SOME OBSERVATIONS ON THE BIOLOGICAL ASSAY OF
GONADOTROPHIC HORMONES.

Thesis

Presented By

Subhas Mukhopadhyay, B.Sc., M.B.B.S.,
D.Phil. (Calcutta).

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Acknowledgement.
The possible existence of an extra-ovarian factor capable of regulating ovarian activity was postulated by Heape in 1897. Foa (1901) observed rapid follicular growth and development in immature ovaries when grafted into adult animals. As early as 1912, Aschner (1912) was able to demonstrate atrophic changes in the genital organs of dogs following hypophysectomy. Enlargement of the ovaries and formation of numerous corpora lutea in normal rats treated with pituitary extracts were reported by Evans and his colleagues during the early 1920's (Evans, 1924). Later these investigators showed that the gonad-stimulating and growth promoting factors in pituitary extracts were different chemical entities (Evans et al., 1933). At about the same time Smith (1926), Zondek and Aschheim (1927) and others, demonstrated that pituitary extracts or implants are capable of influencing the gonads. Subsequently it was found that the administration of pituitary gonad-stimulating extracts produced two distinct types of gonadal reaction: (a) the stimulation of follicular growth in the ovaries and of spermatogenesis in the testis, and (b) maturation of the ovarian follicles with subsequent ovulation and formation of corpora lutea in the ovary; and the assumption of an active secretory role by the interstitial tissue to secrete androgenic hormones in the testis. The 3rd International Conference on the Standardisation of
Hormones, held in Geneva in 1939, decided to adopt the term "gonadotrophin" for the various gonad-stimulating principles.

In 1931 Fevold, Hisaw and Leonard separated pituitary extracts into two components which exhibited different types of hormonal activity, a finding which was subsequently confirmed by other laboratories. These hormones have been designated as the Follicle Stimulating Hormone (FSH) or thylakentrin and interstitial cell stimulating or Luteinizing Hormone (ICSH, LH) or metakentrin. Techniques involving fractional precipitation with organic solvents and inorganic salts combined with pH adjustment have shown that pituitary extracts from sheep and swine contain two gonadotrophin principles (Fevold et al., 1931; Van Dyke and Allen-Lawrence, 1933; Bates et al., 1934; Jensen et al., 1939; Shedlovsky et al., 1940; McShan et al., 1954). Biological and chemical purification of a degree sufficient to determine the physicochemical properties of these two substances have been claimed. A luteinizing hormone preparation of sufficient purity has been obtained, which when administered to hypophysectomised immature female rats had little effect on the ovarian follicles, but which produced interstitial cell repair in these animals. FSH preparations of a high degree of purity have been claimed to cause enlargement of the ovarian follicles without significant effect on the interstitial cells.
Techniques involving differential precipitation by salt solutions and by organic solvents and isoelectric precipitation were used to purify FSH from crude pituitary powder. Recently ion exchange resins and molecular sieve chromatography have been utilized for this purpose. Biethylaminoethyl (DEAE) cellulose column chromatography have been used by McShan et al. (1954), Ellis (1958), Steelman and Segaloff (1959) and Woods and Simpson (1960). Nevertheless even at the present time, the most highly purified preparations of FSH and LH do not fulfill all the criteria of biological and chemical purity when tested by bioassay and by physicochemical techniques.

Luteotrophin.

This principle is believed to be responsible for the maintenance of structural and functional integrity of the formed corpora lutea, and for the secretion of progesterone by the ovaries (Astwood, 1941; 1953; Evans et al., 1941). A specific assay method for the determination of LH activity depending upon the depletion of ovarian ascorbic acid in intact immature pseudopregnant rats was described by Parlow (1958, 1961). It has recently been shown by Guillemin and Sakiz (1965) that the ascorbic acid depletion response to LH in the pseudopregnant rat ovaries cannot be obtained if the animals are hypophysectomised. The altered response to LH following hypophysectomy can be corrected.
by the administration of prolactin shortly before the hypophysectomy operation (Guillemin and Sakiz, 1963). These findings suggest that prolactin has got a certain modifying influence on the gonadotrophic effects produced by LH.

**Chorionic Gonadotrophin.**

In 1927 a gonad-stimulating substance, present in comparatively large amounts in the blood and urine of pregnant women, and capable of producing ovarian follicular maturation, luteinization and haemorrhage into the ovarian stroma in immature female mice was demonstrated by Aschheim and Zondek (1927). Following this important discovery there was much speculation regarding the source of this material. Aschheim and Zondek (1927) put forward in their original view that the gonadotrophic factor was derived from the pituitary. Subsequently it became apparent that, like the adenohypophysis, the chorionic tissue also was capable of elaborating gonadotrophic substances. A number of convincing pieces of experimental evidence have been presented since then to demonstrate that the large amount of the gonadotrophic principle present in the body of the pregnant woman comes from the chorionic tissue and not from the pituitary (Engle, 1929; Philipp, 1930; Collip, 1930, 1934, 1935; Collip et al., 1933; Hamburger, 1933; Deanesly, 1935). Production of gonad-stimulating material *in vitro* was shown to take place in placental tissue culture (Sannicandro, 1934; Gey et al., 1938; Jones and Bucher, 1943; Jones
et al., 1943; Stewart et al., 1948). Histochemical studies by a number of workers (Wislocki and Bennett, 1943; Zilliacus, 1953) and intraocular transplantation experiments (Kido, 1937; Stewart, 1951) confirmed the placential production of the gonadotrophic substance. Since the adoption by the League of Nations of the term “gonadotrophin” for various gonad-stimulating materials, human placental gonadotrophin is generally designated as Human Chorionic Gonadotrophin (HCG). Purified preparations of HCG have been made by Gurin et al. (1939, 1940), Katzman et al. (1943), Claesson et al. (1946) and Landgrebe et al. (1954). Human chorionic gonadotrophin has been obtained in a highly purified form by Bourrillon and his colleagues (Bourrillon et al., 1957, 1959, 1960, 1960a). Morris (1955) claimed to have prepared HCG of remarkably high specific activity of 12,000 i.u./mg. The active hormone is thought to be glycoprotein in nature, which contains galactose as its carbohydrate moiety (Gurin, 1942).

Human Urinary Gonadotrophins (HFG) from Non-pregnant Sources.

Relatively small amounts of gonadotrophic material have been found to be present in human urine from normal adult male and from non-pregnant female subjects. A very small amount of urinary gonadotrophin is believed to be present in the urine of young children of both sexes. It has been generally assumed that the gonadotrophic material excreted
in the urine of non-pregnant human subjects is derived from the pituitary. Crude concentrated extracts of urinary gonadotrophin show both FSH and LH activity in varying proportions. The variation in the ratio of FSH: LH activity in different urinary gonadotrophin preparations may be due to the difference between the various extraction procedures employed for the purpose. Although gonadotrophic material, possessing certain biological resemblance to HCG is secreted sometimes in considerable amounts by certain embryonic neoplastic tumours, the pituitary gland is believed to be the only source of gonadotrophin production in normal non-pregnant human subjects. Human urinary gonadotrophin is generally referred to as HPG (human pituitary gonadotrophin). At present there is considerable difference of opinion regarding the biological and chemical characteristics of HPG. Whether the FSH and LH activities of HPG are present in the same hormonal molecule or whether they are biologically as well as chemically separate entities is not yet known with certainty.

It is interesting to note that in spite of the existing controversy about the differential FSH and LH activity in human urinary gonadotrophins, up till now no clinical situation is known in which assays carried out by an LH specific method or by an FSH specific method or by both give different results from those carried out by general

There is a W.H.O. accepted international reference preparation (I.R.P.) of human urinary gonadotrophin (HMG-24) prepared from pooled postmenopausal urine (HMG). The HMG preparation originally prepared by Donini et al. (1952) and commonly known as Pergonal has been proved to become the new international standard. Before the HMG-24 became the international reference preparation, another human menopausal gonadotrophin (HMG) extract, known as HMG-20A, was used between the years 1954-1957 as an unofficial reference preparation. HMG-24 and HMG-20A are equipotent.

Purification of Pituitary LH.

Earlier investigations on the purification and characterization of pituitary LH, reported during the years 1940-1942, yielded highly active products from sheep (Li et al., 1940, 1940a., 1942) and swine (Chow et al., 1942; Shedlovsky et al., 1942) pituitary glands. The preparations appeared to be homogeneous according to the physical and biological criteria of purity then available. When subjected to electrophoretic, ultracentrifugation and solubility tests they were shown to behave as homogeneous proteins. Sheep LH was found to have a molecular weight of 40,000 and an isoelectric pH of 4.6. The hormone was insoluble in 66% acetone and contained mannose, galactose, galactosamine and
glucosamine in its molecule. Swine LH on the other hand was claimed to have a molecular weight of 100,000 and an isoelectric pH of 7.45. The latter was also found to be insoluble in 60% acetone and contained carbohydrate in its molecule.

The importance of the early ovine and procine preparations is attested by the fact that most of what is known of the biological action of LH was learned from the studies involving the use of these early preparations. It is therefore probably fair to state that due to the lack of a practical method for obtaining the hormone in a state of purity that would satisfy modern criteria, the biological properties attributed to these materials were in fact obtained from experiments using insufficiently pure preparations of LH. These materials were contaminated with other biologically active principles like thyrotropin and FSH. Comparatively recently Li and his associates (Squire and Li, 1958, 1959), Ellis (1958) and Leonora et al. (1958) have described new methods for the purification of LH from sheep pituitary glands. During the course of this procedure Squire and Li (1959) were able to obtain a highly active concentrate of LH by a method involving fractionation with alcohol, ammonium sulphate and sulphasalicylate. Two active fractions were obtained by subsequent purification by chromatography on a resin column. The yield
at this point of purification was found to be 30 times
greater than that reported previously, and the products
showed higher LH activity, although neither fraction was
electrophoretically pure. Further purification by means
of zone electrophoresis on starch and by additional chroma-
tographic steps enabled these workers to obtain LH of a
high degree of homogeneity as indicated by sedimentation
analysis and chromatography on IRC-50 resin. The isoelectric
point of this very highly purified LH preparation was
estimated to be near pH 7.3. The molecular weight calcu-
lated from sedimentation and diffusion data and from assumed
partial specific volume of 0.73 was 30,000. No contami-
nation of this LH preparation with other pituitary hormones
was indicated by biological assays designed to detect such contamination at the level of 0.1 per cent. A total dose
of 0.5 µg of this LH material was found to produce a sig-
nificant increase in the weight of the ventral prostate
of young hypophysectomised rats.

At present there is no international standard or
reference preparation for human or animal pituitary LH.
The highly purified ovine pituitary LH prepared by the
National Institute of Health, Bethesda, Maryland, U.S.A.
(NIH-LH) is widely used as a reference and standard pre-
paration.
Gonadotrophic Substances from Endometrial Tissue.

At one time discussion centred about the question whether or not decidual cells assumed endocrine functions. Nattan-Laurrier (1900) was among the first to regard them as somehow concerned in the elaboration of an internal secretion. Ancel and Bouin (1911) from a study of the changes occurring in the mammary glands of pregnant rabbits, concluded that the glandular development which takes place in the last part of pregnancy is caused by the cells of the "myometrial gland". Hammond (1917) occasionally found these cells in the rabbit's uterus, but he considered that their occurrence was too irregular and uncertain to have the important endocrine functions attributed to them. This is also the conclusion of Mossman (1937) who states that "one is absolutely unjustified, from anything yet known of their cytology, in making assumptions of endocrine functions for them". However, observations have been made in favour of the conclusion that the decidua may be concerned in the secretory process of the placenta. An interesting example of this is seen in the development of the so-called "endometrial cups" in the uterus of the pregnant mare. The endometrial cups are specialised structures to which one can attribute special secretory activity, and in which a decidual transformation of the interglandular tissue has been described by a number of workers (Cole and Goss, 1943;
Amoroso, 1945; Rowlands, 1947), all of whom believe that they have traced to these cups an important source of equine gonadotrophin.

**Pregnant Mares Serum Gonadotrophin.**

Zondek (1930) and Cole and Hart (1930) independently discovered the presence of a potent gonadotrophic material in the serum of pregnant mares. Pregnant mares serum gonadotrophin (PMSG) or otherwise known as equine gonadotrophin have been obtained in very highly purified form (12,000 i.u./mg) by Rimington and Rowlands (1941). It has been observed that even the most highly purified preparations of PMSG exhibit both follicle-stimulating and luteinizing properties. Cole et al. (1940) postulated that both these activities of the hormone most likely resided in the same molecule. The erroneous concept that the gonadotrophic material present in the serum of pregnant mares is not excreted in the urine of these animals has been so prevalent (Albert, 1961) that the term "serum gonadotrophin" has been widely used to mean this particular gonadotrophic substance. Zondek (1931a.) and recently Schmidt-Elmendorff, Loraine, Bell and Wally (1962) have demonstrated the presence of this material in the serum as well as in the urine of pregnant mares.

**Purification of Pituitary FSH.**

Although relatively purified preparations of FSH have
been obtained this hormone has not yet been purified to the extent comparable to highly purified LH preparations. Like LH, sheep pituitary FSH is also believed to be a glycoprotein with a molecular weight of 70,000 and an isoelectric pH of 4.5. The corresponding values for pig FSH are comparable. Recently, methods have been devised for preparation of bovine and ovine LH which have a high specific activity as well as relative freedom from FSH and thyrotrophin (TSH) the major hormonal contaminants of LH (Reichert, 1962; Reichert and Parlow, 1963). The important problem of freeing FSH preparations from their principal and most tenacious hormonal contaminant, LH, has proved to be more difficult. This separation is usually regarded as critical because of the well-known augmenting effect of small amounts of LH on the biological activity of FSH (Greep et al., 1942; Simpson, 1961). Most of the previous efforts to purify FSH have emphasized the importance of attaining a high degree of specific activity. The problem of reducing the degree of LH contamination of FSH did not receive much attention until very recently (Reichert and Parlow, 1963).

Ellis (1958) employed Diethylaminoethyl cellulose (DEAE-C) and zone electrophoresis to produce ovine FSH which was 30 to 40 times more potent than the Armour Standard FSH (264-151X), or approximately 11 to 14 times
more potent than NIH-FSH-S1. The LH activity of this preparation was 0.0075 times that of Armour Standard LH 227-30. Armour Standard LH 227-30 and NIH-LH-S1 are equal in LH potency (Parlow, 1961).

Woods and Simpson (1960) have described purification of ovine FSH using DEAE-Cellulose. The resulting preparation, fraction IV-19F, is 32 times as potent as NIH-FSH-S1, and according to their report, has an FSH:LH ratio which is improved above any previously reported for preparations from sheep pituitary glands.

Reichert and Parlow (1963) prepared an ovine FSH preparation which was approximately 1.4 times more potent than NIH-FSH-S1. Although the specific activity of this last named preparation is less than that of either Ellis's (1958) or Woods and Simpson's (1960), its LH contamination is less than any FSH preparation purified so far. The LH activity of Reichert and Parlow's FSH (FSH-2) has been claimed 0.00010 times that of NIH-LH-S1. It is evident therefore that unlike that of LH the problem of purifying FSH while retaining both its biological and chemical properties to a proportionate degree has not been achieved so far.

At present there is no internationally accepted standard or reference preparation for human or animal pituitary FSH. The relatively purified ovine pituitary FSH prepared by the National Institute of Health, Bethesda, Maryland, U.S.A.
(NIH-FSH) is used in many laboratories as a reference and standard preparation of pituitary FSH.
METHODS OF ASSAY OF GONADOTROPHIC HORMONES.

The methods of determination of gonadotrophic hormones leave much to be desired at present. This is particularly applicable in connection with the routine work of the clinical laboratory. Many of the methods at present used for clinical application suffer from one or other of the defects associated with the reliability and practicability of the assay methods.

Until very recently the bioassays have been the only methods used for assaying gonadotrophic hormones in human body fluids. Very recently attempts have been made to use quantitative immunological techniques for the determination of these hormones.

Bioassays are in general believed to be less reliable than chemical methods. Compared to the chemical methods the reliability criteria of most bioassays, with respect to their accuracy, precision and probably specificity, are generally inferior. Probably the only advantage of certain bioassays over the chemical assays is their remarkable sensitivity. Butenandt (1955) reported a bioassay procedure for Sambyx ecdohormone in which less than $10^{-5}$ µg of the material was found to produce a significant and specific biological response.

Non-Specific Assay Methods.

Certain widely used bioassay procedures for measuring
gonadotrophic activity are often classified as "non-specific" because these methods are claimed to be unable to measure FSH or LH activity specifically (Loraine, 1958). The gonadotrophic activity claimed to be measured by these methods has come to be generally known as "total gonadotrophic activity". Most of the users of these methods agree to the point that "total gonadotrophic activity" does not mean the algebraic summation of FSH and LH activity.

Unfortunately the very common usage of this undefined terminology has created great confusion amongst clinicians not specially familiar with the intricacies of the bioassay procedures. However some authorities still hold the view that the pituitary gonadotrophic hormonal activity (FSH and LH activity) is mediated by a single hormonal entity (Nalbandov, 1961). Nalbandov (1961) is of the opinion that the pituitary gland never secretes or releases either FSH or LH to the exclusion of the other, and that the study of the end-organ effect of these two activities separately using highly purified preparation is tantamount to studying a series of artifacts produced by FSH alone or LH alone which have no counterpart in the normal reproductive performance of animals. In support of this unitarian concept of the two pituitary gonadotrophins up till now no clinical condition is known in which the ratios of excreted FSH and LH activities have, with reasonable accuracy been shown to
differ significantly (Diczfalusy, 1961; Heller, 1961; Segaloff, 1961; Albert, 1961). It is very clear that if the unitarian view is true the alleged non-specificity of these assay methods carries no real significance, and the term "total gonadotrophic activity" becomes obviously meaningful.

Increase in the weight of the uterus of intact immature mice commonly known as the "mouse uterus test" (Klinefelter et al., 1943) is probably the most widely used bioassay procedure performed in clinical investigations. Apart from its disputed specificity, this classical bioassay procedure with its high standard of reliability and practicability is probably still the most satisfactory method described so far. The clinical usefulness of this admirable bioassay is not likely to be properly appreciated until either the biological entity of the gonadotrophic hormone is clearly established or the apparently erratic behaviour of the mouse uterine response to different mixtures of FSH and LH (Brown and Billiwick, 1962; Riley, 1961) is finally settled. An attempt has been made in Section 3 of this thesis to investigate the behaviour of the "mouse uterus test" in response to different gonadotrophin mixtures.

Loraine and Brown (1956) demonstrated that satisfactory reproducible assays can be obtained from human urinary gonadotrophin extracts using the "mouse uterus test" as
the method of bioassay and purified postmenopausal urinary gonadotrophin extract as a standard.

Albert and his associates (Albert et al., 1958; Albert et al., 1958; Albert and Kelly, 1958; Albert and Kelly, 1958; Albert, 1961) used the increasing weight response of the uterus and the ovaries taken together as a measure of gonadotrophic activity from various sources. These workers also demonstrated that the dose-response patterns of the uterine and ovarian responses when plotted together were characteristic for the gonadotrophic materials used.

It seems that as an assay method there is no apparent advantage of the rat uterine weight test over the mouse uterine weight method. The former test is less sensitive than the latter. Being bigger animals, the incidence of lethal toxicity is less commonly encountered when using rats instead of mice. This apparent advantage however does not exclude the possibility of sublethal toxicity of sufficient degree to interfere with the sensitive biological reactions involved in the qualitative and quantitative response pattern of the entire bioassay procedure. Characterisation of the biological properties of various gonadotrophic substances by their ovarian and uterine response patterns plotted together (the "finger print system") as advocated by Albert and his colleagues, appears to have very little specific, quantitative or clinical significance.
because of three main reasons: (a) both the rat ovarian and the rat uterine responses to gonadotrophin are sensitive to either of the pituitary hormones (FSH and LH) and as such either or both of them taken together cannot be used for measuring one by exclusion of the other; (b) the characteristic differences noted between the various gonadotrophins are at the most roughly qualitative rather than quantitative as revealed by the qualitative similarity of dose response patterns of PMSG and HMG; (c) to obtain the full range of the dose response patterns (particularly of the ovary), which is necessary for characterisation, very high dose levels of this hormone, much higher than the normal values clinically encountered, will have to be used.

The total amount of active gonadotrophic material usually obtainable from clinical sources is quite small, very often just sufficient for the performance of the more sensitive "mouse uterus test". Hence the relative insensitivity of the method permitting comparatively smaller numbers of animals which can be used per group for the individual bioassays, increases the possibility of a larger proportion of statistically invalid or less reliable bioassays.

Specific Assay Methods for FSH and LH.

It is generally assumed that any specific bioassay method for pituitary hormones must employ hypophysectomised animals to obviate the possibility of interference by the
hormones produced by the animal's own pituitary (endogenous hormone). Intact prepubertal animals however have been used widely for bioassay of gonadotrophins with the assumption that pituitaries of these animals are likely to secrete very little if any amount of gonadotrophic hormones. Intact animals, treated with specific pituitary inhibiting agents to block the secretion of a particular hormone have been used for a similar purpose. It is generally believed that the hormone elaborated by the end-organ in response to its specific trophic hormone from the pituitary gland, has the property of inhibiting the secretion of the trophic hormone from the pituitary itself. This auto-regulating process, the so-called feed-back mechanism produced experimentally has been utilized to inhibit the pituitary from secreting a particular trophic hormone. Cortisone-treated intact animals instead of hypophysectomised animals have been successfully used for the bioassay of adrenocorticotrophic hormone (Buttle and Hodges, 1953). It is possible that certain pretreatment procedures employed before the animals are used for the assay by themselves stimulate the secretion of end-organ hormones in sufficient quantity to inhibit the pituitary. The pretreatment of assay animals with large doses of PMSG and HCG which is used for Parlow's ovarian ascorbic acid depletion assay (vide infra) may cause inhibition of gonadotrophic function of the animal's own
pituitary due to the production of large amounts of oestrogens from their ovaries.

An added advantage of using intact pre-pubertal animals for bioassay of gonadotrophic hormones in particular is that unlike other hormones of the pituitary the gonadotrophic hormone present in the pre-pubertal animals pituitaries are known to be extremely small. Whether any amount of this extremely small store of gonadotrophic hormone present in the pituitary of the intact immature animals is ever released in the peripheral blood is not known.

Bioassays Claimed to be Specific for FSH.

In general specific methods for measuring FSH activity have depended on observation of the gonads of hypophysectomised male and female rats. Evans et al. (1939) described a technique dependent upon the follicular growth in hypophysectomised immature female rats. This method is usually regarded as highly specific for FSH. The criteria for reliability and practicability of this assay method are not very satisfactory. Paesi et al. (1951) described a test based upon the increase in testicular weight in hypophysectomised immature rats treated with an excess of HCG. Diczfalusy (1953) used this method for measuring the FSH content of placental extracts. Earlier, Simpson et al. (1944) had shown that both FSH and LH are capable of causing
testicular growth in hypophysectomised rats. In view of its low degree of practicability and its questionable specificity Faesi's assay method does not seem to be very satisfactory for specific measurement of FSH.

Steelman and Pohley (1953) described a method depending on the increase in ovarian weight in intact immature female rats treated with relatively large amounts of HCG. Brown (1955) employed intact immature mice in a comparable manner. Despite certain valid objections regarding the simultaneous use of a large amount of HCG, Steelman and Pohley's (1953) and Brown's (1955) assay methods seem to be the most satisfactory available so far. This method commonly known as the "augmentation assay" has been very widely used to measure the FSH content of various gonadotrophins. Apart from its relative insensitivity, the criteria for reliability and practicability of the assay method are satisfactory for routine application.

Assay Methods Specific for LH.

As in the case of FSH most assays for LH have been conducted on hypophysectomised animals. Evans et al. (1939) employed the repair of the interstitial tissue in the ovaries of hypophysectomised immature female rats as a specific parameter of LH activity. Creep, Van Dyke and Chow (1942) designed an assay method for LH depending upon the enlargement of the prostate in hypophysectomised immature male rats.
This method has been widely used as a specific assay procedure for measuring LH activity (Simpson et al., 1943; McArthur, 1952; Loraine and Brown, 1954, 1956). Loraine and Brown (1954) used the weight of the ventral prostate rather than taking the whole organ into account. The index of precision was shown to be usually less than 0.2 by these workers. McArthur (1952) and Loraine and Brown (1954) used the enlargement of seminal vesicles in hypophysectomised immature male rats as a possible parameter for measuring LH. This test was found to be less sensitive than the hypophysectomised rat prostate method.

Schaffenburg and McCullagh, 1951; McCullagh et al., 1953, described a semi-quantitative method depending upon the increase in the activity of the prostatic alkaline phosphatase in hypophysectomised immature male rats. This test, although not satisfactory by itself for the quantitative determination of LH activity, has opened up a new approach for designing future assay methods for measuring this hormone. A chemical rather than a purely physical parameter e.g. weight, colour, etc., was being measured. It is probable that most of the grossly physical changes observed in certain organs following the systemic administration of gonadotrophic hormone must be preceded by alteration of their chemical environment. The large number of chemical reactions which are involved in these reactions
might offer potentially promising specific chemical parameters for designing future bioassay methods. Assay methods designed on this principle are essentially bioassays in the sense that living animals are being used. Yet, there are certain essential differences between these types of assays and the conventional bioassays like the "mouse uterus test". In the former chemical change rather than a gross physical alteration is being used as the essential parameter. In view of this essential difference it is probably desirable to designate these types of assay methods, which use a chemical change rather than a gross physical alteration as the essential parameter, as "biochemical" assays. Many of these chemical parameters are likely to have some of the advantages (cf. sensitivity) and disadvantages (cf. variation) inherent in all bioassay procedures. It is possible that in the future, when our knowledge about these intricate biochemical changes including the specific enzymatic processes occurring at the cellular and subcellular level are more thoroughly clarified, it would be possible to perform these chemical reactions in vitro. Progress along this line is likely to produce not only better assay methods, but also more detailed information about the mechanism of gonadotrophic hormone action in its proper perspective.
A test depending upon the development of ovarian hyperaemia in rats (Farris, 1946) and mice (Lloyd et al., 1949) has been claimed to be specific for LH.

In a series of publications, Mitschi and Segal demonstrated that the characteristic feather reaction in the weaver finch was dependent upon LH, and that the reaction was not influenced by other gonadotrophins, including prolactin (Segal, 1957). It has not been possible to apply this interesting reaction for measuring gonadotrophic activity in human non-pregnant urine on account of the relative insensitivity of the test and due to the toxicity of the urinary extracts often observed in the experimental birds.

In 1958 Parlow (1958, 1961) described a sensitive assay method for the measurement of LH activity depending upon the depletion of ovarian ascorbic acid (OAAD) in intact immature rats pretreated with relatively large doses of PMSG and HCG. Schmidt-Ellendorff and Loraine (1962) established the method and found it to be highly specific and as such suitable for the measurement of LH activity in purified gonadotrophin preparations from human or animal pituitary tissue, from human urine or from serum of pregnant mares. These authors showed however that the method in the form described by Parlow (1958, 1961) was not suitable for clinical application for two main reasons. In the first place toxic effects were frequently encountered in the experimental animals when crude urinary extracts were injected intravenously as described by Parlow (1959, 1961).
Secondly there was a high proportion of invalid assays due to significant deviation from parallelism between the dose-response curves of the standard and the unknown preparations. In view of its hitherto unobtained sensitivity and practicability, attempts have been made to modify the assay technique as described in Section 2 of this thesis.

Ellis (1961, 1961a) devised an interesting variation of the ovarian hyperaemia method by employing radio-iodinated serum albumin for estimation of the hyperaemic response. By this modification the character of the end point was altered from quantal to quantitative assay. Parlow and Reichert (1963) made certain minor modifications of Ellis's (1961, 1961a) original method. After extensive trials they found that, even in its modified form, Ellis's bioradiometric assay method was fifteen times more sensitive than the hypophysectomised rat prostate test but 0.4 times less sensitive when compared to the OOAD assay. The reliability criteria of the OOAD method and Ellis's modified hyperaemic procedure were estimated to be comparable.

**Determination of HCG.**

HCG has been claimed to possess predominantly LH activity. However there is certain histological evidence to indicate the presence of FSH activity in HCG. The methods available at present for quantitative determination of FSH activity (Steelman and Pohley, 1953; Brown, 1955)
involve the simultaneous administration of relatively large quantities of HCG to the test animals. For this reason it has not been possible so far to use these methods to determine the amount of FSH activity, if any, present in HCG.

Lunenfeld et al. (1965) using indirect immunological methods, came to the conclusion that HCG contains a very small but detectable amount of FSH activity which is immunologically identical to the FSH present in HMG.

A large number of methods have been used for assaying HCG, mainly as an aid for diagnosis of pregnancy. In the field of clinical application the determination of HCG has been less difficult because of the relatively large quantities of the hormone produced in the human body. For this reason a number of semi-quantitative bioassay techniques have been used for the detection and determination of HCG in urine, as a diagnostic test for pregnancy.

Being predominantly LH in nature the LH activity of HCG can usually be assayed by specific methods used for measuring LH. Thus using the OAAD method of Parlow (1958, 1961) Schmidt-Elmendorff and Loraine (1962) estimated that the LH activity of 1.0 µg of NIH-LH was equipotent with 1.0 i.u. of HCG. However using the hypophysectomised rat prostate assay it was found that the slope of the regression curves for NIH-LH and HCG were not parallel. It seems probable that the difference in this biological half lives of
various gonadotrophic preparations like HCG and NIH-LH might be responsible for the slope difference when assay methods involving a long period of time (several days) are used instead of less time-consuming procedures, viz. the OAAD method (vide infra).
IMMUNOASSAYS OF GONADOTROPIC HORMONES.

The biological criteria that proved most useful in identifying gonadotrophin action were those associated with acceleration of gonadal growth and development. The bioassay techniques were developed as the basis of target organ reactions. In fact the use of the gonadotrophic preparations in heterologous species have formed the basis for the most investigations of biological properties of gonadotrophins. Highly purified extracts of urine and tissue (pituitary and placental) rich in gonadotrophic materials have been used in current studies in which immunological assays of the gonadotrophic hormones have been attempted.

It has been well established for some time that the prolonged administration of crude ovine pituitary extracts exhibiting gonadotrophic activity to either rats or rabbits may result in the production of antibodies which inhibit the action of gonadotrophins (Collip et al., 1943; Zondek and Sulman, 1942; Deutsch et al., 1950; Ely, 1957; Henry and Van Dyke, 1958). These anti-hormones, as they are described by some workers, have been shown to neutralize the biological activity of the exogenous gonadotrophins as well as to exhibit certain immunological (antigen-antibody) reactions with their homologous antigens.

Immunassay of HCG.

On this basis different preparations of HCG have been
studied in an attempt to develop systems to elicit and evaluate standardized reactions between HCG and its homologous antibody. Such systems were also expected to be of value for characterization, purification, identification and estimation of other protein hormones. For the purpose of developing a quantitative assay method, a number of investigators have made immunological studies of HCG.

Ehrlich (1939) was the first to describe complement-fixing antibodies to gonadotrophic hormone from pregnancy urine. Brody and Karlstrom (1960, 1961) revived the complement-fixation test for the presence of HCG. These workers made no attempt to analyse the antisera for their capacity to neutralize biological activity or for the presence of antibodies to proteins not associated with the biologically active principles.

McKean (1960) described a precipitin test which compared favourably with the Friedman pregnancy test; the antiserum was also found to neutralize the biological activity of HCG. He analysed the antibody content of the antiserum by the agar gel diffusion technique of Ouchterlony (1949) against different serum proteins and samples of urine from non-pregnant individuals. The possibility of using this method to obtain proof of purity is still in dispute.

Wide and Gemzell (1960) assayed HCG in urine, using a haemagglutination inhibition technique. Their quantitative
estimation curves were compared with the biological activity of HCG as determined by the prostatic test (Loraine, 1960). Although the general pattern of the curves were similar, the actual values obtained from the antibody titres were found to be higher than those calculated from their biological activity. The antiserum was not tested for biological neutralization capacity, nor were the antisera and antigens analysed for the presence of non-specific components. Lunenfeld et al. (1962) have shown that post-immunization serum neutralized both in vivo and in vitro biological activity of gonadotrophins and reacted with the antigen in vitro producing haemagglutination giving precipitin arcs in immuno-electrophoresis. These lines were found to be situated in different regions. Only one group of these lines was situated in the region shown to contain biological activity (as glycoprotein).

Brody and Karlstrom (1962) reported the results of 522 immuno-assays of HCG in a study along with 450 samples from pregnant and non-pregnant women who had been tested for HCG with the complement-fixation technique. Using this technique rather than haemagglutination-inhibition (Wide and Gemzell, 1960) they found that the levels of serum HCG during normal pregnancy were of the same magnitude as those previously reported by Wilson et al. (149), Harkins and Sherman (1952), Vermelin et al. (1957), and by Loraine (1957). However
these values were lower than those presented by Jones et al. (1944) as well as those indicated in the Composite Curve of Albert and Berkson (1951) and those presented by Behrman and Nissmann (1955).

**Immunoassay of LH.**

It has been shown that sheep pituitary LH stimulate, in rabbits, the production of precipitating antibodies that are specific for the respective hormones (Hayashida and Li, 1958; Li et al., 1960; Moudgal and Li, 1961). These antibodies have also been shown to fix-complement when allowed to react with the respective antigens (Trenkle et al., 1961). Trenkle et al. (1962) using an immunochemical technique thus claimed to have measured the LH content of relatively purified FSH preparations and of NIH-FSH-S1. NIH-FSH-S1 was found to be contaminated with 0.2 - 0.3% LH.

Schmidt-Elmendorff and Loraine (1962) using the ovarian ascorbic acid depletion method reported that NIH-FSH-S1 contained 1.0% LH activity. It has been found by these observers (Trenkle et al., 1962) that LH present in rat and beef pituitaries cross react with the rabbit antibody to sheep LH in quantitative precipitin and complement-fixation tests. One of the main objections at present against these immunological and immunochemical assay methods is that the immunochemical activity of LH cannot always be correlated with its biological activity. Another fallacy of these methods is that certain chemical
treatments like oxidation with periodate of sheep LH while greatly reducing its biological activity does not appreciably alter its ability to combine with the homologous antibody (Geschwind and Li, 1959).

In view of these and certain other technical difficulties it is generally agreed that the immunoassay of a hormone in biological media, especially in body fluids where the nature of the hormone is not known, must be correlated with biological assay before the immunochemical data can be of biological significance (Trenkle et al., 1962).

Immunoassay of FSH.

A satisfactory assay method for FSH depending upon the immunological response does not exist at present. Immunochemical methods have however been used indirectly to study the nature of the follicle-stimulating activity present in HMG and HCG (Lunenfeld et al., 1963).
SECTION I

EFFECTS OF LUTEINIZING HORMONE ON
RAT OVARIAN CHOLESTEROL - TWO SPECIFIC
ASSAY PROCEDURES FOR LH
The compound now known as Cholesterol was described for the first time in the latter half of the eighteenth century. De Fourcroy (1789) mentions that more than twenty years earlier Poulletier de la Salle had obtained this material from alcohol-soluble part of human gallstones.

Chevrel (1827) isolated cholesterol from human and animal bile, Lecanu (1838) from human blood, Couerbe (1834) from brain, Lecanu (1846) from hens' eggs, Vogel (1843) from atheromatous arteries and Müller (1838) from the tumour cholesteatomes. Cholesterol is now known to be widely distributed in all living mammalian cells.

Berthelot (1859) isolated cholesterol esters from serum in the form of palmitate and oleate.

Reinitzer (1898) published the correct summation formula of cholesterol. Windaus (1919) arrived at a tentative formula which was changed in 1932 to the one now accepted. The new formula was based on X-ray studies by Bernal (1932a., 1932b.) and by the finding of chrysene by catalytic dehydrogenation (Diels and Gadke, 1927) thoroughly worked out by Rosenheim and King (1932a., 1932b.). Windaus (1932) and Wieland and Dene (1932) decided on the final formulation.

**Biosynthesis of Cholesterol.**

Even before the introduction of isotopic tracer techniques, it had been established by balance methods that
animals are capable of cholesterol biosynthesis. Dezani (1913) and later on Dezani and Cattoretti (1914) were the first to show that cholesterol is synthesized in rats reared on a cholesterol free diet. Their findings were confirmed for the same and other species, e.g. in infants, by Gamble and Blackfan (1920), in human adults by Gardner and Fox (1921), in young dogs by Beumer and Lehmann (1923), in rats by Channan (1925) and by Randles and Knudson (1925), in chicks by Dan (1929) and in laying hens by Schoenheimer (1929).

Page and Meuschick (1932) and later Schoenheimer and Breusch (1933) showed that either biosynthesis or degradation could occur, depending upon the cholesterol content of the diet fed, but no clear idea about the rapidity of cholesterol biosynthesis nor of the pathway was gained until tracer methods were applied to this problem. Rittenberg and Schoenheimer (1937) found that deuterium \( H^2 \) present in the body water of a mouse, gradually became incorporated into cholesterol from small units which acquired deuterium atoms during the course of their chemical transformation, an interpretation which was subsequently confirmed by studies with \(^{13}C\) and \(^{14}C\) labelled precursors. Sanderhoff and Thomas (1937) found that deuteracetate was converted by yeast in high yield, and Bloch and Rittenberg (1942) showed it to be utilized by rats and mice for cholesterol synthesis.
Later Bloch et al. (1946) found rat liver slices capable of converting acetate-1-\textsuperscript{14}C into cholesterol, an observation which has stimulated an immense amount of work on many aspects of cholesterol biosynthesis. An important contribution to this field has been the development by Bucher (1953) of a method of preparing cell-free fractions capable of synthesizing cholesterol; only the microsomal and soluble supernatant fractions are required.

Acetate is the principal and probably the only common, simple metabolite from which cholesterol is synthesized. Total synthesis from acetate can be achieved by soluble extracts of liver, but no enzyme involved in this synthesis beyond the part common to fatty acid and cholesterol synthesis has yet been purified or even identified, and many intermediates are still unknown. On the other hand every carbon in cholesterol may be derived from acetate; this was established by the very extensive studies which have been in progress since 1949 in several laboratories with the goal of isolating and identifying the origin of each carbon atom in cholesterol. Wuersch et al. (1952) completed the analysis of the side chain, and Popjak and Cornforth (Cornforth et al., 1953, 1956, 1957; Popjak, 1957) have completed the remarkable feat of a systematic carbon-by-carbon dissection of the whole ring system. Various investigations since that time can be conveniently grouped into two categories,
those with the steps between acetate and squalene, and those concerned with the steps between squalene and cholesterol itself.

Cholesterol biosynthesis occurs in all tissues of the body to a greater or lesser degree. It is interesting but perhaps not surprising that the microsomes are the focus of cellular cholesterol biosynthesis as they are also important sites of protein and ribonucleic acid synthesis.

**Cholesterol in Steroidogenic Tissues.**

The structural relations of cholesterol to certain steroid hormones were determined when ovarian, testicular and adrenal cortical hormones were isolated and their chemistry studied in the 1930's. Ruzicka and co-workers (Ruzicka et al., 1934) prepared androsterone from epicholestanol (cholest-3a-ol) and Fernholz (1934) converted stigmasterol into progesterone. On the assumption that the biosynthetic mechanism utilized to form complex molecules in vivo are implicit in the chemical structures involved, as evidenced by reactivity in the organic reactions of the chemistry laboratory, the pioneers in steroid chemistry suggested biogenetic relationships between cholesterol and the various steroid hormones then known. These efforts were especially frequent before 1940 and a typical scheme of this era is illustrated in the following Figure taken from (Fig.1) Kerrer's text (1938). This embodies hypothetical relation-
ships derived primarily from Ruzicka and Butenandt. It will be seen that one of the hypothetical routes envisaged involved the degradation of the cholesterol side chain to form pregnenolone. This upon oxidation gives rise to progesterone the hormone of the corpus luteum. Progesterone in turn was suggested as a precursor of testosterone, the hormone of the testis which in turn was regarded as the precursor of the ovarian hormones, oestrone and oestradiol. It is interesting to note that it is precisely these reactions, demonstrated only in the past few years, which are now regarded as basically involved in the biosynthesis of steroid hormones. While the problem involved in the biosynthesis of the various steroid hormones is not solved in its entirety, various "patterns" are beginning to emerge and merge; techniques are available for discovery of the remaining missing pieces and the problem now seems near solution.

On the basis of recent studies it is now established that cholesterol is a precursor of oestrogens. Although it was first claimed by Heard et al. (1954) that cholesterol is not a precursor of oestrogens, as evidenced by the failure of $^{14}$C cholesterol administered in the pregnant mare to be incorporated into the urinary oestrogens, subsequent reconsideration of the data has led to the retraction of this view (Heard et al. 1955). It has been shown in humans that
cholesterol is converted to oestrone, in vivo (Werbin et al. 1957). Previously it had been shown in ovarian tissue that cholesterol is converted to progesterone (Solomon et al. 1956); that progesterone is converted by ovarian tissue homogenates to 17-hydroxyprogesterone and androstenedione (Solomon et al., 1956); and that androstenedione is converted to oestrogen in various tissues including the ovary, placenta and the adrenals (Meyer, 1955). The formation of the corpus luteum with its subsequent ability to produce progesterone is brought about by the combined action of the anterior pituitary gonadotrophic hormones (FSH and LH). It is quite probable that the correctly timed action of these two pituitary hormones is essential for the biogenesis of progesterone in the corpus luteum. The latter is probably also true for the biogenesis of oestrogens as well. At just what stage in the biosynthesis these hormones exert their action is not known (Dorfman, 1955).

Turnover of Cholesterol in Steroidogenic Organs.

The term "turnover" may be conveniently defined as the rate of replacement of the molecules of a body constituent or the rate of disappearance due to excretion and metabolic breakdown or conversion into other substances. In this steady state these two rates will be equal. Cholesterol turnover in individual tissues is complicated by movement of labelled molecules from one tissue to another.
Factors influencing biosynthesis, turnover and distribution of cholesterol in tissues, are many. Most of the studies have been made in connection with the turnover rates between the blood and the liver. Amongst the extrahepatic tissues rich in cholesterol the adrenal glands have been studied in some detail. The cholesterol in this organ is claimed to be present in higher concentration and in more labile form than in any other tissue. Various types of experimentally induced stress, including haemorrhage, burns, tourniquet shock, and fasting cause a rapid and marked decrease in adrenal cholesterol concentration, due presumably to an accelerated conversion into adrenocortical steroids. Disappearance of half the adrenal cholesterol in three hours has resulted from non-fatal haemorrhage or the injection of ACTH in rats (Sayers, 1950). It is the esterified cholesterol which decreases in amount; at 15-24 hours after tourniquet shock the esterified fraction was less than one third the control value and the free fraction showed little change (Popjak, 1944). By 48 hours the cholesterol level was again normal.

Although great progress has been made in the field of steroid biosynthesis, our knowledge regarding the question of how steroid biosynthetic sequences are regulated in living cells by specific trophic hormones is less certain. Present understanding in this field is limited not only by insufficient
The available data indicate only that "something more" than is encompassed by the chemical reaction of a sequence (despite the detailed inclusion of enzymes and co-factors) is involved in the regulation and direction of metabolic pathways in the living cells. Further information in relation to this problem is provided by a consideration of the various steroid hormone biosynthetic sequences in the adrenal cortex, testis, corpus luteum and the ovary which produce as end products corticosteroids, androgens, progesterone and oestrogen respectively. Enquiries have revealed that in the various sequences most of the enzyme steps present in each of these endocrine tissues are basically similar, the difference in end products depending on the presence or absence of a few specific hydroxylating or oxidative systems. How then is specificity of trophic hormone action achieved? More particularly, how does ACTH specifically activate corticosteroid hormone production without influencing progesterone secretion from the corpus luteum or testosterone (and androstenedione) secretion from the testis, or oestrogen secretion from the ovary? Similar questions arise in the case of gonadotrophic hormone action directed only at the testis and ovary, and for luteotrophin hormone action specifically upon the corpus luteum. There is no evidence that hormone specificity is the result of
hormone action upon an enzyme specific to the target organ. Indeed, in the case of ACTH action, where our knowledge is most advanced, it is clear that ACTH activates an early step in the sequence between cholesterol and pregnenolone, which normally is the rate-limiting reaction, and this is precisely the area that is common to all of the sequences of steroid hormone biosynthesis. But this is not the only difficulty. Each of the pituitary trophic hormones discussed, in addition to activating specific steroid biosynthetic sequences, also promotes cell growth in its respective target organ. At a minimum growth may be defined as the synthesis of each of the characteristic compounds of specifically differentiated cells. Obviously growth does involve more than merely a collection of biosynthetic reactions. This must mean that in each target organ, the trophic hormone activates every biosynthetic sequence characteristic for the cell (i.e. nucleoproteins, proteins, carbohydrates, lipids, etc.) and it must do this specifically for cells in one endocrine organ but not in the closely related cells of another organ. If the present ignorance of the fundamental mechanism of action of hormones has been emphasized, this is because it seems that the first step towards knowledge in this area is to recognise our need for a concept of sufficient breadth to encompass the problem in its entirety.
Ovarian Cholesterol.

Histochemical methods have been frequently used to explore the very controversial and complex field regarding the distribution, localization and turnover of lipids and fatty substances in the ovary. The histochemistry of the organs which are now known to produce steroid hormones (adrenal, ovary, testis and placenta) dates back well over sixty years. The presence in certain cells of these organs of sudanophil, birefringent, acetone-soluble lipids with properties different from those of ordinary fat has long been known to histologists. Extraction methods showed these droplets to consist of cholesterol esters, phospholipids, triglycerides and fatty acids. The ovarian interstitial gland of rodents and the corpora lutea, strikingly filled with such droplets, have long been linked with hormone formation and secretion. As a consequence, methods used by the organic chemists for characterizing steroid compounds were applied, directly or with modifications, to appropriate organs, first by Bennett (1940) to the adrenal cortex of the cat, and subsequently by Dempsey and Bassett (1943) to rat ovaries. Testing the lipid droplets for a variety of physical and histochemical properties such as birefringence before and after digitonin precipitation, fluorescence in ultraviolet light, phenyl hydrazone formation (indicative of carbonyl groups), colour reactions after treatment with concentrated sulphuric acid (typically given by unsaturated
steroids), sudanophilia and acetone solubility, these workers concluded that the lipids contained a mixture of cholesterol and ketosteroid hormones. The high hope that the actual hormones were being visualized in this way was soon shattered when it was shown that lipid aldehydes, known as plasmasls (or other autoxidation products of unsaturated fatty acid esters of cholesterol) rather than ketosteroids were responsible for the phenyl hydrazine reaction (Gomori, 1942, 1952a; Albert and Leblond, 1946; Claesson and Hillarp, 1947c; Boscott et al., 1946; Boscott and Mandl, 1949). Feulgen's plasmal Schiff-reaction, specific for aldehydes, was shown to parallel the hydrazine carbonyl reaction (Verne, 1929). Whether it is a true plasmal reaction or pseudo plasmal reaction (Cain, 1950; Hack, 1952; Beane and Saligman, 1953) is of secondary importance.

Hence the set of tests which is now frequently being applied to steroid hormone producing tissues reveals not a single substance but various lipoid materials. They may be mixtures, one may be, and often probably is, the vehicle for another, so that several are revealed at the same site. However, it must be emphasized that the simultaneous presence of these different lipids in the same tissue, is to a large extent not fortuitous but indicative of, and conditioned by, the specific steroid metabolic processes occurring therein.

Dempsey and Bessett (1945) concluded from their work
on the rat ovary that it is the theca interna cells which produce the oestrogenic hormones and not the graulosa cells, because the latter did not give any of the various "steroid reactions". Everett (1945) studying the corpora lutea of the cycle in rats, noticed a large accumulation of cholesterol during oestrus in the next youngest set of corpora lutea. He suggested that cholesterol serves as a precursor of progesterone. For storage to occur a special ratio of circulating luteinizing hormone/luteotrophic hormone was found to be all important. In corpora lutea of gestation and lactation no sign of cholesterol storage is seen (Everett, 1947; Claesson and Hillarp, 1948); presumably they are in a state of active progesterone secretion. This agrees with the observations made by Corner (1952) and with subsequent observations of Dawson and Velardo (1955) on rat corpora lutea during pseudopregnancy. However, Everett (1947) showed that experimental intervention could produce storage. Deane's (1952) extensive work covering a succession of controlled oestrus cycles in rats brought out some points of special interest:

(1) the three structures, theca interna, corpora lutea (i.e. of the previous cycle) and interstitial cells, all show evidence of secretion in the immediate preovulatory period;

(2) the lipids of large graafian follicles becoming
atretic at the time of ovulation give the full range of "steroid" tests, suggesting a luteinizing response to gonadotrophin stimulation, whilst follicles becoming atretic at other times of the cycle show merely accumulation of coarse lipid material.

Histochemical evidence indicating that the theca interna represents the predominant site of oestrogen production and the only one in the growing follicle was obtained for a number of species (Dempsey, 1948; Hofliger, 1948; Knigge and Leatham, 1956) including humans (Mackay and Robinson, 1947). Gillman and Stein (1941) in their investigation of human corpora lutea, noted the almost complete absence of visible cholesterol throughout pregnancy. In this connection the earlier work of Skowron and Keller (1934) on rabbits' gestational corpora lutea is of interest. They found fine sudanophil granules to be present in the granulosa lutein cells throughout gestation, indicating continuous secretory activity, which confirms with the well known fact that in this species a functional corpus luteum is indispensable almost up to the end of pregnancy. Deane and Fawcett (1956) noted histochemical evidence of oestrogen production in the transplanted intrasplenic ovarian luteomatas.

The concept of cholesterol being a precursor substance of oestrogenic hormones already mentioned in connection with
the work of Everett (1945) was developed in a series of histochemical studies by Claesson and his colleagues. In 1947 Claesson and Hillarp demonstrated histochemically the presence of a sterol of the cholesterol type in the interstitial glands of the theca interna of the rabbit ovary. They found the amount of this sterol to vary in relation to sexual phase: during oestrus, pregnancy and pseudopregnancy large amounts were present; in immature ovaries or during anoestrus there was very little or none. Chemical extraction methods showed that most of the sterol was present as a biologically inactive ester. After coitus or injection of gonadotrophin the sterol was mobilized from these cells in the ovary parallel with the sign of production and secretion of active oestrogenic hormone. Reduction of cholesterol of the interstitial gland was also found in association with postpartum oestrus. These results strongly suggested that a histochemically detectable sterol is the precursor of the oestrogenic hormones formed in the rabbit ovary. Application of the same methods to ovaries of the rat and guinea pig (Claesson and Hillarp, 1947b., 1949) yielded similar results. In the rats, coitus or graded gonadotrophic stimulation were shown to mobilise an oestrogen precursor from the ovary. Further analysis of the storage mechanism of the oestrogen precursor in the interstitial tissue of the rat ovary (Aldman et al., 1949a., 1949b.;
Claesson, 1954a.) revealed that intense gonadotrophic stimulation results in depletion of the store, while low grade gonadotrophic stimulation with both FSH and LH will lead to storage of the precursor and inhibit its transformation into active hormone.

Claesson extended his work (1954b., 1954c.) and combined this with quantitative biochemical analysis in the interstitial glands of rabbits. Under certain conditions this tissue occupies a large volume of the ovary and is therefore very suitable for biochemical work. Such work had previously shown (Claesson et al., 1948, 1953) that subsequent to gonadotrophic stimulation there is a marked decrease in esterified cholesterol and fatty acids and a great increase in phospholipids, whilst the amount of acetal phospholipids remained unchanged. Claesson then studied the intracellular localization of the lipid fractions by a variety of methods (cytological, histochemical and microbiochemical) before and after gonadotrophin stimulation.

The intensities of both Schultz reactions and birefringence were paralleled by quantitative cholesterol estimations. First teased "living" cells obtained from ovaries before stimulation were seen to be loaded with granules and there was strong birefringence. After stimulation there was a progressive decrease in the birefringence and size of the granules, but not in their number. Analysis of the lipid
extracts from these granules strongly suggested that the double refraction was due mainly to cholesterol esters. The increased phospholipid was largely concentrated in the general cytoplasmic fraction and was thought to be due to newly formed mitochondria. This "precursor" hypothesis is also supported by studies on the lipid histochemistry of the ovarian interstitial tissue of infantile and prepubertal rats (Kennels, 1949, 1951; Dawson and McCabe, 1951; Falck, 1953).
In the following section of the thesis the cholesterol turnover in the ovaries of intact immature female rats following LH stimulation has been investigated.

Depending upon the two important biological actions attributed to LH, two different lines of approach using two completely different experimental conditions have been made. One of the important biological actions of LH on the mature ovarian follicles is its ability to cause ovulation and corpus luteum formation. The cholesterol turnover in the ovaries following exogenously administered LH induced ovulation and corpus luteum formation has been investigated, using rat ovaries containing large numbers of ripe follicles. Simultaneous maturation of a large number of ovarian follicles without subsequent spontaneous ovulation was effected by a single subcutaneous injection of 50.0 i.u. of PMSG. Chapter I deals with this section of the investigation.

Another important function widely attributed to LH is its ability to stimulate steroid hormone production from the corpora lutea. As reviewed already, cholesterol has been shown to be one of the precursors from which ovarian steroid hormones are synthesized. This property, viz. stimulation of steroid hormone production from cholesterol was studied by investigating the cholesterol turnover in the intact immature rat ovaries containing large numbers of corpora
lutea (Chapter II). This latter type of ovary was obtained by pretreating the experimental animals with 50.0 i.u. of PMSG followed 72 hours later by 25.0 i.u. of HCG.

In both these groups of experiments reasonable time was allowed to pass between the time of injection of the hormones for pretreatment and the time of injection of the test doses of LH. Although very little is known at present about the biological half lives of various gonadotrophic preparations, it was reasonably hoped that the time allowed for the hormone used for pretreatment to be used up (or excreted) was sufficient.

The ultimate purpose of both these investigations has been to develop new assay methods for the quantitative and specific determination of luteinizing hormone activity in biological materials from animal and human sources. As already discussed, the available assay methods for the measurement of luteinizing hormone activity in human body fluids are not satisfactory at present.
CHAPTER I

OVARIAN CHOLESTEROL STORAGE IN INTACT IMMATURE RATS FOLLOWING SUPEROVULATION AS A POSSIBLE ASSAY PROCEDURE FOR QUANTITATIVE DETERMINATION OF LUTEINIZING HORMONE ACTIVITY

INTRODUCTION.

Ovulatory response to exogenous gonadotrophin stimulation in intact immature animals has been utilized by a number of investigators as a possible parameter for assaying the luteinizing hormone activity. In spite of its sporadic use over a number of years the technique has not received much attention, and has in general been abandoned by most workers.

Smith and Engle first reported the ovulatory response to gonadotrophins in rats in 1927. The response however could not be properly utilized for developing an assay method for the measurement of LH, mainly due to the fact that suitably purified pituitary hormones were not available until comparatively recently. Zarrow et al. (1958) reported that intact immature female rats pretreated with 30 i.u. of pregnant mares serum gonadotrophin (PMSG) will ovulate when injected intravenously with as little as 0.25 i.u. of human chorionic gonadotrophin (HCG) or 0.1 μg of Armour LCGH.

This method also could not be recommended for quantitative work due to low degree of precision of the test, the mean figure for the index of precision (λ) being 0.53.
More recently Cunningham (1962) using intact immature female mice pretreated with large dosages of PMSG, designed an assay method for luteinizing hormone activity depending upon the percentage of animals ovulating following the administration of a luteinizing hormone (LH) preparation. He was able to obtain a workable dose-response relationship between the amount of LH material used and the percentage of the animals showing ovulation. However, the dose level of LH necessary to obtain a satisfactory dose-response curve was found to be rather large when compared to that used ordinarily for Parlow's ovarian ascorbic acid depletion method (Parlow, 1958, 1961). It is interesting to note, as observed by these workers (Zarrow et al., 1958; Cunningham, 1962) that priming with relatively large doses of PMSG given alone in one single injection failed to induce significant ovulation in the immature rats. Zarrow et al. (1958) noted only one instance of ovulation in a series of more than 200 rats injected with a single priming subcutaneous injection of PMSG. However, contrary to the observation of Howlands (1942) but in agreement with the results reported by Garland and Nelson (1958), ovulation was observed with a second injection of PMSG in rats previously primed with PMSG (Zarrow et al., 1958). This latter effect could simply indicate the presence of LH activity in the PMSG preparation, or the presence of an ovulatory property in the PMSG molecule.
Schmidt-Elmendorff and Loraine using the ovarian ascorbic acid depletion method reported that international standard PMSG had approximately 30% LH type material and 70% FSH type activity (Schmidt-Elmendorff et al., 1962).

It is generally believed that the phenomena of follicle maturation and ovulation are associated with the production of steroid hormones by the ovaries. The relationship of ovarian cholesterol to the ovarian production of steroid hormones has already been reviewed. This chapter deals with the change in the cholesterol content of the ovaries in intact immature female rats, following induced follicle maturation by 50 i.u. of PMSG, and subsequently ovulation and corpus luteum formation by different LH preparations. It was hoped that the change in the cholesterol content of the PMSG-treated ovaries following LH administration (and consequent ovulation and corpus luteum formation) might follow a definite dose-response relationship with the amount of LH used. The object of pretreatment with PMSG was to stimulate the maturation of a large number of graafian follicles to facilitate the subsequent action of the administered LH. It has previously been shown that PMSG when given alone in one injection, even in relatively large doses, stimulates the maturation and enlargement of follicles without ovulation occurring unless an LH preparation is administered subsequently.
The purpose of the present investigation was to investigate the feasibility of using the change in the cholesterol content of the ovaries associated with administered LH induced ovulation and corpus luteum formation as a basis for quantitative determination of LH activity.

MATERIALS AND METHODS.

Animals.

Intact immature Wistar rats from two inbred colonies, varying in age from 25-30 days, and between 35 - 50 gms. weight, were used. For the preliminary experiments animals from one colony only were used. The bioassays proper and the specificity tests were all carried out with animals from a different colony.

Administration of hormones.

The hormones were injected subcutaneously or intraperitoneally as specified. The pretreatment procedure with 50 i.u. PMSG was in all cases performed by subcutaneous injection of 0.5 mls. of normal saline given under the dorsal skin. The test materials were injected in three twelve hourly divided doses of 0.5 ml each, following 72, 84 and 96 hours after the priming injection of 50 i.u. of PMSG. This injection schedule reached arbitrarily was followed throughout and no other factorially designed experiment to investigate the effect of time, number and volume of the injection fluid was undertaken.
estimation of ovarian cholesterol (total cholesterol).

The assay animals were killed by cervical dislocation. At autopsy each ovary was removed by a dorsal approach. The organs were carefully cleaned of all adhering tissues and were weighed on a torsion balance to the nearest 0.1 mg.

The weighed ovaries were ground in a mortar and pestle with a small amount of sand and 4 mls of 50:50 mixture of acetone and ethanol, taking the usual precaution to prevent evaporation. The extract was filtered and 0.4 ml aliquots of the clear filtrate were pipetted out for estimation of the total cholesterol according to the method described by Searcy and Bergquist (1960). Six mls of saturated solution of ferrous sulphate were added to the 0.4 mls of the tissue filtrate and finally 2.0 mls of concentrated 'Analar' sulphuric acid was run into it and mixed very rapidly to prevent charring. The colour developed was read in a Unicam spectrophotometer (S.P. 600) at 500 μ.

Hormones used for the preliminary experiments and for the pretreatment.

1. PMSG (Gestyl) - the commercial pregnant mares serum gonadotrophin manufactured and kindly supplied to us by Messrs. Organon (Newhouse, Scotland).

2. HCG (Pregnynl) - the commercial human chorionic gonadotrophin obtained from the same pharmaceutical firm.
Hormones used for the quantitative assays.

1. Follicle-Stimulating hormone (NIH-FSH-S1) - obtained from the National Institute of Health, U.S.A.

2. Luteinizing hormone (NIH-LH-S1) - obtained from the National Institute of Health, U.S.A.

3. Human menopausal gonadotrophin (HMG), the international reference preparation (I.R.P.). The material is approximately equipotent with the unofficial reference preparation HMG-20A.

4. Human menopausal gonadotrophin (Pergonal). This material has been shown to have a higher specific activity than I.R.P. The original preparation of Pergonal was made by Donini and Marchetti (195) by a method depending on keyolin absorption and elution followed by chromatography on permutit columns. The material tested in this study was put up in ampoules each containing equal amounts of Pergonal and sodium thymonucleinate. Pergonal has been chosen as the future international reference preparation for urinary gonadotrophin.

5. Pregnant mares serum gonadotrophin (PMSG) - international standard.

6. Human chorionic gonadotrophin (hCG) - international standard.

Hormones used in specificity tests.

1. Prolactin (NIH-P-S-4; 15 i.u. per mg) - obtained
from National Institute of Health, U.S.A.


3. Adrenocorticotropic hormone (ACTH). The preparation used was "Cortrophin" manufactured by Organon; 10 i.u./ampoule.

4. Thyroid-stimulating hormone (TSH) - this was the international standard of the hormone.

THE METHOD IN DETAIL.

The whole procedure was conducted in three stages,

(i) experiments to obtain preliminary information regarding the effects of a predominantly luteinizing hormone (HCG) on the behaviour of ovarian cholesterol content, in intact immature female rats pretreated (primed) with 50 i.u. of PMSG; (ii) experiments to obtain the necessary information regarding the dose response relationship between the dosages of luteinizing hormone injected and the amount of ovarian cholesterol in similarly pretreated animals, and (iii) the bioassays proper.

(i) Preliminary experiments regarding the effects of HCG on the behaviour of rat ovarian cholesterol.

In the first experiment altogether 75 animals were used, of which sixty received 50 i.u. of PMSG subcutaneously in one single injection (day 0), fifteen were left as controls without hormone treatment. Seventy-two hours later
(day 3) the sixty animals which received 50 i.u. of PMSG had one subcutaneous injection of 25 i.u. HCG. Five animals from the treatment group were sacrificed each day from the first day after they had their PMSG injection until the 9th day after they had their HCG. On three occasions, i.e. on the day after the treatment group of animals had their injection of PMSG and on the third and the ninth day after these animals had their HCG, five animals from the control group (which did not receive any hormone at all) were also sacrificed. The ovarian weight and ovarian cholesterol were determined. The results are shown in Figures 2 and 3 and in Table 1.

To investigate how far the increasing cholesterol content of the ovaries observed in the last experiment were related to the increasing weight of the organs following HCG administration, the second experiment was carried out. This was designed exactly in the same way as the previous one except for the fact that the control group of animals had 50 i.u. of PMSG pretreatment at the same time as the experimental group, and the HCG injection in the experimental group was administered in three divided intra-peritoneal injections after 72, 84 and 96 hours after they had their PMSG pretreatment. The mean ovarian weights are shown in Table 2 and Figure 4. It may be observed that the ovarian weight in the treatment group (which had HCG) was consider-
ably higher than that in the control group which had the PMSG pretreatment alone.

The augmentation effect of 25 i.u. of HCG on the ovarian weight pretreated with 50 i.u. of PMSG was regarded as rather interesting, and it was thought worthwhile to investigate the effects of other gonadotrophin preparations on similarly pretreated ovaries. Groups of 5 animals from a batch of 35 animals pretreated with 50 i.u. of PMSG were treated with a number of gonadotrophin preparations, using relatively large dosages. The injections were administered in 3 divided doses at 72, 84 and 96 hours after the pretreatment injection. It may be pointed out that the dose levels of all the gonadotrophins obtained from non-pregnant sources used in this experiment had either more LH or more FSH or greater quantities of both activities in them when compared to 25 i.u. PMSG or 25 i.u. HCG (based on data published by Schmidt-Elmendorff and Loraine (1962)). All the animals including the 5 control animals were sacrificed on the fifth day after they had their first injection of the test material. The mean ovarian weights are shown in Table 2.

The next experiment was carried out to examine the effects of much smaller dosages of HCG on PMSG pretreated animals. The dose level of HCG used (2.0 i.u.) was expected not to interfere with the ovarian weight when compared to the
weight of the ovaries of the animals having PMSG alone. 105 animals were used in this experiment. All the animals had the priming injection of 50 i.u. of PMSG on the day zero of the experiment. After this 45 of the animals received 2.0 i.u. of HCG intraperitoneally in three divided doses on the 72nd, 82nd and 94th hours after they had their PMSG. The rest of the sixty animals which did not receive any hormone after the priming injection of 50 i.u. PMSG acted as controls. From day 1 to day 3 after the priming injection 5 animals were sacrificed each day. From day 4 after the priming injection, that is from the day after the treatment group of animals had their 2.0 i.u. of HCG injection, 10 animals, 5 from the treatment group and 5 from the control were sacrificed every day until the end of the experiment. The ovarian weight and ovarian cholesterol (free and total) were determined as shown in Figures 5, and 6 and 7.

(ii) Experiments involving the establishment of the dose-response relationship between the amount of ovarian cholesterol accumulation and the dose level of the administered luteinizing hormone preparations.

Two pre-dominantly luteinizing hormone preparations, i.e. the international standard preparation of HCG and NIH-LH, were used for the purpose. The details of the pre-treatment and the injection of the test material were the
same as in the last experiment. That is all the animals used for the assay were pretreated with one subcutaneous priming injection of 50 i.u. of FMSG, followed 72, 84 and 96 hours later by the injection of the test materials to the animals other than those used as controls. In this experiment with HCG two groups of 40 animals were used; an additional 5 were used as controls. One group of 40 animals received the test dose of the hormones intraperitoneally, the other group received it by subcutaneous injection. Identical dosages were employed for both the groups and 5 animals in either group were used per dose level. The animals were killed and their ovarian cholesterol determined on the fifth day after they had their first dose of the test material.

The graded dosages of HCG used in this experiment were 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 i.u. The results are shown in Figure 8. A progressive increase in cholesterol content with progressively increasing dose levels of HCG was noted.

An identical experiment with the same number of animals was carried out using NIH-LH at dose levels of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 μgs. The results are illustrated in Figure 9. The response is identical to the previous one (Figure 8).

A number of similar experiments were then performed to investigate the steepest and linear part of the dose-response
curve for NIH-LH. The results of four such experiments are shown in Figure 10. In all these experiments and subsequently for all the proper bioassays and the specificity tests, the test materials were given intraperitoneally. For the purpose of avoiding any confusion therefore the bioassay method as performed may be summarised as follows.

Animals are pretreated with 50 i.u. of FMSG given subcutaneously by one injection in 0.5 ml of saline. Following this 72, 84 and 96 hours later the test material is injected intraperitoneally in three divided doses, each with 0.5 ml of normal saline. The group of animals acting as controls are given similar three injections of 0.5 ml of saline only by intraperitoneal injection.

(iii) The bioassays were conducted in the following manner. Groups of animals were pretreated with 50 i.u. of FMSG (Gestyl - Organon). On the third day after they had their FMSG, intraperitoneal injection of the test materials (the standard and the unknown) were given in three divided doses at 72, 84 and 96 hours after the priming, given each time in 0.5 ml of normal saline. The animals were sacrificed for ovarian cholesterol determination on the fifth day after the injection of the test material.

The results of eleven bioassays are shown in Table 3. The dose levels of various gonadotrophin preparations used for these assays are shown in Table 7. The relative
potencies of the various gonadotrophins thus obtained agree with those obtained by Schmidt-Elmendorff and Loraine (1962) using the OAAD method.

**Design of the Assays.**

All the assays performed were pilot assays in order to obtain preliminary data or the dose-response relationship of the various gonadotrophins. A rough estimate of the dose levels was made from the results previously published by Schmidt-Elmendorff and Loraine (1962). Five animals (10 ovaries) were used per dose level of the standard and the unknown materials, and the log dose interval, i.e. the difference between the logarithms of the two neighbouring doses, was log $10^4 (0.602)$.

In all instances the design was a symmetrical four point assay with two dose levels of the standard (S) and two dose levels of the unknown (U) preparations respectively, and with equal spacing of the log doses.

**Validity tests** were performed in each assay following the technique described by Finney (1952) and Gaddum (1953a, b.). Altogether three assays were invalid because of significant deviation from each parallelism of the dose-response curves for the standard and the unknown preparations. The deviation from parallelism was not confined to any particular preparation.

The index of precision ($\lambda$), relative potency and
Fiducial limits of error were calculated by the method recommended by Gaddum (1953a, b.) and by Borth, Diczfaluay and Heinrichs (1957).

Specificity tests.

These were performed in two ways. Firstly anterior pituitary hormones which might interfere with the response to LH were administered alone in dosages as shown below:

- **Prolactin**: 0.1 and 1.0 mg
- **Growth hormone**: 0.1 and 1.0 mg
- **ACTH**: 1.0 and 10.0 i.u.
- **TSH**: 10.0 and 100.0 i.u.

Secondly, these hormones were used in combination with the usual low and high dosages of the standard NIH-LH. Results are shown in Table 6.

**RESULTS.**

**Preliminary Observations.**

Preliminary observations regarding the effect of 25 i.u. HCG on the behaviour of rat ovarian cholesterol show that following the administration of the latter hormone in intact immature female rats pretreated with 50 i.u. of PMSG, there is progressive accumulation of cholesterol in the ovaries of these animals (Figures 2 and 3, and Table 1). It may be seen from the figures that following the administration of HCG, the cholesterol content rises steadily from the first up to the last day of the experiment. Although this effect
is noted in either case, i.e. when cholesterol values are expressed per 100 mg of tissue (Figure 3) or in terms of cholesterol value per ovary (Figure 2), the increase is more marked in the latter. It may also be noted that the ovarian weight also increases markedly, as expected, when compared to untreated (without PMSG or HCG) control ovaries (Figure 2).

In order to investigate how far the ovarian weight under the conditions of the experiment is influenced by HCG in PMSG treated ovaries the next experiment was designed. The results are shown in Table 2 and Figure 4. It may be observed from these figures that the ovarian weight increases significantly in HCG (25 i.u.) treated animals, when compared to those having PMSG pretreatment alone. This effect points towards the possibility that the increasing cholesterol values following HCG administration might be at least partly due to the increasing ovarian weight. It is interesting to note that a comparatively large amount of NIH-FSH, NIH-LH, NIH-FSH plus NIH-LH and Pergonal, does not cause any further increase of ovarian weight.

However it is seen from Figures 5, 6 and 7 that following the administration of 2.0 i.u. of HCG there is no significant change in the ovarian weight when compared to the control ovaries (50 i.u. PMSG alone). Despite the fact that the ovaries did not increase in weight significantly
following the administration of smaller amounts of HCG (2.0 i.u.) there was significant increase in the cholesterol content of the ovaries from animals injected with this hormone. The increase in cholesterol content when compared to the controls was highly significant from the second to the ninth day after the administration of HCG. The difference in ovarian cholesterol was found to be most marked between day 4 to day 6 after the administration of HCG. From Figure 6 it may be seen that the increase in cholesterol is almost entirely due to an increase in the esterified fraction of the sterol, the free cholesterol showing very little alteration.

Experiments involving the investigation of the effect of NIH-LH, NIH-FSH, NIH-LH and NIH-FSH combined, and Pergonal on the PMSG pretreated rat ovarian weight show (Table 2) that these hormones have no significant effect on the weight of these ovaries.

The results of these preliminary experiments have shown therefore that there is a progressive increase of the cholesterol content in the ovaries of the rat following administration of HCG in animals previously pretreated with PMSG.

**Determination of the log dose-response curve for NIH-LH and International Standard of HCG.**

Several assays were performed to provide information
on the region of linearity of the dose-response curve. The result of one such experiment using international standard HCG as the test material is shown in Figure 8, and another using NIH-LH as test material is shown in Figure 9. In the case of NIH-LH the steepest part of the dose-response curve was found to lie between 0.1 and 0.4 µg of the hormone and a "working range" at these dose levels of the preparation was adopted. The results of four such experiments are shown in Figure 10. In all the assays shown, a significant depletion (P < 0.05) over the control value was obtained with 0.05 µg NIH-LH.

Quantitative assays.

A total of eleven assays are presented in Table 3. NIH-LH was used as the standard material and it should be noted that all the gonadotrophins tested showed appreciable LH activity.

Reliability criteria.

1. Precision. Table 3 shows that figures obtained for the index of precision (λ) range from 0.14 to 0.27 but are generally below 0.25.

2. Sensitivity. The dosages of gonadotrophins employed in the quantitative assays are shown in Table 4. The sensitivity of the test for all hormones studied is approximately twice that of the ovarian ascorbic acid depletion method (Schmidt-Elmendorff and Loraine, 1962).
3. **Specificity.** When given alone in dosages stated previously prolactin, growth hormone and ACTH had no effect on the level of ovarian cholesterol. On the other hand TSH produced a marked increase of ovarian cholesterol ($P > 0.001 < 0.01$) at both dose levels tested. This effect is probably due to the contamination of the TSH preparation with luteinizing hormone.

The results obtained when various hormones were given in combination with NIH-LH are shown in Table 5. It will be noted that following the administration of prolactin, ACTH and growth hormone, the fiducial limits of error ($P = 0.95$) include the figure of 0.1 indicating that the materials tested did not interfere with the response of LH. In view of the finding of Parlow (1961) and Schmidt-Elmendorff, that NIH-FSH contains about $1\%$ of LH activity, conventional specificity tests with this hormone could not be performed. The LH content of NIH-FSH is shown in Table 3.

Comparison of ovarian ascorbic acid depletion test with ovarian cholesterol accumulation assay.

The potency of various gonadotrophin preparations when assayed by these two methods are shown in Table 6. In the case of the ovarian ascorbic acid depletion assay the figures are taken from the paper by Schmidt-Elmendorff and Loraine (1962). Indices of discrimination (ovarian cholesterol accumulation/ovarian ascorbic acid depletion assays) were
calculated according to the method of Gaddum (1953a., b.) and are shown at the last column of the Table. It may be seen that in all cases the figures for the indices of discrimination are close to unity. This suggests that both the assay procedures provide an estimate of the same active principle.
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<th>No. of ovaries</th>
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<th>1st day after HCG</th>
<th>2nd day after HFS</th>
<th>2nd day after HCG</th>
<th>3rd day after HFS</th>
<th>3rd day after HCG</th>
<th>4th day after HFS</th>
<th>4th day after HCG</th>
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<th>9th day after HCG</th>
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<td>Control 1</td>
<td>104 ± 19</td>
<td>49.1 ± 3.2</td>
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<td>45.3 ± 6.6</td>
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<td>2260 ± 907</td>
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<td>86.9 ± 19.8</td>
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</tbody>
</table>

* 1st day after PMS
+ 3rd day after HFS
© 9th day after HCG

TABLE 1

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of ovaries</th>
<th>Body weight (g) ± S.D.</th>
<th>Ovarian weight (mg) ± S.D.</th>
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<td>49.1 ± 3.2</td>
<td>61.0 ± 7.1</td>
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<td>9.2 ± 2.2</td>
<td>10.2 ± 3.8</td>
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<td>45.3 ± 6.6</td>
<td>52.6 ± 5.7</td>
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<td>19.8 ± 5.5</td>
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<td>86.9 ± 19.8</td>
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<tr>
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* 1st day after PMS
+ 3rd day after HFS
© 9th day after HCG
<table>
<thead>
<tr>
<th>Number of ovaries</th>
<th>Pretreatment</th>
<th>Total dosages of hormone injected after PMSG pretreatment</th>
<th>Mean ovarian weight ± S.D. mgs</th>
<th>Significant difference in ovarian weight when compared to the control</th>
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<td>NIH-LH 500 μgs</td>
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<td>&quot;</td>
<td>Pergonal 8 μgs</td>
<td>54 ± 9</td>
<td>N.S.</td>
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<td>10</td>
<td>&quot;</td>
<td>PMSG 25 i.u.</td>
<td>82 ± 13</td>
<td>( P &gt; 0.001 ) ( &lt; 0.01 )</td>
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<td>HCG 25 i.u.</td>
<td>80 ± 16</td>
<td>( P &gt; 0.001 ) ( &lt; 0.01 )</td>
</tr>
<tr>
<td>Material tested</td>
<td>Design</td>
<td>Index of Precision (λ)</td>
<td>Relative Potency</td>
<td>Fiducial limits of error (P=0.95)</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>------------------------</td>
<td>------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>HCG (ISF)</td>
<td>2 + 2 0.20 0.00141 0.0012 - 0.0020 0.00152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2 0.18 0.00163 0.0014 - 0.0019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG (ISF)</td>
<td>2 + 2 0.23 0.0005 0.00041 - 0.00082 0.0006</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2 0.25 0.0007 0.00052 - 0.00096</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMG-24 (I.R.P.)</td>
<td>2 + 2 0.27 0.00098 0.00070 - 0.00134 0.0009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2 0.25 0.00082 0.00066 - 0.00091</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH-FSH</td>
<td>2 + 2 0.28 0.00831 0.00530 - 0.0169 0.0093</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2 0.20 0.00884 0.00713 - 0.0152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2 0.27 0.01093 0.00827 - 0.0161</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Pituitary LH</td>
<td>2 + 2 0.20 0.0984 0.0685 - 0.1481 0.0792</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; Assayed by OAAD method</td>
<td>2 + 1 0.25 0.0998 0.0666 - 0.1496 0.0998</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 4

**SENSITIVITY OF OVARIAN CHOLESTEROL ACCUMULATION METHOD USING VARIOUS GONADOTROPHIN PREPARATIONS**

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Minimum effective dose per animal producing significant increase in ovarian cholesterol</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-LH</td>
<td>0.05 μgs</td>
<td></td>
</tr>
<tr>
<td>NIH-FSH</td>
<td>10.6 μgs</td>
<td></td>
</tr>
<tr>
<td>HMG-24 (I.R.P.)</td>
<td>50.0 μgs</td>
<td></td>
</tr>
<tr>
<td>HCG (I.S.)</td>
<td>0.05 i.u.</td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>0.15 i.u.</td>
<td></td>
</tr>
<tr>
<td>Pergonal</td>
<td>0.8 mg</td>
<td></td>
</tr>
</tbody>
</table>
### Table 5

**Specificity Tests for Cholesterol Accumulation Assay. Potency of HIR-LH with Addition of Various Purified Anterior Pituitary Hormone Preparations to the Standards.**

<table>
<thead>
<tr>
<th>Hormone Added</th>
<th>Design</th>
<th>Index of Precision ((\lambda))</th>
<th>Relative Potency</th>
<th>Fiducial limits of error ((P=0.95))</th>
<th>Mean Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin</td>
<td>2 + 2</td>
<td>0.41</td>
<td>0.98</td>
<td>0.520 - 2.411</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.35</td>
<td>1.27</td>
<td>0.756 - 2.308</td>
<td></td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>2 + 2</td>
<td>0.21</td>
<td>0.79</td>
<td>0.506 - 0.994</td>
<td>0.79</td>
</tr>
<tr>
<td>ACTH</td>
<td>2 + 2</td>
<td>0.20</td>
<td>1.04</td>
<td>0.806 - 1.302</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.25</td>
<td>0.641</td>
<td>0.402 - 0.991</td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td>2 + 2</td>
<td>0.24</td>
<td>3.03</td>
<td>2.096 - 4.304</td>
<td>Assay invalid due to significant deviation from parallelism between the 'S' and 'U'</td>
</tr>
<tr>
<td>None</td>
<td>2 + 2</td>
<td>0.21</td>
<td>1.04</td>
<td>0.76 - 1.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.28</td>
<td>1.27</td>
<td>0.41 - 3.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.25</td>
<td>0.85</td>
<td>0.36 - 2.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.18</td>
<td>1.6</td>
<td>0.93 - 3.27</td>
<td></td>
</tr>
<tr>
<td>Hormone</td>
<td>Potency</td>
<td>Index of discrimination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------</td>
<td>-------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OAAD test</td>
<td>Ovarian Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Accumulation Assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH-FSH</td>
<td>90 µg</td>
<td>99 µg</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.R.P.</td>
<td>786 µg</td>
<td>980 µg</td>
<td>1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>0.50 i.u.</td>
<td>0.60 i.u.</td>
<td>1.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCG</td>
<td>1.36 i.u.</td>
<td>1.5 i.u.</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormone</td>
<td>Low dose</td>
<td>High dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>----------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH-LH</td>
<td>0.2 µg</td>
<td>0.8 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIH-PSH</td>
<td>42.4 µg</td>
<td>169.6 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.R.P. (HMG-24)</td>
<td>200.0 µg</td>
<td>800.0 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>0.60 i.u.</td>
<td>2.40 i.u.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCG</td>
<td>0.20 i.u.</td>
<td>0.80 i.u.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 2
PRETREATMENT EXPERIMENT

μg CHOLESTEROL/100 mg OVARY

CONTROL

FIGURE 3
Figure 4
OVARIAN WT(MG)

80

70

60

50

40

30

20

10

500 I.U. PMS-G + 20 I.U. HCG.

500 I.U. PMS-G ALONE

HCG.

FIGURE 5
CHOLESTEROL (u.g.) / 100 MG. TISSUE.

HCG

TOTAL

FREE

FIGURE 6
Figure 8
FIGURE 9
CHOLESTEROL (µG)/100 MG TISSUE.

FIGURE 10
DISCUSSION.

The effect of gonadotrophic hormones on ovarian cholesterol has been previously studied using mainly qualitative methods as reviewed already. On the basis of the present investigation it may be stated that administration of an LH preparation to immature intact rats previously primed with a large dose of PMSG, under the experimental condition described results in a gradual increase of the cholesterol content of the ovaries. Presumably this increase in cholesterol content is associated with the phenomenon of ovulation and corpus luteum formation. Pretreatment with one large dose of PMSG apparently sensitises the ovaries to the action of a comparatively small amount of LH, most likely due to maturation of a large number of graafian follicles ready to undergo the process of multiple ovulation and corpora lutea formation. It seems that during their early stages of life the newly formed corpora lutea are involved in utilizing rather than storing cholesterol, which is probably used up for the synthesis of ovarian steroid hormones. It is probable that this process of cholesterol turnover by the ovary is only possible so long as there is sufficient circulating LH. Once the level of LH falls below a certain level the cholesterol is stored up progressively. The depletion of cholesterol from highly luteinized ovaries in response to LH will be discussed in the next chapter. In the experiments described in this
chapter the administration of an LH preparation administered exogenously presumably stimulated ovulation and subsequently corpora lutea formation. Accumulation of cholesterol in the ovaries then occurred as a result of the gradual disappearance of the injected LH from the circulation. Obviously such a presumption points towards the possibility that in the absence of LH corpora lutea once formed either store up cholesterol or actively synthesize this sterol. It is also probable that the series of changes occurring in the corpus luteum which are associated with the withdrawal of gonadotrophin estimation are generally known as the process of atresia. The significance of cholesterol ester accumulation in the atretic corpora lutea is obscure.

From the point of view of an assay method the accumulation of ovarian cholesterol in suitably pretreated animals can be used as a suitable parameter for quantitative determination of LH activity in relatively purified gonadotrophin extracts.

The method is fairly sensitive, being approximately twice as sensitive as the ovarian ascorbic acid depletion (OAAD) test, and approximately 60 times more sensitive than the hypophysectomized rat prostate assay. The method is not sufficiently sensitive to permit the injection of unextracted urine for the assay of LH activity. Therefore to use this method gonadotrophic material from body fluids
has to be extracted and concentrated. It has been shown that the sensitivity of the assay procedure is considerably increased by injecting the test materials intraperitoneally rather than subcutaneously. Intraperitoneal injection of crude gonadotrophin extract from human urine is often toxic to the experimental animals. This means that some further purification steps for rendering the crude urinary extract non-toxic must be incorporated while concentrating urinary gonadotrophins by the usual methods.

Another advantage of the method is its relatively low degree of precision compared to some other bioassays. The index of precision is usually 0.25, which is slightly less than that commonly observed with ovarian ascorbic acid depletion test.

One of the drawbacks of the method is that the gonadotrophic material from pregnant sources (PMSG and HCG) when used in large doses following PMSG treatment induces further increase of ovarian weight, and the normal dose-response curves which are usually obtained using smaller dose levels of these hormones are significantly flattened. Although not a serious disadvantage in view of the fact that the augmented effect on ovarian weight can be detected by comparison with the controls, and the dose levels accordingly altered, the point has to be taken into consideration.

Further augmentation of PMSG primed ovarian weight,
induced by PMSG and HCG and not by NIH-LH, NIH-FSH, NIH-LH combined with NIH-FSH, Pergonal or HMG following PMSG plus pretreatment seems to be particularly interesting. This could possibly mean that placental and endometrial gonadotrophins possess certain peculiar biological characteristics of their own and the commonly attributed "LH type" and "FSH type" activity to these hormones are too much an oversimplification of the real biological characteristics of these gonadotrophic materials. This might also suggest that purification of gonadotrophins necessarily alters some of their biological properties so that FSH and LH fractions after chemical fractionation cannot reproduce the effects of a naturally combined gonadotrophic substance. Apart from inducing follicular maturation, ovulation, corpus luteum formation and stimulation of ovarian steroid hormone production, gonadotrophins provoke a number of other distinct structural and functional changes in the ovary which might be caused by specific substances of still undiscovered biological behaviour.

Specificity tests have shown that the response is not altered or induced by ACTH, Prolactin and growth hormone. TSH, given alone, caused a significant increase in ovarian cholesterol, given in combination with NIH-LH, the fiducial limits of error for the assays for the standard (NIH-LH alone) and the unknown (NIH-LH combined with TSH) did not
overlap and the relative potency of the unknown was higher than unity. This is most probably due to contamination of TSH with LH.

In view of the relatively longer time required for its completion this assay method is less practicable than the OAAD procedure. The longer time required for completion of the assay is likely to bring about the question of the biological half life of different hormones and the implication of this factor on the final biological reaction produced by them. It has been reported (Schmidt-Elmendorff and Loraine, 1962) that although it is possible to measure the LH activity of two different gonadotrophins (NIH-LH and HMG) of very different degrees of purity by using a short term assay procedure like the OAAD method, the regression slopes obtained from these two same materials using the hypophysectomised rat prostate assay (a long term procedure) are persistently non-parallel. It is quite likely that NIH-LH being a highly purified material is very quickly absorbed and excreted out of the system, whereas more crude preparations like HMG being bound to other less absorbable materials are retained for a longer time. Failure to produce parallel regression slopes usually means difference of biological activity of the materials used. For this reason if the biological variation due to the variation of absorption or excretion rate interferes with a particular
assay method then that particular assay procedure is likely to be less satisfactory than the one which is not interfered with by such a difference. LH activity is associated with many different gonadotrophin preparations. Ideally, for specific measurements of LH, the assay method should not be affected by such factors as biological half life. Short term assay procedures like the OAAD method which are less likely to be affected by the biological half life of the hormones are therefore probably more suitable for measuring biological activity of LH associated with materials of variable purity. It has been reported that although suitable for measuring the LH activity of comparatively crude preparations, the hypophysectomised rat prostate test is unsatisfactory for assaying highly purified material. Failure of parallelism as discussed before has prompted certain workers to use this assay as a quantal method, based on the comparison of the MED (minimum effective dose). It is generally accepted that for quantitative determination, quantitative assay procedures are superior to quantal methods (Perry, 1950).

**SUMMARY.**

A new assay method for Luteinizing Hormone (LH) activity is described and its reliability criteria assessed. The technique depends on the ovarian cholesterol accumulation in intact immature rats pretreated with pregnant mares serum.
gonadotrophin (PMSG).

The main advantage of the ovarian cholesterol accumulation assay is its high degree of reproducibility associated with comparatively high sensitivity. This procedure is approximately two times more sensitive than the OAAD method. The index of precision ($\lambda$) of OCA assay is usually smaller than that obtained with the OAAD method. The chief disadvantage of the technique when compared with the OAAD method is its relatively low degree of practicability.

In view of the difficulty experienced in several laboratories in setting up the OAAD method of assay, the ovarian cholesterol accumulation assay will be a suitable alternative procedure.
CHAPTER II

OVARIAN CHOLESTEROL DEPLETION IN INTACT PSEUDOPREGNANT RATS AS A SENSITIVE ASSAY METHOD FOR LUTEINIZING HORMONE ACTIVITY.

INTRODUCTION.

Over the past three decades many methods have been proposed for the assay of LH activity in anterior pituitary tissue and in body fluids (for references see Loraine, 1958, 1963; Loraine and Schmidt-Elmendorff, 1963). The majority of the older methods, for example the interstitial cell repair test (Evans, Simpson, Tolksdorf and Jensen, 1939) and the ventral prostatic weight test (Greep, Van Dyke and Chow, 1942) depend on observations in hypophysectomised rats and are both time-consuming and laborious. For these reasons they are generally unsuitable for routine application in the clinical field.

In 1956 Parlow, working in Boston, described a new method for the quantitative measurement of luteinizing hormone depending on the depletion of ovarian ascorbic acid in immature rats pretreated with PMS and HCG. Schmidt-Elmendorff and Loraine (1962) established the method and found it to be satisfactory for the estimation of luteinizing hormone activity (LH) in highly purified gonadotrophin preparations prepared from human or animal pituitary tissue, from human urine or from the serum of pregnant mares.

The pretreatment of rat ovaries with PMS and HCG makes
it possible for the first time to obtain a group of animals which, although immature, have enlarged heavily luteinized and metabolically active ovaries. The OAAD method which incorporates this procedure constitutes one of the most successful attempts to measure gonadotrophic activity by the utilisation of a biochemical rather than a gross biological response. It seemed likely that similarly pretreated ovaries might be useful in the investigation of other biochemical responses associated with gonadotrophic activity.

The possible precursor role of gonadotrophic hormones in the production of ovarian steroid hormones from cholesterol has been reviewed. On the basis of certain recent experiments the relation of the gonadotrophic hormones in the production of ovarian steroid hormones has been emphasised. A biosynthetic system capable of high level of oestrogen production has been provided by stimulating normal human ovaries prior to their surgical removal (Ryan and Smith, 1961; 1961a.; 1961b.; Smith and Ryan, 1961). On the basis of an earlier observation of Solomon et al. (1956) regarding the intermediary role of $\Delta^4$-3-ketosteroids in the ovarian synthesis of oestrogens from acetate and cholesterol Smith and Ryan (1961) were able to identify radioactive cholesterol, progesterone, 17$\alpha$-hydroxyprogesterone, androstenedione as well as oestrone and oestradiol 17$\beta$, in experiments with $^{14}$C labelled acetate in normal human subjects.
Conversion steps from cholesterol to progynolone and progesterone have been discussed by Shimizu et al. (1960, 1961). Tamaoki and Pincus (1961) demonstrated that cholesterol could be converted to progesterone by corpus luteum homogenate. They showed however that follicular tissue homogenates showed very little enzymatic activity and in the follicular fluid none was detectable. They postulated that, because corpus luteum free ovaries would have a much higher component of connective tissue than would ovaries carrying corpora lutea, the limited enzymatic activity shown by follicle tissue homogenates may have been due to the smaller amount of secretory tissue available. They also suggested that intact tissue rather than homogenates may be necessary for the gonadotrophic effect or certain specific conditions and addition to incubate may be necessary.

It has been indicated in connection with the research described in the last chapter that once formed corpora lutea are capable of storing cholesterol. It has been shown that the ovarian cholesterol values rise progressively for at least 9 days after withholding gonadotrophin administration.

In view of these findings it was thought that the effect of gonadotrophic hormones on heavily luteinized ovaries as obtained by pretreatment with 50.0 i.u. FMSG and 25 i.u. HCG (as advocated by Parlow for the OAAD assay) might reveal
itself by altering the cholesterol turnover in these ovaries.

This altered ovarian cholesterol content in its turn was expected to provide us with an index of gonadotrophic hormonal activity. The fundamental mechanism involved in the depletion of ascorbic acid from the ovary in response to gonadotrophin stimulation is not well understood, because very little is known regarding the biological role of ascorbic acid in steroidogenesis. On the other hand the precursor-role of cholesterol in ovarian production of steroid hormone is well established. For this reason it was expected that apart from providing a possible assay procedure for quantitative determination of gonadotrophic hormone this investigation might prove to be of some value in elucidating certain fundamental problems relating to the mechanism of action of the gonadotrophic hormones.

MATERIALS AND METHODS.

Hormones used in quantitative assays.

1. Follicle-stimulating hormone (NIH-FSH-S1).
2. Luteinizing hormone (NIH-LH-33).
3. Human menopausal gonadotrophin; International Reference Preparation (I.R.P.). This material has been shown to be approximately equipotent with HMG-20A (Bull. World Hlth. Org. 1960).
4. Pregnant mare's serum gonadotrophin (PMSG); international standard.
5. Human chorionic gonadotrophin (HCG); international standard.

Hormones used in specificity tests.
1. Prolactin (NIH-P-S3, ovine, 15 i.u./mg).
2. Growth hormone (NIH-GH-37).
3. Adrenocorticotrophic hormone (ACTH). The preparation used was 'cortrophin' (Organon Laboratories Ltd. 10 i.u./ampoule).
4. Thyroid-stimulating hormone (TSH). This material was prepared from human pituitary tissue and had a potency of 0.745 i.u./mg. (Butt, Stockell-Hartree and Kirkham, 1963).

The method in detail.
The procedure is conducted in two stages:
(i) pretreatment of animals, and
(ii) bioassay proper.
(i) Pretreatment of animals. Intact immature female Wistar rats bred in a closed colony in the laboratory in Edinburgh were employed. The animals varied in weight from 30 to 50 gm. and in age from 21 to 24 days. Each rat received a subcutaneous injection of 50 i.u. PMSG ('Gestyl', Organon) followed 72 hours later by a subcutaneous injection of 25 i.u. HCG ('Pregnyl', Organon). Both preparations were dissolved in distilled water and the injections were administered in a total volume of 0.5 ml. per animal. It
was noted that as a result of the pretreatment the ovaries became heavily luteinized and varied in weight from 70 to 110 mg.

(ii) Bioassay proper. The material to be assayed was dissolved in 0.5 ml. 0.9% NaCl and was injected intraperitoneally. The animals were killed 5 hours later by dislocation of the cervical spine and the two ovaries were removed using a dorsal approach. The individual ovaries were rapidly cleaned and weighed on a torsion balance to the nearest 1.0 mg. Each ovary was then homogenized in a mortar and pestle with 2.0 ml. of acetone-ethanol (1:1 v/v) and a trace of sand. After thorough grinding a further 2.0 ml. of acetone-ethanol (1:1 v/v) was added and the homogenate filtered through Whatman No.2 filter paper. The total cholesterol content of the filtrate was then determined using the procedure described by Searcy and Bergquist (1962) and previously described in Chapter I. The results were expressed as μg cholesterol/100 mg ovarian weight.

Design of Assays.

The assays performed were of three types:

(i) pilot assays using NIH-LH were conducted in order to determine the dose-response and the time-response relationship. In these assays five animals were employed at each dose level of the hormone and the log dose interval, i.e. the difference between the logarithms of the two neigh-
bouring doses was either $\log_{10}^2(0.301)$ or $\log_{10}^5(0.699)$.

(ii) Quantitative assays using NIH-LH as reference material were conducted in order to estimate the LH activity of various gonadotrophin preparations and to study the specificity of the method. In all instances the design was a symmetrical 4-point assay with two dose levels of the standard and test materials and with equal spacing of the log doses. Five animals (ten ovaries) were used at each dose level of the material to be tested and the log dose interval was $\log_{10}^5(0.699)$ in all assays. In two assays (Table II) only one dose level of the unknown preparation fell within the "working range" of the dose-response curve of the standard material and the results were thus calculated as for a 3-point assay.

(iii) Quantitative assays for measuring the LH excretion in normal children and in normally menstruating women. Samples from 24 hour pooled collections of urine were obtained. The urine sample was diluted as necessary to obtain the dose-response steps for 4-point assays. No attempt was made to concentrate the urinary gonadotrophin by any extraction procedure. For the cyclic women studied it was found necessary to dilute the unextracted urine samples to the extent of at least 1000 times. For the children's urine the dilution varied from 1 : 50 to 1 : >1000, roughly depending upon the age. HMG-24 (I.R.P.) was used as
the standard and the results are expressed in HMG units/24 hours.

Few observations have been made on the LH content of the serum in children. For the purpose of the assay diluted serum without any preliminary treatment was used. The results are expressed in HMG units/100 ml. of serum.

The indices of precision (\(\lambda\)), relative potencies (R.P.) and fiducial limits of error were determined by the method described by Gaddum (1953 a. b.). In none of the 4-point assays was significant deviation from parallelism encountered between the dose-response curves of the standard and test preparations.

**Specificity tests.**

The specificity of the assay procedure was studied in two ways. Firstly, anterior pituitary hormones which might interfere with the response to LH were administered in total dosages at least 10 times higher than those employed by Schmidt-Elmendorff and Lorraine (1962) for the OAAD test. Secondly, combinations of NIH-LH with other anterior pituitary hormones were administered. The hormones tested are shown in Table 8. In view of the finding of Parlow (1961) and Schmidt-Elmendorff et al. (1962) that NIH-FSH contains approximately 1% of LH activity conventional specificity tests in relation to this hormone could not be conducted. The LH content of NIH-FSH is shown in Table 11.
<table>
<thead>
<tr>
<th>Hormone tested</th>
<th>Dosage per animal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Administered alone</td>
</tr>
<tr>
<td>Prolactin</td>
<td>2.0 n*</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>10.0 n</td>
</tr>
<tr>
<td>ACTH</td>
<td>4.0 i.µu</td>
</tr>
<tr>
<td>TSH</td>
<td>14.0 i.µu</td>
</tr>
</tbody>
</table>

* 1 nanogram (n) = 1 x 10^{-9} g.

1 picogram (p) = 1 x 10^{-12} g.

1 i.µu = 1 international milliunit
= 1 x 10^{-3} international units.

1 i.µu = 1 international microunit
= 1 x 10^{-6} international units.
FIGURE 11
Days after PMSG

Days after HCG

PRETREATMENT EXPERIMENT

μg CHOLESTEROL/100 mg OVARY

CONTROL

FIGURE 12
RESULTS.

Pretreatment experiments (Figure 11).

Figure 11 shows the results of an experiment in which 45 animals were divided at random into two groups. 15 animals acted as control and the remainder received 50 i.u. FMS on day 0 followed 72 hours later by 25 i.u. HCG. Groups of 5 animals were killed each day until the 9th day after HCG. The weight of the individual ovaries in mg. and the cholesterol content in μg. is shown. In each case, as in all the following figures, the arithmetic range of the results has been plotted. The ovarian cholesterol content increased slowly at first and then more rapidly, while the ovarian weight increased until the 2nd to 3rd day after HCG, and thereafter was maintained at the same level until the end of the experiment. In terms of ovarian structure this experiment showed the FMSG initiated the growth of a large number of follicles which were luteinized after the administration of HCG. It is interesting to note that on the 8th and 9th days after HCG the range of cholesterol values increased considerably while there was no corresponding increase in the range of the ovarian weight. Throughout the experiment there was little alteration in either the weight or the cholesterol content of the ovaries from rats which received no hormone.

Figure 12.

Figure 12 shows the results of the same pretreatment.
RAT OVARIAN CHOLESTEROL

6th. DAY AFTER HCG

FIGURE 13
RAT OVARIAN CHOLESTEROL
5th. DAY AFTER HCG

FIGURE 14
experiment but with the results expressed as \( \mu g \) cholesterol/100 mg ovary. The value for the control ovaries is very high; however this may be explained by the low weight of the ovaries. Following the administration of PMSG the cholesterol values first fell as the ovaries were stimulated to grow and then slowly increased until the 7th day after HCG. Higher values were noted on the 8th and 9th days, and the range of the individual results increased markedly. At the time the experiment was completed it was not possible to explain this large variation in the range, and in view of the relatively small range of the cholesterol values it was decided to investigate cholesterol turnover in rats which had received HCG either 5 or 6 days previously.

**Figures 13 to 15.**

These Figures show three of the earlier attempts to characterise the dose-response curve.

**Figure 13.**

In this experiment each group consisted of 5 animals and dose levels used ranged from 0.0 to 0.16 \( \mu g \) NIH-LH. Even at the lowest dose level a considerable depletion of cholesterol was obtained.

**Figure 14.**

In the previous experiment the animals were used 6 days after the injection of HCG, while in this one they were left only for 5 days. It should be noted that although the
RAT OVARIAN CHOLESTEROL
6th. DAY AFTER HCG

FIGURE 15
cholesterol has been depleted to the same absolute value in both experiments the results obtained with control rats which received no hormone on the day of the assay were much lower in this experiment than in the previous one. This difference which amounted to about 450 µg indicated that the pretreatment procedure does not always produce the same cholesterol value in each group of animals. This figure also shows that animals which received no injection on the day of the assay had essentially the same ovarian cholesterol value as animals which received an injection of 0.5 ml. saline, thus indicating that the stress effect resultant from an intraperitoneal injection has no marked effect on the level of ovarian cholesterol.

Figure 15.

In this experiment the lowest dose level employed was 39p of NIH-LH and again there was a significant depletion of ovarian cholesterol when compared with ovaries from control animals. It was apparent when the results were calculated that there were two distinct groups of cholesterol values inside the same treatment group. The bottom line shows the mean value of the lower of the two sets of results while the top line represents the mean of all the results with the arithmetic range. This difference in cholesterol content was not related to ovarian weight but it did appear to be associated with ovarian structure in that the ovaries
with the lower cholesterol content were pink and had a smooth surface while those with a high cholesterol content were white and hard.

**Table 9.**

This Table shows the results obtained in two control groups from the same experiment in which there was considerable difference in both the ovarian weight and cholesterol content. All the ovaries were apparently completely luteinized but the ovaries with the lower cholesterol values were pink and soft while the ovaries in the other group were white and of harder consistency. This difference appeared to be related to the ovarian weight, the lighter ovaries containing comparatively larger amounts of cholesterol than the heavier ones. The marked difference in cholesterol content which may occur in ovaries pretreated in a similar manner helps to explain the very large range in ovarian cholesterol content which has been encountered in these experiments.

**Figure 12.** (see page 93)

It may be seen from this Figure that the cholesterol values on the 5th, 6th and 7th days after HCG are rather lower than have been found in subsequent experiments. However, on the 8th and 9th days a rapid rise and increase in range in ovarian cholesterol takes place, presumably associated with the transition from pink luteinized ovaries to white luteinized ovaries with a higher cholesterol content (Table 9). This may possibly be related to the rate of
### TABLE 9

**RAT OVARIAN CHOLESTEROL**

7th DAY AFTER HCG

<table>
<thead>
<tr>
<th>wt (mg)</th>
<th>µg cholesterol/100 mg ovary</th>
<th>wt (mg)</th>
<th>µg cholesterol/100 mg ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>824</td>
<td>97</td>
<td>1907</td>
</tr>
<tr>
<td>88</td>
<td>999</td>
<td>94</td>
<td>2148</td>
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<tr>
<td>79</td>
<td>1214</td>
<td>96</td>
<td>1338</td>
</tr>
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<td>75</td>
<td>1314</td>
<td>84</td>
<td>1488</td>
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<tr>
<td>80</td>
<td>1043</td>
<td>126</td>
<td>1635</td>
</tr>
<tr>
<td>69</td>
<td>1029</td>
<td>124</td>
<td>1635</td>
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<tr>
<td>66</td>
<td>901</td>
<td>129</td>
<td>1194</td>
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<td>70</td>
<td>850</td>
<td>133</td>
<td>1158</td>
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<td>91</td>
<td>1151</td>
<td>84</td>
<td>1702</td>
</tr>
<tr>
<td>87</td>
<td>1174</td>
<td>81</td>
<td>1877</td>
</tr>
</tbody>
</table>

**MEAN S. D.**

<table>
<thead>
<tr>
<th>wt (mg)</th>
<th>µg cholesterol/100 mg ovary</th>
<th>wt (mg)</th>
<th>µg cholesterol/100 mg ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>80.2</td>
<td>1050</td>
<td>104.8</td>
<td>1608</td>
</tr>
<tr>
<td>±10.1</td>
<td>±127</td>
<td>±16.9</td>
<td>±322</td>
</tr>
</tbody>
</table>

**WEIGHT**

\[ t = 3.75 \quad p < 0.01 \quad > 0.001 \]

**CHOLESTEROL**

\[ t = 4.84 \quad p < 0.001 \]
excretion of the relatively large subcutaneous injection of 25 i.u. HCG used in the pretreatment. The importance of gonadotrophins in the production and maintenance of the ovarian vascular tree has been previously stressed by Reynolds (1955). He has also shown that the level of ovarian vascularisation is intimately associated with ovarian activity. Thus in this experiment up to the 6th to 7th day after the injection of HCG the ovaries, presumably still under the influence of this hormone, are pink (hyperactive), soft, indicating increased vascularisation and increased metabolic activity which may well be associated with cholesterol production and utilisation (Table 9). It is possible that as the hormone is excreted the circulating blood level of HCG will decrease until it is below the level which is capable of maintaining the high degree of vascularisation. At the same time it seems likely that the ovarian metabolic activity, and therefore cholesterol utilisation, may decrease, hence the marked increase in cholesterol storage. The large range on the 8th and 9th days after HCG (see Figure 12) is probably due to the ovaries from the 5 animals in each group showing different stages in the transition process between the state of rapid cholesterol utilisation and decreased cholesterol utilisation resulting in cholesterol storage. It was therefore decided to re-in-
FIGURE 16

OVARIAN CHOLESTEROL 
μG/100MG.  

CONTROL

1 HOUR

2 HOUR

3 HOUR

5 HOUR

P NIH-LH

FIGURE 16
RAT OVARIAN CHOLESTEROL
11th DAY AFTER HCG

μg. Cholesterol / 100 mg. ovary

FIGURE 17
vestigate cholesterol depletion in response to LH, using ovaries in which the cholesterol content would be as uniform as possible, namely on the 5th and 11th days after the injection of HCG.

**Experiment (Time Response Curve) - Figure 16.**

In all the previous experiments the animals were killed four hours after the intraperitoneal injection of LH. This is the time suggested by Farlow for the OAAD method. Figure 16 shows the results of a time response curve in which rats were killed from 1 to 5 hours after the injection of either 10 or 50 p of LH. Maximum cholesterol depletion was found to occur at the end of 5 hours. On the basis of these results it was decided to sacrifice the animals after 5 hours after the administration of the test material. This procedure has been adopted in all subsequent experiments.

**Figures 17 to 21.**

These figures show further attempts to characterise the dose-response curve of cholesterol depletion after the administration of LH using rats which were left for 11 days after the HCG injection. Five animals were used at each dose level of hormone and the 10 ovaries obtained have been treated as separate observations.

**Figure 17.**

In this experiment a significant depletion of cholesterol was obtained with 0.27 p of LH. It should be noted that the cholesterol content increased at the higher dose levels.
RAT OVARIAN CHOLESTEROL
11th. DAY AFTER HCG

μg. Cholesterol / 100 mg. ovary

Control 0.0125 0.038 0.11 0.33 1.0 3.0 9.0
p. NIH - LH

FIGURE 18
FIGURE 19
RAT OVARIAN CHOLESTEROL
11th DAY AFTER HCG

μg. Cholesterol / 100 mg. ovary

Control 0-66 2-0 6-0 18-0 54-0 162-0 1640
p. NIH-LH

FIGURE 20
To try to eliminate the possibility of the hormone being adsorbed onto glass 0.1% gelatin was added to the saline used for diluting the hormone. One group of 5 animals received an injection of saline containing gelatin while another group were given saline alone. As would be expected no significant effect on ovarian cholesterol was observed in this last group.

Figure 18.

The lowest dose level used was 0.012 p of LH and although the range of results was very high there was a considerable depletion of cholesterol between 0.056 and 1.0 p. As in the previous experiment the cholesterol content of the ovaries started to increase at the higher dose levels.

Figure 19.

The overall shape of the dose-response curve obtained in this experiment was similar to that in the last experiment over the same dose range. It should be noted that the range of results was again very high.

Figure 20.

In this experiment the depletion of cholesterol which was significant between 0.66 and 6.0 p was followed by an increase at the higher dose levels. The explanation of this increase in cholesterol at higher dose levels is obscure. However, in view of the very high range of the results and the non-uniformity of the ovaries it seems possible that
RAT OVARIAN CHOLESTEROL
11th. DAY AFTER HCG

μg. Cholesterol / 100 mg. ovary

Control 0.0122 0.0366 0.11 0.33 1.0 3.0
p NIH-LH

FIGURE 21
RAT OVARIAN CHOLESTEROL
5th DAY AFTER HCG

μg. Cholesterol / 100 mg. ovary

1100
900
700
500
300

Control 1:0 5:0 25:0 125:0
n. NIH-LH

FIGURE 22
this finding may be an artefact. It should be noted that with a relatively high dosage of hormone the depletion obtained was the same as with 6 p, LH.

**Figure 21.**

This Figure shows only the downward trend of the dose-response curve between 0.11 and 5.0 p. A significant depletion of cholesterol occurred between 0.35 and 3.0 p.

**Figures 22 to 25.**

These Figures show the results of experiments carried out using rats which had been left for five days after the injection of HCG. Again each group consisted of 5 animals. It may be pointed out that at this period of pretreatment ovarian cholesterol values were found to be more uniform than at any other time.

**Figure 22.**

In a preliminary experiment it appeared that on the 5th day after HCG the ovaries were less sensitive to LH than on the 11th day after HCG. Accordingly in this experiment higher dosages of LH ranging from 1.0 to 125.0 n were used. Considerable depletion of ovarian cholesterol was obtained at all dose levels. Two points should be noted, firstly that the range of results is smaller and secondly that the cholesterol value in the control group is lower than when using animals on the 11th day after HCG.
RAT OVARIAN CHOLESTEROL
5th. DAY AFTER HCG

FIGURE 23
RAT OVARIAN CHOLESTEROL
5th. DAY AFTER HCG

\[ \text{\( \mu g \) Cholesterol / 100 mg. ovary} \]

- Control: 1100
- 0.037 NIH-LH: 1000
- 0.11 NIH-LH: 900
- 0.33 NIH-LH: 800
- 1.0 NIH-LH: 700
- 3.0 NIH-LH: 600

\( \text{p NIH-LH} \)
OVARIAN CHOLESTEROL

\[ \mu g/100 \text{ mg.} \]

5th DAY AFTER HCG.

FIGURE 25
Figure 23.

With dosages of hormone ranging from 78 pg. to 10 n, no significant depletion was observed.

Figures 24 and 25.

In these experiments the dose levels of hormone were comparable to those used in dose-response curves carried out on the 11th day after HCG. No significant depletion was observed and it would therefore appear that on the 5th day the response to LH is less sensitive than on the 11th day after HCG.

Conclusions.

The following conclusions may be drawn from the results of the dose-response curves.

1. With rats on the 11th day after HCG the depletion of ovarian cholesterol from the control values ranges from 600 to 1,200 µg.

2. In groups of rats used on the 5th day after HCG the maximum depletion obtainable amounts to not more than 300 µg of cholesterol per 100 mgm of ovary. In view of the finding that the response appears to be less sensitive and even though the range of results is smaller than in animals on the 11th day after HCG, the depletion is too small and the regression slope too flat to provide the steep dose-response curve required for a workable assay method. This latter finding is in conformity with that previously
reported by Parlow (1961).

Table 10.

The dose levels of hormone found necessary to deplete the ovarian cholesterol being found to be very small in comparison with other methods of assay for gonadotrophin it was thought useful to try to show whether or not the depletion of cholesterol seen in intact rats was a true finding. Two experiments have been performed in which one ovary was removed from each of a group of rats before injection of either saline or LH. After 5 hours the rats were killed and the second ovary removed. Table 10 shows the results obtained in one of these experiments for the ovarian cholesterol in μg/100 mg. ovarian tissue for the first and second ovary, the percentage change and the mean percentage change for control and treatment groups. Statistical analysis showed that the control group was significantly different from the 1.0 p group at the 0.05 level, but was not significantly different from the 0.2 p group unless the value in brackets was eliminated. As may be seen this value was obtained from ovaries with an unusually high cholesterol level. The following points should be noted. Firstly, that the range of cholesterol values obtained inside each group was very high, and secondly, that a variable but considerable depletion was observed in the control group. It is possible that this may have been due to
TABLE 10

RAT OVARIAN CHOLESTEROL

11th DAY AFTER HCG

<table>
<thead>
<tr>
<th></th>
<th>1st ovary</th>
<th>2nd ovary</th>
<th>% change</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1685</td>
<td>879</td>
<td>-48</td>
<td>-36</td>
</tr>
<tr>
<td></td>
<td>1789</td>
<td>1033</td>
<td>-43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2141</td>
<td>1826</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2054</td>
<td>1430</td>
<td>-30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2117</td>
<td>800</td>
<td>-62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1545</td>
<td>1302</td>
<td>-18</td>
<td></td>
</tr>
<tr>
<td>0.2 p</td>
<td>1865</td>
<td>672</td>
<td>-64</td>
<td>-53</td>
</tr>
<tr>
<td></td>
<td>1403</td>
<td>587</td>
<td>-58</td>
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<tr>
<td></td>
<td>2174</td>
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<td>-45</td>
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<td></td>
<td>3667</td>
<td>2643</td>
<td>-28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1585</td>
<td>841</td>
<td>-53</td>
<td></td>
</tr>
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<td></td>
<td>1580</td>
<td>570</td>
<td>-64</td>
<td></td>
</tr>
<tr>
<td>1.0 p</td>
<td>1021</td>
<td>486</td>
<td>-52</td>
<td>-56</td>
</tr>
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<td></td>
<td>2015</td>
<td>740</td>
<td>-63</td>
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<td></td>
<td>1421</td>
<td>711</td>
<td>-50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1961</td>
<td>820</td>
<td>-58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3084</td>
<td>1524</td>
<td>-51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1480</td>
<td>567</td>
<td>-62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2939</td>
<td>1367</td>
<td>-54</td>
<td></td>
</tr>
</tbody>
</table>

Control = N.S.  
0.2 p = N.S.  
1.0 p = N.S.  
< 0.02 > 0.05
figure 26
FIGURE 27

TOTAL CHOLESTEROL (µg./100 mg. ovarian weight)

TOTAL DOSE NIH-LH: picograms (LOG SCALE)
the ether anaesthetic used during the removal of the first ovary. Thirdly, that the injection of 1.0 p of LH depleted the ovarian cholesterol of one ovary when compared with that of the other ovary of the same rat. This observation supports the work in intact rats and suggests that the depletion of ovarian cholesterol by LH is a real finding and not an artefact.

In view of this the rest of the investigation was continued with pretreated animals on the 11th day after HCG.

**Determination of log dose-response curve for NIH-LH.**

To provide information on the region of linearity of the dose-response curve a total of 16 assays were performed and the result of a typical experiment is shown in Figure 26. The steepest part of the dose-response curve was found to lie between 0.059 and 0.469 picograms NIH-LH and a "working range" at dose level of 0.06, 0.3 and 1.5 p NIH-LH was adopted. In Figure 27 are shown the data obtained in five typical assays. In the majority of assays a significant depletion (P < 0.05) over control values was obtained with 0.06 p NIH-LH.

**Quantitative assays.**

A total of 16 assays are presented in Table 11. NIH-LH was used as the reference preparation and it should be noted that all the gonadotrophins tested show a low LH activity.
### TABLE II

**POTENCY OF VARIOUS GONADOTROPHINS IN TERMS OF NIH-LH**

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Design</th>
<th>λ</th>
<th>Relative Potency</th>
<th>Fiducial limits (P = 0.95)</th>
<th>Mean Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-FSH</td>
<td>2 + 2</td>
<td>0.31</td>
<td>0.01260</td>
<td>0.00753 - 0.02111</td>
<td>0.00941</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.39</td>
<td>0.00778</td>
<td>0.00363 - 0.01514</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.19</td>
<td>0.00961</td>
<td>0.00706 - 0.01533</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 1</td>
<td>0.38</td>
<td>0.00757</td>
<td>0.00378 - 0.01411</td>
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</tr>
<tr>
<td>I.R.P.</td>
<td>2 + 2</td>
<td>0.37</td>
<td>0.00104</td>
<td>0.00053 - 0.00206</td>
<td>0.00101</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.55</td>
<td>0.00088</td>
<td>0.00027 - 0.00254</td>
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</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.35</td>
<td>0.00114</td>
<td>0.00062 - 0.00214</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.24</td>
<td>0.00099</td>
<td>0.00066 - 0.00149</td>
<td></td>
</tr>
<tr>
<td>PMSG</td>
<td>2 + 2</td>
<td>0.41</td>
<td>0.00071</td>
<td>0.00031 - 0.00142</td>
<td>0.00049</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.24</td>
<td>0.00023</td>
<td>0.00013 - 0.00041</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.51</td>
<td>0.00067</td>
<td>0.00019 - 0.00166</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.47</td>
<td>0.00035</td>
<td>0.00007 - 0.00082</td>
<td></td>
</tr>
<tr>
<td>HCG</td>
<td>2 + 2</td>
<td>0.33</td>
<td>0.00080</td>
<td>0.00042 - 0.00144</td>
<td>0.00082</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.41</td>
<td>0.00086</td>
<td>0.00038 - 0.00180</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.44</td>
<td>0.00055</td>
<td>0.00012 - 0.00140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 + 1</td>
<td>0.13</td>
<td>0.00105</td>
<td>0.00075 - 0.00149</td>
<td></td>
</tr>
</tbody>
</table>
Reliability criteria.

1. Precision. Table 11 shows that figures obtained for the index of precision (\( \lambda \)) range from 0.13 to 0.55 but are generally below 0.45.

2. Sensitivity. The dosages of the gonadotrophin preparations employed in the quantitative assays are shown in Table 12. The most striking feature in this Table is the extraordinary degree of sensitivity of the test which for all the hormones tested is approximately 5,000,000 times more sensitive than the OAAD method.

3. Specificity. When given in the dosages states previously (Table 8) prolactin, growth hormone and ACTH had no effect on the level of ovarian cholesterol. On the other hand TSH produced a marked depletion of ovarian cholesterol (\( p > 0.001 > 0.01 \)) at both dose levels tested but this effect is almost certainly due to the contamination of the TSH preparation with LH.

The results obtained when various hormones were given in combination with NIH-LH are shown in Table 15. It will be noted that following the administration of prolactin, ACTH and TSH, the fiducial limits of error (\( P = 0.95 \)) include the figure of 1.0 indicating that the materials tested did not interfere with the response of LH. In two of the three assays in which growth hormone was added the limits did not include the figure of 1.0.
## Table 12

**Total Dosage of Gonadotrophin Administered Per Animal**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Low dose</th>
<th>High dose</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-LH</td>
<td>0.06</td>
<td>0.3</td>
<td>picograms</td>
</tr>
<tr>
<td>NIH-FSH</td>
<td>6</td>
<td>30</td>
<td>picograms</td>
</tr>
<tr>
<td>I.R.P.</td>
<td>60</td>
<td>300</td>
<td>picograms</td>
</tr>
<tr>
<td>PMSG</td>
<td>0.06</td>
<td>0.3</td>
<td>international microunits.</td>
</tr>
<tr>
<td>HCG</td>
<td>0.06</td>
<td>0.3</td>
<td>international microunits.</td>
</tr>
<tr>
<td>Hormone Added</td>
<td>Design</td>
<td>λ</td>
<td>Relative Potency</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
<td>---</td>
<td>----------------</td>
</tr>
<tr>
<td>Prolactin</td>
<td>2 + 2</td>
<td>0.39</td>
<td>0.714 0.25</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.44</td>
<td>0.58 0.44</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>0.48</td>
<td>0.55 0.42</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>2 + 2</td>
<td>0.35</td>
<td>0.44 0.04</td>
</tr>
<tr>
<td>ACTH</td>
<td>2 + 2</td>
<td>0.43</td>
<td>0.44 0.03</td>
</tr>
<tr>
<td>TSH</td>
<td>2 + 2</td>
<td>0.35</td>
<td>0.48 0.04</td>
</tr>
</tbody>
</table>
Comparison of OAAD test with ovarian cholesterol depletion (OCD) test.

In Table 14 is shown the potency of various gonadotrophins when assayed by the OAAD and OCD tests in terms of NIH-LH. In the case of the OAAD method the figures are taken from the paper by Schmidt-Elmendorff and Loraine (1962). Indices of discrimination (OCD/OAAD tests) were calculated by the method of Gaddum (1955) and are shown in Column 4 of the Table. In all cases the indices are close to unity suggesting that both assay procedures provide an estimate of the same active principle.

Table 15.

This Table shows the result of bioassays performed with the 24-hour pooled urine samples of a normally menstruating, parous, cyclic woman aged 23. The first day of the urine collection was on the second day of menstruation. Appropriately diluted urine samples without any previous extraction were used for the assays. The unextracted urine samples without any preliminary processing had to be diluted to the extent of approximately 1:1,000 or more with normal saline to obtain the slope. HMG-24 was used as the standard preparation, and results have been expressed in HMG units/24 hours.

It may be observed from Table 15 that a detectable but variable amount of LH is excreted through all the different
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Potency</th>
<th>Index of discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-PFS</td>
<td>90 μg</td>
<td>94 μg</td>
</tr>
<tr>
<td>I.R.P.</td>
<td>796 μg</td>
<td>1010 μg</td>
</tr>
<tr>
<td>MSG</td>
<td>0.50 i.u.</td>
<td>0.49 i.u.</td>
</tr>
<tr>
<td>HCG</td>
<td>1.36 i.u.</td>
<td>0.82 i.u.</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Spec. No.</td>
<td>Day of Cycle</td>
<td>Design 24hr. vol.</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>820</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>950</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1650</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>920</td>
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<td>740</td>
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<td>6</td>
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<td>820</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>740</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>820</td>
</tr>
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<td>9</td>
<td>10</td>
<td>1540</td>
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<tr>
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<td>1220</td>
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<td>12</td>
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<td>970</td>
</tr>
<tr>
<td>13</td>
<td>14</td>
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<tr>
<td>14</td>
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<td>15</td>
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<td>520</td>
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<tr>
<td>16</td>
<td>17</td>
<td>890</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>710</td>
</tr>
<tr>
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<td>19</td>
<td>670</td>
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<td>760</td>
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<tr>
<td>21</td>
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<td>710</td>
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<td>1290</td>
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<td>25</td>
<td>26</td>
<td>1440</td>
</tr>
<tr>
<td>26</td>
<td>27</td>
<td>1940</td>
</tr>
<tr>
<td>27</td>
<td>28</td>
<td>690</td>
</tr>
<tr>
<td>28</td>
<td>29</td>
<td>1030</td>
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<td>29</td>
<td>30</td>
<td>660</td>
</tr>
<tr>
<td>30</td>
<td>31</td>
<td>1360</td>
</tr>
</tbody>
</table>
phases of the menstrual cycle. The marked rise in LH excretion on the 14th day of the cycle is noteworthy. Although no conclusive remarks can be made from studying only one cycle, it seems that the transient but sharp rise in LH secretion from the pituitary at the time of ovulation does occur.

**Table 16.**

This Table shows the result of 11 bioassays performed with the samples of 24-hour pooled specimens of urine from children aged three weeks to nine years. The urine samples had to be diluted and used for assay in the same way as in the case of urine from cyclic female subject studied. Results are expressed in HMG units/24 hours.

**Table 17.**

Table 17 shows the result of bioassays performed with the blood plasma of the children studied as shown in the previous Table (Table 16). For children, the urine and plasma samples had to be diluted to approximately the same extent (approximately 1 : 99).

**DISCUSSION.**

The effect of gonadotrophic hormones on ovarian cholesterol has previously been studied by Claesson and Hillarp (1947 a., b.) who employed histochemical techniques and demonstrated that when a very large dose of PMSG was
<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age</th>
<th>24 hour urine volume, mls.</th>
<th>Design of Assay</th>
<th>Index of Precision (λ)</th>
<th>Fiducial limits of error (p = 0.95)</th>
<th>Potency HMG units/24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>3 weeks</td>
<td>210</td>
<td>2 + 2</td>
<td>0.42</td>
<td>0.026 - 0.149</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>14 months</td>
<td>420</td>
<td>2 + 2</td>
<td>0.30</td>
<td>0.077 - 0.265</td>
<td>0.143</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>4 years</td>
<td>360</td>
<td>2 + 2</td>
<td>0.31</td>
<td>0.0043 - 0.172</td>
<td>0.137</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>7 years</td>
<td>360</td>
<td>2 + 2</td>
<td>0.65</td>
<td>0.160 - 0.850</td>
<td>0.131</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>8 years</td>
<td>780</td>
<td>2 + 2</td>
<td>0.61</td>
<td>0.293 - 0.887</td>
<td>0.142</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>9 years</td>
<td>1560</td>
<td>2 + 2</td>
<td>0.42</td>
<td>0.045 - 0.739</td>
<td>0.179</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>10 years</td>
<td>1240</td>
<td>2 + 2</td>
<td>0.35</td>
<td>0.095 - 0.723</td>
<td>0.142</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>11 years</td>
<td>1090</td>
<td>2 + 2</td>
<td>0.58</td>
<td>0.110 - 2.367</td>
<td>0.143</td>
</tr>
</tbody>
</table>
### TABLE 17

LUTEINIZING HORMONE ACTIVITY IN THE PERIPHERAL VENOUS PLASMA OF YOUNG CHILDREN, ASSAYED BY CHOLESTEROL DEPLETION METHOD.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Design of Assay</th>
<th>Index of Precision ($\lambda$)</th>
<th>Fiducial limits of error ($P = 0.95$)</th>
<th>Potency HMG units/100mls.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 weeks</td>
<td>$2 + 2$</td>
<td>0.49</td>
<td>0.014 - 0.353</td>
<td>0.089</td>
</tr>
<tr>
<td>2</td>
<td>14 months</td>
<td>$2 + 2$</td>
<td>0.33</td>
<td>0.029 - 0.124</td>
<td>0.041</td>
</tr>
<tr>
<td>3</td>
<td>4 years</td>
<td>$2 + 2$</td>
<td>0.36</td>
<td>0.100 - 1.601</td>
<td>0.239</td>
</tr>
<tr>
<td>4</td>
<td>7 years</td>
<td>$2 + 2$</td>
<td>0.38</td>
<td>0.007 - 0.063</td>
<td>0.026</td>
</tr>
<tr>
<td>5</td>
<td>8 years</td>
<td>$2 + 2$</td>
<td>0.40</td>
<td>0.181 - 1.500</td>
<td>0.385</td>
</tr>
<tr>
<td>6</td>
<td>9 years</td>
<td>$2 + 2$</td>
<td>0.55</td>
<td>0.011 - 0.139</td>
<td>0.059</td>
</tr>
</tbody>
</table>
administered to rats and rabbits a fall in cholesterol ensued. Some years later Parlow (1961) using rats pretreated with PMSG and HCG and Franchimont and colleagues (Franchimont, 1962; Franchimont and Cawenberge, 1962) using intact immature and adult rats obtained similar results but none of these investigators were able to establish a satisfactory dose-response relationship between the gonadotrophin tested and the ovarian cholesterol level.

In view of the results shown earlier in this chapter of the thesis, it seems that a fairly satisfactory dose-response relationship with relatively steep regression between the falling ovarian cholesterol level and the amount of various LH preparations can be obtained. It is possible that Parlow (1961) was not able to find out this steep regression slope because of the fact that he was dealing with ovaries from animals which had their last pretreatment (HCG) injection between 5 and 8 days previous to the day of the assay. It has been shown that the ovaries are able to accumulate comparatively much smaller amounts of cholesterol at this period after the pretreatment. It has been pointed out previously that the regression curve for the cholesterol depletion assay is considerably flatter when animals are used earlier than the 11th day after they get their HCG pretreatment. It seems that this pretreatment condition is critical for obtaining the comparatively
steeper regression slope necessary for quantitative bio-assays. As will be further discussed later, the final details of the pretreatment procedures are most probably intimately associated with the mechanism of the whole procedure. In view of the apparently different conditions necessary for these two assays (OAAD and cholesterol depletion assays) it appears that the biochemical reactions involved in them are essentially dissimilar.

The main advantage of the OOD test as described herein is its very high degree of sensitivity, the technique being approximately 5 million and 150 million times more sensitive than the OAAD and hypophysectomised rat prostate tests respectively. The extreme sensitivity of the procedure makes it eminently suitable for application to clinical problems. Preliminary evidence indicates that it will no longer be necessary to concentrate the LH activity of body fluids prior to bioassay and that a satisfactory response will be elicited in the experimental animals following the administration of diluted urine or plasma. The high degree of sensitivity of the response may be explained on the basis of biological rebound phenomenon. It is possible that following utilization and excretion of the large amounts of injected PMSG and HCG the ovaries are left eventually with large numbers of corpora lutea but without any gonadotrophic hormone to stimulate this highly reactive luteal tissue.
As explained before, the secretion and/or release of endogenous pituitary gonadotrophin (if indeed any is present in these immature pituitaries) is also most probably completely inhibited by the large amounts of ovarian steroids produced by these animals in response to administered PMSG and HCG. It seems that an experimental condition is produced in which a large amount of highly reactive luteal tissue is present without any (exogenous or endogenous) gonadotrophin to stimulate it. It is probable that comparatively small amounts of an administered gonadotrophic material are able to stimulate the ovaries under this unique experimental condition. The ability on the part of the ovaries of the intact animals which were 36-37 days old on the day they were used for the cholesterol depletion assay, to respond to such a small dose of LH seems to be interesting. It is possible that the pretreatment procedure used before the assay might be involved in suppressing the production and/or release of gonadotrophic hormones from the pituitary by a feed-back mechanism. Pretreatment with very large doses of PMSG and HCG stimulates the production of large amounts of steroid hormones from the ovaries of these animals. It is possible that large amounts of these circulating ovarian steroid hormones completely inhibit the gonadotrophic function of the pituitaries of these immature rats.

The effects of oestrogen on the secretion of gonado-
trophins by the anterior pituitary are complex (Sawyer and Markee, 1959). This group of steroids appear to have a dual capability of stimulating and inhibiting gonadotrophin secretion. For example, oestrogens can inhibit ovarian development in the immature rat by suppressing gonadotrophin release (Leonard et al., 1952; More et al., 1952), whereas on the other hand, injection of oestrogen can induce ovulation in pregnant rats (Everett, 1947) and can advance ovulation in cyclic rats (Parlow, 1959), observations interpreted to mean that a release of LH has occurred. Ovariectomy on the other hand with removal of both ovarian steroids from circulation leads to elevated levels of LH in both plasma and urine, suggesting that removal of the inhibiting effects from ovarian steroids leads to increased rates of synthesis and secretion of LH (Parlow, 1959; Teleisnik and McCann, 1961). McCann and Teleisnik (1961) using the ovarian ascorbic acid assay for LH were able to find detectable quantities of LH in plasma of ovariectomised donor rats. The LH activity of ovariectomised rat plasma was decreased by a single subcutaneous injection of oestradiol benzoate. Horibe et al. (1961) pointed towards the possibility that a part of a steroid compound may be converted into some oestrogen-like substance in the body in amounts sufficient to inhibit gonadotrophin secretion from the pituitary but not enough to affect peripheral target organs.
The main disadvantage of the CCD method is its relatively low degree of precision. The index of precision ($h$) is generally above 0.35 and the procedure is therefore less precise than either the OAAD or hypophysectomised rat prostate tests. The final determination depends on the measurement of the total ovarian cholesterol but it has been shown that the precision of the method cannot be improved by measuring cholesterol in either its free or esterified forms. The main technical difficulty which has been encountered is the lack of uniformity of the ovaries in any given assay in relation both to appearance and to cholesterol content; this necessitates the administration of the standard preparation in all assays. It should be emphasised that the findings reported in the present study were obtained using rats of the Wistar strain bred in a closed colony. Further experience is required to determine whether the procedure will yield reproducible results in animals of various strains bred under different environmental conditions.

Specificity tests have shown that neither ACTH or prolactin affects the response. When TSH was administered a depletion of ovarian cholesterol occurred but it is probable that this was due to the contamination of the TSH preparation used with LH (Butt, Stockell-Bartree and Kirkham, 1963). Growth hormone given alone had no apparent effect but in two of the three assays in which combinations of
growth hormone and LH were administered the fiducial limits of error did not include the figure of 1.0 (see Table 4) suggesting that growth hormone had interfered with the response to LH. It should however be noted that the dosage of growth hormone used was more than 1,000 times greater than that of LH and was therefore grossly in excess of the amounts which might be expected to be present in human body fluids in normal and pathological conditions. FSH preparations appear to affect the OCG test only in relation to their contamination with LH and it is of interest to note that the LH content of NIH-FSH - 0.94% - found in the present study agrees well with the figures quoted by Parlow (1961) and by Schmidt-Imendorff and Loraine (1962) using the OAAD method, and by Trenkle, Li, Sadri and Robertson (1962) using an immunochemical technique.

It is widely believed that sudden release of LH from the pituitary gland is associated with the phenomenon of ovulation. The sharp increase in LH excretion at the mid-cycle (Table 15) supports this view. In the past few years there has been considerable differences of opinion regarding the possible existence of two biologically and chemically distinct separate gonadotrophic hormones in the urine of non-pregnant subjects. Segaloff et al. (1954) suggested that human urine contained a single homogeneous gonado-
trophic factor capable of exhibiting both follicle-stimu-
lating and luteinizing activities. This view is not
generally favoured (Loraine, 1958). Later on Steelman
et al. (1959) and later still Butt, Crooke and Cunningham
(1961) have shown however that it is possible to extract
relatively purified FSH or LH rich materials from human
urine.

A number of investigators including Smith and Smith
(1936), Klinefelter et al. (1943) and Pedersen-Bjergaard
and Tonnerson (1948) investigated the HPG excretion during
the normal menstrual cycle. Heller et al. (1944) and Main
et al. (1943) could not find any persistent single midcycle
peak of HPG excretion in normal cyclic women. Bahn et al.
(1953) stated that HPG excreted by normally menstruating
women at all stages of the cycle was predominantly FSH in
nature. More recently, McArthur (1952) and Loraine and
Brown (1956) using specific tests have demonstrated that LH
activity is present in menstruating female urine at all
stages of the cycle.

Although it is generally agreed now that the HPG in
normal menstruating women possesses both FSH and LH activity
the status of gonadotrophic hormones during the human inter-
menstruum has not been clearly defined so far. There seems
to be certain amount of confusion regarding the biological
nature of the HPG excreted during the midcycle (at the time
of ovulation). Bioassays carried out with methods specific for LH (McArthur et al., 1958, 1961) have shown that there is increased excretion of LH at the time of ovulation. On the basis of non-specific assay methods some of the earlier workers (D'Amour, 1943) claimed that the gonadotrophin peak was the most reliable index of occurrence of ovulation.

Loraine and Bell (1963) reported that midcycle peaks of HPG excretion as found out by the mouse uterus test were demonstrable only in a small percentage of normal ovulatory cycles. It was also reported at the same time that the midcycle peak of gonadotrophin when it occurred, either just post-dated or co-occurred but never preceded the midcycle (follicular) peak of oestrogen excretion. The midcycle peak of oestrogen is generally believed to occur at the time of ovulation.

McArthur et al. (1958) claimed to have shown a qualitative change in the HPG excretion to occur at the midcycle. Segaloff (1961) postulated the existence of a "separable" LH fraction, which was supposed to be only a small fraction of the "total urinary LH activity". However, according to this observer the "separable LH" fraction rather than the rest of it which he believed "to be inherent in the FSH molecule" was more important as a reflection of "circulating effective LH". Segaloff was of the opinion that any assessment of even large shifts in the "separable LH" fraction
had not been possible due to lack of a sufficiently sensitive method for the quantitative determination of LH activity. Purshottam et al. (1962) using immunological techniques reported the possible secretion during midcycle of a gonadotrophic principle immunologically identical to HCG.

The occurrence of increased gonadotrophin excretion following ovulation but not preceding the phenomenon would suggest amongst certain other possibilities either (a) that the pituitary secretion of gonadotrophin is stimulated by the secretion of the steroid hormones from the ovary occurring at the time of ovulation, or (b) that some other ovulation-dependent LH-like gonadotrophic material is secreted from tissues other than the pituitary gland. The presence of an unfertilized ovum in the progesterone prepared uterine cavity has not been so far reported to produce any gonadotrophic principle. In view of Purshottam's finding (Purshottam et al., 1962) that gonadotrophic material immunologically identical with HCG is obtainable from the midcycle specimen of non-pregnant urine, the elaboration of HCG-like material from the secretory endometrium (possibly evoked by the presence of an unfertilized ovum) is a speculative probability. The finding that large amounts of purified pituitary LH would cause depression rather than stimulation of mouse uterine response to urinary
gonadotrophin extracts (see Section III), along with the finding that very low as well as relatively large doses of HCG are capable of stimulating the mouse uterine response to FSH (see Section III) suggests that the gonadotrophic material capable of augmenting the mouse uterine response to midcycle urinary gonadotrophin extracts might have biological identity with HCG. The relatively frequent occurrence of a "gonadotrophin peak" at the time of menstruation may be related to the disintegration of the secretory endometrium. It may be noted that all the specific assay methods used so far for measuring the LH activity are unable to differentiate the LH activity associated with HCG from that associated with other gonadotrophic materials such as HMG, PMSG and purified pituitary gonadotropins.

Very little information is available regarding the excretion of gonadotrophins in young children (Nathanson et al., 1941; Catchpole et al., 1938). Due to the lack of satisfactory assay methods it has not been possible to measure the LH excretion especially in children. The results obtained as shown in Table 16 suggest that LH is excreted in detectable amounts in young children. It is noteworthy that in the child at three weeks of age there is very little but still detectable amounts of LH. It may also be seen from this Table that the urinary excretion of LH gradually increases with age.
Attempts have been made from time to time to measure the gonadotrophic activity of human blood (Loraine and Apostolakis, 1961; McArthur and Antoniades, 1961). Due to the relative insensitivity of the methods and consequently the large volume of blood necessary for even a single estimation, determination of blood gonadotrophin has rarely been used clinically. Using the cholesterol depletion assay method it has been possible to measure the LH content of unprocessed plasma samples from children.

**SUMMARY.**

A new assay method of luteinizing hormone (LH) activity is described and its reliability criteria assessed. The technique depends on ovarian cholesterol depletion in intact immature rats pretreated with pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG).

The main advantage of the ovarian cholesterol depletion (OCD) test is its high degree of sensitivity, the procedure being approximately 5 million times more sensitive than the ovarian ascorbic acid depletion (OAAD) method. The chief disadvantage of the technique is its relatively low degree of precision.

The LH activity of various gonadotrophin preparations has been determined using the OCD test. The results obtained agreed well with those found by the OAAD method.
The extreme sensitivity of the OCD test suggests that it will be eminently suitable for clinical application.
In 1958 Parlow introduced a new method for the quantitative determination of LH activity in anterior pituitary extracts. This method, which employed intact rather than hypophysectomised rats and in which the materials under test were injected intravenously under ether anaesthesia, had as its end point the depletion of reduced ascorbic acid in the ovaries of rats made pseudopregnant by the administration of relatively large quantities of pregnant mares serum gonadotrophin and human chorionic gonadotrophin. The test was claimed by its originator to be both specific and sensitive and it appeared possible that the technique might be suitable for the measurement of LH activity in extracts of human urine.

When experiments involving urinary extracts were conducted it became apparent that the method in the form described by Parlow was unsatisfactory for clinical application for two main reasons (Schmidt-Elmendorff and Loraine, 1962). In the first place toxic effects were frequently encountered in the experimental animals and secondly there was a high proportion of invalid assays due to significant deviation from parallelism between the dose-response curves of standard and unknown preparations. In view of these difficulties it was decided to investigate the possibility of modifying the assay method so as to make it suitable for clinical application. Three possible methods of approach
to this problem were tried.

Firstly it was decided to measure dehydro ascorbic acid and glutathione, along with reduced ascorbic acid. It was hoped that the dehydro-ascorbic acid (and glutathione with which the oxidation of ascorbic acid is closely related) rather than the reduced ascorbic acid would reflect any change in the ascorbic acid metabolism following LH administration. The significance of ascorbic acid in tissue metabolism is obscure at present. Due to its reversibly oxidisable and reversible nature ascorbic acid has been assumed to perform some fundamental role in tissue oxidation. It was thought possible that the ovarian ascorbic acid found to have been depleted following LH administration was involved in the ovarian cellular oxidative processes. In conformity with the events occurring during biological oxidation in other tissues it was assumed that a proportion of the ascorbic acid thus involved in cellular oxidation was likely to be oxidised. The method of ascorbic acid estimation used by Parlow (1958, 1961) and subsequently by Schmidt-Elmendorff and Loraine (1962) measures only the reduced form of ascorbic acid. For this reason it was not possible to determine either of these forms of ascorbic acid specifically. Dehydro-ascorbic acid, the reversibly oxidised, biologically active form of ascorbic acid, does not possess the reducing properties, hence must be reduced by some
reducing agents (e.g. hydrogen sulphide) before it can be
determined with oxidising agents such as 2,6 dichlorophenol-
indophenol.

Secondly, an attempt was made to incorporate two
pituitary gonadotrophin inhibiting agents, stilboestrol
and a dithiocarbamoylhydrazine derivative (compound 33,828,
supplied by the Imperial Chemical Industries Limited) in
the pretreatment procedure. It may be recalled that intact
immature rather than hypophysectomised animals are used for
the OAAD method. The animals are approximately 32 - 35 days
old on the day they are used for the assay. Although
practically nothing is known at present as to whether any
amount of endogenous circulating LH is present in these
animals at the time they are used for the assay, it was
thought possible that the presence of variable amounts of
this hormone was interfering with the precision and sensi-
tivity of the assay method. Hence it was hoped that by
incorporating potent inhibitors of pituitary gonadotrophic
function it would be possible to improve upon the existing
precision, reproducibility and sensitivity of Parlow's
ovarian ascorbic acid depletion assay.

The third method of approach with the view of improving
the existing OAAD assay was aimed at making it more prac-
ticable and suitable for clinical application. It has been
shown by Schmidt-Elmendorff and Loraine (1962) that apart
from its comparatively low degree of precision and repro-
ducibility as a specific method for quantitative estimation of LH activity the OAAD assay is quite satisfactory only for highly purified gonadotrophin preparations but not for relatively crude urinary extracts. It was found by these observers that relatively crude urinary gonadotrophin extracts when injected intravenously as advocated by Parlow (1961) very often produced toxic effects on the experimental animals. With a view to reducing this problem of toxicity it was decided to investigate the possibility of performing the OAAD assay using intraperitoneal rather than intravenous administration of the test material. It is possible that the high percentage of invalid assays obtained by Schmidt-Elmendorff and Loraine (1962) when using the OAAD assay for crude urinary extracts were partly due to toxicity. It was also expected that the comparatively simpler intraperitoneal administration if otherwise satisfactory would greatly increase the practicability of the OAAD procedure.
INTRODUCTION.

Since the original observation by Sayers and Long (1944) that adrenal ascorbic acid decreases in response to stress or ACTH stimulation, ovarian ascorbic acid in intact immature pseudopregnant rats has been shown to decrease following the administration of luteinizing hormone (Parlow, 1958). Slusher and Roberts (1957, 1960) appear to have resolved the controversy as to the fate of the ascorbic acid lost from the adrenal by showing that ascorbic acid lost from this gland can be recovered in adrenal vein effluent. Similar information about the ovarian ascorbic acid is not yet available, and the fate and physiological role of this substance in ovarian and adrenal metabolism has not been established so far. Because of the high concentration of adrenal and ovarian ascorbic acid in the resting gland and the time relationship of its depletion to cholesterol depletion and steroid secretion, it has been suggested that ascorbic acid may play a primary role in steroid biosynthesis (Inoue, 1951; Meikeljohn, 1953). Several investigators have presented evidence which in fact supports quite opposite hypotheses regarding this role (Hayano et al., 1956; Kersten et al., 1958).

It is known that the ovaries contain significant levels
of glutathione and further, that this substance can be oxidised chemically by dehydro-ascorbic acid (Szent-Gyorgyi, 1928, 1931). A parallel relationship between glutathione and ascorbic acid has been reported in adrenal tissue (Goldzieher et al., 1953; Lazarow, 1954). In view of the importance of glutathione to the regulation of protein, disulphide-sulphhydryl interchange reactions (Hopkins, 1925) and the above indicated relationship of glutathione to ascorbic acid, the possibility that changes in ascorbic acid concentration might influence the disulphide-sulphhydryl ratios of the ovarian tissue proteins seemed possible. The purpose of this investigation was to determine the state of oxidation of ovarian ascorbic acid and glutathione, and to use if found suitable the oxidised (dehydro) ascorbic acid and glutathione instead of total or reduced ascorbic acid as a possible parameter for measuring the luteinizing hormone activity in intact immature rats made pseudopregnant by pretreatment with PMSG and HCG. In this way it was hoped that by measuring dehydro-ascorbic acid and glutathione which were more likely to reflect any change in the ascorbic acid metabolism, the precision of the existing OAAD assay will be improved.

MATERIALS AND METHODS.

Estimation of Reduced Ascorbic Acid.

Ovarian ascorbic acid was estimated according to the
method described by Mindlin and Butler (1958).

The weighed ovaries were ground in a mortar and pestle with 10.0 ml. of 2.5 per cent metaphosphoric acid solution. The extract was then filtered. 2.0 ml. of the clear metaphosphoric acid extract was mixed with 2 ml. of standardised 2:6 dichlorophenolindophenol solution in a test tube and read immediately in a spectrophotometer. The dichlorophenolindophenol solution was prepared in the following way. 12.0 mgs. of the dye were dissolved in 40.0 ml. of distilled water which was then mixed with 280.0 ml. of sodium acetate solution (18.16 gms. of sodium acetate in 400 ml. of distilled water - pH of the solution adjusted to pH 7.0 using glacial acetic acid and N/10 sodium hydroxide solution). The dye solution was prepared fresh on the day it was used and the dye concentration adjusted by diluting it with the sodium acetate solution.

The amount of reduced ascorbic acid was determined from the calibration curve prepared with known amounts of pure ascorbic acid solution.

Estimation of Dehydro-Ascorbic Acid.

Dehydro-ascorbic acid, the reversibly oxidised, biologically active form of ascorbic acid, does not possess reducing properties, hence must be reduced before it can be determined with oxidising agents such as 2:6 dichlorophenolindophenol. Hydrogen sulphide has been used most
frequently for this purpose but this reagent has been criticised on the ground that it produces other reducing materials which interfere with the 2,6 dichlorophenolindophenol reaction. This objection is valid when simple titrimetric methods are employed for the determination of ascorbic acid. However, little interference arises from this source when very rapid photoelectric methods are employed (Hawk et al., 1954). Accordingly the photometric method described by Hochberg et al. (1943) was used for the determination of dehydro-ascorbic acid. To 4.0 ml. aliquot of the metaphosphoric acid extract filtrate taken in stoppered test tubes from the samples used for the reduced ascorbic acid estimation a stream of hydrogen sulphide was passed for ten minutes. The tubes were stoppered and allowed to stand overnight in a refrigerator; then the hydrogen sulphide was removed by bubbling nitrogen through the mixture. Each of the tubes was then tested with lead acetate paper to test for any residual hydrogen sulphide. 2.0 ml. aliquots from each tube were then mixed with 2.0 ml. of the standard dichlorophenolindophenol solution. The tubes were read immediately in a spectrophotometer. The difference between the values obtained with and without hydrogen sulphide treatment was regarded as the measure of dehydro-ascorbic acid.
Estimation of Glutathione (GSH).

Glutathione in ovarian tissue was estimated according to the method described by Woodward and Fry (1952).

The weighed tissue was thoroughly ground in a mortar and pestle with 8 ml. of distilled water. 1.0 ml. of molar (22.0 per cent) solution of sulphosalicylic acid was then added and the extract was filtered. 5.0 ml. aliquots of this filtrate were titrated as follows: after the addition of 1.25 ml. of 4% sulphosalicylic acid, 1.25 ml. of 5% potassium iodide containing no trace of free iodine, and one drop of 1% starch solution (1% solution of soluble starch in saturated sodium chloride), 0.001 N potassium iodate solution made up in 2 per cent sulphosalicylic acid was run in with care from a microburette until the first blue colour persisted. During the titration the tube was placed in a beaker of water adjusted to a temperature of 19-20°C. Potassium iodate solution was made in the following manner: 0.1783 gm. of potassium iodate was weighed accurately and was then made up to 1.0 litre with distilled water in a volumetric flask. This gave an 0.005 N solution and could be kept indefinitely. The 0.001 N solution used in the titration was made by combining 50 ml. of this and 22.8 ml. of the molar sulphosalicylic acid and diluting to 250.0 ml. in a volumetric flask. A fresh solution was made from the stock solution at least once a week. The factor was also
determined by comparing the thiosulphate titration of 10 ml.
of the 0.001 N iodate with that of 2.0 ml. of the 0.005 N iodate.

OAAD Method.

These were conducted in the same way as described by
Farlow (1961) and by Schmidt-Elmendorff and Loraine (1962, 1962a.). The method has been described in detail in the
next chapter of this thesis.

RESULTS.

These are shown in Tables 18 and 19. It may be seen
from either of these Tables that there is considerable
depletion of reduced ovarian ascorbic acid in response to
both the dose levels of LH stimulation. It may also be
seen that the ovarian dehydro-ascorbic acid and ovarian
reduced glutathione values following LH administration are
not significantly different from the control.

DISCUSSION.

The result of this investigation indicates that the
depletion of ovarian ascorbic acid in pseudopregnant rats
in response to the administration of NIH-LH is not
associated with any simultaneous alteration of the ovarian
dehydro-ascorbic acid and glutathione. Hence it seems that
under the experimental conditions originally described by
Farlow (1961) the possibility of using ovarian dehydro-
ascorbic acid or ovarian glutathione rather than reduced
ascorbic acid as an index of LH activity, is remote. On
TABLE 18

REDUCED ASCORBIC ACID, DEHYDRO-ASCORBIC ACID AND REDUCED GLUTATHIONE IN PSEUDOPREGNANT RAT OVARIES FOLLOWING ADMINISTRATION OF 0.3 AND 1.5 μg NIH-LH

<table>
<thead>
<tr>
<th>Dosage of NIH-LH administered</th>
<th>Reduced ascorbic acid μg/100mg</th>
<th>Dehydro-ascorbic acid μg/100mg</th>
<th>Reduced Glutathione μg/100mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil (Control)</td>
<td>118.0</td>
<td>10.2</td>
<td>416.8</td>
</tr>
<tr>
<td></td>
<td>100.7</td>
<td>11.7</td>
<td>396.2</td>
</tr>
<tr>
<td></td>
<td>120.2</td>
<td>13.6</td>
<td>430.7</td>
</tr>
<tr>
<td>Mean</td>
<td>108.9</td>
<td>12.6</td>
<td>356.0</td>
</tr>
<tr>
<td>0.3 μg</td>
<td>118.8</td>
<td>10.2</td>
<td>416.8</td>
</tr>
<tr>
<td></td>
<td>100.7</td>
<td>11.7</td>
<td>396.2</td>
</tr>
<tr>
<td></td>
<td>120.2</td>
<td>13.6</td>
<td>430.7</td>
</tr>
<tr>
<td>Mean</td>
<td>108.9</td>
<td>12.6</td>
<td>356.0</td>
</tr>
<tr>
<td>1.5 μg</td>
<td>118.8</td>
<td>10.2</td>
<td>416.8</td>
</tr>
<tr>
<td></td>
<td>100.7</td>
<td>11.7</td>
<td>396.2</td>
</tr>
<tr>
<td></td>
<td>120.2</td>
<td>13.6</td>
<td>430.7</td>
</tr>
<tr>
<td>Mean</td>
<td>108.9</td>
<td>12.6</td>
<td>356.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.3 μg NIH-LH</td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Control</td>
<td>0.3 μg NIH-LH</td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td></td>
<td>Sig.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>0.3 μg NIH-LH</td>
<td></td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td>1.5 μg NIH-LH</td>
<td></td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sig.</td>
</tr>
<tr>
<td>Dosage of NIH-LH administered</td>
<td>Reduced ascorbic acid µg/100mg</td>
<td>Dehydro-ascorbic acid µg/100mg</td>
<td>Glutathione µg/100mg</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>----------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Nil (Control)</td>
<td>92.6</td>
<td>8.9</td>
<td>269.3</td>
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<td></td>
<td>118.4</td>
<td>18.1</td>
<td>362.4</td>
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<td></td>
<td>123.5</td>
<td>8.7</td>
<td>222.3</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>7.1</td>
<td>415.5</td>
</tr>
<tr>
<td></td>
<td>105.1</td>
<td>20.1</td>
<td>401.3</td>
</tr>
<tr>
<td>Mean</td>
<td>107.9</td>
<td>12.5</td>
<td>334.2</td>
</tr>
<tr>
<td>0.3 µg</td>
<td>90.4</td>
<td>7.8</td>
<td>314.0</td>
</tr>
<tr>
<td></td>
<td>87.8</td>
<td>12.3</td>
<td>392.0</td>
</tr>
<tr>
<td></td>
<td>81.5</td>
<td>17.5</td>
<td>462.6</td>
</tr>
<tr>
<td></td>
<td>77.1</td>
<td>15.3</td>
<td>196.5</td>
</tr>
<tr>
<td></td>
<td>78.6</td>
<td>9.8</td>
<td>458.9</td>
</tr>
<tr>
<td>Mean</td>
<td>83.0</td>
<td>12.5</td>
<td>344.8</td>
</tr>
<tr>
<td>1.5 µg</td>
<td>70.5</td>
<td>7.5</td>
<td>514.1</td>
</tr>
<tr>
<td></td>
<td>61.3</td>
<td>18.8</td>
<td>363.8</td>
</tr>
<tr>
<td></td>
<td>52.4</td>
<td>16.2</td>
<td>321.5</td>
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<tr>
<td></td>
<td>51.9</td>
<td>10.1</td>
<td>366.4</td>
</tr>
<tr>
<td></td>
<td>56.0</td>
<td>22.3</td>
<td>286.6</td>
</tr>
<tr>
<td>Mean</td>
<td>58.4</td>
<td>14.9</td>
<td>368.4</td>
</tr>
<tr>
<td>Sig.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

TABLE 19
REDUCED ASCORBIC ACID, DEHYDRO-ASCORBIC ACID AND GLUTATHIONE CONTENT IN PSEUDOPREGNANT RAT OVARIIES FOLLOWING THE ADMINISTRATION OF 0.3 AND 1.5 µg NIH-LH
the basis of these experiments it is not possible to predict the behaviour of dehydro-ascorbic acid or glutathione in response to higher or lower dose levels of LH than used in the experiment (0.3 and 1.5 μg of NIH-LH).

It seems therefore that the mechanism leading to the depletion of ovarian ascorbic acid in pseudopregnant rats is not due to the oxidation of ascorbic acid and consequent oxidation of glutathione by the dehydro-ascorbic acid. It is possible that the observed depletion of reduced ascorbic acid is due to the escape of this substance from the ovary rather than due to being involved in metabolic processes in the organ. It has been shown previously that the ascorbic acid lost from the adrenals in response to ACTH stimulation can be quantitatively recovered from the adrenal venous effluent (Lahiri and Lloyd, 1962). Lahiri and Lloyd (1962) and later on Harding and Nelson (1963) have shown that the depletion of adrenal reduced ascorbic acid in response to ACTH stimulation is not associated with any simultaneous alteration in the adrenal content of dehydro-ascorbic acid and glutathione.

The mechanism of ovarian ascorbic acid depletion in response to LH stimulation is obscure. It is generally postulated (without much experimental evidence) that the depletion of ascorbic acid is somehow related to the mechanism of synthesis or liberation of steroid hormones.
of the ovaries from their precursors. It has been shown in the previous section of this thesis that depletion of ovarian cholesterol occurs from the pseudopregnant rat ovaries in response to very small (0.3 picograms of NIH-LH) dose of LH stimulation. This dose level of NIH-LH necessary to deplete ovarian cholesterol is much smaller than that necessary to deplete the ovarian ascorbic acid under identical experimental conditions. At present it is not possible to predict the fate of the esterified cholesterol found to have been lost from the ovaries in response to very small dose of LH stimulation. Here again it is only likely that the lost esterified cholesterol is utilised for the synthesis of ovarian steroid hormones or some of their precursors.

Apart from cholesterol depletion and ascorbic acid depletion, three other well recognised ovarian phenomena associated with the administration of different dose levels of purified pituitary LH (NIH-LH) have been reported. These are the phenomena of (a) ovulation corpus luteum formation and subsequent deposition of esterified cholesterol in the ovaries described in Chapter I, Section I of this thesis, (b) increased uptake of glucose and oxygen (Armstrong, 1961; Armstrong et al., 1963), and (c) increased rate of blood circulation (Ellis, 1961, 1961a; Parlow and Reichert, 1963). Curiously enough, using all these apparently unassociated phenomena as the parameters, the potency of LH
activity obtained from various gonadotrophic preparations has been found to be very much the same. This observation strongly suggests that the biological activity measured by these apparently unrelated parameters is essentially the same. This also suggests that the biological reactions involved represent a chain of natural events rather than a series of unrelated artefacts as claimed by certain observers (Nalbanyov, 1961). Not only this, in spite of the enormous variability of sensitivity of these different methods, quantitatively each of these very different responses bears a constant relationship to the other. In other words, for example, the amount of cholesterol depleted in response to 0.3 picograms of NIH-LH bears a constant relationship to the amount of ascorbic acid depleted in response to 0.3 µg of NIH-LH. Again the amount of ascorbic acid liberated in response to 0.3 µg of NIH-LH bears a constant relationship to the amounts of increase in the oxygen uptake and glucose utilization in response to the same quantity of the hormone. Again both the amount of esterified cholesterol depleted in response to 0.3 picograms of NIH-LH and the amount of ascorbic acid which can be depleted in response to 0.3 µg of NIH-LH, bears a constant relationship to the amount of increase in ovarian blood circulation brought about by 1.0 µg of NIH-LH. From the point of view of reliability and specificity of bioassays this is rather
convincing. Because, it seems, that in spite of the apparently unrelated and varying nature of these biological reactions used as end points, essentially one and the same material, i.e. LH, is being measured each time.

Perhaps another direct corollary of this interesting observation, viz. the apparently qualitative difference in response brought about by quantitative difference of the stimulus, is that the qualitative difference is more apparent than real. On the basis of what is known about the fundamental relationship between a biological response to a biological stimulus, it seems that all these apparently unrelated biological responses used as end-points of different bioassays for LH, are, in fact, only the different integral parts of a single chain reaction. Further investigations of these individual biological reactions on quantitative bases followed by eventual establishment of their mutual relationship are likely to elucidate the fundamental mechanism of action of luteinizing hormone.

**SUMMARY.**

The results of investigations in relation to the possibility of measuring dehydro-ascorbic acid and glutathione (instead of reduced ascorbic acid) as an index of LH activity, have been described. It has been shown that under the existing experimental conditions there is no alteration of ovarian dehydro-ascorbic acid and ovarian glutathione in response to LH stimulation.
CHAPTER II

MODIFICATION OF PEARLOW'S OVARIAN ASCORBIC ACID DEPLETION ASSAY BY ADDITIONAL PRETREATMENT WITH STEROIDAL AND NON-STEROIDAL PITUITARY INHIBITORS.

INTRODUCTION.

Apart from its comparatively high degree of sensitivity and specificity, one of the advantages of Pearlow's ovarian ascorbic acid depletion assay is that in this, intact immature rather than hypophysectomised rats are used. The high degree of sensitivity of the assay points towards the possibility that very little if any luteinizing hormone is secreted from the pituitary of the assay animals (endogenous LH) by the time they are used for the purpose. However it is possible that the pituitaries in these animals do elaborate some amount of LH, particularly in view of the fact that the animals are approximately 30 - 35 days old at the time they are used for the assay. In view of the remarkable sensitivity of the OAAD response it is possible that the wide range of the ascorbic acid content frequently encountered in a group of identically pretreated animals is due to the interference by endogenous LH. So it was thought that additional pretreatment with a gonadotrophin inhibiting agent, administered at the proper time, might improve the sensitivity and precision of the assay by lowering the range of the ascorbic acid content of the ovaries in a
particular group of animals identically pretreated with PMSG and HCG. It was also postulated that a more uniform ascorbic acid content with resultant smaller standard deviation might be helpful for better statistical treatment of the results of the assays. This might be of help in decreasing the number of invalid assays frequently encountered, resulting from significant deviation from parallelism between the dose-response curves of the standard and the unknown preparations.

For this purpose two groups of pituitary inhibiting agents were used. These materials were administered to the animals two days before they were used for assay. One was stilboestrol the synthetic steroid compound, and the second a dithiocarbamoylhydrazine compound (Compound 33,828 I.C.I.). Stilboestrol has previously been reported to be a potent pituitary inhibitor. The dithiocarbamoylhydrazine derivative investigated (Compound 33,828, Imperial Chemical Industries Ltd., Pharmaceuticals Division) was

\[
\text{CH}_2 = \text{CH} \cdot \text{CH}_{\text{3}} \cdot \text{CH}_{\text{2}} \cdot \text{CH} \cdot \text{NH} \cdot \text{CS} \cdot \text{NH} \cdot \text{CS} \cdot \text{NH} \cdot \text{CH}_{\text{2}}
\]

The compound differs from other pituitary inhibitors previously described in not being a steroid. Previous investigations by Paget et al. (1961) and by Bell et al. (1962) have shown that this compound inhibits pituitary gonadotrophic activity in various animal species and in
postmenopausal women.

MATERIALS, METHODS AND RESULTS.

Details of the OAAD assay method.

On the morning of the day of the assay the animals had the test dose (NIH-LH in this case) of the material intravenously through the femoral vein in 0.5 ml. of normal saline. The procedure was conducted under light ether anaesthesia. Four hours after the injection of the test dose of the material the animals were killed by cervical dislocation. The ovaries were taken out, cleaned and weighed on a torsion balance to the nearest 0.10 mg. Ovarian ascorbic acid was determined according to the method described by Mindlin and Butler (1938). The method of ascorbic acid determination has been discussed in detail in the next chapter.

OAAD assays were performed according to the technique described by Parlow (1961) and Schmidt-Elmendorff and Loraine (1962). A group of intact weanling female rats, weighing 30 - 50 gms. were pretreated with 50.0 i.u. of PMSG ('Gestyl', Organon) followed 72 hours later by 25.0 i.u. HCG ('Fregynl', Organon). The pretreated animals were used on the 7th day after they had their HCG pretreatment injection.
The different experimental groups of pretreatment studied in the present investigation.

As explained this study consisted of comparing the regressing curves obtained by conducting simultaneous OAAD assays with intact immature female rats pretreated with 50 i.u. PMSG followed 72 hours later by 25 i.u. HCG as described by Parlow. The difference in the three different experimental groups consisted of incorporating further additional pretreatment steps given to these identically PMSG and HCG pretreated animals, given shortly before they were used for the assay. The three experimental groups are shown in Table 20.

Experiment 1.

A group of 40 animals were pretreated as usual with PMSG and HCG. Approximately 48 hours before they were due to be used for the OAAD assay 20 of these animals received additional pretreatment in the form of 1.0 mg. "Compound 33,823" per animal, the remaining 20 animals acting as controls. On the morning of the 7th day, counting from the day when all the animals had their HCG pretreatment injection, five animals from either group had 0.3, 0.75 and 1.5 μg of NIH-LH. The remaining five animals in either group acted as controls. Four hours later all the animals were sacrificed and the ovarian cholesterol was determined as described before. Results are shown in Figure 28 and Table 21.
## Table 20

### Pretreatment of the Three Different Groups of Animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Additional Pretreatment</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50 i.u. PMSG followed by 25 i.u. HCG.</td>
<td>nil</td>
<td>On the 7th day after the HCG pretreatment</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>+ Compound 33,828 given in two subcutaneous injections of 0.5 ml. each, 48 and 24 hours before the assay.</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>+ Stilboestrol 0.1mg in oil, given by two subcutaneous injections, 48 and 24 hours before the assay.</td>
<td></td>
<td>&quot;</td>
</tr>
</tbody>
</table>
TABLE 21

EFFECT OF 1-α-allylthiocarbamoyl-2-methylthiocarbamoylhydrazine (Compound 33,828, Imperial Chemical Industries Ltd., Pharmaceuticals Division) ON THE REGRESSION CURVE OF OVARIAN ASCORBIC ACID DEPLETION ASSAY OF PARLOW, FOLLOWING NIH-LH ADMINISTRATION

<table>
<thead>
<tr>
<th>Dose levels of NIH-LH</th>
<th>Pretreatment</th>
<th>Number of Observations</th>
<th>Mean Ovarian Ascorbic Acid Content μg per 100 mg ovary</th>
<th>Standard Deviation Ascorbic Acid/100 mg ovary</th>
<th>Mean Ovarian Weight ± S.D. mgs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30 μg</td>
<td>PMSG + HCG</td>
<td>10</td>
<td>86.4</td>
<td>± 11.57</td>
<td>95.2 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>10</td>
<td>87.9</td>
<td>± 9.29</td>
<td>72.7 ± 14.6</td>
</tr>
<tr>
<td>0.75 μg</td>
<td>PMSG + HCG</td>
<td>8</td>
<td>89.7</td>
<td>± 15.65</td>
<td>81.8 ± 14.0</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>10</td>
<td>74.7</td>
<td>± 5.25</td>
<td>81.3 ± 18.2</td>
</tr>
<tr>
<td>1.5 μg</td>
<td>PMSG + HCG</td>
<td>8</td>
<td>83.4</td>
<td>± 13.47</td>
<td>95.1 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>10</td>
<td>69.4</td>
<td>± 6.43</td>
<td>74.3 ± 4.9</td>
</tr>
<tr>
<td>nil (Control)</td>
<td>PMSG + HCG</td>
<td>10</td>
<td>92.1</td>
<td>± 15.72</td>
<td>93.7 ± 18.6</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>10</td>
<td>98.9</td>
<td>± 7.72</td>
<td>79.6 ± 16.1</td>
</tr>
</tbody>
</table>
Result of Experiment 1.

It may be seen from Table 21 and Figure 28 that in the group pretreated with PMSG and HCG alone there was no significant depletion of ovarian ascorbic acid following the administration of three dose levels of NIH-LH, i.e. the slope of the regression curve was flat. On the other hand the ovarian ascorbic acid in all the groups pretreated with PMSG, HCG and the Compound 33,828 show progressively increasing depletion of ascorbic acid following the administration of progressively higher dose levels of NIH-LH. The slope of the regression curve is considerably steeper when compared to the previous group which had only PMSG and HCG pretreatment. It may also be noted that in this particular experiment comparatively high figures for the standard deviation of the mean ascorbic acid content per 100 mgs. of ovarian tissue were mostly restricted to the group which had only PMSG and HCG but no other additional pretreatment.

Experiment 2.

In this experiment a group of 35 animals were used. All the animals had the PMSG and HCG pretreatment at the same time. Approximately 48 hours before they were due to be used for the OAAD assay one group of ten animals had additional pretreatment in the form of 1.0 mg. of Compound 33,828, a second group of ten animals had additional pre-
treatment with 0.1 mg. stilboestrol, the remaining twenty animals acting as the control group. On the 7th day counting from the day they had their HCG pretreatment injection, five animals from each of the three groups had 0.3 mg NIH-LH, another five from each group had 1.5 mg NIH-LH. The remaining five animals from the control group were left as controls for the assay. All the animals were sacrificed as usual, four hours after they had the intravenous injection of NIH-LH. The results are shown in Figure 29, and Table 22.

Result of Experiment 2.

From Figure 29 and Table 22 it may be observed that the control ascorbic acid value was quite high. At the lower dose levels of NIH-LH there was considerable depletion of ascorbic acid, compared to the control in all the three groups. At higher dose levels of the hormone there was further marked depletion of ovarian ascorbic acid producing a fairly steep slope of the regression curve in all three groups. When statistically analysed the slope of the dose-response curves in the control group and the group which had stilboestrol were parallel. The relative potency of NIH-LH was calculated as 1.4 with the index of precision (λ) 0.18. However the slopes of the dose-response curve obtained from the group of animals which had the additional "Compound 33,329" pretreatment when compared to the standard (obtained
FIGURE 29
TABLE 22

EFFECT OF ADDITIONAL PRETREATMENT WITH 1-\(\alpha\)-ALLYLTHIOCARBAMOYL-2-METHYLMETHIOCARBAMOYLEHYDAZINE (COMPOUND 33,828, I.C.I.) AND STILBOESTROL ON THE NIH-LH DOSE-RESPONSE REGRESSION CURVE OF OVARIAN ASCORBIC ACID DEPLETION ASSAY OF PARLOW

<table>
<thead>
<tr>
<th>Dose levels of NIH-LH</th>
<th>Pretreatment</th>
<th>Number of Observations</th>
<th>Mean Ovarian Ascorbic Acid Content ± S.D. µg/100 mgs ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 µg</td>
<td>PMSG + HCG</td>
<td>10</td>
<td>93.4 ± 9.03</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>8</td>
<td>81.4 ± 3.08</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + Stilboestrol</td>
<td>10</td>
<td>82.3 ± 7.34</td>
</tr>
<tr>
<td>1.5 µg</td>
<td>PMSG + HCG</td>
<td>10</td>
<td>60.6 ± 6.01</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + &quot;33,828&quot;</td>
<td>10</td>
<td>56.8 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>PMSG + HCG + Stilboestrol</td>
<td>10</td>
<td>57.4 ± 8.84</td>
</tr>
<tr>
<td>nil</td>
<td>(Control)</td>
<td>8</td>
<td>121.5 ± 6.66</td>
</tr>
</tbody>
</table>
from the animals which had only FMSG and HCG but no "Compound 33,828" pretreatment) were found to be non-parallel. In this particular experiment the very low figures for mean standard deviation for ovarian ascorbic acid content were confined to the group of animals which had additional "Compound 33,828" pretreatment. The effect produced by stilboestrol appeared to be similar to those of "Compound 33,828".

DISCUSSION.

The results of the experiments have indicated that additional pretreatment of the experimental animals with 1.0 mg. of the "I.C.I. Compound 33,828" might be helpful in improving the OAAD assay method by producing a steeper slope of the regression curve. Schmidt-Elmendorff and Loraine (1962) pointed out that frequent occurrence of non-parallelism between the dose-response curves of the standard and the unknown preparations is one of the drawbacks of Parlow's OAAD method. Recently reports have become available regarding the inconsistencies of the OAAD method, encountered by several workers (Salhanick, 1961; Segaloff, 1961; Sakiz and Guillemin, 1963). Sakiz and Guillemin (1963) have suggested a number of possible improvements on the method including alterations of Parlow's original pretreatment schedule. Expressing the variations
in ascorbic acid contents by comparing first and second ovary in the same animal before and after injection of the test dose of the hormone has been suggested (Parlow, 1958; Courrier et al., 1961; McCann and Taleisnik, 1961; McCann, 1962). It seems that the labour involved in the proper mathematical analysis of the "2-ovaries method" makes it hardly practicable for routine use. Expressing the responses as percentage depletion as originally suggested by Parlow (1958) and currently reported by McCann (1962) (McCann, et al., 1961), probably introduces further complication for the correct mathematical analysis of possible statistically significant differences in the mean responses from one group to another. Sakiz and Guillemin (1962) suggested adjusting the ascorbic acid contents to the ovarian weight by covariance.

Fortunately for the sake of using the OAAD assay method to good purpose, and unfortunately for the fact that it is not readily available for investigation, insufficient regression is not a common occurrence in our laboratory in Edinburgh. Although it has been observed before that the slope of the regression curve is usually flatter when using hMG compared to highly purified LH preparations such as NIH-LH, it is usually possible to obtain mutually "statistically" parallel dose-response curves between hMG and NIH-LH. It is possible that the existing methods of
Statistical analysis for assessment of parallelism are not entirely satisfactory and certain differences of biological specificity cannot always be detected by the existing statistical treatment of results currently used. However, in view of the difficulty in establishing the OAAD method in certain laboratories (Salhanick, 1961; Segaloff, 1961; Sakiz and Guillemin, 1963) due to the flatness of the dose-response curve, it may be advisable to incorporate the additional pretreatment step with "Compound 33,328" in order to obtain more reliable results. It is possible that the alleged strain difference as the cause of the unsatisfactory results is in reality due to the comparatively faster rate of pituitary maturation (and consequent endogenous production of LH) in certain strains of these unsatisfactory animals. It seems likely that some additional pretreatment with specific gonadotrophin inhibitors may be helpful in these cases.

It is generally felt that for the purpose of routine application bioassay results should be satisfactorily reproducible without having recourse to intricate statistical analysis (Sakiz and Guillemin, 1963). Hence it is probably more desirable to alter the pretreatment condition of the animals so as to obtain a more satisfactory reproducible response, rather than to have recourse to intricate statistical methods for analysing basically unsatisfactory experimental data.
As has been pointed out before, the interference by endogenous LH and by the LH from the subcutaneous pretreatment injections still present in the animal about the time of the assay is a possibility. Hypophysectomising the animals before they are used for the assay to obviate the possible interference by the endogenous hormones has been found to be unsuitable (Guillemin and Sakiz, 1963). These observers pointed out that for satisfactory results the presence of the intact pituitary or additional pretreatment with prolactin prior to hypophysectomy is essential for the OAAD assay. This finding is important in connection with the relatively popular concept of using hypophysectomised rather than intact animals for assaying a particular hormone from the pituitary. It is quite possible that a number of biological responses evoked by the pituitary LH need simultaneous interaction from other pituitary hormones. It seems that for designing future bioassays for specific determination of a particular pituitary principle, intact animals treated with specific inhibitors rather than hypophysectomised animals should be used more widely. Hypophysectomised animals seem to be a poor substitute for an animal without any LH in its body.

**SUMMARY.**

The results of investigation of OAAD assays carried out with animals having additional pretreatment with pituitary gonadotrophin inhibiting agents, have been compared with
the results obtained from assays performed with animals having conventional pretreatment alone (PMSG and HCG). The purpose of using pituitary gonadotrophin inhibitors was to prevent the interfering effects of endogenous LH on the ovarian ascorbic acid content. The results obtained thereby suggest that interference by endogenous LH may be a possible factor for the unsatisfactory reproducibility of the OAAD assay. It has also been shown that the additional pretreatment of the experimental animals with pituitary gonadotrophin inhibitors may be helpful in overcoming this interfering effect of endogenous LH.
CHAPTER III

A COMPARISON OF INTRAPERITONEAL AND INTRAVENOUS INJECTION IN PARLOW'S ASSAY FOR LH.

INTRODUCTION.

In 1958 Parlow introduced a new method for the quantitative determination of LH activity in anterior pituitary extracts. This method, which employed intact rather than hypophysectomised rats and in which the materials under test were injected intravenously under ether anaesthesia; had as its end point the depletion of ascorbic acid in the ovaries of rats made pseudopregnant by the administration of relatively large quantities of pregnant mares serum gonadotrophin and human chorionic gonadotrophin. The test was claimed by its originator to be both specific and sensitive and it appeared possible that the technique might be suitable for the measurement of LH activity in extracts of human urine.

When experiments involving urinary extracts were conducted in our laboratory it rapidly became apparent that the method in the form described by Parlow was unsatisfactory for clinical application for two main reasons. In the first place toxic effects were frequently encountered in the experimental animals when the urinary extracts were administered by the intravenous route and secondly there was a high proportion of invalid assays due to significant deviation from parallelism between the dose-response curves of standard and unknown preparations. In view of these diffi-
culties it was decided to investigate the intraperitoneal route of administration and to compare it with the intravenous route. In this way, it was hoped not only to reduce the toxicity of administered materials but also to increase the practicability of the assay method by eliminating the necessity for an anesthetic. The main aim of this chapter is to report a comparison of the intravenous and intraperitoneal routes of injection when a Luteinizing Hormone preparation supplied by the National Institutes of Health in Washington and subsequently designated NIH-LH is administered to the experimental animals.

MATERIALS AND METHODS.

The ovarian ascorbic acid depletion tests were performed in the same way as described by Schmidt-Elmendorff and Loraine (1962). The method has been fully described in Chapter II of this section of the thesis.

The two groups of assays compared in this chapter differed in that in one group the test material was administered intravenously, as originally suggested by Parlow (1961) while in the other group the material was injected intraperitoneally.

The technique of intravenous injection.

The animal is anaesthetised by putting it in a jar containing ether. The margin of safety between the effec-
tive anaesthetic dose (to keep the animal immobile during
the operation) and the lethal dose of the ether has been
found to be very low for the young rats. After anaes-
thesising the animal it is put on its back and the femoral
vein is exposed at the upper part of the thigh. During
the dissection there is sometimes bleeding from the small
vessels lying superficial to the femoral vein. Intravenous
injection is made with a No. 26G ½" needle and the vein
kept pressed by a cottonwool ball before withdrawing the
needle. Even if sufficient care is taken sometimes bleeding
occurs from the punctured vein after some time. The skin
incision is closed by Mitchell's clips.

**Technique of intraperitoneal injection.**

These were performed through the left lower quadrant
of the ventral abdominal wall of the animal, using a very
short bevelled No. 25 1" long needle. It was never found
necessary to anaesthetise the animals for the purpose of
the intraperitoneal injection. In fact it was felt rather
difficult to perform intraperitoneal injection in anaes-
thesised animals because of the very lax abdominal wall.
Precautions were taken to prevent regurgitation of the
injected fluid from the peritoneal cavity by loosening the
grip on the animal prior to withdrawing the needle. It is
usually quite easy to carry out intraperitoneal injections
and one average laboratory technician can usually inject
approximately 60 animals in half an hour, after a few days practice. The entry of the needle can be felt by sudden loss of resistance. Before pushing in the needle it is necessary to hold the animal firmly so as to make its abdominal wall tense. The eddies produced by the dropping fluid inside the peritoneal cavity can usually be felt as a thrill.

Using this technique along with the necessary care to avoid injury to the abdominal viscera, the intraperitoneal injection is remarkably safe and easy to perform. Very occasionally the gut wall has been injured during intraperitoneal administration with resulting injection of the fluid into the lumen of the bowels. This accident is detected immediately by noticing the escape of fluid from the rectum.

As a comparative study it has been observed that three people (one acting as anaesthetist and helping hand for the operator, one operator capable of performing very rapid and efficient intravenous injection, and one assistant for clipping up the wound) would normally take 75 - 90 minutes to inject 60 animals through the femoral or iliac vein. During the operation one should expect to lose roughly about 10% of the animals due to anaesthetic accident or due to bad injection. Often there is considerable haemorrhage (along with the possible escape of some of the injected material) from the skin wound and from the punctured vein.
On the other hand one average technician can conveniently inject 60 animals, single-handed, in 20 - 30 minutes. Loss of experimental animals or occurrence of severe haemorrhage associated with intraperitoneal injection is practically negligible.

**Toxicity experiments.**

**Material.**

Several samples of crude kaolin acetone extracts of urine which proved to be relatively toxic, while performing the 'mouse uterus test' were pooled and used for the toxicity experiment.

**Method.**

This pooled gonadotrophin extract was injected intraperitoneally in 0.4 ml. of saline. On the basis of previous results obtained by the mouse uterus test the amount of extract injected to each animal was expected to contain sufficient LH to cause significant depletion of ovarian ascorbic acid. The animals were killed four hours after the intraperitoneal injection. Altogether 120 animals were used in two separate experiments with 60 animals in each. At the end of four hours after intraperitoneal injection 62 of the 120 animals were dead (approximately 50%). Most of the animals still living at the end of the four hour period on examination looked definitely ill. When left for another hour (5 hours after the intraperitoneal injection) another
Ovarian Ascorbic Acid after Administration
of NIH-LH

FIGURE 30
27 animals were found to be dead.

The conclusion reached from this experiment was that even when given intraperitoneally instead of intravenously, crude kaolin acetone extracts of urine which are toxic to the immature mice produce toxicity in immature rats as well.

RESULTS

Table 23

This Table shows the effect of the intraperitoneal injection of 1.5 µg of LH in different volumes of saline when compared with the intravenous injection of the same amount of LH in 0.5 ml. of saline. The ascorbic acid level in µg/100 mg. ovary ± the standard deviation is shown and the P value indicates that there is no significant difference between the various groups.

Figure 30

Parlow (1961) showed that a four hour period of time was optimal for the maximum depletion of ascorbic acid after the administration of the unknown preparation by the intravenous route. This figure shows the results of a similar experiment after the intraperitoneal injection of NIH-LH. It should be noted that the maximum depletion of ascorbic acid occurred at 4 hours. The circles represent two groups of animals killed at four hours after the administration of 0.3 and 1.5 µg LH intravenously. This finding, taken in
## TABLE 23
COMPARISON OF INTRAVENOUS AND INTRAPERITONEAL ADMINISTRATION
OF 1.5 μg NIH-LH

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>Volume administered</th>
<th>Ascorbic acid μg/100 mg ovary ± S.D.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous</td>
<td>0.5</td>
<td>92.3 ± 5.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>0.5</td>
<td>89.7 ± 8.66</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>99.0 ± 11.90</td>
<td>&lt;0.2  &gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>87.7 ± 9.47</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>
conjunction with the observation in the previous Table (Table 23) indicates that following the injection of 0.5 ml. of NIH-LH the rate of absorption is the same both by the intravenous and the intraperitoneal routes.

Table 24.

This Table shows the level of ovarian ascorbic acid in μg/100 mg. obtained after the administration of 0.3 and 1.5 μg of NIH-LH. In all cases the lower dosage of hormone was sufficient to produce a significant depletion of the ascorbic acid level. The following points should be noted.

1. The absolute levels of ascorbic acid obtained in any one assay are similar both after the intravenous and intraperitoneal injection of the hormone.

2. The slope as indicated by the difference in ascorbic acid levels between the low and high dosages of the hormone varied considerably in the four assays reported.

3. The mean difference in the ascorbic acid levels is very similar for both the intravenous and intraperitoneal groups.

Table 25.

The results of a series of five experiments in which two dose levels of LH injected by the intraperitoneal route were compared with the same dose levels of LH injected by the intravenous route are shown in Table 25. The mean relative potency obtained was 0.97 indicating that the
TABLE 24
OVARIAN ASCORBIC ACID AFTER ADMINISTRATION OF NIK-LH
μg/100 mg.

<table>
<thead>
<tr>
<th></th>
<th>Intravenous Administration</th>
<th>Intraperitoneal Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low dose</td>
<td>High dose</td>
</tr>
<tr>
<td>84.5</td>
<td>52.8</td>
<td>31.7</td>
</tr>
<tr>
<td>101.5</td>
<td>81.7</td>
<td>19.8</td>
</tr>
<tr>
<td>89.0</td>
<td>70.7</td>
<td>18.3</td>
</tr>
<tr>
<td>84.5</td>
<td>61.3</td>
<td>23.2</td>
</tr>
</tbody>
</table>
### Table 25

**Comparison of Intravenous and Intraperitoneal Injection of NIH-LH**

<table>
<thead>
<tr>
<th>Design</th>
<th>R.P.</th>
<th>Fiducial limits (P = 0.95)</th>
<th>Mean R.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 + 2</td>
<td>0.20</td>
<td>0.72 - 1.40</td>
<td></td>
</tr>
<tr>
<td>2 + 2</td>
<td>0.15</td>
<td>0.84 - 1.42</td>
<td></td>
</tr>
<tr>
<td>2 + 2</td>
<td>0.22</td>
<td>0.82 - 1.66</td>
<td>0.97</td>
</tr>
<tr>
<td>2 + 2</td>
<td>0.25</td>
<td>0.45 - 0.98</td>
<td></td>
</tr>
<tr>
<td>2 + 2</td>
<td>0.34</td>
<td>0.56 - 1.55</td>
<td></td>
</tr>
</tbody>
</table>
biological activity of NIH-LH is now altered by using the intraperitoneal instead of the intravenous route of injection. The relative potency obtained in the five experiments varied from 0.68 to 1.17. This range is similar to that previously reported by Schmidt-Elmendorff and Loraine (1962) using intravenous administration and the data show that the reproducibility of the assay method is not affected by the modification employed.

Table 26.

A small number of assays have been performed in which the LH activity of three other gonadotrophic preparations has been compared using the two routes of administration. It should be noted that in the case of each of the five materials tested the mean relative potency was comparable after intraperitoneal and intravenous injection.

DISCUSSION.

In view of the results obtained the original presumption that if the OAAD test could be performed by using intraperitoneal injection instead of intravenous administration, the problem of toxicity and consequently the problem of assaying crude urinary extracts using this method will be overcome, was unfounded. The cause of the toxicity of crude urinary extract is still obscure. It seems possible that further purification of the crude kaolin acetone extracts might
<table>
<thead>
<tr>
<th>Hormone Tested</th>
<th>Relative Potency</th>
<th>Mean R.P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-FSH</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>HCG</td>
<td>0.49</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>FMSG</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.61</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Pergonal</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>HMG-24</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td></td>
</tr>
</tbody>
</table>
yield less toxic urinary extracts, which might be suitable for OAAD assay.

Although even when administered intraperitoneally, crude kaolin acetone extracts produced toxic effects, it has been shown that purified gonadotrophin extracts can be assayed conveniently by using the intraperitoneal route of administration instead of the intravenous injection. From the viewpoint of practicability, particularly when dealing with a large number of animals, intraperitoneal injection is a simpler, more rapid, and less laborious procedure compared to intravenous injection. In view of the fact that the results obtained by either of these two routes of administration are comparable, it is evident that the OAAD assay using intraperitoneal instead of intravenous route of administration is a definite improvement due to the former's better practicability.

SUMMARY:

1. A comparison has been made of the intraperitoneal and intravenous routes of administration in the ovarian ascorbic acid depletion method.

2. The rate of absorption of NIN-LH is the same by the intraperitoneal and intravenous routes.

3. The slopes of the dose-response curves for NIN-LH obtained by the two routes of injection are similar.

4. The LH activity of NIN-FSH, PMSG and HCG appears to be comparable when these hormones are administered by the intraperitoneal or by the intravenous route.
MOUSE UTERINE WEIGHT RESPONSE TO GONADOTROPHIC STIMULATION AS A SPECIFIC INDEX OF PSH AND LH ACTIVITY

INTRODUCTION.

The "mouse uterus test", in other words the test depending on the increase in uterine weight of intact immature mice to gonadotrophin stimulation, is probably the most widely used assay method for evaluation of the pituitary gonadotrophic function in man. This bioassay otherwise admirably fulfills the requirements demanded by an acceptable procedure except for its questionable specificity. "What does the mouse uterus test measure?" has been a matter of considerable controversy over the last two decades. Most of the authorities on this subject at present agree however that this test does not specifically measure either PHS or LH, but does estimate what has been described by the convenient but rather ill-defined terms of "total gonadotrophic activity". At the present state of our knowledge it is not possible to state definitely whether the follicle-stimulating hormonal activity and luteinizing hormonal activity in their natural states are mediated by one or more biological materials secreted by the pituitary gland. Moreover certain extrapituitary gonadotrophins, notably pregnant mares serum gonadotrophin and human chorionic gonadotrophin, even in relatively pure form, retain, to a variable extent, both types of gonadotrophic activity. Indeed if human pituitary
FSH and LH activities are considered to be the two distinct biological effects exhibited by one single chemical entity, the term "total gonadotrophic activity" becomes obviously meaningful. Not only that, it remains then probably the most satisfactory method available for assessment of pituitary gonadotrophic function in its totality. As is well known however, this monophyletic view of pituitary gonadotrophic activity is not generally accepted. Thus it remains for future workers in this field to develop suitable specific assay procedures for the quantitative determination of the follicle-stimulating hormone, luteinizing hormone and probably luteotrophic hormone.

This present work was undertaken primarily to investigate the relative and specific influence of purified FSH and LH materials on the "mouse uterus test" and also if possible to alter the test as it is used to-day in order to measure either or both of the hormones specifically. It was hoped that by investigating the effects produced by interaction of FSH and LH on the mouse uterus test, it would be possible to obtain a correlation between the ovarian and uterine growth in response to gonadotrophic stimulation.

MATERIALS, METHODS AND RESULTS.

The mouse uterus test was performed in the following manner.
FIGURE 31
FIGURE 32
Preparation of the solution.

The total amount of material was dissolved in 2.5 ml. of normal saline. This amount was administered in 5 injections of 0.5 ml. each, the first 72 and the last 24 hours before the animals were sacrificed for the assay.

The uterus was removed from the top of the vaginal vault, cleaned and then slit off longitudinally and finally pressed firmly between two pieces of blotting paper to remove any excess fluid inside or on the surface. The tissue was weighed in a torsion balance to the nearest 0.1 mg.

Animals.

Intact weanling female mice weighing 9 - 11 gms. and 20 - 21 days of age were used for all the work described in this chapter. At least 6 animals were used in each experimental group.

Figure 51 represents a typical dose-response curve using NIH-FSH as the test material. It can be seen that the steepest part of the dose-response curve is obtained by using 50.0 and 100.0 µgs of the hormone.

Figure 52 represents a similar dose-response curve using NIH-LH as the test material. It may be seen that this hormone in dosages up to 4 mgs., i.e. roughly 100 times the dose used for NIH-FSH (Figure 51), results in rather a slight increase in the uterine weight. Moreover the slope of the curve is very flat compared to that obtained by
FIGURE 33
FIGURE 54
FIGURES 33 AND 34 Represent the dose-response curves obtained when a mixture of NIH-FSH and NIH-LH are injected in the amounts as shown. It is evident that the addition of NIH-LH to NIH-FSH in amounts between 10 \( \mu g \) and 30 \( \mu g \) distinctly augments the mouse uterine response to NIH-FSH. It may be further pointed out that NIH-FSH at a dose level of 25 \( \mu g \) and NIH-LH at a dose level of 25 \( \mu g \) either of which by themselves fails to produce any significant increase in uterine weight, do so when used together. This most probably represents a synergistic response. Because NIH-FSH used alone at double this dose produces an almost identical response (Figure 31), while NIH-LH used alone in a much larger dose fails to produce any significant effect (Figure 32), the former may be regarded as the prime mover in this synergistic response. From Figure 34 it is also evident that increasing the dose of added NIH-LH to between 30 \( \mu g \) and 40 \( \mu g \) does not cause further increase of uterine weight than that obtained by the addition of 30 \( \mu g \) NIH-LH. The addition of 50 \( \mu g \) or more of NIH-LH to this mixture decreases the response. These findings are in agreement with those of Brown and Billewicz (1962).

Figure 35 represents a dose-response curve obtained with the international standard for HCG. When compared with the dose-response curve obtained with NIH-FSH it will be seen that
uterine wt. mg.

NIH FSH mg.

- HCG ADDED (IU)
- NO

Figure 36
the shapes of the curves are comparable. The lower dose of the hormone at the steepest part of the dose-response curve produces an increase in uterine weight which is double that of the control uterine weight. Doubling the dose of the hormone results in a further doubling of the uterine weight resulting in a steep slope. In this experiment the steepest part of the dose-response curve obtained with HCG was between 0.4 i.u. and 0.8 i.u. Dose levels of 0.2 i.u. or less of the hormone failed to produce any significant increase of uterine weight over controls.

Figure 36 represents the dose-response curve obtained with NIH-FSH and HCG administered together. The dose levels of HCG used were 0.2 i.u. or less, i.e. dose levels which fail to produce a significant effect on the uterine weight when used alone. It may be seen that the uterine weight response to NIH-FSH is markedly augmented by addition of comparatively small doses of HCG. At the 0.2 i.u. HCG level the steepest part of the dose-response curve lies between 3.125 and 6.25 µg of NIH-FSH, that is at a level 20 times less than that for NIH-FSH alone. The shape of the curve thus obtained is similar to that obtained with NIH-FSH alone. Although considerable controversy exists regarding the true nature of HCG it is widely held that it contains very little FSH activity, which has not been measured properly up till now. The augmenting effect of HCG on NIH-FSH
is most likely due to its LH content.

The mutual augmentation of the response obtained by using mixtures of NIH-FSH and HCG is of considerable interest. From the Figure (Figure 36) it is evident that either purified HCG or purified preparations of FSH can be measured by mixing one of the materials with a very small dose of the other.

**Determination of HCG.**

It is obvious from Figure 36 that using this method as little as 0.05 i.u. of HCG can be conveniently detected by mixing the HCG sample with 25 μgs of NIH-FSH. The sensitivity of this assay method is at least 8 times more than that of the conventional mouse uterus test and about 10 times more sensitive than the OAAD test. For quantitative estimation of HCG in body fluids during pregnancy either the "total rat prostate test" or the "mouse uterus test" is commonly used. For performing any form of "mouse uterus test" with samples from pregnant subjects however it is essential that the biologically active ovarian steroids should be removed from the samples to avoid interference with the assay method. It is doubtful whether any amount of pituitary FSH or LH is secreted during pregnancy. The commonly used tests are based on the assumption that either pituitary hormones are not present during pregnancy or they are present in such small amounts compared to the amount of
HCG that their presence would not interfere with the assay method. It is usually believed that the rat prostate method is responsive to pituitary LH as well as HCG. Similarly the "mouse uterus test" is responsive to pituitary FSH as well as HCG. Therefore if the pituitary FSH or LH is believed not to interfere with the rat prostate assay or with the conventional "mouse uterus test" then the presence of this material should not interfere with this proposed modified "mouse uterus test" using a small dose of NIH-FSH.

Apart from its increased sensitivity this method of assay has the added advantage of producing a comparatively steeper slope of the regression curve when compared to the conventional "mouse uterus test" using HCG alone.

**Determination of FSH.**

It is also obvious from the Figure 36 that by adding very small doses of HCG (amounts which by themselves are not capable of altering the mouse uterine weight) comparatively small amount of purified FSH can be quantitatively assayed. Here again the sensitivity of the method is noteworthy. When using NIH-FSH as the test substance at present it is not possible to measure quantitatively the biological activity of less than 25 - 30 μg of the material: and this again is only possible by using the "augmentation assay" in which a large amount of HCG is superadded to produce an FSH plus HCG augmented response. Compared to the
FIGURE 37
FIGURE 39

ut wt mg.

--- 302 IU HGG
--- CONTROL

HM-G mg.

0.35 0.7 1.4
"augmentation assay" this suggested type of modified "mouse uterus test" is about 10 times more sensitive. It seems possible that when dealing with highly purified FSH or HCG this type of modified or augmented mouse uterus test will be quite suitable as a bioassay.

In view of the marked increased sensitivity of the mouse uterine response to NIH-FSH by HCG, it was hoped that it would be possible to increase the sensitivity of the mouse uterine response to other gonadotrophic materials by the addition of small amounts of HCG. The results of such experiments with Pergonal, PMSG and HMG-20A are shown in Figures 37, 38 and 39. It may be seen that the responses obtained by these materials were not significantly altered by the addition of as much as 0.2 i.u. HCG. The cause of the failure to augment the mouse uterine response obtained by PMSG, Pergonal and HMG-20A by adding 0.2 i.u. HCG was thought to be due to the presence of relatively more LH in these three gonadotrophin preparations than in NIH-FSH. It was argued that the mouse uterine responses obtained by using these three materials were in fact already augmented by the LH present in them. If this is true then this has a certain bearing on the use of highly purified pituitary materials as standard preparations for expressing results obtained from urinary extracts. Because the mouse uterine weight responses to urinary gonadotrophin extracts are
0.2 I.U. HCG AT EACH DOSE: LEVELS

0.25 µG. NIH-LH

2.50 µG. NIH-LH

10.0 µG. NIH-LH

ut.wt.mg.

NIH-FSH (µG.)
FIGURE 42

Graph showing the relationship between NIH-FSH (μG) and uterine wt. mg.

- NIH-lh
- 625 nih lh
- 250 nih lh
- 125 nih lh
- 1000 nih lh

Oil w. HCG at each dose levels
FIGURE 47

UTERINE WT. H.S.

OVARIAN WT. NS.

NIH-LH (U.S.)

NIH-FSH

25 μg NIH-FSH + 0.2 IU HCG AT EACH DOSE LEVELS

IO-UTERINE WT. HS.

IO-OVARIAN WT. NS.

mg.

0 10 20 30 40

0 10
FIGURE 44
FIGURE 45

25 μg NIH-FSH + 0.2 I.U. HCG
AT EACH DOSE LEVELS
FIGURE 46
Figure 47

Graph showing the response of ovulation (OV wt.) and uterine activity (Ut.) to different doses of NIH-FSH (25, 100, 200 μg) in combination with 0.2 IU HCG and 10 μg NIH-LH at each dose level.
mg.

50.

40.

30.

20.

10.

ut. wt.

ov. wt.

ov. wt.

ut. wt.

28.0 µg NIH-FSH at each dose

-22.5 µg

32 64 128 256 512 1024 2048 4096

HCG IU.

FIGURE 47 a
already augmented due to the presence of enough LH in them, while that obtained by purified FSH is still augmentable by the addition of LH materials like HCG. Purified animal pituitary FSH and LH are used in certain laboratories as standards for comparing the results obtained from crude urinary extracts. It seems that there are certain qualitative differences between purified pituitary FSH and urinary gonadotrophin extract, detectable by the mouse uterus test.

**Effects of NIH-LH on various mixtures of gonadotrophins:**

*Figure 40* represents the effect of NIH-LH on Pergonal. It may be seen that NIH-LH added in increasing doses to Pergonal progressively decreases the mouse uterine weight response to this hormone.

*Figure 41* depicts the effect of NIH-LH on the augmented mouse uterine weight response obtained by adding 0.2 i.u. HCG to NIH-FSH.

From both these Figures (Figures 40 and 41) it may be seen that purified LH (NIH-LH) depresses the mouse uterine weight response obtained either by Pergonal or by the combined action of NIH-FSH and HCG. It is interesting to note that the augmenting effect of NIH-LH on the mouse uterine weight response obtained by NIH-FSH alone is not evident in either of these two instances. In view of the fact that NIH-LH will only cause inhibition of mouse uterine weight
obtained by Pergonal alone or NIH-FSH plus 0.2 i.u. HCG alone, it was decided to investigate the possibility of utilizing this decreasing uterine weight as an index of LH activity. *Figures 42 to 46* show the dose-response curves obtained by using various dose levels of NIH-LH on the augmented mouse uterine weight response obtained by NIH-FSH combined with 0.2 or 0.1 i.u. HCG. It may be seen that although the response (uterine weight inhibition by LH) is quantitative the addition of further FSH (NIH-FSH) will inhibit the "uterine weight inhibiting effect" of NIH-LH. The mutual antagonising effects of FSH and LH are clearly revealed in the next experiment. However it may be seen that there is a certain essential difference between the effects produced by NIH-LH on this system when compared to that produced on NIH-FSH alone. In this case (NIH-FSH + HCG) the addition of different dose levels of NIH-LH produces only one demonstrable effect on the uterine weight, viz. that the uterine weight is depressed. This effect is comparable to the effect of NIH-LH on Pergonal.

From *Figure 47* it may be seen that the addition of further NIH-FSH (Figure 47) causes further increase of uterine weight. In fact the inhibition of the uterine weight which was obtained by adding NIH-LH (Figures 42 - 46) was prevented by the addition of NIH-FSH. This effect is however associated with simultaneous increase in the ovarian
weight, a phenomenon not observed in the previous experiments. It was concluded therefore that in this system so long as the available amount of ovarian tissue remains constant the effect produced by NIH-LH on uterine weight is always one of depression. The amount of LH activity present in 25 µg NIH-FSH (roughly equivalent to 0.25 µg of NIH-LH activity) would cause significant depression of uterine weight (Figure 46) when given alone but not when given along with FSH. Because this added amount of FSH results in an increase in ovarian weight presumably due to cellular hypertrophy and hyperplasia and this newly available ovarian tissue will produce enough steroid hormones to disturb the uniformity of response shown by the uterus. In view of this difficulty it would not be possible to use this system to measure either FSH or LH activity specifically in a sample containing both the gonadotrophins. It is clear from this experiment that to obtain uniformity of uterine response from NIH-LH the ovarian weight will have to be kept unaltered. It was argued that the only way of producing this latter condition was to increase the ovarian weight to its maximum limit. It was also thought necessary at the same time that the system would have to be such that in addition to stimulating the ovarian growth to its maximum limit, it will have to produce a relatively high uterine weight. Because, if the uterine weight is very low, further addition of LH would cause at most a very flat regression slope of uterine-
depressing effect. For designing a workable assay such a flat slope will be of very little practical value.

With this result in view, i.e. to produce a system which will stimulate the ovarian growth to its maximum limit, and at the same time will be able to maintain a high uterine weight, a number of gonadotrophins and gonadotrophin mixtures were tested.

The purpose of the following two experiments was to produce the maximum growth of the ovaries associated with high uterine weight.

The "augmentation assay" is known to produce very high ovarian weight. Also in this connection it was thought that a particular mixture of HCG (used for "augmentation assay") and NIH-FSH might be suitable for producing maximum ovarian growth along with high uterine weight. Moreover, it has been shown previously that by using up to 0.2 i.u. of HCG for augmenting the NIH-FSH invoked mouse uterine response, a progressively increasing response can be obtained. On the other hand NIH-LH used in relatively high dosages depressed the otherwise increasing mouse uterine weight response to NIH-FSH. In view of the generally held predominantly LH nature of both NIH-LH and HCG it was decided also to investigate whether, like NIH-LH, at high dose levels HCG also depressed the otherwise increasing mouse uterine weight response to NIH-FSH. The result of this experiment using 25.0 µg NIH-FSH with different concentrations
25.0 μg NIH-FSH at each dose levels

FIGURE 48
of HCG is shown in Figure 48. It may be seen from this figure that a fixed dose of NIH-FSH (25 µgs) was mixed with varying dose levels of HCG. The dose levels of HCG used in this case were much higher than those used in a previous experiment (Figure 56). It will be noted that the uterine weight response to 25 µg of NIH-FSH is augmented at the lower dose levels of the added HCG. Then after reaching a high level the uterine weight falls with the next higher dose level of the HCG. This uterine weight depressing effect of HCG is comparable to the similar effect obtained by NIH-LH. However, after decreasing for a few successively increasing dose levels of HCG the uterine weight again increases with subsequent increasing dose levels of HCG.

Following this the uterine weight is progressively decreased with each succeeding high dose level of HCG. As has been already described in this particularly experiment the dose level of NIH-FSH was kept fixed at 25.0 µg. In other words, increasing dose levels of HCG alternately stimulate and depress the uterine weight induced by NIH-FSH. It will be seen later (Figure 49) that a mixed gonadotrophin (Pergonal) produces similar pattern of mouse uterine and ovarian response. The remarkable feature of this experiment is the increase in the ovarian weight associated with each increasing phase of uterine weight and the constancy of the ovarian weight during the steady or decreasing phase of the
uterine weight. It may also be seen that the lower level of decreasing uterine weight reached during each phase of decline is lower than the previous one. When 50 i.u. of HCG have been used the mean uterine weight is 16 mgs. Further increase of the dose levels of HCG up to 400 i.u. did not alter the uterine weight any further. Here again HCG is behaving like NIH-LH in causing depression of mouse uterine weight. In this part of the graph the ovarian weight may also be found to have reached a steady weight. It may be seen however from this Figure that to produce a high uterine weight and maximum growth of the ovaries further amounts of FSH are needed.

As has been discussed previously, HCG is generally believed to be predominantly LH in nature with very little if any FSH activity in it. In view of the remarkable degree of augmentation of mouse uterine weight obtained by adding small amounts of HCG to NIH-FSH (Figure 36) the augmenting effect of HCG was thought to be quite different from the comparatively weaker degree of augmentation obtainable by the addition of small amounts of NIH-LH to NIH-FSH. Moreover, the alternately increasing and decreasing uterine weight in response to increasing doses of HCG with a fixed amount of NIH-FSH (25.0 μgs) is difficult to explain. It is very likely that the increasing ovarian weight preceding each phase of increasing uterine weight and steady ovarian
weight during each phase of steady or decreasing uterine weight, is the cause of this fluctuation. The uterine weight is augmented, presumably due to the effect of FSH or FSH-like material present in HCG. After the uterine weight reaches a high level further addition of HCG causes depression of the uterine weight, presumably due to its LH activity. So long as the ovarian weight remains constant at a particular level this relationship, i.e. FSH initially causing stimulation and LH eventually causing depression of uterine weight, holds good. Growth of the ovary following the addition of FSH is associated with disturbance of this relationship, but once again at a particular constant level of ovarian weight the uterine weight stimulating effect of FSH and subsequently the uterine weight depressing effect of LH are re-established. It seems as if each new batch of fresh ovarian tissue responds exactly like crops of newly formed tissue in repeating the same series of changes from the beginning to the end over and over again as its predecessors. Hence at a particular fixed level of ovarian weight uterine weight behaves in a more regular fashion. It is almost certain that the uterine weight response to gonadotrophins is mediated by the steroid hormones of the ovaries. It is possible that each phase of increase in ovarian weight is due to fresh follicular development in response to FSH stimulation. This newly formed follicular
tissue secretes follicular hormones which stimulate the uterine weight. Further addition of LH luteinizes this newly formed follicular tissue. Inhibition of uterine weight results either due to inhibition of follicular hormone secretion due to luteinization of the follicular tissue or due to active secretion of luteal hormones from the luteinized ovarian cells. The eventual inhibition of uterine weight is explicable on the ground that all the available follicular tissue has been luteinized. On the basis of this theory the restriction of unlimited growth of the uterus in response to gonadotrophin stimulation (gonadotrophins containing both FSH and LH) is easily explicable. It is conceivable that even when given in huge amounts eventually all the available follicular tissue will be luteinized with consequent restriction of uterine growth. It seems that even in the absence of any other controlling factor LH itself will antagonise the biological effect of FSH on the uterus. The pituitary gland producing its trophic hormones along with their corresponding target organs constitute auto-regulating systems due to the mutual feed-back mechanism. No such feed-back mechanism appears to exist between the ovary and its target organ, the uterus. Nevertheless the interaction of FSH and LH on the ovary is such that the sum total effect of the ovary on the uterus is self-regulating. Marked dissociation of FSH and LH secretion from the pituitary may
theoretically disrupt this regulating system. Such a
dissociated function has not been detected so far, and
whether pure FSH without any amount of LH is capable of pro-
ducing its biological effect is still uncertain.

**Pergonal.**

The uterine and ovarian response curves using a very long range of dosages of Pergonal is shown in Figure 49.
The interaction of FSH and LH as revealed by the simulta-
taneous recording of uterine and ovarian weight is well shown by using the very wide dose range of Pergonal. It is seen clearly that the ovarian growth is continuous and pro-
gressive until it reaches its limit when no further growth occurs. Even at its highest limit the ovarian growth is not inhibited by the addition of further amounts of gonado-
trophin. On the other hand the uterine growth shows three distinct phases, each remarkably resembling the other.
These three phases are closely associated with relatively steep ovarian growth. The curve closely resembles that obtained with the uterine and ovarian weight response curves in relation with the "augmentation assay". It will be noted that even after reaching the maximum growth of the uterus the uterine weight is quite high. This is probably due to the fact that in this case the maximum ovarian growth has been obtained by comparatively larger amounts of FSH material. In the case of the "augmentation assay" curve the maximum growth of the uterus was obtained by using a comparatively
FIGURE 50
FIGURE 51
higher dose of HCG. It seems probable that by adjusting the different proportions of FSH and LH materials this result could be obtained by using a number of different gonadotrophin mixtures. The similarity of the response patterns obtained by HCG and Pergonal suggests that both these substances are of qualitatively similar nature, i.e. HCG like Pergonal contains both FSH and LH activity.

Similar experiments conducted with high dose levels of PMSG have shown that the response is similar to that exhibited by Pergonal.

**Addition of NIH-LH and NIH-FSH to very high dosages of Pergonal and PMSG (Figures 50, 51).**

The purpose of this experiment was to investigate the possibility of producing a uniform uterine response with further addition of LH. As has been explained in previous experiments growth of the ovary results in disturbances of uniformity of uterine response. It has also been shown that it is not practicable to inhibit or suppress the growth of an otherwise growing ovary by using various gonadotrophin mixtures. It was hoped that a uniform uterine response (inhibition of growth in this case) produced by LH would make it possible to measure this gonadotrophin specifically. The results are shown in Figures 50 and 51. In both instances the doses of Pergonal and PMSG used were found to cause maximum ovarian growth even when given by one sub-
cutaneous injection. It was also found that uterine weight was fairly high when maximum ovarian growth was achieved at the smallest effective dose of Pergonal and PMSG, given by one subcutaneous injection. It may be seen from Figures 50 and 51 that the addition of 100 µg of NIH-LH and 100 i.u. HCG causes only inhibition of uterine weight and this inhibitory effect of NIH-LH is not interfered with by NIH-FSH.

Experiment.

It has been shown by Schmidt-Elmendorff and Loraire (1962) that PMSG contains about 30% of LH activity. It has also been shown by Zarrow et al. (1958) that a single injection of PMSG even when given in comparatively large doses, fails to produce any demonstrable LH effect in the ovary as evidenced by absence of ovulation. Zarrow et al. (1958) on the other hand have shown that as little as 0.1 µg of NIH-LH or 0.1 i.u. of HCG given after PMSG is capable of producing ovulation in a significant number of animals. In view of this and the results obtained in the previous experiments it was argued that if sufficient amount of PMSG to induce maximum ovarian growth was given only in the first injection and not subsequently, the effects produced subsequently by injection of LH were likely to be unaffected by contaminated FSH.

It may be seen from these two Figures (Figures 50, 51)
that the NIH-LH causes depression of the mouse uterine weight, and this depressing effect is not inhibited by the addition of NIH-FSH, that is, an experimental condition is produced where the response (depression of mouse uterine weight) is not interfered with by the addition of further NIH-FSH. It is possible that this condition could be utilized for measuring LH activity specifically, by using depression of the 'otherwise increasing mouse uterine weight brought about by PMSG or Pergonal', as the end point.

Figure 52 shows the effect of mouse uterine weight response to one single dose of gonadotrophin administration. As previously postulated by other observers (Zarrow et al., 1958) a single subcutaneous injection of a mixed gonadotrophin-like PMSG produces only FSH effect, it may be seen from Figure 52 that the inhibitory effect of NIH-LH on the otherwise increasing uterine weight response to NIH-FSH is not observed when NIH-FSH and NIH-LH are given together by a single subcutaneous injection. In other words, an experimental condition is produced when a pure FSH effect is obtained following the administration of a gonadotrophin mixture containing both FSH and LH. It is possible that to produce its biological (synergistic or antagonistic) effect LH requires the preliminary priming effect of FSH on the ovarian follicles. When given in one single injection it is very likely that the injected LH is excreted out of the body before enough follicular tissue to act upon is
**DISCUSSION.**

Using the mouse uterine weight as the endpoint the interaction of FSH and LH present in various gonadotrophins and gonadotrophin mixtures have been investigated. In spite of the apparently erratic response of the "mouse uterus test" to different mixtures of FSH and LH, it has been shown that, by using various mixtures of gonadotrophin it is possible to obtain uniform mouse uterine response from either of them. It has been possible to design experimental conditions in which FSH will only stimulate the uterine weight. Similarly experimental conditions have been described when LH will only cause inhibition of the uterine weight. It has also been shown that it is possible to obtain consistently increasing or decreasing mouse uterine weight response by using FSH or LH respectively without cross interference. At present it is not possible to assess how far these methods will be of practical value in designing assay methods for quantitative determination of FSH and LH. The reliability criteria have not been properly studied in any of the possible methods suggested. Nevertheless, it has been shown that using different experimental conditions it is possible to study the opposing effects of FSH and LH in the same way as it is possible to investigate the opposing effects of an antigen and its homologous antibody.
Brown and Dillewicz (1962) investigated the interaction of NIH-FSH and NIH-LH, using mouse uterine weight response as the end point. Hiley (1961) conducted similar experiments using NIH-FSH, "Armour Standard LH" and Pergonal. The result of the experiments described in this Chapter are in accordance with the observations made by these workers.

However, further extension of the work along certain lines not previously reported by other workers makes it difficult to ascertain the validity of the conclusions reached.

The progressive nature of the ovarian growth has been previously discussed by certain earlier observers (Witschi, 1961; Paulsen, 1961; Lunenfeld, 1961; McArthur, 1961). In view of the results discussed earlier in this Chapter it seems that the depression of ovarian weight previously reported by certain observers is almost certainly due to toxicity rather than due to the effects of hormone. McArthur (1961) came to a similar conclusion on the basis of her experiments using very high dosages of Pergonal. It may be pointed out that all the previous workers who reported the depression of ovarian weight, used relatively toxic urinary extracts like HMG rather than comparatively non-toxic materials like Pergonal.

The progressive and uniform nature of the ovarian growth in contradistinction to the erratic behaviour of
uterine growth in response to gonadotrophic stimulation is easily understandable on the grounds that they represent two basically different systems. Ovarian growth represents the ovarian response to pituitary stimulation, whereas the uterine growth reflects the uterine response to ovarian stimulation. It seems that the sum total effect of the interaction of the FSH and LH on the ovarian growth is always synergistic. On the other hand, the ovarian response to FSH and LH stimulation as reflected on the uterine growth may produce a synergistic or an antagonistic effect.

**SUMMARY.**

The effects of interaction of FSH and LH on the mouse uterine weight response have been investigated using various mixtures of purified pituitary FSH (NIH-FSH), purified pituitary LH (NIH-LH), HCG, Pergonal, HMG-20A and PMSG.

Using the mouse uterine weight response as the end point several model approaches for designing possible new assay methods for specific determination of FSH and LH have been suggested.

The factors underlying the behaviour of mouse uterine weight and mouse ovarian weight responses to gonadotrophic stimulation have been discussed.
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ACKNOWLEDGEMENT.

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