ASPECTS OF THE BIOCHEMISTRY OF PROTEIN HORMONES

by

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1. INTRODUCTION

Over the last six decades a large number of investigators have studied the production, mode of action and excretion of the protein hormones elaborated by the anterior pituitary gland and the placenta. For the greater part of this time work has been directed towards the assay and purification of the various hormones. More recently the effect of the administration of the active principles has been extensively studied and numerous workers have investigated the chemical and biological properties of the purified hormone preparations available. In this thesis is presented a series of investigations undertaken to obtain further information about the biological characteristics and the excretion pattern of the gonadotrophic hormones under various experimental and clinical conditions.

As long ago as 1897, Heape postulated the existence of an extra-ovarian factor which he called the "generative ferment" which was thought to be responsible for the regulation of ovarian activity. In 1901 Fox grafted immature ovaries into animals and noted the rapidity of the follicular development in the graft. The significance of this work was not fully appreciated until Long and Evans (1922) showed that the ovary of a young female rat when grafted into a castrated adult female will cause the appearance of oestrus changes more rapidly than in the immature animal. This work again suggested the existence of an extra-ovarian factor for the stimulation and control of some of the reproductive processes. The hypothesis was

supported by the work of Arai (1920 a, b) who demonstrated that in the unilaterally ovariectomised rat the number of follicles at different stages of development in the one remaining ovary is equal to the number in the two ovaries of the control animal. He concluded that the total number of primary follicles which enter into follicular development does not depend on the total number present but rather on some physiological factor outside the ovary.

Site of production of gonadotrophic factors

It was not until 1927 that Aschheim and Zondek showed that the urine or blood of pregnant women contained a substance which when injected subcutaneously into intact immature female mice produced ovarian follicular development and luteinisation. In the years immediately following this discovery there was considerable speculation regarding the sources of the various gonad-stimulating or gonadotrophic substances. It soon became clear that both the anterior pituitary and placenta were capable of elaborating these hormones and that the hormones could be classified according to the site of production.

Aschheim and Zondek had suggested in 1927 that the gonadotrophic material present in pregnancy urine was derived from the pituitary. However, in the early 1930's considerable experimental evidence was advanced to show that the various gonadotrophic factors do not all emanate from this gland. Evans, Meyer & Simpson (1932) stressed the difference in biological response of the recipient animal following the

administration of "prolan" (prepared from pregnancy urine) from that following the administration of rat anterior pituitary gland to hypophysectomised rats. They concluded that "prolan" either provoked the production of the hypophyseal gonad-stimulating hormone or was responsible for the conversion of some inactive component into an active substance. Various other workers including Philipp (1930); Collip (1930, 1934, 1935); Collip, Selye, Anderson & Thomson (1933); Hamburger (1933) and Deanesly (1935) provided further evidence to suggest that the gonadotrophin present in pregnancy urine is produced by the placenta and not by the anterior pituitary.

Conclusive evidence for the placental origin of this hormone was obtained by a number of workers who demonstrated that gonadotrophin was produced <u>in vitro</u> in placental tissue culture (Semmicandro, 1934; Gey, Jones & Hellman, 1938; Jones, Gey & Gey, 1943; Stewart, Sano & Montgomery, 1948). Wislocki and Bennett (1943) and Zilliacus (1953) obtained confirmatory evidence by histochemical studies for the view that most of the gonadotrophin was produced in the Langerhans cells of the chorionic villi.

<u>Human Chorionic Gonadotrophin</u>

In 1939 it was decided by the Third International Conference on the Standardisation of Hormones held in Geneva to adopt the term gonadotrophin for the various gonad-stimulating substances. The hormone produced by the

placenta therefore became known as Human Chorionic Gonadotrophin (HCG). In correspondence with this term the hormones elaborated by the human anterior pituitary are commonly called Human Pituitary Gonadotrophins (HPG) although the term is not yet officially approved. Human Chorionic Gonadotrophin has been obtained in a highly purified form by Bourrillon and Got (1957, 1959, 1960) and by Bourrillon, Got & Maroy (1960). The gonadotrophin is thought to be a glycoprotein the molecule of which contains galactose (Gurin, 1942). Morris (1955) claims to have prepared a material of high specific activity (12,000 i.u./mg.) and this material when administered to intact animals causes changes in the gonads very similar to those observed following the injection of hypophyseal gonadotrophin. However, the effect is indirect and is dependent upon interaction with the test animal's own anterior pituitary hormones. This has been demonstrated by Lamond and Emmens (1961) who showed that when HCG is administered to hypophysectomised mice the ovaries do not grow, although a marked increase in ovarian weight can be obtained under similar experimental conditions in intact animals.

Pituitary Gonadotrophins

As soon as the controversy regarding the site of production of the gonadotrophins was settled it became possible to study in detail the nature of the biologically active hormones elaborated by the anterior pituitary. It

is generally believed that anterior pituitary extracts from sheep and swine contain two distinct gonadotrophic principles (Fevold, Hisaw & Leonard, 1931; van Dyke and Wallen-Lawrence, 1933; Bates, Riddle & Lahr, 1934 and McShan, Kagawa & Meyer, 1954). These substances have been termed "Follicle-stimulating Hormone" (FSH) and "Luteinizing Hormone" or "Interstitial Cell-stimulating Hormone" (LH or ICSH[++]). FSH has been shown to stimulate the growth of follicles in the ovary and in the germinal epithelium in the testis while LH or ICSH causes corpus luteum development and the stimulation of interstitial testicular tissue.

Luteotrophin

It has been suggested that anterior pituitary extracts contain a third gonadotrophic principle which is responsible for the maintenance of the activity of the corpus luteum and for the secretion of progesterone by the ovary (Astwood, 1941, 1953; Evans, Simson, Lyons & Turpeinen, 1941). This substance has been designated "Luteotrophin" and is thought to be identical with prolactin, the lactogenic hormone. At present there is no conclusive evidence of the presence of luteotrophin in biological fluids and its physiological function in man remains obscure. Recently it has been demonstrated by

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[++]Throughout this thesis the abbreviation LH will be used to describe both Luteinising Hormone and Interstitial Cell-stimulating Hormone.

Guillemin and Sakiz (1963) that in the ovarian ascorbic acid depletion test (see p.21), which is dependent upon active corpora lutea, the response of LH in hypophysectomised rats is abolished unless prolactin is given shortly after hypophysectomy. These workers conclude that the function of prolactin is to maintain the corpora lutea which would otherwise regress.

Purification of Pituitary Gonadotrophins

Many claims have been made regarding the biological and chemical purity of FSH and LH preparations from the anterior pituitary. LH preparations have been obtained which when administered to hypophysectomised rats had no effect on follicular growth but produced interstitial cell repair in the ovary. Conversely, highly purified FSH preparations have been shown to cause enlargement of the ovarian follicles without any effect on interstitial tissue. Several preparations have been claimed to be homogeneous when subjected to electrophoretic, ultracentrifuge and solubility tests, but it is possible that these claims are premature. Whereas Li, Simpson & Evans (1940 a, b) reported that sheep ICSH had a molecular weight of 40,000 with an isoelectric point of pH 4.6 a more recent publication from the same laboratory (Squire and Li, 1959) states that sheep ICSH prepared by a different extraction method has a molecular weight of 30,000 and an isoelectric point of pH 7.3.

As in the case of LH, the early attempts to purify FSH

depended on techniques of isoelectric precipitation and differential precipitation by salt solutions and organic solvents; more recently ion exchange chromatography and molecular sieve chromatography have been used. Woods and Simpson (1960) used diethylaminoethyl cellulose in the preparation of a highly purified FSH material. Steelman and Segaloff (1959), McShan, Kagawa & Meyer (1954) and Ellis (1958) have also used techniques of this type to obtain potent FSH extracts from anterior pituitary tissue. Nevertheless, at the present time, even the most highly purified preparations of FSH and LH do not fulfil all the criteria of biological and chemical purity when tested both by bioassay and physico-chemical procedures.

Methods of Assay for Pituitary Gonadotrophins

The measurement of the production of HPG and HCG in clinical conditions has necessarily depended on the assay of the gonadotrophic principle in the most readily available biological fluid, namely, urine. Estimations of HCG were first performed in the clinical field as a means of pregnancy diagnosis and the assay of HPG is also of considerable value to the clinician as an aid to the diagnosis, prognosis and management of many endocrine abnormalities (see Loraine, 1958). The work described in this thesis deals mainly with pituitary gonadotrophins and for this reason the discussion of assay methods will be restricted to the measurement of FSH and LH. One of the outstanding difficulties in the estimation of pituitary gonadotrophins in urine is the absence of an

International Standard. This means that arbitrary units such as "rat" or "mouse" units have had to be used to express the results of clinical assays. The units employed have varied from one laboratory to another but are generally defined as that quantity of gonadotrophin required to produce a given effect in the experimental animal. Because of the large variation in the sensitivity of animal colonies it is generally recognised that the results calculated in this way have little or no quantitative significance (Loraine, 1956). Considerable difficulty has also been experienced in the separation of FSH from LH in human non-pregnant urine and assay methods have been developed to measure either FSH or LH activity separately in extracts containing both materials.

Over the past two decades there has been considerable controversy about the possible existence of two separate gonadotrophins in human non-pregnant urine. In 1954 Segaloff, Gordon, Carabasi, Horwitt, Schlosser & Murison suggested that human urine contained a single gonadotrophic substance with both follicle-stimulating and luteinizing activities. This view is not held by many other workers (see Loraine, 1958) and recently Butt, Crooke & Cunningham (1961) have shown that it is possible to prepare highly purified extracts from human urine which contain predominantly FSH or LH activity.

Methods of Assay for FSH

It is generally agreed that to measure FSH any specific assay method must employ hypophysectomised animals and that

in intact animals the endogenous gonadotrophin will interfere with the response. In general, methods for FSH have depended on observations of the testes or ovaries of hypophysectomised rats after administration of the gonadotrophin extract (Evans, Simpson, Tolksdorf & Jensen, 1939; Paesi, Wijnans & De Jongh, 1951). Because of the difficulty of performing hypophysectomy in rats and mice Steelman and Pohley (1953) investigated the possibility of using intact animals and these workers developed an assay method for FSH in which a standard increase in ovarian weight in intact immature rats is used as the endpoint. This procedure which is called the augmentation test has subsequently been modified for use in mice by P.S. Brown (1955) (see p. 21). Simpson (1961) has compared the augmentation test in rats with a procedure depending on follicular development in the ovaries of hypophysectomised rats. This latter method is thought to be specific for FSH and does not respond to highly purified LH preparations (Evans, Simpson, Tolksdorf & Jensen, 1939). It was shown by Simpson (1961) that when gonadotrophin preparations were assayed by both methods the same potency ratio was obtained suggesting that the augmentation test is also specific for FSH.

Methods of Assay for LH

As in the case of FSH most of the early assays for LH were conducted in hypophysectomised rats (Evans et al 1939; Greep, Van Dyke & Chow, 1942; McArthur, 1952 and Loraine and Brown, 1954). Simpson (1961) compared the ventral prostatic weight test (see p 23) with the method depending on the

repair of interstitial tissue, using hypophysectomised rats in each case. Both assay procedures yielded similar estimates of potency when purified LH preparations were assayed and it was therefore concluded that they were reasonably specific for LH. Recently Parlow (1958, 1961) has described a method for the estimation of LH depending on the depletion of ovarian ascorbic acid in intact immature rats (see p.21). This procedure, which appears to be specific for LH, avoids the use of hypophysectomised animals and is more practicable than many of the early methods.

Non-specific Assay Methods

Apart from these specific methods for the estimation of FSH and LH activities a large number of tests have been developed which measure both FSH and LH and are therefore non-specific in nature. These assays can be divided into two groups according to whether the response is primary or secondary. In the primary group the gonads themselves are inspected while in the secondary group the effects result from the liberation of oestrogens or androgens from the gonads. Many of these methods are not sufficiently sensitive to measure gonadotrophic activity in human non-pregnant urine but one procedure which has gained wide popularity is the technique depending on the increase in uterine weight in intact immature mice following the administration of gonadotrophin (mouse uterus test: see p. 25). This method depends on the liberation of

oestrogens from the ovary and the subsequent growth of the uterus. Most investigators have used the technique as described by Levin and Tyndale (1937) or some modification of this procedure.

In view of the difficulty experienced in the preparation of highly purified FSH and LH extracts it seemed of interest to investigate the biological characteristics of gonadotrophin preparations derived from a variety of sources when assayed both by specific and non-specific bioassay procedures. The results obtained in this study are reported in the third section of the thesis. It was hoped that this investigation would yield further information about the FSH/LH ratio of the preparations tested and at the same time provide data about the reliability of the assay procedures employed.

Extraction Procedures for Urinary HPG

Because of the insensitivity of the bioassay procedures available it has been found necessary to concentrate the HPG in urine before assay. The extraction and concentration of gonadotrophins from human non-pregnant urine has been studied by a number of workers. Since Zondek (1931 b) first used alcohol as a precipitant a variety of different methods has been proposed for the preparation of urinary HPG extracts (Levin and Tyndale 1936; Heller and Heller, 1939; Scott, 1941 and Varney and Kooh, 1942). One of the main drawbacks to some of the extraction procedures is that the extracts frequently show a high degree of toxicity when administered to experimental animals (Borth, Lunsenfeld & Menzi, 1961) and

for this reason a study was undertaken and is reported in section 4 of the thesis to investigate possible improvements to one of the most commonly used extraction procedures, the kaolin acetone method (Loraine and Brown, 1959).

Inactivation of FSH and LH

Another topic which has been studied by numerous workers and which is a direct result of work on the purification of FSH and LH is the effect of a large variety of different chemical agents on the biological activity of various gonadotrophin preparations. It has been shown that some reagents affect the biological activity of either FSH or LH selectively but that FSH is more stable than LH (Fraenkel-Conrat, Simpson & Evans, 1939, 1940; Li, Simpson & Evans, 1939, 1940 a; Légault-Demare, Clauser & Jutisz, 1958; Butt, Crooke, Cunningham & Evans, 1961). Ellis (1961 a, b) showed that 6 M urea denatured pituitary LH preparations without affecting FSH activity and it seemed of interest to extend this study to investigate the effect of urea on purified gonadotrophin preparations obtained from a variety of sources. It was hoped that this series of experiments would provide further information on the selective destruction of LH and FSH in mixtures containing both hormones. The effect of formaldehyde on amino acids has been extensively studied (see French & Edsall, 1945) and because of the action of this materiel on the protein molecule it was decided to investigate the effect of

formaldehyde on the biological activity of a purified LH preparation. The results obtained both with urea and formaldehyde are reported in the fifth section of this thesis.

Pregnant Mare's Serum Gonadotrophin

Apart from the gonadotrophins already described another biologically active material has been extensively studied. This is the Pregnant Mare's Serum Gonadotrophin (PMSG) which was discovered in the serum of pregnant mares independently by Cole and Hart (1930) and Zondek (1930). All the existing preparations of PMSG are luteinizing as well as follicle-stimulating in nature and in 1940 Cole, Pencharz & Goss showed that both activities probably reside in one molecule. One of the most active preparations obtained is that of Rimington and Rowlands (1941) who achieved a potency of 12,500 i.u./mg. The only available source of PMSG is the serum of pregnant mares and apart from a claim on the basis of a single observation by Zondek (1931 a) activity was not demonstrated in the urine of these animals until Schmidt-Elmendorff, Loraine, Bell & Walley (1962) showed that, if the urine was concentrated appropriately, gonadotrophic activity could be detected. This observation made it feasible to study the renal clearance of PMSG in the equidae and because of the previous work on the renal clearance of HPG and HCG such a study was undertaken. The results obtained are reported in the sixth section of this thesis.

Excretion of HPG in Non-pregnant Subjects

In 1955 following the introduction of HMG-20A [+] as an unofficial reference preparation it became possible to obtain a quantitative estimate of the excretion of HPG in non-pregnant subjects and the normal values for both sexes were soon established (see Loraine, 1958). It is generally agreed that the gonadotrophic activity in the urine of children and adolescents is very low (Nathanson, Towne and Aub, 1941). In male urine gonadotrophic activity is present in relatively constant amounts (Schou, 1951) while in premenopausal females fluctuating gonadotrophin levels can be related to the stage of the menstrual cycle (D'Amour, 1943). After the menopause the excretion of urinary HPG rises steeply to levels well above the premenopausal range (Heller and Shipley, 1951). The excretion of HPG in urine during the menstrual cycle has been studied by a number of workers including Brown, Klopper & Loraine (1958); Buchholz (1957) and Loraine (1962). McArthur, Worcester & Ingersoll (1958) studied the excretion pattern of FSH and LH separately during the menstrual cycle and more recently Taymor (1961) has undertaken a similar study.

Although the excretion pattern of HPG throughout the menstrual cycle has been established there is still

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[+] This term stands for Human Menopausal Gonadotrophin and will be used to designate the gonadotrophic activity in the urine of male and non-pregnant female subjects. Such urine extracts contain both FSH and LH activity.

controversy about two points. The first is the existence of a gonadotrophin peak at midcycle and the second is the interrelationship of this peak, if present, with the excretion pattern of the steroid hormones and hormone metabolites. A study has therefore been undertaken to investigate these points and the data obtained are reported in the seventh section of this thesis.

The Effect of Antiovulatory Compounds on HPG Excretion Levels

The clinical use of antiovulatory compounds and their short and long term effect on the urinary excretion of hormones and hormone metabolites from the anterior pituitary gland and the ovary has recently aroused considerable interest and for this reason is worthy of discussion. In 1956 Pincus reported that orally administered progesterone was capable of inhibiting ovulation but that "breakthrough" bleeding occurred in a high percentage of women. It was shown that the addition of oestrogendecreased the frequency of "breakthrough" bleeding but it was not until the synthetic derivatives of testosterone and progesterone were used that the problem was to a large extent overcome. A variety of compounds of the 19-nortestosterone and 17α-hydroxyprogesterone type have been shown to inhibit ovulation when given orally and the clinical effects of such compounds are well documented (Mears, 1961, 1963; Pincus, Rock, Garcia, Rice-Wray, Paniagua & Rodriguez, 1958).

At the present time there is little reliable information on the site or mode of action of the antiovulatory agents.

Recently Brown, Fotherby & Loraine (1962) studied the effect of two of these compounds, norethisterone (17α-ethinyl-19-nortestosterone) and its acetate when administered to normally menstruating women. These workers showed that with adequate dosage of the compound ovulation as judged by the urinary excretion of oestradiol, oestrone, oestriol and pregnanediol was suppressed; no effect was noted on the output of HPG. This observation is in contrast to the findings in postmenopausal women that large dosages of norethisterone oenanthate depress HPG output, indicating that this compound can act as a pituitary inhibitor (Douglas, Loraine & Strong, 1960; Martin and Cunningham, 1961). The failure to reduce HPG excretion during the normal menstrual cycle suggests that the antiovulatory compounds act directly on the ovaries rather than on the pituitary. It should be noted that the mouse uterus test which was employed by Brown et al (1962) for the measurement of urinary HPG is not specific for either FSH or LH activity and a differential inhibition of one or other of these hormones cannot be excluded.

One disadvantage of the use of antiovulatory agents is that they are expensive to manufacture and because of this a considerable amount of work has been undertaken to develop less costly compounds. Paget, Walpole & Richardson (1961) have recently described a dithiocarbamoylhydrazine derivative which was capable of inhibiting pituitary function in rats, dogs and monkeys. When this compound became available for use in human subjects it seemed of interest to study its

effects on hormone excretion because of the possible future use of this substance as an oral contraceptive agent. In the final section of this thesis a study is reported in which the effect of this derivative of dithiocarbamoylhydrazine on hormone excretion in postmenopausal women and women during reproductive life was investigated.

2. MATERIALS AND METHODS

I. HORMONES

The following hormones have been employed during the present study :-

1. Follicle-stimulating Hormone (ovine NIH-FSH-S1).

This is distributed by the Endocrinology Study Section of the National Institutes of Health (Bethesda, U.S.A.) and is contaminated with 0.6% LH as estimated by the ovarian ascorbic acid depletion test (Parlow, unpublished data).

2. Luteinising Hormone (ovine NIH-LH-S1; NIH-LH-S3).

Two preparations of purified luteinising hormone were available for experimental use, namely NIH-LH-S1* and NIH-LH-S3. The potency of these two materials is in the ratio 1.0 : 0.8 as estimated by the ovarian hyperaemia test. (Endocrinology Study Section, National Institutes of Health, unpublished data).

3. Follicle-stimulating Hormone (Clinical Endocrinology Research Unit : Human Preparation).

This was prepared in the laboratory in Edinburgh by Dr. K. C. Hooper using the method of Li, Simpson & Evans (1949). This procedure involves the extraction of gonadotrophin from human pituitary tissue by calcium hydroxide solution and subsequent precipitation with ammonium sulphate.

4. Human Menopausal Gonadotrophin (HMG-20A)

This material was prepared by Dekanski (Organon :Newhouse)

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*Except where stated otherwise the term NIH-LH refers to the preparation NIH-LH-S1.

from postmenopausal urine by a method depending on kaolin adsorption and fractional precipitation with acetone. Tricalcium phosphate was added to an aqueous solution of this precipitate to remove toxic materials and any contaminating HCG. The gonadotrophin was reprecipitated with acetone before being lyophilised. HMG-20A was used as an unofficial reference preparation for a number of years up to 1957. (Bull. World Health Org. 1960).

5. Human Menopausal Gonadotrophin : International Reference Preparation.

This was also prepared by Dekanski (Organon : Newhouse) using an extraction method similar to that employed for the preparation of HMG-20A. An international collaborative study using a variety of assay methods has shown that the International Reference Preparation is approximately equipotent with HMG-20A and by definition both preparations contain equal amounts of FSH and LH activity per mg of the freeze-dried material (Bull. World Health Org. 1960).

6. Human Menopausal Gonadotrophin (Pergonal).

In 1957 it was suggested to the Department of Biological Standards of the World Health Organisation that an International Standard for Human Menopausal Gonadotrophin should be established. For this purpose Donini (Serono : Rome) prepared a batch of gonadotrophin (designated Pergonal) from postmenopausal urine by a method depending on kaolin

adsorption following by chromatography on permutit columns. Each ampoule contains approximately 10 mg. of the freeze dried material consisting of equal amounts of Pergonal and lactone. Rosemberg & Engel (1961) have shown that this material has a higher specific activity than the International Reference Preparation for Human Menopausal Gonadotrophin when assayed by the augmentation and mouse uterus test and the hypophysectomised rat prostate test.

7. Pregnant Mares Serum Gonadotrophin (PMSG).

a) International Standard. This was prepared from the serum of pregnant mares by a method depending on adsorption and fractional precipitation.

b) "Gestyl" (Organon).

8. Human Chorionic Gonadotrophin (HCG).

a) International Standard. Human urine collected during the first three months of pregnancy was used in the preparation of this standard.

b) "Pregnyl" (Organon). Both "Gestyl" and "Pregnyl" are standardised in terms of the respective International Standards and are lyophilised before distribution.

II. BIOASSAY METHODS

A total of six bioassay methods have been employed in the present study; these were as follows :-

A) Augmentation test: This method depends on the increase in ovarian weight in intact immature mice treated with large dosages of HCG (P.S. Brown, 1955), and is claimed to be specific for FSH (Simpson, 1961).

Immature female mice of a Swiss albino strain were bred in a closed colony in the laboratory in Edinburgh. Animals weighing 9 - 11 gm. at 21 - 23 days of age were used in the assay and groups of between 3 and 5 mice were employed at each dose level of the standard and unknown material. The test material, to which 40 i.u. HCG had been added, was injected subcutaneously in three equal doses, each in 0.5 ml. distilled water at 0 hrs. approximately 24 hours and approximately 48 hours. The animals were killed 24 hours after the first injection at which time the ovaries were dissected out and weighed. The combined weight of both ovaries was used as the endpoint of the bioassay and the results of a typical assay are shown in Fig. 1.

B) Ovarian ascorbic acid depletion (OAAD) test in intact immature rats pretreated with PMSG and HCG. This procedure has been shown to be specific for LH activity (Parlow, 1961; Schmidt-Elmendorff & Loraine 1962).

The technique employed was essentially that of Parlow (1958, 1961). The procedure is carried out in two stages, a) pretreatment of animals and b) bioassay of the test preparations.

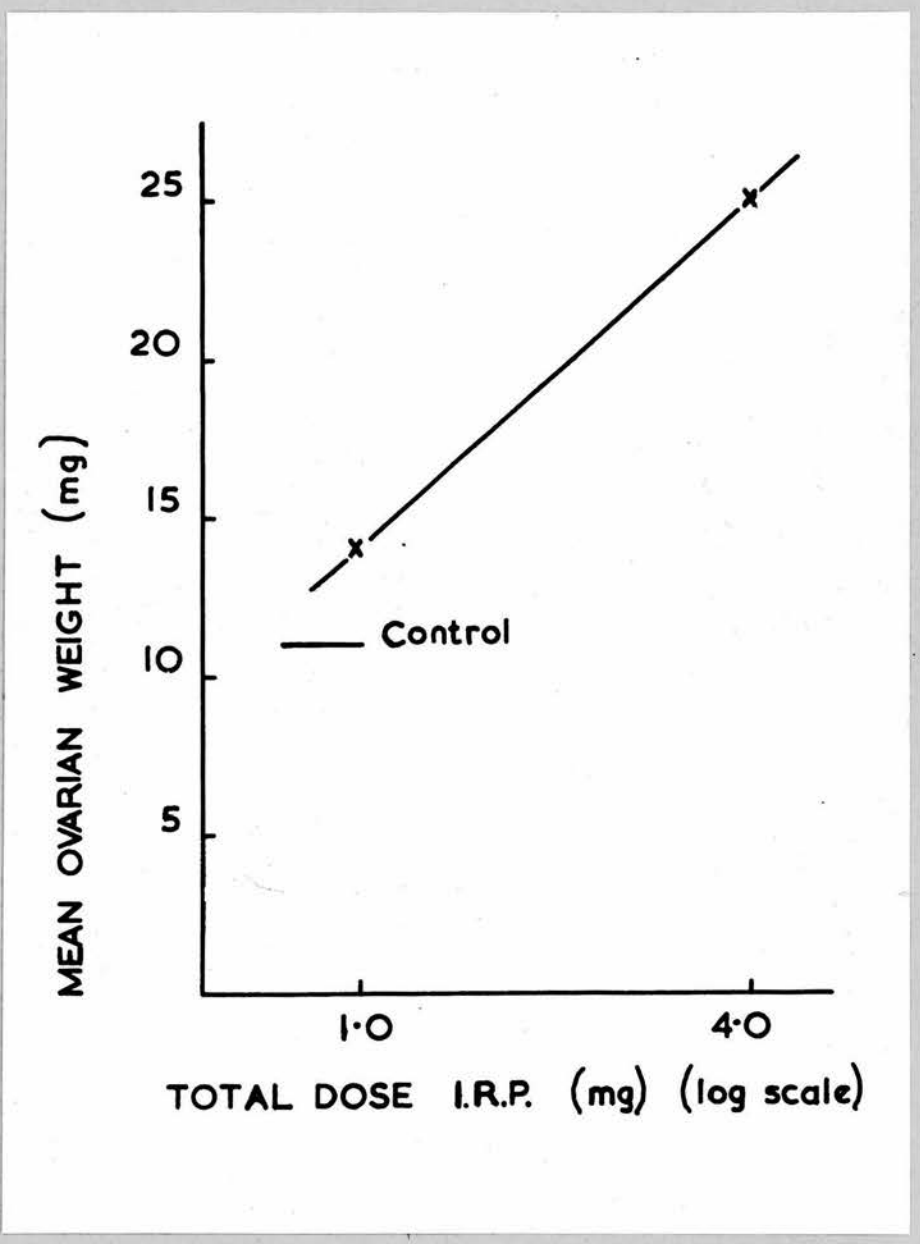

Fig. 1 :- Dose response curve for the International Reference Preparation for Human Menopausal Gonadotrophin - augmentation test (5 animals per dose level).

a) Pretreatment of animals. Intact immature Wistar rats were obtained either from a closed colony maintained in the laboratory in Edinburgh or from the Small Animal Breeding Station, University of Edinburgh. Animals varying in age from 21 - 24 days and in weight from 30 - 50 gm. body weight were used. Each rat received a subcutaneous injection of 50 i.u. PMSG ("Gestyl") followed 72 hours later by 25 i.u. HCG ("Pregnyl"). Both preparations were dissolved in distilled water and administered in a total volume of 0.5 ml. per animal. Following macroscopic examination of the ovaries it was noted that administration of PMSG resulted in marked follicular growth and in the production of ovaries ranging in weight from 50 - 100 mg. After the injection of the HCG luteinization of the ovaries occurred and the increased ovarian weight was maintained for a period of 11 to 13 days.

b) Bioassay. This was performed between the 5th and the 9th day after the injection of HCG. The standard and test materials were dissolved in 0.5 ml. 0.9% NaCl and injected either via the intravenous or intraperitoneal route. In the early assays (Chapter 3) the test preparation was administered via the femoral vein after the animal had been anaesthetised with ether. However, it was subsequently shown that intraperitoneal injection of the standard test material greatly increased the practicability of the assay method without affecting its reliability criteria (Mukerji, Bell & Loraine, 1962). This procedure was subsequently adopted in all later assays.

The animals were killed 4 hours ± 10 min. after the injection by dislocation of the cervical spine and the ovaries were removed using a dorsal approach. The ovaries were rapidly cleaned and weighed on a torsion balance to the nearest 1.0 mg. before being homogenised in a mortar and pestle with 2.5 ml. of 2.5% metaphosphoric acid (freshly prepared) and a trace of sand. After thorough grinding a further 7.5 ml. of 2.5% metaphosphoric acid was added and the homogenate filtered through Whatman No. 2 filter paper. The ascorbic acid content of a 2.0 ml. aliquot of the filtrate was determined by adding 2.0 ml. 0.5% 2.6-dichlorphenolindophenol and reading the resultant colour at 520 mμ in a Unicam Model SP 600 spectrophotometer within one minute of mixing. The ascorbic acid content of each ovary was expressed as μg / 100 mg. ovary. In all assays either four or five animals were used at each dose level of the material to be tested, and the results of a typical assay in which two dose levels of the International Reference Preparation were employed are shown in Fig. 2.

C) Hypophysectomised rat prostate (HRP) test : this method depends on the increase in ventral prostatic weight in hypophysectomised immature male rats (Loraine & Brown, 1954) and is claimed to be specific for LH activity (Loraine & Dicz-Faluay, 1958).

Sprague-Dewley rats of the Charles River CD strain were hypophysectomised at the Charles River Breeding Laboratories, Boston U.S.A. and were transported by air to Edinburgh on the day immediately following the operation. The animals

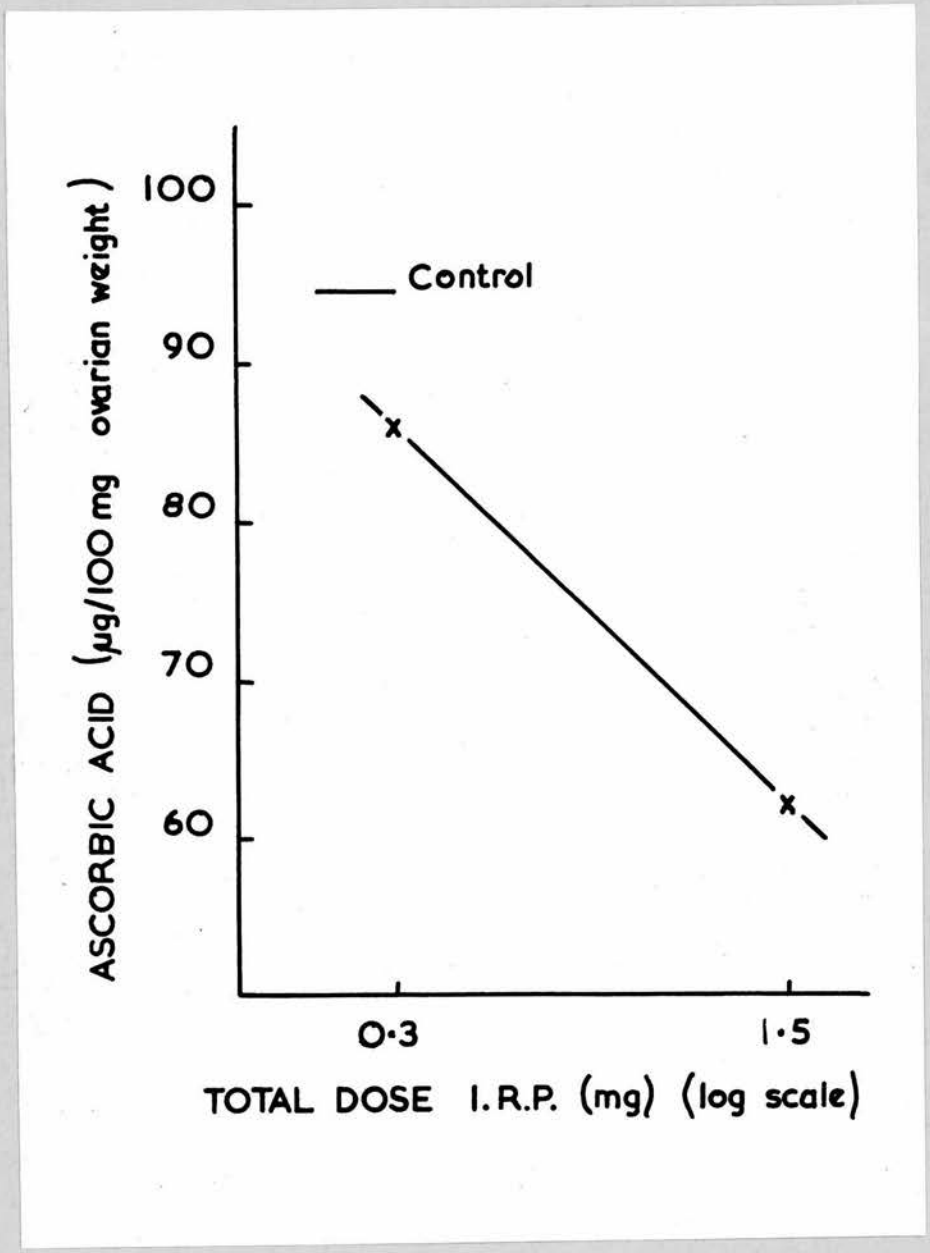

Fig. 2 :- Dose response curve for the International Reference Preparation for Human Menopausal Gonadotrophin - ovarian ascorbic acid depletion test (5 animals per dose level).

were rested for 5 days before the commencement of the injections and groups of 4 animals of body weight 55 - 65 gm. were employed at each dose level. Each rat received a total of 1.0 ml. of the gonadotrophin preparation which was dissolved in distilled water and administered subcutaneously over a period of three days. 0.4 ml. was given on the first day and 0.3 ml. on each subsequent day. The animals were killed on the 5th day at which time the ventral lobe of the prostate was dissected out from the surrounding structures and fixed in Bouin's solution for 24 hours before being weighed.

The following three methods provide a nonspecific estimate of FSH and LH activities :

B) Uterine weight test in intact immature mice (Mouse uterus test)(Loraine & Brown, 1954) :

Swiss albino mice bred in the laboratory in Edinburgh and weighing 9 - 11 gm. at 21 - 23 days of age were employed. The gonadotrophin preparation to be tested was dissolved in distilled water and administered subcutaneously to groups of 3 or 4 mice per dose level over a period of 3 days. Each mouse received 0.4 ml. on the first day and 0.3 ml. on each of the following days. The animals were killed 72 hours after the first injection and the uterus dissected free from connective tissue, dried carefully and weighed on a torsion balance to the nearest 1.0 mg. The results of a typical assay of the International Reference Preparation are shown in Fig. 3.

Because of its practicability the mouse uterus test, although non-specific, is the most convenient method for the routine determination of gonadotrophic activity. Accordingly, the results reported in the sections of this thesis dealing with the application of gonadotrophin assays (see Chapters 4 and 6) were obtained by the mouse uterus test.

E) Uterine weight test in intact immature rats. (Diczfalusy & Loraine, 1955).

Female Wistar rats bred in a closed colony in the laboratory in Edinburgh were used. Groups of 3 or 4 animals ranging in weight from 30 - 40 gm. and 21 - 24 days old were employed at each dose level. The

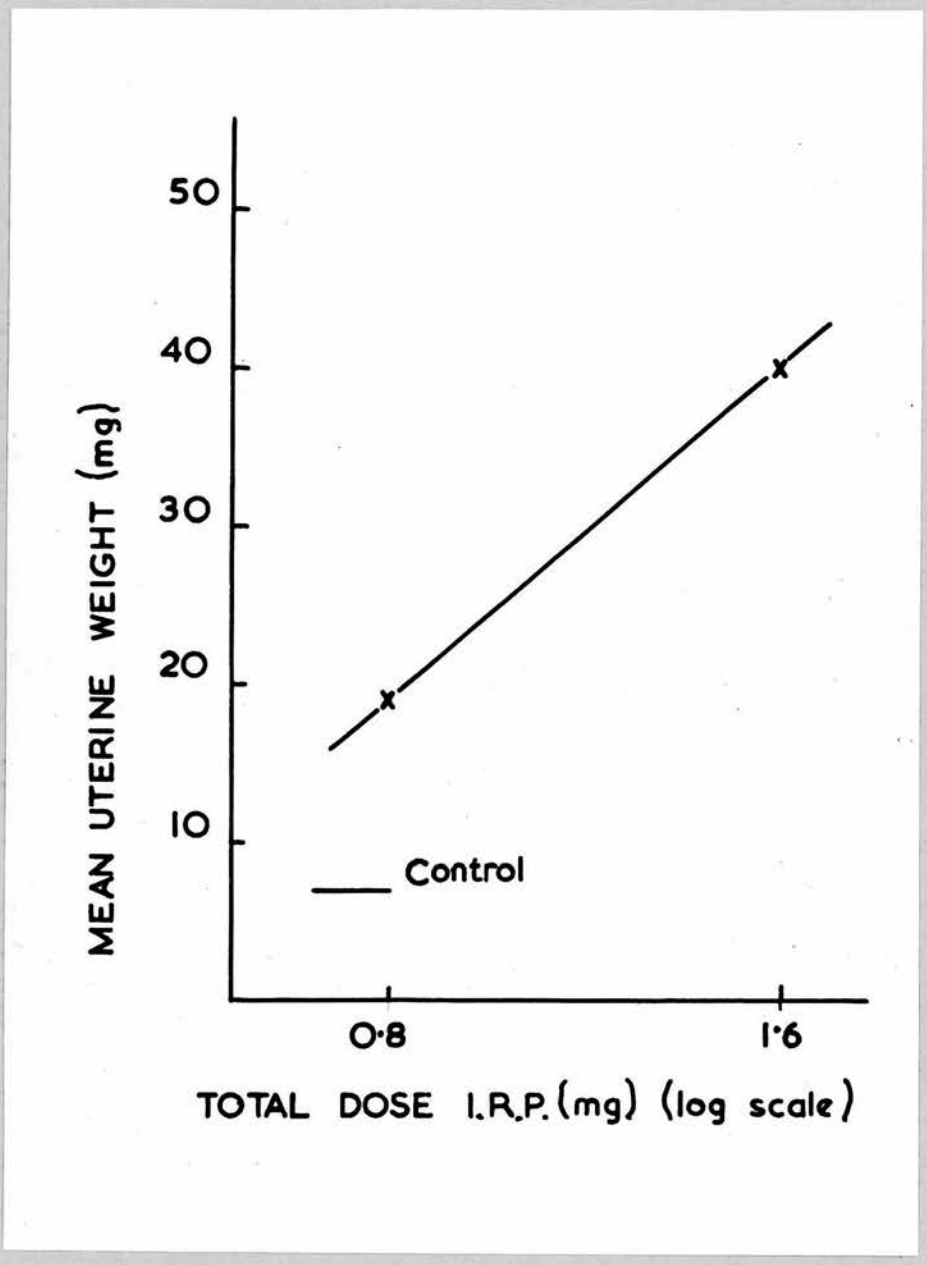

Fig. 3 :- Dose response curve for the International Reference Preparation for Human Menopausal Gonadotrophin - mouse uterus test (4 animals per dose level).

experimental procedure adopted was the same as that described for the mouse uterus test.

F) Ovarian weight test in intact immature mice.(Hamburger & Pedersen Bjergaard, 1937).

The experimental procedure for this essay method is identical to that described for the mouse uterus test. The end point of the assay depends on the increase in the combined weight of the two ovaries.

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III, Summary of Specificity of Assay Methods employed in the present study.

Methods specific for FSH :

1. Augmentation test.

Methods specific for LH :

1. Ovarian ascorbic acid depletion test.
2. Hypophysectomised rat prostate test.

Non-specific methods :

1. Mouse uterus test.
2. Rat uterus test.
3. Mouse ovarian weight test.

IV. Statistical Methods

All the bioassay procedures listed in this section depend on a graded or measured effect and were of a three-point or four-point design as described by Geddum (1953 a). A variety of methods have been described for estimating the precision of bioassay procedures. One of the most convenient of these is to express the error of the various tests in terms of the index of precision (λ). In the calculation of this index, of the relative potency and of the fiducial limits of error the methods of Geddum (1953 a, b) have been used.

The index of precision provides an estimate of the standard deviation of the logarithms of the individual effective doses and may be calculated by dividing the standard deviation of the response (s) by the slope of the line connecting the response with the logarithm of the dose (b)

$$\text{i.e. } \lambda = \frac{s}{b}$$

The precision of a given assay is high when s is small and b is large. Thus the lower the figure for λ the more precise the assay. In practice a figure of 0.2 or less indicates that the assay is very precise, while an assay with a λ figure greater than 0.2 has a low degree of precision.

The statistical methods described by Geddum (1953 a, b) have also been used to demonstrate the absence of a significant difference between the slopes of the log dose-

effect curves of the standard and unknown preperations in any given assay. In assays where a significant difference was observed, i.e. where there was lack of parellelism between the dose response curves of the standard and unknown materials, it was concluded that the two preperations were qualitatively dissimilar and the assays were therefore regarded as invalid.

Throughout the study indices of discrimination were calculated by the method of Gaddum (1955). The index of discrimination is equal to the ratio of the two results which would be obtained if one of the substances were used to assay an unknown extract containing the other. This means that if any given substance is measured by two bioassay methods, and the index of discrimination of the results is unity, then the active principle measured by the two tests is identical. Conversely if the index of discrimination diverges from unity then the quantitative difference between the two materials is present.

3. A STUDY OF THE BIOLOGICAL ACTIVITY OF A NUMBER OF GONADOTROPHIN PREPARATIONS WHEN ASSAYED BY VARIOUS BIOASSAY PROCEDURES

I. INTRODUCTION

In recent years the biological activity of gonadotrophins derived from a variety of sources have been compared by a number of investigators. In the studies conducted by Albert and Kelly (1958 a, b) and Albert, Kelly and Kobi (1958) the biological characteristics of the hormones have been determined by their effects on ovarian and uterine weights in intact, immature rats. Rosemburg and Engel (1961, 1962 a, b) and Schmidt-Elmendorff and Loraine (1962) expressed the gonadotrophic potencies obtained by different assay methods in terms of one or other of the reference preparations currently available. The aim of the present investigation was to extend previous work in this field by the use of highly specific methods, and thereby to gain further information about the FSH and LH content of gonadotrophins derived from human and animal pituitary tissue, from human urine and from the serum of pregnant mares.

II. MATERIALS AND METHODS

All hormones and bioassay methods listed in the section dealing with Materials and Methods (pages 18 to 26) were employed in this study. A total of 60 valid assays are reported, 16 by the augmentation test, 30 by the ovarian ascorbic acid depletion test, 2 by the hypophysectomised rat prostate test, 8 by the mouse uterus test, 2 by the rat uterus test and 2 by the mouse ovarian weight test.

In 58 instances the design was a symmetrical 4-point assay with 2 dose levels of the standard and test preparations, and with equal spacing of the log doses. In the remaining two assays, one by method 3 and one by method 5, it was necessary to calculate results using a 3-point design because only one dose level of the unknown preparation fell within the "working range" of the dose response curve of the standard preparation. In none of the assays reported in Tables 2 to 7 was significant deviation from parallelism encountered between the dose response curves of the standard and test materials.

The total dosages of the materials used in the majority of the assays in the present study are shown in Table 1.

In 45 out of the 60 assays the International Reference Preparation[++] was used as the reference material. In 2 assays by method 6 the reference material was HMG-20A (Table 6). In 3 assays by the augmentation test and in 4 assays by

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[++] Throughout this section the term International Reference Preparation stands for International Reference Preparation for Human Menopausal Gonadotrophin.

Table 1.

Total dosage of test material per animal in different assays

Assay method	Material tested	Low dose (mg)	High dose (mg)
Augmentation test	NIH-FSH	0.02	0.08
	Human pituitary FSH	0.005	0.02
	I.R.P.*	1.0	4.0
	Pergonal	0.0625	0.25
	PMSG	1.0 i.u.	4.0 i.u.
Ovarian ascorbic acid depletion test	NIH-FSH	0.03	0.15
	NIH-LH	0.0003	0.0015
	Human pituitary FSH	0.005	0.025
	I.R.P.	0.3	1.5
	Pergonal	0.08	0.4
	PMSG	0.4 i.u.	2.0 i.u.
	HCG	0.2 i.u.	1.0 i.u.
Hypophysectomised rat prostate test	NIH-LH	0.01	0.04
	I.R.P.	2.5	5.0 or 10.0
	Pergonal	0.5	1.0
Mouse uterus test	NIH-FSH	0.04	0.08
	Human pituitary FSH	0.0125	0.025
	I.R.P.	0.9	1.8
	Pergonal	0.0625	0.125
	PMSG	0.25 i.u.	0.50 i.u.
Rat uterus test	I.R.P.	2.4	4.8
	Pergonal	0.2	0.4
Mouse ovarian weight test	HMG-20A	3.0	6.0
	PMSG	2.0 i.u.	4.0 i.u.

*I.R.P. International Reference Preparation

the ovarian ascorbic acid depletion test the reference materials were respectively NIH-FSH and NIH-LH (Table 4). In these 7 experiments the material tested was the Clinical Endocrinology Research Unit preparation of human pituitary FSH. Pergonal was used in 3 assays as a reference material to estimate the LH activity of HCG (Table 7).

III. RESULTS

A) NIH-FSH.

The biological activity of this material in terms of the International Reference Preparation is shown in Table 2. It will be noted that its FSH activity is approximately 68 times as high as, and its LH activity approximately 29 times as high as that of the International Reference Preparation. The gonadotrophic activity of NIH-FSH as estimated in terms of the International Reference Preparation by the mouse uterus test was found to be considerably lower than that obtained by the other 2 methods.

B) NIH-LH.

The LH activity of NIH-LH by the ovarian ascorbic acid depletion test is shown in Table 3. The results indicate that this material is a highly purified LH preparation, being approximately 2000 times more active than the International Reference Preparation.

When NIH-LH and Pergonal were compared using the hypophysectomised rat prostate test, significant deviation from parallelism occurred over the "working range" of the dose response curves. In this assay the slope of the dose response curve for Pergonal was relatively steep, while that of NIH-LH was very flat. In view of the significant slope difference between the two preparations it was not possible to express the results of the assay on a quantitative basis.

The gonadotrophic activity of NIH-LH as estimated by the

Table 2.

Biological activity of NIH-FSH in terms of the International Reference Preparation using various assay methods.

Bioassay	Design of Assay	Number of animals per assay	λ	Relative Potency	Fiducial Limits (P = 0.95)	Mean Relative Potency
Augmentation test	2 + 2	12	0.28	61.1	27.5 - 177.0	
	2 + 2	14	0.35	105.4	54.1 - 244.6	68.1
	2 + 2	14	0.17	67.0	40.2 - 114.6	
	2 + 2	16	0.26	39.0	14.4 - 80.3	
Ovarian ascorbic depletion test	2 + 2	20	0.45	28.2	19.4 - 94.9	
	2 + 2	20	0.19	40.7	29.7 - 59.8	28.7
	2 + 2	20	0.38	17.2	8.9 - 33.4	
Mouse uterus test	2 + 2	15	0.13	14.4	6.1 - 28.5	15.4
	2 + 2	15	0.10	16.3	10.8 - 23.2	

Table 3.

LH activity of NIH-LH in terms of the International Reference Preparation as measured by the ovarian ascorbic acid depletion test.

Design of Assay	Number of animals per Assay	λ	Relative Potency	Fiducial limits (P = 0.95)	Mean Relative Potency
2 + 2	20	0.29	2670	1362 - 4779	
2 + 2	20	0.36	3190	2200 - 4562	
2 + 2	20	0.19	1361	1007 - 1946	2051
2 + 2	20	0.26	1811	1014 - 2789	
2 + 2	20	0.22	1221	867 - 1721	

mouse uterus test is shown in Figure 4. It will be noted that the slope of the dose response curve is very flat and that very large dosages of the material are required to elicit a response. When NIH-LH was assayed by the mouse uterus test in terms of the International Reference Preparation or IRC-20A, significant deviation from parallelism occurred ($P < 0.001$) over the steepest parts of the dose-effect curves, and the assays were therefore invalid. Present evidence indicates that the mouse uterus test is not a suitable end-point for the estimation of gonadotrophic activity in highly purified LH preparations.

C) Human Pituitary FSH (Clinical Endocrinology Research Unit preparation).

This material is very rich in FSH activity, being 96 times more active than the International Reference Preparation (Table 4). Its LH activity was much lower than that of NIH-LH, using the ovarian ascorbic acid depletion test, but was twelve times higher than that of the International Reference Preparation using the hypophysectomised rat prostate test. The gonadotrophic potency of this preparation, as estimated by the mouse uterus test in terms of the International Reference Preparation lay between its FSH and LH activities.

D) Pergonal.

This preparation has 10 times more FSH and 5 times more LH activity than the International Reference Preparation (Table 5). Estimates of its LH activity obtained by the

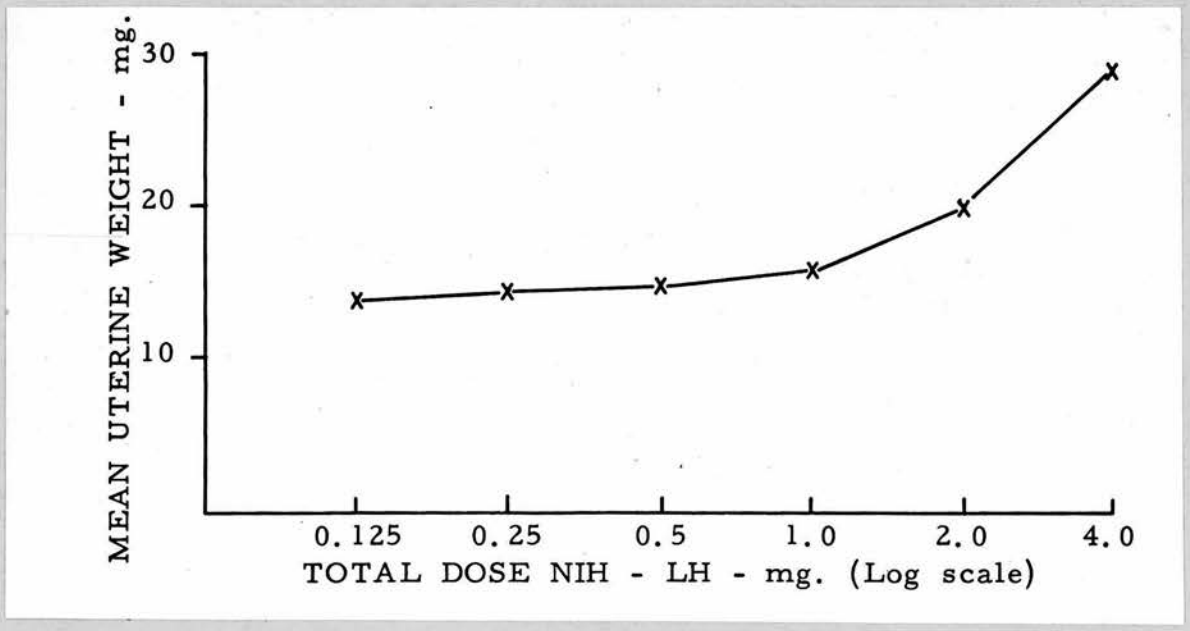

Fig. 4 :- Dose response curve for NIH-LH - mouse uterus test (4 animals per dose level).

Table 4

Biological activity of human pituitary FSH in terms of various reference materials.

Reference Material	Bioassay	Design of Assay	Number of animals per assay	λ	Relative Potency	Fiducial limits (P = 0.95)	Mean Relative Potency
NIH-FSH	Augmentation test	2 + 2	16	0.28	1.27	0.41 - 3.26	
		2 + 2	16	0.20	1.04	0.80 - 1.30	1.38
		2 + 2	16	0.30	1.83	0.15 - 3.95	
NIH-LH	Ovarian Ascorbic Acid Depletion test.	2 + 2	20	0.29	0.016	0.007 - 0.027	
		2 + 2	20	0.27	0.066	0.044 - 0.101	0.054
		2 + 2	20	0.19	0.081	0.079 - 0.083	
		2 + 2	20	0.17	0.052	0.038 - 0.071	
I.R.P.+	Augmentation test.	2 + 2	16	0.23	79.4	39.7 - 159.6	
		2 + 2	16	0.19	93.0	50.4 - 222.9	96.3
		2 + 2	16	0.24	116.6	31.4 - 215.5	
	Hypophysectomised rat prostate test	2 + 2	12	0.12	12.0	6.3 - 18.4	12.0
	Mouse Uterus test	2 + 2	16	0.10	66.0	50.2 - 85.1	69.0
		2 + 2	16	0.12	72.0	51.5 - 98.8	

+ I.R.P. = International Reference Preparation.

Table 5.

Biological activity of Pergonal in terms of the International Reference Preparation using various assay methods.

Bioassay	Design of Assay	Number of animals per assay	λ	Relative Potency	Fiducial limits (P = 0.95)	Mean Relative Potency
Augmentation test	2 + 2	16	0.20	10.5	5.7 - 17.3	
	2 + 2	16	0.18	9.1	5.1 - 14.4	10.05
	2 + 2	14	0.32	10.5	5.8 - 33.1	
Ovarian ascorbic acid depletion test.	2 + 2	20	0.47	3.6	1.60 - 11.1	
	2 + 2	20	0.27	10.9	6.2 - 25.5	
	2 + 2	20	0.18	3.5	2.5 - 5.4	5.3
	2 + 2	20	0.20	3.5	2.7 - 4.9	
	2 + 2	20	0.27	6.1	4.1 - 10.2	
	2 + 2	20	0.33	4.1	2.5 - 7.3	
Hypophysectomised rat prostate test.	2 + 1	12	0.16	5.0	2.3 - 6.6	5.0
Mouse uterus test	2 + 2	16	0.09	11.9	9.1 - 14.9	12.0
	2 + 2	16	0.12	12.1	8.3 - 16.4	
Rat uterus test	2 + 2	12	0.17	11.0	4.3 - 21.7	11.5
	2 + 1	9	0.13	12.0	7.1 - 16.7	

ovarian ascorbic acid depletion and hypophysectomised rat prostate tests agreed well, as did estimates of its gonadotrophic potency by the mouse uterus and rat uterus tests.

E) PMSG.

This material has more FSH and less LH activity than the International Reference Preparation (Table 6). The gonadotrophic potency of PMSG as estimated by the mouse uterus test was higher than either its FSH or LH activities. The potency ratio obtained by the mouse uterus test was approximately double that obtained by the mouse ovarian weight test.

F) HC G.

The LH activity of HCG in terms of the International Reference Preparation and Pergonal is shown in Table 7. It will be noted that the activity was only 1.7 times that of the International Reference Preparation and less than half that of Pergonal. When HCG was assayed by the mouse uterus test in terms of the International Reference Preparation significant deviation from parallelism occurred ($P < 0.001$) and the assay was therefore invalid.

Table 6.

Biological activity of HMG in terms of the International Reference Preparation using various assay methods.

Bioassay	Design of assay	Number of animals per assay	λ	Relative potency	Fiducial limits (P = 0.95)	Mean Relative potency
Augmentation test	2 + 2	12	0.18	1.6	0.9 - 3.2	
	2 + 2	14	0.08	1.3	0.1 - 1.6	1.3
	2 + 2	14	0.25	0.85	0.3 - 2.1	
Ovarian ascorbic acid depletion test	2 + 2	20	0.21	0.58	0.42 - 0.79	
	2 + 2	20	0.23	0.52	0.36 - 0.75	
	2 + 2	20	0.27	0.66	0.43 - 1.02	0.62
	2 + 2	20	0.41	0.98	0.52 - 2.40	
	2 + 2	20	0.40	0.36	0.12 - 0.71	
Mouse uterus test	2 + 2	16	0.08	2.6	2.0 - 3.2	2.9
	2 + 2	16	0.11	3.1	2.3 - 4.2	
Mouse ovarian[+] weight test	2 + 2	16	0.10	1.5	1.2 - 2.0	1.4
	2 + 2	16	0.10	1.3	1.0 - 1.7	

[+]Reference material HMG -20A.

Teble 7.

LH activity of HCG in terms of the International Reference Preparation and Pergonal as estimated by the ovarian ascorbic acid depletion test.

Reference material	Design of assay	Number of animals per assay	λ	Relative potency	Fiducial limits (P = 0.95)	Mean Relative potency
International Reference Preparation	2 + 2	20	0.27	1.27	0.84 - 1.96	1.71
	2 + 2	20	0.21	1.78	1.38 - 2.58	
	2 + 2	20	0.31	2.50	1.55 - 4.75	
	2 + 2	20	0.35	1.27	0.75 - 2.30	
Pergonal	2 + 2	20	0.18	0.53	0.41 - 0.70	0.43
	2 + 2	20	0.28	0.42	0.27 - 0.65	
	2 + 2	20	0.24	0.33	0.23 - 0.47	

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IV. SUMMARY OF BIOASSAY RESULTS

In Table 8 are shown the relative potencies obtained when four of the preparations studied were assayed in terms of the International Reference Preparation by bioassay methods specific for FSH or LH or by a non-specific method (see Tables 2, 4, 5, 6). It will be noted that the results obtained by the non-specific method (mouse uterus test) bear no constant relationship to those obtained either by the augmentation test for FSH or the ovarian ascorbic acid depletion test for LH.

The final column of Table 8 shows the indices of discrimination obtained when the FSH content is compared with the LH content of a given material

i.e. $\frac{\text{Relative potency by augmentation test}}{\text{Relative potency by ovarian ascorbic acid depletion test}}$

In all cases the index was above unity indicating a preponderance of FSH activity in the preparation.

Table 8.

Potencies relative to the International Reference Preparation of a number of gonadotrophin preparations when assayed by different tests.

Gonadotrophin tested	Relative Potencies			Index of discrimination FSH/LH
	Augmentation test (FSH)	Ovarian ascorbic acid depletion test (LH)	Mouse uterus test (non-specific)	
Human pituitary FSH	96.3	12.0+	69.0	8.03
NIH-FSH	68.1	28.7	15.4	2.37
Pergonal	10.1	5.3	12.0	1.90
PMSG	1.3	0.6	2.9	2.10

+Assays by hypophysectomised rat prostate test.

V. DISCUSSION

In the present investigation the biological nature of a number of gonadotrophins have been studied using a variety of assay methods including highly specific tests for both FSH and LH activities. In the great majority of assays the results were expressed in terms of the International Reference Preparation. The use of specific methods to determine separately the FSH and LH content of the test materials appears preferable to the "fingerprinting" system of biological characterisation recommended by Albert and his colleagues (Albert & Kelly, 1958 a, b; Albert, Kelly & Kobi, 1958). In this system non-specific assays in intact animals are employed and it is usually necessary to test the gonadotrophin in question at several dose levels before reliable information on its biological nature can be obtained. Furthermore, the "fingerprinting" system does not give any quantitative information about the FSH and LH content of an unknown gonadotrophin preparation.

The present study has shown that the FSH activity of NIH-FSH is lower than that of material prepared from human pituitary tissue (Clinical Endocrinology Research Unit preparation) and higher than that from human postmenopausal urine (Pergonal). The FSH and LH activities of NIH-FSH were respectively sixty-eight and twenty-eight times higher than those of the International Reference Preparation. It has previously been shown by Schmidt-Elmendorff and Loraine

(1962) that NIH-FSH is contaminated with LH. Thus when this material was compared with the highly purified NIH-LH rather than with the International Reference Preparation by the ovarian ascorbic acid depletion test the figure for its LH activity was very low, the mean potency ratio being 0.00904. This figure compares well with that of 0.006 obtained by Parlow using the same test. (Unpublished observation).

NIH-LH has been shown to be a preparation of high specific activity (Parlow, 1961) and in the present study it was found to be some 2000 times more potent than the International Reference Preparation using the ovarian ascorbic acid depletion test. When the LH activities of NIH-LH and Pergonal were compared using the hypophysectomised rat prostate test significant deviation from parallelism occurred and the assay was therefore invalid.

The human pituitary FSH had the highest FSH activity of any preparation tested and was also richest in its "total gonadotrophic activity" as estimated by the mouse uterus test. The material employed was prepared by the method of Li, Simpson & Evans (1949); a similar method was employed by Gemzell, Diczfalusy & Tillinger (1958) who obtained a material which had a slightly lower FSH activity than the Edinburgh preparation.

The "total gonadotrophic" activity of Pergonal as estimated by the mouse and rat uterus tests was approximately twelve times higher than that of the International Reference

Preparation. Its FSH activity was ten times higher but its LH activity was only five times higher than that of the reference material. In view of the fact that both Pergonal and the International Reference Preparation were prepared from postmenopausal urine it must be concluded that either Pergonal has lost LH or the International Reference Preparation has lost FSH during preparation. The gonadotrophin of pregnant mare's serum (PMSG) is usually stated to be predominantly follicle-stimulating in action (Cole & Hart, 1930; Catchpole & Lyons, 1934; Day & Rowlands, 1940). The present study has shown that this hormone contains a considerable amount of LH activity, the ratio FSH/LH being 2.10. The international standard for HCG appears to be a relatively weak LH preparation, being only 1.71 times as active as the International Reference Preparation and less than half as active as Pergonal.

There is no agreement in the literature regarding the type of gonadotrophic activity estimated by the mouse uterus test. Some investigators, for example Klinefelter, Albright & Griswold (1943), have stated that the test measures mainly FSH activity, while others, e.g. Buchholz (1957) have suggested that the method measures predominantly LH activity. The relative lack of sensitivity of the mouse uterus test should be emphasised and it has been suggested by Loraine (1958) that the material measured by this assay should be designated as the "total gonadotrophic" activity of the

preparation.

The present study has shown (Table 8) that the mouse uterus test does not appear to measure the mean of the FSH and LH potencies of any given preparation, their product or any simple function of the two activities. Thus in the case of NIH-FSH and Pergonal similar "total gonadotrophic" activities as measured by the mouse uterus test are associated with widely differing FSH and LH potencies. It is suggested that in view of the present evidence and that of Brown & Billewicz (1962) the term "total gonadotrophic" activity is inappropriate. The mouse uterus test has been shown to be unsuitable for the assay of highly purified LH materials such as NIH-LH because of the very flat slope of the dose response curve (Fig. 4). This observation is in keeping with the hypothesis that a satisfactory response in the mouse uterus test can only be obtained by the combined actions of FSH and LH.

VI. SUMMARY

The FSH activity of NIH-FSH was 70 times as high, and its LH activity was 30 times as high as that of the International Reference Preparation. The specific activity of NIH-LH was high, the material being approximately 2000 times more potent than the International Reference Preparation. Material prepared from human pituitary tissue (Clinical Endocrinology Research Unit preparation) contained the highest FSH activity of any preparation tested. Its LH activity was, however, considerably lower than that of NIH-LH.

Pergonal had ten times more FSH and five times more LH activity than the International Reference Preparation. Pregnant mares' serum gonadotrophin contained, in addition to its FSH activity, a considerable amount of LH activity, the ratio FSH-LH being 2.10. The international standard for human chorionic gonadotrophin was shown to be a relatively weak LH preparation, being only slightly more active than the International Reference Preparation.

Results obtained by the mouse uterus test did not appear to bear any constant relationship to those obtained by specific methods for either FSH or LH.

4. OBSERVATIONS ON THE KAOLIN-ACETONE METHOD FOR THE EXTRACTION OF PITUITARY GONADOTROPHINS FROM HUMAN URINE

1. INTRODUCTION

In 1941 Scott described a method for the extraction of human chorionic gonadotrophin from pregnancy urine. The procedure depended on adsorption of the hormone on to kaolin at pH4 followed by elution with 0.1 N-NaOH. The extracts prepared by this method were not sufficiently concentrated to permit the estimation of gonadotrophins in non-pregnant urine. More recently a number of workers including Bradbury, Brown and Brown (1949), Dekanski (1949 and Loraine (1950 a) have modified the original kaolin adsorption procedure by precipitating the gonadotrophins with acetone or alcohol after elution with sodium hydroxide. In 1959 Loraine and Brown re-investigated the kaolin-acetone procedure and showed that when this method was applied to the extraction of pituitary gonadotrophins from urine the extracts prepared were satisfactory for the quantitative estimation of HPG in the urine of male and non-pregnant female subjects. The main disadvantages of this method have been shown by Borth, Lunenfeld & Menzi (1961) to be the low specific activity and the relatively high toxicity of the extract when administered to experimental animals.

2. KAOLIN-ACETONE EXTRACTION PROCEDURE

This method as described by Loraine and Brown (1959) is currently employed in the laboratory in Edinburgh for the extraction of HPG from the urine of non-pregnant human subjects. The method is performed as follows :-

1. Complete 24 hour urine specimens were stored without preservative in a refrigerator at 4°C. When the 24 hour volume was less than 1200 ml the urine was diluted with water up to this volume before processing.

2. The urine was adjusted to pH 4.0 with 5N-HCl and 20 gm. acid washed kaolin was added.

3. The mixture was stirred mechanically for one hour and the kaolin was allowed to settle overnight.

4. The supernatant urine was decanted and the kaolin was centrifuged at a relative centrifical force (R.C.F.) of 1,000 for 20 minutes.

5. The kaolin deposit was suspended in 40 ml. distilled water and the suspension adjusted to pH 11.3 with 5N-NaOH. The alkaline suspension was allowed to stand for 20 minutes with occasional stirring before being centrifuged at an R.C.F. of 1,000 for 20 minutes.

6. The pH of the supernatant fluid was adjusted to 4.0 - 5.0 with 5N-HCl and the gonadotrophins were precipitated with 5 volumes of acetone.

7. The precipitate obtained was centrifuged at an R.C.F. of 1,000 for 20 minutes and the supernatant acetone was decanted. The extract was washed with

absolute alcohol and ether before being dried over calcium chloride.

The principle of this method depends on the adsorption of the gonadotrophic material onto kaolin followed by elution with sodium hydroxide. Thus, any improvement to the extraction procedure would entail the purification either of the native urine sample or alternatively of the extract obtained after elution of the gonadotrophin from the kaolin. The aim of this section is to report a series of experiments which have been undertaken to examine these possibilities.

3. RESULTS

A) Reduction in weight of final extract: At the workshop conference on Human Pituitary Gonadotrophins in 1961 Dekanski stated that one of the disadvantages of applying the kaolin adsorption method to bulk extraction of post-menopausal urine was that the alkali employed for the elution of the HMG simultaneously dissolved out silica from the kaolin. He suggested that the gonadotrophin in the final extract might be adsorbed on to silica and that this might lead to a reduction in the apparent potency of the preparation. The following experiments were carried out to investigate this point in relation to the extraction of HPG from small volumes of urine.

Five one litre pools of distilled water were "extracted" by the kaolin acetone method as described on page 51 . Table 9 shows the weight of the precipitates obtained in this experiment. A mean weight of 292.5 mg was obtained and in view of the fact that kaolin was the only solid material employed the final precipitate must therefore consist mainly of this substance. This observation may be explained in two ways, either that some of the kaolin had dissolved during the extraction procedure and was subsequently reprecipitated, or that a portion of the kaolin remained in suspension after being centrifuged following the alkaline elution step. These possibilities were tested in the next experiment.

Five one litre pools of distilled water were "extracted" by the kaolin acetone procedure up to the alkaline elution step. The supernatant fluid obtained after treatment with

alkali was re-centrifuged at an R.C.F. of 2,000 for 20 minuted and the precipitate obtained was dried and weighed (precipitate A). The remaining supernatant was then precipitated with acetone after the pH had been adjusted to 4.0 - 5.0 and washed with alcohol and ether before being dried (precipitate B). The weight of the precipitates obtained are shown in Table 10. It will be noted that the mean of the combined weight of the two precipitates was 321.1 mg. and that only 14.9% (45.5 mg.) could be removed by centrifugation. It is therefore reasonable to conclude that the majority of the kaolin found in the final precipitate was in solution and was re-precipitated by the addition of acetone. This amounts to approximately 1% of the original kaolin and compares favourably with the data reported by Dekanski (1961).

The hypothesis that the kaolin is dissolved by sodium hydroxide was tested in an experiment in which the alkaline elution step was omitted from the kaolin extraction procedure. The results obtained are shown in Table 11. A mean weight of 287.2 mg. was obtained from three samples in which water was "extracted" by the kaolin acetone method while no precipitate was found when the sodium hydroxide elution step was eliminated. This suggests that the kaolin was dissolved by the alkali and subsequently re-precipitated by the addition of acetone.

The kaolin supplied to the laboratory is acid washed but has not been treated with alkali. The effect of washing it with alkali before use in the extraction procedure was

Table 9

Extraction of water by the kaolin acetone method without modification.

Pool No.	Wt. of final precipitate (mg.)
1	269.1
2	276.6
3	307.1
4	337.0
5	272.9
Mean	292.5

Table 10

Extraction of water by the kaolin acetone method with separation of kaolin in suspension (precipitate A) and kaolin in solution (precipitate B) after the alkaline elution step.

Pool No.	Precipitate A(mg.)	Precipitate B(mg.)	Total (mg.)
1	52.2	253.2	305.4
2	57.0	348.7	405.7
3	43.2	270.9	314.1
4	37.9	248.3	286.2
5	38.0	246.9	284.9
Mean	45.7	273.6	319.3

Table 11.

Extraction of water by the kaolin acetone method with and without the alkaline elution step.

Treatment	Pool No.	Wt. of final precipitate (mg.)	Mean (mg.)
Control	1	303.1	
	2	278.2	287.2
	3	280.2	
Alkaline elution	1	0	
step omitted	2	0	0
	3	0	

studied in the following experiment.

160 gm. of kaolin were suspended in 1200 ml. dilute NaOH at pH 12 and allowed to stand overnight before being centrifuged at an R.C.F. of 700 for 20 minutes. The supernatant was discarded, the kaolin washed with distilled water and the pH adjusted to 6.0 with 5N-HCl. The suspension was recentrifuged before being washed with water, centrifuged again and finally dried in a hot air oven. This alkali-washed material was compared with untreated kaolin and the results are shown in Table 12. Ten one litre pools of distilled water were used. Five pools were extracted with untreated kaolin and five pools with alkali washed-kaolin. It will be seen that the weight of the final precipitate was reduced by 73% (from 330.7 mg. to 89.0 mg.) when alkali treated kaolin was used. The conclusion may be drawn that the majority of the alkali-soluble material had been removed from the kaolin.

In the final experiment alkali washed kaolin was compared with the untreated material in the extraction of HPG from normal male urine. The results of two such experiments are shown in Table 13. No difference was observed in the weight of the final precipitate obtained by either extraction procedure. It should be noted that when water was extracted with alkali-washed kaolin a marked reduction in the weight of the final precipitate was obtained, but a comparable decrease was not observed in the case of male urine. For this reason the modification described appears to be of no

Table 12.

Comparison of alkali washed and untreated kaolin in the "extraction" of distilled water.

Treatment	Pool No.	Wt. of final precipitate (mg.)	Mean (mg.)
	1	383.7	
Control:	2	268.4	
Untreated	3	381.5	330.7
Kaolin	4	300.1	
	5	320.0	
Alkali	1	104.2	
Washed	2	96.6	
Kaolin	3	74.6	89.0
	4	84.3	
	5	85.1	

Table 13.

Comparison of alkali washed and untreated kaolin when applied to male urine.

Pool No.	Wt. of final precipitate (mg.)	
	Control: untreated kaolin	Alkali washed kaolin
1	366.9	278.2
2	257.9	280.1
Mean	312.4	279.2

practical value when applied to the extraction of HPG from urine specimens.

B) Effect of filtration of native urine. A small series of experiments have been carried out to investigate the effect of filtering complete 24 hour urine specimens before extraction by the kaolin acetone method. It is frequently not practicable to extract urine specimens immediately after collection and because of this individual samples sometimes contain a heavy precipitate consisting mainly of phosphate. In view of the possible interference of this precipitate with the adsorption of the gonadotrophic material on kaolin, the effect of filtering the urine prior to the extraction has been studied.

In the first experiment a pool of 8 litres of post-menopausal urine was employed. This was divided into 8 one litre aliquots, two of which acted as control and were extracted by the kaolin acetone method without modification. Three aliquots were filtered on a Buchner funnel through Whatman No. 42 filter paper on the day of the extraction, and subsequently treated in the same way as the control specimens. The remaining three aliquots were adjusted to pH 11.3 with 5N-NaOH and were allowed to stand overnight before being filtered through Whatman No. 42 filter paper. All 8 samples were extracted at the same time. Bioassay of the final extract was performed by the mouse uterus test and the results obtained are shown in Table 14. It will be noted that a marked increase in HPG activity over the control value was observed in the specimens which were filtered and a further

Table 14.

Effect of filtration of urine prior to extraction by the kaolin acetone method on HPG activity in postmenopausal urine.

Treatment	Wt. of final extract(mg.)	Design of assay	λ	HMG units/ litre	Fiducial limits (P = 0.95)	Mean R.P.
Control	170.4	2 + 2	0.12	18.3	8.1 - 26.8	18.6
	179.2	2 + 1	0.14	18.8	6.8 - 28.6	
Filtered	173.8	2 + 2	0.16	34.7	22.6 - 75.3	
	159.8	2 + 2	0.10	40.5	30.4 - 54.2	43.5
	149.4	2 + 2	0.15	55.4	36.0 -125.9	
pH 11.3	160.0	2 + 1	0.14	52.3	34.0 -111.8	
	166.4	2 + 1	0.15	64.9	41.5 -116.2	70.7
	163.2	2 + 1	0.14	95.0	64.6 - 218.6	

increase in the level of HPG recovered and noted in the urine samples which were filtered following adjustment of the pH.

This experiment was repeated using a pool of male urine and the results are shown in Table 15. No marked difference in HPG activity was observed and the fiducial limits of the control samples overlap with those of the experimental pools.

In view of the finding that many urine samples become alkaline on standing it was hoped that adjusting the pH of the sample to 11.3 would enhance the precipitation of any material which might interfere at a later stage in the extraction procedure. It was noted that increased precipitation did occur but it was not possible to weigh the material obtained because it was gelatinous in nature.

The finding of increased HPG in post-menopausal urine has subsequently been confirmed, but the explanation of this observation and the difference observed between the two types of urine studied is at present obscure. It is theoretically possible that the increase in gonadotrophic activity observed in post-menopausal urine might be due to a reduction in the toxicity of the final extract rather than to any alteration in the amount of HPG present. Another explanation is that the gonadotrophin in the urine obtained from postmenopausal women is in some way "bound" and that mild alkaline hydrolysis for 24 hours might liberate the gonadotrophic material in a biologically active form. This latter hypothesis was

Table 15.

Effect of filtration of urine prior to extraction by the kaolin acetone method on HPG activity in male urine.

Treatment	Wt. of final extract(mg.)	Design of assay	λ	HMG units/ litre	Fiducial limits (P = 0.95)	Mean R.P.
Control	485.2	2 + 2	0.13	5.9	3.4 - 8.4	6.2
	596.0	2 + 1	0.12	6.5	3.8 - 8.1	
Filtered	369.4	2 + 2	0.11	7.7	5.5 - 10.7	8.7
	448.6	2 + 1	0.19	10.2	4.0 - 20.0	
	387.4	2 + 2	0.15	8.1	4.1 - 12.3	
pH 11.3	333.8	2 + 1	0.14	7.5	3.4 - 11.1	5.4
	377.8	2 + 2	0.13	3.3	0.8 - 5.5	
	435.6	2 + 1	0.11	5.5	2.2 - 8.3	

tested by adsorbing the HPG from a pool of postmenopausal urine on to kaolin (1st extraction) and then re-extracting the supernatant urine after adjusting the pH to 11.3 and allowing the specimen to stand for 24 hours. The results obtained in this experiment are shown in Table 16. It will be seen that less than 3 HMG units per litre was recovered in the supernatant of the 2nd extraction suggesting that the great majority of the active material had previously been adsorbed.

A further explanation of the results obtained in this section is that an alteration in the ratio of FSH to LH had resulted in a higher relative potency of the extracts. As yet no specific assays for either FSH or LH have been carried out to test this supposition.

C) The effect of incorporation of an ammonium acetate-ethanol step into the kaolin acetone method. In 1961 Albert, Kobi, Leiferman & Derner described an ammonium acetate-ethanol purification step for the preparation of highly purified urinary HPG extracts. It was considered that such a step might be of value prior to the bioassay of HPG in urine by the ovarian ascorbic acid depletion test in which the necessity to administer the material to be tested by the intravenous or intraperitoneal route makes it obligatory that the extract be of minimum toxicity. Accordingly the procedure was used as a concluding step in the kaolin acetone method and was applied to the urine of normally menstruating women.

In the procedure of Albert et al (1961) the gonadotrophin

Table 16.

Effect of extraction of HPG from postmenopausal urine followed by re-extraction of the same urine by the kaolin acetone method.

Treatment	Design of assay	λ	HMG units/ litre	Fiducial limits (P = 0.95)
First extraction	2 + 2	0.13	30.6	14.4 - 44.1
Supernatant urine	2 + 2		< 3.0	

was extracted with 10% ammonium acetate in 70% ethanol and then precipitated with 10% ammonium acetate in absolute ethanol. It was noted in this laboratory that in the ovarian ascorbic acid depletion test this purification step did not reduce the toxicity of the extract sufficiently to permit the routine assay of LH in the urine of normally menstruating women. An attempt was then made to incorporate the ammonium acetate-ethanol step into the kaolin acetone method rather than to use it as the concluding step as described by Albert et al (1961).

In the first experiment 10 litres of postmenopausal urine were divided into ten one litre aliquots. Two pools were extracted by the kaolin acetone method without modification and these acted as controls for the remaining pools which were extracted by the kaolin acetone method up to the alkaline elution step. The pH of the eluate was adjusted to 7 and the following additions were made to produce the final concentrations of ammonium acetate and ethanol listed below :-

a) 2 pools : 50% ethanol.

b) 2 pools : 10% ammonium acetate in 30% ethanol.

c) 2 pools : 10% ammonium acetate in 40% ethanol.

d) 2 pools : 10% ammonium acetate in 50% ethanol.

The samples were allowed to stand overnight and the precipitate which formed was centrifuged at an R.C.F. of 1000 for 10 min. (Precipitate A). Two volumes of absolute ethanol were added to the supernatant and the precipitate

obtained (Precipitate B) was centrifuged at an R.C.F. of 1000 for 20 min. It was then washed with ether before being dried over calcium chloride. All bioassays in this experiment and throughout the study were conducted by the mouse uterus test and the results were expressed in terms of the International Reference Preparation as HMG units per litre of urine.

In Table 17 are shown the assay data from this experiment. It will be seen that Precipitate A of the urine specimens extracted by the modified kaolin acetone method contained no measurable gonadotrophin while the activity found in Precipitate B did not differ significantly from that in the control specimens.

In view of the finding that 10% ammonium acetate in 30% ethanol resulted in the smallest final precipitate it was decided to use this concentration of ethanol in the next experiment in which the effect of adding different amounts of ammonium acetate was studied.

12 litres of postmenopausal urine were divided into twelve one litre aliquots. 4 of these were control specimens and the remaining pools were extracted by the kaolin acetone method up to step 6. At this stage ammonium acetate and ethanol were added to give the following concentrations :-

a) 2 pools : 5% ammonium acetate in 30% ethanol.

b) 2 pools : 10% ammonium acetate in 30% ethanol.

c) 2 pools : 15% ammonium acetate in 30% ethanol.

d) 2 pools : 20% ammonium acetate in 30% ethanol.

Table 17.

Effect of incorporation of an ammonium acetate ethanol step into the kaolin acetone method when applied to the extraction of HPG from postmenopausal urine : alteration of ethanol concentration.

Treatment	Precipitate wt. (mg.)		Precipitate A		Precipitate B				
	A	B	Design of Assay	HMG units/ litre	Design	λ	HMG units/ litre	Fiducial Limits (P = 0.95)	Mean HMG units/ litre
Control					2 + 1	0.13	22.9	7.3 - 35.5	24.8
					2 + 2	0.14	26.8	12.1 - 39.9	
50% Ethanol	26.5	343.0	2 + 1	< 3.0	2 + 2		Invalid*		41.4
	22.8	325.5	2 + 1	< 3.0	2 + 1	0.15	41.4	29.0 - 88.6	
10% A.A.+ in 30% Ethanol	304.6	86.3	2 + 1	< 3.0	2 + 2		Invalid*		23.6
	328.7	79.2	2 + 1	< 3.0	2 + 2	0.12	23.6	11.6 - 33.7	
10% A.A.+ in 40% Ethanol	290.0	110.9	2 + 1	< 3.0	2 + 2	0.15	29.7	13.9 - 45.1	24.5
	301.0	118.6	2 + 1	< 3.0	2 + 2	0.12	19.3	7.9 - 28.6	
10% A.A.+ in 50% Ethanol	349.0	110.0	2 + 1	< 3.0	2 + 2	0.09	36.0	27.0 - 46.4	36.0
	355.3	116.3	2 + 1	< 3.0	2 + 2	0.10	36.0	25.9 - 48.2	

* = Lack of parallelism between dose response lines

\+ = ammonium acetate

The specimens were allowed to stand overnight and the resultant precipitate discarded. The gonadotrophin was precipitated by the addition of two volumes of absolute ethanol before being dried. All the powders were assayed by the mouse uterus test and the results obtained are shown in Table 18. In this experiment the activity of the control specimens was below the range normally encountered in post-menopausal women (35 to 158 HMG units/24 hours: Apostolakis & Loraine, 1960). The results are thus very difficult to interpret but it should be noted that the mean relative potency obtained for the experimental pools did not differ markedly from the value for the control specimens. The mean weight of the final precipitate of the four control samples was 211.3 mg. and a reduction of 43% was noted in the two pools to which 10% ammonium acetate was added. In view of the fact that Albert et al (1961) recommended the use of 10% ammonium acetate in their purification step it was decided to use this concentration in future experiments.

The efficiency of the purification step was then tested in postmenopausal and male urine in five experiments (see Tables 19 to 23). The control specimens in each experiment were extracted by the kaolin acetone method without modification, while in the remaining specimens the purification step was introduced after the gonadotrophin had been eluted from the kaolin with sodium hydroxide. At this stage 8 g. ammonium acetate in 40 ml. of 60% ethanol was added to produce a final concentration of 10% ammonium acetate in 30% ethanol. The

Table 18.

Effect of incorporation of an ammonium acetate ethanol step into the kaolin acetone method when applied to the extraction of HPG from postmenopausal urine : Alteration of ammonium acetate concentration.

Treatment	Precipitate wt. (mg.)	Design	λ	HMG units/ litre	Fiducial Limits ($P = 0.95$)	Mean HMG units/ litre
Control	182.8	2 + 1	0.13	8.2	3.2 - 11.8	5.4
	232.0	2 + 2	0.17	4.8	0.3 - 8.9	
	210.0	2 + 2	0.16	4.8	1.7 - 7.3	
	220.4	2 + 2	0.20	4.2	0.2 - 7.4	
5% ammonium acetate	106.0	2 + 2	0.15	4.0	0.9 - 6.6	4.0
	126.0	2 + 2		*Invalid		
10% ammonium acetate	108.0	2 + 2	0.19	12.7	2.3 - 22.6	15.6
	132.0	2 + 1	0.19	18.5	10.2 - 57.7	
15% ammonium acetate	68.6	2 + 2	0.09	11.5	8.3 - 14.6	11.5
	83.4	2 + 2		*Invalid		
20% ammonium acetate	57.4	2 + 1		< 4.0		< 4.0
	64.4	2 + 1		< 4.0		

* Lack of parallelism between dose response lines

Table 19.

Comparison of the kaolin acetone method with and without an ammonium acetate ethanol step when applied to the extraction of HPG from postmenopausal urine.

Pool 1.

Treatment	Precipitate wt. (mg)	Design	λ	HMG units/ litre	Fiducial Limits (P = 0.95)	Mean HMG units/litre
Control	410.0	2 + 2	0.12	25.3	14.2 - 35.2	26.3
	211.6	2 + 1	0.17	33.1	20.2 - 96.6	
	388.0	2 + 2	0.14	22.3	9.6 - 32.7	
	369.0	2 + 2	0.09	24.5	16.4 - 31.9	
Modified Extraction Procedure	170.0	2 + 2	0.08	31.2	24.6 - 39.6	28.8
	160.0	2 + 2	0.11	26.4	16.4 - 35.6	

Table 20.

Comparison of the kaolin acetone method with and without an ammonium acetate ethanol step when applied to the extraction of HPG from postmenopausal urine.

Pool 2.

Treatment	Precipitate wt. (mg.)	Design	λ	HMG units/ litre	Fiducial Limits (P = 0.95)	Mean HMG units/litre
Control	160.0	2 + 1	0.18	15.2	2.7 - 24.9	15.1
	182.9	2 + 2	0.20	15.1	3.1 - 51.5	
	173.1	2 + 2	0.15	15.1	7.4 - 22.8	
Modified Extraction Procedure	99.7	2 + 2	0.14	13.6	6.4 - 20.1	16.0
	99.0	2 + 2	0.11	17.2	11.7 - 23.6	
	101.9	2 + 2	0.13	15.7	9.3 - 22.6	
	101.6	2 + 2	0.21	17.6	2.6 - 46.6	

Table 21.

Comparison of the kaolin acetone method with and without an ammonium acetate ethanol step when applied to the extraction of HPG from male urine.

Pool 1.

Treatment	Precipitate wt. (mg.)	Design	λ	HMG units/ litre	Fiducial Limits (P. = 0.95)	Mean HMG units/litre
Control	-	2 + 1	0.14	14.3	8.3 - 19.2	11.6
	498.3	2 + 2	0.17	12.1	4.4 - 19.9	
	441.0	2 + 2	0.15	8.3	2.2 - 13.0	
Modified Extraction Procedure	11.3	2 + 2		< 2.0		< 2.0
	26.2	2 + 2		< 2.0		
	18.0	2 + 2		< 2.0		
	8.4	2 + 2		< 2.0		
	11.8	2 + 2		< 2.0		
	14.8	2 + 2		< 2.0		

Table 22.

Comparison of the kaolin acetone method with and without an ammonium acetate ethanol step when applied to the extraction of HPG from male urine.

Pool 2.

Treatment	Precipitate (wt. (mg))	Design	χ	HMG units/ litre	Fiducial Limits (P = 0.95)	Mean HMG units/litre
Control	439.4	2 + 2	0.12	7.5	4.8 - 11.4	8.3
	444.2	2 + 2	0.09	12.2	9.2 - 19.4	
	513.2	2.+ 2	0.16	5.1	1.9 - 7.9	
Modified Extraction Procedure	23.0	2 + 2		< 2.0		< 2.0
	16.4	2 + 2		< 2.0		
	14.2	2 + 2		< 2.0		

Table 23.

Comparison of the kaolin acetone method with and without an ammonium acetate ethanol step when applied to the extraction of HMG from male urine.

Pool 3.

Treatment	Precipitate wt. (mg.)	Design	λ	HMG units/ litre	Fiducial Limits P = 0.95	Mean HMG units/litre
Control	463.4	2 + 1	0.15	10.9	6.3 - 18.2	10.3
	452.4	2 + 2	0.08	9.1	6.8 - 12.3	
	485.4	2 + 2	0.08	9.6	6.3 - 12.5	
	447.4	2 + 2	0.08	11.7	9.0 - 15.0	
Modified Extraction Procedure	29.8	2 + 2		< 2.0		< 2.0
	59.6	2 + 2		< 2.0		
	47.8	2 + 2		< 2.0		
	-	2 + 2		< 2.0		

precipitate which formed was removed and the gonadotrophin was precipitated by the addition of 2 volumes of absolute ethanol.

In Tables 19 and 20 are shown the bioassay results obtained when aliquots from 2 pools of postmenopausal urine were extracted by the kaolin acetone method and modification described above. No significant difference was noted between the biological activity of the control and experimental pools. There was however, a decrease in the weight of the final precipitate when the urine specimen was extracted by the modified kaolin acetone method.

The bioassay data obtained when aliquots from 3 pools of male urine were extracted in a similar manner are shown in Tables 21, 22 and 23. The gonadotrophic activity observed in the control specimens was within the range normally encountered in male subjects (Loraine 1958). There was however no detectible gonadotrophin present in any of the pools extracted by the modified procedure and in view of the finding that no HPG could be detected in the first precipitate formed after the addition of the ammonium acetate and ethanol it must be concluded that the majority of the gonadotrophin had been denatured and was no longer biologically active. Because of the apparent inactivation of the gonadotrophic activity in male urine it was decided not to employ the ammonium acetate-ethanol step in the kaolin acetone method for the routine extraction of urinary HPG. It should however be noted that the modification to the kaolin acetone method described in this section was not applied to the extraction of gonadotrophins from the urine of women during reproductive life.

4. DISCUSSION

Over the past three decades a large number of methods have been described for the extraction of HPG from urine. The procedures differ considerably in nature and complexity but a common feature of many of these methods is that the gonadotrophin is adsorbed onto an inert material in the first step of the extraction technique. The substances used have included benzoic acid, aluminium hydroxide, permutit and attapulgite (Silicious clay) as well as kaolin. The kaolin acetone procedure as described by Loraine and Brown (1959) has been shown by Loraine and Mackay (1961) to provide a yield of gonadotrophin comparable to that obtained with several of the other extraction procedures currently in use. It can be seen from the results obtained in the present series of experiments that this method is not entirely satisfactory.

Three points of interest emerge from the data presented: (i) Kaolin was found in the final precipitate when water was extracted by the kaolin acetone method. (ii)An increase in the biological activity of the HPG recoverable from post-menopausal but not from male urine was obtained by filtration of the specimen prior to extraction. (iii) The incorporation of an ammonium acetate ethanol step into the kaolin acetone method resulted in a reduction in the weight of the final precipitate without loss of biological activity in the case of postmenopausal urine. When male urine was treated in a similar manner no HPG could be detected in the final precipitate.

In the experiments reported in this section there are thus two examples of an apparent difference between the HPG of male and postmenopausal urine. Thus, the gonadotrophin present in male urine was more readily inactivated following treatment with ammonium acetate and ethanol. It was also noted that filtration of the samples resulted in an increase in HPG activity of the urine obtained from postmenopausal but not from male subjects. Because of the lack of specificity of the mouse uterus test it is difficult to explain the differences observed when male and postmenopausal urine samples were treated in a similar manner. It is however theoretically possible that the procedures employed had resulted in an alteration of the ratio of FSH/LH activity which could not be measured by the bioassay procedure employed. Further information on this point will only be obtained by the separate determination of FSH and LH activities in urine.

5. SUMMARY

The kaolin acetone extraction procedure for HPG was described in detail and a number of possible modifications to the method were investigated.

Following the extraction of water by this technique it was shown that approximately 1% of the kaolin used in the adsorption stage was found in the final extract. It was also noted that the weight of the final precipitate could be reduced by washing the kaolin with alkali. When this modification was applied to urine specimens the procedure was found to make no difference to the weight of the final extract.

After the filtration of the urine prior to extraction by the kaolin acetone method an increase in the gonadotrophic activity recoverable from postmenopausal but not from male urine was observed.

The incorporation of an ammonium acetate ethanol purification step into the kaolin acetone method resulted in a decrease in the weight of the final extract without loss of biological activity from postmenopausal urine. When applied to male urine this modification resulted in the inactivation of all the gonadotrophic activity.

5. APPLICATION OF ASSAY METHODS TO EXPERIMENTAL PROBLEMS

I. THE EFFECT OF 6 M UREA AND FORMALDEHYDE ON THE BIOLOGICAL ACTIVITY OF VARIOUS GONADOTROPHIN PREPARATIONS

1. INTRODUCTION

Over the past three decades a number of workers have shown that the biological activity of the gonadotrophic hormones is diminished by the action of various reagents. The effect of oxidation, reduction, acetylation and enzymic hydrolysis have been extensively studied and certain differences in the relative stability of FSH and LH have been noted.

Fraenkel-Conrat, Simpson & Evans (1939, 1940) and McShan and Meyer (1940) showed that reduction with cysteine diminished the activity of sheep FSH, while Li, Simpson & Evans (1940 a) and Fraenkel-Conrat *et al.* (1940) demonstrated that the same material completely destroyed sheep LH. Similar results were obtained after acetylation with ketene by Li, Simpson & Evans (1939). Chow, Greep & van Dyke (1939) found that under certain conditions hog FSH was stable to tryptic digestion and was only partially destroyed by chymotrypsin. Hog LH on the other hand was completely destroyed by both enzymes.

More recently reactions with salt solutions have been studied. Légault-Demare, Clauser & Jutisz (1958) purified a preparation of pregnant mare's serum gonadotrophin by

removing the insoluble material precipitated with barium chloride and Anntoniades, Pennell, McArthur, Ingersoll, Ulfelder & Oncley (1957) have used zinc sulphate and ethanol to precipitate gonadotrophins from human plasma. Butt, Crooke, Cunningham & Evans (1961) have also studied the effect of a number of reagents on the activity of human and pituitary gonadotrophin and noted that sodium borohydride destroyed LH more readily than FSH.

This section describes a study undertaken in order to obtain further information about the inactivation of gonadotrophins by various materials. Data are presented on the biological activity of a number of gonadotrophin preparations following incubation with either urea or formaldehyde.

2. THE EFFECT OF 6M UREA ON THE BIOLOGICAL ACTIVITY OF VARIOUS GONADOTROPHIN PREPARATIONS

The aim of this section of the study was to extend the observation made by Ellis (1961 a, b) who showed that when pituitary gonadotrophic extracts were incubated with 6M urea, luteinizing hormone activity was greatly reduced, while follicle-stimulating hormone activity was virtually unaffected. To this end, the effect of 6M urea on the LH, FSH and "total gonadotrophic" activities of preparations from a number of different sources have been investigated.

A. Materials and Methods

Three bioassay procedures were employed in this study. The augmentation test, the ovarian ascorbic acid depletion test and the mouse uterus test. (See pages 21 to 26).

Incubations were carried out using both 6M urea and distilled water. The hormones were dissolved in 2.0 ml. 6M urea or in 2.0 ml. distilled water, in stoppered tubes fitted with "cold finger" stoppers, and were then incubated in a water bath at 40 ± 1°C for 24 hours. In no experiment did the concentration of hormone exceed 1%. After incubation the hormones were diluted with saline in the case of the ovarian ascorbic acid depletion test, and with water for the augmentation and mouse uterus tests. The quantities of hormones incubated were as follows :-

1. Follicle-Stimulating hormone (ovine NIH-FSH-S1) 15.0 to 20.0 mg.
2. Luteinising hormone (NIH-LH-S1). 1.0 to 2.0 mg.
3. Human Menopausal Gonadotrophin (Pergonal). 2.6 to 9.0 mg.
4. Pregnant Mare's Serum Gonadotrophin (PMSG) International Standard. 100 to 200 i.u.
5. Human Chorionic Gonadotrophin (HCG) International Standard. 100 to 300 i.u.
6. NIH-FSH + NIH-LH. 2.0 + 2.0 mg.

Dialysis of the incubated material was not performed before assay.

In all, 41 assays are reported, 21 by the ovarian ascorbic acid depletion test, 4 by the augmentation test and 16 by the mouse uterus test. (See p.21 to p.26). In the assays by the ovarian ascorbic acid depletion test (Tables 24 and 25) NIH-LH was used as the reference preparation, and the assays of NIH-LH after incubation with either urea or water are direct comparisons of the incubated and non-incubated materials. In the case of HCG, PMSG and Pergonal the "residual activity percent" was obtained by a two-stage procedure. First, NIH-LH was compared with the non-incubated material, and then in the second assay the incubated material was assayed in terms of NIH-LH. The fiducial limits of error and indices of precision reported in Tables 24 and 25 are calculated from the second assay. The residual activity represents a percentage difference

between the relative potency of the incubated and non-incubated materials in terms of the reference preparation NIH-LH. Assays by the augmentation and mouse uterus tests (Tables 26 to 28) represent direct comparisons in which the incubated and non-incubated materials were compared without the use of a specific reference preparation. All the assays reported were of a symmetrical 4-point design. In 16 cases it was possible to calculate the results on a 4-point basis, and in none of these was significant deviation from parallelism observed. In the remaining 25 assays only one dose level of the incubated material fell within the working range of the dose response curve of the non-incubated preparation, and the results were therefore calculated as for a 3-point assay. In most assays by the ovarian ascorbic acid depletion test the total dosage per animal of the incubated material was ten times that of the non-incubated material.

B. Results

1) Ovarian Ascorbic Acid Depletion Test

In Tables 24 and 25 is shown the remaining LH activity when the hormones were incubated with 6M urea and distilled water respectively. In the study with urea (Table 24) it will be noted that NIH-LH and NIH-FSH, either alone or in combination, lost more than 94% of their original LH activity. In the case of Pergonal and HCG a comparable

Table 24.

Residual LH activity following incubation with 6 M urea-ovarian ascorbic acid depletion test.

Materials tested	Design of assay	λ	Residual activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
NIH-LH	2 + 2	0.29	4.4	2.1 - 8.7	5.7
	2 + 1	0.12	7.1	5.8 - 8.6	
NIH-FSH	2 + 2	0.34	0.2	0.04- 0.9	4.1
	2 + 1	0.16	8.0	7.0 - 9.0	
NIH-FSH + NIH-LH	2 + 1	0.45	1.8	0.2 - 13.2	1.8
HCG	2 + 1	0.12	6.8	5.3 - 8.7	3.6
	2 + 2	0.14	0.5	0.4 - 0.5	
PMSG	2 + 1	0.13	56.0	42.5 - 73.4	46.2
	2 + 2	0.15	36.4	26.2 - 49.5	
Pergonal	2 + 1	0.45	10.5	4.2 - 28.3	6.4
	2 + 1	0.33	2.4	1.6 - 3.4	

Table 25.

Residual LH activity following incubation with water-ovarian ascorbic acid depletion test.

Materials tested	Design of assay	λ	Residual activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
NIH-LH	2 + 2	0.21	104	73 - 148	106
	2 + 2	0.19	107	69 - 169	
NIH-FSH	2 + 2	0.26	66	41 - 108	82
	2 + 2	0.23	97	53 - 174	
HCG	2 + 2	0.13	82	64 - 105	100
	2 + 1	0.31	118	65 - 212	
PMSG	2 + 1	0.11	102	83 - 126	101
	2 + 2	0.20	100	68 - 159	
Pergonal	2 + 2	0.18	36	25 - 51	41
	2 + 2	0.25	46	32 - 67	

amount of LH activity was destroyed, but with PMSG a mean figure of 46% of the activity remained. In the incubations with distilled water involving NIH-LH, NIH-FSH, HCG and PMSG (Table 25) the losses of LH activity were very small, and in all experiments the fiducial limits of error (P = 0.95) included the figure of 100%. In the case of Pergonal more than 50% of the activity was destroyed and this suggests that the LH activity of Pergonal is less stable than that of the other gonadotrophins studied.

2) Augmentation Test

In Table 26 is shown the remaining FSH activity when NIH-FSH and Pergonal were incubated with 6M urea. It was apparent that in the case of NIH-FSH very little activity was lost, the mean figure for the remaining activity being 91%. With Pergonal 93% of the FSH activity was destroyed.

3) Mouse Uterus Test

Tables 27 and 28 show the remaining "total gonadotrophic" activity when the hormones were incubated with 6M urea and with distilled water. In the studies involving 6M urea (Table 27) it will be noted that the degree of inactivation was greater with NIH-FSH than with PMSG. With Pergonal more than 90% of the activity was lost. In the incubation studies with distilled water (Table 28) neither NIH-FSH nor PMSG lost appreciable activity. In the two experiments involving Pergonal the residual activities were, respectively, 49 and 91%.

Table 26.

Residuel FSH activity following incubation with 6 M urea - augmentation test.

Materials tested	Design of assay	λ	Residual activity (%)	Fiducial limits (%) (P = 0.95	Mean residual activity (%)
NIH-FSH	2 + 1	0.14	117	81 - 254	91
	2 + 1	0.17	65	35 - 142	
Pergonal	2 + 1	0.17	7	2 - 11	7
	2 + 1	0.18	6	3 - 10	

Table 27.

Residual "total gonadotrophic" activity following incubation with 6 M urea - mouse uterus test.

Materials tested	Design of assay	λ	Residual activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
NIH-FSH	2 + 2	0.10	28	20 - 40	
	2 + 2	0.09	54	44 - 68	
	2 + 2	0.11	52	46 - 72	50
	2 + 1	0.13	43	30 - 70	
	2 + 1	0.12	74	51 - 152	
PMSG	2 + 1	0.12	37	26 - 77	67
	2 + 1	0.10	97	65 - 198	
Pergonal	2 + 1	0.12	9	7 - 11	
	2 + 1	0.10	7	5 - 9	8
	2 + 1	0.12	7	5 - 9	

Table 28.

Residual "total gonadotrophic" activity following incubation with water - mouse uterus test.

Materials tested	Design of assay	λ	Residual activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual Activity (%)
NIH-FSH	2 + 1	0.09	76	58 - 122	88
	2 + 1	0.13	100	71 - 168	
PMSG	2 + 2	0.09	120	94 - 160	113
	2 + 1	0.08	105	81 - 128	
Pergonal	2 + 1	0.17	49	32 - 87	70
	2 + 1	0.10	91	49 - 214	

C. Discussion

It has been known for several years that urea is capable of causing changes in the physical characteristics of proteins by a process of involving cleavage of hydrogen bonds in the molecule (Sterman & Foster, 1956; Kauzmann, 1955; Fruton & Simmonds, 1958). Stark, Stein & Moore (1960) have pointed out that some of the effects of urea solutions on proteins were due to the cyanic acid which was produced by tautomeric changes in the urea solution on standing.

The present investigation, which represents an extension of the original work of Ellis (1961 a, b), has demonstrated that when various gonadotrophins are incubated with 6M urea at 40°C their LH activity was almost completely destroyed. This was noted with hormones derived from sheep pituitary tissue (NIH-FSH and NIH-LH), from human pregnancy urine (HCG) and from human postmenopausal urine (Pergonal). In the case of PMSG the degree of inactivation was less than that observed with the other preparations tested. When the hormones were incubated with distilled water instead of 6 M urea an appreciable loss of LH activity was found only in the case of Pergonal, thus indicating the relative instability of this preparation.

In agreement with the finding of Ellis (1961 a, b) the present study has shown that when NIH-FSH was incubated with 6 M urea at 40°C little or no loss of FSH activity occurred. However, when a similar experiment was conducted with Pergonal, the greater part of the FSH activity was destroyed.

The "total gonadotrophic" activity of NIH-FSH as estimated by the mouse uterus test was reduced by 50% after incubation with 6 M urea, while in the case of PMSG the degree of inactivation was smaller. When NIH-FSH and PMSG were incubated with water no appreciable loss of activity was observed. The "total gonadotrophic" activity of Pergonal, like its FSH and LH activities was almost completely destroyed by incubation with 6 M urea. After incubation with water Pergonal lost approximately 70% of its "total gonadotrophic" activity.

D. Summary

Following incubation with 6 M urea the LH activities of NIH-LH, NIH-FSH, HCG and Pergonal were almost completely destroyed, while the LH activity of PMSG was reduced to a smaller extent.

The FSH activity of NIH-FSH was little affected by this form of treatment, but in the case of Pergonal a considerable reduction in FSH activity occurred.

The "total gonadotrophic" activity of NIH-FSH, PMSG and Pergonal was reduced after incubation with 6 M urea, the degree of inactivation being greatest in the case of Pergonal.

After control incubations with water no appreciable loss of biological activity was observed with any hormone other than Pergonal.

3. THE EFFECT OF FORMALDEHYDE ON THE BIOLOGICAL ACTIVITY OF NIH-LH

The action of formaldehyde on proteins and amino acids has been studied in considerable detail by a large number of investigators. (See French and Edsall, 1945). It has been shown that formaldehyde can combine with any one of a number of different functional groups found in protein molecules. Thus, reactions can occur with the peptide linkages, the aromatic rings and the hydroxyl and carboxyl groups as well as with various other parts of the protein. Moreover, when steric conditions are favourable, it can react with two groups to form a methylene bridge between them.

The aim of this section is to report a series of experiments designed to investigate the effect of varying concentrations of formaldehyde on the biological activity of NIH-LH-S3*.

A) Materials and Methods

Incubations were carried out with 4%, 0.8% and 0.16% formaldehyde solution (lab. grade) as well as with distilled water. NIH-LH (1.0 - 3.0 mg) was dissolved in either formaldehyde solution or water at a concentration of 0.5 mg./ml. in stoppered tubes and the incubations were carried out at room temperature (21 ± 1°C) for 12, 16, 20 or 24 hours. The hormone was then diluted with saline before

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*The preparation NIH-LH-S3 was employed throughout this study.

being assayed in such a way that the final formaldehyde concentration never exceeded 1.0% and was generally below 0.1%. Dialysis of the incubated material was not performed before assay.

In all, 33 assays were conducted with the ovarian ascorbic acid depletion test (see p. 21). All the assays were of a symmetrical 4-point design and represented a direct comparison of the incubated and non-incubated materials. In 26 cases it was possible to calculate the results as a 4-point assay and in none of these assays was significant deviation from parallelism of the dose response lines observed. In 4 instances the results obtained indicated that more than 99.9% of the biological activity had been destroyed and it was therefore not possible to calculate the precision or fiducial limits of error of these assays. The remaining 3 assays were calculated on a 3-point basis because only one dose level of the incubated material fell within the working range of the dose response lines of the non-incubated material. In the majority of assays the total dosage of the test preparation administered to each animal was more than ten times that of the standard.

B) Results

1) 4% formaldehyde: In Table 29 is shown the remaining LH activity when NIH-LH was incubated with 4% formaldehyde. It will be noted that in all assays the incubated material lost more than 95% of its biological activity and that the

Table 29.

Residual LH activity of NIH-LH following incubation with 4% formaldehyde.

Time of incubation (hours)	Design of assay	λ	Residual Activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
24	2 + 2	0.31	0.5	0.03 - 0.6	< 0.7
	2 + 2	0.28	2.1	1.30 - 3.5	
			< 0.5		
20	2 + 2		< 0.5		< 0.5
	2 + 2		< 0.5		
	2 + 2		< 0.5		
16	2 + 2	0.54	0.9	0.1 - 2.7	1.0
	2 + 2	0.31	1.0	0.4 - 1.8	
	2 + 2	0.35	1.0	0.3 - 1.8	
12	2 + 1	0.26	3.2	1.9 - 5.5	2.7
	2 + 2	0.29	2.1	1.3 - 3.5	

inactivation was complete in 12 hours.

2) 0.8% formaldehyde: The remaining biological activity when NIH-LH was incubated with 0.8% formaldehyde is shown in Table 30. Approximately 90% of the biological activity was destroyed at the end of an incubation period of 16, 20 or 24 hours. However, it will be seen that at 12 hours a mean of only 80% of the activity of the incubated material had been inactivated.

3) 0.16% formaldehyde: Table 31 shows the results obtained with 0.16% formaldehyde. It should be noted that the percentage inactivation of NIH-LH after an incubation period of 16, 20 or 24 hours was similar to that observed with 0.8% formaldehyde. In the case of the 12 hour incubation a mean of only 70% of the biological activity had been destroyed.

4) Distilled water: In the study involving 6 M urea it was shown that when NIH-LH was incubated with water at 40°C for 24 hours no loss of biological activity occurred (see p.86). For this reason only two incubations with water were carried out in the present investigation. These are shown in Table 32 where it will be seen that the fiducial limits of error include the figure of 100%, thus suggesting that little or no destruction of the LH activity had occurred under the experimental conditions employed.

5) Relationship between formaldehyde concentration and inactivation of LH: If the formaldehyde was in great excess its concentration would not be affected by reaction

Table 30.

Residual LH activity of NIH-LH following incubation with 0.8% formaldehyde.

Time of incubation (hours)	Design of assay	λ	Residual Activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
24	2 + 2	0.33	4.7	2.7 - 8.1	7.2
	2 + 2	0.29	9.7	6.0 - 15.5	
20	2 + 2	0.19	8.9	5.7 - 14.0	11.4
	2 + 2	0.52	13.9	6.1 - 47.4	
16	2 + 2	0.55	20.8	8.5 - 142.8	11.2
	2 + 2	0.20	10.0	7.1 - 13.9	
	2 + 2	0.59	9.0	2.6 - 25.0	
	2 + 2	0.30	4.1	2.0 - 8.3	
12	2 + 2	0.58	18.3	7.0 - 136.5	19.4
	2 + 2	0.36	14.7	8.4 - 26.1	
	2 + 2	0.41	25.2	13.1 - 69.8	

Table 31.

Residual LH activity of NIH-LH following incubation with 0.16% formaldehyde.

Time of incubation (hours)	Design of assay	λ	Residual Activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
24	2 + 2	0.23	20.0	13.4 - 30.0	14.6
	2 + 2	0.20	9.2	6.8 - 12.4	
20	2 + 2	0.30	21.4	13.3 - 34.7	17.2
	2 + 2	0.26	13.0	8.6 - 19.8	
16	2 + 1	0.29	10.1	6.0 - 17.0	12.0
	2 + 1	0.27	15.0	7.2 - 31.5	
	2 + 2	0.28	10.7	6.9 - 16.8	
12	2 + 2	0.34	25.0	14.3 - 50.8	29.9
	2 + 2	0.30	34.7	20.0 - 76.0	

Table 32.

Incubation of NIH-LH with water

Time of incubation (hours)	Design of assay	λ	Residual Activity (%)	Fiducial limits (%) (P = 0.95)	Mean residual activity (%)
24	2 + 2	0.14	129.3	98.8 - 165.5	102.3
	2 + 2	0.22	75.2	68.9 - 145.0	

with the hormones. Thus

$$-\frac{dy}{dt} = ky$$

where y is the hormone concentration at time t and k is dependent on the formaldehyde concentration

This gives :- $\frac{dy}{y} = -kt$

and, on interpretation $\log_e y = -kt + c$.

where c is a constant.

Since y = yo, the initial hormone concentration, when t = o

$$c = \log_e yo \text{ and :-}$$

$$\log_e \frac{yo}{y} = kt$$

In the assays reported herein y is expressed as P, a percentage of yo thus :-

$$\log_e \frac{100}{P} = kt$$

$$\text{or} \quad \log_{10} \frac{100}{P} = k^1 t$$

$$\text{or} \quad k^1 = \frac{2 - \log_{10} P}{t}$$

The values for k^1 obtained when this equation was applied to the present study are shown in Table 33. It will be seen that the mean k^1 value increases with increasing formaldehyde concentration but as shown in Fig.5 when the mean k^1 is plotted against the total formaldehyde concentration a straight line relationship is not obtained. However, if the concentration of formaldehyde present as the monomer (see Auerbach & Barschall, 1905) is plotted instead of the total concentration, a straight line relationship is obtained.

Table 33.

Relationship between formaldehyde concentration and inactivation of NIH-LH

Formaldehyde concentration		Time of incubation (hours)	k^1	Mean k^1
Total	Monomer			
4%	3.8%	12	0.523	0.512
		16	0.500	
0.8%	0.7%	12	0.237	0.213
		16	0.238	
		20	0.187	
		24	0.190	
0.16%	0.16%	12	0.141	0.144
		16	(0.230)*	
		20	0.153	
		24	0.139	

*Eliminated before calculation of mean k^1.

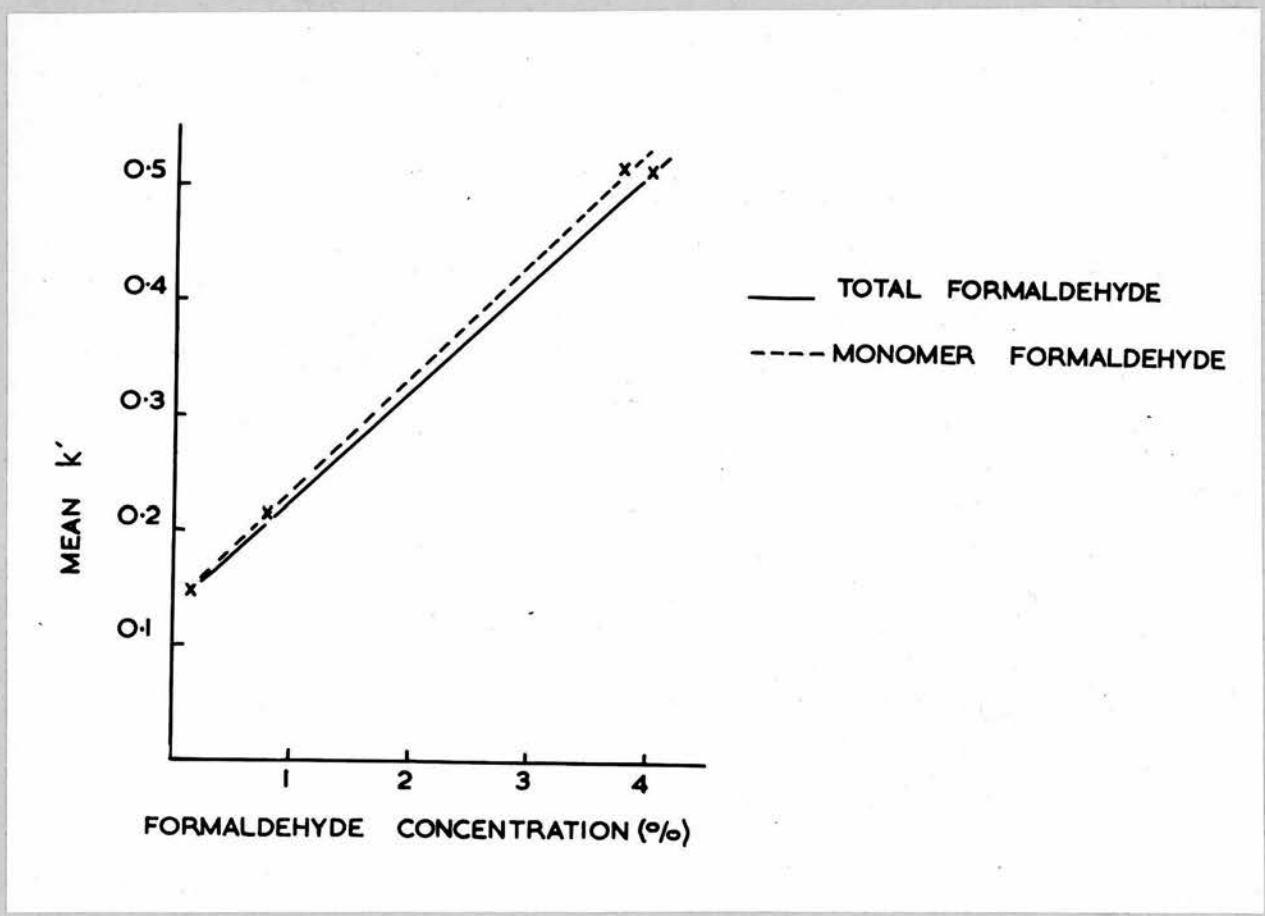

Fig. 5 :- The relationship between formaldehyde concentration and a constant (Ḱ) relating formaldehyde concentration and inactivation of NIH-LH.

4. DISCUSSION

The present investigation has demonstrated that when NIH-LH was incubated with formaldehyde a considerable reduction in the biological activity of the hormone takes place. The data obtained are summarised in Fig. 6 in which it can be seen that the degree of inactivation was more marked at the higher concentrations of formaldehyde. In view of the finding that both with 0.16% and 0.8% formaldehyde complete inactivation had not occurred within 12 hours it seems likely that the reactions involved were fairly slow. The interpretation of the changes in the gonadotrophin molecule resulting in biological inactivation is rendered very difficult because of the multiplicity of the reactions which may occur. It is of interest to note that a straight line relationship can be obtained between the inactivation of LH and the concentration of formaldehyde present as the monomer. This indicates that, although in many assays the fiducial limits of error of the bioassay data were very wide, useful quantitative data can be obtained by the ovarian ascorbic acid depletion test.

One point which must be considered both in relation to this study and that involving urea is that the action of the reagent used might not be on the hormone itself but on a contaminating substance which would interfere with the bioassay of the test material. Thus it has been shown that the potency of NIH-LH cannot be measured by the mouse uterus test and one explanation of this observation is that

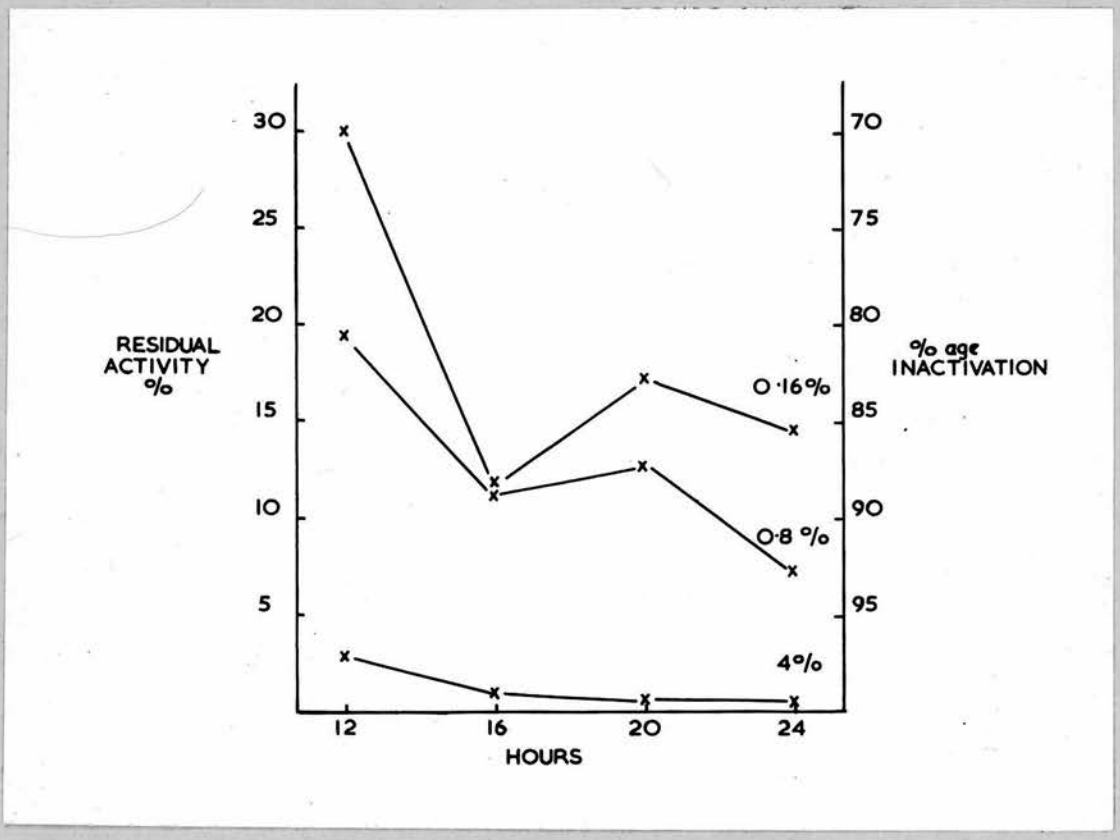

Fig. 6 :- The effect of formaldehyde on NIH-LH showing the relationship between the time of incubation and the residual LH activity at various concentrations of formaldehyde.

there is not enough FSH activity present in the material to initiate oestrogen production by the ovary. It might therefore be argued that an apparent decrease in the biological activity of any given gonadotrophin preparation was due to an alteration in the ratio FSH/LH rather than to inactivation of the hormone present in the largest amount in the test material. This difficulty can largely be overcome by the use of specific methods and the purest available hormones.

A study of the reactions described in this section may eventually lead to a greater knowledge of the chemical nature of those groups in the protein molecule that are necessary for gonadotrophic activity. However, quantitative data on a mode of action of urea and formaldehyde on the gonadotrophin molecule will only be of value when pure preparations are available. At the present time it is difficult to assess the nature of the processes which result in biological inactivation and it cannot be stated with any certainty whether the chemical or configurational changes in the molecule are of more importance.

5. SUMMARY.

Following incubation of NIH-LH with 0.16%, 0.8% and 4% formaldehyde considerable reduction in LH activity as measured by the ovarian ascorbic acid depletion test was noted.

It was shown that the degree of inactivation increased with the length of incubation and the concentration of formaldehyde.

After control incubations with water no appreciable loss of biological activity was observed.

II. THE RENAL CLEARANCE OF EQUINE GONADOTROPHIN

1. INTRODUCTION

The urinary and serum levels of HCG throughout pregnancy have been studied extensively by a number of workers including Wilson, Albert & Randall (1949); Johnson, Albert & Wilson (1950); Loraine (1950 b); Albert & Berkson (1951) and Haskins & Sherman (1952). It has been shown that characteristic fluctuations in hormone levels occur and presumably these represent alterations in the rate of formation, in the rate of excretion or in the rate of destruction of the hormone. In an attempt to gain further information on the subject Gastineau, Albert & Randall (1949) studied the renal clearance of HCG and found a mean value of 0.36 ml/min. in normal pregnancy. These workers also found that the clearance in the first trimester of pregnancy did not differ significantly from that obtained in the 2nd and 3rd trimesters.

The renal clearance of the pituitary gonadotrophins has been studied by Apostolakis & Loraine (1960) who showed that as in the case of HCG the mean clearance value was less than 1 ml/min. The serum levels of PMSG have been studied by a number of workers including Cole & Hart (1930); Day & Miller (1940); and Day & Rowlands (1947) but it was only recently (Schmidt-Elmendorff, Loraine, Bell & Walley, 1962) that gonadotrophic activity was detected in urine. In this section a study of the renal clearance of equine gonadotrophin in the horse, the pony and the donkey is described.

2. METHODS

Unextracted serum was employed in all assays. Urine samples were precipitated with 5 volumes of acetone at pH 6. The precipitate was washed twice with ether and once with ethanol before being dried over calcium chloride. The use of acetone as a precipitating agent and the subsequent washing of the precipitate with ether makes it reasonably certain that the final extracts were not contaminated with oestrogens. All assays in serum were performed using the test depending on the enlargement of the ovaries in intact immature mice (see p. 26). In urine samples the mouse uterus test was employed (see p. 25). The results of all the assays were expressed in terms of the International Standard for PMSG in international units per litre of serum or urine.

Clearance values were calculated by the formula $\frac{UV}{P}$ and were expressed in millilitres per minute :-

Where U = concentration of hormone in urine.

V = volume of urine excreted per minute.

P = concentration of hormone in plasma.

A total of 72 assays are reported, 44 by the ovarian weight test and 28 by the mouse uterus test. All the assays were of a symmetrical four point design and in 38 instances it was possible to calculate the results on a 4-point basis. In 34 assays only one dose level of the unknown material fell within the "working range" of the dose response curve of the standard preparation and the results were therefore

calculated as for a 3-point assay. Three assays were invalid (Tables 34 and 35); in one of these significant deviation from parallelism of the dose response curves of the test and standard materials was observed and in two the fiducial limits of error were infinite ($g > 1.0$).

In all, 44 serum samples and 28 urine samples were collected from two ponies, two donkeys and one horse. During the period of study the ponies and donkeys were housed on the premises of the University of Glasgow Veterinary School while the horse was stabled at the Macclesfield Laboratories of Imperial Chemical Industries Limited (Pharmaceutical Division). The urine samples from the ponies and donkeys were obtained by catheterization of the bladder, while the horse was kept in a metabolism cage during the period of urine collection.

3. RESULTS

A) Ponies and Donkeys

In Table 34 and Figure 7 are shown the serum levels obtained during the period of study. The levels in the two donkeys were much lower than in the two ponies. However, the excretion pattern was similar in all four animals with the exception of one pony in which the serum levels were still rising at the end of the study. Table 34 also shows the urinary gonadotrophin values in these animals and it will be noted that these are much lower than the serum levels. A total of 7 complete 24 hour urine collections were obtained from the four animals and the renal clearance values obtained are shown in the last column of the Table. These vary from 0.0006 to 0.41 ml per minute with a mean of 0.1008 ml per minute.

B) Horse

A more comprehensive study has been carried out in one horse and the results of the assays in serum and urine are shown in Table 35 and Figure 8. This animal was studied from day 102 to day 157 of pregnancy and the assay results obtained are in the range previously reported by other workers (see Schmidt-Elmendorff, Loraine, Bell & Walley, 1962). A total of 18 complete urine specimens were collected and the renal clearance values are shown in the final column of Table 35. It is of interest to note that, despite the marked changes in both urinary and serum levels during the period of study, the renal clearance of pregnant

Table 34.

Serum and Urinary Gonadotrophin Values in ponies and donkeys.

Animal	Date	Day of pregnancy	SERUM				URINE				Renal clearance ml/min
			Design	λ	i.u./litre	Fiducial limits P = 0.95	Design	λ	i.u./litre	Fiducial limits P = 0.95	
Pony	4/8/62	35	2 + 1	0.08	4216.6	3078.1 - 5312.9					
	9/8	40	2 + 2	0.28	12337.6	$\varepsilon > 1.0$	2 + 1	0.12	29.4	18.8 - 41.2	-
	14/8	45	2 + 2	0.23	60621.5	13942.9 -712908.8					
	18/8	49	2 + 2	0.16	122880.0	76185.6 -411648.0	2 + 2	0.10	43.5	24.8 - 61.8	0.0006
	23/8	54	2 + 2	0.20	226086.9	128869.5 -604437.2					
	30/8	61	2 + 2	0.08	93920.0	73136.0 -117400.0					
Pony	5/8	55	2 + 2	0.14	26489.6	15364.0 - 38939.7					
	12/8	62	2 + 2	0.17	25037.3	9764.5 - 53329.4					
	15/8	65	2 + 2	0.12	62176.0	36683.8 - 90776.9	2 + 1	0.07	60.3	48.8 - 74.8	0.0027
	24/8	74	2 + 2	0.12	71904.0	47457.4 -102822.7					
Donkey	6/8	35	2 + 1	0.08	3789.3	2956.0 - 4888.8					
	11/8	40	2 + 2	0.08	752.5	541.8 - 963.2					
	15/8	44	2 + 1	0.10	2352.0	1693.4 - 3081.1	2 + 1	0.18	36.3	12.0 - 62.4	0.020
	21/8	50	2 + 2	0.10	13388.0	8702.2 -17672.2					
	26/8	55	2 + 2	0.10	9429.3	6317.6 -12446.7	2 + 1	0.13	72.8	48.0 - 112.8	0.017
	31/8	60	2 + 2	0.13	13260.0	9414.6 -22807.2					
	5/9	65	2 + 1	0.09	8697.1	6348.9 -11654.1	2 + 2	0.16	46.4	22.3 - 84.5	0.027
Donkey	5/8	40	2 + 1	0.08	8432.0	6661.3 -10708.5					
	10/8	45	2 + 1	0.12	1300.0	832.0 - 1911.0	2 + 1	0.13	103.5	70.4 - 162.5	-
	15/8	50	2 + 2	0.20	1414.0	791.8 - 8059.8					
	20/8	55	2 + 1	0.09	12368.0	9152.3 -16078.4					
	24/8	59	2 + 1	0.10	7412.0	5114.3 - 9683.8					
	30/8	65	2 + 1	0.21	42865.0	23575.7 -513951.6					
	5/9	71	2 + 2	0.15	1224.8	575.6 - 1861.7	2 + 2	0.09	164.2	108.4 - 215.1	0.310
	17/9	83	2 + 1	0.19	340.7	98.9 - 555.3	2 + 1	0.17	57.8	21.4 - 101.7	0.410

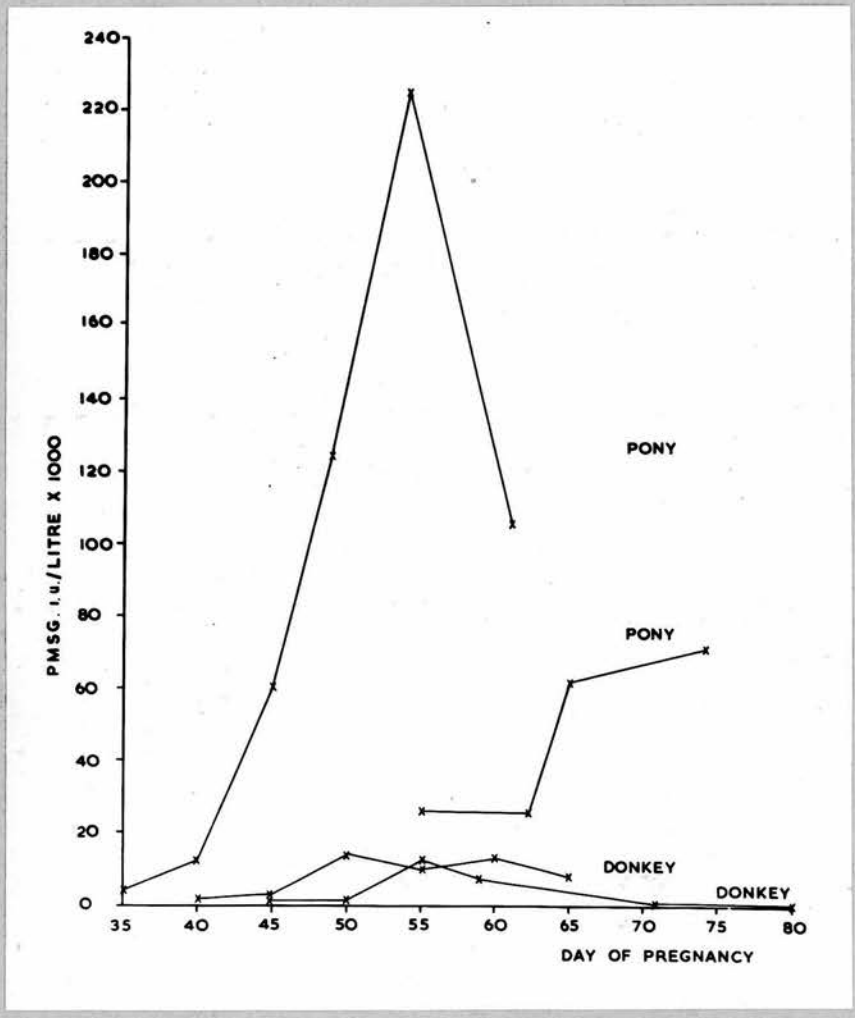

Fig. 7 :- Serum levels of pregnant mare's gonadotrophin from day 35 to day 80 of pregnancy in two ponies and two donkeys.

Table 35.

Serum and Urinary Gonadotrophin levels in the horse.

Date 1962	Day of pregnancy	SERUM Design	SERUM λ	SERUM i.u./litre	SERUM Fiducial limits (P = 0.95)	URINE Design	URINE λ	URINE i.u./litre	URINE Fiducial limits (P = 0.95)	Renal clearance ml/min
13/10	102	2 + 1	0.20	79960.0	44777.6 - 470964.4	2 + 1	0.13	176.80	113.20 - 285.10	0.0084
15/10	104	2 + 1	0.16	49226.5	25105.5 - 80239.2	2 + 1	0.06	102.10	85.80 - 121.50	0.0083
23/10	112	2 + 2	0.07	22080.0	18326.4 - 26716.8	2 + 2	0.27	100.20	g > 1.0	0.0044
30/10	119	2 + 2	0.10	15253.3	11439.9 - 21659.7	2 + 1	0.05	6.41	5.38 - 7.63	0.0184
6/11	126	2 + 2	0.11	10362.7	6943.0 - 13886.0	2 + 2	0.10	30.28	20.89 - 41.48	0.0040
8/11	128	2 + 1	0.10	6184.6	4452.8 - 81634.0	2 + 2	0.18	14.19	6.81 - 26.53	0.0060
10/11	130	2 + 1	0.07	1585.7	1300.3 - 1934.6	2 + 1	0.06	24.84	20.61 - 29.81	0.0255
13/11	133	2 + 1	0.08	1667.7	1334.2 - 2067.9	2 + 2	0.10	9.10	6.46 - 12.29	0.0113
15/11	135	2 + 1	0.09	4690.8	3424.3 - 6051.1	2 + 2	0.20	15.51	7.76 - 57.39	0.0038
17/11	137	2 + 1	0.10	4690.8	3283.6 - 6238.8	2 + 2	0.08	19.64	15.52 - 24.94	0.0030
19/11	139	2 + 1	0.10	5218.3	3757.2 - 6940.3	2 + 2	0.10	18.36	9.73 - 24.24	0.0035
21/11	141	2 + 1	0.10	4216.6	2740.8 - 5692.4	2 + 2	0.12	10.06	6.34 - 13.78	0.0024
23/11	143	2 + 1	0.09	4216.6	3078.1 - 5270.8	2 + 2	0.09	12.45	9.71 - 15.93	0.0040
26/11	146	2 + 2	0.15	1341.0	898.5 - 2682.0	2 + 2	0.16	13.89	8.33 - 27.64	0.0201
28/11	148	2 + 2	0.08	2188.0	1750.4 - 2735.0	2 + 2	0.18	9.34	3.18 - 17.37	0.0050
30/11	150	2 + 2	0.09	2160.0	1598.4 - 2786.4	2 + 2	lines	not parallel		-
3/12	153	2 + 2	0.05	1211.3	1041.7 - 1405.1	2 + 1	0.06	6.33	5.25 - 7.60	-
5/12	155	2 + 2	0.12	2341.0	1545.1 - 3394.5	2 + 1	0.14	4.19	2.05 - 6.41	0.0046
7/12	157	2 + 1	0.13	481.6	322.7 - 765.7	2 + 1	0.06	2.34	1.99 - 3.42	0.0164
									Mean	
									± S.D.	± 0.0088 ± 0.0071

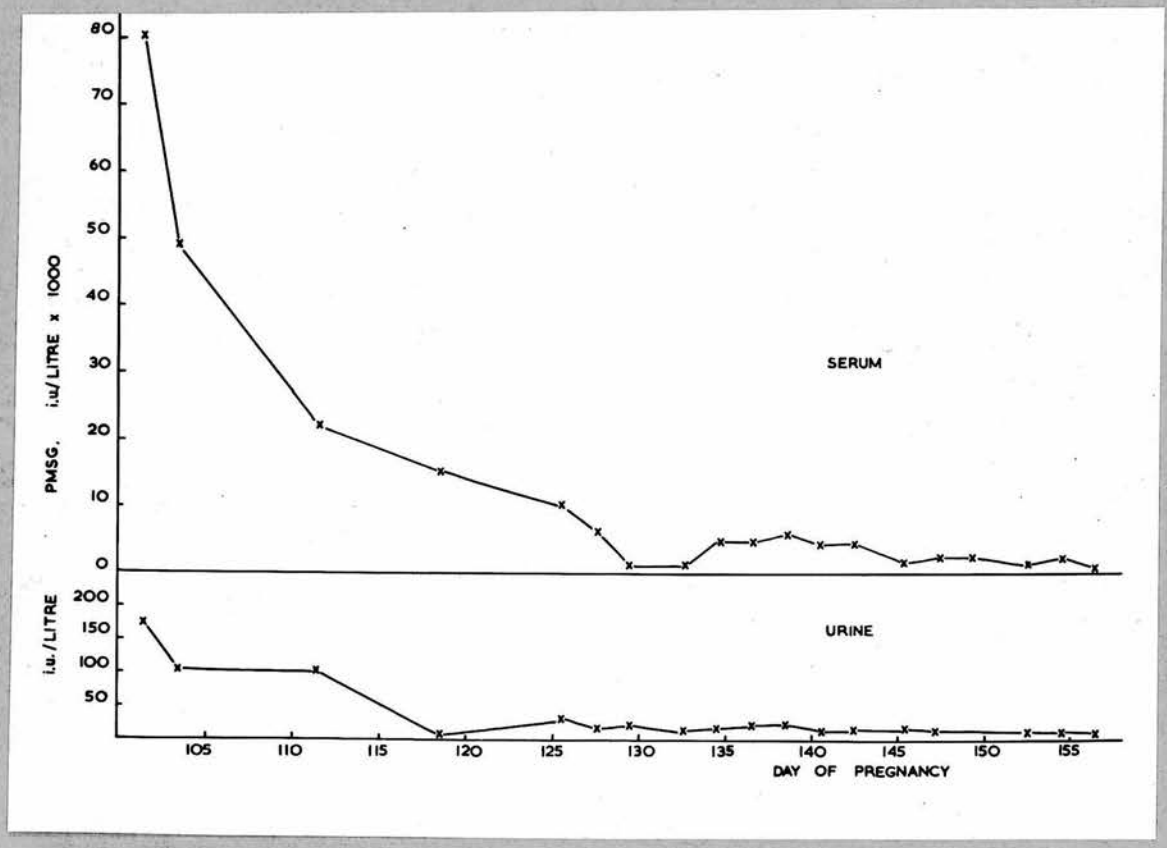

Fig. 8 :- Serum and urine levels of pregnant mare's gonadotrophin from day 100 to day 160 of pregnancy in one horse.

mare's gonadotrophin did not appear to alter in a similar manner. This indicates that a fixed proportion of the serum gonadotrophin is excreted in the urine. In this study a mean of 0.045% (arithmetic range 0.002 - 0.16%) of the gonadotrophin present in serum was detectable in the urine.

4. DISCUSSION

In the determination of the renal clearance of gonadotrophins one fundamental assumption must be made; namely, that the bioassay methods employed measure the same material in both serum and urine. Thus, it has been assumed that the extraction method used for urine provides a high yield of gonadotrophin and that the bioassay methods would show whether or not there was a qualitative difference between the gonadotrophic activity present in serum and in urine.

The first assumption has been tested by extracting serum by the same method as that employed for urine. It was shown that there was no significant difference in the increase in ovarian weight following the administration of either extracted or unextracted serum. Because of the low levels of gonadotrophin present in urine it was not possible to carry out a similar experiment, but it may be inferred from the results obtained using serum that the extraction procedure used when applied to urine would provide a high yield of gonadotrophin.

The international standard for PMSG was used as a reference preparation throughout the study and in view of the finding that only one out of the 74 assays reported was invalid because of significant deviation from parallelism of the dose response curves it seems likely that the gonadotrophin present in both urine and serum is qualitatively similar.

In Table 36 is summarised some of the previous work on the renal clearance of gonadotrophins. Clearance values

Table 36.

Summary of previous work on the renal clearance of gonadotrophins.

Hormone	Source	Number of cases.	Number of observations	Renal clearance in ml/min		Reference
				Mean value	Arithmetic range	
Human pituitary gonadotrophins	Post-menopausal women	50	67	0.18 ± 0.02*	0.04 - 0.43	Apostolakis & Loraine (1960)
Human chorionic gonadotrophin	Normally pregnant women	12	32	0.95 ± 0.03*	0.69 - 1.19	Loraine (1950)
Human chorionic gonadotrophin	Normally pregnant women	4	20	0.38 ± 0.03*	0.24 - 0.79	Gastineau, Albert & Randall (1949)
Human chorionic gonadotrophin	Normally pregnant women	5	5	0.47	0.18 - 0.99	Johnson, Albert & Wilson (1950)
Pregnant pony and donkey gonadotrophin	Ponies and donkeys	4	8	0.10	0.0006 - 0.41	-
Pregnant horse gonadotrophin	Horse	1	17	0.0088 ± 0.0017*	0.0024 - 0.0255	-

*Standard error

are given for postmenopausal and pregnant women as well as those reported in the present study. The close agreement between the renal clearance of gonadotrophins in humans and the equidae should be noted. In humans the glomerular filtration rate has been shown to be closely correlated to the surface area of the body (see Smith, 1951). In the present study no attempt has been made to correct the data for surface area and this may in part account for the somewhat lower results obtained in the equidae. The large arithmetic range observed in the small series of samples from ponies and donkeys cannot be explained in the same way but this may to some extent be a reflection of the difficulty of collecting urine specimens from such animals.

At present no reliable information is available on the chemical nature of the circulating gonadotrophin in human or animal blood. McArthur & Antoniades (1961) have shown that following fractionation of human postmenopausal plasma by 'Method 6' (a cold ethanol procedure) of Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor (1946) the gonadotrophic activity was associated with fractions II and III. These fractions were further purified with sodium tetrametaphosphate and the gonadotrophic activity was recovered by lyophilisation. Paper electrophoresis was then performed and the fractions corresponding to the albumin a_2 and β_1 globulins were shown to contain gonadotrophic activity.

Two hypotheses may therefore be advanced to explain

the low levels of Pregnant Mare's Gonadotrophin in urine. First that the gonadotrophin is filtered through the glomerulus but is reabsorbed in the renal tubule. Second that the molecules are either bound to plasma proteins or are themselves macromolecules and are too large to be filtered through the glomeruli. At present no evidence can be offered for the validity of either hypothesis but it is possible that a study of the ultrafiltration of pregnant mare's serum might provide useful information on this point. It should however be stated that although the chemical and biological characteristics of human pituitary and placental gonadotrophins and pregnant mare's gonadotrophin are known to be dissimilar the renal handling of these proteins by the equidae and humans does not appear to differ to any marked extent.

5. SUMMARY

The renal clearance of pregnant mare gonadotrophin has been studied in two ponies, two donkeys and one horse.

The clearance values obtained were all less than 1 ml/min. and are thus similar to data previously reported for the clearance of chorionic and pituitary gonadotrophins in human subjects.

It is suggested that the low clearance rate may be associated with protein binding of pregnant mare gonadotrophin in serum.

6. APPLICATION OF ASSAY METHODS TO CLINICAL PROBLEMS

I. HORMONE EXCRETION DURING THE NORMAL MENSTRUAL CYCLE.

1. INTRODUCTION

In recent years a number of investigators have studied the pattern of hormone excretion during the normal menstrual cycle (for references see Brown, Klopper & Loraine, 1958; Loraine & Schmidt-Elmendorff, 1963). In the great majority of these studies only one hormone or hormone metabolite has been estimated and no attempt has been made to perform parallel assays of a number of hormones in the same individual. Thus, the urinary output of HPG has been determined by Buchholz (1957) and by Loraine (1962). Pregnanetriol excretion has been estimated by Fotherby (1962) while urinary oestrogens and pregnanediol have been measured by J.B.Brown (1955 a) and by Klopper (1957) respectively.

The aim of this section is to provide information on the hormonal interrelationships throughout the menstrual cycle by comparing the excretion pattern of one hormone with that of another. Assays of urinary oestrogen and pregnanediol have been performed in 15 ovulatory menstrual cycles, and in 11 and 6 of these respectively excretion values for HPG and pregnanetriol were also measured.

2. MATERIALS AND METHODS

Complete 24 hour urine samples were collected continuously throughout 15 menstrual cycles from 10 women, 8 of whom were nulliparous while the remaining 2 were para 2 and para 3 respectively. All subjects continued their normal activities during the period of investigation and in none was there a previous history of menstrual abnormalities. The age range was from 18 to 37 years. Five of the subjects collected urine throughout 2 menstrual cycles. In two of these consecutive cycles were studied; in one there was an interval of one month and in two an interval of two years between the collection periods.

Assays were performed on either 24 or 48 hour pools of urine and the results were expressed per 24 hour urine sample. HPG was extracted from urine by the method of Loraine and Brown (1959) as described on page 51 and the extracts were assayed by the mouse uterus test. All results were expressed in terms of the International Reference Preparation for Human Menopausal Gonadotrophin as HMG units/24 hours. Urinary oestrone, oestradiol and oestriol were determined by the method of J.B. Brown (1955 b). The methods of Klopper, Michie and Brown (1955) and of Fotherby and Love (1960) were used for the estimation of urinary pregnanediol and pregnanetriol respectively.

3. RESULTS

Typical ovulatory menstrual cycles in two of the subjects included in the present study are shown in Figs. 9 and 10. It is generally agreed that peaks of oestrogen and gonadotrophin excretion occur at or about mid cycle and that in the luteal phase of the cycle there is a second oestrogen peak associated with a rise in the output of pregnanediol and pregnanetriol. Thus in Fig. 9 the mid-cycle and luteal phase peaks of oestrogen excretion together with the luteal phase rise in pregnanediol and pregnanetriol output are well demonstrated. The increase in pregnanetriol excretion commenced at the time of the midcycle oestrogen peak and levels reached their maximum on the 23rd and 24th days of the cycle. In the case of pregnanediol the highest values were obtained between the 21st and 28th day. In this subject the midcycle HPG peak coincided with the oestrogen peak on days 15 and 16.

In Fig. 10 the pattern of hormone excretion was very similar to that in Fig. 9. It should, however, be noted that the midcycle HPG peak was less pronounced in this subject and that it postdated the oestrogen peak by a period of 48 hours.

The data obtained from the 15 cycles studied have been used to construct a composite curve for the hormone excretion during the menstrual cycle; this is shown in Fig. 11. The curve was constructed in such a way that the day of the midcycle peak of excretion of oestrone and oestradiol was

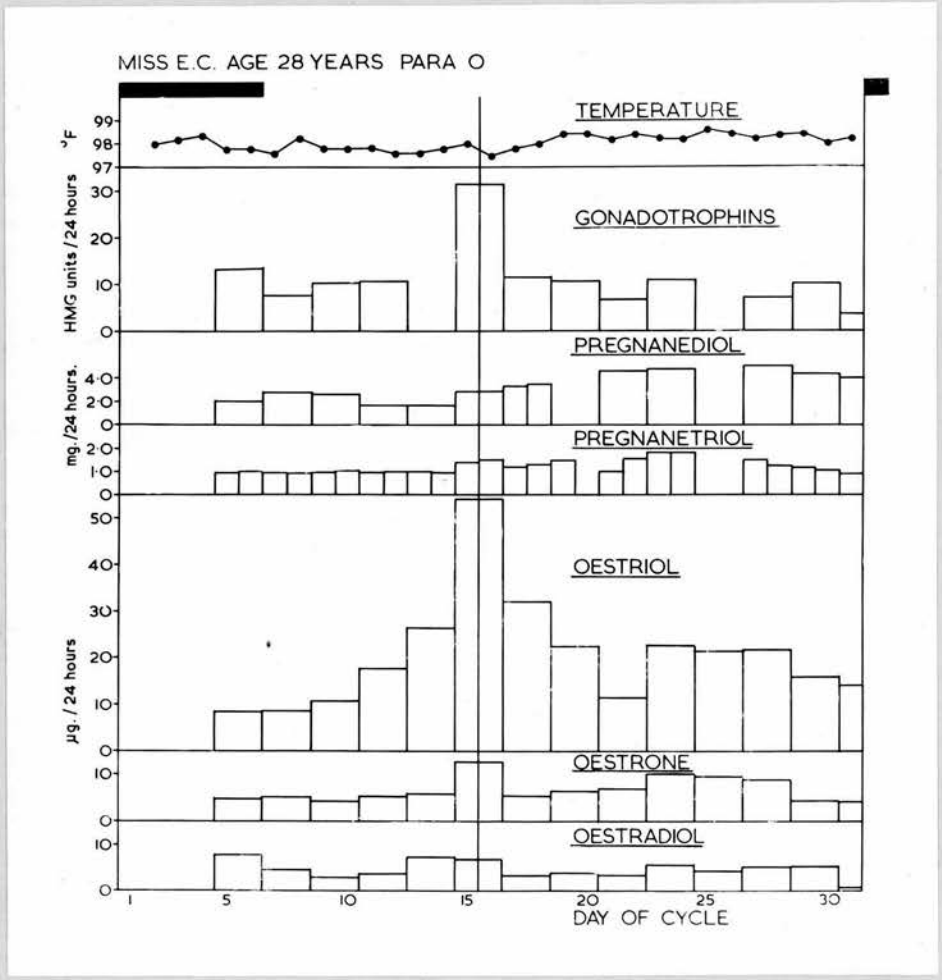

Fig. 9 :- The excretion pattern of gonadotrophins, pregnanediol, pregnanetriol, oestriol, oestrone and oestradiol during the menstrual cycle in a normally menstruating woman. Shaded areas indicate periods of menstrual bleeding.

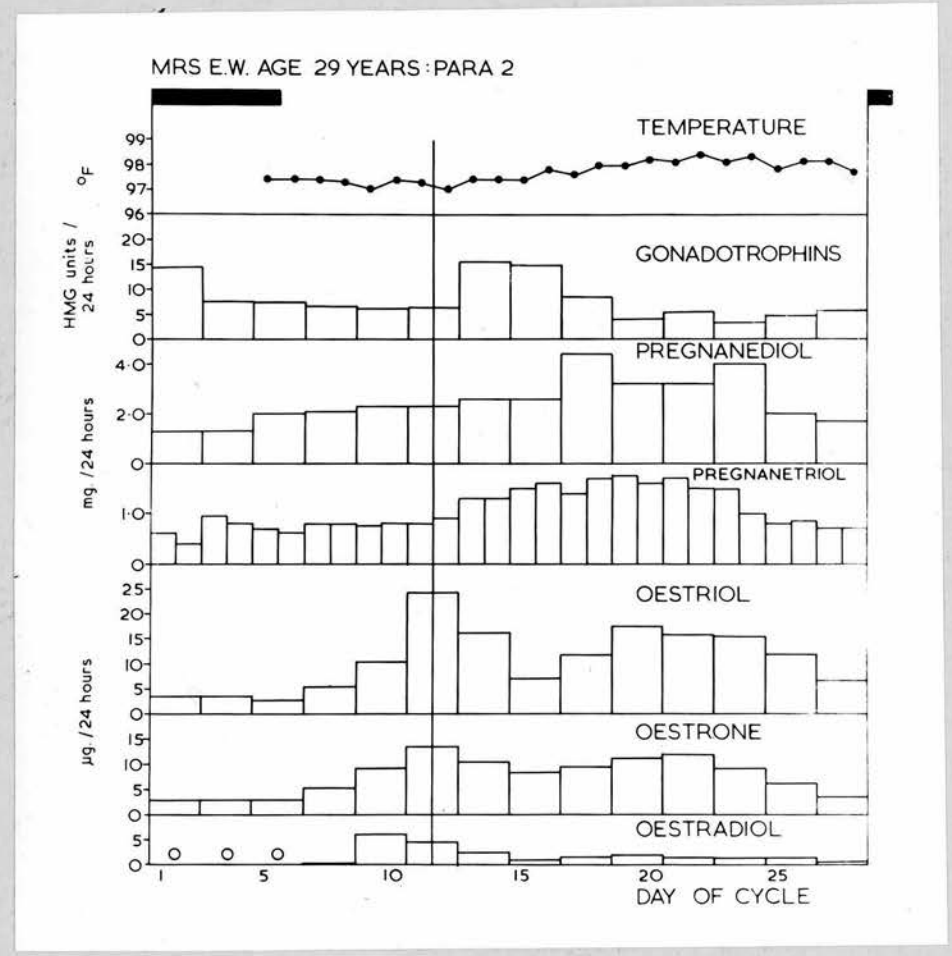

Fig. 10 :- The excretion pattern of gonadotrophins, pregnanediol, pregnanetriol, oestriol, oestrone and oestradiol during the menstrual cycle in a normally menstruating woman. Shaded areas indicate periods of menstrual bleeding.

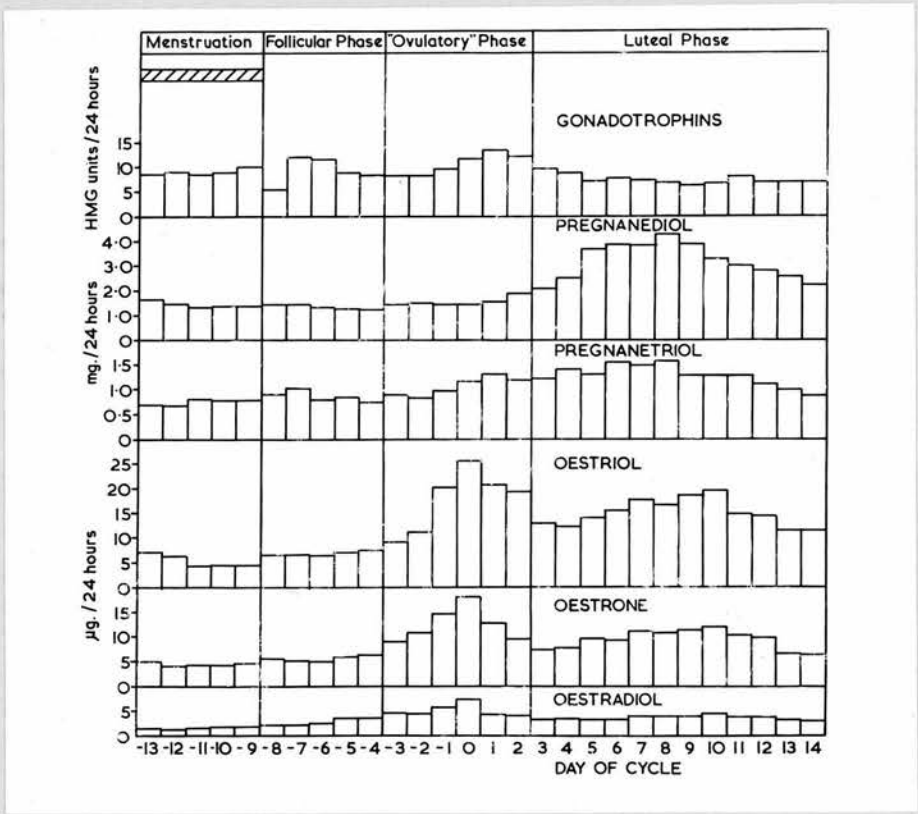

Fig. 11 :- Mean hormone excretion during the normal menstrual cycle of gonadotrophins, pregnanediol, pregnanetriol, oestriol, oestrone and oestradiol.

designated day 0. The average length of the 15 cycles was 28 ± 1.9 days (S.D.). The composite cycle was divided into 4 phases designated respectively menstruation, follicular phase, "ovulatory" phase and luteal phase. For the purposes of the investigation the ovulatory phase was considered to extend over a period of 6 days. It included the day of the oestrone/oestradiol peak and incorporated data on 3 x 48 hour pools of urine. The division of the cycle into 4 phases as described resulted in the elimination of 5% of the readings obtained in subjects in whom either the follicular or luteal phase was longer than 5 or 12 days respectively.

The pattern of oestrogen excretion in Fig. 11 shows the characteristic midcycle peak of day 0; this is followed some 7 to 10 days later by the luteal phase rise in oestrogen output. In 4 of the menstrual cycles studied the peak of oestriol excretion occurred one day after the oestrone/oestradiol peak, while in the remaining cycles the maximum values for oestriol output coincided with those of oestrone and oestradiol. Urinary pregnanediol values rose to a maximum on day 8 during the luteal phase of the composite cycle; the output of urinary pregnanetriol began to rise at the time of ovulation and reached its highest levels on the 6th, 7th and 8th days after the ovulatory peak of oestrogen excretion. These findings in relation to steroid excretion are in keeping with those of previous investigators (J.B.Brown 1960; Fotherby 1962).

A peak of HPG excretion was observed in only 3 menstrual

cycles. In 2 of these the peak postdated while in the third it coincided with the midcycle oestrogen peak. The mean HPG excretion rose to a maximum of 13.2 HMG units per 24 hours on day 1 of the composite cycle (Fig. 3) but no significant difference was found when the mean HPG values for the 4 phases of the cycle were compared.

In Table 37 are shown the mean, minimum and maximum values of the 4 stages of the cycle for the six substances measured. The table shows the large variations in hormone excretion from one individual to another and indicates that the variation tends to be greater in the "ovulatory" and luteal phases of the cycle. This is further illustrated in Table 38 in which the mean, minimum and maximum values are given for the six hormones and hormone metabolites measured during the "ovulatory" phase of the cycle.

Table 37.

Hormonal excretion during the normal menstrual cycle.

Hormone	Units	Menstruation			Follicular Phase			"Ovulatory" Phase			Luteal Phase		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
H.P.G.	HMG units/ 24 hours	9.0	3.0	15.7	9.2	2.8	30.4	10.5	2.8	31.6	7.4	2.9	14.9
Pregnanediol	mg/24 hours	1.43	0.3	2.6	1.33	0.5	2.8	1.54	0.4	3.8	3.16	0.7	6.6
Pregnanetriol		0.73	0.40	1.10	0.87	0.36	1.40	1.06	0.45	2.00	1.28	0.57	2.80
Oestriol	µg/24 hours	5.4	2.1	12.5	6.8	1.5	17.6	17.8	2.7	73.8	15.0	4.0	58.7
Oestrone		4.5	1.2	11.0	5.7	1.4	18.0	12.6	2.5	24.6	9.4	2.2	23.6
Oestradiol		1.7	0	7.7	2.9	0	13.0	5.2	1.0	23.0	3.6	0	13.2

Table 38.

Hormone excretion during the "ovulatory" phase of the menstrual cycle.

Hormone	Units		Day of "Ovulatory" Phase					
			-3	-2	-1	0	+1	+2
HPG	MMG units /24 hours	Mean	8.2	8.1	9.6	11.6	13.2	12.3
		Min.	2.8	2.9	2.9	5.8	10.0	3.9
		Max.	15.0	12.5	31.6	31.6	25.4	25.4
Pregnanediol	mg/24 hours	Mean	1.43	1.50	1.43	1.45	1.58	1.87
		Min.	0.50	0.50	0.40	0.50	0.50	0.50
		Max.	2.80	3.80	3.80	2.70	3.30	3.60
Pregnanetriol	mg./24 hours	Mean	0.88	0.81	0.97	1.17	1.32	1.19
		Min.	0.48	0.48	0.45	0.45	0.80	0.80
		Max.	1.25	1.30	1.60	2.00	2.00	1.75
Oestriol	µg/24 hours	Mean	9.3	11.3	20.1	25.7	20.9	19.4
		Min.	2.7	4.0	8.0	9.8	4.9	8.0
		Max.	26.4	35.0	54.0	64.8	73.8	45.9
Oestrone	µg/24 hours	Mean	9.2	11.0	14.8	18.2	12.8	9.6
		Min.	2.5	3.5	6.4	7.8	4.0	5.3
		Max.	15.0	18.0	20.4	24.6	23.0	17.0
Oestradiol	µg/24 hours	Mean	4.8	4.5	5.9	7.8	4.3	4.1
		Min.	2.0	1.9	3.0	3.0	2.1	1.0
		Max.	9.0	8.4	8.4	23.0	9.0	10.0

4. DISCUSSION

The present investigation has shown that a peak of HPG excretion at midcycle occurred in only 3 out of the 11 menstrual cycles studied. The finding that the midcycle HPG peak is a relatively rare occurrence is in keeping with the observation of Borth, Lunenfeld & de Watteville (1957) but in contrast to those of other investigators, e.g. D'Amour (1943), McArthur, Worcester & Ingersoll (1958)who claimed that the peak is almost invariable and can be used as a reliable indication of ovulation.

Three possibilities must be considered in order to explain these apparently divergent findings. The first concerns the specificity of the bioassay method for HPG. The procedure employed in the present study and in that of Borth et al.(1957) was the mouse uterus test which is not specific for either follicle-stimulating or luteinizing hormone activities (Loraine, 1958) and, for this reason, a differential rise in one or other of the pituitary gonadotrophins at midcycle cannot be excluded. The second possibility is associated with the generally accepted fact that the peak of HPG excretion is of short duration. Accordingly, its detection may not be possible when assays are conducted in urine samples collected over periods of 24 hours or longer. The final possibility is that the age and parity of the subject may affect the HPG excretion throughout the menstrual cycle and may in particular

influence the midcycle peak. At present no reliable information on this question is available although preliminary evidence (Loraine, 1962) suggests that the HPG excretion tends to be higher in parous than in non-parous women.

The findings in the present investigation confirm those of a previous study (Brown et al. 1958) in that the HPG peak at midcycle does not precede the oestrogen peak, but either coincides with it or postdates it. The reason for this observation, which is not in agreement with generally accepted views on the hormonal control of the cycle, is at present obscure. One possibility might be that the gonadotrophin is reabsorbed in the renal tubule and that there is a considerable lag between change in secretion rate and change in excretion rate. Thus if it were practicable to conduct HPG assays in plasma throughout the cycle, the peak concentration in blood might be found to occur earlier than that in urine. An alternative explanation is that, in the initiation and maintenance of the menstrual rhythm in humans, the ovary is more autonomous than has previously been supposed, and that changes in gonadotrophin secretion are the result, rather than the cause, of alterations in the production of ovarian hormones.

The data presented in Tables 37 and 38 show firstly that at any given phase of the cycle there is considerable individual variation in hormone excretion and secondly that, especially in the case of the oestrogenic hormones, large fluctuations in output occur over relatively short periods of time. This latter point is well illustrated by the

level of oestriol excretion during the "ovulatory" phase of the cycle where it will be noted that the output varied from 2.7 to 73.8 µg/24 hours with a mean value of 17.8 µg/24 hours. These figures indicate that isolated hormone assays at widely spaced intervals during the cycle are unlikely to be meaningful, and that worthwhile information on the pattern of hormone excretion in individual subjects will only be obtained if serial determinations are performed.

5. SUMMARY

The urinary excretion of oestrogens, pregnanediol, pregnanetriol and gonadotrophins has been studied throughout the normal menstrual cycle, and the hormonal inter-relationships at the various phases of the cycle have been investigated.

The peak of gonadotrophin excretion at midcycle occurred rarely but when present did not precede the oestrogen peak.

Considerable variation in hormone excretion was noted from one individual to another and it is suggested that isolated hormone assays during the menstrual cycle are unlikely to be of clinical value.

II. THE EFFECT OF A DERIVATIVE OF DITHIOCARBAMOYLHYDRAZINE ON HORMONE EXCRETION IN HUMAN SUBJECTS

1. INTRODUCTION

Paget, Walpole & Richardson (1961) have shown that derivatives of dithiocarbamoylhydrazine act as selective inhibitors of pituitary gonadotrophic function in rats, dogs and monkeys. These workers demonstrated that in female rats treated with such compounds oestrus cycles ceased and the number of maturing follicles in the ovaries was markedly reduced; in male rats similarly treated atrophy of the prostate and seminal vesicles occurred and spermatogenesis was arrested. In view of these actions in experimental animals it was obviously of interest to determine whether such compounds exhibited similar pituitary-inhibiting properties in man. The aim of this section is to report the results obtained when one of the derivatives of dithiocarbamoylhydrazine was administered to human subjects. A small section is included to describe the effect of the same material on the biological activity of a number of gonadotrophin preparations *in vitro*.

2. MATERIALS AND METHODS

A) Compound administered: The derivative of dithiocarbamoylhydrazine investigated (Compound 33,828) was 1-x-methyl-allylthiocarbamoyl-2-methyl-thiocarbamoylhydrazine.

$$CH_2 = CH.\underset{\underset{CH_3}{|}}{CH}.NH.CS.NH.NH.CS.NH.CH_3$$

The compound was supplied by Imperial Chemical Industries Ltd., and it should be emphasised that in contradistinction to many pituitary inhibitors previously described this material is not a steroid.

B) Methods of assay: The methods employed for the measurement of the urinary excretion levels of the various hormones and hormone metabolites are listed below :-

1. Oestriol, oestrone and oestradiol. J.B.Brown (1955b). Brown, Bulbrook & Greenwood (1957).
2. Pregnanediol. Klopper, Michie & Brown (1955).
3. Pregnanetriol. Fotherby & Love (1960).
4. Total 17-hydroxycorticosteroids. Appleby, Gibson, Norymberski & Stubbs (1955).
5. Total 17-oxosteroids. Vestergaard (1951).
6. Dehydroepiandrosterone. Fotherby (1959).
7. Human Pituitary Gonadotrophins(HPG). Loraine & Brown (1959).

The endpoint of the bioassay was the mouse uterus test and the results were expressed in terms of the International Reference Preparation for Human Menopausal Gonadotrophin as HMG units/24 hour urine sample.

3. THE ADMINISTRATION OF COMPOUND 33,828 TO POSTMENOPAUSAL WOMEN

A) Patients: Subject 1; J.S. age 75 years. Body weight 60 kg. Mild cerebro-vascular insufficiency. Subject 2; A.S. age 63 years. Body weight 54 kg. Ischaemic heart disease.

B) General design of investigation: Both subjects were hospitalised during the period of investigation but neither showed any endocrine abnormality. No hormonal therapy was given to either patient during or immediately prior to the study. The two subjects collected 24 hour urine samples continuously. Following a control period of 8 days, Compound 33,828 was administered at dose levels of 10 mg./kg. body weight/day and 2.5 mg./kg./day for 14 days to subjects 1 and 2 respectively. Urine specimens were collected for a further control period during which no treatment was given.

C) Results: The hormone assay data obtained from the two subjects are shown in Figs. 12 and 13. In both the patients studied "total oestrogen" excretion (oestriol + oestrone + oestradiol) was below 10.0 µg./24 hours in the control period. The values obtained are thus in the range normally encountered in postmenopausal women (see McBride, 1957; Brown, Kellar & Matthew, 1959). The administration of Compound 33,828 did not appear to affect the output of these hormones in either patient. As in the case of the oestrogens, urinary excretion values for 17-hydroxycorticosteroids and 17-oxosteroids lay within the normal range. No

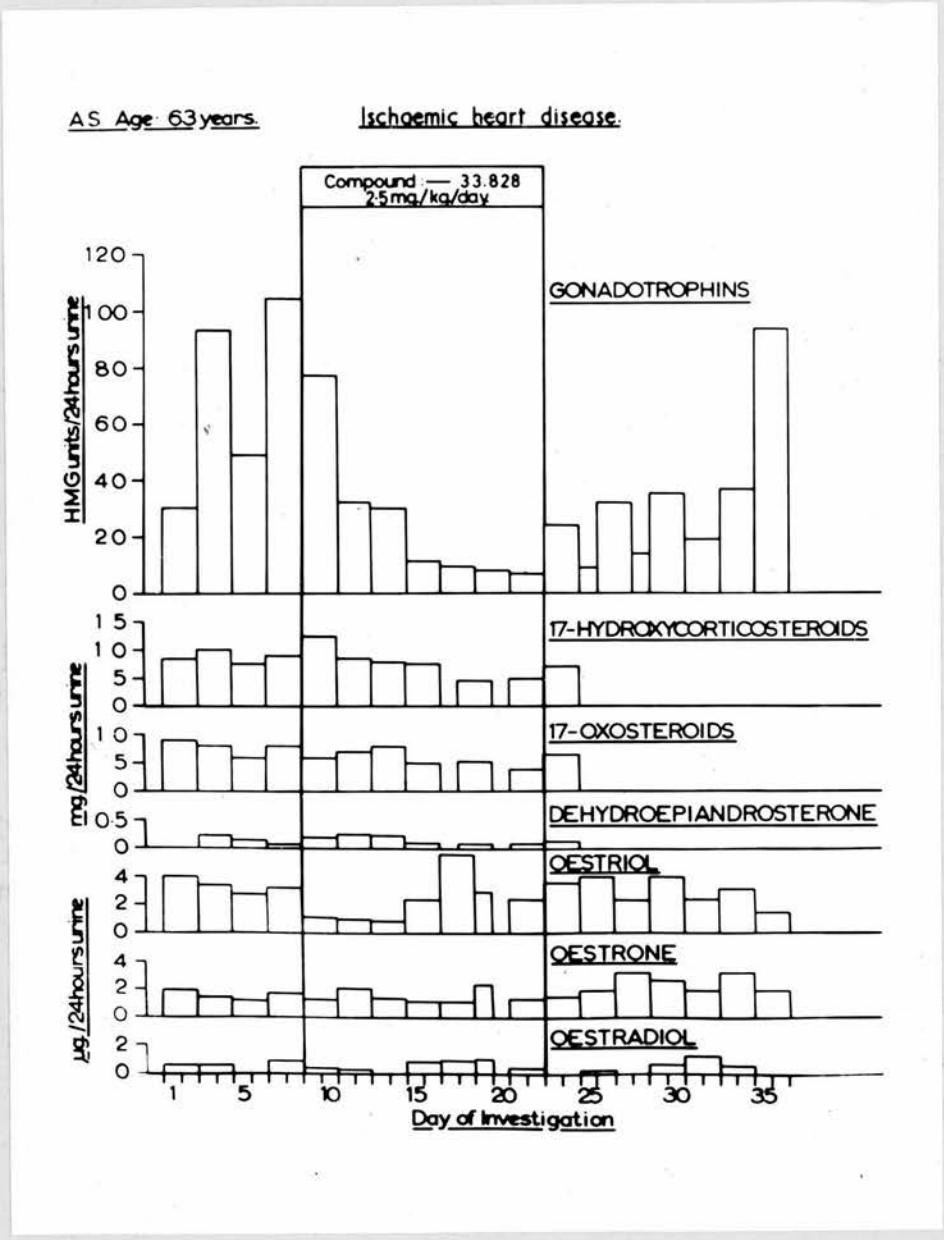

Fig. 12 :- Subject 1 : the effect of Compound 33,828 on hormone excretion in a postmenopausal woman.

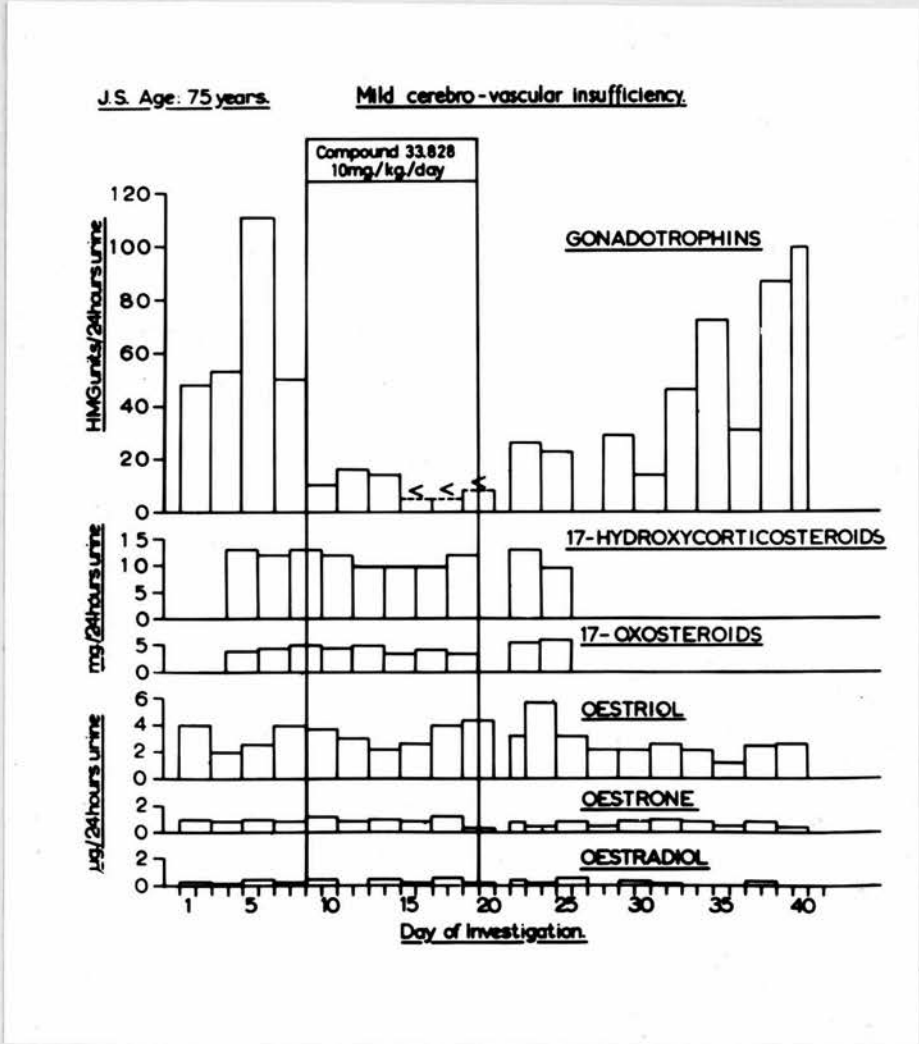

Fig. 13 :- Subject 2 : the effect of Compound 33,828 on hormone excretion in a postmenopausal woman.

change in the output of these hormones occurred during therapy. In the one subject (A.S.) in whom estimations of dehydroepiandrosterone were made excretion values were at the low end of the normal range throughout the period of investigation.

In all, 8 HPG assays were conducted in the two subjects before treatment. Seven of these estimations lay within the 95% fiducial range normally encountered in normal postmenopausal subjects i.e. 38 - 158 HMG units/24 hours (Apostolakis & Loraine 1960) and only one reading in patient A.S. lay outside this range.

In Subject 1 (Fig. 12) the mean HPG excretion before treatment with Compound 33,828 was 66 HMG units/24 hours. During therapy a marked fall in HPG excretion occurred, the lowest value obtained being less than 5 HMG units/24 hours. In the post-treatment period urinary HPG levels rose slowly and values in the control range were found 13 days after cessation of treatment. Side effects consisting of anorexia, nausea, lethargy and drowsiness were noted during the period of drug administration but the symptoms subsided rapidly when the drug was withdrawn.

In Subject 2 (Fig. 13) the mean HPG excretion in the pretreatment period was also 66 HMG units/24 hours. Following the administration of Compound 33,828 a marked depression in urinary HPG excretion occurred but the fall in output was more gradual than in Subject 1 who received

the 10.0 mg./kg. dose. The lowest value obtained during therapy was 7.5 HMG units/24 hours. After withdrawal of the drug a rise in HPG output occurred and a reading similar to those in the control period was found on the 13th day after treatment. No side effects of the drug administration were noted in this subject.

4. THE ADMINISTRATION OF COMPOUND 33,828 TO WOMEN DURING REPRODUCTIVE LIFE.

A) Patients: Subject 3. E.C. age 26 years. This subject was nulliparous and had a previous menstrual history of regular 30-day cycles with menstruation lasting 5 days.

Subject 4. E.W. age 29 years. This subject was multiparous and had a previous menstrual history of regular 28-day cycles with menstruation lasting 5 days.

B) General design of investigation: Both subjects collected 24-hour urine samples continuously throughout the period of study. Basal temperature and any episodes of bleeding were also recorded. Subject 3 (Fig. 4) did not receive the compound during the first cycle, which acted as a control. On the 5th day after the start of menstruation (day 36 of the investigation) the adminstration of Compound 33,828 was begun: the compound was given by mouth in two divided doses, morning and evening, 100 mg. daily being given for 3 days and 50 mg. daily for the following 18 days. In the last menstrual cycle included in the study the compound was given during the luteal phase at the lower dose level. It was hoped that this procedure would demonstrate the effect, if any, of the compound on the activity of the corpus luteum. Subject 4 (Fig. 15) also did not receive the drug during the first cycle. During the second cycle, Compound 33,828 was given by mouth, in a single daily dose of 50 mg. taken in the morning from the 5th day after the start of menstruation for the following 20 days (days 33 - 52 of the investigation). No further treatment was given

during the remainder of the study which included a complete menstrual cycle.

C) Results: Subject 3. (Fig. 14).

The first cycle showed the pattern of a normal ovulatory menstrual cycle. In the second cycle treatment with compound 33,828 was begun on day 5 at a dose of 100 mg. daily. After only three days the dose had to be reduced to 50 mg. daily because the subject complained of nausea, lethargy and drowsiness; when the dose had been reduced the side-effects disappeared. The compound produced a rise in body temperature when given at the higher dose level, but no such effect was noted at the lower level.

During the 21 days on which the compound was administered, the oestrogen, pregnanediol and pregnanetriol excretion remained uniformly low, showing that ovarian activity and ovulation had been suppressed. In spite of this, the mean HPG excretion did not differ significantly from that in the control cycle. 15 days after the compound was discontinued, urinary oestrogen levels rose to an ovulatory peak; this was followed by a rise in oestrogen, pregnanediol and pregnanetriol levels characteristic of the luteal phase, and finally by menstrual bleeding, showing that ovarian activity had been resumed. During this time, two HPG peaks occurred - the first two days prior to and the second two days after the oestrogen peak. The rise in basal temperature between days 59 and 61 of the study was probably due to a mild intercurrent infection.

Compound 33,828 was administered during the luteal

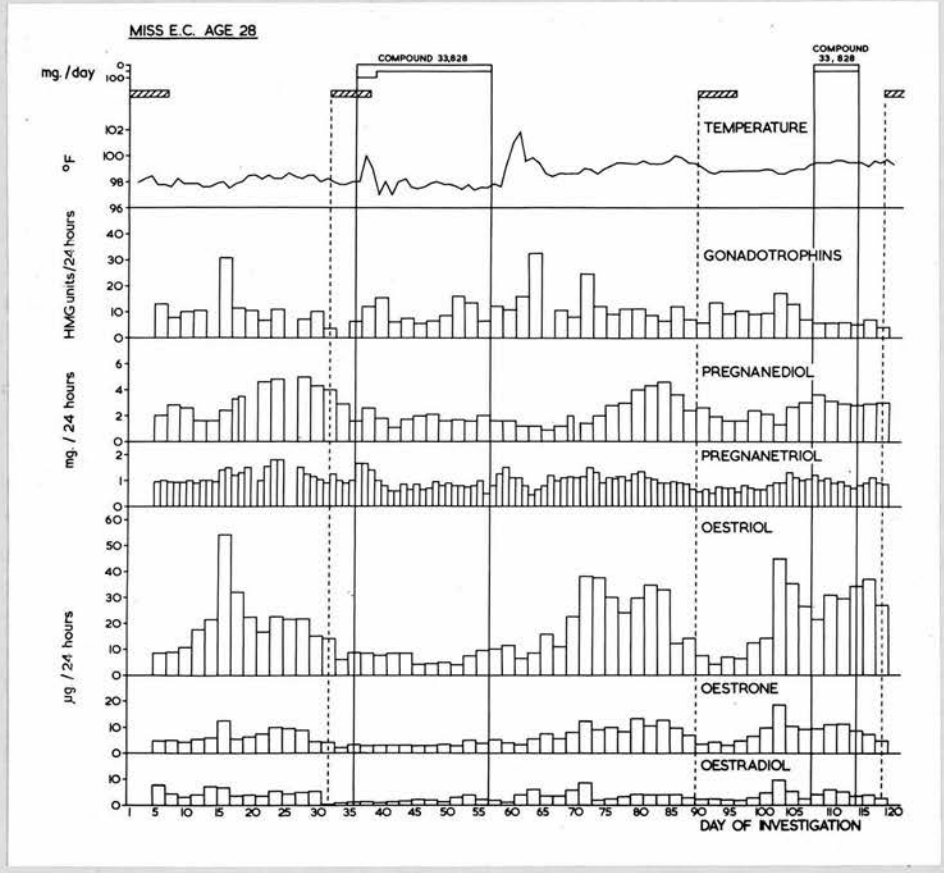

Fig. 14 :- Subject 3 : the effect of Compound 33,828 on hormone excretion in a normally menstruating woman. Shaded areas indicate periods of menstrual bleeding.

phase of the third cycle (days 106 - 114 of the investigation) and no effect on hormone excretion was observed. In this cycle the normal ovulatory pattern of oestrogen, pregnanediol and pregnanetriol output was found, the temperature chart was biphasic, and the subject menstruated at the expected time.

Subject 4 (Fig. 15).

The first cycle of the study showed the pattern of a normal ovulatory cycle. During the administration of the compound (days 33 - 52) at a dose level of 50 mg. daily, no side effects were noted and the amounts of pregnanediol and pregnanetriol excreted remained low. During the last two days of the administration, the oestrogen levels (which were initially low) began to rise, suggesting the start of follicular activity. This suggestion was confirmed by an ovulatory peak of oestrogen excretion 2 days after the withdrawal of the compound, and by a subsequent luteal phase rise of oestrogen, pregnanediol and pregnanetriol excretion; menstruation began on day 70 of the investigation. The final cycle showed the same pattern of hormone excretion as the initial control cycle.

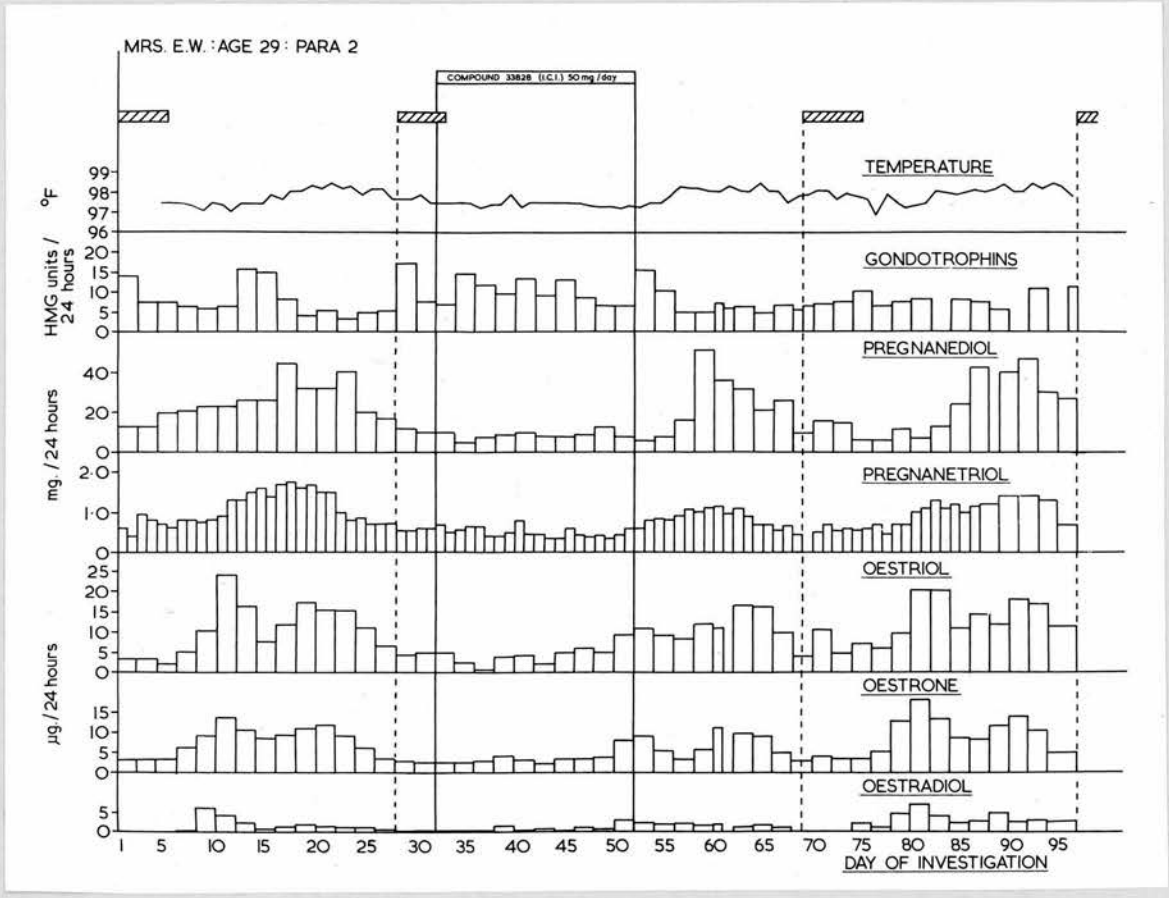

Fig. 15 :- Subject 4 : the effect of Compound 33,828 on hormone excretion in a normally menstruating woman. Shaded areas indicate periods of menstrual bleeding.

5. THE EFFECT OF COMPOUND 33,828 ON THE BIOLOGICAL ACTIVITY OF A NUMBER OF GONADOTROPHIN PREPARATIONS IN VITRO

In section 3 of this chapter it was shown that Compound 33,828 is capable of causing depression or urinary HPG excretion in postmenopausal women. It is theoretically possible that the compound might act by depressing the production of the gonadotrophin by the pituitary or by some direct effect on the circulating hormone after release. In order to obtain further information about this latter point it was decided to incubate Compound 33,828 with various gonadotrophin preparations in vitro.

A) Materials and Methods: Four hormone preparations were tested, these were NIH-LH, NIH-FSH, Human Menopausal Gonadotrophin (Pergonal) and the International Standard for PMSG. Assays were carried out by the augmentation test (see p. 21) and the ovarian ascorbic acid depletion test (see p. 21) for FSH and LH activities respectively.

A stock solution of Compound 33,828 was prepared by dissolving 200 mg. in 0.9 ml. 1N-NaOH with the addition of one drop of octyl alcohol as "wetting" agent. The resultant solution was diluted to 200 ml. with distilled water and had a final concentration of 1 mg./ml. The hormone to be incubated was dissolved in 2.0 ml. of diluted solution at a concentration not exceeding 1%. Incubations were carried out in a water bath at 40 ± 1°C for 24 hours in stoppered tubes fitted with "cold-finger" stoppers. After incubation the hormones were diluted with water in the case of the

augmentation test and with saline for the ovarian ascorbic acid depletion test. Dialysis of the incubated material was not performed before assay.

In all, 8 assays are reported, 2 by the ovarian ascorbic acid depletion test and 6 by the augmentation test. All the assays represent a direct comparison of the incubated and non-incubated materials and were of a symmetrical 4-point design. In 4 cases it was possible to calculate the results as for a 4-point assay and in none of these assays was significant deviation from parallelism observed. In the remaining 4 assays (all by the augmentation test) only one dose level of the incubated material fell within the working range of the dose response curve of the non-incubated preparation and the results were therefore calculated on a 3-point basis.

B) Results: In Table 39 is shown the remaining biological activity when the various hormones were incubated with Compound 33,828. It will be seen that in the case of NIH-LH as measured by the ovarian ascorbic acid depletion test and NIH-FSH and the International Standard for PMSG as estimated by the augmentation test no loss of biological activity was noted and the fiducial limits of the assays included the figure of 100%. When Pergonal was incubated with Compound 33,828 the FSH activity of this preparation as measured by the augmentation test was considerably reduced and a mean residual activity of only 11.8% was recorded.

Table 39.

Incubation of various gonadotrophic preparations with Compound 33,828 (I.C.I.)

Assay Method	Materials tested	Design of assay	λ	Residual activity (%)	Fiducial Limits (%) (P=0.95)	Mean Residual Activity (%)
Ovarian ascorbic acid depletion test	NIH-LH	2 + 2	0.29	100.0	49.5 - 285.0	112.5
		2 + 2	0.31	125.0	62.5 - 356.2	
Augmentation Test	NIH-FSH	2 + 1	0.08	87.0	68.7 - 106.1	93.5
		2 + 1	0.10	100.0	77.1 - 144.2	
	PMSG	2 + 2	0.15	78.2	49.3 - 126.7	99.1
		2 + 2	0.09	120.0	92.4 - 146.4	
	Pergonal	2 + 1	0.08	13.6	10.7 - 16.6	11.8
		2 + 1	0.13	10.0	7.7 - 16.3	

6. DISCUSSION

The present study has shown that urinary HPG excretion is decreased in postmenopausal women by the administration of Compound 33,828 and returns to pretreatment values on withdrawing the drug. In neither of the subjects studied did the dithiocarbamoylhydrazine derivative affect adrenocortical function as judged by the excretion of 17-hydroxycorticosteroids, 17-oxosteroids and the three oestrogens, oestrone, oestradiol and oestriol. The effect on HPG excretion was more marked in the case of Subject 1 who received 10 mg./kg. and the urinary HPG levels fell to below 5 HMG units/24 hours. Compound 33,828 appears to be less active as a pituitary inhibitor than is the synthetic oestrogen stilboestrol. Numerous investigators including Heller, Chandler & Myers (1944), Smith & Albert (1955) and Loraine (1958) have shown that when stilboestrol is administered orally to postmenopausal and oophorectomised subjects for periods ranging from 1 to 6 weeks at dose levels varying between 1.0 and 10.0 mg./day a marked decrease in HPG excretion occurred to levels below those normally found in premenopausal subjects.

It has also been shown that when Compound 33,828 is given early in the menstrual cycle in a daily dose of 50 mg. it can supress ovarian activity and ovulation without affecting HPG excretion. In subject 4 the supression of ovarian activity was not as complete as in subject 3 and

this difference may have been due to the timing of the dose; whereas Compound 33,828 was given to subject 4 in a single morning dose, subject 3 received the same total amount of the drug in two divided doses, morning and evening. When given during the luteal phase only, the compound had no apparent effect on the normal function of the corpus luteum.

The effect of Compound 33,828 in normally menstruating women resembles that of the progestational compounds norethisterone (17α-ethynyl-19-nortestosterone) and norethisterone acetate. These compounds are also capable of supressing ovarian activity and ovulation without affecting HPG excretion when given for about 20 days from the 5th day of the menstrual cycle (Brown, Fotherby & Loraine, 1960, 1962) and have no apparent effect on the function of the corpus luteum when given during the luteal phase only. The progestational and oestrogenic effects of norethisterone and its acetate generally result in withdrawal bleeding a few days after they are discontinued. Compound 33,828 possesses no such hormonal properties and bleeding therefore does not occur on its withdrawal.

The total dosage of Compound 33,828 administered to the postmenopausal women was 600 mg. and 125 mg./day for subjects 1 and 2 respectively. In subjects 3 and 4 on the other hand, the daily dosage was 50 mg. with the exception of the first three days of treatment when subject 3 received 100 mg./day. A possible explanation of the finding that urinary HPG excretion was depressed in subjects 1 and 2 but

not in subjects 3 and 4 is that at the relatively low dose level given to the premenopausal women the compound supressed ovarian but not pituitary activity while at the higher dose level it also supressed the pituitary.

As noted on p. 25the mouse uterus test is not specific for either FSH or LH activity. It would therefore be desirable to determine these hormones separately in urinary extracts but this will not become feasible until extraction and bioassay techniques have been improved.

No information on the mode of action of Compound 33,828 in human subjects can be obtained from the present study. It is, however, of interest to note that when this material was incubated at 40°C for 24 hours with a number of gonadotrophin preparations no effect on the biological activity of NIH-LH, NIH-FSH or PMSG was observed suggesting that Compound 33,828 has no direct action on the gonadotrophin molecule. When a similar experiment was carried out with Pergonal a marked reduction in the activity of the hormone was noted. This observation may be explained in part by the finding that when Pergonal was incubated with water under the same conditions (see p. 86) approximately 60% and 30% of the LH and "total gonadotrophic" activity of the material was destroyed. The fiducial limits of these latter assays did not overlap with those obtained in the present study; thus indicating that under the experimental conditions used Compound 33,828 appears to have inactivated a greater proportion of the Pergonal than when the hormone was incubated with water.

Compound 33,828 and a closely related but biologically less active member of the same series (Compound 22,365) do not appear to have any inhibitory action on placental gonadotrophic function. Thus, in pregnant mares where the gonadotrophin found in large quantities in early pregnancy is produced by the placenta and not by the pituitary (see Amoroso, 1955), relatively large doses of both compounds, ranging from 10 to 25 mg./kg. body weight/day had no pronounced effect on serum or urinary gonadotrophin levels (Schmidt-Elmendorff, Loraine, Bell & Walley, 1962). Similarly, in a female subject 15 weeks pregnant who was scheduled to have a therapeutic abortion the administration of large doses of Compound 22,365 (14.8 g. over 9 days) failed to influence the pregnancy or to affect placental function as judged by serum levels of human chorionic gonadotrophin (HCG) and urinary excretion values for HCG, oestriol, oestrone, oestradiol and pregnanediol (Loraine, unpublished observations.)

The fact that derivatives of dithiocarbamoylhydrazine are capable of inhibiting pituitary gonadotrophic function in postmenopausal women and that supression of ovarian function in women during reproductive life is temporary and completely reversible suggests that these compounds might be of value as oral contraceptive agents. Further clinical trials will, however, be necessary before any conclusion can be drawn as to whether such substances are likely to produce any long term endocrinological effects or whether they are potentially harmful to the pituitary, the ovary or both.

7. SUMMARY

The effect of the dithiocarbamoylhydrazine derivative, Compound 33,828, on hormone excretion has been studied in two postmenopausal and two normally menstruating women.

Compound 33,828 was shown to be a potent inhibitor of pituitary gonadotrophic function in postmenopausal women without affecting the excretion of 17-hydroxycorticosteroids, 17-oxosteroids and oestrogens.

When given early in the menstrual cycle the compound supressed ovarian activity and ovulation, as judged by the assay of steroids in the urine. The excretion of HPG was not affected in these subjects.

It has been shown that when Compound 33,828 was incubated with NIH-LH, NIH-FSH and PMSG no loss of biological activity occurred suggesting that this material exerts no direct effect on the gonadotrophin molecule. When Pergonal was incubated with Compound 33,828 considerable loss of FSH activity was noted.

7. DISCUSSION

Thirty six years have now elapsed since Aschheim and Zondek (1927) showed that the blood or urine of pregnant women contained a substance with gonad-stimulating properties. During this period a considerable amount of work has been carried out to investigate the nature and mode of action of the gonadotrophic hormones. There is however still considerable controversy about a number of fundamental points and much of this has arisen because of the difficulties associated with the preparation of highly purified hormones. It can readily be seen that the assessment of the biological role of an impure material is very difficult because of the presence of contaminating substances. Likewise the assay of such preparations can never be entirely satisfactory because it is impossible to demonstrate whether or not any given method is specific.

The present investigation has shown (Chapter 3) that NIH-LH was the most active LH preparation tested and that this hormone was very inactive when administered to the intact immature mouse. All the other hormones tested showed both FSH and LH activity, as estimated by the augmentation and ovarian ascorbic acid depletion tests respectively. When the potencies of these preparations was measured by the mouse uterus test no clear relationship could be established between these results and those obtained by the augmentation and ovarian ascorbic acid depletion tests. This finding may be explained in one of two ways; either that the augmentation and ovarian ascorbic acid depletion tests are

not entirely specific or that the mouse uterus test is influenced by the FSH to LH ratio of the preparation tested.

With the existing bioassay methods it is possible to obtain an accurate assessment of the gonadotrophic activity of a purified preparation but because of the low sensitivity of the methods it is not possible to measure FSH and LH separately in addition to the steroid hormones oestradiol, oestrone, oestriol and pregnanediol on the same 24 hour urine sample from a human subject. For this reason non-specific methods such as the mouse uterus test have been used to provide some indication of the total gonadotrophin level. Thus in the studies reported herein on hormone excretion during the normal menstrual cycle and on the effect of a derivative of dithiocarbamoylhydrazine on hormone excretion in human subjects the mouse uterus test was employed to measure urinary HPG. The evidence presented in Chapter 3 of this thesis suggests that results obtained by this test cannot readily be correlated with data obtained by specific methods for FSH and LH. However, no alternative to the use of non-specific assay methods such as the mouse uterus and mouse ovarian weight tests will become available until very sensitive procedures are developed for the specific determination of FSH and LH activities in human body fluids.

Most of the assay procedures for FSH depend either upon histological changes or an increase in the weight of the ovary of the experimental animal, but a more sensitive

growth is initiated by the combined action of FSH and LH acting together through the ovary. Brown and Billewicz (1962) have shown that a marked increase in uterine weight is obtained when NIH-LH and NIH-FSH are given together to intact immature mice in dosages which if given alone would have had no uterotrophic effect. This type of work has been stimulated by the preparation of highly purified gonadotrophins and because of the availability of these materials a considerable amount of work on their biological role is currently in progress.

With the development of more sensitive assay procedures for gonadotrophins such as the method depending on the depletion of rat ovarian cholesterol it is possible that it may no longer be necessary to prepare urinary extracts of HPG. However at the present time a number of methods are available for the extraction of HPG from urine and Loraine and Mackay (1961) have shown that there is no marked difference in the yield of gonadotrophin obtained by six of the most commonly used extraction procedures. All the techniques suffer from the common disadvantage that the final extract contains material which is toxic when administered to experimental animals and in Chapter 4 of this thesis is described a series of experiments undertaken to try to reduce the toxicity of the final extract obtained by the kaolin acetone procedure for the extraction of HPG from human urine. None of the modifications employed resulted in a marked improvement in the specific activity of the extract and for this reason a new approach to the problem might be advantageous.

It is possible that the use of such materials as diethylaminoethyl cellulose might provide a more satisfactory method of extracting HPG from urine.

Following the incorporation of an ammonium acetate-ethanol step into the kaolin acetone method all the gonadotrophic activity in male urine was destroyed without any effect on the HPG recovered from postmenopausal urine. This selective destruction of one type of gonadotrophic activity in the presence of another may yield valuable information about the structure of the gonadotrophin molecule. Thus in Chapter 5 of this thesis it has been shown that 6 M urea selectively destroys LH without markedly affecting FSH activity. Work at present in progress in this laboratory indicates that when the urea concentration is reduced to 2 M little or no destruction of FSH is observed but at least 90% of the LH activity is lost. It seems unlikely that at this concentration the urea acts in the way in which it normally denatures proteins and the effect may therefore be due to the cyanate content of the urea solution (Stark, Stein & Moore, 1960). The reactions involved appear to be fairly rapid in nature because with a six hour incubation period at 40°C virtually all the LH activity was destroyed by 2 M urea. In the case of FSH the degree of inactivation was observed to increase with the time of incubation and with the concentration of urea. If however the urea is responsible for inactivating the LH then it is questionable whether or not a true picture of the LH content of a urine specimin with a

high urea concentration can ever be obtained.

Although it is probable that urea acts at various sites on the LH molecule it could be argued that the functional group responsible for biological activity constituted only a small part of the hormone and that this could readily be inactivated. It has been shown that in order to obtain a reduction in the activity of FSH it is necessary to use a higher concentration of urea than that required for LH suggesting that the active site of the FSH molecule is either protected from or is more resistant to the action of the reagent.

The work reported in this thesis on the effect of formaldehyde on NIH-LH shows that a marked destruction of LH activity can be obtained with low concentrations of formaldehyde but that the reaction is not complete within six hours at room temperature. The effect of formaldehyde on NIH-FSH is currently being studied and preliminary results indicate that this material is more resistant to the action of formaldehyde than is NIH-LH.

The present study has confirmed the observation that FSH was less affected by chemical treatment and enzymic action than were LH preparations (Fraenkel-Conrat, Simpson & Evans, 1939; Li, Simpson & Evans, 1939; 1940), and it is hoped that further work of this type will provide a means of destroying the biological activity of LH. This would be of considerable use as a means of testing the specificity of new assay procedures and in investigating the effect of pure hormones on the response obtained in non-specific assay

procedures such as the mouse uterus or mouse ovarian weight tests.

The renal clearance of HCG has been extensively studied and recently Apostolakis and Loraine (1960) have obtained data on the clearance of HPG. It was therefore of interest to extend this study to include the investigation of the renal handling of equine gonadotrophin. Very low values were obtained for the clearance of pregnant mare gonadotrophin in two ponies, two donkeys and one horse. It should however be noted that the clearance rates were in general similar to those observed for HCG and HPG. Further information on the renal handling of protein hormones may be obtained by studying the ultrafiltration of such materials and thus assessing whether or not the gonadotrophins are freely filtered through the glomerulus. If the excretion of LH and FSH is entirely determined by passive glomerular filtration then it will be possible to follow changes in the serum concentration. But if excretion is the resultant of passive filtration plus active tubular reabsorption then changes in urinary excretion may be due to alteration in renal activity rather than to alteration in plasma concentration. In view of this and of the possible effect of endogenous urea on LH it would therefore be desirable to have assay methods that can readily be used directly on blood plasma.

Fundamental work on the clinical application of gonadotrophin assays is rendered very difficult by the lack of specific and highly sensitive assay methods. However, despite this it is still possible to obtain useful data and

in the present study the interrelationships of various hormones and hormone metabolites throughout the menstrual cycle have been described. A number of investigators have stated that the output of HPG during the menstrual cycle is low in the follicular and luteal phases and that a peak of excretion occurs at midcycle coinciding approximately with the time of ovulation (Smith and Smith, 1936; Klinefelter, Albright & Griswold, 1943 and Pedersen-Bjergaard and Tønnensen 1948). However, Main, Cox, O'Neal & Stoeckel (1943) reported that in a proportion of subjects a second gonadotrophin peak may occur just before the onset of menstruation while Heller, Farney, Morgan & Myers (1944) found that the pattern of gonadotrophin excretion was very variable from one subject to another. Both Main et al (1943) and Heller et al (1944) concluded that the stage of the menstrual cycle and the time of ovulation could not be predicted by estimating the output of urinary gonadotrophins.

It is probable that the lack of uniformity in the results reported by earlier investigators is dependent to some extent on the absence of serial determinations and the unsatisfactory bioassay methods employed. Brown, Klopper & Loraine (1958) using more reliable assay methods have studied the relationship between the midcycle gonadotrophin and oestrogen peaks in normally menstruating women. They observed a rise in gonadotrophin excretion in seven out of the nine patients studied but noted that there was no correlation between the height of the gonadotrophin and oestrogen peaks. In none of the subjects did the rise in gonadotrophin excretion precede

the oestrogen peak and these workers concluded that the increase in oestrogen output could not have been initiated by the rise in urinary HPG levels. The present investigation using the mouse uterus test and taking samples in general at intervals of 48 hours has shown that in only three out of the eleven cycles studied was there a peak of gonadotrophin excretion and that in these patients the maximum occurred at midcycle. In the remaining eight subjects no fluctuation in the urinary HPG levels was noted. It is theoretically possible that the assay methods employed were too insensitive or that because of the short duration of the gonadotrophin peak it will only be observed if urine specimens collected over a short period of time are studied.

Valuable information of the effect of antiovulatory agents on pituitary function as judged by the assay of HPG in urine has been obtained in the present study. Thus in the two postmenopausal women studied urinary HPG output was depressed following the administration of a derivative of dithiocarbamoylhydrazine (Compound 33,828) while in two women with normal ovulatory menstrual cycles a smaller dosage of the same compound resulted in an inhibition of ovulation without any effects on urinary HPG excretion. The interpretation of the results obtained following the administration of this compound is made very difficult because the mode and specific site of action of the gonadotrophic hormones and their role in the regulation of ovarian function is only partially understood.

The mechanism by which the anti-ovulatory agents induce sterility in human subjects is uncertain but in experimental animals it appears that these compounds act via the pituitary. Goldman, Epstein & Kupperman (1957) and Epstein, Kupperman & Cutler (1958) showed that in parabiotic rats the release of gonadotrophin following orchidectomy was inhibited by anti-ovulatory compounds. Holmes and Mandl (1962 a) observed an increase in pituitary weight without any alteration in the number of basophil cells in rats treated with norethynodrel while Saunders and Drill (1958) showed that the simultaneous administration of norethisterone and exogenous gonadotrophin did not influence the ovarian response to the latter.

The evidence for the supression of pituitary activity in experimental animals appears fairly conclusive but there is no reason to suppose that this work is directly applicable to human subjects. As noted in the Introduction (see p. 16) Douglas, Loraine & Strong (1960) and Martin and Cunningham (1960) showed that derivatives of norethisterone depress HPG levels in postmenopausal women while Brown, Fotherby & Loraine (1962) found that similar compounds had no effect on gonadotrophin excretion in normally menstruating women. Recently Buchholz, Nocke & Nocke (1962) have stated that the anti-ovulatory agents abolish the midcycle of HPG excretion while Taymor (unpublished observations) noted that norethisterone and norethisterone acetate depressed the LH and "total gonadotrophin" levels in urine as measured by the

increase in ventral prostatic and testicular weight in hypophysectomised rats.

The derivative of dithiocarbamoylhydrazine studied herein appears to have an effect both on the ovary and the pituitary in different experimental conditions, but this may well be a phenomenon of the dosage employed. Alternatively the possible effects of urea and active tubular reabsorption on urinary HPG levels previously described may have been in part responsible for the results obtained. It is clear from the data presented in this thesis that the development of sensitive assay procedures is of great importance to the furtherance of work in this field. This is made more apparent by the considerable confusion which exists at the present time about the site of action of anti-ovulatory compounds in human subjects and further information on this topic will only be obtained by the careful study of pituitary and ovarian function.

8. SUMMARY

1. The biological activity of a number of gonadotrophin preparations has been studied using specific and non-specific bioassay procedures. It was found that there was no constant relationship between the results obtained by the mouse uterus test and by specific methods for either FSH or LH.

2. The kaolin acetone extraction procedure for HPG was described in detail and the effect of filtration of urine prior to extraction and of incorporation of an ammonium acetate-ethanol step into the method were investigated. Neither modification was found to be suitable for application to the routine extraction of urinary HPG.

3. Incubation of various gonadotrophin preparations with 6 M urea was shown to result in destruction of the greater part of the LH activity without markedly affecting the FSH activity.

4. After incubation of NIH-LH with formaldehyde the degree of biological inactivation as estimated by the ovarian ascorbic acid depletion test was shown to be more closely related to the length of incubation and the concentration of formaldehyde than to the temperature employed.

5. The renal clearance of pregnant mares gonadotrophin was found to be less than 1 ml/min. and is thus in the same range as the values previously reported for HPG and HCG.

6. Hormonal interrelationships have been studied throughout the menstrual cycle in 11 normally menstruating women. It was shown that the peak of gonadotrophin excretion at midcycle occurred rarely but when present did not precede the oestrogen peak.

7. The dithiocarbamoylhydrazine derivative (Compound 33,828) was found to be a potent inhibitor of pituitary gonadotrophic function in postmenopausal women. When administered at a lower dose level to two women during reproductive life the compound inhibited ovarian activity and ovulation as judged by steroid assays without any effect on urinary HPG output. No loss of biological activity was noted when four gonadotrophin preparations were incubated in vitro with Compound 33,828.

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PUBLICATIONS

1. Studies on the biological activity of various gonadotrophin preparations (with H. Schmidt-Elmendorff and J.A.Loraine) J. Endocrin (1962), 24, 349.

2. The effect of 6 M urea on the follicle-stimulating and luteinising hormone activities of various gonadotrophin preparations (with H. Schmidt-Elmendorff and J.A.Loraine) J. Endocrin (1962), 24, 153.

3. Hormone excretion during the normal menstrual cycle (with J.A.Loraine) Lancet (1963) i, 1340.

4. The effect of derivatives of dithiocarbamoylhydrazine on hormone excretion in postmenopaugal women (with J.B.Brown, K.Fotherby, J.A.Loraine and J.S.Robson) J. Endocrin (1962), 25, 221.

5. Effect of a derivative of dithiocarbamoylhydrazine on hormone excretion during the menstrual cycle (with J.B.Brown, K.Fotherby and J.A.Loraine) Lancet (1962), ii, 528.