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Bioassay of the sex hormones

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INTRODUCTION

Much has been written about the history of the development of the science of endocrinology, from the observations of the ancients up to the work of Brown-Sequard in the early years of this century, from which time all of the more important knowledge of the subject has accumulated. However, not much was done until after 1932 when the discovery of insulin and its dramatic results in the treatment of diabetes seemed to stimulate experimental investigation in all branches of endocrine science. It is not the purpose of this paper to review the history of endocrinology, nor even of the sex hormones; this has been adequately accomplished in such books as those of Frank, Allen, Doisy, Fieser, etc.

According to Doisy (26)"the investigations of an endocrine gland fall naturally into four stages: (1) the recognition of the gland as one producing an internal secretion; (2) the methods of detecting the internal secretion, which, of course, leads to a survey of possible sources of the autacoid; (3) the preparation of extracts and the purification of the hormone; (4) the isolation of the pure hormone,

determination of its structure and its synthesis." We might add a fifth, that of intermediary metabolism of the hormone, a field that has opened within the last two or three years.

We are concerned in this paper first with Stage III. "Without a biological reaction that may be used as a quantitative measure, advance can not go on. Rapid progress can not be made unless a reasonably fast and accurate method of bio-assay is available. Given such a procedure the biochemist can proceed with the preparation of crude extracts and the separation of the mixture into its components, each of which must be assayed. Each step must be tested by a determination of the potency of the product if the investigator wishes to avoid working in the dark. Time consuming bio-assay processes have retarded the isolation of the vitamins, and many of the hormones."

Next, we are concerned here with Stage IV in that we will discuss some of the chemical nature of the female sex hormones, especially their relation to each other and to some of the common phenanthrene substances.

Before going into the chemistry and bioassay

procedures of the female sex hormones a brief discussion of their physiology in the female economy seems indicated.

The functional value of the ovary has to do with the formation and rupture of the Graafian follicles (whereby an ovum is liberated) and with the subsequent formation of corpora lutea. The primordial follicles consist of an ovum surrounded by a layer of follicular epithelium. Beginning at puberty, and continuing throughout the period of active sexual life, some of these primordial follicles develop into mature Graafian follicles and by growth come to lie near the surface of the ovary. The change consists in a proliferation of the follicular epithelium and the formation of a serous liquid, the liquor folliculi, between the layers of this epithelium. In the matured follicle there is a connective tissue covering, the theca folliculi, formed from the stroma of the ovary and consisting of two coats or tunics - the external and the internal. The cells of the internal tunic develop a yellowish pigment as the follicle grows, and are sometimes designated as lutein cells. Within the capsule formed by the

internal tunic there is a layer of follicular cells known as the membrana granulosa and attached to one side is a mass of the same cells, called the cumulus oophorus, which contains the ovum. As the follicular liquid continues to accumulate the peripheral portion of this structure is stretched to a very thin almost avascular membrane; at full material the graafian follicle ruptures at this point and the egg, together with the surrounding follicular cells of the cumulus are extruded into the peritoneal cavity to be received into the open end of the Fallopian tube. After the rupture of the follicle its walls collapse and from the ruptured thecal vessels there is hemorrhage into the central cavity, the structure now being designated as the corpus hemorrhagicum. Subsequently, due to proliferation of the cells of the follicular epithelium, the vesicle becomes filled with cells containing a yellow pigment (lutein), and the early corpus luteum is formed.

The period of duration of the corpus luteum is dependent upon whether or not fertilization occurs. If it does not occur, as is the case in the usual monthly periods, the corpus luteum reaches its maximum

size in a week or two, then retrogressive changes set in rapidly and the structure is finally absorbed, leaving a scar, the corpus atreticum. Such is the fate of the corpus luteum spurium. In the event of conception the life of the corpus luteum is much prolonged. Instead of undergoing retrogressive changes at the end of a few days it continues to increase in size by proliferation and hypertrophy of the follicular epithelium, and does not begin to show retrogressive changes until the sixth month of pregnancy or later; indeed, the very existence of the pregnancy is dependent on the function of this corpus luteum verum, as will be explained later.

The anterior lobe of the pituitary gland is the master gland of the endocrine system. Ten different groups of effects have been identified with this part of the pituitary. Six of these have been shown to be due to six separate hormones, namely, the growth hormone, and two gonadotropic hormones. Other effects of the anterior lobe for which definite hormones have not been demonstrated are the ketogenic, hyperglycemic, parathrotropic and pancreatropic effects.

We are interested here in the two gonadotropic hormones. Several observations in the past have pointed

to the anterior pituitary as exerting on influence upon sexual development, notable among which are the gradual atrophy and suppression of the sex functions in diseases of the anterior lobe such as acromegaly and Frohlich's syndrome. Subsequently it was shown that in immature animals, or animals, that had been subjected to hypophysectomy, the effect of administration of extracts of anterior lobe was to produce typical estrus, a phenomenon to be observed normally only in mature intact animals. Furthermore it was observed that the ovaries of such animals contained a large number of follicles which ripened and discharged a "shower" of ova (superovulation), following which numerous typical corpora lutea developed. This was shown to be a dual effect (the details will be discussed in a later section), the effect being to stimulate luteinization with the formation of a mature functioning corpus luteum. While these two gonadotropic principles have never been separated and isolated, the follicle-stimulating principle may be obtained by acid extraction of the anterior lobe, and two names have been applied. Prolan A is the name for the pituitary gonadotropic principle responsible for follicle maturation in the

ovary; Prolan B is the name applied to the principle responsible for luteinization. Their action is as follows:

Prolan A initiates sexual activity. The ovaries, stimulated by this anterior lobe hormone, bring about the psychic phenomena of puberty and of the estrous cycles of postpuberal life, as well as the physical changes in the accessory organs such as the vagina and uterus. It stimulates the development of the ova and granulosa cells of the follicles, and is finally responsible for the ripening of follicles. The liquor folliculi of these mature follicles contains one of the estrogenic hormones, estrin.

Prolan B on the other hand depresses the first part of the ovarian cycle as described above and encourages the second or luteal phase. It acts specifically upon the cells of the theca and granulosa with the production of a specific hormone, the action of which is shortly to be discussed.

The final expression of the influence of the sex hormones is the series of changes through which the female reproductive organs pass every 28 days or so.

This constitutes the menstrual cycle, its counterpart in lower mammalian forms being periodic estrus or "heat". It is generally believed that rhythmical variations in the activity of the anterior pituitary (i.e., in production of the gonadotropes) are primarily responsible for the regular recurrence of the menstrual cycle, the ovary playing the role of intermediary. The uterine changes of the entire human cycle may be divided into four stages:

(1) Repair Stage (post menses): During this stage the epithelium of the endometrium which was shed during the menstrual flow is restored. The mucous membrane is thin, the uterine glands are short and straight, the cells are cuboidal with centrally placed nuclei. This phase occupies about six days.

(2) Resting Stage (early proliferative): The tubular glands are now longer, are wavy and cork-screw shaped. The cells of the glands and of the surface epithelium have become more columnar and have more centrally placed nuclei. The stroma cells do not change much in appearance. This phase also occupies about 5 or 6 days.

(3) Premenstrual Stage (pregravid, secretory): This stage commences some 12 to 14 days before the

onset of the menstrual flow. The endometrium becomes much thicker, the glands larger, deeper and much more convoluted. The lining cells are columnar with their nuclei down toward the basement membrane. The cells are full of mucin and give a positive stain for glycogen. The stroma cells are round instead of elliptical (the precursors of decidual cells if pregnancy intervenes)

(4) Menstrual Stage: Pregnancy not having intervened the hypertrophied endometrium breaks down, hemorrhage occurs, and the superficial layers of the mucosa are shed. The flow lasts about four days following which repair begins and another cycle is started.

Now we can correlate the interplay of the gonadotropic and estrogenic hormones with the structural changes as described.

Prolan A has its maximum concentration around the 4th to the 11th day of the cycle. As indicated above, it is responsible for the stimulation of follicular growth; the follicular fluid contains the estrogenic hormone estrin which in turn is responsible for early endometrial proliferation (and for vaginal opening and cornification and estrus in other mammals).

Prolan A appears in the non-pregnant female only in the intermenstrual phase; it is present in castrate and in menopausal, but not pregnant women.

Prolan B is most active from the 16th to the 28th days of the cycle. It is responsible for the luteinization of follicular and thecal cells, and formation of the mature corpus luteum. This structure in turn produces another hormone, progestin, which is the substance directly responsible for continued endometrial proliferation and for the secretory phase of the menstrual cycle. For this reason it is sometimes given the name of "the progestational hormone".

Summarizing the ovarian hormones, their properties may be briefly outlined as follows: (61)

I. THE FOLLICULAR HORMONE: Synonyms are oestrin, estrin, theelin, folliculin, estrone, estrogin, theelol, progynon, feminin, ovarin, oophorin, thelykinin, menformin, ovarian sex hormone.

1. Physiologic Action

   A. Effects uterine growth and vascularization

   B. Incites estrus inimmature laboratory animals, with premature growth and cornification of the vaginal epithelium.

   C. Acts in developing secondary sex characteristics.
      a) From birth to puberty it develops femininity.

b) After puberty it aids in impregnation and lactation.

D. The effects on the ovary and pregnancy are antagonistic.
   a) Inhibits follicular growth, may induce abortion.

E. Allays autonomic symptoms of the menopause.

F. Estrin content of urine is increased in pregnancy.
   a) Pregnancy urine for various pregnancy tests.

2. Source and Quantitative Distribution

A. According to Zondek the mature human graafian follicle contains 2-3 cc. of fluid which possesses 8-12 mouse units estrin; it is constantly produced by the follicles.

B. The amount secreted varies at different times of the month.
   a) Greatest soon after ovulation, between 12th and 28th days after the last menstruation.

C. Corpus luteum also secretes follicular hormone, accounting for the premenstrual and pregnancy concentrations.

D. Placenta is thought to be the main source during the latter part of pregnancy.

E. Blood estrin is low because of low renal threshold. Large amounts are excreted in the urine.

3. Extra-ovarian Location of Follicular Hormone

A. Placentae of humans contains large quantities. It may be manufactured there or merely stored there.

B. The urine is a constant vehicle.
   a) 5-20 MU/liter in all humans (Zondek)
   b) Postmenstrual urine contains 50 MU/liter.
   c) Intermenstrual urine has 300 MU/liter.
   d) Urinary hormone is increased with menopause.
   e) During pregnancy blood and urine concentrations go up.
   f) Urinary estrin increases from moment of conception
      1. Second month - 2000 - 3000 MU/liter.
      2. Fifth month - 10,000 MU/liter
      3. Ninth month - 50,000 or more MU/liter
      4. Concentration is even greater in cases of hydatiform mole and chorionepithelioma.

C. Also found in males, vegetations and minerals.

4. Occurrence of Estrin in the Male

A. 50-200 mouse units/liter of male urine.

B. In stallions the estrin is secreted by the testes. According to Zondek, 1 kilo of stallion testes contains 66,000 units, 500 times more than the mature mare ovary.

5. Occurrence in Plants and Carbon Compounds

A. Estrus principles are found in alder leaves, willow catkins, sprouted oats, rhubarb leaves, palms, etc.

B. Aschheim reports its presence in petroleum, coal tars, coal and bitumen from moors.

6. Crystalline Estrin

A. Isolation in pure crystalline state as a keto-hydroxy estrin was accomplished by Doisy in 1929 and by Butenandt, and it was called theelin.

B. Other workers isolated a similar substance having one more molecule of water and called it theelol. It is less potent than theelin but has the advantage of being active by mouth.

C. Commercially the estrogenic hormone is usually obtained from the urine of pregnant mares.

II. THE CORPUS LUTEUM HORMONE: Synonyms are progestin, corporin, progesterone, and luteosterone.

1. Physiologic Action

A. Frankel, in 1903, showed that cauterization of the corpus luteum during early pregnancy was followed by abortion.

B. Loeb showed that the corpus luteum secretes a uterine sensitizing substance responsible for the formation of decidual tissue.

C. Ovulation and estrus in guinea pigs can be suppressed by injecting aqueous solutions of corpus luteum.

D. Weichert in 1928 produced placentomata in unmated rats with the lutein hormone.

E. Lutein hormone appears only after ovulation, so as to prepare the uterine mucosa to receive the ovum properly and to assist in development of the functioning mammae. This was proved by Hisaw and Leonard (Am. J. Physiol. 92:574, 1930) and by Corner and Allen (Am. J. Physiol. 88:326, 1929).

2. Multiple Fractions of the Lutein Hormone

A. Relaxin A substance, present in almost pure state in the corpora lutea of the sow, responsible for marked relaxation of the pelvic ligaments. (Hisaw: Physiol. Zool., 2:59, 1929)

B. The Mucification Factor Responsible for the mucification of the vaginal mucosa, according to Hisaw, Leonard, et al.

C. The Two Crystalline Fractions
   a) A - Progesterone - melting point 128 degrees, produces decided hyperemia of the uterine mucosa.
   b) B - Progesterone - melts at 121 degrees, produces characteristic transformation of the endometrium.

3. Sources and Quantitative Distribution

A. The lutein hormone is recoverable only in small quantities. 100 grams of ovary (from 10 sows) yield only one rat unit of the hormone. It is even more scarce in the human female.

CHEMISTRY OF THE SEX HORMONES

The chemistry of the sex hormones is a strictly modern problem. Not until 1929 when Doisy and his co-workers (28) isolated estrone in pure crystalline form was the interesting problem of structure made possible. The history of the problem since 1929 has been remarkable in many ways, not the least of which being that in the short space of ten years sex hormones of three important types were isolated, their complicated molecular structures were completely elucidated, and methods were developed for making the pure materials available for clinical use and for biologic experimentation.

"Preceding the chemical researches, and furnishing the necessary foundation for this phase of the work, there was a considerably longer period of biological experimentation leading to the recognition of the existence of the hormones and to the definition of their specific functions. By purely biological methods it was established that all sexual processes of the organism proceed under the influence of certain chemical substances recognizable by specific biological tests and known as the sex hormones. The chemical

work has established the compositions and the structural formulas of the principal members of the group, namely, the follicular hormones oestrone and oestradiol (dihydro-oestrone), progesterone, the hormone of the corpus luteum, and the male hormones androsterone and testosterone. Each hormone exists in various polymorphic forms, and each is accompanied by certain related substances, of which some are inactive and some have the same kind of physiological activity as the principal hormone of the group. Androsterone, testosterone, and all related male hormones have qualitatively similar physiological properties, but the actions of oestrone and progesterone in the female organism are entirely different. In discussing the physiological functions it is convenient to refer to the three principal groups of hormones rather than to specific members which have been isolated in a pure, crystalline condition. The term "oestrin" is applied to the group of which the pure substances oestrone and oestradiol are the typical representatives, and "progestin" designates progesterone and its possible companions of similar physiological properties

present in the corpus luteum". (31)

Pituitary-Gonad Relationship: Before entering into the discussion of the chemical nature of the sex hormones referred to above it seems advantageous to consider some of the more elementary facts about them, and the role played by the pituitary gland. The sex hormones control the growth and the physiological functioning of the reproductive organs and, according to their nature, promote the development of either male or female secondary sex characteristics. The male hormones control the development of the genital organs (and the accessory organs) and have a direct influence on the longevity and motility of the sperm. Corresponding to the greater complexity of the female organism, at least two kinds of hormones seem to be necessary to control the various processes in the estrus cycle and during pregnancy. The first type "oestrin", thought to be formed in the graafian follicles, has its effect on the uterus and vagina and is responsible for the characteristic and well-known changes in the estrus cycle. The second type, "progestin", is formed by the corpus luteum, and, acting in conjunction with

oestrin, controls the so-called "uterine cycle", which in the human is seen as two phases in the periodic preparation for pregnancy. During the first phase, which is under the hormonal influence of oestrin, there is proliferation of the uterine endometrium preparing a functional bed for the gravid ovum. In the event of fertilization of the ovum progestin now exerts its effect on the endometrium, and is responsible for the successful nidation of the ovum. If there is no fertilization the corpus luteum is only transitory, the supply of progestin is shut off, the uterine mucosal bed sloughs off, and along with the accompanying hemorrhage, constitutes the menstrual flow.

However, these sex hormones which have their origin in the ovary are not in complete, primary control of these processes for they owe their origin to the stimulating action of still another kind of sex hormones. These hormones have their origin in the adenohypophysis, and acting directly on the ovary, are responsible for the formation of the follicular and corpus luteum hormones; hence, they are called the gonadotropic hormones.

Historically, the functions of the anterior pituitary were elucidated in three ways. The earlier experiments were firstly clinical observations, secondly a study of the effects of hypophysectomy, and thirdly, the effect of administration of anterior lobe extracts.

That the pituitary gland as a whole was involved in certain general diseases was established many years ago. Marie (25 a) in 1866, established a connection between the gland and acromegaly (although his interpretations were absolutely wrong) and Simmonds, (25 b) in 1914, established further relationships between the gland and other organs of the body by studying the disease which now bears his name.

Paulesco, (25 c) in 1908, demonstrated that death of the animals follows complete hypophysectomy, and this was confirmed by Cushing (25 d and e) and his co-workers. Cushing (25 f) was also able to demonstrate that partial hypophysectomy resulted in adiposity and cessation of sexual activity. As a result of these researches these workers could definitely refer sexual control to the pituitary gland.

Following perfection of methods of hypophysectomy in small animals great advances were made and Smith (25 g and h) in 1927, was able to demonstrate persistent infantilism in rats operated before puberty and cessation of the oestrus cycle and atrophy of the ovaries in rats operated after puberty. There is now an extensive literature indicating the pituitary-gonadal interrelationship.

Next came studies on implants of the gland. In 1926, Aschheim and Zondek (25 i), and independently Smith (25 j) published papers giving the following results:

(1) Implantation of pituitary into a hypophysectomized animal induces follicle growth and corpus luteum formation.

(2) Implanatation of pituitary into immature normal females induces oestrus and its characteristic changes.

The final proof of the relation of the pituitary gland to the sex organs was demonstrated by the preparation of gonadotropic hormones from extracts of the anterior lobe of the pituitary. The first active extracts were prepared by Evans in 1928.

It was shown that these extracts were capable of inducing hypertrophy of the ovary and puberty in the immature female animal.

The above facts are the most important in establishing the historical foundation for the doctrine of pituitary control of the sexual cycle, yet, as presented here, the story is far from complete. The whole problem of the chemistry of the gonad-stimulating hormones still awaits solution for the active principles have not been isolated in a pure condition.

The Estrogenic Hormones: As stated before, the isolation of the estrus-producing hormone in a pure crystalline state was accomplished by Doisy (28) and his co-workers at the St. Louis University School of Medicine in 1929, also independently by Butenandt at Gottingen (16). In each case the material was obtained from pregnancy urine. When such urine is shaken with an immiscible solvent such as ether, butanol, or benzene a considerable portion of the estrogenic material is extracted, for the hormone is readily soluble in all organic solvents and sparingly soluble in water. A better yield is obtained if the urine is acidified and

submitted to hydrolysis before the solvent extraction. Fresh urine is not required, as the hormone does not appear to deteriorate rapidly. In the first step of Doisy's original process the urine was acidified to pH 4, allowed to stand for several days, and extracted with olive oil. The hormone was then extracted with alcohol.

Treatment of human pregnancy urine with acid markedly increases the yield of hormone which can be extracted with solvents, and for the highest yield hydrolysis under drastic conditions is required. According to Fieser, it is not a problem of liberating oestrone and its reduction products from their simple phenolic salts, but of decomposing stable combinations of the substances with constituents of the urine, possibly glucuronic acid. Cohen and Marrian (18, 20) studying the factors influencing hydrolysis, found that for accurate assay of estrone and estriol in pregnancy urine, the urine must be hydrolyzed under conditions which combine maximum liberation of the hormones from the ether-insoluble forms with minimum destruction of the liberated substances. They recommend

that the urine be adjusted to pH 1, further acidified by the addition of 3.3 cc. of 12 N. Hydrochloric acid per 100 cc. of urine, and autoclaved at 120 degrees for two hours. In this work Cohen and Marrian assayed the hormones quantitatively by a colorimetric method based on the Kober test, which depends upon the development of a red color when the hormones are heated with phenolsulfonic acid followed by the addition of water. The results seem to be in good agreement with bio-assays.

The following properties of estrone are known: its empirical formula is $C_{18}H_{22}O_2$, its corrected melting point is 259 degrees, the optical activity is plus 158.5 degrees (dextrorotatory), it is soluble in water to the extent of 2.1 mgm. per liter; its dissociation constant is 0.44×10^{-9}, and its physiological activity is said to be 8 to 10 million mouse units per gram. Microchemical observations of the melting point indicated that estrone can exist in no less than three polymorphic forms, melting at 254, 256, and 259 degrees. Structurally, the estrogenic hormone contains the cyclopentenophenanthreene carbon skeleton. Among the large number of interesting naturally occurring compounds which possess this

carbon skeleton are four groups of hormones: the estrogens of the ovary, the andorgens of the testis, the progestational hormone of the corpus luteum, and the "life-maintaining" hormones of the adrenal cortex. Marrian (65) has suggested that "steroid" be used as a group name for all substances containing this basic skeleton. Other closely related compounds, having essentially the same basic structure are the cardiac glycosides, the heart poisons secreted by toads, certain of the hemolytic saponins, the bile acids, certain antirarchitic agents, alkaloids of the morphine and aporphine groups, the acids obtainable from resins of conifers, the triterpenoid saponins, and certain of the carcinogenic hydrocarbons. This last relationship opens up fascinating speculations in regard to the possible role of the steroid hormones in the production of carcinoma. The story of the elucidation of the structural configuration of the sex hormones is a long one, and of general interest only to the biological chemist. For an excellent review of the detailed chemistry the reader is referred to "Chemistry of Natural Products Related to Phenanthrene" by Fieser, Reinhold Publishing Co., New York, 1936.

The estrogenic hormone has been known by a number of different names, including theelin, the name given by Doisy, the progynon, the name given by Butenandt. The English nomenclature (1) has been adopted by the League of Nations Conference because it indicates the ketonic character of the hormone and its most important physiological action; these terms are oestrone, oestradiol for the alcohol resulting from the reduction of the hormone, and oestriol for the companion substance having three hydroxyl groups.

The average estrogenic activity of pregnancy urine is about 10,000 mouse units per liter, corresponding to about 1 mgm. of the pure oestrone per liter; only a fraction of this can be obtained by ordinary laboratory technic. In 1930 Zondek discovered a new and better source of the hormone in the urine of pregnant mares. the average activity of which he found to be 100,000 mouse units per liter. This abundant source greatly expedited the work of isolation, although it is present in an ether-insoluble form and only 10-25% is directly extractable. Further investigation of the occurrence of the hormone by Zondek revealed an even richer

source. The paradox of a higher excretion of the female sex hormone by the male than by the female animal he found in (and only in) the equines. For example, he found that stallion urine contains, on the average, 170,000 mouse units per liter. Cartland, (17) et al., found this a superior source, the hormone being relatively free from closely related substances found in mare urine. The urine was covered with one-third its volume of butanol, acidified strongly, and refluxed for four hours. The aqueous layer was extracted twice with butanol at room temperature by shaking and the combined butanol solutions were extracted with dilute soda solution and concentration in vacuum under nitrogen. These workers found a yield of 16 mgm. of almost pure crystalline oestrone per liter of urine.

Another discovery, almost as surprising as the above, is that oestrin occurs in the vegetable kingdom. It has been observed that extracts from various flowers, as well as from certain lower animals and bituminous substances, possess definite oestrogenic activity, and in two cases there is definite proof that the active principle was present. Pure oestrone has been isolated from a palm kernel extract, and a

crystallizate of pure oestriol has been obtained from female willow flowers.

Oestriol: Shortly after the discovery of oestrone by Doisy and Butenandt another crystalline substance was obtained (from the urine of pregnant women) that had a distinctly higher melting point than oestrone. This was discovered by Marrian (63) and was subsequently found to be fairly active as judged by the Allen-Doisy test. The nature of the substance was not clear, but investigation by Marrian indicated that the formula was $C_{18}H_{24}O_3$ and this suggested that it might be a hydrate of the hydroxketone (estrone). This hypothesis was later verified by Butenandt who was able to prepare oestone by heating Marrian's substance with potassium hydrogen sulfate and distilling it in a high vacuum. Doisy and his co-workers independently discovered oestriol sometime later while trying to perfect a process for the isolation of oestrone. (27) They found sufficient difference in the acidic strength of the two phenols to permit a nearly quantitative separation. Oestriol alone is extracted by 0.1N NaOH solution from an ethereal solution of the mixture, and oestrone then can be extracted with normal alkali.

This method yields about 03. mgm. of oestrone and 1.3 mgm. of estriol per liter of human pregnancy urine. In view of the finding of this new oestrogen Doisy suggested at this time that the name "folliculin" be dropped.

The properties of oestriol are as follows: its formula has been given as $C_{18}H_{24}O_3$, its corrected melting point is 280 degrees, while that of the triacetate is 127, and that of the methyl ether 159 degrees. Its optical activity is plus 30 degrees, and its dissociation constant 0.77×10^{-9}. The physiological activity is said to be 75,000 mouse units per gram of the pure crystals. The oestrogenic potency of the hydrate is only about one-hundredth that of the ketonic hormone, and its action has been found to be somewhat more protracted. The triacetate is about 10 times as active as oestriol. Structurally, oestriol differs from oestrone only in that one molecule of water has been split off C16 and C17, leaving a double bone oxygen at C17.

Of particular interest is the fact that a series of unsaturated hydroxy ketones, entirely similar to oestrone in structure and action, have been isolated from the urine of pregnant mares.

These are equilin, hippulin and equilenin. Equilin and hippulin are isomeric, containing two atoms of hydrogen less than oestrone, and possessing one alicyclic double bond in addition to those present in a benzene nucleus.

Oestradiol: According to the Allen-Doisy test, oestrone is the most potent of the oestrogenic compounds that occur naturally. However, the hormone is surpassed in activity by certain substances which can be obtained from it by chemical transformations. Most important of these is the dihydro compound oestradiol, which is prepared by the reduction of oestrone at the carbonyl group. The phenolic benzoate has a particularly high potency, and like the other esters, is characterized by a protracted and persistent action. Oestradiol monobenzoate is available clinically as "Progynon B" and is regarded as a particularly valuable substance.

The Corpus Luteum Hormone: As indicated before, the corpus luteum has a direct effect on the uterine mucosa in preparing it for the reception of the fertilized ovum. If conception occurs the corpus luteum persists and serves to suppress ovulation,

maintain a condition in the uterus essential to the development of the embryo, inhibit uterine motility, and, in conjunction with oestrin, induces mammary gland development.

That the corpus luteum had a hormonal action was first suggested by Fraenkel and others who observed that in rabbits the removal of the corpora lutea shortly after ovulation terminates pregnancy. This was further substantiated by Corner and Allen, (21, 22, 23) 1928 when they showed that after progestational changes have been prevented by ablation of the early corpus luteum, activity can again be restored by the use of extracts of corpora lutea. Furthermore, pregnancy can be maintained after castration by the use of such extracts. This work not only opened the way for chemical studies but also provided a convenient assay method which will be described later.

Allen (2) and Hisaw (49) shortly devised methods for the preparation of crystallinizates from corpus luteum. These were found to be physiologically active but were not pure enough to permit much in the way of chemical study, and chemical characterization of this substance was very difficult because of the

expense of the starting material and the great sensitiveness of the hormone to alkalies and to oxidizing agents. However, in 1934, independent preparation of the pure corpus luteum hormone was announced from four different laboratories, three of which were in Germany. Allen and Wintersteiner (7) working in this country, announced the preparation of substances melting at 128, 121, and 190 degrees. Two of these were active diketones, and crystallographic studies showed that they were closely related in chemical behavior; they were both shown to have the same empirical formula, and their polymorphism was demonstrated by interconversion of the two forms. These were designated as alpha and beta-progesterone, but the term progestin has been retained to be used in a general sense similar to oestrin.

Allen and Meyer (5) developed procedures for the preparation of purified extracts which are in general use, with modifications, by all of the investigators. The extraction processes involve solvent extraction and various partitions between solvents. After partial concentration in petroleum ether, practically all of the active material can be

precipitated as the very sparingly soluble semicarbazone. Following hydrolysis, further purification can be accomplished by sublimation in high vacuum, by selective adsorption, and by crystallization. The inactive hydroxyketone with the high melting point is best removed as the acetate.

Progesterone is very sparingly soluble in water and readily soluble in the usual organic solvents. The pure material is colorless and does not deteriorate on storage. Its formula is given as $C_{21}H_{30}O_2$, and its optical activity is 192 degrees of dextrorotation. Its physiological activity is given as one rabbit unite per 0.5-1.0 mgm.

The observation that progesterone ($C_{21}H_{30}O_2$) and the inactive hydroxyketone pregnanolone ($C_{21}H_{34}O_2$) differ in composition only by four hydrogen atoms suggested that the latter is a hydrogenation product of the former, or at least that they are closely related. A further obvious inference was that they bore some relationship to prenganediol ($C_{21}H_{36}O_2$), a non-estrogenic compound discovered by Marrian (64) while searching for the follicular hormone. The structural formula was largely worked out by Butenandt who found its reactions to be entirely similar to those

of the bile acids and other steroids. This inactive alcohol (pregnanediol) is thought to represent an intermediate stage in the degradation of sterols or bile acids in the organism to the hormones, or else it arises in a closely related degradative process. It is found only in human pregnancy urine. Continuing the comparison of progesterone with other known compounds, and studying it further by means of the ultraviolet absorption band and X-Ray, complete proof was available, six months after its isolation, of its structure.

Some interesting observations have been made recently in regard to some of the biochemistry of progesterone. In 1936, Venning and Browne (74) extracted sodium pregnanediol glucoronide from the urine of pregnant women, using butyl alcohol. Further studies by Venning and his co-workers (75,76) associated the urinary excretion of this compound with the progestational phase of the menstrual cycle. Later (77) they concluded that, in common with the other sex sterols, progesterone is excreted only in the hydrosoluble conjugated form. Just recently Hamblen, Ashley and Baptist (45) investigated the

relation of pregnanediol and pregesterone and came to the following conclusions:

"Four factors are concerned in the metabolism of progesterone and in subsequent urinary excretion of sodium pregnanediol glucuronide: a)ovarian, involving the formation of progesterone by post-ovulational corpora lutea and possibly from the marginal granulosal luteinization of follicles; b) endometrial, concerned with the alteration of progesterone into pregnanediol; c) hepatic, which brings about the conjugation of pregnanediol with glucoronic acid; and, d) renal, involving the excretion of sodium pregnanediol glucoronide."

The excretion of this compound indicates the functional capacity of all of these factors; the absence of the compound from the urine indicates the functional failure of one or more of these factors, but is not conclusive evidence that ovarian function is inadequate. Furthermore, its excretion is not evidence that an endometrium is undergoing progestational proliferation, but according to Stover and Pratt (72) "evidence is accumulated to indicate that excretion of pregnanediol signifies luteal activity" but that "on account of the variation in pregnanediol

excretion in normal cases, it is premature to draw conclusions of abnormal states from single determinations of pregnanediol."

EXTRACTION AND ASSAY OF ESTRONE

I. URINE

(A) Method of Kurzrok and Ratner - The method for extraction and assay of follicular hormone in the twenty-four hour urine specimen as utulized by Kurzrok and Ratner (55) is as follows:

"The twenty-four hour urine specimen is measured and 700 c.c. placed in a one-liter flat-bottomed flask (A) This is made weakly acid with acetic acid. The hormone is more easily extracted from an acid solution. The urine is then saturated with sodium chloride which decreases the solubility of ethyl acetate in the urine. The treated urine is then covered with ethyl acetate halfway up to the neck of the flask and the flask connected as shown in Fig. 1. The second flask (B) of 300 c.c. capacity is filled with 250 c.c. of ethyl acetate. The steam bath (C) is turned on and the ethyl acetate distills over through the upper side arm, is condensed and drops to the bottom of the flask A from which it returns through the lower side arm to flask B. The drops of ethyl acetate in passing upwards through the urine

extract the hormone. This provides continuous extraction with pure ethyl acetate. The extraction is continued for twenty-four hours. It is automatic and does not need watching. The extracted urine is then discarded, and the ethyl acetate extract, which contains the hormone, is concentrated by vacuum distillation. The distilling flask (A) 300 c.c., is filled one-third full with the ethyl acetate extract and connected with the condenser (B), a receiving flask (C), a second receiving flask (D), a trap (E) and finally an aspirator type suction pump (F). After the distilling flask has been heated in a steam bath, the suction is turned on. The remaining extract is allowed to drop into flask (A) through the thistle tube (G) which is provided with a stopcock, at about the same rate as the ethyl acetate distills over. When all the extract is finally added, it is concentrated further to about 30 or 40 c.c. and the 10 c.c. of olive oil is slowly admitted through the thistle tube as the distillation progresses. The distillation is continued until all the ethyl acetate has been removed. If bumping occurs, the suction should be reduced with the screw clamp at (H). The oil now contains the hormone

originally present in the 700 c.c. or urine.

This 10 c.c. of oil, prepared as described above, is used for the biologic assay. Two groups of castrated rats are selected containing three animals in each group . They are injected with the oil, subcutaneously, in the back, at 9 A.M. and 5 P. M. of the first day, and 9 A.M. of the second day. Group 1 received 0.5 c.c. of oil at each injection. Group 2 received 0.25 c.c. of the oil at each injection. Vaginal smears are taken forty-eight, fifty-six, and seventy-two hours after the first injection. A No. 13 dental spatula is very convenient for obtaining the vaginal secretion, which is then spread in a drop of saline on a glass slide. If no fewer than two animals of a group show the cornified cells characteristic of estrus, the injected marerial may be considered active. Mucus, leucocytes and epithelial cells must be absent to establish a positive result. The least amount of oil necessary to produce a positive smear is considered as containing one rat unit.

The hormone content of a liter of urine expressed in terms of rat units, may be calculated from the following formula.

$$\frac{10}{y} \times \frac{1000}{700} = \text{Rat unit per liter of urine,}$$

when y equals the total amount of oil injected into a single rat from the group receiving the smallest amount necessary to produce positive smears.

For example: If Group 1 should have positive smears and Group 2 negative smears, then there would be 9 rat units per liter of urine, while if both Groups 1 and 2 showed positive smears, there would be at least 19 rat units present. Larger amounts of hormone may be determined by using smaller quantities of oil for injection, or by making the necessary dilutions. The calculations may also be made for the total amount of urine excreted in twenty-four hours by substituting this amount in the equation in place of 1000."

(B) Bergen and Smelser Modification of Estrone Extraction: Kurzok (54) has outlined a previously unpublished modification of the above method by Bergen and Smelser. Briefly the method is as follows: A twenty-four-hour urine specimen is collected and 750 c.c. of it is poured into a 2-liter flask. After making the urine slightly acid with acetic acid, 50

grams of sodium chloride then 750 c.c. of ethyl acetate are added. After vigorous shaking for three minutes, the urine fraction is drawn off by means of a separatory funnel. The ethyl acetate fraction is allowed to stand a few hours, then is drawn off and distilled according to the method described above. The biologic assay is the same as above. If an emulsion results during the shaking one should allow the ethyl acetate fraction to stand over night, then it should be transferred to a clean flask. The specimen should be centrifuged if the emulsion is very heavy.

"The variable factors which may affect the standardization of estrone fall into the following main groups: (a) individual variation in sensitivity of the test animals, (b) variation in the condition of the animals, (c) difference in the method of administration, (d) difference in criteria of activity." (66)

That great care must be taken in interpreting the results of assaying estrone by the original method (graduated doses, considering the unit as the least amount required to produce estrus in an ovariectomized animal) has been pointed out by Coward and Burn. (24)

These authors have pointed out that individual variation in sensitivity and variation in the same animal from time to time may result in a given amount being inactive in one animal and a smaller amount active in another. They concluded that the administration of graduated amounts is of value for accurate assay only when comparatively large batches of animals are used. Winton (78) has shown that when plotting curves for the results of this type of work, the curves tend to be much steeper for more homozygous material. Coward and Burn suggested that the unit should be defined as the amount required to bring 50% of a batch of 20 ovariectomized animals into estrus, but Allan, Dickens, Dodds and Howitt (3) claim that the use of 20 animals to the batch is insufficient to overcome individual variation even when very homozygous (Wistar) rats are used. These authors observed quite considerable variation in response of groups of 20 to the injection of the same amount. However, for practical purposes it seems that the method of assay as given above gives satisfactory results, especially in the hands of experienced workers. Rarely is it possible for the average laboratory to work with such large numbers

of animals, as the expense and time involved prohibit it.

In regard to the condition of these test animals, weight and time after ovariectomy seem to affect the sensitivity most. Variation in weight, by causing variation in amount given per gram, might well be supposed to cause variation in response, and Bugbee and Simond (14) correct their results to allow for variation in weight. Other workers have attached variable significance to this factor; Coward and Burn (24) failed to find that this factor had any influence. The time after ovariectomy may be of importance for two reasons, viz., the ovariectomized animal very rapidly lays on fat, and progressive atrophy of the uterus and vagina follow operation. Coincident with this atrophy there is a decline in sensitivity.

The optimum site for administration of the test substance is quite universally agreed to be subcutaneous, because by reason of the rather slow absorption rate this most nearly simulates the events in the normal animal and is most nearly physiologically sound. Since the stimulus responsible for the production of estrus in the normal animal is undoubtedly

exerted over a period of 24 hours before the appearance of estrus it is necessary to arrange for the absorption of a continuous supply of the hormone over at least a similar period. With the old fatty extracts absorption was slow and one injection would give this continuous supply. However, Dodds and others, working with water soluble preparations give the dose in six injections over 48 hours. Marrian and Parkes investigating these factors concluded that, for routine testing, the optimum method is to give four injections at 12 hour intervals, i.e., to cover 36 hours.

Variations in results among the different workers using the above method of assay are partly due to variations in the criteria of estrus. Some workers consider that the mere disappearance of leucocytes from the smear is sufficient to indicate a positive reaction (66, a,b) while others insist on complete cornification of the vaginal contents (66 c,d). Obviously, reasonably accurate and consistent results may be obtained in any given clinic by adopting and adhering to a given smear as an end point.

The use of the weight of the immature rodent uterus in the assay of gonadotropic substances has

been established. Levin and Tyndale (59) worked out a method using mice and Heller, Lauson and Sevringhaus (47) employed rats. Lauson, Heller, Golden and Sevringhaus (56) investigating the mechanism of the uterine reaction worked out a similar assay for estrogens and used it for comparing the actions of estrone and its reduction compounds estradiol (dihydroxyoestrin) and estriol (trihydroxyestrin).

The method employed was as follows: "Female albino rats of the Sprague-Dawley strain, 22 to 23 days old and 34 to 39 grams in weight, were used in all of the experiments. Aqueous solutions of crystalline estradiol, estrone, and estriol were injected subcutaneously twice daily for three days. One-half c.c. was given at each injection. On the morning of the fourth day (72 to 75 hours after the first injection) body weight and vaginal opening were noted, and the animals were killed by rapid decapitation. The uteri were separated from the vaginas by cutting through the cervix, the surrounding tissue was stripped off and the uterotubal junction severed. The uteri were weighed immediately after the intrauterine fluid had been pressed out on moistened

blotting paper. Weights were read to tenths of a mgm. on a Roller-Smith torsion balance. Weights of the uteri before fluid was expressed and of the vaginas and ovaries were also recorded, but since they have been found to have no particular significance to this paper, they will not be included."

These workers, employing a large number of animals, prepared standard dosage-response curves for estradiol and estrone, the usefulness of which was tested by a large series of assays. In these test assays small numbers of rats were used (usually one to four) and then from the uterine weights obtained with unknown solutions they were able to read off (within practical limits) the amount of estrogen present, the results checking quite closely. The chief disadvantages of their method are two in number: first, a dependable supply of immature rats is needed; and, second, the animals can be used only once. However, several advantages are apparent: there is a completely objective endpoint, no castrating, priming or other laborious standardization procedures are required, the range of reactivity is large, greatly reducing the number

of preliminary assays required and finally, the uniformity of response makes possible consistent relative accuracy with very few animals per assay. Since the material available clinically is often very limited in amount this last fact becomes most important.

The principles upon which assays of this type are based are not new since Frank (35) in 1929 devised a quantitative test for estrin based on the old observation that estrin stimulates growth of the rabbit uterus. Astwood (12) studied these effects of estrogens in the immature rat and found that during the first few hours following an injection of estrogen the uterus undergoes a rapid change, accompanied by an increase in weight, due almost entirely to an accumulation of water. The quantitative aspects of this response have calibrated by Astwood and a simple method for accurate assay of estrogens devised. (13)

Working with a large number of animals he found that a single injection of estrogen caused a maximum response in 6 hours, followed by a decrease in weight, then a second increase during the next 15 hours. Investigation showed that the first response

was purely a hydroptic change, with maximal imbibition of water in the endometrial stroma, and that the second represents a true increase in protoplasm. Results obtained over a 6 months period were collected amd a standard curve produced from which it was apparent that a direct logarithmic relationship exists between dosage and uterine response, between certain dose levels. Astwood showed that the maximal dose in the reactive range is 16 times greater than the minimal. "This wide range of dosage over which the uterine response is proportional greatly facilitates assay, for the number of dilutions of an unknown solution which may be tested need by very few. For example, if an unknown solution be diluted in multiples of 4, and each dilution injected into five animals, at least two of these dosages will fall within the reaction range, and from them the estrogenic potency can be read directly. A very close approximation to the true value can thus be obtained in six hours."

Comparing Astwood's method with that of Lauson, et al, it seems that the former should yield more accurate results if larger numbers of animals are used, but if small groups were used in both assays,

the latter would yield more consistently useful results.

The methods of assay of which the above are representative, namely, the Allen-Doisy vaginal spread and the organ weight methods, are the two most commonly employed at present, both for clinical assays and for standardization procedures. There are as many variations in technic as there are workers in the field but most of these variations are relatively minor. For reviews of the various technics and their respective merits the reader is referred to the papers of Allen and Doisy (4) Marrian (62), Dorfman, et al (29), Bulbring and Burn (15), and McPhail (67). Hain and Robson have compared the assay of estrin in the rat and the mouse and have reviewed a good portion of the foreign literature.

In the literature of the last four or five years has appeared a number of procedures for the determination of estrogens by other than biologic assay. A few of these will be briefly mentioned.

Cohen and Marrian (18), and also Kober (53) have devised a method for the colorimetric determination of estrin in urine. Alcoholic solutions

of estrogenic residues prepared from urine by phenolic fractionation are analysed in a Lovibond Tintometer and the weights of estrone (or estriol) present in an aliquot sample are read from the appropriate standarization curve.

Allen, Smith, and Gardner (6) described a short test for estrogenic substances, completed in 10 to 16 hours as compared with 48 to 56 hours for the vaginal smear test. It substitutes recognition of early growth produced by the estrogenic hormone in the vaginal wall for the complete estrous growth as required by the original test. This is made possible without sacrificing the clear-cut nature of the end-point, by the use of colchicine with the estrogenic material to be tested. Active estrogenic material starts mitotic division of the basal cells of the vaginal epithelium. Colchicine arrests the mitoses, holding the dividing cells in metaphase, thus accumulating the evidence of growth. Biopsy specimens of vagina are used instead of vaginal smears for the determination of the end-point.

Venning, Evely, Harkness and Browne (76) make use of a photoelectric colorimeter. Estrin compounds extracted from 300 c.c. of urine with butyl

alcohol are hydrolyzed by autoclaving with concentrated HCl, and reextracted with peroxide-free ether. The residue is dissolved in alcohol and an aliquot taken for the determination. The estrin solution is evaporated to dryness and treated with a mixture of 3.6 parts of phenol and 5.6 parts of concentrated sulphuric acid and the tube placed in a boiling-water bath for 20 minutes. Readings are taken through blue and green filters in the photoelectric colorimeter. The amount of estrin is determined by the use of a normogram. According to the authors between 500 and 5000 micrograms of estrin per liter of urine can be determined with a maximum error of 10%.

II. BLOOD

(A) Method of Fluhmann - Fluhmann, in an attempt to devise a simpler test for estrogens in the blood, worked out the following procedure. It is dependent upon the formation, in the vaginal mucosa of recently spayed mice, of tall columnar cells secreting mucus (mucification), a change which precedes cornification as described in the original Allen-Doisy technique. It has been accepted by the Stanford University Gynecological Laboratory

as presented here.

25 to 40 c.c. of venous blood are collected in a sterile test tube for tansmission to the laboratory. The sample is centrifuged, the cells discarded, and the clear serum kept sterile and refrigerated.

The test animals employed are recently spayed adult female mice. It is important that oophorectomy should be done not shorter or longer than seven days prior to assay to avoid unusual changes difficult of interpretation.

A total of 4.5 c.c. of serum is given each test animal by three daily injections, subcutaneous, of 0.5 c.c. for three days. The sites of injection should be varied as much as possible to facilitate absorption. On the fourth morning the animal is sacrificed. The vagina is carefully dissected free and, after formalin fixation, sections at different levels are made and stained with hematoxylin-eosin.

Six reactions, of variable intensity, are recognized by Fluhmann:

Reaction 0 - Atrophy of the vagina, the mucosa showing two layers of cuboidal epithelium.

Reaction 1 - Vaginal mucosa shows two layers, a basal layer of low cuboidal, and a superficial layer of tall columnar cells. A few leucocytes are present.

Reaction 2 - The superficial cells are high, there is beginning stratificiation and mucus secretion. There is a well marked increase in white cells.

Reaction 3 - The vaginal mucosa is multi-layered, the superficial cells are mucified. Leucocytes are numerous. Rapid growth may produce a characteristic folding-in, or festooning, of the mucosa.

Reaction 4 - The mucosa has 6-12 rows of cells, the superficial cells are mucified, the lower cells resemble those of the basal layer of squamous epithelium. Leucocytes are diminished. Early cornification may be present.

Reaction 5 - The vaginal lining is fully developed squamous epithelium with cornified cells. There are no white cells.

Since individual biologic variation is present here as in all biologic procedures, accuracy is increased as the number of test animals per specimen is

increased. Two or three mice should be the absolute minimum. The reactions of each animal are estimated the numbers totaled and the total is divided by the number of mice used to give a final reaction value. For example, reactions of 3, 2, and 2 in three mice would be interpreted as a reaction of 2.3 for the specimen.

In order to be able to interpret such results on a quantitative basis, Fluhman worked out the average reaction for known gradient doses of estrogenic substance and tabulated them. His control results run from an average reaction of 1.1 with 1/32 of a mouse unit to an average reaction of 5.0 with two mouse units. His tabulated results would indicate an increase in probable error as the amount of estrogenic substance decreases.

(B) Modified Method of Frank and Goldberger

Frank and his co-worders published numerous papers up to 1926 giving technics for the demonstration of female sex hormone in the circulating blood of both animals and the human female. (See Frank, et al, 36,37, 38) In 1926 they discovered that the hormone could be detected in the blood of gravid women as early as the 6th to 8th week and subsequently published a standardized

procedure. (39) These were alcohol extraction methods. Sometime later the procedure was modified in that ether instead of alcohol was used for extraction, (40) but advance in the knowledge of the chemistry of the estrogens showed that some of the estrogenic compounds were ether insoluble. In consequence, Frank and Goldberger returned to a modification of their earliest technic in which alcohol extraction was used, (41) with a resultant great increase in the estrogenic activity of the extract.

The new technic requires 50 c.c. of venous blood. This is dehydrated in anydrous sodium sulphate, following which it is extracted twice with 200 c.c. of 95% ethyl alcohol. The alcohol fractions are combined then evaporated to dryness on a water bath. The residuum is taken up in 5 c.c. of olive oil and injected into spayed mice. The bio-assay is carried out according to the Allen-Doisy method as previously described.

(C) Neustaedter Modification - Neustaedter, working at the New York Post-Graduate Medical School and Hospital, used the Frank-Goldberger method in the Endocrinology Laboratory of the Gynecology Department on a large number of women and found the

test satisfactory, with one exception: according to him it is not always possible to emulsify the lipoid residue with water. (68) At times he overcame this difficulty by rubbing the residue with acacia. Subsequently, however, he modified the procedure somewhat. 40 c.c. of blood are anhydrated with anhydrous sodium sulphate, then twice extracted with ether. (See previous comment under B.) The lipoid residue is then taken up in 6 c.c. of benzene to which 0.6 c.c. of olive oil is added, following which the benzene is allowed to evaporate. This then is used for Allen-Doisy assay. Using this procedure in 44 normal, fertile menstruating women Neustaedter obtained results practically identical with those of Frank and Goldberger.

In a recent article, Taylor (73) summarizes the present status of the bio-assay of estrogenic substance in the blood and urine, especially as regards its practicability for routine hospital use. He says, "The assay of a single specimen of urine for estrin involves several chemical steps and the use of perhaps twenty castrated mice or rats. It must also be remembered that the excretion of estrin is quite variable throughout the menstrual cycle, and that the report on a single specimen may

give a quite erroneous idea of the patient's ovarian function. Even four specimens taken at weekly intervals may accidentally strike only the peaks or the depressions in the irregular curve of estrin excretion. It is scarcely possible to avoid, then, some system of continuous study, such as the assay of consecutive 72 hour specimens, throughout the cycle. Such a routine is confining to the patient and the actual cost of the test to the laboratory cannot be less than a hundred dollars. Test made upon the blood are even less satisfactory, for a mouse unit is rarely present in less than 40 c.c. The amount of blood required practically precludes repeated tests or a quantitative assay.

The indications for such an assay of the estrogenic hormones in practice are at present few. If a patient menstruates, it may be assumed that a definite amount of estrogenic substance is being excreted. Furthermore, enough data have not yet accumulated upon urinary assays to define the range which should be considered normal. Only if very large or very small amounts are excreted can the figure be given much significance. In amenorrhea, the test may be of use in determining prognosis and

possibly in avoiding the mistake of treating a rare case of hyperhormonal amenorrhea with estrogenic substance."

EXTRACTION AND ASSAY OF GONADOTROPIC HORMONES

Most of the present day methods of the demonstration of the gonadotropic substances in the blood and urine depend on the changes in appearance of the reproductive system (usually of uterus or ovary) of the immature rodent. The basis for these procedures was laid in 1921 when Evans and Long (30) succeeded in producing very definite changes in the reproductive system of the white rat by the intraperitoneal injection of an alkaline preparation of bovine anterior hyophyseal substance. Some years later Smith and Engle (71) in this country and Aschheim and Zondek (9) in Germany observed that injections of fresh pituitary gland tissue into immature mice and rats leads to a precocious sexual maturity. In 1928 Aschheim and Zondek (10,11) described their "pregnancy test" and showed that the urine of pregnant women contains large amounts of some hormone which has the property of inducing changes in the ovaries of laboratory animals. A standard procedure for the detection of gonadotropic hormones thus became available, and they were found in many body

tissues and fluids under varying conditions, in gravid and post-climacteric women, and in the urine of people with certain tumors, especially chorionepithelioma and teratoma of the testicle.

As indicated before, there is considerable evidence pointing to duality of "the gonadotropic hormones", but the chemical separation of follicle-stimulating from luteinizing fractions (Prolan A from B) has proven very difficult, and they have not been isolated in pure forms, if they exist. It has never been decided, furthermore, whether the urinary prolans and those of the adenohypophysis are identical. The consensus of opinion is that the hormones are similar but not identical. Therefore, it seems logical that any attempt to assay one or the other, at the present, would be of experimental value only. However, Zondek (79) and Loeser (60) have described in the German literature methods for assay of the luteinizing hormone. Suffice it to say that the following are representative of methods of assay of the gonadotropic hormones.

I URINE

(A) Modification of Zondek's Method - The

procedure, as given here, is the one worked out by Kurzrok at Columbia University. For the extraction, 47 c.c. of morning urine are made slightly acid with dilute acetic acid then placed in a 250 c.c. centrifuge tube. 200 c.c. of 95% ethyl alcohol are added then the mixture is allowed to stand for 2 to 24 hours. After centrifuging the supernatant fluid is discarded. To remove the estrogenic substances 20 c.c. of ethyl ether are added and the mixture thoroughly shaken to break up the precipitate, which is then separated by centrifuging. The supernatant fluid is discarded. The ether is allowed to evaporate until the precipitate is dry following which 7 c.c. of distilled water are added and the mixture is centrifuged again, and the supernantant fluid, which now contains the follicle stimulating hormone, is collected.

For the assay, three immature female mice weighing from 6 to 8 grams are injected twice daily for three days. Each mouse received 0.25 c.c. at each injection, subcutaneously in the back, using a 1 c.c. tuberculin syringe and a No. 24 hypodermic needle. The mice are not injected on the fourth day but are autopsied 100 hours after the first injection.

A macroscopic examination of the ovaries and uterus is made. A positive test shows enlarged, reddened ovaries showing large follicles, and an enlarged and distended uterus and an open vagina. A negative test shows small white ovaries and a threadlike uterus. The extract should be kept refrigerated. Each mouse receives the equivalent of 10.5 c.c. of urine. If the test is positive it means that there are at least 100 mouse units of the follicles stimulating hormone per liter. By diluting the remaining hormone solution it is possible to determine, more or less accurately, the amount of hormone present.

Aschheim (8) described this test in the American literature to be used qualitatively for the early diagnosis of pregnancy, chorionepithelioma, and hydatiform mole. Reinhart and Scott (69) and Schneider (70) following the work of Friedman (43) greatly simplified the test for the demonstration of excess of this hormone during pregnancy by using a single, non-pregnancy rabbit weighing not less than 4 pounds. 10 to 15 c.c. of freshly passed urine are injected into the marginal ear vein. Twenty-four hours later celiotomy is done and the ovaries and

uterus are examined macroscopically. A positive reaction is evidenced by enlargement of the ovaries to two or three times normal size, and by minute yellowish protrusions of corpora lutea, or cyanotic protrusions which are due to hemorrhages into a follicle or a corpus luteum. There is also often swelling and hyperemia of the uterus. The advantages of this procedure over the original test are obvious; a single injection is made and the animals can be used repeatedly.

(B) Method of Levin and Tyndale - Levin and Tyndale (57,58) while attempting to concentrate the gonadotropic activity of the urine of post-menopausal (or castrate) women, found that the active material could be practically quantitatively converted into non-toxic extracts by the use of tannic acid. Their procedure is as follows:

The urine is preserved with chloroform, chilled, siphoned from any sediment, and brought to pH 5.0 with acetic acid. To each liter of urine 20 c.c. of fresh aqueous 10% tannic acid are added, forming an immediate precipitate. The heavy precipitate is collected by centrifuging and is then extracted repeatedly with 95%, later 80% ethyl

alcohol; this is removed by several washings with acetone and the residue is made acetone-free by reduced pressure. This alcohol-acetone treatment, in addition to removing considerable inert material, removes any estrogens present.

This dry tannate is quite stable (lasts for a year or more) and each liter of urine yields 100-200 mgm. It contains from 75 to 100% of the original gonadotropic activity as indicated by parallel assays of the raw urine.

For assay, 20-22-day-old mice (8-12 grams body weight) are used. The mouse unit, as established by Levin and Tyndale, is defined as that amount which, when administered in three divided doses on three successive days by subcutaneous injection, will cause:

1. Vaginal canalization.
2. At least a 200% increase in the weight of the uterus drained of fluid (controls 4-7 mgm.)
3. A slight increase in ovarian weight as compared to uninjected controls.

These criteria were adopted because, with minimal doses, ovarian weight alone is quite unreliable. They claim the unit is quite sensitive and accurate in the

absence of estrogens.

These authors have described purification methods for these crude tannates which yield 1015 mgm. for each liter of pooled urine and in which the original activity of the urine is concentrated 10 to 20 thousand times; these purified extracts are non-toxic even in large doses.

(C) Method of Katzman and Doisy - These authors (51) described a method for the quantitative determination of small amounts of the gonadotropic material based upon tungstic acid precipitation and removal of the tungstate by means of barium, but because the preparation was often decidedly toxic, a later modification using solid brucine instead of barium and prepipitation by benzoic acid followed by filtration was presented. The activity of the resulting preparation was recognized by its ability to produce vaginal canalization and cornified vaginal epithelium (estrus smear), and recovery experiments indicated that the extraction was adequate for measuring small amounts (52).

(D) Method of Drips and Osterberg - These authors, investigating the urine of women suffering from menopausual vasomotor manifestations found that

methods previously in use (including most of those mentioned above) very often gave negative results where the urinary prolan concentration was less than 66 rat units per liter. Wishing to be able to differentiate ovarian failure from decreased pituitary function (in which instance very little or no prolan would be present) they evaluated a number of procedures and the method as adopted by them seems to give the best results obtainable to date.

The technic is that of Frank, Salmon and Friedman (42) which is a modification of the original Aschheim-Zondek precipitation (79) method, using acetone instead of alcohol as acetone makes a more stable precipitate. Instead of beginning with 60 c.c. of urine, 40 c.c. is used and in the final amount for biologic assay (8 c.c.) 2 c.c. represents 100 c.c. of urine.

Four hundred cubic centimeters of urine are taken from a fresh 24 hour specimen and acidified with concentrated acetic acid to pH 3.5 (Congo Red). Four volumes of cold acetone are added to the urine, shaken vigorously, and allowed to stand over night in the refrigerator. The supernatant fluid is poured off and the precipitate extracted with weak sodium hydroxide. The pH of the mixture is adjusted to

between 8 and 8.5, the residual precipitate stirred thoroughly, the mixture centrifuged, and the final precipitate discarded as the supernatant fluid contains the gonadotropic principle. The fluid is then adjusted to pH 7 with dilute acetic acid.

In making the biologic assay, varying amounts of the extract are injected into immature animals, (22-24 gram rats) in a series of injections over 2 or 3 days, as is usual. The smallest amount of injected material which gives a positive reaction represents the largest amount of prolan present in the urine and vice versa. For example, a positive reaction with 0.2 c.c. represents 100 rat units, a positive reaction with 2 c.c. of extract represents 10 rat units, and a positive reaction with 4 c.c. represents 5 rat units per liter of urine.

Albino rats are used; the uteri and ovaries are examined grossly and weighed; the ovaries are then fixed in Bouin's fluid, embedded in paraffin and after cutting and staining in the usual manner, are observed for evidence of follicle stimulation and luteinization.

The Levin assay made use of uterine weight and Levin found out that the uterus showed more change

at the end of 72 hours than at the usual period of 96 hours. However, the microscopic changes in the ovaries are more reliable than uterine weight (according to Drips and Osterberg) and the ovarian changes at 96 hours are more definite than those at 72 hours, so that is when necropsy is best done. Litter mates are used and one rat of each litter is kept as the control.

Microscopically, the ovaries of those animals giving positive reactions contai n one or two very large, well-developed follicles; the other follicles are small and undeveloped, as though these one or two follicles had developed at the expense of the other. In other "positive" ovaries there is more or less uniform increase in the number and size of follicles throughout the whole ovary, and this is considered to be just as significant as the presence of one or two large follicles. Corpora lutea, when present, appear as normally formed structures.

Drips and Osterberg, using the above procedure, studied some 248 women, of whom 13 were normal controls. Some had functional menstrual disturbances, others were suffering from menopausal symptoms. The amount of prolan in the urine helped to substantiate the

diagnoses and in the case of menopausal vasomotor phenomena the severity of the symptoms paralleled the urinary prolan concentration.

II. BLOOD

(A) Method of Fluhmann - The technic of this test is again based on the "pregnancy test" of Aschheim and Zondek, and has been described in two publications by the author. (33,34) 15 to 20 c.c. of venous blood from the patient to be examined are centrifuged; 3 to 5 c.c. of the clear serum are injected subcutaneously, twice daily in from 0.5 to 1 c.c. doses, into an immature female white mouse (between seventeen and twenty-two days of age). In the case of a positive result, the vaginal introitus of the mouse is established by the fourth or fifth day and the animal is then killed and its ovaries fixed in Zenker's solution. After embedding in paraffin serial sections are made and studied. The conditions which indicate the presence of anterior pituitary hormone are grouped into three categories by Aschheim and Zondek, as follows: Anterior pituitary reaction one, (APR I), the "ovulation" reaction, is denoted by the presence of follicles, and these

consist of normal developing follicles or small cysts lined with lutein cells. There is a striking absence of follicles showing chromatolytic degeneration, as seen with anterior lobe preparations. "APR-II" consists of hemorrhages into follicles or lutein cysts, and may be seen grossly as "Blutpunkte", but they do not occur as readily in rats as in mice. "APR-III" is indicated by corpora lutea, with or without imprisoned ova. It is worthy of note that these structure occur much sooner than normal corpora lutea would be formed following ovulation, and have been seen as early as forty-eight hours following the first injection of serum from a patient with an early pregnancy. In a later publication, Fluhmann states that it is not necessary to concentrate the blood serum for this test, but rather in doing quantitative tests it is usually necessary to dilute the original specimens. Further (32) he states that at least three rats should be used for each dosage level.

The two main obstacles which must be overcome to make possible an accurate assay of the gonadotropic hormones are: (a) a reliable assay end-point, and (b) a satisfactory concentration method. The first obstacle

has been satisfactorily removed as shown by the thorough investigation of the immature rat uterus and ovary as end-points carried out by Heller, Lauson, and Sevringhaus (47). As indicated by the fore-going procedures this is the assay end-point most widely used.

Overcoming the second obstacle presents a much different problem, particularly in the case of the urinary prolans. Reports of efforts in this direction are legion, indicating general dissatisfaction with concentration methods now extant. According to Heller and Heller (46) "for the routine assay of large numbers of urine specimens from many different patients, any urine concentration method must meet the following prerequisites: (a) it must be simple in order that large quantities of individual specimens may be handled; (b) it must concentrate the active fraction many times; because of the low content of some urines, the gonadotropic activity of from 500 cc. to 1000 cc. of urine must be concentrated into an amount small enough to inject into one rat; (c) it must retain a large percentage of the original activity; (d) it must meet all the above requirements without being toxic to the test animals."

Most of the concentration methods in the literature were developed for intensive study of the product of any given concentration procedure and were not designed for daily routine clinical procedures. Many were designed for pregnancy urines, hence, due to differences in urinary prolan concentration were not applicable to normal or menopausal urines.

According to Heller and Heller, only the alcohol-ether precipitation method, the tannic acid method and its modifications, and their own saturated ammonium sulphate method showed any promise of meeting the above requirements. The last was discarded after thorough investigation because of its complexity and toxicity. These authors investigated the other two methods with the above prerequisites in mind, using 500 c.c. aliquots of pooled urine samples, and assaying with 21 day old female rats. Uterine and ovarian weights and vaginal opening as found at necropsy on the fourth day were the criteria. They found the tannic acid method undesirable because of the toxicity of the tannates (as evidenced by soreness and hematomata at the site of injection) and several modifications tried by them failed to reduce the

toxicity. Furthermore, routine assays of individually run urines showed that responses produced by tannic acid at 500 c.c. equivalents could also be produced by the alcohol-ether concentrates at the 200 c.c. equivalent level. Finally, of the concentration procedures, the alcohol-ether method gives the greater amount of recovery, although raw urine controls showed that this cannot be considered to be 100 per cent.

ASSAY OF THE CORPUS LUTEUM HORMONE

I. Method of Corner and Allen: Many functions are attributed to the corpus luteum but the best attested of these is the production of a special state of the uterine endometrium, called "progestational proliferation" by Corner and Allen (22) which follows ovulation and corpus luteum formation. In this state the uterus becomes enlarged and hyperemic; its epithelium, both superficial and glandular, undergoes mitotic proliferation, and the crypts and glands increase their complexity of ramification, until in cross-section a very characteristic picture is produced. This condition occurs only in the presence of recent corpora lutea in the ovaries and can be prevented if both ovaries or all the corpora lutea are removed soon after ovulation. Corner (21) in 1928 proved that surgical ablation of the corpora lutea 14 to 20 hours after mating causes total failure of progestational proliferation and death of the embryos on reaching the uterus on the fourth day. These observations indicated that the influence of the corpus luteum

was not only necessary for implantation but also for survival of the free blastocysts during the four days between arrival in the uterus and nidation, and further indicated clearly that the corpus luteum is an organ of internal secretion, acting upon the endometrium in behalf of the embryos. Finally, these observations placed in the hands of these workers a standardized method for testing extracts of corpus luteum from which has evolved a method of assay.

An adult doe, preferably one which had been isolated for one month or more, is mated to one or two bucks until insemination has been proved by the discovery of spermatozoa in vaginal smears made immediately after mating. The animal is now again isolated until the experiment is completed. Some 18 hours later the animal is anesthetized, the abdomen is opened under strict aseptic technic, and the ovaries removed (if ruptured follicles are found, as expected). This is accomplished by clamping the ovarian pedicle tightly in a small artery clamp, then transfixing and ligating the pedicle with black silk, following which the ovary can be excised distally. Bleeding points are carefully

controlled by ligature and one is careful to avoid kinking of the fallopian tube. Essential to the accuracy of the test is complete ablation of the ovaries to assure that no corpus luteum tissue is left behind. A portion of the uterus near the left cornu is now excised between ligatures, after the corresponding mesometrial vessels have been tied, and the cut ends of the uterus are approximated by tying the two uterine ligatures. This portion of the uterus is preserved in Bouin's fluid and is used as the histologic control. The abdomen is now closed. Immediately after operation the extract is administered subcutaneously in the back and continued once daily thereafter until five doses have been given. Necropsy is performed on the fifth day and the genital organs are removed for examination. The ovarian beds are carefully searched for residual ovarian tissue and any suspicious tissues are best removed for histologic examination. The uterine cornua and the fallopian tubes can be washed out with saline into watch glasses to obtain the embryos, after which they are fixed in Bouin's solution for sectioning. A fairly accurate provisional estimate of the result can be obtained without waiting

for paraffin sections, by examining with a dissecting microscope in a strong light the surfaces of razor cuts made through the specimens after a few hours hardening in the fixing fluid. The result of the test is measured by the degree of progestational proliferation induced in the uterus, according to a standard described below.

The test is considered fully positive if at the time of necropsy the endometrium has attained throughout both cornua a condition similar to that of the uterus at the 8tg day of normal pregnancy (as seen in sections taken from the undilated portions between the implantation sites.).

A "rabbit unit" is the minimum dose of an extract which suffices, when divided into five daily doses, to alter the uterus of a doe weighing three to four kilograms, under the specified experimental test, to a state equal to that described above.

II. Method of Clauberg: Clauberg (quoted in Kurzrok) has modified the above procedure in that a similar animal is injected with 10 M.U. of estrone daily for eight days, following which the corpus luteum extract is injected and the uterus subjected

to microscopic study exactly as described above. However, one must be careful in interpreting results because there is a great difference between the Corner-Allen and the Clauberg units, the former being about twice as large as the latter.

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