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

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

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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.

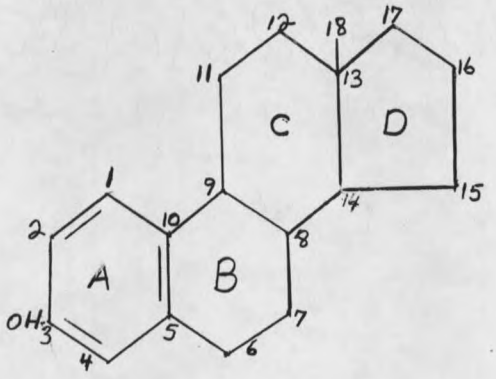
REVIEW OF LITERATURE

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).

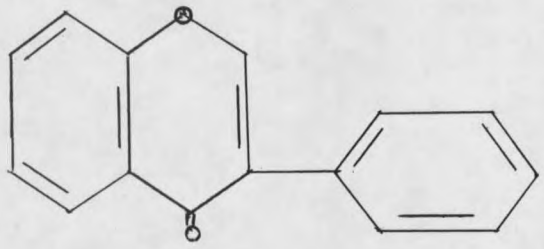
STRUCTURE OF ESTROGENS

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:

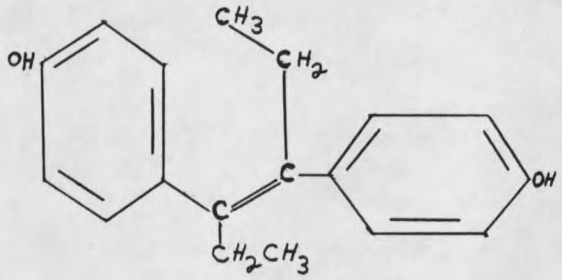


Ring A is phenolic which is different from other steroid sex hormones and there is a single methyl group at position 13. Estriol is considered to be a "strong" phenol while estrone and estradiol are "weak" phenols (60, 85).

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.