PLASMA BOUND ESTROGENS DURING PREGNANCY IN THE DEER (*Odocoileus virginianus texanus*)

by

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

INTRODUCTION

With the discovery by Frank and Rosenbloom (1915) of a substance taken from the placenta and corpus luteum, and by Allen and Doisy (1923) of a substance from the ovary that demonstrated a physiologic activity on the reproductive tract, the study of the estrogenic hormones was begun. At about this time, Stockard and Papanicolau (1917), Allen (1922), and Long and Evans(1922) demonstrated cyclic changes in the reproductive tract during the estrous cycle. These studies, along with the first findings of a hormonally active compound from the ovary, accelerated interest in this field and led quickly to the isolation and identification of the classic estrogens: estrone (Butenandt, 1929; Doisy, Veler, and Thayer, 1929), estriol (Marrian, 1929; Doisy et al., 1929), and estradiol-17β (MacCorquodale, Thayer, and Doisy, 1935; and Wintersteiner, Schwenk, and Whitman, 1935). Since that time, several more estrogens have been isolated from various animals and the metabolic fate of these estrogens within the animal system has been investigated by several research workers.

Not long after the isolation of the first estrogenic hormone, several reports appeared which showed that estrogens are found circulating in the sulfate-conjugated fraction (Schachter and Marrian, 1938; and Jensen, Lariviere and Elie, 1945), the glucuronic acid-conjugated fraction (Cohen and Marrian, 1936; Muhlbock, 1937; and 1939) as well as in protein-bound form

(Rakoff, Paschkis, and Cantarow, 1943; Roberts and Szego, 1946; Szego and Roberts, 1946; 1947; 1953; 1955). This shed a new light on the study of circulating estrogens and their metabolism. A new look had to be taken at the quantitation of these estrogens and the metabolic function of conjugation and protein binding. Extensive reviews on the binding of estrogens to plasma proteins exist (Sandberg, Slaunwhite, and Antoniades, 1957; Daughaday, 1959) as well as reviews on conjugated forms of the estrogens (Schneider and Lewbart, 1959; Katzman, Straw, Buehler, and Doisy, 1954).

Earlier methods of quantitating estrogens depended upon bioassay methods. These techniques suffered in that mixtures of estrogens could not be assayed in terms of any one specific estrogen; furthermore, large numbers of test animals were needed and the variability of injection technique was very large. To measure accurately small amounts of estrogen-containing fluid or tissue there was a need for a specific and efficient technique.

The discovery in 1931 by Kober of a sensitive and highly specific color reaction for estrogens was an event which led to the development of many useful methods for the colorimetric determination of estrogens from biological sources. A modification by Brown (1955) of the Kober technique increased the sensitivity and accuracy of the technique but it still lacked the extreme sensitivity required for measuring estrogenic steroids. In addition, interfering colors produced during the reaction

made quantitation difficult to achieve. Recently, phosphoric or sulfuric acid fluorescence has become the method of choice for quantitating estrogens. Introduced by Jailer (1948) and modified by Bates and Cohen (1950a; 1950b), the method has been adapted to many extraction techniques for routine assay of small biological samples. Essentially, the reaction involves an activation of the A-phenolic ring of the steroid. When activated by a 436 mμ mercury lamp, the steroid will give a characteristic fluorscence at about 480 mμ. The sulfuric acid method is distinguished by extreme sensitivity. Using the optical conditions and reagents recommended by Preedy and Aitken (1961), as little as 0.005 μg of estrone can be measured with accuracy. Armed with the newer extraction techniques and chemical assays, much work has been carried out on the determination of circulating levels of estrogens in the blood of both pregnant and non-pregnant animals. By far the most work has been done with the human. Only Roy, Harkness, and Kerr (1965) have determined systematically estrogen levels in the circulation throughout a complete pregnancy although several investigators (Roy and Brown, 1960; Roy and Mackay, 1962; Preedy and Aitken, 1957; Diczfalusy and Magnusson, 1958; and Veldhuis, 1953) have added data on estrogen levels in the plasma during isolated stages of pregnancy. In non-pregnant humans, circulating levels of estrogens have been determined by Preedy and Aitken (1957), Roy and Brown (1960), and Roy et al., (1965). In other than human species only Metzler, Eleftheriou, and Fox (1966), Eleftheriou,

Boehlke, and Tiemeier (1966), and Eleftheriou, Boehlke, Zolovick, and Knowlton (1966) have determined systematically estrogen levels in the dog during the estrous cycle and pregnancy, the catfish at different periods before spawning, and the deer during pregnancy, respectively. In all the above reports only the free fraction of estrogens was determined with no report on conjugated and protein-bound estrogen levels. To this time only Rakoff et al., (1943) has studied conjugated estrogens in blood, this being in the human and only in late pregnancy.

It was with the above facts in mind that the present study was undertaken, to determine the conjugated and protein-bound estrogens in plasma systematically throughout pregnancy in the deer (Odocoileus virginianus texanus). The animal was chosen because of the ability to obtain large plasma samples, the availability of a controlled herd at the Welder Wildlife Game Preserve at Sinton, Texas, and because of the interest in obtaining such information by officials at the preserve as well as in this laboratory. It was felt that any information obtained about circulating levels of plasma-bound and conjugated estrogens during pregnancy in any animal would be a contribution to the knowledge about estrogen hormones.

MATERIALS AND METHODS

Collection of plasma

Blood was collected from 29 adult female, white-tailed deer by rope restraint and in some cases by shooting for herd conservation. Blood was collected by jugular puncture. The blood was heparinized, centrifuged immediately and the plasma frozen for later analysis. The samples were collected during estrus (determined by vaginal smear, with knowledge of the ideal time of year for ovulation, and mating habits) and from six to twenty-three weeks of pregnancy. The number of animals in each experimental group were as follows: estrus, 2; pregnancy, 8 weeks, 3; 12 weeks, 3; 14 weeks, 6; 16 weeks, 5; 18 weeks, 4; and 22 weeks, 3. The length of gestation of each animal was determined by measuring the length of the fetus (Winters, Green, and Comstock, 1942). Where multiple foetuses were found, the average length was used. All of the experimental animals were maintained at the Welder Wildlife Game Preserve at Sinton, Texas. Samples were collected and sent to Kansas State University by Dr. F. F. Knowlton, Bureau of Sport Fisheries and Wildlife, San Antonio, Texas.

Extraction procedures

Extraction and purification of the estrogenic steroids in the plasma was carried out using modifications of several techniques (Migeon, Wall, and Bertrand, 1959; Sandberg and

Slaunwhite, 1956; and Veenhuizen, Erb, and Gorski, 1960). Aliquots of plasma (50 ml) were extracted 3 times with 2 volumes of chloroform to remove the unconjugated free plasma estrogens. The residue was then extracted 2 times with 2 volumes of ethanol, centrifuged each time and the ethanol extracts were pooled and taken to dryness under nitrogen in vacuo at 38° C. This ethanol extract contained all of the bound estrogens in the sample. The dried residue was dissolved in 40 ml of acetate buffer (pH 4.7; 12 ml acetic acid to 28 ml sodium acetate) and incubated with β-glucuronidase (300 units/ml) for 48 hours at 37° C and constant stirring in a Dubnoff metabolic shaker. Following incubation the solution was extracted 3 times with equal volumes of chloroform (figure 1, step 1, page 8). The use of β-glucuronidase was for the disassociation of the glucuronic acid bond to the estrogen thus making it a free estrogen which was readily extracted by chloroform. Hereafter, the glucuronic acid-conjugated fraction will be referred to as the glucuronide fraction. Following the above extraction with chloroform, the aqueous residue was adjusted to a pH of 0.8 to 1.0 with 65% sulfuric acid and extracted continuously with 150 ml of ether for 48 hours with the use of a magnetic stirrer to insure complete mixing of the organic and aqueous phases (figure 1, step 2, page 9). The adjustment to acidic pH and continuous ether extraction liberated the hydrolyzable conjugated fraction or sulfate-bound fraction. After the continuous extraction step, the ether was drawn off

and the aqueous reside was made 5% with respect to sulfuric acid, refluxed for 45 minutes, cooled and extracted 3 times with equal volumes of ether (figure 1, step 3, page 9). The acid treatment with subsequent refluxing and ether extraction yielded the protein-bound fraction. Each of the above fractions was radioactively labelled with C^{14}-4-estrone following extraction, and the fraction taken to dryness at 37° C in vacuo. The radioactive label was used to calculate steroid recovery following extraction. After each fraction was taken to dryness, it was transferred to a 40 ml centrifuge tube with 10 ml of toluene and washed 3 times with 5 ml of 5% NaOH each time. Each wash was shaken for 30 seconds and centrifuged at 500 x G for 5 minutes. The three NaOH fractions were pooled and washed again with 5 ml of toluene, shaken, centrifuged as above and the toluene aspirated and discarded. The NaOH was then neutralized with 65% sulfuric acid (pH 7.0 to 7.5) and extracted 3 times with 20 ml of benzene. The benzene was taken to dryness in vacuo at 37° C, the residue dissolved in a small portion of chloroform-methanol (v/v) and prepared for thin-layer chromatography. A flow sheet diagram for the extraction of the unknown estrogens appears in figure 1, page 8.

Chromatography and identification procedures

Thin-layer chromatography of the estrogenic compounds was carried out by following the general procedure according to Stahl (1958). Glass plates, 20 x 20 cm, were coated with a thin layer (0.3 mm) of silica gel G (Merck, Germany) using the Desaga

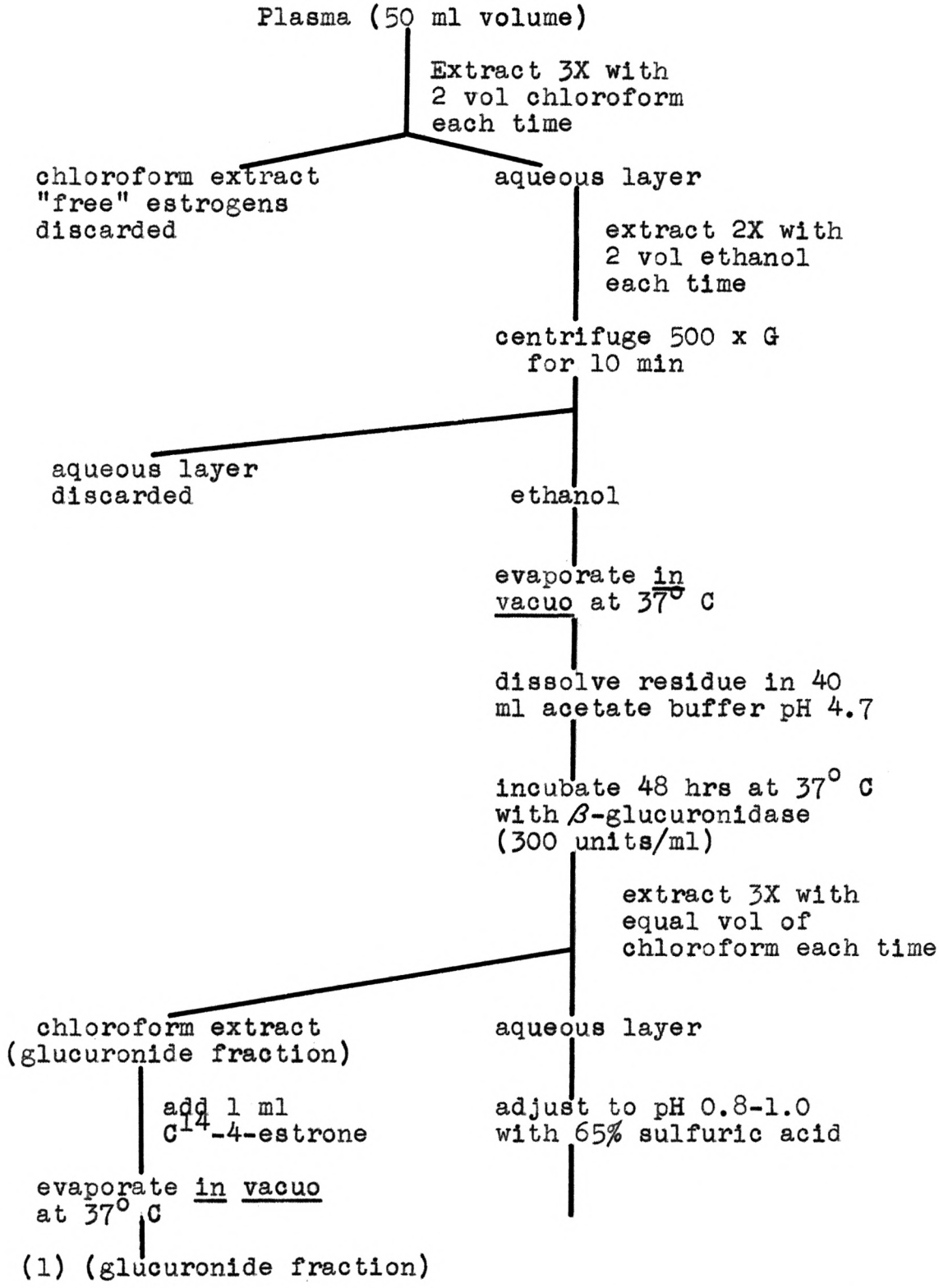


Fig. 1. Flow diagram for bound estrogen extraction procedure.

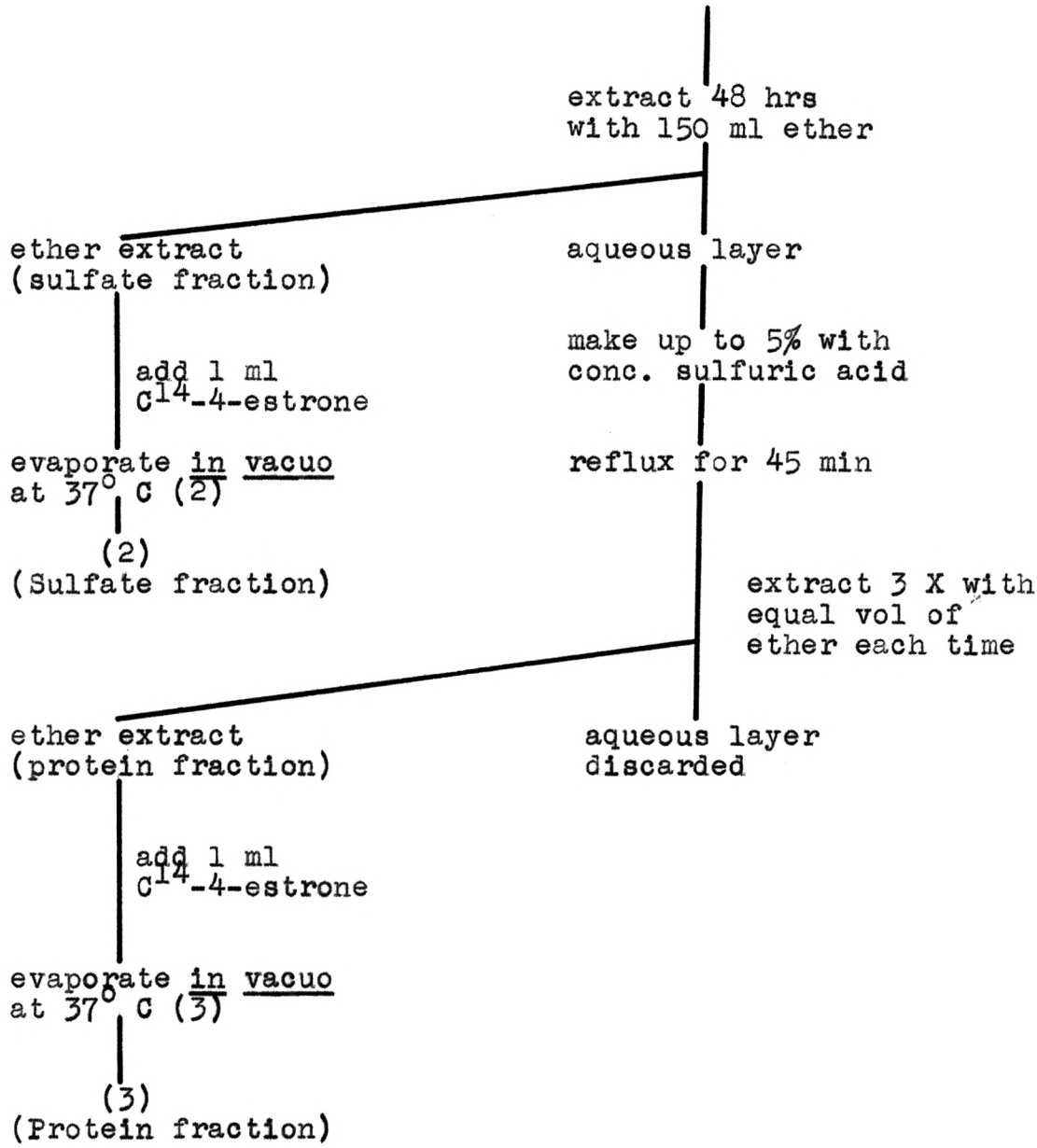


Fig. 1. (contd). Flow diagram for bound estrogen extractiom procedure.

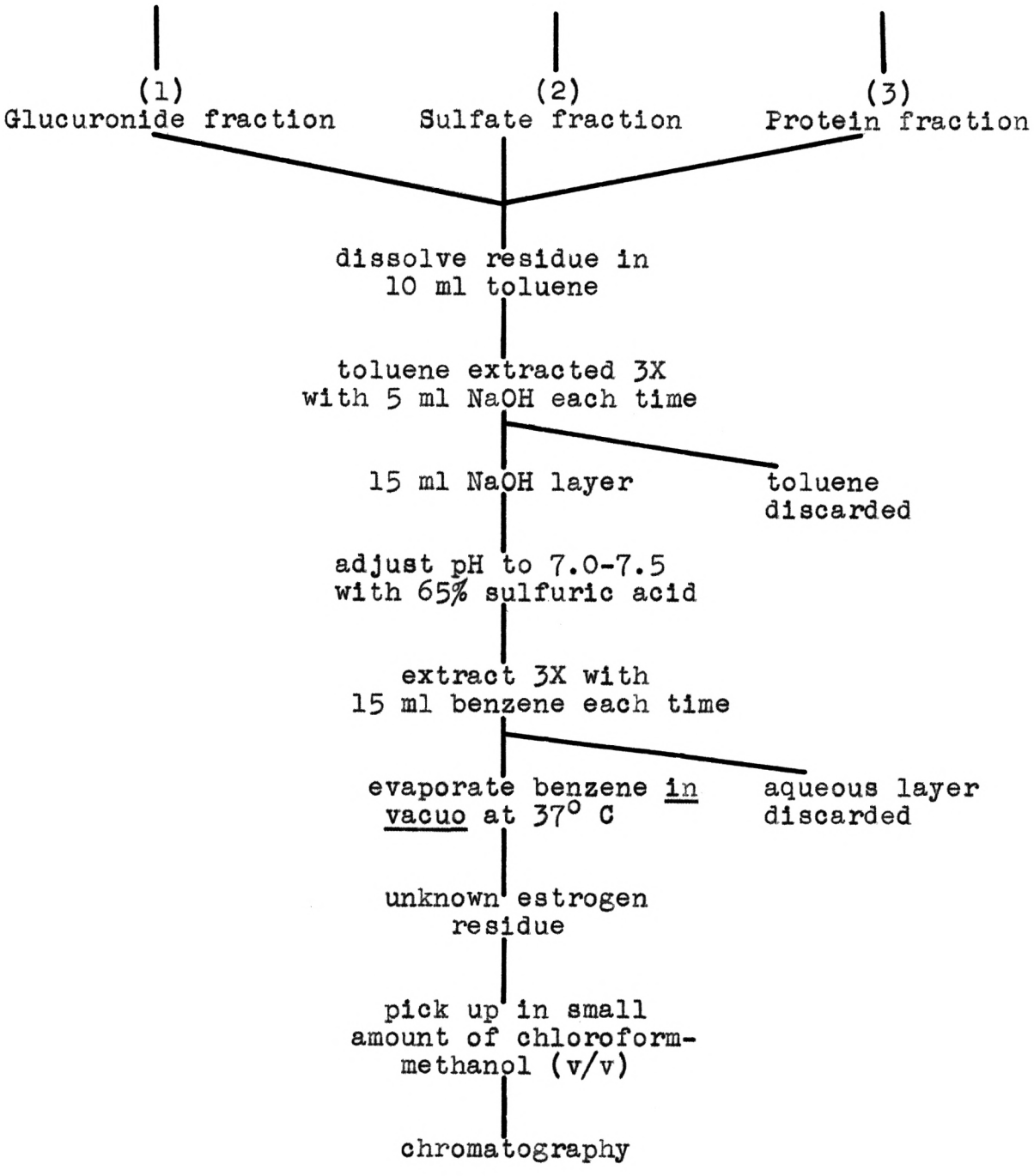


Fig. 1. (contd). Flow diagram for bound estrogen extraction procedure.

coating apparatus. The chromatograms then were allowed to air dry after which they were activated in an oven at 120° C for 30 minutes. The chromatograms then were removed and allowed to cool at room temperature. Either the unknown estrogenic compounds or appropriate estrogen standards were then applied (in a chloroform-methanol vehicle) to one corner of the plate after which the plates were developed two-dimensionally in a hexane-ethyl acetate (v/v) solvent system. Following chromatographic development, the plates were allowed to air dry to evaporate completely any solvent remaining on the plate. The chromatograms containing standard estrogens were visualized by spraying the plates with a 10% ethanolic phosphomolybdic acid solution and heated at 120° C for 5 minutes which caused estrogens to color dark blue on a yellow background. In addition, chromatograms were sprayed with a reagent consisting of 20 ml of concentrated phosphoric acid and 80 ml of ethanol (Jaminet, 1952) and then heated at 100° C for 20 minutes, which gave various specific <u>in situ</u> color reactions for each of the various estrogens both in daylight and under short ultra violet light. In some cases, the unknown chromatograms were also visualized by the above methods and the relative mobilities of the unknown spots were compared to standard estrogens. Rf values for the various estrogens in the solvent system used were as follows: estriol - 0.059±0.012; 16-epiestriol - 0.195±0.026; 16-ketoestradiol-17β - 0.362±0.044; estradiol-17β - 0.507±0.39; estradiol-17α - 0.581±0.046; and estrone - 0.707±0.060.

In the initial identification procedure visualization of the unknown compounds on the chromatograms was carried out by spraying them with the phosphomolybdic acid or the phosphoric acid solutions and then comparing the relative mobilities of the unknown compounds to those of standard known estrogens. With the phosphoric acid spray, the estrogens showed specific color reactions both in daylight and under short ultra-violet light. Acetylation (Bush, 1961), co-chromatography and, in some instances, U. V. absorption in sulfuric acid (Zaffaroni, 1950) was also used to identify further the estrogenic steroids. A Beckman spectrophotometer, model DK-2A, was used to determine absorption spectra. When acetylation was used, the unknown extract was chromatographed as above and areas corresponding to standard estrogens were scraped off the chromatogram with a razor blade, eluted with chloroform to separate the estrogens from the gel, and the chloroform eluate taken to dryness in vacuo. Acetylation of this extract was carried out using the fast method of Bush (1961) and then rechromatographed and mobilities of the unknown estrogenic compounds were compared to acetylated standards with phosphomolybdic acid spraying and phosphoric acid spraying.

Quantitative procedures

After extraction, the unknown extracts were chromatographed in two dimensions, as outlined previously for the qualitative procedures, with standard estrogens being chromatographed simultaneously on another chromatogram in the same tank. Following

development of the chromatograms, the standard chromatogram was sprayed with phosphomolybdic acid and heated to visualize the estrogens. A tracing of the plate was made outlining the front, origin, and positions of each of the appropriate standard estrogens as well as the boundaries of the plates. Using a short U. V. light, the front, origin and all fluoresent areas were traced on the unknown chromatogram. Then, using the tracing from the standard chromatogram with overlap of the solvent fronts and extract origins, the standard estrogen spots were traced on the unknown chromatogram. These areas were enlarged slightly to a uniform area and these areas were scraped off the chromatogram with a razor blade. A blank area of the plate was also scraped off and used as an internal silica gel blank. When scraping off the unknown areas, care was taken not to remove any short-U. V. fluorescing areas. These silica gel scrapings were then eluted with chloroform and each chloroform eluate was dried in vacuo. To each fraction to be quantitated was added 0.1 ml of absolute ethanol followed by 0.7 ml of 90% sulfuric acid. The tubes were heated at 80° C for 20 minutes, cooled and 4.3 ml of 65% sulfuric acid was added. After shaking well the acidic unknown solution was poured into cuvettes and measured fluorometrically using a Turner model 110 filter fluorometer with a primary filter system consisting of a Kodak number 47B plus a number 2A, and a Kodak number 2A-12 plus a 10% neutral density filter for the secondary system. Fluorometric values obtained for each estrogen were extrapolated from their appropriate

standard curve and the values corrected to 100 ml and 100% recovery. Recovery was calculated by removing 0.01 ml from the estrone fraction, to which was earlier added a C^{14}-4-estrone label, before 0.7 ml of sulfuric acid was added for quantitation. To this 0.01 ml ethanol aliquot was added 10 ml of toluene scintillation fluid and radioactivity measured using a Packard Tri-Carb model 331 liquid scintillation counter.

RESULTS

Qualitative analysis

One- and two-dimensional thin-layer chromatography of unsubstituted estrogen unknown samples resulted in five spots corresponding to the standard estrogens estriol, 16-epiestriol, ketoestradiol-17β , estradiol-17α , estradiol-17β , and another spot approximating the mobility of estrone and equilin. Figure 2, page 16 shows a typical chromatogram with the various estrogens visualized in situ.

Color reactions using the phosphoric acid spray of Jaminet (1952) tended to confirm the earlier observations above with estrone being the estrogen of choice for the last spot. Table 1, page 17 gives Rf values and color reactions with phosphoric acid in situ of the unknown and standard estrogens observed.

U. V. absorption in sulfuric acid (Zaffaroni, 1950) also ruled out equilin as being the last spot observed, as equilin gives a very characteristic absorption spectra as compared to estrone (estrone having only one absorption peak, at 455 mμ, equilin having several peaks).

Co-chromatography, and acetylation of the estrogens also tended to confirm the observations above. Table 1, page 17 also gives Rf values of acetylated derivatives for the unknown and standard estrogens.

On the basis of the above data as well as the published results on the "free" estrogen fraction in the deer (Eleftheriou

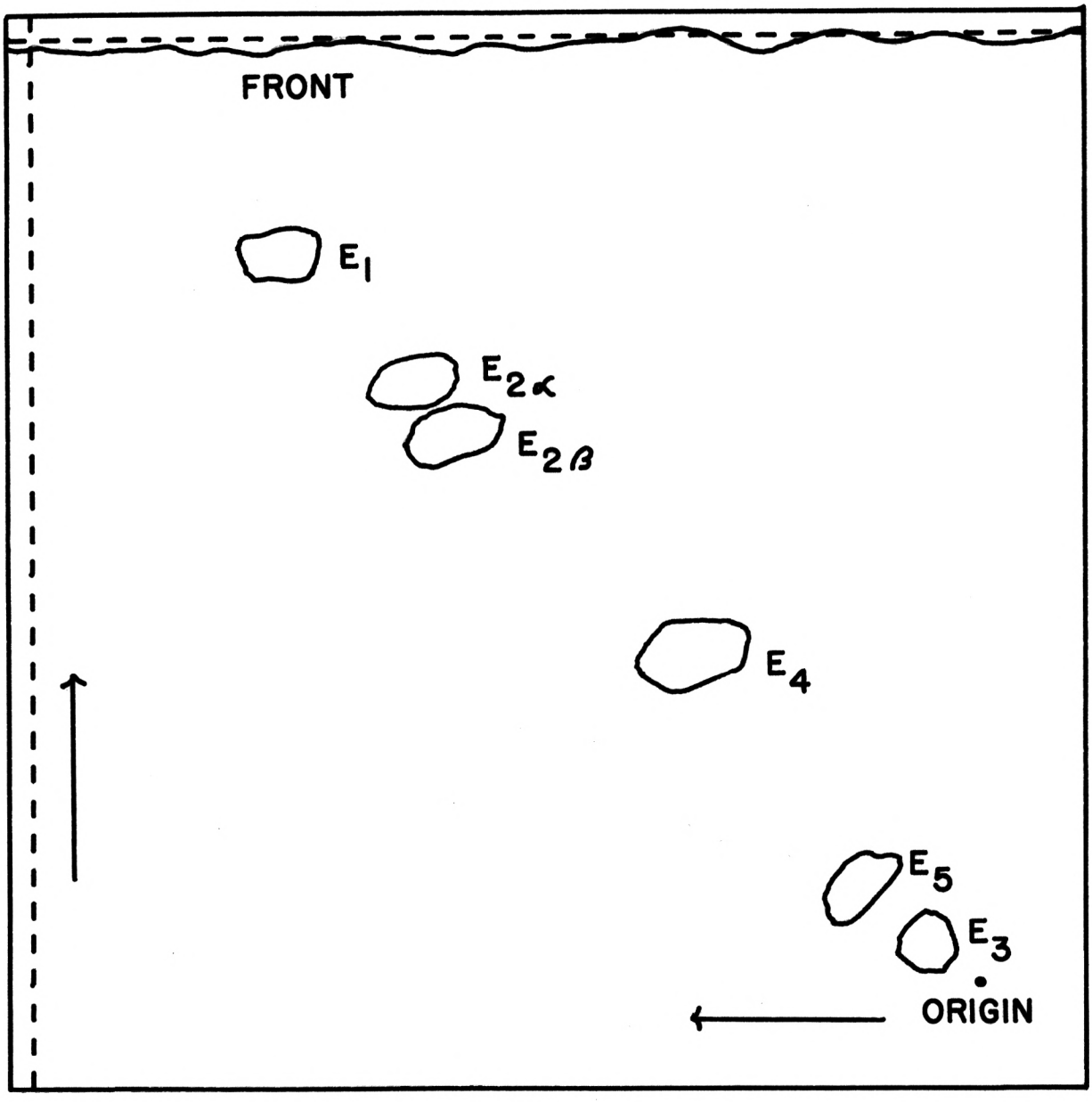


Figure 2. Two-dimensional TLC of estrogen standards. Both dimensions were run in hexane-ethyl acetate (v/v) solvent system. Running time approx. 70 min. each direction. Designations: E_3-estriol, E_5-16-epiestriol, E_4-ketoestradiol-17β, $E_{2\alpha}$-estradiol-17α, $E_{2\beta}$-estradiol-17β, and E_1-estrone.

Table 1. Rf values and color reactions in phosphoric acid of the estrogens identified in the bound fraction of deer plasma.

Estrogen	Rf value 2-D TLC Chrom. Std.	Rf value 2-D TLC Chrom. Unk.	Rf value Acetylation Std.	Rf value Acetylation Unk.	Phosphoric Acid Daylight Std.	Phosphoric Acid Daylight Unk.	Phosphoric Acid U.V. light Std.	Phosphoric Acid U.V. light Unk.
Estriol	0.059 ±.012	0.058 ±.012	0.342 ±.010	0.334 ±.011	BrLi	BrLi	OrBr	Br
16-epi-	0.195 ±.026	0.183 ±.022	0.349 ±.003	0.350 ±.021	Pu	Pu	OrR	ROr
16-keto-estradiol-17β	0.362 ±.044	0.360 ±.031	0.379 ±.013	0.382 ±.018	YOr	Y	Y	Y
Estradiol-17α	0.581 ±.046	0.580 ±.033	0.452 ±.022	0.444 ±.011	Car	R	Or	Or
Estradiol-17β	0.507 ±.039	0.502 ±.059	0.403 ±.002	0.400 ±.011	Br	Br	YG	YG
Estrone	0.720 ±.012	0.722 ±.021	0.919 ±.010	0.921 ±.011	PR	PR	Y	Y

Bl---Blue
Br---Brown
Car--Carmine
G----Green
Gr---Grey
Li---Lilac
Or---Orange
P----Pale
Pu---Purple
R----Red
Y----Yellow

et al., 1966), the bound estrogens were found to be estriol, 16-epiestriol, ketoestradiol-17β, estradiol-17α, estradiol-17β, and estrone. All these estrogens were found in the glucuronide, sulfate, and protein fractions of the plasma.

Due to the extremely low levels in the plasma and the difficulty of separating adequately and recovering estradiol-17β and estradiol-17α, these two estrogens were not quantitated in the plasma in this study.

Quantitative analysis

Recovery values for the total bound estrogens ranged from 0.59% to 79% with an overall average of 37.12%. The glucuronide fraction recovery averaged 40.70%, sulfate fraction recovery average was 40.50%, and the protein fraction recovery averaged 30.14%. It was observed that most of the radioactivity was lost during the toluene-sodium hydroxide wash; often as much as 30% of the label was lost in the toluene. Possibly the high pH of the sodium hydroxide served to degrade the estrogens. A more efficient purification procedure to replace the toluene-sodium hydroxide step would aid greatly in obtaining a more desirable recovery. Checking for radioactive recovery after each step in the procedure gave recoveries of greater than 85% in all but the toluene-sodium hydroxide step.

To test the repeatability of the procedure, two identical samples of pregnant deer plasma were run through the complete technique. Table 2, page 20 shows results of this test. Also, a plasma sample taken from a male hog was tested in the same

manner and the areas corresponding to the standard estrogens pertaining to this experiment scraped off and quantitated as above (Table 2, page 20). Table 2 also shows the results of running distilled water samples through the complete technique, to check the accuracy and specificity of the technique. As one can see in Table 2, page 20, the reproducibility of the technique is very reliable and that one gets only traces of non-specific fluorescence when distilled water is run through the technique. The somewhat larger values evident in the hog plasma sample probably are due to lipid material having a chromatographic mobility similiar to the individual estrogens. This material may or not exist in deer plasma.

Standard curves were obtained for estrogen fluoresence in sulfuric acid and are presented in Figure 3, page 21. Each curve was plotted from readings of 9-10 concentrations and repeated several times. Dilutions were made from a 0.4 μg/ml dilution with the crystalline estrogens dissolved in chloroform-methanol (v/v).

Glucuronide fraction

The results for the glucuronide fraction are summarized in Table 3, page 22. Initially there was an increase in total glucuronide estrogens analyzed from estrus (13.05 μg/100 ml) to 8 weeks of pregnancy (14.84 μg/100 ml) followed by a 700% decrease in estrogen levels between 8 and 16 weeks of pregnancy (14.84 μg/100 ml to 2.65 μg/100 ml). There follows a small peak at 20 weeks of pregnancy (7.52 μg/100 ml) followed by a decline

Table 2. Results of running duplicate deer plasma samples, male hog plasma, and distilled water through the complete technique of extraction, chromatography and quantitation. (Fractions in μg/100 ml).

Fraction	Deer Plasma 1	Deer Plasma 2	Male hog Plasma	Distilled Water
Glucuronide				
E_1	0.61	0.66	0.08	0.01
E_3	2.87	2.66	0.30	0.10
E_4	3.02	2.97	0.11	0.00
E_5	0.86	0.81	0.07	0.01
Sulfate				
E_1	0.38	0.35	0.00	0.07
E_3	0.10	0.08	0.03	0.01
E_4	1.20	1.18	0.21	0.08
E_5	0.12	0.07	0.00	0.10
Protein				
E_1	0.37	0.40	0.01	0.10
E_3	0.63	0.65	0.00	0.01
E_4	1.76	1.74	0.20	0.03
E_5	0.33	0.29	0.04	0.00

E_1 = estrone

E_3 = estriol

E_4 = 16-ketoestradiol-17β

E_5 = 16-epiestriol

Figure 3. Sulfuric acid-fluorescence curves for the following standard estrogens: Estrone, estradiol-17α, estradiol-17β, estriol, 16-epiestriol, and ketoestradiol-17β. Each point represents the means of 5-9 runs.

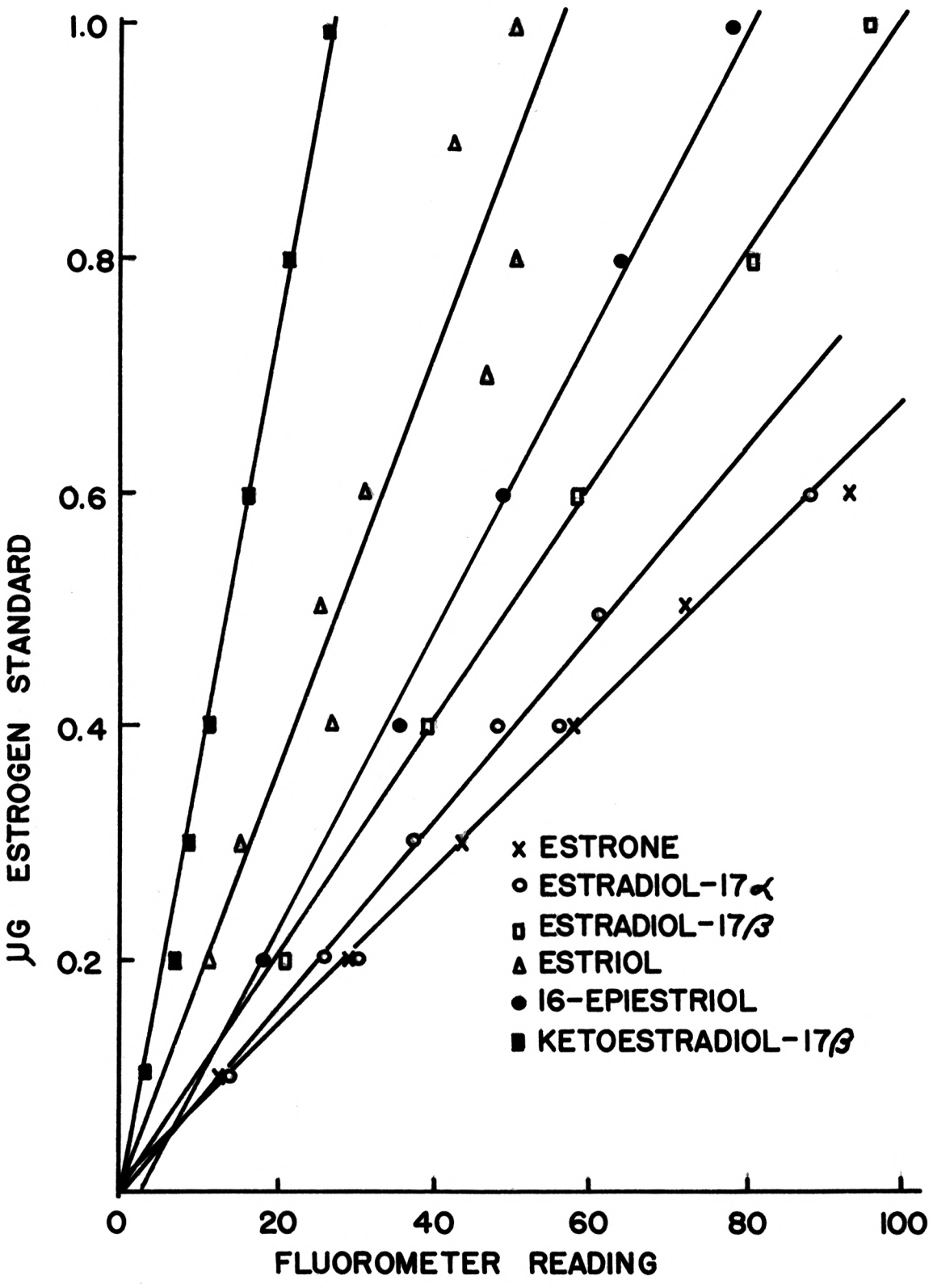

1.0
0.8
0.6
0.4
0.2
µG ESTROGEN STANDARD
0
20
40
60
80
100
FLUOROMETER READING
x ESTRONE
o ESTRADIOL-17α
□ ESTRADIOL-17β
△ ESTRIOL
● 16-EPIESTRIOL
■ KETOESTRADIOL-17β

Table 3. Means of individual estrogens in µg/100 ml plasma in the glucuronide fraction during pregnancy.

Week of Pregnancy	No. of Samples	E_1	E_3	E_4	E_5	Total Average
ESTRUS	2	3.17	2.17	5.75	1.96	13.05
8	3	1.15	5.18	4.70	3.81	14.84
12	3	0.89	3.80	7.21	0.11	12.01
14	6	0.62	1.64	1.46	0.78	4.50
16	5	0.65	0.59	1.09	0.32	2.64
18	4	0.46	1.89	1.38	1.45	5.18
20	3	0.62	1.97	1.88	3.05	7.52
22	3	0.39	2.78	0.48	0.55	4.20

E_1 = estrone

E_3 = estriol

E_4 = 16-ketoestradiol-17β

E_5 = 16-epiestriol

at 22 weeks. This second peak at 20 weeks of pregnancy is not great enough to be considered an actual change in estrogen levels and thus a plateau in estrogen levels is found between 14 and 22 weeks of pregnancy. Due to the small population of experimental animals in each group it was decided that any statistical analysis of the results would serve no useful purpose and, therefore, no tests of significance were undertaken.

The individual estrogens, as shown in Table 3, page 22, exhibit the same initial rise from estrus to 8 weeks with the same 5-fold decrease at 14 weeks of pregnancy with a plateau in levels to 22 weeks. Ketoestradiol-17β, the major estrogen in the glucuronide fraction shows the initial rise from estrus (5.75 μg/100 ml) to 12 weeks (7.21 μg/100 ml) and the decrease and plateau at 14 weeks (1.46 μg/100 ml) to 22 weeks (0.48 μg/100 ml). Estriol, 16-epiestriol, and estrone in order of decreasing concentration show essentially the same rise from estrus to 8 weeks, a significant decrease from 8 to 14 weeks and a plateau 14 to 22 weeks during pregnancy.

Sulfate fraction

Table 4, page 24 summarizes the results for the sulfate fraction. There is observed a gradual and very small increase in total sulfate estrogens from estrus (2.14 μg/100 ml) to 12 weeks of pregnancy (6.27 μg/100 ml) followed by a decrease at 14 weeks (1.82 μg/100 ml) which remains essentially unchanged throughout pregnancy except for a small peak at 20 weeks (4.37 μg/100 ml). The peaks observed in the sulfate fraction

Table 4. Means of individual estrogens in μg/100 ml plasma in the sulfate fraction during pregnancy.

Week of Pregnancy	No. of Samples	E_1	E_3	E_4	E_5	Total Average
ESTRUS	2	0.35	0.61	0.64	0.54	2.14
8	3	0.21	0.47	1.42	0.19	3.29
12	3	1.03	3.17	1.22	0.85	6.27
14	6	0.31	0.32	1.02	0.17	1.82
16	5	0.08	0.73	0.33	0.32	1.46
18	4	0.24	0.46	0.19	0.54	1.43
20	3	1.89	0.42	1.82	0.24	4.37
22	3	0.96	0.58	1.18	1.47	4.09

E_1 = estrone

E_3 = estriol

E_4 = 16-ketoestradiol-17β

E_5 = 16-epiestriol

are not great enough to indicate a trend and thus the sulfate fraction must be seen to remain essentially unchanged throughout pregnancy.

Ketoestradiol-17β, the greatest in concentration in the sulfate fraction, exhibits the same rise from estrus (0.64 μg/100 ml) to 8 weeks of pregnancy (1.42 μg/100 ml) and a drop at 14 weeks (0.17 μg/100 ml) with the slight rise at 20 weeks (1.82 μg/100 ml). Estriol is second in concentration while 16-epiestriol and estrone follow in order of decreasing concentration. Each of the individual estrogens reflects the exact increases and decreases as observed in the total sulfate fraction.

Protein fraction

Protein fraction estrogens are presented in Table 5, page 26. There was observed a considerable variation in the total protein fraction estrogens with a 900% decrease from estrus (10.73 μg/100 ml) to 8 weeks of pregnancy (1.96 μg/100 ml) followed by a 12-fold increase at 12 weeks (12.05 μg/100 ml). Throughout the rest of pregnancy the protein fraction followed closely the trend of the glucuronide and sulfate fractions, having a drop in estrogen concentration from 12 weeks to 14 weeks of pregnancy (12.05 μg/100 ml to 2.40 μg/100 ml) and the plateau continuing throughout pregnancy. The waxing and waning of the protein fraction was due mainly to the great variability in the individual estrogens. Ketoestradiol-17β, again the highest in concentration was typical of the waxing and waning of the individual estrogens. There was observed

Table 5. Means of individual estrogens in the protein fraction in μg/100 ml plasma during pregnancy and grand total of all bound estrogens during pregnancy.

Week of Pregnancy	No. of Samples	E_1	E_3	E_4	E_5	Total Average	Grand Total
ESTRUS	2	0.33	5.83	2.30	2.27	10.73	25.92
8	3	0.09	1.57	0.19	0.11	1.96	20.09
12	3	3.39	3.62	3.17	1.87	12.05	30.33
14	6	0.45	0.68	0.96	0.31	2.40	8.73
16	5	2.09	1.38	1.05	0.40	4.92	9.03
18	4	0.39	0.76	3.32	2.87	7.34	13.95
20	3	2.35	0.48	5.60	0.89	9.32	21.21
22	3	0.49	2.31	0.30	0.65	3.75	12.04

E_1 = estrone

E_3 = estriol

E_4 = 16-ketoestradiol-17β

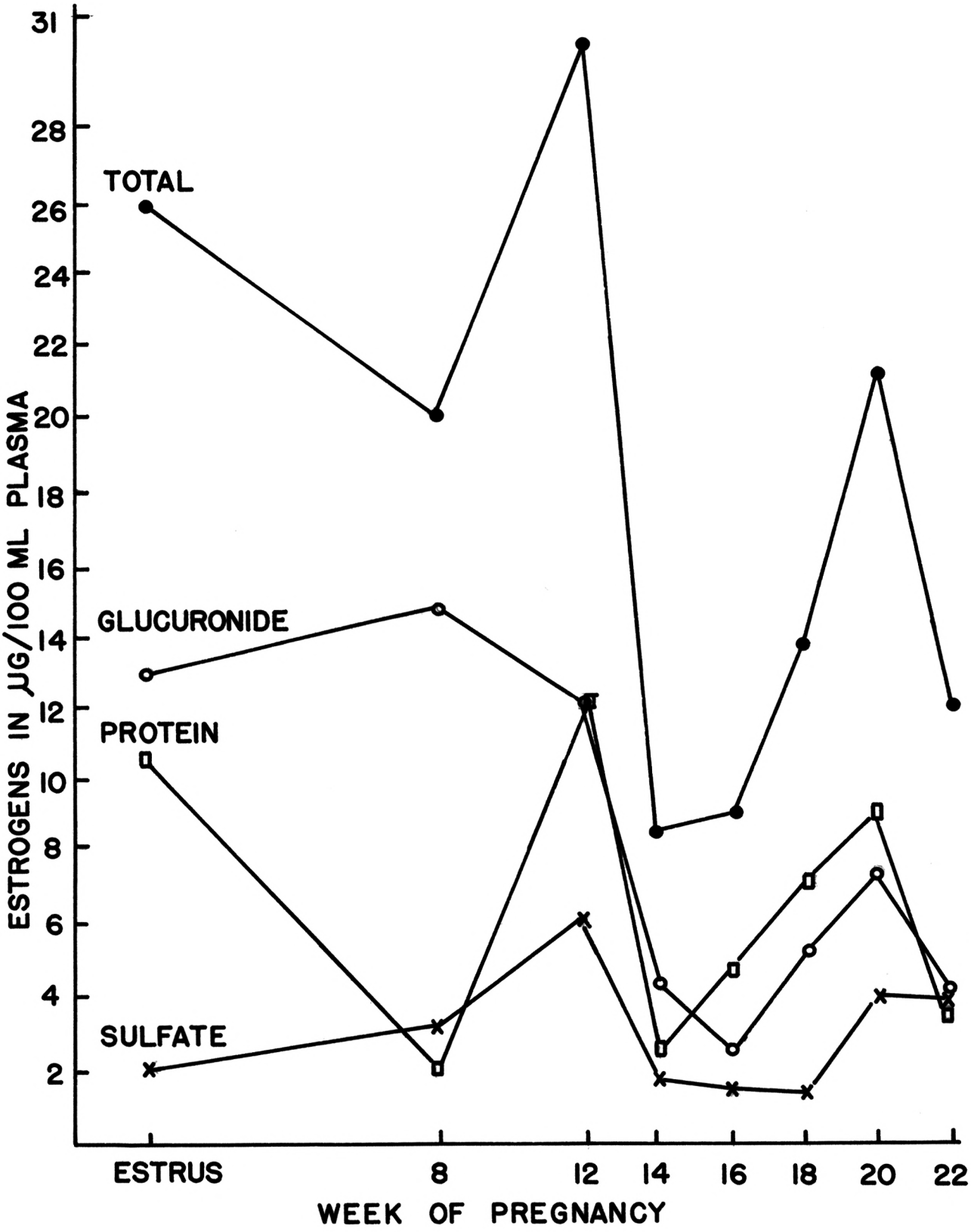
E_5 = 16-epiestriol

a decrease from estrus to 8 weeks of pregnancy (2.30 μg/100 ml to 0.10 μg/100 ml) followed by a 30-fold increase at 12 weeks. A decrease in concentration was seen again at 14 weeks (0.96 μg/100 ml) and a peak in concentration at 20 weeks (5.60 μg/100 ml). Estriol, 16-epiestriol, and estrone, in order of decreasing concentration are seen to exhibit identical waxing and waning.

Values for total bound estrogens are summarized in Table 5, page 26. Also Figure 4, page 28 shows the graph of the glucuronide, sulfate, and protein fraction total estrogens as well as the graph of total bound estrogens from estrus throughout pregnancy. There was observed a decrease from estrus to 8 weeks of pregnancy (25.92 μg/100 ml to 20.09 μg/100 ml) followed by an increase and peak at 12 weeks (30.33 μg/100 ml). There was then a 200% decrease at 14 weeks (8.72 μg/100 ml) where a plateau is maintained until 20 weeks after which a rise was observed (21.21 μg/100 ml) followed by a slight decrease at 22 weeks (12.04 μg/100 ml). This final peak was not considered to be large enough to represent a major change in estrogen secretion.

The sulfate fraction exists in the smallest concentration within the bound portion of the plasma. Glucuronide and protein bound estrogens were in approximately equal proportions in the bound fraction and have levels 3 times greater than the sulfate fraction. When compared to the free estrogen fractions (Eleftheriou et al., 1966) one observes that free estrogens were greater by 30% than the glucuronide and protein-bound fractions and 5 times larger than the sulfate conjugated fract-

Figure 4. Levels of glucuronide, sulfate, and protein fraction estrogens throughout pregnancy. Also the total conjugated and bound estrogen fraction is presented.


31
28
26
24
22
20
18
16
14
12
10
8
6
4
2
TOTAL
GLUCURONIDE
PROTEIN
SULFATE
ESTROGENS IN µG/100 ML PLASMA
ESTRUS
8
12
14
16
18
20
22
WEEK OF PREGNANCY

ion. Compared to the total bound fraction, the free fraction is 50% lower. Thus the total bound estrogens were in greater concentration than the free fraction in plasma during pregnancy.

DISCUSSION

The extraction procedure was found to be at least 85% efficient in all steps following addition of the radioactive label. This agrees closely with that reported by Metzler et al., (1966), and Veenhuizen et al., (1960) who used the same technique to purify similar fractions for chromatography. It was not possible to determine the recovery of each estrogen fraction from the plasma extraction, but Migeon et al., (1959), and Sandberg et al., (1956) found quantitative recovery of each conjugated and bound fraction in their work using the same plasma extraction and hydrolysis procedure. Therefore, one must assume total quantitative recovery of all estrogens up to preparation for purification of each fraction at which point the radioactive label was added and recovery followed closely. The low recovery values for some samples was concluded to originate in chromatographing the sample and eluting the estrogen from the silica gel. During the identification process it was observed that occasionally the steroid "spots" would not migrate in a normal manner but would become distorted and display trailing of the steroid. This probably is due to some contamination of the sample with material which was not removed during extraction and purification. This contamination was assumed to be of a lipid nature and thus could not be completely removed from the sample. As this lipid material would migrate along with the steroids occasionally, this tended to affect the mobilities of

the estrogenic steroids and sometimes an area would be scraped off the chromatogram for elution which did not include the estrogen under examination.

There also was observed the presence of non-specific fluorescing materials on the plates when observed under a short-U. V. (2537 Å) lamp. These were traced around and not eluted from the plate. The possibility exists that the unknown estrogen overlapped partially to this fluorescing spot and thus again would have not been eluted completely. Slaunwhite and Sandberg (1959), and Veldhuis (1953) reported such fluorescing contamination in their procedures, thus this problem seems to be a common source of error in many estrogen assays. If such contamination were eluted and quantitated by sulfuric acid fluorsence, extremely high values would be observed. When such extreme values were observed during the course of the assay, the values were discarded. Veldhuis (1953) also reported fluorescent contamination in many reagents used in the technique.

Qualitative analysis

Migeon et al., (1959), and Sandberg et al., (1956) have found the initial extraction procedure with enzymatic and sulfuric acid hydrolysis to be an acceptable technique. In this laboratory, the extraction method was found to be fast, efficient and easily manageable. Combined with the estrogen purification procedure of Veehhuizen et al., (1960), and Metzler et al., (1966) the extraction and purification of estrogens was carried out routinely. The only undesirable part

of the procedure was the frequent high loss of estrogens during the NaOH-toluene "wash" step. Here a new innovation should be added. Probably the estrogens were affected by the extreme pH of the NaOH solution which, as a 5% solution, is highly alkaline.

The use of thin-layer chromatography to separate the estrogens has been found to be much more efficient and desirable than paper chromatography. This is due to the shorter equilibrating time and the shorter time for development of the chromatogram (about 70 minutes for thin-layer, between 5 and 15 hours for paper chromatography). Thin-layer chromatography has drawbacks in that the unknown sample must be very pure before applying it to the chromatogram to insure proper development and the fact that the estrogen must be eluted from the gel, thus neccessitating extreme care in washing the gel before use. Also, the gel must be applied carefully to the glass plate to avoid bubbles, uneven areas of gel and broken areas where the gel falls off the plate. With proper practice and technique, very acceptable chromatograms can be made routinely.

Results from TLC analysis, <u>in situ</u> color reactions with phosphoric acid, acetylation, and co-chromatography showed that estriol, ketoestradiol-17β, estradiol-17α, estradiol-17β, and 16-epiestriol were present in the bound and conjugated plasma fraction of the deer. Estrone identification was based mainly on a process of elimination. Equilin, which has a chromatographic mobility identical with estrone was eliminated by its

U. V. absorption in sulfuric acid as well as by *in situ* color reactions. 2-Methoxyestrone, 16α-hydroxyestrone, and 16-oxo-estrone were eliminated by their specific *in situ* color reactions. The spot was decided to be estrone after laborious extraction and chromatographic analysis. The spot "almost" had the same phosphoric acid color reaction *in situ*, C^{14}-labeled estrone corresponded exactly with the unknown spot, co-chromatography with standard estrone and the unknown spot gave only one spot, and the acetylated products corresponded well with standard acetylated estrone.

Gorski and Erb (1959) and Veenhuizen *et al.*, (1960) isolated estrone, estradiol-17α, and estradiol-17β from placental and fetal cotyledons in the cow while Norman, Eleftheriou, Spies, and Hoppe (1967) found estrone, estradiol-17β, and keto-estradiol-17β in the non-pregnant ewe. As these two animals are the only close relatives of the deer yet studied, very little comparison can be made. More metabolites are evident in the deer plasma probably because these estrogens are already somewhat degraded. Possibly only one or two parent compounds are synthesized in the ovary and placenta and then these compounds are metabolized to many other products. The non-pregnant ewe is notorious for having very few types of estrogens and these in extremely low levels (Wright, 1962a; 1962b).

Quantitative analysis

Unless the unknown estrogen fractions were purified sufficiently during the procedure, gross contamination of the

chromatograms was observed. This contamination was in the form of a dark, oily residue which spotted on the chromatograms very slowly. Although most of the contamination present either remained at the origin or traveled in the front, some contamination was observed to form spots on the chromatograms. These spots would give very high fluorescent readings and were avoided when the estrogens were scraped off the plate. Nandi and Bern (1963) cite this as one of the drawbacks of using TLC for quantitative analysis.

Nandi et al., (1963), Randerath (1962), and Siegel and Dorfman (1963) report that silica gel impurities will give erroneous quantitative values when they are eluted along with the unknown estrogen. Therefore, extensive washing with methanol of the silica gel was used to remove impurities. This was found to be quite satisfactory in cleaning the gel.

Although there has been very little actual data published with which to compare this work, one can make some general statements concerning estrogens during pregnancy. Roy et al., (1962), and Roy et al., (1965) reported that in the human total estrogen levels increase progressively between the 6th to the 40th week of pregnancy. They also reported some fluctuation in individual estrogens during this period. Metzler et al., (1966) reported that in the dog there is a great increase in total free estrogens at the 3-4 week period of pregnancy. He also reports that this period is the time when placental development is completed. Following this 3-4 week

rise, the estrogen levels drop back to the estrus period levels and remain there throughout pregnancy. This same trend is followed almost exactly in the free estrogen fraction in the deer (Eleftheriou et al., 1966) with the high estrogen level occuring at the 6-8 week period. This 6-8 week period may correspond with the 3-4 week period in the dog.

The levels observed for the total bound estrogen fraction are somewhat confusing in that a drop in total estrogen concentration occurs from estrus to 8 weeks of pregnancy with the peak estrogen levels coming at 12 weeks of pregnancy. This drop in estrogen levels at 8 weeks of pregnancy is due to the protein-bound fraction which drops at an extreme rate from estrus to 8 weeks. This can be explained by moving the period of placenta formation to the 12th week, where the placenta can function to carry pregnancy through without help from the ovary. The great rise in free estrogens in the deer (Eleftheriou et al., 1966) at 8 weeks can be due to the switching of protein-bound estrogens to free estrogens for utilization by the mother and fetus as their demands increase. Glucuronide fraction estrogens showed a high level at 8 weeks thus demonstrating increased inactivation of estrogens at this time. As the placenta takes over the job of producing estrogens for pregnancy there will be a drop in maternal blood estrogens as the ovary slows down production and as the fetal circulation and placenta utilize more and more estrogens. The sulfate fraction was seen to be by far the lowest fraction in estrogen levels but its trend mirrors the

glucuronide fraction in increasing inactivation of estrogens at 12 weeks followed by the decrease from 12 weeks to 18 weeks of pregnancy. The sulfate fraction is probably the fraction of least consequence to the animal due to its small quantity in the circulation. The sharp rise in estrogen levels in all three fractions studied at the 20th week of pregnancy is probably indicative of a "jump" in estrogen requirements for the fetus and continued pregnancy. This jump is also observed in the free estrogen fraction in the deer at this same time (Eleftheriou et al., 1966) and to a slight extent in the dog before parturition (Metzler et al., 1966). The increase could be due to a compensatory adjustment in the progesterone:estrogen ratio which delicately controls pregnancy.

Based on the comparatively high levels, the glucuronide and protein-bound fractions are seen to be the most important fractions to the system, the first representing metabolised, inactivated steroid in preparation for excretion while the protein-bound fraction represents potentially active and "useable" steroid being transported to various parts of the body. The great variation within this protein fraction causes the overall variability within the total bound estrogen fraction due to the tremendous decreases and increases throughout pregnancy. This variation has been observed in estrogen levels throughout pregnancy by other workers (Slaunwhite et al., 1959) in total plasma estrogens in humans and in free plasma estrogens in the deer (Eleftheriou et al., 1966). During pregnancy

binding protein increases to high levels in the plasma. The observed fact that protein binding of estrogens decrease during the middle and latter half of pregnancy may be due to the selective binding of other steroid molecules as well as thyroxine which also increase during pregnancy. Due to this preferative binding of other than estrogen molecules, the estrogens remain in the free state and less are protein-bound.

It is interesting to note that urinary estrogens in the pregnant mare increase from the third to the eighth month of pregnancy and then decrease (Gaudry and Glen, 1960) while in the cow and goat there seems to be a continuous rise in estrogen excretion throughout pregnancy (Nelson and Smith, 1963; and El-Attar and Turner, 1957; and Klyne and Wright, 1957). In the pregnant ewe and the bitch, urinary excretion of estrogens is very low or nonexistent throughout pregnancy (Beck, 1950; and Kristoffersen and Uelle, 1960). Of the animals studied, the deer is more closely related to the cow and the goat and, if urinary excretion of estrogens in the deer is similiar to these two animals, then it would indicate that the increased excretion of estrogens in the urine occurs at the expense of circulating glucuronic acid and sulfuric acid conjugated estrogens, causing the decrease in systemic levels of these fractions. An analysis of the urinary estrogens in the deer during pregnancy possibly would clear up many unanswered questions about estrogen levels in the deer.

SUMMARY

Enzymatic and sulfuric acid hydrolysis techniques accompanied by thin-layer chromatography and sulfuric acid fluorometry led to the identification and quantitation of estriol, 16-epiestriol, ketoestradiol-17β, estradiol-17β, estradiol-17α, and estrone as the primary bound estrogens during estrus and from six to twenty-three weeks of pregnancy in the Texas white-tailed deer (Odocoileus virginianus texanus). Because of extremely low quantities and poor recovery, no measurements were made for estradiol-17β and estradiol-17α. During pregnancy, there was observed an initial rise in total bound estrogens from estrus to 12 weeks of pregnancy (25.92 μg/100 ml to 30.33 μg/100 ml) followed by a decrease in total estrogen concentration at 14 weeks (8.73 μg/100 ml) where a plateau in estrogen levels between 14 and 18 weeks of pregnancy was evident. A peak of estrogen levels was again seen at 20 weeks (21.21 μg/100 ml) with a return to lower levels at 22 weeks (12.04 μg/100 ml). The individual fractions (glucuronide, sulfate, and protein) followed the same pattern as the total bound fraction with the glucuronide and protein-bound fractions being essentially equal in concentration while the sulfate fraction was the minor fraction having only one-third the concentration of estrogens as either the glucuronide or protein-bound fractions.

Several hypotheses are discussed to explain the fluctuations observed. These involve the maternal system, the

placental-fetal metabolism and increased inactivation as observed by the protein-bound fraction shift to glucuronide-sulfate conjugated inactivation of the estrogens.

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PLASMA BOUND ESTROGENS DURING PREGNANCY IN THE DEER (*Odocoileus virginianus texanus*)

by

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B. A., Kansas State University, 1966

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Protein-bound and conjugated estrogens were identified and measured quantitatively in the Texas white-tailed deer (*Odocoileus virginanus texanus*) in estrus and from six to 23 weeks of pregnancy. Due to the extreme lack of data on estrogen metabolism *in vivo*, especially in infrahuman species, it was felt that this work would lend a contribution to the field of estrogen biochemistry.

Combining enzymatic and sulfuric acid hydrolysis techniques to free the protein-bound and conjugated estrogens with thin-layer chromatographic separation of the individual estrogens, the various estrogens were quantitated using sulfuric acid fluorometry and radioisotope labeling.

Three plasma fractions studied were the glucuronic acid conjugated, the sulfuric acid conjugated, and the protein-bound. The glucuronic acid conjugated fraction was hydrolysed by use of β-glucuronidase, after which the free estrogens were extracted with ether and further purified. The sulfate conjugated and protein-bound estrogens were hydrolysed by sulfuric acid treatment with selective extraction of the freed estrogens from each fraction by ether. A C^{14}-4-estrone label was added to calculate recovery of each fraction following ether extraction.

Following additional purification, the estrogens were spotted on thin-layer chromatograms and developed in a hexane:ethyl

acetate (v/v) solvent system. Chromatography, co-chromatography, acetylation, in situ color reactions with phosphoric acid or phosphomolybdic acid, U. V. absorption in sulfuric acid all led to the identification of estriol, 16-epiestriol, ketoestradiol-17β, estradiol-17α, estradiol-17β, and estrone as the main bound and conjugated estrogens in pregnant deer plasma. Due to low recovery and extremely low plasma levels, estradiol-17α and estradiol-17β were not quantitated.

Quantitation was carried out by first purifying the sample extracts with the 2-dimensional thin-layer chromatographic systems mentioned above. Areas corresponding to standards were eluted with a methanol:chloroform (v/v) mixture. The eluate was then taken to dryness, the residue taken up in 0.1 ml ethanol and a 0.01 ml aliquot taken for purposes of counting radioactivity. To the remainder of the ethanol is added 0.7 ml 90% sulfuric acid and then heated for 20 minutes at 80° C. After cooling, 4.3 ml of 65% sulfuric acid was added. The fluorescence of the mixture was measured with a fluorometer using a primary combination of 47B plus 2A and a secondary combination of 2A-12 plus a 10% neutral density Kodak Wratten filter. After correcting for recovery rate and original sample volume, values for estrogen in μg/100 ml plasma were obtained.

The glucuronide fraction and the sulfate fraction, both of which are the fractions that represent estrogenic inactivation and excretion physiologic processes show an initial rise from

estrus to 8-12 weeks of pregnancy followed by a decline that remains persistent from 14 to 18 weeks of pregnancy. A rise was seen at 20 weeks and 23 weeks of pregnancy.

In the protein-bound fraction, considerable waning and waxing was observed with high peaks at 12 and 20 weeks of pregnancy. At other times the estrogen levels are low. The sulfate fraction was observed to be the lowest quantitatively throughout pregnancy and appeared to be of the least consequence to the animal of the three fractions analyzed.

The major peak observed at 8-12 weeks of pregnancy was interpreted as a result of increased placenta growth and increased steroid synthesis. The second smaller peak at 20 weeks is rather confusing and hard to interpret but may reflect changing progesterone:estrogen levels as pregnancy draws to a close.