling frequency and duration will maximize the contraceptive effects of breast feeding.

Although the contraceptive effect of breast feeding is a major demographic importance in developing countries (Rosa, 1975) there has been a tendency to view its value with scepticism because it does not protect against pregnancy in all cases (Kamal *et al.*, 1969). It would, therefore, be of great practical value if it could be established what were the minimum suckling requirements to protect against pregnancy so that mothers could be

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guided as to when it was necessary to start contraception. In the present study, twenty-seven mothers recorded suckling duration and frequency throughout lactation and had the resumption of ovulation determined by urinary pregnanediol estimation. In this way, it was possible to determine the infant feeding patterns at the time of first ovulation. In addition, it was also possible to examine which patterns of infant feeding were associated with the most effective suppression of ovulation.

## SUBJECTS AND METHODS

The subjects of this report were twenty-seven breast feeding mothers who were studied longitudinally from delivery until the resumption of ovarian activity and ovulation. The age and parity of the mothers and the method of their recruitment to the study have been previously described (Howie *et al.*, 1982), as were the methods of clinical recording and the methods of hormone assays.

## RESULTS

### *Suckling patterns at first ovulation*

Of the twenty-seven breast feeding mothers, fourteen had discontinued lactation before ovulation returned. Thirteen mothers ovulated while still breast feeding but, at first ovulation, all were suckling fewer than six times/day, for less than 60 min/day and had

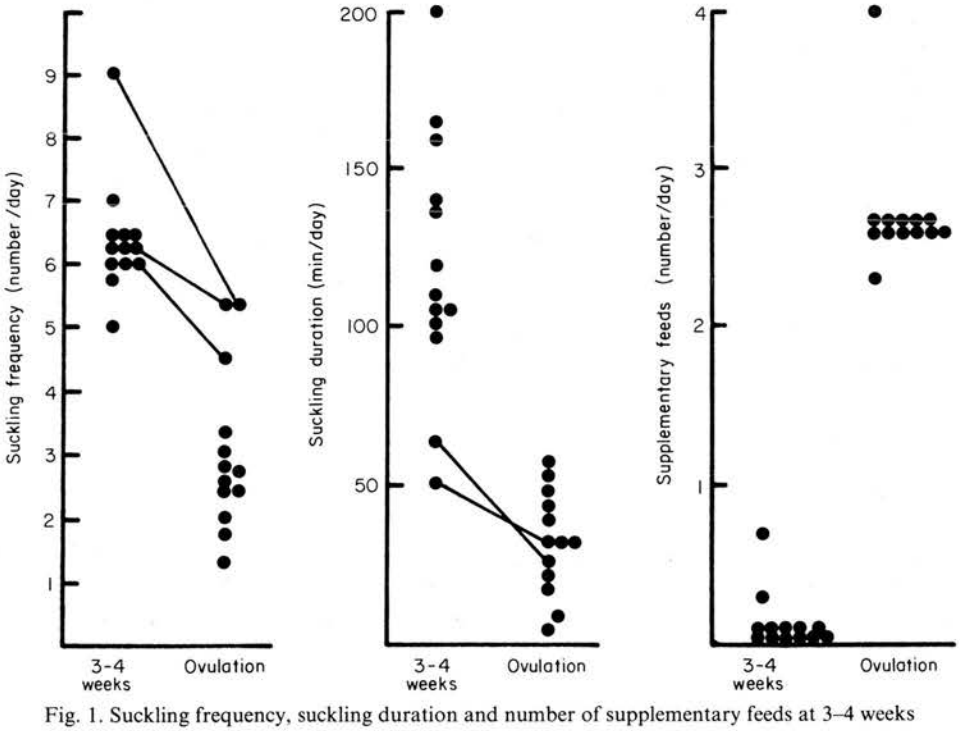



Fig. 1. Suckling frequency, suckling duration and number of supplementary feeds at 3–4 weeks post-partum and at first post-partum ovulation in thirteen mothers who ovulated during lactation.

Table 1. Age, time to first ovulation and duration of breast feeding (mean ± SE) in early, middle and late ovulation groups

| | Ovulation group | | |
|---|---|---|---|
| | Early ($n=7$) | Middle ($n=9$) | Late ($n=9$) |
| Time to first ovulation (weeks) | | | |
| mean | 21·8 | 34·3 | 50 |
| Duration of breast feeding (weeks) | | | |
| mean | 24·4 | 37·3 | 53·3 |
| SE | 5·6 | 2·4 | 5·8 |
| Age (years) | | | |
| mean | 30·3 | 28·5 | 30·0 |
| SE | 1·6 | 1·8 | 1·3 |
| Ovulating during lactation | 4/7 | 5/9 | 4/11 |

introduced two or more supplementary feeds (Fig. 1). Compared with the time of maximum suckling 3–4 weeks partum, suckling duration and suckling frequency had been reduced in all mothers at the time of first ovulation.

*Basal PRL levels at first ovulation*

At the time of first ovulation the mean basal PRL levels were 245 $\mu$U/l ± 42 SE in the mothers who ovulated after lactation and 367 $\mu$U/l ± 63 SE in the mothers who ovulated during lactation. All mothers, except one, had basal PRL levels below 600 $\mu$U/l at first ovulation. The remaining mother ovulated during lactation with a basal PRL level of 1250 $\mu$U/l.

*Comparison of early, middle and late ovulation groups*

On the basis of the time to first ovulation, seven mothers were allocated to an early group (ovulation before 30 weeks), nine to a middle group (ovulation between 30 and 40 weeks) and nine to a late group (ovulation after 40 weeks). The two mothers who did not ovulate during the period of study were excluded from this part of the analysis. The mean ages of the mothers and the proportions ovulating while still lactating did not differ significantly between the groups (Table 1). The mean time to first ovulation increased progressively from the early to middle and late groups (Table 1). The total duration of breast feeding was 53·3 weeks (± 5·8 SE) in the late group compared with 37·3 (± 2·4 SE) in the middle group and 24·4 (± 5·6) in the early group.

*Comparison of infant feeding patterns.* (Fig. 2) Results are presented as the mean values (± SE) at 4, 10, 20, 30 and 40 weeks post-partum. In the early group, results are not given at 40 weeks because all but two mothers in this group had stopped breast feeding by this time.

*Suckling duration and frequency*

Suckling duration was the first factor to discriminate the early ovulation group from the other two groups; at 4 and 10 weeks post-partum, the early ovulation group were

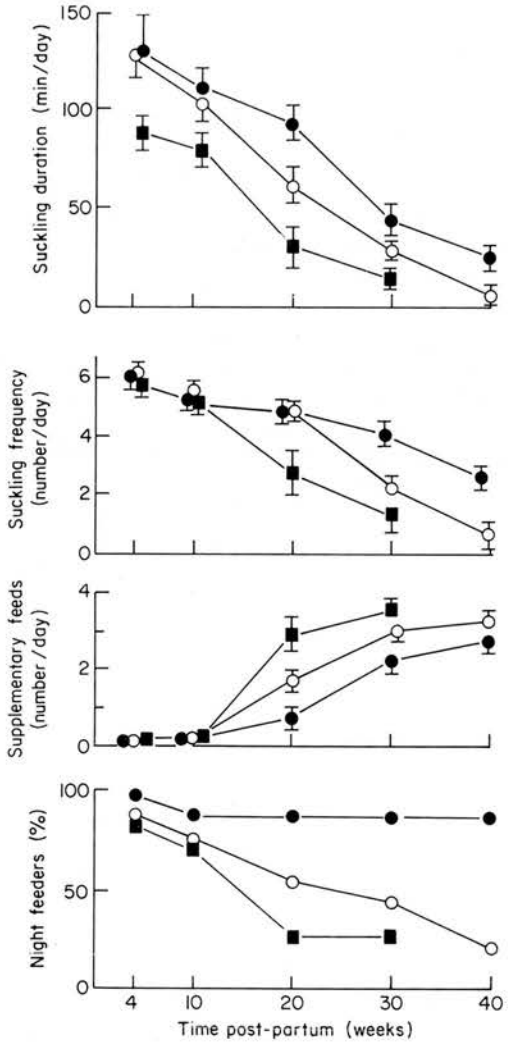



Fig. 2. Comparison of suckling duration (mean ± SE), suckling frequency (mean ± SE), supplementary feeds and number of night feeders in mothers who ovulated in the late (> 40 weeks, ●), middle (30–40 weeks, ○) or early (< 30 weeks, ■) post-partum period.

suckling for a shorter duration each day ($P < 0{\cdot}05$) despite having suckling frequencies which were similar to the middle and late ovulation groups. From 20 weeks post-partum onwards, suckling duration was different across the groups, being longest in the late ovulation and shortest in the early ovulation group. At 20 weeks post-partum, suckling frequency was significantly less in the early ovulation group than in the late ovulation group ($P < 0{\cdot}02$) and, at 30 weeks post-partum, suckling frequency was less in the middle group than in the late group ($P < 0{\cdot}01$).

*Supplementary feeds*

Supplementary food (in the form of formula milk or solid food) was introduced in the

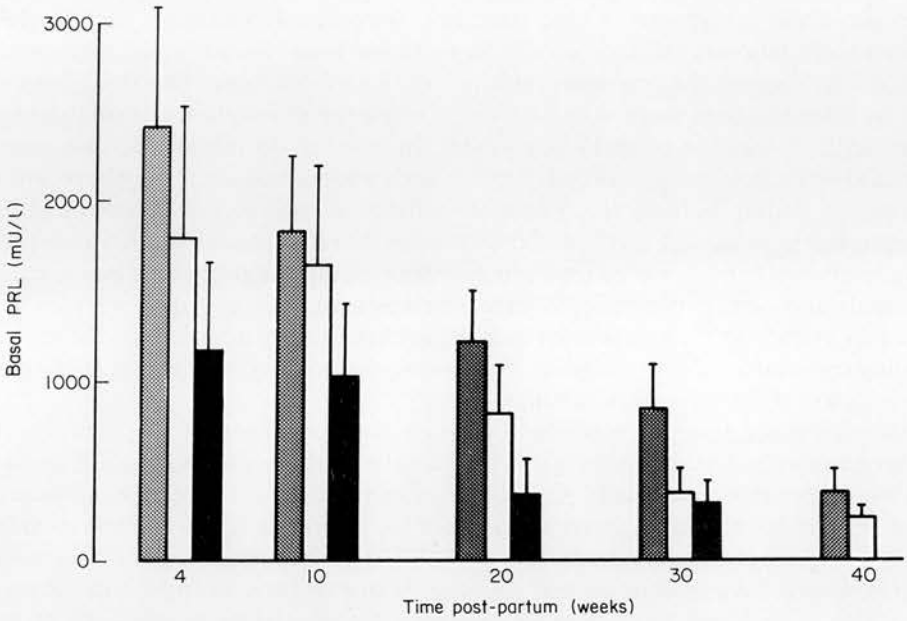



Fig. 3. Comparison of basal PRL levels (mean ± SE after log transformation) in mothers who ovulated in the late (>40 weeks, ▩), middle (30–40 weeks, □) or early (<30 weeks, ■) post-partum period.

early, middle and late ovulating groups at means of 12·5 weeks (±2·1 SE), 17·2 weeks (±0·9 SE) and 20·7 weeks (±1·2 SE) respectively. At 20 weeks post-partum, the early ovulation group were giving significantly more supplements than either the middle group ($P<0·05$) or the late group ($P<0·01$). At 30 weeks post-partum the middle group were giving more supplementary feeds than the late group ($P<0·01$).

*Night feeds*

At 40 weeks post-partum, eight out of nine mothers (89%) in the late ovulation group were still giving one or more night feeds (defined as a suckling episode between midnight and 08.00 h), at 30 weeks, this incidence of night feeds in the late group was significantly more than in the other two groups combined ($\chi^2=6·61$, $P=0·02$).

*PRL levels (Fig. 3)*

There was a gradient in the basal PRL levels across the groups, being highest in the late ovulating group and lowest in the early group. The mean PRL levels had fallen to the non-pregnant range at 20 weeks in the early group, at 30 weeks in the middle group and at 40 weeks in the late group.

## DISCUSSION

It would be of great practical value, if simple guidelines could be developed to indicate to nursing mothers the minimum suckling necessary to inhibit ovulation during lactation.

The *WHO Collaborative Study on Breast Feeding* (1979) which investigated twenty-

seven economic sub-groups in nine countries, were unable to supply such guidelines because their data was collected on a cross-sectional basis. They rejected a longitudinal study design because 'the practical problems were insurmountable'. Our study has shown that, by a longitudinal study, it is possible to relate the resumption of ovulation to the frequency and duration of suckling episodes. In order to do this reliably two essential criteria must be met; firstly, suckling duration and suckling frequency must be recorded at the time of feeding because recall data are unreliable and secondly, there must be an accurate and reproducible method of determining the return of ovulation. In the present study, every mother was visited regularly to ensure that feeding data were being recorded accurately and urinary pregnanediol excretion was used as an objective measure of first ovulation. Although the numbers in this study are insufficient to define fully the minimum suckling required to inhibit ovulation, it will be possible to do so by studying larger groups of mothers in different cultural settings.

From this study, however, it is clear that when ovulation occurs during lactation, there has been a sharp reduction in suckling as compared with the levels at the time of maximum suckling in the early puerperium. At first ovulation, two mothers were suckling more than five times/day but all mothers were suckling for less than 60 min/day and had introduced two or more supplementary feeds. It is probably that the relatively poor contraceptive effect of breast feeding in developed countries is due to early weaning and subsequent reductions in suckling. In developing countries, the contraceptive efficiency of breast feeding falls as mothers move from a rural to an urban setting (Bonte *et al.*, 1974). One explanation, which needs further investigation is that this is due to a change in suckling frequency. This pattern of suckling contrasts sharply with that of the !Kung hunter gatherers who practise short but frequent bouts of nursing and achieve inter-birth intervals of up to 4 years (Konner & Worthman, 1980). The !Kung hunter gatherers also sleep with their babies at night and, in our own study, the mothers who suckled at night were those who postponed ovulation for longest. It may be that a long interval during the night without a suckling episode may enable the hypothalamic pituitary ovarian axis sufficient time to recover its normal function and resume ovulation. The relationship of night-time suckling to ovarian activity requires further study.

At the time of first ovulation, all of the mothers, in the present study, had introduced supplementary feeds which have the effect of reducing suckling frequency and duration (Howie *et al.*, 1981). This is in keeping with the reports that have shown that menses, and possible ovulation, are more likely to return during partial as compared with full breast feeding (McKeown & Gibson, 1954; Perez *et al.*, 1972). Gioiosa (1955) reported that out of 500 birth intervals in breast feeding mothers, forty-six (9·2%) conceived during lactation but that forty of these (87%) conceived when the weaning process was taking place. Perez *et al.* (1972), however, reported that fourteen out of 200 mothers ovulated during the period of full nursing and that two of them conceived before menstruation. This emphasizes that full breast feeding is not a guarantee against pregnancy but Perez *et al.* (1972) did not define suckling frequency, suckling duration or the incidence of night feeding in those mothers who ovulated during lactation. It is, however, quite clear from our own study that the early and abrupt introduction of supplementary food was associated with the early return of ovulation. The nutritional necessity of early supplements is a matter of controversy (Waterlow & Thomson, 1979; Jeliffe & Jeliffe, 1979), but it must be a matter of some concern that, in most developing countries, 50% of mothers are giving supplements by 2–3 months post-partum. If the introduction of

supplements led to a sharp reduction in suckling this practice could seriously undermine the important contraceptive effect of breast feeding in those countries where the use of artificial contraception is low.

The changes in basal PRL supported the hypothesis that reduced suckling was associated with an increased probability of ovulation. In their cross sectional studies in Zaire, Delvoye *et al.* (1978) found that PRL levels related both to the frequency of suckling and to the duration of lactational amenorrhoea. By extrapolation, their data are consistent with the view that the strength of the suckling stimulus is the variable which controls the return of post-partum menstruation and ovulation. It is not yet known whether PRL *per se* inhibits ovulation during lactation (McNeilly, 1979) but the PRL acts as a useful objective marker of suckling intensity. Badraoui & Hefnawi (1979) compared the use of sulpiride and placebo in nursing mothers and found that sulpiride delayed the resumption of menses post-partum. At 9 months, the number of pregnancies were lower in the sulpiride treated mothers but this difference had disappeared at 1 year. More information is required to evaluate this approach to contraception during lactation and, in particular, it will be necessary to know whether a dopamine antagonist would have any effect on the baby (McNeilly *et al.*, 1980).

Apart from theoretical interest, the contraceptive effect of breast feeding is still of great practical importance in developing countries. In the developed world, some mothers dislike the currently available methods of artificial contraception and would prefer to choose breast feeding as a method of birth spacing if they had a method of defining its reliability. It is, therefore, a matter of urgency that further studies should be undertaken to define the practical information upon which mothers could base their decision of when they should introduce post-partum contraception. From the present study, however, it is clear that those infant feeding practices which encourage suckling will maximize the contraceptive effect of breast feeding.

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# FERTILITY AFTER CHILDBIRTH: ADEQUACY OF POST-PARTUM LUTEAL PHASES

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SUMMARY

Normal ranges of urinary pregnanediol and total urinary oestrogen were determined from weekly estimations in twenty-seven cycles from seven normally menstruating control women and compared with the levels in the cycles of twenty-seven breast feeding and ten bottle feeding mothers. During lactation, the luteal phase pregnanediol levels were within normal limits in thirteen of forty-nine (27%) cycles, the remainder of the luteal phases being deficient (31%) or absent (42%). The proportion of normal luteal phases remained low during first cycles after lactation in six of twenty-three (26%) but rose to twenty-four of thirty-one (77%) in subsequent cycles. In bottle feeding mothers, the luteal phases of first post-partum cycles were deficient in two of ten (20%) and absent in eight of ten (80%). In second post-partum cycles, the majority of luteal phases were deficient (eight of ten, 80%) and it was not until third post-partum cycles that seven of eight (88%) had luteal phase pregnanediol levels in the normal range. Cycles during lactation and first cycles after lactation had significantly lower mean urinary pregnanediol and total urinary oestrogen levels than both the control cycles and the later cycles after lactation. Similarly, first post-partum cycles in bottle feeders had lower urinary pregnanediol and total urinary oestrogen levels than controls. This study shows an increased frequency of abnormal luteal phases during the early post-partum menstrual cycles of both breast and bottle feeding mothers which may be associated with defective development of the follicle.

In a longitudinal study of breast and bottle feeding mothers, many of the post-partum menstrual cycles appeared to be anovular (McNeilly *et al.*, 1980; Howie *et al.*, 1982) and it

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was of interest to determine whether some of the apparently ovular cycles were characterized by defective luteal phases.

These cycles were being monitored by weekly urinary pregnanediol levels and total urinary oestrogens and, in the present paper, a series of cycles in normal non-pregnant women has been studied in a similar way. This has enabled a comparison to be made between the luteal phases in normal control women with those in mothers who are either breast or bottle feeding. The objective of this study was to define more closely the frequency and the type of luteal phase deficiency in post-partum women.

## PATIENTS AND METHODS

Ten bottle feeding and twenty-seven breast feeding mothers were studied longitudinally and their ages, parities and method of recruitment have been previously described (Howie *et al.*, 1982). Seven regularly menstruating, non-pregnant, women (aged 23–41 years), who were not using steroidal contraception, acted as controls. Weekly 24 h urine samples were collected in the manner previously described for the bottle and breast feeding mothers (Howie *et al.*, 1982).

Urinary pregnanediol and total urinary oestrogens were measured as previously described. (Brown *et al.*, 1968; Chamberlain & Contractor, 1968).

### *Number of cycles studied*

In the non-pregnant controls weekly urinary pregnanediol and total urinary oestrogen were measured during twenty-seven cycles. Urine samples were also collected from breast feeding women in whom forty-nine cycles were completed during lactation, twenty-three cycles were classified as first cycles after lactation and thirty-one as later cycles after lactation. In 14% of the first cycles after lactation, menstrual bleeding started within 4 weeks of stopping lactation so that the follicular phase may have started while breast feeding was continuing. In the bottle feeding mothers, weekly urine samples were collected during ten first post-partum cycles, ten second post-partum cycles and eight third post-partum cycles. Weekly urinary samples were also collected in the twenty-seven breast feeding mothers during a total of 779 weeks prior to the resumption of menstrual cycles.

### *Statistics*

Comparisons between groups were made using Student's $t$ test after logarithmic transformation of the data.

## RESULTS

### *Definition of ovulation*

In the twenty-seven normal control cycles, the urinary pregnanediol levels collected during the follicular phase (i.e. samples collected between 15 and 28 days prior to menstruation) fell within the range of 0·1 and 0·87 mg/24 h. The urinary pregnanediol levels invariably fell within this resting range as defined by the follicular phases levels of the normal controls, during the follicular phases of all 103 cycles in lactating women, twenty-eight cycles of bottle feeding women and during the 779 weeks prior to the return of menstruation in breast feeding women. From these data, it was assumed that a urinary

pregnanediol above the follicular phase levels of controls was indicative of corpus luteum activity. Allowing for the error of assay, a urinary pregnanediol above 1·0 mg/24 h was taken to indicate presumed ovulation.

*Definition of follicular activity*

Only one of the twenty-seven breast feeding women menstruated during the first 12 weeks post-partum and rising levels of total urinary oestrogen were obtained from the values between weeks 3 and 5 post-partum in the remaining twenty-six breast feeders. During this time, total urinary oestrogens ranged from below the limit of sensitivity ( < 1 μg 24 h) to 9·6 μg/24 h. During the twenty-seven menstrual cycles in control women, the 103 cycles in lactating women and the twenty-eight cycles in bottle feeding women, one or more of the total urinary oestrogens collected in the four weeks prior to menstruation was invariably greater than 10 μg/24 h. From these considerations, it was assumed that a total urinary oestrogen of greater than 10 μg/24 h was presumptive evidence of follicular activity.

*Adequacy of luteal phase*

The adequacy of the luteal phase was assessed on the basis of the two urinary pregnanediol levels collected in the 2 weeks prior to menstruation. If both levels were less than 1 mg/24 h, the luteal phase was considered to be absent.

In the twenty-seven cycles from normal controls, the luteal phase was absent in one cycle. In the remaining twenty-six cycles, the higher of the two values of urinary pregnanediol collected between 1 and 14 days before menses fell in the range 1·3–6·8 mg/24 h and the lower value in the range 0·5–2·4 mg/24 h. These values in the control women were considered to represent the normal range. A luteal phase was classified as deficient if either **1** the peak luteal phase urinary pregnanediol fell between 1·0 and 1·3 mg/24 h or **2** the lower luteal phase urinary pregnanediol fell below 0·5 mg/24 h (i.e. outside the non-pregnant normal range).

*Adequacy of luteal phases in breast and bottle feeding mothers (Table 1)*

The luteal phases in the breast and bottle feeding mothers were classified as absent, deficient or normal as defined above. During lactation, thirteen of forty-nine (27%) of the

Table 1. Type of luteal phases encountered in menstrual cycles of breast feeding mothers before and after lactation and of bottle feeding mothers

| | Type of luteal phase | | |
|---|---|---|---|
| | Normal | Deficient | Absent |
| *Breast feeders* | | | |
| During lactation ($n=49$) | 13(27%) | 15(31%) | 21(42%) |
| First cycle after ($n=23$) | 6(26%) | 10(44%) | 7(30%) |
| Late cycle after ($n=31$) | 24(77%) | 2(6%) | 5(16%) |
| *Bottle feeders* | | | |
| First cycle ($n=10$) | — | 2(20%) | 8(80%) |
| Second cycle ($n=10$) | 1(10%) | 8(80%) | 1(10%) |
| Third cycle ($n=8$) | 7(88%) | 1(12%) | — |

cycles were classified as normal, fifteen of forty-nine (31%) as deficient and twenty-one of forty-nine (42%) as absent. In the first cycle after lactation, the number of cycles with normal luteal phases remained at six of twenty-three (26%) and it was not until later cycles after lactation that twenty-four of thirty-one (77%) of the luteal phases could be classified as normal.

Similarly, in the bottle feeding mothers, the luteal phases of first postpartum cycles were absent in eight of ten (80%) and deficient in two of ten (20%) and it was not until third post-partum cycles that seven of eight (88%) had been classified as normal.

*Urinary pregnanediol and total urinary oestrogens*

*Comparison between breast feeding mothers and controls.* The mean values (lognormal distribution) of urinary pregnanediol and total urinary oestrogen are shown in Fig. 1 for the cycles in breast feeding and control women. The results are grouped according to whether they were collected between days 1–7, 8–14, 15–21 or 22–28 prior to menstruation. The mean levels of urinary pregnanediol in the forty-nine cycles during lactation were significantly less than the control at days 1–7 and 8–14 prior to menses ($P < 0{\cdot}001$ at both times). In the twenty-three first cycles after lactation, luteal phase urinary pregnanediol levels were still significantly less than those of the control at both days 1–7 and 8–14 before menses ($P < 0{\cdot}01$ in both cases) but in the thirty-one later cycles

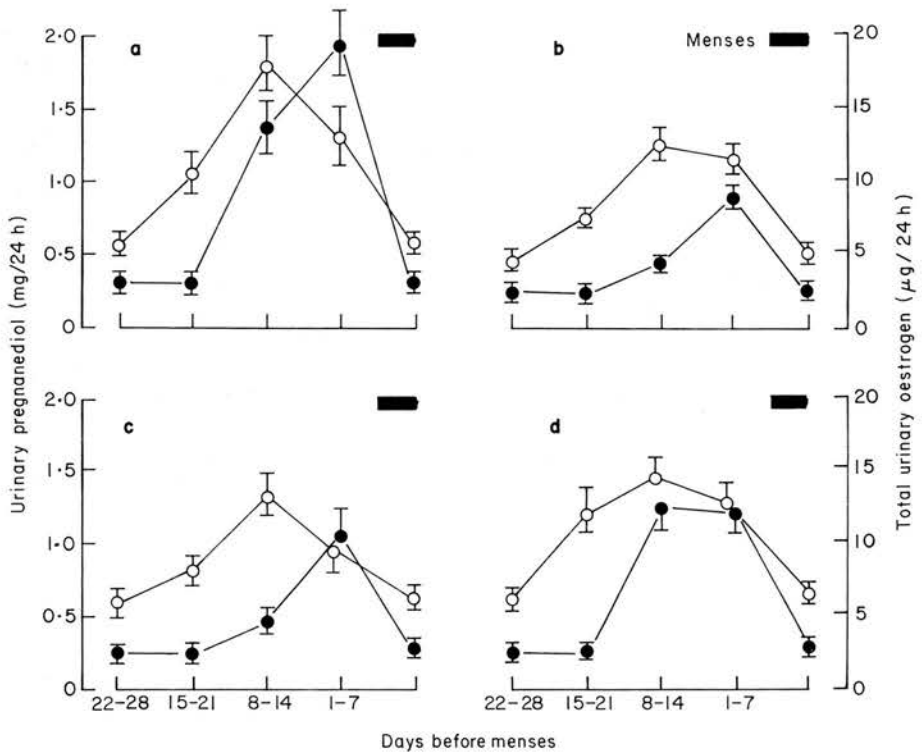



Fig. 1. Urinary pregnanediol (●) and total urinary oestrogen (○) levels (lognormal mean levels ± SE) from non-pregnant controls (**a**, $n = 27$) and from breast feeding mothers in cycles during lactation (**b**, $n = 49$), in first cycles after lactation (**c**, $n = 23$) and in later cycles after lactation (**d**, $n = 31$). Data are grouped in 7-day periods prior to the onset of menstruation.

after lactation, they were significantly less than the controls only in the days 1–7 prior to menstruation ($P < 0{\cdot}05$).

Total urinary oestrogens in the cycles during lactation were significantly less than the controls at days 22–28 ($P < 0{\cdot}05$), days 15–21 ($P < 0{\cdot}05$) and days 8–14 ($P < 0{\cdot}01$) prior to menses but not at days 1–7 before menses. In first cycles after lactation, total urinary oestrogens were significantly less than the controls only at days 8–14 prior to menses ($P < 0{\cdot}01$); in later cycles after lactation total urinary oestrogens showed no significant differences from control at any stage of the cycle.

*Comparison of cycles during lactation and first cycles after lactation with later cycles after lactation.* Compared with later cycles after lactation, mean urinary pregnanediol levels were significantly lower in cycles during lactation at days 8–14 ($P < 0{\cdot}001$) and days 1–7 ($P < 0{\cdot}05$) before menses, and were significantly lower in first cycles after lactation at days 8–14 ($P < 0{\cdot}01$) before menses.

Compared with later cycles after lactation, total urinary oestrogen levels were significantly lower in cycles during lactation at days 22–28 ($P < 0{\cdot}05$) and days 15–21 ($P < 0{\cdot}01$) before menses and were significantly lower in first cycles after menses at days 15–21 ($P < 0{\cdot}05$) before menses.

*Comparison between bottle feeding mothers and controls.* Mean urinary pregnanediol levels during the first post-partum cycles of the bottle feeding mothers (Fig. 2) were

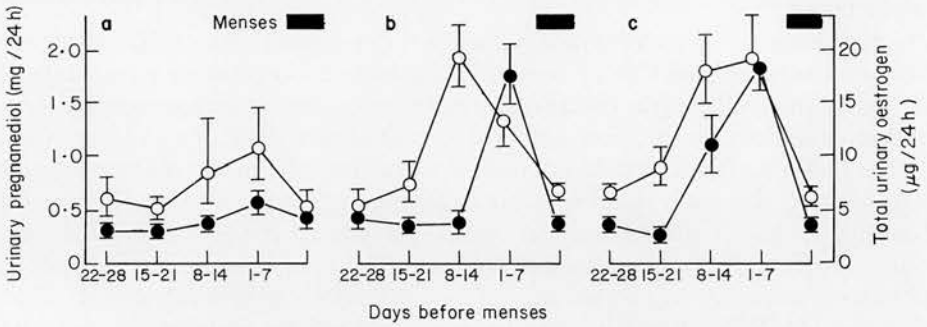



Fig. 2. Urinary pregnanediol (●) and total urinary oestrogen (○) levels (lognormal mean levels ± SE) from bottle feeding mothers in first (**a**, $n = 10$), second (**b**, $n = 10$) and third (**c**, $n = 8$) post-partum cycles.

significantly less than controls at days 1–7 ($P < 0{\cdot}01$) and 8–14 ($P < 0{\cdot}01$) before menses. In the second post-partum cycles, urinary pregnanediol levels were significantly less than controls at days 8–14 before menses ($P < 0{\cdot}01$) but not at days 1–7. In the third post-partum cycles, urinary pregnanediol levels did not differ significantly from the controls.

During the first post-partum cycles, total urinary oestrogen levels were significantly less than controls at days 15–21 ($P < 0{\cdot}01$) and 7–14 ($P < 0{\cdot}001$) before menses. There were no differences in total urinary oestrogen between controls and bottle feeders in second and third post-partum cycles.

## DISCUSSION

In a previous paper, we reported that ovulation failed to occur in 42% of menstrual cycles during lactation (Howie *et al.*, 1982). In that report, diagnosis of ovulation was based on a

urinary pregnanediol excretion of greater than 1 mg 24 h in at least one of two samples collected in the 14 days before menstruation. This analysis of the same data shows that an arbitrary division into ovular and anovular cycles fails to define fully the nature of the luteal phase defect in menstrual cycles during lactation. When the adequacy of the luteal phases during lactation were compared with control cycles, the urinary pregnanediol levels fell below the normal range in more than half the cases which had significant elevations of urinary pregnanediol above base-line values. There is evidence that defective luteal phases are associated with reduced fertility in non-lactating women (Andrews, 1979) and it is likely that the defective luteal phases during lactation contribute to the reduction in fertility which persists during lactation after the return of menstrual cycles (Chen *et al.*, 1974).

It is not possible from measurement of urinary pregnanediol to determine whether the reduced excretion was due to defective corpus luteum function or represented secretion from luteinized follicles, and this requires further investigation. There have been few studies of luteal sufficiency in breast feeding mothers but Reyes *et al.* (1972) reported defective progesterone secretion in the first post-partum cycles of two lactating women. Using a cross-sectional study design, Delvoye *et al.* (1980) found that mean serum progesterone levels were lower in cycles during lactation than in normal cycles, suggesting a high number of defective luteal phases. Similarly, Gross *et al.* (1980) have found that defective luteal function is common in Australian women returning to menstruation during lactation.

A high incidence of defective luteal phases during lactation may explain why Cronin (1968) found a short plateau phase of raised basal body temperature in menstrual cycles during lactation. Similarly, the failure of normal coordination of the secretory patterns in the endometrium during lactation may be due to abnormal ovarian steroid secretion.

The reason for the frequent failure of normal corpus luteum function during lactation is not explained by this study. Cycles during lactation, first cycles after lactation and first post-partum cycles in bottle feeders had evidence of reduced urinary oestrogen excretion in addition to the defective pregnanediol excretion. This finding would be consistent with the possibility that defective corpus luteum function has its origins in subnormal follicular development (McNeilly, 1980). In cycles not associated with the post-partum period it has been suggested that deficient corpus luteum function resulting in either short or inadequate luteal phases is associated with a reduction in the FSH–LH ratio around the time of menses i.e. days $-10$ to $-15$ before ovulation (see DiZerega & Hodgen, 1981). However, no apparent deficiency in the levels of FSH have been found in the present studies of women post-partum but levels of LH do appear to be somewhat lower than normal (McNeilly *et al.*, 1980; McNeilly & Howie, unpublished observations). It is also possible that the raised levels of PRL associated with lactation may act directly at ovarian level to interfere with the normal development of the pre-ovulatory follicle (McNatty, 1979; McNeilly, 1980; McNeilly *et al.*, 1982). Further studies are required to determine the factors which operate on the ovary to influence corpus luteum function during lactation and such studies might point towards new methods of controlling fertility during lactation.

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# FERTILITY AFTER CHILDBIRTH: PREGNANCY ASSOCIATED WITH BREAST FEEDING

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SUMMARY

During studies on the resumption of fertility postpartum in 12 breast feeding mothers who were using no contraception, eight pregnancies occurred. In seven cases these pregnancies occurred while the mothers continued to breast feed while in one it occurred within 2 weeks of weaning. In two cases pregnancy occurred prior to first postpartum menstruation but followed an abrupt decline in suckling frequency and duration resulting in resumption of follicular development and ovulation. In the remaining six cases, pregnancy was preceded by between 1 and 7 menstrual cycles, the majority of which (13/19) had deficient luteal phases or were anovular (4/19).

In all mothers a significant decrease in the suckling frequency and duration observed during lactational amenorrhoea had occurred prior to the resumption of ovulation and conception. No mother conceived with a suckling frequency of greater than three times per day although some mothers ovulated without conceiving when suckling four times per day.

The results suggests that if a breast feeding mother wishes to rely upon the infertility associated with lactational amenorrhoea, she must suckle at least five times per day with a total suckling duration of more than 65 min per day (more than 10 min per feed). Any reduction below either of these limits may result in a return of fertility.

In previous reports we have shown that breast feeding is associated with a prolonged period of infertility consisting firstly of a period of amenorrhoea and then, in many cases a series of inadequate luteal phases which are presumed to be infertile (McNeilly *et al.*, 1980, 1982; Howie *et al.*, 1980, 1982a, 1982b). Nevertheless, in spite of this and the clear

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demographic evidence of the important contraceptive action of breast feeding (Rosa, 1975) there is a tendency to view this with scepticism because it does not protect against pregnancy in all cases (Kamal *et al.*, 1969). It is clear however, that even when menstrual cyclicity resumes in mothers who continue to breast feed, fertility is markedly reduced (see Howie & McNeilly, 1982).

In the course of our longitudinal study of 70 breast feeding mothers (Howie *et al.*, 1980, 1982a, 1982b; McNeilly *et al.*, 1980, 1982; Glasier *et al.*, in preparation) eight pregnancies occurred in association with breast feeding. Here we report the circumstances which culminated in the return to fertility in these breast feeding women and conclude that, in all cases, breast feeding had been reduced to a level that, from our previous studies, were known not to provide reliable contraception.

## PATIENTS AND METHODS

From a total of 70 mothers participating in our longitudinal study of breast feeding, 12 mothers, who used no contraception during lactation were hoping to conceive and were having regular unprotected intercourse. These mothers were studied longitudinally until next pregnancy or, in the absence of conception, until 4 weeks after complete weaning of the baby. Ten of the mothers were studied from the immediate puerperium onwards while two mothers volunteered to participate from 36 and 38 weeks postpartum. At their time of recruitment, these latter two mothers were still amenorrhoeic and had not conceived. The mothers in this study had an age range of 21–40 years and a fertility range of 1–5 previous children.

As previously described (Howie *et al.*, 1982a), mothers kept a record of the frequency and duration of suckling and of the times of menstrual bleeding. Weekly 24 h urine samples were collected and urinary pregnanediol and total urinary estrogens measured as described previously (Howie *et al.*, 1982a).

On the basis of urinary steroid levels, it was possible to determine the times at which there were resumptions of ovarian follicular activity (totally urinary oestrogens > 10 $\mu$g/24 h) and ovulation (urinary pregnanediol > 1 mg/24 h; see Howie *et al.*, 1982a). The adequacy of the luteal phase was defined as described in detail previously (McNeilly *et al.*, 1982) using urinary pregnanediol values in the two samples collected between 1 and 14 days before menses. A normal luteal phase was defined by the higher of the two values being greater than 1·3 and the lower greater than 0·5 mg/24 h. A luteal phase was deficient if either the peak luteal phase pregnanediol fell between 1·0 and 1·3 mg/24 h or the lower luteal phase level fell below 0·5 mg/24 h. The times of conception were indicated by a rise in urinary pregnanediol levels which were maintained for three weeks or more; in all cases, the presence of a pregnancy was subsequently confirmed by pregnancy test and/or ultrasonic examination.

## RESULTS

### *Pregnancies during lactation*

Of the 12 mothers who used no contraception during lactation, seven conceived during lactation and one (No. 3, Figs. 1 and 2) conceived in the first cycle after complete weaning. The remaining four mothers, who breast fed for 20, 37, 42 and 39 weeks, had not conceived within 4 weeks of complete weaning.

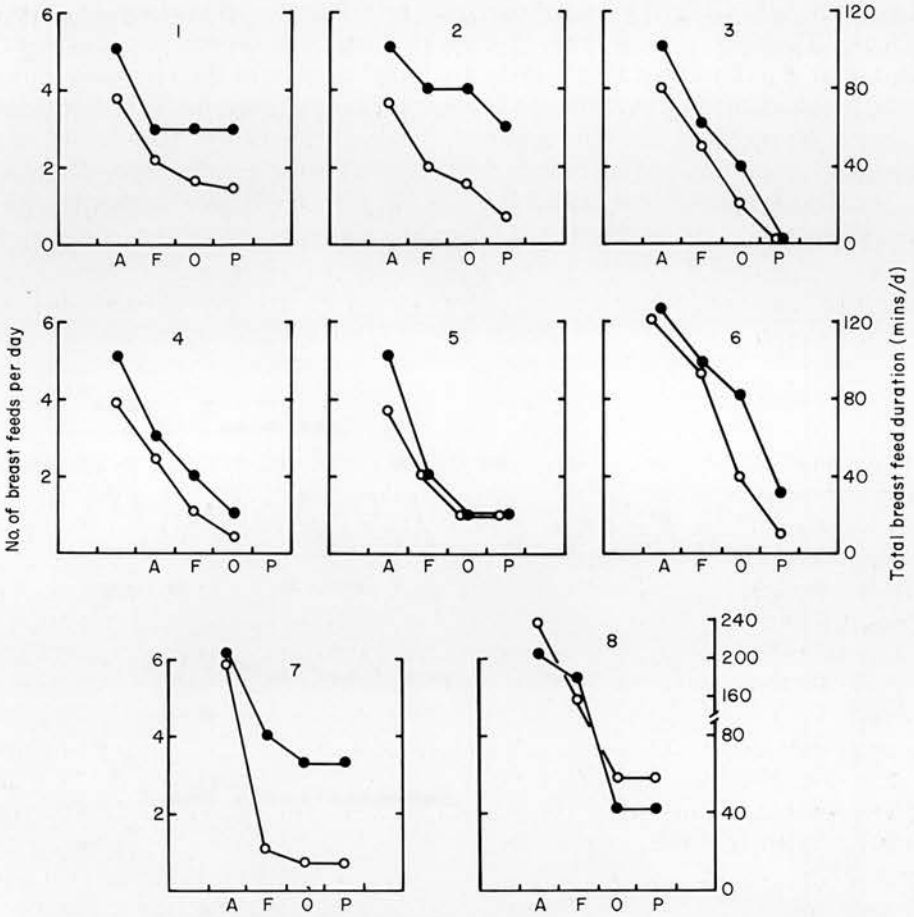



Fig. 1. Changes in the number (●) and duration (○) of breast feeds per day during amenorrhoea (A) and at the time of resumption of follicular activity only (F), ovulation (O) and at the time of conception (P) on eight occasions in seven breast feeding women.

*Breast feeding patterns in relation to ovarian activity and pregnancy*

In Fig. 1, the suckling patterns are shown for the eight mothers who conceived either during or immediately after lactation: the number of suckling episodes and the duration of suckling are shown at the time of pregnancy conception (P), at first ovulation (O) and at the resumption of follicular activity (F) and contrasted with the minimum level of suckling during the period of lactational amenorrhoea (A).

In all eight mothers, suckling frequency and duration were highest during lactational amenorrhoea and showed progressive falls before there was a return of ovarian follicular activity and ovulation. No mother conceived when suckling more than three times per day although two mothers ovulated when suckling four times per day. In seven out of the eight mothers, suckling duration had fallen to 30 min/d or less at the time of conception and to 40 min or less at first ovulation; the exception was subject No. 8, who ovulated and conceived while suckling for 60 min/d. This mother suckled for at least 30 min for every

feed and, during lactational amenorrhoea, had a total daily duration of suckling in excess of 200 min. Follicular activity resumed when suckling duration was 160 min per day, a much longer duration than that seen in any other mother at the return of follicular activity. Much of this suckling time was comfort suckling and resulted in inappropriately low levels of prolactin for the apparent suckling stimulation (Glasier *et al.*, in preparation). Ovulation and conception only occurred, however, after sharp decreases in both suckling frequency and duration from the high levels seen during lactational amenorrhoea and at the resumption of follicular activity.

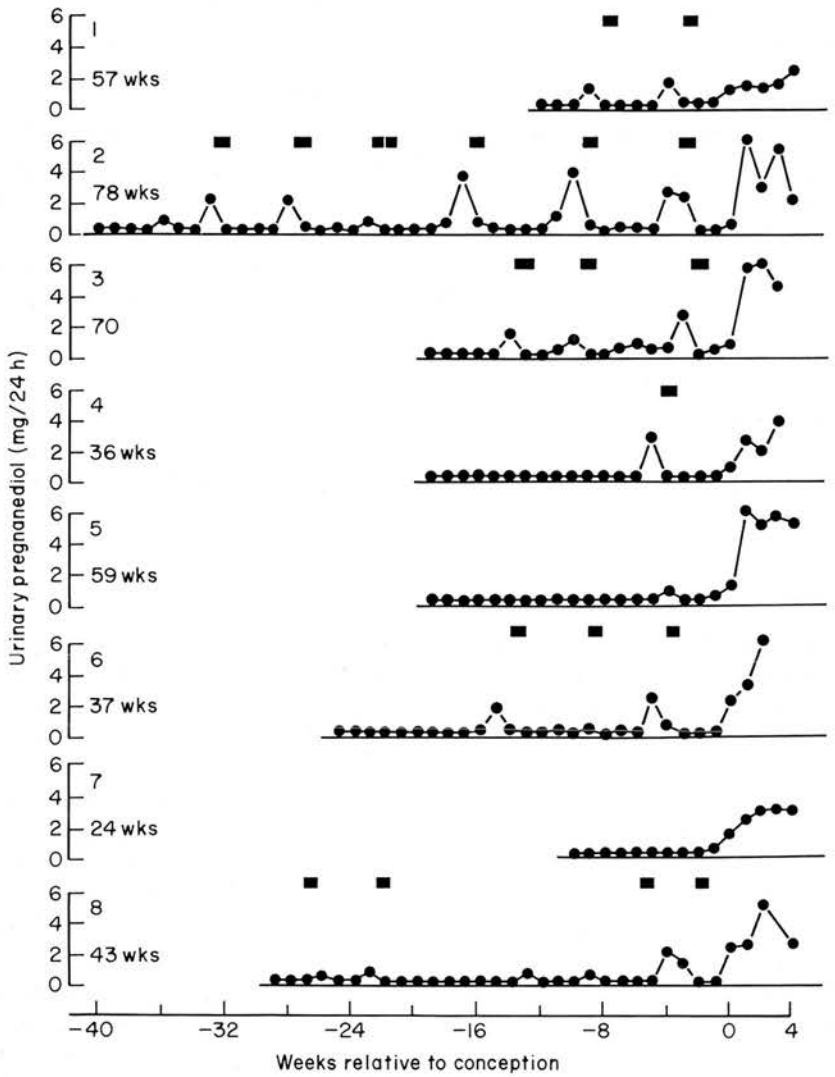



Fig. 2. Changes in urinary levels (●) and Menses (■) prior to conception in seven breast feeding women. Changes during lactation were recorded after successive pregnancies (Nos. 4 & 5) in the same woman. The time of conception postpartum in weeks postpartum is also indicated.

*Conception and menstruation*

Two mothers conceived during lactation, before first menstruation. In one case, (No. 5) suckling duration and frequency had remained unaltered for 49 weeks postpartum; over a period of 10 weeks, suckling frequency and duration fell rapidly and there was evidence of follicular activity without menstruation at 51 weeks (urinary total oestrogens > 10 $\mu$g/24 h); ovulation and conception occurred 8 weeks later at 59 weeks postpartum.

In the other case (No. 7) suckling frequency and duration remained unaltered for 18 weeks postpartum. During week 19, two suckling episodes per day were omitted and suckling frequency fell drastically. This resulted in an immediate resumption in follicular activity and ovulation and conception occurred within 3 weeks. In the other six mothers, pregnancy occurred in the 2nd, 3rd, 4th ($n=2$), 5th and 7th menstrual cycles.

*Adequacy in corpus luteum function in non-conception cycles in mothers who conceived*

A total of nineteen non-conception cycles occurred in the eight mothers who conceived during the study (Fig. 2). Four of the nineteen cycles were anovular and thirteen had pregnanediol levels indicating deficient corpus luteum function; only two non-conception cycles, cycle 4 of subject No. 8 and cycle 6 of subject No. 2, were associated with normal luteal phases. The mean intermenstrual interval in the five mothers with more than one cycle before pregnancy was 37 d ($\pm$4 SEM, range 21–51 d) and in no case was cycle length regular.

*Menstrual cycles in mothers who did not conceive*

Three of the four mothers who did not conceive remained amenorrhoeic and did not ovulate while breast feeding. The fourth mother, however, had several ovulatory cycles but did not conceive. The intermenstrual interval was variable (29–47 d, $n=5$) as a result of variation in the length of the follicular phase (16–23 d).

## DISCUSSION

This paper describes for the first time the changes in suckling patterns and menstrual cyclicity which occurred before, and at the time of conception, in eight of twelve mothers who became pregnant while breast feeding. In two mothers (2/12) pregnancy occurred without an intervening menstrual bleed. Epidemiological evidence suggests that 2–10% of mothers conceive during the period of lactational amenorrhoea (Badraoui & Hefnawi, 1979) but no endocrinological or physiological explanation has been available to explain this. In both cases reported here, follicular development occurred in response to a rapid decline in both suckling frequency and duration and conception occurred after ovulation of the follicles developed during this phase. In one (No. 5) the total time from resumption of follicular development to ovulation was 8 weeks. During this time a small rise in pregnanediol occurred around 4 weeks prior to conception suggesting that a corpus luteum or luteinized follicle may have been formed but secreted inadequate amounts of progesterone. Even though oestrogens preceeding this increase in pregnanediol were within normal follicular phase levels, it must be presumed that the amount of progesterone secreted and its withdrawal was not sufficient to cause menses. In the other mother (No. 7), follicular development was followed by ovulation 4 weeks later at which time conception occurred.

In the other six mothers conception occurred only after at least one menstrual period giving clear warning of the return of potential fertility. As described previously (McNeilly *et al.*, 1980, 1982), the large majority (17/19) of these menstrual cycles were associated with anovulation or deficient luteal phases and intermenstrual intervals were very variable. The follicular phase varied in length between 10 and 44 d making it almost impossible to assess the time of ovulation in the absence of endocrinological data. Clearly, therefore, once menstrual cycles have returned in women who continue to breast feed, the use of contraceptive methods relying on timing of ovulation are hazardous.

Sexual intercourse occurred throughout the majority of menstrual cycles at times when endocrine data suggested that conception would normally occur and also occurred at similar times to those in the cycle in which conception did occur. This supports the suggestion that deficient luteal phases both in lactation (McNeilly *et al.*, 1982) and in pathological situations (Andrews, 1979) are associated with reduced fertility. However, this will need further clarification.

Of considerable importance in terms of the contraceptive effect of breast feeding, is our finding that pregnancy occurred only after both suckling frequency and duration had been dramatically reduced from that prevailing during the prolonged period of lactational amenorrhoea. In the present study, pregnancy did not occur until suckling frequency fell to three times per day. However, in some mothers there was evidence of ovulation, with the potential for pregnancy, at a suckling frequency of four times per day so that the minimum frequency required to guarantee against conception is not less than five times per day (more than 10 min per feed). Similarly, the total suckling time must be at least 65 min/d. It is of interest that a recent longitudinal study by Andersen *et al.* (1982) has suggested a similar figure of 5 feeds per day as the minimum required to reliably protect against pregnancy. This figure is supported by our previous (Howie *et al.*, 1980, 1982a, 1982b) and more recent studies (A. Glasier, B. Alder, A. S. McNeilly and P. W. Howie, unpublished observations) after longitudinal tracking of more than 70 breast feeding women. Once breast feeding is reduced below either of these limits the possibility of ovulation and pregnancy, in the absence of a menstrual period, arises. The first menstrual cycle is more likely to have a normal luteal phase, and therefore potentially fertile if the reduction in the suckling stimulus is relatively abrupt. A more gradual decline in suckling often associated with the introduction of solid food to the baby (Howie *et al.*, 1980) is more likely to result in resumption of menstrual cycles with defective luteal phases.

Thus, it is clear from the present studies that, although breast feeding can provide a high degree of protection against pregnancy, it will only do so if the suckling frequency and duration is maintained at a sufficient level. In Western Society, a suckling frequency of five time per day is close to the maximum number of suckling episodes adopted by many mothers. This may explain why the contraceptive efficiency of breast feeding has been low in developed countries.

ACKNOWLEDGEMENTS

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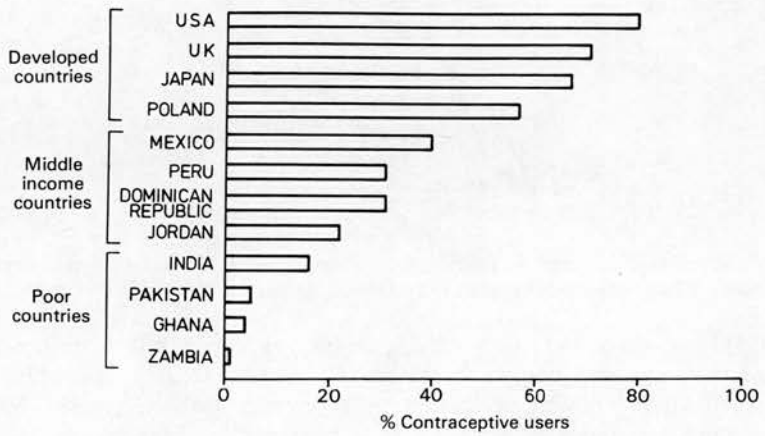
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# Effect of breast-feeding patterns on human birth intervals*

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An adequate inter-birth interval is of great importance because it enables a mother to recover her physical and emotional strength between pregnancies and confers upon the child advantages of better health and development (Morley, 1977). Breast feeding is the naturally evolved method of ensuring an adequate inter-birth interval but the contraceptive effect of breast feeding has been largely dismissed in developed countries on the grounds that it is an unreliable method of family planning for individual mothers (Kamal *et al.*, 1969). The use of artificial contraception varies very widely in different countries, being much higher in developed nations than in the poorer developing countries (Text-fig. 1). Where the use of artificial contraceptives is low, breast feeding is the most widely used method of birth spacing and assumes major demographic importance. It is important, therefore, that the factors controlling lactational infertility should be understood so that guidelines can be developed which will allow mothers to maximize the birth-spacing effect of breast feeding to their own advantage. In developed countries, few mothers choose to rely on breast feeding for family planning although an increasing minority may find breast feeding to be an attractive method of spacing their families, thereby avoiding the potentially adverse side-effects of currently available contraceptive methods. A better understanding of how breast feeding mediates its birth-spacing effect may lead to the development of new contraceptive methods which will try to imitate this naturally evolved method of fertility control (McNeilly, 1979).




**Text-fig. 1.** Contraceptive usage by married women aged 15–44 years in different countries by economic status. Source: Population Reference Bureau Inc. Washington D.C., U.S.A.

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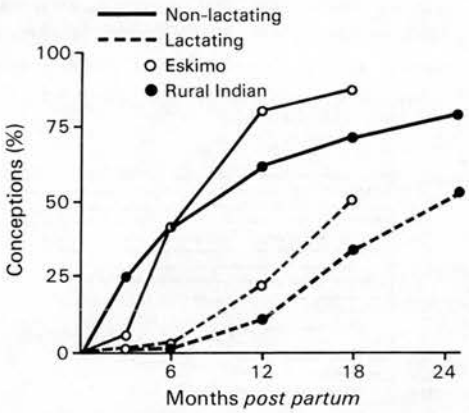
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This paper will describe the methods which have been used to investigate lactational infertility and discuss how different breast-feeding patterns influence human birth intervals.

## Methods of assessing lactational infertility

Lactational infertility can be measured in three different ways: (1) by the duration of the interbirth interval, (2) by the duration of lactational amenorrhoea and (3) by the return of ovulation. These methods present different problems of interpretation and a complete picture of lactational infertility can only be achieved by considering all the information which has been gathered from these different sources.

*Interbirth interval.* The ultimate test of fertility is pregnancy and several reports have demonstrated that breast feeding increases the interval between pregnancies (for summary, see van Ginneken, 1977). For example, two studies compared the time to next conception in nursing and non-nursing mothers from Alaskan Eskimo (Berman, Hanson & Hellman, 1972) and rural Indian populations (Potter, New, Wyon & Gordon, 1965). Despite the wide differences in climate and culture, the conception rates were similar in the two populations and conception occurred sooner in the non-lactating than lactating mothers (Text-fig. 2). Considering the time until 50% of the mothers conceived again, breast-feeding conferred an additional fertility protection of 14 months in the Indian women and 10 months in the Eskimo women; assuming that these intervals repeated themselves between successive pregnancies, this would mean that non-nursing women would have almost double the number of children than the breast feeding women over the same time period.




**Text-fig. 2.** Cumulative conception rates since last pregnancy in lactating and non-lactating mothers. Data for Eskimos from Berman *et al.* (1972), Indian rural from Potter *et al.* (1965).

Rosa (1975) has attempted to quantify the contraceptive effect of breast feeding in developing countries and has compared its contribution to fertility protection with that of artificial contraceptives. Assuming additional fertility protection of 8 months in rural mothers and of 4 months in urban mothers, Rosa (1975) estimated that, in the course of 1 year, breast feeding contributed over $31 \times 10^6$ couple years of contraceptive protection; by comparison, the known sales of artificial contraceptives contributed $24 \times 10^6$ couple years of fertility protection over the same time period. Knodel (1977) has pointed out that the demographic consequences of breast feeding are complex because the contraceptive effects of breast feeding on population growth are reduced, at least to some extent, by the lower mortality rates among breast fed babies. There can be no doubt, however, that breast feeding has profound implications in developing countries both for the individual mother and for the population as a whole.

Despite the clear evidence that breast feeding is associated with prolonged interbirth intervals, it cannot be assumed that breast feeding *per se* is directly responsible for this effect. In many cultures, particularly in Africa, sexual taboos are imposed on nursing mothers and reduced frequency of intercourse could explain, at least in part, the fertility-inhibiting effect of breast feeding. Contraceptive practices during lactation could also influence the interbirth interval and the use of coitus interruptus, for example, cannot be monitored on the basis of contraceptive marketing figures. An example of how behavioural factors can influence the interbirth interval was demonstrated by Chen, Ahmed, Gesche & Mosley (1974) during their detailed prospective study in Bangladesh. They found that the conception rates after the return of post-partum menses were lower in fishermens' wives than in farmers' wives, probably because the fishermen were absent from home for longer periods. It is essential, therefore, to know what behavioural variables might influence the interbirth interval before the epidemiological evidence of more prolonged birth spacing can be directly attributed to breast feeding itself.

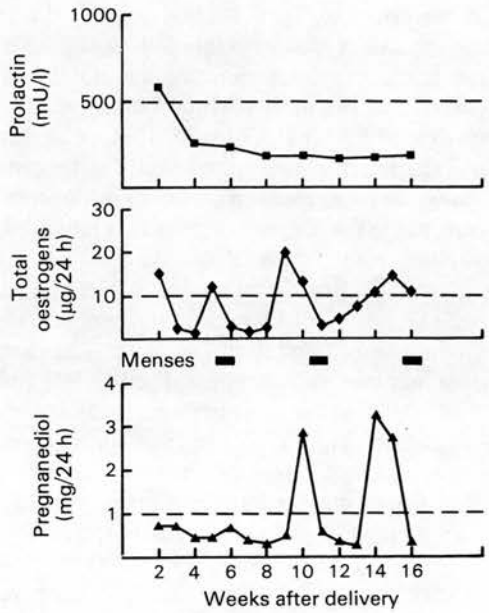
*Lactational amenorrhoea.* The return of menstruation *post partum* has been used in many studies as an indirect index of resumed ovulation (see van Ginneken, 1977). This method is convenient because it is easy to measure and can be applied to large populations. Many studies have shown that the duration of post-partum amenorrhoea is longer in nursing than non-nursing mothers (for summary, see Buchanan, 1975). In non-nursing mothers, the duration of post-partum amenorrhoea averages about 3 months, but among nursing mothers it may last for more than 2 years. It is also clear that fertility is markedly reduced during the period of lactational amenorrhoea. Chen *et al.* (1974) calculated that, during 18 months of lactational amenorrhoea in Bangladesh, the pregnancy rate was less than 5 per 100 women years of exposure which compared well with many artificial contraceptive methods. The duration of lactational amenorrhoea is much longer in rural than in urban communities (van Ginneken, 1977) because rural mothers breast feed more intensively and for a longer time.

Much valuable information has been gathered from the study of lactational amenorrhoea but its validity depends upon the assumption that the capacity of breast feeding to prevent pregnancy is limited to the duration of lactational amenorrhoea. It is well known that breast feeding does not postpone pregnancy indefinitely and 2–10% of mothers conceive during the period of lactational amenorrhoea (Badraoui & Hefnawi, 1979). There is also evidence (see below) that the fecundity of breast feeding women does not return to normal after the return of menses *post partum.* It is, therefore, desirable to have information from studies which directly measure the resumption of ovulation during the post-partum period.

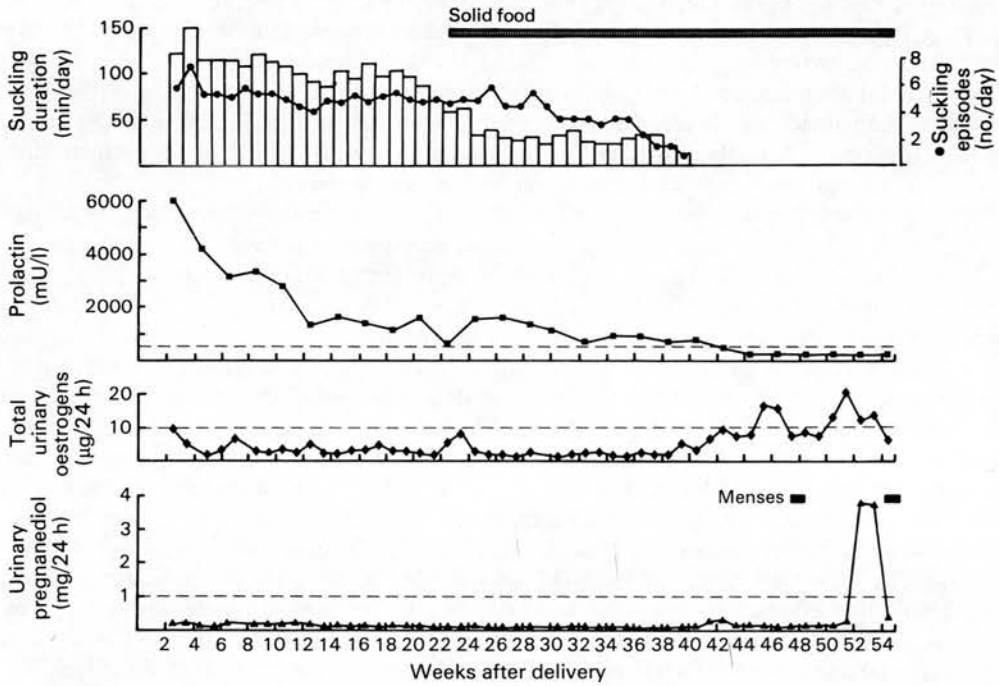
*Detection of ovulation.* Most studies which have attempted to define the timing of ovulation after childbirth have used endometrial biopsy (Lass, Smelser & Kurzrok, 1938; Topkins, 1943; Udesky, 1950; El-Minawi & Foda, 1971), basal body temperature (Sharman, 1951; Cronin, 1968), cervical mucus or vaginal cytology (Perez, Vela, Masnick & Potter, 1972) as markers of ovulation but these methods present problems of interpretation. During lactation, the histology of the endometrium may show inadequate co-ordination of maturation signs characteristic of the normal menstrual cycle, making it difficult to determine whether ovulation has occurred (El-Minawi & Foda, 1972). Ideally, endometrial biopsies should be taken in the pre-menstrual phase and, in the absence of regular menstrual cycles, the correct timing of biopsies can be difficult to achieve. Biopsies taken on the first day of bleeding, as happened in several studies, may be unreliable markers of ovulation. Temperature charts during lactation may show a much shorter plateau in the pre-menstrual phase (Cronin, 1968) making precise interpretation difficult.

As an alternative to these methods, the use of plasma or urinary steroid concentrations provides objective evidence of ovulation and enables a quantitative estimate of menstrual cycle adequacy. Rolland, Lequin, Schellekens & de Jong (1975) used plasma steroid levels to show that ovulation returned more quickly in mothers who discontinued breast feeding early in the puerperium but the studies were not continued beyond 100 days *post partum.* Urinary total oestrogen and pregnanediol excretion can be used to monitor ovarian follicular activity and

ovulation (McNeilly, Howie & Houston, 1980) and this non-invasive method has enabled us to monitor 10 bottle-feeding and 27 breast-feeding mothers from delivery until first ovulation.




**Text-fig. 3.** Return of ovarian activity (urinary total oestrogens $>10$ μg/24 h) and ovulation (urinary pregnanediol $>1$ mg/24 h) in the early puerperium in a bottle feeding mother.




**Text-fig. 4.** Delayed return of post-partum ovarian activity and ovulation in a breast-feeding mother.

The inhibitory effects of breast feeding are illustrated by the pattern of endocrine events leading to the resumption of ovulation in a typical bottle feeding (Text-fig. 3) and a typical breast feeding (Text-fig. 4) mother. In the bottle-feeding mother, basal prolactin levels had returned to the non-pregnant range within 4 weeks *post partum* and this was followed by an early resumption of ovarian follicular activity (total urinary oestrogens > 10 μg/24 h) and menstruation. Ovulation (urinary pregnanediol > 1 mg/24 h) did not occur during the first cycle but resumed in the second and third cycles. In the breast-feeding mother, basal prolactin levels fell progressively as suckling duration was reduced but remained above the non-pregnant range until the cessation of breast feeding at 40 weeks *post partum*. Ovarian activity and ovulation were suppressed until 44 weeks *post partum* when the first anovulatory cycle was followed by menstruation at 47 weeks. After the first post-partum cycle, there was an interval of 7 weeks before the next menstruation which was followed by a normal ovulatory cycle.

Considering the time of first ovulation in the total group of 10 bottle-feeding and 27 breast-feeding mothers, the bottle feeders ovulated between 6 and 15 weeks *post partum* compared with 15–68 weeks in the breast feeders. This finding is consistent with previous reports (Udesky, 1950; Sharman, 1951; Cronin, 1968; Perez *et al.*, 1972) and indicates that a major part of the antifertility effect of breast feeding can be attributed to the inhibition of ovarian follicular development and ovulation.

## Components of the interbirth interval

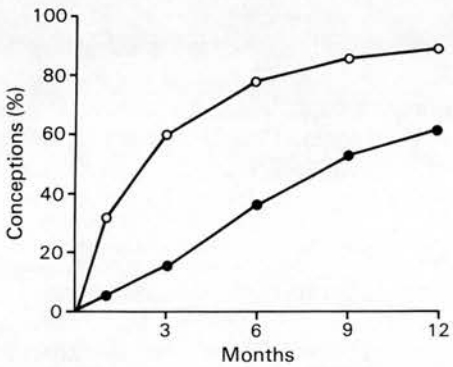
The interbirth interval consists of three phases, the phase of lactational amenorrhoea, the menstruating interval (which is the interval between the return of post-partum menstruation and next conception) and the length of gestation itself. Only the period of gestation is relatively fixed, the durations of lactational amenorrhoea and the menstruating interval varying widely between populations and between individuals within these populations. It is, therefore, important to consider the timing and frequency of ovulations during lactational amenorrhoea and during the menstruating interval.

*Ovulation during lactational amenorrhoea.* As previously discussed, fertility is substantially reduced during the phase of lactational amenorrhoea but between 2 and 10% of nursing mothers conceive before first post-partum menstruation. This is an important practical point because it means that the return of menstruation cannot be used as an indication of when to resume contraception.

Using endometrial biopsy as a marker of ovulation, Udesky (1950) reported that ovulation during lactation was followed either by menses or by pregnancy. This finding has been supported by our own studies (Howie, McNeilly, Houston, Cook & Boyle, 1981) and indicates that recurrent ovulations do not take place throughout the phase of lactational amenorrhoea. There is some dispute about the frequency of ovulation in the cycle before the end of lactational amenorrhoea, the estimates varying from 14% (Udesky, 1950), 23% (Cronin, 1968) and 33% (Howie *et al.*, 1981) to 75% (Perez *et al.*, 1972). The different estimates of ovulation frequency during lactational amenorrhoea may be partly explained by the different methods used to assess ovulation but are more likely to be due to the different suckling patterns of the populations studied. It has been shown that the longer that the first menses is delayed during lactation, the more likely is the first cycle to be ovulatory (Perez *et al.*, 1972; Howie *et al.*, 1981). The impact of different suckling patterns upon post-partum ovulation is discussed below.

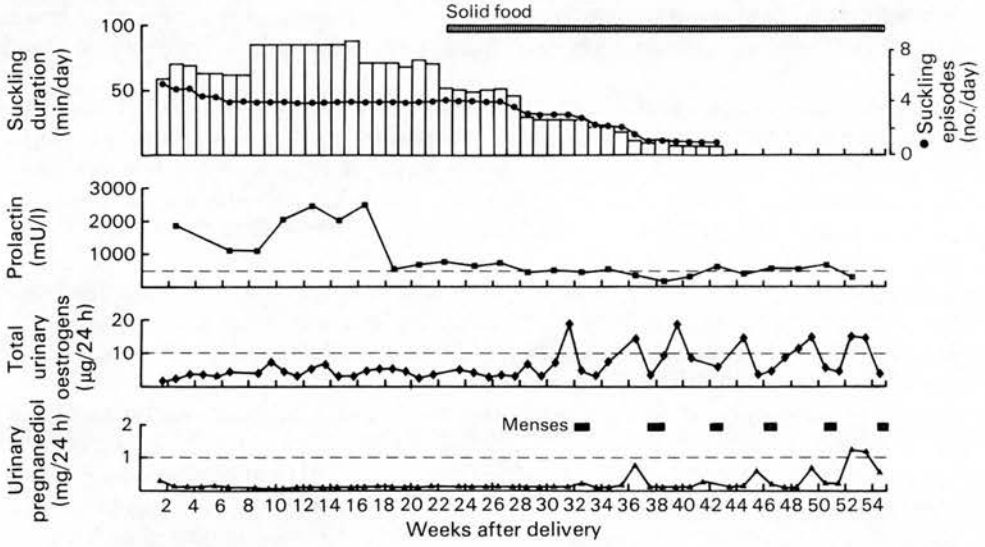
*Ovulation during the menstruating interval.* There is less information about the frequency of ovulation in breast feeding mothers who have resumed menstruation and it has been suggested that the capacity of prolonged breast feeding to prevent pregnancy is limited to the period of lactational amenorrhoea. Several strands of evidence suggest, however, that fecundity is reduced

during the menstruating interval. Chen *et al.* (1974) studied the conception rates after the return of post-partum menses in nursing mothers and found that they were much lower than would be expected in a non-nursing group of women (Tietze, 1968) who were not using contraception (Text-fig. 5). Similarly, Potter *et al.*, 1965) found pregnancy rates of 22 and 32% in nursing mothers at 3 and 6 months after first menses compared with corresponding figures of 39 and 47% in mothers who experienced a neonatal death. During the menstruating interval, the frequency of anovulatory cycles increased, being estimated at 55% (Lass *et al.*, 1938) and 42% (Howie *et al.*, 1981). Reduced fecundity amongst nursing mothers is further supported by the increased intervals between menstrual periods, compared with cycles in non-nursing mothers (Sharman, 1951; Berman *et al.*, 1972; Howie *et al.*, 1981), which suggests a disturbance of normal rhythms during lactation.



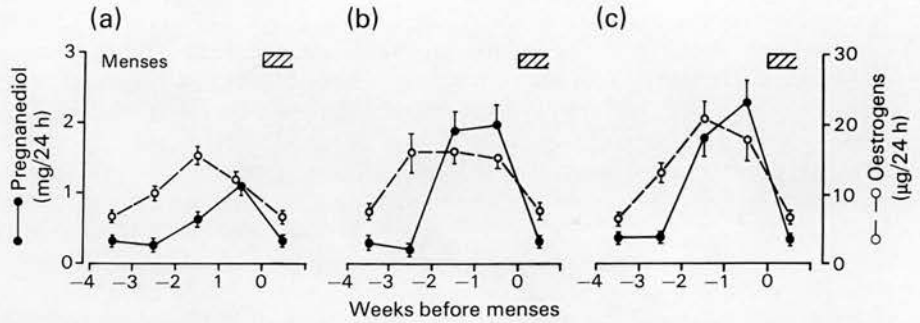

**Text-fig. 5.** Conception rates in (●) nursing mothers after the return of post-partum menses (Chen *et al.*, 1974) and in (○) non-nursing women using no contraception (Tietze, 1968).

Using plasma steroid concentrations as a marker of ovulation, Duchen & McNeilly (1980) found a lower incidence of ovulatory cycles during lactation than would be expected in a normal population. An example of an individual mother who had recurrent anovulatory cycles during lactation is shown in Text-fig. 6. After the introduction of solid food, basal prolactin levels




**Text-fig. 6.** Anovulatory cycles in a breast-feeding mother during lactation.

returned to the pregnant range and there were 5 anovulatory cycles (urinary pregnanediol $<1$ mg/24 h) before ovulation resumed after complete weaning. We have studied the levels of urinary steroid excretion in 54 cycles during lactation and compared them with the levels in 30 cycles after lactation and 27 cycles in non-pregnant controls (Text-fig. 7). During lactation, the mean ($\pm$s.e.m.) level of urinary pregnanediol in the luteal phase were $0{\cdot}93 \pm 0{\cdot}09$ mg/24 h which was significantly lower than the mean level of $1{\cdot}62 \pm 0{\cdot}15$ mg/24 h in cycles after lactation and $2{\cdot}13 \pm 0{\cdot}83$ mg/24 h in the non-pregnant controls. These results suggested a high incidence of cycles which were either anovular or characterized by inadequate luteal phases. Levels of total urinary oestrogen excretion (calculated as the mean of the 4 levels throughout the cycle) were $10{\cdot}3 \pm 1{\cdot}0$ µg/24 h in cycles during lactation, $12{\cdot}2 \pm 1{\cdot}3$ µg/24 h in cycles after lactation and $14{\cdot}1 \pm 1{\cdot}7$ µg/24 h in cycles from non-pregnant controls. This was consistent with the hypothesis that the defective luteal function might have its origins in abnormal follicular development (McNeilly *et al.*, 1980).




**Text-fig. 7.** Excretion of urinary total oestrogen and pregnanediol in menstrual cycles (a) during lactation ($n = 54$), (b) after lactation ($n = 30$) and (c) in non-pregnant controls ($n = 27$). Values are mean $\pm$s.e.m.

The combined evidence suggests that the menstruating interval is associated with a reduction of fecundity which, however, is less complete than it is during the phase of lactational amenorrhoea.

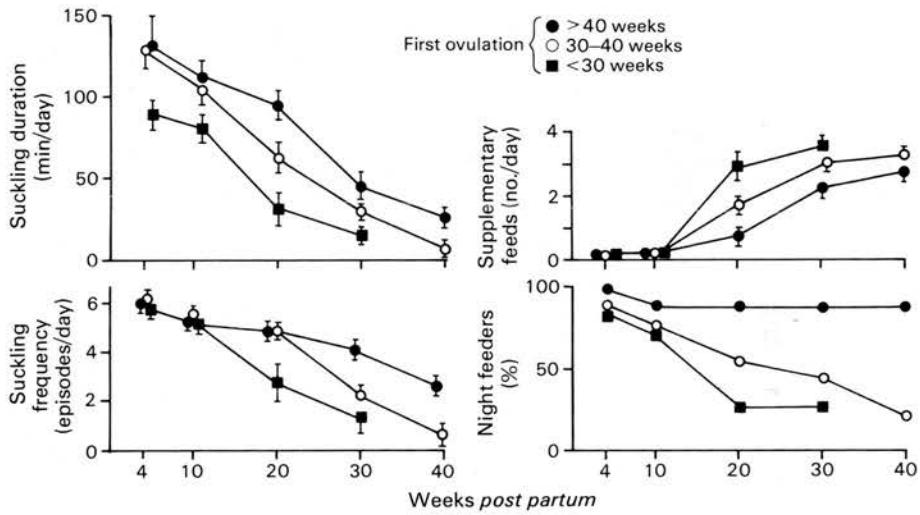
## Effect of suckling upon fertility

It is important to understand the effect which different suckling patterns have upon the restoration of fertility so that appropriate advice can be given to nursing mothers who wish to prolong the interbirth interval for as long as possible. It is well known that the phase of lactational amenorrhoea is much shorter during partial as compared with full (unsupplemented) breast feeding (Sharman, 1951; McKeown & Gibson, 1954; Perez *et al.*, 1972; Chen *et al.*, 1974). Gioiosa (1955) reported that, out of 500 birth intervals in mothers who breast fed, 46 (9·2%) conceived during lactation but 40 of these (87%) conceived when the weaning process was taking place. Gioiosa (1955) concluded that the fertility protection associated with breast feeding lasted for 9 months or more, provided that no additional supplementary or complementary formula was used.

It is clear, therefore, that suckling is a major variable in the control of post-partum ovulation but relatively few studies have attempted to measure the suckling stimulus. Konner & Worthman (1980) have reported that the prolonged interbirth intervals of up to 4 years among the nomadic !Kung hunter gatherers are associated with very frequent suckling bouts, which last for only 2 min every 15 min. This suggests that very frequent suckling exerts a profound inhibitory effect upon reproduction.

In Rwanda, rural women breast feed on demand and 50% conceive again within 23 months after delivery; by contrast, in urban women, breast feeding is operated on a more rigid schedule with fewer feeding episodes and 50% of mothers have conceived again within 9 months *post partum* (Bonte, Akingeneye, Gashakamba, Nbarutso & Nolens, 1974). In a series of cross-sectional studies, Delvoye, Badawi, Demaeged & Robyn (1978) found that prolactin levels were related both to the duration of lactational amenorrhoea and to the frequency of suckling. By extrapolation, these data suggest that the duration of lactational amenorrhoea is related to the pattern of suckling frequency. These various studies point to the profound effect which different suckling patterns exert upon post-partum fertility.

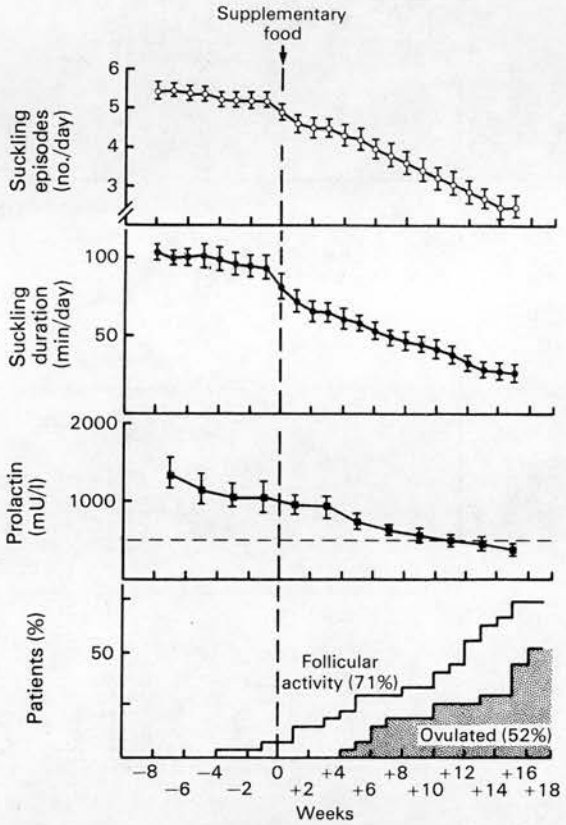
*Infant feeding patterns and the time of first ovulation.* From our own prospective studies, the infant feeding patterns were compared between mothers who ovulated before 30 weeks *post partum*, those who ovulated between 30 and 40 weeks *post partum* and those who ovulated after 40 weeks *post partum*. The mothers who postponed ovulation for longest (>40 weeks) had a longer total duration of breast feeding than did those in the other two groups, but there were other differences between the groups as illustrated in Text-fig. 8; the mothers who ovulated after 40 weeks maintained suckling duration and frequency at the highest levels, introduced supplementary food most slowly and maintained night feeds for longest. The maintenance of night feeds (defined as a suckling episode between midnight and 08:00 h) may be important because a long interval without suckling could allow the hypothalamic–pituitary–ovarian axis sufficient time to recover and resume ovulation. This study suggested strongly that there was an inverse relationship between the use of supplementary foods and the strength of the suckling stimulus, and that the early and abrupt introduction of supplements might play an important part in relation to the resumption of post-partum ovarian activity and ovulation.




**Text-fig. 8.** Comparison of infant feeding in mothers ovulating before 30 weeks (N = 7), between 30 and 40 (N = 9) and after 40 weeks (N = 9) *post partum*. Values are mean ± s.e.m.

*Effect of supplementary food on suckling and ovulation.* The critical importance of supplementary food was emphasized by centring the levels of suckling frequency and suckling duration round the time at which supplements were introduced (Text-fig. 9). Suckling duration and suckling frequency for the whole cohort of breast-feeding mothers remained relatively constant until supplements were given, after which both fell abruptly. These changes in suckling were reflected in the basal prolactin concentrations and, before the introduction of supplements, no mother had ovulated although four had evidence of ovarian follicular development. After

supplements, progressively more mothers had evidence of ovarian follicular development and 52% had ovulated within 16 weeks of introducing supplementary food.

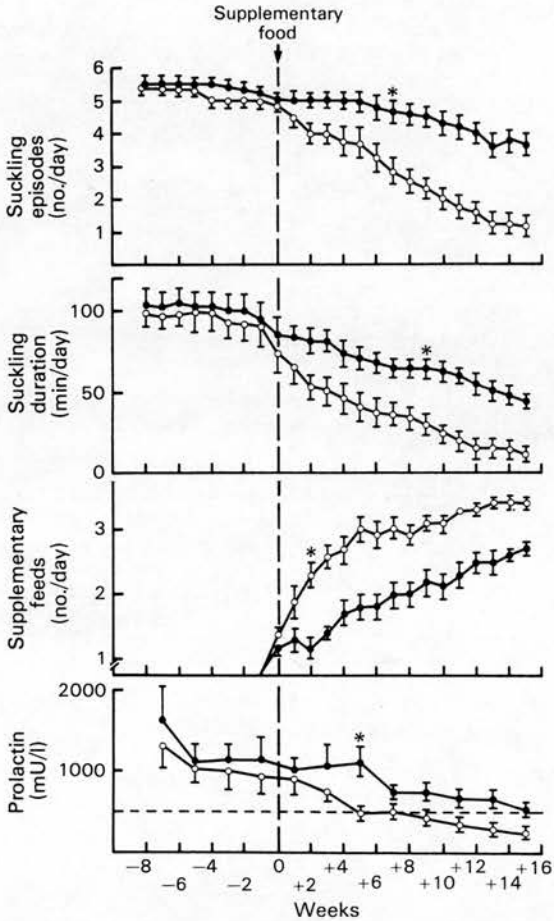



**Text-fig. 9.** Mean suckling duration, suckling frequency, basal prolactin and ovarian activity in women (N = 27) before and after the introduction of supplementary food. Values are mean ±s.e.m. Broken line indicates upper limit for non-pregnant women. (From Howie *et al.*, 1981.)

In Text-fig. 10, the feeding patterns in the 52% who ovulated within 16 weeks of introducing supplements are compared with the 48% who continued to suppress ovulation over that time. The mothers who ovulated reduced suckling more rapidly and weaned more abruptly. The stronger suckling stimulus amongst those who continued to suppress ovulation was reflected in higher mean basal prolactin levels which remained above the non-pregnant range.

## Sequence of events leading to post-partum ovulation

From the data presented in this paper, it is possible to formulate a hypothesis of the events leading to the return of post-partum ovulation. In bottle-feeding mothers, plasma prolactin concentrations return to normal within a few weeks of delivery and there is an early resumption of ovarian activity and ovulation. In breast-feeding mothers, prolactin values are elevated and ovarian function is suppressed, the extent of that suppression being dependent upon the strength and duration of suckling; the suckling stimulus is undermined by the use of supplementary food which indirectly encourages the resumption of ovarian activity. In their detailed study, Perez *et al.* (1972) emphasized that full (unsupplemented) breast feeding was not a guarantee against




**Text-fig. 10.** Comparison of infant feeding patterns and basal prolactin between (○) mothers ovulating within 16 weeks (N = 14) and (●) mothers suppressing ovulation (N = 13) after introduction of supplementary food. Values are mean ±s.e.m. The broken line indicates the upper limit for non-pregnant women. * $P < 0{\cdot}01$. (From Howie *et al.*, 1981.)

**Table 1.** Percentage of breast-feeding mothers giving supplements (occasional or regular) in economically advantaged, urban poor and rural groups of different countries (from W.H.O. Collaborative Study on Breast Feeding, 1979)

| Country | Group | 2–3 months | 6–7 months | Country | Group | 2–3 months | 6–7 months |
|---|---|---|---|---|---|---|---|
| Hungary | All | 46·8 | 96 | Chile | Advantaged | 60·0 | 90·0 |
| | | | | | Poor | 59·0 | 100·0 |
| Sweden | All | 30·7 | 100 | | Rural | 59·0 | 95·5 |
| Ethiopia | Advantaged | 67·8 | — | Guatemala | Advantaged | 93·3 | — |
| | Poor | 49·2 | 38·6 | | Poor | 51·9 | 87·1 |
| | Rural | 49·3 | 85·3 | | Rural | 13·8 | 65·4 |
| Nigeria | Advantaged | 86·1 | 100 | Philippines | Advantaged | 84·7 | — |
| | Poor | 63·5 | 91·7 | | Poor | 36·1 | 90·0 |
| | Rural | 35·0 | 86·3 | | Rural | 41·9 | 95·0 |
| Zaire | Advantaged | 38·3 | 84·4 | India | Advantaged | 52·3 | 82·3 |
| | Poor | 36·0 | 95·8 | | Poor | 5·5 | 22·3 |
| | Rural | 49·0 | 76·0 | | Rural | 1·8 | 12·4 |

ovulation or pregnancy but they did not define the suckling frequency which constituted full breast feeding in their population. Nevertheless, it is clear that the early and regular use of supplementary food will have a detrimental effect on the contraceptive effect of breast feeding and it must be a matter of concern that the W.H.O. Collaborative Study on breast feeding found such a high use of supplementary foods among mothers from developing countries within 2–3 months *post partum* (Table 1).

## Other factors influencing lactational infertility

It has been suggested that malnutrition may have an impact on the duration of lactational amenorrhoea, independent of breast feeding itself (Thomson, Hytten & Black, 1975). Bongaarts (1980) has reviewed the evidence and concluded that chronic malnutrition had a relatively minor effect on fertility; for example, the median durations of amenorrhoea in low, medium and high nutrition groups from Bangladesh were 21·2, 20·4 and 20·2 months respectively (Huffman, Chowdhury, Chakbarty & Mosley, 1978). Extreme food deprivation during famine, however, exerts a large but temporary reduction in fertility (Bongaarts, 1980).

Lunn, Prentice, Austin & Whitehead (1980) compared two groups of breast-feeding Gambian mothers, one receiving a calorie supplement, the other receiving no such supplement. The mothers receiving the calorie supplement had lower prolactin concentrations and menstruated more quickly. This evidence suggested that maternal nutrition might have an effect on fertility but interpretation of data is made difficult by the tendency of poorly nourished mothers to breast feed for longer and suckle more frequently than well nourished mothers (Prema, Naidu & Kumari, 1979). Future studies which investigate the effects of nutrition upon the contraceptive effects of breast feeding should have objective measures both of suckling and nutritional status.

Age has also been implicated as a factor of importance, because the duration of lactational amenorrhoea tends to be longer in older than in younger women (Jain, Hsu, Freedman & Chang, 1970).

## Practical implications of lactational infertility

*Reliability of breast feeding as a contraceptive method.* It has to be recognized that breast feeding cannot be relied upon as a guarantee against pregnancy for individual mothers. The place of breast feeding in fertility control has to be considered in the context of all the other factors which are pertinent to a particular population and to each of the individuals within that population. The importance of breast feeding as a method of birth spacing will depend upon the availability of alternative contraceptive methods, the willingness of the mothers to use the methods and the average duration of use of any particular method. These factors vary widely in different communities and breast feeding should be regarded as a method of fertility control which should be exploited along with, rather than instead of, alternative methods of contraception.

Nevertheless, breast feeding continues to have an important role in birth spacing throughout the world, and enthusiastic breast-feeding, characterized by frequent and energetic suckling, can exert a high degree of fertility protection, particularly during the phase of lactational amenorrhoea.

*Birth spacing in developing countries.* The main importance of breast feeding as a contraceptive method is in developing countries where alternative methods of family planning are used infrequently. It is clear that the modern methods of infant feeding, with the early and frequent use of supplements, will have profound implications on fertility and population growth. If there is a continuation of the trend towards early weaning in order to 'emancipate' mothers

from the burdens of childbearing, this must be matched by increased availability and use of alternative contraceptive methods. The abandonment of prolonged breast feeding, without alternative contraception, will shorten interbirth intervals and greatly increase the burdens on individual mothers and community resources.

Breast-feeding practices have implications for infant growth, neonatal infection and birth spacing. Infant feeding policies should be designed only after recognizing the importance of all these competing factors.

*Strategies of contraception.* Potter, Masnick & Gendell (1973) have pointed out that, in many communities, a contraceptive method may be used only for a finite period of time. If a contraceptive method is begun immediately after parturition, there will be an overlap between the time when the contraceptive is used and the period of post-partum anovulation when protection is not needed. By delaying the introduction of the contraceptive method, the extent of the overlap is reduced, and the potential contraceptive efficiency increased. For example, in Bangladesh, Potter *et al.* (1973) calculated that if the use of artificial contraception were delayed until 6 months *post partum*, only 1 in 200 women would have conceived by that time. More studies are required in different communities to develop strategies which exploit post-partum infertility and contraception to the best advantage.

*Birth spacing in developed countries.* In developed countries the majority of breast-feeding mothers will use a contraceptive method. Some of these mothers, however, dislike the inconvenience and the potential side-effects of the various methods and regard the possibility of using the birth-spacing effect of breast feeding as an attractive alternative. These mothers will have to breast feed enthusiastically and accept that the method is not absolutely reliable. More work is required to define the minimum suckling frequency and duration which are necessary to inhibit ovulation reliably but a number of guidelines can be tentatively suggested to those who wish to maximize the contraceptive effect. Mothers should feed on demand and use supplementary bottles as infrequently as possible. When it becomes nutritionally necessary for solid food to be given to the baby, this should be introduced gradually and not abruptly. Mothers should be encouraged to breast feed at night and avoid long intervals between suckling.

These guidelines lay the emphasis on suckling and require much enthusiasm on the part of the mother. Breast feeding is a complex physiological process with functions beyond those of infant nutrition alone. It is important that, in the future, the advice and support which is offered to mothers should be formulated with full realization of all the implications which breast feeding has for both maternal and child health.

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# Failure of Lactation to Have a Consistent Effect on Interbirth Interval in the Common Marmoset, *Callithrix jacchus jacchus*

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**Key Words.** Marmoset · Lactation · Interbirth interval · Abortion

**Abstract.** Analysis of intervals between successive births, or of the interval between spontaneous or induced abortion and subsequent birth, indicate that unlike most primates for which information is available, lactation does not consistently influence the ability of the common marmoset to conceive during the early post-partum period.

## Introduction

In the human female, lactation is associated with a period of amenorrhoea and infertility [see McNeilly, 1979, for review]. Although the exact mechanisms involved in inhibition of reproduction during the post-partum period have not yet been elucidated, both elevated levels of prolactin and the suckling stimulus itself would appear to be involved.

Lactationally-induced infertility has been recorded in a number of non-human primates, the overt reasons for failure of conception being inhibition of ovulation, and reduction or abolition of sexual activity. There is good evidence of lactational infertility in the great apes [gorilla, S.J. Hall, R.V. Short and A.S. McNeilly, unpublished observations; chimpanzee, Douglas and Butler, 1970; Clegg and Weaver, 1972; Tutin, 1980] and lesser Old World anthropoids such as the baboon [Rowell, 1974], various species of macaque [Kaufman, 1965; Koford, 1965, 1966; Fujiwara, Honjo, Imaizumi and Imanichi, 1967; Vandenberg and Vessey, 1968; Weiss et al., 1973; Drickamer, 1974; Varley and Vessey, 1977], and members of the genus *Cercopithecus* [Mallinson, 1971; Rowell, 1974]. Among the New

World primates an effect of lactation on reproduction has been observed in spider [Dempsey, 1939; Wolf et al., 1975], howler [Carpenter, 1934], and squirrel monkeys [Travis and Holmes, 1974; Coe and Rosenblum, 1978].

The aim of the present study was to examine interbirth intervals in a captive colony of common marmosets, *Callithrix jacchus jacchus* and to relate these intervals to the occurrence of lactation.

## Materials and Methods

The animals were housed at the MRC primate colony which was established in 1973; details of colony management were provided by Hearn et al. [1975]. Interbirth interval data were collected for the 7-year period January, 1973 to December, 1979 inclusive. Additional data were analysed for the time elapsed between a spontaneously occurring abortion in which the conceptual products were recovered and a subsequent birth, or for the interval following a pregnancy which was terminated by surgically induced abortion and a subsequent birth. The data were classified as follows:

*Group A* (interbirth intervals). Category 1: No young raised beyond the day of birth. Category 2: No young raised beyond 1–10 days of age. Category 3: No young raised beyond 11–20 days of age. Category 4: No young raised beyond 21–50 days of age. Category 5: No young raised beyond 51–200 days of age. Category 6: At least one offspring reared beyond 200 days of age.

*Group B* (intervals between spontaneous or induced abortion and subsequent birth). Category 1: Interval between spontaneous abortion and subsequent birth. Category 2: Interval between induced abortion and subsequent birth.

## Results and Discussion

A total of 181 interbirth intervals was available for analysis; however, 29 of these were eliminated because it was considered that they had been artificially lengthened, e.g. by death of the male partner without immediate replacement; by severe loss of condition of the female post-partum; or by the use of experimental procedures which interfered with fertility.

There were 5 instances where the date of spontaneous abortion was known with certainty, allowing the interval between abortion and the following birth to be calculated. 17 hysterectomies were performed at various stages of pregnancy and the time which elapsed between operation and the next parturition was recorded.

The results obtained for the 152 interbirth intervals and the 22 post-abortion intervals are given in table I.

**Table I.** Analysis of interbirth and abortion to subsequent birth intervals in the common marmoset

| Group | Category | n | Mean ± SD, days | Median, days | Range, days |
|---|---|---|---|---|---|
| A | 1 | 5 | 174.8 ± 38.8 | 156.0 | 151–243 |
| | 2 | 15 | 189.3 ± 49.4 | 158.0 | 151–274 |
| | 3 | 6 | 187.7 ± 26.6 | 193.5 | 157–227 |
| | 4 | 6 | 164.0 ± 18.8 | 156.5 | 154–202 |
| | 5 | 6 | 179.0 ± 39.5 | 155.5 | 154–248 |
| | 6 | 114 | 212.6 ± 57.7 | 206.0 | 147–441 |
| Total | | 152 | 204.9 ± 55.2 | 186.5 | 147–441 |
| B | 1 | 5 | 172.2 ± 29.7 | 163 | 151–223 |
| | 2 | 17 | 181.1 ± 28.2 | 180 | 153–246 |
| Total | | 22 | 179.1 ± 28.1 | 175 | 151–246 |

See 'Materials and Methods' for details of classification.

In the common marmoset, gestation lasts for between 20 and 21 weeks [see inter alia, Rothe, 1975; Stevenson, 1976; Hiddleston, 1978; Chambers and Hearn, 1979]. Suckling has been recorded for up to 129 days post-partum [Box, 1975], and although the duration of lactation, whether established by observations of suckling or by expression of milk from the nipple, appears to be variable, the young usually suckle for up to 80–100 days [Fitzgerald, 1935; Epple, 1967; Ingram, 1975; Rothe, 1977; Lunn and Hearn, 1978].

The mother begins to actively discourage suckling at about 70–77 days post-partum [Ingram, 1975], although lactation may end before this [Fitzgerald, 1935; Epple, 1967], however, young marmosets have been known to survive without human intervention following the loss of their mother at 35 days post-partum [Mallinson, 1969]. The latter report may reflect the relatively young age at which the young start to eat solid foods in addition to suckling, weaning commencing at about 3 weeks of age [Lucas et al., 1927; Epple, 1967; Ingram, 1975, 1977; Stevenson, 1976]. If lactation in the marmoset influences reproductive events by delaying conception post-partum, then the interbirth interval should be significantly lengthened beyond the 20–21 weeks of a normal gestation period. The results of the present study suggest that this is not necessarily the case.

The degree to which lactation occurred was assessed on the basis of offspring survival; thus in group A, category 2, all of the young born at a particular birth died between days 1 and 10, lactation presumably ceasing at an earlier stage than in group A, category 4, where at least one offspring survived to between 21 and 50 days of age.

The mean interbirth intervals for categories in group A ranged from 164.0 ± 18.8 days (category 2) to 212.6 ± 57.7 days (category 6) and there was a considerable overlap between the range of values obtained for animals in which no young survived the day of birth (category 1) compared with those in which full lactation occurred (category 6). The median values varied between 155.5 days in category 5 and 206 days in category 6.

The results for group A suggest that there may be a prolongation of interbirth intervals in animals in which full lactation occurs; however, this is not invariably so, since in one-third of the cases in category 6 this period was less than 3 weeks longer than the gestation period. The minimum interval between post-partum mating, as judged by the first recovery of sperm at vaginal lavage and subsequent birth, was 145 days in a category 6 animal who delivered full-term twins (on the bases of birth weights and appearance) and reared these successfully to adulthood.

In group B it was assumed that any trauma associated with spontaneous abortion or surgery would not influence subsequent conception, and that lactation would not occur. As a result, the animals in this group would act as non-lactating controls and it was expected that mean values for interbirth intervals would resemble those recorded for group A, categories 1–3 where lactation was absent, or impaired in terms of duration. This was found to be the case, interbirth intervals in group B ranging between 151 and 246 days. These results differ from those reported by Wolfe et al. [1972] for the tamarin (*Saguinus* sp.) where the interval between abortion and subsequent birth was apparently longer than the interval between successive births when the young were removed for hand-rearing within 48 h post-partum. Intervals between hysterotomy and subsequent birth have been provided by Phillips [1976], values being similar to those reported here. In addition, he noted the similarity between interbirth intervals in control animals and those from operation to subsequent parturition in his experimental series. Chambers and Hearn [1979] also recorded that the interval from birth to post-partum ovulation was the same as that between hysterotomy and ovulation.

In all categories interbirth intervals were recorded which were only marginally longer than the gestation period, suggesting that lactation in the

common marmoset does not invariably infuence the length of the period between successive births. This finding is in keeping with the reports of others who have recorded interbirth intervals of between 151 and 330 days [Epple, 1967; Mallinson, 1969; Rothe, 1975, 1977; Phillips, 1976; Stevenson, 1976; Ingram, 1977; Hiddleston, 1978]. The slightly longer interbirth intervals recorded in some instances in the present study may reflect the fact that this is an experimental, rather than a breeding colony, and would support the findings of Eckstein and Kelly [1966] who showed superior performance in macaque colonies in which there was minimal experimental interruption.

In this marmoset colony, daily vaginal lavage revealed that post-partum mating occurred on day 7.6 ± 6.7 (mean ± SD, n = 54, median = day 7, range = 0–47) irrespective of lactational status [Lunn, 1979, unpubl. observation]. Of 31 animals, post-partum ovulation occurred in 23 (74%) within 3 weeks after birth (mean ± SD = 10.5 ± 0.7 days, range = 5–17 days) [Chambers and Hearn, 1979], and 76% of these ovulations resulted in conceptions. Such conceptions occurred irrespective of whether or not the animal was lactating, and indeed some 72% of the animals in which fertile matings occurred were lactating [P.C. Chambers, 1980, personal commun.].

In summary therefore, it appears that post-partum mating, ovulation and conception can occur in the presence of lactation in the marmoset. This is underlined by the fact that in the present study interbirth intervals were found to be inconsistently affected by lactational status. The reason for the failure of lactation to influence post-partum conception may lie in the rapid growth rate of the young with its early onset of weaning, despite continued lactation, since in the human it appears likely that initiation of supplemental feeding may trigger menstruation post-partum in the presence of continued suckling [McNeilly et al., 1980]. The early onset of weaning is followed by early puberty and the short generation time typical of a small mammal. In fact, comparison of the life history and reproductive events of the common marmoset would suggest that this callitrichid is an r rather than a K strategist [see Daly and Wilson, 1978], and as such it is advantageous to have a lactational/weaning system which does not interfere with a requirement for a short interbirth interval. However, *C. jacchus* may differ from other callithrichid monkeys in this respect, since it has been reported that in the genus *Saguinus,* mating and ovulation do not occur for 2½ months post-partum [Wendt, 1964] and lactation delays subsequent pregnancy [Wolfe et al., 1972; D. Abbott and C. Snowdon, 1980, personal commun.].

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# EFFECTS OF INHIBITING PROLACTIN SECRETION ON THE MAINTENANCE OF EMBRYONIC DIAPAUSE IN THE SUCKLING RAT

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SUMMARY

Treatment of rats with bromocriptine between days 5 and 8 after the post-partum mating resulted in suppression of serum prolactin levels and caused luteal regression. Although this treatment led to embryonic resorption when suckling was prevented by removing litters soon after birth, the diapausing embryos of animals nursing a litter of eight pups were unaffected by the treatment. These results suggest that the high levels of prolactin and progesterone in the circulation during lactation are not responsible for maintenance of the diapausing state.

## INTRODUCTION

The gestation period of the rat may be extended considerably by concurrent lactation. This effect is due to lowered receptivity of the uterus to the nidatory stimulus and entry of blastocysts into a metabolically quiescent state. These embryos will emerge from this state and implant after treatment with oestrogen or gonadotrophic hormones which increase oestradiol production (Psychoyos, 1973; Raud, 1974; Maneckjee & Moudgal, 1975).

These observations have led to the conclusion that embryonic diapause results from ovarian follicular quiescence and oestrogen deficiency during lactation. Follicular quiescence has, in turn, been attributed to suppression of the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Rothchild, 1960; Raud, 1974). Comparable patterns of gonadotrophin and ovarian steroid secretion have been found in lactational anoestrus (Amenomori, Chen & Meites, 1970; Raud, 1974; Lu, Chen, Huang, Grandison, Marshall & Meites, 1976; Smith, 1978). In both states the suckling stimulus may inhibit gonadotrophin secretion by a common pathway, that is by decreasing hypothalamic luteinizing hormone releasing hormone (LH-RH) secretion.

An alternative hypothesis is that the increased circulating levels of prolactin which result from the suckling stimulus act on the ovary to inhibit follicular growth and/or steroidogenesis or act centrally to decrease FSH secretion (Smith, 1978). Since prolactin is a luteotrophic hormone during the first half of pregnancy in the rat (Tomogane, Ôta & Yokoyama, 1969, 1975; Morishige & Rothchild, 1974; Yoshinaga, 1974; Ford & Yoshinaga, 1975) it may also contribute to the maintenance of embryonic diapause through the action of progesterone on the uterus (Dickmann, 1973). It has, furthermore, been suggested that prolactin has a direct action on the uterus (Armstrong & King, 1971).

Since the maintenance of high levels of prolactin in the circulation during lactation depends on suckling, the occurrence of implantation after withdrawal of the nursing litter does not distinguish the two hypotheses outlined above. Prolactin secretion can, however, be inhibited by ergot alkaloids without interruption of the suckling stimulus (Tomogane *et al.* 1975; Smith 1978; Flint & Ensor, 1979). We have therefore used bromocriptine in the present

study to determine whether a high concentration in the circulation of prolactin and, concomitantly, of progesterone are required for the maintenance of embryonic diapause. Since suppression of prolactin secretion leads to luteal regression it was expected that supplementary progesterone would be necessary for the continuation of pregnancy if nidation occurred.

## MATERIALS AND METHODS

The study comprised two groups of pregnant rats. Group 1 was used to establish whether pregnancy could continue during treatment with bromocriptine and, if not, whether supplementary progesterone would reverse the abortifacient action of the drug. Group 2 animals comprised the main experiment in which bromocriptine and progesterone were administered simultaneously during concurrent lactation and pregnancy to ensure that pregnancy would be supported by sufficient progesterone if embryonic diapause was interrupted (as was indicated in group 1 animals).

Virgin Sprague–Dawley female rats weighing 200–250 g and about 4 months old were maintained in temperature-controlled ($23 \pm 1$ (range) °C) and artificially lit rooms (14 h light/day from 05.00 h) and food pellets and water were available *ad libitum*. They were mated with males of the same strain and mated again at the next post-partum oestrus. Conception was inferred from the presence of sperm in vaginal smears (day 1 of pregnancy).

After delivery of the litter the mothers were randomly and equally allocated to one of two groups: in group 1 the litters were removed within 48 h of birth and in group 2 litters were adjusted to eight pups per nursing rat within the same period *post partum*. Each group was further divided into four sub-groups based on a factorial experimental design. Each of the sub-groups was treated with one of the following: (1) progesterone and bromocriptine; (2) progesterone and vehicle; (3) bromocriptine and vehicle; (4) both types of vehicle. Progesterone (Sigma, London) was prepared by dissolving the dose of 4 mg in 0·2 ml arachis oil containing benzyl benzoate, 10% by volume. The 1 mg dose of bromocriptine (2-bromo-α-ergocryptine methanesulfonate, CB-154, Sandoz Ltd, Basel, Switzerland) was dissolved in 0·1 ml ethanol to which was added 0·1 ml 0·9% saline. These doses or the equivalent volume of vehicle were injected subcutaneously at 12.00 h daily on days 5–8 inclusive of the second pregnancy. Since galactopoiesis is suppressed by bromocriptine (Flückiger & Wagner, 1968), the litters were removed, weighed and exchanged between animals receiving and those not receiving this substance. Litters were exchanged at 09.00 and at 17.30–19.00 h on each of the 4 days of treatment.

Animals were anaesthetized with ethyl carbamate (1·75 g/kg, i.p., injected as a 25% solution (w/v); BDH Ltd, Poole, Dorset) between 10.00 and 12.00 h on day 9 of pregnancy. Suckling rats were fitted with an atrial catheter for the withdrawal of blood and subsequent determination of serum concentrations of prolactin and progesterone. The volume of blood removed (1·5 ml) was replaced by an equal volume of a solution of Evan's blue dye (1% (w/v) 0·9% saline; Gurr, London). All of these steps were carried out while the animals were unconscious and they were completed by 12.30 h.

The animals were killed by cervical dislocation 15 min after injection of dye. The ovaries were inspected *in situ* and dissected for histology. Uterine horns were examined for implantation swellings or incipient implants (blue spots). Where evidence of implantation was absent, uterine horns were dissected and flushed with 0·9% saline solution. The flushings were then searched for unimplanted embryos with a dissecting microscope (magnification × 25) using transmitted illumination.

An additional group of eight animals with simultaneous pregnancy and lactation received similar injections to those described above but they were not killed until day 16 or 20 of pregnancy. On day 9, the litters were removed and further drug treatment ceased except in the case of some animals treated with bromocriptine plus progesterone which continued to receive 4 mg progesterone daily until autopsy.

### *Histology*

Ovaries and uterine tissue were fixed by immersion in aqueous Bouin's fluid and prepared as paraffin-wax sections cut serially at 10 μm. The mounted sections were stained with haematoxylin and eosin. These preparations were used for morphological observation and counting of the number of medium- and large-sized ovarian follicles. In each ovary of the right side all the follicles above 100 μm maximum diameter (i.e. antral follicles) were measured. This was carried out by projecting an enlarged image ( × 40) of the sections onto a screen with a 150 mm lens (Leitz); the mean maximum diameter of each follicle was then assigned to one of the three size classes by using a simple measuring device with points 4, 8 and 16 mm apart (equivalent to 100, 200 and 400 μm).

### *Radioimmunoassays*

Serum levels of prolactin were measured by a previously described method using reagents provided by NIAMDD, Maryland, U.S.A. (McNeilly, Sharpe, Davidson & Fraser, 1978). The results are expressed in terms of the NIAMDD reference preparation RP-1. The levels of progesterone were measured by a specific radioimmunoassay (Baird, Burger, Heavon-Jones & Scaramuzzi, 1974). Samples were analysed in a single assay having an intra-assay coefficient of variation of 7 and 9% for prolactin and progesterone respectively.

### *Statistics*

The proportions of animals with implanted embryos and the numbers of ovarian follicles in each treatment group were analysed by Chi-squared test and ANOVA respectively. Since the form of the statistical distribution obtained from hormone measurements and weighing of litters was uncertain, the results were expressed by their median and range and analysed by non-parametric methods, namely, Wilcoxon's test and the Kruskal–Wallis method of analysis of variance of ranks.

## RESULTS

### *Group 1: effects of bromocriptine on pregnancy in non-lactating rats*

Conspicuous implants were present in the uterine horns on day 9 of pregnancy in animals whose pups were removed soon after birth and the post-partum mating (Table 1). Implantation had evidently occurred directly or at least shortly after the normal pre-attachment period of non-lactating rats; supplementary progesterone had no effect on the numbers of implants (Table 1). Pregnancy was terminated when removal of the litter was combined with treatment with bromocriptine. The embryos had died soon after implantation in these rats because small, equally spaced patches of scar or trophoblastic tissue were found during histological examination. This abortifacient action of bromocriptine was completely overcome by simultaneous treatment with progesterone. These results indicated that progesterone would be necessary during bromocriptine treatment of group 2 animals in order to support any implanted embryos.

Table 1. *Effects of treatment with bromocriptine and progesterone on implantation in rats lacking suckling stimuli*

| | Number of animals | | | |
|---|---|---|---|---|
| Treatment | Total | With barren uteri | With implants | Implants/pregnancy (mean ± S.E.M.) |
| Control (both vehicles) | 9 | 1 | 8 | 15·0 ± 0·8 |
| Progesterone + vehicle | 9 | 0 | 9 | 13·0 ± 1·4 |
| Bromocriptine + vehicle | 10 | 10* | 0 | — |
| Bromocriptine + progesterone | 10 | 1 | 9 | 14·6 ± 0·8 |

* $P < 0{\cdot}001$ compared with all other groups ($\chi^2$ test).

Table 2. *Effects of treatment with bromocriptine and progesterone on simultaneous pregnancy and lactation in rats*

| Treatment | Number of animals | | | | Median % change of litter weight during suckling (range) | Median (range) serum hormone (ng/ml) | | No. of follicles in right ovary (mean ±S.E.M.) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | With barren uteri | With implants | With unimplanted embryos‡ | | Prolactin | Progesterone | 100–200 (μm) | 201–400 (μm) | >400 (μm) | Total (>100 μm) |
| Control (both vehicles) | 8 | 0 | 0 | 8 | 8·9 (7·0, 25·0) | 140 (22, 380) | 64 (60, 67) | 43±4 | 38±4 | 14±2 | 95±7 |
| Progesterone + vehicle | 8 | 0 | 1 | 7 | 9·0 (4·4, 28·7) | 130 (17, 408) | — | 42±5 | 39±2 | 13±1 | 94±6 |
| Bromocriptine + vehicle | 8 | 1 | 0 | 7 | −2·7 (−7·8, 7·6)* | 11 (9, 68)* | 21 (5, 30)* | 40±4 | 27±3† | 14±1 | 81±6 |
| Bromocriptine + progesterone | 8 | 1 | 1 | 6 | −1·6 (−5·7, 3·6)* | 22 (11, 69)* | — | 42±3 | 31±2† | 17±2 | 90±4 |

*$P<0·01$ compared with groups not treated with bromocriptine (Wilcoxon's test and Kruskal–Wallis analysis of variance of ranks); † $P<0·05$ compared with groups not treated with bromocriptine (ANOVA). ‡ There was no significant difference between proportions of animals with unimplanted embryos in the various groups ($\chi^2$).

*Group 2: effects of bromocriptine on pregnancy during lactation*

Implantation was prevented by concurrent lactation in vehicle-treated animals (Table 2). There was no evidence of interruption of pregnancy by treatment of nursing rats with bromocriptine; neither was there any suggestion that this treatment initiated implantation, even when the declining levels of progesterone were restored by supplementation (Table 2). That serum prolactin levels had been suppressed by bromocriptine was shown unequivocally by radioimmunoassay and the failure of litters to gain weight when suckled by bromocriptine-treated rats. The exceptional animals that either had implanted embryos or no embryos of any kind can be attributed to failure of suckling or mating respectively. The majority of animals of all groups suckling litters yielded blastocysts of normal morphological appearance in their uterine flushings. These embryos were elongated and had hatched from their zonae pellucida. Further experiments showed that they retained their viability during treatment since the expected number of implanted embryos were found on days 16 or 20 when suckling treatment had been suspended on day 9. However, implants were only obtained in animals previously treated with bromocriptine when injections of progesterone were given after day 9: all three such animals bore implants (14 or 15 in each animal).

Corpora lutea were prominent at autopsy in the ovaries of both suckling and non-suckling animals, even when bromocriptine had induced luteal cell involution. The lutein cells of bromocriptine-treated animals were shrunken and vacuolated with heteropyknotic nuclei; the neighbouring blood vessels were often congested. These cellular changes paralleled the reduction of progesterone concentration in the circulation (Table 2).

Similar populations of medium- to large-sized follicles were found in the ovaries of the various groups of suckling animals (Table 2). Despite the absence of any significant differences in total number of antral follicles, a small but significant ($P < 0{\cdot}05$) reduction in number of follicles in the size range 201–400 μm was found in the two groups of animals receiving bromocriptine. This difference was not, however, reflected in adjustments of numbers of follicles in the smaller or larger size categories and may be without biological significance.

## DISCUSSION

The effectiveness of bromocriptine treatment in terminating lactation and pregnancy without lactation has been confirmed. Bromocriptine inhibited prolactin secretion and thereby suppressed milk production and caused embryonic resorption during the early phase of implantation development (Flückiger & Wagner, 1968; Morishige & Rothchild, 1974; Flint & Ensor, 1979). The drug had not interfered with the initiation of implantation as residual products of conception were seen on day 9. Since the abortifacient activity of bromocriptine was fully reversed by supplementary progesterone to replace the lost function of the corpus luteum, it was thought to be necessary also to provide replacement hormone for lactating pregnant animals.

Bromocriptine lowered serum prolactin and progesterone levels in lactating pregnant rats. Since administration of bromocriptine with or without progesterone did not interrupt embryonic diapause it is concluded that neither the raised circulating levels of prolactin nor progesterone normally found during lactation are required for delaying implantation. The hypothesis that prolactin has direct or indirect inhibitory effects on ovarian follicular growth or steroidogenesis is thus refuted because removal of the inhibition would have been expected to permit ovo-implantation since this has a small but essential requirement for oestrogen in the rat (Psychoyos, 1973). As additional evidence for this conclusion we found little change in the profile of the ovarian follicle population with declining prolactin levels. However, our conclusion that the effect of the suckling stimulus on the process of pregnancy is not exerted through the action of prolactin is in apparent conflict with several studies. In particular, the studies of Smith (1978) on lactational anoestrus and Maneckjee & Moudgal (1975) and Flint & Ensor (1980) on lactation-induced delay of implantation directly

implicate prolactin as at least part of the mechanism for the maintenance of gonadotrophin suppression.

The effects of bromocriptine treatment on embryonic viability differed according to whether or not the embryos had implanted. But in both cases there was evidence of luteal regression and lowered progesterone secretion. It is interesting that diapausing embryos apparently remained viable while progesterone levels were substantially reduced by bromocriptine treatment. There is disagreement about the need for progesterone replacement in ovariectomized rats for sustaining diapausing biastocysts (Powell, Bennett & Cochrane, 1979). Our results imply that the residual amounts of progesterone from ovaries of bromocriptine-treated rats together with the adrenal contribution (Labhsetwar, 1971) are sufficient for embryonic viability. The reduction of progesterone secretion by bromocriptine did not, however, bring about a resumption of development as a result of an altered balance of oestrogen and progesterone action on the uterus. Although the progesterone in the circulation during lactational pregnancy seems to be in excess of the requirements of a uterus carrying diapausing embryos, it may serve an essential role in priming the uterus in anticipation of the event of implantation (Martin & Finn, 1969; Dickmann, 1972). It is also likely that the progesterone requirement rises rapidly during early implantation development and this is perhaps shown by the abortifacient effect of prolactin suppression in the non-lactating pregnancies.

We therefore agree with an earlier conclusion that the suckling stimulus is the primary cause of gonadotrophin suppression during lactation in the rat (Rothchild, 1960; Raud, 1974; Lu *et al.* 1976), although a facilitating action of low levels of prolactin cannot be ruled out. Further studies are required to elucidate the mechanisms whereby the neural stimulus is translated into reduced gonadotrophin release; the hypothetical inhibitory effect on hypothalamic neurones containing LH-RH, first suggested by Rothchild 20 years ago, is still a plausible explanation.

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# Prolactin and human reproduction

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CONTROVERSY over the very existence of human prolactin, which was not finally resolved until 971, hindered advances in our knowledge of the role f prolactin in the human. In other species, however, rolactin had been identified and shown to possess a ultitude of actions ranging from osmoregulation in eleost fish to hepatic lipogenesis in birds (Nicoll and Bern, 1972). In mammals interest in prolactin has entred on its essential role in lactation and to a lesser xtent on its involvement in the formation and main-enance of the corpus luteum during the oestrous ycle. With the advent of radioimmunoassays for uman prolactin (Greenwood et al, 1973; McNeilly, 973) it has become possible to investigate and valuate the role of prolactin in man.

This article will review the present state of nowledge of the role of prolactin in human repro-uction under four headings: control of prolactin ecretion, control of ovulation and menstruation, regnancy, and lactation.

## Control of prolactin secretion

Unlike the gonadotrophins (LH and FSH), prolac-n secretion from the pituitary appears to be under nhibitory control from the hypothalamus (Meites, 973). Thus when the pituitary stalk is damaged or ut, secretion of prolactin increases while that of both onadotrophins decreases. A distinct prolactin nhibitory factor (PIF) has not yet been identified and ne possibility of the control of prolactin secretion by releasing factor (PRF) cannot be ruled out.

It has recently been demonstrated that nyrotrophin-releasing hormone (TRH) releases not nly TSH but also prolactin (Bowers et al, 1971), and nat the threshold of response is similar for both hor-nones (Bowers et al, 1973). Although it has been uggested that TRH may be a physiological regulator f both TSH and prolactin release (Bowers et al, 1973), many observations indicate that TSH release usually does not parallel prolactin release (Meites, 1973) and that in situations in which prolactin release occurs, such as suckling, TSH levels remain unchanged. In addition, in porcine and rat hypothalami at least, TRH and PRF are separate entities (Valverde et al, 1972).

Several drugs and biogenic amines may either elevate or suppress prolactin release (Meites, 1973). Although in animals oestrogen usually stimulates prolactin release, in man only high doses of oestrogen are effective in stimulating prolactin release. The physiological significance of this remains in doubt, since changes in oestrogen levels during the menstrual cycle are not reflected by changes in prolactin levels. In contrast, release of LH and FSH during the menstrual cycle is related to the positive feedback effects of oestrogens.

## Control of ovulation and menstruation

The best documented action of prolactin on the ovary in animal species is its essential role in the luteotrophic complex. In sheep, for instance, Denamur et al (1973) have shown that both prolactin and LH are necessary for the maintenance of the corpus luteum; prolactin alone has little effect and LH alone has no effect. Whether a similar situation exists in man is uncertain. The well known clinical occurrence of the amenorrhoea-galactorrhoea syndrome demonstrates clearly that prolactin is related to gonadal function.

A specific role for human prolactin in the control of the menstrual cycle could not be ascertained until information was available on both the circulating level of prolactin during the cycle and the effects of reduction of prolactin levels by pharmacological agents. Direct measurement by radioimmunoassay of prolactin in the circulation during the menstrual cycle has,

however, given rise to controversial data. L'Hermite et al (1972) and Robyn et al (1973) have reported significant increases in serum prolactin levels at mid-cycle and during the luteal phase, and that oestrogens and prolactin follow a similar pattern. They have suggested that the changes in circulating levels of prolactin are closely related to the morphological changes observed in the human mammary gland during the menstrual cycle (Robyn et al, 1973).

In contrast, however, several groups of workers have been unable to demonstrate a consistent change in circulating levels of prolactin (Hwang et al, 1971; Ehara et al, 1973; Jaffe et al, 1973; McNeilly et al, 1973; Tyson and Friesen, 1973; McNeilly and Chard, 1974). Although a peak of prolactin occasionally coincided with that of LH, in the majority of cycles prolactin levels did not parallel the LH/FSH secretion pattern. It was also apparent that prolactin levels on single daily samples were not related either to levels of oestrogens or progesterone (Ehara et al, 1973; McNeilly et al, 1973; McNeilly and Chard, 1974), or to menstruation (McNeilly and Chard, 1974).

Analysis of short-term changes in LH and FSH revealed the expected pulsatile release of LH, whereas prolactin levels were more erratic and did not change in response to the pulses of LH (McNeilly et al, 1973). In addition, when rapid serial samples were taken during the follicular, midcycle, and luteal phases, both LH and FSH levels varied by up to 200-fold at the different phases of the cycle and by up to 10-fold during the short-term sampling periods. Prolactin levels and changes, however, were remarkably similar at all stages of the cycle (McNeilly et al, 1973; McNeilly and Chard, 1974) and were similar to the pattern of secretion seen in normal male subjects (McNeilly et al, 1974).

The present evidence on circulating levels of human prolactin indicates little or no significant change during the menstrual cycle and no direct relation to LH, FSH, or steroid levels at different stages of the cycle.

The existence of a relationship between prolactin and gonadotrophins is suggested by the effects of administration of a specific pharmacological agent that blocks prolactin secretion—bromocriptine (CB 154)—in patients with amenorrhoea-galactorrhoea syndromes. These syndromes are usually, but not always, associated with raised circulating prolactin levels and low urinary oestrogen levels in the face of apparently normal levels of LH and FSH (Thorner et al, 1974). Since both LH and FSH responses to exogenous gonadotrophin-releasing hormone (LH-RH) are normal or excessive (Mortimer et al, 1973; Thorner et al, 1974), the amenorrhoea appears to be caused by a failure of cyclic release of LH and FSH which results in anovulation. Bromocriptine appears to affect only prolactin secretion, and reduction of elevated prolactin levels to within the normal range in these patients results in cessation of milk secretion and, in the majority of cases, return to gonadal function with cyclic release of LH and FSH and regular menstruation (Besser et al, 1972; Thorner et al, 1974).

A similar situation occurs during puerperal lactation, which is usually accompanied by anovulation. The return of normal menstruation occurs much more rapidly in women who do not lactate postpartum (Minairi and Foda, 1971). During lactation, however basal levels of both LH and FSH are normal in the face of increased prolactin levels (Reyes et al, 1972; Jaffe et al, 1973). The underlying reason for anovulation in both puerperal lactation and amenorrhoea-galactorrhoea syndromes appears to be the failure of cyclic release of gonadotrophins related to elevated levels of prolactin. Thus, a specific relationship between prolactin and gonadotrophin secretion does seem to exist although it is not apparent in changes of blood levels during the menstrual cycle.

This apparent inverse relationship may reside at the hypothalamic-pituitary level, although recent short-term studies have shown no apparent relationship between prolactin and gonadotrophin secretion despite episodic releases of these hormones (Ehara et al, 1973; McNeilly and Chard, 1974). Injection of TRH while causing an immediate increase in blood prolactin levels, does not affect levels of LH or FSH, and injection of LH-RH has no effect on prolactin levels (Mortimer et al, 1973). In addition, neither the prolactin nor gonadotrophin responses are affected when these releasing hormones are given together either in normal males (Mortimer et al, 1973) or in females at different stages of the menstrual cycle (McNeilly and Hagen, 1974), indicating absence of inhibition of action of releasing hormones at the level of the pituitary.

Similarly, stimulation of gonadotrophin secretion by clomiphene or inhibition of secretion by long-term oestrogen therapy (McNeilly et al, unpublished observations) in normal male subjects is not associated with any change in circulating levels of prolactin. In physiological situations such as lactation, prolactin levels are high, while LH and FSH levels are normal. In postmenopausal women, despite high levels of gonadotrophins, prolactin levels and responses to TRH are normal (McNeilly et al, unpublished observations). It appears unlikely, therefore, that any relationship between prolactin and LH/FSH resides at the hypothalamic-pituitary level.

It has recently become apparent that prolactin may act directly on the ovary, and that the relationship between gonadotrophins and prolactin may be mediated by the ovarian steroids (McNatty et al, 1974). In subjects with elevated circulating prolactin levels associated with puerperal lactation (Zarate et al, 1972) or galactorrhoea (Thorner et al, 1974), the ovarian steroid response to gonadotrophin stimulation is reduced. In cases of galactorrhoea, the steroid response to gonadotrophins becomes normal (Thorner et al, 1974) after reduction of prolactin levels with bromocriptine. In the male, HCG-induced

stosterone output by the testis was reduced in the ce of elevated circulating prolactin levels and re- rned to normal when prolactin levels were reduced horner et al, 1974).

McNatty et al (1974) have shown that although a w level of prolactin is necessary for steroidogenesis the human ovary in vitro, high levels of prolactin hibit progesterone synthesis even with levels of LH d FSH normally sufficient to induce maximum roidogenesis. In addition, it has been shown that e levels of prolactin in the fluid of developing llicles in the human ovary remain constant until just ior to ovulation (McNatty et al, 1974). At this stage vels of prolactin decrease markedly while those of th LH and progesterone increase. Whether this flects active removal of prolactin from the follicle or ilization of prolactin in the formation of steroids is t known. The levels of prolactin in the follicular uids bear little or no relation to circulating levels of olactin in the normal menstrual cycle, but are rectly related to events within the follicles, where olactin appears to play a permissive role for nadotrophin-induced steroidogenesis.

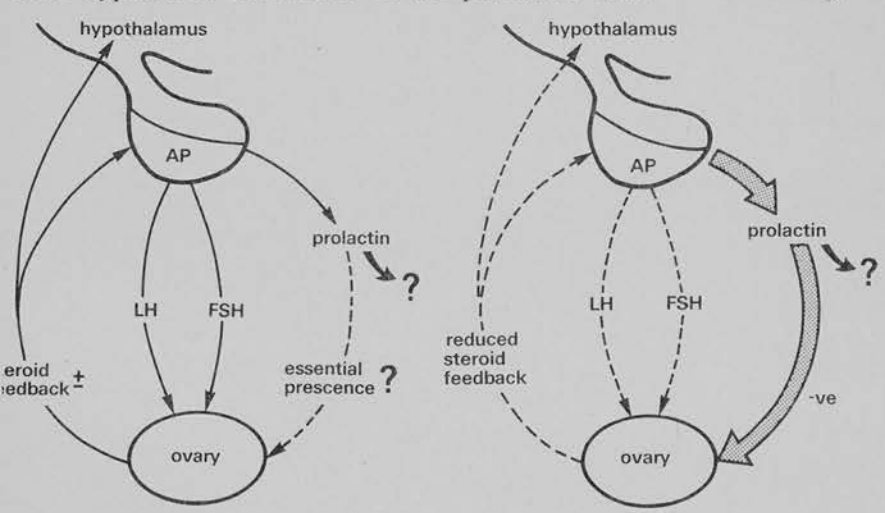
This view is compatible with the demonstration of rmal menstrual function in women treated with omocriptine, in whom prolactin levels are sup- essed throughout the complete menstrual cycle (Del ozo, 1973), since the important factor for prolactin uring the normal menstrual cycle would appear to be e concentration within the developing ovarian llicle. Thus anovulation that occurs with elevated rculating levels of prolactin could be explained by e inhibitory effect of prolactin on steroidogenesis, moval of the normal positive steroid feedback on the pothalamus, and thus failure of cyclic discharge of H and FSH (**Fig. 1**).

This hypothesis fits much of the published data on prolactin/gonadotrophin interrelationships and suggests that while a low level of prolactin is an essential factor for the steroidogenic actions of LH and FSH on the ovary and testis, high levels are inhibitory and induce what may be termed "prolactin anovulation".

### Prolactin in pregnancy

In contrast to all other animal species, including the monkey, circulating levels of prolactin during pregnancy in women show a gradual rise to reach a maximum about term (Jaffe et al, 1973; Robyn et al, 1973; Tyson et al, 1973; Evans et al, 1974). At present the reason for this increase in prolactin levels is unknown. In addition, prolactin concentrations in amniotic fluid in early pregnancy are several times greater than in the maternal circulation, and these levels decline towards term. The origin of this prolactin remains obscure although it appears to be similar in character to pituitary prolactin (Friesen et al, 1973; Robyn et al, 1973).

Although in the early stages of pregnancy prolactin levels in the fetus are low (Evans et al, 1974), at term levels in cord blood are comparable to those in the maternal circulation (Hwang et al, 1971; Evans et al, 1974). No arteriovenous difference in levels was found, but a highly significant linear correlation has been found between levels of prolactin in the mother and the fetus at term (Evans et al,1974). The reason for this close correlation between maternal and fetal levels is unknown. It seems unlikely that the fetal prolactin is maternal in origin, since isotopically labelled prolactin failed to cross the placental barrier from mother to fetus in the monkey (Friesen et al, 1972). This implies that the stimulus resulting in increased secretion of prolactin in both mother and fetus may be similar and could be related to the relatively high oestrogen secretion during gestation.




**ig. 1.** *Diagrammatic representations of normal steroid feedback control of LH and SH secretion* **(a)** *and effects of elevated levels of circulating prolactin* **(b)**. *In the ormal situation* **(a)** *oestrogen and/or progesterone are secreted during the follicular hase of the menstrual cycle feedback on the hypothalamus-pituitary to cause release of H-RH, LH, and FSH, ovulation, and regular menses. In prolactin anovulation* **(b)** *evated prolactin levels inhibit steroidogenesis at the level of the ovary; the positive edback of oestrogen/progesterone is prevented, and cyclic discharge of LH-RH, LH, d FSH fails with resulting anovulation and amenorrhoea.*

### Prolactin and lactation

The maintenance of lactation is the best known action of prolactin. In most species prolactin is essential for milk synthesis and secretion, and the human appears to be no exception. At term, prolactin levels in the maternal circulation are elevated by 3—10 times normal and gradually return to normal basal levels over the first 2 weeks postpartum (Reyes et al, 1972; Jaffe et al, 1973). Suckling results in a large increase in the secretion of prolactin throughout most of the period of lactation.

Prolactin appears to exert two actions on mammary tissues: a mammotrophic action and a lactogenic action. Failure of

lactation during pregnancy, despite greatly elevated levels of both prolactin and human placental lactogen, suggests that the lactogenic action of these hormones is inhibited by the high levels of oestrogens and progesterone that circulate during pregnancy. At term, loss of the fetoplacental steroids removes this inhibition and prolactin exerts a lactogenic effect with resulting milk secretion.

Pharmacological reduction of circulating levels of human prolactin with bromocriptine is directly associated with cessation of milk secretion, both in galactorrhoea (Besser et al, 1972) and puerperal lactation (Del Pozo and Flückiger, 1973; Rolland and Schellekens, 1973), and indicates that prolactin is essential for milk secretion in the human and may be the major factor. The cause of the well known lactation amenorrhoea has been discussed earlier, and may reflect peripheral inhibition of ovarian steroidogenesis by the elevated prolactin levels associated with lactation.

## CONCLUSION

Prolactin appears to play an active role in human reproduction. No consistent pattern of prolactin secretion is seen during the menstrual cycle, and circulating levels are unrelated to changes in LH, FSH, oestrogen, or progesterone. Within the follicular fluid, however, prolactin levels change in relation to follicular development. It appears that basal levels of prolactin are essential for ovarian steroidogenesis. contrast, elevated levels appear to inhi steroidogenesis at the level of the ovary, resulting failure of the normal positive feedback to LH a FSH secretion, anovulation, and amenorrhoea.

During pregnancy maternal levels of prolactin r gradually towards term. High levels of prolactin a found in amniotic fluid, and levels in the fetus at te are elevated and related to those found in the moth On the maternal side prolactin probably forms part the mammogenic complex, whereas the function the fetal and amniotic fluid prolactin is unknown.

During the puerperium prolactin levels are elevat due to suckling, and the initiation of milk secreti appears to depend on the removal of inhibition fetoplacental steroids. A direct effect of prolactin the ovary probably explains the anovulation th accompanies lactation.

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# Lactation and the physiology of prolactin secretion

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**Summary**

**Prolactin in man appears to form an essential part of the complex of hormones necessary for milk secretion and lactation. Levels of prolactin during pregnancy gradually increase towards term, and remain elevated for up to 6 weeks post partum. The lactogenic effects of prolactin appear to be blocked at the mammary gland by the elevated levels of fetoplacental steroids secreted during pregnancy. The immediate decline in steroid levels at delivery removes this block to prolactin and milk secretion ensues.**

**The amenorrhoea associated with both puerperal lactation and galactorrhoea appears to reflect failure of cyclical discharge of gonadotrophins and anovulation. This appears to be due to peripheral inhibition of steroidogenesis by the elevated levels of prolactin associated with these situations. No consistent changes in circulating levels of prolactin occur during the menstrual cycle, but changes in prolactin levels within the follicular fluid of the developing ovarian follicle indicate a specific and permissive role of prolactin in steroidogenesis.**

## Introduction

Although in animal species over eighty-two actions for prolactin have been suggested, a major function in mammals, as suggested by its name, is in lactation. Much work has been carried out in animals to investigate those factors which contribute to milk secretion; in several species a well defined complex of hormones, including prolactin, has been elucidated. By contrast, in man, little is known of the hormonal control of either mammary growth (mammogenesis), or milk secretion (lactogenesis), and much of the information available has been derived from indirect evidence. A major stumbling block in our understanding of human lactation has been the controversy over the existence of a distinct prolactin molecule in the human. Despite pioneering work by Pasteels in the 1960's, human prolactin in pure form was not isolated until 1970. Since then, with the introduction and widespread use of specific radioimmunoassays, a wealth of data has been accumulated from man which sheds light not only on the role of prolactin in lactation, but also on its role in reproduction.

## Prolactin and lactation

### *Mammary growth*

Whether prolactin plays a role in the growth of the mammary gland is uncertain. There is no evidence to suggest that prolactin levels alter at puberty. It is apparent, however, that prolactin forms part of a complex of hormones necessary for normal mammary development. Thus, Contesso, Ceriani and Nata (1972) have shown that growth and differentiation of human female mammary tissue in culture is dependent not only on prolactin, but also requires growth hormone, insulin, oestrogen and progesterons. Whether growth hormone is an important part of thie complex is uncertain, since mammary ductal growth occurred after ethinyl oestradiol treatment in hypopituitary men without measurable growth hormone (Frantz, 1972).

### *Prolactin and milk secretion*

The role of prolactin in the control of milk secretion in animals is now reasonably well understood. It is apparent that prolactin forms part of a complex of hormones (including growth hormone and insulin) necessary to maintain milk secretion; only in the rabbit can milk secretion be maintained by prolactin alone (see Cowie and Tindal, 1972). At the level of the secretory alveolar cell in the mammary gland, prolactin binds to specific receptor sites at the plasma membrane (Falconer, 1972; Turkington, Frantz and Majumder, 1973; Shiu, Kelly and Friesen, 1973), and induces formation of RNA and subsequently the formation of the characteristic milk proteins and secretion into the ductal system of the gland (Turkington *et al.*, 1973). The action of the alveolar cell appears to be mediated via the surface receptor, since Turkington (1970) has demonstrated that prolactin coupled to sepharose beads, which would prevent entry into the cell, can stimulate isolated mammary epithelial cells.

Whether prolactin plays a similar role in promoting milk synthesis and secretion in the human is uncertain, but seems likely since human prolactin, in common with prolactin from other species, binds to mammary receptors (Shiu *et al.*, 1973) and can induce milk secretion in explants of mammary tissue (see review by Forsyth and Parke, 1973).

*Circulating levels of prolactin and lactation*

In the traditional view, prolactin release was thought to begin in the early puerperium and to be responsible for milk secretion in the breast which had previously been prepared by other factors, in particular the fetoplacental steroids during pregnancy. But direct measurement of prolactin levels reveals a different picture. During pregnancy, circulating levels of prolactin show a gradual rise to reach a maximum at term (Tyson *et al.*, 1972; Jaffe *et al.*, 1973; Robyn *et al.*, 1973; Evans, McNeilly and Chard, 1975). After delivery, prolactin levels fall but basal concentrations do not reach the non-pregnant range until at least 2 weeks post partum.

Milk secretion begins only at the time of delivery, in spite of the high levels of circulating lactogenic hormones, prolactin and human placental lactogen, during pregnancy. This suggests that the lactogenic activity of prolactin is opposed by the high levels of oestrogens and progesterone which circulate during the latter part of pregnancy. Immediately after delivery, the levels of fetoplacental steroids decline rapidly, while those of prolactin are maintained (Reyes, Winter and Faiman, 1972); the removal of steroid inhibition thus permits prolactin to exert its normal action on the stimulation of milk secretion. Jaffe *et al.* (1973) have shown that administration of oestradiol valerate and testosterone enanthate (deladumone) for suppression of lactation does not alter the secretion of prolactin, further indicating steroid mediated suppression of prolactin at the breast.

During puerperal lactation, prolactin levels fluctuate widely (Reyes *et al.*, 1972; Jaffe *et al.*, 1973) owing to release in association with suckling (Hwang, Guyda and Friesen, 1971).

The response to suckling may vary with time after delivery, and Tyson *et al.* (1972) have shown that as lactation advances, the amount of prolactin released in response to suckling decreases. These changes are unrelated to the amount of milk produced, since milk production remains reasonably constant despite wide fluctuations in the amount of prolactin released. The reason for the decrease in prolactin release is not known. It does not appear to reflect a reduction in the releasable pool of prolactin in the pituitary gland, since the amount of prolactin released in response to thyrotrophin-releasing hormone (TRH) during puerperal lactation does not alter despite the reduction of release in response to suckling (Tyson *et al.*, 1972).

*Control of prolactin secretion in lactation*

Prolactin secretion by the pituitary appears to be controlled predominantly by a prolactin inhibiting factor (PIF) secreted by the hypothalamus (see Meites, 1973). It has been suggested that a prolactin-releasing factor (PRF) may also exist and the release of prolactin as well as TSH in response to TRH had led to the suggestion that TRH may be the physiological PRF (Bowers, Friesen and Folkers, 1973).

The rapid rise in prolactin levels during suckling could represent either cessation of PIF secretion, or release of PRF, both of which would result in an increase in prolactin levels. It appears unlikely that the suckling-induced prolactin release is mediated by release of TRH, since it is not accompanied by release of TSH; injection of TRH induced release of both TSH and prolactin in lactating subjects (Gautvik *et al.*, 1973).

The exact nature of the stimuli associated with suckling which cause prolactin release is not known. It seems probable, however, that the release results from a neural reflex associated with stimulation of the nipple and alveolar area of the breast during suckling. Thus, prolactin release comparable with that seen during suckling may occur after manual breast stimulation in non-pregnant, non-lactating women and even in men (Noel, Suh and Frantz, 1972; Kolodny, Jacobs and Daughaday, 1972). On the other hand, psychological factors associated with breast feeding, in particular anticipation of nursing, do not appear to play an important role in prolactin release since these stimuli do not release prolactin in nursing mothers (Noel *et al.*, 1972).

Although there is no direct quantitative relationship between prolactin release and milk secretion, there is good evidence that prolactin is an essential part of the complex of hormones necessary for milk secretion in the human. Thus, reduction of levels of prolactin by Bromocriptine (2α-bromergocryptine, CB 154), which affects only prolactin release by the pituitary, is effective in suppression of puerperal lactation (Rolland and Schellekens, 1973; Del Pozo and Flückiger, 1973) and galactorrhoea (Besser *et al.*, 1972; Del Pozo *et al.*, 1972). Prolactin levels are reduced and are maintained within the normal range. On the other hand, growth hormone does not appear to form an essential part of the hormonal complex for lactation, since normal lactation occurs in the complete absence of human growth hormone (Rimoin *et al.*, 1968).

*Prolactin and inappropriate lactation*

The well known clinical situation of the galactorrhoea-amenorrhoea syndromes or inappropriate lac-

tation is normally, but not always, associated with elevated levels of prolactin (Besser *et al.*, 1972; Jacobs and Daughaday, 1973; Frantz, Kleinberg and Noel, 1972; Tolis *et al.*, 1974). Frantz *et al.* (1972) have reported that patients with galactorrhoea and normal menses have prolactin concentrations within the normal range. It is apparent that milk secretion in these patients is dependent upon enhanced sensitivity of the breast tissue. Secretion is maintained by the elevated levels of prolactin, but lactation will only occur in face of these elevated levels if the mammary tissue has been appropriately primed. Thus, the hormonal control of mammary development on the one hand and milk secretion on the other are not the same, and both require further investigation in man.

**Lactational amenorrhoea—prolactin anovulation**

In addition to an essential role in lactation, prolactin in mammals is also concerned with the maintenance of luteal function of the ovary. It has been generally accepted that when prolactin secretion is high, gonadotrophin secretion is reduced and *vice versa*. Such a relationship between prolactin and the gonadotrophins (LH and FSH) appears to exist in man. It is well known that anovulation often accompanies normal lactation and return to menstruation occurs much more rapidly in women who do not lactate post partum (El Minairi and Foda, 1971). A similar relationship occurs in the well known clinical occurrence of the amenorrhoea-galactorrhoea syndromes. Many cases of galactorrhoea (Thorner *et al.*, 1974a) and normal puerperal lactation (Reyes *et al.*, 1972; Jaffe *et al.*, 1973) are associated with raised circulating prolactin levels and low urinary oestrogen levels in the face of apparently normal LH and FSH levels. Amenorrhoea seems to result from a lack of cyclical release of both FSH and LH, resulting in anovulation. This failure of cyclical discharge of gonadotrophins may result from inadequate release of gonadotrophin-releasing hormone (GnRH) since, in galactorrhoea, normal or excessive LH and FSH responses to GnRH are seen (Mortimer *et al.*, 1973).

Specific block of prolactin secretion by Bromocriptine in patients with amenorrhoea-galactorrhoea syndromes (Besser *et al.*, 1972; Thorner *et al.*, 1975) and in puerperal lactation (Rolland and Schellekens, 1973) results not only in cessation of milk secretion (see above) but, in the majority of cases of galactorrhoea and in all studied cases of puerperal lactation, a rapid return to gonadal function with cyclical release of LH and FSH and regular menstruation.

*Prolactin and the menstrual cycle*

The relationship between prolactin and gonadotrophin secretion suggests that prolactin may play a role in the control of the menstrual cycle and ovulation. L'Hermite *et al.* (1972) and Robyn *et al.* (1973) have reported a peak of prolactin at mid-cycle and higher levels during the luteal than the follicular phases. The peak of prolactin was attributed to the concomitant rise of endogenous oestrogens occurring at this time.

In contrast, several groups of workers have been unable to show consistent changes in prolactin levels during the cycle (Hwang *et al.*, 1971; Tyson and Friesen, 1973; Jaffe *et al.*, 1973; McNeilly, Evans and Chard, 1973; Ehara *et al.*, 1973; McNeilly and Chard, 1974). Although on rare occasions a peak of prolactin occurred coincident with the mid-cycle peak of LH, daily prolactin levels showed wide fluctuations. No relationship appeared to exist between prolactin and the levels of LH and FSH, oestrogens, progesterone (McNeilly *et al.*, 1973; Ehara *et al.*, 1973; Jaffe *et al.*, 1973), or menstruation (McNeilly and Chard, 1974). Short-term changes of prolactin and gonadotrophins during the day at different stages of the menstrual cycle were also unrelated (Ehara *et al.*, 1973; McNeilly and Chard, 1974), and prolactin secretion appeared to occur in a spike-like manner, similar to that seen in normal male subjects (McNeilly *et al.*, 1974). These results indicate that no specific changes in prolactin levels are associated with events of the menstrual cycle and if there is a relationship between prolactin and gonadotrophins it is not apparent in changing blood levels of these hormones.

The relationship observed during normal or abnormal lactation might operate at the hypothalamic pituitary level. However, injection of TRH, while causing release of prolactin, is not associated with release of either LH or FSH and injection of luteinizing hormone-releasing hormone (LHRH), while releasing LH and FSH, does not affect prolactin release (Mortimer *et al.*, 1973; McNeilly and Hagen, 1974). In addition, no changes in prolactin levels occur when gonadotrophins are elevated in postmenopausal women (McNeilly, Ormston and Hall, unpublished observations), after clomiphene in normal men (Thorner *et al.*, 1974), or when LH and FSH levels are reduced by long-term treatment with oestrogen (McNeilly, Anderson, Fisher, Mortimer and Thorner, unpublished observations). It appears unlikely, therefore, that any relationship between prolactin and gonadotrophins would reside at the hypothalamic or pituitary level.

*Prolactin and the ovary—effects on steroidogenesis*

It has recently become apparent that prolactin may act directly upon the ovary in women, and the relationship between prolactin and the gonadotrophins may be mediated by ovarian steroids (McNatty, Sawers and McNeilly, 1974). Stimulation of ovarian steroidogenesis by gonadotrophin (Pergonal) in the face of high circulating levels of prolactin in either puerperal lactation (Zarate *et al.*, 1972), or galactorrhoea (Thorner *et al.*, 1974) results in a reduced

steroid response, reflecting apparent ovarian refractoriness. In galactorrhoea, reduction of prolactin levels with Bromocriptine is associated with return of a normal response to Pergonal stimulation (Thorner *et al.*, 1974a), indicating removal of a peripheral block to steroidogenesis. Since Bromocriptine specifically affects only the secretion of prolactin, it would appear that elevated levels of prolactin inhibit steroidogenesis at the site of the ovary. This has been confirmed by McNatty *et al.* (1974) who have shown that while low levels of prolactin are essential for steroidogenesis, inhibition of progesterone secretion by human granulosa cells in culture occurs when high levels of prolactin are included in the incubation medium.

That a similar situation might occur during the normal menstrual cycle is suggested by changes in prolactin levels within the fluid of the developing ovarian follicle (McNatty *et al.*, 1974). During development, follicular fluid levels of prolactin remain similar to normal circulating blood levels. Just before ovulation, prolactin levels decrease markedly while those of both LH and progesterone increase. Whether these changes reflect active removal of prolactin from the follicular fluid, or utilization of prolactin for steroidogenesis is unknown, but results indicate that the levels of prolactin within the follicular fluid are not directly related to circulating levels.

Anovulation during lactation and galactorrhoea can thus be explained by the peripheral block of steroidogenesis at the ovary by elevated levels of prolactin resulting in failure of normal steroid feedback to the pituitary and hypothalamus and thus loss of cyclical discharge of LH and FSH (McNeilly, 1974). Reduction of elevated prolactin levels results in removal of this inhibition, return to normal steroidogenesis, cyclical discharge of LH and FSH, ovulation and normal menstruation. In puerperal lactation, this normally occurs either at weaning or at late stage lactation, when release of prolactin in response to suckling is negligible.

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**Intragonadal control of ovarian function.** By A. S. McNeilly. *Departments of Obstetrics, Gynaecology and Reproductive Physiology, St Bartholomew's Hospital Medical College, London, EC1A 7BE*

There is considerable evidence that pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are required for the later stages of follicular growth, maturation of the oocyte and ovulation (see Baker, 1972; Greep, 1973; Schwartz, 1974). Although the changes in blood levels of these gonadotrophins can be correlated to the output of steroids from the ovary, the precise action of LH and FSH on the follicle has remained obscure. Recent studies in man on hormone levels within the follicular fluid of developing Graafian follicles have demonstrated that the microenvironment of the follicle plays a critical role in all phases of follicular growth and development (Channing, 1970; Edwards, 1974; McNatty, Sawers & McNeilly, 1974; McNatty, Hunter, McNeilly & Sawers, 1975*b*).

The appearance of FSH in the follicular fluid of the small antral follicle is critical for the growth of that follicle (Baird, Baker, McNatty & Neal, 1975; McNatty *et al.* 1975*b*). These developing follicle(s) secrete increasing amounts of oestradiol into the fluid (Smith, 1960; Short & London, 1961; Sanyal, Berger, Thompson, Taymor & Horne, 1974; Baird & Fraser, 1975; McNatty *et al.* 1975*b*). This is reflected in an increase in peripheral blood oestradiol levels which, in turn, inhibits FSH secretion, leading to a fall in peripheral blood levels of FSH in the mid-follicular phase of the cycle (Midgley & Jaffe, 1968). Exposure of the follicle to high levels of both oestradiol and FSH within the follicular fluid stimulates both mitotic activity of the granulosa cells and an increase in the number of LH receptors (Channing & Kammerman, 1974; Zeleznik, Midgley & Reichert, 1974) in preparation for steroid secretion.

High levels of oestradiol within the follicular fluid may sensitize the cells to both FSH and LH (Goldenberg, Vaitukaitis & Ross, 1972). The entry of LH into the follicle in the preovulatory period (McNatty *et al.* 1975*b*) inhibits mitosis and promotes progesterone secretion resulting in high levels in the follicular fluid (Sanyal *et al.* 1974; McNatty *et al.* 1975*b*) and release into the peripheral circulation (Johansson & Wide, 1969; Yussman & Taymor, 1970). Prolactin concentration within the follicular fluid while being high in early follicular and late luteal phase follicles is low in preovulatory follicles when progesterone secretion is maximal (McNatty *et al.* 1974). This may represent utilization of prolactin to maintain an adequate pool of precursor for progesterone synthesis within the follicle.

After ovulation, both prolactin and LH are necessary for maximal steroid secretion by the corpus luteum. In the luteal phase despite an early increase in the number of follicles developing, presumably in response to the mid-cycle peak of FSH and LH, in the mid and late luteal phase follicular growth is arrested and follicles become atretic, possibly as a result of the low levels of FSH and LH in the circulation at this time (Midgley & Jaffe, 1968).

In addition, although the follicular fluid of these follicles contains FSH and LH, only low levels of oestradiol and progesterone are found (Baird & Fraser, 1975; McNatty *et al.* 1975*b*), suggesting a functional failure of these follicles. These data suggest that the sequence of hormonal changes within the follicular fluid is critical for the full expression of steroidogenic activity of the granulosa cells and subsequent corpus luteum.

McNatty & Sawers (1975) have demonstrated that the ability of granulosa cells in culture to undergo mitosis and secrete progesterone is predetermined by the hormonal environment within the follicle from which they were harvested.

Maximal biosynthetic capacity of granulosa cells harvested from early follicular phase follicles, is only achieved after continual exposure to FSH and oestradiol for 8–10 days (McNatty & Sawers, 1975) a time similar to that necessary *in vivo* (Bertrand, Coleman, Crooke, Macnaughton & Mills, 1972).

These results suggest that the hormonal sequence of events within the follicle predetermines follicular and corpora luteal development and the following sequence of events may be suggested. During the early follicular phase, FSH enters the small follicles thus promoting development. By the mid-follicular phase some of these have developed into large follicles under FSH stimulation and one will contain both FSH and high concentrations of oestradiol in the follicular fluid. The remainder have become atretic due to the low levels of FSH in the circulation. This exposure to FSH and oestradiol promotes the mitotic activity of the granulosa cells and increases the number of LH receptors in preparation for eventual steroidogenesis. Just before ovulation, LH enters the follicle, inhibits mitosis and together with prolactin initiates and maintains progesterone synthesis and secretion. After ovulation, secretion of progesterone from the corpus luteum is maintained together with FSH by both LH and prolactin (from McNatty *et al.* 1974; Baird *et al.* 1975; McNatty, Bennie, Hunter & McNeilly, 1975*a*; McNatty *et al.* 1975*b*; McNatty & Sawers, 1975).

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# *Chapter 13*

# Nature of Prolactin and Its Measurement

H. G. Friesen and A. S. McNeilly

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## I. INTRODUCTION

Studies on the purification and characterization of human prolactin began with the recognition that there is a separate and distinctive prolactin in man as in other species. Although there was considerable clinical evidence to favor this view, the important studies by Frantz and his colleagues (Frantz and Kleinberg, 1970; Frantz *et al.*, 1972) provided direct experimental evidence to support the hypothesis. They showed that, in a variety of clinical conditions, the circulating levels of prolactin measured by bioassay were far in excess of those which could be accounted for by radioimmunoassayable growth hormone in the serum. Concurrently, careful histological studies using special staining techniques established the presence of specific prolactin cells which increased dramatically in

number during late pregnancy and lactation. Shortly thereafter, immunochemical studies established the separate identity of primate prolactin and growth hormone. This led to the development of specific heterologous and finally homologous radioimmunoassays for primate prolactins which subsequently proved to be invaluable in the identification and separation of prolactin and growth hormone in pituitary extracts.

## II. PURIFICATION AND CHARACTERIZATION

Several procedures have been employed for the purification of prolactin from pituitary extracts. They include methods for the purification of prolactin from frozen glands, from side fractions obtained in the purification of growth hormone using Raben's procedure, and from acetone dried glands. In addition to pituitary glands, amniotic fluid has also been used as a source of prolactin. For a detailed reference list see Horrobin (1974, 1975).

Estimates of the pituitary content of prolactin vary between 100 and 500 $\mu$g per gland. There is a considerable range in pituitary prolactin content but, more importantly, the nature of the material which is extracted varies considerably. In our experience in alkaline extracts of pituitary glands approximately 50% of the prolactin upon gel filtration appears to be of large molecular weight, that is, greater than 50,000, and this form represents aggregated hormone. The purification procedures that have been developed employ standard methods including isoelectric precipitation, ion-exchange chromatography, and gel filtration. The final products obtained by different methods appear comparable if not identical with each other.

The purified prolactin preparations have been characterized by gel filtration, electrophoresis, bioassay, and receptor assay. A limited amount of chemistry has been carried out on each of the preparations. Acrylamide gel electrophoresis reveals considerable heterogeneity in most of the preparations that have been described. The major component of prolactin has an isoelectric point which is somewhat higher than that of growth hormone and hence upon acrylamide gel electrophoresis the mobility of prolactin is less than that of growth hormone. Sophisticated studies using isoelectric focusing revealed even greater degrees of heterogeneity (Hummel *et al.*, 1975). Rogol and Chrambach (1975), using this technique, have identified at least six isohormone species in both pituitary and amniotic fluid prolactin preparations. The molecular weight (MW) of human prolactin is very similar to that of human growth hormone—in the neighborhood of 21,000 MW, although careful gel filtration studies of crude extracts of pituitary (Guyda, 1975) as well as of serum (Suh and Frantz, 1974) have revealed that several molecular size forms of prolactin can be identified in both. The principal one is the monomeric form which constitutes somewhere in the neighborhood of

75–80% of the total prolactin. A second molecular weight species (MW ≈ 40,000) is also found in the circulation and in pituitary extracts to an extent of 10–15% of the total. In some cases a very high molecular weight form referred to as "big-big" prolactin has been identified but in most situations this forms less than 5% of the total. Some tumors appear to secrete mainly the high molecular weight form.

## A. Chemistry

Peptide maps and amino acid sequence analysis of prolactin indicate considerable homology between human and other prolactins (Seavey *et al.*, 1973). For example, of the first 23 residues of human prolactin 13 are identical with those found in ovine prolactin (Niall *et al.*, 1973). Approximately 90% of the amino acid sequence of human prolactin has been established. Sequence analysis of one preparation of amniotic fluid prolactin has also been carried out and of the 25 N-terminal residues all are identical with those in the same positions for pituitary prolactin.

## B. Biological Effects

The biological characterization of human prolactin has been relatively limited. In those cases in which comparisons have been made with ovine prolactin preparations, human prolactin exhibits similar effects as those that have been noted with the best preparations of ovine prolactin. Thus, human prolactin in the classic pigeon crop sac assay has a potency in the neighborhood of 25–30 IU/mg. There appears to be very little growth-promoting activity in human prolactin in a body weight gain or tibia assay and one can presume that even the majority of this activity is due to contaminating amounts of human growth hormone in the preparation. Radioreceptor assays also have been utilized to characterize human prolactin (Shiu *et al.*, 1973). In these assays human and ovine prolactin preparations are equipotent, whereas in a growth hormone receptor assay, using either rabbit liver or human liver, prolactin exhibits very little, if any, growth-promoting activity (Carr and Friesen, 1976). Thus, we believe there are separate prolactin and growth hormone receptors in human tissues. Although we know that human prolactin does not bind to the human growth hormone receptor, it is not clear whether human growth hormone binds to the prolactin receptor in human tissues, but there is preliminary evidence that it may do so. Unlike the human growth hormone receptor the human receptor for prolactin does not appear to be species specific.

In summary, human prolactin has been purified from both pituitary and amniotic fluid sources. The purified hormone preparations which have been obtained using several different methods appear to be very similar if not identical to each

other. Human and ovine prolactin share a great deal of structural homology. Data on the complete amino acid sequence of human prolactin is awaited eagerly so that full comparisons of the homology among prolactin preparations can be made and also to facilitate structure–function studies. The biological effects which have been ascribed to ovine prolactin also appear to be produced by human prolactin.

## III. MEASUREMENT OF PROLACTIN

Prolactin and lactogenic hormones may be measured by bioassay, radioimmunoassay, and radioreceptor assay. The sensitivity and specificity vary greatly and the method utilized depends on the primary purpose of the estimation. Each method will be described briefly and the usefulness discussed.

### A. Pigeon Crop Sac

The original method for measuring prolactin utilized the ability of prolactin to stimulate proliferation of the pigeon crop sac epithelium (Riddle *et al.*, 1933). This has become the classic bioassay for prolactin. It was initially applied to the measurement of prolactin in serum (Lyons and Page, 1935), but nonspecific interference of serum even after extraction renders the results uninterpretable. Despite improvements in sensitivity and precision (Nicoll, 1967) this technique remains cumbersome and is not sufficiently sensitive to measure serum levels of prolactin. Moreover, preparations of human growth hormone and human placental lactogen are active in stimulating the pigeon crop. This assay remains the classic method for the comparison and standardization of the biological potency of purified prolactin and lactogenic hormones.

### B. Stimulation of Mammary Tissue

Intraductal injection of prolactin into the mammary glands of pseudopregnant rabbits results in marked development of the mammary tissue. This could be quantified and used as an *in vivo* bioassay for prolactin (Chadwick, 1963). This technique lacks sensitivity but forms the basis of four methods of *in vitro* bioassay for prolactin. These depend on the ability of prolactin to induce quantifiable changes in mammary tissue maintained *in vitro* for 3 to 5 days. Two of these methods have a histological end point using an arbitrary grading system of the amount of secretion accumulating in the alveolar lumina. These methods use mammary tissue obtained from either a midpregnant mouse (Frantz and Kleinberg, 1970) or a pseudopregnant rabbit (Forsyth and Myres, 1971). Two further

methods have biochemical end points and use the midpregnant mouse mammary gland. These depend either on the incorporation of $^{32}P$ into casein (Turkington, 1971) or the induction of the enzyme *N*-acetyllactosamine synthetase (the galactosyltransferase of lactose synthetase) (Lowenstein *et al.*, 1971). These assays are sensitive (2 to 10 ng prolactin/ml of culture medium). When applied to serum this represents a sensitivity of 20 to 100 ng/ml depending on the amount of serum used. The most serious drawback is the lack of specificity of these techniques. They detect lactogenic activity and both human growth hormone and human placental lactogen exhibit marked activity in these systems. To overcome this problem the human growth hormone or human placental lactogen in serum samples may be neutralized by the addition of specific antibodies to these hormones prior to assay. Alternatively, the levels of human growth hormone and human placental lactogen may be estimated by specific radioimmunoassays and the prolactin level estimated by the difference. Normal male serum has been used as a diluent for standards and results in an apparent improvement of both sensitivity and precision. Since male serum contains variable amounts of prolactin this casts doubt on the validity of this approach.

Nevertheless, these bioassays allow the demonstration of prolactin activity in serum. However, they require specialized facilities for the setting up of *in vitro* cultures, are time-consuming, often show poor precision, and have a low throughput of samples.

### C. Radioimmunoassays

Difficulties in developing specific radioimmunoassays (RIA's) for human prolactin were directly related to the unavailability, until recently, of purified human prolactin. To circumvent these problems, several modifications of the RIA techniques were utilized. The RIA's developed for human prolactin may be defined as of two types. The first is homologous, in which prolactin of human origin was used for immunization, for labeling, and as standard. The second are heterologous RIA's in which the antiserum used reacts with a prolactin of one species while the tracer is prolactin from another species.

The first RIA for human prolactin used material prepared from tissue culture medium of fetal pituitary glands. This was used for immunization and as labeled hormone (Bryant *et al.*, 1972). Using such systems, some indication of the changes in prolactin secretion was obtained but the assay did not appear to measure the endogenous hormone alone (see Greenwood *et al.*, 1973).

Mixed heterologous RIA's were subsequently developed which were suitable for the measurement of prolactin in serum. These involved the use of antiserum to sheep prolactin and either sheep prolactin or porcine prolactin as tracer hormone. A further group of assays utilized primate prolactin or human prolactin as

tracer and antibodies to sheep or monkey prolactin or, in one case, human growth hormone (see L'Hermite, 1973). These RIA's were sufficiently sensitive to measure circulating levels of prolactin. Because a standard preparation of human prolactin was unavailable early reports of serum levels of prolactin were often defined in $\mu$l or mU of a laboratory standard of serum containing high levels of prolactin. With the purification of human prolactin, homologous RIA's were developed where purified human prolactin was used for immunization, standard and for labeling. The heterologous and homologous RIA's are specific for prolactin and show no cross-reaction with human growth hormone or human placental lactogen.

### D. Radioreceptor Assays for Prolactin

In the past radioimmunoassay procedures have been criticized because they do not measure the biological activity of the circulating hormone. Recently radioreceptor assays have been developed in which the binding agent is the physiological hormone receptor on the cell membrane of target tissues. These receptors bind only biologically active hormone and are extracted from the mammary glands of late pregnant or suitably primed pseudopregnant rabbits (Shiu *et al.*, 1973). The radioreceptor assay for prolactin recognizes only the biologically active lactogenic activity of the hormone and is not species specific. Thus, it can be used to detect lactogenic hormones besides prolactin in other species and has been instrumental in detecting placental lactogens in several species. The sensitivity of this assay is similar to that of the radioimmunoassay and it may be used to determine the levels of biologically active hormone in serum. However, primate growth hormones, human growth hormone, and human placental lactogen show equal activity to human prolactin in this system. Nonspecific serum interference in the assay system also requires that samples be diluted before assay, thus reducing the effective sensitivity of the method.

Nevertheless, the radioreceptor assay offers substantial improvements over the other available methods of bioassay, is simple, and offers great advantages in precision and reproducibility.

## IV. THE FORM OF PROLACTIN IN SERUM

As mentioned, gel filtration studies have demonstrated that prolactin in serum is heterogeneous. The proportions of these different forms in serum vary under different physiological and pathological states. In normal situations the major component is of small molecular weight, comprising more than 80% of the total prolactin activity. The highest amounts of big prolactin (10–30%) are found

**TABLE I**

**Normal Serum Concentrations of Prolactin Measured by Various Radioimmunoassays**

| Authors | Radioimmunoassay system[a] | | | Prolactin levels (ng/ml) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Mean | | Range | |
| | Antiserum | Labeled hormone | Standard | Male | Female | Male | Female |
| Hwang *et al.* (1971) | hGH | mPRL | hPRL | | | 0–28 | 0–30 |
| Friesen *et al.* (1972) | hPRL | hPRL | hPRL | 7 | 11 | 3–25 | 3–25 |
| Jacobs *et al.* (1972) | oPRL | pPRL | Plasma | 6 | 9 | ±0.6[b] | ±0.6[b] |
| Sinha *et al.* (1973) | hPRL | hPRL | hPRL | 13 | 14 | 6–24 | 7–18 |
| McNeilly and Hagen (1974) | hPRL | hPRL | hPRL | 11 | 15 | 8–25 | 8–25 |
| Aubert *et al.* (1974) | hPRL | hPRL | hPRL | 5 | 9 | 1–12 | 4–21 |
| Schmidt-Gollwitzer and Saxena (1975) | hPRL | hPRL | hPRL | 14 | 21 follicular, 25 luteal | ±0.9[c] | ±1.5[c] ±1.0[c] |

[a] hGH, human growth hormone; oPRL, ovine prolactin; hPRL, human prolactin; mPRL, monkey prolactin; plasma, plasma pool standardized against oPRL by bioassay.

[b] SE.

[c] SD.

during pregnancy and in some hyperprolactinemic states. The significance of these findings is not apparent.

The immunological and biological activities of these components under most circumstances appear to be equal (Guyda, 1975), suggesting that both forms are equally active. Rare tumors have been described in which the predominant circulating form ("big-big") appears to have low biological activity despite great elevations in radioimmunoassayable levels. It is possible that big prolactin represents a storage form of prolactin that appears in the circulation only when hypersecretion of prolactin occurs, e.g., during pregnancy or from a tumor. RIA's for prolactin do not appear to discriminate between the various molecular weight forms of prolactin seen in serum. Thus, the values of prolactin in serum obtained by radioimmunoassay will reflect the total immunological prolactin activity present.

## V. COMPARISON OF METHODS

It is apparent that with the very large throughput of clinical samples in routine laboratories several factors require consideration when comparing bioassays, tissue receptor assays, and RIA's. These include specificity, sensitivity, precision, reproducibility, time, cost, and ease with which these assays can be performed. It is apparent that the currently available bioassays are not specific but measure human growth hormone and human placental lactogen. They require specialized facilities and are impracticable for large numbers of samples. RIA's and radioreceptor assays have a greater sensitivity, and a higher precision and reproducibility. Unlike the radioreceptor assay, which measures human growth hormone and human placental lactogen, the RIA's are specific for prolactin and large numbers of samples can be handled with considerable ease. The major drawback of the RIA is its failure to discriminate between biologically active hormone and immunologically active, biologically inactive prolactin products.

In the case of prolactin this is not a major consideration. Extensive comparisons between serum levels of prolactin estimated by RIA, bioassay, and radioreceptor assay show a high degree of correlation. It is apparent that for routine clinical application the radioimmunoassay is the method of choice.

Although all the specific RIA's used to measure serum levels of prolactin yield generally similar physiological and pathological findings, the absolute values in terms of nanograms hormone per milliliter serum vary. This variation suggests that each RIA may detect a slightly different immunoreactive part of the prolactin molecule. Even using the same reagents different laboratories may report different normal ranges (see Table I). Standardization of reagents in all laboratories should minimize the small variation seen in quantitative basal values.

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**REVIEW**

Horm. Metab. Res. 11 (1979) 533–546

## Prolactin: Assessment of its Role in the Human Female

H.G. Bohnet and A.S. McNeilly*

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REVIEW

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## I Introduction

Although prolactin (PRL) had been purified from sheep and cow pituitaries in the early 1930's it was not until the early 1970's that human PRL (hPRL) was finally identified and purified (*Hwang, Guyda* and *Friesen* 1971; *Lewis, Singh* and *Seavey* 1971). Human PRL is of similar molecular weight to human growth hormone (hGH) and the large structural homology between hPRL and hGH resulting in the equipotent lactogenic activity of hPRL and hGH in the bioassays used during purification procedures contributed to the delay in isolation of hPRL (*Friesen* and *McNeilly* 1978). Subsequently homologous radioimmunoassays for the determination of hPRL have been developed (*Hwang, Guyda* and *Friesen* 1971; *Sinha, Selby, Lewis* and *Vanderlaan* 1973; *McNeilly* 1973) culminating in a tremendous increase in knowledge concerning physiological and pathological secretion of this hormone. An attempt is made to review the present state of research and to delineate biological actions of hPRL.

## II Patterns of secretion of hPRL

1) *Short term variations*

Basal serum PRL levels show random fluctuations and short term variations of the hormone (*Ehara, Siler, Vandenberg, Sinha* and *Yen* 1973; *Parker, Rossman* and *Vanderlaan* 1973; *McNeilly, Sturdy, Evans* and *Chard* 1974; *Bohnet, Dahlén* and *Schneider* 1974; *Bohnet, Wiest, Dahlén* and *Schneider* 1975; *McNeilly* and *Chard* 1976). Variations of baseline PRL values about the mean are generally less than 20% and do not reach levels considered to be pathologically elevated (*Leighton, McNeilly* and *Chard* 1978; *Jaquet, Grisoli, Guibout, Lissitsky* and *Carayon* 1978).

While circannual variations of PRL secretion are lacking (*Gala, van de Walle, Hoffmann, Lawson, Pieper, Smith* and *Submaranian* 1977), circadian periodicity was shown by *Nokin, Vekemans, L'Hermite* and *Robyn* (1973) and related to sleep (*Sassin, Frantz, Weitzmann* and *Kapen* 1972, 1973). PRL peaks occurring during sleep exceed wake increments in quantity and frequency (*Parker, Rossman* and *Vanderlaan* 1973, 1974; *Osterman* and *Wide* 1975) and tend to be higher during the luteal than during the follicular phase of the menstrual cycle (*Bohnet*, unpublished observations). Ergot alkaloids suppress both basal and sleep related increases of serum PRL (*Rozencweig, Heuson, Bila, L'Hermite* and *Robyn* 1973; *Mendelson, Jacobs, Reichman, Othmer, Cryer, Trivedi* and *Daughaday* 1975), while chronic administration of L-Dopa failed to abolish diurnal rhythms of PRL secretion (*Malarkey, Cyrus* and *Paulson* 1974). Nocturnal PRL elevations shift with changes of sleep periods occurring also during daytime and are foreshortened by early awakening. Darkness itself does not lead to an increase of serum PRL, although in some individuals PRL release is observed before sleep during evening hours (*Robyn, Delvoye, Nokin, Vekemans, Badawi, Perez-Lopez* and *L'Hermite* 1973). Measurements of PRL in cord serum at birth suggested that circadian changes of PRL may also occur in late fetal life (*Badawi, van Exter, Delogne-Desnoeck, van Meenen* and *Robyn* 1977). In physiological (*Boyar, Finkelstein, Kapen* and *Hellman* 1975; *Robyn, Delvoye, van Exter, Vekemans, Caufriez, De Nayer, Delogne-Desnoeck* and *L'Hermite* 1977) as well as in pathological hyperprolactinemia the amplitude of sleep induced increase of serum PRL is reduced and a rise is frequently not seen (*Jacobs* and *Daughaday* 1973; *Boyar, Kapen* and *Finkelstein* 1974; *Kapen, Boyar, Freeman, Frantz, Hellman* and *Weitzmann* 1975; *L'Hermite, Degueldre, Caufriez, Delvoye* and *Robyn* 1975; *Boyar, Kapen, Weitzmann* and *Hellman* 1976). In obese women the acrophase of circadian PRL release shifts from night time to early daytime and is restored to normal during fasting (*Copinschi, de Laet, Brion, Leclerque, L'Hermite, Robyn, Virasoro* and *van Cauter* 1978). In male volunteers short term fasting resulted in significantly diminished increase of PRL after TRH stimulation (*Vinik, Kelk, McLaren* and *Paul* 1975), while fasting of obese women for weeks accompanied by considerable weight loss had no significant influence on PRL secretion (*Carlson, Drenick, Chopra* and *Hershman* 1977). Consistent rises of serum PRL observed at about 1 and 6 p.m. were suggested to depend on meals (*Sassin* et al. 1973).

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Girls with amenorrhea due to weight loss and chronic starvation were shown to have PRL levels within normal ranges during daytime (*Beumont, Friesen, Gelder* and *Kilakowska* 1974), but reduced at night and during sleep (*Kalucy, Crisp, Chard, McNeilly, Chen* and *Lacey* 1976). In such emaciated patients the increase of PRL after metoclopramide stimulation was also significantly reduced, possibly due to diminished circulating estrogen and gonadotropin levels (*Bohnet, Hanker, Liermann* and *Schneider* 1978).

Psychic as well as physical stress leads to an increase in serum PRL concentrations (*Charters, Odell, Thompson* 1969; *Sowers, Raj, Hershman, Carlson* and *McCallum* 1977). The magnitude of this increase is higher in females than in males (*Hwang, Friesen* and *Hardy* 1971; *Noel, Suh, Stone* and *Frantz* 1972; *Noel, Suh* and *Frantz* 1974) and is exaggerated in neurotic patients (*Mijabe, Asato* and *Mizushima* 1977). Injuries, in particular of the chest also increase serum PRL (*Morley, Dawson, Hodgkinson* and *Kalk* 1977) probably mediated by irritation of nerve-endings near the nipples (*Tyson* 1977). In some men and in the majority of non-lactating women nipple stimulation induces a rise in serum PRL levels (*Kolodny, Jacobs* and *Daughaday* 1972; *Noel, Suh* and *Frantz* 1974) being exaggerated in women with mammary hypertrophy (*Archer* and *Josimovich* 1975). Sexual intercourse without stimulation of the breast does not necessarily induce an increase of serum PRL in women or in men (*Stearns, Winter* and *Faiman* 1973; *Noel, Suh* and *Frantz* 1974).

2) *Long term variations*

*a) Childhood.* At birth serum PRL levels are 10 to 20 times normal and are similar in either sex comparable to the raised maternal levels at term (*Hwang, Guyda* and *Friesen* 1971; *Badawi* et al. 1977). TRH stimulation results in an increase in serum PRL levels suggesting that at birth the pituitary gland contains a relatively large store of releasable PRL. Neither L-Dopa nor pyrodoxine, both inhibitors of PRL secretion in the adult, have any effect in the newborn possibly reflecting an immaturity of the hypothalamo-pituitary axis in the newborn (*Delitala, Meloni, Masala, Alagna, Devilla* and *Corti* 1978). During the first weeks of life a gradual decrease of PRL is observed (*Guyda* and *Friesen* 1973; *Aubert, Grumbach* and *Kaplan* 1975) and remain at low levels until puberty.

*b) Sex differences.* While first reports on basal PRL levels did not show significant differences between children and adults as well as between males and females (*Friesen* and *Hwang* 1973) more detailed studies revealed clear sex differences (*Jacobs, Mariz* and *Daughaday* 1972; *Nokin* et al. 1973). In prepubertal children a greater release of PRL was seen in girls than in boys after metoclopramide stimulation (*Dammaco, Rigillo, Chetri, Torelli, Frezza, Mastrangelo* and *Zuccaro* 1977). Girls with retarded puberty, who show an adult type LH response after LH-RH stimulation, release greater amounts of PRL after metoclopramide administration than those exhibiting an impaired LH release (*Bohnet,* unpublished observations). In boys with gynecomastia stimulation of PRL secretion by sulpiride resulted in a greater release than in normal boys (*d'Agata, Ando, Gulizia, Condorelli, Paci, Scapagnini* and *Polosa* 1977). These reports suggest that sex differences in PRL secretion appearing around puberty are probably due to changes in sex steroids' secretion, in particular due to the increase in ovarian estrogens already occurring before menarche (*Jacobs, Mariz* and *Daughaday* 1972; *Ehara, Yen* and *Siler* 1975; *Lee, Xenakis, Winer* and *Matzenbaugh* 1976). This is underlined by the observation that not only estrogens and PRL correlate with each other, but they also correlate with the stage of breast development (*Thorner, Round, Jones, Grooms, Butcher* and *Thompson* 1977).

In males basal serum PRL levels remain stable from childhood to adulthood and senium, while during the reproductive lifespan in females PRL levels are higher than after menopause (*Robyn* et al. 1977). Several investigators have shown that the PRL response to TRH (*Jacobs, Snyder, Utiger* and *Daughaday* 1973; *Franchimont, Dourcy, Legros, Reuter, Vrindts-Gevaert, Van Cauwenberge, Remacle, Gaspard* and *Colin* 1976), metoclopramide (*Bohnet* et al. 1978b) and cimetidine (*Bohnet, Greiwe, Hanker, Aragona* and *Schneider* 1978) is higher in cycling than in menopausal women, the latter being similar to that observed in men. Indirect confirmation of reports on sex differences was also obtained by extraction of pituitary PRL from either sex (*Yamaji, Shimamoto, Ishibashi, Kosaka* and *Orimo* 1976).

*c) Menstrual cycle.* In 1972 *Vekemans* and coworkers (*Vekemans, Delvoye, L'Hermite* and *Robyn* 1972) described a biphasic secretion pattern of PRL during the menstrual cycle with a midcycle peak and elevated levels during the luteal phase. This was not supported by other investigators (*Hwang, Guyda* and *Friesen* 1971; *Tyson* and *Friesen* 1973; *Jaffe, Yuen, Keye* and *Midgley* 1973; *McNeilly* and *Chard* 1974). Some reports showed that PRL elevations around midcycle occur (*Ehara, Siler, Vandenberg, Sinha* and *Yen* 1973). More recent studies have demonstrated a trend towards higher PRL levels during the luteal than during the follicular phase of the menstrual cycle (*Franchimont* et al. 1976; *Vekemans, Delvoye, L'Hermite* and *Robyn* 1977; *Bohnet, Hanker, Horowski, Wickings* and *Schneider* 1979). In addition, PRL secretion in response to TRH (*Fran-*

*chimont* et al. 1976; *Reymond* and *Lemarchand-Béraud* 1977; *Boyd* and *Sanchez-Franco* 1977), cimetidine (*Bohnet* et al. 1978a) and metoclopramide (*Bohnet* et al. 1978b) was greater during the luteal than during the follicular phase of the cycle. Although still a matter of discussion such changes of PRL secretion probably reflect an altered hypothalamic neurotransmission which could explain psychic lability throughout the menstrual cycle such as mid-cycle mood elevation and premenstrual dysphoria (*Carroll* and *Steiner* 1978). Indeed, there is good evidence that patients complaining of premenstrual discomfort have elevated PRL levels (*Halbreich, Assael, Ben-David* and *Borstein* 1976). PRL suppression with Bromocriptin does not only result in patients' relief of such symptoms, but also in normalisation of the most frequently observed inadequate luteal progesterone secretion (*Benedek-Jaszmann* and *Hearn-Sturtevant* 1976).

*d) Pregnancy.* It is well established that serum PRL increases progressively during pregnancy towards term to levels 5 to 20-fold normal (*Hwang, Guyda* and *Friesen* 1971; *L'Hermite* and *Robyn* 1972). This increase may occur just after implantation and is paralleled by the increase of circulating luteal and placental estrogens (*Barberia, Abu-Fadil, Kletzky, Nakamura* and *Mishell* 1975; *Robyn* et al. 1977; *Rigg, Lein* and *Yen* 1977; *Hertz, Andersen* and *Larsen* 1978). The role of estrogens in mediating the rise of PRL has been demonstrated by investigations in the rhesus monkey, where serum PRL remains low until late pregnancy. Then both estrogens and PRL rise in synchrony (*Josimovich, Weiss* and *Hutchinson* 1974). The weight of the pituitary gland increases throughout pregnancy and is greater in multi- than in primiparae reflecting hyperplasia of the lactotrophs (*Erdheim* and *Stumme* 1909; *Pasteels* 1972). Despite elevated basal PRL levels TRH stimulation provokes further increments of PRL, the net increase being similar to that observed in non-pregnant women. This suggest that total pituitary PRL content is increased. There is however no significant difference regarding parity (*Bohnet, Heuberger, Dahlén* and *Schneider* 1975).

The amniotic fluid contains the greatest amount of PRL when compared to other compartments (*Friesen* and *Hwang* 1973). Until week 10 of pregnancy concentrations are similar to those in maternal serum, but rise thereafter dramatically until week 20, when a gradual decrease is observed (*McNeilly, Gilmore, Jefferey, Dobbie* and *Chard* 1977; *Robyn* et al. 1977). The total amount of PRL however does not decrease (*Clemens, Reyes, Winter* and *Faiman* 1977; *McNeilly* et al. 1977). Thus, the decrease in amniotic fluid PRL is still unknown. While some investigators favoured maternal (*Josimovich, Weiss* and *Hutchinson* 1974) or fetal origin (*Fang* and *Kim* 1975; *Clemens* et al. 1977), others suggested that amniotic PRL is gained from both sources (*McNeilly* et al. 1977). *Del Pozo* and co-workers (*del Pozo, Hiba, Lacranjan* and *Künzig* 1977) reported that in two women and their babies serum PRL was markedly suppressed by Bromocriptin therapy throughout pregnancy, whereas amniotic fluid PRL was unaffected. A similar result was obtained in a hypophysectomised woman in whom PRL levels remained at the lower limit of normal non-pregnant range throughout pregnancy. Despite this, foetal cord levels and amniotic fluid levels of PRL were normal (*McNeilly* and *Baird,* unpublished observations). It has been suggested that amniotic fluid PRL may be derived from an independent chorionic source (*Friesen* et al. 1972; *del Pozo, Lancranjan* and *Künzig* 1977). Support for this has come recently from short term *in vitro* cultures of term human amnion and chorion where the release of PRL into the medium during culture was greater than the total content of the membranes (*Bohnet* and *Friesen,* unpublished observations). More recently PRL has been localized in the decidua (*Riddick* and *Kusmick* 1977) and apparent *de novo* synthesis of PRL by the chorionic trophoblast has recently been reported (*Healy, Muller* and *Burger* 1978; *Golander* et al. 1978). Binding sites for PRL have been found on the amniotic epithelial cells (*Healy, Muller* and *Burger* 1977) from whence it can presumably be released into the amniotic fluid as appears to be the case in the rhesus monkey (*Josimovich, Merisko* and *Boccela* 1977).

The role of amniotic fluid prolactin remains to be fully explained but PRL can influence water and ion flux across the amnion thus affecting the volume and composition of amniotic fluid (*Holt* and *Perks* 1975; *Leontic* and *Tyson* 1977; *Josimovich, Merisko* and *Bocella* 1977). In the rhesus monkey, amniotic fluid PRL also appears to protect the foetus from dehydration (*Josimovich, Merisko* and *Bocella* 1977).

PRL is detectable in the serum of the human fetus from the 12th week of gestation steeply increasing after week 24 (*Aubert, Grumbach* and *Kaplan* 1955; *Clemens* et al. 1977, *McNeilly* et al. 1977). This is paralleled by an increase of pituitary PRL content as well as by the increase of maternal serum PRL. The regulating mechanisms underlying these changes are not clear, but the augmentation of pituitary PRL is suggested to be mediated by a direct stimulatory action of endogenous estrogens on the pituitary glands and not by hypothalamic factors (*McNeilly* et al. 1977) as judged from investigations in anencephalic fetuses (*Aubert, Grumbach* and *Kaplan* 1975). This hypothesis might be supported by the observation that fetal and maternal serum PRL concentrations correlate significantly with each other (*Aubert, Grumbach* and *Kaplan* 1975; *Robyn* et al. 1977).

*e) Lactation.* The endocrine changes occurring in women who are breastfeeding are different from those who are not (See *McNeilly* 1977). Overall serum PRL levels decrease more rapidly in women not breastfeeding, while in nursing mothers PRL is elevated for as long as 18 months post partum (*Delvoye, Demaegd, Uwayitu-Nyampeta* and *Robyn* 1978). This is probably due to the repeated and frequent suckling episodes which result in maintained PRL secretion. Severance of nipple nerve supply as well as nipple anesthesia abolish nursing induced PRL release, but not milk ejection, suggesting dissociation between oxytocin and PRL release (*Tyson* 1977). The increase of PRL probably depends not only on the intensity of the nipple stimulation, i.e. automanipulation, breast pump (*Noel, Suh* and *Frantz* 1974) and suckling of one or two babies (*Tyson* 1977) but also on the size of pituitary PRL pools. We observed a wide variation of PRL secretion following TRH stimulation in lactating individuals on distinct post partum days (*Bohnet* et al. 1975b). On the other hand suckling also resulted in different PRL output varying in time and degree of elevation of PRL (*Bohnet* et al. 1975a). The different PRL response behaviour to such stimuli were suggested to resemble milk yield (*Aono, Shioji, Skoda* and *Kuwachi* 1977). Chronic administration of TRH resulted in an increase in milk yield in previously insufficient lactation, but not if milk production was normal (*Tyson, Perez* and *Zacur* 1976).

Initiation of milk secretion is considered to occur only after clearance of placental sex steroids, in particular of estrogens (for references see *Hiba, del Pozo, Genazzani, Pusterla, Lancranjan, Sidiropoulos* and *Gunti* 1977; *McNeilly,* 1979). These hormones inhibit the action of PRL directly at the breast and subsequently production of milk composites such as $\alpha$-lactalbumin (*Kleinberg, Todd* and *Niemann* 1978). Recently, we could demonstrate that mammary PRL receptors decreased when lactating rats were treated with relatively high amounts of estrogens (*Bohnet, Gómez* and *Friesen* 1978). On the other hand priming of the mammary gland by estrogen is required for lactation (*Rabello, Snyder* and *Utiger* 1974) and for induction of PRL receptors (*Bohnet, del Pozo* and *Gómez* 1978). While estrogens inhibit milk secretion without profound changes of serum PRL (*Bohnet* et al. 1975b; *Hiba* et al. 1977), antiestrogens decrease milk yield probably by both, the inhibition of PRL secretion (*Masala, Delitala, Lo Dico, Stoppelli, Alagna* and *Devilla* 1978) as well as by interference with the induction of PRL receptors, a mechanism similar to that by which ergolines decrease milk production (*Brun del Re, del Pozo, de Grandi, Friesen, Hinselmann* and *Wyss* 1973; *Bohnet* et al. 1978).

### III Puerperal infertility

It is generally accepted that resumption of normal cyclical feedback mechanisms and menstrual bleeding after parturition is considerably delayed in nursing mothers when compared to nonlactating women (*Keettel* and *Bradbury* 1961; *El-Minawi* and *Foda* 1971; *Perez, Kela* and *Masnick* 1972; *Delvoye* et al. 1978). Lactation has been suggested to be the central control of reproduction (*Short* 1976) and the relation between lactation and reproductive function has been reviewed recently (*McNeilly* 1979) It is unclear whether the elevated levels of PRL during lactation are directly responsible for the period of amenorrhoea. In the initial postpartum week pituitary serum levels of both LH and FSH may be lower than normal (*Rolland* and *Schellekens* 1973; *Bonnar, Knott, Franklin* and *McNeilly* 1975; *de la Lastra* and *Llados* 1977). Previous reports also suggested that the gonadotropin response to LH-RH stimulation was impaired to a greater degree and for a longer period in lactating than in non-lactating women (for references see *Bohnet* and *Friesen* 1976; *Tyson* 1978; *McNeilly* 1979). More recent and detailed investigations showed that the refractory period of the pituitary to LH-RH stimulation is similar in both groups (*Jeppsson, Rannevik Thorell* and *Wide* 1977; *Tyson, Carter, Andreassen, Huth* and *Smith* 1978). In addition, serum FSH as well as pituitary FSH are higher in lactating than in non-nursing mothers (*Delvoye, Demaegd, Uwayitu-Nyampeta* and *Robyn* 1978; *Tyson* et al. 1978). Pulsatile LH release is absent at least during the first three weeks postpartum (*Bohnet* et al. 1975b) and resumes earlier after PRL suppression than in fully lactating women (*Tyson* et al. 1978). Thus, while in lactating women there appears to be adequate LH and FSH, the ovaries remain unstimulated. The reason for this remains unclear and whether PRL *per se* is responsible for this inhibition of ovarian activity perhaps by a direct inhibitory action of PRL on the ovary (*McNatty, Sawers* and *McNeilly* 1974) as well as an action at the hypothalamus remains to be answered. It has recently been demonstrated, however, that oestrogen fails to evoke a positive discharge of LH in lactating versus non-lactating women (*Baird, McNeilly, Sawers* and *Sharpe* 1979). This suggest that even if ovarian follicular development with consequent oestrogen secretion was to occur, ovulation would not result and the women would not show normal ovarian cyclicity with a corpus luteum.

### IV. Pathological Hyperprolactinaemia

*Hyppocrates* wrote that "if a woman has milk, who is not with a child, nor has brought forth, her menses are obstructed". That non-puerperal milk secretion (galactorrhoea) was often associated with amenorrhoea was not re-emphasized until *Chiari,*

*Brown* and *Spaeth* (1852) and *Frommel* (1882) described amenorrhoea related to persistence of puerperal galactorrhoea in the absence of nursing. *Ahumada* and *del Castillo* (1932) described amenorrhoea associated with non-puerperal galactorrhoea while *Forbes* and co-workers (*Forbes, Hennemann, Griswald* and *Albright* 1954) first showed that such disorders may be related to the existence of pituitary tumours.

After the development of reliable methods to measure PRL it became apparent that this hormone is the common link between these syndromes (*Besser, Parke, Edwards, Forsyth* and *McNeilly* 1972). While galactorrhoea is not always accompanied by hyperprolactinaemia and vice versa (*Frantz* 1978; *Kleinberg, Noel* and *Frantz* 1977), elevated PRL levels commonly interfere with reproduction. The degree of the menstrual disorders however varies widely including corpus luteum insufficiency as well as premenstrual discomfort, oligo- and amenorrhoea. Therefore those classifications appear to be based upon eponyms and are unsatisfactory; they may evolve through various syndromes (*Greenblatt, Carmona* and *Haegler* 1956; *Levin, Daughaday* and *Levy* 1956; *Levin, Daughaday* and *Levy* 1959; *Bercovici* and *Ehrenfeld* 1967; *Young, Bradley, Goldzieher, Meyer* and *Lecocqu* 1967; *Maas* 1967; *Sandler* and *Gardner* 1973; *Bergh, Nillius* and *Wide* 1978). This led to the proposal to designate such disturbances as "*Hyperprolactinaemic Anovulatory Syndromes*" (*Bohnet, Dahlén* and *Schneider* 1975; *Bohnet* et al. 1976). The incidence accounts for about 15 to 30% of women with secondary amenorrhoea (*Bohnet, Dahlén*, and *Schneider* 1975; *Bohnet* et al. 1976; *Franks, Murray, Jequier, Steele, Thomson, Nabarro* and *Jacobs* 1975; *Rjosk, von Werder* and *Fahlbusch* 1976; *Bergh, Nillius* and *Wide* 1977; *Shearman* and *Fraser* 1977). Galactorrhoea is present in about 50% of the cases (*Bohnet, Dahlén* and *Schneider* 1975; *Franks* et al. 1975) but may account for up to 90% in some reports (*Guitelman, Apraricio, Mancini, Encina, Levalle* and *Schally* 1977; *Jaquet* et al. 1978). In male hypogonadism, galactorrhoea is less frequent (5% *Kleinberg, Noel* and *Frantz* 1977) and the incidence of hyperprolactinaemia may be as low as 0.5% (*Skakkebaek*, personal communication). This is probably due to the lack of priming of the mammary gland by oestrogens. Such priming by either pregnancy or oral contraceptives may also account for the association of hyper- and normoprolactinemia with or without galactorrhoea. We and others have shown the close relationship of the regulation of lactogen and oestrogen receptors in the rat mammary gland (for references see *Bohnet, Gómez* and *Friesen* 1977). On the other hand, lack of galactorrhoea could also be explained by the observation that the PRL receptors do not equally recognize the heterogeneous molecules of PRL, i.e. big-big, big, small (*Aubert, Garnier, Kaplan* and *Grumbach* 1975). Serum concentrations of PRL do not determine the occurrence of galactorrhoea (*Friesen* and *Tolis* 1977).

The etiology of hyperprolactinaemia is diverse (for references see *Hwang* and *Friesen* 1973; *Bohnet* and *Friesen* 1976; *Frantz* 1978) but can be reduced to the following principles: firstly, hypothalamic derangement resulting in an alteration in secretion of neurotransmitters resulting in either decreased secretion of PRL inhibiting factor (PIF) or increased secretion of PRL releasing factor(s); secondly, a pituitary tumour, either PRL secreting (autonomously) or a non-secreting pituitary tumour pressing upward on the hypothalamus or pituitary stalk occluding the hypophysial portal vessels and preventing PIF from reaching the pituitary and inhibiting PRL secretion. Indeed, pituitary adenomas were found in up to 75% of hyperprolactinemic disorders (*Franks* et al. 1975; *Rjosk, von Werder* and *Fahlbusch* 1976; *L'Hermite, Caufriez* and *Robyn* 1977; *Kleinberg, Noel* and *Frantz* 1977; *Mornex, Orgiazzi, Hugues, Gagnaire* and *Claustrat* 1978). Although there is a wide range of levels of serum PRL in hyperprolactinaemia it is well established that increasing levels of PRL increase the probability that a pituitary tumour is present, even in the absence of obvious anatomical lesions (*Gómez, Reyes* and *Faiman* 1977; *Tolis* and *Friesen* 1977; *Frantz* 1978). Concentrations of more than 100 μg/l are strongly suggestive of organic lesions and are most commonly associated with amenorrhoea. PRL levels in the cerebrospinal fluid are not indicative of a pituitary tumor, but correlated with serum concentrations (*Assies, Schellekens* and *Touber* 1978) and dissociation of serum and cerebrospinal PRL heterogeneity does not give further information (*Jordan* and *Kendall* 1978). Levels under 30 μg/l are compatible with a "normal" menstrual cycle, but inappropriate luteal progesterone secretion may occur in up to 50% (*Bohnet, Mühlenstedt, Hanker* and *Schneider* 1977; *Seppälä, Lehtovirta, Laatikainen, Rante, Hirvonen* and *Arjomar* 1977).

The most common dynamic test for evaluation of PRL secretion is the TRH stimulation test. In hyperprolactinaemic disorders TRH induced secretion of PRL is blunted (for references see *L'Hermite* et al. 1977) but is restored for instance after removal of an adenoma (*Jaquet* et al. 1978). It was suggested that lactotrophes may have lost receptor sites for PRL releasing factor (s) or that endogenous stimulation is already at maximum (*Kleinberg, Noel* and *Frantz* 1977). With one of the most potent stimulators of PRL secretion, namely metoclopramide (*McNeilly, Thorner, Volans* and *Besser* 1974), we have been able to stimulate PRL secretion in patients with a demonstrable pituitary adenomas (see Fig. 1) questioning the autonomy of such tumors.

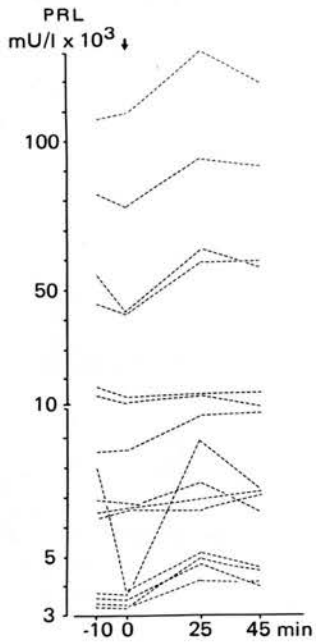



Fig. 1 Serum PRL levels (40 mU/l MRC-standard 71/222 account for approximately 1 μg/l) before and after metoclopramide stimulation (at time 0 – arrow – , 10 mg were given intravenously) in patients suffering from a PRL secreting pituitary adenoma, which had been demonstrated by sella x-ray

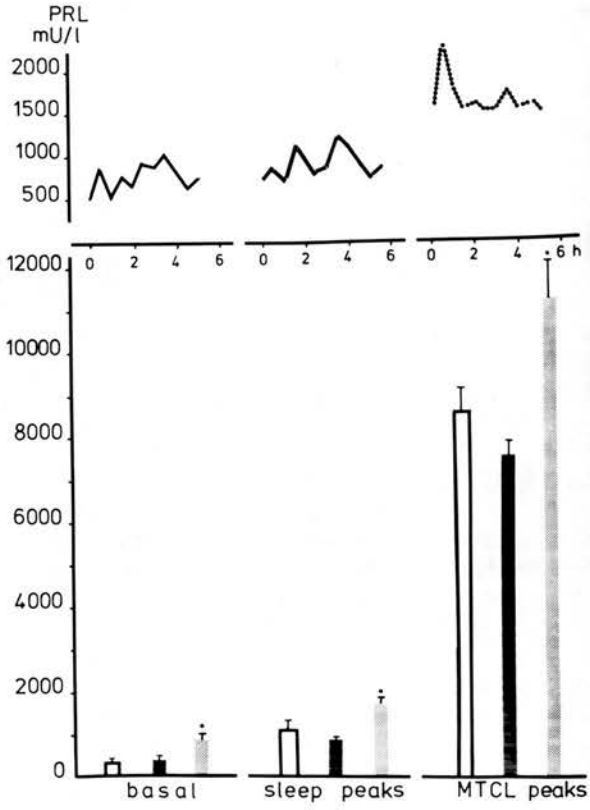



Fig. 2 Serum PRL levels (40 mU/l MRC-standard 71/222 accounts for approximately 1 μg/l) in normally ovulating women (n = 7 open bars) and in infertile patients with normoprolactinemic (n = 6 hatched bars) and hyperprolactinemic (n = 8, dotted bars) corpus luteum insufficiency. Basal concentrations represent mean values of times – 10 and 0 minutes (see Fig. 1). Sleep peaks represent highest PRL levels observed during a night sleep period of at least 2 hours (upper panel). Serum PRL levels observed 25 minutes after metoclopramide (MTCL) stimulation are also shown. * Significantly different from controls at $P < 0.05$

TRH tests performed in different physiologic and pathologic states of hyperprolactinemia reveal that with increasing basal PRL levels the net increment of PRL decreases (*Bohnet* et al. 1975b). Similar mechanisms were reported for sleep-related PRL secretion (*Boyar, Kapen* and *Finkelstein* 1974; *Boyar* et al. 1975, 1976; *L'Hermite* et al. 1977; *Jaquet* et al. 1978). In a group of patients with short luteal phase we found that peak PRL levels after metoclopramide stimulation correlated with peaks occurring during sleep such that an augmented response to metoclopramide was seen in patients, who had higher night-time levels than controls (Fig. 2). Basal PRL levels were normal or only slightly elevated. Such findings could explain why some investigators observe an increased luteal progesterone secretion after PRL suppression in "normoprolactinemia" shortened luteal phases and normalization of oestrogen secretion (*Tolis* and *Naftolin* 1976; *van der Steeg* and *Coellingh-Bennink* 1977). Baseline gonadotropin levels are normal or decreased and a decrease of LH precedes that of FSH. LH-RH stimulation tests more frequently reveal an impaired or absent LH response than a normal one, while the pituitary FSH pools are rather stable (*Tyson, Andreasson, Huth, Smith* and *Zacur* 1975; *Zárate, Canales, Soria, Villalobos, Soria, Jacobs, Kastin* and *Schally* 1975; *Bohnet* et al. 1975; 1976; *Bohnet* and *Schneider* 1977). However, there are reports showing that basal gonadotropin levels may also be elevated and the response to LH-RH is excessive (*Thorner, McNeilly, Hagen* and *Besser* 1974; *L'Hermite* et al. 1977; *Thorner* and *Besser* 1977; *Rolland* and *Corbey* 1977). In our opinion, the contradictory results may reflect different definitions of normal levels and the different dosages used in the LH-RH stimulation tests. In more detailed studies it was shown that with increasing PRL levels there was a progressive decline in pituitary LH sensitivity as well as reserve while the sensitivity and reserve for FSH appeared to increase resulting in a marked reduction of the LH/FSH ratio (*Lachelin, Abu-Fadil* and *Yen* 1978). This suggests that in hyperprolactinaemia endogenous LH-RH secretion is reduced reflecting the diminished functional status of the gonadostat (*Bohnet* et al. 1976) similar to that in prepubertal children (*Roth, Kelch, Kaplan* and *Grumbach* 1972; *Job, Garnier, Chaussain,*

*Binet, Rivaille* and *Milhaud* 1972). The pulsatile release of LH is absent in most cases of hyperprolactinaemia but can be observed in shortlived hyperprolactinemia. We were recently able to study a postmenopausal woman aged 57 years suffering from a PRL secreting adenoma. The history revealed menstrual disorders from her early thirties after she had given birth to a child and menopause occured at the age of 46. While PRL levels were excessively elevated, gonadotropins were within normal ranges (not postmenopausal) and lacked pulsatility. Adequate PRL suppression resulted in an increase of gonadotropin secretion as well as in restoration of normal pulsatile release (unpublished observations). Thus the pattern of gonadotropin secretion may reflect the degree of hypothalamic dysfunction (*Bohnet, Dahlén* and *Schneider* 1974, 1975; *Bohnet* et al. 1976; *Boyar* et al. 1975). Estrogen mediated gonadotropin release has been reported to be impaired in hyperprolactinemic syndromes suggesting a defective positive feedback control due to hypothalamo-pituitary dysfunction (*Glass, Shaw, Butt, Edwards* and *London* 1975; *Aono, Miyake, Shioji, Kindigasa, Onishi* and *Kuraghi* 1976). This is consistent with the findings that clomiphene treatment fails to induce ovulation in the majority of hyperprolactinemic patients (*Bohnet* et al.1977; *Bergh, Nillius* and *Wide* 1978). Nevertheless, as shown in Fig. 3 an LH increase can be provoked in some cases by exogenously given estrogens even in the presence of a pituitary adenoma. Thus, hyperprolactinemic patients may represent heterogenous groups with different degrees of hypotahlamic disorder which may account for the different findings of several investigators. In hyperprolactinemic anovulatory syndromes serum estradiol levels are found to be similar to those observed in the early follicular phase and gestagen withdrawal bleedings can be induced in the majority of hyperprolactinemic women (*Bohnet, Dahlén* and *Schneider* 1975; *Bohnet* et al. 1976; *Bohnet* and *Schneider* 1977; *Lachelin, Abu-Fadil* and *Yen* 1977; *Bergh, Nillius* and *Wide* (1978), if sufficient gestagen is given. This is consistent with the findings that in hyperprolactinemic women exogenously given human menopausal gonadotropins (HMG) will result in increased estradiol secretion (*Aono, Yasuda, Shioji, Kondo* and *Kurachi* 1978) and ovulation (*Bergh, Nillius* and *Wide* 1977), which is somewhat contradictory to results obtained in puerperal women (*Zárate, Canales, Soria, Ruiz* and *MacGregor* 1972; *Jeppsson, Rannevik* and *Kullander* 1974; *Nakano, Mori, Kayashima, Washio* and *Tojo* 1975). However, the large amounts of HMG given may overcome a desensitisation of the ovary by PRL (*McNatty, Sawers* and *McNeilly* 1974). In conclusion, alterations of the hypothalamo-pituitary-gonadal axis in the postpartum period are similar to those observed in pathological hyperprolactinemia and drug induced elevations of PRL (*L'Hermite* et al. 1977,

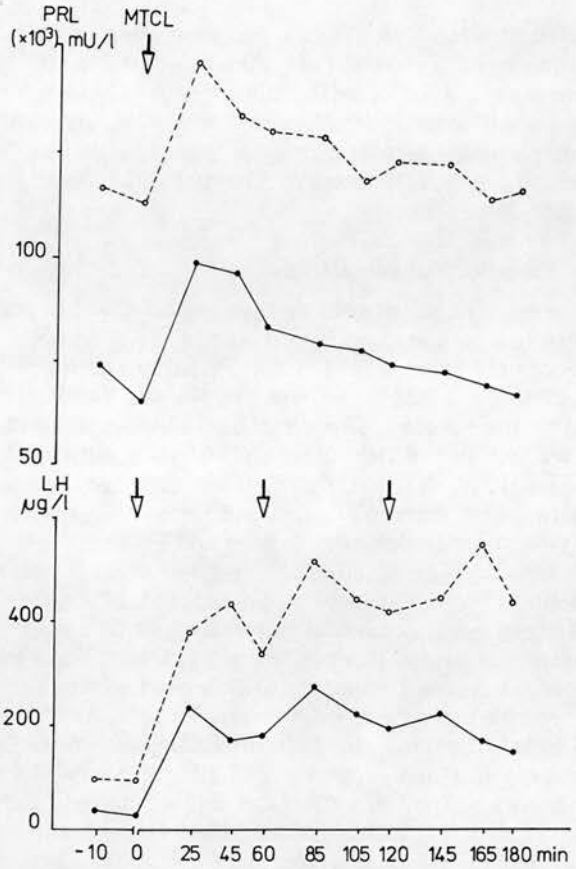



Fig. 3 Amplified PRL (upper panel) and LH response (lower panel) following estradiol benzoate treatment for 6 days (5 µg/kg, bid, broken lines) in a patient suffering from a PRL secreting pituitary adenoma. PRL release was stimulated by metoclopramide (MTCL; 10 mg i.v.) at time 0, while LH was stimulated by 25 µg LH-RH at hourly intervals as indicated by the 3 arrows

1978; *Aono, Shiomi, Kinngasa, Onishi* and *Kurachi* 1978). The inconsistent findings of several authors may be due to the lack of standardization of the investigations and due to different degrees of dysfunction which may be influenced by the concentration of PRL and by the time hyperprolactinemia exists. Concerning puerperal hyperprolactinemia the intensity of lactation may influence the findings and elevated sex steroid levels during pregnancy may be responsible for the differences observed between lactational and pathological hyperprolactinemia. Despite the apparent wide-ranging differences in hyperprolactinemic states preferential suppression of PRL with ergot alkaloids (for references see *Besser* et al. 1972; *Thorner* et al. 1974; *Bohnet* et al. 1976; *Bohnet* and *Friesen* 1976; *Bergh* et al. 1977; *L'Hermite* et al. 1977; *Franks* and *Jacobs* 1977; *Frantz* 1978) or removal of the PRL secreting adenoma (*Healy, Pepperell, Stockdale, Bremner* and *Burger* 1977; *Gómez, Reyes* and *Faiman* 1977; *Jaquet* et al. 1978) will restore normal cyclical feedback mechanisms resulting in ovulation and curing

infertility. Most pregnancies occurring after normalisation of PRL secretion are uneventful even in the presence of adenomas (for references see *Fahlbusch* and *von Werder* 1978; *Mornex* et al. 1978), although complications such as bitemporal hemianopsia can arise (*Lamberts, Seldenrath, Kwa* and *Birkenhäger* 1977).

## V. Prolactin and adrenal function

The association between amenorrhea-galactorrhea and hirsutism, obesity and hypercorticism has long been recognized (*Forbes* et al. 1954; *Bercovici* and *Ehrenfeld* 1963). Later hyperprolactinemia was identified to be the etiology (*Thorner* et al. 1974; *Seppälä* and *Hirvonen* 1975). Dehydroepiandrosterone and its sulphate (DHA-S) (*Bassi, Giusti, Borsi, Catteneo, Giannotti, Forti, Pazzagli, Vigiani* and *Serio* 1977; *Carter, Tyson, Warne, McNeilly, Faiman* and *Friesen* 1977) as well as 17-keto-steroids, their major urinary metabolites (*Thorner* et al. 1974; *Gómez* et al. 1977), are elevated in many cases of both male and female hyperprolactinemia. However, both DHA and DHAS levels in hyperprolactinemic patients overlap with normoprolactinemic controls (*Vermeulen* et al. 1977). This might explain the different findings of several investigators that suppression of PRL to normal does not always result in a decrease of those steroids (*Carter* et al. 1977; *Kandeel, Rudd, Butt, Edwards* and *London* 1978; *Parker, Chang* and *Odell* 1978). Chronic but not short-lived drug induced hyperprolactinemia results in hypersecretion of DHA-S; during pregnancy this could not be observed (*Vermeulen* and *Ando* 1978). Correlations between serum PRL levels in the fetus and weight of the fetal adrenal gland, however, have been established (*Winters, Colston, MacDonald* and *Porter* 1975) suggesting that ACTH and PRL exert a synergistic stimulatory effect on the adrenals. Moreover, ACTH stimulation induced a greater release of DHA-S in hyperprolactinemic than in control subjects; other steroids such as cortisol, 17-β-androgens were not affected (*Vermeulen* and *Ando* 1978; *Kandeel* et al. 1978). These findings favour the view that the 3-β-ol-dehydrogenase converting $\Delta^5$ to $\Delta^4$ steroids is impaired in hyperprolactinemia, a result which was shown in animal experiments (*Mathno, Bartke* and *Weiß* 1975). This is underlined by the observation of *Kandeel* et al. 1978) that androstenedione showed a transient rise after PRL suppression and was found to be lower in hyperprolactinemic male hypogonadism (*Hanker,* personal communication).

## VI. Prolactin and osmoregulation

The role of PRL in the regulation of salt and water metabolism is well established in teleost fishes, amphibians and birds (for references see *Carey, Johanson* and *Seif* 1977). In subjects undergoing chronic hemodialysis elevated PRL levels were found in relation to both gynecomastia and galactorrhea (*Nagel, Freinkel, Bell, Friesen, Wilber* and *Metzger* 1973). Such elevations of PRL suggested that this hormone may be involved in human osmoregulation. However, changes in PRL secretion occur only in about 20% of anephric patients (*Chirito, Gonda* and *Friesen* 1972) and may reflect a general disturbance of the hypothalamo-pituitary axis (*Ramirez, O'Neill, Bloomer* and *Jubiz* 1977), since the secretion of other proteo-hormones may also be altered (*Ølgaard, Hagen* and *McNeilly* 1975). Nevertheless, serum PRL and creatinine concentrations show significant correlations and both parameters become normal after successful renal transplantation (*Cowden, Ratcliffe, Dobbie* and *Kennedy* 1978). Horrobin and colleagues (*Horrobin, Lloyd, Lipton, Burstyn, Durkin* and *Muiruri* 1971) reported striking renal actions of exogenous ovine PRL including reduced excretion of water, sodium and potassium, but this may be attributable to the contamination of the PRL preparations with vasopressin (*Carey, Johanson* and *Seif* 1977). Water loading and sodium excretion of hypo- and hyperosmolar saline solutions were suggested to alter PRL secretion (*Buckman* and *Peak* 1973), but this was not supported for either normo- or hyperprolactinemic subjects (*Archer* and *Josimovich* 1975; *Adler, Noel, Wartofsky* and *Frantz* 1975; *Baumann, Marynick, Winters* and *Loriaux* 1977). Suppression of PRL secretion by Bromocriptin was shown to inhibit the increase of serum aldosterone after furosemide treatment seen in normal volunteers (*Edwards, Miall, Hanker, Thorner, Al-Dujaili* and *Besser* 1975). Recently metoclopramide was shown to increase aldosterone serum concentrations in hypophysectomized patients. This suggests that both, Bromocriptin as well as metoclopramide interfere with aldosterone secretion by a direct action on dopaminergic neurons (*Norbiato, Bevilacqua, Raggi, Micossi* and *Moroui* 1977) at the kidney (*Ølgaard, Hagen, Madsen* and *Hummer* 1977). Thus, effects of PRL on mineralcorticoid secretion should be viewed with caution as should the apparent effects of PRL on both renal function and osmoregulation.

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EDITORIAL

# Lactational amenorrhoea, prolactin and contraception

The development of new contraceptive methods which are safe and effective remains a research area of high priority. The most efficient contraceptive is the combined oestrogen–progestagen oral contraceptive pill which acts by suppressing ovulation. Unfortunately, oestrogens have widespread metabolic effects which lead to numerous, and sometimes dangerous, side-effects [1]. It would be of great advantage if a contraceptive could be devised which interrupted ovulation without producing the unwanted metabolic effects of the pill.

Lactational amenorrhoea is nature's own contraceptive, when prolonged periods of infertility occur without demonstrable side effects. Following the recognition of human prolactin and the development of specific radioimmunoassay methods for its measurement, understanding of the mechanisms underlying lactational amenorrhoea have advanced rapidly in the last few years and, in the long-term, could lead to the evolution of new approaches to contraception.

In Western societies, the contraceptive effect of lactational amenorrhoea has been largely dismissed on the grounds that it is unreliable because ovulation often occurs before the return of menstruation [2]. Data from developing countries, however, indicate the profound effect which breast feeding can have on fertility [3]. In the !Kung hunter gatherers of the Kalahari, breast feeding without supplementation continues for 3–4 years and, in the total absence of contraception, this results in an average birth interval of 35.4 months [4]. When the !Kung move from a nomadic life to settle in an agricultural community, breast feeding is supplemented with grain meal and cow's milk and the birth interval falls to below 3 years. Similarly, in Rwanda, rural women breast feed on demand and 50% conceive again within 23 months after delivery; by contrast, in urban women, breast feeding is operated on a more rigid schedule with fewer feeding episodes, and 50% of women have conceived again within 9 months post-partum [5]. This evidence suggests that traditional patterns of frequent suckling without supplementation play an important role in maintaining the birth spacing effect of prolonged lactation. It is well known that suckling leads to the release of prolactin from the anterior pituitary and it has been shown in prolonged lactation that the basal prolactin levels are related both to the frequency of suckling episodes [6] and to the duration of lactational amenorrhoea [7]. Hyperprolactinaemic states are associated with infertility and when this is corrected by bromocryptine (CB 154), fertility returns to normal [8]. It is possible, therefore, that suckling-induced prolactin release is the initiating factor in the control of puerperal infertility.

If prolactin has an important role, it is not clear whether it acts directly on the ovary, on the hypothalamus, or on both. Throughout pregnancy, levels of oestrogen and progesterone are high but fall rapidly after delivery of the placenta [9]. Follicle stimulating hormone (FSH) is suppressed by the high level of oestrogens during pregnancy but increases after delivery to reach the normal follicular phase range by 15–20 days post partum. In non-lactating women, oestradiol levels rise about day 17 post partum, indicating a resumption of follicular activity when prolactin levels have returned to normal [9]. By contrast, oestradiol levels are suppressed in lactating women and this absence of ovarian follicular development may persist for many months despite normal FSH levels [10]. *In vitro* studies have shown that raised prolactin levels inhibit progesterone production by granulosa cells so that, during normal lactation, the ovary may be refractory to gonadotrophins when prolactin levels are raised [11]. On the other hand, evidence against a direct inhibiton at the ovarian level has come from the studies in which gonadotrophins or luteinizing hormone releasing hormone (LHRH) have been used to stimulate ovarian activity in puerperal women. After an initial refractory phase for the first 7–15 days post partum, gonadotrophins, whether given exogenously or secreted endogenously in response to LHRH, are capable of stimulating ovarian follicular development and oestrogen secretion [12]. This indicates that, with adequate stimulation, it is possible to overcome any direct inhibitory effect that prolactin might have on the gonad.

The alternative pathway in which prolactin could act is through an effect on the hypothalamic–pituitary axis. In pathological hyperprolactinaemic states, the pulsatility of luteinizing hormone (LH) secretion is absent [13] and it is possible that a similar mechanism operates in the maintenance of lactational infertility. For the first 15 days after delivery it is not possible to measure LH because of cross reaction with human chorionic gonadotrophin in the radioimmunoassays used. It is probable that LH levels are low because, in late pregnancy, the LH content in the pituitary is 1% of the normally cyclic woman [14]. In lactating women, LH levels have reached low normal by 15–20 days post partum, but there is no evidence of the pulsatility of secretion, which is seen in normally cycling women [15]. LH levels rise when weaning occurs and prolactin levels fall [16].

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The absence of LH pulsatility in lactating women suggests a reduced hypothalamic secretion of LHRH. When LHRH is administered to lactating women after the initial refractory phase, there is, at 6–8 weeks post partum, a normal FSH and a diminished LH response [12]. In prolonged lactation of over a year, LHRH induces an exaggerated FSH release and an LH response similar to that found in the luteal phase. This evidence indicates that, during lactation, the pituitary is capable of releasing gonadotrophins but that the LH response to LHRH lags behind the FSH response.

During the normal menstrual cycle the mid-cycle peak of LH at ovulation is induced by the progressively rising levels of oestrogen during the follicular phase. If oestrogen is injected there is initially a negative feedback effect which suppresses LH and FSH levels followed by a positive feedback effect when both LH and FSH are released from the pituitary. In a recent study, oestrogen was given to lactating and non-lactating women and the negative feedback suppression of FSH and LH was more marked in the lactating women [10]. Furthermore, there was a failure of positive feedback effect in the breast-feeding group. From this evidence, it seems likely that a failure of a normal hypothalamic response to oestrogen may be the central feature in maintaining lactational amenorrhoea and it is possible that this effect is dependent on raised prolactin levels.

In summary, therefore, a hypothesis concerning the mechanisms of lactational amenorrhoea can be offered. The raised prolactin levels induced by suckling inhibit the normal positive feedback effect of oestrogen on the hypothalamus. This leads to diminished LHRH production and loss of LH pulsatile secretion from the pituitary which prevents ovulation from taking place.

Many questions, however, remain to be answered. It is not certain whether it is prolactin itself that inhibits ovulation or whether it simply acts as a marker of disordered hypothalamic activity. It is possible, for example, that the suckling stimulus *per se* is the afferent input which alters hypothalamic sensitivity to steroid feedback during lactation and that hyperprolactinaemia is of secondary importance. It would also be of interest to know whether drugs which stimulate prolactin secretion could be used to reinforce the contraceptive effects of lactation. If so, they might be a more attractive alternative method of contraception for lactating women than the commonly used progestagen-only contraceptive pills. Sulpiride is one drug which can induce hyperprolactinaemia and has been used to induce amenorrhoea in women who were not breast feeding. Sulpiride induces galactorrhoea which limits its potential use as a contraceptive in non-nursing women. It would be of great importance, therefore, if a drug could be developed which had the contraceptive activity of prolactin without the milk-secreting effects.

From the more immediate practical viewpoint, it should be recognized that lactational amenorrhoea is of immense importance in developing countries where alternative methods of contraception are either not available or not acceptable. Indeed, it has been suggested that, in the world as a whole, breast feeding prevents more pregnancies that all other methods of contraception put together [17]. This should be borne in mind when infant feeding programmes are introduced which interfere with traditional feeding practices. More research is required which relates suckling frequency to prolactin levels and the events which control ovulation. Such work might point the way towards new, effective and safe methods of contraception.

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# Prolactin and the control of gonadotrophin secretion in the female

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It is not intended that this paper should present an exhaustive review of the literature concerning the interaction between prolactin and gonadotrophin release. Rather it is hoped that it will present a more critical analysis of some of the areas where prolactin appears to be involved or at least associated with alterations in the secretion and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). To this end, it is first necessary to review briefly the hypothalamic control of prolactin and gonadotrophin secretion followed by an appraisal of the relationship between prolactin and gonadotrophin secretion during the normal oestrous and menstrual cycle. Finally, consideration will be given to the role of hyperprolactinaemia in gonadotrophin secretion, an area which has recently received much attention.

## Hypothalamic control of prolactin secretion

Many experiments confirm that the major hypothalamic influence over prolactin secretion from the anterior pituitary is inhibitory (see MacLeod & Lehmeyer, 1972; Meites, Simpkins, Bruni & Advis, 1977; Meites, 1977; Lancranjan & Friesen, 1978). While it was initially expected that the prolactin inhibitory factor (PIF) would be a small peptide, a growing body of evidence supports the concept that the PIF is dopamine. Using histochemical and immunochemical methods, Fuxe, Goldstein, Hökfelt, Jonsson & Lidbrink (1974) and Hökfelt *et al.* (1975) identified a dopaminergic pathway within the tubero-infundibular neurones which appears to control prolactin secretion. This pathway originates mainly from the arcuate nucleus and the ventral part of the anterior periventricular nucleus and ends in the external layer of the median eminence. Here the dopamine nerve terminals are adjacent to the hypophysial portal capillaries which carry the hypothalamic regulatory factors to the anterior pituitary. Cuts made in the hypothalamus which maintained the tubero-infundibular neurone–median eminence intact but isolated it from the rest of the brain had no effect on serum levels of prolactin, whereas increased secretion of prolactin occurred if this connection was severed (Butler, Krey, Lu, Peckham & Knobil, 1975). Dopamine *in vitro* and *in vivo* inhibits prolactin secretion from the pituitary (see MacLeod & Lehmeyer, 1974, for references). More recently, dopamine has been detected in hypophysial portal vessels at levels which will inhibit prolactin secretion by a direct action on the pituitary lactotrophs (Ben-Jonathan, Oliver, Weiner, Mical & Porter, 1977; Gibbs & Neill, 1978; Plotsky, Gibbs & Neill, 1978; Gudelsky & Porter, 1979; Ben-Jonathan, 1980). Finally, specific dopamine receptors have been demonstrated on the cells of the anterior pituitary (Brown, Seeman & Lee, 1976; Caron, Drouin, Raymond, Kelly & Labrie, 1976), further reinforcing the argument that dopamine is a major, if not the only, PIF.

There is no doubt, however, that agents other than dopamine are involved in the secretion of prolactin. No particular agent has yet been identified as a specific prolactin-releasing factor (PRF). While thyrotrophin-releasing hormone (TRH) releases prolactin as well as thyroid-

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stimulating hormone (TSH), the physiological role of TRH in the control of prolactin secretion remains uncertain. In many physiological situations, such as suckling, sleep and the response to cold stress, prolactin and TSH responses occur independently. Support for the role of TRH came from the demonstration that passive transfer of antiserum to TRH into rats caused a 50 and 70% decrease in serum levels of prolactin and TSH respectively (Koch *et al.*, 1977). However, such dramatic effects on prolactin secretion were not seen in ewes actively immunized against TRH, even though thyroid function had been reduced by over 50% (H. M. Fraser, P. Osman & A. S. McNeilly, unpublished observations).

It is possible that the serotoninergic pathway within the brain is involved in the stimulation of prolactin release. Serotonin (5-hydroxytryptamine) and the serotonin precursor 5-hydroxytryptophan can increase the secretion of prolactin via an action on the hypothalamus (Kamberi, Mical & Porter, 1971a; Clemens, Sawyer & Cerimele, 1977) and the suckling-induced release of prolactin is related to an increase in 5-hydroxytryptamine turnover (Mena, Enjalbert, Carbonnell, Priam & Kordon, 1976).

A most recent development is the demonstration that opiate receptor agonists are powerful releasers of prolactin. Thus both methionine- and leucine- enkephalin and β-endorphin cause an increase in circulating levels of prolactin (Dupont, Cusan, Caron, Labrie & Li, 1977; Cusan *et al.*, 1977; Rivier, Vale, Ling, Brown & Guillemin, 1977) and β-endorphin has been implicated in both stress- and suckling-induced release of prolactin (Ferland, Kledzik, Cusan & Labrie, 1978). It has been suggested that β-endorphin may stimulate prolactin secretion by reducing the release of dopamine from the tubero-infundibular neurones (Van Loon, Ho, Kim, De Souza & Shin, 1979).

Oestrogens have been shown to increase the secretion of prolactin by an action at both the pituitary and the hypothalamus (see Lancranjan & Friesen, 1978, for references) and oestradiol can block the inhibitory action of dopamine on the pituitary lactotrophs (Labrie, Baulieu, Caron & Raymond, 1978). If a similar action was to occur in the hypothalamus, there would be a reduction in the effectiveness of dopamine to inhibit prolactin secretion, resulting in increased release.

Of physiological importance is the auto-regulatory short-loop feedback control of prolactin secretion. High circulating levels of prolactin have been shown to inhibit prolactin release by the pituitary *in situ* (Meites & Clemens, 1972; MacLeod, 1974), and increases in the circulating levels of prolactin increase dopamine turnover in the tubero-infundibular neurones of the hypothalamus (Fuxe *et al.*, 1974; Hökfelt *et al.*, 1975; Gudelsky, Simpkins, Mueller, Meites & Moore, 1976; Advis, Hall, Hodson, Mueller & Meites, 1977). Since it may be assumed that dopamine is a PIF (Ben-Jonathan, 1980), then the increase in dopamine turnover probably also reflects an increase in dopamine release and therefore will cause the suppression of prolactin secretion by the pituitary *in situ*. A protein immunochemically similar to prolactin has been detected in the hypothalamus of the rat (Fuxe, Hökfelt, Eneroth, Gustafsson & Skett, 1977). It is not yet clear whether this material is prolactin, and it may be synthesized in the hypothalamus or represent a binding of prolactin to receptors there. Oliver, Mical & Porter (1977) have demonstrated that while levels of prolactin in the peripheral circulation of normal rats were around 25 ng/ml, values of nearly 5000 ng/ml were found in the long hypophysial portal vessels. Oliver *et al.* (1977) suggested that since there appeared to be countercurrent flow of blood within the long portal vessels, then the high levels of pituitary hormones (including prolactin) reaching the hypothalamus by this route could modulate neurotransmitter synthesis and release. It is apparent that an autoregulatory, short-loop negative feedback operates for prolactin and appears to act by increasing the dopamine turnover of the tubero-infundibular neurones.

Since modulation of dopamine output is intimately involved in the control of prolactin secretion, it is important to understand the role of dopamine in the control of gonadotrophin secretion. Initial experiments by Schneider & McCann (1969) suggested that dopamine stimulated the release of luteinizing hormone-releasing hormone (LHRH) and hence LH. How-

ever, subsequent studies strongly suggest that dopamine is inhibitory to LHRH release, while brain-stem noradrenergic neurones appear to facilitate LHRH release (see Fink, 1979, for references).

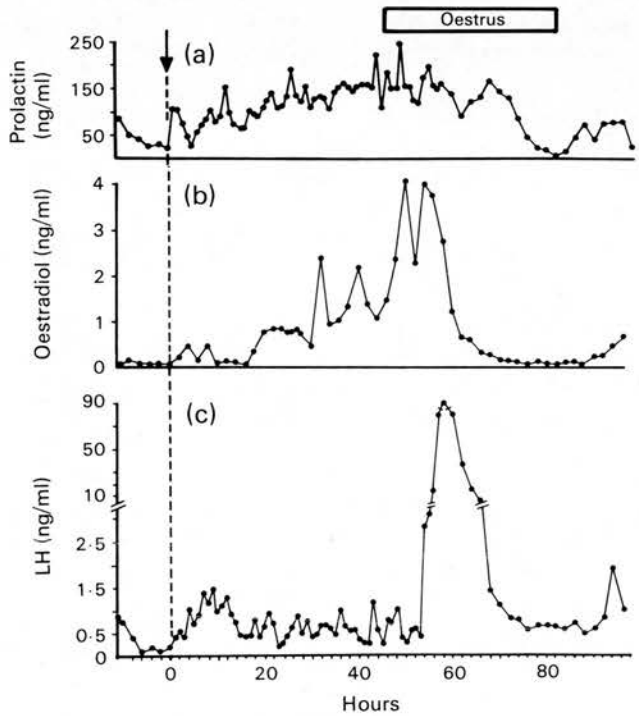
In the rat, infusions of dopamine (Vijayan & McCann, 1978) or stimulation of dopamine receptors (Drouva & Gallo, 1977) inhibit the pulsatile secretion of LH. In men and women, short-term infusions of dopamine suppress serum levels of LH (Leblanc, Lachelin Abu-Fadil & Yen, 1976; Lachelin, Leblanc & Yen, 1977; Judd, Rakoff & Yen, 1978), while administration of a dopamine antagonist, metoclopramide (McNeilly, Thorner, Volans & Besser, 1974), resulted in an acute increase in serum levels of LH and FSH in hyperprolactinaemic patients (Quigley, Judd, Gilliland & Yen, 1979). While these data clearly suggest an action of dopamine at the hypothalamus, dopamine or the dopamine agonist CB154 may slightly reduce the LH response to LHRH (Judd *et al.*, 1978; McNeilly & Land, 1979), suggesting an effect directly on the gonadotrophs, although this effect is small (Kamberi *et al.*, 1971a, b). It is unclear whether increased dopamine turnover alone can maintain the suppression of LH and FSH secretion. When dopamine turnover was increased by increased circulating levels of prolactin secreted from donor pituitary grafts in ovariectomized female rats, the suppression of LH pulsatility was not sustained beyond 10 days (Beck, Engelbart, Gelato & Wuttke, 1977; Beck & Wuttke, 1977). The increase in hypothalamic dopamine turnover in these animals appeared to be sustained for at least 14 days in spite of elevated prolactin levels (Löfström Jonsson, Wiesel & Fuxe, 1976). In the absence of the gonads, therefore, the hypothalamus appears to become less sensitive, in terms of LHRH secretion, to the inhibitory effects of the increase in dopamine turnover, as demonstrated by Beck & Wuttke (1977). In the presence of the gonads, however, suppression of LH and FSH levels is maintained, clearly suggesting an interaction between increased hypothalamic dopamine turnover and the negative feedback effects of gonadal steroids (McNeilly, Sharpe, Davidson & Fraser, 1978).

## Prolactin and the normal pattern of gonadotrophin release

The most dramatic change in circulating levels of LH and FSH occurs around the time of the preovulatory surge of LH. Before this surge, there is an increase in the pulse frequency of release of LH (Midgley & Jaffe, 1971; Yen, Lasley, Wang, Leblanc & Siler, 1975; Baird, 1978), presumably reflecting an increase in the release of hypothalamic LHRH (see Fink, 1979; Lincoln, 1979). The increase in secretion of LH stimulates increased secretion of oestrogens from the developing ovarian follicle(s) and this increase precipitates the preovulatory surge of LH. A peak of prolactin coincident with this surge of LH occurs in the rat (Neill, Freeman & Tillson, 1971), mouse (Kwa & Verhofstad, 1967) and hamster (Bast & Greenwald, 1974). The increase in prolactin in the rat can be prevented by blockade of the pro-oestrous rise in oestradiol by injection of antiserum either to oestradiol (Neill *et al.*, 1971) or LH (Freeman, Reichert & Neill, 1972). Since oestradiol increases prolactin secretion in the rat (Amenomori & Meites, 1970), it seems probable that the pro-oestrous rise of prolactin in rats occurs as a result of the increase in pro-oestrous oestradiol secretion.

In sheep, a rise in serum prolactin occurs after luteal regression and continues until the end of the ovulatory LH surge (Reeves, Arimura & Schally, 1970; Cumming, Brown, Goding, Bryant & Greenwood, 1972; Kann & Denamur, 1974; Text-fig. 1). Since administration of oestradiol will result in an increase in circulating levels of prolactin (Fell, Beck, Brown, Cumming & Goding, 1972), it has been suggested that the pro-oestrous rise in prolactin in the sheep, as in the rat, is related to the increasing levels of oestradiol during the periovulatory period. However, a closer examination of the time course of release of prolactin in relation to the secretion of oestradiol suggests that the increase in prolactin occurs before any dramatic change in circulating levels of oestradiol (A. S. McNeilly & D. T. Baird, unpublished observations; Text-

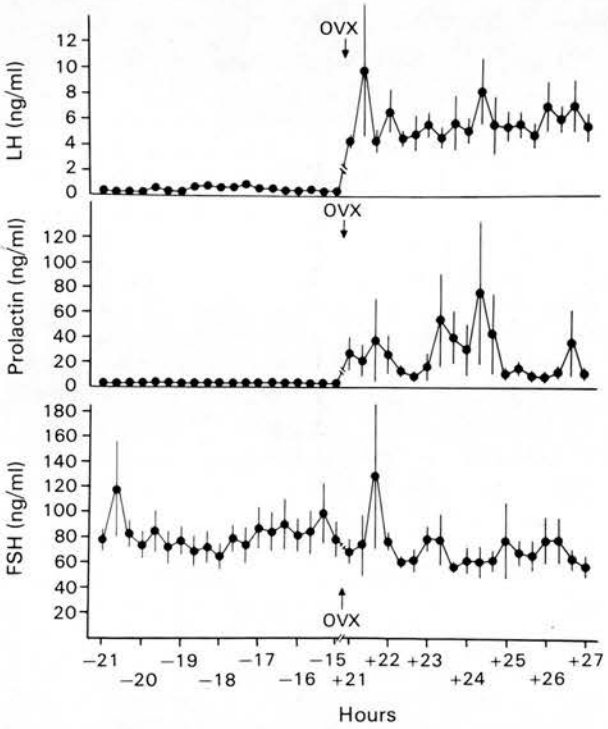
fig. 1). This suggests that a factor other than oestradiol may be responsible for the initial increase in release of prolactin. During this initial periovulatory period, the pulse frequency of LH (and by implication LHRH) increases around the same time as the increase in prolactin. Since dopamine appears to be inhibitory to LHRH release, an increase in LHRH release may require a decrease in hypothalamic dopamine turnover. Thus, both the increase in pulse frequency of LH and the increase in circulating levels of prolactin may reflect a decrease in hypothalamic dopamine and the change in prolactin occurs as a consequence and not as a cause of this decreased turnover. That prolactin *per se* is not implicated in the change in levels of LH is suggested by the fact that, in the cyclic ewe, LH levels, ovulation and luteal function are not affected by suppression of prolactin levels with ergocornine hydrogen maleinate or CB154 (2α-bromo-ergocryptine) (Louw, Lishman, Botha & Baumgartner, 1974; Niswender, 1974).




**Text-fig. 1.** Changes in serum levels of (a) prolactin, (b) oestradiol-17β and (c) LH after induction of luteal regression with a prostaglandin analogue (ICI 80,996: arrow) in a ewe with an ovary transplanted to the neck. Prolactin and LH were measured in jugular and oestradiol in ovarian venous blood samples. Note the increase in prolactin concentration after the analogue injection followed by a further increase before any significant increase of oestradiol concentrations occurred. (A. S. McNeilly & D. T. Baird, unpublished observations.)

If increased pulsatile release of LHRH requires a decrease in hypothalamic dopamine turnover, then a concomitant increase in prolactin levels should also be expected. After ovariectomy in the ewe, LH pulse frequency increases to 15–30 pulses/24 h. This increase is accompanied by an increase in the circulating levels of prolactin (I. J. Clarke & A. S. McNeilly, unpublished observations; Text-fig. 2). A similar situation arises after ewes are actively immunized against LHRH. Because of the neutralization of LHRH by antibody in the circulation, the secretion of both LH and FSH from the pituitary is dramatically reduced and ovarian follicular development is attenuated. In the absence of gonadal steroids, it may be expected that the hypothalamus responds to an apparent castrated situation with an increase in the secretion of LHRH.

It is of considerable interest, therefore, that in these ewes circulating levels of prolactin were 4–10 times higher than in controls (Clarke, Fraser & McNeilly, 1978). These results suggest the possibility that the rise in plasma prolactin seen at oestrus in the ewe may be related not only to the rise in circulating oestradiol but also to an initial decline in the hypothalamic dopamine turnover. Oestradiol may amplify such an effect by further blocking the action of dopamine at its receptor (Labrie *et al.*, 1978).




**Text-fig. 2.** Changes in serum levels (mean ± s.e.m.) of LH, prolactin and FSH before and after ovariectomy (OVX) in 8 ewes. (I. J. Clarke & A. S. McNeilly, unpublished results.)

In the chimpanzee (Reyes, Winter, Faiman & Hobson, 1975), rhesus monkey (Butler *et al.*, 1975), common marmoset (A. S. McNeilly, D. H. Abbott, S. F. Lunn, P. Chambers & J. P. Hearn, unpublished results) and man (McNeilly & Chard, 1974), no consistent changes in circulating levels of prolactin have been found throughout the cycle, although prolactin concentrations may be higher at midcycle in women (Robyn *et al.*, 1973). A direct relationship between the levels of oestrogen and prolactin have been reported by some (Robyn *et al.*, 1973; Franchimont *et al.* (1976) but not by other (McNeilly & Chard, 1974; Epstein, McNeilly, Murray & Hockaday, 1975) authors. It is clear that in man, as in sheep, there is an increase in LH pulse frequency before the ovulatory surge of LH (Yen *et al.*, 1975). In the periovulatory period of the menstrual cycle, infusion of dopamine into normal women caused a dramatic decrease in the levels of LH, an effect which was not dependent on the concentration of oestradiol but was related to the increased basal level of LH in the women (Judd *et al.*, 1978). Dopamine infusion earlier in the menstrual cycle was without effect. While basal levels of prolactin were not significantly different between early and mid-cycle, the inhibition of prolactin release by dopamine was correlated with endogenous oestradiol levels. Judd *et al.* (1978) conclude that "the selective hypersensitivity of both LH and FSH to DA observed on the day

before the mid cycle LH peak is consistent with a reduction in LHRH neuronal inhibition by tubero-infundibular DA neurones at this time."

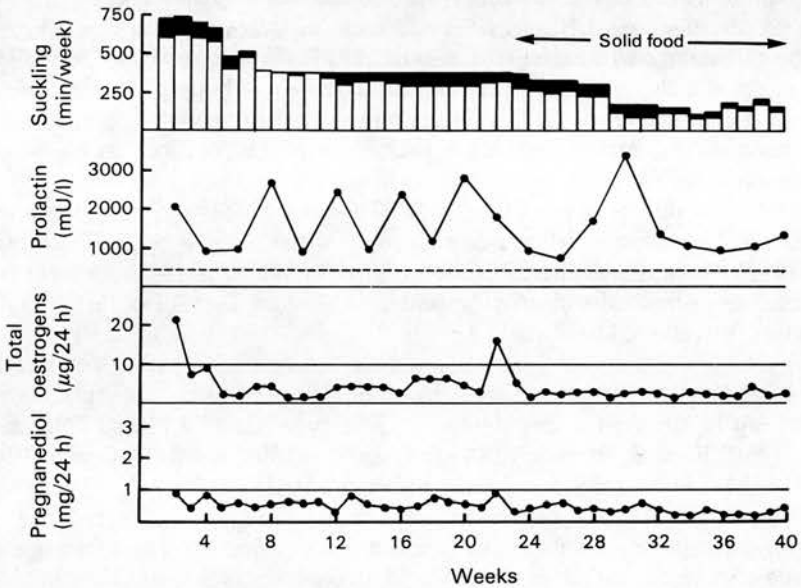
It is clear that in women and sheep the reduced hypothalamic turnover of dopamine in the periovulatory period is similar. It is probable, therefore, that the change in prolactin, if it occurs around the time of the LH surge, is related not only to the increased secretion of ovarian oestradiol, but also to a decrease in dopamine secretion, and occurs as a consequence and not as a cause of the changes occurring around this time.

## Hyperprolactinaemia and lactational amenorrhoea

Hyperprolactinaemia is associated with lactational anoestrus and amenorrhoea, pathological amenorrhoea and in sheep with seasonal anoestrus. It is not proposed to discuss the latter in this review as this topic has been dealt with by Karsch, Goodman & Legan (1980). The role of prolactin in lactational amenorrhoea has recently been reviewed (for references, see McNeilly, 1979) and will only be discussed briefly here.

During pregnancy in women, circulating concentrations of prolactin rise to reach values by term that are 4–20 times those in non-pregnant women. This increase in prolactin probably occurs in response to the increasing levels of placental oestrogens during pregnancy. The high steroid concentrations are probably responsible for the reduction in circulating FSH values and pituitary content of LH at term. After delivery, prolactin concentrations in non-lactating women decline to normal over a 15–20-day period, LH and FSH levels return to normal within about 28 days, with resumption of ovarian follicular development, ovulation and corpus luteum function within 30–40 days (for references, see McNeilly, 1979). In contrast, circulating concentrations of prolactin are maintained by suckling in lactating women, and hyperprolactinaemia may last up to 2 years (Delvoye, Demaegd, Delonge-Desnoek & Robyn, 1977; Duchen & McNeilly, 1980). During lactation in women FSH increases to high normal values within 20 days of delivery and remain at this level until ovarian activity resumes. In contrast serum concentrations of LH increase to low normal values by 20 days but remain low with absent or reduced pulsatility during the period of lactational amenorrhoea. If an adequate suckling frequency and duration is maintained in lactation, pulsatile secretion of LH remains inadequate to stimulate ovarian follicular development and oestradiol secretion. As the suckling stimulus is reduced in the later stages of lactation, LH secretion increases sufficiently to stimulate follicular development with an increase in circulating oestradiol levels. Nevertheless, during the first 100 days of lactation, women do not show a positive feedback surge of LH in response to this oestradiol (Baird, McNeilly, Sawers & Sharpe, 1979) and they are more sensitive to the negative feedback effects of oestrogen. Thus, while ovarian follicular development with increased oestrogen can occur during lactation, provided that the suckling stimulus is sufficient and hyperprolactinaemia is maintained, ovulation and normal luteal function will not take place (Duchen & McNeilly, 1980; A. S. McNeilly, P. W. Howie & M. Houston, unpublished observations; Text-fig. 3). The pituitary gland during this time contains adequate stores of LH and FSH, as assessed by responses to LHRH (see Jeppson, Rannevik & Kullander, 1974; Keye & Jaffe, 1976; Delvoye, Badawi, Demaegd & Robyn, 1978). It is apparent that high circulating levels of prolactin are related to the maintenance of lactational amenorrhoea (Delvoye *et al.*, 1978; Duchen & McNeilly, 1980). Maintenance of these levels depends on the maintenance of an adequate suckling stimulus, with long-term hyperprolactinaemia being maintained provided the suckling frequency is more than 6 times per day (Delvoye *et al.*, 1978). Our own data suggest that the prolactin concentration is also related to the total time of the suckling stimulus over a 24-h period (A. S. McNeilly, P. W. Howie & M. Houston, unpublished observations; Text-fig. 3). Thus, even at a stage of lactation when each suckling episode may not release prolactin, the stimulus is adequate to maintain hyperprolactinaemia (Delvoye *et al.*, 1977, 1978) and inhibit

the normal release pattern of LH required for resumption of normal menstrual cyclicity. That the inhibition is readily reversible is demonstrated by the resumption of full ovarian activity with ovulation occurring 14–30 days after weaning, when there is an immediate drop in blood concentrations of prolactin (Rolland, Lequin, Schellekens & de Jong, 1975). Thus, in women at least, lactation is associated with a period of anovulation, this period being up to or more than 2 years in some instances. Since hyperprolactinaemia in lactation is dependent upon the maintained suckling stimulus, it is unclear whether it is the neural suckling stimulus, the elevated prolactin levels, increased sensitivity to gonadal steroid negative feedback, or a combination of these which is important for the maintenance of lactational anovulation.




**Text-fig. 3.** Post-partum changes in urinary total oestrogens and pregnanediol in relation to the total weekly suckling time and serum levels of prolactin in a breast-feeding woman. The total duration of the night-time feed is also indicated (solid columns). (A. S. McNeilly, P. W. Howie & M. Houston, unpublished results.)

Pathological hyperprolactinaemia in women is associated with amenorrhoea which appears similar to that of lactation. There is no ovarian function, reduced or absent pulsatility of LH secretion, and an absence of positive feedback response to oestrogen (Besser, Parke, Edwards, Forsyth & McNeilly, 1972; Glass, Shaw, Butt, Logan-Edwards & London, 1975; Bohnet, Wiest, Dahlen & Schneider, 1975). When prolactin levels are reduced to normal by the removal of a prolactin-secreting microadenoma or treatment with the dopamine agonist bromocriptine, normal ovarian cyclicity returns. While the use of a dopamine agonist might be interpreted as a repletion of depleted hypothalamic dopamine reserves, removal of a discrete microadenoma secreting only prolactin clearly implicates prolactin *per se* as a major factor in maintaining the anovulatory state.

Selective pharmacological elevation of prolactin in women with dopamine receptor blocking drugs, e.g. Sulpiride (Robyn *et al.*, 1976) or TRH (Bohnet & Schneider, 1977), can induce a loss in pulsatile secretion of LH and of the positive feedback of oestrogen on LH and FSH and an increase in sensitivity to the negative feedback effects of oestrogen. This further suggests that prolactin may be the causal agent. Nevertheless, in the absence of the gonads, even in the presence of elevated levels of prolactin, gonadotrophin concentrations may be elevated to within the normal post-menopausal range. This suggests that while prolactin may play a vital role, this

may be to sensitize the hypothalamus to the negative feedback effects of low levels of gonadal steroids.

Evidence from the sheep would support a major role for hyperprolactinaemia in suppressing gonadotrophin secretion during lactational anoestrus. Kann & Martinet (1975) and Kann, Martinet & Schirar (1977) showed that the resumption of oestrous activity in sheep was delayed by lactation. Suppression of prolactin secretion either by treatment of ewes with bromocriptine or severance of the neural pathway for the suckling stimulus resulted in an earlier onset of ovarian activity. After neural pathway severence, both the release of prolactin and the neural response to suckling were absolished, while bromocriptine treatment resulted in maintenance of the suckling stimulus but not of the suppression of gonadotrophin secretion. The positive feedback effect of oestrogen on LH and FSH secretion in lactating ewes is suppressed or absent during the first 21 days *post partum* (Kann *et al.*, 1977; Wright, Findlay, Geytenbeck & Clarke, 1979), an effect which can be induced in ewes rendered hyperprolactinaemic by repeated injections of TRH (Kann *et al.*, 1977). These results, while not conclusive, suggest that prolactin rather than the suckling stimulus plays the major role in the post-partum inhibition of gonadotrophin secretion in the ewe.

Data for the cow do not support the above observations. It is well recognized that the return to oestrus in suckling cows is delayed compared with that of cows milked only twice daily (see Lamming, 1978). Since there is no difference in serum levels of prolactin in these two situations, the neural suckling stimulus is clearly implicated in the maintenance of lactational anoestrus in the cow (Peters, Vyvoda & Lamming, 1979).

Over the past few years, many investigators have concentrated efforts on understanding the mechanisms responsible for maintaining lactational anoestrus in the rat. During lactation, ovarian cyclicity is inhibited and serum and pituitary levels of LH and FSH are suppressed (Rothchild, 1960; Ford & Melampy, 1973; Hammons, Velasco & Rothchild, 1973; Smith & Neill, 1977). The LH and FSH response to both LHRH (Lu, Chen, Grandison, Huang & Meites, 1976a; Smith, 1978a) and oestrogen (Smith, 1978b) are also reduced, suggesting a reduction in hypothalamic stimulation of gonadotrophin secretion. The suckling stimulus alone, in the absence of elevated prolactin levels, led to an earlier resumption of ovarian activity (Lu *et al.*, 1976b), suggesting that prolactin alone plays an important part in the suppression of gonadotrophin secretion. The relative contributions of the suckling stimulus and the increased prolactin level resulting from this stimulus have been further investigated by measuring the serum LH and FSH responses to ovariectomy during lactation. In the rat the neural stimulus from suckling does not independently suppress LH secretion (Smith, 1978c). During the early stages of lactation the suckling stimulus contributes more than prolactin to the suppression of the post-castration rise in LH but has little effect on FSH. During the second half of lactation the suckling stimulus declines in effectiveness while the relative contribution of prolactin in inhibiting the LH and FSH response to castration increases (Smith & Neill, 1977; Smith, 1978c).

A complicating feature of lactation in the rat and other rodents is the maintenance of corpora lutea secreting substantial amounts of progesterone. This does not occur in ruminants and primates, and because progesterone has a facilitatory role in the positive feedback effects of oestrogen on LH and FSH secretion during lactation in the rat (Smith, 1978b), interpretations based on data from the rat must be applied with caution to results from other species.

Our own studies have concentrated on investigating the effects of hyperprolactinaemia in the adult male rat. This was chosen because elevation of endogenous prolactin could be achieved by placing pituitary grafts under the kidney capsule, and the male was chosen to overcome the difficulties associated with the ovarian dependence on prolactin in the female rat. Hyperprolactinaemia in the intact adult male rat results in a maintained suppression of serum and pituitary levels of LH and FSH with no change in the circulating levels of testosterone. The pattern of suppression of LH and FSH was highly suggestive of a reduction in the hypothalamic secretion of LHRH (McNeilly *et al.*, 1978). Removal of the adrenals was without effect on these

responses (A. S. McNeilly & R. M. Sharpe, unpublished observations), whereas removal of the gonads resulted in an immediate increase in the serum concentrations of LH and FSH to levels found after castration (McNeilly, Sharpe & Fraser, 1979). This result clearly suggested that prolactin acted by increasing the sensitivity of the hypothalamus to the negative feedback effects of gonadal steroids, a feature identical to that observed in lactational amenorrhoea in women (Baird *et al.*, 1979) and during seasonal anoestrus in sheep (see Karsch *et al.*, 1980), a time when serum concentrations of prolactin are elevated (Walton, McNeilly, McNeilly & Cunningham, 1977).

**Table 1.** Effects of testosterone on the post-castration increase in the mean ± s.e.m. serum levels of LH (ng/ml) 10 days after castration in control and pituitary-grafted (hyperprolactinaemic) adult male rats (no. in parentheses)

| | | Castrated | | |
|---|---|---|---|---|
| | | | Testosterone implant* | |
| | Intact | No treatment | 10 mm | 30 mm |
| Control | 72 ± 9 (4) | 508 ± 64 (4) | 12 ± 2 (8) | 344 ± 50 (8) |
| With pituitary grafts | 16 ± 4 (4) | 463 ± 67 (4) | 9 ± 1 (8) | 20 ± 6 (8) |

* Silastic implants (10 or 30 mm) of testosterone were placed s.c. 1 day before castration.

The LH castration response in adult male rats can be prevented by implants containing testosterone but the amount required is three times less than that needed in control castrated rats if the rats also had pituitary grafts maintaining hyperprolactinaemia (A. S. McNeilly & R. M. Sharpe, unpublished observations; Table 1). This clearly demonstrates that prolactin has increased the sensitivity of the hypothalamo–pituitary axis to the negative feedback effects of gonadal steroids. The mechanism behind this effect remains unknown but may be related to the increase in hypothalamic dopamine turnover induced by the hyperprolactinaemia and indicated by the highly significant decrease in the prolactin content of the pituitary *in situ*, presumably resulting from increased hypothalamic release of dopamine (PIF).

## Conclusions

During the normal oestrous and menstrual cycles, changes in serum levels of prolactin occur around the time of increased gonadotrophin secretion about ovulation. It is probable that the increase in oestrogen secretion during this periovulatory period plays an important role in increasing prolactin secretion. Nevertheless, since it appears that a decrease in hypothalamic dopamine turnover is necessary to facilitate the required increase in LHRH secretion, the rise in prolactin may merely reflect this decrease in the hypothalamic secretion of dopamine (PIF). The increased secretion of prolactin is probably not directly involved in the normal secretion of LH and FSH at ovulation.

During lactation, high circulating levels of prolactin are associated with an inhibition of gonadotrophin secretion and anoestrus or amenorrhoea result. In women, it is not yet possible to distinguish between the relative roles of the suckling stimulus and hyperprolactinaemia associated with lactational amenorrhoea. Clinical observations in pathological hyperprolactinaemia clearly suggest, however, that elevated serum levels of prolactin alone are sufficient to maintain inhibition of LH and FSH secretion and anovulation. Our recent

observations with hyperprolactinaemic male rats strongly suggest that elevated levels of prolactin may act by sensitizing the hypothalamus to the negative feedback effects of gonadal steroids.

It is clear, however, that our understanding of the mechanisms behind such an enhancement of sensitivity is very limited and this is an area which requires much more investigation.

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frequency radio waves, and sferics are not observed by satellites. But there are special circumstances where energy can propagate through in the so-called 'whistler mode'. These conditions, as measured by other instruments on the Orbiter, are thought to occur fairly frequently on the night side of Venus. When they do, the plasma-wave detector sees impulsive signals of a kind not normally encountered at orbital altitude. The instrument would need much better time resolution to obtain the length or shape of the pulses, but they are consistent with an origin from lightning and no other explanation has been suggested. Taken all together, the evidence for lightning on Venus is fairly convincing.

Ksanfomaliti also suggests that lightning could generate a mean nightside brightness $10^{-4}$ that of the day side. This source is put forward as an explanation of the 'ashen light', a phenomenon reported by many visual observers who see the whole disk faintly illuminated when the planet is in a crescent phase. It remains to be seen whether such suggestions and reports can be reconciled with the brightnesses observed from vehicles close to Venus.

The indications are that lightning is likely to occur in any substantial planetary atmosphere. Theories of electrification are faced with the need to explain its presence under a wide variety of circumstances and atmospheric compositions. □

# Paradoxical prolactin

*from Alan S. McNeilly*

LACTATION in many mammals is associated with suppression of ovulation as a result of the failure of ovarian follicles to develop to the appropriate stage. The widely varying suckling patterns in different societies (from very frequent short periods, for example in the !Kung peoples of the Kalahari, to infrequent longer periods of suckling) are all associated with suppression of normal gonadotropin secretion and of ovarian activity, and with raised basal levels of prolactin which increase further in response to the suckling stimulus (see McNeilly *Br. Med. Bull.* **35**, 151; 1979). As ovarian activity normally resumes once suckling ceases, either the suckling stimulus itself and/or the raised levels of prolactin may be responsible for the suppression of gonadotropin secretion and the consequent failure of normal follicular development.

The best evidence that the prolactin level itself may be responsible for ovulatory inhibition comes from cases of pathological hyperprolactinaemia which is the apparent cause of 20% of secondary amenorrhoeas. Unlike lactation, the raised prolactin levels in this condition may be due to an alteration in hypothalamic catecholamine turnover. The way in which prolactin itself may play a part has received scant attention; it may act on the hypothalamic-pituitary axis to suppress gonadotropin secretion, or may act directly on the ovary to suppress follicular development.

Evidence that prolactin is implicated in normal follicle development has been obtained from studies of progesterone secretion from human granulosa cells *in vitro* (McNatty, Sawers & McNeilly *Nature* **250**, 653; 1974). Levels of prolactin equivalent to those seen during the normal menstrual cycle were essential to maintain LH-stimulated progesterone secretion. In contrast, high levels of prolactin equivalent to those present in lactation and pathological hyperprolactinaemia inhibited progesterone secretion, an inhibition which could not be overcome by the addition of gonadotropins (LH or FSH). A similar situation was apparent in the mouse where follicular progesterone secretion *in vitro* was also inhibited by high levels of prolactin (McNatty, Neal & Baker *J. Reprod. Fertil.* **37**, 155; 1976).

But how prolactin can have both an obligatory permissive role in progesterone secretion and an inhibitory effect remains unexplained. Studies by Veldhuis and Hammond (this issue of *Nature*, page 262) go some way to resolving this dilemma. Using short term *in vitro* studies of progesterone secretion from porcine granulosa cells they found that oestradiol 17$\beta$ seems to modify the action of prolactin.

Previous studies suggest that development from a primordial to a Graafian (preovulatory) follicle is initially under the control of FSH and oestradiol. LH stimulates oestradiol and androgen secretion from the thecal layer of cells surrounding the developing follicle; FSH stimulates aromatisation of these androgens to oestrogens by the granulosa cells within the developing follicle. Both FSH and oestradiol are required for the proliferation of granulosa cells and antrum formation within the developing follicle and induce an increase in LH receptors on these granulosa cells. Prolactin is detectable within follicular fluid of all follicles and receptors have been detected on granulosa cells. Veldhuis and Hammond found that progesterone secretion from granulosa cells *in vitro* taken from small immature pig follicles could be inhibited by prolactin. Short-term oestradiol treatment also suppressed progesterone secretion. However prolonged treatment with oestrogens (for 48 h) resulted in increased progesterone secretion and reversed the action of prolactin from inhibition to synergistic stimulation of progesterone. The authors conclude therefore that "oestrogens may regulate the divergent actions of prolactin in the mammalina ovary."

The basis of the oestrogen requirement for proliferation and maturation of granulosa cells is as yet unknown. Thus the change in response to prolactin seen in the present experiments may simply reflect the maturation of granulosa cells: prolactin will stimulate progesterone secretion from granulosa cells taken from large mature pig follicles. In this respect the effects of prolactin in the pig follicle may be different from those in the human where high levels of prolactin inhibit progesterone secretion from granulosa cells regardless of the stage of follicular development.

The importance of the direct inhibitory effects of high prolactin levels on steroidogenesis by granulosa cells in follicles before ovulation is emphasised by the fact that the rare cases of ovulation in hyperprolactinaemia, in particular that associated with lactation, is associated with an inadequate corpus luteum capable of secreting only limited amounts of progesterone (McNeilly, Howie & Houston, unpublished data). This suggests that prolactin-mediated inhibition of normal granulosa cell development before ovulation may explain the inadequacy of luteal function. This is supported by the observation that drug-induced hyperprolactinaemia in the luteal phase has only a slight effect on progesterone secretion from the developed corpus luteum whereas hyperprolactinaemia induced during the follicular phase results in the formation of an inadequate corpus luteum (Delvoye *et al. C. r. Seanc. hebd. Acad. Sci. (Paris)* Ser D. **279**, 1463; 1974). McNatty (*Fertil. Steril.* **32**, 433; 1979) has provided more direct evidence in support of this. Raised levels of prolactin in plasma and ovarian follicular fluid were associated with a reduced number of granulosa cells and a marked reduction in intrafollicular steroidogenesis, not always apparent from the levels of circulating oestrogens.

Thus it seems that prolactin may have an important regulatory role in granulosa cell development within the follicle. Although present reports suggest that oestrogens may modulate the action of prolactin, the species difference in this action has yet to be resolved. The importance of understanding these mechanisms is apparent since hyperprolactinaemic blockade of steroidogenesis may in part explain the natural contraceptive effect of lactation in women and many other mammals. □

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