T H E S I S

A PRACTICAL METHOD OF PREPARING AN OVARIAN EXTRACT FOR USE IN SOME OF THE LOWER ANIMALS

Submitted by

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I

Introduction

The lack of a simple practical method for the production of an ovarian hormonal extract for use in some of the lower animals furnished the impetus for the work presented in this paper. Then too, the vast amount of sterility existing in the domestic animals is significant of the tremendous economic loss thus incurred. Valuable blood lines are often interrupted or entirely lost because of the failure of the female of the species to propagate and continue her strain.

Because of these factors and the work of the early investigators and of the hope that the future may bring forth even more valuable information, there seems little reason to doubt that a large part of the present economic loss due to sterility may be prevented. Also, any adjunct to present methods in the treatment of sterile animals will be eagerly accepted for the reason of the hazards now existent in such therapy.

The fact that the hormonal substance has been demonstrated to exist in the liquor of the follicle of the ovary, in the corpus luteum, in the blood stream during oestrus and in the placenta of the pregnant female; but not in the blood stream of the adult castrate female, points to the ovary as the nidus of its production. Also, the results obtained by Knauer, Landau, Mainzer and others of feeding by mouth of

ovarian substances and the results gained by Adler, Fraenkel and Frank by injections of extracts of the ovary and placenta even more strongly indicate the latent possibilities of this method of so-called replacement therapy.

Many cases of sterility give definite symptoms of abnormal ovarian function as, irregularity of oestrus, oestrus of very short duration or the entire absence of oestral phenomena and failure to conceive. These are the conditions in which it is believed that ovarian replacement therapy gives most promise.

The frequency with which sterility of a functional nature is met in the practice of veterinary medicine places the production of a readily prepared and easily available ovarian extract of low cost, in the light of an important subject.

II

A Brief Treatise of the Early Development Made in the Science of Endocrinology

The birth of the idea of certain of the ductless glands furnishing to the blood a secretion or incretion of their own production occurred in the year of 1749.

In that year Theophile de Bordeu arrived in Paris, coming from his home in Montpelier. His exceptional brilliance in medicine soon gained for him a membership by election to the Royal Academy of Sciences. Rapidly he gained a practice among the aristocracy, and even Louis XV became one of his patients.

The sexual side of men and women aroused his interest and by experiments upon spayed animals and capons he formulated the conception of sexual secretions being absorbed into the blood. This work apparently settled the question of the reason for male and female characterists.

A few years later the cell was recognized as being the unit of the structure of body tissues and organs of the body. Upon this basis the glands were studied and those having no apparent channel for the removal of their secretions, were accordingly called ductless glands.

The first quarter of the nineteenth century produced the concept of the classification of the glands into those of internal and external secretion, but the importance of the internal secretions to the body was not known. Some

of the most advanced physiologists asserted that the glands of internal secretion were of no importance and might be removed at will with no serious consequences, as they were of no real significance to the functions of the body.

Until the nineteenth century nothing of importance was done in experimental research on the internal secretions. Later A. A. Berthold of Gottingen transplanted the testes of four roosters under the skin. He thus showed that there was an internal secretion which played a part in the male body functions. He is accordingly given credit as the first experimenter to prove the existence of an internal secretion and the power it exercised over the body. He demonstrated the presence of two kinds of cells, one for internal secretions and one for external secretions. This all occurred ten years before Darwin's Origin of Species made its appearance.

Forty years went by with nothing of importance having been contributed to the study of the internal secretions. Then in 1855 Claude Bernard of the College of France coined and established the designations internal and external secretions.

In the following year Addison of England described a fatal disease of the suprarenal glands stating that something necessary to the body was lost by their destruction and said that it was an internal secretion. The disease was then called Addison's Disease.

Brown-Sequard, a Frenchman, in 1863 duplicated Addison's Disease experimentally. He removed the adrenals and noted the results, he transfused the blood of normal subjects to these and found that he could prolong life.

In a paper on The Physiology of the Thymus by Friedleben published in 1856, he mentioned that in the young without a thymus, softening of the bones and a physical and mental deterioration occurred. Next, Moritz Schiff of Frankfort on the Main showed that the excision of the thyroid in dogs was fatal. Notice was given, at this time, to goitre and its associated symptoms but no particular attention was given to the disease.

Sir William Gull in 1878 described cretinism and gave to the condition the name myxedema. Shortly after, in 1882, the first surgery of the thyroid was performed by Theodore Kocker, a Swiss surgeon, who accomplished the removal of a thyroid gland. The same symptoms as described by Schiff were noted, and the term operative myxedema was given the condition by J. L. Reverdin of Geneva.

Then in 1884 Schiff, by a series of cases, showed that the symptoms could be allayed by and prevented by a graft under the skin of a piece of the thyroid or an intravenous injection of the juice of the thyroid or by injection of the raw thyroid. Mobius, a German neurologist, next explained the qualitative and quantitative changes in

the secretion of the thyroid and the ailments resulting from the same.

Experimental removal of the pituitary was not successfully accomplished until 1908 when Paulesco of Bucharest performed the operation by trepanning the skull. His proof consisted of the fact that this gland was essential to life. Harvey Cushing of Johns Hopkins next demonstrated its relation to growth and obesity. Pierre Marie, of Paris, discovered the cause of acromegely and showed that the enlargements of the extremities were due to an hypertrophy of the pituitary.

Now comes the birth of the present interesting subject of the testicular secretions and their effects. Docent Berthold by autografting of testes in roosters showed the fact that retention of male characterists was possible. Later, Brown-Sequard, at the age of seventy-two at the Agassiz Laboratory on Long Island, reported to the Society of Biology of Paris the rejuvenating effects in man of hypodermic injections of testicular juices of monkeys and of dogs.

Paris ridiculed him, but many of the Parisians soon became his patients. In 1891 he sums up his work by saying that such juices had a definite energy-mobilizing effect, or dynamogenic action upon the subject, stimulating the general health, muscular power and mental activity.

A field of gigantic magnitude was thus opened to further investigation, and research began in earnest. From the time of Brown-Sequard to our present day vast and important contributions have been made to the science of endocrinology. Among the problems of the early workers in this field was the task of coining new terms for their discoveries. Bayliss and Starling were the first to consider this problem of nomenclature and are responsible in a large measure for our present designations of the physiological actions and products of the ductless glands.

Thus the term "internal secretion" is used to apply to those products of glandular tissue which are taken up directly by the blood stream from the gland substance itself and are not delivered to the blood by way of ducts. These glands are often called the endocrin system, as they are related in a chemical sense, one to another. That is, an over secretion or an under secretion on the part of one gland interrupts the pluriglandular balance and causes a symptom complex commensurate with the degree of existing dysfunction.

Schafer (1) calls attention to the fact that some hormones inhibit functional activity while others act as chemical stimulants. The term "homone" (homao, I excite), etymologically considered, is not applicable to the former class. He suggests, therefore, the general term "autocoid substances" for both groups to indicate their drug-like ac-

tion (acos, a remedy), and sub-divides them into two groups according as they stimulate or inhibit as follows:

Autocoid substances { (Hormones (I stir up) stimulating action
(Chalones (I make slack) inhibitory action.

III

Present Knowledge Relative to the Physiology of the Mammalian Ovary.

The ovary contains the Graafian follicles with their ova, follicular epithelium and liquor folliculi, and later the corpora lutea. These are imbedded in a highly vascular stroma formed of a peculiar connective tissue, firm in texture and containing numerous spindle-shaped cells. In most animals the stroma contains groups of cells of a different appearance from the ordinary stroma cells. They have been named the interstial cells. They are not as distinct in the human ovary as in many animals.

Like the interstitial cells of the testicle, they are regarded as the source of the internal secretions of the gland which regulates the development of secondary sex characters.

The conditions in the ovary as regards internal secretion are more complex than those in the testicle, since the cells of the Graafian follicles, the liquor folliculi, the corpora lutea, the interstitial cells, and the cells constituting the stroma, all play a part in the endocrine functions of the organ.

The Graafian follicles are derived from the epithelium of the germinal ridge; this epithelium grows into the substance of the ovary in the form of epithelial cords.

The cells of these cords are first alike, but after a time some of them become enlarged to form the primitive ova. The cords presently break up into small nests of cells, each of which eventually contains an ovum with a layer of flattened epithelial cells surrounding it. After a time these surrounding cells proliferate and form two layers, which soon become multiple. The stroma of the ovary is differentiated around each follicle into two layers, termed the internal and the external thecae: the internal theca is very vascular and contains besides the ordinary stroma cells, which are spindle-shaped and of connective tissue nature, large epithelium-like cells, known as theca cells; these are probably of the nature of interstitial cells.

With the approach of sexual maturity the Graafian follicles which become greatly enlarged, partly by multiplication of their epithelial cells, partly by the formation of liquor folliculi within them, during their enlargement have sunk deeply in the substance of the ovary, now reach the surface, where they form bulging cyst-like prominences projecting beyond the general surface. On rupture of one of these mature cysts the liquor folliculi escapes, and with it the ovum surrounded by a mass of the epithelial cells of the follicle (discus proligerus, cumulus), leaving behind in the follicle the remaining epithelial cells, which

form a layer several cells deep, lining the folliclar wall (membrana granulosa).

In the natural course of events this bursting of a mature follicle and extrusion of its ovum (ovulation) occurs spontaneously; in the cow thirty to sixty-five hours after the cessation of the manifestation of oestrum (2). The extruded ovum is caught on one of the fimbriae of the Fallopian tube, and is carried along the tube by the action of its cilia to the horn of the uterus. Here, nidition occurs if the ovum has been fertilized. But whether there is or is not a fertilized ovum, the uterine mucous membrane has become modified in such a manner as to facilitate attachment. These modifications in the uterine mucous membrane are preceded by partial disintegration of its substance, with desquamation of the epithelium and mucous secretions, which escape by the vagina. If the ovum is not fertilized it also becomes discharged and lost, and the mucous membrane of the uterus instead of proceeding to the formation of the embryonic covering attachments undergoes a process of restitution and reacquires its normal character. These changes in the uterus form what is termed the oestrus cycle, which is commonly divided into the period of increased growth and vascularity of the uterine mucosa, generally accompanied by the escape of secretions (proestrus), the period of proliferation of the cells of the

mucosa, or oestrus proper, and the period of the return of the mucosa to its interoestral condition (metoestrus). Proestrus is always characterized by extreme vascularity of the whole generative apparatus, both external and internal, whether there is an escape of secretions or not. A proliferation and desquamation of epithelium occurs in the vagina also; the extent of this has been used to determine the commencement and progress of the oestrus cycle and unterine changes.

After discharge of the ovum the emptied Graafian follicle undergoes a remarkable change. Its epithelium (membrana granulosa) becomes greatly thickened by enlargement of the cells and deposition within them of a yellowish lipoid material. These cells now resemble the epithelium of a secreting gland and collectively form a spheroidal or oval mass known as a corpus luteum. Very soon the mass becomes vascularized by the ingrowth from the theca interna of stroma tissue containing blood-vessels; these ingrowths are accompanied by the large "theca cells" before mentioned as derived from the interstitial cells. The theca cells are also transformed into lipoid-containing cells and become indistinguishable from the original cells of the corpus luteum, which are derived from the membrana granulosum. The strands of lutea cells and blood vessels converge towards a scar-like hilum which is formed at the surface of the ovary at the place where the ovum was originally extruded.

If the ovum remains unfertilized the further development of the corpus luteum ceases relatively early (in the cow about seventeen or eighteen days after oestrum), and about this time undergoes regressive changes, resulting in its eventual disappearance. Such a corpus luteum is known as a corpus luteum spurium. The sharp distinction between it and the ovarian stroma becomes lost, its cells appearing to migrate into the adjacent stroma. Here they disappear as a distinct tissue, many probably becoming converted into interstitial cells. What is left of the corpus luteum takes the form of a fibrous structure in the stroma of the ovary, known as the corpus albicans.

If the ovum becomes fertilized and fixed in the uterus, so that pregnancy supervenes, the corpus luteum undergoes considerable development. It persists in a mature form, becoming greatly enlarged, and forming a large solid prominence at the surface of the ovary, known as a corpus luteum verum or corpus luteum of pregnancy. In most animals this appears as a vascular globular mass of yellowish color, with (in section) strands of cells alternating with sinus-like capillaries. The corpora lutea of pregnancy are, like the corpora lutea of oestrum, ultimately absorbed or merged in the ovarian stroma, but the scar on the surface of the ovary which marks the place where they had been formed long persists.

The corpus luteum appears responsible for the preoestral changes in the uterine mucosa, and, if fertilization of the ovum takes place, directs the early welfare of the embryo. (3)

The above account of the mode of development of the corpus luteum was originally due to Bischoff (1842) and follows that given by Marshall who has made a special study of the subject. (4)

The physiology of the ovary of mammals and particularly the domestic animals, has been studied extensively by able investigators. In the mammalian group there is a fair degree of uniformity in the female sex life. The outstanding variations being the absence of the phenomena of menstruation in mammals below the primates, the varying frequency of oestrus, and such anatomic characters as horns being sex distinctive in some species and not in others . (5)

The complexity of the physiologic processes in the mammalian female that depend, directly or indirectly, on ovarian functions is indicated by the following outlines.

I. Preadolescent Period.

A. Fetal sex differentiation.	This depends on hormones from the anlage of the gonads.
B. Infancy nutrition.	Continued growth and nutrition of the uterus, mammae, and female pelvis differentiation. These, in some way depend on the ovaries.

C. Growth — Inhibition of long bone growth controlled in part by the ovaries.

II. Normal Sex Life.

A. Continuous function.

1. Nutrition of uterus, mammae, and genital tract.
2. Development of female pelvis.
3. Basal Metabolic Rate. High during entire active sex life. (Lower in castrates.)
4. Sex behavior.
5. Growth Control. Regression of the thymus, and of cell groups responsible for the outcropping of the male characters which may appear following removal or atrophy of the ovaries.

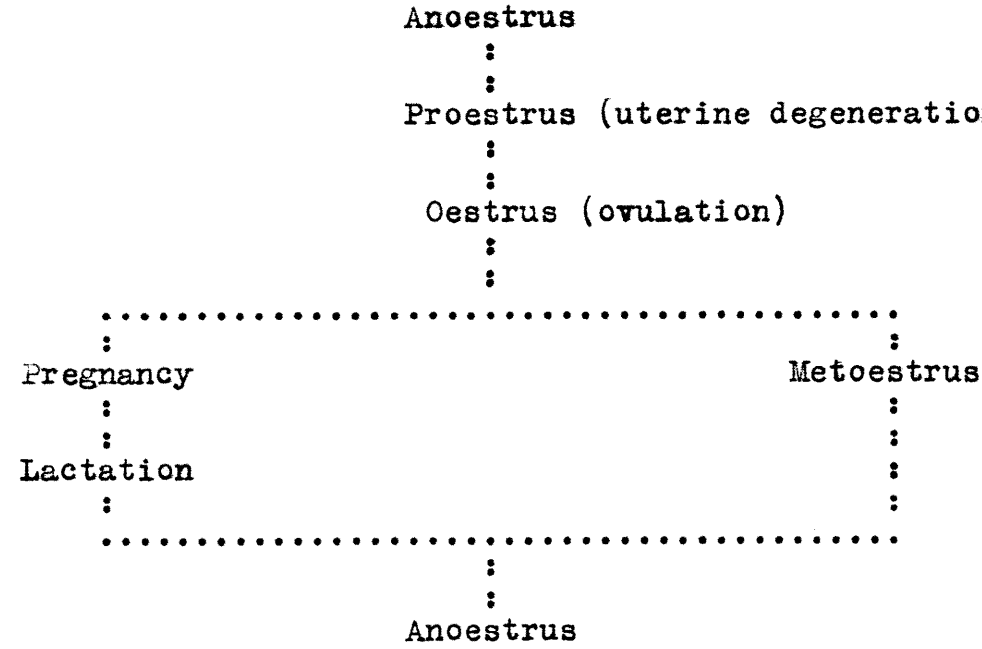
B. Periodic Function.

1. The Oestrus Cycle. Uterine mucosa changes, oestrus, sex urge.
2. Pseudopregnancy. Uterine mucosa growth and mammary hyperplasia.
3. Pregnancy. With suppression of ovulation. Growth and uterine sensitization.

III. Climacteric Phenomena. These are principally depression of metabolism, tendency to obesity, disappearance of oestrus, atrophy of genitalia.

IV. Physiologic and Pathologic Deviations. Sterility, anoestrus genitalia hyperplasia, nymphomania.

Swale Vincent (6) quoting the work of Hill and Donoghue, gives the following diagramatic scheme of the cylical physiological changes occurring in the genital tract.



Interruptions in the cycle due to ovarian causes frequently lead to a condition of functional sterility which evidences an endocrine imbalance of a physiological nature.

It has been common knowledge for centuries that removal of the ovaries in animals results in a decided change in the growth and metabolism of the body. Thus, female animals have been castrated for the purpose of modifying body form and temperament. This practice has been much in vogue but the physiological reasons explanatory of the body changes were not attempted until Knauer (7), submitted the first good experimental evidence that the ovaries con-

trolled the phenomena of the oestral cycle, and the body changes after castration could be prevented by transplanting the ovaries from their normal position to the mesometrium. Also, that if the ovaries were removed prior to sexual maturity, the uterus remains infantile, the mammary glands do not develop, the oestrus cycle is not inaugurated and the individual develops a neuter body type. If castration is performed after sexual maturity, the individual retains the feminine body type but the oestrus cycle ceases, the uterus and mammary glands undergo atrophy. Marshall and Jolly, amplifying the work of Knauer, demonstrated that normal nerve connections were not essential to the ovaries in their functional control over metabolism, but that a secretory product of the ovaries, an internal secretion, played the active part responsible for the development of the uterus and the oestrus cycle of sexual maturity in the female. This evidence is conclusive of the fact that the ovaries are the seat of production of the controlling force and that the specific substance is an internal secretion.

Experiments conducted by Loewy and Richter (8) and published in 1899, showed that a decrease in the oxidative processes occurred in the castrates of both sexes. Upon the administration of ovarian substance in the female and orchitic substance in the male, this modified oxidative change could be returned to normal. Two years later L. Fräenkel concluded that the corpus luteum was the gland

of internal secretion and that it controlled the nidition of the ovum and brought about oestrum, that the corpus luteum was responsible for uterine hyperplasia and the uterine changes necessary for the nidition of the ovum. Deciduomata could be produced experimentally, according to Loeb (9), in the uterine mucosa of the guinea pig by making cross scarifications in a manner to break the tissue continuity. The same reaction could be produced by the introduction of a foreign body into the uterine cavity. This reaction could only be obtained at a certain period of the oestral cycle and was due to the action of the corpus luteum. Other workers in the field report contradictory results from the use of expressed juices or aqueous extracts. Next Iscovesco used fractions obtained by lipoid solvents and found that these fractions contained a substance which produced a hypertrophy of the uterus in rabbits. Fellner definitely proved the work of Iscovesco by inducing uterine and vaginal hyperplasia thru lipoid fractions of the ovary and the placenta. He further showed the lipoid to be soluble in ether, acetone and ethyl alcohol and to be thermostable.

Frank (10) in 1917 reported premature sex development in virgin rabbits following very early the injections of lipoid extracts of the ovarian follicular fluid of pregnant and nonpregnant cows.

In 1923 Allen and Doisy (11) introduced the vaginal smear method of assaying ovarian extracts in spayed rats.

This work was based on the findings of Long and Evans (12), and Stockard and Papanicolaou (13). The method is based on the cytographical microscopic picture obtained from light scrapings of the vaginal mucosa smeared upon slides and stained with hemotoxylin and eosin. By this procedure the oestral cycle may be traced in a definite manner and information poignant to the reaction of ovarian lipoid injections accurately measured. The basis for the comparison of the activity of extracts by this method is classified as follows:

1. Negative ..... The presence of luecocytes only.
2. Plus 1 reaction .... The presence of leucocytes and a very few nucleated squamous epithelial cells.
3. Plus 2 reaction .... The presence of a few leucocytes and a few nucleated squamous epithelial cells.
4. Plus 3 reaction .... The absence of leucocytes and a decreasing number of nucleated and increasing number of non-nucleated squamous epithelial cells.
5. Plus 4 reaction .... The presence only of non-nucleated squamous epithelial cells.

By the above method of assay Allen and Doisy (14) were able to confirm the findings of Frank (15) as to the potency of liquor folliculi and further that the active

principle is found in the lipoid fraction. They were unable to demonstrate the active principle by their method, from extracts of the corpus luteum. The rat and mouse were used as test subjects because of their brief period of oestrum and ease in handling. Castrates were used again and again under this method of assay and each time it was shown that injections of the follicular hormone caused growth in the vagina as well as in the uterus of these animals.

A degree of growth equivalent to the maximum occurring in the normal animal was obtained by such injections. This reaction can be easily followed by serial observations on the living animal, while formerly criteria of activity of extracts depended on a single observation at necropsy. Furthermore, these tests can be rigidly controlled.

IV

A Review of Previous Investigations of the Ovarian Hormone.

It is thru the experiments of Brown-Sequard with subcutaneous injection of testicular extracts that our knowledge not only of the internal secretions of the sexual glands, but of internal secretions in general, began. It is well known that the experiments of Brown-Sequard have been much disputed, but notwithstanding all the objections which have been made, new scientific principles and new knowledge were established on disputed, or even really erroneous data.

After Brown-Sequard, repeated attempts were made to obtain from the gonads the chemical substances corresponding to the sexual hormones. The determination of the characters of these substances by chemical analysis or the establishment of their chemical constitution was not attempted until later. Several investigators, however, tried to isolate the hypothetical hormones from the female gonad, but the results have been somewhat conflicting, due possibly to the various methods employed in the isolation processes.

The pioneer work of Iscovesco (16) in 1912 (quoting from Lipschütz after Herrmann and Stein, 1916) "first drew attention to the action of lipoid containing extracts of endocrine organs. He succeeded in producing a very marked increase of the volume of the uterus by injecting an ex-

tract prepared from the corpus luteum by treating it with alcohol and then desiccating the alcoholic extract and treating with acetone, ether and chloroform." The experimental animals used by Iscovesco were normal virgin rabbits and dogs.

Fellner (1913)(17) used castrate animals as experimental subjects and followed the method of Iscovesco in the preparation of extracts of placenta, of ovaries of pregnant females containing corpora lutea, and of ovaries of non-pregnant animals. He used for extraction a solution of sodium chloride, alcohol and ether. He stated that injection of extracts from pregnant animals caused in rabbits a hyperemia and a thickening of the muscle layer of the uterus. The epithelium thickened and the number of uterine glands increased. On the contrary, extracts of ovaries of non-pregnant animals had no effect. Fellner thinks that the specific substance which causes the above mentioned effects is possibly a lipoid present in the corpus luteum in greater quantity than in other organs, since extracts of other organs had no such effect.

In recent papers Fellner (1917 (18) 1920 (19) puts forward the view that the lipoid which he isolated from the corpus luteum is really the specific sexual hormone or possibly one of the sexual hormones secreted by this organ.

Later experiments by Fellner (20) in which he investigated the presence of the same substance in the inter-

stitial cells of the ovary by preparing an extract from the ovary of the pregnant cow which contains no corpus luteum, but in which the interstitial tissue is highly developed during pregnancy, showed a very marked effect on the uterus. Such an ovary differs from the ovary of a non-pregnant animal only in the fact that the interstitial tissue is hypertrophied. He states that an extract of a corpus luteum of a non-pregnant animal has quantitatively the same effect as that of a corpus luteum of a pregnant animal.

In the above mentioned experiments the augmentation of the uterus was made use of as a quantitative test of the hormonic effect of a given extract. By the same method Fellner arrived at the conclusion that the extract of a placenta is equivalent to that of an extract of forty corpora lutea.

An experimental comparison between the action of extracts of the corpus luteum and that of the hilum ovarii was undertaken by Itagake (21) in the laboratory of Schafer. He stated that the action of these extracts upon the movements of the living uterus of the rat are antagonistic to one another, the first causing a distinct increase of tone, the second causing inhibition. But the uterus of other animals reacts differently; in the rabbit, cat and guinea pig extracts of both the corpus luteum and the hilum produce an

increase of tone. Sometimes, however, the extract of corpus luteum may produce inhibition. Itagaki suggests that this difference of effect is possibly due to a difference in the samples of corpus luteum. He points out that there are apparently two principles in the corpus luteum having an antagonistic action upon the uterus. Both these principles can be extracted, according to Itagaki, by Locke's solution; the principle causing inhibition of the uterine contractions is soluble in alcohol, the principle causing increase of tone is soluble in water. Athias (22) has recently confirmed the stimulating action of the ovary and of the corpus luteum on the uterus.

Herrmann (23) investigated the effects of extracts prepared by ether. He made a comparative study of extracts of corpora lutea excised from ovaries, of extracts of ovaries without corpora lutea and of extracts of the placenta. By different chemical methods Herrmann obtained a thick yellow oil which became solid when cooled. This substance showed the characteristic reactions of carbon, hydrogen and oxygen. Thus, Herrmann was the first investigator to give chemistry to the hormone and designate it as a CHO compound. According to Herrmann it is possibly a derivative of cholesterine, soluble in alcohol, ether, petrolether, acetone and benzol, but insoluble in water. He examined the physiological effects of all the different fractions obtained in pre-

paring the above mentioned oily substance. The experiment was made on fully grown castrated female rabbits and on young animals eight weeks old. By injecting the extracts the atrophy of the uterus which normally follows castration was inhibited, and the changes characteristic of normal "heat" took place in the uterus, very striking results were obtained on young animals. After three injections an extraordinary development of the uterus began: there were changes in the mucosa and in the muscle layer. The infantile uterus increased enormously and the blood vessels were very much enlarged.

The ovary is also influenced by the extract. The ovary of the young rabbit consists mainly of primary follicles, but there are also some follicles just entering upon development. In the ovary of an animal which received five injections numerous ripening follicles were to be found; some of them had even already attained maturity.

From these experiments it seems that the extract prepared by Herrmann may cause in animals of about eight weeks, a development of sex characters such as is found at an age of about six to seven months. The genital organs may even develop so far as to become like those during heat or at the beginning of pregnancy. So far as is known, Herrmann obtained all these changes by injecting preparations of the corpus luteum and the placenta, but not by prepara-

tions from ovaries from which the corpora lutea had been excised. Allen and others (24) in 1924 report preparations from placenta, liquor folliculi, and ovaries from which the liquor folliculi have been removed, are active; from embryos and corpora lutea, negative. Their method of preparation of the extract follows the scheme of lipoid extraction in general. Extraction of the lipoids with hot alcohol (95 %) is followed by saponification with sodium hydroxide and evaporated to dryness. The hormone is subsequently extracted from the soaps with chloroform or ether. It is soluble in lipoid solvents but insoluble in water. It is stable toward dilute boiling acids and alkali dissolved in oil, its activity is not destroyed by autoclaving at 15 pounds pressure for 15 minutes.

The methods of Herrmann in pursuing the chemical means of obtaining the female sex hormone in concentration are elaborate and beset with failures. However, credit is due him for his thoroughness in amplifying the work of Iscovesco and Fellner in placing the method of lipoid extraction on a clearer basis.

His method consisted of chopped up corpora lutea which were treated twice with a large volume of acetone for 24 hours. Then after filtering off the watery acetone in a Soxhlet apparatus, both the acetone extractions were united, distilled and a part of the residue shaken separately with

petroleum ether, alcohol and acetone; each representing its respective fraction. After concentration by evaporation the materials were tested by injection into castrate females and the hormonal reactions recorded as stated above. Fränkel and Fonda (25) have gone further using Herrmann's method and state that the empirical formula of both the placental and corpus luteum hormone is $C_{32}H_{52}O_2$.

Frank (26) in 1917 obtained premature sex developments to the degree of pregravid changes in four immature, virgin rabbits, with injections of follicle fluid. Frank and Gustavson, working on the chemistry and isolation of the ovarian hormone, have confirmed the previous findings of Iscovesco, Herrmann and Fränkel and Fonda. They state the active principle is soluble in lipoid solvents and that purified extracts do not contain phosphorus or nitrogen as reported by Giesy (27).

Giesy (28) found that the activity was not destroyed by digestion with soy bean lipase. Frank and Gustavson (29) have saponified the active lipoid fraction with half normal potassium hydroxide, sodium methylate, sodium ethylate, and sodium butylate for as long a period as forty-eight hours without loss of activity. When the saponified mixtures are evaporated to dryness and extracted with ether, they find that these extracts are physiologically active. In other words, it is either a nonsaponifiable material or,

if it is an ester, the activity resides in the high molecular weight alcohol liberated by the saponification process. These findings also indicate that the failure to get a physiological response when extracts are given by mouth is not due to the action of digestive enzymes as is the case with insulin. The failure is probably due to an inability of absorption.

The active substance showes a very high degree of thermostability. All workers are agreed on this property. Herrmann (30), Fränkel and Fonda (31), and Frank and Rosenbloom (32) have attempted purification by distillation under high vacuum at temperatures ranging from 190° to 250°C without loss of activity. In this respect the active principle differs markedly from vitamins and enzymes.

Allen, Pratt and Doisy (33) in 1925 reported negative results from injections of extracts of corpora lutea. Corpora lutea of both oestrus and pregnancy from pig ovaries grouped according to their stage of development were carefully excised, and extracts of amounts varying from twenty to several hundred grams tested. To date, more than thirty tests of different preparations of fully formed corpora have been negative. More than twelve tests of separate preparations of corpora from cows have also been negative. From the above it appears that the presence of the active substance or the female sex hormone in the corpus luteum remains an open question.

No such controversy exists regarding the presence of an active substance in the follicular fluid of mature or nearly mature Graafian follicles. The presence of the genitalia growth-inducing factor in the follicles of the ovary, in the placenta and even in the blood stream is recognized by all investigators. Its source and compositior is still to be learned. Allen and Doisy (34) later report positive reactions from injections of extracts of human corpora lutea. (Quoting Allen and Doisy (35)) "The first corpus luteum to be tested displaced only 1.4 cc of fluid. It was excised two weeks following the onset of menstruation. The extract in full strength returned a positive test, but in 1:3 dilution proved negative. The majority of corpora so far tested have contained some of the follicular hormone. The amounts being less than our best results from tests of follicular fluid. These results indicate that there is a functional difference in corpora lutea in different species of animals; that the human corpus luteum, unlike that of the pig and cow continues to secrete the follicular hormone in appreciable amounts for a considerable period following ovulation."

"The method of Allen for the production of the follicular hormone consists of adding a double volume of 95 % alcohol to a given quantity of fresh liquor folliculi (obtained by aspirating the follicles with a hypodermic needle into a vacuum bottle) and allowing it to stand for

twenty-four hours or until all the proteins are coagulated. The coagulum is then filtered off. The filtrate, which is almost free of protein, contains the active constituent. Further extraction of the coagulated protein with boiling alcohol yields an additional amount of the hormone. The alcohol is distilled off and the residual aqueous suspensions extracted with ether. The ether extract is evaporated and the solids dried in a desiccator. The residue is dissolved in a minimal amount of ether and a double volume of acetone is added. The precipitate, which consists of lipoids (lecithin and cephalin) shows no activity in test animals. The filtrate is evaporated and the residue dried. By boiling out the solid material with 95 % alcohol, the active substance is obtained from protein but contaminated with a little free fatty material. The alcohol is evaporated off and the minute yield of oily residue taken up in purified corn oil. This constitutes a partially purified preparation of the follicular hormone, the subcutaneous injection of which does no harm."

Gustavson (36) prepared a partially purified follicular hormone by macerating minced placenta in a double volume of 95 % alcohol for twenty-four hours and after filtering out the solids reduced the filtrate by evaporation of a fraction of the original volume, which was further extracted with benzine. The residue, which is an oily sub-

stance of a light yellow color and turpentine-like odor, represents the partially concentrated hormone and is positive in reaction in castrate animals in initiating oestrus-like uterine changes.

Murphey (37) in his work upon the oestrus cycle of the domestic cow, prepared a dilute sterile follicular-fluid solution. He aspirated the liquor from mature follicles and diluted with four parts of physiological sodium chloride solution, which he filtered thru asbestos wool and thru a Berkefeld filter into sterile tubes, which were immediately placed in refrigeration. The dose of this material was established as the amount to be obtained from one ovary. Favorable reactions are reported by Murphey from the use of this material.

Allen and associates (38) report only negative results from the use of several commercial extracts in test animals. The work done on this phase of the subject by Frank and Gustavson (39) is more exhaustive and is quoted as follows: "Using the rabbit's uterus as a test object, the following ovarian preparations obtainable on the market were examined. Positive results would be signified by visible hyperplasia after injecting twenty-five milligram doses on alternate days and examining the uterus on the tenth day after the first injection. Using potent preparations prepared by methods described in the preceding part of this

paper an enormous hyperplasia is the result. The dosage employed with commercial preparations was from six to ten times that dose and was given from six to ten times by injection, or orally depending on the preparation. The following table shows the results obtained.

Armour's ovarian substance ..... Negative
Hynson, Westcott's Lutein tablets ..... Negative
Burroughs, Welcom "Varium" ..... Negative
Parke, Davis ampoules corpus luteum ..... Negative
Parke, Davis ovarian substance ..... Negative
Lederle's corpus luteum sol ..... Negative
Sistomensin "Ciba" ..... Negative
Agomensin "Ciba" ..... Negative
Iscovesco's Gynocrinol, Hypo ..... Negative
Iscovesco's Gynocrinol, Mouth tablets ..... Negative

Further investigation of other materials was carried on by Gustavson who reports the following:

Material	Test Used	Amount Injected	Result
Olive oil	h. u.	4 cc	Negative
Oleone	h. u.	200 mg	"
Liver	h. u.	200 mg	"
Butter	h. u.	200 mg	"
Histomine	h. u.	6 mg	"
Tenosin	h. u.	6 mg	"
Aolan	h. u.	6 mg	"

Material	Test Used	Amount Injected	Result
Adrenalin	h. u.	6 mg	Negative
Histamine	v. s.	6 mg	"
Tenosin	v. s.	6 mg	"
Aolin	v. s.	6 mg	"
Adrenaline	v. s.	6 mg	"
Brain	h. u.	100 mg	"
Brain	v. s.	100 mg	"
Liver	v. s.	75 mg	"
Liver	v. s.	82 mg	"
Testes	h. u.	100 mg	"
Testes	v. s.	100 mg	"
Adrenal cortex	h. u.	150 mg	"
Adrenal cortex	v. s.	150 mg	"
Adrenal medulla	v. s.	75 mg	"
Thymus	v. s.	100 mg	"
Linseed Oil	v. s.	100 mg	"

h. u. Indicates hypertrophy and hyperplasia of the uterus in young immature virgin rabbits.

v. s. Indicates the vaginal smear test introduced into this type of research by Allen and Doisy.

The above evidence is strongly confirmatory of the hypothesis regarding the specificity of action of the female

sex hormone. Lipschuts (40) inclines to the belief that there is but one hormone: that it derives in part from the cells of the membrana granulosa, in part from the cells of the theca interna, and that these cells originate from atrophic or from normally ripening follicles. Berkeley (41) states that whether there be one or more than one ovarian hormone is disputed on scientific grounds, on clinical grounds distinctions have not been conclusively made. Chemically there is but an indefinite empirical formula existing to designate the composition of the hormone and its physical nature lends itself to lipoid extraction. Physiologically it is apparently sex specific and non-specie specific in the mammalia. It is of ovarian origin, but its specific source and mode of production is still a question for future solution.

Bugbee and Simond (42) by using rats of approximately uniform weight (140 grams) and allowing but little individual variations, have found that a relation exists between the weight of the test animal and the amount of the ovarian extract necessary to bring about typical induced oestrus. They followed the method of Allen and Doisy in the preparation of the ovarian extract and its assay.

The fact that the ovary is concerned with sexual characteristics and function has been long recognized, but the work of Knauer (43) in 1900 amounted to the first defi-

nite experimental evidence that the ovary played an important part in the phenomena of oestrus, and that the results of spaying an animal could be in part overcome by an ovarian graft.

About the same time Landan (44) tried the feeding of dried ovaries by mouth for relieving symptoms of the eliмacteric and double oophorectomy. His work was repeated by Mainzer (45) in 1903, and the cases so treated were reported to have been greatly benefitted. Mainzer prepared his substance by drying minced ovaries from cows and pigs and making the dried substance into the form of tablets, which represented the whole of the ovarian tissue. The improved method of defatting the ovarian tissue amounts to the removal of what is now believed to be the active principle.

The work of Knauer was later fully confirmed by Marshall and Jolly (46) and Fraenkel (47) reported good results from the administration of corpus luteum. These preparations were given by mouth except that of Federoff (48) which was given subcutaneously and intravenously as an extract.

It is surprising that the early literature on the subject contains no accounts of any animal experimental work having been done before the administration of ovarian substance or extract to human beings.

However, in 1912 Adler (49) injected some animals with ovarian preparations in an attempt to observe and measure gland therapy. He states that he was able by repeated injections of extracts of the whole ovary to produce typical signs of oestrus in animals. He later confirmed his work by histological investigations. The work of Adler seems to be the first scientifically controlled series of investigations up to 1912.

Zondek and Aschheim (50) but very recently conclude from their experiments that the water soluble ovarian hormone enhances the growth of the uterus and causes premature puberty as well as inducing the estrus. Also, that it is superfluous to assume the presence of several hormones in the ovary.

To date sufficient animal experimentation has been done to show the physiologic and pharmacodynamic action of the ovarian sex hormone. Now comes the problem of the preparation of an extract for veterinary use having a sufficient amount of the principle of the ovarian secretion to act in such a manner as to partially replace the hormonal production of the ovary and to assist the physiological process of oestrus in the lower animals.

V

The Preparation of an Ovarian Extract for Veterinary Use.

The evidence produced by laboratory investigations established the fact that the active substance of the follicular fluid and the placenta are identical and may be obtained by lipoid extraction.

Commercial processes for the production of ovarian extracts make use of lipoid extractives only to remove the fats or degrease the ovarian tissues before utilizing the residue thus obtained for the final commercial product. The lipoid fraction is then discarded as useless when in fact the hormone is really contained therein.

Comercial ovarian preparations present a nice looking product but for the most part are probably inert as determined by Gustavson, Frank and Allen.

Since most of the ovarian products on the market were intended for human use their results when administered to animals have been of a very diverse nature and consequently highly unsatisfactory. For this reason as well as the fact that commercial preparations contain little or none of the female sex hormone it was considered desirable to attempt the production of a preparation especially designed for veterinary use and in so doing demonstrate the hormonal content.

An investigation into the methods used in the production of commercial ovarian preparations resulted in the following information.

The general methods used in the production of commercial extracts follows somewhat the work of the very early investigators of the subject of apotherapy who prepared tissue juices by simple expression or who fed the raw tissue itself. More recent commercial methods make use of non-selected ovaries from cows and hogs which are minced and desiccated under vacuum at 100°F and later degreased. The dried substance is then dissolved in physiological sodium chloride solution in which one cubic centimeter of the solution represents five grains of the desiccated substance. This watery solution is then placed in ampoules and preserved by the addition of sufficient phenol to make one-tenth of one per cent concentration. Pills and tablets are also prepared for oral administration by simple mixture of the desiccated ovarian substance and a pilular base substance. These tablets are quantitatively expressed in terms of grains of the desiccated substance they contain. Capsules and powders are likewise prepared from the desiccated substance. The commercial preparations available are extracts of the whole ovary, corpus luteum, the ovarian residue, and combinations of varying amounts and compositions with other products of the ductless glands. Still another process is used in which

the whole ovaries containing the corpus luteum, the corpus luteum alone, and the ovary after removal of the corpus luteum are placed in a hot air oven until perfectly dry, the dried material is then finely comminuted and degreased by washing with lipoid solvents. The residue after a second drying is made up into an aqueous solution, tablets, pills or powders.

For convenience and economy in the preparation of an extract containing the female sex hormone human placentae were secured for extraction purposes. The method used to extract the hormone consisted of washing the fresh placenta in water and removing the umbilical cord. The placenta was then minced in a food grinder and the mass transferred to flasks to which a double volume of 95 percent alcohol was added. After macerating for thirty-six hours the solids were filtered out thru several thicknesses of outing flannel and the filtrate placed in tightly stoppered flasks. This fluid was then placed in divided portions into a distillation apparatus and the alcohol removed. The residue consists of a watery sludge of a yellowish, reddish-brown turbid appearance.

To the sludge was added an equal volume of benzol and the mixture was shaken frequently for several hours. The whole was then placed in a separatory funnel and the lower aqueous portion removed. The upper benzol fraction

was put into an evaporation dish and placed on a hot water bath where the benzol was evaporated leaving a yellowish thick oily mass which became solid on cooling.

The placentae thus treated gave a total combined hormone yield of twelve grams by weight. An average of 1.2 grams of hormone for each placenta represents the average for the ten placenta used.

Sufficient hot 2 percent camphorated olive oil was added to one-half the hormone to represent 50 milligrams of hormone to each cc of oil. To the remaining one-half the hormone sufficient hot 2 percent camphorated olive oil was added to give a concentration of 25 mg of hormone to each cc of oil. The whole was then placed, while still hot, into sterile vaccine bottles and tightly stoppered and sealed.

Several one cc ampules of this stock containing 25 mg to the cc of olive oil were sent to Dr. R. G. Gustavson, Denver University, for assay. He, however, sent it to Dr. R. T. Frank in New York City, who assayed the preparation by injections into a castrate rat. The following is a report of his assay as given by Dr. Gustavson.

1/4 cc injections were negative.

1/2 cc injections were two plus.

3/4 cc injections were positive.

Thus 18.75 milligrams of hormone turned a positive reaction in a castrate female rat. This material gave beneficial re-

sults in several cases of functional sterility in cows when injected in larger varying amounts.

Two heifers weighing 610 and 630 pounds respectively were spayed and allowed one month to recover from the operation. Both animals showed sexual maturity by the presence of a corpus luteum in one of the ovaries at the time of operation.

These heifers were injected one month after the removal of their ovaries with one cc of placental extract of 25 mg hormone content three times a day for three days. Close observation of the vulvae and vaginae disclosed no reaction.

Three weeks elapsed before the next injections were made. At this time the amount of the injections was doubled but with the same failure of reaction resulting. At the end of the second three-weeks period heifer No. 1 received two seven and one-half cc injections of 50 mg per cc placental hormone extract injected six hours apart. Reactions were again entirely absent, as far as external manifestations of oestrum were concerned and it was noted that the oily suspension was not readily absorbed but remained under the skin for four or five days.

At the end of the third three-weeks period a full gram of placental hormone in 15 cc of 2 percent camphorated olive oil was injected into heifer No. 1 at one dose. Absorption was again very slow. Vaginal smears were taken for

five days previous to the injection and continued for nine days after injection. The smears were obtained by inserting a 14" long and 3/4" diameter heavy glass tube into the vagina and thru the lumen of which was passed a platinum loop to the anterior vagina where the smear material was obtained in the loop and smeared on a slide. After drying the smear spontaneously the slide was immersed in 1 percent alum solution for two minutes, then in hemotoxylin for three minutes, washed in H_2O and stained with 1 percent aqueous eosin for one minute, washed in water and mounted in balsam. This method gives a well defined differentiation of cell types and nuclear stain affinities. From the examination of the slides it was shown that an oestrus-like reaction had occurred.

The apparent necessity for a more rapid absorption of the preparation led to the production of a colloidal emulsoid. One gram of the hormone as obtained by the previously described method was added to 60 cc physiological saline solution containing 5 percent of equal parts of borax and boric acid and the whole shaken until an emulsion-like product was obtained. The colloidal emulsoid was then placed in sterile vaccine vials and stoppered. The last method seems to meet some of the requisites of a commercial extract for veterinary use in that it can be emulsified in an alkaline aqueous solution, is readily absorbed and can be measured accurately for unitage. It is readily standardized for potency and re-

mains stable for an indefinite period when not exposed to light or air. This preparation was not tried in the larger animals.

However, the extract has the objection of being too complicated and expensive of production for ordinary veterinary use wherein many animals of not very high value are to be treated or where the animal is even less valuable to the owner in the event treatment fails. Thus, it seems that a simpler method of extraction would be of greater general veterinary utility, less arduous of processing, and far less expensive, and within the limits of the average practitioner's ability for production.

Three kilograms of bovine ovaries having matured or nearly matured follicles were obtained from the packing plants at Detroit, Michigan. The follicle fluid was aspirated from the follicles by means of a five cubic centimeter hypodermic syringe and a small gauge needle. To 300 cc of this follicle fluid was added a double volume (600 cc) of 95 percent alcohol and the mixture shaken for several minutes in a flask. The flask was tightly stoppered and placed in a cool atmosphere for twenty-four hours. The alcohol coagulated and precipitated the proteins which for the most part settled to the bottom of the flask leaving the alcohol somewhat cloudy due to a small amount of suspended protein particles. The alcohol possessed a yellowish color when viewed thru the flask.

The precipitated proteins were removed from the alcoholic extract by filtering thru several thicknesses of outing flannel. The filtrate appeared clear and free of suspended matter. By placing the filtrate in a flask the lower part of which was immersed in a hot water bath and connecting a condensing apparatus to the flask stopper, it was found that the alcohol could be removed entirely by distillation leaving a viscid aqueous fluid residue. This material which possessed a distinctly yellow color and a not unpleasant odor represents an aqueous suspension of the follicular hormone. The tissue fats and other diluents were not removed. Formalin was then added as a preservative and the material placed in sterile vaccine vials for the potency test.

The test for the potency of this material was carried out on five adult female castrate white laboratory rats. Subcutaneous injection of varying amounts were made for two days in the abdominal region and the reactions determined by vaginal smears made on the third day following the first injections. Positive reactions were secured in each case as determined by the vaginal smear method.

In this assay a change was made from the smear method of Stockard and Papanicolaon and Allen and Doisy. It became apparent that the irritant action of even a carefully controlled delicate wire loop or a cotton swab was

sufficient to produce a change in the cytographical picture. Instead of the wire loop or a small cotton swab a cone cubic centimeter pipette was used to introduce a minute amount of sterile physiological sodium chloride solution into the vagina. After this was allowed to remain in the vagina for about a minute, the region over the lower abdominal region was then lightly compressed with the thumb of the hand holding the rat forcing the solution toward the external vaginal orifice where it is readily taken up by the pipette. This method produces no injury to the vaginal mucosa and is preferable to other methods in that it precludes any injury to the genital tract and the animals readily tolerate the procedure and evidence no pain.

The remaining portion was diluted with an equal volume of ether, was vigorously shaken and allowed to stand for six hours. The whole was then placed in a separatory funnel and the lower aqueous layer was drawn off and discarded. The ether fraction was then evaporated spontaneously leaving an oily residue. The residue, which was a yellowish viscid solid was taken up in hot olive oil and placed in sterile vaccine vials. This oily suspension when injected into cows and dogs suffering from functional sterility gave very gratifying results and a condition of normal function restored in about sixteen cases so treated.

A direct deviation from the process employed in the previous preparation was made by macerating in a covered flask in physiological sodium chloride solution finely minced connective tissue-denuded, non-selected fresh ovaries from cows. These ovaries were collected at random from the animals slaughtered at one day's killing. To each minced ovary five cubic centimeters of slightly acidified (HCl) physiological sodium chloride solution was added to make up the macerating medium and the whole placed in a refrigerator. After the mass had soaked for eighteen hours it was placed over the flame and boiled for ten minutes to coagulate the proteins. The solids were then filtered out thru several thicknesses of outing flannel. The filtrate was allowed to cool and a sufficient amount of a ten percent formalin solution added as a preservative to give a formalin concentration of one-tenth of one percent. The extract was then placed in vaccine bottles of a fifteen cubic centimeter capacity and tightly stoppered.

This material possessed but a slight flesh-like odor and was a little more viscid than water. There was a slight opacity but it was otherwise colorless. The opacity was due to a small amount suspended proteins.

The assay of this aqueous extract to determine its potency value was made in castrate adult female rabbits. Into each of the three castrate adult female rab-

bits one cubic centimeter of the extract was injected daily for four days, a fourth castrate adult female rabbit was used as a control. On the morning of the fifth day post-mortem examination revealed no signs of uterine hyperplasia in any of the injected subjects. The uteri resembled, in all respects, the uterus of the control which showed anemia and atrophy. The extract was accordingly considered without hormone content or possessing so small a quantity of hormone as to have no effect.

The next method of the production of an extract followed somewhat the above except that ovaries having matured or nearly matured follicles were selected and any corpora lutea present were removed. The ovaries were then minced in a meat grinder and to each ovary thus comminuted five cubic centimeters of physiological sodium chloride solution was added. The mass was allowed to macerate at ordinary room temperature for eighteen hours. Upon expiration of that time the solids were filtered out thru several thicknesses of outing flannel and the filtrate reduced by evaporation on a steam bath to one-fourth of its original volume. Formalin was added as a preservative and the extract sealed in sterile vaccine bottles.

The potency test was carried out on two adult female castrate rabbits. No control being available. Injections of five cubic centimeter doses were made into the ab-

dominal musculature twice a day for four days and the animals were examined postmortem the morning of the fifth day. The uteri of both rabbits showed a pronounced hypertrophy which was of such a degree as to be indicative of the action of the hormone in the extract. This preparation was accordingly considered to possess an ovarian hormone content. At this point in the work a quantitative assay of the hormone content of the extract was made. The method of Stockard and Papanicolaon, 1917, as modified by Allen and Doisy, 1923, for following the uterine changes in the rat by the microscopic examination of vaginal smears as described previously was adopted. Five adult female laboratory white rats were ovarectomized and allowed six days in which to recover from the operation. Injections of graded quantities were made subcutaneously in the abdominal region three times a day for two days and the vaginal smear readings made on the fourth day. The amount being the same at each injection for the same rat. The results were both positive and negative showing clearly the necessity of passing the threshold point of concentration in the blood stream to induce oestrus. After a period of six days had elapsed the same five rats were again injected but with larger amounts of this extract. The reactions were all strongly positive as shown by the vaginal smear readings.

The qualitative assay of this extract having been determined it was deemed advisable to make a quantative determination of the preparation. Several vials were emptied into a flask and a double volume of benzol added. The mixture was vigorously shaken and allowed to stand at room temperature for eighteen hours. The whole was then placed in a separatory funnel and the lower aqueous portion removed. The benzol fraction was then placed in an evaporating dish and placed in a hot water bath where the benzol was removed leaving the hormone. The quantity of the material thus secured showed the extract to contain considerable hormone. In physical appearance the hormone was identical with that obtained from human placentae and from the aspirated follicular fluid. When taken up in olive oil and injected into spayed rats a positive reaction was given as determined by the smear method.

A large quantity of this preparation was sent to practicing veterinarians for their use in the treatment of cases of sterility of a functional nature. Their reports have shown that the extract was well adapted to veterinary therapy and that the results they obtained in over one hundred cases from its use were very gratifying. How many of these cases might have returned to normal function without treatment cannot be hazarded.

The last method seems a desirable procedure for the preparation of an ovarian hormone for veterinary use

in some of the lower animals. The large number of cases of sterility that have been treated with this extract and the success obtained in the return to normal function of a large percentage of these cases points to its usefulness and practicability. It also seems that a large part of the present tremendous economic loss to the livestock industry could in part be prevented by the use of this product.

VI

Conclusions.

An aqueous extraction of selected bovine ovaries yeilds the female sex hormone.

The extract has a pronounced dynamic oestrus-inducing effect upon the castrate and uncastrate female (rat and cow) as evidenced by laboratory and clinical observations.

Such a dyamogenic action upon the genitalia of the female suggest future possibilities for this product in veterinary opotherapy.

The simplicity of the method denotes the practicability and economy of the process of preparation.

VII

Explanatory Note.

The lack of specific data which includes reaction tables, charts, photographs, individual case reports, and photomicrographs of the biological assays of the extracts is due to their having been consumed in the fire that destroyed the veterinary buildings. Consequently, the work as hereinafter presented is very brief and devoid of the details necessary to a well balanced scientific paper. The author realizes this fact and recognizes the inexactitude that must occur if memory alone were depended upon for the lost records.

Only that part of the work which can be dealt with in a more or less general descriptive manner is given.

Intermittent periods of work covering about three years was spent on this problem. No less than one hundred twenty-five animals were used in making the observations. During the procedure photographs and photomicrographs were made of some of the reactions obtained from injections of the various extracts, these were accompanied by charts, tables and case histories, and the results obtained. It is thus apparent that much time and travel was necessitated in gaining this information.

It is to be regretted that the loss of this material was sustained for it would have supplied the exact data so poignant to a paper of this nature.

VIII

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