UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION
RESEARCH BULLETIN 150

A Study of the Estrus Producing Hormone in the Urine of Cattle During Pregnancy

(Publication Authorized August 25, 1930)

COLUMBIA, MISSOURI
SEPTEMBER, 1930

TABLE OF CONTENTS

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE

Agricultural Experiment Station

STATION STAFF, SEPTEMBER, 1930

WALTER WILLIAMS, LL.D., Acting President

F. B. MUMFORD, M.S., D. Agr., Director S. B. SHIRKY, A. M., Asst. to Director

MISS ELLA PAHMEIER, Secretary

AGRICULTURAL CHEMISTRY

A. G. HOGAN, Ph.D.
L. D. HAIGH, Ph.D.
W. S. RITCHIE, Ph.D.
A. R. HALL, B.S. in Agr.
E. W. COWAN, A. M.
ROBERT BOUCHER, JR., A.M.
L. V. TAYLOR, A.B.
ROBERT PILCHER, M.S.
LUTHER W. RICHARDSON, A.M.
U. S. ASHWORTH, A.B.

AGRICULTURAL ECONOMICS

O. R. JOHNSON, A.M.
BEN H. FRAME, A.M.
F. L. THOMSEN, Ph.D.
PRESTON RICHARDS, A.M.
C. H. HAMMAR, Ph.D.

AGRICULTURAL ENGINEERING

J. C. WOOLEY, M.S.
MACK M. JONES, M.S.
R. R. PARKS, B.S. in Agr. Eng.
D. D. SMITH, B.S. in A.E.

ANIMAL HUSBANDRY

E. A. TROWBRIDGE, B.S. in Agr.
L. A. WEAVER, B.S. in Agr.
A. G. HOGAN, Ph.D.
F. B. MUMFORD, M. S., D. Agr.
D. W. CHITTENDEN, A.M.
H. C. MOFFETT, A.M.
J. E. COMFORT, A.M.
F. F. McKENZIE, Ph.D.

BOTANY AND PHYSIOLOGY

H. W. RICKETT, Ph. D
I. T. SCOTT, Ph.D.

DAIRY HUSBANDRY

A. C. RAGSDALE, M. S.
WM. H. E. REID, A.M.
SAMUEL BRODY, Ph. D.
C. W. TURNER, Ph.D.
HARRY HERMAN, B.S. in Agr
WARREN GIFFORD, A.M.
E. R. GARRISON, A. M.
M. E. POWELL, A.M.

ENTOMOLOGY

LEONARD HASEMAN, Ph.D.
T. E. BIRKETT, B. S. in Ed.

FIELD CROPS

W. C. ETHERIDGE. Ph.D.
C. A. HELM, A. M.
L. J. STADLER, Ph.D.
R. T. KIRKPATRICK, A. M.
B. M. KING, A.M.
MISS CLARA FUHR, M.S.*
W. R. TASCHER, Ph. D.
S. F. GOODSELL, A.M.

HOME ECONOMICS

MABEL CAMPBELL, A. M.
JESSIE A. CLINE, A. M.
MARGARET C. HESSLER, Ph.D.
ADELLA EPPEL GINTER, M.S.
SYLVIA COVER, A.M.

HORTICULTURE

T. J. TALBERT, A.M.
H. G. SWARTWOUT, A.M.
J. T. QUINN, A.M.
A. E. MURNEEK, Ph.D.

POULTRY HUSBANDRY

H. L. KEMPSTER
E. M. FUNK, A.M.

RURAL SOCIOLOGY

E. L. MORGAN, A.M.
HENRY J. BURT, A.M.
MISS ADA C. NIEDERMEYER, A.M.
WALTER BURR, A.M.
HOWARD E. JENSEN, Ph.D.
GEORGE A. GEMMELL, A.M.

SOILS

M. F. MILLER, M.S.A.
H. H. KRUSEKOPF, A.M.
W. A. ALBRECHT, Ph. D.†
HANS JENNY, Ph.D.
GEORGE Z. DOOLAS, M.S.
LLOYD TURK, A.M.
HAROLD F. RHOADES, B.S.A.
JAMES F. LUTZ, B.S.A.

VETERINARY SCIENCE

J. W. CONNAWAY, D.V.M., M.D.
O. S. CRISLER, D.V.M.
A. J. DURANT, A.M., D.V.M.
ANDREW UREN, D.V.M.
ELLMORE F. SANDERS, D.V.M.

OTHER OFFICERS

R. B. PRICE, B.L., Treasurer
LESLIE COWAN, B.S., Sec., of Univ.
A. A. JEFFREY, A.B., Agricultural Editor
J. F. BARHAM, Photographer
LEON WAUGHTEL, Ass't Photographer
JANE FRODSHAM, Librarian

*In service of U. S. Department of Agriculture. †On leave of absence.

ABSTRACT

In a study of the rate of secretion of the estrus producing hormone in the urine of pregnant cows, considerable variation was observed at all stages of gestation. On the average, however, the hormone was found to be secreted at a low level during the early stages of pregnancy but gradually increases until the time of parturition. Following parturition the secretion of the hormone declined rapidly. Of the animals included, little difference in the rate of secretion of the hormone by Holstein and Jersey cattle occurred. However, in comparison with beef cattle it was found that the former begin to show a very noticeably higher level of secretion after 100 days of gestation. In view of the experimental work indicating that the estrus producing hormone plays a part in the normal growth of the mammary gland, reasons are advanced for the belief that the increasing secretion of the hormone during pregnancy is required for the development of the gland.

A Study of the Estrus Producing Hormone in the Urine of Cattle During Pregnancy

C. W. Turner, A. H. Frank, C. H. Lomas, and C. W. Nibler*

It has long been realized that there is a very close relation between the development of the mammary glands and the cycle of reproduction. However, it has only been during the last thirty or forty years that definite experimental work has been conducted to determine the mechanism which governs the growth of the mammary gland and the secretion of milk at parturition.

The prevailing opinion of physiologists previous to 1900 was that the development of the mammary gland was under the influence of the nervous system. To determine the relation between the nervous system and the development of the mammary gland, two types of eexpriments were tried.

One type, illustrated by the work of Goltz and Ewald (1896) was to sever the nervous connections passing to the pelvic organs. They found that in such cases normal pregnancy was accompanied as usual by mammary gland development and lactation.

A second type of experiment consisted in transplanting mammary gland tissue of small animals into the skin of the ear. In these cases Ribbert (1898) and Pfister (1901) observed that normal growth of the gland occurred. It seems evident, therefore, that the growth of the mammary gland is not dependent on the normal nervous connections.

On the other hand, it is well known that the ovary has a very marked influence on the mammary gland. The removal of the ovaries stops all further development of the glands and results in definite signs of involution. That this is not due to the severing of the nervous connection between the ovary and mammary glands was proven by the experiments of Grigorieff (1897), Halban (1900), Knauer (1900), and others, who showed that the ovaries could be grafted successfully into the wall of the peritoneum as well as intramuscularly. Here again the normal nervous connections were demonstrated to be unnecessary, for the organs of reproduction remained normal and the mammary glands developed the same as when the ovaries were in the normal site.

These observations led investigators to the view that the growth of the mammary gland and initiation of milk secretion were under the control of substances elaborated by the organs of reproduction, and possibly by the products of conception, which were carried by the blood stream and stimulated the growth observed.

*This paper is a summary of two years of experimental work conducted under the direction of the senior author. The data presented formed parts of the theses of C. W. Nibler, 1929, A. H. Frank, and C. H. Lomas, 1930, presented in partial fulfillment of the requirements for the degree of Master of Arts in the graduate school of the University of Missouri.

The classic experiments of Lane-Claypon and Starling (1906) were the first to determine the possible presence in the organs of reproduction of substances which would stimulate the growth of the mammary gland. While they failed to produce material development of the mammary gland, the hormone theory was widely accepted and a new era of scientific research was begun.

Reports of the effectiveness of water soluble extracts were made by Foa (1908), Biedl and Königstein (1910), Aschner and Grigoriu (1911) Frank and Unger (1911), and others. In 1912 Iscovesco found that the lipoid solvents were more effective in extracting the active principle. The extensive experiments of Fellner (1913) in which lipoid extracts were used marks the beginning of the successful use of isolated extracts of the reproductive organs.

With the introduction of the rat unit test for the estrus producing hormone by Allen and Doisy (1923-24), it became possible to further study the factors producing the growth of the mammary glands, especially the role of the estrus producing hormone. Two types of experiments may be made. It is possible to study the development of the mammary gland following the injection of potent extracts of the estrus producing hormone. For a review of this work the reader is referred to a previous paper in this series by Turner and Frank (1930).

The second method of indicating the relation of the estrus producing hormone to the mammary gland would be the demonstration of the estrus producing hormone in the blood during the period of active growth of the mammary gland. To Loewe (1925) goes the credit for discovering the presence of this hormone in the blood during the menstrual cycle. This was followed by its discovery in the blood during pregnancy by Fels (1926). It was soon discovered (Loewe and Lange, 1926), that the path of elimination of the hormone from the blood was by way of the kidney into the urine. As a result of this discovery, a study was initiated at the Missouri Experiment Station to determine the amount of the estrus producing hormone in the urine of pregnant cows during the advance of the stage of gestation. Two short preliminary reports of this work have been published (Nibler and Turner 1929). This work has been continued during the past year. The purpose of the present report is to summarize the results of these two years of work.

REVIEW OF LITERATURE

The Biological Assay of the Estrus Producing Hormone.—As noted above, the discovery of the rat unit test has made possible the rapid biological assay of the estrus producing hormone. It is necessary, there-

fore, to review the literature covering the development and recent progress in this important field.

The Estrus Cycle.—In spite of the ease with which small animals can be kept for study in the laboratory, it was not until comparatively recently that a complete, detailed study of the estrus cycle in the rat was reported. Early workers were handicapped by the difficulty of external diagnosis of estrus, and were compelled to use observed copulation as their starting point. The discovery, however, that a very definite change occurred in the epithelial cells of the vaginal mucosa in the guinea pig by Papanicolaou and Stockard (1917) has made it possible to analyze completely the length of the estrus cycle in other test animals. Since the rat was used as a test animal, in a study to be reported later, a brief description will be given of the changes taking place in the normal estrus cycle of this animal. In view of the excellent work of Long and Evans (1922), on the estrus cycle of the rat, it will suffice to give a summary of their work.

As a result of this investigation, the length of the estrus cycle of the rat was concluded to be about four days, which is divided into the following stages.

Diestrus Interval. This period covers one-half of the entire cycle; nucleated epithelial cells and leucocytes constitute most of the vaginal smear during this stage. The smear tends to be fairly fluid.

Stage 1. *Proestrum.* During this stage the leucocytes disappear altogether from the vaginal contents and the smear is made up entirely of nucleated epithelial cells. During this stage females will usually not accept copulation. Average length, twelve hours in the normal rat.

Stage 2. *Early Estrum.* This is the period of mating. The small, nucleated, and somewhat granular appearing epithelial cells characteristic of Stage 1 are rather quickly replaced by large, thin, transparent, non-nucleated scale-like elements, the cornified cells. Average length of this stage is approximately twelve hours.

Stage 3. *Late Estrum.* This stage is similar to Stage 2 but differing in the respect that animals at this time will usually no longer accept coitus. The average combined length of Stages 2 and 3 would appear to be about thirty hours.

Stage 4. *Metestrum.* This stage is inaugurated by the appearance in the vaginal smear of leucocytes among the cornified cells and ends with the disappearance of the latter. Finally, nucleated epithelial cells appear, and the smear becomes of the normal diestrus type. This stage generally lasts six hours.

Allen (1922) made a similar study of the mouse. He found the entire cycle to last, on an average, for a period extending from four to six days. Likewise, he divided the cycle into four stages: the diestrus interval, proestrum, estrus period, and metestrum. The cellular changes

occurring during each period are practically the same as outlined by Long and Evans. General conclusions from his observations are as follows:

1. That external signs were unreliable criteria for estrum and the vaginal smear method was fairly accurate.
2. That before ovulation there is a rapid growth of the vaginal epithelium and the formation of a stratum corneum. After ovulation there is an infiltration of leucocytes caused by a degeneration and replacement of the stratum corneum.

A representation of the estrus cycle, as determined by the vaginal smear method, is shown in Figure 1.

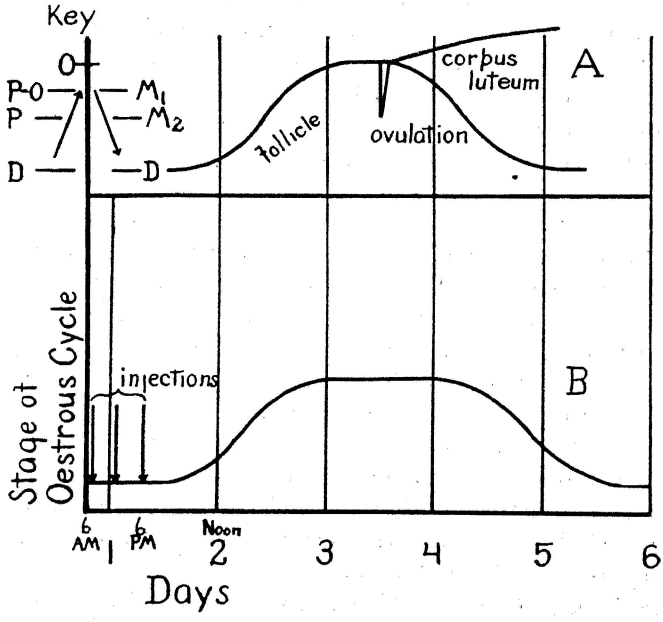

Figure 1. Representation of estrus cycles as determined by the vaginal smear method. Allen (1924). Explanation of key: A. Normal cycle in an unoperated animal. B. Experimental cycle in a spayed animal induced by three injections of follicular hormone. D. Diestrum P. Proestrum O. Oestrum M^1. Metestrum (early) M^2. Metestrum (late).

The Rat Unit Test of the Estrus Producing Hormone.—Any biological test for the activity of the estrus producing hormone must clearly depend on the production of estrus changes when they would not otherwise occur. Thus, immature or ovariectomized animals must be used, as there is danger of spontaneous ovarian activity. Ovariectomized animals are, therefore, highly desirable and are generally used. Any method used in testing the potency of the extract, to be desirable, must give an abrupt and definite reaction on the test animals.

The rat unit test devised by Allen and Doisy (1924) has given a simple method of determining, in a qualitative way, the potency of hormone extracts. The rat unit has been defined as: "The minimum amount of potent substance necessary to produce a full change from the negative to the estrual vaginal spread in a castrate rat of approximately one hundred and forty grams (± 20 grams) in weight".

Allen and his co-workers (1924) reported that the changes in the vaginal smear of the castrated animal could be detected forty to forty-eight hours after injection. They further state that for certain physiological reasons, three subcutaneous injections are made at four-hour intervals.

Suggested Modifications of the Allen-Doisy Test.—As a result of the widespread use of this test, a number of investigators have suggested certain modifications and improvements. Thus Coward and Burn (1927) say that the rat unit will vary one thousand per cent. They re-define the rat unit as the amount which will produce characteristic estrus in fifty per cent of rats injected. For titration, Laquer (1927) recommends the use of a minimum of twelve test animals. Of these at least seventy-five per cent should show the characteristic estrus changes.

Bugbee and Simond (1926) found that the rat unit varied directly with the weight of the test animal. They concluded that the following formula will obviate inaccuracies due to the effect of direct proportion of dosage to weight:

$$\text{R. U. per c. c.} = \frac{W}{140\,Q}$$

in which W is the weight of rat in grams, and Q is the minimum c. c. of hormone extract which will produce estrus.

To eliminate certain criticisms offered, and to obviate any individual variation of the rat unit test, Kant and Doisy (1928) have outlined a further procedure for the vaginal smear method of assay of the estrus producing hormone. In brief they propose that:

1. Daily smears to be made for two or three weeks before ovariectomy, and only those animals with normal cycles should be used.
2. Make daily smears for two weeks after operation but before the beginning of injection. Discard any animal showing estrus or proestrus smears later than the second day after operation.
3. Prime the animals with two rat units.
4. One week later test the reaction of each animal by the injection of one and three-tenths rat units. If the smears are negative, discard the animals.
5. A week later test the reaction to seven-tenths rat unit; if positive smears occur, discard the animal.

6. Use each animal for no longer than four months.

7. In attempting accurate assays, prime all animals by injecting one and five-tenths rat units, if the reaction of the preceding week was negative.

8. Use a sufficient number of animals.

If seventy-five per cent of the animals injected with the same volume give a positive reaction, consider that the amount injected contains one rat unit.

Methods of Administration.—Several methods of administration have been tried in an effort to determine through what entrance to the body the hormone is most effective. Zondek and Aschheim (1925) have used a method of intra-muscular implantation. This technique consists in the implantation into the muscle of a small piece of the tissue to be tested. This method is used for testing pieces of tissue too small to extract, and also to save chemical treatment. It was by this method that the ovary stimulating properties of the anterior pituitary body was discovered.

Intra-venous injections have not been found satisfactory with the crude fat-soluble extracts used in the past. According to Parkes (1929), however, this method may become of importance with the assay of the comparatively pure water-soluble preparations now available. More frequent injections, however, would be required to keep the continuous action which is necessary for positive results.

Allen (1924) reports uniformly negative results of the oral administration of the estrus producing hormone. Loewe, Lange, and Faure (1926) found that twenty-fold larger doses were required by oral administration than by subcutaneous injections. Without regard to the species the dose required was proportional to the weight of the animals used. Hannan (1928) found a ratio of one to sixty-one necessary to produce oestrus by oral administration. Brown (1930) reports that twenty units are required to produce estrus in twenty-five per cent of the test animals.

The fate of the hormone in the alimentary canal does not seem to be known. Frank (1929) offers the hypothesis that the hormone after ingestion is absorbed into the portal system, taken up by the liver and stored in the bile. It, therefore, does not reach the general circulation, but is excreted in the urine and feces.

The most common method of administration is by subcutaneous injections. The behavior of the preparation injected by this method varies greatly according to the nature of the extract. Preparations containing large amounts of impurities cause granulation and subsequent sloughing of the skin. However, with the somewhat purified extracts, injections by this method are very effective and no disturbance occurs at the point of injection.

In test animals intraperitoneal administration has not been found to have any advantages over subcutaneous injections. Coward and Burn (1927) were unable to detect greater efficiency, and Evans and Burr (1926) even report a decreased activity from intraperitoneal injections.

Pratt and Smeltzer (1929) obtained characteristic changes of estrus by applying estrus producing hormone to the mucous membranes of the eyes, nose, and vagina of rats. However, it was found that the smallest dose which gave a positive test was double the amount necessary to give the same result when the material was injected hypodermically. Powers, Varley and Morrell (1929) prepared the estrus producing hormone in the form of a gelatin pessary for vaginal administration. The characteristic signs of estrus were induced in spayed albino rats with the preparation. The ratio between the pessary dose and the subcutaneous dose was probably in the neighborhood of three to one.

Sources of the Estrus Producing Hormone.—The progress in the isolation of the estrus producing hormone may be divided into two more or less distinct periods. Early workers were largely concerned with the preparations of water-soluble ovarian extracts.

Jentzer and Beuttner (1900) were among the first to attempt to determine, in an experimental way, the action of ovarian extracts. Their saline extracts were not effective. Marshall and Jolly (1906) produced estrus changes in an anestrus bitch by the injection of saline extracts of ovaries. Iscovesco (1912) by the use of lipoid solvents, extracted a substance which caused rapid hypertrophy of the uteri of adult animals; the uteri of the treated animals being three or four times as heavy as the controls. Fellner (1912) analyzed the effect of lipoid extracts of various reproductive organs. The effectiveness of these extracts on the uterus, vagina, and mammary gland was observed. The advances made by Fellner were chiefly the use of ovariectomized animals, and the careful examination of the effects produced.

Since the advent of the Allen-Doisy rat unit test the studies of the estrus producing hormone, which up to this time had stagnated to a certain degree, were again taken up with zeal. The extracts were prepared in much the same way as those of previous workers (the use of lipoid solvents). At the same time, various elaborations were being introduced in an effort to further purify the product.

As has been shown, early workers were primarily interested in the effect of the activating substance on test animals. This simple and easily performed test stimulated investigators to seek new sources, as well as to study its effect on the reproductive organs.

With the simultaneous isolation of the estrus producing hormone from the follicular fluid of sow's ovaries and the perfection of the rat unit

test by Allen and Doisy (1923), investigators began to seek new sources of the hormone. Some of the more important sources which have been discovered and tested by the vaginal smear technique are given in Table 1.

TABLE 1.—SOURCES OF THE ESTRUS PRODUCING HORMONE AS TESTED FOR BY THE VAGINAL SMEAR TECHNIQUE

Source of Extract	Yield per kilogram or liter	Investigator
Whole Ovaries		
Cow	293 m. u.	Parkes and Bellerby (1926)
Pig	120 r. u.	Doisy, Ralls, Allen and Johnston (1924)
Pig	219 m. u.	Parkes and Bellerby (1926)
Hog	220 r. u.	Morrell, McHenry and Powers (1930)
Sheep	Positive	Allen and Doisy (1923)
Sheep	203 m. u.	Parkes and Bellerby (1926)
Hens	Positive	Allen, Whitsett, Hardy & Kneibert (1924)
Human	Positive	Allen, Pratt, and Doisy (1925)
Liquor Folliculi		
Human	433-7000 r. u.	Allen, Pratt, and Doisy (1925)
Cow	Positive	Allen and Doisy (1923)
Cow	37-788 m. u.	Parkes and Bellerby (1926)
Sheep	Positive	Allen and Doisy (1923)
Horse	133 m. u.	Parkes and Bellerby (1926)
Mare	Positive	Hart, DeJongh, Laquer, Wysenbeck (1925)
Sow	2100 r. u.	Allen and Doisy (1923)
Pig	167 r. u.	Dickens, Dodds, and Wright (1925)
Pig	600-1600 m. u.	Laquer, Hart, DeJongh, and Wysenbeck (1925)
Pig	23-75 m. u.	Parkes and Bellerby (1926)
Pig	878 r. u.	Ralls, Jordan and Doisy (1926)
Hen	Positive	Allen (1924)
Corpora Lutea		
Human	3700 r. u.	Allen, Pratt and Doisy (1925)
Hog	170 r. u.	Frank and Gustavson (1925)
Pig	8-25 r. u.	Allen and Doisy (1927)
Cow	16-184 m. u.	Parkes and Bellerby (1927)
Placenta		
Human	400-700 r. u.	Doisy, Ralls, Allen & Johnston (1924)
Human	150-300 m. u.	Loewe (1926)
Human	1500 m. u.	Aschheim (1926)
Human	192-2123 m. u.	Parkes and Bellerby (1927)
Human	52-270 r. u.	Allen, Dickens, Dodds and Howitt (1928)
Cow	Positive	Allen (1925)
Cow	143-4300 m. u.	Parkes and Bellerby (1928)
Sheep	183-308 m. u.	Parkes and Bellerby (1927)
Blood of Females		
Human (during menstrual cycle	Positive	Frank, Frank, Gustavson & Weyerts (1925)
Human	Positive	Loewe (1925)
Human (during) pregnancy)	1000 m. u.	Aschheim (1926)
Human (during pregnancy)	200 m. u.	Fels (1927)
Hogs	Positive	Frank (1925)
Sow	43 r. u.	Morrell, McHenry, and Powers (1930)
Cow	40 r. u.	Morrell, McHenry and Powers (1930)

TABLE 1.—SOURCES OF THE ESTRUS PRODUCING HORMONE AS TESTED FOR BY THE VAGINAL SMEAR TECHNIQUE (CONTINUED)

Source of Extract	Yield per kilogram or liter	Investigator
Urine of Females		
Human	Positive	Loewe (1925)
Human (during menstrual cycle)	Positive	Loewe and Lange (1926)
Human (during pregnancy)	2-7000 m. u.	Laquer (1927)
Human (during pregnancy)	1100 m. u.	Aschheim and Zondek (1927)
Human (during pregnancy)	470-1240 r. u.	Veler and Doisy (1928)
Human (during pregnancy)	300-500 m. u.	Frank (1929)
Cow (during pregnancy)	Positive	Aschheim and Zondek (1927)
Cow (during pregnancy)	650 r. u.	Hisaw and Meyer (1929)
Cow (during pregnancy)	12-148 r. u.	Nibler (1929)
Cow (during estrus cycle)	11 r. u.	Nibler (1929)
Cow (during pregnancy)	2-400 r. u.	Frank (1930)
Fetal Fluid		
Cow (amniotic)	87 r. u.	Morrell, Powers, and Varley (1930)
Cow (allantoic)	87 r. u.	Morrell, Powers, and Varley (1930)
Feces		
Human	30,000 m. u.	Dohrn and Faure (1928)
Hen	Positive	Frank (unpublished)
Milk		
Goat (during estrus)	1000 m. u.	Frank and Gustavson (1929)

Following the isolation of the estrus producing hormone from liquor folliculi by Allen and Doisy (1923), the next important source was the discovery of its presence in the placenta by Allen and Doisy and co-workers (1924), Laquer and collaborators (1925), Loewe (1926), and Parkes and Bellerby (1927).

Since it is obvious that the hormone must pass from its site of origin to its site of activity by way of the circulating blood, one would expect to be able to detect its presence in the blood. Also, since its action is cyclic it might be expected that periodic variation in amount in the blood could be demonstrated.

The presence of the estrus producing hormone in the blood of women during the menstrual cycle was demonstrated by Loewe (1925). A little later Frank and collaborators (1925) announced the recovery of the active

substance from the blood of estrus sows and its absence in the non-estrus animal. The amount of the hormone varied with the stage of the cycle. According to Frank (1929), the estrus producing hormone is present in greatest amounts in the circulating (venous) blood during the five days previous to menstruation. In the menstrual blood it is present in greatest amounts during the first day, after which it rapidly decreases. However, Fels (1926) found the hormone in the circulation only during pregnancy.

Following the isolation of the estrus producing hormone from the circulating blood during menstruation, Fels (1926), Zondek and Ascheim (1927), and Smith (1927) discovered its presence in human blood during pregnancy. Fels describes it as increasing rapidly during the advance of pregnancy. Morrell, Mc Henry, and Powers (1930) report the presence of this hormone in the circulating blood of cows and sows during pregnancy.

Since the estrus producing hormone was demonstrated to be present in the blood, its probable source of elimination would be by way of the kidneys, in the urine. This idea was verified by Loewe and Lange (1926), who demonstrated its presence in the urine of women during the menstrual cycle. Later its presence was demonstrated in the urine of pregnant cows by Aschheim and Zondek (1927). Hisaw and Meyer (1929), Nibler (1929), Frank (1930), and Lomas (1930) in their studies on the estrus producing hormone in the urine of pregnant cows, showed very clearly that the hormone was present at a low level during early pregnancy, but gradually increased as pregnancy continued.

EXPERIMENTAL PROCEDURE

Our studies of the estrus producing hormone content of urine have included individuals of the four breeds of dairy cattle (Ayrshire, Guernsey, Holstein and Jersey) and three breeds of beef cattle (Angus, Hereford and Shorthorn). We are indebted to J. E. Comfort of the Department of Animal Husbandry of the Station for his cooperation, which made possible the collection of urine from the beef cattle.

At the beginning of this investigation the cows were watched continuously to obtain samples of the urine. Before our experiments had progressed very far one of us (A. H. F.) observed that the act of micturition could be induced by a method which will be described later. In all of the work to be reported this method was followed, collection of the urine being made at two hour intervals.

In the preliminary experiments, the length of the period of urine collection necessary to obtain a representative sample of the urine and

the estrus producing hormone was determined by comparing the secretion by twelve and twenty-four hour periods. The average of 12 cows from which urine was collected for forty-eight hours indicate that although the cows show a large variation in many instances from day to day the average variation of all the cows is negligible.

As to a comparison of day and night collections (8 A. M. to 8 P. M. and 8 P. M. to 8 A. M.) an average of 26 cows in all stages of gestation showed that there were 1117 c. c. or 13 per cent more urine, and 29 rat units or 6 per cent more estrus producing hormone secreted during the night collections. It was concluded from these data that a 24-hour collection period was the minimum period during which a representative sample of urine could be obtained. Therefore, uniform collections of urine covering periods of twenty-four hours were made in all subsequent work. Each two-hour collection of urine was measured in a graduated cylinder and poured into cans, from which the estrus producing hormone was extracted.

While there have been published complicated methods of extracting the estrus producing hormone from urine, Doisy, Veler, and Thayer (1929) found that olive oil has an affinity for the hormone found in human urine and would absorb a large proportion of the hormone present in the urine. The same plan was found to operate successfully in the extraction of the hormone from the urine of cattle.

Because of the lack of information as to the efficiency of the method of extraction, considerable preliminary work was necessary. It was found that when 10, 15, or 20 c. c. of oil were added to two liters of urine practically the same number of rat units were extracted. In our studies from 20 to 50 c. c. of oil were added to 2000 c. c. of urine.

After the addition of the oil to the urine the samples were stirred for one hour to obtain a thorough mixture. The stirring apparatus consisted of a battery of six winged-shaped stirring rods driven by an electric motor. The urine and oil mixture was then poured into separatory funnels and allowed to stand until the oil had separated from the urine. The urine was then drawn off and discarded. The oily residue was drawn into beakers and transferred to test tubes for clarification in a centrifuge. The cleared oil was pipetted off and placed in bottles for biological assay of its estrus producing hormone content.

Since the beginning of this investigation, Doisy, Veler, and Thayer (1930) have reported a study on the efficiency of extracting the estrus producing hormone from urine by this method. They find that greater yields are obtained by acidifying the urine to be extracted with hydrochloric acid to a pH of four and allowing the uric acid to settle out before extraction. They also determined the percentage of hormone that was obtained by a single extraction.

A one liter sample of acidulated urine from pregnant women was extracted with five 50 c. c. volumes of olive oil. The first extraction contained 712 rat units, the second 143, the third 125, the fourth 125, and the fifth 143. Another sample of urine treated similarly gave in successive extracts 1250, 167, 230 and 250 rat units. This gives an efficiency of 75 per cent and 73 per cent, respectively, for the first extraction.

It seems from these data that a single extraction does not recover all the hormone present in the urine, but by following a uniform procedure a comparative determination may be made.

Method of Testing the Potency of the Extract.—The Allen-Doisy rat unit method was used in testing the potency of the extracts from the urine of each cow.

Mature, spayed white rats were used as test animals throughout this investigation. The rats were kept in groups of six in wire cages. The rats were fed daily, their diet consisting of a grain mixture and milk. Cod liver oil was mixed in the grain to supplement the vitamin content of the feed.

The subcutaneous method of administration was used for the reasons previously discussed in this paper. Injections of the previously cleared oil were divided into three doses and administered at four-hour intervals. At the time of the first injection vaginal smears were made to be sure that the rats were in the diestrus interval as shown in Figure 2. Any rats showing signs of ovarian activity were discarded.

Vaginal smears were made from the injected rats forty-eight and fifty-six hours after the time of injection. These slides were fixed in an open flame and stained in Delafield's hematoxylin and eosin for microscopic examination.

Smears showing all cornified cells as previously described and shown in Figure 4 were considered positive reactions. The minimum amount of the extract required to produce this condition was considered a rat unit.

Smears showing all nucleated epithelial cells as elsewhere described and shown in Figure 3 indicate that some action had taken place but not enough to bring the test animal into full estrum.

Smears showing a predominance of leucocytes were designated as negative, indicating that too little of the hormone had been injected to bring the test animal into the full estrus condition (see Figure 5).

As it requires a period of five or six days after injection for the test animal to return to the diestrus interval condition, the same animal was not injected more often than at weekly intervals.

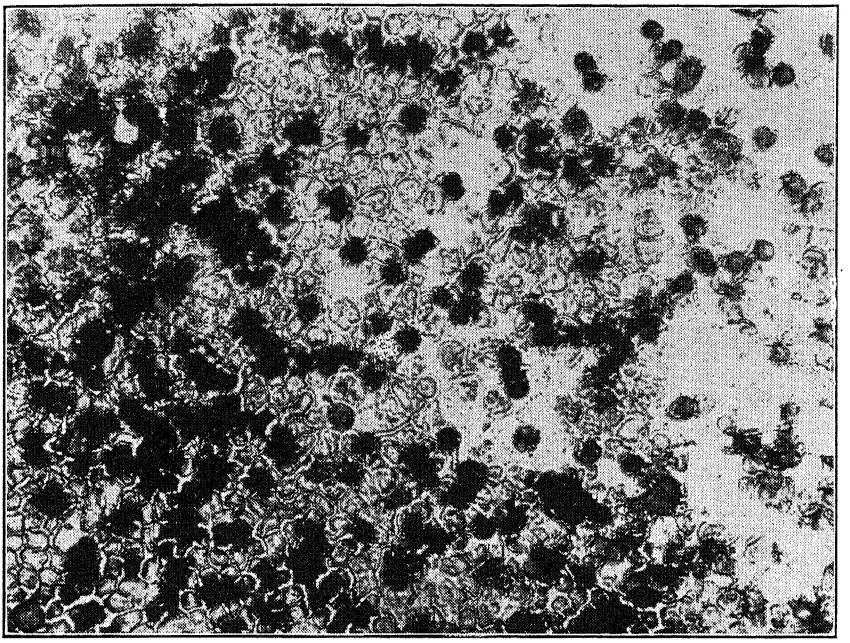

Figure 2. A representative of a negative smear showing leucocytes and epithelial cells. This indicates the spayed rat is in the diestrus interval condition.

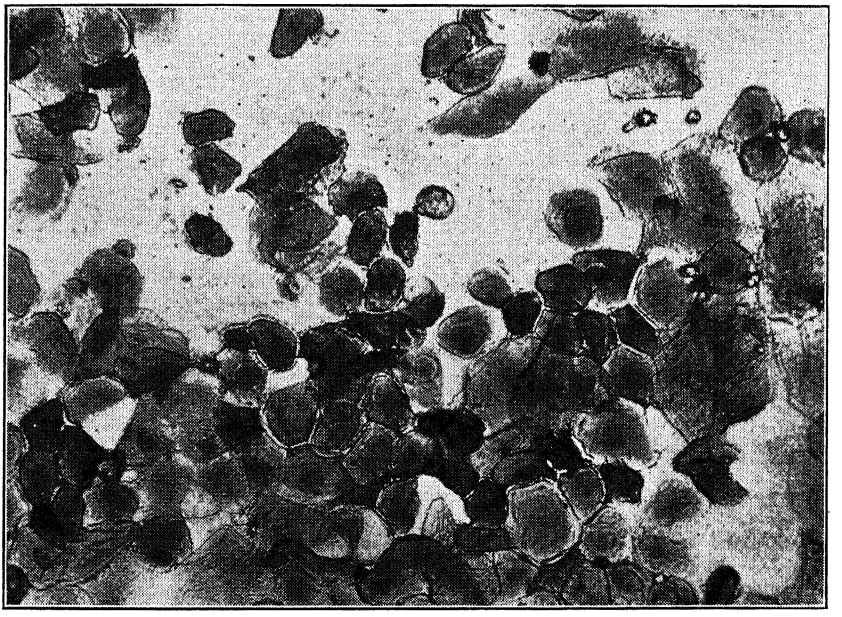

Figure 3. Smear showing all nucleated epithelial cells. This smear indicates partial reaction, but not enough for full estrum condition.

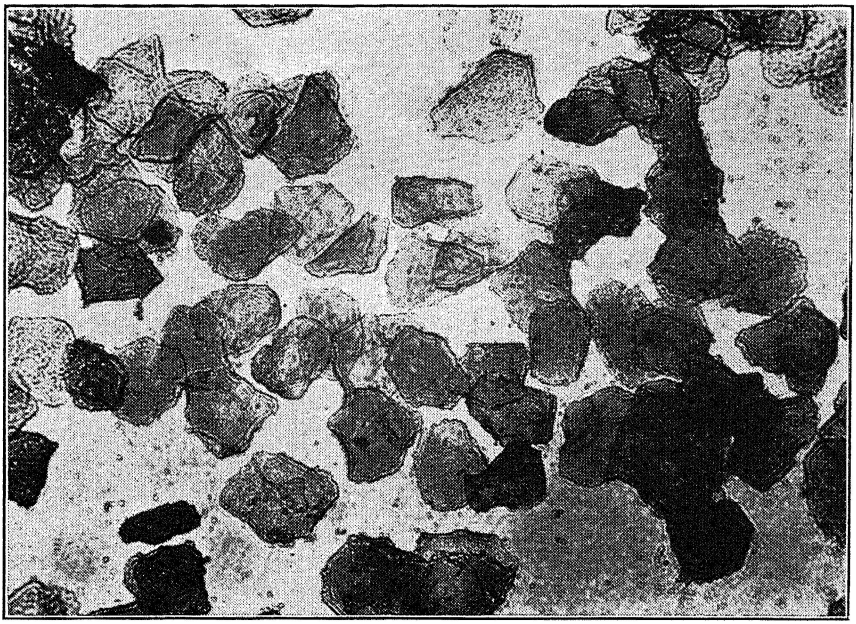

Figure 4. A representative of a positive smear showing cornified cells. This indicates the spayed rat is in full estrum.

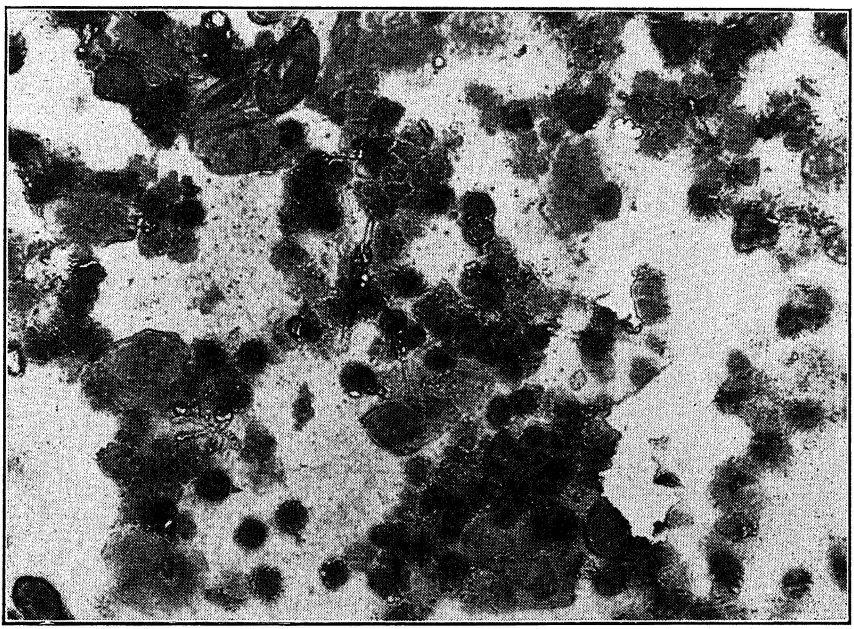

Figure 5. A representative smear of the metestrum stage, showing the infiltration of leucocytes.

PRESENTATION OF EXPERIMENTAL DATA

Certain of the preliminary experiments which dealt with the method of extraction of the estrus producing hormone from the urine of pregnant cattle have already been reported.

As indicated in the introduction, the primary object of our work has been the determination of the physiological mechanism regulating the growth of the mammary gland and the initiation of milk secretion. The hormone theory is the logical development of the early experimental work. The proof of the theory in the case of the development of the mammary gland must rest upon two types of observations. First, the development of the mammary gland must be produced synthetically by the injection of a hormone or hormones which will produce the complete growth of the mammary gland similar to that observed at the end of pregnancy. Second, the hormone or hormones must be isolated from the circulating blood in amounts sufficient to produce the observed results.

While some progress has been made in the synthetic development of the mammary gland, little progress has been made in the determination of the hormones present in the blood. Frank (1929) has determined the presence of the estrus producing hormone in the blood of pregnant women but the amount of blood which may be obtained does not permit more than a rough approximation of the hormone content. As the same amount of blood was used in each of his tests, the only indication of an increase in the hormone content of the blood with the advance of pregnancy was the increasing frequency of positive tests.

The discovery that the path of elimination of the hormone from the blood into the urine was believed to offer the opportunity of determining the rate of the secretion of the estrus producing hormone during the advance of the period of gestation in cattle.

Many interesting problems concerning the variation in the size of the mammary glands and the secretion of large yields of milk are possible of study as a result of this discovery. Thus an understanding of the underlying mechanism of the inheritance of milk yield may be advanced by the determination of the relation between the potentialities localized in the anlage of the mammary gland and the variation in the concentration of the hormone or hormones acting on the anlage of the gland.

As the amount of the estrus producing hormone excreted in the urine could be measured the determination of the influence of this hormone on the anlage of the mammary gland seemed possible of realization. Thus if little or no variation in the rate of secretion of the estrus producing hormone was observed in cattle of varying productive ability, it would appear that the potentialities of the mammary gland were largely centered in the anlage of the gland and the function of the hormone was simply to bring to maturity those potentialities.

On the other hand, if the secretion of the estrus producing hormone varied significantly with the development of the mammary gland and the yield of milk, there would then be an experimental basis for the theory that part of the mature development of the mammary gland was dependent upon the concentration of the hormone or hormones in the blood during pregnancy.

In our initial experiment samples of urine from dairy cows in all stages of gestation were analyzed for their estrus producing hormone content. Two interesting observations of these studies have served as a basis of further work. It was found that during the early period of pregnancy the rate of secretion was rather low, but that the rate increased with the advance of gestation. It was also observed that at the same stage of gestation great variation occurred in the rate of secretion of the hormone.

In continuing these studies it was decided to trace the rate of secretion of the estrus producing hormone in the same cows during pregnancy. A quantitative analysis was begun at the time of breeding and continued at regular intervals up to parturition, and in some cases for a time during lactation.

As beef cattle are usually distinctly inferior to dairy cattle in the size and capacity of their mammary glands, a comparison of these two types of cattle as to the rate and total secretion of the hormone at various stages of pregnancy should furnish evidence as to the relation of this hormone to the extent of the growth of the mammary gland.

Variation in the Secretion of the Hormone by Dairy Cattle.—About 215 samples of urine from dairy cattle in all stages of gestation have been analyzed for their content of the estrus producing hormone. These data are presented in Table 2 and Fig. 6. It will be noted that in all stages of gestation considerable variation in the daily secretion of the hormone by different cows occurs. However, this is especially true during the latter stages of gestation. Undoubtedly, much of this variation is due to the method of biological assay of the hormone which is not yet completely standardized. It is believed, however, that part of the variation is due to distinctly different levels of secretion of the hormone by various cows. While this variation does not prove that the level of hormone secretion influences the extent of the development of the mammary gland, it at least indicates that such may be the case and is not contrary to such a theory.

The figure also indicates a gradual increase in the daily secretion of the hormone reaching high levels toward the close of the gestation period. The rate of increase will be dealt with in more detail later.

TABLE 2.—AVERAGE DAILY SECRETION OF THE ESTRUS PRODUCING HORMONE

Stage of Gestation	Holstein		Jersey		Ayrshire		Guernsey		All Dairy		All Beef		Same 10 Cows
	Number of Tests	Average Daily Hormone Secretion	Number of Tests	Average Daily Hormone Secretion	Number of Tests	Average Daily Hormone Secretion	Number of Tests	Average Daily Hormone Secretion	Number of Tests	Average Daily Hormone Secretion	Number of Tests	Average Daily Hormone Secretion	Average Daily Hormone Secretion
		r. u.		*r. u.*		*r. u.*		*r. u.*		*r. u.*		*r . u.*	*r. u.*
Preceding conception	7	51	3	72					10	57			
0- 19	2	103	4	123					6	116			116
20- 39	5	112	7	77					12	92	4	66	88
40- 59	6	206	3	194					9	202	6	122	245
60- 79	5	99	6	133					11	117	7	125	88
80- 99	6	249	6	73					12	161	5	118	215
100-119	7	337	8	231					15	281			300
120-139	6	439	8	433	1	225			15	421	2	54	432
140-159	8	768	8	604	1	395			17	669	2	205	694
160-179	8	1117	8	552	5	370			21	724	8	122	830
180-199	7	871	6	916	4	438			17	785	6	185	861
200-219	7	1235	6	1091	1	509			14	1121	5	262	1622
220-239	5	2988	7	1518	2	1255			14	2006	4	418	2353
240-259	3	1302	9	900	2	1205	1	2562	15	1132	7	559	1562
260-	9	2596	9	1289	1	1303	3	1392	22	1838	3	425	2911
Following parturition	5	209	1	186					6	206			

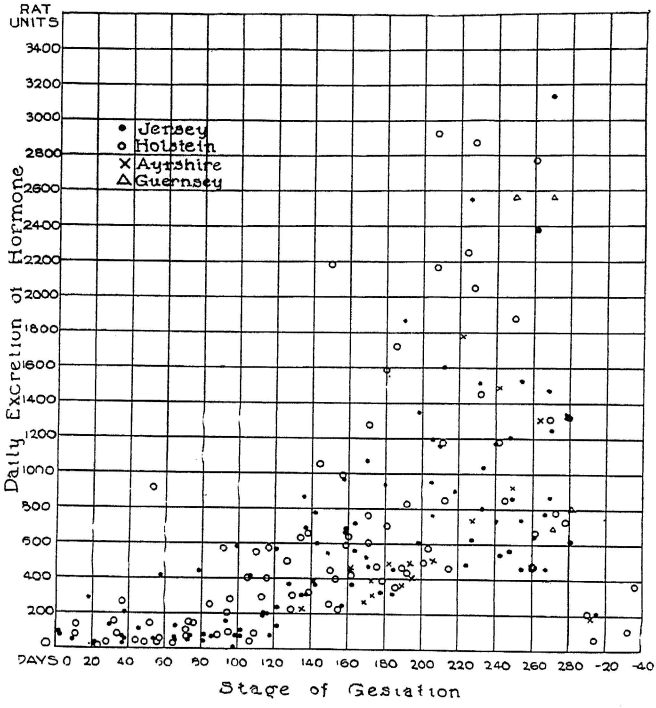

Fig. 6. The average excretion of the estrus producing hormone during the advance of the stage of gestation. There are included in the chart the results of the biological assay of the estrus producing hormone from over 200 twenty-four hour samples of urine obtained during all stages of gestation. Included are 95 samples from Jersey cattle, 84 from Holstein, 17 from Ayrshire, and 4 from Guernsey cattle. It will be noted that during the early stages of gestation the hormone content is rather low but increases rapidly with the advance of the stage of gestation.

It is interesting to note the relation between the average rate of secretion of the hormone of the Holstein and Jersey cattle with the advance of the stage of gestation. (Fig. 7). During the early period of pregnancy little or no difference can be observed. However, during the last 80 days of gestation the Holstein cows average somewhat higher than do the Jerseys.

Secretion of the Hormone by Beef Cattle.—In order to determine the variation in the secretion of the estrus producing hormone during various stages of pregnancy by these distinctly different types of cattle, fifty-nine samples of urine covering periods of 24 hours each were collected from 32 different beef cows. In cases where more than one collection was made from the same cow, a period of 21 days passed before the next collection period.

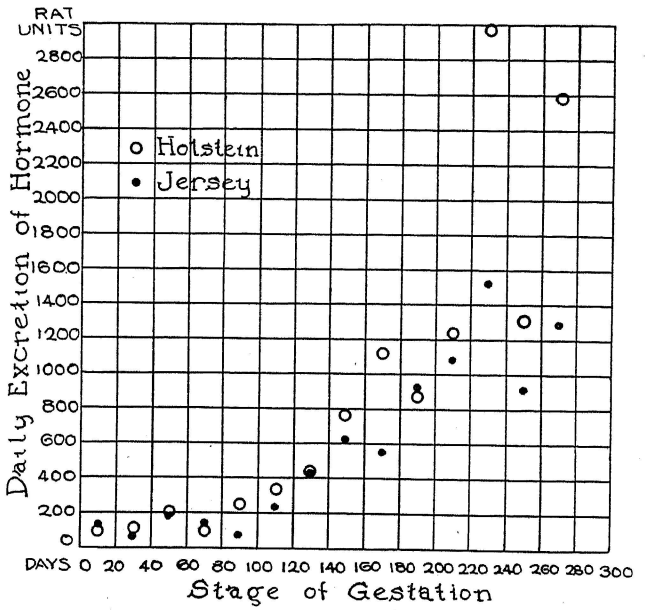

Fig. 7. The relation between the excretion of the estrus producing hormone by Holstein and Jersey cattle. The figure indicates that there is little difference between these two breeds in the excretion of the hormone during the early stages of gestation. There appears, however, a tendency for the Holstein cattle to exceed the Jerseys during the latter stages of gestation.

At the beginning of the gestation period there was no noticeable difference in the rate of secretion of hormone between beef and dairy cattle (Table 2). For about 100 days the secretion of hormone from these two types of cattle continued at a similar rate. After this period the dairy cows began to show a very noticeably higher level of secretion of the hormone as compared to the beef cows. After the first 100 days and until parturition the production of the estrus producing hormone by the dairy cattle was, on an average, approximately 100 per cent higher than that secreted by the beef animals.

Individual cows of both dairy and beef type showed considerable variation in the excretion of the hormone. It will be observed, however, that the beef cattle in most cases are below the dairy cattle. (Fig. 8).

As indicated in the discussion of the factors influencing the volume of urine secreted, it was found that the average volume of urine secreted by dairy cows is distinctly higher (9299 c. c.) than the average for the beef cows (5416 c. c.).

As a consequence of the distinctly lower level of urine secretion of the beef cattle, comparison of the hormone content per liter of urine between the two types of cattle shows less difference than the daily

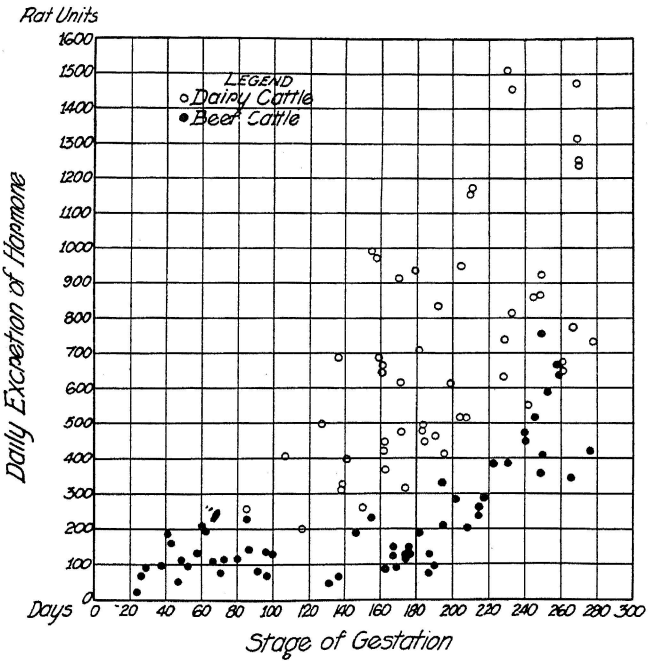

Figure 8. The variation in the excretion of the estrus producing hormone as extracted from the urine of pregnant beef and dairy cows. It can be noted that the increase in the dairy cows begins about the one hundredth day, whereas there is no noticeable increase in the beef cows until about the one hundred and eightieth day.

secretion. It seems probable that the elimination of the hormone is dependent on its concentration in the blood and not on the daily volume of urine secreted. The total daily elimination of the estrus producing hormone, is, therefore, believed to be the best measure of the concentration of the hormone in the blood.

The Rate of Secretion of the Estrus Producing Hormone.—Of more than usual interest from a physiological standpoint is the rate of secretion of the estrus producing hormone during pregnancy. During the recurring estrus cycles, it seems reasonably certain that this hormone originates in the mature follicles of the ovary. However, during pregnancy with the presence of the corpora lutea, this source is probably eliminated or at least greatly decreased. What then is the source of the increasing amount of hormone during the course of pregnancy? It is true that the placenta is also a rich source of the hormone, but it seems questionable whether it is the point of origin.

While experimental evidence as to the source of the hormone is not available at this time, the rate of secretion of the hormone as measur-

ed by its elimination in the urine may indicate something of the changes in the structure which is the source of the secretion.

The rate of increase of the hormone in the two principal breeds of dairy cattle studied is rather low during the initial stages of pregnancy. Gradually the curve of the daily hormone secretion increases at an ever increasing slope until the time of parturition. After parturition the hormone found in the urine rapidly decreases again. Similarly the curve combining all tests of the hormone shows the same general tendency (Fig. 9).

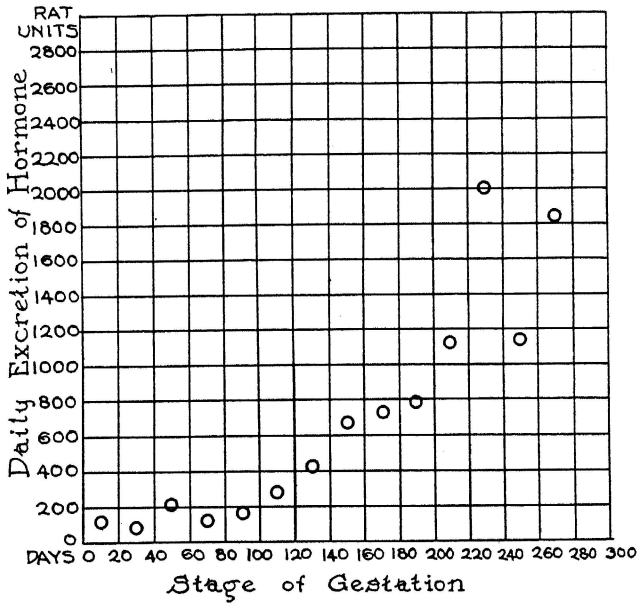

Fig. 9. The variation in the average daily excretion of the estrus producing hormone as extracted from cow's urine during all stages of gestation. There are over 200 determinations included.

In these figures cows in all stages of gestation have been included but the same group of cows were not traced throughout the gestation period. One group of about 10 cows was started at the first service period and continued throughout gestation. From this group a more representative curve indicating the average change in hormone secretion has been obtained.

It was found that an equation of the form $H=Ae^{kt}$ could be fitted with considerable accuracy to these data. The graphic method described by Brody (1927) was used in determining the parameters A and k. The fit of the equation $H=93.0e^{\cdot 0126t}$ is shown in Figure 10. In this equa-

tion H is the daily hormone secretion at any day during pregnancy t, A (93.0) is the initial daily level of hormone secretion, k (.0126) is the rate of increase of the hormone, and e the base of natural logarithms.

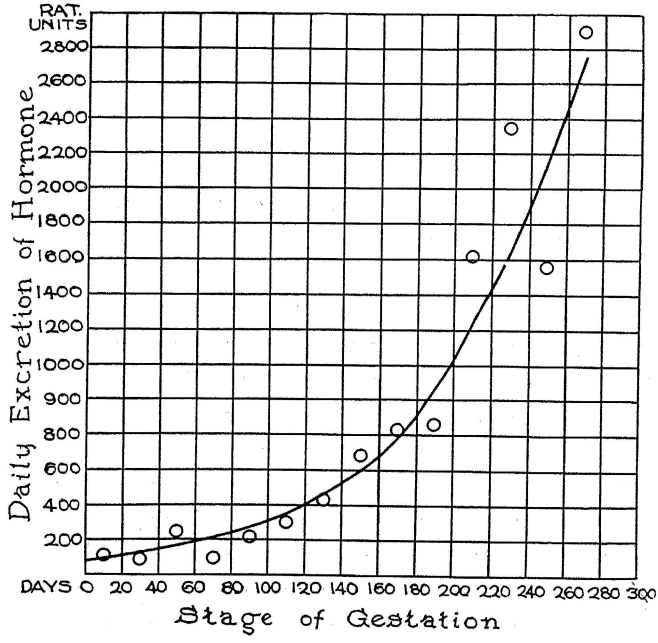

Fig. 10. The average secretion of the estrus producing hormone during the advance of the stage of gestation of the same group of cows. The continuous line passing through the points has been fitted by an equation of the form $H = Ae^{kt}$. The paremeters were determined by the graphic method desscribed by Brody (1927) as $H = 93.0e^{.0126t}$ in which H is the daily hormone secretion at any time t, A (93.0) is the initial daily level of hormone secretion, k(.0126) is the rate of increase of the hormone, and e the base of natural logarithms.

It is this equation which Brody (1927) has found to represent the rate of growth of animals previous to the point of inflection of the growth curve. This segment of the growth curve extends from the beginning of growth until about one-third to one-half of the mature weight is reached. Thus in the case of the rat, the growth of the fetus from 14 days after conception to birth is accurately represented by an equation of the same form.

The fact that the rate of hormone secretion may be represented by an equation of this form is therefore of considerable interest because it may give some insight into the mode of secretion. If the equation is rational the value for A must have a definite meaning. The parameter A in the equation represents the daily level of hormone secretion at the

beginning of pregnancy. The value 93.0 rat units of hormone secreted daily may seem high but as a matter of fact 10 tests of non-pregnant cows showed an average 57 rat units of hormone per day. These animals were bred but later it was found had not conceived. As they were in various stages of the estrus cycle it was quite possible that cows in estrus would be secreting at least 90 rat units per day.

The increase in the secretion of the hormone similar to the growth of the fetus is not necessarily taken to indicate that the fetus is the source of the hormone but rather that some structure associated with the fetus increases in size at a similar rate. The increase in size of this secretory structure (uterine glands etc.) would parallel the rate of secretion of the estrus producing hormone.

The rate of secretion of the estrus producing hormone observed may be considered from another point of view. What is the function of the increasing amounts of the hormone during the course of pregnancy? As indicated in another paper of this series (Turner and Frank, 1930), the estrus producing hormone undoubtedly plays a part in the growth of the mammary glands. Does the increasing secretion of the hormone during the later stages of pregnancy indicate an increasing need of the hormone to properly develop the mammary glands or are there other functions of the hormone in the pregnant animal? So far as the writers are aware, no other functions have been suggested for the hormone during pregnancy. It would seem reasonable to assume, therefore, that the proper development of the mammary glands require an increasing concentration of the estrus producing hormone.

As indicated above the growth of the fetus may be represented by the same type of increase as that observed for the hormone. It seems reasonable to assume that the development of organ systems would follow a similar course. Thus the mammary gland system normally would be expected to increase in size and weight during pregnancy following a course which could be represented by an equation of the same form. It therefore seems logical to believe that the increasing secretion of the hormone during the advance of the stage of pregnancy is needed for the increasing growth of the mammary gland.

The Induction of Micturition in Cattle.—In connection with the studies on the excretion of the estrus producing hormone in the urine of cattle during pregnancy, it became necessary to make complete collections of the urine over periods of 24 to 48 hours.

At the beginning of this investigation it was necessary to watch the cows continuously to obtain the urine. This method was not found entirely satisfactory for a number of reasons. One of the most serious objections to this method for our investigation was that the urine was not obtained covering a definite time interval such as 24 hours. Further,

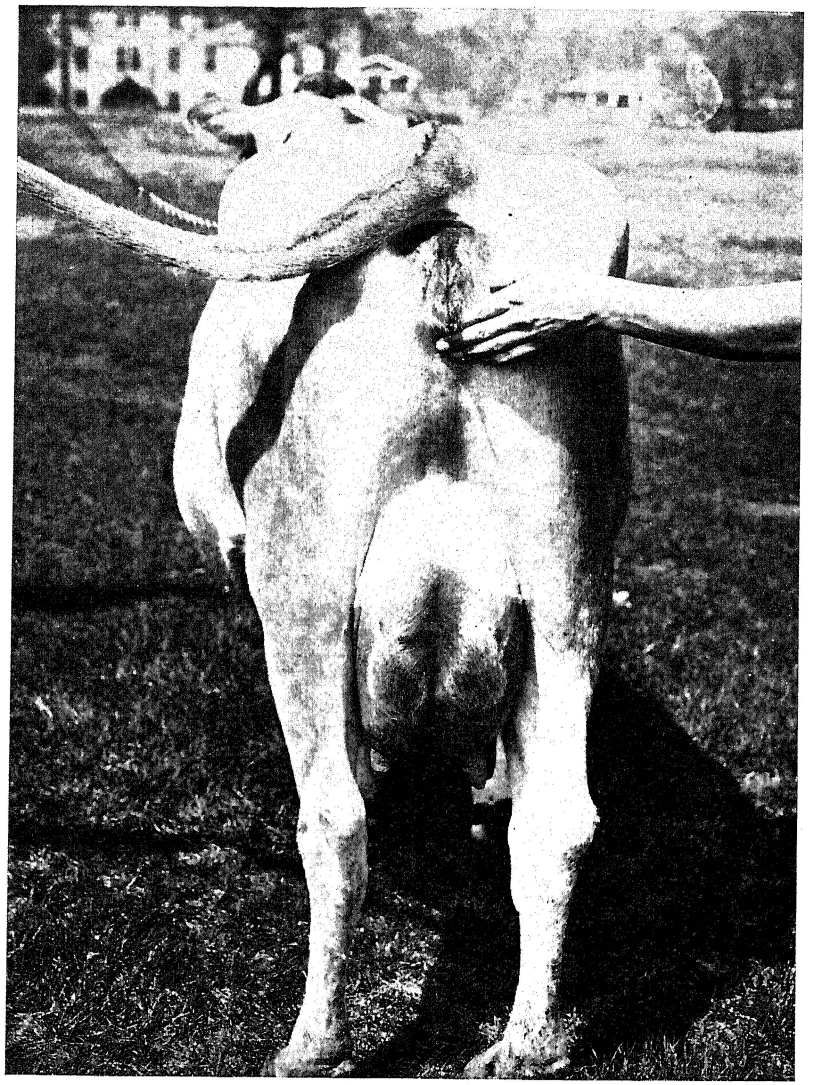

Fig. 11. The position of the hand below the ventral commissure of the vulva, where it is placed at the start of the massage which causes the cow to urinate. From this position the hand takes its course upward and laterally, terminating beside the labium vulva.

there was frequently a slight loss before reaching the cow. The quick movement of the collector or the rattle of the pail might also frighten the cow in the act of urination, and cause a retention of the urine.

When cows urinate at will, the urination of one cow frequently causes several others to quickly follow. In such cases several men would be required to make a complete collection or the cows would have to be kept in fairly close quarters. At best the number of cows which can be observed by a single collector is very limited by this system.

The Induction of Micturition.—Before our experiments had progressed very far, one of us (A. H. F.) observed that the act of micturition could be induced by a gentle massage starting below the ventral commissure of the vulva and taking its course upward and laterally terminating beside the labium vulva. As a result of this massage immediate micturition is induced. A visual demonstration of the method is presented in Fig. 11. Micturition was also induced in a Guernsey steer by a gentle massage below the anus.

The physiology of the act of micturition indicates a duality of control. Thus Stewart (1921) states that the series of coordinated movements resulting in micturition can be started by impulses passing to a center in the spinal cord from above or from below, from the brain or from the bladder. He cites Goltz to the effect that in dogs with the spinal cord divided at the upper level of the lumbar region, micturition takes place regularly when the bladder is full, and can be excited by such a slight stimuli as sponging of the skin around the anus.

The act of emptying the bladder is primarily dependent upon rhythmic muscular contractions which may be controlled by the peripheral neurons in the muscular wall. After the nerves have been sectioned the bladder can be emptied by the peripheral mechanism until the pressure within is less than the contraction pressure of the sphincter muscle. Not only is the contraction of the bladder walls necessary but the relaxation of the sphincter trigonalis muscle surrounding the neck of the bladder is necessary also for its emptying. Presumably this is presided over by the nervous mechanism in the wall of the bladder.

The path of the reflex stimulus in cattle may be traced through the posterior mesenteric plexus. Sisson (1914) states that two or more nerves proceed from the posterior mesenteric ganglion and enter the pelvic cavity ventral to the large vessels, anastomose with each other and with branches from the sacral nerves, especially the 3rd and 4th, and ramify on the pelvic viscera. The peripheral plexuses derived therefrom are named according to the organs which they supply.

In tracing the path of stimulation it is suggested that an afferent impulse is stimulated by lightly massaging the skin below the vulva. The stimulus is transmitted by the clitoris rami of the posterior mesenteric ganglion, from which an efferent impulse passes by way of the vesical rami to the bladder stimulating the neurons of the wall. Thus rhythmic muscular contractions are initiated which induces the act of micturition.

Advantages of the Method.—One of the most important advantages of the method outlined as it applied to our studies was our ability to obtain the volume of urine secreted during a definite time interval.

At the start of a collection period, all the cows were massaged and stimulated to urination. This urine was discarded. At two-hour intervals, the collection of urine was repeated, and continued for 24 hours. Thus the exact volume of urine secreted in 24 hours was obtained. It is true that occasionally a cow will urinate at shorter intervals, but such cases were very infrequent.*

In practice it was found that losses caused by several cows urinating at the same time could be prevented by making a collection just before the cows were to be disturbed. At such times each cow is quietly caused to get up and urinate before milking or feeding is begun. Thus one man can collect from a large number of cows and be assured of obtaining practically all of the urine secreted.

This system further permits the collection of the urine under practically normal herd conditions. It is not necessary to keep them in close quarters or even in the barn. During hot weather the cows may be turned outside into convenient lots.

The induction of micturition may be of value for other purposes such as might arise in the treatment of certain pathological conditions, in nutrition experiments, and where occasional samples are desired. In the latter case our experience has indicated that a convenient time to make such collections is just before and after milking.

Even where other methods of collection are available the value of having collection periods cover definite intervals in nutrition experiments is made possible by the induction of micturition at the beginning and close of the experimental period.

Factors Influencing the Volume of Urine.—During the past two years, in connection with the experimental work reported in the first section of this bulletin, collections have been made of the urine of a large number of beef and dairy cattle. In all 326 collections covering 24-hour periods were available for study. The animals were normal in every particular and kept under normal conditions of feeding and management. The larger proportion of the animals, however, were in various stages of gestation. While these data are not as extensive as might be desired, it seemed worthwhile to make a preliminary study to determine if possible some of the factors influencing the rate of urine secretion in cattle.

Urine Secretion of Various Animals.—Before taking up the study of the urine secretion of cattle it is interesting to note the amounts secreted by

*Dr. S. Brody of this Department found the method unsatisfactory in collecting urine from calves. Apparently, the complete evacuation of the bladder was not obtained, as many of the calves would urinate shortly after the induction of micturition.

other animals. Colin (1888) quotes Boussingault to the effect that the cow daily produces 7.2 kgs. (15.9 pounds) of urine and the pig at 9 months 3 kgs. (6.6 pounds). He states further that Sacc gives 7 to 9 kgs. (15.4-19.8 pounds) as the daily excretion of cattle and 9 to 10 kgs. (19.8-22 pounds) for the horse.

From data compiled by Van Slyke (1915) the daily urine secretion of domestic animals per 1000 pounds live weight is computed as follows: pig 33 pounds, cow 22 pounds, sheep 11 pounds, horse 10 pounds.

Storer (1916) gives the yearly production of urine of the horse and cow reported by Boussingault. Converted to a daily yield they are 7.1 pounds for the horse and 18.7 pounds for the cow.

Fish (1929) reports the following range in the urine secretion of various domestic animals. In the horse the average amount is about 3000 to 4000 c. c. (6.7 to 9.0 pounds*), although it may go as high as 7000 or 9000 c. c. (15.7 to 20.2 pounds). In the ox a still greater quantity is secreted, the usual limits being from 4500 c. c. to 19000 c. c. (10.1 to 42.7 pounds), while in sheep from 250 to 700 c. c. (0.6 to 1.6 pounds), and in the dog from 200 c. c. to 900 c. c. (0.4 to 2.0 pounds), depending upon the size of the animal.

Relation of the Breed to Volume of Urine Secreted.—The variation in the volume of urine secreted by the various breeds of cattle has not been extensively studied. Fuller (1928) has determined the water consumption, milk production, and urine secretion for a period of three days for a group of 4 cows of each of the dairy breeds. His results are summarized in the following table.

TABLE 3.—VOLUME OF URINE SECRETED BY DAIRY CATTLE (FULLER)

Breed	Average Weight lbs.	Average Daily Water Consumption lbs.	Average Daily Milk Production lbs.	Average Daily Urine lbs.	Average Daily Feces lbs.
Ayrshire	1280	105.3	16.7	38.6	64.8
Guernsey	988	111.6	27.0	28.2	62.1
Holstein	1403	163.1	43.8	31.8	88.4
Jersey	1000	83.6	10.0	26.8	60.8
Average	1167	121.7	27.8	31.3	69.0

Storer (1916) states that Heiden determined the amount of urine voided per diem, per head of 1000 pounds live weight in winter and in summer in a stable of 30 head of cattle as follows: in winter 7 pounds, in summer 16 pounds, on very salty food 33 pounds.

Keitt (1916) has determined the daily urine excretion of 3 cows and 3 heifers for 8 days. The cows secreted 17.8 pounds of urine per day while the heifers yielded 12.7 pounds.

*One pound equals 445 cubic centimeters.

TABLE 4.—FREQUENCY DISTRIBUTION OF DAILY URINE SECRETION OF CATTLE

Volume of Urine Mid-class Value	Holstein Breed		Jersey Breed		All Dairy Cattle		All Beef Cattle	
	Frequency Distribution	Percentage Distribution	Frequency Distribution	Percentage Distribution	Frequency Distribution	Percentage Distribution	Frequency Distribution	Percentage Distribution
c. c.								
2500	0		0		0		3	4.6
3500	0		0		0		10	15.4
4500	1	1.0	1	0.8	3	1.1	21	32.3
5500	2	2.0	5	3.9	9	3.4	13	20.0
6500	7	6.9	13	10.1	28	10.7	7	10.8
7500	11	10.9	21	16.3	36	13.8	4	6.2
8500	16	15.8	20	15.5	40	15.3	4	6.2
9500	8	7.9	18	14.0	30	11.5	3	4.6
10500	20	19.8	17	13.2	39	14.9		
11500	11	10.9	7	5.4	18	6.9		
12500	9	8.9	9	7.0	21	8.0		
13500	6	5.9	6	4.7	14	5.4		
14500	3	3.0	8	6.2	11	4.2		
15500	1	1.0	1	0.8	2	0.8		
16500	2	2.0	0		3	1.1		
17500	1	1.0	1	0.8	2	0.8		
18500	1	1.0	0		1	0.4		
19500	1	1.0	1	0.8	2	0.8		
20500	0		0		0			
21500	1	1.0	0		1	0.4		
22500			1	0.8	1	0.4		
Total	101		129		261		65	
Mean	10,470.3±101.78		9,127.5±87.01		9,298.8±62.69		5,415.5±72.11	
S. D.	1,516.6±71.97		1,465.5±61.52		1,501.0±44.32		861.7±50.98	
C. V.	14.48±.70		16.05±.69		16.14±.49		15.91±.96	

In the experiments of Larsen and associates (1917) comparison was made of the effect of watering at 8 hour and 24 hour intervals. The experiment was conducted in January. The average daily urine excretion of 4 cows watered at 8 hour intervals was 10.5 pounds and at 24 hour intervals 11.1 pounds. The animals were grade cows. The average of 17 cows which were watered once or more in 24 hours was 12.5 pounds of urine per day.

In order to indicate the variation in the volume of urine secreted by the various breeds and types of cattle, our data has been studied statistically. The results of this study are presented in Table 4. It will be noted that the dairy cattle represented by the Jersey and Holstein breeds vary greatly in their daily urine secretion. The range in volume of urine for the dairy cattle was from 4500 c. c. to 22,500 c. c. The Holstein and Jersey cattle were found to be quite similar in this respect. The mean and the mode for the Jerseys was somewhat smaller than for the Holsteins.

The range in volume of urine of the beef cattle was much less than that of the dairy cattle, from 2500 to 9500 c. c. As a result the mean volume of urine and the standard deviation is about half that of the dairy cattle. It is interesting to note, however, that the coefficient of variation of the beef cattle and dairy cattle is quite similar.

In the tabulation of the urine secretion by breeds (Table 5), it was found that the Holstein breed stood first with the Jersey and Ayrshire cattle only slightly below. The Guernsey cattle are distinctly below the others due probably to the lack of numbers to give a fair sample and also to the fact that the animals included were young cows. The entire group of dairy cattle averaged 20.9 pounds of urine per day (9200 c. c.). Of the beef cattle, the Hereford, Shorthorn, and Angus were represented and yielded quite similar amounts averaging 12.2 pounds (5416 c. c.) per day. It will be noted that the average daily secretion of urine by beef cattle is distinctly less than dairy cattle.

TABLE 5.—RELATION BETWEEN THE BREED AND DAILY URINE SECRETION

Breed	Number of 24-hour periods	Average Secretion of Urine	
		Volume	Weight*
		c. c.	*lbs.*
Holstein	101	10,470	23.5
Jersey	129	9,128	20.5
Ayrshire	24	9,177	20.6
Guernsey	7	6,707	15.1
Hereford	26	5,454	12.3
Shorthorn	25	5,310	11.9
Angus	14	5,189	11.7
All Dairy	261	9,299	20.9
All Beef	65	5,416	12.2
All Cattle	326	8,805	19.8

*One pound equals 445 cubic centimeters.

Body Weight.—As cattle increase in body weight, their feed and water consumption undoubtedly increases. It would be expected as a consequence that the volume of urine secreted per day would increase as body weight increases. The tabulation of the data of the dairy cattle of all breeds into groups according to their body weight is shown in Table 6 and Figure 12. It will be noted that there is a fairly regular increase in the secretion of urine as body weight increases. A straight line was fitted by the method of least squares and the following equation was obtained.

$$D.\ U.\ V. = 3758 + 568.4\ Wt.$$

in which D. U. V. is the daily urine volume in cubic centimeters, and Wt. is the body weight of the cow. For each 100 pounds increase in body weight there is an average increase of 568.4 c. c. of urine secreted per day.

TABLE 6.—RELATION BETWEEN THE BODY WEIGHT AND URINE SECRETION

Body Weight	Number of 24-hour periods	Average Secretion of Urine		Urine Calculated Volume*
		Volume	Weight	
lbs.		*c. c.*	*lbs.*	*c. c.*
700	6	7,727	17.4	7,737
800	37	9,419	21.2	8,305
900	34	8,082	18.2	8,873
1000	46	8,321	18.7	9,442
1100	44	10,496	23.6	10,010
1200	46	11,291	25.4	10,579
1300	23	9,723	21.8	11,147
1400	29	12,120	27.2	11,715
1500	4	12,665	28.5	12,284
Total	269			

*Equation fitted by the method of least squares D. U. V. = 3758 + 568.4 Wt., where D. U. V. is the daily urine volume in cubic centimeters, and Wt. is the body weight of the cow.

Age.—Considering the relation between weight and production, it would be expected that the daily secretion of urine would increase with age. However, the tabulation of the data shown in Table 7 does not indicate such a relation. The reason for the irregularity is not known.

Milk Production.—The studies at the South Dakota Station by Larsen and associates (1917) indicate that about 15 per cent of the water consumed was eliminated in the milk. The percentage, however, increased with high producing cows.

The data tabulated here is rather irregular and does not indicate any clear cut relation between milk production and the rate of urine secretion (Table 8). The decline in the volume of urine secreted by the heavy producers may not be representative as the number of animals included is limited.

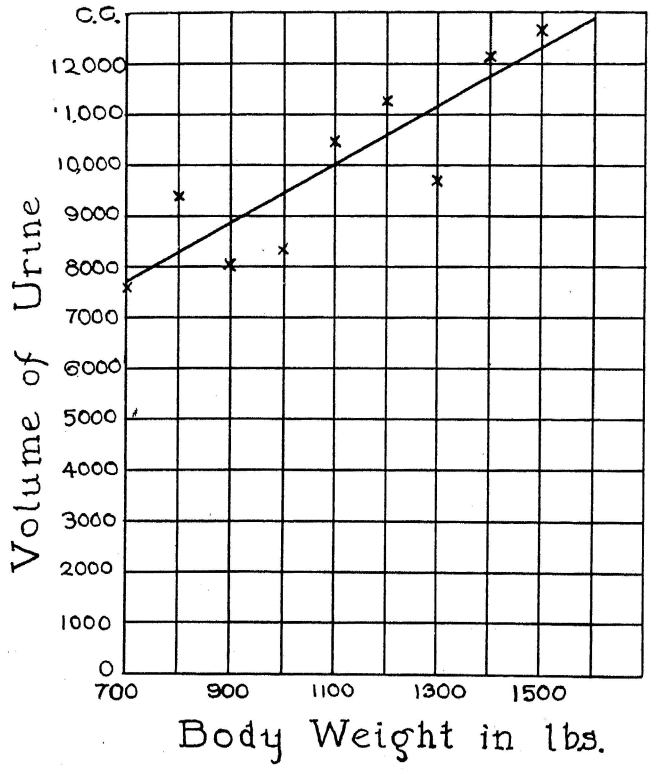

Fig. 12. The relation between the average daily urine secretion of dairy cattle and increase in body weight. The straight line passing through the observed values was fitted by the method of least squares and the following equation was obtained: D. U. V. = 3758 + 568.4 wt. in which D. U. V. is the daily urine volume in cubic centimeters and wt. is the average body weight of the cow. For each 100 pounds increase in body weight there is an average increase of 568.4 cc. of urine secreted per day.

TABLE 7.—RELATION BETWEEN AGE AND URINE SECRETION

Age	Number of 24-hour periods	Average Secretion of Urine	
		Volume	Weight
yrs.		*c. c.*	*lbs.*
1- 2	1	10,540	23.7
2- 3	77	9,946	22.4
3- 4	42	8,624	19.4
4- 5	17	9,771	21.9
5- 6	13	6,757	15.2
6- 7	14	9,442	21.2
7- 8	24	10,453	23.5
8- 9	40	9,906	22.3
9-10	5	12,682	28.5
10-11	0	----	----
11-12	2	12,115	27.2
12-13	13	11,437	25.7
13-14	20	12,217	27.5
Total	268		

TABLE 8.—RELATION BETWEEN DAILY MILK PRODUCTION AND URINE SECRETION

Daily Milk Production	Number of 24-hour periods	Average Secretion of Urine	
		Volume	Weight
lbs.		*c. c.*	*lbs.*
Heifers	71	9,987	22.4
Dry Cows	97	9,178	20.6
6-10	18	10,882	24.5
11-15	20	10,102	22.7
16-20	13	11,203	25.2
21-25	18	10,828	24.3
26-30	6	9,583	21.5
31-35	4	10,772	24.2
36-40	8	12,260	27.6
41-45	3	11,480	25.8
46-50	8	8,624	19.4
51-55	1	7,400	16.6
56-60	1	8,300	18.7
61-65	0	----	----
66-70	1	6,860	15.4
Total	269		

The Water Intake.—The most important factor in regulating the output of urine in man is said to be the water intake in various forms. In lactating animals,in addition to the elimination of water by way of the urine, feces, breathing, and perspiration, there is a large withdrawal of water from the body in the milk as about 85 per cent of the milk is composed of water.

In studies at the South Dakota Station, Larsen, Hungerford, and Baily (1917) found that about 12 per cent of the total water drunk was eliminated through the skin. This was true during the winter under barn conditions. During the summer (August) about 27 per cent was so eliminated. About 13 per cent of the water taken in was excreted in the form of urine. Another 15 per cent of the water was eliminated in the milk. However, the percentage increased with high producing cows. Over 56 per cent of the water drunk was eliminated in the feces.

Unfortunately, in our studies the feed and water intake was not measured, so that nothing can be contributed to this phase of the problem.

Temperature and Season.—The environmental temperature may affect the volume of urine in two ways. Less urine may be formed because of increasing amounts of water passing off in perspiration. Thus the volume of urine during the summer months might be expected to be less. On the other hand, the water intake might be increased sufficiently to offset the greater loss in the form of perspiration.

Larsen (1917) and associates found that 12 per cent of the water was eliminated by evaporation from the body surface and from breathing during the winter while this increased to 26 per cent during the sum-

mer. However, the percentage of the water excreted in the urine also increased slightly (11.6 to 15.0) during the summer at the expense of the water in the milk and feces.

The data available in our studies were classified according to the month the urine was collected as shown in Table 9. Only dairy animals were included in this tabulation as the period during which the urine from the beef cattle was collected included only the fall and winter months. While many of the animals differ from month to month about 12 were carried through the entire gestation period.

TABLE 9.—RELATION BETWEEN SEASON AND URINE SECRETION

Month	Number of 24-hour periods	Average Daily Secretion of Urine	
		Volume	Weight
		c. c.	*lbs.*
January	36	10,006	22.5
February	36	9,941	22.3
March	12	10,628	23.9
April	----	----	----
May	----	----	----
June	22	9,354	21.0
July	13	10,018	22.9
August	24	9,353	21.0
September	14	10,581	23.8
October	31	11,172	25.1
November	37	9,200	20.7
December	39	9,699	21.8
Total	264		

As little if any seasonal trend can be noted, it is concluded that the volume of urine is not greatly influenced by the season of the year.

TABLE 10.—RELATION BETWEEN STAGE OF GESTATION AND URINE SECRETION

Stage of Gestation	Average Daily Secretion of Urine					
	Dairy Cows			Beef Cows		
	No. of Animals	Volume	Weight	No. of Animals	Volume	Weight
		c. c.	*lbs.*		*c. c.*	*lbs.*
Open	33	10,052	22.6			
1- 30	22	10,105	22.7	3	4,460	10.0
31- 60	22	9,215	20.7	7	6,284	14.1
61- 90	18	9,928	22.3	9	6,585	14.8
91-120	26	11,017	24.8	3	6,730	15.1
121-150	21	11,013	24.7	3	6,056	13.6
151-180	33	10,430	23.4	9	5,563	12.5
181-210	25	10,025	22.5	8	4,591	10.3
211-240	22	9,769	22.0	7	3,009	6.8
241-270	32	8,568	19.3	9	5,422	12.2
271-300	15	8,352	18.8	1	4,200	9.4

Stage of Gestation.—It is interesting to note that the average daily volume of urine increases during the first one hundred days of the gestation period. After this time there is a gradual decline in the average daily volume of urine secreted until parturition. (Table 10).

Although the average daily volume of urine from the dairy cows is approximately one hundred per cent greater than that from the beef cows, at any particular stage of the gestation period, it can be noted that the curve shows the same general shape for both types of cattle (Figure 13).

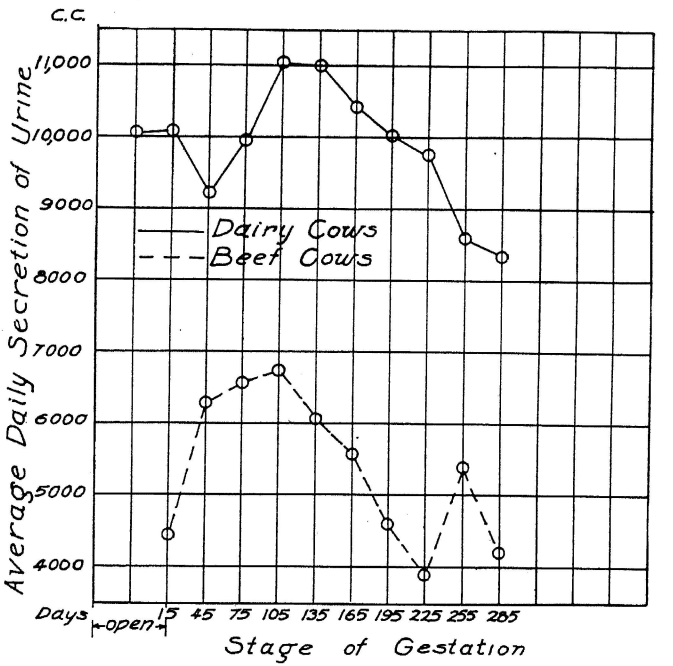

Fig. 13. Influence of stage of gestation on the average daily volume of urine secreted by dairy and beef cows.

SUMMARY AND CONCLUSIONS

1. A study is reported of the rate of secretion of the estrus producing hormone in the urine from 45 dairy cows including 216 individual 24 hour samples and from 32 beef cows including 59 samples at various stages of gestation.
2. The urine was collected at two hour intervals by inducing micturition by a method described.
3. It was concluded from a preliminary study that a 24-hour collection period was the minimum period during which a representative sample of urine could be obtained.
4. A simple method of extracting the estrus producing hormone from the urine of pregnant cattle is described.
5. The hormone extracted from the urine was assayed by the biological method described by Allen and Doisy.
6. It was observed that during the advance of the stage of gestation, the daily secretion of the hormone began at a low level. Gradually the curve of hormone secretion increases at an ever increasing slope until the time of parturition. It was found possible to represent the course of hormone secretion during pregnancy by an equation of the form $H=Ae^{kt}$. The significance of the rate of secretion observed was discussed. It was suggested that the structure secreting the hormone probably grows at a parallel rate. Reasons for believing that the increasing secretion of the hormone during pregnancy is required for the normal development of the mammary glands are also advanced.
7. Of the animals included little difference in the rate of secretion of the hormone by Holstein and Jersey cattle occurred. However, in a comparison of dairy and beef cattle it was found that the dairy cows begin to show a very noticeably higher level of secretion of the hormone after 100 days of gestation.
8. In a study of some of the factors influencing the daily volume of urine secreted it was found that the dairy cattle yield a significantly greater volume than do beef cattle, that there is an increase in urine of 568 cc. per day for each 100 pounds increase in body weight of dairy cattle, and that after 100 days of gestation there is a gradual decline in the average daily volume of urine secreted until parturition. Changes in age and milk production did not appear to influence the volume of urine secreted perceptibly.

REFERENCES

Allen, E. 1922 *The oestrous cycle in the mouse.* Am. Jour. of Anat., Vol. 30, pp. 297-346.

Allen, E. 1926 *The ovarian follicular hormone: A study of variation in the pig, cow, and human ovaries.* Proc. Soc. Exper. Biol. Med., Vol. 23, pp. 383-387.

Allen, E., Colgate, C. E., Francis, B. F., Johnston, C. G. Robertson, L. S., Doisy, E. A. Gilson, H. V. and Kountz, W. B., 1924. *The hormone of the ovarian follicle; its localization and action on test animals, and additional points bearing upon the internal secretion of the ovary.* Am. Journ. of Anat., Vol. 34, pp. 133-181.

Allen E. and Doisy E. A., 1924 *The Induction of a sexually mature condition in immature females by injection of the ovarian follicular hormone.* Am. Jour. Physiol. 69, p. 577.

Allen, E., Pratt, J. P., and Doisy E. A. 1925 *The ovarian follicular hormone. The distribution in human genital tissues.* Jour. Am. Med. Assn., Vol. 85, pp. 399-404.

Allen E., Whitsett, J. W., Hardy, J. W. and Kneibert, F. L. 1924 *The follicular hormone of the hen ovary.* Proc. Soc. Exper. Biol. and Med., Vol. 21, pp. 500-503.

Aschheim, S. 1926 *Hormon und Schwengerschaft.* Med. Klin., Vol 22. pp. 2023-2025. (Hormone of pregnancy.)

Aschheim S., and Zondek, B. 1927 *Hypophysenvorderlappenhormon und ovarialhormon im Harn von Schwangeren.* Klin. Wchnschr., Vol. 6, p. 1322. (Hormone of the anterior lobe of the hypophysis and ovarian hormone in the urine of the pregnant).

Aschner, B. and Grigoriu C., 1911 *Placenta, Fëtus u. keimdrüse in ihrer wirkungauf die milchsekretion.* Arch. f. Gynak. 94, pp. 766-793. (Placenta, fetus, and ovary in their influence on milk secretion).

Biedl, A. and Konigstein, H. 1910 *Untersuchungen über das brustdrüsen-hormon der gravidat.* Ztschr. f. Expr. Path. u. Therap., Vol. 8, pp. 358-373. (Study of the mammary gland hormone of pregnancy).

Brody, S. 1927 *Growth and development. III. Growth rates their evaluation and significance.* Mo. Agr. Expt. Sta., Res. Bul. 97.

Brown, J. H., 1930 *The errors of biological assay.* Physiol. Reviews, Vol. 10, pp. 146-169.

Bugbee, E. P. and Simond, A. E. 1926 *Standardization of preparations of ovarian follicular hormone.* Endocrinology, Vol. 10, pp. 191-200.

Colin, G., 1888 *Traite de Physiologie Comparee des Animaux.* Vol. 2, J. B. Bailliere et Fils, Publisher, Paris. (Comparative Physiology of Animals).

Coward, K. H., and Burn, J. H., 1927 *The variation in the unit of the oestrus-producing hormone.* Jour. Physiol. Vol. 63, pp. 270-279.

Dickens, F., Dodds, E. C., and Wright, S. 1925 *Observations upon the preparation and standardization of the ovarian hormone.* Biochem, Jour., London, Vol. 19, pp. 853-859.

Dohrn, M., and Faure, W., 1928 *Uber die Ausscheidung des Weiblichen Sexualhormons* Klin. Wchnschr., Vol. 7, p. 943. (The excretion of the female sexual hormone).

Doisy, E. A., Ralls, J. O., Allen, E., and Johnson, C. G., 1924 *The extraction and some properties of an ovarian hormone.* Jour. Biol. Chem., Vol. 61, pp. 711-727.

Doisy, E. A., Veler, C. D., and Thayer, S., 1929 *Folliculin from urine of pregnant women.* Abstracts of Comm. XIII International Physiclogical Congress. Amer. Jour. Physicl., Vol. 90, pp. 329-330.

Doisy, E. A., Veler, C. D., and Thayer, S. 1930. *The preparation of the Crystalline ovarian hormone from the urine of pregnant women.* Jour. Biol. Chem. Vol. 86, pp. 499-509.

Evans, H. M., and Burr, C. O. 1926 *Increased efficiency of subcutaneous when compared with intraperitoneal administration of the ovarian hormone.* Am. Jour. Physiol., Vol. 77, pp. 518-521.

Fellner, O. O. 1912 *Experimentelle erzeugte Wachstumsveranderungen am Wweiblichen Genitale de Kaninchen.* Centralbl. f. Allg. Path. u. Path. Anat., Vol. 23, pp. 673-676. (Experimental production of growth in the female genitalia of the rabbit.)

Fellner, O. O., 1913 *Experimentalle Untersuchungen uber die Wirkung von Gewebsextrakten aus der Plazenta und den Wweiblichen Sexualorganen auf das Genitale.* Arch. f. Gynäk., Vol. 100, pp. 641-719. (Experimental examinations of the effect of tissue extracts from the placenta and the female sexual organs on the genitalia).

Fels, E., 1926 *Untersuchungen über das ovarialhormon im Blute Gravider und Nichtgravider.* Klin Wchnschr., Vcl. 5, pp. 2349-2352. (Ovarian hormone in the blood of pregnant and not pregnant.)

Foà, C., 1908 *Sui fattori che determinano L' accrescimento e la funzione della ghiandola mammaria.* Archiv. di fisiol. 5, pp. 520. (On the factors influencing the growth and function of the mammary gland.)

Fish, P. A. 1929 *The examination of the urine of the horse and man.* 4th ed. Slingerland-Comstock Pub. Co., Ithaca, N. Y.

Frank, R. T. 1929 *The Female Sex Hormone*, Springfield, Ill., Charles C. Thomas, publisher.

Frank, A. H., 1930 *A study of the estrus producing hormone in its relation to the development of the mammary gland.* Master's Thesis, Univ. of Mo.

Frank, R. T., Frank, M. L., Gustavson, R. G., and Weyerts, W. W., 1925 *Demonstration of the female sex hormone in the circulating blood.* Jour. Amer. Med. Assn., Vol. 85, p. 510.

Frank, R. T., and Gustavson, R. G. 1925 *The female sex hormone and the gestational gland.* Jour. Amer. Med. Assn., Vol. 84, pp. 1715-1719.

Frank, R. T., and Unger A. 1911 *An experimental study of the causes which produce the growth of the mammary gland.* Archiv. Int. Med., Vol. 7, pp. 812-838.

Fuller, J. M. 1928 *Some physical and physiological activities of dairy cows.* N. H. Agr. Exp. Sta. Tech. Bul. 35.

Goltz, F., and Ewald, J. R., 1896 *Der Hund mit verkurzten Ruckenmark.* Pfluger's Arch., Vol. 63, pp. 362-400. (A dog with sectioned spinal cord).

Grigorieff, W., 1897 *Die Schwangerschaft bei der Transplantation der Eierstocke.* Centralbl. f. Gynäk., Vol. 21, pp. 663-668. (Pregnancy after transplantation of the ovary.)

Halban, J., 1900 *Uber den Einfluss der Ovarien auf die Entwicklung des Genitales.* Monats. f. Geburtsh Gynäk., Vol. 12 pp. 496-506. (The influence of the ovary on the development of the genitalia.)

Hannan, J. H. 1928 *Observations on the oral administration of the estrus producing autocoid.* Endocrinology, Vol. 12, pp. 193-198.

Hart, C. P., de Jongh, S. E., Laquer, E. and Wijsenbeek, I. A., 1925 *Uber das Hormon des Ovariellen Zyklus.* Deutsch. med. Wchnschr., 51:1700. (On the hormone of the ovarian cycle).

Hisaw, F. L., and Meyer, R. I. 1929 *The estrus producing hormone in the urine of pregnant cows.* Proc. Soc. Exper, Biol. and Med., Vol. 26, pp. 586-588.

Iscovesco, H., 1912 *Morcellement des Differents Lipoides Constitutifs des Organes.* Technique General. Comp. Ren. des Seances de la Soc. de Biol., Vol. 71, pp. 858-860. (Separation of the different lipoid constituents of the organs).

Iscovesco, H., 1912 *Les Lipoïdes de l'ovaire.* Compt. Ren. des Seances de la Soc. de Biol., Vol 73, pp. 16-18. (The lipoids of the ovary).

Iscovesco, H., 1912 *Le Lipoide Utero-Stimulant de l'ovaire.* Proprietes Physiologique Compt. Ren. des Seances de la Scc. de Biol., Vol. 73, pp. 104-106. (The lipoid uterine stimulant from the ovary. Physiological properties.)

Iscovesco, H., 1912 *Les Lipoids du Corps Jaune—Leur Role dans l'involution Post-puerpérale de l' Uterus.* Compt. Ren. des Seances de la Scc. de Biol., Vcl. 73, pp. 189-191. (The lipoids of the corpus leuteum).

Jentzer A. and Beuttner O., 1900 *Experimentelle Untersuchungen ur Frage der Castrations atrophie.* Ztschr. f. Geburtsh. u. Gynäk, Vol. 42, pp. 66-103. (Experimental research on the question of castration atrophie.)

Kahnt, L. C., and Doisy, E. A. 1928 *The Vaginal Smear Method of Assay of the Ovarian Hormone.* Endocrinology, Vol. 12, pp. 760-768.

Keitt, T. E. 1916 *The influence of the ration on the composition of urine of dairy cows.* S. C. Sta. Rpt. pp. 37-48.

Knauer, E., 1900 *Die Ovarientransplantation,* Arch. F. Gynäk, Vol. 60, pp. 322-376. (Ovarian transplantation.)

Lane-Claypon, J. E., and Starling, E. H., 1906 *An experimental inquiry into the factors which determine the growth and activity of the mammary glands.* Proc. Roy. Soc. B. 77, pp. 505-522.

Laquer, E. 1927 *Uber Weibliches Sexualhormone, im besonderen Menformon.* Klin. Wchnschr., Vol. 6, pp. 390-396. (Female sex hormone in various menformon.)

Laquer, E., Hart, P. C., De Jongh, S. E., and Wijsenbeek, I. A., 1925 *On the preparation of the hormone of the estrus cycle, and its chemical and pharmacological properties.* Proc. Acad. Sci., Amsterdam, Vol. 28, pp. 890-899.

Larsen, C., Hungerford, E. H., and Bailey, D. E. 1917 *The role of water in a dairy cow's ration.* S. D. Agr. Expt. Sta. Bul. 175.

Loewe, S., 1925 *Nachweis Brunsterzeugender Stoffe in Weiblichen Blute.* Klin. Wchnschr. Vol. 4, p. 1407. (Proof of estrus producing substances in female blood).

Loewe, S., and Lange, F. 1926 *Der Gehalt des Frauenharnes an Brunsterzeugenden Stoffen in Abhängigkeit vom Ovariellen Zyklus.* Klin. Wchnschr., Vol. 5, pp. 1038-1039. (The presence in female urine of the estrus producing substance dependent upon ovarian cycle.)

Loewe, S., Lange, F., and Faure, W., 1926 *Uber Weibliche Sexualhormone.* III. Die Wirksamkeit des Zyklus-hormons bei peroraler Zufuhrung. Deut. Med. Wchnschr., Vol. 52, pp. 310-313. (On the female sex hormone.)

Lomas, C. H., 1930 *A quantitative study of the estrus producing hormone in the urine of cattle.* Master's thesis, Univ. of Mo.

Long, J. A., and Evans, H. M. 1922 *The estrus cycle in the rat and its associated phenomena.* Univ. Calif. Press, Berkeley, Cal.

Marshall, F. H. A. and Jolly, W. A., 1906 *Contributions to the physiology of mammalian reproduction.* Part. II. The ovary as an organ of internal secretion. Phil. Trans. Roy. Soc. B. 198, pp. 123-141.

Morrell, J. A., McHenry, E. W., and Powers, H. H. 1930 *Distribution and preparation of the ovarian follicular hormone.* Endocrinology, Vol. 14, pp. 25-31.

Nibler, C. W. 1929 *A study of an ovarian hormone found in the urine of cattle and its influence on the mammary gland.* Master's thesis, Univ. of Mo.

Nibler, C. W., and Turner, C. W. 1929 *The ovarian hormone content of pregnant cow's urine.* Jour. of Dairy Sci. Vol. 12, pp. 491-506.

Nibler, C. W., and Turner, C. W. 1929 *Ovarian hormone content of pregnant cows urine.* Proc. of the Soc. for Exp. Biol. and Med., 26, pp. 882-884.

Papanicolaou, C. N., and Stockard, C. R., 1917 *The existence of a typical oestrous cycle in the guinea pig with a study of its histological and physiological changes.* Amer. Jour. Anat., Vol. 22, pp. 225-265.

Parkes, A. S., 1929 *The Internal secretions of the ovary.* Longsman, Green and Co., Publishers. 55 Fifth Ave., New York City

Parkes, A. S., and Bellerby, C. W. 1926 *Studies on the internal secretions of the ovary. I.* The distribution in the ovary of the estrus producing hormone. Jour. Physiol., Vol., 61, pp. 562-575.

Parkes, A. S., and Bellerby, C. W. 1927 *Studies on the internal secretions of the ovary.* IV. The significance of the occurrence of oestrin in the placenta. Jour. Physiol., Vol. 62, pp. 385-396.

Pfister, M., 1901 *Ueber die reflektorischen Bezichungen zuischen Mammae und Genitalia Muliebria.* Beitrage zur Geb. und Gynäk, Vol. 5, pp. 421-445. (On the reflex relations between the female Mammae and genitalia.)

Powers, H. H., Varley, J. R., Morrell, J. A. 1929 *Preliminary note on the assay of the follicular hormone by vaginal administration.* Endocrinology, Vol. 13, pp. 395-398.

Pratt, J. P., and Smeltzer, M., *Nasal spray method of administering hormones of the ovary and pituitary glands.* Endocrinology, Vol. 13, pp. 320-326.

Ralls, J. O., Jordon, C. N., and Doisy, E. A., 1926 *An improved procedure for the extraction of the ovarian hormone and some chemical properties of the product.* Jour. Biol. Chem., Vol. 69, pp. 357-380.

Ribbert, H., 1898 *Ueber Transplantation von Ovarium, Hoden und Mamma.* Arch. f. Entwcklngsmechn., Vol. 7, pp. 688-708. (On the transplantation of the ovary, testicle and mammae.)

Sisson, S., 1914 *The anatomy of the domestic animals.* 2d Ed., W. B. Saunders Co., Phil.

Smith, M. G., 1927 *A study of the ovarian follicular hormone in the blood of pregnant women.* Bul. Johns Hopkins Hosp., Vol. 41, pp. 62-66.

Stewart, G. N. 1921 *A manual of Physiologie.* Eighth Ed. New York City, William Wood and Company.

Storer, F. H., 1916 *Agriculture in some of its relations with chemistry.* Vol. II. 7th Ed. Charles Scribner's Sons, New York.

Turner, C. W., and Frank, A. H. 1930 *The effect of the estrus producing hormone on the growth of the mammary gland.* Mo. Agr. Exp. Sta. Res. Bul. 145.

VanSlyke, L. L. 1915 *Fertilizers and Crops.* Pub. Orange Judd Co., p. 294.

Veler, C. D., and Doisy, E. A. 1928 *Extraction of ovarian hormone from urine.* Proc. Soc. Exper. Biol. Med., Vol. 25, pp. 806-808.

Zondek, B., and Aschheim, S., 1925 *Experimentelle Untersuchungen über die Funktion und das Hormon des Ovariums.* Klin. Wchnschr., Vol. 4, pp. 1388-1390. (Research on the function of the hormone on the ovary.)

Zondek, B., and Aschheim, S., 1927 *Hypophysenvorderlappenhormon und ovarialhormon in Harn von Schwangeren.* Klin. Wchnschr., Vol. 6, p. 1322. (Hormone of the anterior lobe of the hypophysis and ovarian hormone in the urine of the pregnant.)