MONTANA STATE UNIVERSITY

Urinary excretion of estrogenic substances by the bovine during estrus and at different stages of gestation

by Dennis W Nelson

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Dairy Production
Montana State University
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Abstract:
Twenty-four urine samples were collected from thirty-five Holstein cows and analyzed for alpha-estradiol, beta-estradiol, and estrone. The urine was collected from cows during estrus, and at 50, 100, 200, and 275 days of gestation. The calculations were determined as the number of micrograms "per" 100 pounds of body weight per 24 hour period. The urine was extracted chemically and measured by fluorometric assay, Kober reaction using a Beckman "B" spectrophotometer, and bioassay using the four day immature' mouse uterine weight method® Separation of alpha-restradiol, beta-estradiol, estrone, and the urine contaminants was carried out by use of a descending paper chromatographic system employing normal-hexane and benzene as the mobile solvents and formamide impregnated in filter paper as the stationary solvent® A comparison of Kober color reaction and fluorometry with bioassay when measuring alpha-estradiol, beta-estradiol, and estrone from urine extract's gave significant correlation coefficients in all cases (P < .01). The coefficient of correlation of the Kober reaction and bioassay was 0®73U9 for alpha-estradiol, 0.8452 for beta-estradiol, and 0.6456 for esrone® When comparing the fluorometric assay and the bioassay the correlation coefficients were 0.7481 for alpha-estradiol, 0.9454 for beta-estradiol, and 0.6542 for estrone® As determined by the bioassay method the least amount of all three estrogens was excreted during the collection period of 50 days after conception® The two periods, estrus and 100 days of gestation, had nearly the same total estrogen excretion rates although the alpha-estradiol rate was higher at estrus® From 100 days of gestation to parturition, the increase" in estrogen in estrogen excretion becomes much more pronounced® The alpha-estradiol excretion shows the most noticeable increase with estrone "next" and then beta-estradiol® All the increases from the low. point (50 days of gestation) to the high point (275 days of gestation) were significant (P< .01) ® None of the differences between estrus and 50 days and 5)0 days and 100 days of-gestation were significant® The alpha-estradiol increase whs significant from 100 days to 200 days (P < .05) and from 200 days to 275 days (P < .01). The estrone increase was dignificant from 100 to 200 days (P < .01) but not from 200 to 275 days® When the total estrogen is' considered, the increase is significant from 100 to 200 days and from 200 to 275 days (P < .0l)*

URINARY EXCRETION OF ESTROGENIC SUBSTANCES BY THE BOVINE DURING ESTRUS AND AT DIFFERENT STAGES OF GESTATION

by

DENNIS W. NELSON

83

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Chairman, Examining Committee

Dean, Graduate Division

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Twenty-four urine samples were collected from thirty-five Holstein cows and analyzed for alpha-estradiol, beta-estradiol, and estrone. The urine was collected from cows during estrus, and at 50, 100, 200, and 275 days of gestation. The calculations were determined as the number of micrograms per 100 pounds of body weight per 24 hour period. The urine was extracted chemically and measured by fluorometric assay, Kober reaction using a Beckman "B" spectrophotometer, and bioassay using the four day immature mouse uterine weight method.

Separation of alpha-estradiol, beta-estradiol, estrone, and the urine contaminants was carried out by use of a descending paper chromatographic system employing normal-hexane and benzene as the mobile solvents and formamide impregnated in filter paper as the stationary solvent.

A comparison of Kober color reaction and fluorometry with bioassay when measuring alpha-estradiol, beta-estradiol, and estrone from urine extracts gave significant correlation coefficients in all cases ($P < .01$). The coefficient of correlation of the Kober reaction and bioassay was 0.7349 for alpha-estradiol, 0.8452 for beta-estradiol, and 0.6456 for estrone. When comparing the fluorometric assay and the bioassay the correlation coefficients were 0.7481 for alpha-estradiol, 0.5494 for beta-estradiol, and 0.6542 for estrone.

As determined by the bioassay method the least amount of all three estrogens was excreted during the collection period of 50 days after conception. The two periods, estrus and 100 days of gestation, had nearly the same total estrogen excretion rates although the alpha-estradiol rate was higher at estrus. From 100 days of gestation to parturition, the increase in estrogen in estrogen excretion becomes much more pronounced. The alpha-estradiol excretion shows the most noticeable increase with estrone next and then beta-estradiol. All the increases from the low point (50 days of gestation) to the high point (275 days of gestation) were significant ($P < .01$). None of the differences between estrus and 50 days and 50 days and 100 days of gestation were significant. The alpha-estradiol increase was significant from 100 days to 200 days ($P < .05$) and from 200 days to 275 days ($P < .01$). The estrone increase was dignificant from 100 to 200 days ($P < .01$) but not from 200 to 275 days. When the total estrogen is considered, the increase is significant from 100 to 200 days and from 200 to 275 days ($P < .01$).

INTRODUCTION

Reproductive failure in dairy cows remains an important problem even though much has been done to reduce loss by this means in the past decade. Research emphasis has been placed on developing disease-free herds thereby lowering reproductive problems. There are still many instances of infertility which cannot be traced to disease or to damage or abnormalities of the various reproductive organs. Many of these cases are thought to be due to hormonal imbalances.

Values for hormone levels have been established in humans but little work has been done on these values in the cow. Since considerable quantities of the female sex hormone excretion are found in the urine and as chemical extraction methods have been worked out for urine, measurement of this estrogen is presently believed to be the most feasible means of establishing values for estrogenic levels of the cow. Biological assays and chemical assays have been used to estimate the urinary estrogenic activity but all the methods developed and used so far have certain disadvantages.

The purpose of this study was to compare various methods of assay in an attempt to find the most reliable and accurate method for estimating the estrogenic content of urine. The levels of alpha and beta-estradiol and estrone excretion were measured at estrus and 50, 100, 200, and 275 days of gestation to attempt the establishment of the normal excretion trends of these compounds during gestation and at estrus.

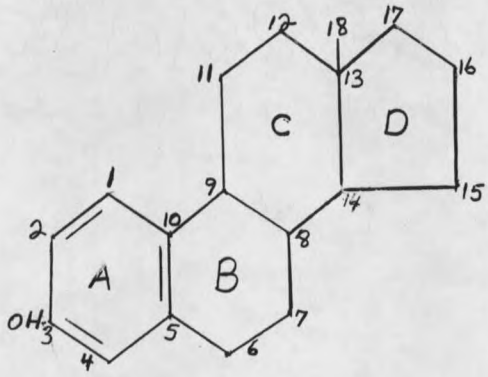
REVIEW OF LITERATURE

DEFINITION OF ESTROGENS

In a broad sense, the term "estrogen" refers to any substance which will induce cornification in the vagina of the adult mouse (39). Under this definition are included the natural estrogens of plant and animal origin and the synthetic estrogens of which the more important are diethylstilbestrol, dienestrol, and hexestrol (156). In a more limited sense, estrogen refers to the female sex hormone produced by the Graafian follicle of the ovaries. In most animals, the estrogenic hormones include alpha and beta-estradiol (dihydrotheelin), estrone (theelin), and estriol (theelol), but in the equine the estrogenic hormones produced include equilin and equilenin (6). In the older literature, the more active estradiol was referred to as "alpha". However, in present day literature it is called beta-estradiol (84).

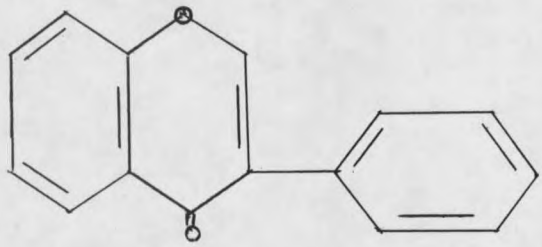
STRUCTURE OF ESTROGENS

So far, all of the natural estrogens that have been isolated are steroids posessing a cyclopentaphenanthrene ring system. The phenolic grouping seems to be important for estrogenic activity. The following is the basic structural formula for estrogen:

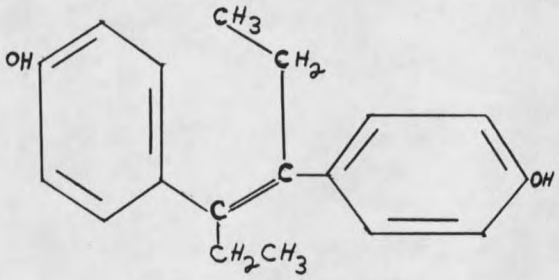


Ring A is phenolic which is different from other steroid sex hormones and there is a single methyl group at position 13. Estriol is considered to be a "strong" phenol while estrone and estradiol are "weak" phenols (60, 85).

The various isoflavones which exhibit estrogenic activity have the following general formula and are quite closely related to diethylstilbestrol which is also shown.



Isoflavone



Diethylstilbestrol

Formononetin has one "OH" group, genistein and Biochanin A have two, and daidzein three "OH" groups which may account for their relative activity as this is how they are ranked according to increasing estrogenic activity (22,45,46).

POTENCY OF THE ESTROGENS

There is a considerable amount of disagreement as to the activity exhibited by the various estrogens with even the order of activity of the three main estrogens being changed by different workers. This, however, can be explained by the confusion which has existed as to which is alpha and which is beta-estradiol. The activity varies considerably with different species of animal and with the biological assay method used.

Thayer et al (190) in 1944 measured the potency of alpha and beta-estradiol and estrone by five different bioassay methods with the results summarized in the following table.

TABLE I. ESTRONE AND ALPHA-ESTRADIOL POTENCIES EXPRESSED AS A PERCENTAGE OF THE BETA-ESTRADIOL POTENCY

Estrone	Alpha-Estradiol	Animal Used
3.5	2.0	mouse
22.9	11.4	mouse
10.0	5.0	mouse
11.7	1.2	rat
70.0	14.0	rat

Pearlman (138) reports the following potencies using two different biological assays.

TABLE II. RELATIVE ACTIVITY OF DIFFERENT ESTROGENS USING SPAYED RAT METHOD AND IMMATURE MOUSE UTERINE WEIGHT METHOD OF ASSAY

Compound	Spayed Rat Method	Mouse Method
Estrone	100	100
Beta-estradiol	1,000	1,000
Alpha-estradiol	10	7.5
Estriol	20	40

Hisaw et al (94) in 1954 conducted an experiment using 200 gram spayed rats to determine the dose of various estrogens needed to bring about maximum response of the uterus. They found that this was accomplished by a daily dose of 1.0 micrograms of estradiol-17β and 10.0 micrograms of estrone with the treated uteri weighing about the same.

Estriol at the maximal response dose of 20.0 micrograms did not stimulate the uteri nearly as much as the other estrogens. The control uteri weighed 125 milligrams, estrone and estradiol-17 β stimulated--300 milligrams, and estriol treated--240 milligrams.

Other workers (134) found that estriol injected subcutaneously into female rats was 1/100 to 1/1000 as potent as estradiol when judged by the cornification of the cervix and vagina, disappearance of leukocytes from the vagina, and hypertrophy of the uterine horns. However, estriol was almost as potent as estradiol when judged by the development of stratified epithelium in the cervix and vagina. It is believed that estriol has certain specific actions, especially on the uterine cervix and vagina. Puck and Hubner (152) compared the physiological effects of estradiol and estriol on the genital organs of female rabbits and guinea pigs and found that estriol has more marked effects than had been recognized before. Hisaw et al (94) suggest that estriol is primarily a primate hormone of pregnancy during which time it assists in the maintenance of a steady endocrine balance by modulating the actions of the stronger estrogens.

A number of workers have found α-estradiol to be more potent than β-estradiol (61,166). Emmens and Paskis (61) state that estrone in urine is one-fourth to one-twelfth as active as α-estradiol and that estriol is one-third to 1/250 as active as estrone which means that α-estradiol was probably considered more potent than β-estradiol.

Esterification has a favorable effect on the duration of action of the estrogens. In one particular experiment (103), the duration of

action was determined by measuring the duration of vaginal cornification in spayed female rats after a single injection. The intensity of effect was measured by weighing the uteri. It was found that by increasing the length of the esterifying fatty acid in position 17 the duration of action is increased. With some of the shorter chained acids, the threshold dose for estrus is somewhat lowered when compared to free estradiol. With the longer acting esters it may be raised as much as five to ten times. The intensity of effect is also increased with the 17-esters. Estradiol 3-esters act similarly to the estradiol 17-esters but are somewhat inferior with respect to the intensity and duration. The estradiol 3,17-diesters have a higher threshold dose but generally act longer than the 17-esters.

The same amount of hormone needed to bring about a certain response is also needed to maintain the response which implies a definite quantitative balance between the hormone and the level of metabolic activity for establishing and keeping a reaction in physiological equilibrium for extended periods (94).

SOURCES OF ESTROGENIC SUBSTANCES

Ovaries--Early workers found the main estrogen produced by the ovaries (secretion takes place in the theca interna cells of the Graafian follicle) to be alpha-estradiol although considerable amounts of estrone have also been isolated from ovarian tissue. This nomenclature is according to the early terminology of the estradiols. Freed and Soskin (73) stimulated rat ovaries and obtained evidence of estrogen formation. They found one substance formed in the theca cells which was incomplete in its action and one in the granulosa cells which was complete and resembled

estrone. It has been found that the estrone content of urine is definitely decreased when the ovaries are removed during pregnancy. The latest information states that the main estrogenic ovarian hormone is beta-estradiol (2,7,60,73,85,94,138,198,199). Ingram and Mandl (98) found that the uterus involutes more rapidly in ovariectomized than in hypophysectomized rats. After hypophysectomy, uterine involution will be hastened by removal of the ovaries. The inference is that the ovaries produce small amounts of estrogen in the absence of the pituitary. This is believed the case rather than a gradual depletion of the stores since the uterus remains heavier than would reasonably be expected even beyond the two and one-half month hormone storage depletion period. In humans, at least, the ovarian estrogen excretion varies considerably from month to month indicating variability in ovarian activity (31).

Placenta--Estrone, α-estradiol, and estriol have all been isolated from human placenta (60,85). Pearlman (138) states that the placenta is one of the chief sources of estrogen.

Adrenals--The adrenals produce estrogen but in much smaller quantities than either the ovaries or the placenta (138). Estrone has been isolated from the ox adrenals (60). The adrenal cortex in man is believed to produce nearly 20 per cent of the total estrogen present (121). There is an increase in estrogenic secretion of the adrenal cortex when the ovaries are removed (207).

Testes--Stallion testes have long been known as one of the richest sources of estrogen. Zondek (210) in 1934 found that stallion urine contained as much estrogen as that excreted by the pregnant mare. As

castrated and immature horses contained very little estrogen in their urine, the testes were believed to be the source. Later, Beall (20) extracted 28 kilograms of horse testes for estrogen and isolated alpha-estradiol as di-α-naphthoate at the rate of 0.21 milligrams per kilogram of testes and estrone as estrone 3:5-dinitrobenzoate at the rate of 0.36 milligrams per kilogram. This was the first time that α-estradiol had been isolated from tissues or fluids other than those of female origin. In man, the testes account for about 80 per cent of the estrogen excretion. The estrogen is produced by the Leydig cells along with androgen and is a good indication of Leydig cell function (115,121).

Urine and Feces--Leven (117) collected feces from cows during the last few weeks of pregnancy and found estrogen present in concentrations of 0.9 to 1.4 milligrams per kilogram of dry weight when calculated as estradiol. Between 73 and 96 per cent of the active substance was believed to be α-estradiol as most of the activity was found in the weakly phenolic, non-ketonic fraction of extracts.

Beta-estradiol, estrone, and estriol have all been isolated from human urine and α and β-estradiol and estrone from pregnant mare's urine (56). Klyne and Wright (110) extracted estrogen from cow's urine, collected during the last two months of pregnancy, with toluene. They identified estrone in the phenolic ketone fraction and found it in concentrations of about 0.2 mg per liter of urine. The phenolic non-ketonic fraction yielded equol (iso-flavone-7:4-diol) and estradiol-17α with the estradiol present in concentrations of 0.1 mg per liter of urine. Using the Kober reaction and acetic acid-sulfuric acid reaction, they could find

no detectable amounts of estradiol-17β. They also (109) isolated alpha-estradiol as estradiol-17α 3-methyl ether from pregnant goat urine in concentrations of 0.1 mg per liter. Pope et al (149) isolated estrone in concentrations of 0.3 mg per liter of acid hydrolyzed urine from cows in late pregnancy. They found that there was at least one more hydrophilic estrogen present which they did not identify. Velle (197) reports 0.10 to 0.15 mg per liter of urine and 0.8 to 1.0 mg per 24 hours for estrone and 0.15 to 0.20 mg per liter and 2.0 to 2.5mg per 24 hours for estradiol-17α from cows in the ninth month of pregnancy. Gorski et al (79) tested for estrogenic activity in the urine of pregnant, non-pregnant, and ovariectomized cows. The estrogenic activity of composite samples of urine from estrual cycles ranged from 73 to 173 micrograms per day of estrone equivalent with the values for pregnant cows considerably higher and that of an ovariectomized cow considerably lower. The fact that urinary estrogen values increase in pregnancy has been known for many years. In 1929, Nibler and Turner (132) found the amount of hormone excreted small during the first part of pregnancy and that it increased as pregnancy advances. Others (172,173,192) have found the levels to be low the first 50 to 100 days at which time excretion begins to increase quite rapidly. At parturition, estrogen excretion in the urine falls off quite rapidly. Asdell and Mixner (10) state that the cow excretes estrone and estradiol and an estriol-like substance without biological activity. Using several chromatographic and spectrophotometric methods, estrone and estradiol-17β have been identified in the urine from adult normal boars. The estriol fraction gave a positive reaction to the Kober reagent but only

insignificant amounts were present. Also, recording of the absorption spectra in the ultra violet region indicates the substance is different from estriol (196). Velle (201) reports that significant amounts of estrone and estradiol-17α are excreted with the urine of the calf during the first few days of life.

Liver, Blood, and Bile--Pearlman (139) in 1947 found estrogen present in the bile of pregnant cows. The major estrogen was estrone which was present in amounts of about 600 micrograms per liter of gallbladder bile. If the assumption is made that the activity of non-ketonic weakly acid phenols is due to α-estradiol, there was 70 micrograms of this estrogen present per liter. No estriol was detected in the bile. The estrogen from the bile is believed to be the source of fecal estrogen.

Liver extracts have been shown to contain estrogen and it is believed to be the pathway of absorption of oral estrogen. It has been suggested that the liver removes the estrogen from the blood and concentrates it in the bile (10,83,149).

Widely variable results have been obtained in attempting to extract estrogen from blood. Asdell and Mixner (10) obtained good recoveries of estrogen added to drawn blood and found detectable amounts in the blood of cows that had been injected with estrogens. However, the same method failed to detect estrogen in the blood of pregnant cows. Others (30) have found estrogenic activity in blood but could not get very reproducible results. In cattle and humans, Bitman and Sykes (29) found estrogen in whole blood present at the rate of three to eight micrograms per liter.

Meconium--Meconium was taken from normal, healthy, new-born calves of both sexes and the material examined for contents of both free and conjugated estrogens. A strongly reacting Kober chromogen was found in both the free and conjugated estradiol fractions but no traceof Kober chromogens could be detected in either the estrone or estriol fractions. In subjecting the estradiol fraction to the Kogi Miescher test and infrared spectroscopy, it was found to be identical to estradiol-17α. The quantities present were 20 milligrams per kilogram in the unconjugated form and 25 to 35 milligrams per kilogram in the conjugated form. As there were no differences in sex, it is believed that the source was maternal (195). Other workers (108) have isolated estriol in both the free and conjugated forms from human meconium. They could not isolate estradiol or estrone but they could possibly have been present in very small amounts.

Colostrum--Pope and Roy (150) found estrogenic activity in bovine colostrum that was about equal to that of human colostrum, human pregnancy urine, and bovine urine. In one cow, they found concentrations of 5.2 micrograms estradiol-17 β equivalent per liter. No estrogenic activity could be detected in normal milk.

Plants--Many different compounds exhibiting estrogenic activity have been isolated from a large number of different plant species. Probably the richest and most widely known source is that of the subterranean clover grown in Australia. This plant has been found responsible for many of the sheep breeding problems in that country. An isoflavone derivative, genistein, which has been isolated from the chloroplast fraction

of the plant, is believed to be the compound responsible. Genistein has a very low estrogenic potency which is about 1/50,000 that of diethylstilbestrol and as a result is considered as a proestrogen. Three other isoflavone compounds occurring in plants, formononetin, daidzein, and Biochanin A, are known to have estrogenic activity (26,45,46,54,169).

Other workers (118,142,143) have found estrogenic activity in alfalfa, ladino clover, red clover, birdsfoot trefoil, wheat, wheat germ oil, rye, oats, and soybean oil meal.

Another estrogenic hormone, coumestrol, has been isolated from Ladino clover and has also been found to be present in alfalfa and strawberry clover. It has been found to have about 30 times the activity of genistein (5).

An estrogenic substance was isolated from the female willow flower as long ago as 1933 and was later identified as estriol. Estrone has been isolated from palm kernel extract (56,171).

There are many factors which seem to effect the estrogenic content of plants. Most of the activity is found in the leaves which results in seasonal variations. In testing various silages ensiled by various methods, it has been concluded that fermentation increases estrogenic activity. Wilting of legumes results in decreased activity as does an increase in maturity of the plant (1,116,142,143,158).

ESTROGENIC FORMS IN VIVO

There are several terms which refer to the forms in which estrogen may be found in the animal body. Reversible binding refers to the combination of a steroid with a protein molecule in which association or

dissociation occurs spontaneously and rapidly. Chemically bound is when hydrolytic procedures are needed for dissociation of the steroid and protein. Unbound is the same as dissociated. Conjugated estrogens refer to those that are attached to sulfuric or glucuronic acid and form water soluble compounds while the unconjugated are free estrogens. At physiological pH the free estrogens are uncharged while the conjugated are charged (85,164).

Binding of steroids to plasma proteins is probably used as a means of hormone transport in the body. There seems to be very little evidence pointing toward the presence of chemically bound steroid complexes in the blood but rather most of the binding seems to be of a weak and reversible nature. Both free and conjugated steroids seem to be involved. The major plasma fractions that seem to be involved are IV-1 which consists of lipoproteins, gamma globulin, and cholestrol; IV-4 which includes alpha and beta globulin and the metal containing globulins; and V which is the albumin fraction (85,164). Roberts and Szego (157) found that most of the estrogenic activity of the blood was present in the lipoprotein fraction III-0 (a Beta globulin). Blood estrogen will pass through a colloidal membrane with the dialyzate containing all the estrogen originally present in the blood. They conclude that the estrogen and protein are in equilibrium in the blood and that dialysis results in progressive dissociation of the estrogen-protein complex. The estrogen is present in the dialyzate in an esterified form and can be released by acid hydrolysis. Rakoff et al (153) and Szego and Roberts (183) have reported that probably 50 to 70 per cent of the estrogen in the peripheral body is bound in some way

to plasma-proteins.

The estrogens are excreted in the urine both in the free form and in the conjugated form as sulfates and glucuronides with the glucuronide forms the main conjugate. These forms are much more water soluble than the free estrogens (69,84).

INGESTION, METABOLISM, AND EXCRETION OF ESTROGEN

Although the estrogenic activity of the isoflavones found in plants is quite low, the large amounts of feed consumed by farm animals may result in an important influence upon their physiological functions by these compounds (47,118). These isoflavones are believed to be produced in the chloroplasts of the plants as chloroplasts precipitated from plant juice have been shown to cause nearly all the estrogenic activity in animals. Early occurrence of the estrogenic materials in the leaf and the fact that plants are most potent during the green growing season tend to support this (23,54,116,142). If this chloroplast extract is treated with alkali, it yields a second estrogen which is at least ten times more active. This factor and the structural similarity between some of the isoflavones and diethylstilbestrol suggest that they may be precursers of estrogen. It is believed that these isoflavones such as genistein are changed into more active minor metabolites (26,46,54).

Cholestrol due to its similarity in structure is thought possibly to be a precurser of estrogen and androgen. Estrone and β-estradiol are synthesized by processes which begin with cholesterol, ergosterol, or diosgenin (73,204). There is considerable evidence that the liver is the most important organ in the metabolism of estrogen although it is not the

only place as the kidneys, reproductive organs, intestine, and probably other tissues to a limited extent play a definite part. It seems that the liver removes estrogen from the blood and concentrates it in the bile. Also, there is the theory that the natural estrogens are converted to a common form or mixture in the liver as when estrone, β-estradiol, and estriol are given by intravaginal route they have a mutually antagonistic action but when given subcutaneously they behave as simple dilutions of one substance. The liver must remove the estrogens at varying rates according to the concentration in the blood as the level is low even during pregnancy when estrogen production is maximal. Also, intravenously injected estrogen disappears rapidly from the blood stream. Indications are that the liver is capable of hydrolyzing conjugated estrogen as bile has a higher ratio of free to combined estrogen than does the liver (27, 47,89,137,139,157,162,211). However, others (84,183) feel that conjugation of estrogens takes place here. Roberts and Szego (157) and others (204) state that the liver is the site of formation or combination of the estrogen-protein complex. The liver seems to be capable of deactivating some compounds and activating others. Segaloff (166) ranks the following chemical degradation products of estrogen in the following order of decreasing estrogenic activity: 3-methyl ether of bis-dehydro-doisynolic acid > sodium bis-dehydro-doisynolate > alpha-estradiol > Westerfield's lactone acetate > beta-estradiol > estrololactone acetate. His experimental results indicate that the liver activates the first two compounds and inactivates the last four. These compounds vary greatly in activity and are handled differently by the liver which is quite interesting as

they can all be produced by rupturing the five membered ring of estrone. To explain these two functions of the liver it seems that the liver inactivates estrogen and is essential for "chemical activation" of estrogen. Also, the liver may furnish some metabolite necessary for the uterine response (144). Evans et al (64) tested the blood entering and leaving the liver of nine patients receiving large amounts of water soluble estrogens intravenously for biological activity and found the blood leaving the liver to have less estrogenic activity. In another experiment, other workers (122) induced vaginal cornification in a spayed female parabiotically combined with a gonadectomized partner of either sex with a single injection of estrogen which was 16 times the minimal dose. Hepatectomy of the non-injected parabiont enhanced its own vaginal response while hysterectomy had no specific effect. Hysterectomy of the injected female partner gave an actively increased response in the other. Zondek (211) conducted tests in which estrogen was incubated with liver pulp. His results showed a loss of estrogenic potency if the liver was not heated but if it was the potency remained the same which indicated the destruction was due to an enzyme . He also found some inactivation of estrogen by the kidney and spleen (212). Dorfman and Unger (56) report that the following enzymic actions take place in the liver: 17 β-dehydrogenase changes β-estradiol to estrone and 17α-hydrogenase changes estrone to α-estradiol. Also, 17β-hydrogenase will change estrone to β-estradiol in human tissue while 16α-hydrogenase acts on 16-keto estradiol-17β and 16-keto estrone to from estriol. Ryan and Engel (163) found that rat liver slices will interconvert estrone and estradiol under

both aerobic and anaerobic conditions. Also, they found that the hormone concentration is a critical factor in this interconversion as well as in further changes of the compounds.

Kidney slices have been found to partially inactivate α-estradiol, while estrone was completely inactivated by rabbit liver and partially by rabbit kidney and rat liver. Estriol was only partially inactivated by any type of tissue slices. These results show that there is quite a difference in inactivation of different estrogens and by different species of animals (89).

It seems that the intestinal flora is capable of converting estrone to α-estradiol as most of the feces estrogen has been found to be α-estradiol while the source of feces estrogen contains considerable amounts of estrone (117,138,139).

Uterine tissue will modify the structure of the estrogen molecule, but most of the evidence indicates that the uterus and ovaries are not essential for the chemical transformation which estrogens are known to undergo. Alpha-estradiol (old nomenclature) is converted to estrone in both normal and ovariectomized female and in male guinea pigs. In rabbits, α-estradiol (old system) is converted to estrone and β-estradiol by normal and ovariectomized, hysterectomized animals. The estrone is believed to be an intermediate product. However, these organs probably play a part in normal animals (67,68,138).

Ryan and Engel (162) in studying interconversion of estrone and estradiol by various human tissue slices found that all but tissue from the testes converted estradiol to estrone at a greater rate than the reverse.

They found that placental tissue had the greatest activity of any of the tissues and yielded 11 to 38 per cent estrone from estradiol. None of the tests resulted in conversion of either estrone or estradiol to estriol nor was estriol converted to either estrone or estradiol. Velle (198,199,200) found significant increases in urinary estradiol-17α and estrone following intramuscular injection of estradiol-17β to young calves. The conversion of β-estradiol to α-estradiol appears to be quite rapid. When estrone was injected into young calves the major part of the estrogen was recovered as α-estradiol although there was also a distinct rise in the estrone fraction. He also found injected α-estradiol is partially converted to estrone which indicates a common metabolic pathway. Velle concludes that the conversion of estradiol-17β to estrone is not reversible and that the conversion of estradiol-17β to estradiol-17α goes via estrone in cattle. In neither of his experiments could he detect the presence of estriol in the urine which indicates that estriol is not an estrogen metabolite in the bovine species. However, in pregnant women estriol appears to be one of the main pathways of estrogenic metabolism (76).

Experiments show that metabolism is responsible for the rapid destruction or inactivation of injected estrogen as only ten per cent or slightly more of that injected is accounted for by urine excretion (137).

The following seems to fairly well summarize the reactions occurring in mammalian tissue, although estriol formation seems to be characteristic of the human only (144).

The nutritional state of the animal has a definite effect on its estrogenic metabolism, particularly that which takes place in the liver. Biskind and Shelesnyak (28) found that vitamin B complex deficiency resulted in the liver losing its capacity to deactivate estrogen. A later experiment (27) with rats on a vitamin B complex deficient diet caused a protracted state of estrus. Riboflavin and thiamin added to the diet brought the animals back to normal. Singher et al (170) found liver tissue from riboflavin and thiamin deficient rats was incapable of inactivating estradiol while livers from those on the same diets plus riboflavin and thiamin would. Estrogens administered intrasplenically in rats maintained on an adequate diet are relatively ineffective. However, a purified diet free of B-complex factors gives expected potency and can result in cessation of estrus. The addition of thiamine and riboflavin restores the animals capacity to inactivate estrogen (167,168). Others (208) found that female rats maintained on rations with a borderline deficiency of riboflavin gave birth to fetuses that had normal growth but not sex differentiation. If the amount of riboflavin is lowered further sterility or embryonic death results. The protein intake has also been found to be critical since 50 per cent food restriction in rats resulted in anestrus in 11 days indicating cyclic production of estrogen was stopped and that it was destroyed by the liver (193).

A considerable amount of the estrogen excretion is found in the urine as has been mentioned previously in this paper. However, there is also a substantial amount excreted in the feces. In an experiment with dogs, 1.3 to 8.0 per cent of the injected estrone was recovered in bile excreta

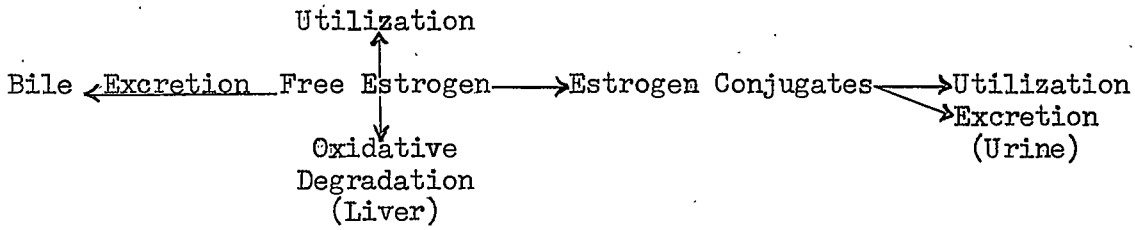
while 6.4 to 13.5 per cent was recovered in the urine (120). Hanahan et al (83) in 1953 showed that the main pathway of absorption of oral estrogen in rats is the portal system and that 50% of administered radioactivity was present in the feces. Hunt et al (97) found radioactive estrogen injected into an 18 month old dairy heifer resulted in large quantities in the urine and feces for the first 24 hours. Only a small amount in the urine (less than 2%) was in the free form while 60 to 80 per cent of the radioactive estrogen was in the conjugated form. Subcutaneously administered estrogens in contrast to oral estrogens do not seem to pass through the liver. Brown (37) injected estrone, β-estradiol, and estriol intramuscularly into humans and found that 27 to 59 per cent of the estrogen recovered in the urine was estriol when estrone and estradiol were injected and was 100 per cent estriol when it was injected. Estriol excretion reached its peak when estrone or estradiol was injected oneday later than the other two estrogens which indicates a conversion takes place. The level of the estradiol excreted was never more than one-half of the estrone.

When animals are fed synthetic estrogen, there is some retention in the tissues which varies with the estrogen fed. Early work revealed no estrogenic activity by these tissues, but later, samples taken from liver, kidney, kidney fat, muscle, and intestinal tissue of cattle showed estrogenic residues from diethylstilbestrol in all the tissues and in all but muscle tissue with dienestrol. Hexestrol retention was found only in the kidney and kidney fat (151,181). Lambs placed on a diet containing one milligram or two milligrams of diethylstilbestrol per day resulted in 80

per cent recovery in the urine and feces with the major portion in the feces (180).

A small amount of estrogenic metabolites is excreted in colostrum but not in normal milk (150).

The following summarizes how estrogen is eliminated (69).



PHYSIOLOGICAL ACTIONS OF ESTROGEN

Estrogen has a wide variety of actions on many different functions and organs of the body. The primary functions of estrogen are the development and maintenance of the accessory sex organs and the development of the secondary sex characteristics. In addition, it is important in regulating growth rate and type of growth and affects many important body functions. Many of these physiological actions of estrogen were first revealed when the ovaries were removed from females and the physiological changes observed. Cessation of estrus, termination of pregnancy--in most animals, and atrophy of the sex organs are some of the effects that have been known for some time. The estrogenic functions at the cellular level and on enzyme systems have only been studied during the last few years so many aspects have probably not been discovered (39,145,156).

The fact that excess estrogen has many deleterious effects was brought to the attention of many research workers when it was discovered that an estrogenic material present in subterranean clover was causing

infertility, lactation of unbred ewes and whethers, failure to deliver lambs, and many times eventual death of the animal in many bands of sheep in Australia (24,26,169).

ESTROGENIC EFFECTS ON DIFFERENT SYSTEMS AND ORGANS

Embryonic Gonads and Mullerian and Wolffian Systems--Estrogenic effects on embryos have been observed only in the later stages of pregnancy as estrogen administration early in pregnancy will either prevent nidation of the ova or death of the blastocysts.

One of the specific functions of estrogen is to preserve and stimulate development of the mullerian system and a slight inconstant inhibitory effect on the wolffian system (80,154). In 1939, Raynaud (154) injected estradiol into pregnant mice and found that in the male offspring the mullerian system was preserved, a uterus--occassionally highly developed--was present, and in a few cases oviducts were found opening against the walls of the testes. The wolffian ducts were either normal or partially atrophied. Green et al (81) noticed similar inhibitions in rats as well as development of a vagina in some of the males. Normally, the two systems of ducts develop equally for a while with one subsequently lagging in development and finally being represented only by vestigial remains while the other continues to develop (73). Green et al (81) also found that the female mice offspring had grossly enlarged uteri and nipples larger than normal. In male offspring, there was an inhibitory effect on the vas deferens, seminal vesicles, and epididymis.

Injections of hormones, including estrogen, in pregnant cows and other animals have failed to alter the gonads in any way (9,127).

Gonads after Birth--Estrogen given to a female animal shortly after birth will retard development of the ovary and prevent follicles from ripening. However, this arrestment is not permanent as discontinuance of injections will allow the ovary to return to normal (106,147). The arrested condition is believed due to the estrogenic inhibition of gonadotrophic production by the pituitary.

Estrogen will effect the mature ovary nearly the same as the immature but a larger dose over a longer period is needed. Noble (133) in 1938 implanted diethylstilbestrol crystals into adult rats with atrophy of the ovaries resulting. Assay of the pituitary indicated a deficiency of gonadotrophin. The removal of the estrogen allows the ovaries to return to normal.

However, estrogens in certain circumstances exercise an effect comparable to that caused by gonadotrophin. In hypophysectomized rats, it was found that different estrogens caused enlargement of the ovaries with numerous closely packed follicles present. It is believed that this enlargement is due to a synergism between estrogen and gonadotropes (140, 206).

It appears that development of new egg cells in the ovarian cortex by mitosis in the germinal epithelium is dependent on estrogen (39).

An excess of estrogen will prevent the maturation of follicles by checking the supply of gonadotrophin from the pituitary. As a result, no corpora lutea will form and the ovaries will atrophy. However, estrogen increases the output of LH and will thus cause any corpora lutea present to hypertrophy and remain in an active condition which will cause

enlargement of the ovary (25,95).

All of the effects of estrogen on the ovary seem to be indirect in that it acts on the pituitary to alter the supply of gonadotrophin (39).

Estrogen given to young male animals will prevent maturation of the testes and descent into the scrotum. In older animals, the function will be arrested and the testes will atrophy (39).

Growth--Estrogen seems to have a depressing effect on growth as in most species the male is larger than the female. Steinach and Holzknecht (177) in 1916 found that a spayed female with a grafted testes grew to an unusually large size, even larger than normal males, while a castrated male with a grafted ovary was of extremely small size. Since then many other workers have found that injections of estrogen will inhibit growth. However, like many hormonal effects, estrogen in small amounts will increase growth rate in male and female, castrated or intact sheep and cattle and will increase the fat deposition and carcass quality in chickens (156). In a very recent experiment (1958), Sullivan and Smith (182) injected estradiol subcutaneously into immature male rats for several weeks and obtained a depression in body weight and food intake proportional to the dose. If the controls were fed the same amount of feed as the experimental animals, the growth curves were the same which indicates part of the depressing effect on growth is due to decreased appetite. Also, estrone and estradiol implants in the spleen inhibited growth of rats on normal diets and accentuated the loss of rats on high fat diets (141). It is believed that the inhibitory effect of high estrogen doses is due to a depressing effect on the secretion of growth hormone from the anterior

pituitary and a closure of the epiphyseal junctions of the long bones (156). Growth of bones ceases when the epiphysis unites with the shaft which union occurs early in females and in estrogen treated males. In addition to this change, there is also increased calcification of the skeleton (39).

The characteristic distribution of fat in the normal female is a result of estrogenic activity as is the thinner skin of females which is due to inhibitory action on growth of the epidermis (39).

Pituitary and Its Secretions--Estrogen has an inhibitory effect on the gonadotrophic activity of the pituitary as has been mentioned previously. This is supported by the fact that removal of the gonads is followed by an enlargement of the anterior lobe of the pituitary and an increase in its gonadotrophic potency (39).

Haterius and Nelson (86) found that the histological changes in the pituitary which occurred in castrated male rats could be prevented or if present the condition reversed by means of ovarian implants. Another experimenter (131) found that the basophile cells in the pituitary of castrated rats were changed to chromophobes upon injection of estrogens and that the number of basophiles and eosinophiles were reduced.

Badowi and Soliman (16) found that estrogen injections interfered with the synthesis and release of gonadotrophic hormone but increased the rate of release of thyrotropic hormone.

Ovarian grafts in the anterior pituitary do not inhibit gonadotrophic activity (FSH output). Ovarian grafts (1 mm in size) in the paraventricular region of the hypothalmus bring about a significant decrease in

uterine weight as compared with normal rats or ones with grafted hepatic tissue. It is postulated that estrogen effects the gonadotrophic function of the hypophysis through a nervous mechanism. It is assumed that the functional state of nervous elements localized somewhere in the region of the paraventricular nuclei is modulated by the blood level of estrogens and thus regulates the secretion of gonadotrophic hormone (70).

External Genitalia--Estrogen given to the mother shortly before parturition may restrict the growth of the penis and scrotum in the male (113). In the female, the only noticeable effect early in life is hypertrophy of the folds of the vulva (119). After puberty, estrogen causes hypertrophy of the vulva in the female and atrophy of the musculature of the penis in the male as well as scrotal hernia occasionally (39).

Vagina--A very important action of estrogen is cornification of cells in the vagina as this effect is used as an assay to detect minute quantities of estrogen. The condition of the vaginal epithelium is also indicative of the stage of the estrual cycle and has wide application in clinical studies (156).

The fact that cornification occurs in the vagina has been known since 1917 when it was discovered by Stockard and Papanicolaou (179).

Estrone given in an effective dose to a spayed mouse will produce a change in the vagina within 24 hours. An early result is multiplication of epithelial cells followed by keratinization of the cells. If injections are not continued there is a leucocytic infiltration of the epithelium and the cells desquamate. If they are continued the vagina will become filled with a keratinous mass (39). The estrogenic effect is

detected by obtaining the large scaly cells which have sloughed off on the inner side of the vagina and studying them microscopically (156). An excess of estrogen will keep mucin from being formed in the vagina of the cow.

This metaplastic response of the vaginal epithelium is not confined to animals of any certain age as it effects the foetus, newborn and old. Estrone given to pregnant guinea pigs will cause the young to be born with open vulva and keratinized vagina (50). The effect of estrogen on the vagina is believed to be direct as there is no difference in intact rats and hypophysectomized and ovariectomized rats with regard to the response elicited (174).

Estrogen also causes a general hyperaemia and moderate hypertrophy of the muscular and fibrous stroma (39).

Estrogen causes a more acid condition of the vagina possibly by the formation of lactic acid from glycogen present in the vaginal epithelium. This reduction in alkalinity of the vaginal secretion promotes liquification of the vaginal seal (7,82,119). An increase in phosphatase and riboneucleic acid may also affect the pH of the vagina (137). Glycogen granules have been found in the vaginal epithelial cells of humans and monkeys in concentrations second only to the liver with estrogen apparently responsible (137).

Uterus--Estrogen is responsible for the well-being and functional utility of the uterus as spaying of the animal will result in atrophy of the uterus. Development of the uterus is initiated by estrogen and then completed by progesterone. Uterine activity is at a maximum during estrus

due to the high estrogenic concentrations present (156). The response of the uterus to administered estrogen is very rapid and occurs in normal or spayed animals. This response is used as the basis for a biological assay of estrogenic activity. There are two types of response--one due to increased water retention and the other due to protoplasmic growth--which will be described in detail later in the paper.

Estrogen causes a hypertrophy and congestion in the uterus; the epithelial cells become swollen and rounded; the submucosa is thickened, hyperaemic, and edematous, and penetrated by uterine glands (51).

Estrogen also causes hypertrophy of the cells of the myometrium and appears to be the limiting substance in the synthesis and maintenance of the final contractile system of the myometrium (53,137) which accounts for the rythmic contractions of the uterus. Low estrogen titers will increase the length of uterine muscle cells but high levels will not (9).

Paesi and co-workers (135,136) found that the uterus does not receive high concentrations of estrogen due to the nearness of the ovary as transplantation of the ovary did not make any difference. They found that subcutaneous administration of estrogen to spayed rats in sufficient quantities to produce normal uteri would result in larger than normal adrenals and hypophyses. Ligation of the fallopian tubes in intact animals showed this was not the mode of transportation. They thought that the ovary may produce some substance which either enhances the influence of estrogen on the uterus or diminishes its effect on the hypophysis and adrenals.

Mammary Development--In most species of animals, estrogen is responsible for the development of mammary ducts and in some it developes both

the alveoli and ducts. However, later experimentation has shown that, at least with guinea pigs, progesterone acts in synergism with the estrogen as it greatly increases the rate of lobulo-alveolar growth when added to estrogen administrations. Also, the DNA and RNA concentrations are greatly increased as is the rate of respiration of the tissue all of which indicate increased lobular development.

Many workers have found that estrogen increases the size of the nipples and teats but only to a certain point with direct applications being the most effective. It appears that other hormones act with estrogen to develop the mammary system although estrogen is believed initially responsible (39,192). Excess amounts of estrogen can have a depressing effect on mammary development. It has also been found that estrogen has an effect on the secretion of milk which is quite noticeable in some species during estrus. Many workers have found that estrogen in fairly large amounts will have an inhibitory effect on lactation while small amounts tend to increase fatty and non-fatty solid concentrations in milk (72).

It is believed that elimination of the placenta at parturition allows lactation to ensue as the placenta produces a considerable amount of estrogen especially towards the end of the gestation period (39).

Mammary Cancer--Even before the identification of estrogen, the ovary was thought to be an agent in the development of mammary cancer. Sir George Beatson in 1896 stated that in the female we must look to the ovaries as the seat of the exciting cause of carcinoma (21). Since then, many workers have induced cancer in mice and rats by injections of various estrogens. The level of estrogen and the age of the animal affect the

development of mammary cancer with the young being more susceptible. However, a non-genic agent, probably a virus, transmitted through the mothers milk and a genic Mendelian factor apparently have to be present to make the animals readily susceptible as these two and estrogen seem to work together (39).

Estrus--Estrogen induces estrus when it reaches a certain level in the animals system. In ovariectomized heifers, Asdell et al (9) found that 600 rat units of estradiol benzoate injected daily for three days would bring the animals into estrus. The estrus period would last only one day even though the injections were continued for a considerable length of time. This low threshold, especially when considering the size of the animal, is apparently reached early in the development of the Graafian follicle in the normal cow at which time an "estrous block" in the nervous system sets in so that the cow is out of heat before ovulation. Injection of 0.255 mg of diethylstilbestrol will also bring an ovariectomized heifer into estrus. Intramuscular injections of estradiol cyclopentlproprionate is effective in inducing estrus in cattle but will not always establish a normal cycle (59). In gilts intramuscular injection of diethylstilbestrol was given on the 6th, 11th, and 16th days of the estrual cycle. Injections on the 6th day had no apparent effects; those on the 11th day lengthened the estrual cycle probably due to release of small amounts of LH from the anterior pituitary during mid cycle and subsequent luteinization of the follicles; and those on the 16th day usually caused a shortening of the estrual cycle (107).

Robinson (159) in a study on ewes also found that small amounts of

estrogen (as low as 25 micrograms) would inhibit ovulation in some cases by premature luteinization of the follicle through release of LH from the anterior pituitary.

Brown (36) in humans found two peaks of estrogen excretion in the menstrual cycle--one on the 13th day (ovulation peak) and one on the 21st day (luteal peak). He found the estrone and estradiol ratios to remain quite constant throughout the cycle but the estriol quantity varied considerably.

In the non-pregnant cow, certain phases of the activity of estrogen seem to occur at midestrus. Alkaline phosphatase is found in uterine tissues in the greatest quantities during mid-cycle. In the vaginal smear, cornified cells are most frequent at mid-cycle instead of at estrus. Both of these are increased with an increase of estrogen which also indicates two peaks (10).

A low threshold may have a bearing on the free-martin condition in cattle. If the threshold is low for male hormones, a small amount in circulation may effect the twin heifer (9).

Pregnancy--During the first 100 days of pregnancy in the cow the estrogen excretion is quite low. However, beginning at about the 50th day of gestation there is a definite increase in estrogenic excretion which continues until parturition at which time there is a rapid decline. The excretion rate returns to the non-pregnant state after two to three days. It has long been known that removal of the ovaries during pregnancy results in a definite decrease in the estrone content of urine (2,132,172, 192,201).

Estrogens prepare the uterus for nidation of the fertilized ovum which is supplemented by the effects of progesterone. However, pregnancy will follow only if the supply of estrogen ceases or is much reduced. Excess doses of estrogen early in gestation will result in abortion or death of the fetuses. This fact has been known for many years although the reason for it was not.

An investigation conducted to determine reasons for infertility in repeat breeding cows and heifers showed that embryonic deaths during the first month of pregnancy account for a large number of cases of infertility, in this case embryonic deaths accounted for 54.1 %. In repeat breeding cows 39.7 % indicated failure of fertilization. Prior to 34 days post breeding 39.2 % of the cases had embryonic abnormalities and mortality and at 34 days 21.1 % had normal embryos (187,188).

These figures indicate that there very possibly may be a hormonal imbalance, probably estrogen, which has caused this abortion or embryonic death.

Enzyme Systems--Estrogen is believed to stimulate a DPN-linked isocitric dehydrogenase. Studies suggest that estrogen converts an inactive form of the enzyme to an active one by forming a steroid-enzyme complex. Estrone and estradiol-17β are believed bound to the enzyme at the same locus. These two are bound 200 times more strongly than stilbestrol and have twice as great an ability to activate the enzyme. Estradiol-17α and stilbestrol both seem to be inhibitors and thus act as competitors with β-estradiol and estrone. An increase in the activity of the isocitric dehydrogenase permits a general speeding up of the whole Krebs

cycle as evidenced by increased oxygen consumption of tissues under estrogen influence both in vivo and in vitro. The end result is an increase in the energy supply available to the tissues for the accomplishment of the various physiological effects known to be induced in the whole animal by the injection of estrogen. All the data found by these workers (81, 203) support the hypothesis that estrogen combines with an inactive protein to give an active enzyme.

Estrogen injected in ovariectomized mice is followed by a marked increase of alkaline phosphatase in the uterine glands and luminal epithelium and in the circular layer of the myometrium. A direct ratio between the level injected and the response was found with the response peak and decline occurring at the same rate as other morphological changes produced by estrogen. It is possible that the mobilization of alkaline phosphatase is a link in the mechanism by which estrogen affects lipid and carbohydrate metabolism (13). Asdell and Mixner (10) found the amount of alkaline phosphatase in the uterus to vary inversely with the amount of glycogen.

Intracellular and Extracellular Contents--Very little work has been done on this estrogenic effect and most of that only within the last year or two. The first information on this subject was revealed as a result of the study of estrogenic effects on the uterus. Thorn and Engel (191) in 1938 found injections of estrone or estradiol caused increased sodium and chloride ion retention in the uterus. In 1940, Talbot et al (186) found that six hours after estrogen injection the concentration of sodium ions had risen significantly and the chloride ion concentration slightly. The

amounts of phosphorus and potassium increased but the concentrations were lowered. They found the following increases in amounts: H_2O--67 %, Na--116 %, Cl--63 %, K and Mg--33 %, P--12 %, and solids--27 %. The sodium and chloride ions are considered as extracellular electrolytes and potassium and phosphorus as intracellular. It is believed that estrogen changes the cell permeability. A later experiment (104) showed peak increments of sodium and chlorine four hours after estrogen injections. The increase in sodium as compared to chlorine could not be accounted for on the basis of accumulated plasma ultra-filtrate or on the basis of leakage of plasma protein. Kalman and Lowenstein (105) injected certain radioactive materials into ovariectomized rats treated with estrogen. The uterine content of H_2O, $Na_2HP^{32}O_4$, NaI^{131}, and $Na^{22}Cl$ was significantly increased two hours after treatment but not at one hour. The uptake of I^{131} labeled serum albumin was higher than controls at one hour and continued to show an accelerated rate of influx through the third hour.

Frieden and Bates (74) found injection of 20 micrograms of estradiol to ovariectomized rats over a two day period stimulated the incorporation of labeled glycine into uterine protein by 230 per cent. Szego and Roberts (184) also found that estrogen enhanced the rate at which amino acids are incorporated into uterine proteins during active growth of the uterus which is indicative of protein synthesis. Other workers (32,161, 189) have found that estrogen causes an increase in glycogen concentrations of the uterus in both the myometrium and endometrium. They postulated that the changes are mediated by a series of enzyme systems.

Elftman (59) found that estrogen decreases the basal lipid of the

cells in the uterus of ovariectomized rats. There is an increase in the size of the Golgi apparatus which implies an increase in apical phospholipid as it contains an appreciable amount. It is possible that a migration takes place but a more immediate metabolic use of the phospholipid is likely.

Mueller and Herranen (128) conducted an experiment which indicated that estrogen affects the metabolism of formate into protein, acid soluble serine, nucleic acid, purines, and crude lipid fractions of uterine segments. It is believed that an early effect of estrogen is a shift in the reducing potential of the uterine cell or of certain loci within the cell to a balance more favorable to reductive synthetic pathways providing precursers for new growth. Such an environment may also facilitate the release or activation of protein forming the enzymatic machinery for the growth process.

Brody and Westman (33) injected estradiol benzoate into castrated rabbits and observed marked increases in pentose nucleic acids and proteins in both the endometrium and myometrium of the uterus.

Miscellaneous Estrogenic Effects--There are also a number of other known effects of estrogen in the female some of which are quite important. Estrogen causes an increase of the cervical mucus with a lower viscosity which favors sperm migration, motility, and longevity (137).

Estrogen splenic implants in weanling rats maintained on high fat (40 %) and low protein (5 %) diets show that estradiol has a lipotrophic effect as the controls had 32 % fat in the liver, estrone--30 %, and estradiol--17 %. The estrogens also caused an accentuated loss of weight

in these animals. Part of the lipotropic effect is believed due to choline sparing of growth inhibition but part is probably a true lipotropic effect (141).

Estrogen acts as an automatic check on its own production by inhibiting the production of gonadotrophin from the pituitary (39). Later evidence indicates that this is accomplished through a nervous mechanism. It is believed that the functional state of nervous elements localized somewhere in the region of the PV nuclei is modulated by the blood level of estrogen and thus regulates the secretion of gonadotrophic hormone (70).

Estrogen increases the oxygen consumption of castrated rats if the thyroids are intact (176). Telfer and Hisaw (189) found the rate of oxidative metabolism is also increased in the rabbit.

Since estrogen changes the endometrium of the uterus to increase its resistance to infection, it is used to treat uterine infections. It also is used in veterinary medicine for cows in weak labor, and to help remove mummified fetuses and retained placenta (52).

Estrogenic Interactions--Relaxin will increase the weight of the uterus by increasing the water content. Estrogen has to be present as ovariectomized rats are not affected unless estrogen is also given. Maximum increase is at six hours with a return to normal at 24 hours (99,209).

Estrogen has a number of interactions with progesterone. Progesterone apparently is necessary for appearance and disappearance of leucocytes from vaginal smears of guinea pigs and ewes (160). In male castrated rats, estrogen increases the release of thyrotrophic hormone while

progesterone decreases it. Estrogen interfered with the synthesis and release of gonadotrophic hormone while progesterone increased the level (16). Progesterone and estrogen are both needed for maximal lobulo-alveolar growth in the mammary gland (175). Estrone in the presence of progesterone is converted to estriol (148).

Ershoff et al (63) found that concurrent feeding of dried alfalfa could largely counteract the deleterious effect of α-estradiol on the ovaries of immature rats. The protective factor(s) is apparently distinct from any known nutrient and is present in dried alfalfa juice and water washed alfalfa pulp remaining after the extraction of the juice. The quantity varies with the sample. All known nutrients were used to supplement the basal ration with no effect. The factor may be an orally active gonadotrophin, a factor which is effective in promoting the synthesis and secretion of pituitary gonadotrophin, or an unknown nutrient(s) which may be part of an enzyme system concerned with estrogen inactivation.

CHEMICAL ASSAYS OF ESTROGEN

A number of different chemical assays have been suggested for the quantitative measurement of estrogen but only two have proven to be of much value. These methods work fine for pure estrogens but if any brown pigments from urine are present considerable trouble developes in the accuracy.

Kober Reaction--Probably the Kober reaction developed by Kober (111) in 1931 is the most important chemical assay. Kober discovered that estrin heated with concentrated sulfuric acid gives rise to a yellow color.

The addition of water and reheating converts the yellow to a highly specific pink. The yellow color first developed had a green fluorescence which was eliminated by the use of phenol which also intensified the red color. Kober used a solution of acid cresol red (10 mg/100 ml) as a standard and found that five micrograms of the hormone gave a color of the same intensity as 25 micrograms of the acid cresol red.

Cohen and Marrian (49), however, found that it was impossible to duplicate Kober's results using any concentration of cresol red. They also found that heating the solution decreased the yellow color component and increased the red. Also, the addition of various amounts of water was found to alter the color.

Later (1938) Kober (112) substituted β-napthol for the phenol. However, most workers found the phenol to be satisfactory (85).

The Kober reagent has also been found to react with other substances in urine extracts to give a brown color which masks the pink. Some of the factors affecting the Kober reaction are: 1. The composition of the reagent and the amount used; 2. The length of time that estrogen is heated with the Kober reagent to develop the yellow color; 3. Amount of water added to the yellow mixture; and 4. The time of reheating required to convert the yellow color to the specific pink (202).

Bachman (15) in 1939 modified the Kober reaction by adding one volume of water to the original solution and heating at 150°C. instead of 100°. He found that the reaction with estriol was faster in certain dilute solutions than with the other estrogens. Also, if a 2:1 solution of sulfuric acid to water was used a pink color was developed without heating.

However, when heated this solution gives rise to the usual yellow-brown color and then changes back to the pink as do the other estrogens. This indicates quite clearly that estriol goes through phases not observed with the other estrogens. Marrian (126) states that the basic red pigment is the same (an oxonium salt of sulfonated 16,17-diketoestratrienol 1-3) and that the immediate precurser of this pigment is also formed in the metabolism of estrogen.

Later, an attempt was made to modify the Kober reaction by omitting the phenol but it was found to be accurate only within the range of 10 to 16 micrograms. It has not been tried with urine extracts (48).

In 1950, Allen (4) developed a simple equation to permit analysis of mixed absorption curves providing the curves of the contaminants approximate a straight line which has been found to be the case in the study of urinary extracts with the Kober test. The equation is:

$$CDX_x = OD_x - \frac{OD_a + OD_b}{2}$$

where OD_x is the observed density at the absorption maximum wave length, OD_a and OD_b are the observed densities at wave lengths a and b equidistant from x, and CDX_x is the calculated density due to substance X at wave length x.

Bauld (19) verified the use of this correction formula and found it to be satisfactory between 480 and 550 millimicrons at which range the reagent blank is linear. He also found the Kober reaction to be valuable due to its great specificity and stability. He used wave length settings of 480, 512.5, and 545 millimicrons for estriol and estrone and 480, 515,

and 550 millimicrons for estradiol.

Bauld (18) also modified the reaction by using ethanol as a solvent and adding quinol and aqueous sulfuric acid. The ethanol was evaporated either by heating in a stream of air at 90 to 95°C. or under reduced pressure in a stream of nitrogen. He found no significant differences in the two methods providing the ethanol used was aldehyde free. However, he found that the color produced was decreased 15 to 40 per cent by solvent residues.

Brown (35) in 1955 found that the estrogen methyl ethers should be evaporated to dryness in the absence of air as he found jets of air or impure nitrogen might cause losses due to impurities of the solvents which partly oxidize during evaporation and interfere with development of the Kober color. He found the absorption peaks of estriol and estrone to be 513 mμ and that of β-estradiol to be 514 mμ.

Essentially the Kober reaction is a two stage process in which the initial formation of a yellow compound with a green fluorescence is followed by its conversion into a pink non-fluorescent complex on reheating in more dilute sulfuric acid. This pink color fades if heated in more dilute solutions. There are thus three possible forms of interference with the final color produced which are as follows: 1. Failure to form the initial yellow complex; 2. Failure to convert the yellow complex fully into the pink; and 3. Instability of the pink complex (fading) (18).

Hydroquinone is used in the Kober reagent as a reducing agent. If this substance becomes sulphonated, its reducing power is much lowered.

However, if solvent residues are not present its ability to produce color is still adequate. Ageing of the reagent did not interfere with color production by estrone and estradiol in the presence of solvent residues (18). Brown (34) found that estrone does not require a reducing agent in the first stage of the Kober reaction which accounts for the above.

Estriol appears to undergo at least two different reactions when heated in sulfuric acid the first of which is oxidation (this is not undergone by estrone and estradiol) and the second is that it requires a reducing agent and is a much slower reaction than reduction of estrone (15,43,125).

The fact that estriol requires longer heating and higher acid concentrations suggests that in the first stage of the Kober reaction estriol is dehydrated to estrone. This hypothesis is also supported by the similarity of the absorption spectra (185). Possibly the reducing agent is needed to prevent the alternative oxication of estriol and permit quantitative dehydration to estrone.

Color development of the first stage has been found to be aided by sunlight. The conversion of yellow to pink is believed to be an oxidative process. Hydrogen peroxide will convert the pink into a colorless compound (18).

Thpe I inhibition thus is eliminated by adding hydroquinone at the start of the color reaction (this applies only to estriol).

Type II inhibition is controlled by using more concentrated sulfuric acid.

Type III inhibition is controlled by the use of a slightly modified

Kober reaction which stabilizes the color.

Marlow (124) in testing various estrogenic compounds found that maximum color was developed when the compound contains a carbonyl group at position 17 and a methylenic carbon at position 16. If an OH group is at position 17 or both 16 and 17, the value was found to be about 50 per cent that of the compound with a carbonyl group at 17. Methoxyestrone gives more color while the benzyloxy derivative gives less color than estrone. Carbonyl groups at position 16 or both 16 and 17 gave no color. A hydroxyl group at 17 and a carbonyl group at 16 show as much transmission activity as a carbonyl at 17 with an adjacent methylenic carbon.

Fluorometric Assay--This chemical method also uses the reaction of estrogen when it is heated with sulfuric acid (194). Natural estrogens show a characteristic absorption in ultraviolet light due to the presence of a phenolic ring (144).

Jailor (100,101) used 60 to 70 per cent sulfuric acid and methyl alcohol and found that the yellowish-green fluorescence produced by heating could be measured quantitatively. He found the method to be especially useful in measuring very small quantities of estrogen. Amounts as small as 0.05 micrograms of estrone could be detected. Estriol has about 1/20 as much fluorescence as estrone and estradiol. He found that urine extracts had a whitish tinge in addition to the yellowish-green fluorescence. Results from urine extracts were somewhat higher than with bioassay which has also been found by a number of other workers (173). Finkelstein (66) found it to be more sensitive, accurate, and specific than biological or colorimetric tests. However, Bauld (19) states that

it is more susceptible to instability and quenching by solvent or urinary residues which makes the Kober reaction more valuable. Methanol and hydrochloric acid quench fluorescence. Fluorescence of high titer urines is due almost entirely to estrone, estradiol-17β, and estriol but there is an overestimation in low titer urines (17,62). Japanese workers (129) found that values obtained by fluorescence of estrogen of blood and urine were lower than the true values.

Bates and Cohen (17) found that oxidizing agents such as hydrogen peroxide and various nitrates will destroy the fluorescence that has developed or prevent its appearance if added first. They also found that the sulfuric acid concentration could vary from 70 to 85 per cent and the temperature as much as five degrees without altering the results. If the heated sample is diluted with 65 per cent H_2SO_4, the fluorescence will remain for several days. The various estrogens all have different values. With a 3389 filter values were as follows: Estrone--100, α-estradiol--83, β-estradiol--94, and estriol--34.

BIOLOGICAL ASSAYS OF ESTROGEN

As with the chemical assays, a number of biological assays have been developed but only a few are of much importance. Probably the most important at the present time are those based on the increase in uterine weight. Methods of administration of estrogen are peroral, subcutaneous, percutaneous, intravenous, and intravaginal. The most useful vehicle for injection of natural or synthetic estrogens is an oil. If free substances are used they are absorbed from the site of the injection within a day or two and thus exert their effects only transiently (60).

Uterine Weight Assays--Injections of estrogen in immature female rats or mice result in two distinct curves in plotting the total weights of the uteri at different time intervals. The first rise reaches a maximum at about six hours after which there is a drop and a subsequent second rise which reaches a peak at about 30 hours and at a maximum point higher than the first rise (11,186).

Astwood's (11) six hour test is based on this first rise and is believed due primarily to an increased water retention. Estrogen seems to cause a rapid change in the permeability of the uterus as evidenced by an increase in uterine water (186). As a result of this increase, there is a hypertrophy of the epithelial linings and endometrial glands, an enhancement of the uterine blood supply and an increase of the uterine capillaries (11,87,88,123). Pincus and Graubard (146) found that excised rat uteri six hours after estrogenic injections lost weight when placed in an isotonic solution while those of normal animals gained weight.

Astwood (12) used female albino rats 25 to 49 grams in weight and gave one injection of 0.1 milliliter of sesame oil containing the estrogen. After six hours the rats were killed and the uteri dissected out by cutting at the utero-tubal junctions and at the cervix. The mesometria was then stripped off and the uteri blotted and weighed. Water determinations were then made by desiccating in an oven at 110°C. and weighing again.

The four day uterine weight assay is based on the second peak of the response curve which is believed due primarily to true protoplasmic growth. The rate of incorporation of radioactive phosphorus is an index

of tissue growth. Several workers have noted an increase of phosphorus in the uterus following estrogen administration. Szego and Roberts (184) found increased uterine solids and tissue growth. After 30 hours, the uteri of injected rats were much larger than controls but no longer edematous and the electrolyte pattern had returned to normal concentrations although the total weights of each were higher (186). Carbohydrate incorporation and protein synthesis are both at an increased rate all of which results in the increased uterine weight (74,184).

Lawson et al (114) in 1939 developed a four day test in which immature female rats were injected twice daily for three days with 0.5 milliliter of an aqueous solution and killed on the fourth day. The uteri were dissected out, trimmed, blotted, and weighed.

In 1941, Evans and co-workers (65) experimented with a four day mouse uterine weight method for the assay of estrogens and found it to be very sensitive and relatively consistent in uterine weight responses. Extracts have to be prepared free of gonadotrophins.

Wicks and Segal (205) found that uterine response to estradiol in immature female mice is inhibited significantly by concurrent injections of estriol with the amount of inhibition proportional to the amount of estriol added. In no case was there a positive additive action. Both wet and dry weights gave the same conclusion. It appears that estriol competes at a physiological site essential for the development of the usual estradiol stimulation of the uterus. This inhibition of estriol on estradiol also takes place in the rat uterus (94).

Drasher (57) administered one gamma of estradiol diproprionate to

castrated mice for three consecutive days. He found a considerable difference in the response of various strains as well as a difference in the speed of regression after injections ceased.

Various methods of estrogen administration have been used in the uterine weight response which is due to protoplasmic growth. Probably the best method is the subcutaneous injection of 0.05 ml (for mice) of some oil solvent for four consecutive days (44,65). Another method is the use of certain quantities of feed containing estrogen which is fed for ten days (142,143).

Szego and Roberts (184) postulate two possible alternatives for the estrogenic action on the uterus. The first is that the activity of specific enzyme systems are modified. The second is that the permeability of the target cell is altered by orientation at the surface of active molecules or by interaction with metabolic systems responsible for maintaining the state of the cell membrane. Possibly the action is due to a combination of the two.

Vaginal Cornification Assays--This type of assay which was developed by Allen and Doisy in 1923 has been used to a considerable extent for the accurate detection of minute quantities of estrogen (3,156). It is based on the fact that estrogen causes a multiplication of the epithelial cells followed by keratinization of the cells (39). Spayed animals (rats, mice, and guinea pigs) are normally used and are primed one week before the actual injections of estrogen to be tested. The assay is made by taking vaginal smears and examining them microscopically for cornified epithelial cells (60). Administration of the extrogens may be peroral, subcutaneous,

percutaneous, intraperitoneal, intravenous, or intravaginal. The intravaginal method has been found to be the most sensitive means of administration and may be accomplished by local application of estrogens, use of aqueous media, or use of intravaginal pellets (60).

Another assay is based on the duration of vaginal cornification in spayed mice. It is used to test long acting estrogens but is time consuming and uses large numbers of animals (55).

Other Biological Assays--Other tests for estrogen include the degree of vaginal opening at different time intervals (60,114) and an *in vitro* assay in which estrogen is incubated with DPN for 20 minutes. This results in reduction of the DPN to DPNH which is measured on a DU spectrophotometer at 340 millimicrons (77).

CHROMATOGRAPHY OF ESTROGEN

Several different types of chromatography have been tried to obtain separation of the various estrogens. One of the first methods used was to obtain fractionation with a column of activated alumina and different concentrations of methanol-benzene filtrate (178). Another early method was the use of Celite chromatography in which sodium hydroxide adsorbed on the Celite was the stationary phase and benzene the moving phase. In this method, the estrogens are obtained in certain parts of the filtrate (29). Recently, a number of workers have experimented with paper chromatography.

PAPER CHROMATOGRAPHY

There are essentially two different types of solvent systems which have been used. The first is the Zaffaroni system which is any solvent

system using a paper impregnated with a high boiling polar solvent as the stationary phase and a non-polar solvent saturated with the stationary phase as the mobile phase. Formamide-benzene and propylene glycol-toluene are two solvent sets that are used. This type of system permits the separation of large quantities of material. The development time is longer than the other system but it can be removed from the system for ultraviolet scanning and put back. The use of a volatile substance such as methanol with the stationary phase reduces the amount of this phase and thus speeds up and sharpens the resolution (155).

The other main system is the Bush system which employs a stationary phase such as water and methanol which is preferentially adsorbed onto the paper from the vapor of both phases during equilibration. The mobile phase consists of various non-polar solvents. A slightly elevated temperature is usually desirable. This system is faster in developing with the resolution achieved with the solvent front still on the paper (41, 42,155).

Theoretically, ascending, descending, and radial development of paper chromatograms should give equivalent results but practically, descending chromatography separates closely situated bonds better, ascending is technically simpler, while two dimensional gives better resolution (92).

Reineke (155) used toluene, Skellysolve C, methanol, and water in a 5:5:7:3 ratio and benzene, Skellysolve C, methanol, and water in a 333:667:800:200 ratio and obtained separation of estrone and estradiol. He used a phosphomolybdic acid reagent (sensitive above 16 micrograms) to detect the spots. The spots were bluish to black on a green background.

Burton et al (40) used benzene or toluene and formamide or propylene glycol with the mobile phase saturated with the stationary phase and descending chromatography. They lined the tank with filter paper and placed excess solvent in the bottom of the tank to saturate the atmosphere. Whatman No. 1 filter paper was used and cut into strips one centimeter wide which were dipped into the stationary solvent and excess liquid removed by blotting between filter paper. After separation, the strips were developed by use of alkaline silver nitrate, sodium thiosulfate, iodine reagent, or potassium permanganate.

Heftman (90) used a method involving ascending chromatography and states that the difference in distribution of the estrogens is due to the number of hydroxyl groups. By using a solvent system of petroleum ether, toluene, ethanol, and water in a 20:10:10:9 ratio, he also obtained good separation of alpha and beta-estradiol (91).

Beginning at the starting point the estrogens appear in the following order with the distance traveled dependent upon the solvents used: estriol, β-estradiol, α-estradiol, and estrone (90,91,93,155).

A number of the detection methods such as a ten per cent antimony chloride-chloroform solution (93) result in different colored spots for the different estrogens. Other detection methods are fuming sulfuric acid, antimony pentachloride, iodine in light petroleum, and bromine solution (14,41,42,93).

With many of the methods it is difficult to measure the estrogens quantitatively partly due to the fact that it is difficult to obtain samples from chromatograms that are free from contaminants. Steroids will

decompose when exposed to air or oxygen and to ultraviolet light as well as other forms of radiant energy (92,165).

The estrogenic extracts used in paper chromatography must be quite concentrated and purified (42,92).

The steroids may be eluted from the chromatograms by using absolute methanol, 95 % ethanol, or other suitable substance. If the quantitative measurement is made with a spectrophotometer, the eluate from a comparable piece of filter paper must also be measured and the proper adjustments made (92).

ESTROGEN EXTRACTION

Acid hydrolysis has normally been used on urine samples to break estrogenic compounds but Asdell and Mixner (10) found enzyme hydrolysis better than either acid or alkaline hydrolysis. Napp (130) used 15 volume per cent of concentrated HCl in hydrolyzing human urine for 10, 30, 60, 120, and 240 minutes and found that the most estrogen was obtained after two hours. Although the amounts of estrone and estradiol increased only up to 30 minutes that of estriol reached its maximum at two hours. He found no decrease of Kober chromogens by boiling up to four hours. Probably this was due to liberation and destruction of estrogen occurring at the same rate. Crystalline estrogen in alcohol added to water and boiled with HCl for up to two hours showed no loss. However, Velle (197,201) reports that refluxing cow urine with 15 volume per cent HCl for two hours leads to total destruction of estradiol although the estrone fraction remained about the same. Alpha-estradiol is almost totally destroyed in 30 minutes. He suggests using three volume per cent HCl for a period of 30

minutes.

Brown (35) found that 10 to 20 per cent of the estrogenic activity is lost during the acid hydrolysis of human urine. If the hydrolysis of conjugated estrogens is assumed complete, there is an overall recovery rate of 60 to 75 per cent for the whole extraction process. Brown et al (38) later obtained recovery rates from acid hydrolyzed urine varying from 75 to 90 per cent. For duplicate determinations, the lowest levels in micrograms per 24 hours distinguishable from zero using chemical methods are 0.64 for estrone, 0.60 for estradiol, and 0.84 for estriol. They also found that to have less than 25 per cent error for duplicate determinations 2.5 micrograms of estrone were needed, 2.4 micrograms for estradiol, and 3.3 for estriol.

Velle (198) added estradiol-17β and estradiol-17α to different samples of urine and then hydrolyzed with acid but found no conversion to estrone by the hydrolysis.

EXPERIMENTAL PROCEDURE

This study was initiated to determine the trends in the normal urinary estrogenic excretion rates of Holstein cows during estrus and at different stages of gestation. Quantitative data was obtained by use of bioassay, Kober color reaction, and fluorometry and the results compared. Thirty-five Holstein cows from the herd at Montana State College, Bozeman, Montana, were used. Urine was collected for 24 hours by use of a modification of the apparatus used by Smith et al (173). Collections were made at estrus and at 50, 100, 200, and 275 days after conception. The collections taken during gestation were made no more than five days either way from these dates. A 500 milliliter aliquot of each 24 hour urine collection was refrigerated, then analyzed within 24 hours. The urine was extracted in duplicate using a modification of the methods of Friedgood (75) and Stimmel (178). The procedure entails the following steps:

1. Two hundred and fifty milliliters of urine were filtered through glass wool into a flask.
2. Fifteen volume per cent of concentrated HCl was added and the mixture was hydrolyzed with a Glas-col heater for 18 minutes (boiling time at 5000 foot altitude). After removing from the heat, it was cooled in an ice bath.
3. The mixture was then extracted three times with 80 milliliters of ether (ether washed with one per cent $FeSO_4$ 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

nine per cent $NaHCO_3$ 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 water which was added to the KOH. The CCl_4 phase was discarded.
8. The KOH was washed once with 20 milliliters of ether and the ether discarded.
9. The KOH was acidified to pH-3 with six normal H_2SO_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 per cent $NaHCO_3$, two times with 30 milliliters of water, five times with 20 milliliters of two and one-half per cent of Na_2CO_3, and two times with 30 milliliters of water.
12. The ether was distilled to dryness under vacuum.
13. The residue was taken up in ten milliliters of 95 per cent ethanol and refrigerated.

As part of the study was to determine the quantities of α-estradiol, β-estradiol, and estrone excreted in the urine at the different stages of gestation and at estrus, it was necessary to obtain a separation of the three estrogens and eliminate as many contaminants as possible from the

urine extract.

To accomplish this separation, the paper chromatographic method developed by Burton et al (40) and modified by Axelrod (14) and Gorski (78) was used with a few modifications. In this procedure, the filter paper is treated with formamide and methanol with the formamide acting as the stationary solvent. Hexane and benzene in a 1:1 ratio equilibrated with formamide acts as the mobile phase with descending chromatography used. The detection method used was that of Jellinek (102). The chromatographic method used is as follows:

1. Whatman No. 1 filter paper was cut in strips three centimeters wide with a three millimeter strip removed between.
2. The paper strips were marked at six and eight centimeters from the top.
3. The strips were boiled in absolute methanol for one-half hour and then air dried.
4. The paper strips were next treated with formamide-methanol (1:1) by running through a trough containing about three milliliters of solution for each strip to be treated.
5. The strips were then blotted between sheets of filter paper to remove the excess liquid.
6. The urine extract in ethanol was divided into 0.8 milliliter portions and the ethanol evaporated.
7. The estrogenic residue was applied to the starting line (8 cm from the top) on paper strips by dissolving in three drops of ethyl acetate:methanol (5:1) solution once and two drops twice.

Application was made by the use of glass capillaries. Each application was made as a narrow line with the solvent allowed to dry thoroughly between applications.

8. The prepared strips were placed in a tank saturated with normal-hexane and benzene by lining the tank with filter paper and placing the solvents in the bottom. The strips were hung level and with the six centimeter line on the glass rod located over the edge of the trough.

9. Benzene and normal-hexane (1:1) equilibrated for several hours with formamide was used as the mobile solvent in the descending chromatographic procedure.

10. The separation time used was eleven hours.

11. The strips were dried overnight using a cold air fan.

12. The spots were developed by running the strips through a solution of one per cent ferric chloride and one per cent potassium ferric cyanide (1:1) on a watch glass.

13. The developed strips were rinsed in five per cent HCl and in water to remove the background material.

The position of the different estrogens on the chromatograms would vary slightly from one strip to another and would also vary from one time to the next. Probably these differences were due to slight differences in application and changes in atmospheric conditions. In the urine samples with lower quantities of estrogen, the spots were usually quite faint. In order to locate these spots as accurately as possible, reference strips containing added estrogen were included each time. By using

these strips and a one centimeter portion from each of those to be used for quantitative measurement the corresponding spots on the undeveloped portions were located. Each corresponding spot was then cut out and those in the alpha and beta-estradiol positions were eluted off with ten milliliters of absolute methanol. Each corresponding estrone spot was eluted with 20 milliliters of absolute methanol as the area was considerably larger. The methanol was evaporated from each sample in a water bath and under vacuum. The residue was taken up in about ten milliliters of absolute methanol with the flask being rinsed several times. The sample was divided into three parts for quantitative determinations.

Quantitative determinations on one part were made using the biological assay method developed by Evans et al (65). This is the four day immature mouse uterine weight method based on protoplasmic growth of the uterus. The mice used in this experiment were all received from a colony at the college and were kept in a small animal laboratory under controlled temperature and humidity. The mice were approximately ten grams in weight and two and one-half weeks of age when received. They were kept in groups of five or six to a cage. All the mice were fed ground Purina Laboratory Chow.

In developing the response curves, the increase in uterine weight expressed as a per cent of the body weight was used. This method was also used for "unknowns". To find the points on each curve, known quantities of crystalline estrogen dissolved in ether was added to peanut oil, mixed with the oil, and the ether evaporated off. The estrogen in the peanut oil was then injected subcutaneously into the mice. Each

mouse was injected with 0.05 milliliter of peanut oil for four consecutive days. On the fifth day, approximately 24 hours after the last injection, they were killed with chloroform, weighed, and the uteri dissected out, trimmed of extra tissues, and put into Bouin's solution. Twenty-four hours later the uteri were taken out of the solution, further trimmed, pressed between filter paper, and weighed on an analytical balance. The uterine weight was calculated as a per cent of the body weight. With the "unknowns" the methanol was evaporated off and the residue taken up in ether. The above procedure was then followed.

The control group for the response curves consisted of 74 mice while each point on the curves was determined by at least 12 mice and most of the points over 20. The quantity of each unknown sample was determined using about ten mice per group.

The second portion of the sample was measured quantitatively using the Kober color reaction as modified by Hunt et al (97). In using this assay, curves were established using known quantities of estrogen ranging from two to sixteen micrograms. The various reagents and chemicals used in the determinations were treated as follows: Absolute ethanol was purified by refluxing for 12 to 14 hours with zinc dust (50 g/liter of ethanol) and NaOH pellets (50 g/liter of ethanol) and distilling twice. The hydroquinone solution was made by dissolving 0.2 grams of hydroquinone in ten milliliters of purified ethanol. One liter of the Kober reagent for estrone was made by adding 660 milliliters of H_2SO_4 to 422 milliliters of water. Ten milligrams of $NaNO_3$ was added and dissolved and 20 milligrams of quinone which was dissolved by gentle warming. In the last step, 20

grams of hydroquinone is added and dissolved by warming and shaking. The solution was cooled, filtered through glass wool, and stored in a brown bottle. The estradiol Kober reagent was prepared in the same way except that 600 milliliters of H_2SO_4 and 482 milliliters of H_2O were used. The Kober reagent was always filtered through glass wool before being used and fresh hydroquinone solution was made the day it was to be used. In order to find the absorption peaks of estrone and estradiol, pure crystalline estrogen was added to purified ethanol and by using the method described below the samples were prepared for reading on the Beckman "B" spectrophotometer. The absorption peak for estrone was found to be 512 mμ for estrone and 515 mμ for α and β-estradiol.

Method of analysis using the Kober color reaction is as follows:

1. Two-tenths milliliter of hydroquinone solution added to the sample.
2. The solvent was evaporated from the sample in a water bath at 85 to 90°C.
3. Upon evaporation 2.6 milliliters of estradiol Kober reagent was added to the estradiol samples and 3.0 milliliters of estrone Kober reagent was added to the estrone samples. The tubes were capped with aluminum foil and placed in a boiling water bath for 20 minutes.
4. The tubes were shaken after two minutes and five minutes to effect solution and mixing.
5. The tubes were then placed in cold tap water for five minutes.
6. Seven-tenths milliliter of estradiol Kober reagent was added to

the estradiol samples and 0.3 milliliter of water to the estrone samples to bring the final volume to 3.3 milliliters and 60 per cent sulfuric acid.

7. After capping and shaking the tubes, they were placed in a boiling water bath for 15 minutes.

8. The tubes were then cooled in a water bath at 15°C. for 5 to 10 minutes and read at room temperature.

9. Readings for estrone were taken at 544, 512, and 480 mμ for estrone and at 548, 515, and 482 mμ for α and β-estradiol. Sixty per cent H_2SO_4 was used in the solvent cell.

In using the Kober reaction, one or two tubes were always included as reagent blanks as well as tubes representing certain portions of the filter paper which were included when unknowns were assayed from filter paper. This was done to take into account the effects of the filter paper on the spectrophotometric readings. Allen's (4) correction formula was applied to all the determinations.

The third portion of the sample was used for the fluorometric assay. The procedure is as follows:

1. The solvent was evaporated from the samples and ten milliliters of 75 per cent H_2SO_4 (freshly diluted) was added to each. The test tubes were then heated in a water bath at 80°C. for 30 minutes.

2. The fluorescence of the samples was measured with a Coleman Electronic Photofluorometer Model 12C with a lamp filter transmitting at a wave length of 436 mμ (accomplished by a Corning

#3389 and Corning #5113 glass filters) and a photo cell filter transmitting at 525 mμ (accomplished by a Baird 525 mμ interference filter and a Corning #3486 glass filter).

3. The fluorescence of the extract was then compared with the fluorescence given off by a standard of estrogen added to some of the tubes and the amount of estrogenic material present calculated.

As the fluorescence of estrogen is influenced by solvents used and by impurities, the samples to be measured fluorometrically were handled as follows to account for as many of these factors as possible. The sample to be measured fluorometrically was divided into six equal parts. Three were kept as the unknowns while known quantities of estrone or estradiol were added to the other three. This was done to account for as much of the internal quenching as possible. The effect of the filter paper was also accounted for in the final calculations.

The determinations for the two chemical methods were compared with the biological assay for each estrogen and the correlation of the values determined.

RESULTS

Response curves based on the increase in uterine weight of immature female mice expressed as a percent of the body weight were developed for alpha-estradiol, beta-estradiol, and estrone. Figure 1 shows the six point response curve developed for alpha-estradiol using pure crystalline alpha-estradiol.

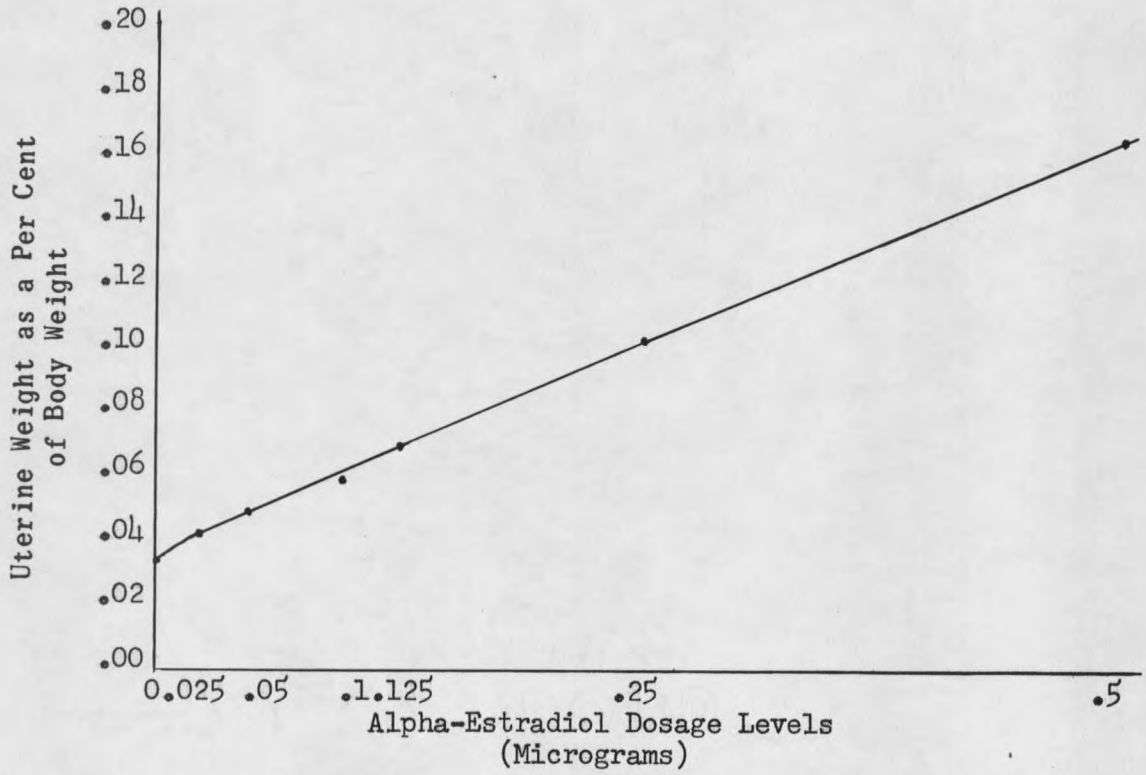


Figure 1. Uterine Weight Response Curve Expressed as a Per Cent of Body Weight of Immature Female Mice Given Six Different Dosages of Pure Crystalline Alpha-Estradiol in Peanut Oil Injection

A three point response curve was developed for beta-estradiol using pure crystalline beta-estradiol. This curve is shown in Figure 2.

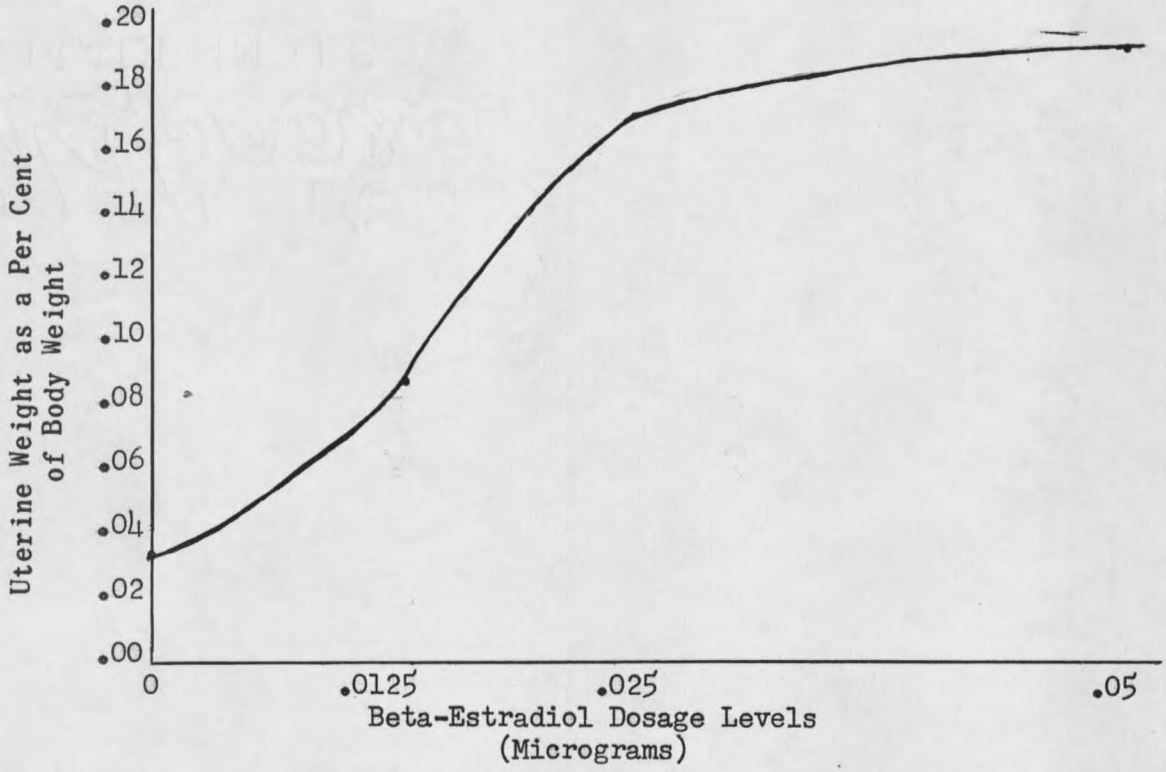


Figure 2. Uterine Weight Response Curve Expressed as a Per Cent of Body Weight of Immature Female Mice Given Three Different Dosages of Pure Crystalline Beta-Estradiol in Peanut Oil Injection

A three point response curve was developed for estrone using pure crystalline estrone. This curve is shown in Figure 3.

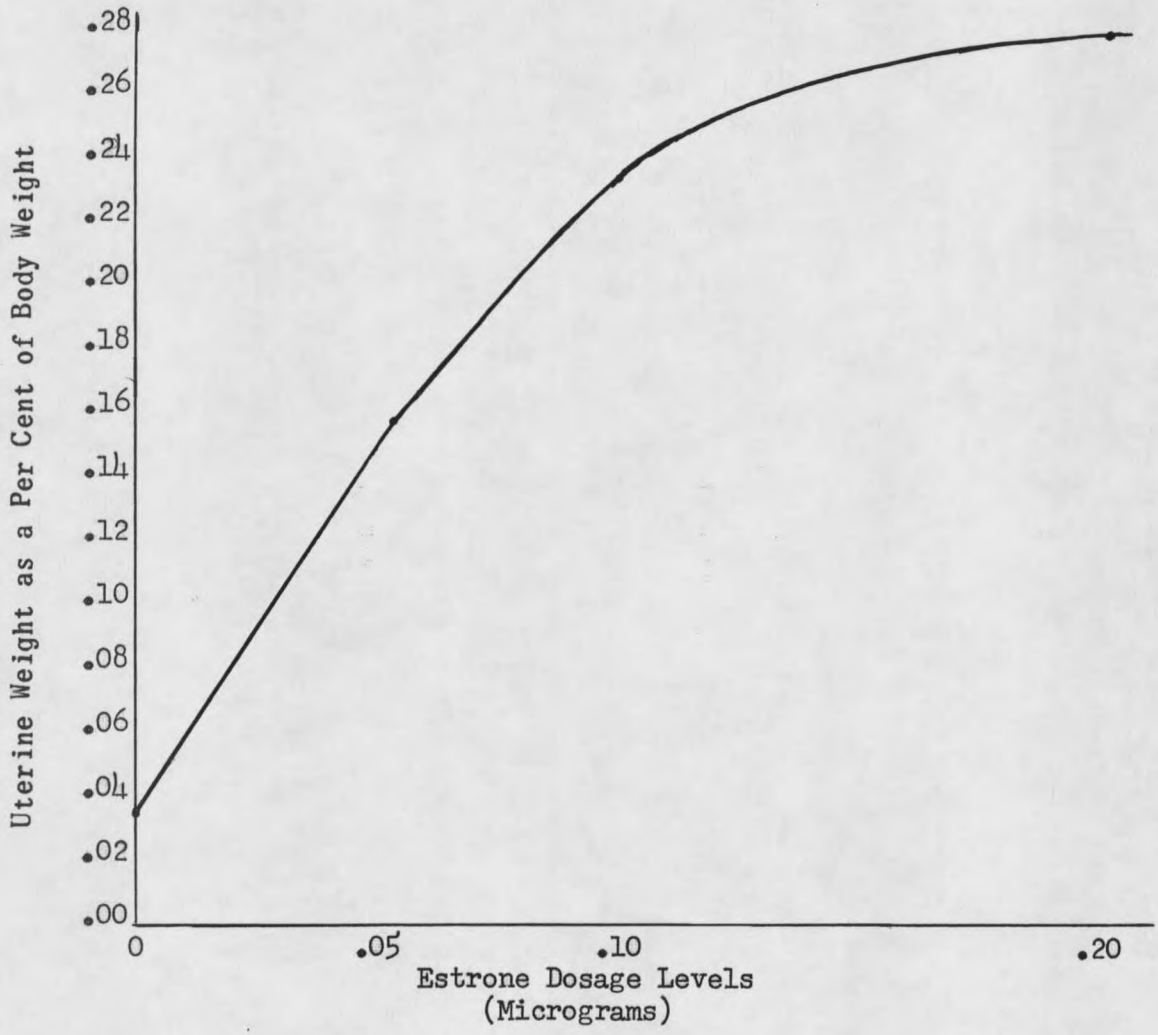


Figure 3. Uterine Weight Response Curve Expressed as a Per Cent of Body Weight of Immature Female Mice Given Three Different Dosages of Pure Crystalline Estrone in Peanut Oil Injection

Standard Curves were also established for estrone, alpha-estradiol, and beta-estradiol for the Kober reaction using the pure crystalline substances. These curves are shown in Figure 4.

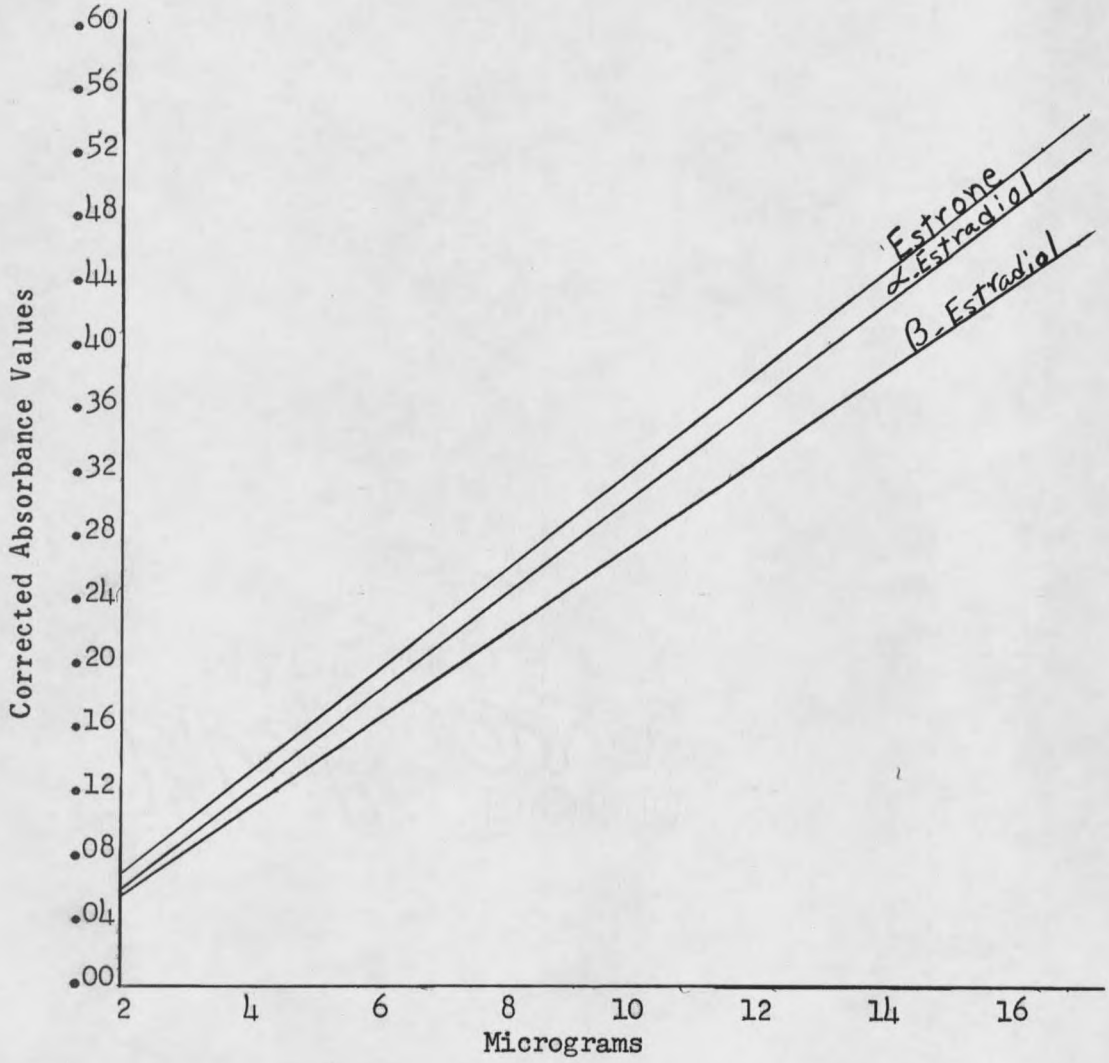


Figure 4. Corrected Absorbance Readings of Pure Crystalline Estrogens Using the Beckman "B" Spectrophotometer

In developing the paper chromatographic separation of the estrogens, a number of different solvents were tried. Toluene on propylene-glycol treated paper was tried but it did not give very good separation or good resolution in separating estrogen from the urine impurities. Petroleum ether and benzene on formamide treated paper gave quite fast separation (about five hours) but there was a considerable amount of tailing of the spots. Hexane and benzene on formamide treated paper gave good separation and resolution and, although the slowest of those tried (eleven hours), was the system used.

In all the work with the urine extracts, the spots corresponding to the alpha and beta-estradiol and estrone spots on the reference strips were eluted with the estrogen in the eluate measured by the Kober reaction, fluorometric assay, and bioassay.

In measuring the different estrogens, it was found that there was an individual variation between cows. The following three tables show the amounts of estrone, α-estradiol, and β-estradiol excreted per 100 pounds of body weight per 24 hour period for each cow using the three methods of assay.

TABLE III. MICROGRAMS OF ESTROGEN EXCRETED PER 100 POUNDS OF BODY WEIGHT PER 24 HOUR PERIOD AS DETERMINED BY BIOASSAY

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
540	Estrus	3.1	2.6	1.9	7.6
237	"	11.7	0.9	4.4	17.0
561	"	21.0	6.6	5.8	33.4
249	"	23.6	1.0	6.4	31.0
508	"	14.1	2.0	9.1	25.2
394	"	0.0	0.5	0.7	1.2
484	"	95.8	1.8	4.4	102.0
Mean		24.2	2.2	4.7	31.1
Standard Deviation		30.3	1.9	2.6	31.0
525	50 days	15.5	3.4	2.9	21.8
499	"	0.0	0.0	1.7	1.7
434	"	24.6	2.3	4.8	31.7
400	"	8.4	0.0	2.0	10.4
630	"	2.6	0.8	1.8	5.2
291	"	0.0	0.9	7.6	8.5
618	"	11.9	0.8	4.9	17.6
Mean		9.0	1.2	3.7	13.9
Standard Deviation		8.4	1.2	2.0	9.7
471	100 days	12.0	0.8	8.4	21.2
605	"	2.9	0.7	0.8	4.4
382	"	37.1	2.1	10.7	49.9
508	"	7.9	1.5	12.8	22.2
456	"	9.2	4.0	3.8	17.0
612	"	28.8	1.5	24.4	54.7
623	"	23.4	1.0	2.5	26.9
Mean		17.3	1.7	9.1	28.1
Standard Deviation		11.7	1.1	7.5	16.6

TABLE III. (CONTINUED)

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
448	200 days	93.1	6.7	44.4	144.2
503	"	23.8	1.1	36.3	61.2
353	"	45.7	9.3	46.3	101.3
599	"	108.1	24.3	22.0	154.4
602	"	15.4	3.8	70.5	89.7
545	"	69.6	3.3	62.2	135.1
383	"	24.3	1.4	26.8	52.5
Mean		54.3	7.1	44.1	105.5
Standard Deviation		33.9	7.5	16.4	37.4
530	275 days	180.2	24.5	179.4	384.1
501	"	301.8	15.5	54.4	371.1
581	"	104.7	29.0	29.5	163.2
419	"	417.8	14.3	68.3	500.4
536	"	188.6	6.6	68.9	264.1
548	"	71.5	12.0	81.0	164.5
383	"	224.3	17.3	75.9	317.5
Mean		212.7	17.0	79.6	309.3
Standard Deviation		109.1	7.0	43.7	113.6

TABLE IV. MICROGRAMS OF ESTROGEN EXCRETED PER 100 POUNDS OF BODY WEIGHT PER 24 HOUR PERIOD AS DETERMINED BY THE KOBER REACTION

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
540	Estrus	23.2	4.2	67.2	94.6
237	"	23.6	6.1	15.8	45.5
561	"	31.1	15.8	263.7	310.6
249	"	7.3	0.9	52.9	61.1
508	"	3.9	5.5	242.1	251.5
394	"	0.5	0.0	83.5	84.0
484	"	5.2	4.2	117.7	127.1
Mean		13.5	5.2	120.4	139.1
Standard Deviation		11.2	4.8	88.7	94.2
525	50 days	11.5	2.5	65.5	79.5
499	"	4.0	6.6	54.5	65.1
434	"	0.0	1.3	79.6	80.9
400	"	6.2	0.0	15.1	21.3
630	"	9.5	5.7	54.5	69.7
291	"	11.2	8.3	169.9	189.4
618	"	2.4	2.7	67.7	72.8
Mean		6.4	3.9	72.4	82.7
Standard Deviation		4.2	2.8	44.0	47.4
471	100 days	11.8	6.7	126.7	145.2
605	"	2.2	0.9	7.9	11.0
382	"	13.6	4.6	118.6	136.8
508	"	5.3	2.6	119.6	127.5
456	"	2.6	1.7	18.7	23.0
612	"	11.0	2.1	26.9	40.0
623	"	3.0	3.4	49.6	56.0
Mean		7.1	3.1	66.9	77.1
Standard Deviation		4.5	1.8	48.9	53.3

TABLE IV. (CONTINUED)

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
448	200 days	55.9	14.0	213.1	283.0
503	"	28.8	5.0	107.3	141.1
353	"	38.6	10.7	253.1	302.4
599	"	30.6	29.3	47.0	106.9
602	"	36.8	7.0	264.8	308.6
545	"	16.4	2.2	96.6	115.2
383	"	25.4	0.5	29.5	55.4
Mean		33.2	9.8	144.5	187.5
Standard Deviation		11.5	9.1	90.5	98.8
530	275 days	129.0	52.3	311.8	493.1
501	"	169.9	13.6	225.5	409.0
581	"	371.3	23.4	232.5	627.2
419	"	269.6	15.8	340.3	625.7
536	"	217.9	6.3	331.5	555.7
548	"	160.6	11.1	281.9	453.6
383	"	127.9	14.1	90.8	232.8
Mean		206.6	19.5	259.2	485.3
Standard Deviation		81.9	14.2	80.4	128.4

TABLE V. MICROGRAMS OF ESTROGEN EXCRETED PER 100 POUNDS OF BODY WEIGHT PER 24 HOUR PERIOD AS DETERMINED BY FLUORESCENCE

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
540	Estrus	20.0	13.6	80.4	114.0
237	"	8.8	8.1	30.9	47.8
561	"	30.1	8.7	49.2	88.0
249	"	10.4	23.4	29.1	62.9
508	"	14.2	14.2	51.0	79.4
394	"	0.6	2.4	13.3	16.3
484	"	0.0	2.8	4.2	7.0
Mean		12.0	10.5	36.9	59.4
Standard Deviation		9.9	6.8	23.8	35.8
525	50 days	3.8	11.9	12.8	28.5
499	"	0.0	0.0	3.1	3.1
434	"	18.1	8.0	34.1	60.2
400	"	9.4	7.8	22.1	39.3
630	"	1.0	4.2	1.2	6.4
291	"	4.7	11.2	28.6	44.5
618	"	0.0	4.9	12.2	17.1
Mean		5.3	6.9	16.3	28.5
Standard Deviation		6.1	3.9	11.5	19.4
471	100 days	17.9	16.6	2.4	36.9
605	"	0.0	4.9	2.1	7.0
382	"	21.4	21.0	40.6	83.0
508	"	22.2	11.3	50.0	83.5
456	"	18.2	7.9	37.2	63.3
612	"	10.3	6.4	31.3	48.0
623	"	3.2	5.5	9.4	18.1
Mean		13.3	10.5	24.7	48.5
Standard Deviation		8.3	5.7	18.3	27.8

TABLE V. (CONTINUED)

Cow Number	Period	Alpha-Estradiol	Beta-Estradiol	Estrone	Total
448	200 days	28.9	8.9	79.1	116.9
503	"	14.4	4.0	44.3	62.7
353	"	15.0	5.8	358.8	379.6
599	"	16.7	28.9	31.7	77.3
602	"	13.6	8.5	86.6	108.7
545	"	6.2	4.1	27.8	38.1
383	"	57.7	19.4	121.1	198.2
Mean		21.8	11.4	107.1	140.3
Standard Deviation		15.9	8.7	107.4	108.6
530	275 days	No Data			
501	"	72.4	20.1	149.0	241.5
581	"	227.2	22.3	112.6	362.1
419	"	415.4	34.3	302.4	752.1
536	"	100.3	11.8	207.9	320.0
548	"	43.4	9.9	169.2	222.5
383	"	19.4	8.1	66.6	94.1
Mean		146.4	17.8	168.0	332.2
Standard Deviation		137.4	8.4	74.6	205.9

A comparison of the figures from the preceding three tables gives the correlation coefficients and regression coefficients presented in Table VI.

TABLE VI. CORRELATION COEFFICIENTS AND REGRESSION COEFFICIENTS OF THE THREE METHODS OF ESTROGEN ASSAY USED

Compound	Methods Compared	Correlation Coefficient	Regression Coefficient*
Alpha-Estradiol	Bioassay & Kober Reaction	0.7349**	0.6786
" "	Bioassay & Fluorometric	0.7481**	0.6311
Beta Estradiol	Bioassay & Kober Reaction	0.8452**	1.1100
" "	Bioassay & Fluorometric	0.5494**	0.6099
Estrone	Bioassay & Kober Reaction	0.6456**	1.7881
"	Bioassay & Fluorometric	0.6542**	2.0661

* Regression of Chemical Assay on Biological Assay
** Significant ($P < .01$)

Figures 5, 6, and 7 show the changes in urinary estrogen excretion during gestation and the excretion during estrus as measured by the three methods of assay used. The means from Tables III, IV, and V were used to determine the points for each period. All three methods show the same trend except for the quantities of urinary estrogen excreted during the 50 day and 100 day periods as determined by the Kober reaction.

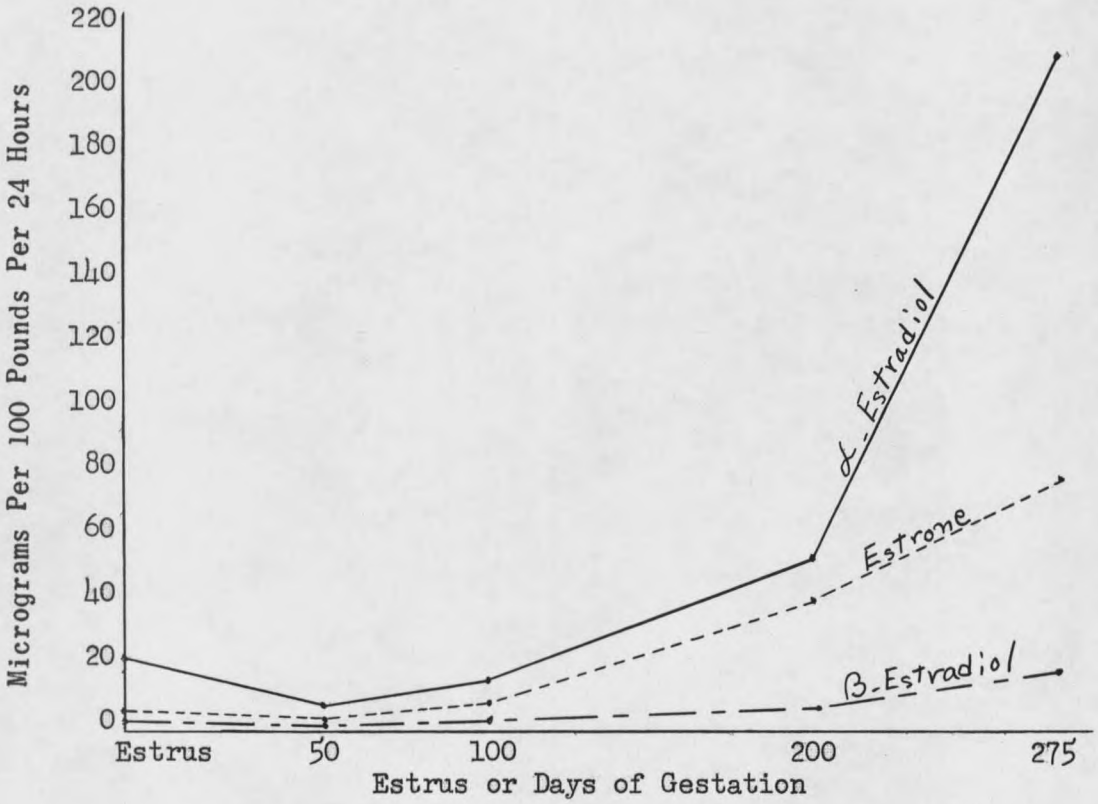


Figure 5. Pattern of Urinary Estrogen Excretion During Gestation and at Estrus as Determined by Bioassay

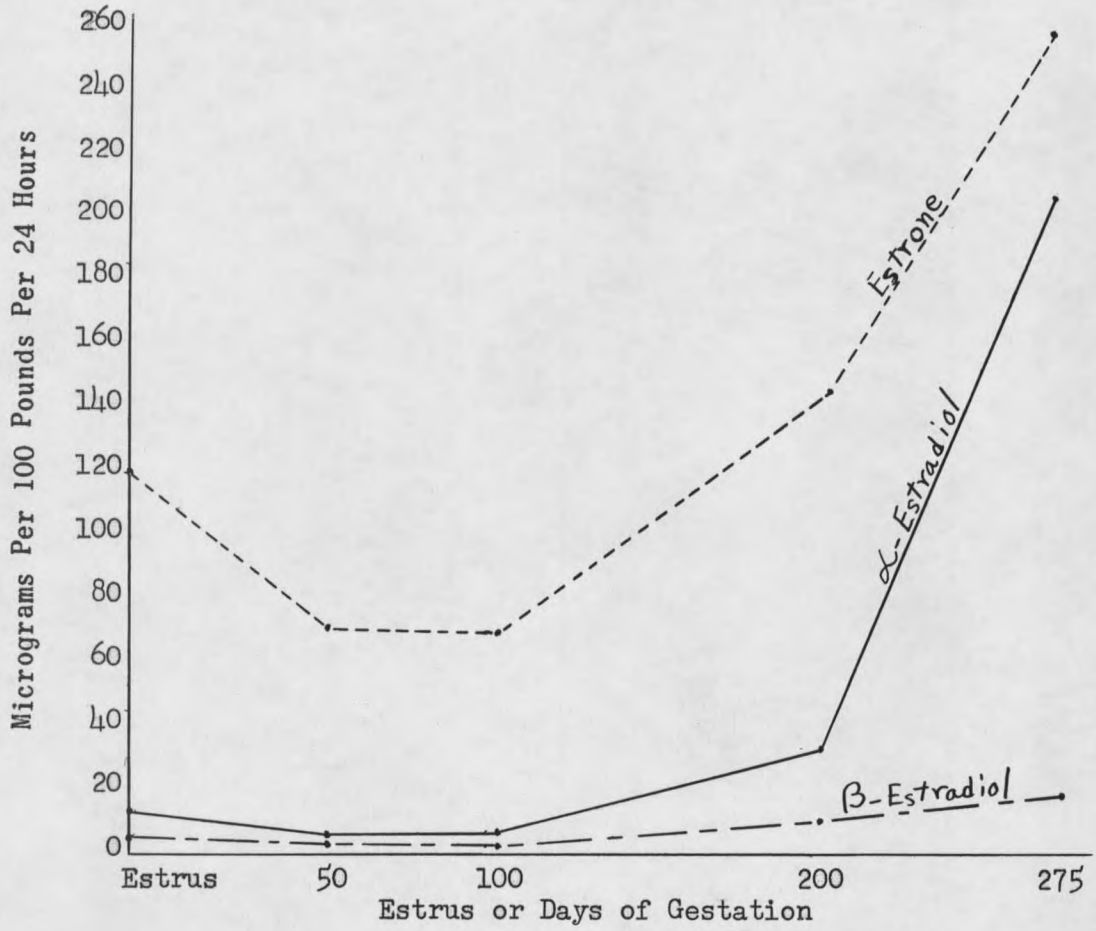


Figure 6. Pattern of Urinary Estrogen Excretion During Gestation and at Estrus as Determined by the Kober Reaction

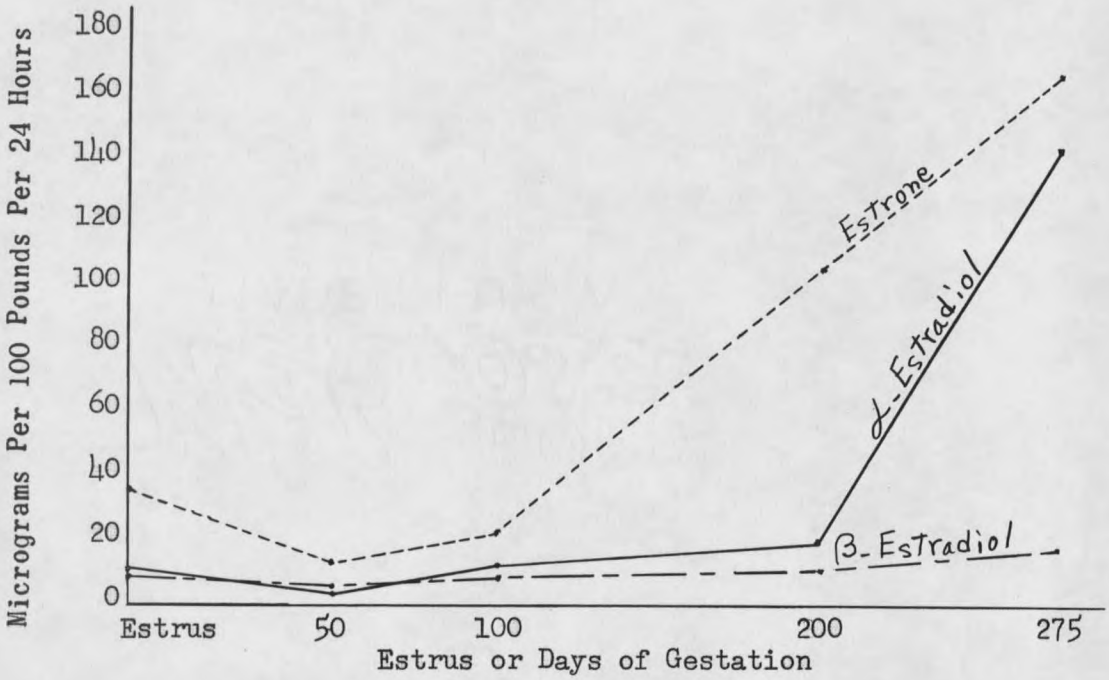


Figure 7. Pattern of Urinary Estrogen Excretion During Gestation and at Estrus as Determined by Fluorometric Analysis

Of the periods measured, 50 days of gestation seems to be the period at which the least quantity of estrogen is excreted while the estrogen excretion at 100 days of gestation and at estrus are quite similar. There is a very large increase in estrogen excretion, particularly α-estradiol, during the last 70 to 80 days of gestation.

Table VII shows the average amount of each estrogen excreted during the different periods on a per liter basis as determined by bioassay while Table VIII shows each estrogen excretion average for a 24 hour period also as determined by bioassay.

TABLE VII. MICROGRAMS OF EACH ESTROGEN EXCRETED PER LITER DURING DIFFERENT STAGES OF GESTATION AND AT ESTRUS AS DETERMINED BY BIOASSAY

Period	Alpha-Estradiol	Beta-Estradiol	Estrone
Estrus	21.4	1.9	4.2
50 days	8.8	1.1	3.6
100 days	16.5	1.6	7.9
200 days	73.7	10.9	49.9
275 days	191.3	17.0	70.5

TABLE VIII. MICROGRAMS OF EACH ESTROGEN EXCRETED PER 24 HOURS DURING DIFFERENT STAGES OF GESTATION AND AT ESTRUS AS DETERMINED BY BIOASSAY

Period	Alpha-Estradiol	Beta-Estradiol	Estrone
Estrus	284.9	25.0	57.6
50 days	108.7	14.3	46.7
100 days	207.5	21.7	107.5
200 days	757.6	98.0	619.3
275 days	2,871.4	231.2	1,066.2

DISCUSSION AND CONCLUSIONS

The part of this study concerned with the quantities of estrogen excreted during different periods of gestation and at estrus indicated that the quantities excreted are low at 50 days with only a slight increase from then until 100 days. This probably is to allow for the process of implacentation. In the cow, a loose attachment of the ovum to the uterine wall begins to take place at about the 12th day after ovulation. Placental plates begin to appear at about 30 days and firm attachment is thought to begin at this time. Villi begin to develop on the chorionic plates between 30 and 35 days with attachment occurring at about the 36th day (8). This attachment period is quite critical to the developing fetus as it is known that excess amounts of estrogen during the early stages of gestation will prevent nidation of the ovum, cause fetal death, or cause abortion depending upon when the estrogen imbalance occurs (187,188).

After 100 days of gestation, the increase in the excretion of estrogen in the urine became much more pronounced with a very sharp increase occurring sometime around 200 days or shortly thereafter. Most of the increase seems to be in the metabolic products of estradiol-17β ; that is estradiol-17α and estrone. Secretion of estrogen by the placenta probably begins to occur around this 100 day period to account for much of the increase. Part of the increase is probably due to increased ovarian activity as removal of the ovaries during pregnancy results in lowered estrogen excretion (2,145).

The differences between the excretion of the various estrogens at estrus and at 50 days of gestation were not significant which was also

true of the differences between 50 and 100 days of gestation. The alpha-estradiol increase in urine was significant from 100 to 200 days ($P < .05$) and from 200 to 275 days ($P < .01$). The beta-estradiol excretion rate increase was significant from 200 to 275 days ($P < .05$). The increase in estrone excretion was significant from 100 to 200 days ($P < .01$) but not from 200 to 275 days. When considering the total estrogen the differences between the 100 and 200 day periods and the 200 and 275 day periods were significant ($P < .01$). Even though some of the means between the non-significant groups were quite different, they were not significant due to the large amount of variation between animals.

From the experimental results, it seems there are two possible theories as to why estrone and α-estradiol rates of excretion have more pronounced changes than the β-estradiol excretion rate. First, it is possible that the metabolic processes whereby α-estradiol is changed to the less potent estrogens--estrone and α-estradiol--are speeded up as the placenta begins secreting estrogen. Velle (198) states that β-estradiol is rapidly changed to α-estradiol and as the metabolic change probably goes via estrone this could account for the large increases of estrone and α-estradiol. Second, it could be that much of the estrogen produced by the placenta is in the form of estrone and/or α-estradiol. Both α-estradiol and estrone have been isolated from human placenta (85) while estrone has been isolated from the placenta of the cow (138).

The rate of excretion on a per liter basis at the 275 day period was 0.19 mg of α-estradiol, 0.02 mg of β-estradiol, and 0.07 mg of estrone. When the computation is put on a 24 hour basis, the excretion rate was

2.87 mg for α-estradiol, 0.23 mg for β-estradiol, and 1.07 mg for estrone. These are the results using the biological assay and substantiate those of Velle (197) for α-estradiol and estrone excretion of the cow during the ninth month of pregnancy. He found 0.10 to 0.15 mg of estrone and 0.15 to 0.20 mg of α-estradiol per liter of urine and when put on a 24 hour basis 0.8 to 1.0 mg of estrone and 2.0 to 2.5 mg of α-estradiol.

Nearly all of the chromatograms which were developed in this study revealed spots in positions corresponding to α and β-estradiol and estrone spots on the reference chromatograms. In the chromatographic separation of the estrogens from the urine extract, identification of the estrogens was made by the corresponding positions on the chromatogram. By using various combinations of known estrogens, it was found that the estrogen added to a strip could be predicted very accurately by determining the spot at which it appeared on the chromatogram. Beta-estradiol appeared near the top closely followed by alpha-estradiol with estrone about two-thirds of the way down the strip. Another factor which supports the idea that the spots on the strips with the urine extracts corresponding to the α and β-estradiol on the reference strips were actually α and β-estradiol is that the values as determined by bioassay and by the Kober reaction agreed quite closely. This would not have been the case if they had been the same compound since there are pronounced differences in uterine response to the two estradiols. Only the urine samples taken from cows in the early stages of gestation or at estrus failed to show all three spots and this was probably due to the small quantities of estrogen present or the fact that there was none at all. As there was always a

definite uterine response when the spots were fairly visible, it seems quite evident that all of the three estrogens are normally present in the urine of cows in late gestation and usually during estrus and in the early stages of gestation.

In developing the bioassay curves, the non-injected mice (control group) were found to have a uterine weight that was 0.033 per cent of the body weight. The mice injected with three levels of pure estrone gave the following per cents (uterine weight/body weight): .05 micrograms--.155 %, .10 micrograms--.231 %, and .20 micrograms--.267 %. The mice injected with pure α-estradiol gave the following results: .025 micrograms--.041 %, .05 micrograms--.045 %, .10 micrograms--.058 %, .125 micrograms--.065 %, .25 micrograms--.102 %, + .50 micrograms--.168 %. Injection of pure β-estradiol into the mice gave the following precentages: .0125 micrograms--.087 %, .025 micrograms--.172 %, and .05 micrograms--.190 %. When the uterine response reached .037 % of the body weight, the response was significant. ($P < .05$).

The other part of this study involved the comparison of the Kober reaction and fluorometric analysis with bioassay of estrogen in an attempt to find a chemical method that might be suitable for measuring urinary estrogens. All of the comparisons made had significant correlations. A comparison of the Kober reaction with bioassay on β-estradiol gave the best correlation--0.8452 while fluorometric analysis was the lowest, probably due to the fact that more of the urinary contaminants were present in the β-estradiol eluate due to its nearness to the starting line on the chromatogram. Fluorometric assays are affected considerably by

urinary contaminants (17,19,62,129). The spectrophotometric readings and bioassay did not give as high a correlation for α-estradiol (0.7349) as β-estradiol probably due to two facts: One is that there is much more chance for error in using this bioassay curve as only a small change in uterine weight per cent of the body weight results in a large change in the dosage due to the small response. Another factor that might cause more error here is that the spot usually was a little larger resulting in more contaminants from the filter paper in the eluate. The lower correlation values for both chemical assays when compared to bioassay for estrone is due partly to the large area of filter paper that was eluted to obtain the estrone and thus the possibility of more error in the calculation of the filter paper contaminants. Also, in the evaporation of the solvent there was always some that would not evaporate probably due to the filter paper contaminants. It has been reported (96) that incomplete evaporation of the solvent blanks before development of the Kober color cause erroneously high readings, as much as 40 per cent for 0.05 milliliter of solvent remaining. This would also account for the fact that the bioassay determinations were always lower for estrone than the determinations from the Kober reaction. The α-estradiol determinations by bioassay were usually a little higher than those made by the Kober reaction which might have been due to small quantities of β-estradiol in the α-estradiol eluate. Due to the greater potency of β-estradiol, this would cause the α-estradiol determinations to be too high.

Although the bioassay method was used as the standard and the chemical assays compared to it, this method also is susceptible to error as

are all biological assays. There is variation among individual animals due to genetic factors, environment, age, and weight. If larger numbers of animals are used, the accuracy of bioassay methods is increased.

As was mentioned in the literature review, the more active form of estradiol is now known as beta-estradiol, whereas in the older literature it was known as alpha-estradiol (84). Part of the contradiction in the literature as to the potency and effects of these two estrogens is probably due to confusion as to which is the alpha and which is the beta form. One research worker (78) found it necessary to order from four different chemical distributors before he obtained pure α-estradiol. They received β-estradiol from three of the firms instead of the α-estradiol.

SUMMARY

Aliquots of twenty-four hour urine collections from cows during estrus and at 50, 100, 200, and 275 days of gestation were extracted chemically for estrogen. The extracts were assayed by bioassay using the four day immature mouse uterine weight method and by two chemical methods--fluorometric assay and the Kober reaction. Estrogenic potency for each estrogen was calculated as micrograms per 100 pounds of body weight per 24 hour period.

Separation of the three estrogens was accomplished by using descending paper chromatography with normal-hexane and benzene as the mobile solvents and formamide as the stationary solvent.

Comparison of the two chemical assays with bioassay for the three estrogens (α-estradiol, β-estradiol, and estrone) gave significant correlations ($P < .01$) for all comparisons.

Of the periods measured, estrogen excretion of the three estrogens was at the lowest point at 50 days of gestation. The excretion rates at 100 days and at estrus were nearly the same with the amounts approximately twice that of 50 days. After 100 days of gestation, the increase in urinary estrogen excretion becomes more pronounced and is at quite an accelerated rate during the last stages of pregnancy. The most pronounced increase is in the alpha-estradiol excretion although estrone excretion is also considerably higher. Beta-estradiol excretion becomes higher as gestation progresses but the increase is at a much less rapid rate than with the other two estrogens. The increase of excretion of each estrogen was significant ($P < .01$) from the low point of 50 days to 275 days of

gestation. The differences between 200 and 275 days for α-estradiol and 100 to 200 days for estrone were highly significant ($P < .01$). The increase of α-estradiol excretion from 100 to 200 days and β-estradiol from 200 to 275 days was significant ($P < .05$). None of the other increases between periods were significant.

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