STUDIES ON THE PATHOPHYSIOLOGY OF GYNAECOLOGICAL ENDOCRINE DISORDERS

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Statement as to the author's participation in the work submitted

The composition of this thesis is that of the author. The methods described for radioimmunological measurement of 17β-oestradiol and 17α-ethinyloestradiol in biological fluids were both developed solely by the author. Many studies described in this thesis have been carried out in collaboration with other workers and their contributions have been duly acknowledged. The author has been personally involved in the design, organisation, collection of samples and data-analysis of all these studies and has acted as principal investigator for the studies on positive feedback in normal men, adolescent and perimenopausal dysfunctional uterine bleeding and for those on hypothalamic-pituitary function in secondary amenorrhoea. All 17β-oestradiol and 17α-ethinyloestradiol measurements reported were performed personally by the author.

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Publications

The following publications have been produced in connection with this thesis.

Abstracts

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Full papers

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Abstract of thesis

The functional organisation of the hypothalamic-pituitary-ovarian axis is reviewed.

A description is given of two newly developed radioimmunoassays for the measurement of 17β-oestradiol and 17α-ethinyloestradiol in peripheral plasma. The characteristics of these assay methods qua sensitivity, specificity, accuracy and reproducibility are described.

The effects of exogenous oestrogen administration on pituitary gonadotrophin and gonadal steroid secretion have been studied in normal women during the early-mid follicular phase of the cycle, in intact adult men and in male pseudohermaphrodites (testicular feminization, XY pure gonadal dysgenesis). Unlike in normal women and XY pure gonadal dysgenesis, oestrogen treatment did not stimulate LH release in intact adult men or patients with testicular feminization. Evidence is presented which suggests that oestrogen may directly inhibit testicular testosterone secretion in normal men.

In adolescents with anovulatory dysfunctional uterine bleeding it was demonstrated that the failure to ovulate in this condition may be attributed to an inability of these patients to release an adequate amount of LH in response to oestrogen.

Long-term serial measurements of pituitary gonadotrophins and ovarian steroid hormones in perimenopausal women indicated that dysfunctional uterine bleeding in this age group may have a variety of causes. Several abnormalities in pituitary gonadotrophin secretion were found including failure to release LH in response to endogenous or exogenous oestrogen, monotropic elevation of FSH during ovulatory cycles with short follicular phase, and increases in both FSH and LH during anovulatory cycles with long intermenstrual intervals. It is suggested that these endocrine abnormalities during the menopausal transition may arise from a change in hypothalamic-pituitary sensitivity to steroid feedback or from a progressive decline in the ovarian secretion of a hypothetical FSH-release inhibiting substance (FRIS) produced by the growing follicle.

In patients with polycystic ovary syndrome it was demonstrated that positive and negative feedback are intact in this condition and that pituitary LH secretion under basal conditions and in response to LRF was influenced by the pattern of ovarian activity during the 4 to 6 week period which preceded the measurement of this hormonal parameter.

In patients with secondary amenorrhoea elevated basal 17-fluorogenic corticosteroid and androstenedione levels were found. In addition, underweight patients had elevated basal growth hormone levels, markedly suppressed basal gonadotrophin levels and impaired pituitary FSH and LH release after LRF-injection. Growth hormone and prolactin secretion in response to insulin-induced hypoglycaemia were also impaired in these patients. Low basal 17β-oestradiol levels were found in patients with low FSH and LH but also in women with elevated prolactin levels who had normal peripheral gonadotrophin levels. Clomiphene responsiveness was related to the basal gonadotrophin and prolactin concentrations. It is hypothesised that the abnormalities in hypothalamic-pituitary function in women with secondary amenorrhoea may be a result of selective neurotransmitter deficiencies.

CHAPTER ONE

BACKGROUND TO THE STUDY

INTRODUCTION

Although scientific interest in the working of the reproductive processes dates from pre-classical times, the development of our understanding of reproduction has been much slower than that of any other bodily function. The reasons for this, not all of them of a purely scientific character, were multiple but two are undoubtedly outstanding. The very nature of the mammalian gametes, i.e. the egg and the sperm, excluded their recognition as germinative elements until the invention of suitable visual aids and the formulation of the cell theory. Also, the complex way in which reproductive processes are controlled, the number and nature of the structures involved in this control, and the method of control by hormones have long been such formidable obstacles that the delay in our knowledge of reproductive physiology is far from surprising on this account alone.

The lack of hard facts however never deterred biologists, philosophers and lay-people alike from having a lot of ideas on the subject, some of which survived in popular belief until recent times.

The Greeks, not surprisingly, were very active in this respect, particularly during and after the reign of Alexander the Great when contact with other civilised cultures from the Far and Middle East was intense (Tarn, 1930). From this period (i.e. fourth century B.C.) dates the first ever recorded treatise solely dedicated to the subject of reproduction: Aristotle's "Generation of animals" (De generatione animalium). Partly philosophical and partly based on empirical observations of himself and his contemporaries, Aristotle's views were to dominate the concept of the working of reproductive processes, and indeed of most other biological processes, for the next two thousand years. Considering the limited range of data at his disposal, Aristotle's conception was quite logical. Since menstruation ceases during pregnancy the foetus must arise from menstrual fluid which has been activated by seminal fluid. These "residues" in males and females, semen and menstrual fluid respectively, are concocted blood and hence derived from all parts of the body ("pangenesis"). The concoction is a result of the heat, the main source of which is the heart. The brain is not involved in the regulation of reproduction to any larger extent than any other part of the body; in fact "the brain itself has very little heat" (Aristotle, op. cit., p. 525). Castration of the male does not necessarily result in infertility since the testes are merely plummets which keep the excretory tubes from becoming kinked.

The Greeks of Aristotle's age had no knowledge of the existence of the ovaries in mammals. Credit for their discovery is given by Galen (c. 131-200 A.D.) (Claudii Galeni Opera Omnia, Vol 4, p. 536) to Herophilus of Chalcedon, an anatomist working in Alexandria at the beginning of the third century B.C. Indoctrinated with Aristotle's "pangenesis" theory, Herophilus however did not appreciate their true role but regarded these "female testes" merely as the functional equivalents of the male gonads.

After the decline of the Greek Alexandrine school little else could be recorded until Fallopius (1523-1562) described the tubes that bear his name. From then onwards the study of reproductive organs and their functions finally gained momentum.

The discovery of the corpus luteum is attributed (Solomons and Gatenby, 1924) to Volcherus Coiter (1573), but there is evidence that Vesalius had observed a corpus luteum in the ovary of a young girl some thirty years previously (Harrison, 1948). In 1667 the anatomist Steno first suggested that Herophilus' "female testes" were in reality the mammalian equivalents of the ovaries of egg-laying animals. A few years later Regnier de Graaf (1672) described the Graafian follicle which he suggested to represent the egg. The development of the microscope by van Leeuwenhoek and his description of spermatozoa (van Leeuwenhoek,1679) marked the beginning of a new era in the study of reproduction and the early stages of embryonic development. Yet another one and a half century would elapse before the mammalian ovum eventually would be discovered (von Baer, 1827).

Although as early as 1849, Berthold drew attention to the fact that transplantation of the testes upon the intestines maintained the secondary sex characteristics and male sexual behaviour in castrated roosters, interest in the endocrine function of the gonads was not very lively before the beginning of this century.

Soon after the formulation of the hormone theory (Bayliss and Starling, 1904), Marshall and Jolly (1905) reported that ovarian grafts and extracts were capable of inducing oestrus in ovariectomized dogs. However, the crudeness of these first extracts made interpretation of the results often difficult and controversial while the lack of suitable biological assays capable of estimating the relative potencies of various extracts, hampered substantially the search for the active principle(s). Progress was made when Stockard and Papanicolaou (1917) published their observations on the histological and physiological changes during the oestrous cycle of the guinea pig. Similar studies in the mouse (Allen, 1922) and the rat (Long and Evans, 1922) soon followed. These studies showed that microscopic examination of vaginal smears gave reliable information on the oestrous condition of the intact animal and, consequently, that changes in vaginal smears of spayed animals injected with ovarian or placental tissue extracts could be used as a specific index of the oestrous-inducing activity present in these extracts. Shortly afterwards, Allen and Doisy (1923) demonstrated that cell-free extracts obtained from follicular fluid of sows' ovaries were capable of inducing vaginal cornification in spayed mice, thereby proving that the active principle is a chemical substance. Isolation of this oestrogenic substance from tissue extracts however proved to be difficult since the organic solvents extracted a considerable amount of inert material in addition to the hormone. Little progress towards isolation was made until 1927 when Aschheim and Zondek discovered that the urine of pregnant women contained considerable quantities of oestrogenic material. Apart from their higher biological potency, organic extracts of pregnancy urine offered the additional advantage of containing far less contaminating material, making separation of the active material less cumbersome. Isolation of oestrone (Doisy, Veler and Thayer, 1929; Butenandt, 1929) and oestriol (Marrian, 1930) from urine duly followed. A few years later Doisy's group succeeded in isolating the parent hormone 17β-oestradiol from swine ovarian tissue extract (MacCorquodale, Thayer and Doisy, 1935).

The endocrine nature of the corpus luteum and its role in maintaining pregnancy first suggested by the work of Fraenkel (1903), Loeb (1907) and Bouin and Ancel (1909 a,b) was convincingly established in 1929 when Corner and Allen demonstrated that injections of corpus luteum extracts into rabbits, ovariectomized shortly after mating, resulted in the development of a progestational condition similar to that observed in early pregnancy. The subsequent intensive search for the active component of these extracts was successful in 1934 when no less than four laboratories announced independently the isolation of pure corpus luteum hormone (Butenandt and Westphal, 1934; Allen and Wintersteiner, 1934; Slotta, Ruschig and Fels, 1934; Hartmann and Wettstein, 1934).

An androgenic function of the ovary was suspected from the work of Parkes (1937) who demonstrated that atrophy of the hen's comb after spaying can be prevented by administration of male hormone but not of oestrone or oestradiol, but it was not until 1960 that androgens were identified in follicular fluid and corpora lutea (Zander, 1958; Short, 1960).

The existence of a functional interrelationship between the pituitary gland and the ovary was first recognised at the beginning of this century when Fichera (1905) reported his observations on pituitary hypertrophy following castration in the rat. This was soon followed by the work of Crowe , Cushing and Homans (1910) who demonstrated that hypophysectomy in the dog resulted in atrophy of the reproductive organs. Direct evidence for the gonad-stimulating properties of the anterior pituitary was obtained in 1926 by Smith and by Zondek and Aschheim. Following Smith's (1927) observation of a follicular response to pituitary implants, and Evans and Simpson's (1928) finding that aqueous extracts of pituitaries induced luteinization, the existence of two separate gonadotrophic hormones was postulated (Weisner and Crew , 1930) and subsequently confirmed (Fevold, Hisaw and Leonard, 1931). In 1928 Stricker and Grueter produced evidence for the presence of an additional pituitary hormone which influences milk secretion. It was partially purified by Riddle, Bates and Dykshorn (1932 a,b; 1933) who gave it the name prolactin.

The gonad-stimulating activity of human pregnancy urine was discovered by Aschheim and Zondek in 1927, who assumed it to be produced in the hypophysis. This opinion was soon challenged however in view of the differences in biological activity of this urinary gonadotrophin and anterior pituitary extracts (Evans and Simpson, 1929), and the absence of gonadotrophic activity in the pituitary of pregnant women (Philipp, 1930 a, b). A few years later the placental origin of "PROLAN" (Aschheim and Zondek, 1927) was conclusively demonstrated both in-vivo (Kido, 1937) and in-vitro (Gey, Seegar and Hellman, 1938.)

Thus in the early 1930's, the role of the anterior pituitary in regulating gonadal function through its secretion of gonadotrophic hormones was fairly well established. Partial separation of the pituitary gonadotrophins had been achieved thus providing the first, albeit still crude, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) preparations. Moreover, human pregnancy urine and pregnant mare serum (Cole and Hart, 1930) proved to be abundant sources of gonadotrophic material with LH- and FSH- like activity respectively, which made it no longer necessary to rely upon the limited supply of pituitaries for isolating hormones with gonadotrophic activity.

At about the same time however, evidence was accumulating that hypophyseal gonadotrophin secretion was not autonomous but influenced by a variety of endogenous and exogenous factors. Thus it was demonstrated that gonadal steroids (Moore and Price, 1932; Hohlweg and Junkmann, 1932) and environmental stimuli (Marshall, 1936) may affect pituitary gonadotrophin secretion.

In 1937 Harris showed that electrical stimulation of the hypothalamus would cause the female rabbit to ovulate, an event which in this species normally requires the external stimulus of copulation (Heape, 1905). Since no neural connections between the hypothalamus and the anterior pituitary could be demonstrated, Harris (1944) tentatively suggested that hypophyseal gonadotrophin secretion was regulated by neurohumoral substances secreted by the median eminence of the hypothalamus. The confirmation of the cranio-caudal bloodflow (Green 1947;

Green and Harris, 1949) in the hypophyseal portal vessels (Popa and Fielding, 1930), the subsequent demonstration of the presence of LH-releasing factor (LRF) activity (McCann, Taleisnik and Friedman, 1960) and FSH-releasing factor (FRF) activity (Igarashi and McCann, 1964 a; Mittler and Meites, 1964) in hypothalamic extracts and, finally, the isolation of LRF (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk and White, 1971) eventually removed from Harris' concept the realm of hypothesis.

THE HYPOTHALAMO-PITUITARY-OVARIAN AXIS

1. The pituitary-ovarian axis

1.1 The gonadotrophic role of the anterior pituitary

Historically, the existence of gonadotrophic substances i.e. substances affecting growth of the gonads, was first proposed in 1905 when Heape postulated that the activity of the ovaries was regulated by an extra-ovarian factor which he gave the provisional name "generative ferment". Support for Heape's concept had earlier been produced by Foa (1901) who demonstrated that the ovary of an immature animal grafted into an adult host undergoes rapid development and assumes adult function long before it would normally have done so. It was likely therefore that normal ovarian function required the presence of some trophic extra-gonadal factor(s) which was absent in immature animals. Neither Foa nor Heape however implicated the pituitary as the source of this (these) hypothetical substance(s).

More direct evidence for the existence of a functional interrelationship between the pituitary gland and the gonads was obtained during the first decade of this century when Fichera (1905) reported his observations on pituitary hypertrophy following castration. A few years later Crowe, Cushing and Homans (1910) and Aschner (1912) demonstrated that hypophysectomy in the dog inhibited genital development in young animals which indicated that the hypophysis might be essential for the normal development of the gonads at puberty. The question however as to whether the hypophysis was also required for the maintenance of gonadal function in adult animals remained largely unanswered. It should be remembered that in those early days the technique of hypophysectomy was far from perfect and substitution therapy unknown. Animals which survived the surgical intervention without too much brain damage were few and those which survived, quite often did so for only a few days or weeks. As a result, conclusions derived from some of this earlier work were occasionally rather misleading or erroneous as, for instance, the statements of Aschner (1912) that hypophysectomy in adult dogs caused no ovarian atrophy. The discovery of a parapharyngeal approach to the hypophysis in rats (Smith, 1927, 1930) however gave new impetus to the study of the effects of hypophysectomy. Since then most animal species have been subjected to this procedure (Jacobsohn, 1966). The inhibition of gonadal development in young animals, reported by Crowe and his co-workers (1910) and Aschner (1912) has been confirmed, and the regressive changes in the gonads and accessory reproductive organs in the adult hypophysectomized animal have been described.

The clearest piece of evidence that a particular organ, such as the anterior pituitary, is an endocrine gland is that the organ, when transplanted to a remote site in the body, will still maintain its normal function. Indeed, successful transplantation of a gland in this manner implicates that the normal function of the gland is controlled by a substance (or substances) reaching it via the blood stream and, secondly, that the gland itself exerts its effects through secretions passed into the blood stream. The aforementioned hypophysectomy studies left little doubt as to the endocrine nature of the anterior pituitary. Yet, prior to 1926, all attempts to obtain functionally active pituitary transplants yielded only negative or but questionably positive results. One of the earliest attempts to transplant pituitary tissue was reported by Crowe, Cushing and Homans (1909). These workers made autotransplants into the rectus muscle, bone marrow and cerebral cortex of dogs but although the transplanted tissue became revascularised and remained histologically identifiable, restoration of its functional activity was in most instances not apparent.

From 1926 onwards however, two important changes in the design of this type of experiments were introduced. Prior to that date most workers favoured transplantation of a single gland and generally preferred adult animals for this purpose. Beginning with 1926, a number of investigators started using hypophysectomized or normal immature rats or mice as recipients. The practice of transplanting one single gland was largely abandoned in favour of the use of repeated, often daily, administration of pituitary tissue or pituitary extracts. In fact, already a few years earlier Evans and Long (1922) had demonstrated that daily intraperitoneal injections of pituitary gland extracts resulted in luteinization of the ovaries in the rat but these observations were inconclusive since intact rather than hypophysectomized animals had been used.

In 1926 Smith reported that daily administration of pituitary tissue to immature female rats produced precocious sexual maturity and superovulation. Similar results were obtained by Zondek and Aschheim (1926) after transplantation of bovine or human pituitaries into infantile mice and by Smith and Engle (1927) in rats and mice. In the adult hypophysectomized female, pituitary implants induced follicular growth and corpora lutea formation, although regular cycles did not appear and follicular cysts were frequent (Smith, 1927). Many of the effects observed in immature animals, such as the premature rupture of the vaginal closing membrane, the appearance of cornified cells in the vaginal smear and the premature development of the uterus were similar to those described in infantile mice and rats injected with follicular fluid extracts (Allen and Doisy, 1923). However, in contrast to the pseudo-pubertas praecox induced by "folliculin" injections, administration of pituitary tissue provoked a true precocious puberty by stimulating follicular growth in the infantile ovary to the point where enough endogenous "folliculin" was produced to cause the characteristic secondary changes in the uterus and vagina. Clearly, the anterior pituitary by virtue of its action on the gonads and, indirectly through the gonads, its influence on the accessory reproductive organs could truly be called "der Motor der Sexualfunktion" (Zondek and Aschheim, 1927).

Speculation as to whether the anterior pituitary secretes one or two gonadotrophic hormones arose coincidentally with the demonstration of the gonad-stimulating properties of pituitary transplants and extracts. Smith (1927) observed that the early ovarian response to pituitary implants was chiefly follicular in character with very little luteinization. Evans and Simpson (1928) reported that alkaline extracts of pituitary tissue were more strongly luteinizing than acid extracts while Fluhmann (1929, 1930) found that the blood of women in the post-climacteric period, after castration and in certain cases of amenorrhoea contained a gonad-stimulating substance which elicited mainly follicular growth in the ovaries of immature rats. It was likely therefore that the anterior pituitary secreted two different gonadotrophic substances: one factor concerned with the induction of follicular growth ("Prolan A") and a second one responsible for inducing luteinization ("Prolan B") (Zondek, 1930 a, b). Wiesner and Crew (1930) proposed the terms "Rho 1" and "Rho 2" for these two substances and postulated that either Rho 1 alone or the combined action of Rho 1 + Rho 2 was required to initiate the first phase of ovarian activity during which the ovaries produce the "α-substance" (i.e. oestrogen). Rho 2 alone subsequently induces the second phase of ovarian activity, which is characterised by the ovarian secretion of a "β-substance" (i.e. progesterone). The existence of two distinct gonadotrophins was subsequently confirmed, and partial chemical separation of the FSH- and LH- activity of pituitary extracts effected, by Fevold, Hisaw and Leonard (1931).

Evidence for the existence in mammals of a lactogenic hormone of pituitary origin was first produced by Stricker and Grueter (1928). Riddle and Braucher (1931) subsequently reported that a similar hormone of pituitary origin stimulated the crop gland of pigeons, a discovery which led to a favourite method of assay. It was partially purified by Riddle, Bates and Dykshorn (1932 a, b; 1933) who introduced the term "prolactin". Later it was shown that in hypophysectomized rats, injections of prolactin could produce functional corpora lutea (Astwood, 1941) and induce pseudopregnancy with formation of deciduomata after uterine traumatization (Evans, Simpson, Lyons and Turpeinen , 1941). In view of this luteotrophic action in the hypophysectomized rat, prolactin (luteotrophin, LTH) was subsequently added to the list of gonadotrophic hormones. The justification of this classification however is still a major subject of controversy. While there is little doubt that prolactin forms part of the luteotrophic complex in the rat (Astwood, 1941; Evans <u>et al</u>, 1941), mouse (Dresel , 1935; Bruce and Parkes, 1960), rabbit (Spies, Hilliard and Sawyer, 1968), hamster (Greenwald, 1967), ferret (Donovan, 1963) and ewe (Moore and Nalbandov, 1955 ; Denamur, Martinet and Short, 1966), the gonadotrophic role of prolactin in other mammalian species is far from clear. In the human it has been suggested that the presence of prolactin in physiological concentrations may be required for the production of progesterone by human granulosa cells <u>in-vitro</u> (McNatty, Sawers and McNeilly, 1971). Supraphysiological levels of circulating prolactin on the other hand may have an antigonadotrophic action by making the gonads refractory to pituitary gonadotrophin stimulation (Reyes, Winter and Faiman, 1972).

1.2 The pituitary gonadotrophins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH)

1.2.1 Chemistry of the pituitary gonadotrophins

After the first attempt by Fevold and his co-workers (1931) to separate the two gonadotrophic substances from pituitary extracts, several laboratories, including their own, set to work in the 1930's trying to isolate and identify the active components. For the most part, methods of extraction and purification were initially applied to pituitary glands from those species that could be obtained in large numbers, i.e. from bulls and cows (referred to in the literature as bovine), from sheep (ovine), pigs (porcine) and horses (equine). Thus the first highly purified gonadotrophin preparations were obtained from sheep and swine pituitaries; both were luteinizing hormone preparations. Follicle-stimulating hormone proved to be more difficult to obtain in sufficient quantities for chemical studies because of the low FSH-content of the pituitary gland in most species and the high lability of FSH.

(a) Luteinizing hormone

Purification

Sheep pituitaries were one of the first sources from which LH was isolated. Purified LH preparations with high biological activity were obtained from sheep pituitaries by Li, Simpson and Evans (1940). In the same year, Shedlovsky, Rothen, Greep, van Dyke and Chow (1940) reported the preparation of purified LH from swine pituitaries. Both preparations were considered to be homogeneous by all the available physico-chemical criteria, yet they differed in several respects. The molecular weight (MW) of sheep LH, estimated at 40,000, appeared to be less than half that of porcine LH (MW 100,000). Both preparations had different iso-electric points, the ovine being more acidic (iso-electric point at pH 4·6) than the nearly neutral porcine hormone (iso-electric point at pH 7·4). Both preparations contained carbohydrate in their molecule but this was present in different quantities. Neither preparation however stood the test of time in terms of chemical homogeneity.

Later, in 1959, two groups working independently reported the isolation of ovine LH (Squire and Li, 1958, 1959; Ward, McGregor and Griffin, 1959). Both these preparations had a biological activity 30 times greater that previously reported and were comparable in their physico-chemical properties. Contamination with other pituitary hormones was reported to be less than 0·1%.

Reichert (1962) first described the purification of bovine LH. Highly purified preparations of equine LH with a biological activity five times greater than that of the ovine NIH-LH-S_1 standard were obtained by Braselton and McShan (1970). Fairly pure LH- preparations have also been isolated from rat (Ward, Reichert, Fitak, Nahm, Sweeney and Neill, 1970) and monkey pituitaries (Monroe, Peckham, Neill and Knobil , 1970).

The wide interest in the clinical uses of human pituitary gonadotrophins has sparked intensive efforts to obtain highly purified preparations suitable for such purposes as well as for biochemical analysis. The first detailed account of the isolation of human LH was provided by Squire, Li and Andersen (1962). Since then numerous other studies have appeared describing the preparation of highly active human LH (e.g. Hartree, Butt and Kirkham, 1964; Parlow, Condliffe, Reichert and Wilhelmi, 1965; Rathnam and Saxena, 1970; Reichert and Parlow, 1964).

Physico-chemical properties

Estimates of the molecular weight of LH determined by sedimentation analysis in alkaline solutions are very similar in all species studied, ranging from 26,000 (human LH) to 34,000 (porcine LH) with the exception of equine LH for which a value of 44,500 has been reported. In acidic solution however, the sedimentation coefficient for LH is much lower and corresponds to a molecular weight of about 16,000. Li and Starman (1964) were the first to recognise this fact for ovine LH and from this they suggested that the ovine LH molecule in acidic solution dissociated into two monomers of equal size. The importance of this observation is discussed below.

One of the problems in isolating glycoprotein hormones has been the electrophoretic heterogeneity that has been encountered in purified preparations considered to be homogeneous on the basis of many other physico-chemical criteria. Thus ovine LH, while behaving as a single component when subjected to electrophoresis on cellulose acetate paper (Papkoff, Gospodarcwicz, Candiotti and Li, 1965), distributed into several, biologically active fractions on electrophoresis on polyacrylamide gels or cellulose columns (Justisz and Squire, 1958, 1961). A similar polymorphism on electrophoresis has also been observed in purified, "homogeneous" LH preparations from other species, including the human (Squire, Li and Andersen, 1962; Peckham and Parlow, 1969 b). Several explanations have been put forward to account for this heterogeneity. Ward, McGregor and Griffin (1959) who observed two active components in ovine LH and Squire, Li and Andersen (1962) who found three distinct, biologically active, human LH fractions concluded that heterogeneity may arise from interactions of LH with other inactive proteins. Polymorphism may also result from proteolytic action on the LH molecule during the period between death of the donor and collection of the pituitary, during subsequent storage of the gland or in the course of the extraction process. The peptidase activity of the pituitary gland has been recognised to be very high (Adams and Smith, 1951). In fact, proteolytic activity has been detected in highly purified human LH (Reichert and Parlow, 1964), and has been shown to be responsible for the observed heterogeneity of the β-subunit of ovine LH (Ward and Liu, 1972). The detailed structural studies on ovine LH have also indicated that the carbohydrate composition may be heterogeneous (Sairam, Papkoff and Li, 1972; Sairam, Samy, Papkoff and Li, 1972), due either to incomplete biosynthesis or to the destructive action of carbohydrases that might be present in the pituitary. Though some of the factors responsible for heterogeneity of the isolated LH preparations may be eliminated by appropriate methods, the question as to whether a completely homogeneous LH fraction will ever be isolated remains speculative, particularly in view of the suggestion that genetic polymorphism within the species may be involved in the observed heterogeneity of bovine LH (Maghuin-Rogister, Closset and Hennen, 1971).

In terms of amino acid composition no major differences in LH from ovine, bovine and human origin have been reported, suggesting that their primary structures are very similar (ovine LH: Papkoff, Gospodarowicz, Candiotti and Li, 1965; bovine LH: Papkoff and Gan, 1970; human LH: Hartree, Thomas, Braikevitch, Bell, Christie, Spaull, Taylor and Pierce, 1971). The total number of amino acid residues also appears to be very similar.

Likewise, a great deal of similarity exists in the carbohydrate composition of LH but for a somewhat lower sugar content and the absence of sialic acid in the bovine hormone (Kathan, Reichert and Ryan, 1967). This latter sugar forms an integral part of the human hormone, although there has not been general agreement as to the amount, with estimates varying from 0·7 to 2·0%. Similar variations in sialic acid content have been reported for purified preparations of human chorionic gonadotrophin (hCG) (Bell, Canfield and Sciarra, 1969) and are probably due to loss of terminal residues during purification.

Structure

In 1964 Li and Starman, while studying the molecular weight of ovine LH by sedimentation analysis, observed that in acidic solution the LH molecule apparently dissociated into two monomers of equal size. Shortly afterwards Ward, Fujino and Lamkin (1966) demonstrated that these two glycopeptide monomers had a different chemical composition and suggested that ovine LH consisted of two non-identical, noncovalently bound subunits. Unequivocal proof for the subunit nature of ovine LH was obtained by Papkoff and Samy (1967) who achieved successful separation of the subunits by countercurrent distribution. These workers also showed that the individual subunits possessed only about 7 to 8% of the biological activity of the parent hormone. Recombination of the subunits however restored the biological activity. The subunit nature of LH was subsequently confirmed and separation of the subunits achieved for LH from several other species including the bovine (Reichert, Rasco, Ward, Niswender and Midgley, 1969), rat (Ward, Reichert, Fitak, Nahm, Sweeney and Neill, 1970), porcine (Hennen, Prusik and Maghuin-Rogister, 1971) and the human (Reichert and Ward, 1969).

With the recognition of the subunit structure of thyroid-stimulating hormone (TSH) (Liao and Pierce, 1970), of FSH (Papkoff and Ekblad, 1970) and hCG (Swaminathan and Bahl, 1970) and their structural interrelationships (Pierce, Liao, Carlsen and Reimo, 1971) a uniform nomenclature for the subunits of these four glycoprotein hormones became imperative. Accordingly, the terms α and β subunit proposed by Pierce and his co-workers to designate the subunits of bovine TSH, were accepted to differentiate between the subunits of all glycoprotein hormones.

The term α-subunit is employed to designate the subunit which is common or similar to all glycoprotein hormones. Thus the α-subunit of human FSH (hFSH-α) is virtually identical, both in terms of amino acid composition and linear amino acid sequence to the α-subunit of human LH (hLH-α), human TSH (hTSH-α) and hCG (hCG-α) (Shome and Parlow, 1974 a). Evidently, the α - subunit is not responsible for conferring the specific hormonal activity to the molecule, e.g. the α-subunit of LH can be replaced by either FSH-α or hCGα to give a hybrid molecule possessing LH- activity (Saxena and Rathnam, 1972).

The term β-subunit is used to indicate the other subunit which is hormone specific. Thus the primary structures (linear amino acid sequences) of the β-subunits of human FSH (hFSH-β), human LH (hLH-β) and human TSH (hTSH-β), though showing some degree of structural homology probably indicative of their common ancestral origin, are essentially different (Shome and Parlow, 1974 b).

(b) Follicle-stimulating hormone

Purification

As mentioned earlier, purification of FSH has been substantially hampered by the lability and low pituitary content of this glycoprotein. Moreover, virtually all initial purified "homogeneous" FSH preparations possessed some LH-activity in biological assays (e.g. ventral prostatic weight assay) which even led to the suggestion that this LH-activity was intrinsic to the FSH-molecule (Papkoff, 1965).

Isolation of ovine FSH was first reported in 1949 (Li, Simpson and Evans, 1949) but this preparation was later shown to be electrophoretically heterogeneous. Far more potent and homogeneous ovine preparations have since been described (Hashimoto, McShan and Meyer, 1966; Papkoff, Gospodarowicz and Li, 1967; Sherwood, Grimeck and McShan, 1970). Physicochemically fairly pure FSH has also been isolated from porcine (Steelman and Segaloff, 1959),bovine (Reichert and Jiang, 1965), equine (Braselton and McShan, 1970) and rhesus monkey pituitaries (Yamaji, Peckham, Atkinson, Dierschke and Knobil, 1973) but the yield in these species has usually been rather low.

After the initial attempt by Li (1958) to purify human FSH of pituitary origin, preparations of increasing biological specific activity have been reported among others by Roos and Gemzell (1964), Amir, Barker, Butt and Crooke (1966), Saxena and Rathnam (1967), Roos (1968) and Peckham and Parlow (1969 a).

Physicochemical properties

The molecular weight of FSH as determined by sedimentation analysis is about 30,000. As with LH, the sedimentation coefficient is lower at low pH values due to dissociation of the FSH- molecule into its two subunits, each of which has a molecular weight of about 16,000.

The number of amino acid residues in FSH is somewhat lower than that of LH due to a shorter chain length of the β-subunit of FSH (Shome and Parlow, 1974 b).

Considerable differences in the carbohydrate content of FSH isolated from the same species by different laboratories have been reported (Papkoff, Mahlmann and Li, 1967; Reichert, Kathan and Ryan, 1968). These discrepancies may be due either to impurities or to partial degradation by carbohydrases during purification. As with LH (Braunstein, Reichert, Van Hall, Vaitukaitis and Ross, 1971) removal of the sialic acid from the carbohydrate moiety of the molecule impairs in-vivo biological activity due to an increase in the rate of disappearance of the desialylated products from the circulation (Van Hall, Vaitukaitis, Ross, Hickman and Ashwell, 1971).

Structure

The lowering of the sedimentation coefficient of FSH in acidic solutions and in the presence of guanidine hydrochloride (Ryan, Jiang and Hanlon, 1970) suggested that FSH, like the other glycoprotein hormones, consisted of two subunits. Successful separation of the subunits of ovine FSH (Papkoff and Ekblad, 1970) and human FSH (Saxena and Rathnam, 1972) soon followed. Recently, the amino acid sequences of both the α and β subunit of human FSH have been determined and the postulated structural identity of the former with the α -subunit of LH has been confirmed (Shome and Parlow, 1974 a, b).

1.2.2 Measurement of gonadotrophins

For more than 35 years, after the initial observations of Smith (1926) and Zondek and Aschheim (1926) that pituitary gonadotrophins could induce premature sexual maturity when administered to immature rats or mice, many investigators attempted to develop specific and sensitive assay methods for measurement of gonadotrophins using a biological parameter as end-point of the assay. Thus between 1930 and the mid 60's, several bioassays for the estimation of total gonadotrophic, FSH or LH-activity in suitably prepared extracts of pituitary tissue or biological fluids were described (for review see Loraine and Bell, 1971). Biological assays however invariably suffer from the unavoidable and ubiquitous problems inherent in the use of whole, living animals and which are reflected predominantly in the lack of sensitivity and the relatively poor precision.

In the late 1960's the use of bioassays was largely abandoned in favour of immunological and particularly radioimmunological assay methods which made it possible, for the first time, to measure both gonadotrophins in a small volume of plasma or serum with a precision hitherto undreamed of. Subsequent methodological advances such as the use of purer gonadotrophin preparations to minimize cross-reactivity of the other structurally related glycoproteins with the obtained antiserum, and, more recently, the development of radioimmunoassays (RIA) using antibodies raised against the hormone-specific β-subunits, further secured that the measurement of gonadotrophins by RIA is not only the most practical, sensitive and precise but also the most specific method available to date.

Radioimmunoassays however have their limitations, related to the fact that they measure immunological rather than biological activity. It is now clear that biologically inactive hormone fragments, e.g. the α or β-subunit, may appear in the circulation and can react in the same way as the intact hormone in RIA. A similar disparity in gonadotrophic activity as measured by RIA and bioassay could result from the presence of hormone precursors that may react with the antibody but have a much reduced biological activity. One recent development that may help to reduce such discrepancies is the radioreceptor assay.

A problem common to all types of gonadotrophin assays whether biological or immunological, is the lack of readily available, suitable hormone preparations for use as standards in the assay. It is evident that the employed reference preparation should be homologous, i.e. obtained from the same species for which the gonadotrophin assay is used. It is also clear that, within the species, the standard used should be derived from the same biological material as that used in the assay, e.g. urinary gonadotrophin concentrations should be expressed in terms of a gonadotrophin standard obtained from urine. However, since reference preparations derived from plasma or serum are not available, gonadotrophin levels measured by RIA in serum or plasma are commonly expressed in terms of a pituitary gonadotrophin standard. Caution should therefore be exercised when interpreting such results, not least because in these instances gonadotrophin concentrations, as determined with an immunological method, are expressed in units of a standard whose numerical value is based on its biological potency.

(a) Bioassays

Biological assays in living animals are based on the ability of gonadotrophins to induce some specific change in the gonads or in some other organ which is responsive to gonadal steroid secretion. The assay is made quantitative by comparing the response observed following administration of the "unknown" gonadotrophin-containing preparation with those induced by known amounts of a suitable standard. Bioassays for gonadotrophins are generally conducted in rats and mice which are more sensitive to stimulation by these hormones than other animal species such as rabbits, guinea pigs or amphibia (Loraine and Bell, 1971). Interference from endogenously produced gonadotrophins is minimized by using immature and/or hypophysectomized animals. The majority of bioassay methods are not sensitive enough to detect FSH and LH with any regularity in blood, but they can be employed for measuring gonadotrophins in urine samples which have been extracted and concentrated. Examples of the most popular bioassays are listed in Table 1.1. Recently, more sensitive and precise in-vitro bioassays for hLH and hCG have been developed utilizing the production of testosterone by collagenase dispersed interstitial cells of the rat testis (Dufau, Mendelson and Catt, 1974).

SPECIFICITY	TEST ANIMAL	ASSAY END-POINT	REFERENCE
FSH + LH	intact, immature female mice	uterine weight	Klinefelter, Albright and Griswold (1943)
FSH	hypophysectomized female rats	follicular growth	Evans, Simpson, Tolksdorf and Jensen (1939)
	hypophysectomized, immature male rats	testicular weight	Paesi, Wijnans and de Jongh (1951)
	intact, immature, female rats (hCG-treated)	ovarian weight	Steelman and Pohley (1953)
	intact, immature female mice (hCG-treated)	ovarian weight	Brown (1955)
	intact, immature female mice (hCG-treated)	uterine weight	Igarashi and McCann (1964b)
	hypophysectomized female mice (hCG-treated)	uterine weight	Lamond and Bindon (1966)
LH	hypophysectomized, immature female rats	ovarian interstitial tissue	Evans, Simpson, Tolksdorf and Jensen (1939)
	intact, immature male rats	seminal vesicles	Fevold (1937)
	hypophysectomized, immature male rats	seminal vesicles	McArthur (1952)
	hypophysectomized male rats	ventral prostate	Greep, Van Dyke and Chow (1941)
	intact, immature female rats (PMSG-hCG primed)	ovarian ascorbic acid	Parlow (1958)

Table 1.1: <u>In-vivo</u> biological assays for gonadotrophins

(b) Radioimmunoassays

Radioimmunoassay of the native molecules

The widespread application of the RIA technique (Yalow and Berson, 1960) for measuring pituitary and placental glycopeptide hormones was substantially hampered for many years because of the difficulty in obtaining a sufficient amount of highly purified hormone for use as immunogen. It may not be surprising therefore that immunological (Wide and Gemzell, 1960) and radioimmunological (Paul and Odell, 1964) assay methods were first applied to the measurement of hCG which was readily available in a relatively pure form, free from contamination with pituitary glycoproteins. Fortunately, because of the close immunological resemblance between hCG and hLH (Wide, Roos, and Gemzell, 1961), most antisera raised against hCG cross-reacted with hLH and could therefore be used in the development of RIA's for LH (e.g. Franchimont, 1966; Midgley, 1966; Odell, Ross and Rayford, 1966, 1967; Saxena, Demura, Gandy and Peterson, 1968). In fact, only few workers (e.g. Faiman and Ryan, 1967 b; Stevenson, 1967; Schalch, Parlow, Boon and Reichlin, 1968) attempted initially to raise antisera against hLH itself. RIA techniques for FSH using antisera raised against homologous or heterologous pituitary FSH preparations, have been described by various investigators including Franchimont (1966), Midgley (1967), Faiman and Ryan (1967 a), Saxena, Demura, Gandy and Peterson (1968) and Donini and Donini (1969).

In most RIA systems a highly purified homologous gonadotrophin-preparation is used for radioiodination, which is commonly achieved by procedures based on the chloramine-T method described by Greenwood, Hunter and Glover (1963). More recently however, several alternative iodination procedures have been proposed such as the enzymatic iodination of tyrosyl groups in the tracer hormone using lactoperoxidase (Thorell and Johansson, 1971) and the conjugation labelling (Bolton and Hunter, 1972, 1973). In general, tracers prepared by these new methods, aimed at reducing the iodination damage, appear to have a higher immunoreactivity than those obtained with the conventional method and thus permit the achievement of a higher assay sensitivity.

Several procedures for the separation of free from antibody bound hormone have been employed in RIA's for gonadotrophins, e.g. chromatoelectrophoresis (Saxena, Demura, Gandy and Peterson, 1968), starch gel electrophoresis (Franchimont, 1966), charcoal adsorption (Neill, Johansson, Datta and Knobil, 1967) and the double antibody technique (Odell, Ross and Rayford, 1967). More recently, attention has been directed towards the development of solid-phase RIA methods in which the antibody, rather than being free in solution as in conventional liquid-phase RIA's, has been adsorbed to polystyrene or polypropylene discs or tubes (Catt, 1969) or coupled to activated dextran or cellulose particles (Wide, 1969). Apart from simplifying the separation of free from antibody bound hormone, solid-phase methods offer the additional advantage of being less time-consuming since the binding of antigen to the immunoadsorbent is virtually irreversible and "equilibrium" between free and bound antigen is therefore rapidly established. Solid-phase separation using an immunoadsorbent consisting of antigen coupled to a solid phase has also been successfully applied to immunoradiometric assay methods for FSH and LH (Miles, 1971).

Radioimmunoassay of the α and β-subunits

Specificity has always been one of the major problems in RIA of gonadotrophins. Admittedly, the use of incompletely purified hormone preparations as immunogen could be blamed to a certain extent for the observed cross-reactivity of the pituitary glycoproteins, but the problem of specificity is not solely one of contamination. The observation of Odell, Wilber and Paul (1965) that antisera raised against pituitary thyroid-stimulating hormone (TSH) cross-reacted with hCG which, being of placental origin, was unlikely to be a major contaminant of pituitary TSH preparations, indicated that the problem of contaminated immunogens was compounded by the close immunological resemblance between these glycopeptide hormones.

The subsequent recognition that FSH, LH, TSH and hCG consist of two subunits, an α-subunit which is immunologically (Ross, Vaitukaitis and Robbins, 1972) and structurally (Shome and Parlow, 1974 a) identical in all glycoprotein hormones and a hormone-specific β-subunit, provided a conceptual framework for understanding the cross-reaction between these hormones, and revived the hope of achieving absolute specificity by raising antisera against the hormone-specific β-subunits. Yet, although the specificity of so generated antisera has markedly improved, even to the extent that antibodies raised against hLH discriminate between native hLH and hCG (Vaitukaitis, Ross, Reichert and Ward, 1972), absolute specificity has not been achieved and antisera against hFSH still cross-react with hTSH and, to a lesser extent, with hLH(Vaitukaitis, Ross, Pierce, Cornell and Reichert, 1973). A chemical explanation for this residual a-specificity has recently been provided by the elucidation of the primary structure of hFSH-β whose amino acid sequence shows considerable homology with hLH-β and even more so with hTSH-β (Shome and Parlow, 1974 b). It is likely that, by generating antibodies against hormone-specific peptide fragments of the β-subunits, specificity of gonadotrophin RIA's may be increased even further in the not too distant future.

(c) Radioreceptor assays (radioligand-receptor assays)

The measurement of gonadotrophins by RIA suffers from the fact that immunological, rather than biological activity is measured and these two activities do not necessarily coincide. In general, RIA's tend to overestimate the potency of pituitary or urinary extracts in comparison with bioassay even when the appropriate reference standards are employed (Ryan, 1969; Stevens, 1969). In recent years it has become increasingly clear that the observed discrepancies between RIA and bioassay estimates of gonadotrophin concentrations may be attributed, at least partially, to the presence of "prohormones" or hormone fragments which are inactive in the conventional bioassays but react immunologically in the same way as the native hormones. Free α and β -subunits of LH have been found in urine and sera from normal subjects and postmenopausal women (Laburthe, Dolais and Rosselin, 1973; Hagen and McNeilly, 1975 a) and in human pituitary tissue (Hagen and McNeilly, 1975 b). In addition to the free subunits of LH, a large molecular species of immunoreactive LH which may represent a prohormone, has been demonstrated in pituitary tissue extracts (Prentice and Ryan, 1975). As for FSH, the failure to demonstrate free FSH-β in pituitary extract, menopausal urine and serum (Talas, Midgley and Jaffe, 1973) may explain the earlier observations that the disparities in potency estimates obtained by RIA and bioassay are on the whole not so great for FSH as for LH.

Since the primary event in the action of gonadotrophic hormones at end-organ level is now believed to involve a membrane-bound receptor site of the target cell which interacts stereospecifically with the hormone, the substitution of the antibodies of a conventional RIA by such a hormone-specific receptor would provide an assay method which measures biological rather than immunological hormonal activity. Such in-vitro bioassays, commonly referred to as "radioreceptor assays" (Lefkowitz, Roth and Pastan, 1970), incorporate the advantages of the RIA technique and usually show greater precision and sensitivity than do the conventional bioassays.

Several systems of radioreceptor assay for hLH and hCG have been developed using tissue homogenates of rat testes (Catt, Dufau and Tsuruhara, 1971, 1972; Leidenberger and Reichert, 1972 a, b) and partially purified membrane-bound receptor fractions from bovine corpora lutea (Gospodarowicz, 1973). Potency estimates of purified pituitary gonadotrophin preparations obtained with such assays are usually somewhat lower than those obtained by in-vivo bioassays (e.g. OAAD or VPW tests). The application of radioreceptor assays to the measurement of biological LH-activity in serum or plasma however has generally been rather unsuccessful due to interference by non-hormonal serum factors with the uptake of labelled hormone (Reichert, Leidenberger and Trowbridge, 1973) and, even despite recent improvements, large discrepancies in plasma LH estimates obtained with RIA and RRA still exist (Leidenberger, Willaschek, Pahnke and Reichert, 1976).

Radioreceptor assays for FSH using homogenates of rat or bovine testes tubules have been described by Reichert and Bhalla (1974) and Cheng (1975) respectively. The method developed by Cheng can be applied to the measurement of serum FSH concentrations for which values similar to those obtained by RIA have been reported (Cheng, 1975).

Although from a physiological point of view, radioreceptor assays are undoubtedly superior to conventional radioimmunoassays, it would be misleading to regard them as ideal substitutes of in-vivo biological assays, particularly in view of the finding that the desialated forms of hLH and hCG (Catt, Dufau and Tsuruhara, 1971) and of hFSH (Reichert and Bhalla, 1974), though biologically inactive, bind equally well to their respective receptors as the native hormones.

(d) Reference preparations

1. Urinary gonadotrophin preparations

The first unofficial reference material to be introduced was derived from the urine of postmenopausal women and was termed "human menopausal gonadotrophin HMG-20A" (Dekanski, Organon). One HMG unit was defined as the gonadotrophic activity contained in 1 mg HMG-20A (Loraine and Brown, 1959).

A similar preparation, HMG-24, was distributed by the World Health Organization as the "first international reference preparation for human menopausal gonadotrophin (1st IRP-HMG)"

The second international reference preparation for human menopausal gonadotrophin (2nd IRP-HMG), formerly known as Pergonal 23, was prepared by Donini (Serono, Rome) from postmenopausal urine. Each ampoule of this standard material contains, by definition, 40 i.u. of human urinary FSH and 40 i.u. of human urinary LH.

2. Pituitary gonadotrophin preparations

Several pituitary preparations of various purity have been made available through the National Pituitary Agency (NPA) of the National Institute of Arthritis, Metabolism and Digestive Diseases (USA) and the National Institute for Biological Standards and Control of the Medical Research Council (MRC) of the UK. However, there are still no internationally agreed reference preparations for RIA of serum gonadotrophins.

The most commonly employed reference preparations of pituitary gonadotrophins are LER 907, a partially purified material of human origin containing 24 i.u. LH and 10 i.u. FSH per ampoule, and its derivative MRC 69/104, described by Bangham, Berryman, Burger, Cotes, Furnival, Hunter, Midgley, Mussett, Reichert, Rosemberg, Ryan and Wide, (1973). FSH and LH measurements reported in this thesis are expressed in terms of the FSH standard MRC 68/39 (33 i.u. FSH per ampoule) and LH standard MRC 68/40 (77 i.u. LH per ampoule) respectively.

1.2.3. The physiological effects of pituitary gonadotrophins

The morphological effects of gonadotrophins at tissue or organ level have been known for a considerable time and have formed the basis of the various in-vivo bioassays discussed in the previous section. In general, FSH promotes gonadal development and maturation of the germinal epithelium, while LH stimulates gonadal steroid secretion and thus influences indirectly the accessory reproductive organs. These gonadotrophin-induced changes in the gametogenetic and endocrine function of the gonads are the result of a chain of metabolic events in the target-organ cell sparked off by the binding of the gonadotrophin molecule to its cellular receptor.

(a) Follicle-stimulating hormone

Morphological actions

The physiological actions of FSH have been studied in a number of species including the rat (Greep, van Dyke and Chow, 1942), sheep (Hammond, Hammond and Parkes, 1942), monkey (Simpson and van Wagenen, 1953; Knobil, Kostyo and Greep, 1959) and man (Gemzell, Diczfalusy and Tillinger, 1958).

In females, FSH acts on the ovary to promote the growth of multiple follicles particularly of those which have already reached the antral stage of development. Whether FSH is also required for the earlier stages of follicular development, i.e. before antral cavity formation begins, remains still very much an open question. Earlier evidence tended to suggest that growth of pre-antral follicles was independent of pituitary FSH stimulation. Smith (1930) for example, reported that 4 days after hypophysectomy in the rat, no normal medium-sized follicles were present, but primordial follicles were undergoing development continually and atresia invariably occurred not later than the stage of cavity formation. However, as the interval between hypophysectomy and examination of the ovaries increases, it becomes apparent that follicular development is retarded at a much earlier stage (Ingram, 1953; Paesi, 1949), suggesting that, at least in the rat, development of the growing follicle beyond the two-layered granulosa stage requires pituitary support.

The processes involved in the more advanced development of follicles i.e. the mitotic proliferation of granulosa cells and the secretion of follicular fluid, leading to the formation of vesicular follicles are without any doubt FSH-dependent. Likewise, the transformation and aggregation of stromal cells surrounding the developing follicle into a layer of thecal cells can be attributed to the action of FSH but oestrogen secretion from these cells does not become apparent unless LH is given concurrently (Greep, van Dyke and Chow, 1942). Large doses of FSH preparations however, given to hypophysectomized animals, were reported not only to stimulate follicular growth but also to produce some repair of the interstitial cells, luteinization of the theca and corpus luteum formation and some secretion of oestrogen (van Dyke, P'an and Shedlovsky, 1950; Simpson, Li and Evans, 1951; Steelman and Segaloff, 1959). It is likely that these latter biological effects are due to LH contamination of the employed FSH material rather than to an intrinsic LH activity of the FSH molecule. Indeed, administration of purified FSH, devoid of any LH activity, does not induce oestrogen-secretion from vesicular follicles in mice (Eshkol and

Lunenfeld, 1967) nor does it lead to follicular rupture in the majority of amenorrhoeic women (Gemzell, 1961). FSH nevertheless facilitates both events by sensitizing the growing follicle to the biological actions of LH.

Several investigators have presented evidence which suggests that oestrogens, in addition to their direct effect on the growing follicle, will enhance gonadotrophin-induced follicular maturation. Oestradiol-benzoate, given in conjunction with exogenous hCG to hypophysectomized rats, produces larger vesicular follices than hCG alone (Paesi, 1952). Similarly, the local application of oestradiol or stilboestrol to one ovary of intact or hypophysectomized rats leads to a greater increase in ovarian weight and a greater responsiveness to exogenous or endogenous gonadotrophins (Bradbury, 1961). Recently, Reiter, Goldenberg, Vaitukaitis and Ross (1972) confirmed these observations using a specific anti-oestradiol antiserum to investigate the role of endogenously produced oestrogen in the ovarian response to exogenous gonadotrophins. As anticipated, the antiserum inhibited the increase in uterine weight following FSH and hCG. In addition, however, the increase in ovarian weight and follicular maturation were significantly reduced by the antiserum. Although the physiological significance of this synergistic effect of oestrogen on FSH-induced follicular growth is not yet fully understood, it seems not unreasonable to assume that oestrogen may play an important role in the selection mechanism of the pre-ovulatory follicle(s) by making the more developed follicle(s) (i.e. follicle(s) with higher rate of oestrogen production) less dependent on further FSH stimulation (Speroff and Vande Wiele, 1971).

Cellular actions

Being a hormone primarily concerned with division of follicular cells, FSH can be expected to affect cellular metabolism and synthesis of DNA, RNA and proteins. A number of investigators have demonstrated stimulatory effects of FSH on the synthesis of DNA both in-vivo (Lunenfeld and Eshkol, 1968) and in-vitro (Crooke and Ryle, 1968). Administration of diethylstilboestrol potentiates this effect of FSH (Goldenberg, Vaitukaitis and Ross, 1972). A single injection of pure FSH will also increase labelled uridine incorporation into RNA which, after leaving the cellular nucleus, aggregates to form ribosomes. Soon afterwards, an increase in protein-synthesis can be detected (Lunenfeld and Eshkol, 1968). Apart from inducing the follicular cells to undergo mitosis, the FSH-induced changes in cellular metabolism may also play a role in sensitizing the follicle for LH e.g. by increasing the number of specific LH-receptors in the cell membrane (Channing, 1974).

It is of interest to note that desialytion of FSH does not affect its ability to stimulate DNA synthesis in-vitro (Ryle, Chaplin, Gray and Kennedy, 1970), which suggests that the loss of activity of desialated FSH in conventional in-vivo bioassays is due to its higher metabolic clearance rate (Van Hall, Vaitukaitis, Ross, Hickman and Ashwell, 1971) rather than to impairment of the intrinsic biological activity of the molecule.

(b) Luteinizing hormone

Morphological actions

It is now well established that pure LH stimulates the development of thecal cells, induces luteinization of theca and granulosa and stimulates steroid production in follicular and luteinized cells. For these changes to occur, preliminary or simultaneous exposure of the ovary to FSH is essential. In the absence of FSH, LH-injections are incapable of stimulating the ovaries of immature rats or inducing premature rupture of the vaginal membrane (Fevold, Hisaw and Leonard, 1931). Similarly, moderate doses of LH given to hypophysectomized animals are not followed by an increase in ovarian weight or a significant secretion of oestrogen. Their only salient effects are repair and stimulation of interstitial cells and enlargement of thecal cells but without follicular maturation (Greep, van Dyke and Chow, 1942).

Cellular actions

The biological actions of LH at the cellular level have been studied most intensively in granulosa cells of animal or human origin since these cells can be harvested quite easily from growing follicles and represent a uniform population. There is however no reason to believe that the same principles may not apply to other LH target-cell types such as the theca interna.

As with any other hormone, the initial step in the activation of a sensitive target-cell involves an interaction of the LH-molecule with a specific membrane-bound receptor. The presence, in the ovary, of receptors with high affinity for LH (and hCG) has been demonstrated by a number of groups (e.g. Van Hall, Catt and Ross, 1972; Dufau, Catt and Tsuruhara, 1971; Kammerman, Canfield, Kolena and Channing, 1972; Lee, Coulman, Jiang and Ryan, 1973) and use of such receptors has been made to develop radioreceptor assays (see p. 27). During follicular maturation the number of specific LH-receptors on granulosa cells increases (Kammerman and Ross, 1975) presumably under the influence of FSH (Channing, 1974).

The binding of LH to its receptor activates the membrane-bound adenyl cyclase system (Savard, Marsh and Rice, 1965; Marsh and Lemaire, 1974) which converts adenosine-triphosphate (ATP) to adenosine-3'5'-monophosphate or cyclic-AMP (c-AMP). Similar activation systems employing c-AMP as "second messenger" have been described for several other endocrine organs such as adrenals, pancreas and pituitary. The central role of c-AMP in the steroidogenic action of LH has been illustrated by work of Butcher and Sutherland (1968) who showed that LH increases c-AMP levels within the cells of the corpus luteum before leading to an increased synthesis of progesterone. Moreover, it can be demonstrated that exogenous c-AMP stimulates the synthesis of progesterone in corpora lutea and interstitial cellular tissue in a number of species (for a recent review see e.g. Marsh, 1976).

Studies on bovine adrenal cortex have shown that c-AMP stimulates steroid-synthesis by increasing the activity of cholesterol esterase, the enzyme which converts cholesterol ester into free cholesterol Trzeciak and Boyd, 1974). The transport of this free cholesterol into the mitochondria and the subsequent conversion to pregnenolone are also enhanced in the presence of c-AMP (Hall and Koritz, 1965). It seems not unreasonable to assume that stimulation of steroidogenesis through activation of cholesterol-esterase by c-AMP is a common feature of all steroid producing cells, the nature of the resulting secreted steroid being dependent on the specific enzymatic apparatus of the cell. Thus, in the ovary, the follicular granulosa and the granulosa lutein cells, when stimulated by LH, produce mainly progesterone since these cells have only weak 17α-hydroxylase and little or no 17,20-desmolase activity (Fig. 1.1). Production of oestrogens by granulosa cells however

BIOSYNTHESIS OF STEROID HORMONES

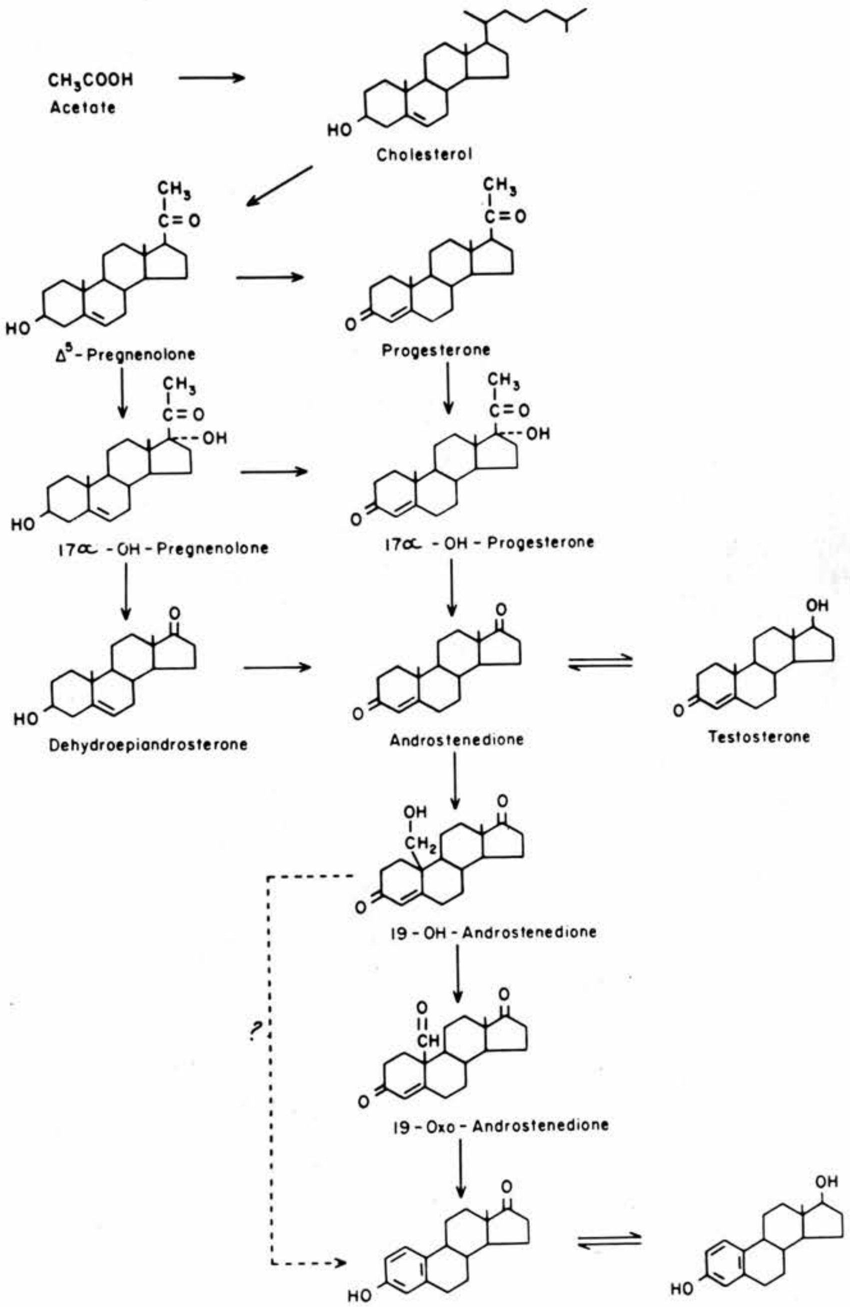


Figure 1.1: Steroid biosynthesis in the ovary

is possible if the cells are provided with androstenedione or testosterone. The follicular theca interna cells and the theca lutein cells, on the other hand, are the main source of 17β-oestradiol which is synthesized through the Δ^5 pathway (Short, 1962, 1964; Ryan and Short, 1965).

1.2.4 The pattern of secretion of pituitary gonadotrophins

Although biological assays provided some idea on the fluctuations in urinary excretion of FSH and LH during the menstrual cycle (Stevens, 1967), their relative insensitivity substantially hampered more detailed studies of urinary excretion patterns and, evidently, excluded their application to the measurement of plasma concentrations. The development of RIA's for pituitary gonadotrophins and, subsequently, for steroid hormones however, resulted in a major breakthrough in our knowledge and understanding of the changes in circulating gonadotrophin levels and their intricate relationships with the fluctuations in plasma steroid levels. Moreover, as the clearance rates of FSH (Coble, Kohler, Cargille and Ross, 1969) and LH (Kohler, Ross and Odell, 1968) appear to be concentration independent, changes in the circulating levels of these hormones may be considered to reflect fluctuations in their rate of excretion from the pituitary gland, rather than variations in the rate of their peripheral disposal.

From a dynamic point of view it would appear that, at any given moment, the rate of pituitary gonadotrophin secretion can be considered as the net result of at least four superimposed biological rhythms, each with its own characteristic secretion pattern. The relative importance of each of these secretion rhythms will of course depend upon several factors, e.g. animal species, age, sex, reproductive status etc.

(a) Circhoral rhythm

It is now well established that gonadotrophins are secreted from the pituitary in a pulsatile fashion. This pulsatile release is most conspicuous in sexually mature animals or humans in whom gonadal steroid secretion is minimal or completely absent, e.g. in ovariectomized sheep (Reeves, O'Donnell and Denorscia, 1972) or rhesus monkeys (Atkinson, Bhattacharya, Monroe, Dierschke and Knobil, 1970), in women after the menopause (Yen, Tsai, Naftolin, Vandenberg and Ajabor, 1972) or in patients with ovarian dysgenesis (Yen, Tsai, Vandenberg and Rebar, 1972). In these instances both amplitude and frequency of the episodic release are maximal with bursts of secretion occurring fairly regularly at 60 to 180 minute intervals, hence the term "circhoral" introduced by Dierschke, Bhattacharya, Atkinson and Knobil (1970). In general, episodic secretion of LH and FSH occur simultaneously, but the pulsatile FSH fluctuations are usually less well defined than those of LH. This may partly be due to the significantly longer half-life of the FSH-molecule (Coble , Kohler, Cargille and Ross, 1969).

In the intact human female, episodic gonadotrophin release can be seen at all stages of the menstrual cycle, but both amplitude and frequency of the release are markedly reduced as compared to e.g. postmenopausal women. Moreover, significant variations in amplitude and frequency of the pulses occur throughout the cycle. During the luteal phase of the cycle, frequency appears to be reduced as compared to the follicular phase, particularly the early part of it. Amplitude on the other hand, is smaller during the late follicular phase than during the luteal phase or peri-ovulatory period (Midgley and Jaffe, 1971; Yen, Vandenberg, Tsai and Parker, 1974).

The mechanism(s) involved in the generation and regulation of pulsatile gonadotrophin release are still rather obscure; several factors have been implicated. There is little doubt that steroid hormones, acting at the hypothalamic and/or pituitary level, can modify episodic secretion. Thus, in the ovariectomized rhesus monkey, administration of 17β-oestradiol rapidly interrupts pulsatile LH-release and depresses basal LH-levels (Yamaji, Dierschke, Bhattacharya and Knobil, 1972). Similar results were obtained in women with ovarian dysgenesis (Yen, Tsai, Vandenberg and Rebar, 1972). Progesterone alone appears to be ineffective in suppressing pulsatile LH-release in the rhesus monkey but under certain conditions, simultaneous administration of oestrogen and progesterone may have a synergistic action (Knobil, 1974). Similar effects resulting from changes in the hormonal environment may cause the variations in pulsatile release observed at different stages of the menstrual cycle.

Since other rhythms of gonadotrophin-secretion (i.e. diurnal, lunar and annual rhythms) either originate in or are mediated through the hypothalamus, it has been suggested that pulsatile release may also be under hypothalamic control. Indeed, the presence or absence of episodic release is to a large extent related to the degree of gonadotrophic activity of the hypothalamus. Instances in which this hypothalamic activity is generally assumed to be low are characterised by low basal gonadotrophin levels with very little or no pulsatile secretion e.g. during anoestrus in sheep (Yuthasastrakosol, Palmer and Howland, 1975; Martensz, Baird, Scaramuzzi and Van Look, 1976) or prepubertally in children (Boyar, Finkelstein, Roffwarg, Kapen, Weitzman and Hellman, 1972). Intensive activity of the hypothalamus on the other hand such as occurs following ovariectomy or after the menopause, is associated with intense episodic gonadotrophin secretion. However, the question as to whether in these latter situations the pulsatile release of gonadotrophins from the pituitary is a result of pulsatile changes in hypothalamic LRF secretion or, alternatively, may be attributed to an intrinsic property of the pituitary to release gonadotrophins in a pulsatile fashion, remains largely unanswered. Bhattacharya, Dierschke, Yamaji and Knobil (1972) have shown that in the ovariectomized rhesus monkey α -adrenergic blockers such as phentolamine or phenoxybenzamine, inhibit pulsatile LH-release and concluded therefore that pulsatile secretion is under the control of the central nervous system. These results could not be confirmed in the human (Yen, Vandenberg, Tsai and Parker, 1974), but the possibility that this discrepancy results from differences in the dosage employed cannot be excluded. Evidence for episodic secretion of bioassayable LRF-activity in human peripheral plasma (Seyler and Reichlin, 1974) and radioimmunoassayable LRF in sheep peripheral plasma (Nett, Akbar and Niswender, 1974) has been presented, but the observed fluctuations in LRF-activity are seemingly unrelated to episodic changes in LH secretion.

It should be remembered however that the pituitary response to pulsatile LRF-stimulation may vary according to the functional status of the gonadotrophs, the prevailing hormonal environment (Lasley, Wang and Yen, 1975) and the extent of "priming" of the pituitary by previous exposure to LRF (Aiyer, Shiappa and Fink, 1974). Other factors, such as a certain degree of refractoriness of the gonadotroph to repeated pulses of LRF occurring in quick succession (Schneider and Dahlen, 1973) would tend to further obscure any causal relationship between episodic LH-release and hypothalamic pulsatile LRF-secretion. More direct evidence to support a hypothalamic mechanism for pulsatile gonadotrophin release has recently been obtained by Carmel, Araki and Ferin (1975) who observed a pulsatile pattern of LRF in the portal blood of the rhesus monkey. In addition, Osland, Gallo and Williams (1975) demonstrated that pulsatile LH release from superfused isolated rat pituitaries can be obtained only by pulsed delivery but not by constant infusion of LRF.

Alternatively, Vande Wiele and Ferin (1974) have suggested that "the pulsatile secretion of FSH and LH may be dependent upon pituitary mechanisms rather than upon a pulsatile stimulatory input from the central nervous system". This conclusion was reached by the authors, following their observation that chronic administration of LRF to an ovariectomized rhesus monkey resulted in a pulsatile secretion of LH even after complete transsection of the pituitary stalk. Obviously, additional proof will be required to substantiate this hypothesis.

(b) Diurnal rhythm

There is little evidence to suggest that major changes in pituitary gonadotrophin secretion related to sleep-wake activity do occur in man. Earlier claims e.g. by Faiman and Ryan (1967 d), Saxena, Leyendecker, Chen, Gandy and Peterson (1969) and by Faiman and Winter (1971), of such a diurnal rhythm in FSH and/or LH secretion have not been confirmed by subsequent work (e.g. Krieger, Ossowski, Fogel and Allen, 1972; Naftolin, Yen, Perlman, Tsai, Parker and Vargo, 1973). Notable exceptions however occur during puberty (Boyar, Finkelstein, Roffwarg, Kapen, Weitzman and Hellman, 1972) and in the remission stage of anorexia nervosa (Boyar, Katz, Finkelstein, Kapen, Weiner, Weitzman and Hellman, 1974) when marked, sleep-synchronized increases in LH-secretion can be seen. In adult cycling women studied during the early follicular phase of the cycle the occurrence of a transient but consistent fall in plasma LH concentrations during the initial hours of nocturnal sleep has also been reported (Kapen, Boyar, Perlow, Hellman and Weitzman, 1973). It is not clear however whether this decrease in LH-levels is related to sleep rather than to posture since a comparable decline in LH has been observed by us on several occasions in supine patients who were fully awake.

(c) Menstrual rhythm

But for minor variations related to species differences in the length of the cycle and its component parts, changes in gonadotrophic secretion during the oestrous cycles in animals are basically similar to those observed during the menstrual cycles of higher primates and man. These changes will therefore not be discussed separately in this thesis.

The pattern of FSH and LH secretion during the menstrual cycle has been described by numerous investigators (e.g. Midgley, 1966; Bagshawe, Wilde and Orr, 1966; Franchimont, 1966; Faiman and Ryan, 1967 c; Midgley and Jaffe, 1968; Taymor, Aono and Pheteplace, 1968; Swerdloff and Odell, 1968; Saxena, Demura, Gandy and Peterson, 1968; Abraham, Klaiber and Broverman, 1969; Cargille, Ross and Yoshimi, 1969).

In summary (Figure 1.2), FSH-levels are relatively high during the early follicular phase of the cycle. They then decline to lower values until just prior to ovulation when a peak in FSH values occurs. This mid cycle FSH-peak is followed by a decline during the luteal phase to peripheral levels which are lower than those seen during the follicular phase. At the end of the luteal phase and a few days prior to menstruation, FSH levels start to rise again. Variations in LH are quite similar to those outlined for FSH. The only differences are that the plasma level of LH does not decline prior to the pre-ovulatory LH-surge and that the magnitude of the pre-ovulatory discharge is much larger for LH than for FSH.

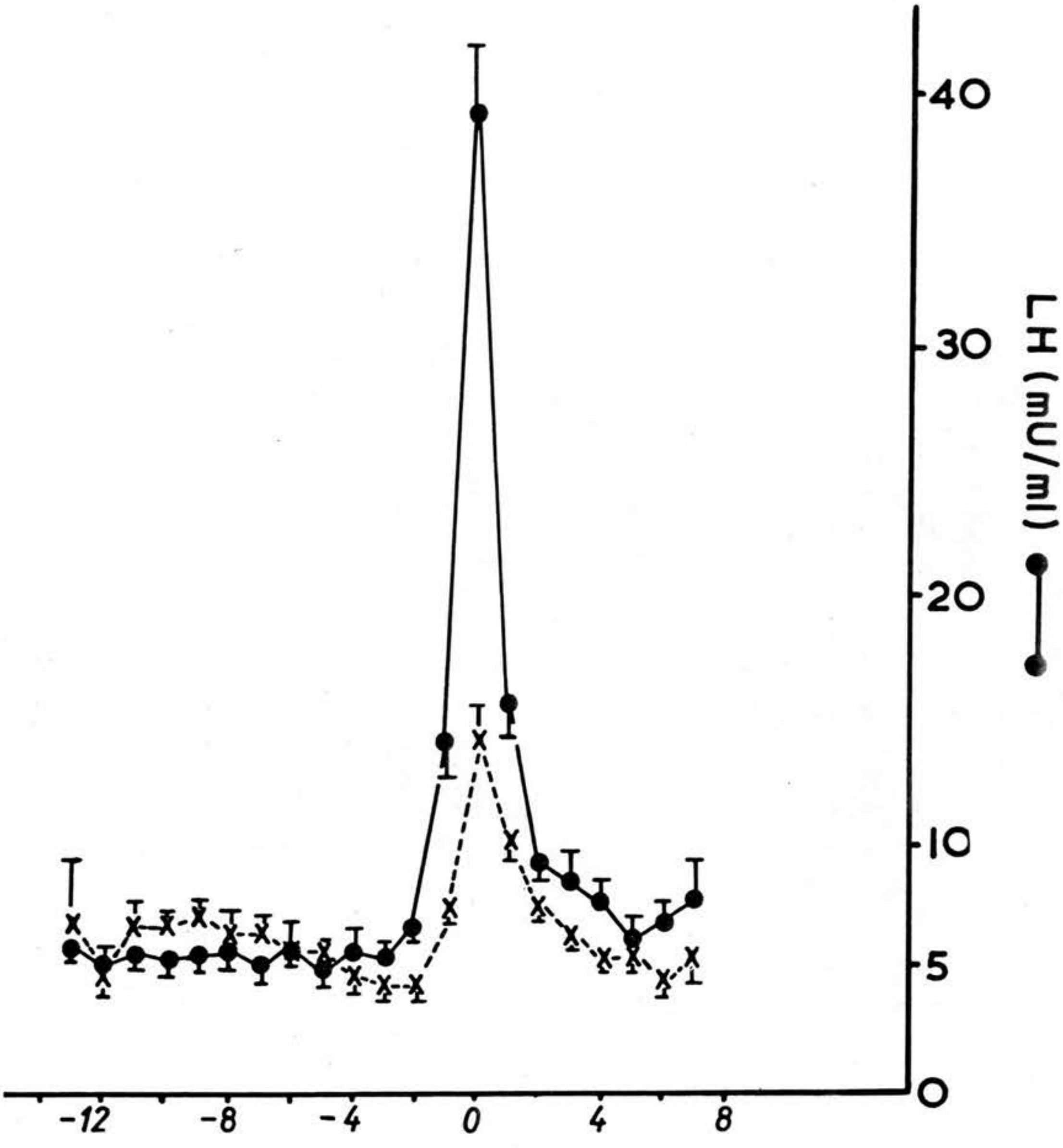


Figure 1.2: FSH and LH levels (means ± SEM) during the follicular and early luteal phase of the menstrual cycle

(reproduced with permission of Dr. W.H. Hunter, unpublished)

It cannot be doubted that the described changes in FSH and LH during the oestrous or menstrual cycle result from positive and negative feedback actions exerted by ovarian steroids at the hypothalamic and pituitary level. These feedback actions and the relationships which exist between gonadal steroid excretion and pituitary gonadotrophin release will be discussed more fully in the section on ovarian steroids and their feedback effects. (p.93)

(d) Breeding rhythm

Most animal species have a distinct breeding season during which mating and conception occur. Linked to environmental factors such as changes in the length of daylight or alterations in temperature or food intake, breeding seasons in mammalian species have usually, though not always, an annual rhythm (a notable exception is the grey squirrel which has two breeding seasons per year) (Sadleir,1972). Some species of wild animals (e.g. wild rabbit and certain tropical mammals), several domesticated (e.g. cow and pig) and laboratory animals (e.g. rat, mouse) on the other hand can breed nearly continuously. Their breeding "season", as that of man, occupies the larger part of their life-span, starting at puberty and extending into old age.

Although there is little direct evidence, it is generally assumed that the onset of the mating season in seasonal breeders, as the onset of puberty in continuous breeders, results from a decrease in hypothalamic sensitivity for the negative feedback effects of gonadal steroids (for reviews see Davidson, 1974; Grumbach, Roth, Kaplan and Kelch, 1974). In seasonal breeders, the reverse mechanism i.e. an increase in hypothalamic sensitivity, would terminate the mating season. As for the continuously breeding species, the reasons for reproductive failure in old age appear to be more diversified. In female rats, the decline of reproductive capacity in older animals has been attributed to a hypothalamic-pituitary failure of gonadotrophin secretion with secondary ovarian failure. Indeed, the ovaries of such acyclical elderly females still contain a considerable number of oocytes (Mandl and Shelton, 1959) and, when transplanted to ovariectomized young recipients, are capable of restoring regular oestrous cycles in the recipient. Conversely, when young ovaries are transplanted into old ovariectomized females, regular oestrous cycles are not re-established (Aschheim, 1964-1965). In contrast, in the human female, reproductive failure after the menopause is a direct result of primary ovarian failure, due to depletion of the oocyte stock (Block, 1952).

1.3 Prolactin (pituitary luteotrophic hormone, LTH)

Following the initial observations of Stricker and Grueter (1928) and Riddle and Braucher (1931) that extracts of anterior pituitary tissue could induce milk-production in pseudopregnant rabbits and pigeons respectively, the existence of a separate pituitary hormone involved in lactogenesis was strongly suspected. Subsequent work by Riddle, Bates and Dykshorn (1933) demonstrated that the same adenohypophysial principle was responsible for the milk production by the pigeon's crop sac as well as for milk secretion in mammals. The same authors also achieved partial purification and isolation of this lactogenic hormone which they called "prolactin".

It soon became clear however that the action of prolactin was not solely confined to its lactogenic effects in birds and mammals. In 1933, Riddle and Bates reported that purified prolactin induced remarkable suppression of the gonads in male and female pigeons. The same group found prolactin to be responsible also for the induction of broodiness in fowl (Riddle, Bates and Lahr, 1935) and maternal behaviour in rats (Riddle, Lahr and Bates, 1935). Other early reported effects of prolactin included the lengthening of the oestrous cycle of rats (Lahr and Riddle, 1936) and mice (Dresel, 1935) and the induction of brooding behaviour in fish (Noble, Kumpf and Billings, 1936). In 1941, prolactin joined the ranks of the pituitary gonadotrophins in view of its luteotrophic effect in rats (Astwood, 1941; Evans, Simpson, Lyons and Turpeinen, 1941). Since then numerous other actions of prolactin have been described. A recent review (Nicoll and Bern, 1972) lists not less than 85 diverse and distinct effects among the vertebrates. Most of these effects have recently been discussed in detail e.g. by Horrobin (1973, 1974) and Nicoll (1974) and will not be considered here.

Despite the overwhelming body of evidence derived from animal work, the existence of human prolactin as a separate pituitary hormone has long been a subject of intense controversy, which was rooted in the observation that even the most purified human growth hormone (hGH) preparations had intrinsic prolactin activity when bioassayed against a purified ovine prolactin standard (Li and Liu, 1964; Forsyth, 1968). Moreover, since animal prolactins have most of the metabolic actions of hGH in human subjects (Blizzard, Drash, Jenkins, Spaulding, Glick, Weldon, Powell and Raiti, 1966; McGarry and Beck, 1972), several investigators (e.g. Li, 1968) took the view that in man and other primates, growth hormone and prolactin are one and the same molecule. And even though several lines of experimental and clinical evidence (reviewed e.g. by Pasteels, 1973) challenged this view and supported the concept of a separate human prolactin, distinct from human growth hormone, the existence of human prolactin was not generally accepted until its isolation in pure form from human hypophyses was finally achieved (Hwang, Guyda and Friesen, 1972; Lewis, Singh and Seavey, 1972).

1.3.1 Chemistry of prolactin

Following the landmark report of Riddle, Bates and Dykshorn (1933) on the identification of "a new pituitary hormone" several laboratories started work on the further purification of these initially crude pituitary extracts. Most of these early studies on extraction and isolation of prolactin were carried out on ovine and bovine pituitaries because of their higher content of prolactin activity.

(a) Purification

At the present time, homogeneous, physicochemically pure prolactin preparations have been isolated from the pituitaries of a number of species including the sheep (Cole and Li, 1955), cattle (White, Bonses and Long, 1942), pig (Eppstein, 1965), rat (Ellis, Grindeland, Nuenke and Callahan, 1969), dog (Saluja and Kwa, 1972), monkey (Guyda and Friesen, 1971) and man (Hwang, Guyda and Friesen, 1972; Lewis, Singh and Seavey, 1972). One of the main difficulties encountered in the isolation of human prolactin is the relative inefficiency of the employed extraction procedures which generally yield only 10-25% of the prolactin that radioimmunoassay indicates is actually present in fresh pituitary glands. This may be due to proteolytic alteration of prolactin during prolonged storage of the gland before extraction (Lewis and Singh, 1973) and to aggregation of the molecule after extraction to high molecular weight forms which are difficult to purify (Pensky, Murray, Mozaffarian and Pearson, 1972).

Apart from the anterior pituitary, amniotic fluid has proved to be a major source of a prolactin molecule which immunologically, electrophoretically and chromatographically appears to be similar to pituitary prolactin but is quite distinct from human placental lactogen (hPL; human chorionic somatomammotropin, hCS) (Fournier, Desjardins and Friesen, 1974).

(b) Physicochemical properties and structure

Prolactin, as placental lactogen (hPL) and growth hormone (hGH), is a pure protein without carbohydrate moiety in the molecule. All three hormones have molecular weights of the same order (22,000-23,000). In view of the considerable overlap in their biological activities, it is not surprising that the three molecules also show considerable homology in their linear amino acid sequence suggesting that they may have evolved from a common primordial precursor (Niall, Hogan, Sauer, Rosenblum and Greenwood, 1971). When the primary structures of human prolactin, hPL and hGH are compared (Niall, Hogan, Tregear, Segre, Hwang and Friesen, 1973), hPL and hGH are found to differ in only 32 of the 191 amino acid residues. In contrast, human prolactin and hGH are structurally much further apart. There appears to be much greater similarity between human and other mammalian prolactins than between human prolactin and either growth hormone or human placental lactogen.

1.3.2 Measurement of prolactin

Methods for the measurement of prolactin in biological fluids include in-vivo and in-vitro biological assays, radioimmunological and radioreceptor assays. For each type of assay, the general principles qua sensitivity, specificity, practicability and biological relevance of the assay result are similar to those outlined for the measurement of pituitary gonadotrophins (p. 20). In summary, biological assays in general are often laborious, relatively insensitive and not very precise. In addition, bioassays for prolactin, particularly those based upon the lactogenic activity of the molecule, are relatively non-specific since they respond also to growth hormone and placental lactogen. Radioimmunoassays have the advantages of high practicability, sensitivity and specificity, but suffer from the fact that they detect immunological rather than biological activity. Finally, the present radioreceptor assays for prolactin, though far more sensitive and precise than classical bioassays, still lack the necessary specificity to make them the ideal substitute for both bio- and radioimmunoassays.

(a) Bioassays

In view of the multitude of physiological actions produced by the hormone it may not be surprising that a vast range of methods employed for the bioassay for prolactin have been described. A detailed discussion of these methods is outside the scope of this thesis but can be found in Horrobin (1973).

(b) Radioimmunoassays

In the radioimmunological field prolactin undoubtedly occupies a unique position in that RIA's capable of detecting human prolactin in peripheral plasma were described before its separate identity from hGH was generally accepted and its isolation from human hypophyses was achieved. In fact, without the aid of radioimmuno- and radioreceptor-assays for monitoring prolactin-concentration during the course of its purification from pituitary extracts, isolation of human prolactin might yet not have been achieved. This rather unusual situation where peripheral concentrations of a hormone in various physiological and clinical conditions were reported before its very existence was generally admitted, arose from the fact that prolactins from various mammalian species are immunologically (and presumably structurally also) very similar to each other and will therefore cross-react in each other's assays (Nicoll and Bryant, 1972). Thus the homologous ovine prolactin assay (Bryant and Greenwood, 1968) could be successfully applied to the measurement of human prolactin (Jacobs, Mariz and Daughaday, 1972; L'Hermite, Delvoye, Nokin, Vekemans and Robyn, 1972; Midgley and Jaffe, 1972). Likewise, antisera raised against monkey prolactin bound human prolactin and could be used for the development of a RIA (Hwang, Guyda and Friesen, 1971). Bryant, Siler, Greenwood, Pasteels, Robyn and Hubinont (1971) developed an assay system based on material isolated from normal and tumourous human pituitaries cultured in-vitro, but this technique as well as the aforementioned heterologous systems have now been largely replaced by homologous RIA's similar to those developed by Friesen, Hwang, Guyda, Tolis, Tyson and Myers (1972) and Sinha, Selby, Lewis and Vanderlaan (1973).

As with gonadotrophins no general agreement has yet been reached on the use of a common standard to express assay results obtained by different laboratories, although efforts in that direction have been made (Cotes, 1973).

As for the biological relevance of concentration estimates obtained by RIA, the situation for prolactin appears to be more favourable than that for FSH and LH. Indeed, in an extensive series Frantz, Kleinberg and Noel (1972) were able to obtain a very good correlation between the results of bioassay and immunoassay of prolactin in individual human plasma samples. Similar results were reported by Besser, Parke, Edwards, Forsyth and McNeilly (1972).

(c) Radioreceptorassays

The ability of the prolactin molecule to interact with specific membrane-bound receptors has been employed in the development of several radioreceptor assays (RRA) using cell membrane fractions isolated from pregnant rabbit mammary glands (Friesen, Tolis, Shiu and Hwang, 1973), from female rabbit liver (Parke, 1973) and from mouse mammary glands (Turkington, Frantz and Majumder, 1973). Their application as assay methods for routine use however appears unlikely because of the high degree of cross-reactivity with hGH and hPL (Friesen et al.,1973). Moreover, since in conditions where hGH is low, the results obtained by RRA and RIA correlate very well, RRA's appear to offer no real advantage to the highly specific RIA's currently in use. However, they may be favourably employed to detect lactogenic activity in situations where specific radioimmunoassays are not available or to detect lactogenic activity other than that due to prolactin if the latter can be measured by a homologous RIA (Shiu, Kelly and Friesen, 1973).

1.3.3 Physiological effects of prolactin

When looking for a "common denominator" among the various actions claimed for prolactin, Nicoll and Bern (1972) concluded that prolactin appeared to be "the jack-of-all-trades of the pituitary gland, a versatile hormone that did not become specialized to subserve any single function or group of functions". Indeed, the authors were able to compile from the literature not less than eighty-odd different effects attributed to prolactin, including seventeen which had been demonstrated in mammals. Of the latter, about eleven are more or less directly related to reproduction. A detailed account of them as well as of the other reported actions of prolactin has been given by Nicoll (1974).

However, as emphasised by the latter author as well as by Horrobin (1974), not all of the reported actions of prolactin may have biological significance and several of them may need re-evaluation in view of more recent findings. Indeed,virtually all studies on the effects of prolactin in mammalian species have been performed using ovine prolactin preparations. Heterologous use of hormones however may lead to erroneous interpretations (Nicoll, 1974). Secondly, work by Meier's group and others (reviewed by Nicoll, 1973) has indicated that tissue responsiveness to prolactin may vary depending on the time of day the prolactin-preparation is administered, or, more precisely, depending on the temporal relationship between the time of prolactin injection and the peak level of endogenously secreted or exogenously administered corticosteroids. Since endogenous prolactin, like adrenal corticosteroids,has a diurnal secretion pattern (see further, p62), it seems not inconceivable that similar interactions between prolactin and adrenal corticosteroids (or, for that matter, any other hormone with a cyclical secretion pattern) may also occur under normal physiological conditions.

Thus, the temporal relationships among the secretion patterns of prolactin and other hormones, i.e. the "phasing" of their secretion, may be of major significance in determining the eventual response of target organs. Consequently, the possibility must be considered that pathological conditions could arise not only from an excess or deficiency of prolactin, but also from improper "phasing" of hormonal secretion.

Finally, when studying the function of prolactin, it should be borne in mind that experimental results obtained with the use of supraphysiological amounts of this hormone may not necessarily be relevant. In the past, research-workers tended to start a study on the effects of prolactin, and indeed of any other hormone, by giving large doses and continuing only if these yielded positive results. However, this practice may be hazardous in the prolactin field. Indeed, recent work by McNatty, Sawyers and McNeilly (1974) would indicate that, at least as far as progesterone production by human granulosa cells in-vitro is concerned, prolactin may have a biphasic effect: low levels are required for normal progesterone production, high levels, on the other hand, have quite the opposite effect and inhibit progesterone-synthesis. It is evident that this finding, if confirmed in other situations, would have important implications for future studies on prolactin physiology.

Of the various actions of prolactin, only those related to female reproduction in mammals will be briefly considered here.

(a) Physiological effects at tissue level

Mammotrophic effect

Since the mammotrophic and lactogenic effects of prolactin provided the first clues as to the very existence of this hormone, it may not be surprising that this aspect of prolactin physiology has received most of the attention. It would be misleading however to regard prolactin as the only hormone involved in the development and secretory activity of the mammary gland, This was already recognised by Stricker and Grueter (1928) who noticed that their lactogenic pituitary extracts did not stimulate mammary growth or initiate lactation in immature animals. Subsequent studies e.g. by Lyons and co-workers (see e.g. Lyons, Li and Johnson, 1958) in hypophysectomized, ovariectomized and adrenalectomized rats have amply demonstrated that prolactin forms only part of a "lactogenic complex" involving several hormones. Thus, development of the mammary duct system at puberty requires the synergistic action of at least four hormones: oestrogens, growth hormone, insulin and corticosteroids. Subsequent lobuloalveolar growth such as occurs during pregnancy, is the result of synergism between oestrogen, progesterone, insulin and prolactin. Growth hormone, corticosteroids and placental lactogen may facilitate this process. The initiation of secretory activity from the fully developed gland requires prolactin, insulin and corticosteroids and may be further facilitated by growth hormone and thyroxine.

Although the hormonal requirements for mammary growth and initiation of lactation have not been investigated as extensively in other species as they have been in the rat, the information available in other species (reviewed e.g. by Denamur, 1969) would indicate that major qualitative differences do not exist. There is however considerable disagreement as to the role played by prolactin in the maintenance of lactation. Data in the rat (Simpson, Simpson, Sinha and Schmidt, 1973) as well as in ruminants (e.g. sheep: McNeilly, 1972 and cattle: Ingalls, Convey and Hafs, 1973) indicate that both basal prolactin levels and the prolactin response to suckling, after having been high in the early postpartum period, fall progressively as lactation proceeds, which would suggest that maintenance of full secretory activity of the mammary gland may become less dependent on intensive prolactin stimulation once lactation is established. Consistent with this view are the findings that neither elevation of prolactin levels in lactating ewes by treatment with perphenazine or haloperidol (Morag, Shani, Sulman and Yagil, 1971) nor the abolition of suckling-induced prolactin surges with 2-bromo-α-ergocryptine in goats (Hart, 1973) affect lactational performance. Since the half-life of prolactin in the mammary gland may be hours or even days, it has been suggested (Horrobin, 1974) that once the mammary gland has been saturated with prolactin in the early post-partum period and maximal secretory activity has been reached, only minimal amounts of the hormone would be further required to maintain full saturation of the receptors. Normal lactation could thus be maintained despite low levels of prolactin, the secretion of which could be regulated by a negative feedback system.

In the human, the pattern of prolactin secretion during lactation is similar to that described in animals (Hwang, Guyda and Friesen, 1971). As in animals, prolactin appears to be essential for the initiation of lactation since deficient prolactin secretion, such as is present in Sheehan's syndrome (Fournier, Desjardins and Friesen, 1974) is associated with a failure to lactate. Inappropriate, non-puerperal lactation (i.e. galactorrhoea) on the other hand is often accompanied by abnormal prolactin secretion (Turkington, 1972). Inhibition of prolactin release with 2-bromo-α-ergocryptine effectively suppresses lactation in puerperal women (Varga, Lutterbeck, Pryor, Wenner and Erb, 1972; del Pozo, Brun del Re, Varga and Friesen, 1972; del Pozo, Friesen and Burmeister, 1973; Rolland and Schellekens, 1973) and in patients with galactorrhoea (Lutterbeck, Pryor, Varga and

Wenner, 1971; Besser, Parke, Edwards, Forsyth and McNeilly, 1972; Varga, Wenner and del Pozo, 1973). Like in ruminants, the maintenance of fully established milk secretion may become less dependent on prolactin since normal levels of prolactin in peripheral plasma are found during the later stages of puerperal lactation (Tyson, Hwang, Guyda and Friesen, 1972) as well as in several cases of nonpuerperal lactation (Tolis, Somma, Van Campenhout and Friesen, 1974; Malarkey, 1975). However, further suppression of these basal levels in lactating women with 2-bromo-α-ergocryptine inhibits milk production (Brun del Re, del Pozo, de Grandi, Friesen, Hinselmann and Wyss, 1973), suggesting that even when lactation is fully established, its maintenance requires continuing, albeit minimal, prolactin stimulation.

Luteotrophic effect

Although prolactin appears to be essential for normal luteal function in a few selected species (for references see p.12), its role as a luteotrophic hormone in most other mammalian species is not so well established. In the human, several investigators have suggested that prolactin may not be involved in the control and function of the corpus luteum, but the presented evidence has usually been rather inconclusive. Speroff and Vande Wiele (1971) observed that highly purified hLH alone was capable of supporting luteal function in a hypophysectomized woman in whom follicular development and ovulation was induced with hMG and hLH. They therefore concluded that LH is the only essential luteotrophic stimulus in the human female. A synergistic role for prolactin cannot be excluded from these studies however since prolactin levels (which were not measured in this patient) do not necessarily become undetectable after hypophysectomy (Friesen, Webster, Hwang, Guyda, Munro and Read, 1972). Suppression of the basal levels and the nocturnal prolactin rise in normally cycling women treated with 2-bromo-α-ergocryptine did not affect follicular development, ovulation or luteal function (del Pozo, Goldstein, Friesen, Brun del Re and Eppenberger, 1975), but prolactin levels never became undetectable during treatment.

Thus at the present time, it cannot be excluded that low levels of prolactin may be required for normal function of the human corpus luteum. A similar view has been expressed by McNatty, Sawyers and McNeilly (1974) who demonstrated that maximal progesterone production by human granulosa cells cultured <u>in-vitro</u>, could only be obtained when physiological amounts of prolactin were present in the culture medium.

Luteolytic effect

The luteolytic action of prolactin in the hypophysectomized rat was first demonstrated by Malven (1969) using an ovine prolactin preparation and has subsequently been confirmed with homologous rat prolactin (MacDonald, Yoshinaga and Greep, 1973). The physiological significance of this effect has been revealed by work of Wüttke and Meites (1971). These authors showed that inhibition of the prolactin surge in the pro-oestrous rat by injection of ergot drugs does not interfere with normal oestrous cycles and ovulation. However, the old set of corpora lutea which was not activated to a functional state during the previous cycle, failed to degenerate. After several cycles the ovaries were filled with many corpora lutea from previous cycles. A similar luteolytic action of prolactin has been described in the mouse (Grandison and Meites, 1972).

The relevance, if any, of these observations for other mammalian species is not clear. In the human, plasma levels of prolactin fluctuate widely during the course of the menstrual cycle (see e.g. McNeilly, Evans and Chard, 1973) and there is no indication of increased secretion at the time of luteal regression. In addition, suppression of prolactin levels by means of 2-bromo-α-ergocryptine in normally cycling women does not prolong the length of the cycle (del Pozo, 1973). Thus it would appear that physiological prolactin concentrations do not have a luteolytic action in the human female. The possibility however that abnormally elevated prolactin levels might be luteolytic cannot be excluded. Indeed, Calaf (1973) reported that sulpiride, a psychotropic drug which is known to stimulate prolactin secretion (L'Hermite, Delvoye, Nokin, Vekemans and Robyn, 1972), could shorten the cycle and affect luteal function as evidenced by monophasic basal body temperature and low urinary pregnanediol excretion, when administered to normally cycling women during the peri-ovulatory period. Similar findings were reported by Delvoye, Taubert, Jürgensen, L'Hermite, Delogne and Robyn (1974), who also drew attention to the fact that the low progesterone levels observed during the treatment cycles were associated with low FSH and LH-levels.

It is likely therefore that in both instances the deficient luteal function is primarily a result of the decrease in gonadotrophin secretion which itself may be due either to a direct action of the administered compound at the hypothalamic level or to a "short-loop" negative feedback effect of the elevated prolactin levels (Varga, Wenner and del Pozo, 1973). However, the possibility that the manifestation of this luteal deficiency may be facilitated by a direct luteolytic action of the elevated prolactin levels must also be considered in view of the aforementioned observations by McNatty, Sawyers and McNeilly (1974). It is noteworthy in this respect that recently reported data shows that short luteal phases and low progesterone secretion may be associated with elevated plasma prolactin concentrations in some infertile women. Prolactin suppression with 2-bromo-α-ergocryptine was found to restore normal luteal function and fertility in these patients (del Pozo, Wyss and Varga, 1975).

Antigonadotrophic (antigonadal) effect

In recent years much attention has been devoted to the question as to whether the elevated prolactin levels found in lactating women and in some forms of male and female hypogonadism, are causally related to the hypofunctional status of the gonads in these conditions, and, if so, whether the hypogonadism results from a direct inhibitory effect of prolactin on pituitary gonadotrophin secretion or from an antagonism between prolactin and gonadotrophin in their gonadal effects. However, the alternative explanation that both hypogonadism and elevated prolactin secretion may be manifestations of one and the same underlying defect in hypothalamic function must also be considered.

In puerperal women, it is well known that the duration of post-partum amenorrhoea tends to be related to the duration of full breast feeding. In non-lactating women ovulation is unlikely to occur during the first six weeks after delivery while in lactating women this period is extended to about nine weeks. (Perez, Vela, Masnick and Potter, 1972). During this phase the ovaries appear relatively insensitive to gonadotrophin stimulation. Indeed ovarian activity as reflected by vaginal smears (Keettel and Bradbury, 1961) or peripheral 17β-oestradiol levels is minimal and this despite the fact that FSH-levels are normal or even slightly elevated (Jaffe, Lee and Midgley, 1969; Reyes, Winter and Faiman, 1972; Rolland, Lequin, Schellekens and De Jong, 1975). Moreover, administration of exogenous gonadotrophins to lactating women in the early post-partum period does not give rise to an increase in urinary total oestrogen excretion (Zarate, Canales, Soria, Ruiz and MacGregor, 1972). Since the duration of this phase of ovarian refractoriness is apparently related to the period of hyperprolactinaemia, it has been suggested (Reyes et al, 1972; Zarate et al, 1972) that prolactin may exert an antigonadotrophic effect at the level of the ovaries. This view is seemingly supported by the observation that the duration of post-partum amenorrhoea is shorter in women treated with 2-bromo-α-ergocryptine for suppression of lactation than it is in untreated, lactating controls (Rolland, De Jong, Schellekens and Leguin, 1975). However, del Pozo, Varga, Schulz, Künzig, Marbach, Lopez del Campo and Eppenberger (1975) recently challenged the concept of an antigonadotrophic action of prolactin at the ovarian level. By administering exogenous gonadotrophins to lactating women, to non-lactating women in whom prolactin secretion and lactation had been suppressed with 2-bromo-α-ergocryptine and to patients with amenorrhoea, galactorrhoea and elevated prolactin levels, these workers were able to demonstrate that the ovaries in both groups of puerperal women are refractory to gonadotrophin-stimulation regardless of their prolactin levels. This would indicate that the antigonadotrophic effect of prolactin may not be exerted at gonadal level, a view which was further substantiated by their finding that the ovaries of the galactorrhoeic patients with elevated prolactin levels, in contrast to those of puerperal women, did respond to exogenous gonadotrophin therapy. Incidentally, male patients with galactorrhoea due to a prolactin-secreting tumour, also show a normal or near normal rise in plasmatestosterone following hCG treatment (Friesen, Tolis, Shiu and Hwang, 1973). It would appear therefore that in puerperal women, ovarian refractoriness to gonadotrophins cannot solely be attributed to an antigonadotrophic effect of prolactin at the gonadal level.

An alternative explanation has been put forward by Varga, Wenner and del Pozo (1973) who suggest that the antigonadotrophic action of prolactin may be exerted at hypothalamic level through a short-loop negative feedback system. Such mechanism might account for the impaired secretion of LH in postpartum women, the levels of which remain scarcely detectable throughout the period of hyperprolactinaemia (Rolland, De Jong, Schellekens and Lequin, 1975). Alternatively, both the elevated prolactin secretion and the suppression of LH-secretion may be manifestations of an inhibition of hypothalamic activity effected by neurogenic (e.g. suckling stimulus) and/or hormonal pathways (e.g. hypothetical substance(s) secreted by lactating mammary gland).

(b) Cellular actions

In view of the variety of its effects, including actions on such basic physiological processes as osmoregulation, Nicoll and Bryant (1972) have suggested that a role of prolactin of major significance may be that of modifying the responsiveness of various target organs to the trophic influence of other hormones. At the cellular level, such an interaction could take place under several forms (Horrobin, 1974) e.g. modification of the binding of the trophic hormone to its cellular membrane receptor, interference with the production of a second messenger such as c-AMP or modification of c-AMP dependent protein-kinase activity which is the preliminary step to phosphorylation and activation of several enzyme systems (Krebs, 1972). Some evidence would suggest that this latter mechanism may be involved in mediating the mammotrophic effect of prolactin. In contrast to other trophic hormones such as LH, ACTH etc., prolactin-binding to its cellular mammary receptor does not activate the adenyl-cyclase system. It does stimulate the induction of cyclic-AMP dependent protein kinase activity however and could thus potentiate the effects of other mammotrophic hormones which act primarily on the formation of c-AMP (Turkington, Frantz and Majumder, 1973).

1.3.4 The pattern of secretion of prolactin

As with the other two gonadotrophins the secretion pattern of prolactin at any given moment can be considered as the algebraic sum of several intrinsic biological rhythms each with its own characteristic secretion pattern.

(a) Circhoral rhythm

Studies of prolactin levels in plasma samples taken at frequent intervals has revealed that prolactin, like LH and FSH, is secreted in a pulsatile fashion (Sassin, Frantz, Weitzman and Kapen, 1972; Sassin, Frantz, Kapen, and Weitzman, 1973; Parker, Rossman and Vanderlaan, 1973). Spurts of secretion appear to occur every 30 to 180 minutes and are seemingly unrelated to the stage of the menstrual cycle (McNeilly and Chard, 1974). As might be expected amplitude, but not so much frequency, of the pulsatile secretion is more pronounced in patients with hyperprolactinaemia (Boyar, Kapen, Finkelstein, Perlow, Sassin, Fukushima, Weitzman and Hellman, 1974; Kapen, Boyar, Freeman, Frantz, Hellman and Weitzman, 1975) and during pregnancy (Boyar, Finkelstein, Kapen and Hellman, 1975).

The mechanism(s) involved in the generation of pulsatile prolactin secretion is (are) still obscure. On the basis of their observations in patients with hyperprolactinaemia Boyar and his co-workers (Boyar et al, 1974; Kapen et al, 1975) have suggested that pulsatile prolactin release may be regulated by a hypothalamic dopaminergic control mechanism. As will be discussed further (p. 84) pituitary prolactin secretion in mammals appears to be controlled predominantly through hypothalamic secretion of a prolactin release-inhibiting factor (PIF). The release of this latter substance is apparently dependent upon a dopaminergic mechanism since the injection of dopamine into the third ventricle of rats causes a rise in PIF-activity in the hypophyseal portal circulation (Kamberi, Mical and Porter, 1970). Activation of a similar dopaminergic control system will also cause a rise in LRF-activity in the portal vessels (Kamberi, Mical and Porter, 1969). Thus there appears to exist an inverse relationship between the secretion of FSH and LH on the one hand and that of prolactin on the other hand. Since Boyar and his co-workers observed that in some of their patients with elevated prolactin levels, a similar temporal reciprocal relationship existed between the initiation of prolactin secretion and the cessation or decrease in the pulsatile secretion of LH, the authors suggested that a hypothalamic dopaminergic mechanism may be involved in episodic LH and prolactin secretion. Evidently, this hypothesis will have to await further confirmation.

(b) Diurnal rhythm

The initial observations of Nokin, Vekemans, L'Hermite and Robyn (1972) on the existence of a marked diurnal variation in plasma prolactin concentrations (with highest values occurring during sleep), have been amply confirmed and refined by several workers. Within 60 to 90 minutes after sleep-onset, a clear increment in prolactin concentration can be demonstrated and this is followed by a series of larger secretory episodes resulting in progressively higher baseline plasma concentrations towards the end of the sleep period (Sassin, Frantz, Weitzman and Kapen, 1972; Parker, Rossman and Vander Laan, 1973). Although the nocturnal release of prolactin is similar to that of hGH with regard to sleep-dependency, the actual secretion patterns are quite different since hGH is secreted predominantly in the early period of sleep (Sassin, Frantz, Kapen and Weitzman, 1973).

There are no apparent qualitative differences in sleep-related prolactin secretion at different stages of the menstrual cycle (McNeilly, Evans and Chard, 1973). Quantitatively, however, nocturnal prolactin-secretion might be increased at midcycle as compared to the follicular or luteal phases of the cycle (Ehara, Siler, Vanden Berg, Sinha and Yen, 1973).

In most forms of pathological hyperprolactinaemia the normal sleep-wake difference in prolactin concentrations is lost; levels during sleeping and waking periods are elevated and equal and no further sleep-related augmentation of prolactin secretion occurs (Boyar, Kapen, Finkelstein, Perlow, Sassin, Fukushima, Weitzman and Hellman, 1974). In physiological hyperprolactinaemia, on the other hand, such as is present during pregnancy, the hypothalamic sleep-related secretory "program" is maintained in a qualitative manner, albeit at a higher set-point (Boyar, Finkelstein, Kapen and Hellman, 1975). It is worth noting in this respect that in a patient with persistent postpartum amenorrhoea and galactorrhoea (Chiari-Frommel syndrome) the 24-hour prolactin-profile was similar to that observed during pregnancy with preservation of the sleep-associated rise in prolactin levels (Kapen, Boyar, Freeman, Frantz, Hellman and Weitzman, 1975).

(c) Menstrual rhythm

In several mammalian species serum prolactin levels appear to follow a specific pattern during the oestrous cycle. In the rat, prolactin levels are low at all stages of the cycle except for a brief, 12-hour period on the afternoon and evening of pro-oestrus when a surge in prolactin secretion occurs with peak values as much as 25-fold higher than basal values (Gay, Midgley and Niswender, 1970; Neill, 1972; Wüttke and Meites, 1970). This increase in prolactin on the afternoon of pro-oestrus is directly linked to the secretion of oestrogen. Indeed, administration of oestradiol-antibodies on di-oestrus 2 completely abolishes the surge of prolactin secretion at pro-oestrus, but this effect can be reversed by the simultaneous administration of diethylstilboestrol (to which the antibodies do not bind) (Neill, Freeman and Tillson, 1971). In the sheep (Reeves, Arimura and Schally, 1970) and cow (Raud and Odell, 1971) the profile of prolactin secretion during the oestrous cycle is similar to that described in the rat.

In view of the characteristic, well-defined pattern of prolactin secretion in other mammalian species and its intimate relationship with ovarian oestrogen secretion, it seems rather surprising that most workers (e.g. Hwang, Guyda and Friesen, 1971; Midgley and Jaffe, 1972; Friesen, Hwang, Guyda, Tolis, Tyson and Myers, 1972; McNeilly, Evans and Chard, 1973) fail to detect any significant change in serum prolactin during the human menstrual cycle. However, Vekemans, Delvoye, L'Hermite and Robyn (1972) and Delvoye, Hasan, L'Hermite, Neumann and Robyn (1973) reported significant increases of prolactin at mid-cycle and during the luteal phase, which coincided with those of plasma oestradiol. At the present time, it is not clear what factors may account for the reported discrepancies in prolactin profiles.

On theoretical grounds one might expect that prolactin levels during the menstrual cycle would parallel those of oestradiol as they do in other mammals. Indeed several data indicate that prolactin release in the human, as in animals, can be stimulated by oestrogens. During pregnancy, the increase in plasma prolactin (Hwang, Guyda and Friesen, 1971; L'Hermite, Stavzic and Robyn, 1972; Jacobs, Mariz and Daughaday, 1972) correlates very well with the rise in maternal plasma oestradiol. Furthermore, administration of exogenous oestrogens to normally cycling women (Robyn, Delvoye, Nokin, Vekemans, Badawi, Perez-Lopez and L'Hermite, 1973), to normal men (Frantz, Kleinberg and Noel, 1972), or hypogonadal patients (Yen, Ehara and Siler, 1974) will elevate prolactin levels. Finally, serum prolactin levels in normally cycling women are significantly higher than those found in men (Ehara, Siler, Vanden Berg, Sinha and Yen, 1973) or postmenopausal women (Vekemans and Robyn, 1975).

Thus, from the clinical and experimental evidence presented it seems not unreasonable to conclude that, in the human, oestrogens exert a positive feedback effect on pituitary prolactin secretion. The failure to detect a similar effect during the menstrual cycle is therefore rather puzzling. Since most studies on changes in prolactin concentrations during the cycle have been based on daily samples, subtle changes in episodic or nocturnal release may have escaped a measurable effect. Alternatively, this positive feedback effect of oestrogen on prolactin release may not be operative during the menstrual cycle when several other steroid hormones secreted by the growing follicle are present and could exert an inhibitory effect.

(d) Breeding rhythm

Recent evidence would suggest that in rams (Pelletier, 1973) and ewes (Walton, McNeilly, McNeilly and Cunningham, 1975) the secretion of prolactin may be under photoperiodic control. During the non-breeding season levels of prolactin are high but they decline towards the beginning of the breeding season. Obviously this pattern of prolactin secretion is inversely related to that of FSH and LH, which raises the intriguing question whether the suppression of FSH and LH secretion during the non-breeding season is due to the elevated prolactin levels acting via a short-loop negative feedback system rather than to a direct photoperiodic effect. Experimental suppression of the elevated prolactin levels during the non-breeding season might provide an answer to this question. It is noteworthy, in this respect that in several avian species prolactin has been shown to inhibit photostimulated gonadal growth (Dusseau and Meier, 1971).

2. The hypothalamic-pituitary axis

2.1 The hypophysiotrophic role of the hypothalamus in the regulation of pituitary gonadotrophin secretion

For many years it has been known that changes in the external environment can influence reproductive processes in a variety of animal species (see e.g. Marshall, 1936), but the way in which external stimuli produce these effects has long been poorly understood. Some of these exteroceptive factors such as the presence (or absence) of adequate food supplies or the prevailing metereological conditions have such profound effects on nearly every biological function in living organisms that their influence on reproductive function could easily be explained in terms of their generalized metabolic effects. Such a concept, however, is clearly inadequate to account for e.g. the effects of seasonal changes in day length on ovarian activity or for the phenomenon of reflex induction of ovulation through copulation. Since in these instances exteroceptive stimuli apparently produce their effects through activation of specific neurogenic sensory receptors, it seems not unreasonable to assume that some part of the central nervous system might be involved in the control of pituitary-ovarian activity. Moreover, since in the course of embryogenesis the anterior pituitary migrates from the roof of the mouth to become attached to the floor of the third ventricle, it would seem likely, on teleological grounds, that the hypothalamus is the part of the central nervous system most directly concerned with the regulation of anterior pituitary activity. Several lines of evidence substantiate this concept of hypothalamic control of gonadotrophin secretion and a brief outline of the various types of approaches used is given below.

2.1.1 Electrical and electrochemical stimulation of the hypothalamus

The basic experiment dealing with nervous stimulation and gonadotrophin secretion was made by Marshall and Verney (1936) who demonstrated that diffuse electrical stimuli applied to the head or lumbar spinal cord of rabbits resulted in ovulation and pseudopregnancy. Similar results were then obtained in rats (Harris, 1936) in which pseudopregnancy could be induced by electrical stimulation of the head.

In an attempt to delineate more closely the neural structure(s) involved in these responses, Harris (1937) and Haterius and Derbyshire (1937) subsequently illustrated that stimulation of the tuber cinereum and preoptic area (Figure 1.3) was equally effective in evoking ovulation in the anaesthetized rabbit. Direct electrical stimulation of the pituitary gland on the other hand, did not result in ovulation unless there were signs of spread of the stimulus (Markee, Sawyer and Hollinshead, 1946). Similar findings have also been obtained in the conscious rabbit (Harris, 1948), in the rat in which spontaneous ovulation has been blocked with pentobarbital (Critchlow, 1958) or by neonatal administration of testosterone (Barraclough and Gorski, 1961) and in the monkey (Anand, Malkani and Dua, 1957).

In the rat, the duration of "electrochemical" stimulation of the preoptic area needed to produce ovulation, varies with the stage of the oestrous cycle, being shortest at pro-oestrus (Holsinger and Everett, 1970). This suggests that hormonal factors may be important in modifying the sensitivity of the hypothalamic-pituitary axis to electrical stimulation.

Until a few years ago, virtually all the experiments on hypothalamic stimulation relied on ovulation as biological end-point, and

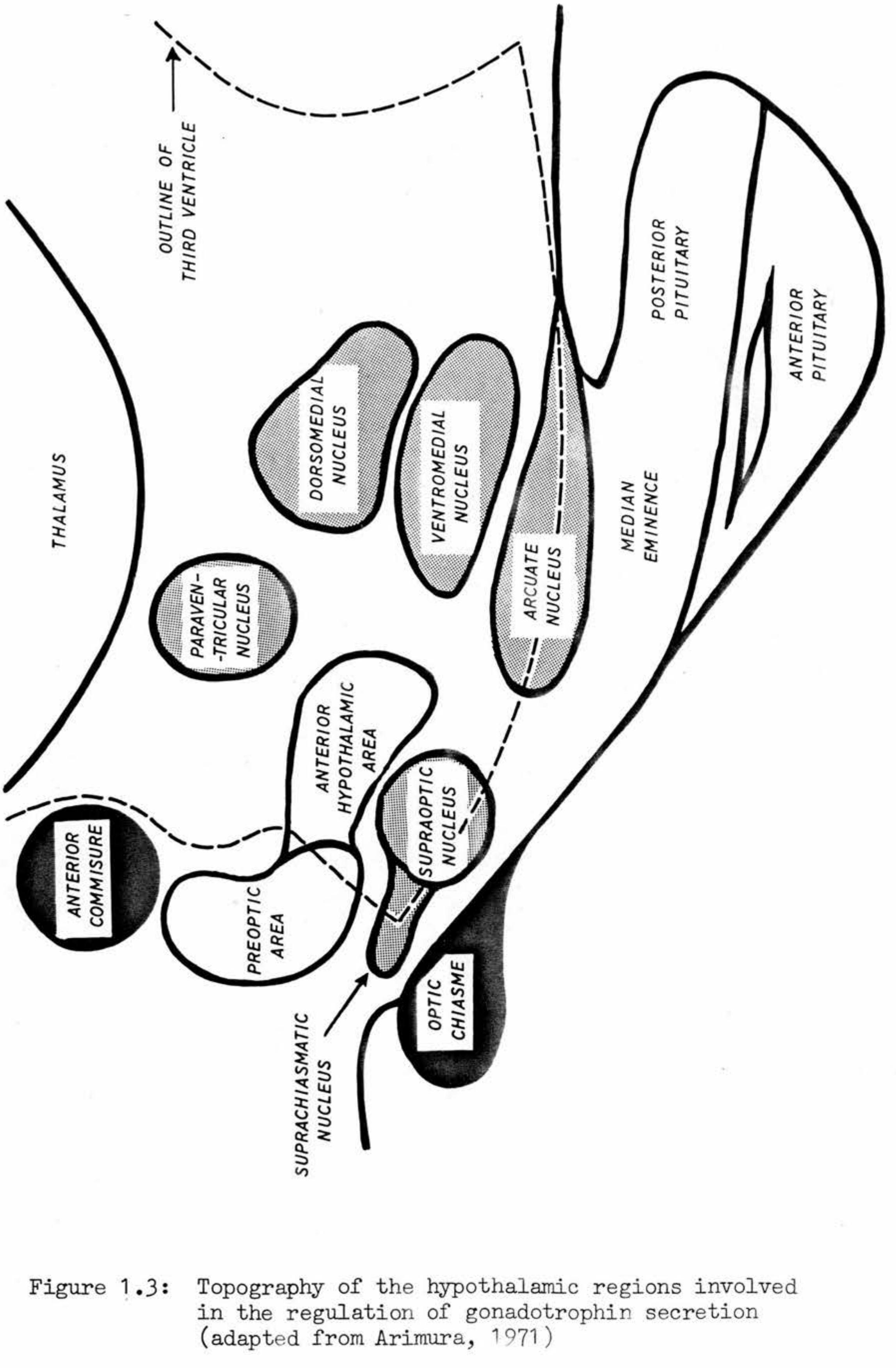


Figure 1.3: Topography of the hypothalamic regions involved in the regulation of gonadotrophin secretion (adapted from Arimura, 1971)

hence suffered from the fact that false negative results could be encountered if the follicles in the ovary of the test-animal were not competent to respond to the induced ovulatory LH-surge. The advent of RIA's however has obviated this problem and has made it possible to further refine our knowledge on the neural structures involved in the release of LH and/or FSH. Thus (Figure 1.3), stimulation anywhere within a broad band of tissue extending from the preoptic region rostrally to the arcuate-median eminence region caudally is capable of evoking marked elevations in plasma LH in the rat. Stimulation outside this medial basal region is ineffective. Plasma FSH elevations occur after stimulations which are located slightly more caudally in this region (Kalra, Ajika, Krulich, Fawcett, Quyada and McCann, 1971; Cramer and Barraclough, 1971).

2.1.2 Hypothalamic lesions

Although stimulation studies point to the existence of a functional link between the hypothalamus and the anterior pituitary gland, they do not prove that this is the normal pathway of hypophysial activation. More direct evidence for the central role of the hypothalamus in the regulation of pituitary gonadotrophin secretion has been provided by several clinical and experimental observations on the effects of hypothalamic lesions.

In 1920, Camus and Roussy first drew attention to the fact that experimental destruction of the hypothalamus in dogs resulted in gonadal atrophy. Similar findings were reported by Bailey and Bremer (1921) who further demonstrated that the gonadal atrophy following hypothalamic destruction could not be accounted for by simultaneous unintentional lesioning of the pituitary. Subsequent work in the guinea-pig (Dey, 1943) and the rat (Hillarp, 1949) showed that, depending on the site of the hypothalamic lesion, either of two syndromes can be produced.

In the first, rostral lesions placed bilaterally in the region just over the optic chiasme (Figure1.3) result in a state of constant oestrus. The ovaries of animals in this condition are filled with multiple large follicles which are actively secreting oestrogens as evidenced by the presence of continuous vaginal cornification. Apparently, animals in constant hypothalamic oestrus secrete relatively constant amounts of FSH and LH which initiate regular follicular development and oestrogen secretion, but the cyclic LH-surge required for ovulation is missing. A similar condition can be found after exposure of female rats to constant light (Browman, 1937; Daane and Parlow, 1971) or following neonatal treatment of females with certain androgens and oestrogens (Gorski, 1971). In these instances the permanent oestrous condition is also believed to result from the inactivation of a hypothalamic cyclic centre located in the preoptic-suprachiasmatic region and which is essential for the initiation of the preovulatory LH-surge in the female (Barraclough and Gorski, 1961; Wagner, Erwin and Critchlow, 1966).

The second syndrome results from a much more profound interference with gonadotrophin secretion which follows lesions involving the basal tuberal hypothalamus and particularly the median eminence, the junction between the hypothalamus and the pituitary stalk (Figure 1.3). Lesions placed in this region result in gonadal atrophy (Dey, 1943; Bogdanove, 1957), decrease the pituitary content of LH and FSH (Davidson, Contopoulos and Ganong, 1960) and prevent the rise in pituitary gonadotrophin secretion that normally follows ovariectomy

(Taleisnik and McCann, 1961; Bishop, Krulich, Fawcett and McCann, 1971). In contrast to the marked atrophic changes observed in the follicular apparatus, lesions of the median eminence in rats favour the development of existing corpora lutea which appear to be fully functional (McCann and Friedman, 1960). The presence of elevated prolactin levels under these conditions (Bishop, Krulich, Fawcett and McCann, 1971) adds further support to the luteotrophic role of prolactin in the rat and indicates that, in the normal animal, the hypothalamus exerts a tonic inhibitory control over prolactin release (see further, p. 84).

2.1.3 Pituitary transplantation and pituitary stalk section

Although anterior pituitary tissue, when transplanted to a distant site in the body, will not maintain any of its normal functions apart from its luteotrophic action (Everett, 1954), quite different results are obtained if the pituitary gland is removed from the sella turcica so that all its vascular and neural connections are interrupted, but is then replaced in the sella turcica or in an adjacent site.

Greep in 1936 was the first to report that such auto- or homografts of anterior pituitary tissue in the sella could become fully functional as evidenced by the restoration of normal body growth and the presence of regular oestrous cycles, pregnancy and lactation in the hypophysectomized recipients. Harris and Jacobsohn (1950, 1952) subsequently showed that restoration of normal functional activity of such grafts could only be observed if the grafted tissue had acquired rich vascular connections with the hypophysial portal vessels of the median eminence. Indeed, if pituitary tissue was grafted a few millimetres laterally from the median eminence, below the temporal lobe of the brain so that it became revascularized by blood vessels of this temporal lobe, little, if any, anterior pituitary function could be discerned. It was likely therefore that the hypophysiotrophic function of the hypothalamus was mediated through humoral substances liberated from nerve fibres of the hypothalamus into the primary plexus of the hypophysial portal vessels, which transmitted these substances to the adenohypophysis. This assumption was further supported by experiments involving section of the pituitary stalk.

Before the 1950's several workers had studied reproductive activity following pituitary stalk section but highly discordant results had been obtained (for review see e.g. Harris, 1955). However, in 1954 Donovan and Harris convincingly demonstrated that these variable results of pituitary stalk section could be explained in terms of variable degrees of portal vessel regeneration across the site of section. If regeneration of these vessels was prevented e.g. by inserting a paper plate between the hypothalamus and pituitary gland after sectioning of the stalk, restoration of anterior pituitary function did not occur even though the tissue remained well-maintained.

In conclusion, from these and many other studies it became apparent that the hypothalamus is intimately concerned with the regulation of anterior pituitary gonadotrophin release through secretion of neurohumoral substances into the hypophysial portal system. The validity of this brilliant hypothesis proposed by Harris (1944, 1955) was proved, some years later, by the actual demonstration of the existence in hypothalamic extracts, of specific neurohormonal material capable of stimulating LH (McCann, Taleisnik and Friedman, 1960) and FSH- release (Igarashi and McCann, 1964 a; Mittler and Meites, 1964) from anterior pituitary cells.

2.2 The hypothalamic hypophysiotrophic hormones

Hypophysiotrophic hormones can be defined as "blood-borne chemical agents that accelerate or retard the secretion and/or synthesis of the hormones of the hypophysis" (Saffran, 1973). The hypothalamic area of the brain is believed to be the major source of these hormonal substances, although their presence has also been detected in other areas of the brain e.g. in the pineal gland (White, Hedlund, Weber, Rippel, Johnson and Wilber, 1974).

Depending on whether they stimulate or inhibit anterior pituitary hormone secretion, hypophysiotrophic factors can be divided into "releasing factors" and "release-inhibiting factors". In man, as in most other animal species, the secretion of LH appears to be controlled exclusively by a releasing factor (LRF) while the secretion of prolactin seems to be regulated predominantly by a release-inhibiting factor (prolactin release-inhibiting factor, PIF). As for the control of FSH-secretion, the observations that natural and synthetic LRF preparations cause the release of both LH and FSH, have casted some doubt upon the existence of a separate follicle-stimulating hormone-releasing factor (FRF), distinct from LRF.

2.2.1 Luteinizing hormone releasing-factor (LRF)

(a) Isolation, determination of structure, synthesis

Following the demonstration that hypothalamic extracts from rats, domestic animals and humans were capable of stimulating LH and/or FSH release (McCann, Taleisnik and Friedman, 1960; Courrier, Guillemin, Jutisz, Sakiz and Aschheim, 1961; Schally and Bowers, 1964; Mittler and Meites, 1964; Igarashi and McCann, 1964; Kuroshima, Ishida, Bowers and Schally, 1965), attempts were made by various laboratories to try and isolate the active substance(s). But the technical difficulties involved in extracting and purifying several thousands of ovine or porcine hypothalami were so manifold that the number of entrants in the race dropped steadily until eventually, at the end of the 1960's, only a small handful of biochemists, concentrated in the laboratories of A. V. Schally and R. Guillemin, remained.

After a series of unsuccessful efforts (reviewed by Schally, Kastin and Arimura, 1972), Schally's group, working with porcine hypothalami, obtained an active substance in a relatively high state of purity, which stimulated the release of LH as well as FSH in animals and humans (Kastin, Schally, Gual, Midgley, Bowers and Diaz-Infante, 1969). The substance, henceforth designated LRF, was subsequently isolated in a homogeneous state by two different methods of purification (Schally, Arimura, Baba, Nair, Matsuo, Redding, Debeljuk and White, 1971; Schally, Nair, Redding and Arimura, 1971). Parallel chemical studies established that the isolated material was a decapeptide, the structure of which was subsequently elucidated (Matsuo, Baba, Nair, Arimura and Schally, 1971; Baba, Matsuo and Schally, 1971). Synthetic work further confirmed that the proposed structure was correct. The synthesis of LRF was carried out by the solid-phase method and the resulting synthetic product was shown to possess identical physicochemical properties as natural porcine LRF (Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk and White, 1971; Matsuo, Arimura, Nair and Schally, 1971).

A few months after the isolation of porcine LRF, the purification of LRF from sheep hypothalami was reported (Amoss, Burgus, Blackwell, Vale, Fellows and Guillemin, 1971). As expected, the amino acid sequence of this ovine LRF corresponded exactly to that of porcine LRF (Burgus, Butcher, Ling, Monahan, Rivier, Fellows, Amoss, Blackwell, Vale and Guillemin, 1971). Once the difficult task of establishing the structure of LRF was accomplished, several other groups of peptide chemists quickly synthesized it, allowing various workers all over the world to study the biological activity of synthetic LRF in a wide range of animal species and under a large variety of experimental conditions. In view of the anticipated diagnostic and therapeutic significance of LRF in clinical medicine numerous attempts have also been made to synthesize analogues of LRF with higher biological activity (Fujino, Kobayashi, Obayashi, Fukuda, Shinagawa, Yamazaki, Nakayama, White and Rippel, 1972; Arimura, Vilchez-Martinez, Coy, Coy, Hirotsu and Schally, 1974) or antagonistic action (Coy, Vilchez-Martinez, Coy, Arimura and Schally, 1973).

(b) Measurement of LRF

Since the discovery of LRF a variety of tests have been developed to detect and quantitate LRF-activity in tissue extracts or biological fluids.

Bioassays

Biological assay methods for the measurement of LRF activity are based on the ability of LRF to release LH. This evidently implies that all methods will also incorporate in them the variability intrinsic in the measurement of LH. Consequently, before the advent of RIA's for this hormone, few of the tests could strictly speaking be classified as bioassays since dose-response relationships could in most instances not be demonstrated (McCann,1970). Notable exceptions were:

(1) in-vivo assays

- elevation in plasma LH in the ovariectomized, oestrogen-progesterone blocked rat (Ramirez and McCann, 1963)
- elevation in plasma LH in castrated male rats, pretreated with testosterone (Schally, Carter, Arimura and Bowers, 1967)
- elevation in plasma LH in immature female rats, pretreated with gonadotrophins (McCann, 1962)
- elevation in plasma LH and the induction of ovulation in pro-oestrous female rats (Nikitovitch-Winer, 1962) or rabbits (Campbell, Feuer and Harris, 1964) after intra-pituitary infusion of the test substance

(2) in-vitro assays

- stimulation of LH release from short-term incubated pituitaries of normal male (Piacsek and Meites, 1966) and castrated male rats (Samli and Geschwind, 1967) and of ovariectomized, oestrogen-progesterone blocked female rats (Schally and Bowers, 1964).

After the introduction of radioimmunoassays for LH, however, the number of potential in-vivo bioassays for LRF has increased considerably since a dose-response relationship between the increase in plasma LH and the administered amount of LRF has been demonstrated in virtually every species, including man (Kastin, Schally, Gual, Midgley, Arimura, Miller and Cabeza, 1971).

Radioimmunoassays

The availability of sufficient amounts of the synthetic LRF decapeptide made it possible to generate antisera against LRF and establish radioimmunoassays for this hormone in blood (Nett, Akbar, Niswender, Hedlund and White, 1973; Jeffcoate, Fraser, Gunn and Holland, 1973; Arimura, Sato, Kumasaka, Worobec, Debeljuk, Dunn and Schally, 1973) and urine (Bolton, 1974).

(c) Physiological effects of LRF

Administration of either natural or synthetic LRF will elevate the plasma level of LH and, in most instances, of FSH. The increase in the peripheral concentration of both gonadotrophins is a result of a direct stimulatory action of LRF on the pituitary gland (Schally, Kastin and Arimura, 1971). At the cellular level, this stimulatory effect of LRF appears to involve stimulation of both release and de-novo synthesis of FSH and LH in the pituitary gonadotrophs (Redding, Schally, Arimura and Matsuo, 1972).

Physiological actions at the tissue level

Since studies on the effects of synthetic LRF in various physiological and pathological situations will be discussed more fully in relation to our own clinical studies described in this thesis, only a few relevant points will be summarized here.

Although LRF is capable of releasing both gonadotrophins, their secretion pattern has different characteristics depending on the endocrine status of the individual. Prior to puberty, single or repeated injections of LRF lead mainly to an increase in FSH and little or no increase in LH. During pubertal development the LH response increases progressively while the FSH release does not differ significantly from that seen before puberty (Roth, Kelch, Kaplan and Grumbach, 1972).

Normal adult men and women show a large variation in their responsiveness to LRF, but a few generalizations can be made:

(1) LH-release occurs at lower doses of LRF than those required for FSH- release.

(2) LH- release is within certain limits directly proportional to the dose of LRF injected; this is not so for FSH-release

(3) the time at which the maximum level is achieved is shorter for LH than it is for FSH

(4) the return to basal levels is slower for FSH than for LH

(5) the amplitude of the response is always greater for LH than for FSH

(6) in normal women, LH-release is more or less correlated with the level of circulating oestrogens, and is highest at midcycle and greater during the luteal phase of the cycle than during the early or mid-follicular phase.

Cellular actions

At the cellular level, LRF stimulates both the release and synthesis of LH and FSH. However the primary action of LRF appears to be on the release process, since release can still occur when de-novo synthesis is blocked (McCann, 1971). Although a complete description of the physicochemical events involved in LRF-induced gonadotrophin release is not yet at hand, several lines of evidence (recently reviewed by Jutisz, Bérault, Kerdelhué and Théoleyre, 1975 and Kraicer, 1975) indicate that the first steps in the sequence of events leading to hormone release involve the binding of LRF to a membrane-bound specific receptor with subsequent activation of the adenyl-cyclax system and formation of cyclic AMP. Since there is little doubt that Ca^{2+} is involved in the release process, a tentative model recently proposed by Kraicer (1975) suggests that the mechanism of hormone release may be similar to that of muscle contraction. According to this model, c-AMP and Ca^{2+} would activate the phosphorylation of contractile protein elements in a cytoskeleton-vesicle complex which, when activated, leads to contraction with subsequent extrusion of hormone-containing granules.

(d) Secretion-patterns

Since at the present time, experimental techniques which would permit repeated determinations of LRF concentrations in hypophysial portal blood have not yet been developed, information on the actual secretion-pattern of LRF under various physiological conditions is very scarce. Obviously the pattern of peripheral LH-levels does not necessarily reflect changes in LRF secretion since other factors such as hormonal steroids or previous exposure to LRF can modulate the sensitivity of the pituitary gonadotroph and hence influence pituitary LH-release. More direct information on the changes in hypothalamic LRF secretion has been obtained from studies on LRF content of portal venous or peripheral blood and from the measurement of LRF concentrations in hypothalamic tissue.

Circhoral (or episodic) secretion rhythm

Recent observations on LRF concentrations in the hypophysial portal circulation of the rhesus monkey suggest that LRF may be secreted in a pulsatile manner (Carmel, Araki and Ferin, 1975). Similar findings have been reported by Seyler and Reichlin (1974) who observed short-term fluctuations in bioassayable LRF activity in human peripheral plasma, and by Nett, Akbar and Niswender (1974) who detected a pulsatile LRF-pattern in sheep peripheral plasma.

Diurnal secretion rhythm

To our knowledge no systematic studies on diurnal changes in hypothalamic LRF secretion have been reported.

Oestrous and menstrual secretion rhythm

In mature female rats, Chowers and McCann (1965) and Ramirez and Sawyer (1965) observed that hypothalamic LRF content is highest late in di-oestrus and declines at pro-oestrus, suggesting that a release of LRF into the portal vessels may occur prior to and/or simultaneous with the ovulatory LH-peak on the afternoon of pro-oestrus. However, direct measurement of LRF in rat portal venous blood on the day of pro-oestrus did not reveal any significant increase in LRF concentrations (Fink and Jamieson, 1976). Nett, Akbar and Niswender (1974) were also unable to detect a massive increase in the peripheral levels of LRF on the day of oestrus in sheep.

In the human female, Malacara, Seyler and Reichlin (1972) reported that peripheral LRF levels as measured by bioassay, increased on the day of the midcycle LH-peak. Arimura, Kastin and Schally (1974), using RIA, have confirmed this finding. Most other workers however fail to detect any significant increase in peripheral LRF-levels at midcycle (Nett, Akbar, Niswender, Hedlund and White, 1973; Keye, Kelch, Niswender and Jaffe, 1973).

Breeding rhythm

Although no direct information is available, studies by Pelletier and Ortavant (1968) on the light-induced changes in hypothalamic LRF content of the sheep indicate that during the non-breeding season LRF content of the hypothalamus is higher than during the breeding season. These results could be interpreted as being indicative of an increased secretion of LRF during the breeding season.

2.2.2 Follicle-stimulating hormone releasing-factor (FRF)

Shortly after the discovery of LRF, a FSH-releasing action of hypothalamic extracts was demonstrated (Igarashi and McCann, 1964). In the initial report, hypothalamic extracts were shown to elevate FSH activity in plasma as measured by the mouse uterine weight augmentation bioassay in animals with median eminence lesions and in animals that had been treated with gonadal steroids. Subsequent work using the Steelman-Pohley assay confirmed this initial observation (Igarashi, Nallar and McCann, 1964). Proof that hypothalamic FRF acted directly on the pituitary gland to stimulate FSH release was obtained by Mittler and Meites (1964). Several workers subsequently reported that FRF could be separated from LRF by gel filtration on Sephadex and further purification of bovine (Schally, Saito, Arimura, Müller, Bowers and White, 1966), ovine (Dhariwal, Naller, Batt and McCann, 1965; Jutisz and de la Llosa, 1967) and porcine hypothalamic extracts (Schally, Saito, Arimura, Sawano, Bowers, White and Cohen, 1967). The FSH-releasing substances thus purified were able to deplete the pituitary FSH-stores of rats both <u>in-vivo</u> and <u>in-vitro</u>. For a time it seemed as if the problem of the identity of FRF was solved when it was reported that the ability of hypothalamic extracts to deplete pituitary stores of FSH could be accounted for by polyamines such as histamine, putrescine, spermidine, spermine and the amino-acid lysine, that were present in these preparations (White, Cohen, Rippel, Story and Schally, 1968). However, further work by Schally, Mittler and White (1970) showed that the FSH-releasing capacity of polyamines could only be demonstrated <u>in-vivo</u>, but not <u>in-vitro</u>. The polyamines were therefore judged to act as releasers of FRF from the hypothalamus instead of acting as releasers of FSH from the pituitary. The identify of FRF once more became an enigma.

At the same time work was proceeding which showed that purified porcine LRF could stimulate the release of FSH as well as LH (Kastin, Schally, Gual, Midgley, Bowers and Diaz-Infante, 1969). It was not known whether this FSH-releasing activity of porcine LRF, was an intrinsic property of the molecule or whether it could be accounted for by contamination with FRF. The dilemma was solved after the isolation, determination of structure and synthesis of LRF (for references, see p. 74) when it was found that natural and synthetic materials stimulated the release of both LH and FSH. Since a separate releasing factor for FSH has not been isolated, it has been suggested that LRF is the only hypothalamic gonadotrophin-releasing factor responsible for the secretion of both FSH and LH (Schally, Arimura and Kastin, 1973). Yet there are several experimental and clinical conditions in which FSH and LH are apparently not released together (e.g. monogonadotrophic elevation of FSH in men with germinal aplasia, Rosen and Weintraub, 1971), indicating that the control system for FSH is dissimilar to that for LH. These observations however are not necessarily inconsistent with the concept of a single releasing factor controlling the secretion of both FSH and LH. Indeed, recent evidence would suggest that gonadal steroids through a direct action at the pituitary level, can modulate hypophysial sensitivity to LRF and could thus be responsible for adjusting preferential release of LH and FSH from the pituitary in accordance with the requirements of the target organ. The as yet still unidentified inhibin-like substance(s) supposed to be secreted by the germinative compartment of the gonad, could conceivably have a similar action. Recently, additional support for the concept of a single releasing factor has been presented by Shahmanesh and Jeffcoate (1976) who demonstrated that treatment of rat median eminence extract with various doses of an antiserum raised against synthetic LRF reduced LH-releasing and FSH-releasing potencies of the extract to a similar extent.

2.2.3 Prolactin-release inhibiting factor (PIF)

Several lines of evidence indicate that in mammalian species the hypothalamus has an inhibitory influence on the synthesis and release of prolactin (Meites, 1972). Prolactin-release inhibiting activity has been demonstrated in hypothalamic extracts from a variety of species, including sheep, cow, pig and man (Schally, Kastin, Locke and Bowers, 1967). Chemical characterization of PIF however has not yet been reported, although it appears to be a small molecule (MW 5,000) with the solubility properties of a peptide (Dhariwal, Grosvenor, Antunes-Rodrigues and McCann, 1968). Catecholamines, such as noradrenaline and dopamine, when infused into the portal vessels of rats, will also suppress pituitary prolactin release, but it is unlikely that they represent the true PIF (Takahara, Arimura and Schally, 1974).

Although no direct information on the secretion-pattern of PIF is available, indirect evidence, derived from studies on changes in hypothalamic PIF-activity in response to a variety of stimuli, appears to confirm the view that PIF-secretion is inversely related to prolactin release. Thus, hypothalamic PIF activity in rats, as measured by an in-vitro method (Kragt and Meites, 1967) is reduced and prolactin levels elevated in response to suckling, administration of oestrogen, progesterone, testosterone, chlorpromazine, haloperidol, α-methyldopa and amphetamine (Meites and Clemens, 1972). Conversely, hypothalamic PIF activity is increased after administration of agents which decrease serum prolactin concentrations such as L-DOPA (Lu and Meites, 1971), ergot drugs (Wuttke, Cassell and Meites, 1972) or prolactin itself (Voogt and Meites, 1971).

The cellular mechanism involved in the inhibitory action of PIF on prolactin release is still obscure. Calcium-ions have been shown to be essential for prolactin release by the pituitary in-vitro (Parsons, 1970), and it has therefore been suggested (Nicoll, 1971) that PIF acts on the prolactin-secreting cell membrane to prevent its spontaneous depolarization thus inhibiting Ca^{2+} influx into the cell. Recent work by Kidokoro. (1975) appears to support this hypothesis.

2.2.4 Prolactin-releasing factor (PRF)

Although the presence of prolactin-releasing activity in avian hypothalami is fairly well established (Meites and Nicoll, 1966), no substantial evidence for the existence of PRF-activity in mammalian hypothalami has been presented. Indeed, most of the earlier work (e.g. by Mishkinsky, Khazen and Sulman, 1968) which suggested the presence of PRF in crude hypothalamic extracts from mammals must be interpreted with caution in view of the more recent finding that another hypothalamic releasing hormone, i.e. the tripeptide thyrotrophin-releasing factor (TRF), induces rapid release of prolactin in addition to TSH (Bowers, Friesen, Hwang, Guyda and Folkers, 1971). This latter observation has raised the question as to whether TRF is a physiological releasor of prolactin. In favour of this view are the observations that even minute doses of TRF cause a significant increase in serum prolactin (Bowers, Friesen and Folkers, 1973). However, under physiological conditions, most of the stimuli known to release prolactin do not release TSH and vice versa (Meites, 1973).

More recently, Valverde, Chieffo and Reichlin (1972) reported the presence of PRF distinct from TRF in hypothalamic extracts but rather little information is available on its nature and the factors which control its secretion.

3. The regulation of pituitary gonadotrophin secretion by steroid hormones

Although the regulation of pituitary gonadotrophin secretion has been intensively studied since the discovery of these hormones in pituitary extracts, our knowledge of the mechanisms involved has been slow to accumulate because of methodological problems (McCann, 1973). In early work it was necessary to rely upon biological end-points such as occurrence of ovulation or changes in weight or histological appearance of pituitary and gonadal target organs following various interventions. The subsequent development of specific bio-assays made it possible to measure more accurately changes in pituitary content of FSH and LH but since the sensitivity of these methods did not permit, except in a few instances, to measure gonadotrophin titres in peripheral plasma, doubt still remained as to whether the observed changes in pituitary gonadotrophin content might be interpreted as reflections of concomitant parallel changes in pituitary gonadotrophin secretion. The advent of radioimmunoassays which allowed repeated determinations of peripheral gonadotrophin concentrations changed all this. As a result, little doubt now remains that pituitary gonadotrophin release is a result of stimulatory and inhibitory effects of steroid hormones acting at the hypothalamic and pituitary level.

In the following paragraphs the discussion of negative and positive feedback effects of steroids on gonadotrophin secretion will deal primarily with data obtained in female primates. However, where appropriate, relevant findings in other species will also be incorporated. The possible role of oestrogens in the regulation of pituitary prolactin secretion has been discussed before (pp. 63) and will therefore not be considered here.

3.1 Steroid hormones which may be involved in feedback regulation of gonadotrophin secretion and their site of origin

Theoretically, every single steroid hormone which is present in the peripheral circulation can affect pituitary gonadotrophin secretion provided it has some intrinsic oestrogenic, androgenic or gestagenic activity. Such steroids may be directly secreted by the gonads and/or adrenals, or they may arise from peripheral conversion of precursors, which themselves can be of gonadal or adrenal origin. Thus, the adrenal glands could, at least in theory, have a significant role in the regulation of gonadotrophin release, particularly because the adrenal production rates of several steroids are quite important in quantitative terms and do often exceed ovarian production rates. However, most of the sex steroid hormones secreted by the adrenals have only weak intrinsic biological activity, and though they can serve as precursor hormones for conversion in peripheral tissues to more potent androgens, oestrogens or gestagens, the extent of these conversions is usually rather small. It seems likely therefore that under physiological conditions only those steroids which are secreted exclusively or at least predominantly by the ovaries will be most intimately involved in the regulation of gonadotrophin secretion. Furthermore, since gonadotrophic hormones are concerned with the control of gonadal function, one might expect, on teleological grounds, that the feedback control of this trophic hormone secretion is most likely to originate from the target organ. Also, the observation that suppression of adrenal steroid secretion with dexamethasone does not affect pituitary gonadotrophin secretion to any significant degree (Abraham, 1974; Kim, Hosseinian and Dupon, 1974) adds further support to the view that the involvement of adrenal steroids in feedback is likely to be minimal.

The intact, mature human ovary is known to secrete virtually all the steroid hormones on the biosynthetic pathway from pregnenolone to 17β-oestradiol. Pregnenolone, progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, oestrone and 17β-oestradiol have all been identified in higher concentration in ovarian than in peripheral venous plasma, indicating direct ovarian secretion (Baird, 1974). Of these steroids, 17β-oestradiol and progesterone show the most marked changes during the menstrual cycle (Figure 1.4) and hence are probably the most important with respect to feedback control of gonadotrophins.

In women of reproductive age circulating 17β-oestradiol is almost exclusively derived from direct ovarian secretion. In the absence of bilateral ovulation, the "active" ovary i.e. the ovary containing the pre-ovulatory follicle or corpus luteum, produces approximately 95% of the total blood production rate of 17β-oestradiol (Baird and Fraser, 1974). The remaining 5% arises from extraglandular conversion of oestrone (Baird, Horton, Longcope and Tait, 1969) since no 17β-oestradiol is secreted by the adrenal glands (Baird, Uno and Melby, 1969). Although about 0·2% of plasma testosterone is converted to circulating 17β-oestradiol at extraglandular sites (Longcope, Layne and Tait, 1968), the female produces only small amounts of testosterone (200-300 μg/day). In contrast, after the menopause, most if not all 17β-oestradiol production can be accounted for by extraglandular conversion. The postmenopausal ovary secretes only minimal amounts of 17β-oestradiol (Judd, Judd, Lucas and Yen, 1974) and oophorectomy in postmenopausal women is not followed by a decrease in 17β-oestradiol production (Barlow, Emerson and Saxena, 1969).

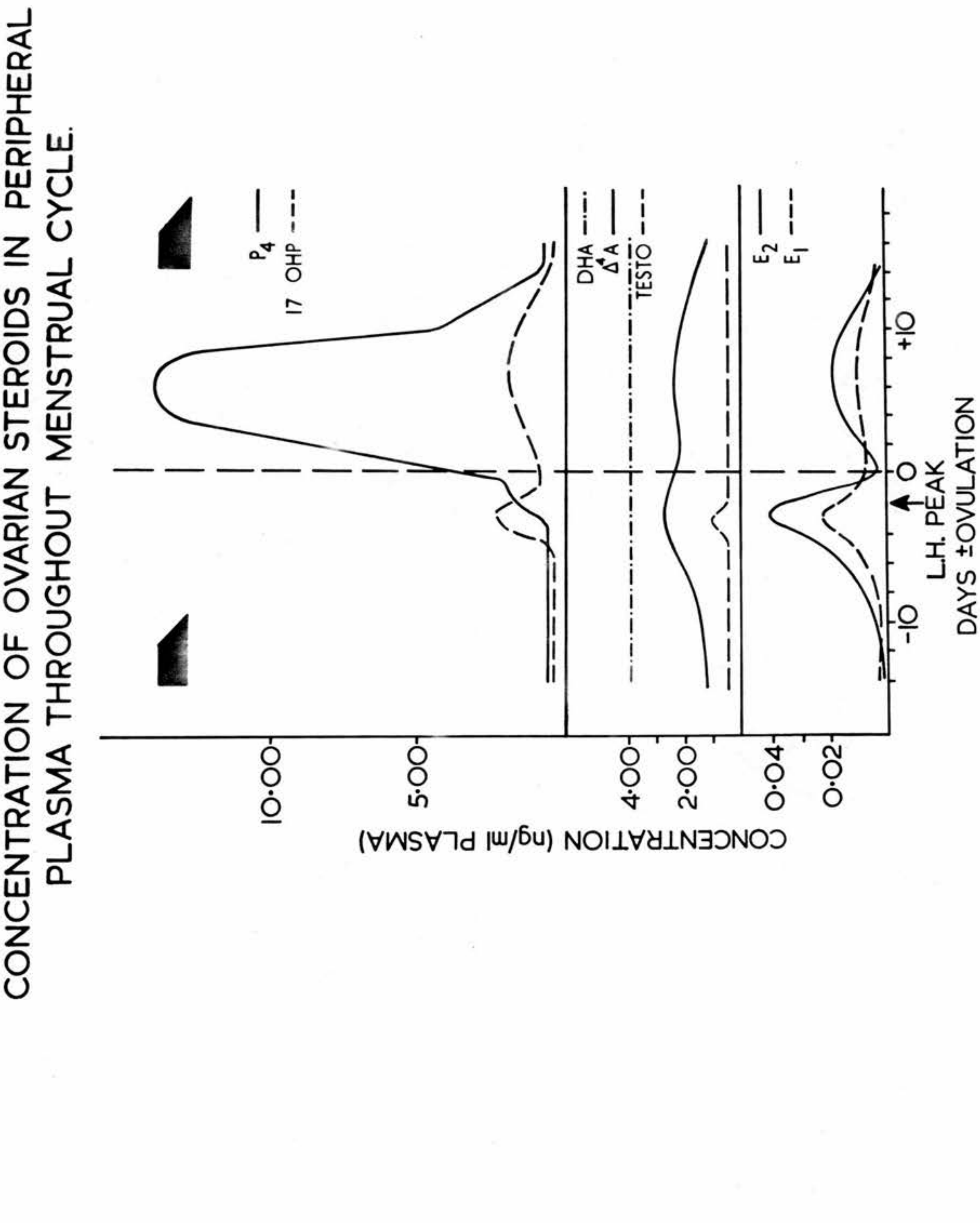


Figure 1.4: The concentration of ovarian steroids in peripheral plasma throughout the menstrual cycle (taken from Baird, 1974 with permission of the author)

During the early follicular phase of the cycle, circulating progesterone arises from at least 3 sources: direct ovarian and adrenal secretion, and extraglandular conversion of pregnenolone. The progesterone production rate at this stage of the cycle has been estimated at 1-2 mg/24 hours (Lin, Billiar and Little, 1972). Concomitant with the pre-ovulatory LH peak and prior to ovulation, peripheral progesterone levels increase (Yussman and Taymor, 1970; Johansson and Wide, 1969). This rise probably represents increased ovarian production since the granulosa cells of the pre-ovulatory follicle show morphological signs of luteinization before follicular rupture occurs (Hertig, 1967). During the luteal phase, nearly all the progesterone is secreted by the ovary which contains the corpus luteum, the secretion from the contralateral ovary being negligible (Lloyd, Lobotsky, Baird, McCracken, Weisz, Pupkin, Zanartu and Puga, 1971). Adrenal secretion and extraglandular production of progesterone contribute but very little to the total luteal progesterone production rate of 20-30 mg per 24 hours (Little and Billiar, 1969).

Little is known about the physiological role, if any, of the other steroid hormones secreted by the ovary, and few of them have been investigated with respect to possible feedback effects at hypothalamic and/or pituitary level.

Abraham, Odell, Swerdloff and Hopper (1972) have suggested that 17α-hydroxyprogesterone may play a role in the mid-cycle gonadotrophin surge. During the early follicular phase, most of the circulating 17α-hydroxyprogesterone is derived from peripheral conversion of 17α-hydroxypregnenolone which is almost exclusively of adrenal origin (Strott, Bermudez and Lipsett, 1970). This constant adrenal contribution to circulating 17α-hydroxyprogesterone becomes proportionally less significant during the final stages of follicular development when a rise in peripheral 17α-hydroxyprogesterone levels concomitant with the increase in plasma LH concentrations can be detected (Thorneycroft, Mishell, Stone, Kharma and Nakamura, 1971). During the luteal phase of the cycle almost all circulating 17α-hydroxyprogesterone is derived from direct ovarian secretion.

In the castrated rat, 20α-dihydroprogesterone has been shown to facilitate oestrogen-induced LH release (Swerdloff, Jacobs and Odell, 1972). It is uncertain whether this steroid might have a similar action in the human. Ovarian secretion of 20α-dihydroprogesterone has been demonstrated in some women during the luteal phase but not during the follicular phase or after the menopause (Mikhail, 1970).

At least three androgens: androstenedione, testosterone and dehydroepiandrosterone are secreted by the ovary. Relative to adrenal secretion, the ovarian contribution to circulating dehydroepiandrosterone is small (less than 10-20%) and does not seem to vary throughout the menstrual cycle (Abraham, 1974) which makes it unlikely that this steroid should be involved in the regulation of gonadotrophin secretion to any significant extent. Circulating testosterone in women is largely derived from extraglandular conversion of dehydroepiandrosterone and in particular androstenedione, although smaller amounts (about 30% of total production rate) are secreted directly from the ovary and adrenal gland (Tait and Horton, 1966). The ovarian contribution to plasma testosterone reaches maximum levels at midcycle (Abraham, 1974), but the total blood production rate even at this stage of the cycle is probably too small to have any significant effect on gonadotrophin release. It is of interest to note in this respect that passive immunization against testosterone did not affect pre-ovulatory LH release or ovulation in female rats (Gay and Tomacari, 1974).

In contrast to dehydroepiandrosterone, the concentration of peripheral androstenedione shows significant variations throughout the cycle with a rise occurring at midcycle (Judd and Yen, 1973). It can be calculated that during the early follicular phase of the cycle, about 70% of the total production rate (estimated at about 1-2 mg a day) arises from adrenal sources (direct secretion and extraglandular conversion of dehydroepiandrosterone), but that the relative adrenal contribution to circulating androstenedione decreases to less than 40% at midcycle (Baird, Burger, Heavon-Jones and Scaramuzzi, 1974). Circulating androstenedione may play a role in the control of preovulatory gonadotrophin release since active immunization against this steroid abolishes the LH-surge induced by moderate doses of exogenous oestrogen in anoestrous sheep (Martensz, Baird, Scaramuzzi and Van Look, 1976). Conversely, chronic administration of androstenedione to sheep facilitates the LH release in response to subthreshold doses of oestrogen (Van Look and Scaramuzzi, unpublished). A similar mechanism may account for the abnormalities in gonadotrophin secretion observed in patients with polycystic ovarian disease (see further this thesis). At the present time however it is not yet clear whether androstenedione influences gonadotrophin secretion directly rather than indirectly, through its conversion to oestrone at peripheral and/or hypothalamic sites.

In women of reproductive age, between 10 to 50% (depending on the stage of the cycle) of circulating oestrone may arise from peripheral utilization of androstenedione (Siiteri and MacDonald, 1973). The extent of this conversion increases with advancing age (Hemsell, Grodin, Brenner, Siiteri and MacDonald, 1974) and is related to body weight (Siiteri and MacDonald, 1973). The extra-ovarian origin of oestrone explains the fact that the peripheral oestrone level changes less throughout the cycle than 17β-oestradiol, resulting in significant alterations in the ratio of 17β-oestradiol to oestrone (Baird and Guevara, 1969).

In summary, therefore, it seems likely that 17β-oestradiol and progesterone are probably most important with regard to feedback regulation of gonadotrophin release, but the possibility that other ovarian and adrenal steroids may have a permissive action cannot be excluded.

3.2 Feedback effects of gonadal steroids

Shortly after the recognition that gonadal hormones could suppress pituitary gonadotrophin secretion (i.e. negative feedback) (Moore and Price, 1932; Hohlweg and Junkmann, 1932), evidence was presented that under certain conditions steroid hormones may also promote gonadotrophin release (i.e. positive feedback) (Hohlweg, 1934). Subsequent work, mainly in the rat, showed that negative and positive feedback involved suppression, respectively activation, of separate hypothalamic centres: a "tonic" centre located in the median basal hypothalamus which forms part of the negative feedback loop, and a "cyclic" centre in the preoptic/suprachiasmatic region which is involved in phasic LH-release. In the rat, the latter centre may be rendered non-functional following its exposure to the organizing actions of testicular steroids during a critical period in neonatal life (see e.g. Gorski, 1966; Flerkó, 1966; Halász, 1969 and Gorski, 1971 for reviews). Thus the regulation of gonadotrophin secretion involves two separate control mechanisms: a negative feedbacksystem for the regulation of tonic secretion of FSH and LH, and, supernumerary to this, a positive feedbackmechanism for cyclic LH (and FSH) release. The activity of the latter feedbacksystem however may be suppressed under certain conditions.

3.2.1 Negative feedback

The secretion of FSH and LH, at times other than during the pre-ovulatory gonadotrophin surge in the female, appears to be controlled by a classical negative feedbackloop involving the hypothalamus, the gonadotrophs of the anterior pituitary and the gonads.

That gonadal hormones may suppress pituitary gonadotrophin secretion was perhaps first recognised by Moore and Price in 1932. Following a series of experiments in castrated rats treated with gonadal steroids, pituitary extracts or a combination of both, the authors concluded: "Gonad hormones, of either sex, exert a depressing effect upon the hypophysis which results in a diminished amount of the sex-stimulating factor available to the organism." However, already the same year, Hohlweg and Junkmann (1932) challenged this view and advanced the concept that gonadal hormones exert their negative feedback effect on gonadotrophin release not through direct action on the pituitary gland, as suggested by Moore and Price, but rather indirectly through suppression of a hypothetical "Sexualzentrum" in the brain. It is worth noting that the same authors (Hohlweg and Junkmann, 1932) also postulated that changes in the sensitivity of this "sexual centrum" might be responsible for the hormonal changes associated with such phenomena as the onset of puberty or psychogenic amenorrhoea.

(a) Maturation and changes in sensitivity of the negative feedback mechanism

The pattern of change in the concentrations of immunoreactive pituitary FSH and LH and serum FSH suggests that in the human foetus the negative feedbackmechanism becomes operative by the third trimester of gestation. Before that period, and beginning from the second or third month of gestation, pituitary concentrations of FSH and LH and serum levels of FSH rise steadily to reach peak values between 20-25 weeks, followed by a decline that persists through the remainder of gestation (Grumbach and Kaplan, 1973). The initial high pituitary and serum concentrations may be indicative of relatively unrestrained, autonomous secretion of FSH and LH during the first two trimesters of intra-uterine life, while their decline in the third trimester may perhaps be attributed to progressive maturation of the inhibitory negative feedbackmechanism (Grumbach, Roth, Kaplan and Kelch, 1974). The secretion of androgens by the foetal Leydig cells (Reyes, Winter and Faiman, 1973) may advance this maturation process as evidenced by lower pituitary FSH and LH and lower serum FSH concentrations in male foetuses as compared to females.

A similar sex-difference persists during the first 2-3 years of extra-uterine life. In the male infant, a brisk rise in plasma LH, FSH and testosterone occurs during the first 6 months of life (Forest, Sizonenko, Cathiard and Bertrand, 1974). In female neonates, LH rises but to a lesser degree than in male neonates. Plasma FSH, in contrast, shows a striking and prolonged rise with a gradual decline into the normal prepubertal range by 3 years (Faiman and Winter, 1971). These findings are consistent with the notion that complete maturation of the negative feedbackmechanism is attained between 2-3 years of age (somewhat earlier in boys than in girls) and confirm the earlier work which suggested that the negative feedbackloop is fully functional in prepubertal children but at a low level of activity, i.e. low levels of circulating steroid hormones are capable of suppressing hypothalamic-pituitary gonadotrophin secretion.

The ample, early evidence that inhibitory feedback exists long before puberty has been reviewed by Donovan and van der Werfften Bosch (1965). It includes the observation that castration of one member of parabiotic infantile rats induces precocious puberty in the other intact animal, a reaction which can be prevented by the administration of small doses of oestrogen (Kallas, 1930), and the finding that the appearance of castration-cells in the pituitary of prepubertal castrates can be prevented by the administration of gonadal steroids (Dohrn and Hohlweg, 1931). Additional evidence for a functional negative feedback system prepubertally is the occurrence of compensatory hypertrophy in the descended testis of unilateral cryptorchid prepubertal boys (Laron and Zilka, 1969), the suppression by oral ethinyl oestradiol of urinary and serum gonadotrophins in prepubertal children (Kelch, Kaplan and Grumbach, 1973), and the presence of slightly elevated serum FSH and LH levels in prepubertal girls with gonadal dysgenesis (Penny, Guyda, Baghdassarian, Johanson and Blizzard, 1970). The absence of markedly elevated gonadotrophin levels in the latter condition suggests that sex steroids derived from other sources than the gonads may participate in the suppression of gonadotrophin secretion before puberty. Additional support for this concept has been provided by work of Dierschke, Karsch, Weick, Weiss, Hotchkiss and Knobil (1974) in the rhesus monkey. Following castration of immature male and female animals, the authors were unable to detect any significant increase in either FSH or LH until somatic development had proceeded to the stage where the first pubertal changes could be detected in intact animals. These findings in the monkey and the human differ from those reported in prepubertal rats (Goldman, Grazia, Kamberi and Porter, 1971), sheep (Foster, Cook and Nalbandov, 1972) and cattle (Odell, Hescox and Kiddy, 1970). In these species the time course of the postcastration increase in plasma gonadotrophin concentrations in sexually immature animals does not differ markedly from that found in adults.

There is good evidence to support the hypothesis (Hohlweg and Junkmann, 1932) that the initiation of puberty is associated with a decrease in sensitivity of the hypothalamic negative feedback receptors. As a consequence, low levels of sex steroids are no longer capable of suppressing gonadotrophin secretion in pubertal children (Kelch, Kaplan and Grumbach, 1973). Similarly, clomiphene citrate, an anti-oestrogen with weak oestrogenic activity (Wood, Wrenn and Bitman, 1968), is no longer effective in suppressing urinary FSH and LH secretion in children at the time of puberty (Kulin, Grumbach and Kaplan, 1972). The findings that in prepubertally castrated rhesus monkeys (Dierschke, Karsch, Weick, Weiss, Hotchkiss and Knobil, 1974) and children with gonadal dysgenesis (Conte, Grumbach and Kaplan, 1975) plasma gonadotrophin levels rise at the age of expected puberty, indicate that this pubertal change in hypothalamic negative feedback sensitivity occurs independently of the presence of functional gonads. It appears however to correlate with the attainment of a critical level of skeletal maturation (which reflects general somatic maturity) and with a critical body weight (Frisch and Revelle, 1971). Adrenal sex steroid secretion may also be involved in facilitating the decrease in sensitivity of the negative feedbackmechanism since true precocious puberty often follows initiation of glucocorticoid treatment in children with congenital adrenal hyperplasia, in whom diagnosis and treatment was delayed (Wilkins and Cara, 1954; Reiter, Grumbach, Kaplan and Conte, 1975).

Once an "adult" level of negative feedback sensitivity is attained, cessation of gonadal function is associated with an increase in plasma levels of FSH and LH due to the "escape" of hypothalamic negative feedback receptors from the suppressive action of gonadal hormones. In postmenopausal women (Franchimont, 1966) as well as in surgically castrated women (Ostergard, Parlow and Townsend, 1970) or postpubertal patients with gonadal dysgenesis (Reiter, Grumbach, Kaplan and Conte, 1975) the elevation in plasma FSH is usually more pronounced than that of LH. The difference may be related to differences in the metabolic clearance rate of FSH and LH (Kohler, Ross and Odell, 1968; Coble, Kohler, Cargille and Ross, 1969) or to a residual suppressive effect of adrenal steroids on LH secretion. Although most of the sex steroid hormones secreted by the adrenal such as androstenedione and dehydroepiandrosterone, have little intrinsic biological activity, they may act as prehormones which can be converted peripherally to more active compounds (Baird, Horton, Longcope and Tait, 1968). Conversions of androgens to oestrogens have been demonstrated to occur in the anterior hypothalamus (Ryan, Naftolin, Reddy, Flores and Petro, 1972) and in peripheral fat tissue (Nimrod and Ryan, 1975). The quantitative importance of these conversions is further illustrated by the fact that virtually all oestrogenic activity present in the peripheral circulation of postmenopausal women is derived from peripheral aromatization of androgens to oestrogens (Grodin, Siiteri and MacDonald, 1973). The continued secretion of androgens by the postmenopausal ovary (Judd, Judd, Lucas and Yen, 1974) may also contribute in the formation of this circulating androgen precursor pool.

Little information is available on possible changes in hypothalamic sensitivity to negative feedback once gonadotrophin secretion occurs in the "open loop mode". In postmenopausal women, a decline of the elevated levels of both FSH and LH has been noted in subjects over

60 years of age as compared to those aged between 50 and 60 years (Wide, Nillius, Gemzell and Roos, 1973). The reason for this decline is not clear. It may be a result of ageing processes affecting hypothalamic-pituitary function, similar to those seen in old female rats (Aschheim, 1964-65), or a consequence of the greater ability of older women to convert androstenedione to oestrone (Hemsell, Grodin, Brenner, Siiteri and MacDonald, 1974). Alternatively, the decline may result from an increase in hypothalamic negative feedback sensitivity such as is seen in castrated female rhesus monkeys (Karsch, Weick, Hotchkiss, Dierschke and Knobil, 1973).

(b) Dynamics of the negative feedbackmechanism

Of the eight steroids secreted by the ovary, 17β-oestradiol and, under certain conditions, progesterone appear to be most intimately involved with negative feedback regulation of pituitary gonadotrophin secretion. Evidence to support this conclusion has been discussed earlier (p. 87).

Negative feedback effects of 17β-oestradiol

In ovariectomized rhesus monkeys, intravenous injection or infusion of 17β-oestradiol leads to a prompt cessation of the pulsatile LH-discharges and a decline in the mean concentration of the hormone. The duration of this suppression persists for several hours beyond the time when oestrogens cease to be detectable in peripheral plasma (Yamaji, Dierschke, Bhattacharya and Knobil, 1972). The reason for this apparent discrepancy is not clear, although it is worth noting in this respect that, in the ovariectomized rat, the hypothalamus and pituitary bind tritiated 17β-oestradiol for approximately 6 hours after a single injection of the labelled steroid (McGuire and Lisk, 1968). If plasma oestradiol concentrations characteristic of those observed in the mid to late follicular phase of the menstrual cycle (100-150 pg/ml) are maintained in ovariectomized rhesus monkeys for several days, mean gonadotrophin levels which are consonant with this stage of the cycle are achieved. Lower levels of circulating oestradiol however fail to prevent the rise in circulating LH and FSH following ovariectomy (Karsch, Weick, Hotchkiss, Dierschke and Knobil, 1973).

In the human female, intramuscular, intravenous (Tsai and Yen, 1971) or oral administration (Yen, Martin, Burnier, Czekala, Greaney and Callantine, 1975) of 17β-oestradiol is also followed by a prompt decline in peripheral FSH and LH levels and an interruption of the pulsatile LH-release (Yen, Tsai, Vandenberg and Rebar, 1972).

Negative feedback effects of synthetic oestrogens

Small doses (20 µg a day) of orally active synthetic oestrogens such as 17α-ethinyloestradiol or its 3-methylether (mestranol) exert an almost specific inhibitory effect on FSH-secretion. Their effect on LH is slight and very often delayed (Franchimont, Legros and Meurice, 1972). At higher dose levels (50-60 µg a day) both steroids produce a concomitant reduction in FSH and LH, the levels of which remain depressed throughout the period of treatment (Franchimont, Legros and Meurice, 1972; Leyendecker, Wardlaw and Nocke, 1972). However, if the daily dose of either steroid is further increased their suppressive effect on peripheral gonadotrophin levels, and particularly on LH, becomes less uniform due to the concomitant activation of the positive feedbackmechanism. Thus, Swerdloff and Odell (1969) found multiple LH peaks in women during the oestrogenic phase (80 µg of mestranol a day) of oral sequential contraceptive therapy. In surgically castrated women, administration of 100 µg mestranol a day for 14 days led to a progressive decline in FSH. The LH levels on the other hand decreased also during the first days but later showed peaks at the end of treatment (Wallach, Root and Garcia, 1970). Yen and Tsai (1971) reported similar results in postmenopausal women taking 400 µg a day of ethinyloestradiol.

Negative feedback effects of progesterone

In contrast to the inhibitory efficacy of small physiologic increments in circulating oestrogen, supraphysiological levels of progesterone, even when maintained for prolonged periods, are inactive in influencing LH secretion in ovariectomized rhesus monkeys (Yamaji, Dierschke, Bhattacharya and Knobil,1972). Likewise in postmenopausal women, the intramuscular injection of 10-100 mg of progesterone produced no change in FSH or LH levels (Franchimont and Legros, 1970; Nillius and Wide, 1971).

Negative feedback effects of synthetic gestagens

Although natural progesterone when given alone, has no inhibitory effect on gonadotrophin release, several synthetic gestagens such as 19-nortestosterone or medroxyprogesterone derivatives are potent inhibitors of gonadotrophin release when administered in relatively large doses (Franchimont, Cession, Ayalon, Untsers and Legros, 1970). Low doses of the same preparations although ineffective in affecting basal gonadotrophin levels, are capable of blocking cyclic or oestrogen-induced LH-release (see further p.109).

Negative feedback effects of oestrogen-progesterone (gestagen) combinations

In the ovariectomized rhesus monkey, physiological amounts of progesterone can synergize with subthreshold levels of 17β-oestradiol in the inhibition of tonic LH secretion (Karsch, Weick, Hotchkiss, Dierschke and Knobil, 1973). A similar synergistic action of synthetic oestrogens and gestagens in suppressing pituitary gonadotrophin secretion can be demonstrated in agonadal women (Wallach, Root and Garcia, 1970). In normal women under non-sequential contraceptive treatment, the combined action of oestrogens and gestagens suppresses the early rise of FSH during the follicular phase and the peaks of FSH and LH at midcycle (Ross, Odell and Rayford, 1967; Cargille, Ross and Rayford, 1968).

3.2.2 Positive feedback

Although the negative feedback of gonadal steroids can account for the low gonadotrophin levels observed during the larger part of oestrous and menstrual cycles, it cannot account for the cyclic gonadotrophin discharge observed at midcycle. Consequently, workers early began to search for possible stimulatory effects of gonadal steroids on gonadotrophin secretion. Kaufmann (1933) was the first to describe such an effect. Following treatment of women with secondary amenorrhoea with oestradiol benzoate the author observed withdrawal bleedings from endometria which showed secretory changes, a finding which led him to suggest that oestrogen may cause luteinization of follicles through stimulation of the pituitary gland. This view was subsequently substantiated by Hohlweg (1934) who demonstrated that oestrogen could induce corpora lutea formation in immature female rats. Since the pituitary gland of the treated animals showed marked histological changes, it seemed likely that this organ was involved in this "positive" feedback effect of oestrogens. Subsequent work by Everett (1948) demonstrated that, under certain conditions, administration of oestrogens or progesterone could advance ovulation in the rat, which led Everett, Sawyer and Markee (1949) to the suggestion that oestrogens acting on the central nervous system initiate the ovulatory release of LH. The formal demonstration however that oestrogens through their stimulatory effect on gonadotrophin secretion, do play an essential role in the control of ovulation had to await the development of radioimmunoassays.

In 1969 Ferin, Tempone, Zimmering and Vande Wiele reported that the administration of an antiserum to 17β-oestradiol blocked ovulation in the rat but that this effect could be overcome by diethylstilboestrol, a synthetic oestrogen which did not cross-react with the antibodies. This observation, together with the nearly contemporaneous findings that parenteral administration of oestrogens to sheep (Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969), rats (Caligaris, Astrada and Taleisnik, 1971) or women (Vande Wiele, Bogumil, Dyrenfurth, Ferin, Jewelewicz, Warren, Rizkallah and Mikhail, 1970) led to an increase in plasma LH resembling the spontaneous midcycle LH-surge, provided unequivocal proof that the preovulatory rise in circulating 17β-oestradiol (Baird and Guevara, 1969) represents the critical stimulus for the initiation of the ovulatory gonadotrophin surge.

(a) Maturation of positive feedbackmechanism

In rodents, it is now well recognized that differentiation of the neural mechanism which controls oestrogen-induced cyclical gonadotrophin secretion occurs during the perinatal period. In the neonatal male rat, testicular activity during this critical period is held responsible for the permanent suppression of the cyclic centre while in the female, the absence of gonadal activity allows the hypothalamus to remain in the undifferentiated or cyclic state. Consequently, adult female rats or male rats castrated immediately after birth will respond positively to an injection of oestrogen by a surge of gonadotrophin secretion while adult intact males or females treated neonatally with testosterone will fail to respond (see Gorski, 1971 for review).

Though these observations indicate that testosterone plays an important role in the process of sexual differentiation of the hypothalamus, recent evidence tends to suggest that this organising action of testosterone on neural differentiation may be mediated through oestrogens. Indeed, treatment of neonatal female rats with oestrogens will also permanently suppress cyclicity (Gorski, 1963; Dörner, Döcke and Hinz, 1971). Neonatal administration of nonaromatizable androgens such as dihydrotestosterone on the other hand, does not inhibit cyclicity in the female rat (Luttge and Whalen, 1970; McDonald and Doughty, 1974). The findings that the hypothalamus of several species is capable of converting androgens to oestrogens (Ryan, Naftolin, Reddy, Flores and Petro, 1972) and that in the rat this aromatizing system is present in the neonatal period (Reddy, Naftolin and Ryan, 1974) add further credence to the notion that oestrogens may play an important role in the process of sexual differentiation of the rat hypothalamus. A similar mechanism may be responsible for the suppression of cyclicity observed in the male partners of several other species of rodents (Barraclough, 1973) and sheep (Short, 1974). The critical period however during which testicular androgens exert their organizing effect on hypothalamic sexual differentiation may vary between species, being e.g. prenatal (between 20th-60th days of gestation) rather than neonatal in sheep.

In contrast to the situation in rodents and sheep, attempts to masculinise the hypothalamus of female primates either by androgen treatment of animals carrying female foetuses (Goy and Resko, 1972) or by neonatal androgen administration to newborn females (Treloar, Wolf and Meyer, 1972) have been uniformly unsuccessful. Moreover, unlike in rats (Ying, Fang and Greep, 1971) and sheep (Land, Thimonier and Pelletier, 1970) in which oestrogen-induced gonadotrophin-surges can be evoked in immature animals, maturation of the positive feedback mechanism in primates appears to be a late pubertal maturational event. In the rhesus monkey oestrogen-induced gonadotrophin surges are not demonstrable until 4 to 8 months after menarche and spontaneous cyclic gonadotrophin discharges which eventuate in ovulation can only be detected approximately one year after menarche (Dierschke, Weiss and Knobil, 1974). Likewise, in the human female, the ability to respond to parenteral oestrogen exposure with a rise in circulating plasma LH is not acquired until mid to late puberty (Reiter, Kulin and Hamwood, 1974). The observation by the latter authors and by Yen, Tsai, Vandenberg and Rebar (1972) that postpubertal girls with gonadal dysgenesis will release LH following oestrogen administration suggests also that the maturation of this positive feedback mechanism is independent of gonadal steroid secretion. A similar conclusion was reached by Dierschke, Weiss and Knobil (1974) who were unable to advance maturation of positive feedback by chronic treatment of prepubertal female rhesus monkeys with oestradiol or progesterone.

Since pre- or neonatal androgen-exposure of females in both monkey (Treloar, Wolf and Meyer, 1972; Goy and Resko, 1972) and human species (Wilkins, 1965) appears not necessarily to affect normal ovarian cyclicity in later life, it has been suggested that, unlike in rodents (Caligaris, Astrada and Taleisnik, 1972) or sheep (Karsch and Foster, 1975), the ability of the hypothalamic-pituitary unit to discharge LH in response to oestrogen may not be a sexually dimorphic characteristic in primates. In the rhesus monkey, Karsch, Dierschke and Knobil (1973) have shown that although intact adult males do not show positive feedback when given oestrogen, castrated males can be made to release LH either by pretreating them with oestrogen or by giving larger doses of oestradiol than those required to elicit a gonadotrophin surge in ovariectomized females. The sex difference in hypothalamic differentiation may therefore be quantitative rather than qualitative in the rhesus monkey.

In the human, a positive feedback effect of oestrogens in intact adult men cannot be demonstrated (see further, this thesis p. 184). However, progesterone administration to oestrogen-primed castrated adult men has been shown to elevate gonadotrophin levels (Stearns, Winter and Faiman, 1973). Similar progesterone-induced LH releases have been demonstrated in oestrogen pre-treated castrated or post-menopausal women (Odell and Swerdloff, 1968; Nillius and Wide, 1971 a; Leyendecker, Wardlaw and Nocke, 1972). The relevance of these observations however is questionable particularly in view of the finding that progesterone-induced LH release can only be demonstrated in ovariectomized rhesus monkeys but not in intact animals (Clifton, Steiner, Resko and Spies, 1975). Recently, claims have been made that oestrogen may be capable of inducing LH-release in normal (Kulin and Reiter, 1976) and homosexual men (Dörner, Rohde, Stahl, Krell and Masius, 1975) but the observed gonadotrophin rises are neither in timing nor magnitude comparable to those seen in normal women.

(b) Dynamics of positive feedback mechanism

There is little doubt that the preovulatory rise in circulating 17 β-oestradiol represents the triggering stimulus for the initiation of the midcycle gonadotrophin-surge. Data obtained in agonadal subjects suggests also that this positive feedback effect of oestrogen may be facilitated by progesterone. However, the physiological significance, if any, of this latter effect in the regulation of the spontaneous pre-ovulatory LH-peak remains to be determined.

Positive feedback effect of oestrogens

In contrast to the ewe, in which a single (10 µg) intramuscular injection of 17β-oestradiol induces LH-surges 8 to 12 hours later (Goding, Catt, Brown, Kaltenbach, Cumming and Mole, 1969), activation of the positive feedback mechanism in primates requires a more prolonged exposure of the hypothalamic-pituitary unit to oestrogen. Moreover, in order to be effective, the peripheral level of circulating oestrogen must exceed a certain threshold concentration.

Thus single intramuscular or subcutaneous injections of 17β-oestradiol given to intact female rhesus monkeys during the early follicular phase of the cycle and which resulted in large but short-lived (less than 12 hours) elevations in plasma oestrogen concentrations did not elicit an LH-surge (Yamaji, Dierschke, Hotchkiss, Bhattacharya, Surve and Knobil, 1971). Subthreshold plasma oestrogen levels of less than 100 pg/ml are equally ineffective even when maintained for as long as 120 hours. However, slightly higher plasma oestrogen levels exceeding a threshold of approximately 150 pg/ml and which are sustained for at least 36 hours will consistently induce LH-release (Karsch, Weick, Butler, Dierschke, Krey, Weiss, Hotchkiss, Yamaji and Knobil, 1973). Observations by the same authors also indicate that the oestrogen stimulus, in order to be effective, must be applied until the response actually begins. Furthermore, unlike in rats (Legan, Coon and Karsch, 1975; Legan and Karsch, 1975) or hamsters (Norman and Spies, 1974) the initiation of the LH surge in the monkey is not coupled to the diurnal light-dark cycle. This suggests that in primates as in sheep (Jackson and Thurmon, 1974) the ovary is the "Zeitgeber" or "clock" for the LH surge and hence ovulation (Karsch, Dierschke, Weick, Yamaji, Hotchkiss and Knobil, 1973).

Although the dynamics of the positive feedback mechanism have not been investigated in the human female to any such extent as they have been studied in the rhesus monkey, several separate observations suggest that the minimal requirements for an oestrogen stimulus to be effective may be comparable in the two species. Following a brief (4 to 12 hours) intravenous infusion of 17β-oestradiol into regularly cycling women, only a modest increase in serum LH concentrations occured (Tsai and Yen, 1971). Sustained elevations of peripheral oestrogen levels, on the other hand, achieved either by repeated intramuscular injections of oestradiol-benzoate (Monroe, Jaffe and Midgley, 1972) or by oral administration of ethinyloestradiol (Yen and Tsai, 1972; this thesis p.184) consistently evoke an LH discharge from the pituitary 3 to 4 days after the initiation of treatment. In contrast, single intramuscular injections of oestradiol-benzoate or 36 hours infusions of 17β-oestradiol into normal women are usually less effective (Yen and Tsai, 1972) unless a large dose of oestradiol-benzoate is employed (Nillius and Wide, 1971 b).

Several factors can inhibit the stimulatory action of oestrogen on LH and FSH release. Elevated plasma progesterone concentrations, achieved by parenteral administration of natural progesterone, block the positive feedback action of oestrogen in the intact rhesus monkey (Dierschke, Yamaji, Karsch, Weick, Weiss and Knobil, 1973; Spies and Niswender, 1972; Clifton, Steiner, Resko and Spies, 1975), the sheep (Scaramuzzi, Tillson, Thorneycroft and Caldwell, 1971), rat

(Labhsetwar, 1971) and human (Netter, Gorius, Thomas, Cohen and Joubinaux, 1973). Continuous treatment of regularly cycling women with low doses of several synthetic gestagens can also block the spontaneous preovulatory LH-surge (Franchimont, Cession, Ayalon, Untsers and Legros, 1970; Larsson-Cohn, Johansson, Wide and Gemzell, 1970; Weinter, Johansson and Wide, 1976). This inhibitory effect of progesterone on oestrogen-induced LH-release may account for the failure to demonstrate positive feedback during the luteal phase of the menstrual cycle (Dierschke, Yamaji, Karsch, Weick, Weiss and Knobil, 1973).

Since testosterone can inhibit LH release in response to oestrogen stimulation in the female rat (Klawon, Sorrentino and Schalch, 1971), Knobil's group (Karsch, Dierschke and Knobil, 1973) has suggested that a similar blocking action of testosterone may be responsible for the apparent inability of intact male primates to release LH following oestrogen-exposure. Our own (limited) observations in testosterone-insensitive patients with the syndrome of testicular feminization (Morris, 1953) however appear not to support this hypothesis (see further, p. 226).

Systemic injections of synthetic glucocorticoids have been shown to possess anti-ovulatory properties in rats (Hagino, Watanabe and Goldzieher, 1969) and baboons (Hagino, 1972). In the rat, dexamethasone blocks the spontaneous pre-ovulatory LH-surge due to a direct effect at the hypothalamic (Hagino, Watanabe and Goldzieher, 1969) and/or pituitary level (Baldwin and Sawyer, 1974). Recently, Cunningham, Caperton and Goldzieher (1975) have presented evidence that a single dose of triamcinolone acetonide given on day 1 or 2 of the menstrual cycle may similarly delay follicular development and inhibit ovulation in women. Dexamethasone (Abraham, 1974) and paramethasone acetate (Cortés-Gallegos, Gallegos, Tovar, Cervantes and Parra, 1975) on the other hand were ineffective in blocking the midcycle LH-peak and ovulation.

Finally, studies by Glass, Shaw, Butt, Logan-Edwards and London (1975) have shown that women with amenorrhoea-galactorrhoea and elevated plasma prolactin levels failed to release LH in response to an intramuscular injection of oestradiol-benzoate. It is not clear however if the failure of the positive feedback mechanism in these conditions is a result of the hyperprolactinaemia per se rather than another manifestation of the underlying hypothalamic-pituitary dysfunction. It is of interest to note in this respect that the stimulatory action of oestrogen on LH release cannot be demonstrated in lactating female rhesus monkeys (Yamaji, Dierschke, Hotchkiss, Bhattacharya, Surve and Knobil, 1971).

Positive feedback effect of progesterone

Progesterone injected intramuscularly into oestrogen-suppressed agonadal or postmenopausal women (Odell and Swerdloff, 1968; Leyendecker, Wardlaw and Nocke, 1972) and in oestrogen-suppressed castrated men (Stearns, Winter and Faiman, 1973) induces gonadotrophin release within 24 hours after its administration. In the ovariectomized rhesus monkey the simultaneous administration of progesterone significantly advances the onset of oestrogen-induced LH release (Clifton, Steiner, Resko and Spies, 1975). From these and similar observations in other species it has been suggested that progesterone may facilitate the stimulatory action of oestrogen on gonadotrophin release by lowering the sensitivity of the positive feedback centre. The relevance of these observations in agonadal subjects for the mechanism governing the initiation, regulation and termination of the spontaneous midcycle LH surge is questionable however.

Studies by Ferin and his colleagues (Ferin, Tempone, Zimmering and Vande Wiele, 1969) indicate that this progesterone effect is certainly not essential for the initiation of the pre-ovulatory LH peak since administration of anti-progesterone antibodies did not block ovulation in the rat. Furthermore, in contrast to ovariectomized rhesus monkeys, simultaneous administration of progesterone and oestrogen to intact females did not advance but inhibited oestrogen-induced LH release (Clifton, Steiner, Resko and Spies, 1975). This blocking effect of progesterone remains present even when the hormone is introduced 12 hours after the initiation of oestrogen treatment (Dierschke, Yamaji, Karsch, Weick, Weiss and Knobil, 1973).

It would appear therefore that the presence of elevated progesterone levels in the peripheral circulation of intact animals has an inhibitory rather than stimulatory effect on the initiation of the pre-ovulatory gonadotrophin-surge. Clearly, this does not exclude that, following the induction of the LH-peak by 17 β-oestradiol, the rising levels of progesterone might facilitate the further development of the LH peak. The importance of this effect however is likely to be minimal since the LH-release induced by exogenous oestrogen administration during the mid-follicular phase of the menstrual cycle is often, both in terms of magnitude and duration, comparable to the spontaneous pre-ovulatory LH-surge, even despite the fact that the former is not associated with ovulation and corpus luteum formation (Yen and Tsai, 1972).

In summary, there seems to be very little evidence to support the concept that progesterone in the intact animal has any effect other than its inhibitory action on oestrogen-induced LH-release. Obviously, this conclusion raises the question as to the physiological relevance of studies on positive feedback utilizing progesterone administration to oestrogen-primed subjects.

3.3 Sites at which steroids exert their feedback effects

There is little doubt that the main sites of action of gonadal steroids involved in feedback regulation of gonadotrophin secretion are located within the hypothalamus. However, recent observations on gonadotrophin responses after parenteral administration of synthetic LRF clearly indicate that part of their feedback effects may also be exerted at the pituitary level through modulation of the sensitivity of the pituitary gland to hypothalamic neurohumoral stimulation.

3.3.1 Hypothalamus

The vast amount of data accumulated during studies on the effects on gonadotrophin secretion of procedures such as deafferentation of the hypothalamus or the implantation of gonadal steroids into the hypothalamic-pituitary region have left little doubt that, in the rat, positive and negative feedback effects of steroid hormones involve activation, respectively suppression of different hypothalamic regions (see e.g. Szentágothai, Flerko, Mess and Halasz, 1972 for review). Sites responsive to the negative feedback effects of gonadal steroids appear to be concentrated primarily in the medial basal hypothalamus; the preoptic-suprachiasmatic region on the other hand is involved in oestrogen-induced release of LH. Dopamine and noradrenaline have been implicated as synaptic transmitters of inhibitory and stimulatory feedback effects respectively from these hypothalamic regions to the releasing factor-secreting neurons. In primates, the hypothalamic regions concerned with feedback regulation of gonadotrophin secretion have not been investigated to any such extent as in the rat. Preliminary observations tend to suggest that major differences may exist, particularly with respect to the neural control of the oestrogen-induced midcycle LH-surge. As in the rat, most hypothalamic sites responsive to the negative feedback action of oestrogens appear to be located in the mediobasal hypothalamus and include the suprachiasmatic, infundibular, ventromedial and mamillary complex nuclei (Ferin, Carmel, Zimmerman, Warren, Perez and Vande Wiele, 1974). However, in contrast to the rat, complete deafferentation of the median basal hypothalamus did not effect spontaneous ovulation in the rhesus monkey, suggesting that in primates the ovulatory LH-discharge might not require a signal generated by the preoptic area of the brain (Knobil, 1974).

3.3.2 Pituitary

Both in-vitro and in-vivo studies on the effects of gonadal steroids on the pituitary gonadotrophin response to synthetic LRF support the concept that steroid hormones may alter gonadotrophin secretion by a direct action on the pituitary. The existence in the anterior pituitary of specific receptor sites for gonadal steroids has been demonstrated on several occasions (see e.g. Stumpf, Sar and Keefer, 1975 for recent review).

In-vitro, small doses of 17β-oestradiol decreased the release of FSH and, to a smaller extent, of LH from anterior pituitary tissue incubated with LRF. Progesterone was ineffective in this respect but the combination of progesterone with 17β-oestradiol suppressed both basal release of FSH and LH and the stimulatory effect of LRF on gonadotrophin release (Schally, Redding and Arimura, 1973).

In-vivo, rapid increases in circulating oestrogen concentrations achieved by constant infusion of 17β-oestradiol into hypogonadal (Yen, Vandenberg and Siler, 1974) or eugonadal women (Keye and Jaffe, 1974) induced a prompt and marked diminution in pituitary responsiveness to

LRF, indicating a direct inhibitory action of oestradiol on pituitary sensitivity to LRF. A more prolonged exposure of the pituitary to elevated 17β-oestradiol concentrations on the other hand enhances pituitary gonadotrophin secretion following LRF (Jaffe and Keye, 1974). An increase in pituitary sensitivity to LRF has also been demonstrated following progesterone administration (Shaw, Butt and London, 1975). These observations indicate that changes in ovarian steroid secretion during the menstrual cycle may alter pituitary sensitivity to hypothalamic LRF-stimulation. This would explain why the pituitary gonadotrophin response to a constant dose of synthetic LRF varies depending on the stage of the cycle (Nillius and Wide, 1972). It also offers an explanation for the rather puzzling observations that the enhanced pituitary gonadotrophin secretion during the pre-ovulatory period in rats and sheep is apparently not associated with an increase in hypophysial portal, respectively peripheral LRF levels (Fink and Jamieson, 1976; Nett, Akbar and Niswender, 1974).

4. Hypothalamic-pituitary-ovarian relationships during the menstrual cycle

The newly acquired information on the dynamic relationships between the gonadotrophic and gonadal hormones has led to a better understanding of the hormonal changes observed during the menstrual cycle (Figure 1.5). Taking into account the observations made on the morphological changes in the ovary during the menstrual cycle (see e.g. Koering, 1969) and on the physiological effects of FSH and LH on ovarian morphology and function (for references see p. 29), the hormonal events during the menstrual cycle can be summarized as follows (see e.g. Speroff and Vande Wiele, 1971 for review).

At the end of the cycle, the regression of the corpus luteum and the concomitant decline in peripheral 17β-oestradiol and progesterone levels enhance pituitary gonadotrophin secretion through activation of the negative feedback mechanism. This rise in FSH and to a lesser extent LH levels, will stimulate development of a new crop of gonadotrophin-sensitive follicles from the pool of primordial follicles present in the ovaries. As follicular maturation progresses, steroid synthesis and secretion gradually increase and peripheral 17β-oestradiol levels progressively rise. The increase in circulating oestrogen concentrations reduces pituitary FSH secretion through negative feedback action. This decline in circulating FSH ensures that no other follicle(s) develop(s) except that (those) which is (are) most active in oestrogen synthesis and secretion and which is (are) therefore less dependent on further FSH-stimulation. The remaining follicles become atretic due to lack of gonadotrophic support. Once circulating 17β-oestradiol levels reach a minimum threshold concentration of about 150-200 pg/ml for a minimum period of about 36 to 48 hours, the positive feedback loop is activated and a LH-surge is initiated. The subsequent decline in 17β-oestradiol (and the increase in progesterone levels?) resulting from the luteinization of the follicle may facilitate the further development of the midcycle LH-peak. Following ovulation and corpus luteum formation, the synergistic negative feedback effects of 17β-oestradiol and progesterone suppress pituitary gonadotrophin secretion to its lowest level. Follicular development is thus prevented. The blocking action of progesterone on the positive feedback effect of oestrogen further ensures that the rising levels of 17β-oestradiol secreted by the corpus luteum will not stimulate gonadotrophin release during the luteal phase of the cycle.

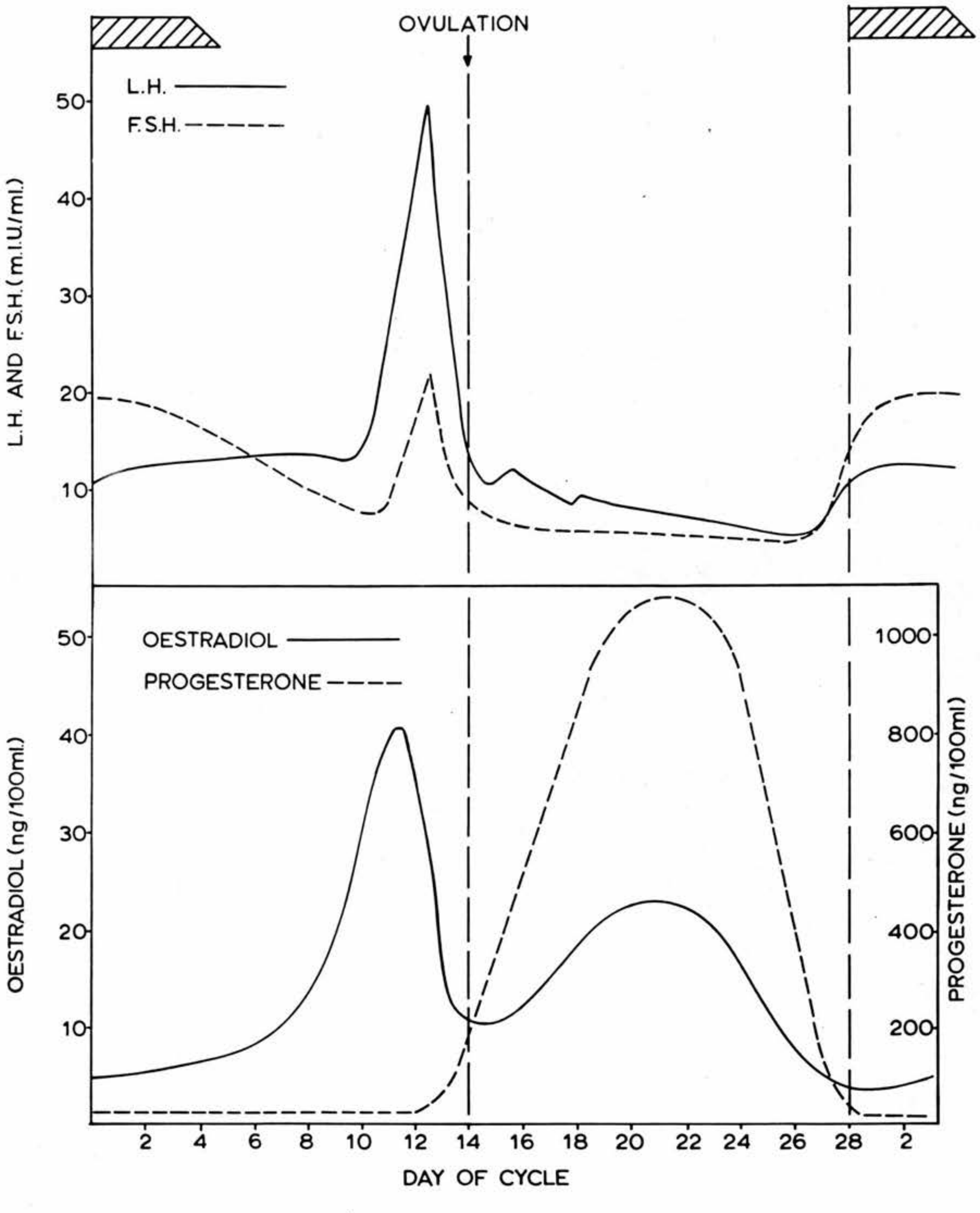


Figure 1.5: Peripheral FSH, LH, 17β-oestradiol and progesterone levels during the human menstrual cycle (from Baird, 1974 with permission of the author)

5. Factors which may influence hypothalamic-pituitary-ovarian relationships

The occurrence of regular follicular development and ovulation is dependent upon the undisturbed interplay between hypothalamus, anterior pituitary and ovaries, which, together with their secretory products, constitute the hypothalamic-pituitary-ovarian (H.P.O.) axis. In the absence of interfering factors the activity of this axis is essentially cyclical, the mode of the cycle being determined by the inherent time lags in adaptability of its component parts to changes in the hormonal environment (Short, 1974).

A variety of factors may influence this intrinsic cyclical activity of the H.P.O. axis but a detailed discussion of them is outside the scope of this thesis. In Table 1.2 an attempt has been made to classify some of the more important factors which may affect H.P.O. function in physiological or pathological conditions on the basis of their (assumed) site of action. A detailed discussion of the conditions listed in Table 1.2 can be found in Baird, Van Look and Hunter (1976).

TABLE 1.2: Factors which may influence H.P.O. relationships

SITE OF INTERFERENCE	TYPE OF INTERFERENCE	EXAMPLES
HYPOTHALAMUS	STRUCTURAL	TUMOURS, ENCEPHALITIS, GRANULOMEN, TUBERCULOSIS, LESIONS etc. of the hypothalamus
	FUNCTIONAL	
	(a) neurogenic	ENVIRONMENTAL STIMULI (stress, photoperiod, temperature, lactation, nutrition, etc.)
	(b) humoral	ULTRA-SHORT FEEDBACK LOOPS (LRF → LRF) SHORT FEEDBACK LOOPS (LH → LRF) (FSH → LRF) (prolactin → LRF, PIF) DRUGS, OTHER ENDOCRINE DISORDERS
HYPOTHALAMIC-PITUITARY AXIS	STRUCTURAL	SECTION OR LESIONS of pituitary stalk
	FUNCTIONAL	LRF-DEFICIENCY (Kallmann's syndrome, congenital hypopituitarism, LRF antibodies) LRF-EXCESS (ectopic production ?; exogenous administration)
PITUITARY	STRUCTURAL	TUMOURS, INFARCTION GRANULOMEN, TUBERCULOSIS etc. affecting anterior pituitary
	FUNCTIONAL	DRUGS
PITUITARY-OVARIAN AXIS	FUNCTIONAL	GONADOTROPHIN-DEFICIENCY (isolated FSH deficiency, gonadotrophin antibodies) GONADOTROPHIN-EXCESS (pregnancy, hydatiform mole, chorio-carcinoma, ectopic gonadotrophin production, exogenous gonadotrophin administration)

TABLE 1.2 (continued)

OVARY	STRUCTURAL	PRIMARY OVARIAN FAILURE (gonadal dysgenesis, oopheritis, menopause etc.)
	FUNCTIONAL	?PROLACTIN ?PROSTAGLANDIN F2 ?DRUGS (e.g. glucocorticoids)
OVARIAN-HP AXIS	FUNCTIONAL	STEROID-DEFICIENCY (17β-oestradiol antibodies) STEROID-EXCESS (pregnancy, oestrogen or androgen producing tumours of ovary or adrenal; extraglandular conversion of androgens to oestrogens; exogenous steroid administration)

CHAPTER TWO:

AIMS OF THE PRESENT STUDY

Since the introduction of radioimmunoassays for pituitary gonadotrophins and, subsequently, for steroid hormones our knowledge of the physiology of the hypothalamic-pituitary-ovarian (H.P.O.) axis has considerably increased. The secretion patterns of pituitary gonadotrophins and ovarian steroid hormones, and their interrelationships during the normal menstrual cycle have been elucidated. The existence of negative and positive feedback mechanisms has been confirmed and their dynamics explored.

When we started our studies in 1973, very little information however was available on H.P.O. relationships in pathological conditions. Until then most of the endocrine studies in gynaecological patients had been concerned with spontaneous fluctuations of peripheral gonadotrophin and steroid levels. These studies provided very valuable information on the endocrine pathology (i.e. the symptoms) of various disorders of the H.P.O. axis. In most instances however, measurement of peripheral hormone levels under basal conditions alone does not permit the elucidation of the underlying pathogenesis. In every feedback system, and the H.P.O. axis is no exception in this respect, a functional failure of any component of the system will have repercussions on the function of the other components. Often such a failure will lead to loss of the intrinsic cyclical activity of the H.P.O. system and the establishment of a "steady-state" condition. Under these circumstances measurement of basal hormone concentrations alone usually can yield but little information on (a) the site of the primary defect and (b) the functional integrity of the individual components of the H.P.O. axis.

Within recent years however it has become possible to investigate the various components of the H.P.O. axis into somewhat greater detail by using a variety of "dynamic tests". If properly applied and interpreted, these test-procedures should, at least theoretically, permit the evaluation of the functional capacity of the different constituents of the H.P.O. axis and could thus prove very useful in the diagnosis and treatment of H.P.O. disorders.

Abnormalities of the H.P.O. axis present clinically as disorders of menstruation and/or infertility and are usually classified on the basis of their presenting symptom e.g. primary or secondary amenorrhoea. Such classification, albeit clinically useful, is however rather unsatisfactory not only from a pathophysiological but also from a therapeutic point of view. It is well known for example that some patients complaining of secondary amenorrhoea and infertility respond favourably to treatment with clomiphene for induction of ovulation while others consistently fail to ovulate or even to grow a follicle when treated with this drug. Obviously, this variability in responsiveness to clomiphene is likely to be a reflection of the extent of H.P.O. failure. Provided a simple, reliable method can be found to assess the degree of functional integrity of the H.P.O. axis in those instances, it goes without saying that a considerable amount of frustration and disappointment on the side of both the infertile woman and the medical practitioner could be prevented.

In the present study it was intended to investigate H.P.O. relationships using dynamic tests in a few selected gynaecological endocrine disorders. By doing so, it was hoped to contribute a little to the elucidation of the pathophysiological mechanisms underlying these disorders. It is evident that any study of this kind is not a one-man effort but involves a number of collaborating clinicians and laboratory staff. The nature and the extent of involvement of these co-workers in the studies reported in this thesis have been duly acknowledged. The radioimmunoassays for 17β-oestradiol and 17α-ethinyloestradiol, developed by myself during the course of these studies, are described in the chapter on hormone assay techniques (Chapter 5.)

CHAPTER THREE:

GENERAL PRINCIPLES AND EXPERIMENTAL APPROACH OF CLINICAL STUDIES

3.1 Classification of clinical studies

Menstrual bleeding occurring at regular 4-weekly intervals is usually the obvious clinical manifestation of cyclical follicular development and ovulation. The former event, i.e. the presence of regular co-ordinated follicular growth, is dependent upon appropriate stimulation by pituitary gonadotrophins, the secretion of which is regulated by a classical negative feedback mechanism. This mechanism ensures that under normal circumstances only one follicle develops into a pre-ovulatory Graafian follicle. The second event, i.e. the rupture of this pre-ovulatory follicle, is also under ovarian control since it is the increasing follicular 17β-oestradiol secretion which triggers the midcycle LH-peak by stimulating the positive feedback mechanism. Thus the presence of regular ovulatory cycles requires functional integrity of both positive and negative feedback.

In the absence of recognisable local or systemic disease, abnormalities in menstrual bleeding pattern are often indicative of an upset in the function of the H.P.O. axis. Such functional H.P.O. disorders can be classified into two groups depending on whether there is an absence of regular follicular development (i.e. negative feedback failure) or anovulation (i.e. positive feedback failure).

(a) Negative feedback failure is usually associated with infrequent or absent menstruation and is hence most commonly encountered in patients presenting with polycystic ovarian disease (P.C.O.) and in those with amenorrhoea but without evidence of P.C.O. Although ovarian activity in both instances is a-cyclical, previous studies have indicated that the hormonal environment in terms of circulating gonadotrophin and steroid levels is quite different. This suggests that the degree of H.P.O. dysfunction in these conditions may also be different. To test this view, it was decided to investigate hypothalamic-pituitary function in patients with P.C.O. and in women with secondary amenorrhoea.

(b) In the rat (and probably in primates also) the hypothalamic centre involved in positive feedback (or "cyclic" centre) is anatomically and functionally distinct from the negative or "tonic" feedback centre. Because of this functional differentiation at the hypothalamic level, one might theoretically expect that negative feedback can continue to operate in the absence of positive feedback. In the female, such a situation would give rise to the occurrence of anovulatory ovarian cyclicity. Anovulatory cycles in the human are particularly common immediately after the menarche and before the menopause. To test the hypothesis that the failure to ovulate in these women and particularly in those with a prolonged history of persistent anovulation, is due to a failure of the positive feedback mechanism, ovarian activity and hypothalamic-pituitary function were investigated under basal conditions and using dynamic tests e.g. oestrogen provocation tests. The latter test was also performed in intact genetic males (normal men and male pseudohermaphrodites) to compare the dynamics of pathological (anovulatory patients) and physiological (normal men) positive feedback failure.

3.2 General outline of the experimental approach

3.2.1 Studies on positive feedback failure

Failure to ovulate in the presence of apparently normal follicular development suggests the presence of a defect in the mechanism whereby oestrogen normally evokes an LH discharge from the pituitary at mid-cycle. In our studies on patients with anovulatory cycles we have tried to detect such defect in two ways:

(1) by making daily measurements of plasma gonadotrophins and ovarian steroids in a few selected cases

(2) by performing an oestrogen provocation test.

Results of the latter test were compared to those obtained in normal women and men.

A failure to release LH in response to an adequate oestrogen stimulus indicates a functional defect of the positive feedback loop. Since this loop consists of two components i.e. hypothalamic cyclic centre and the anterior pituitary, such a defect must necessarily reside in either of these two. To exclude the anterior pituitary as possible cause, a LRF-test was performed subsequent to the oestrogen provocation test in all the patients as well as in the control women (but not in the normal men).

3.2.2 Studies on negative feedback failure

In patients with proven P.C.O. daily measurements of plasma gonadotrophins and steroid hormones were made in a few selected cases. The functional capacity of the individual components of the negative feedback loop was further investigated using clomiphene, oestrogen provocation tests and LRF-tests.

In women with secondary amenorrhoea but without clinical evidence of P.C.O. the integrity of the negative feedback loop was tested with clomiphene and the response evaluated on the basis of

(1) changes in pituitary gonadotrophin secretion during treatment

(2) the subsequent ovarian response as reflected by changes in urinary total oestrogen and pregnanediol excretion.

Hypothalamic-pituitary function in these women was also investigated using LRF, TRF and insulin. A group of normal women underwent the same tests and served as controls.

3.3 Statistical analysis

Data were analysed for statistical significance according to the principles and using the formulae described by England (1975). Differences were considered significant if p was less than 0·05.

CHAPTER FOUR:

EXPERIMENTAL CLINICAL PROCEDURES

4.1 Selection of participating subjects

Informed consent was obtained from all subjects studied.

(a) Seven normal men were recruited among staff members of the MRC Unit of Reproductive Biology. All of them were in good health and without any evidence of endocrine disease.

(b) A total of 17 normal women volunteered to serve as controls. Three of them however had to be excluded either during or after completion of the studies for the following reasons: intervening pregnancy (1), use of β-adrenergic medication (1) and failure to take clomiphene on the indicated days (1). The volunteers were selected at random from women attending the Gynaecological Out-Patients' Department with a request for tubal sterilization. All selected women had a history of regular menstrual cycles and were of proven fertility.

(c) Three male pseudohermaphrodites participated in the study on positive feedback in this condition. Two siblings with the syndrome of testicular feminization were brought to our attention by Dr. J. Scrimgeour. The third patient attended the Gynaecological Endocrine Clinic because of primary amenorrhoea.

(d) A total of 20 patients (9 adolescents and 11 perimenopausal women) suspected of having persistent anovulation were recruited from the Gynaecological Endocrine Clinic. Selection of these women was based on their menstrual history and/or the presence of a persistent proliferative or cystic glandular hyperplastic endometrium at diagnostic curettage.

(e) Women with polycystic ovarian disease numbered twelve. Diagnosis of this condition was based on clinical and endocrine parameters.

In all but one of these women the presence of poly- or sclerocystic ovaries was confirmed by diagnostic laparoscopy.

(f) From December 1973 onwards, all women presenting at the Gynaecological Endocrine Clinic with secondary amenorrhoea of at least 6 months' duration but without clinical evidence of P.C.O. were asked to participate in the study on hypothalamic-pituitary function in this condition. At the beginning of 1976 and after excluding patients with primary ovarian failure, a total of 50 women had been studied and the results on these are reported in this thesis.

4.2 Urine-sampling technique

Twenty-four hour urine specimens were collected either daily, once a week or three times a week depending on the type of the study. Patients were instructed to ensure that a complete 24-hour collection was made and to notify the laboratory of any accidental loss. Urine samples were usually assayed on the day each collection was completed or were stored at 4°C over the weekend.

4.3 Blood-sampling technique

Daily blood samples were collected in the patient's home or at her place of work usually between 8.00 and 10.00 A.M. Samples were taken from an antecubital vein after thoroughly cleansing the overlying skin with a sterile disposable isopropyl alcohol swab (Sterets^{R}, Prebbles Medical Ltd., Liverpool). Blood was withdrawn using a sterile disposable 10-ml polypropylene syringe (Plastipak, B-D) fitted with a 21- or 23-G needle, and the sample was placed in a 10-ml heparinized container (LH/10, Searle). The plasma was separated within

1 to 2 hours of collection following centrifugation at 2,500 rpm for 15 minutes at 4°C, and separate plasma aliquots for gonadotrophin and steroid-assays were stored at -20°C.

Tests involving frequent blood-sampling were performed in the hospital with the patient lying down for the whole duration of the test. A suitable antecubital vein was selected and the skin was cleansed with a Sterets^{R} swab. A 19 or 21-G Butterfly^{R} (Abbott Ireland Limited, Sligo) was inserted and fixed to the skin with adhesive plaster. The tubing was connected to a disposable syringe containing heparinized saline which was used to prevent clotting. Blood samples were withdrawn using disposable syringes but the first 1 to 2 ml of each collection were discarded. Samples were placed in heparinized containers and left in ice until centrifugation (usually within 2 hours). If hormones were injected through the butterfly, care was taken to flush the tubing thoroughly with heparinized saline after injection.

4.4 Dynamic tests

4.4.1 Oestrogen provocation test

Blood samples were collected at daily intervals for 10 consecutive days. In women, the test was performed during the early follicular phase of the cycle (i.e. when urinary total oestrogen excretion was less than 12 µg/24 hours). Ethinyloestradiol (4 x 50 µg per day) was administered orally for 3 days starting on the morning of the fifth day. Twenty-four hours before and 24-hours after the start of treatment (i.e. on the mornings of the 4th and 6th day of the test respectively), blood samples were also collected at 15 minute intervals for a period of 3 hours to detect changes in pulsatile gonadotrophin secretion. All samples were assayed for FSH and LH. Daily samples were also assayed for 17β-oestradiol and 17α-ethinyloestradiol and, where indicated, for progesterone, testosterone, oestrone and androstenedione.

4.4.2 LRF-test

Daily samples were collected for 5 consecutive days during the early follicular phase of the cycle, but the LRF-test proper was performed on the fourth day. Fifty microgram LRF (Hoechst Pharmaceuticals) was injected at time zero, and blood samples collected at 15 minute intervals from -180 to -30 min., at 5 minute intervals from -30 to +30 min., at 15 minute intervals from +30 to -180 min, and hourly thereafter until +360 minutes. Samples were assayed for FSH, LH and 17β-oestradiol. In some instances, testosterone, oestrone and androstenedione were also measured on daily collections.

4.4.3 Clomiphene stimulation test

Clomiphene (Clomid^{R}, Merrell) was administered in a dose of 100 mg a day for 5 days and blood samples collected at 15 minute intervals for one hour and a half before and on the last day of treatment. FSH and LH was measured in all the samples and 17β-oestradiol in every second sample.

4.4.4 Combined hypothalamic-pituitary function test

This test involved measurement of FSH, LH, TSH, hGH, prolactin, glucose and 17-fluorogenic steroids before and after injection of TRF (200 µg), LRF (50 µg) and insulin (0·15 or 0·10 U/kg body weight). Hormones were injected through separate syringes at time zero and blood samples collected at -30, -5, +20, +30, +60 and +90 minutes. To minimise the influence of stress upon basal hormone secretion, the butterfly was inserted two and a half hours before the -30 min. blood sample was withdrawn.

CHAPTER FIVE:

HORMONE ASSAY TECHNIQUES

The measurements of urinary total oestrogen and pregnanediol excretion were made in the Clinical Laboratory, SMMP by Mrs. E. Michie, Miss H. Lothian, Mrs. M. Leith and Mr. H. Boyle. Urinary gonadotrophin assays were done in the Hormone Laboratory by Dr. B. Hobson and his staff. Plasma gonadotrophins were assayed by the MRC Radioimmunoassay Team (director Dr. W. Hunter) and plasma TSH and hGH by the Regional Hormone Laboratory (director Dr. E. Cameron). Prolactin was measured by Drs. A. McNeilly and O. Thorner, St. Bartholomew's Hospital, London. Steroid assays were done in the MRC Unit of Reproductive Biology: androstenedione by Mr. I. Swanston, progesterone by Mrs. E. MacDonald, oestrone and testosterone by Mr. D. Davidson, and 17β-oestradiol and 17α-ethinyloestradiol by myself. Estimations of plasma glucose, 17-fluorogenic steroids and thyroxine were made in the Clinical Chemistry Department, RIE.

5.1 Urinary measurements

5.1.1 Total oestrogens

Urinary "total oestrogens" were measured by fluorimetry using the method described by Brown, MacLeod, Macnaughtan, Smith and Smyth (1968) with minor modifications.

Urine samples (usually $^1/1000$th of the total 24-hour volume) were diluted to 6 ml with distilled water and hydrolysed by boiling with concentrated hydrochloric acid for 30 minutes. The acid-hydrolysed urine was transferred to a semi-automatic Paton-Brown partitioning extractor (Paton Industries, Stepney, South Australia) and sodium chloride was added to improve the extraction of oestriol. The urine was then extracted with ether and the lower aqueous phase was discarded. The acidic fraction was subsequently removed by extracting the ether with carbonate solution (pH 10·5) which was discarded. After adding petroleum ether to improve the extraction of oestrone, the phenolic steroids were extracted with N sodium hydroxide and the upper organic phase, containing the neutral fraction, was discarded. Sodium bicarbonate was added to the alkali extract and the oestrogens were subsequently extracted with ether. The ether extract was evaporated and the oestrogens measured fluorimetrically by the Kober-Ittrich procedure using an Aminco-Bowman spectrophotofluorimeter (American Instrument Company, Inc., Maryland, U.S.A.) After correcting for aspecific fluorescence, "total oestrogen" concentrations were calculated by direct comparison with the corrected fluorescence reading of an oestriol standard and expressed in µg/24 hours. No correction for procedural losses was made.

The method measures oestriol and oestrone together with the minor Kober-chromogenic and alkali stable oestrogens, including oestradiol.

5.1.2 Pregnanediol

Urinary pregnanediol excretion was measured by a gas-liquid chromatographic method based on that described by Chamberlain and Contractor (1968).

Urine samples (usually 10 ml) were hydrolysed by boiling with concentrated hydrochloric acid in the presence of toluene. After cooling, the hydrolysed urine was transferred to the Paton-Brown partitioning extractor and the lower aqueous layer was discarded. The acidic and phenolic fractions were extracted from the toluene with 25% sodium chloride in N sodium hydroxide and were discarded. After washing with water, the toluene was evaporated to dryness and the dried extract acetylated with acetyl-chloride and benzene. The volatile components were removed by heating under nitrogen stream, cholesterol (20 µg in 0·2 ml of ethanol) was added as internal standard and 10 µl of this solution was used for chromatography on a Pye 104 series gas chromatograph (Pye and Co. Ltd., Cambridge). Pregnanediol concentrations were read from a calibration curve which was run with each assay, and were multiplied by the appropriate correction factors to give the 24-hour excretion value which was expressed in mg/24 h.

5.1.3 Gonadotrophins

Urinary FSH excretion was measured using the commercially available FSH-nosticon^R kit obtained from Organon Teknika, Oss, Holland. The assay, described by Schuurs and van Wijngaarden (1975), is based on a modified haemagglutination-inhibition reaction using sheep erythrocytes sensitized with hFSH of urinary origin and an antiserum raised in rabbits against the same gonadotrophin preparation. The assay procedure used was identical to that outlined in the instruction leaflet accompanying each kit, except for the fact that the sensitivity of the method (2 iu 2nd IRP-HMG per litre according to the makers) was checked for each new batch of reagents. Batches for which the limit of sensitivity did not conform to the indicated value of 2iu/l were discarded. Results were expressed in iu of the 2nd IRP-HMG standard per 24 hours. In normal women of reproductive age, urinary FSH excretion per 24 hours ranges from 2-10 iu during the pre-and postovulatory phases of the cycle, and from 8-26 iu round the time of ovulation.

Urinary LH excretion was measured using the Luteonosticon^R kit supplied by Organon Teknika, Oss, Holland, and which is based on a modified haemagglutination-inhibition test using hCG-coated sheep erythrocytes and an anti-hCG antiserum (Schuurs and van Wijngaarden, 1970). The assay procedure followed was identical to that described in the instruction manual supplied with the kit. The 2nd IRP-HMG preparation was used as the standard. The sensitivity of this method is 0·15 iu per tube or 25 iu per litre of urine, and this was regularly verified for each new batch of reagents. With this method, urinary LH excretion during the follicular and luteal phases of the cycle ranges between 20-100 iu/ 24 hours, and between 150-600 iu/ 24 hours at midcycle.

5.2 Plasma measurements

5.2.1 Pituitary hormones

5.2.1.1 Pituitary gonadotrophins

(a) Follicle-stimulating and luteinizing hormones

Plasma FSH and LH were measured by specific double antibody radioimmunoassays as described by Hunter, Edmond, Watson and McLean (1974).

The assays employed antisera raised in guinea pigs to the LH preparation DEAE-2 from Dr. Anne Stockell Hartree and to the crude FSH-containing fraction CM-1 from Dr. W. R. Butt. LH for iodination was preparation IRC-2 (Hartree) distributed in micro-ampoules 71/53 by MRC National Institute for Biological Standards and Control (NIBSC). FSH for iodination was fraction CPDS/6 from Dr. Butt. The standards used were, for the FSH assay, preparation MRC 68/39 (assuming a potency of 32·8 U/ ampoule), and for LH, preparation MRC 68/40 (assuming a potency of 77 U/ampoule) both from NIBSC. The concentrations of FSH and LH reported in this thesis are expressed as mU/ml plasma, where 1 mU LH = 11·6 ng LER 907 and 1 mU FSH = 44·6 ng LER 907. The within-assay precision was 11·8% for the FSH and 15·4% for the LH assay, and the respective values for between-assay precision using a pool of normal male plasma were 10·2% and 11·5%. Plasma FSH and LH levels as measured with these assays during the normal menstrual cycle are illustrated in Figure 1.2. Samples were run in duplicate, usually at 2 or more dilutions.

(b) Prolactin

Plasma prolactin was measured by a double antibody radioimmunoassay method employing highly purified human prolactin (supplied by Dr. H. Friesen) for standard and iodination, and an antiserum raised against an impure preparation of human prolactin. Details of the method can be found in McNeilly (1973) and McNeilly and Hagen (1974). Results were expressed in ng of Friesen's prolactin per ml of plasma (1 ng Friesen prolactin = 18·5 µU MRC prolactin standard A, 71/222, assuming 10 mU per ampoule). The assay has a sensitivity of 1·5 ng/ml and within- and between-assay coefficients of variation of 6 and 8% respectively. No significant cross-reactions were encountered with any of the other pituitary hormones. In normal women during the menstrual cycle, basal levels range between 3-17 ng/ml.

5.2.1.2 Other pituitary hormones

(a) Human growth hormone

Growth hormone levels in peripheral plasma were measured by radioimmunoassay using antiserum raised in rabbits and human growth hormone preparation MRC 66/217 (assuming 0·35 iu per ampoule) for iodination and as standard (Hunter, 1976). Separation was by double antibody technique. Samples were run in duplicate at three dilutions, and results are expressed in mU/ml of the aforementioned standard. Within- and between-assay coefficients of variation over the workable range of the standard curve (0·5 - 2·7 mU/ml) were both less than 10%.

(b) Thyroid-stimulating hormone

The radioimmunoassay of plasma TSH was carried out as described by Toft, Seth, Kirkham, Marshall and Irvine (1973) with minor modifications. The assay employed a hCG-absorbed antiserum raised in guinea-pigs, purified human TSH (preparation DE-32-3, Dr. A. Stockell Hartree) for iodination, and MRC human TSH preparation 68/38 (assuming 147 mU/ampoule) as standard. Separation of bound and free hormone was by double antibody technique. Samples were run in duplicate at two dilutions and results were expressed in μU/ml (normal range, mean ± SD: 3·8 ± 3·6 μU/ml). Within-and between-assay variation were less than 10%.

5.2.2 Steroid hormones

5.2.2.1 17β-Oestradiol assay

Using a highly specific 17β-oestradiol antiserum, a radioimmunoassay for the hormone which does not require a chromatographic step, was developed and validated.

(a) Solvents

Analytical grade chloroform, acetone, methanol, toluene, hexane and ethylacetate (BDH) and ethanol (Burroughs Ltd.) were used without further purification. Analytical grade diethylether (BDH) was washed with 50% ($^g/v$) ferrous sulphate (BDH) in 5% sulphuric acid (BDH) and distilled water, and redistilled within 24 hours before use.

The assay buffer was a 0·1 M phosphate buffer (pH 7) (8·6g anhydrous di-sodium hydrogen orthophosphate, BDH and 6·08g sodium-dihydrogen orthophosphate, BDH per litre distilled water) and contained 0·15M (9g/1) sodium chloride (Hopkin and Williams) and 0·1% gelatin (Sigma Chemical Company). Sodium azide (BDH) (0·1% w/v) was added to the buffer as a preservative. The assay buffer, henceforth referred to as PBSG, was stored at 4°C.

The dextran-charcoal mixture used for separation of free and antibody-bound hormone was prepared just prior to use by suspending 250 mg activated charcoal Norit A (Sigma Chemical Company) and 25 mg dextran T 70 (Pharmacia Fine Chemicals) in 100 ml of PBSG. The suspension was kept in ice-water and was continuously stirred while samples were being removed for the assay.

The scintillation fluid was prepared by dissolving 10g 2,5-diphenyloxazole (PPO, Nuclear Enterprises Ltd.) and 750 mg 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP, BDH) in 2·5 l of sulphur-free toluene (A. and J. Beveridge Ltd.), which was then added to 1·250 l of Triton X-100 (A. and J. Beveridge Ltd.) and mixed thoroughly until a homogeneous solution was obtained.

(b) Steroids

Non-radioactive 17β-oestradiol was purchased from Sigma Chemical Company and stored at 4°C as a 500 ng/ml stock solution in ethanol. Standard solutions of 1 and 10 ng/ml for use in the assay were prepared from this stock solution by further diluting with ethanol, and were also kept at 4°C.

Radioactive 17β-oestradiol (6,7-^{3}H-17β-oestradiol) with a specific activity of 45 to 50 Ci/mM was obtained from New England Nuclear and stored at 4°C. The tracer was further diluted with ethanol to obtain stock solutions containing approximately 10-20 µC/ml and 0·02 µC/ml for use in the assay and for determination of recovery respectively. At the time of the assay an aliquot of the former stock solution was dried down under nitrogen stream and redissolved in PBSG to give a solution containing approximately 15,000 dpm of 6,7-^{3}H-17β-oestradiol (i.e. 35-40 pg) per 0·1 ml of buffer.

The other non-radioactive steroids used in specificity studies were purchased from Sigma Chemical Company or Steraloids Inc. and were stored at 4°C in ethanol at concentrations ranging from 1 to 100 µg/ml.

(c) Materials

Four ml stoppered glass tubes (MF 24/0 Quickfit Ltd) were used for the extraction of plasma samples, and disposable glass test tubes (7·6 x 0·9 cm, GWS) for the assay. Organic solvents were evaporated on an electric Dri-Block DB-3 (Techne) with thermostatic control. Liquid scintillation counting was performed on a Packard Tri-carb 2450 (Packard) using glass counting vials of the low background type (Packard Instrument Company). In the system described the counting efficiency was ± 35%. Samples were counted for sufficient time (4 - 10 min) so that the counting error was less than 2%, and background (15 - 20 cpm) was subtracted automatically.

(d) Antiserum

Two antisera kindly provided by Drs. H. Lindner (antiserum L) and P. Dean (antiserum D) were used during the course of these studies. Both antisera were raised in rabbits against a 17β-oestradiol-6-(O-carboxymethyl)-oxime-bovine serum albumen conjugate. The preparation of the immunogen, the immunization-schedule employed and some of the characteristics of these antisera, particularly with respect to specificity, have previously been described (Lindner, Perel, Friedlander and Zeitlin, 1972; Dean, Exley and Johnson, 1971; Exley, Johnson and Dean, 1971). Aliquots of both antisera diluted with PBSG to 1/100 ($^v/v$) were kept frozen at -20°C until required.

Antiserum L was used during the initial stages of this work (until February 1975), but was later replaced by antiserum D after the supply had run out. It is extremely unlikely that this (compulsory) change of antiserum should bias the results reported here since both antisera were virtually similar in terms of specificity and gave similar results when the same plasma samples were assayed in both systems (see below).

(e) Extraction procedure

Replicate aliquots (usually 0·5 ml) of plasma or deionised water were extracted with 2 ml of diethylether by thoroughly mixing on a vortex shaker for 1 min. After separation, the lower aqueous layer was quick-frozen in a methanol-dry ice bath and the upper ether layer was decanted into a disposable glass assay tube. The ether was then evaporated to dryness under a stream of nitrogen and the dried residue was taken up in 0·1 ml of PBSG using a Repette^{R} (Chance Brother Ltd.) for dispensing the buffer. The tubes were mixed briefly on a vortex shaker and allowed to stand at room-temperature for at least 1 hour.

At least four separate plasma-aliquots to which 50 µl (2000 dpm) of the recovery-tracer stock solution was added, were extracted in the same way. However, after decanting and evaporating the ether, the residue was taken up in 0·3 ml of PBSG. The tubes were mixed and left at room-temperature until transferred with the other assay tubes to the frigidaire for overnight incubation at 4°C. Analysis of duplicate 0·1 ml aliquots of these samples provided an estimate of the mean procedural loss of the extraction procedure.

(f) Standard curve

Duplicate aliquots of the standard stock solutions of 17β-oestradiol (corresponding to 5, 10, 20, 30, 50, 100, 200 and 300 pg) or of the other steroids to be tested were pipetted with a Hamilton-syringe

(Micromeasure N.V.) into assay tubes and dried under nitrogen. The residue was taken up in 0·1 ml of PBSG, the tubes were mixed, left at room-temperature for at least one hour and further processed with the assay tubes containing the plasma extracts.

(g) Radioimmunoassay

For each assay an appropriate aliquot of the 1/100 antiserum dilution was further diluted with PBSG. Antiserum L was used at an initial dilution of 1/1,000 (final dilution 1/3,000) and antiserum D at an initial dilution of 1/7,000 (final dilution 1/21,000).

Using a Repette^R a 0·1 ml aliquot of the antibody solution was added to each incubation tube. The tubes were mixed and allowed to stand at room-temperature for 1 hour. One hundred µl of the tritiated 17β-oestradiol solution was then added and the tubes again shaken briefly and incubated overnight at 4°C. The next day, separation of bound and free hormone was performed at 4°C by the addition of 1 ml of the dextran-coated charcoal suspension. After mixing, the tubes were left to stand for 15 minutes (antiserum L) or 35 minutes (antiserum D) before centrifugation at 2,500 rpm for 15 minutes at 4°C on a Mistral 6L (MSE) centrifuge. The supernatant containing the antibody-bound steroid was then decanted into scintillation vials, 10 ml of scintillator was added and the contents mixed. The vials were allowed to equilibriate in the scintillation counter at 4°C for at least 1-2 hours prior to counting.

Apart from the standards and unknowns, quadruplicate tubes containing 0·2 ml of PBSG and 0·1 ml of tracer solution were run with each assay. Two of these were used as estimates of the average amount of radioactive 17β-oestradiol added to each tube, and to them 1 ml of

PBSG instead of 1 ml of dextran-charcoal was added. The other two served as estimates of the "non-specific binding" i.e. the amount of tracer present in the supernatant after charcoal separation in the absence of antibody.

Since in most instances 50 - 100 duplicate plasma samples were assayed at the same time, separation of antibody-bound and free hormone was performed in separate batches of 24 - 48 incubation tubes to minimize "stripping" effects (see below). To each batch, 2 buffer control or "zero" tubes containing 0·1 ml of PBSG and 0·1 ml of both antibody and tracer solution, were added to estimate the amount of tracer bound by the antibody in the absence of unlabelled steroid.

(h) Calculations

After subtraction of the non-specific binding, the amount of antibody-bound tracer (in cpm) in standards and unknowns was expressed as a percentage of the amount of antibody-bound tracer (in cpm) in the "zero" tubes which was taken as 100%. The concentration of 17β-oestradiol in aliquots of the unknown samples was then calculated by interpolation on the standard curve. After correcting for procedural losses, results were expressed in pg/ml.

(i) Results

Extraction procedure

Since the antisera used in the assay were specific enough to allow measurement of 17β-oestradiol in simple ether extracts of plasma without prior chromatography, procedural losses during preparation of the plasma samples were minimal and, more important, highly consistent. The reproducibility of the extraction procedure was further increased by careful timing of each extraction which was performed mechanically on a vortex mixer. In 42 consecutive extractions the mean recovery of tritiated 17β-oestradiol from plasma averaged 72·09 ± 0·75% (mean ± SEM; coefficient of variation, C.V. 6·87%). Since the extraction procedure was highly reproducible, recovery determinations on individual samples were omitted, and samples within each assay were corrected for procedural losses using a mean recovery estimate based on at least 4 separate samples to which recovery-tracer had been added. This simplification was perfectly valid since the variation in recovery estimates could largely be attributed to the inevitable errors associated with the required additional pipetting. Further proof for the validity of using a mean recovery estimate was obtained by comparing 17β-oestradiol levels as measured with the present assay with those obtained in a different radioimmunoassay system which required chromatography of the plasma extracts prior to assay and hence necessitated recovery determinations on individual samples (see below).

Selection of antibody dilution

For both antisera a titer-curve was constructed by incubating (overnight at 4°C) duplicate 0·1 ml aliquots of serially diluted antiserum (in PBSG) with 0·1 ml of tracer solution and 0·1 ml of either PBSG or nonradioactive ("cold") 17β-oestradiol in PBSG. Incubation tubes were centrifuged 15 minutes after adding the dextran-charcoal suspension.

In the absence of cold 17β-oestradiol, a 1/1,000 dilution of antiserum L bound approximately 60% of the added tracer, 70% of which was displaced by the addition of 250 pg cold 17β-oestradiol. This dilution was therefore chosen for use in the assay. Antiserum D could be used at a higher dilution and appeared to have a somewhat higher affinity since a 1/7,000 dilution bound 49% of the tracer in the absence of cold 17β-oestradiol, but only 11% (i.e. 77% displacement) when 200 pg 17β-oestradiol was added (Figure 5.1).

Antibody-titers were determined immediately after receipt of the antisera. The antisera were then diluted to 1/100 with PBSG and aliquots (0·5 or 2 ml depending on the titer) were pipetted into small glass vials which were kept frozen at -20°C. Before assay, one (or more) vial(s) were thawed and the contents further diluted with PBSG to the appropriate dilution. Remaining antiserum was stored at 4°C and used within one week or otherwise discarded. Using these precautions no appreciable loss of tracer-binding activity was encountered.

Effect of the dextran-charcoal suspension on the dissociation of antibody-antigen complex ("stripping")

The stripping-effect of the dextran-charcoal suspension on the antibody-antigen complex was evaluated by measuring the percentage of radioactive 17β-oestradiol remaining in the supernatant (i.e. antibody bound 17β-oestradiol) at various time intervals (1 - 60 min) after the addition of the dextran-charcoal suspension. The study was performed at 4°C and at 3 different concentrations of antibody-tracer complex: maximal tracer-binding (zero-tubes without cold 17β-oestradiol), intermediate (tubes containing 50 pg cold 17β-oestradiol) and absent tracer-binding (non-specific-binding tubes without antibody). The results obtained with antiserum D are illustrated in Figure 5.2. At all three concentrations of antibody-tracer complex, the percentage of tracer remaining in the supernatant decreased in a non-linear fashion as a function of time. The stripping-rate, i.e. the percent decrease in antibody-bound tracer per unit of time, was highest during the first 5 minutes (3·55%, 2·28% and 1·10% per minute at time 0 for 0,50 pg

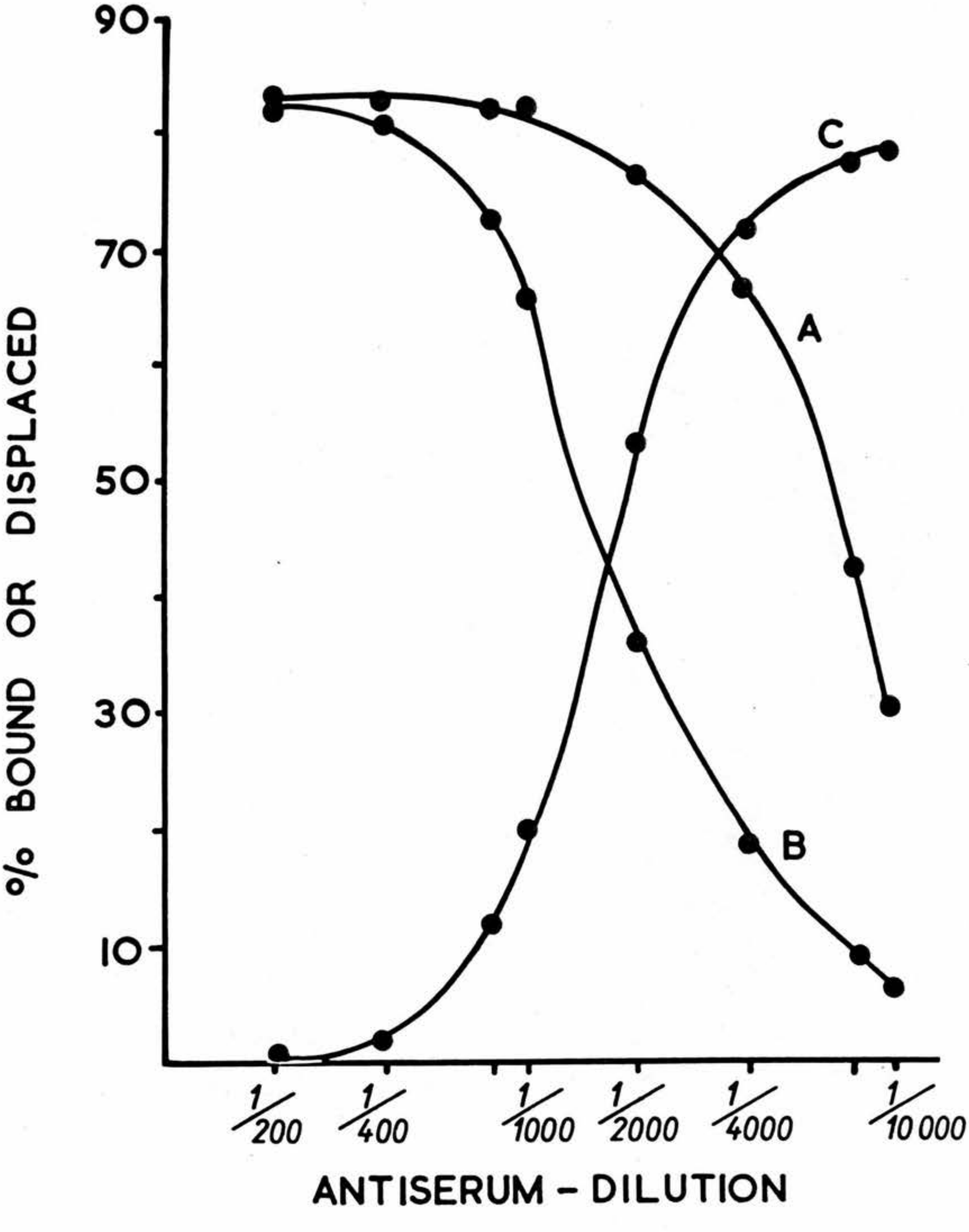


Figure 5.1: Titer- and displacement curves for antiserum D
A: titer-curve in the absence of non-radioactive 17β-oestradiol
B: titer-curve in the presence of 200 pg of non-radioactive 17β-oestradiol per tube
C: displacement curve ($\frac{A-B}{A} \times 100$)

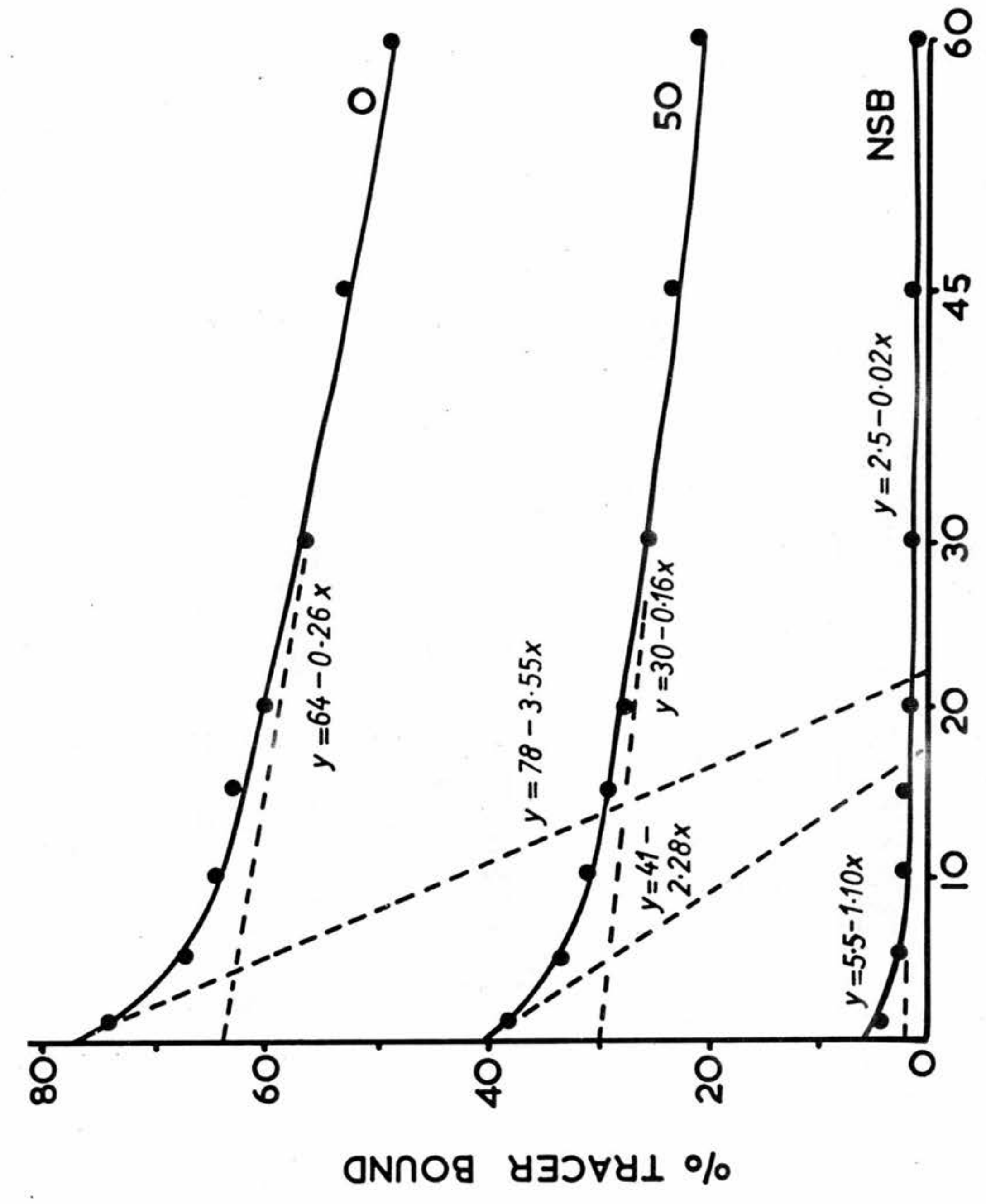


Figure 5.2: Effect of dextran-charcoal suspension on dissociation of antibody-tracer complex. Solid lines represent the percentage of antibody-bound tracer remaining in the supernatant of buffer control (0), 50 pg standards (50) and non specific binding (NSB) tubes. The slopes of the broken lines obtained by extrapolation, are an index of the stripping-rates at times zero and 30-60 min.

and NSB tubes respectively), but then gradually declined to become constant after approximately 30 minutes. From then onwards the percentage antibody-bound tracer remaining in the supernatant decreased at a constant rate of about 0·26, 0·16 and 0·02% per minute for 0, 50 pg and NSB tubes respectively. It is evident that in order to minimize this stripping effect as much as possible, centrifugation of the incubation tubes had to be delayed for at least 30 minutes after adding dextran-charcoal suspension. In all assays using antiserum D, tubes were therefore allowed to stand at 4° for 35 minutes before centrifugation.

The characteristics of the stripping curves for antiserum L were very similar to those of antiserum D. In the absence of cold 17β-oestradiol (zero tubes), the percentage of antibody-bound tracer decreased at a rate of 1·83% per minute during the first 5 minutes. However, after 5 minutes the rate of stripping declined very rapidly and became virtually constant at 0·35% per minute after 10 minutes. Accordingly, centrifugation was delayed for 15 minutes when using this antibody.

Effect of temperature and length of incubation on tracer-binding

In order to establish the optimum incubation time and temperature, the percentage of tracer bound to the antibody in the absence of cold hormone, was determined for various lengths of incubation (from 30 min to 24 hours) at 4°C and at room-temperature. At both temperatures, the antibody-tracer reaction reached equilibrium within 30 minutes and no significant alteration in binding was observed during the remainder of the observation period (24 hours). In practice, however, incubations were always performed overnight at 4°C.

Sensitivity of the assay and assessment of blanks

The means (± SD) of 20, respectively 30 consecutive standard curves obtained with antiserum L and D are illustrated in Figure 5.3. Results were plotted with mass of cold 17β-oestradiol on a logarithmic scale to see how closely they might approximate to straight lines. The amount of tracer bound in the absence of unlabelled steroid (56·8±5·2%, mean±SD, in the case of antiserum L and 50·6 ± 4·2% when using antiserum D) was taken as 100%.

The two standard curves were slightly sigmoid in shape but were virtually parallel straight lines over the largest portion of the mass range, suggesting that the specificity of both antisera was apparently directed against the same antigenic determinants. The limit of sensitivity, defined as the amount of unlabelled 17β-oestradiol required to displace 10% of the tracer bound in the zero tubes, was significantly lower for antiserum D (4·98 ± 1·02 pg/tube, mean ± SD) than for antiserum L (7·30 ± 1·66 pg/tube).

Several types of "blank" material were examined for possible effects on the antibody-tracer binding. Neither the dried residue of 100 µl of ethanol or that of 2 ml of diethylether had any significant, consistent effect on sensitivity or shape of the standard curves. Interference with the tracer-binding by non-specific plasma factors present in the crude diethylether extracts was excluded by comparing 17β-oestradiol levels in chromatographed and unchromatographed extracts (see below) and by measuring 17β-oestradiol in ether extracts of different volumes of the same plasma pool. In the latter study, which was performed on 2 separate occasions, duplicate aliquots of 0·25, 0·50, 0·75 and 1·00 ml of a female plasma pool were extracted with 4 volumes of ether and 17β-oestradiol measured in the crude dried

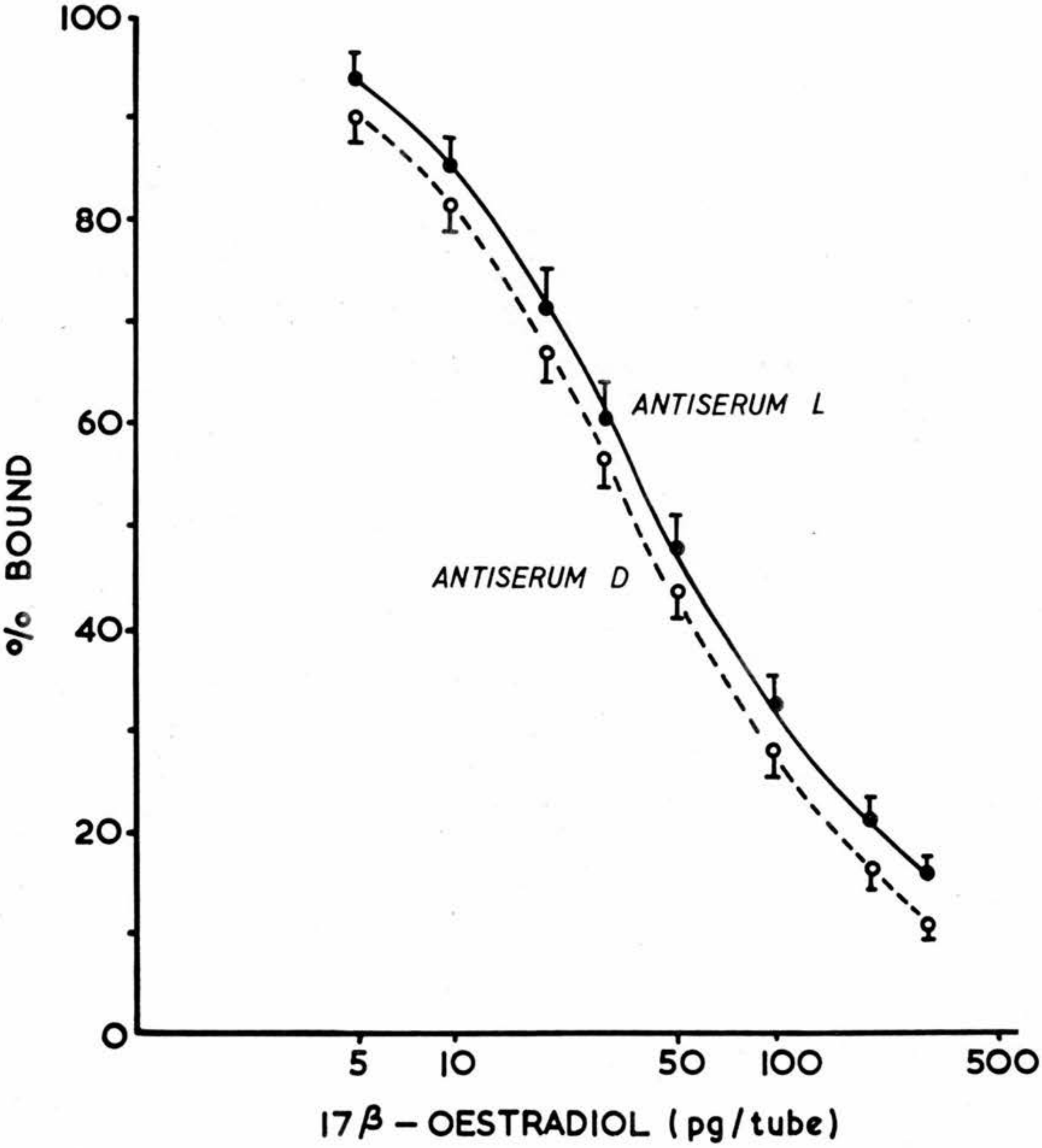


Figure 5.3: Standard curves for 17β-oestradiol using 2 different antisera. Illustrated are means and SD (n = 20 for antiserum L and n = 30 for antiserum D)

extracts. As shown in Table 5·1, the volume of plasma extracted did not significantly influence the results, which fell within the range expected on the basis of inter-assay variability.

Table 5.1: 17β-Oestradiol levels in ether extracts of different volumes of the same plasma pool

VOLUME EXTRACTED (ml)	AMOUNT FOUND* (pg/ml)
1·00	135·0 ± 6·4
0·75	115·0 ± 9·1
0·50	115·3 ± 7·7
0·25	124·0 ± 5·5
All volumes	122·3 ± 4·7

*mean ± SEM (n = 4)

Deionised water was used routinely to evaluate the method blank of the assay. The tracer-displacing activity present in water, when handled as a sample, was always well below the limit of detection, and usually within the 95% confidence interval of the zero tubes except on one occasion when samples, unrelated to the studies reported in this thesis, were assayed. Likewise, no tracer-displacing activity could be detected in 0·5 ml aliquots of peripheral plasma obtained from women using the combined contraceptive pill.

Specificity

Specificity of the antisera was tested by examining the relative potency of a wide variety of steroids in this assay. Results are summarized in Table 5.2. No cross-reactivity was encountered with any of the non-phenolic steroids tested, and, more important, none of these steroids showed any significant tracer-displacing activity when present in concentrations such as may be found under physiological conditions.

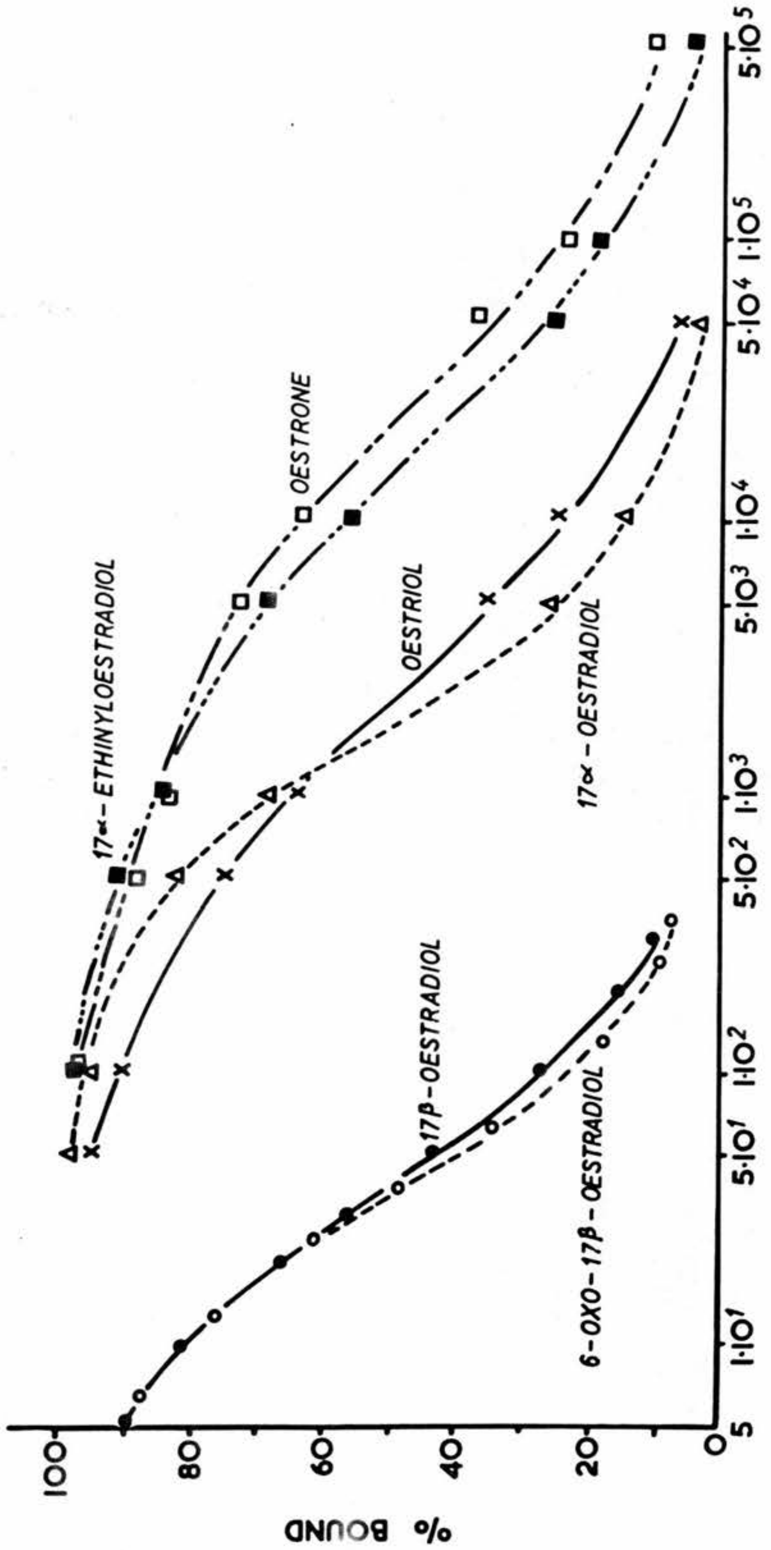
As anticipated 6-oxo-17β-oestradiol, the hapten used for generating the antibodies, showed complete competition with 17β-oestradiol for the antibody-binding sites. The naturally occurring oestrogens, oestrone and oestriol, however showed only minor cross-reactivity and, as illustrated in Figure 5.4, did not cause a significant decrease in tracer-binding at physiological concentrations. It is of interest to note that in contrast to 6-oxo-17β-oestradiol and 17α-oestradiol, the inhibition curves obtained with oestrone and oestriol were not parallel to the 17β-oestradiol standard curve. This would suggest that the latter two steroids probably competed with the tracer for different antigen-binding sites (or different antibodies) than those involved in the specific binding of 17 β-oestradiol, its 6-oxo-derivative or of 17α -oestradiol. As indicated in Table 5.2 and Figure 5.4, 17α-ethinyloestradiol, the synthetic oestrogen employed in the clinical studies on positive feedback, showed only weak cross-reactivity, and at the concentrations employed in the clinical work, was unlikely to interfere to any significant extent with 17β-oestradiol measurements.

Additional evidence of specificity was obtained by measuring 17β-oestradiol levels in chromatographed and unchromatographed ether extracts of plasma samples collected throughout a normal menstrual cycle.

Table 5.2: Specificity of 17β-oestradiol antisera

STEROID TESTED	CROSS REACTION (%)*	
	ANTISERUM L	ANTISERUM D
17β-Oestradiol	100·0	100·0
6-oxo-17β-Oestradiol	94·8	106·0
17α-Oestradiol	2·1	2·0
Oestriol	1·5	1·7
Oestrone	0·3	0·2
17α-Ethinyloestradiol	0·03	0·3
Diethylstilboestrol	< 0·002	< 0·002
Progesterone	< 0·002	< 0·002
Pregnenolone		
Pregnanediol		
Testosterone	< 0·002	< 0·002
Androstenedione		
Dehydroepiandrosterone		
Cholesterol	< 0·002	< 0·002
Cortisol		
Corticosterone		

*Defined, according to Abraham, Odell, Edwards and Purdy (1970) as $\frac{x}{y}$ x 100 where x is the mass of unlabelled 17β-oestradiol and y the mass of the heterologeous compound to produce 50% inhibition of the binding of 35 pg tritiated 17β-oestradiol by the antibody.



<u>Figure 5.4</u>: Cross-reactivity of oestrogens in the radioimmunoassay for 17β-oestradiol (antiserum D)

Chromatography was performed on Celite-colums as described by Abraham, Tulchinsky and Korenman (1970). This system effectively separates the three naturally occurring oestrogens, the only known contaminant of the oestradiol-fraction being 17α-oestradiol. Following chromatography, oestradiol concentrations were measured with an antiserum raised against a 17β-oestradiol-17-hemisuccinate-BSA conjugate (Baird, Burger, Heavon-Jones and Scaramuzzi, 1974), and the results were corrected individually for procedural losses using the percentage recovery in the column eluates of 2,000 dpm of tritiated 17β-oestradiol which had been added to the samples before extraction. The unchromatographed extracts were assayed using antiserum L. Linear regression analysis of the results (Figure 5.5) revealed a highly significant correlation ($r = 0{\cdot}9931$, $n = 20$, $p < 0{\cdot}001$) with a regression coefficient (1·01) close to unity and intercept with y-axis (0·99) not significantly different from zero, thus adding further proof that (a) the assay was specific for 17β-oestradiol and (b) that non-specific factors present in the crude ether extracts did not interfere with the antibody-hapten reaction.

Good agreement also existed between results obtained with antisera L and D when the same plasma samples were measured ($y = -0{\cdot}05 + 0{\cdot}968\,x$, where x is the value found with antiserum L and y that obtained with antiserum D; $r = 0{\cdot}9964$; $n = 10$; $p < 0{\cdot}001$).

Accuracy and precision

The accuracy of this radioimmunoassay system was checked by adding known amounts of 17β-oestradiol (from 5-200 pg) to duplicate 0·5 ml aliquots taken from a female plasma pool which had a mean concentration of 120 pg 17β-oestradiol per ml. Agreement between the

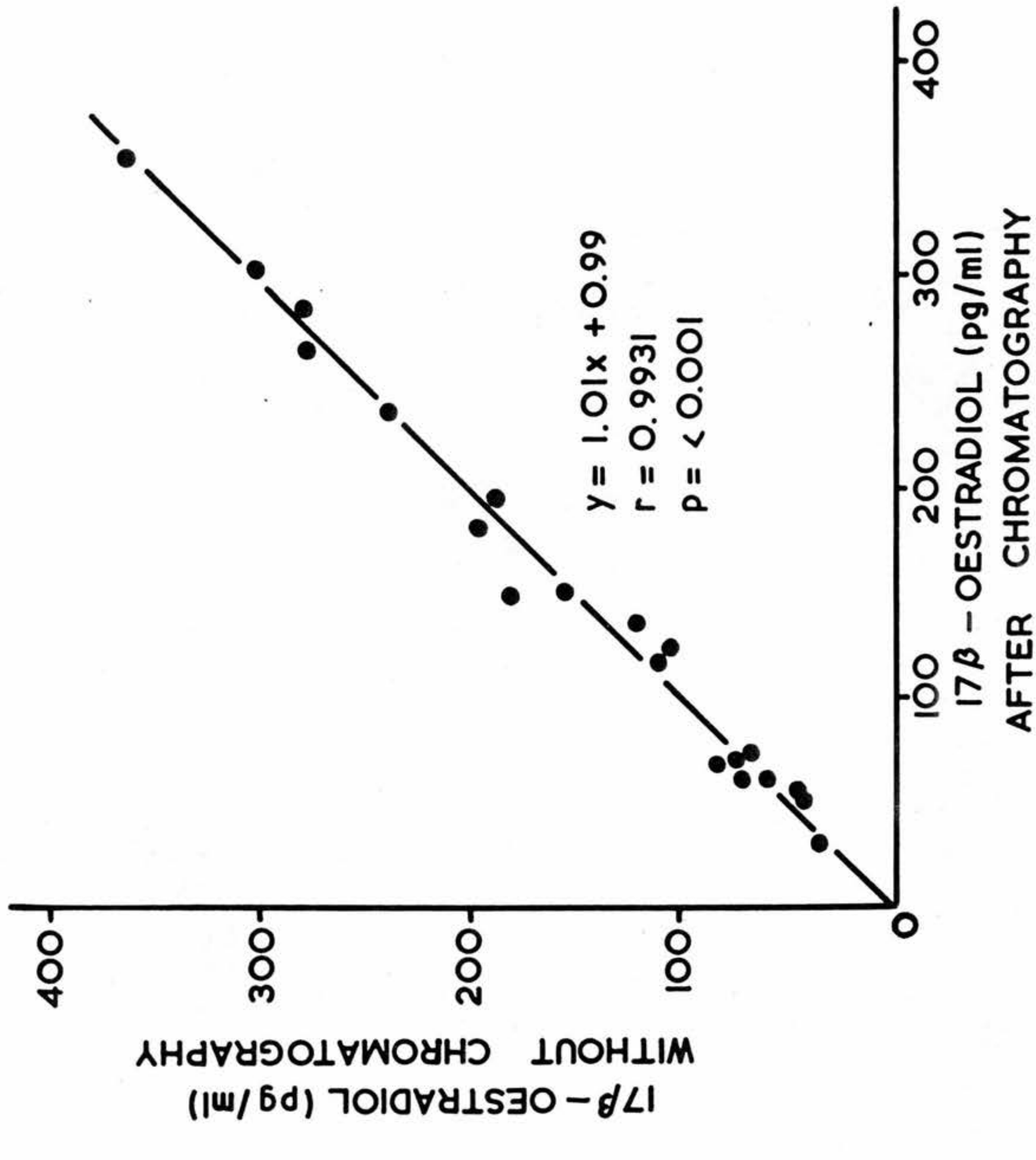


Figure 5.5: Comparison of 17 β-oestradiol levels obtained with (x-axis) or without (y-axis) chromatography of ether extracts of the same plasma samples.

amount of 17β-oestradiol added and that recovered (Table 5.3) was good with correlation coefficient of 0·9947 ($p < 0{\cdot}001$), regression coefficient of 0·9759 and y-intercept of 3·5 pg/ml.

The between-assay precision of the method was evaluated by including aliquots of several plasma pools in a number of different assays (Table 5.4). A total of six different pools with concentrations ranging from 28 to 405 pg/ml were used during the course of these studies, and aliquots from at least 2 pools were run with each assay. Three of the pools were used with both antiserum D and L. As shown in Table 5.4 estimates obtained with the two different antisera did not differ significantly. Between-assay precision, expressed as coefficient of variation, ranged from 6·1 to 13·3% and tended to be somewhat lower when using antiserum D.

Within-assay precision expressed as the coefficient of variation of the difference between duplicate measurements of 71 plasma samples with 17β-oestradiol concentrations ranging from 30-400 pg/ml

$$CV = \sqrt{\frac{\Sigma (\frac{d}{\bar{x}} \times 100)^2}{2n}}$$

(where d = difference between duplicate measurements, $\bar{x}$ = mean of duplicate measurements and n = number of duplicate pairs) was 9·3%.

(j) Discussion

Following the demonstration that steroid hormones could be made antigenic by coupling to a peptide carrier and could thus be used for the generation of antibodies (see e.g. Thorneycroft, Tillson, Abraham, Scaramuzzi and Caldwell, 1970 for review), a number of radioimmunoassays for the measurement of 17β-oestradiol in biological fluids have

Table 5.3: Recovery of known amounts of 17β-oestradiol added to 0·5 ml aliquots of a female plasma pool. Values are the means of duplicate measurements

MASS ADDED (pg)	MASS FOUND (pg/0·5ml)	MASS RECOVERED (pg)	RECOVERY (%)
0	63	0	
5	68	5	100
10	75	12	120
20	84	21	105
50	114	51	102
100	179	116	116
200	255	192	96

TABLE 5.4: Between-assay precision. Values given are the means ± S.E.M. in pg/ml and, between brackets, the number of observations and coefficient of variation (in %).

	ANTISERUM L	ANTISERUM D	ALL ESTIMATES
POOL A	35·9 ± 1·7 (8; 13·2)	38·0 ± 0·7* (11; 6·1)	37·1 ± 0·8 (19; 9·7)
POOL B	51·6 ± 2·0 (12; 13·3)	50·0 ± 1·1* (18; 9·5)	50·6 ± 1·0 (30; 11·1)
POOL C	416·1 ± 8·8 (18; 9·0)	396·9 ± 5·3* (27; 6·9)	404·6 ± 4·9 (45; 8·1)
POOL D	70·0 ± 2·5 (12; 12·1)	-	70·0 ± 2·5 (12; 12·1)
POOL E	-	28·0 ± 0·7 (22; 11·9)	28·0 ± 0·7 (22; 11·9)
POOL F	-	239·6 ± 6·4 (14; 10·0)	239·6 ± 6·4 (14; 10·0)

* values not significantly different from those obtained with antiserum L.

been described. In earlier studies (e.g. Ferin, Zimmering, Lieberman and Vande Wiele, 1968; Midgley and Niswender, 1970; Thorneycroft et al. 1970), coupling of 17β-oestradiol to the carrier protein was invariably made through one of its functional groups in the A or D ring (usually position 3 or 17). As a result, these groups were masked or modified and the produced antisera failed to discriminate between structurally related compounds which differed only in composition or orientation of the functional group through which the linkage had been made. In the case of 17β-oestradiol, this implied that antibodies raised against a 17β-oestradiol-17-hemisuccinyl conjugate showed considerable cross-reactivity with oestrone, and necessitated the use of various chromatographic procedures prior to the actual radioimmunoassay if reliable estimates were to be obtained (Abraham, Odell, Edwards and Purdy, 1970).

Speculation that more specific antisera might be produced if coupling to the protein was effected at a site which left the structurally unique regions of the A and D ring available for recognition by the antibody subsequently led to the preparation of antisera to conjugates in which the carrier protein was linked to 17β-oestradiol through carbon 6 of the B-ring. This new approach proved successful and several reports dealing with the preparation of the conjugate and the characteristics of the produced antisera appeared (Exley, Johnson andDean, 1971; Jeffcoate and Searle, 1972; Lindner, Perel, Friedlander and Zeitlin, 1972). These authors however were mainly concerned with the question of specificity of such antisera and little with their practical application to the measurement of 17β-oestradiol in biological fluids. That this was nevertheless feasible was suggested by preliminary observations of Cameron and Jones (1972) although the latter authors pointed out that in order to obtain accurate results with these antisera chromatography of the plasma extracts was required.

In the present study, two antisera raised against 17β-oestradiol-6-O-(carboxymethyl)-oxime bovine serum albumin conjugate and kindly provided by Drs. H. Lindner and P. Dean, were used to develop and validate a radioimmunoassay for 17β-oestradiol which has been successfully applied to the measurement of this hormone in a variety of body fluids including peripheral, ovarian and testicular vein plasma, and follicular and peritoneal fluid.

The earlier work on the specificity of these antisera (Lindner et al. 1972; Exley et al., 1971) which was confirmed by our own observations, had demonstrated that cross-reactivity with the other naturally occurring oestrogens was minimal, suggesting that the use of such an antiserum in radioimmunoassays may obviate the need for chromatographic separation of the extracted plasma samples. For this to be true, however, it is essential (a) that the procedure used in the preparation of the samples for assay does not give rise to the presence of "blank" material, which otherwise would have been removed by the chromatographic step, and (b) that non-specific factors present in the crude organic plasma extracts do not interfere with the binding of the ligand by the antibodies. Also, conclusions about the specificity of an antiserum based on determinations of cross-reactivity of related steroids, may not necessarily hold true when the antiserum is tested under actual assay conditions. Indeed, cross-reacting steroids often give inhibition curves which are not parallel to the standard curve obtained with the hormone to which the antiserum has been raised (see e.g. the inhibition curves of oestrone and oestriol in Figure 5.4). An apparently insignificant percentage cross-reaction, measured at the 50% tracer-displacement level, does therefore not always guarantee that low concentrations of the cross-reacting steroid will not show any degree of tracer-displacing activity.

However, the experiments described in the foregoing pages appear to indicate that the radioimmunoassay developed for the measurement of 17β-oestradiol is specific for this hormone and does not require chromatographic separation of the extracted plasma samples. This conclusion is based on the following observations. Firstly, the blank of the method which was evaluated by using deionised water as well as peripheral plasma from women using combined oral contraception, was always well below the limit of detection of the assay, and in most instances, the percentage of tracer bound in the tubes containing the blanks was not significantly different from that in the zero tubes. For this to be achieved,it was found essential to repurify and redistill the diethylether used for the extraction procedure. Secondly, the antibody-tracer reaction was not affected by non-specific plasma factors since neither changes in the amount of inert material present (Table 5.1) nor the removal of this material by chromatography (Figure 5.5) had any detectable effect on the results. Finally, steroids which are structurally related to 17β-oestradiol do not interfere in the assay since (a) none of these steroids showed any significant tracer-displacing activity when present in concentrations likely to be encountered in physiological circumstances (Figure 5.4), and (b) similar results were obtained when plasma samples were assayed with or without chromatographic separation of these steroids (Figure 5.5).

We could therefore not confirm the observations of England, Niswender and Midgley (1974) that 17β-oestradiol levels as measured with an antiserum raised against a 6-BSA derivative of the hormone, were consistently lower when the extracted samples were subjected to preparatory Sephadex LH-20 chromatography. These workers however provided no details as to the specificity of their antiserum, and any direct comparison of our results is hence not possible. England and his colleagues suggested that the discrepancy in their results between chromatographed and nonchromatographed samples may have arisen from the presence of 6-substituted derivatives of 17β-oestradiol which can be expected to exhibit marked cross-reactivity with the antiserum. The existence of such derivatives has been demonstrated in human pregnancy urine (Diczfalusy and Mancuso, 1969) but their relative importance appears to be rather small and little information is available on their concentration in peripheral plasma during the normal menstrual cycle. 6-Oxo-17β-oestradiol, the metabolite which is structurally most similar to the hapten, is unlikely to be of any significance since peripheral plasma levels of this hormone are undetectable (less than 1·2 pg/ml) in men and women during the luteal phase of the cycle (Bolton and Rutherford, 1976).

Apart from being specific, the present assay also seems to satisfy the criteria of accuracy since 17β-oestradiol can be recovered quantitatively when known amounts are added to plasma (Table 5.3). Our results are thus at variance with those obtained by Cameron and Jones (1972) who, using a similar antiserum, could not achieve accurate recovery of added 17β-oestradiol unless chromatography was employed. The discrepancy is probably related to the fact that the dried diethyl-ether residue interfered with the binding of labelled 17β-oestradiol to the antiserum used by Cameron and Jones, a finding which we did not observe in our assay.

The unique properties of these antisera with respect to specificity and the absence of interference by non-specific "blank" material made it possible to employ a simple but efficient extraction procedure which, by virtue of its simplicity, was extremely reliable. As a result, recovery-determinations on individual samples could be omitted without any appreciable loss of precision as evidenced by the excellent agreement between results obtained with the present method and those of an established assay involving individual recovery estimations (Figure 5.5). The fact that within and between-assay precision (Table 5.4) are comparable to those of recently reported methods using similar antisera but individual recovery-determinations (see e.g. Korenman, Stevens, Carpenter, Robb, Niswender and Sherman, 1974) adds further proof to our belief that the omission of the recovery-estimations on individual samples did not adversely affect the precision of our assay.

In summary, the method described appears to give an accurate, sensitive and precise estimate of 17β-oestradiol concentrations in biological fluids, and is sufficiently specific to allow the use of a simple diethylether extract of plasma without subsequent chromatography.

5.2.2.2 17α-Ethinyloestradiol assay

Using a specific antiserum to 17α-ethinyloestradiol, a radioimmunoassay for the measurement of this synthetic oestrogen in peripheral venous plasma, was developed and validated.

(a) Solvent and materials

The solvents and materials employed were similar to those described for the radioimmunoassay of 17β-oestradiol, except for the charcoal-suspension which contained 500 mg of activated charcoal per 100 ml of PBSG.

(b) Steroids

Unlabelled 17α-ethinyloestradiol was obtained from Steraloids, Inc. and was stored at 4°C in ethanol at concentrations of 100 ng/ml (stock solution) and 10 and 1 ng/ml (standard solutions for use in the assay).

6,7-^{3}H-17α-Ethinyloestradiol with a specific activity of 45 Curies/mM was purchased from New England Nuclear and stored at 4°C in benzene:ethanol (9:1, $^v/v$). After checking the radiochemical purity by thin-layer chromatography on Silica gel in the system benzene: ethanol (9:1, $^v/v$), an aliquot was further diluted with ethanol to obtain a stock solution of approximately 20 µC/ml, which was kept at 4°C.

(c) Antiserum

The antiserum, provided by Dr. R. Nieuweboer, was produced in a rabbit after immunization with 17α-ethinyloestradiol coupled to bovine serum albumin at position 6 of the steroid molecule. It was stored under conditions similar to those described for the 17β-oestradiol antisera. Titer and displacement curves (Figure 5.6) performed upon receipt, showed that the antiserum, when used at an initial dilution of 1/4,000 (final dilution 1/12,000), would bind approximately 35% of 15,000 dpm tritiated 17α-ethinyloestradiol (i.e. approximately 35-40 pg).

(d) Extraction procedure and standard curve

The extraction method was basically similar to that outlined for the 17β-oestradiol assay, except on the following points. The extraction with diethylether was performed on duplicate 0·2 - 0·5 ml aliquots of the plasma sample to be assayed. Volumes which were smaller

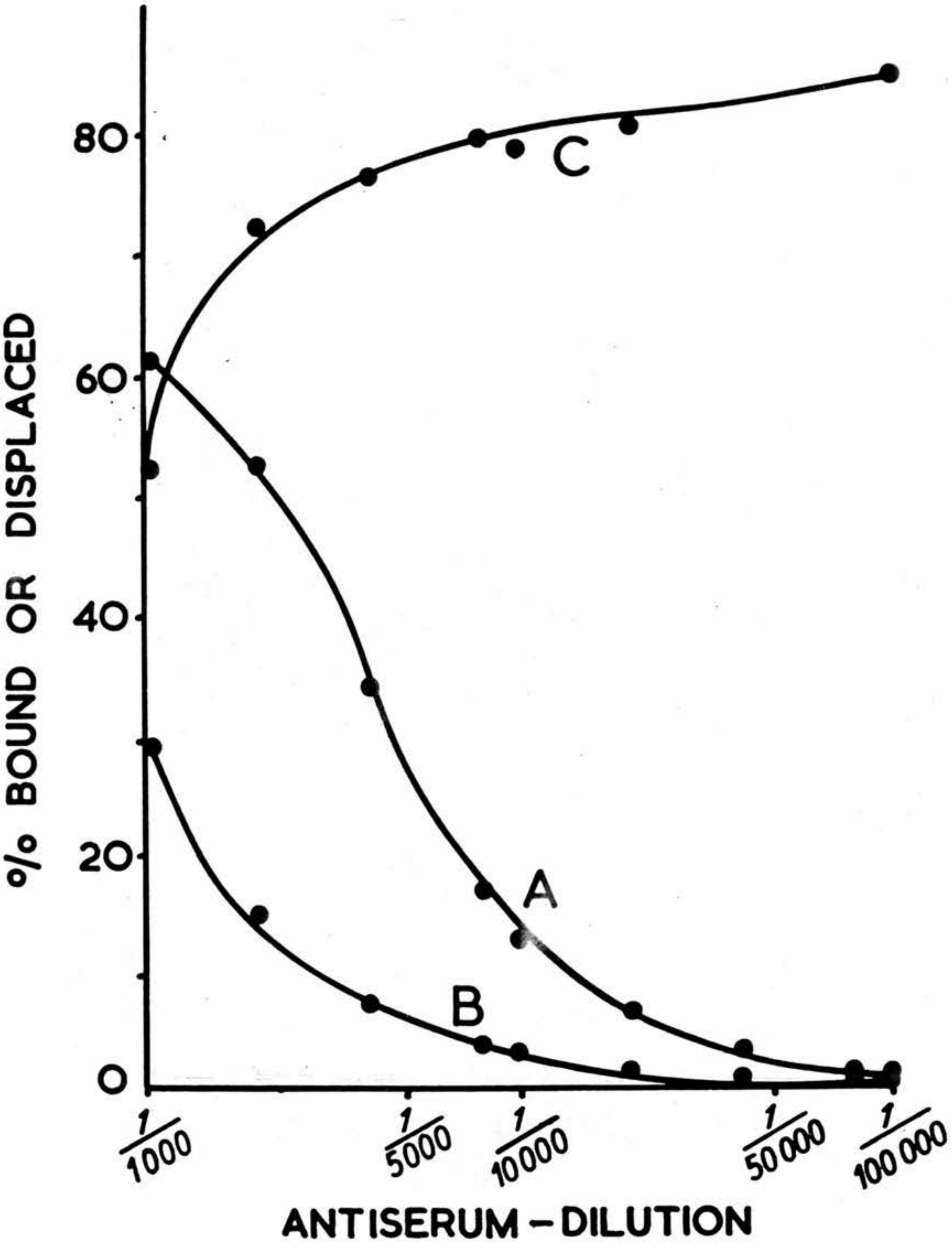


Figure 5.6: Titer and displacement curves for 17α-ethinyloestradiol antiserum

A: titer curve in the absence of unlabelled 17α-ethinyloestradiol

B: titer curve in the presence of 200 pg of unlabelled 17α-ethinyloestradiol

C: displacement-curve $(= \frac{A-B}{A} \times 100)$

than 0·5 ml were made up to 0·5 ml with blank plasma from a male plasma pool. Standard amounts of unlabelled 17α-ethinyloestradiol (corresponding to 0,5, 10, 25, 50, 100 and 200 pg) were added to duplicate 0·5 ml aliquots of the same pool. The extraction tubes containing the standards were mixed briefly and allowed to equilibrate for 1 hour at room temperature (or, alternatively, overnight at 4°C). Unknowns and standards were then extracted with 2 ml of redistilled diethylether and the extracts prepared for assay as described for the 17β-oestradiol assay.

(e) Radioimmunoassay

The assay procedure was identical to that outlined for 17β-oestradiol. The antibody was used at an initial dilution of 1/4,000 and the labelled tracer-solution contained 15,000 dpm of tritiated 17α-ethinyloestradiol per 0·1 ml of PBSG. After an overnight incubation at 4°C, bound and free hormone were separated with 1 ml of a 0·5% ($^{W}/v$) charcoal suspension in PBSG, and the tubes were left to stand for 35 minutes before centrifugation at 2,500 rpm for 15 minutes. 17α-Ethinyloestradiol concentrations in the unknown plasma samples were calculated as described for the 17β-oestradiol assay but no correction for procedural losses was made.

(f) Results

Effect of time and temperature of incubation, stripping-effect

After having selected the antibody-dilution (1/4,000; Figure 5.6) and "stripping-time" (35 minutes) according to the principles outlined for 17β-oestradiol, zero tubes containing 0·1 ml PBSG and 0·1 ml of both antiserum and tracer-solution were incubated at 4°C and at room-temperature for various lengths of time in order to establish the optimum incubation conditions. At both temperatures, maximum binding of the tracer was achieved within 2 hours, but, in practice, incubations were always performed overnight at 4°C for convenience.

Specificity

A number of steroids most likely to interfere in the assay was tested for their relative tracer-displacing activity. As illustrated in Table 5.5 and Figure 5·7, the antiserum proved to be highly specific and none of the steroids tested showed any significant degree of tracer-displacing activity when present in physiological concentrations. Specificity of the assay was further checked by testing for parallelism between the dose-response curves of the standard and of dilutions of an unknown sample. For this purpose, quadruplicate aliquots (0·1 - 0·5 ml) of a plasma pool obtained from a woman treated with ethinyloestradiol (200 µg a day) were made up to 0·5 ml with blank plasma and assayed for 17α-ethinyloestradiol content as described. The results, shown in Figure 5.8 further confirmed that the assay was specific for 17α-ethinyloestradiol since both standard and endogenous hormone reacted in the same way with the antibody as illustrated by their parallel inhibition curves.

Sensitivity of the assay and assessment of blanks

The mean (± SD) of 4 consecutive standard curves is shown in Figure 5.8. In the absence of unlabelled hormone the antibody bound 38·1 ± 0·8 (SD)% of the added tracer (15,000 dpm, i.e. 30 - 40 pg) and this was taken as 100%. Plotted on a semi-logarithmic scale, as in Figure 5.8, the dose-response curve was virtually linear over the range between 25 to 200 pg and the variation at each point of the standard curve, expressed as the coefficient of variation, was less than 5%.

TABLE 5.5: Specificity of 17α-ethinyloestradiol antiserum

STEROID TESTED	CROSS REACTION (%) *
17α-Ethinyloestradiol	100·0
17β-Oestradiol	0·2
Oestriol	0·03
Oestrone	$<$ 0·006
17α-Oestradiol	0·007
Progesterone Pregnenolone Pregnanediol	$<$ 0·001
Testosterone Androstenedione Dehydroepiandrosterone	$<$ 0·001
Cortisol Corticosterone Cholesterol	$<$ 0·001

* Defined as in Table 5.2

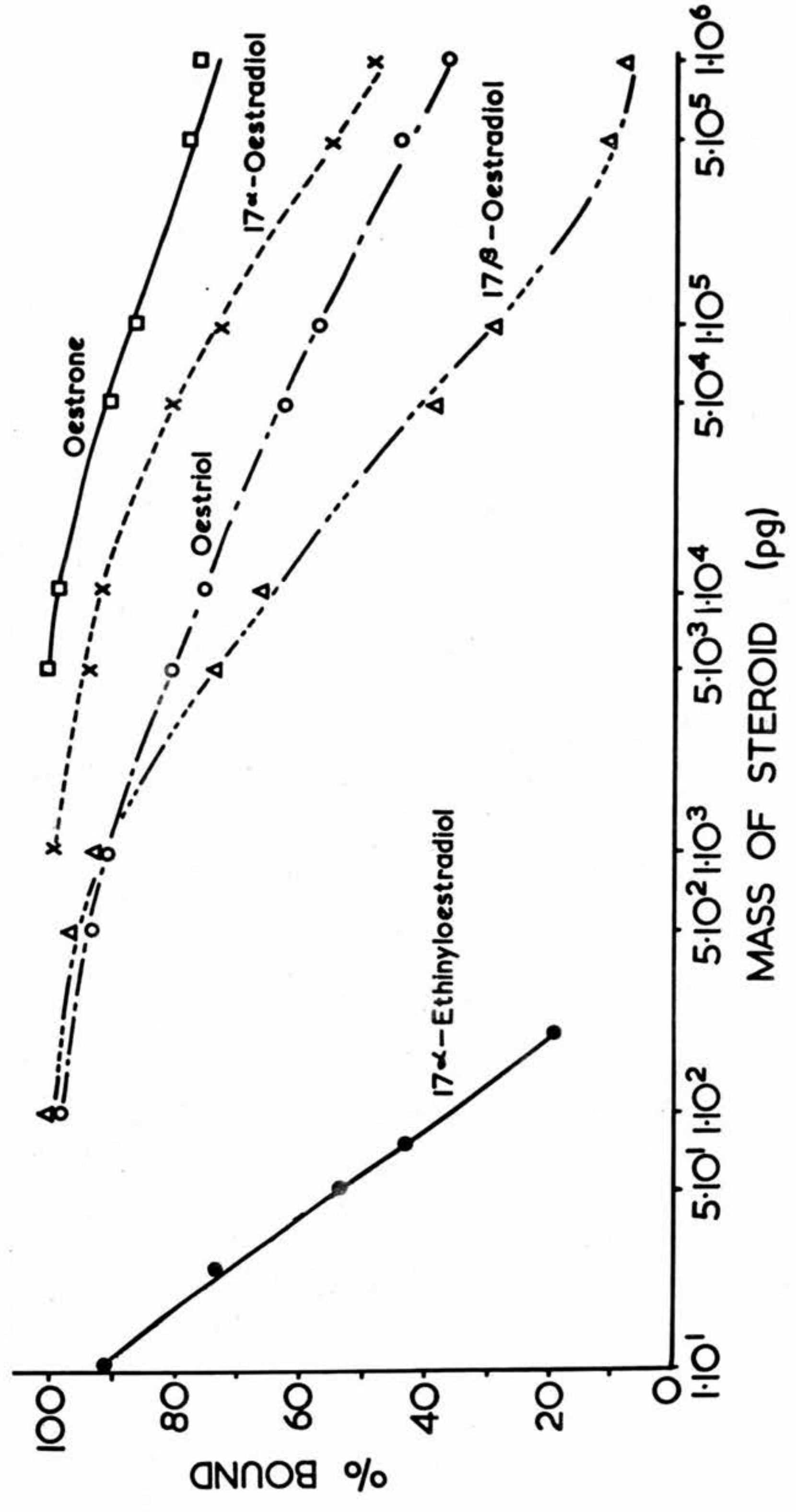


FIGURE 5.7: Cross-reactivity of oestrogens in the radioimmunoassay for 17α-ethinyloestradiol

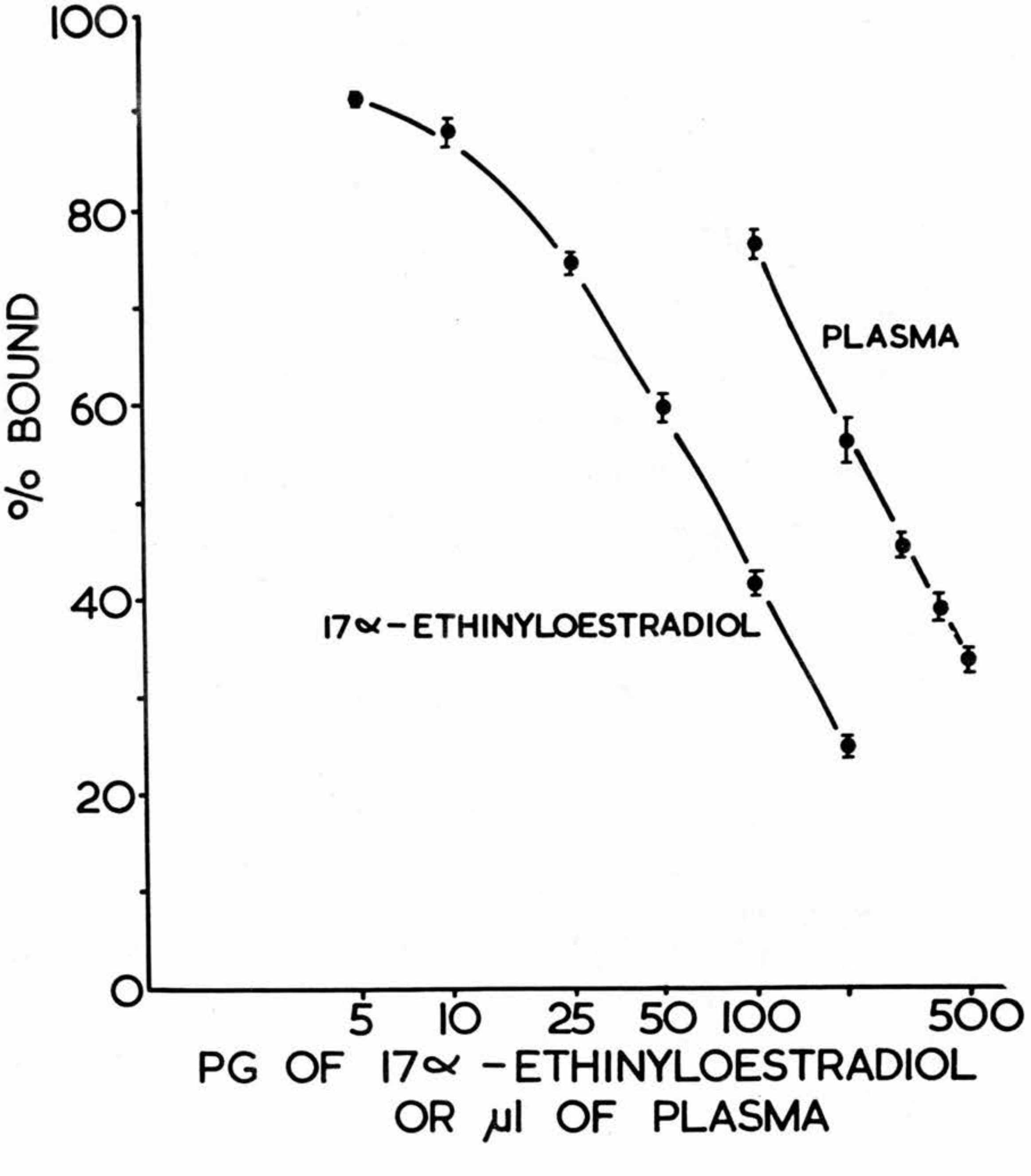


FIGURE 5.8: Dose response curves of 17α-ethinyloestradiol standard and of serial dilutions of an unknown plasma sample. Each point represents the mean (± S.E.M.) of 8 (standard) or 4 (plasma) measurements.

The sensitivity of the assay, defined as the amount of unlabelled hormone required to displace 10% of the tracer bound in the zero tubes, was 8·5 ± 2·5 (SD) pg, and the blank of the method using peripheral venous plasma of subjects not taking ethinyloestradiol was always zero.

Accuracy and precision

The accuracy of the method, evaluated by adding known amounts of 17α-ethinyloestradiol to aliquots of a blank plasma pool, which were then assayed in the usual way, was considered to be satisfactory since the added hormone could be quantitatively recovered ($y = 1{\cdot}03x - 2{\cdot}8$ where y is the amount of 17α-ethinyloestradiol recovered and x the amount which was added; $x = 0{\cdot}9983$; $p < 0{\cdot}001$). The within-assay precision, expressed as the coefficient of variation of the difference between duplicate estimates was 8·8% ($n = 31$), and the between-assay precision was 9·8% for a plasma pool with a mean 17α-ethinyloestradiol concentration of 46·4 pg/ml.

(g) Discussion

Since in our clinical studies on positive feedback orally administered ethinyloestradiol was used to induce pituitary LH release, it seemed not inconceivable that differences between individuals in the degree of gastro-intestinal absorption or in the rate of metabolism of this compound could give rise to false negative results in the oestrogen provocation test (i.e. failures to release LH not due to hypothalamic-pituitary dysfunction but to an insufficient strength of the provided oestrogen stimulus). In order to exclude this possibility, a radioimmunoassay was developed which permitted the measurement of circulating unconjugated 17α-ethinyloestradiol in peripheral venous plasma.

Because the antiserum was raised to a BSA-conjugate coupled to the hormone through carbon atom 6 of the B-ring, thus leaving the functional groups of the A and D ring unmasked for antibody recognition, a high degree of specificity could be expected. That this was the case is illustrated by the negligible cross-reactivity of any of the other structurally related steroid hormones (Table 5.5). Moreover, since none of the cross-reacting steroids showed any significant degree of tracer-displacing activity at physiological concentrations (Figure 5.7), it seemed likely that the high specificity of the antiserum would permit the measurement of 17α-ethinyloestradiol levels directly in simple ether extracts of plasma without prior chromatography. Under these conditions, the only steroids which might interfere with the assay would be 6 substituted unconjugated metabolites, but previous <u>in-vitro</u> work on the metabolism of 17α-ethinyloestradiol by human liver tissue had shown that such derivatives are of minor importance (Bolt, Kappus and Käsbohrer, 1974). Sulphate conjugates, the principal circulating form of 17α-ethinyloestradiol (Reed, Fotherby and Steele, 1972), would not be measured with our assay since these compounds are not extracted from plasma with diethylether (Boilert, Edqvist, Johansson, Lindberg and Martinsson, 1973).

As discussed earlier, the use of nonchromatographed extracts for radioimmunoassay requires that the inert, non-specific material which inevitably is present in the extracts, does not interfere with the antibody-ligand reaction, or, if it does, that the effect can be compensated for. Neither the addition of dried ether or ethanol residue had any detectable effect on the 17α-ethinyloestradiol standard curve, but, as illustrated in Figure 5.9, blank plasma residue markedly affected tracer-binding at low concentrations of unlabelled hormone. However, since

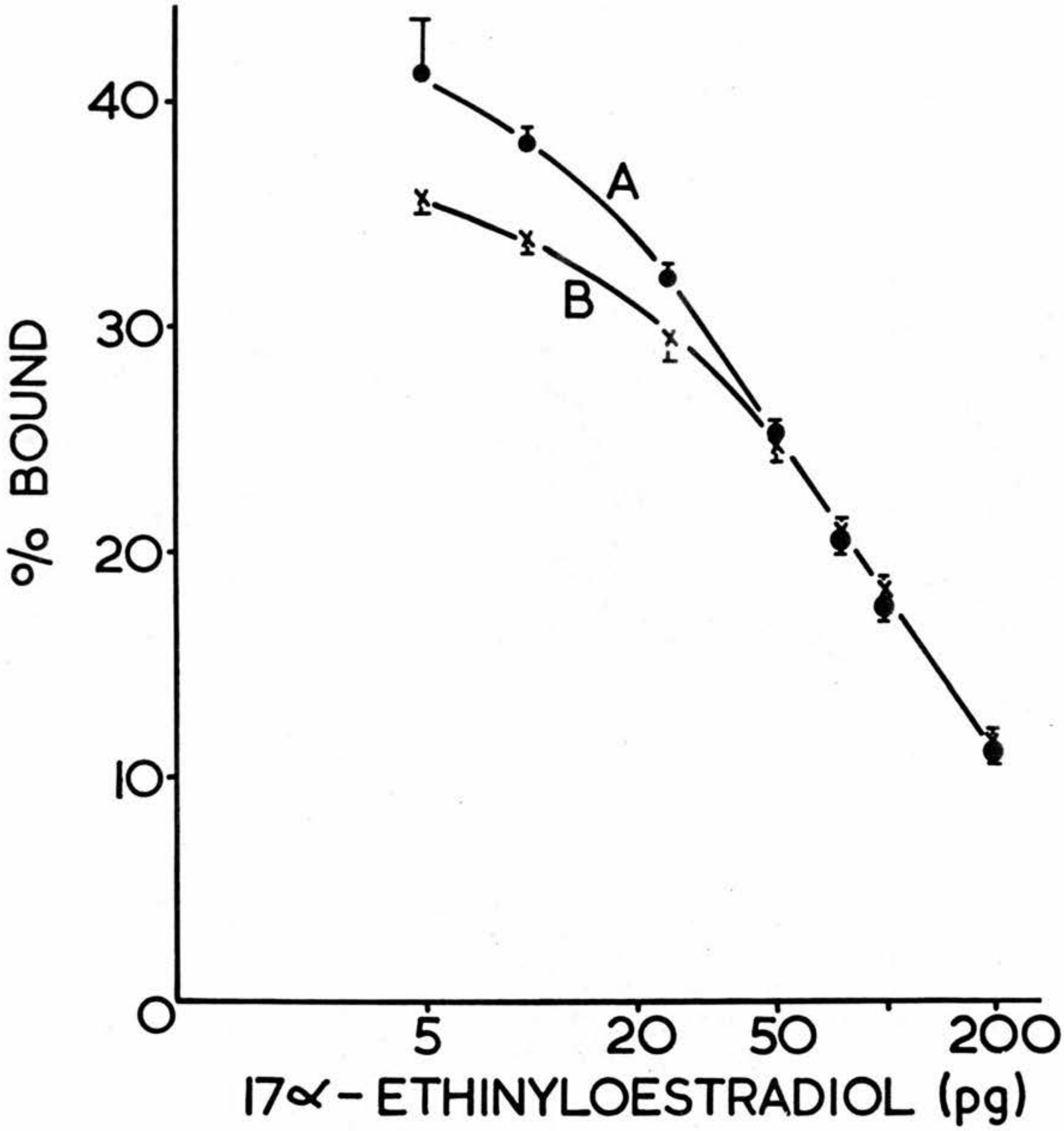


Figure 5.9: The effect of blank plasma on the dose response curve of 17α-ethinyloestradiol

A: Dose response curve in the absence of blank plasma residue

B: Dose response curve after adding dried plasma residue to the standards

17α-ethinyloestradiol is not usually present in the circulation, it was possible to compensate for this blank effect. Accordingly, standard curves were constructed by adding known amounts of 17α-ethinyloestradiol to aliquots of blank plasma which were then extracted in the same way as the unknown plasma samples. This method of compensating for the blank effect of plasma was preferred to the possible alternative of adding blank plasma residue to the incubation tubes containing the standards (in ethanol) since it simplified the preparation of the standards and obviated the need for correcting the unknowns for procedural losses. Evidently, the validity of this approach was entirely dependent upon the reliability of the extraction procedure, which was assumed to be highly consistent. However, the good agreement between duplicate measurements (within-assay coefficient of variation: 8·8%) and the reproducibility of the dose-response curves (less than 5% variation at each point; Figure 5.8) indicate that this assumption was correct. Also, the fact that peripheral 17α-ethinyloestradiol levels as measured with this method are similar to those of previously reported studies using orally administered ^{14}C-ethinyloestradiol (Reed, Fotherby and Steele, 1972) suggests that the method described gives an accurate, specific and precise estimate of circulating, unconjugated 17α-ethinyloestradiol concentrations.

5.2.2.3 Progesterone

Progesterone was measured by radioimmunoassay using an antiserum raised in sheep to progesterone-11α-hemisuccinate coupled to bovine serum albumin. The specificity of this antiserum (91920/9) as well as details of the assay technique have been described by Scaramuzzi, Corker, Young and Baird (1975).

In brief, 0·1-0·2 ml aliquots of plasma were extracted with 2 ml of redistilled "Analar" grade petroleum ether (b.p. 40°-60°) (BDH) by thoroughly mixing on a vortex mixer for 2 minutes. After quick-freezing the aqueous phase in a mixture of dry ice and methanol, the upper organic phase was decanted, evaporated under nitrogen stream and the residue dissolved in 0·1 ml of PBSG. A 0·1 ml aliquot of antiserum-dilution ($^1/5$,000, initial dilution) and 30,000 dpm of 1,2,6,7^{3}H-progesterone (S.A. 84 Ci/mM, The Radiochemical Centre, Amersham) in 0·1 ml of PBSG were then added, the tubes mixed briefly and incubated overnight at 4°C. Free and bound hormone were separated at 4°C by adding dextran-coated charcoal (0·25 mg dextran and 2·5 mg charcoal per tube in 1 ml of PBSG), followed 15 minutes later by centrifugation at 2,500 rpm for 10 minutes. The standard curve was virtually linear over the range of 0·02 to 1·00 ng, and the blank of the method, using deionised water, was 0·14 ± 0·03 ng/ml (mean ± S.E.M.; n = 5).

5.2.2.4 Testosterone

The assay for testosterone, described by Corker and Davidson (1976), was based on the method of Furuyama, Mayes and Nugent (1970) except that the chromatographic step after extraction was found to be unnecessary when male plasma samples were assayed.

The antiserum (E01), supplied by Dr. S. A. Tillson, Aliza Corporation, Palo Alto, U.S.A.) was raised in a goat immunised with testosterone-3-(O-carboxymethyl)-oxime coupled to bovine serum albumin, and used at an initial dilution of 1/6,000. Cross-reactions of other steroids were: 5α-dihydrotestosterone (25%), 17β-oestradiol (0·20%) and androstenedione (0·08%). Plasma-samples (0·1-0·2 ml) were extracted with 10 volumes of hexane and ether (4:1, v/v) by mixing for

30 seconds on a vortex mixer. The aqueous layer was frozen in dry-ice and methanol mixture and the organic layer transferred to a 30 x 5 mm alumina column or, if no chromatography was required, to a glass incubation tube. Before application of the sample, the alumina columns had been washed sequentially with 3·2 ml ethanol, 6·4 ml methanol, 4·8 ml methanol and dichloromethane (1:1, v/v) and 4·8 ml dichloromethane. The sample was allowed to run through the column and washed in with a further 1·6 ml of hexane and ether (4:1, v/v). After eluting the columns with 6·4 ml of 0·4% ethanol in hexane, the testosterone fraction was collected in 3·2 ml of 1·0% ethanol in hexane. The organic solvents were taken to dryness under nitrogen and the residue redissolved in PBSG.A 0·05ml aliquot was taken for recovery and aliquots of the remainder were used for assay as described by Corker and Davidson (1976).

5.2.2.5 Androstenedione

The antiserum used for the assay of androstenedione was raised in rabbits immunised with androstenedione-11α -hemisuccinate (supplied by Dr. W. Schopman, Rotterdam, Holland) coupled to bovine serum albumin. Of the steroids tested, only 11 α -hydroxyandrostenedione (36%), andrenosterone (43%) and testosterone (0·3%) cross-reacted to any significant extent with the antiserum.

The assay for androstenedione in peripheral plasma differed from that described by Baird, Burger, Heavon-Jones and Scaramuzzi (1974) only in the method of extraction and preparatory chromatography of the samples. For estimation of androstenedione, 0·1 ml aliquots of plasma to which 2,000 dpm (approximately 5 pg) of 1,2-^{3}H androstenedione (S.A. 40 Ci/mM, NEN) was added, were extracted with 20 volumes of hexane and ether (4:1,v/v). After quick-freezing the lower aqueous layer in dry ice, the upper organic layer was transferred to a 28 x 8 mm alumina column, which had been previously washed sequentially with 3·2 ml ethanol, 6·4 methanol, 4·8 ml dichloromethane and methanol (1:1 v/v) and 4·8 ml dichloromethane. The sample was allowed to run through the column which was then washed with a further 4·0 ml of hexane and ether (4:1, v/v) and 6·4 ml of 0·1% (v/v) ethanol in hexane. The androstenedione fraction was collected after adding 3·2 ml of 1·0% ethanol in hexane to the column. The samples were then evaporated to dryness under nitrogen and redissolved in 0·5 ml of PBSG. An aliquot (0·1 ml) was taken for estimation of procedural losses and aliquots of the remainder were assayed as described by Baird et al., (1974).

5.2.2.6 Oestrone

For the assay of oestrone in peripheral plasma, an antiserum raised in sheep to 17β-oestradiol-17-hemisuccinyl-BSA was used. The characteristics of this antiserum, which showed 35% cross-reactivity with oestrone, have been described previously (Baird et al., 1974). For the oestrone-assay, the antiserum was used at an initial dilution of $^1/100,000$ (final dilution $^1/300,000$).

Aliquots (0·5-4 ml) of the samples to be assayed were made up to 4·0 ml with distilled water and extracted three times with 4 ml of diethylether. The pooled ether extracts were evaporated to dryness and the residue dissolved in 0·5 ml iso-octane which was transferred to Celite columns prepared as described by Abraham, Tulchinsky and Korenman (1970). After washing the columns with a further 5·5 ml iso-octane, the oestrone was eluted with 4 ml ethylacetate and iso-octane (35: 65, v/v). The organic solvents were evaporated to dryness and the residue dissolved in 0·5 ml of PBSG. A 0·1 ml aliquot was used to estimate the recovery of a tracer amount (3,000 dpm, i.e. 7 pg) of tritiated oestrone which had been added to the samples before extraction, and aliquots of the remainder were used for assay.

5.2.3 Miscellaneous measurements

Plasma glucose was measured by an enzymatic (glucose-oxidase) method and plasma fluorogenic corticosteroids by the method of Mattingley (1962).

Plasma total thyroxine (T_4) was measured by solid-phase radioimmunoassay as described by Seth, Toft and Irvine (1976).