



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
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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.

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BY THE BOVINE IN THE ESTROUS CYCLE

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ROBERT KAYE BERGMAN

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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.

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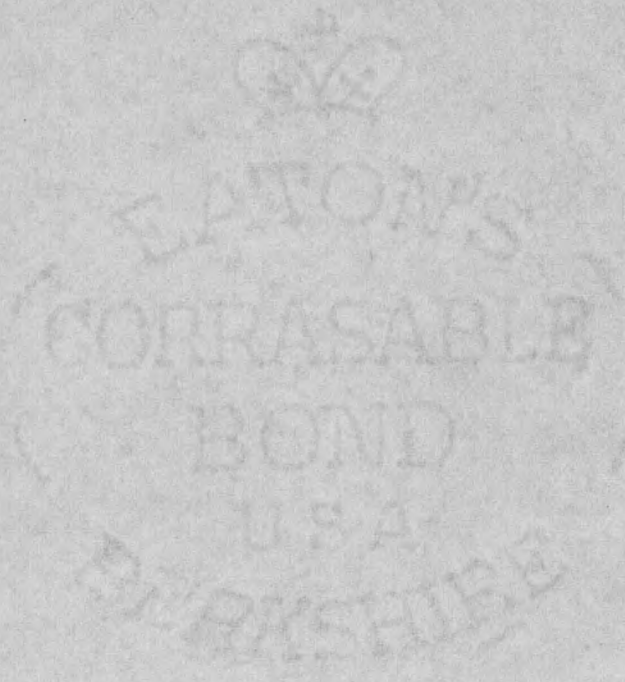
INTRODUCTION

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

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

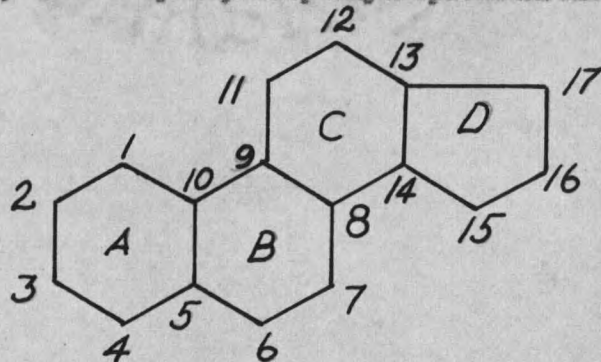
small, however, the stallion testes does produce large amounts of estrogen. In cases of carcinomatous or tumorous growth of the testes or adrenals, there has been noted increases in the excretion of estrogens. This secretion of estrogenic substances from malignant growths of the adrenals can become high 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 alcohol, 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. Kemens (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. Fearlman *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,

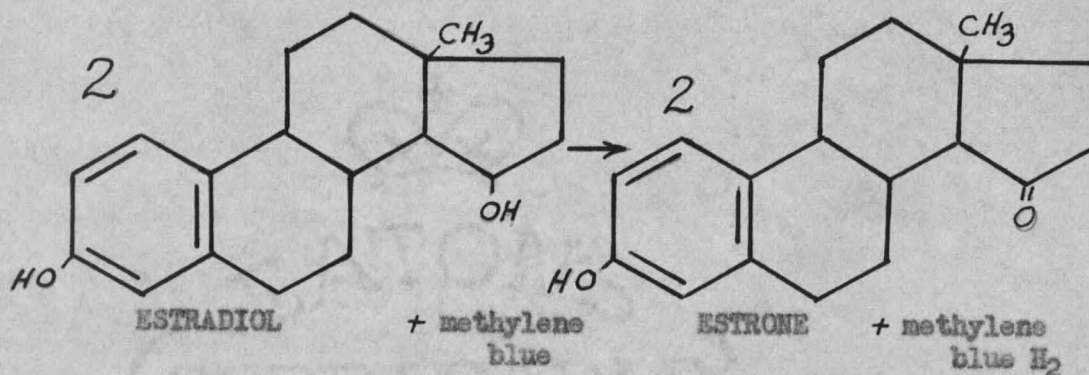
guinea pigs, and dogs. Monkey liver is the only one known that cannot deactivate estrogens.

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 Meio (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

Singer (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.

