



Urinary excretion of estrogenic substances by the bovine in the estrous cycle
by Robert Kaye Bergman

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
of Master of Science in Dairy Production at Montana State College

Montana State University

© Copyright by Robert Kaye Bergman (1957)

Abstract:

The urine of sixteen open cows from the college herd was collected and analysed for estrogenic substances in terms of micrograms of estrone per hundred pounds of body weight. It was found that the urinary estrogen excretion of the cow which did not conceive was higher than that of the cows which did conceive. Method of analysis was a chemical extraction of the urine for the estrogenic substances and a measurement of the fluorescence of the urine extract compared with the fluorescence of a standard of estrone.

In order to gain greater accuracy in measuring only estrogenic substances of the urine extracts, samples of urine extracts were passed through a Celite chromatographic column. By passing pure crystalline estrone and estradiol through the Celite columns it was found that the estrone was eluted out in the first 10 milliliters of benzene and estradiol in the next 140 milliliters of benzene. The addition of crystalline estrone and estradiol to the urine of a bilaterally ovariectomized cow with subsequent extraction of the urine by the chemical process and passing 0.1 milliliter of urine extract through the Celite column gave the same recovery pattern. This was also true when the urine of cows in late pregnancy (250 days) was analysed by the same procedure.

The urine of eleven virgin heifers was extracted for estrogenic substances and chromatographed. In some cases half of the eluate was bioassayed with immature female rats and the results compared with the fluorimetric assay of the other half of the eluate. In all cases, injection of rats with extracts of eluates gave an estrogenic effect on a crude uterine weight basis. The coefficient of correlation between the two methods on estrone was 0.4483 while on estradiol it was -0.133. This difference in the correlations was probably due to the carry over of some estrone into the estradiol fraction. Estrone gives a higher fluorescence than does estradiol, but it is less potent in its estrogenic activity. Therefore, a small amount of estrone in the estradiol fraction would give a false high measurement of estradiol by fluorimetric assay which would not be proved out in bioassay.

The results of the fluorimetric assays of the urine from the heifers gave results quite similar to those of the cows. The estrogen excretion of the heifers which conceived was lower than was that of the heifers which did not conceive. These results are taken to support the theory that some of the infertility problems in dairy cattle are caused by hormone imbalances. It is felt that the high estrogen level in the cattle which did not conceive was partially responsible for preventing pregnancy.

195

URINARY EXCRETION OF ESTROGENIC SUBSTANCES
BY THE BOVINE IN THE ESTROUS CYCLE

by

ROBERT KAYE BERGMAN

A THESIS

Submitted to the Graduate Faculty

in

partial fulfillment of the requirements

for the degree of

Master of Science in Dairy Production

at

Montana State College

Approved:


Head, Major Department


Chairman, Examining Committee


Dean, Graduate Division

Bozeman, Montana
June, 1957

RECEIVED
MONTANA STATE COLLEGE
LIBRARY
JUN 20 1957

N378
B454u
cop. 2

1091

- 2 -

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS	4
LIST OF TABLES	5
ABSTRACT	6
INTRODUCTION	7
REVIEW OF LITERATURE	9
ISOLATION AND ASSAY OF ESTROGENS	9
DEFINITION OF ESTROGENS	9
STRUCTURE OF ESTROGENS	10
EXTRACTION OF ESTROGENS	11
METHODS OF BIOASSAY	12
CHEMICAL METHODS	15
METABOLISM OF ESTROGENS	16
FATE OF ESTROGENS	17
<u>IN VITRO</u> STUDIES ON TISSUE SLICES	18
METABOLISM IN THE LIVER	19
PROGESTERONE INTERACTION ON ESTROGEN DEACTIVATION	23
INTESTINAL METABOLISM	23
EFFECT OF NUTRITION	23
CARBON-TETRACHLORIDE FEEDING EFFECTS ON ESTROGEN METABOLISM	24
PHYSIOLOGICAL ACTIONS OF ESTROGENS	25
SEX ORGANS AND SECONDARY SEX CHARACTERISTICS	26
EFFECT OF ESTROGEN TO BRING ON "HEAT"	27

TABLE OF CONTENTS (CONTINUED)

VAGINAL CELLULAR CHANGES IN ESTROUS CYCLE	28
EFFECT OF ESTROGENS ON PREGNANCY	29
INFLUENCE OF ESTROGEN ON UTERINE MUSCLE	31
ESTROGEN AND MAMMARY DEVELOPMENT	32
ESTROGEN AND MAMMARY CANCER	32
ESTROGEN AND GROWTH	33
EXPERIMENTAL PROCEDURE	36
RESULTS	43
DISCUSSION AND CONCLUSIONS	56
SUMMARY	58
LITERATURE CITED	59

LIST OF ILLUSTRATIONS

<u>Figure</u>		<u>Page</u>
1	Chromotography of Estrone From Celite Chromatographic Columns	45
2	Chromotography of Estradiol from Celite Chromatographic Columns	46
3	Chromotography of Estrone and Estradiol from Celite Chromatographic Columns	46
4	Chromotography of Estrone Added to Urine of Ovariectomized Cow. Urine Extract Added to Celite Chromatographic Column	47
5	Chromotography of Estradiol Added to Urine of Ovariectomized Cow. Urine Extract Added to Celite Chromatographic Column	48
6	Chromotography of Estrone and Estradiol Added to Urine of Ovariectomized Cow. Urine Extract Added to Celite Chromatographic Column	48
7	Fluorescence Obtained from Urinary Extracts Added to Celite Chromatographic Columns	49
8	Crude Uterine Weight Response Curve of Immature Female Rats Given Four Different Dosage Levels of Pure Crystalline Estradiol in Peanut Oil Injection	51
9	Crude Uterine Weight Response Curve of Immature Female Rats Given Three Different Dosage Levels of Pure Crystalline Estrone in Peanut Oil Injection	52

LIST OF TABLES

<u>Number</u>		<u>Page</u>
I	THE EFFECT OF INTERCHANGING THE GONADS BETWEEN THE TWO SEXES ON THE ULTIMATE BODY WEIGHT OF GUINEA PIGS	33
II	THE EFFECT OF OVARIAN IMPLANTS ON THE LENGTH OF BONES IN THE CASTRATED MALE GUINEA PIG	34
III	FLUORESCENCE PRODUCED BY URINARY EXTRACTS OBTAINED DURING THE ESTROUS CYCLE OF COWS THAT CONCEIVED	43
IV	FLUORESCENCE PRODUCED BY URINARY EXTRACTS OBTAINED DURING THE ESTROUS CYCLE OF COWS THAT FAILED TO CONCEIVE	44
V	COMPARISON OF THE RESULTS OF FLUORIMETRIC ASSAY WITH BIOASSAY ON URINE EXTRACTS OF VIRGIN HEIFERS WHERE ESTIMATED POTENCY OF INJECTION WAS MADE BY FLUORIMETRIC DETERMINATION	53
VI	FLUORESCENCE PRODUCED AFTER CHROMATOGRAPHIC SEPARATION OF URINARY EXTRACTS FROM VIRGIN HEIFERS WHICH CONCEIVED AFTER BREEDING	54
VII	FLUORESCENCE PRODUCED AFTER CHROMATOGRAPHIC SEPARATION OF URINARY EXTRACTS FROM VIRGIN HEIFERS WHICH DID NOT CONCEIVE AFTER BREEDING	55

ABSTRACT

The urine of sixteen open cows from the college herd was collected and analyzed for estrogenic substances in terms of micrograms of estrone per hundred pounds of body weight. It was found that the urinary estrogen excretion of the cows which did not conceive was higher than that of the cows which did conceive. Method of analysis was a chemical extraction of the urine for the estrogenic substances and a measurement of the fluorescence of the urine extract compared with the fluorescence of a standard of estrone.

In order to gain greater accuracy in measuring only estrogenic substances of the urine extracts, samples of urine extracts were passed through a Celite chromatographic column. By passing pure crystalline estrone and estradiol through the Celite columns it was found that the estrone was eluted out in the first 110 milliliters of benzene and estradiol in the next 140 milliliters of benzene. The addition of crystalline estrone and estradiol to the urine of a bilaterally ovariectomized cow with subsequent extraction of the urine by the chemical process and passing 0.1 milliliter of urine extract through the Celite column gave the same recovery pattern. This was also true when the urine of cows in late pregnancy (250 days) was analyzed by the same procedure.

The urine of eleven virgin heifers was extracted for estrogenic substances and chromatographed. In some cases half of the eluate was bioassayed with immature female rats and the results compared with the fluorimetric assay of the other half of the eluate. In all cases, injection of rats with extracts of eluates gave an estrogenic effect on a crude uterine weight basis. The coefficient of correlation between the two methods on estrone was 0.4488 while on estradiol it was -0.133. This difference in the correlations was probably due to the carry over of some estrone into the estradiol fraction. Estrone gives a higher fluorescence than does estradiol, but it is less potent in its estrogenic activity. Therefore, a small amount of estrone in the estradiol fraction would give a false high measurement of estradiol by fluorimetric assay which would not be proved out in bioassay.

The results of the fluorimetric assays of the urine from the heifers gave results quite similar to those of the cows. The estrogen excretion of the heifers which conceived was lower than was that of the heifers which did not conceive. These results are taken to support the theory that some of the infertility problems in dairy cattle are caused by hormone imbalances. It is felt that the high estrogen level in the cattle which did not conceive was partially responsible for preventing pregnancy.

INTRODUCTION

Infertility is an important problem in the dairy industry, however there is little specific information available as to the causes of bovine infertility. One important cause is thought to be hormone imbalances, but there has been little research done to determine the normal hormone levels of cattle. Such information would be valuable in diagnostic work and in therapeutic treatment of cattle infertility.

Various methods have been devised to measure hormone levels in animals, but many of them are time consuming and subject to inaccuracies. The measurement of the estrogen excretion in the urine is thought to be the best way to determine estrogen levels in dairy cattle. It is recognized that some of the estrogen will be excreted through feces and in unidentifiable forms. Conjugated forms of estrogen are excreted, this necessitates that the estrogen be broken from the conjugate in order for it to be measured. Bioassay methods have been used to measure estrogenic potency, but these are subject to large variations. This has initiated the search for a sound chemical method of assay for estrogenic substances.

It is the purpose of this study to measure by chemical procedures urinary excretion of estrogenic substances during estrous cycles in dairy cattle. Bioassay was used to compare results obtained by chemical assay. A comparison was made on urinary excretion of estrogenic

- 8 -

substances in dairy cattle which conceived and those that did not.
A correlation is drawn with failure to breed and high urinary
estrogen excretion.

REVIEW OF LITERATURE

ISOLATION AND ASSAY OF ESTROGENS

The search for methods has indeed been an interesting one. Much of the interest of isolating and assaying estrogens has centered around the desirability of finding pregnancy tests for the human female. A great deal of the estrogen work has been done in diagnosing hormone imbalances in the human female. Very little work of this type has been done with farm animals. However, it would be desirable to have a great deal more of this data available to use in problems of non-infectious sterility of farm animals. For instance, the cow, needs low amounts of estrogen but the exact amounts needed, or amounts in excess that lead to infertility are not known.

DEFINITION OF ESTROGENS

In the strictest sense, estrogens refer to the group of hormones produced by the ovarian follicle in the ovaries. These hormones are responsible for bringing the female into estrus, the development of secondary sex characteristics and the partial development of the mammary system. In a broader sense, estrogen is a word used to designate any substance which will induce cornification in the vagina of the adult mouse as occurs in natural estrus. These substances are found in the ovaries, other animal organs, certain plants, and have been synthesized.

Estrogens have been isolated from ovaries, testes, placentae, and adrenals (5,6,11), and extracts of liver, bile, blood, and urine. The amounts produced in the testes and adrenals in most cases is rather

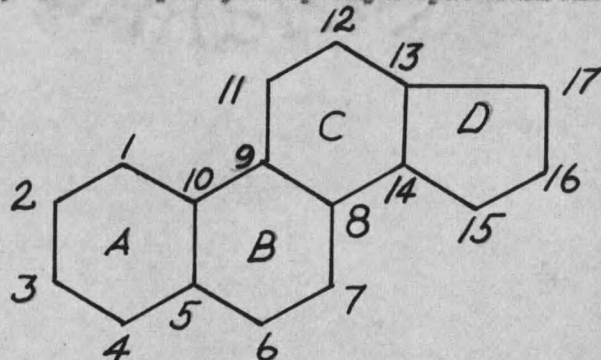
small, however, the stallion testes does produce large amounts of estrogen. In cases of carcinomatous or tumorous growth of the testes or adrenals, there has been noted increases in the excretion of estrogens. This secretion of estrogenic substances from malignant growths of the adrenals can become high enough to cause marked feminism in males (11).

Relatively high titers of estrogenic substances have been reported in plant materials. The cause of infertility and fetal death in Australian sheep is thought to be due to the high content of estrogens found in a strain of early-subterranean sweet clover in that country (18).

A number of very potent compounds not found in nature are known to possess estrogenic activity. The most important of these are diethyl-stilbesterol, hexestrol and dienestrol, all of which have similar activity and a potency which falls between injected estrone and α -estradiol.

STRUCTURE OF ESTROGENS

Thus far, all of the natural estrogens that have been isolated are steroids, with a cyclopentoperhydrophenanthrene ring system:



This structure is similar to that found in the androgens and progesterone except that ring A is phenolic and there is a single methyl group at position 13.

The main ovarian estrogen seems to be α -estradiol (estratriene - 3, 17 - diol) which is the most potent of all the estrogens. However, it is not certain that no other estrogen is produced by the ovaries. The stereoisomer, β -estradiol is found in pregnant mare's urine, but is much less potent. The other estrogens are found in the urine and are therefore, thought to be metabolic products of α -estradiol. Estrone, (estratriene -3-ol-17-one) is found in urine, but has also been isolated from ox adrenals and placentae. It is not as potent as α -estradiol, which is rated from one-fourth to one twelfth as active. Estriol (estratriene -3, 16, 17, - triol) is found in the urine but is much less active than estrone. Estimates of the estrone: estriol potency ratio vary from 1:1 to 250:1. Estrone and estriol are excreted in the conjugated forms as sulfates and glucuronides (18).

Equilin (estratetrene -3-ol-17-one) and equilenin (estrapentene - 3-ol-17-one) are two estrogens found in the urine of pregnant mares. They differ from estrone in that they have double bonds in positions 7,8; 6,7, and 8,9 (18).

EXTRACTION OF ESTROGENS

Estrogens are found in two different forms in the body i.e. in the free and the conjugated form. Those in the free state can be extracted with a lipid solvent such as alcoholol, ether, or benzene. Those in the

conjugated form must be hydrolyzed by heating with an acid. Estrogens found in the various body organs are probably in the free state so that by pulping the tissue and extracting with a lipid solvent, usually ether, the estrogens can be removed. In extracting estrogens from urine, it is necessary to hydrolyze the urine usually with hydrochloric acid, because the estrogens are present in the conjugated form (23,24, 26,31,43,46). The hydrolyzed urine is then washed with a lipid solvent. The extracts are then taken through a series of steps to purify them and then the estrogen is taken up in sesame oil or some other vehicle if it is to be used in bioassay or it may be taken up in some other compound if the assay is to be made chemically.

METHODS OF BIOASSAY

A number of methods of bioassay have been developed and though the results are variable, they are still considered to be the most accurate methods of estrogen assay. A brief discussion of chemical methods of assay will follow later. The individual differences of laboratory animals such as rats and mice, and their reactions to climatic changes, often make the results of bioassay work hard to interpret. However, a number of bioassay tests have been developed which give some indication of estrogenic activity. A discussion of some of the major tests follow (18).

Allen and Doisy found that there was vaginal cornification in mice during estrus and they adopted a method of using this phenomenon to assay estrogens on castrated female mice. Stock solutions of

of estrogens are made up of absolute alcoholol, with the estrogen in it, into saline, oil or distilled water. The methods of administration vary, but the peroral or the subcutaneous have probably been the most successful. Other methods that have been used are the percutaneous, intraperitoneal, intravenous, and intravaginal. Peroral administration is made by the use of a feeding tube or an elastic stomach tube. Subcutaneous injections are made with a small diameter short hypodermic needle under the skin on the back (18).

It has been found that mice should be given a priming dose before they are put on tests. Sensitivity as well as uniformity of response is better after the mice have been given an injection of 0.5 micrograms to 1 microgram of estrone or diethylstilbesterol. In a typical Allen-Daisy test the mice are given two subcutaneous injections of estrogen in arachis oil at 10:00 a.m. on Monday and Tuesday. Kuenens (18) recommends that at least two groups be used on the known and unknown and that each group contain not fewer than 20 animals.

Smears are taken Wednesday afternoon, 11:30 p.m. Wednesday, and 10:00 a.m. and 4:00 p.m. Thursday. The smear taken Wednesday afternoon is discarded, but this makes reading the other smears easier. Smears are taken with a metal spatula from the dorsal vaginal wall as gently as possible. The smears are transferred to slides and stained with methylene blue for ten to fifteen minutes and then washed, dried, and scored. A positive smear contains nucleated or cornified epithelial cells, but no leucocytes. If any of the three smears from an animal

is positive, the animal is considered to be positive. By comparing the results of the known with the unknown, a determination of the potency of the unknown, can be made. There are several modifications to this test, but it is still often used in making estrogenic assays (18).

It has been found that by applying estrogens locally to the vaginal tissues that vaginal cornification will result. For instance, if estrogens are made up in blood pellets, or fifty percent aqueous glycerol and placed in the mouse vagina, cornification will result as in natural estrus. Smears are taken and read in much the same way as in the Allen-Delisy tests. This method however, does not seem to be very accurate for assay methods (18).

This principal that estrogens cause an increase in uterine weight has been used in a number of tests developed to assay estrogens. In general, the tests are carried out as follows. Intact immature female rats are injected with estrogens and then after a definite period of time they are killed and the uteri dissected out. The uterus is stripped of its outside tissue and weighed after expressing the inter-uterine fluid. One uterine weight test took four days to complete, but the results from it were quite variable.

Astwood (4) developed a six-hour test which was based on the fact that the rapid increase in weight after an estrogen injection was based on water retention. He gave single doses of estrogen in sesame oil to rats subcutaneously and after six hours sacrificed them. The uteri were removed by cutting at the utero-tubal junctions, stripping

off the endometria and trimming the vagina off at the cervix. The uteri are then blotted on absorbent paper and quickly weighed on a damped analytical balance. Determination of the water content was made by desiccating the weighed uteri in an oven at 110° C. Certain corrections must be made for rats of different sizes, but the method seems to be reasonably reliable (18).

CHEMICAL METHODS

A number of colorimetric methods have been used to assay estrogens, but in general these methods have not proven to be too accurate, especially with assay of urine extracts. The brown color, which is non-estrogenic, interferes with the readings in colorimetric methods. Some work has also been done with spectropotometry and fluorescence. The opinion seems to be that chemical assays are not very reliable except where the amounts of estrogens are very high, (46) however workers are continuing to develop more accurate chemical methods. Chromatographic columns are used in the separating of estrogens into the three main fractions; estradiol, estrone, and estriol, and improvements are being made in this technique (19).

If a better correlation between the results of bioassay and chemical methods could be worked out, it would greatly simplify the problem of assaying estrogens and cut down on the expense. As yet, there is no completely reliable method of assaying estrogens because of the large number of variables which enter into the determinations.

METABOLISM OF ESTROGENS

The question of what happens to the estrogens both endogenous and injected is a complex question. The exact mechanism of estrogen deactivation and the end products of such processes is not clearly understood. A great deal of research has been done, some of which has resulted in conflicting reports. No doubt there are complex interactions which would make several of the reports correct. If the answers to these questions could be found, it might be possible to determine how progesterone takes an animal out of heat, or to measure more accurately the endogenous production of estrogens by measuring the excretion of the metabolites. Thus, it would be possible to diagnose fertility problems more accurately. It would also be interesting to know if environmental factors would alter the percentage excretion of the various metabolites.

As would be expected, the greatest majority of the estrogen metabolism studies have been bioassays on humans, rats, guinea pigs, monkeys, rabbits and others. This brings on differences in results, because of the natural variation in individuals. Also, different species of animals do not react in the same way to various treatments. Many of the studies have been carried out in vitro. These studies give indications, but do not give an accurate picture of what happens in the animal's body.

Briefly, some of the places where estrogen metabolism is known or thought to take place are: liver, reproductive organs, digestive system, and placental membranes (32). No doubt, a certain amount of metabolism takes place in all the tissues of the body. Of all the tissues, the liver is the most important in deactivating the estrogens (15,40). Other factors which affect estrogen metabolism are: nutritional state of the animal, hepatectomy, and certain poisons such as carbon-tetrachloride and cyanide.

FATE OF ESTROGENS

In an attempt to determine the end products of estrogen metabolism, various experiments have been carried out in which known doses of an estrogen such as α -estradiol or estrone were injected. Then the amounts and kinds of estrogens excreted in the urine, feces, and bile were determined. Pearlman et al gave massive dosages of estrone as the acetate intramuscularly to three bile fistula dogs (32). They recovered a small quantity of estrone and α -estradiol in the pooled bile specimens of the three dogs. By comparison, there was much less estrogen in the feces and urine as determined by bioassay. Most of the estrogenic substance administered could not be accounted for in the excreta.

Heard and Hoffman (22) administered a total of 250 milligrams of purified α -estradiol intramuscularly to a normal male, to ascertain the nature of the urinary excretion products. They recovered unchanged

9.8 milligrams (3.9 percent) of α -estradiol and found that 16.2 milligrams (6.4 percent) had been oxidized to estrone. No estriol or β -estradiol was obtained. These workers came to the conclusion that the remaining 90 percent of unaccounted for estradiol must undergo chemical changes (beyond simple conjugation) to a point where physiological potency is destroyed.

IN VITRO STUDIES ON TISSUE SLICES

Although in vitro studies do not give a true picture of what is happening in an animal's body, they do give indications of capabilities and possibilities of the role of tissues in metabolism. Ryan and Engel (34) conducted a study on tissues of the digestive tract, reproductive organs, and endocrine system incubated in vitro with estrone, estradiol, and estriol. They found that these tissues can convert estradiol to estrone. Eighteen to 58 percent of the administered estrogen could not be accounted for. This estrogen was considered to be changed to unknown metabolites and lost during the extraction and analysis (approximately 10 percent of starting material). It was found that estriol could almost be quantitatively recovered after incubation with testes and term placenta. However, four month placenta gave a lower recovery of estriol. In none of the experiments, was estradiol or estrone found after incubation of estriol.

Szego and Samuels (48) incubated aerobically samples of viable endometrium from bovine during various stages of pregnancy, from a

three months pregnant woman, and from pregnant rabbits with estrone and estradiol. During no stage of the estrous cycle would the bovine endometria either destroy estrone significantly or convert it to estradiol. The same was found to be true of the endometrium of the pregnant woman. Conversely, the endometria of pregnant rabbits appeared to almost completely convert estrone to estradiol.

METABOLISM IN THE LIVER

As was mentioned earlier, the liver seems to be the most important single organ in the deactivation and metabolism of the estrogens. A number of experiments have been carried out on the liver to determine the exact mechanism and end products of estrogen metabolism. Various experiments (26,36,37) have shown the powerful effect of the liver in deactivating estrogens. It is found that when estrogens are administered in such a manner so as to enter the systemic circulation (usually intramuscularly) the female animal will show signs of estrus. If however, the estrogen is administered so it will enter the hepatic circulation (usually intrasplenically) the animal will show no estrus. This is true even when quite large amounts of estrogens are given. Kirgis and Rothchild (26) in an experiment on women found that estradiol absorbed into the hepatic-portal system gave very little estrogenic effect. When estradiol was absorbed directly into the systemic circulation it gave definite evidences of estrogenic activity. In this respect human liver is much the same as the liver of rats, rabbits,

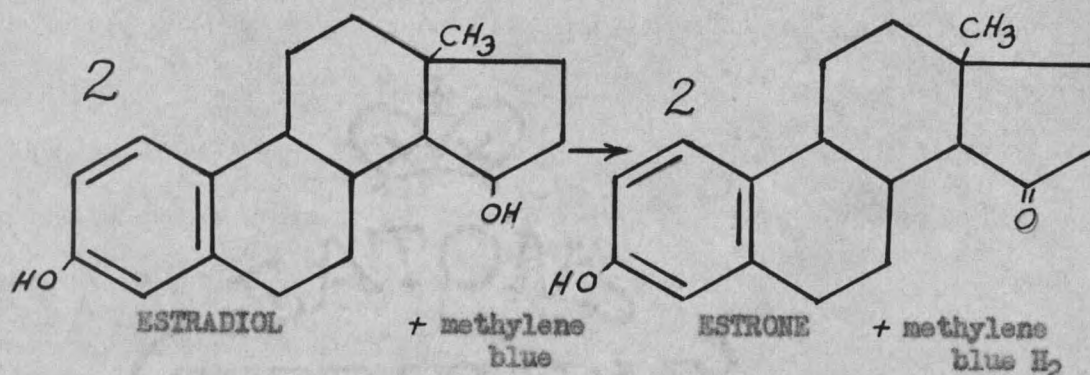
guinea pigs, and dogs. Monkey liver is the only one known that cannot deactivate estrogens.

Bernstorf (8) showed that the liver does not completely deactivate the estrogens. In his experiment, the uterine and vaginal weights were determined on I. spayed mice, II. spayed mice with an autoplastically grafted ovary in the spleen, and III. unoperated mice. The organs of the graft bearing mice weighed significantly less than those of the controls. In turn the organs of the castrates weighed significantly less than the graft-bearing mice. If there was complete estrogen deactivation, there would be little difference between the organs of the castrates and graft-bearing mice. Histological examination of the uterus and vagina of the graft-bearing mice also supported the conclusion that the liver does not completely inactivate estrogen.

Ryan and Engel (35) showed in in vitro studies on rat liver slices that there is an interconversion between estrone and estradiol under aerobic and anaerobic conditions. The extent of conversion of estradiol to estrone by normal and cirrhotic rat livers and hepatoma nodules depends on the hormone concentration.

De Maio (15) made a study to determine some of the aspects of liver deactivation of estrogens. They stated that 95 percent of the biological activity of estrone disappears when administered to man or incubated with rat liver. Previous workers have shown that liver slices and "brei" will inactivate estrogens. The process must be enzymatic inasmuch as boiled liver slices show no such activity. The

transformation is probably oxidative in nature as it does not occur in a nitrogen gas phase. An indication that the process at least partially contains a dehydrogenating mechanism is indicated by the fact that methylene blue re-establishes in part the activity of slices incubated anaerobically. It is believed that methylene blue acts as a hydrogen acceptor, and under these conditions, estradiol may be converted to estrone.



Not all of the liver's activity is deactivating, since there are some estrogenic materials which are more activated by the liver. These materials are called "progestrogens". Segaloff (36) injected a proestrogen, triphenylchloroethylene subcutaneously and intrasplenically into spayed female rats. The estrogenic response, as judged by vaginal estrus, was increased when the material had to pass through the liver before entering the systemic circulation. This increase in activation was further increased by partial hepatectomy, contrary to what would be expected. A possible explanation of these results is as follows: the liver can change a phenyl radical to a phenol radical, which will increase estrogenic activity by adding hydroxyl groups. The liver also

oxidizes or conjugates the formed estrogens. Thus we have two competing processes going on within the liver, one increasing estrogenic activity and the other decreasing it. Now we must assume that more tissue is needed (whether it be oxidation or conjugation) in the liver to carry on the destruction process. Thus hepatectomy would cause a greater effect on the "decrease potency process" than it would on the "increase potency process."

Segaloff (37) also determined the liver's action on several estrogen degradation products and α -estradiol. The degradation products could be placed in the following descending order of estrogenic potency: 3 methyl ether of bis-dehydro doisyolic acid > sodium bis-dehydro doisyolate > α -estradiol > Westerfeld's lactone acetate > β -estradiol > estrololactone acetate. It was found that 43 times more α -estradiol, 12 times more β -estradiol, 17 times more Westerfeld's lactone acetate, and three times more estrololactone acetate was needed when injected intrasplenically rather than subcutaneously to produce vaginal estrus. On the other hand, only half as much 3-methyl ether of bis-hydro doisyolic acid and sodium bis-dehydro doisyolate was required when injected intrasplenically as when given subcutaneously to produce vaginal estrus. These results indicate that the liver deactivates the first four and activates the latter two. It is interesting to note that rupture of the five membered ring in estrone can lead to compounds of such varying estrogenic potency which are handled by the liver in totally different ways.

PROGESTERONE INTERACTION ON ESTROGEN DEACTIVATION

Segaloff (38) found that in rats, the in vivo deactivation of estradiol by the liver is reduced when 0.5 milligrams of progesterone is given each day. At the same time though, the progesterone seems to lessen the sensitivity of the vaginal mucosa to α -estradiol as vaginal cornification is reduced by the injections of progesterone. Progesterone does seem to lessen the liver's power of deactivating α -estradiol. This does not seem to hold true with rabbits though. When Heard et al (21) gave progesterone to rabbits simultaneously with α -estradiol there was no change in the end products from those that received only α -estradiol. In all cases β -estradiol and estrone were obtained in the proportion four-five to one. No estriol was found in any of the work. These findings fail to substantiate the hypothesis that estriol formation from α -estradiol or estrone takes place in the uterus under the influence of progesterone.

INTESTINAL METABOLISM

Levin (27) found that during the last two weeks of pregnancy, cows excrete 5,000-10,000 rat units of estrogenic substance per kilo of dry feces. Calculated as α -estradiol this would amount to 0.9 to 1.4 milligrams of α -estradiol. It is not known if it is secreted into the gut as such or is converted from some other estrogen.

EFFECT OF NUTRITION

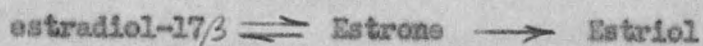
Singher (39) found that liver slices from riboflavin and thiamine deficient rats were unable to deactivate estradiol under conditions

that livers from rats on same diet, but with adequate thiamine and riboflavin could. The loss of deactivating ability paralleled the change of thiamine and riboflavin content in the liver. Pyridoxine, pantothenic acid, biotin, and vitamin A deficiencies had no effect on liver deactivation of estrogens. It is thought that thiamine and riboflavin may be related to estrogen metabolism through an oxidative enzyme system. Other workers, Jailer, Vanderline, and Westerfeld (25,53) say that it is not the B vitamins which are the critical factor in liver deactivation of estrogens, but that it is the protein intake of an animal. There is probably an interaction here which makes both of these factors critical.

CARBON-TETRACHLORIDE FEEDING EFFECTS ON ESTROGEN METABOLISM

Furlong et al (20) found that by feeding carbon-tetrachloride, the following changes could be made in the excretion of estrogen by the guinea pig. During the first 50 days the level of estrogen excretion increased to a peak 350 times normal. During the second 50 days a fairly constant level of estrogen excretion is maintained at approximately 3.7 times normal. It is suggested that the sequence of effects of carbon-tetrachloride administration might be as follows: (1) an impaired inactivation of estrogens resulting in an increased excretion, followed by (2) either an inhibition in the production of endogenous estrogens or loss of activation of estrogens resulting in decreased estrogen excretion.

In brief it is believed that the conversion between the estrogens is about as follows in humans (17).



Estriol has only been isolated from human sources. There are many other estrogenic substances and metabolites to be found in other animals such as α and β -estradiol, equilin, equilenin, etc., but the metabolism of these estrogens is not clearly understood.

There are a number of metabolites which are not identified into which the estrogens are transformed.

PHYSIOLOGICAL ACTIONS OF ESTROGENS

When speaking of the functions of the estrogens it is commonly understood that they are responsible for the normal growth and maintenance of the accessory sex organs and the development of the secondary sex characteristics. It is recognized that the estrogens bring the female into heat and are necessary for the development of the mammary duct system. These functions were discovered by spaying females and watching the physiological changes which took place and then grafting ovaries in the spayed animal and observing the recovery which followed. This work was greatly augmented by the isolation and synthesis of purified estrogens such as estradiol, estrone, and stilbesterol. It became possible to treat spayed females with pure estrogens and observe the physiological results which followed.

Until recently, the exact function of estrogens at the cellular level was not known. This branch of the field is only beginning to be

opened up and a great deal of research remains to be done. The fact that the estrogens have a profound effect on general body growth and skeletal development is not commonly realized. The effect that estrogens have on enzyme systems is now beginning to be investigated.

SEX ORGANS AND SECONDARY SEX CHARACTERISTICS

It has long been known that removal of the ovaries from a female will cause the fallopian tubes, uterus and vagina to atrophy and become degenerate. Allen in 1932 (2) reported that treating ovariectomized rats or mice with estrone leads to considerable growth in the vaginal wall within 24 hours. In the uterus, there is active mitotic division in the surface epithelium and glands followed by an extensive increase in the number of cells. Then a clear fluid is secreted into the lumen and uterine contractions increase in amplitude. The fallopian tubes are repaired from their degenerate condition. The degenerations of the mammary glands also become repaired.

The secondary sex characteristics such as color of feathers, thickness of skin, pitch of voice, distribution of body hair, and size of pelvic girdle can all be changed or altered by ovariectomy in females. Ovariectomized human females grow beards and get deeper voices. Castrate hens develop a more male-like comb and plumage (12). All of these can be alleviated by the use of ovarian implants or treatment with estrogens.

The question might be asked, what is the role of estrogens in sex differentiation in the embryo. At present, it is thought that the sex

hormones have no part in determining the sex of an embryo. It is thought to be a genetic action. Some evidence of hormonal control over sex differentiation has been brought forward, but the evidence is inconclusive (10). Moore (29) says that the sex hormones are responsible for the development of the accessory sex organs and secondary sex characteristics, but they have nothing to do with sex differentiation and very little to do with the sex duct systems. Treatment of the gonads with sex hormones has given little, if any specific effect. It is also pointed out that the gonads do not start producing sex hormones until after the gonad is well formed. However, he is not able to explain the case of the "freemartin" in cattle.

EFFECT OF ESTROGEN TO BRING ON "HEAT"

It is known that unless enough estrogen is being secreted in the female to bring on the phenomena of "heat" or estrus she will not accept the male in copulation. "Heat" is not necessary for ovulation, but unless it occurs, there is no chance of fertilization in such animals that have "heat" periods. Ovulation without "heat" is known to occur in such animals as cattle and sheep. Asdell et al (3) conducted an experiment on ovariectomized heifers to determine what levels of estrogen were necessary to bring them into normal "heat". Normal "heat" being the acceptance by the heifers of a bull in copulation. They found that cattle have a much lower threshold value for estrogens in comparison with their size than do other animals. They found that by using an average level of 600 rat units of estradiol benzoate per

day for three days on ovariectomized heifers that heat would follow. Duration of the heat was usually less than one day even when injections of the estrogen were continued. They explained that the cow probably has an "estrous block" in the central nervous system which sets in when the threshold value for estrogen is reached. This threshold value is probably reached early in the development of the Graffian follicle in a normal cow and this is the reason she is out of heat when ovulation occurs, i.e. due to the "estrous block" in the central nervous system.

VAGINAL CELLULAR CHANGES IN ESTROUS CYCLE

In 1917, Stockard and Papanicolaou (47) published a report describing the cyclical cellular changes which occur in the walls of the vagina of a guinea pig during the estrous cycle. By taking vaginal smears and examining them under a microscope the exact period of the estrous cycle can be determined. Smears taken in diestrus show leucocytes in a stringy mass. During proestrus and estrus the mucus fluid contains an abundant mass of cells, of a squamous type and showing considerable plasmolysis with bent and wrinkled cell membranes. The nuclei are very small and pyknotic; the protoplasm has degenerated and does not stain well. Towards the end of this stage and at the beginning of metestrus there are present some elongate, cornified cells without nuclei; which are desquamated from the more external portions of the vagina. They stain decidedly red with haematoxylin and eosin while the commoner types appear merely gray. During metestrus, the enormously increasing number of cells in the fluid causes its cheese-like

consistency. These cells, from the vaginal wall, are healthy epithelial cells. The nuclei show only slight signs of degeneration and the protoplasm stains well. In late metestrus and early diestrus the epithelial cells become separated from each other and each is surrounded by a number of leucocytes. The leucocytes appear to dissolve or digest the epithelial cells and the fluid becomes more serous. Different workers have examined vaginal smears of mice, rats, monkeys, opossums, cows, and rabbits and found a strikingly uniform correlation between the cellular composition of the smear and the period in the estrous cycle (12,13).

EFFECT OF ESTROGENS ON PREGNANCY

Some of the effects of estrogens on pregnancy have long been known and studied. The ancients recognized the use of estrogens to induce abortions in women. Ortus Sanitatis a pharmacopeia by an unknown author published in 1494, stated that the residue from evaporated horses urine will cause pregnant women to abort. Stallions' urine is very high in concentration of estrogen (10). Numerous experiments have shown that estrogens will prevent or terminate pregnancy. However, it is important to point out that implantation of a fertilized ova can only take place in a uterus that has first been under the effects of estrogen and then progesterone. In other words, estrogen is necessary for pregnancy too (10).

It is thought that estrogen imbalances are responsible for many cases of infertility and embryonic death. Tanabe et al conducted an

investigation on repeat breeding cows and heifers to learn the cause of infertility. Their investigation on heifers showed that the fertilization rate was about 66.7 percent. Embryonic death seemed to be the greatest cause of infertility as 54.1 percent of the embryonic deaths occur in the first month. Genital abnormalities occurred in 13.5 percent of the heifers studied and were not a significant cause of breeding failures (50). The investigation on repeat breeding cows showed that there was a failure to fertilize in 39.7 percent of the cows. Embryonic abnormalities and mortality before 34 days post breeding was found in 39.2 percent of the cases. In 21.1 percent of the cases it was found that the embryos were still normal at 34 days post breeding (51). These results would indicate that possibly estrogen is coming into an imbalance and thus stopping pregnancy or causing embryonic death.

Investigation into estrogen levels during pregnancy have shown that the estrogen level starts out low at 50-100 days of pregnancy and increases steadily until parturition at which time it falls off rapidly (10,30,42). It is thought that the major source of this estrogen is the placental membranes. However, in the case of a human female it was found that a bilateral oophorectomy during pregnancy definitely decreased the estrone content of the urine without causing abortion (1). It would seem that estrogen has a very definite effect on parturition. Depending on the time in pregnancy when an excess of estrogen occurs it may cause either an abortion or parturition (10). Smith, Smith and

Pincus (44) noted that the estrogen level continues to rise right up until delivery with a very sharp increase right before the event.

INFLUENCE OF ESTROGEN ON UTERINE MUSCLE

As was mentioned earlier, the dosage of estrogens to an ovariectomized animal will increase the amplitude of the uterine contractions. In one experiment, a rat uterus was excised and placed in Ringers solution at 37° C. Follicular fluid from a mare's ovary was added to the Ringers solution. If the uterus was motionless, the addition of the follicular fluid caused the uterus to undergo rhythmic contractions. If the uterus was already undergoing rhythmic contractions the amplitude was increased (10).

Before describing the effects of estrogen on the individual muscle cells, it is best that a description of the muscle cells and what is responsible for the tension be developed. Szent-Gyorgyi (49) et al have shown in skeletal muscle that the final contractile system of myofibrils is the fibrous protein complex actomyosin, the high-energy phosphate compound ATP and ions. Attempts to show differences between the contractile systems of skeletal and uterine muscle have failed. Therefore it is assumed that the contractile systems are the same. Now to look at the effects of estrogen on the myometrium. Three weeks after ovariectomy in rabbits in natural estrus, an 80 percent decrease both in actomyosin content and in maximum tension was found. A seven day treatment with estrogen resulted in the recovery of both actomyosin and maximum tension (33). It had been found earlier that it was the

plasma space containing the actomyosin filaments which decreases on the withdrawal and increases on the administration of estrogen. It has also been noted that the high-energy compound creatine phosphate disappears, and ATP decreases by 50 percent after ovariectomy and is returned to status quo with about two days of estrogen treatment. This implies that metabolism is corrected first by estrogen before its effect on protein metabolism becomes maximal. Pregnancy tends to increase the actomyosin concentration, maximum tension, and length of the cell about twofold over conditions in natural estrus.

ESTROGEN AND MAMMARY DEVELOPMENT

It has long been known that the estrogens have a definite effect on the development of the mammary gland and especially on the mammary duct system. Numerous experiments have been carried out showing the profound effect and increase in growth of the nipples and mammary gland (10,52). Some work also has shown that too large a dose of estrogen will impede the development of the mammae. It is also recognized that other factors must be present such as progesterone for optimal growth in the mammary gland.

ESTROGEN AND MAMMARY CANCER

In 1896 Sir George Beaston (7) said that we must look to the ovaries in females as the seat of the exciting cause of cancer especially in the mammae and in female organs generally. Numerous experiments on mice seem to prove this out. Removal of the ovaries in mice before six months of age was very effective in reducing the incidence of mammary

cancer. Cori in 1927 (14) ovariectomized mice belonging to a strain in which 78 percent of the females suffered from spontaneous mammary carcinoma. Removal of the ovaries before 6 months of age reduced the incidence of cancer to 10 percent. Mammary cancer is rarely if ever seen in male mice, but when ovaries were grafted into 16 male mice cancer appeared in nine of them. Pure estrogen is also influential in causing mammary cancer in male mice (10).

ESTROGEN AND GROWTH

In most mammals the male is larger than the female and it has been shown that this effect is due to estrogen. Steinach and Holaknecht in 1916 (45) interchanged the gonads of young male and female littermate guinea pigs, implanting testes into spayed females and ovaries into castrated males. The males failed to attain the size of normal females and the females grew to an unusual size.

TABLE I. THE EFFECT OF INTERCHANGING THE GONADS BETWEEN THE TWO SEXES ON THE ULTIMATE BODY WEIGHT OF GUINEA PIGS (Steinach and Holaknecht, 1916)

<u>Subject</u>	<u>Body Weight (g)</u>
Normal female	845
Normal male	1,002
Spayed female with grafted testis	1,200
Normal male	980
Normal female	808
Castrated male with grafted ovary	516

There is little doubt that these differences in body weight are due to differences in bone growth. Bones in females attain their growth earlier than they do in males. Steinach and Holaknecht in 1916 (45) demonstrated that the difference between the two sexes was due to ovarian action. They grafted ovaries into male guinea pigs from female litter mates and measured the length of various bones after full growth was attained. Some of the measurements are shown in Table II.

TABLE II. THE EFFECT OF OVARIAN IMPLANTS ON THE LENGTH OF BONES IN THE CASTRATED MALE GUINEA PIG (Steinach and Holaknecht, 1916)

MEASUREMENTS OF BONES			
Part of skeleton	Normal Brother (m.m.)	Normal Sister (m.m.)	Castrated brother grafted with ovary (m.m.)
Tibia	44	40	38
Femur	36	33	31
Ulna	33	31	29
Humerus	31	28	26
Vertebral Column	160	148	136
Pelvis	47	43	38

The pituitary is known to control the general body growth and secretes the growth hormone, somatotropin. It is known that estrogen inhibits secretions of the pituitary gland and this is thought to be in part responsible for the growth inhibition by estrogen (10). Another aspect is that estrogens may control growth by an influence on enzyme

systems. This aspect is only beginning to be explored. The influence on hormones may be as follows: (a) by means of changes in tissue enzyme concentrations, (b) by the hormone functioning as a component of an enzyme system, and (c) by direct or indirect effect on accelerators and/or inhibitors of enzyme systems (16). It has been shown that steroid hormones have an effect on brain metabolism and that estrogens in mice (Wistar strain) cause an increase in glucuronidase content of secondary sexual tissue such as the uterus and mammary gland.

EXPERIMENTAL PROCEDURE

Sixteen cows from the college herd, including both Jerseys and Holsteins were used in the study to establish the urinary estrogen excretion of mature cows during their estrous cycles. All the cows were open and palpated by the college veterinarian and declared to be free of genital abnormalities; however, pyometra was detected at a later date in one of the cows that failed to conceive. Most of the cows were bred artificially. Breeding was done on the day of heat at mid to late heat. Twenty-four hour collections of urine were taken after breeding on the day of heat, at seven days after breeding, at fourteen days after breeding, and at the twenty-first to twenty-fifth day after breeding if the cow did not return in heat. The urine was collected by a modification of the apparatus used by Smith (41). The total twenty-four hour excretion of urine was measured, mixed, and a sample of at least 250 milliliters saved in a bottle under refrigeration. The urine was then extracted in duplicate. The method of analysis was a modification of the methods of Friedgood (19) and Stimmel (46). The method of analysis consisted of the following steps:

1. Two hundred and fifty milliliters of urine were filtered through glass wool into a round bottom flask.
2. Fifteen volume percent of concentrated HCl was added and the mixture was hydrolyzed with a glass-coil heater for 18 minutes (boiling time) under low vacuum. After removing from the heat it was immediately cooled in cold water or an ice bath.

3. The mixture was then washed three times with 80 milliliters of ether (ether washed with 1 percent Fe SO_4 and distilled water). The washing was done in a separatory funnel and the ether portion saved.
4. The ether extract was washed four times with 30 milliliters of 9 percent Na HCO_3 and two times with 30 milliliters of distilled water.
5. The ether extract was then distilled nearly to dryness under vacuum.
6. The residue was taken up in six milliliters of ether and 108 milliliters of CCl_4 .
7. The organic phase was extracted three times with 50 milliliters of one normal KOH and one time with five milliliters of H_2O .
The H_2O was put in with the KOH and the CCl_4 phase was discarded.
8. The KOH phase was washed once with 20 milliliters of ether and the ether discarded.
9. The KOH was then acidified to pH-3 with six normal $\text{H}_2 \text{SO}_4$.
10. The aqueous phase was then extracted three times with 80 milliliters of ether.
11. The ether was saved and washed three times with 30 milliliters of nine percent NaHCO_3 , two times with 30 milliliters of H_2O , five times with 20 milliliters of two and one-half percent Na CO_3 , and two times with 30 milliliters of H_2O .
12. The ether was then distilled to dryness.

13. The residue was taken up in 10 milliliters of 95 percent ethanol. The ethanol was allowed to stand in the flasks for a few hours under refrigeration.
14. A 0.1 aliquot of the ethanol was pipeted into a test tube and ten milliliters of 75 percent H_2SO_4 (freshly diluted) was added. The test tube was then heated in a water bath at $80^{\circ}C$. for 30 minutes.
15. The fluorescence of the extracts was measured with a Coleman Electronic Photofluorometer Model 12 C with a lamp filter transmitting at a wave length of 436 m μ . (accomplished by a Corning #3385 and Corning #5113 glass filters) and photo cell filter transmitting at 525 m μ . (accomplished by a Baird 525 m μ . interference filter and a Corning #3385 glass filter). The fluorescence of the extract was compared with the fluorescence given off by a standard of estrone and the amount of estrogenic substance present calculated in terms of estrone.

Since it was apparent that non-estrogenic compounds might be producing fluorescence which was being measured as estrogenic compounds, it was desirable to attempt to separate the estrogenic compounds from the urine extracts. It was also desirable to separate the estrogens into fractions of estrone and estradiol for the purpose of knowing which was responsible for the estrogenic activity of the urine. It was also noted that there were often compounds in the urine extracts which reacted with the sulfuric acid to give a dark color which seemed to mask the

fluorescence. To overcome these problems, it was decided to use chromatographic separation. The technique developed by Bitman and Sykes (9) was used. In this procedure, a Na OH solution absorbed on Celite forms the stationary phase and benzene the moving phase. It was found that when estrone and estradiol were added at the top of the Celite column, they were eluted out of the column in different fractions of the benzene as it came out of the column. Estrone exhibits the greatest rate of elution and appears in the eluate after a forerun of 30 milliliters of benzene. It is recovered in the following 80 milliliters of benzene. A clear zone of ten milliliters appears and then the estradiol appears in the following 90 milliliters of benzene.

Since the internal diameter of the columns used by Bitman and Sykes was 10.8 millimeters and ours was 9.0 millimeters it was necessary to characterize our columns to determine what kind of a "recovery pattern" was obtained with estrone and estradiol. Varying amounts of pure crystalline estrone and estradiol dissolved in benzene were added directly to the Celite columns and the eluate collected in ten milliliter aliquots. The estrogenic potency of each ten milliliter aliquot was determined by fluorometric assay. The benzene in each aliquot was evaporated and ten milliliters of 75 percent H_2SO_4 added. The fluorescence was developed by heating in a water bath at $80^{\circ}C$. for 30 minutes and the fluorescence measured with the Coleman Electronic Photofluorometer Model 12 C.

Next, varying amounts of pure crystalline estrone and estradiol were added to the urine of a bilaterally ovariectomized cow. This urine was extracted by the same chemical procedure as was described previously and one-tenth milliliter of the extract passed through the chromatographic column. The "recovery pattern" was determined as described previously.

For comparison, the urine of cows in late pregnancy (over 250 days) was analyzed by the same procedure and the "estrogen recovery pattern" developed and compared with that of urine from the spayed cow with and without estrogens added.

Virgin heifers were used for the determination of urinary estrogen excretion by the bovine during the next part of the study. It was considered that they would be more nearly normal and there would be less chance of infertility problems. Twenty-four hour collections of urine were taken from ten heifers in the college herd. Collections were made at one, eight, and fifteen days post breeding. If they did not return in heat, a collection was made on the twenty-fifth day post heat. The urine was handled in the same way as was that of the cows in the earlier trial. After the chemical extraction of the urine for its estrogenic content, a one-tenth milliliter aliquot of the extract was added to a chromatographic column and the benzene collected in two portions. The first 110 milliliters was considered to contain estrone and the next 140 milliliters was considered to contain the estradiol fraction, because the pure hormones were eluted out in these fractions.

In some cases, each collection of benzene was halved, the quantity in the one half estimated fluorimetrically and the quantity in the other half determined with bioassay. In all other cases the entire eluate was assayed only by fluorimetric methods.

In the bioassay work, rats were received from the Holtzman Company at 19-21 days of age and 40-45 grams body weight in the early part of the study, and 35-40 grams body weight later in the study. They were kept in a small animal laboratory where the temperature and humidity were held constant by electronic controls. The rats were kept in cages in groups of from five to ten and fed Purina Laboratory Chow.

The six-hour test of Astwood (4) was used and is based on the fact that estrogens cause an increase in water content of the uterine tissues of the rat and this is most pronounced at six hours after treatment. In this work the increase in crude uterine weight was used as the assay principle. The most sensitive weight range of the rats to estrogen treatment was determined by giving rats in various weight classes the same dosage of estrogen. The most sensitive weight range was found to be 40 to 50 grams body weight. Rats in this weight range were used to determine response curves to "knowns" and for assay of unknowns. A four point "response curve" was developed for estradiol and a three point "response curve" developed for estrone. Ten rats were used for each point determination and 34 were used as controls. Injections of "knowns" were made by dissolving pure crystalline estrone or estradiol into ether and then diluting with ether to the desired

potency. Peanut oil was added to the ether and the ether evaporated off. This left the estrogen in the oil and ready for subcutaneous injection into the rats.

For an estimation of the potency of the substance which was to be injected into the rats for bioassay, the results of the fluorimetric assay was used. This was necessary in order to be sure that an approximate dose of estrogenic substance was injected which would give a response that would fall on the dosage response curve. This adjustment of the concentration of the injection was accomplished by the amount of peanut oil which was added to the half of the eluate saved for bioassay. For example, if half of the eluate gave a fluorimetric assay of two micrograms of estradiol, the other half of the eluate was evaporated to dryness and the residue taken up in ten milliliters of ether. Then ten milliliters of peanut oil were added to the ether, mixed, and the ether evaporated off. This left a concentration of 0.2 micrograms of estradiol per milliliter of oil. When the rat was injected with 0.1 milliliters of this oil it meant that she was receiving 0.02 micrograms of estradiol as calculated by the fluorimetric assay. This was a dosage which would fall on the dosage response curve. Five to six rats were used for each determination of an unknown. The estimation of the potency of the injection as determined by bioassay was compared with the estimation by fluorimetric assay and the correlation of the two estimates calculated.

RESULTS

The cows which were used on the initial estrogen study were divided into two groups, those which apparently conceived and those which apparently did not conceive. It was found that there was considerable variation in the fluorescence of urine extracts between individual cows that conceived. The fluorescence of the urine extracts measured in terms of estrone is given in Table III.

TABLE III. FLUORESCENCE PRODUCED BY URINARY EXTRACTS OBTAINED DURING THE ESTROUS CYCLE OF COWS THAT CONCEIVED

Cow	Days after heat and breeding			
	0	7	14	23
Micrograms of estrone per 100% body weight				
388	377.9	365.2	403.9	395.8
249	125.9	168.9	183.5	missing data
227	152.4	185.9	178.0	290.5
418	176.0	152.3	missing data	263.9
67	346.0	157.6	285.6	304.5
291	311.4	329.2	369.1	364.0
410	144.9	256.0	278.2	296.8
306	232.6	232.6	202.0	250.6
341	399.2	436.7	385.6	390.7
394	537.3	516.7	253.5	178.6
342	287.3	205.2	243.0	290.8
317	266.7	196.4	168.4	331.4
Mean	281.0	266.9	268.2	305.2
Standard deviation	123.8	119.1	85.6	63.9
Range	125.9-537.3	152.3-516.7	168.4-403.9	178.6-390.7

The cows which did not conceive showed a much higher fluorescence of urine extracts and there was more variation between individuals. The fluorescence of urine extracts from cows which failed to conceive is shown in Table IV.

TABLE IV. FLUORESCENCE PRODUCED BY URINARY EXTRACTS OBTAINED DURING THE ESTROUS CYCLE OF COWS THAT FAILED TO CONCEIVE

Cows	Days after heat and breeding			
	0	7	14	23
	Micrograms of estrone per 100# body weight			
370	522.0	161.3	124.6	193.3
370	193.3	234.4	296.0	262.8
370	262.8	171.5	332.7	615.9
370	615.9	350.2	290.2	967.9
370	967.9	304.7	321.5	790.0
370	790.0	1188.0	255.6	303.8
370	303.8	393.9	306.2	838.6
388	375.4	151.4	315.7	377.9
249	137.4	228.8	236.6	125.9
71	141.1	143.3	missing data	855.0
71	855.0	1150.0	missing data	280.9
277	686.8	707.6	505.9	430.4
351	933.0	550.0	628.1	485.2
317	599.8	939.0	567.0	237.3
317	237.3	362.7	700.9	273.9
317	273.9	403.7	204.0	266.7
Mean	493.5	465.0	363.2	456.6
Standard deviation	249.9	349.2	169.6	855.7
Range	137.4-967.9	143.3-1188.0	124.6-700.9	125.9-967.9

As was pointed out earlier, it was certain that some of the fluorescence in the urine extracts was caused by compounds other than estrogenic substances, it was hoped that chromatographic separation

would give a method whereby only estrogenic substances would be measured. Also, there were often compounds in the urine extracts which reacted with the sulfuric acid to give a darker color and thus mask some of the fluorescence. Before attempting to chromatograph urine, it was decided to characterize the chromatographic columns with varying amounts of estrone and estradiol. Nine trials were conducted in which varying amounts of pure crystalline estrone were added to the Celite chromatographic columns. The pattern of the recovery of the estrone is shown in Figure 1.

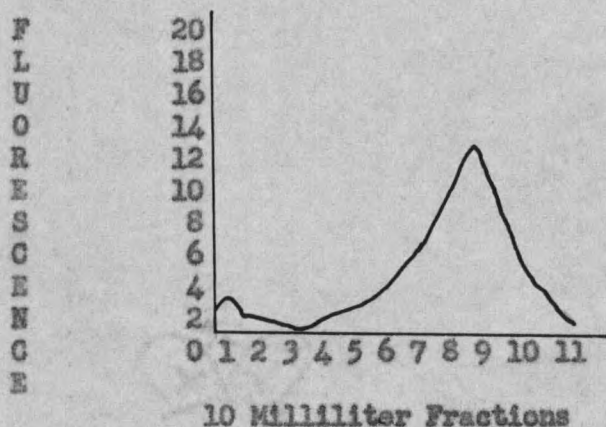


Figure 1. Chromatography of Estrone From Celite Chromatographic Columns

Nine trials were conducted in which varying amounts of pure crystalline estradiol were added to the Celite columns. The pattern of recovery of the estradiol is shown in Figure 2.

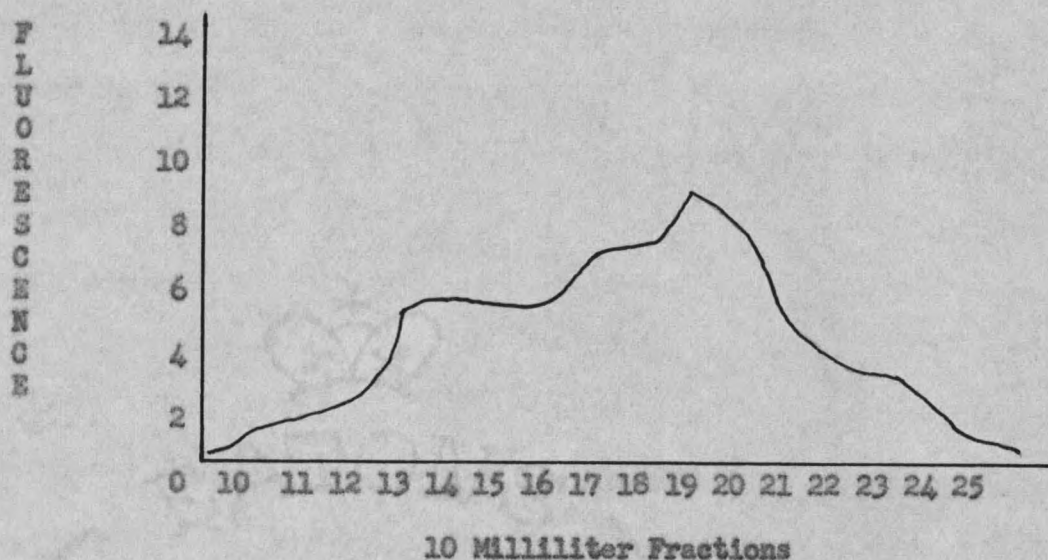


Figure 2. Chromatography of Estradiol from Celite Chromatographic Columns

Nine trials were conducted in which varying amounts of both estrone and estradiol were added to the Celite columns. The pattern of their recovery is shown in Figure 3.

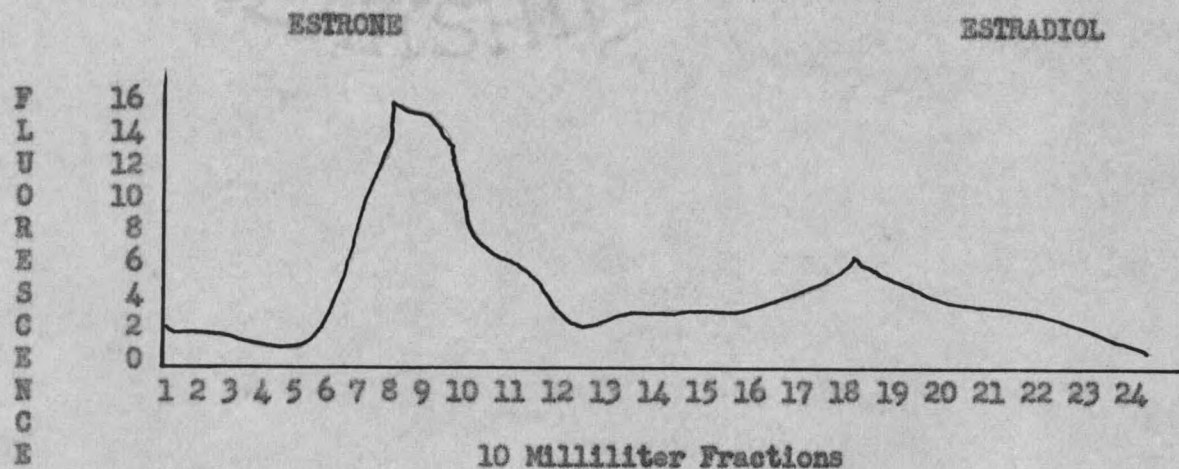


Figure 3. Chromatography of Estrone and Estradiol from Celite Chromatographic Columns

After it was seen how the estrogens were eluted out of the chromatographic column when added directly to the column it was decided to add varying amounts of estrone and estradiol to the urine of an ovariectomized cow and see if the same peaks were found in extracts of the urine. Six trials were conducted in which crystalline estrone was added to the urine of the ovariectomized cow. The urine was then subjected to the same chemical procedure previously used in this study. The urine extracts were then added to the Celite chromatographic columns. The average recovery pattern is shown in Figure 4.

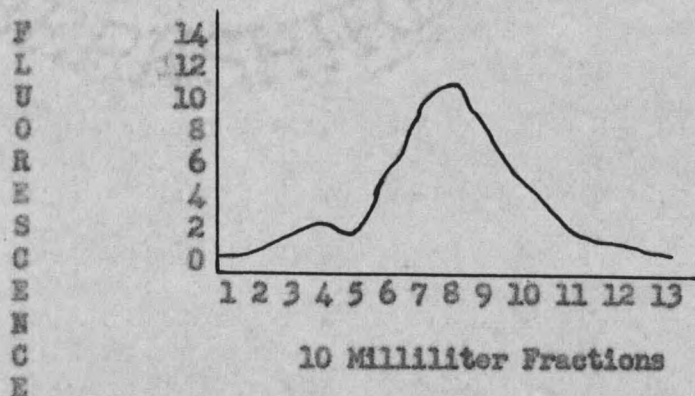


Figure 4. Chromatography of Estrone Added to Urine of Ovariectomized Cow. Urine Extract Added to Celite Chromatographic Column

Six trials were conducted in which crystalline estradiol was added to the urine of the ovariectomized cow. The urine was then subjected to the same chemical procedure and the urine extracts added to the Celite chromatographic column. The average recovery pattern is shown in Figure 5.

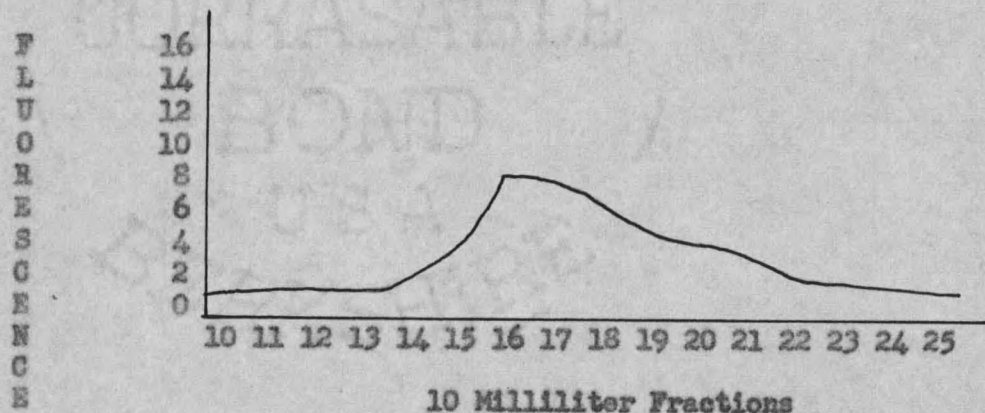


Figure 5. Chromatography of Estradiol Added to Urine of Ovariectomized Cow. Urine Extract added to Celite Chromatographic Column

Six trials were conducted in which both crystalline estrone and estradiol were added to urine of the ovariectomized cow. The urine was then subjected to the same chemical procedure and the urine extracts added to the Celite chromatographic column. The average recovery pattern is shown in Figure 6.

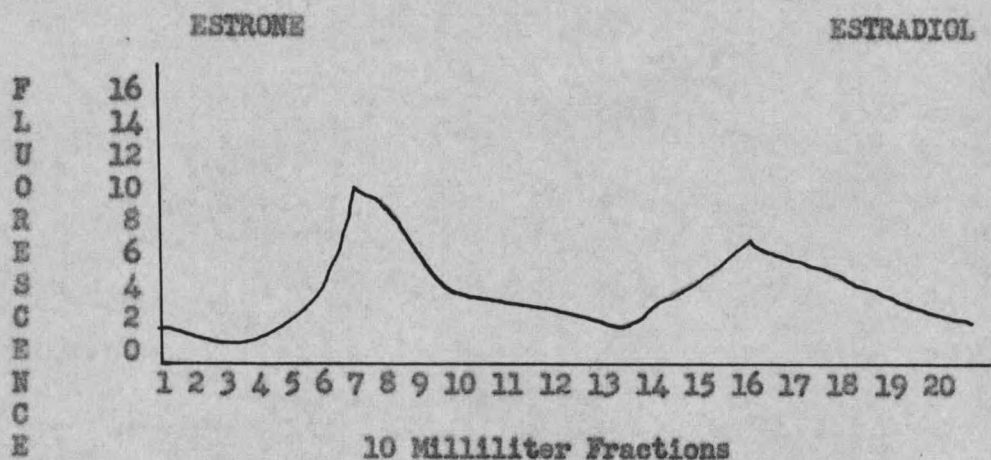


Figure 6. Chromatography of Estrone and Estradiol Added to Urine of Ovariectomized Cow. Urine Extract Added to Celite Chromatographic Column.

When it was found that the peaks were very much the same, it was decided to try urine which was very high in natural estrogen and compare it with the urine of the ovariectomized cow. Six trials were conducted in which the urine of cows (at least 250 days pregnant) was subjected to the chemical procedure previously used in this study. The urine extracts were then added to the celite chromatographic columns. The pattern of the fluorescence of the fractions obtained from the column is shown in Figure 7. Ten trials were conducted in which the urine from an ovariectomized cow was subjected to the chemical purification procedure. The urine extracts were added to the Celite chromatographic columns. The pattern of the fluorescence of the fractions obtained from the column is also shown in Figure 7.

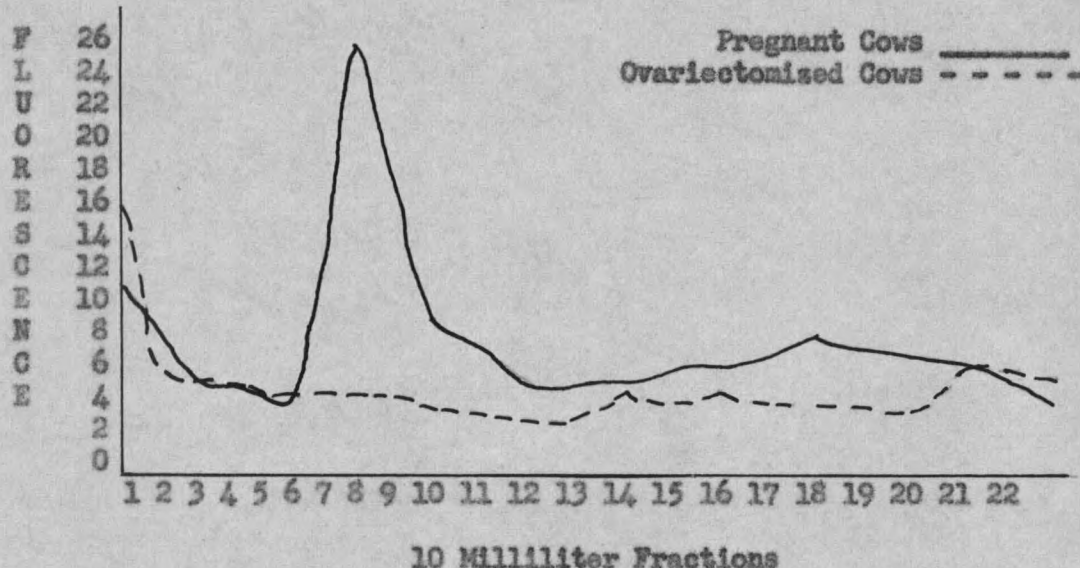


Figure 7. Florescence Obtained from Urinary Extracts Added to Celite Chromatographic Columns.

The next step was to prove if the material which was giving the peaks in fluorescence in natural urine was estrogenic. For this purpose, bioassay with immature female rats was used. As was pointed out previously, the urine of virgin heifers was extracted for estrogens by the same chemical procedure as used earlier and chromatographed in Celite columns. Half of the eluate from the column was measured for estrone and estradiol fluorometrically and the other half was used in bioassay.

A four point "response curve" based on the crude uterine weight of the rats uteri was developed for estradiol using pure crystalline estradiol. This curve is shown in Figure 8.

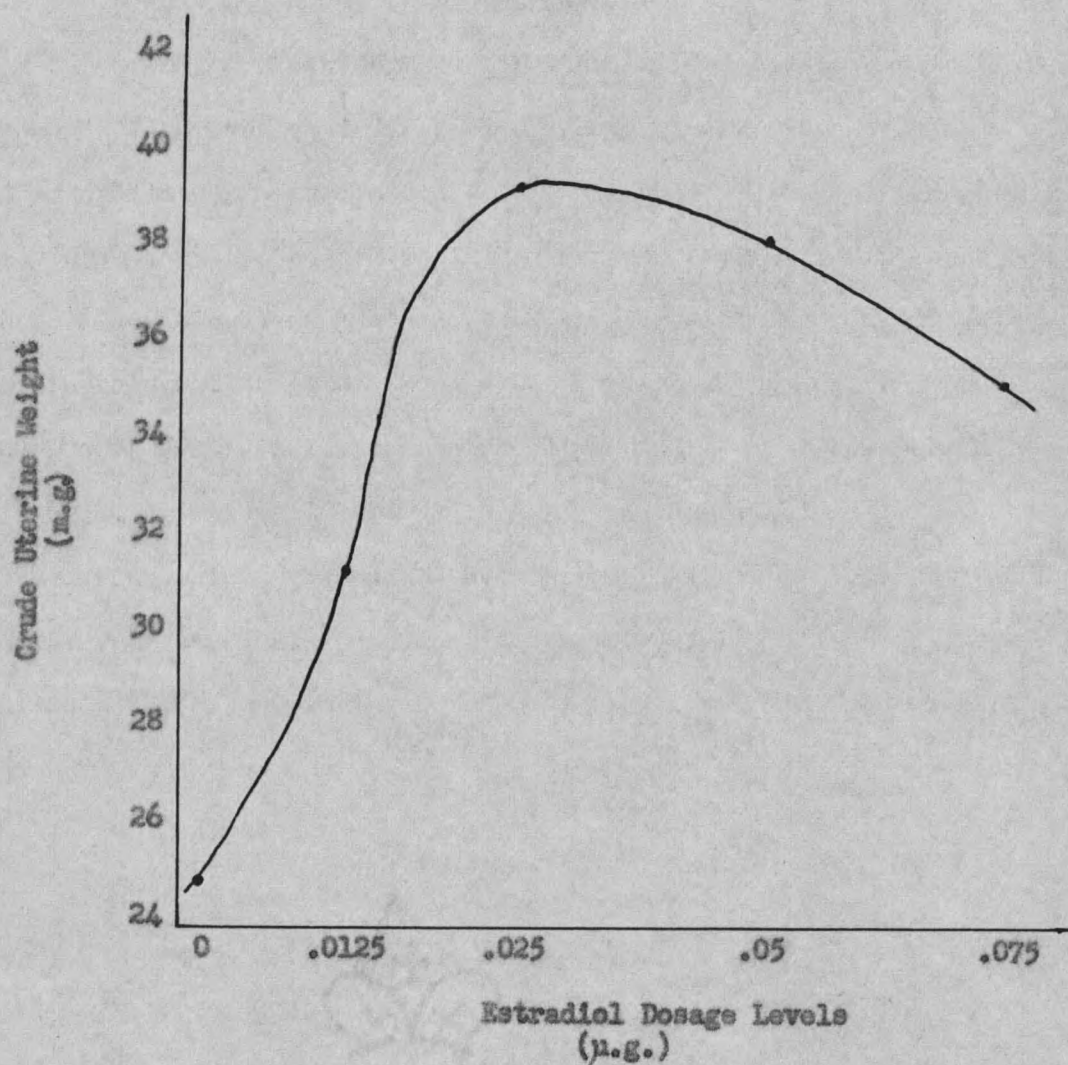


Figure 8. Crude Uterine Weight Response Curve of Immature Female Rats Given Four Different Dosage Levels of Pure Crystalline Estradiol in Peanut Oil Injection

A three point "response curve" based on crude uterine weight of the rat's uteri was developed for estrone using pure crystalline estrone. This curve is shown in Figure 9.

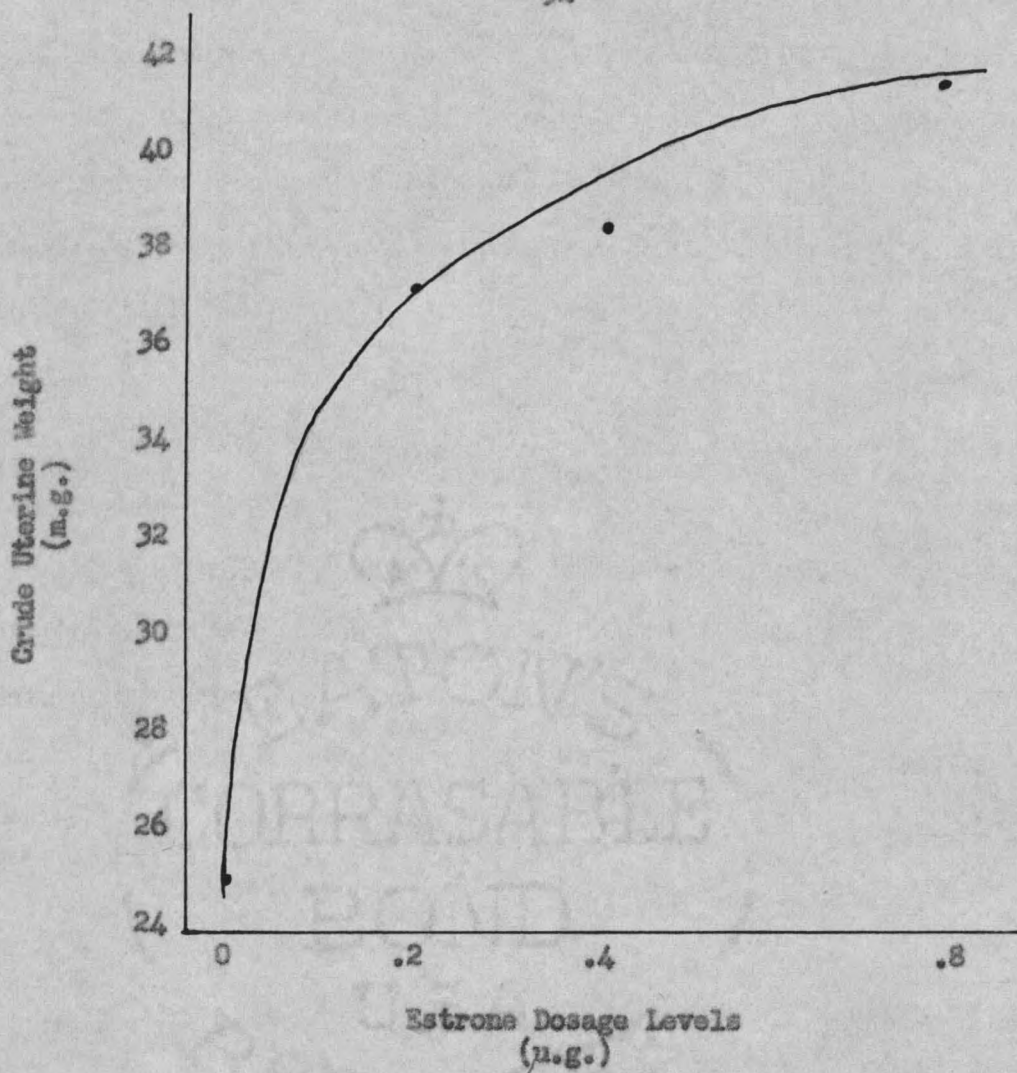


Figure 9. Grude Uterine Weight Response Curve of Immature Female Rats Given Three Different Dosage Levels of Pure Crystalline Estrone in Peanut Oil Injection

A comparison of the results of the fluorimetric assay with the results of the bioassay on urine extracts of virgin heifers is shown in Table V.

TABLE V. COMPARISON OF THE RESULTS OF FLUORIMETRIC ASSAY WITH BIOASSAY ON URINE EXTRACTS OF VIRGIN HEIFERS WHERE ESTIMATED POTENCY OF INJECTION WAS MADE BY FLUORIMETRIC DETERMINATION

Heifer Number and date urine was collected	Fluorimetric Estimation of potency of injection into rats for bioassay		Bioassay estimation of potency of injection into rats. (Crude uterine weight basis)	
	Estrone (u.g.)	Estradiol (u.g.)	Estrone (u.g.)	Estradiol (u.g.)
521 2/8/57	0.028	0.023	0.04	0.005
521 2/28/57	0.05	0.023	0.045	0.00562
521 3/11/57	0.069	0.048	0.05	0.006875
526 2/6/57	0.033	0.017	0.03	0.005625
526 2/15/57	0.02	0.014	0.03	0.00563
526 3/1/57	0.037	0.031	0.03	0.005625
530 2/23/57	0.039	9.0074	0.085	0.005
531 2/27/57	0.029	0.0062	0.042	0.0075
532 3/14/57	0.056	0.029	0.0775	0.006875
536 3/16/57	0.049	0.034	0.035	0.004375
540 3/7/57	0.07	0.057	0.06	0.005

It can be seen that in all cases there was an estrogenic effect produced by injections of the urine extracts, on a crude uterine weight basis. However, it must be noted that the correlation between the measurements of estrone is larger than of estradiol. The correlation coefficient of estrogen is 0.4488 and of estradiol it is -0.133. Neither is statistically significant.

The results of chromatographic separation of the urine extracts and then assaying them by fluorimetric procedures gave results quite similar to the results obtained on the cows in the earlier work. Again the heifers which did not conceive showed a considerably higher excretion of estrogen, both estrone and estradiol, than did those heifers which

did conceive. The chromatographic columns did seem to remove most of the material in the urine extracts which reacted with the sulfuric acid to give a dark masking color. This gave greater confidence in the readings of the photofluorometer in the fluorimetric assays.

Table VI shows the fluorimetric assays of the urine extracts for estrone and estradiol from the virgin heifers which conceived. It will be noted that the means and standard deviations of the heifers was not quite as high as was those of the cows.

TABLE VI. FLUORESCENCE PRODUCED AFTER CHROMATOGRAPHIC SEPARATION OF URINARY EXTRACTS FROM VIRGIN HEIFERS WHICH CONCEIVED AFTER BREEDING

Heifer Number	Days After Heat and Breeding							
	1		8		15		25	
	Es- trone	Estra- diol	Es- trone	Estra- diol	Es- trone	Estra- diol	Es- trone	Estra- diol
Micrograms of Estrone and Estradiol per 100# body weight								
521	124.44	84.89	data missing		266.67	122.67	153.33	213.77
526	93.18	48.00	64.94	47.06	data missing		176.00	147.76
529	data missing		81.50	140.75	159.50	86.50	108.75	62.50
530	90.25	345.00	117.62	85.37	77.63	29.62	105.38	49.00
538	102.87	57.25	71.75	36.62	50.00	182.62	86.12	39.87
540	140.00	114.37	103.62	209.00	77.63	75.00	81.62	237.25
547	142.33	87.00	104.67	232.33	84.00	40.67	95.33	36.67
$\bar{y}$	115.51	122.75	90.68	125.19	119.24	89.51	115.22	112.40
s	23.22	111.41	20.95	82.80	81.06	56.50	35.72	86.23
range	90.25	48.00	64.94	36.62	50.00	29.62	81.62	36.67
	to 142.33	to 345.00	to 117.62	to 232.33	to 266.67	to 182.62	to 176.00	to 237.25

Table VII shows the fluorimetric assays of the urine extracts for estrone and estradiol from the virgin heifers which did not conceive.

Again it is noted that the means and standard deviations are considerably larger than are those of the heifers which conceived.

TABLE VII. FLUORESCENCE PRODUCED AFTER CHROMATOGRAPHIC SEPARATION OF URINARY EXTRACTS FROM VIRGIN HEIFERS WHICH DID NOT CONCEIVE AFTER BREEDING

Heifer Number	Days After Heat and Breeding							
	1		8		15		25	
	Es- trone	Estra- diol	Es- trone	Estra- diol	Es- trone	Estra- diol	Es- trone	Estra- diol
Micrograms of Estrone and Estradiol per 100# body weight								
521	105.00	80.67	54.89	228.22	90.31	465.77	197.67	810.67
526	2,424.47	280.00	94.12	474.35	856.47	533.65		
529	92.37	92.37	data missing		112.37	316.75		
531	66.67	113.33	292.33	57.66	48.33	31.33		
531	80.00	305.00	103.33	117.67	75.33	35.00		
532	80.86	43.00	128.85	662.86	120.71	52.57		
539	74.25	130.25	94.88	80.87	91.37	76.63		
539	69.63	135.00	120.00	31.75	62.37	36.25	75.00	309.87
$\bar{y}$	374.16	147.45	126.91	236.20	182.16	193.49	136.34	560.27
s	828.55	94.42	76.66	241.76	273.52	212.03	86.74	354.12
range	66.67 to 2,424.47	43.00 to 280.00	54.89 to 292.33	31.75 to 662.86	48.33 to 856.47	31.33 to 533.65	75.00 to 197.67	309.87 to 810.67

DISCUSSION AND CONCLUSIONS

The theory that some of the infertility problems of dairy cows are caused by hormone imbalances would seem to be partially substantiated by this study. According to Burrows a high amount of estrogen in the system will prevent pregnancy, cause fetal death, or abortion, depending upon when the imbalance occurs (10). Since in both cases, with the cows and the virgin heifers, there was a higher estrogen excretion in those estrous cycles where pregnancy did not result, it would seem quite possible that the higher estrogen level was preventing pregnancy.

One factor which could have a very pronounced effect on the results and conclusions of this work is the breeding system used at estrus and the infertility of the semen. It may be that in some cases the cow or heifer was normal in her estrogen secretion but due to the fact that she was bred too early or too late or with semen which was of low fertility, she did not become pregnant. Her estrogen excretion data would be lower in values than the data from animals which did not conceive because of high estrogen excretion, but both cases would be considered the same and averaged together. This may be the reason why the data for the cows and heifers which did not conceive had a larger standard deviation than the data of the animals which did conceive. If this variable in breeding could be removed, the difference in the data between those animals that conceive and those that did not conceive, might be much wider and more pronounced.

CONFIDENTIAL
BOND
USA

The results of the work with the chromatographic Celite columns and the bioassay gives definite indication that the material which came through the Celite columns and gave fluorimetric readings was estrogenic in its properties. The correlation between the fluorimetric assay and the bioassay on estrone was higher than it was on estradiol. An explanation of why the fluorimetric assay and the bioassay were not so closely correlated on estradiol might be explained by a factor of carry over of estrone into the estradiol fraction. It was found in the laboratory that estrone had a higher fluorescence than estradiol, but it is known that estradiol has a higher estrogenic activity than does estrone (4, 18). If some estrone came out in the estradiol fraction it would cause a higher than true calculation of the estradiol potency as determined by fluorimetric assay. Thus, the potency of the estradiol fraction as determined by bioassay would not be as high as that expected by fluorimetric assay. This, in part, may explain why the bioassay always gave a determination of estradiol potency somewhat less than that expected by fluorimetric assay.

SUMMARY

The urine of open cows in normal estrus was extracted chemically for estrogen and assayed by fluorimetric methods for estrogenic potency in terms of micrograms of estrone per 100 pounds of body weight. It was found that those cows which had conceived had a lower excretion of estrogen in their urine than did those cows which did not conceive.

Varying amounts of estrone and estradiol were added to chromatographic Celite columns and the recovery of the hormones from the column was determined by fluorimetric means. It was found that the estrone appeared in the first 110 milliliters of benzene and the estradiol in the following 140 milliliters. Estrone and estradiol added to the urine of an ovariectomized cow, extracted chemically and passed through the Celite column gave about the same recovery picture.

The urine of virgin heifers was extracted chemically and 0.1 milliliter aliquots of the extracts were added to chromatographic columns. These were assayed by fluorimetric means and some of them by bioassay with immature female rats. The correlation coefficient between estrone by fluorimetric assay and bioassay was 0.4488 while the correlation coefficient of estradiol was -0.133. Neither of these coefficients was statistically significant.

It was also found that the heifers which did conceive had a lower excretion of estrogens than did those which did not conceive. As with the cows, it was also noted that those heifers which did not conceive had a larger standard deviation than did those which conceived.

LITERATURE CITED

1. Allan, H., Dodd, E. C. --- "Hormones in the Urine Following Oophorectomy During Pregnancy" *Biochem. J.* 29: 285-287; 1935.
2. Allen, E. --- SEX AND INTERNAL SECRETIONS, Chpt. IX, Williams and Wilkins, Baltimore, 1932 (as quoted by Cameron (12) --- original not seen.
3. Asdell, S. A., DeAlba, J., Roberts, J. S. --- "The Levels of Ovarian Hormones Required to Induce Heat and Other Reactions in the Ovariectomized Cow" *J. An. Sci.* 4: 277-284; 1945.
4. Astwood, E. B. --- "A Six-Hour Assay For the Quantitative Determination of Estrogen" *Anat. Record* 70: (Suppl. No. 3) 5; 1938.
5. Beal, D. --- "The Isolation of Progesterone and 3, 20, Allopregnanolone From Ox Adrenals" *Biochem. J.* 32: 1957; 1938.
6. Beall, D. --- "The Isolation of Alpha-Oestradiol and Oestrone From Horse Testes" *Biochem. J.* 34: 1293-1298; 1940.
7. Beaston, G. --- as quoted by Burrows (10) p. 348 - original not seen.
8. Bernstorff, E. C. --- "Incomplete Hepatic Inactivation of Hormone Produced By the Intrasplenically Grafted Ovary in the Mouse" *Endocrinology* 49: 302-309; 1951.
9. Bitman, J., Sykes, J. F. --- "Chromatographic Separation of Estrone, Estradiol, and Estriol" *Science* 117: 356; 1953.

10. Burrows, H. -- BIOLOGICAL ACTIONS OF SEX HORMONES, Cambridge at the University Press, Cambridge, Eng.; 1945.
11. Burrows, H., Cook, J. W., Roe, E. M. F., and Warren, F. L., -- "Isolation of 3:5-Androstadiene -17- one From the Urine of a Man With Malignant Tumor of the Adrenal Cortex" Biochem. J. 31: 950; 1937.
12. Cameron, A. T. -- RECENT ADVANCES IN ENDOCRINOLOGY, P. Blakiston's Son & Co., Inc. 2nd Ed, Philadelphia; 1935.
13. Cole, H. H., Miller, R. F. -- "Changes in the Reproductive Organs of the Ewe with Some Data Bearing on Their Control" Am. J. of Anatomy; 57: 39-97; 1935.
14. Cori, C. F. -- as quoted by Burrows (10) p. 348 -- original not seen.
15. DeMeio, R. H., Rakoff, A. E., Cantarow, A., Paschkis, K. E. -- "Mechanism of Inactivation of Estradiol by Rat Liver In Vitro" Endocrinology 43: 97-104; 1948.
16. Dorfman, R. I., Goldsmith E. D. (Conference Chairmen) -- "The Influence of Hormones on Enzymes" New York Academy of Sciences Annals; V. 54, Art. 4, pp. 531-728.
17. Dorfman, R. I., Ungar, F. -- METABOLISM OF STEROID HORMONES, Burgess Publishing Co.; Minneapolis, 1953.
18. Emmens, C. W. -- HORMONE ASSAY, Academic Press Inc., New York, 1950.

19. Friedgood, H. B., Garst, J. B., and Haagen-Smith, A. J. -- "A New Method for the Separation of Androgens from Estrogens and for the Partition of Estriol From the Estrone-Estradiol Fraction" J. Biol. Chem. 135: 801-809; 1940.
20. Furlong, E., Krichesky, B., Glass, S. J. -- "Effect of Carbon-Tetrachloride Feeding on Estrogen Excretion in the Normal Female Guinea Pig" Endocrinology 45: 1-9; 1949.
21. Heard, R. D. H., Bauld, W. S., Hoffman, M. M. -- "Steroids V Estradiol and Progesterone Metabolism" J. of Biol. Chem. 141: 709-710; 1941.
22. Heard, R. D. H., Hoffman, M. M. -- "Steroids IV. The Fate In Man of Injected α -Estradiol" J. of Biol. Chem. 141: 329-342; 1941.
23. Heard, R. D. H., Hoffman, M. M. -- "The Isolation of 5,7,9, Estratrienol -3-one-17 From the Urine of Pregnant Mares" J. Biol. Chem. 135: 801-809; 1940.
24. Heard, R. D. H., and Hoffman, M. M. -- "The Isolation from Equine Pregnancy Urine of 5,7,9, Estratrienol 3-one-17" J. Biol. Chem. 138: 651-656; 1941.
25. Jailer, J. W. -- "The Effect of Inanition on the Inactivation of Estrogen by the Liver" Endocrinology 43: 78-81; 1948.
26. Kirgis, H., Rothschild, I. -- "The In Vivo Inactivation of Estradiol by the Human Liver" Endocrinology 50: 269-270; 1952.
27. Levin, L. -- "The Fecal Excretion of Estrogens by Pregnant Cows" J. of Biol. Chem. 157: 407-411; 1945.

28. Longwell, B. B. and McKee F. S. -- "The Excretion of Estrogens in the Bile and Urine After the Administration of Estrone." J. of Biol. Chem. 142: 757-564; 1942.
29. Moore, C. R. -- "Gonad Hormones and Sex Differentiation" Am. Naturalist 78: 97-130; 1944.
30. Nibler, C. W., Turner, Chas, W. -- "The Ovarian Hormone Content of Pregnant Cows Urine" J. Dairy Sci. 12: 491-506; 1929.
31. Pearlman, W. H., Rakoff, A. E., Cantarow, A., and Raschkis, K. E. -- "The Isolation of Estrone from the Bile of Pregnant Cows" J. Biol. Chem. 170: 173; 1947.
32. Pearlman, W. H., Rakoff, A. E., Paschkis, K. E., Cantarow, A., Walkling, A. A. -- "The Metabolic Fate of Estrone in Bile Fistula Dogs" J. of Biol. Chem. 173: 175-183; 1948.
33. Pincus, G. -- RECENT PROGRESS IN HORMONE RESEARCH, XII (1956), Academic Press Inc., New York; 1956.
34. Ryan, K. J, Engel, L. L. -- "The Interconversion of Estrone and Estradiol by Human Tissue Slices" Endocrinology 52: 287-291; 1953.
35. Ryan, K. J., Engel, L. L. -- "The Interconversion of Estrone and Estradiol- 17β by Rat Liver Slices" Endocrinology 52: 277-286; 1953.
36. Segaloff, A. -- "The Effect of Partial Hepatectomy on the In Vivo Activation of Triphenylchloroethylene" Endocrinology 42: 472-475; 1948.

37. Segaloff, A. -- "The Metabolism of Some Chemical Degradation Products of Estrogens: Westerfeld's Lactone, Bis-Dehydro Doisyolic Acid, Estrololactone and β - Estradiol" *Endocrinology* 42: 15-19; 1948.
38. Segaloff, A. -- "The Sparing Effect of Progesterone on the Hepatic Inactivation of α - Estradiol" *Endocrinology* 40: 44-46; 1947.
39. Singher, O. H., Kensler, G. J., Taylor, H. C., Jr., Rhoads, C. P., Klaus, Unna; -- "The Effect of Vitamin Deficiency on Estradiol Inactivation by Liver" *J. of Biol. Chem.* 154: 79-86; 1944.
40. Smith, E. P. -- "Review of Literature C. Intermediary Metabolism of Estrogens" Ph. D. Thesis, Washington State College.
41. Smith, E. P., Dickson, W. M., and Erb, R. E. -- "Fluorimetric Estimation of Estrogens In Bovine Urine" *J. Dairy Sci.* 39: 162-170; 1956.
42. Smith, E. P., Dickson, W.M. -- "Urinary Estrogen Excretion During the Gestation Period of the Bovine" *J. Dairy Sci.* 36: 586; 1953.
43. Smith, G. V. S., Smith, O. W., Huffman, M. N., MacCorquodale, D. W., Thayer, S. A., Doisy, E. A. -- "The Isolation of Dihydrotheelin from Human Pregnancy Urine" *J. Biol. Chem.* 130: 431-432; 1939.
44. Smith, G. V. S., Smith, O. W., Pincus, G. -- as quoted by Burrows (10) p. 323 -- original not seen.

45. Steinach and Holzkencht --- as quoted by Burrows (10) pp. 368-371
--- original not seen.
46. Stimmel, B. F. --- "The Fractionation and Photometric Estimation
of the Estrogens In Human Pregnancy Urine" J. Biol. Chem.
162: 99-105; 1946.
47. Stockard, in Cowdry's SPECIAL CYTOLOGY, 2nd Ed. Vol. III, Chpt.
XI, Hoeber, New York, 1932 --- as quoted by Cameron (12) ---
original not seen.
48. Szego, C. M., Samuels, L. T. --- "The Metabolism of Estrone In
Surviving Rabbit, Bovine, and Human Endometrium" J. of Biol.
Chem. 151: 599-605; 1943.
49. Szent-Gyorgyi, A. --- CHEMICAL PHYSIOLOGY OF CONTRACTION IN
BODY AND HEART MUSCLE, Academic Press, New York, 1953 --- as
quoted by Pincus (33) --- original not seen.
50. Tanabe, T. Y., Alaquist, J. O. --- "Some Causes of Infertility
in Dairy Heifers" J. Dairy Sci. 36: 586; 1953.
51. Tanabe, T. Y., Casida, L. E. --- "The Nature of Reproductive
Failures in Cows of Low Fertility" J. Dairy Sci. 32: 237-246; 1949
52. Turner, C. W., Nibler, C. W. --- "The Relation of the Oestrous
Hormone to Mammary Gland Growth" Mo. Agr. Exp. Stat. Bull. 285
(1930) p. 60.
53. Vanderline, R. E., Westerfeld, W. W. --- "The Inactivation of Estrone
by Rats in Relation to Dietary Effects on the Liver"
Endocrinology 47: 265-273; 1950.

MONTANA STATE UNIVERSITY LIBRARIES



3 1762 10012906 1

N378

B454u

cop.2

123706

Bergman, R. K.

Urinary excretion of estrogenic substances by the bovine in the estrous cycle.

NAME AND ADDRESS

5-17-71

Don Capen Set 5H

11-11-72

Holly Hoagli

N378

B454u

cop.2

123706