URINARY EXCRETION OF ESTROGENIC COMPOUNDS DURING ESTRUS AND GESTATION BY THE BOVINE AS DETERMINED BY THREE ASSAY METHODS

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SUMMARY

Aliquots of 24-hr urine collections from cows during estrus and at the 50th, 100th, 200th, and 275th days of gestation were extracted and assayed for the estrogenic content by the Kober color reaction, fluorometrically, and with bioassay. The bioassay procedure gave results for estradiol-17α that averaged 1.19 times higher than those obtained by the Kober color reaction and 1.74 times higher than the fluorometric results. However, in measuring estradiol-17β, the bioassay results were 0.7 times greater than the Kober results and 0.54 times greater than the fluorometric results. The greatest differences between the methods were revealed in measuring urinary estrone, in that the bioassay results averaged only 0.21 times greater than the Kober results and 0.42 times higher than the fluorometric results.

Excretion of the three estrogens was lowest at 50 days, but after 100 days of gestation an increase in urinary estrogenic excretion occurred and it was at an accelerated rate during the last stages of pregnancy. The most pronounced increase was in the excretion of estradiol-17α, although estrone excretion was also considerably higher during the latter part of gestation. The urinary excretion of estradiol-17β increased at a much slower rate than did that of two other estrogens.

Reproductive failure in dairy cows remains an important problem, even though much has been done in the past decade to reduce loss from this cause. There are many instances of infertility that cannot be traced to disease or to abnormalities of the reproductive organs. It is possible that many of these cases are due to hormone imbalances.

Though bovine fetal cotyledons contain measurable quantities of estrone, estradiol-17α, and estradiol-17β (20), at the present state of our knowledge bovine urine appears to be the best material from which an assessment of estrogen levels at various reproductive states can be made. Estrone and estradiol-17α have definitely been identified (13, 15, 21, 22) and estradiol-17β is probably present (14). Recently, Hunt et al. (11) have presented data which lend encouragement to the use of urinary values. These workers injected 16-C14 estrone into a luteal-phase heifer using rapid I.V. injection of 5 mg in one experiment and 0.4 mg over a 2-hr period in a second experiment. In each experiment, the ratio of excreted radioactive estrogens in feces and urine was similar. This suggests that at physiological levels one may expect a near-constant ratio between feces and urine and that urinary estrogens may provide a reliable means of evaluating rates of estrogen metabolism during different reproductive states.

Though methods are not yet entirely satisfactory, considerable progress has been made relative to extraction, purification, and separation of the estrogens (1, 2, 4, 7, 10, 14, 16, 17). Previous work has consistently shown the presence of estrogenic activity in extracts of cow urine, with levels increasing markedly during pregnancy. In 1930, Turner et al. (19) reported that they had found 11 rat units per liter in nonpregnant cow urine. Gorski et al. (9), using bioassay, observed from 73 to 173 µg of estrone equivalent in 24-hr urine samples from nonpregnant cows.

Levels in late pregnancy urine are 10 to 15 times higher (16, 22). Velle (21) reported that the estrogenic activity of cow urine begins to increase about the 80th day of gestation.
He found an average of 0.10 to 0.15 mg of estrone and 0.15 to 0.20 mg of estradiol-17α per liter of urine near the end of pregnancy. This amounted to a 24-hr excretion rate of 0.8 to 1.0 mg of estrone and 2.0 to 2.5 mg of estradiol-17α.

It was the purpose of this study to compare the Kober color reaction, the fluorometric, and the bioassay methods of assay to determine the quantities of estradiol-17α, estradiol-17β, and estrone excreted via the urine by the cow at estrus and at various times during gestation.

**EXPERIMENTAL PROCEDURE**

Twenty-four-hour urine samples were collected from seven Holstein cows at time of estrus and at 50, 100, 200, and 275 days of gestation. The urine samples were collected by an apparatus similar to that described by Goriski et al. (8). At the end of each 24-hr collection period, the total quantity of urine voided was measured and a 500-ml aliquot taken to the laboratory for analysis. The urine samples were extracted in duplicate, using the methods of Friedgood (7) and Stimmel (17), as outlined by Smith et al. (16).

After the extraction was completed the urine extract was separated into fractions corresponding to estradiol-17α, estradiol-17β, and estrone. This was accomplished by use of the paper chromatographic method of Burton et al. (4), modified by Axelrod (2). The detection method used was that of Jelinek (12). To locate the spots containing the estrogen, reference strips containing added estrogen standards were included in each trial. The reference strips and a portion 1 cm wide from the length of each strip containing the unknown estrogenic substances were developed to locate the position of the estrogenic compounds. The areas of the undeveloped strip that corresponded to the estrogenic compounds on the developed portions were cut out and the estrogen eluted from the filter paper with absolute methanol. The eluate was divided into three parts for quantitative measurement of the estrogens by means of bioassay, the Kober color reaction, and by fluorometry.

The bioassay method was the four-day immature mouse uterine weight method developed by Evans et al. (6). The uterine weight was expressed as a per cent of the body weight. The estrogen to be measured by bioassay was taken up in peanut oil and injected subcutaneously. Standard curves containing four points for the two estradiols and six points for estrone were used to determine the quantities of the unknown samples. There were twelve mice per dosage level of the standards, as well as for each of the unknowns.

The portion developed by the Kober reaction as modified by Hunt et al. (10) was measured quantitatively with a Beckman B spectrophotometer. Absorption readings for estrone were made at 544, 512, and 480 μμ. Readings for estradiol-17α and estradiol-17β were made at 548, 515, and 482 μμ. Allen’s (1) correction formula was applied to all determinations. Effects of the filter paper on the estrogens were also considered.

Fluorescence was measured with a Coleman Electronic Photofluorometer Model 12C, using Corning #3389 and Corning #5113 glass filters for the lamp filter and a Baird 525 m/z interference filter and a Corning #3486 glass filter for the photo-cell filter. The internal Standard method was used to account for the quenching effect.

**RESULTS AND DISCUSSION**

Results of this study are summarized in Table 1 and the trends in urinary estrogenic excretion as the gestation period progresses can be seen in Figures 1, 2, and 3.

**Assay of estradiol-17α.** Figure 1 shows the...
results of measuring estradiol-17α by the three different methods. There was some difference in magnitude of the results, but essentially the same trends persist. The bioassay procedure gave results in measuring the quantities of estradiol-17α that averaged 1.19 times higher than the results obtained by the Kober color reaction \((r = 0.74)\) and 1.74 times greater than the fluorometric results \((r = 0.75)\).

Assay of estradiol-17β. Figure 2 shows the results of measuring estradiol-17β by the three different methods. The levels of this compound were relatively low and some differences were noted in the magnitude of the results, but again essentially the same trends are shown by the three methods of analysis. The bioassay procedure gave results in measuring the quantities of estradiol-17β that averaged 0.70 times higher than the results obtained by the Kober color reaction \((r = 0.85)\) and 0.54 times greater than the fluorometric results \((r = 0.55)\).

Assay of estrone. Figure 3 shows the results of measuring estrone by the three different methods. A greater difference was noted in measuring estrone than in measuring the two estradiol isomers. Although similar trends are shown, the bioassay method of measurement yielded results that averaged only 0.21 times higher than those results obtained by the Kober color reaction \((r = 0.64)\) and 0.42 times higher than the fluorometric results \((r = 0.65)\).

Recovery trials. Four trials, in which known amounts of estrone standards were added to the urine at the time of extraction, resulted in an average recovery rate of 81, 80, and 77% by the Kober color reaction, fluorometric, and bioassay methods, respectively.

Undoubtedly, all three of the methods of assay have their advantages and shortcomings. Some contaminating compounds will add to the fluorescence, whereas other contaminants will have a quenching effect. Hunt (10) reported that incomplete evaporation of solvent blanks

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**TABLE 1**

<table>
<thead>
<tr>
<th>Estradiol-17α</th>
<th>Estradiol-17β</th>
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</tr>
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<td>275 days</td>
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</table>

**Figure 2.** Urinary estradiol-17β excretion expressed as micrograms of excretion per 100 lb. Body weight per day as measured by three assay methods.
before development of the Kober color causes erroneously high readings. She stated that the values will be as much as 40% too high for every 0.05 ml of solvent remaining. Likewise, bioassay analysis has several factors which might cause the resulting values to be in error. Turner (18) discussed the neutralizing effects of anti-hormones and emphasized that certain substances may be capable of neutralizing the effects of an administered hormone but may or may not give the typical precipitin test. Edgren and Calhoun (5) found that certain steroids will inhibit the effects of estrone. In every case in which they found a steroid to inhibit the effects of estrone, the estrone was blocked at intermediate levels. Barth and Watterville (3) compared bioassay analysis with chemical analysis of estrogens excreted by the human female. They stated: "Considering all the points and counterpoints, we have come to believe that the balance is strongly in favor of the chemical estimation."

Levels of excretion during gestation. Data contained in Table 1 indicate that the quantities of estrogenic substances excreted via the urine are low at 50 days of gestation, with only a slight increase from then until 100 days of gestation. After 100 days of gestation, the increase in the excretion of estrogen in the urine became much more pronounced, with a sharp increase occurring sometime around 200 days. Most of the increase was in quantities of estradiol-17α and estrone. Secretion of estrogenic substances by the placenta probably begins to occur around this 100-day period and accounts for much of the increase. Estrone, estradiol-17α, and estradiol-17β were identified in fetal cotyledons of two cows in advanced pregnancy in 1959 (20).

There are at least two theories to help account for the more pronounced excretion rate of estrone and estradiol-17α. First, it is possible that the metabolic processes in which estradiol-17β is transformed to the less potent estrogens (estrone and estradiol-17α) are speeded up as the placenta begins secreting estrogenic substances. Velle (22) stated that estradiol-17β is rapidly changed to estradiol-17α. Since the metabolic pathway is thought to go via estrone, this could account for the large increases in quantities of estrone and estradiol-17α excreted. It is also possible that much of the estrogen produced by the placenta is in the form of estrone and/or estradiol-17α. Estradiol-17β, estradiol-17α, and estrone have been isolated from the placenta of the cow, with estradiol-17α found in largest quantities in the term placenta (20).

Rate of excretion on a per-liter basis and as determined by the Kober color reaction at the 275th day of gestation was 0.19 mg of estradiol-17α and 0.02 mg of estradiol-17β. Rate of excretion of estrone as determined by bioassay was 0.07 mg per liter. When the computation is put on a 24-hr basis, the excretion rate was 2.87 mg of estradiol-17α, 0.23 mg of estradiol-17β, and 1.07 mg of estrone. These results are similar to those of Velle (21), who found an average of 0.15 to 0.20 mg of estradiol-17α and 0.10 to 0.15 mg of estrone per liter of urine. When his results were computed to a 24-hr basis, the average excretions of 2.0 to 2.5 mg of estradiol-17α and 0.8 to 1.0 mg of estrone were noted.

CONCLUSIONS

Because the same trends of excretion were revealed (Figures 1 and 2), and because the magnitude of the differences was not great in measuring estradiol-17α and estradiol-17β by the three different methods, it would indicate that any one of the three methods could be used to measure these two substances in com-
parative studies. However, the magnitude of the differences between the three methods was larger when measuring estrone (Figure 3). To be most conservative, one should use the bioassay procedure in measuring urinary estrone. If facilities for bioassay were not available, one could use either one of the chemical methods but should recognize that results will be higher than if they had been determined by bioassay.

The average quantities of estrogenic substances excreted via the urine at various stages of gestation compared very favorably with the values reported by Velle (21).

REFERENCES