THE QUALITATIVE AND QUANTITATIVE DETERMINATION OF FREE ESTROGENS IN DOG PLASMA DURING THE ESTROUS CYCLE AND PREGNANCY

by

FRED METZLER, JR.

B. A., University of Maine, 1963.

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1966

Approved by:

Dr. B. E. Eleftheriou

Major Professor

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

INTRODUCTION

Just after the turn of the century it was noted for the first time that biologically active extracts of ovaries were of lipoidal nature (Iscovesco, 1912; Fellner, 1913; Frank & Rosenbloom, 1915). These findings along with the historic studies of Stockard and Papanicolau (1917), Long and Evans (1922) and Allen (1922) dealing with changes in the reproductive tract during the estrous cycle gave the study of ovarian hormones its initial impetus. This acceleration quickly led to isolation in crystalline form of the so-called classical estrogens: estrone (Butenandt, 1929; Doisy et al., 1930), estradiol-17β (MacCorquodale et al., 1935; Wintersteiner et al., 1935) and estriol (Marrian, 1930; Doisy et al., 1930). In turn numerous other estrogens were, and still are being, isolated from biological fluids and tissues.

Prior to the mid-1950's most estrogen determinations employed the use of bioassays. Biological tests such as the vaginal smear test in ovariectomized mice (Allen & Doisy, 1923), increase in uterine weight in intact immature mice or rats (Astwood, 1938) and increased weight of the oviduct in immature chickens (Dorfman & Dorfman, 1948) were more than adequately sensitive when large amounts of test material were available and these tests were reportedly specific for estrogens. However, the usual drawbacks of a bioassay were inherent in all such tests: the large number of subjects and, therefore, the large quantity of test material required, test animal variability, poor reproducibility and test material dependency upon other variable factors. In addition, the use of a bioassay gave no indication as to which particular estrogen was initiating the response and how much of each estrogen was involved. Resolution of these two problems

was accomplished with the advent of sensitive and specific chemical methods.

In 1930, Kober published a short paper on a colorimetric technique for estrual hormone (Brunsthormon). Although Cohen and Marrian (1934) first attempted quantitation of estrone and estradiol-17β in human pregnancy urine with a Kober modification, it was not until much later (Brown, 1955) that appropriate modifications were made and the method used to accurately measure estrogens in urinary extracts. In addition to Brown (1955) several other investigators (Diczfalusy & Lindkvist, 1956; Ittrich, 1958; Roy & Brown, 1960; Roy & Mackay, 1962; Roy et al., 1965) have used variations of the original Kober reaction for both urinary and blood estrogens.

The sulfuric acid-fluorescence method for determining estrogens was first reported by Bates and Cohen (1947) for pure crystalline estrogens and by Jailer (1948) for estrogens in pregnancy urine. Later work by Bates and Cohen (1950) established the optimal conditions for this reaction. Since then numerous estrogen determinations have employed the sulfuric acid-fluorescence procedure. Engel et al. (1950) for example combined a countercurrent distribution purification with fluorometry to investigate the possibilities of quantitating estrogens in urine. Diczfalusy (1953) measured the free estrone, estradiol-17β and estriol in early and full-term human placental tissue. Mitchell and Davies (1954) followed this study with a similar one of their own which also measured the conjugated and protein-bound estrogens. Slaunwhite et al. (1953) re-evaluated the Bates and Cohen (1950) study and concluded that after excitation at 436 mμ, estrone, estradiol-17α and -17β, and estriol possessed fluorescence maxima between 478 and 487 mμ. These maxima were shorter than had previously been reported. Veldhuis (1953) also used

fluorometry to quantitate the free estrogens in the plasma of pregnant humans. Veldhuis (1953) reported that non-specific fluorescing material which interferred with the accuracy of the fluorometric technique came from the reagents and to a lesser extent from the plasma extracts. A method for the routine clinical determination of urinary estrogens was subsequently developed by McAnally and Hausman (1954). Preedy and Aitken (1957) combined acid hydrolysis, solvent partitioning, column chromatography and fluorometry to measure the total estrogen content in plasma of humans during late pregnancy. This method was again applied to determine the total estrogen levels in fetal and maternal plasma at parturition (Aitken et al., 1958), levels in both urine and plasma of pregnant humans (Preedy & Aitken, 1961), the interrelationships of estrogen concentration in fetal and maternal circulations and maternal urine (Manner et al., 1963) and the renal clearance of estrogens (Brown et al., 1960 & 1964). Veenhuizen et al. (1960) adapted the use of solvent partitioning, paper chromatography and fluorometry to identify and measure the free estrogens in bovine fetal cotyledons.

By combining the technical information gained from the above investigations with improved methods of isolation (eg., thin-layer and gas-liquid chromatography) the determination of micro amounts of estrogen in small volumes (2-5 ml) of plasma has now become feasible. Thus far the only investigation of estrogen levels in circulation throughout a complete pregnancy has been that for the human by Roy et al. (1965) with additional data procured from studies by Roy and Brown (1960), Roy and Mackay (1962), Preedy and Aitken (1957), Diczfalusy and Magnusson (1958) and Veldhuis (1953). Similar data for other species are completely lacking. Circulating

estrogen levels in non-pregnant subjects have also been investigated only in the human (Preedy & Aitken, 1957; Roy & Brown, 1960; Roy et al., 1965); the levels being considerably lower than those during pregnancy.

The results of some early studies indicated the absence of detectable amounts of estrogen in urine of pregnant dogs (Helm, 1931; Küst, 1931) and others (Lesbouyries et Berthelon, 1936; Finck, 1936; Stiasny, 1937; Nilsson, 1948) reported positive findings in urine. Recently, Kristoffersen and Velle (1960) have reported finding small (0 - 2.2 µg/l) quantities of estrogen in the urine of pregnant bitches. Siegel et al. (1962) have identified urinary metabolites of injected tritium-labelled estradiol-17β in the dog and concluded that while estradiol-17α was a major urinary metabolite, estriol was not.

After administrating estrone to dogs, Longwell and McKee (1942) found evidence of estrogens in both bile and urine, but none in control animals. In somewhat similar studies by Paschkis et al. (1943 & 1944), who injected androgens instead of estrogens, it was noted that estrogens were excreted in urine as well as in bile. Another group of workers (Pearlman et al., 1945, 1947 & 1948) also injected estrogen into dogs and examined the excreta. It was found that most estrogenic material was present in bile, a smaller amount in urine and an even smaller quantity in feces. Cantarow et al. (1943) injected dogs with human chorionic gonadotropin (HCG) and pregnant mare serum (PMS) and concluded that relatively large amounts of endogenous estrogens are excreted in bile.

After an extensive review of the literature only one reference has been found dealing specifically with estrogens in dog blood. Paschkis and Rakoff (1950) have reported that very little estrogenic activity

could be found in blood from either ovarian or femoral veins after inducing estrus in the dog by injecting PMS.

The trend in chromatography, particularly of steroids, has been to reduce running times and to develop simplified methods for separating minute quantities of material. This progress has presently culminated in two basic types of chromatography, gas-liquid and thin-layer. The latter has been widely applied to many substances since its introduction (Stahl, 1958). Recently Lisboa and Diczfalusy (1962) published a paper on the separation and characterization of a number of estrogens by means of thin-layer chromatography. Though initially used as a rapid and sensitive means of qualitative analysis, thin-layer chromatography has since been applied to steroid analysis as a quantitative tool. Ladany and Finkelstein (1963) have separated estrone, estradiol-17β and estriol on silica gel plates with a benzene:methanol (9:1, v/v) and chloroform:ethanol (9:1, v/v) systems and quantitated them by phosphoric acid-fluorometry.

Considering the dearth of knowledge on the circulating levels of estrogens in infrahuman species it was thought that any information obtained along this line would be a worthwhile contribution. It was first necessary to standardize quantitative procedures employing thin-layer chromatography and fluorometry so that these convenient and sensitive methods could be applied to the present investigation.

The dog was chosen as the subject with which to work since a well-established purebred colony was available and because little of the reproductive physiology of this popular laboratory animal at the molecular level is known.

MATERIALS AND METHODS

The dogs used in this study were all purebred adult female animals which were part of a colony maintained at the Hamilton Station, Jackson Laboratory, Bar Harbor, Maine. They consisted of three pregnant and two cycling basenjis, two pregnant beagles, one pregnant cocker spaniel and one pregnant wirehaired fox terrier. Plasma samples of about 5 ml each were obtained at various intervals ranging from the onset of the estrous cycle to two-three weeks after bleeding had ceased. In the case of pregnant dogs, samples were taken before fertilization (ie., early estrus) and after parturition as well as at intervals throughout gestation. Approximately 12 samples were obtained from each animal. All plasma samples were immediately frozen and stored at -20°C until extracted.

The chemical extraction procedure for free estrogens was essentially that of Veenhuizen et al. (1960). Frozen samples of about 5 ml were allowed to melt at room temperature and to each was added 1.0 ml estrone-16-^{14}C and 0.5 ml estradiol-17β-4-^{14}C in benzene (11,100 disintegrations per minute each). The sample was then measured to the nearest 0.1 ml. The volume of tracer added (ie., 1.5 ml) was subtracted and the net volume recorded. The sample was then extracted three times with equal volumes of anesthesia-grade diethyl ether, the ether extracts pooled, dried over anhydrous sodium sulfate, and evaporated in vacuo in a 38-40°C water bath. The lipid-like residue was dissolved in 10 ml toluene and transferred to a 45 ml ground glass-stoppered centrifuge tube. The toluene solution was washed three times by shaking with 5 ml 5% NaOH and centrifuged at 600 x G for five minutes. The NaOH phases (bottom layers) were pooled and back-washed with 5 ml toluene. The toluene layers were then discarded. The

pH of the pooled NaOH layers was adjusted to 7-8 with 6N sulfuric acid. This "neutralized" fraction was transferred to a separatory funnel and washed three times with 15 ml benzene to remove the estrogens from the aqueous phase. The benzene was evaporated under vacuum and nitrogen in a water bath at 38°C. The tube was flushed with nitrogen, capped and kept refrigerated (about 6°C) until chromatographed.

The qualitative study was initiated by subjecting several (wirehaired fox terrier for the most part) sample extracts to either one- or two-dimensional thin-layer chromatography (TLC) in an effort to discern the identity of dog plasma estrogens. The TLC procedure and solvent systems are described below.

Extracts from six cocker spaniel samples equivalent to 32.3 ml plasma were pooled and subjected to descending paper chromatography according to the method of Veenhuizen et al. (1960).

Whatman No. 1 paper strips were first cleaned by submerging in boiling absolute ethanol for 30 minutes, and after drying were then saturated with a 50% formamide solution in absolute methanol. The sample was taken up with a chloroform:absolute ethanol (1:1, v/v) mixture containing a few drops of formamide and was streaked with a capillary pipette. The strip was run in a glass chromatography tank whose atmosphere was saturated with benzene. Another strip spotted with 20-30 µg each of pure estrone, estradiol-17α, estradiol-17β and estriol was run simultaneously in the same manner. An equilibration period of at least two hours was allowed before adding a fresh 1:1 (v/v) mixture of Skellysolve B:benzene saturated with formamide. After addition of the mobile phase, chromatography was carried out for 16 hours at room temperature in subdued light. Upon completion of

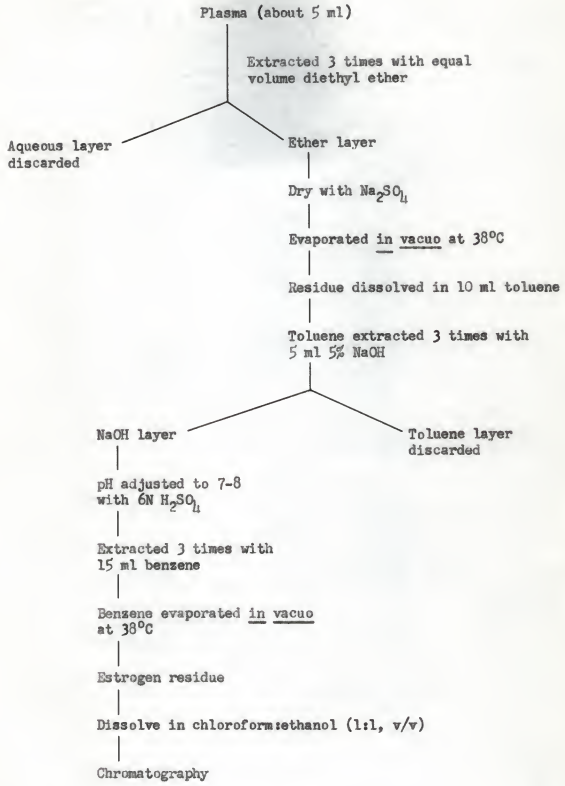

Fig. 1. Flow sheet of estrogen extraction procedure.

chromatography a hair dryer was used to hasten the drying time which took between 6-12 hours. The dried standard strip and a 6 mm portion of the sample strip were treated successively with 1% $FeCl_3$:1% KCr_2O_7 (1:1, v/v), 5% HCl and water (Veenhuizen et al., 1960). Blue areas indicated the presence of estrogens. The sample strip was also counted in a strip counter (Atomic Accessories, Inc. model RSC-5A) in order to locate the estradiol-17β-4-^{14}C and to see if the estrone-16-^{14}C had run off the strip into a collecting beaker. Taking the above into consideration, corresponding areas were cut from the sample strip and eluted with absolute methanol.

Solvent containing the estrone, which had been eluted into a collecting beaker during chromatography, was quantitatively transferred to a test tube. The residue was rechromatographed as above, but for only six hours. After drying, the estrone-16-^{14}C on the rechromatographed strip was located with the strip counter and this information, in addition to the unlabelled standard strip developed with the $FeCl_3$:KCr_2O_7 mixture, was used to determine the area to be eluted.

The eluate from the area on the original chromatogram corresponding to the standard estriol was rechromatographed on Whatman No. 1 paper for five hours in an ethyl acetate:toluene (15:85, v/v) system saturated with methanol (Mellin, 1964). In addition to the standard strip, a 6 mm strip from the sample strip was developed as above. Since it was found that the standard had remained principally at the origin with some streaking (to a distance of 11 cm), the standard strip was interpreted with caution. The 6 mm strip gave some indication of a blue area 1 cm from the bottom of the strip. It was decided that instead of eluting just one area, three

sections would be eluted separately. The first section included the origin and the first 11 cm, the second section comprised the next 10 cm and the third section contained the last 10 cm of the strip. These eluates were labelled E_3 'a', 'b', and 'c', respectively.

Infrared spectrophotometry was attempted on the estrone eluates, but was unsuccessful due to insufficient material.

A reaction to form methyl ethers (Bush, 1961) was carried out on half of the eluate from each area mentioned above as well as on pure estrone, estradiol-17α, estradiol-17β, estriol and 16-epiestriol standards. Another fourth of the eluates and standards were acetylated according to Bush (1961).

Both the methyl ether and acetylated derivatives were two-dimensionally chromatographed on thin-layer plates. For this and all subsequent two-dimensional TLC the first direction was run in chloroform:ethyl acetate (2:1, v/v) and the second direction in an ethyl acetate:n-hexane (1:1, v/v) system. Preperation of TLC plates consisted of coating 20 x 20 cm glass plates to a thickness of about 0.30 mm with a slurry of silica gel G (Merck) purified according to Randerath (1963). The slurry was made by thoroughly mixing 2 ml double-distilled water with 1 g silica gel G. Plates were activated for 30 minutes at 120°C and spotted when cool.

A sample for quantitation was taken up in successive aliquots of chloroform:methanol (1:1, v/v) and each portion applied to the same corner spot of the plate, employing a stream of nitrogen to hasten evaporation of the solvent. Another plate was spotted with about 20 μg each of estrone, estradiol-17α, estradiol-17β, estriol and 16-epiestriol standards in the same manner.

In most cases the sample extracts contained a substance, or substances which were soluble in organic solvents, but did not readily evaporate. This substance had a tendency to diffuse out from the spot on the plate. Since this spreading was accelerated when the plate was suspended in the tank, the equilibration time for the first direction was generally reduced to around one minute.

The time for the front to go 18-19 cm was about 90 minutes for the first system and about one hour for the second.

After chromatography, the standard plate was sprayed with 10% phosphomolybdic acid in absolute ethanol and heated for about 10 minutes at 100°C. Such treatment shows estrogens as varying hues of blue. The standard estrogen distribution, as well as the origin and front, were traced on paper. After scanning the sample plate with a short UV (2537 Å) Mineralight lamp to check for possible fluorescing impurities, the standard tracing was laid over the sample plate. The origins were aligned and the short UV fluorescing impurities and the sample plate fronts were added to the tracing. In addition, while the paper was still on the sample plate the standard spots were lightly traced so as to leave impressions of the spots on the sample plate. Taking into consideration the relative front movements and the amount of impurities (note: impurities tended to cause distortion of normal mobilities), measured areas were marked off around the corresponding standard spots. These areas were generally rectangular in shape and were about 16-21 cm^2. The short UV fluorescing areas were excluded as much as possible. A clear area was similarly marked off to serve as a blank. It was necessary to have approximately equal areas eluted from each plate since the blank varied with the amount of gel eluted

and made a difference in the fluorometric readings.

The areas were scraped off the glass plates with a stainless steel razor blade, transferred onto waxed weighing paper and then quantitatively transferred to glass-stoppered centrifuge tubes. Although Randerath (1963) suggested using methanol for eluting steroids from silica gel and most investigators commonly use methanol for eluting paper chromatograms, it was thought that at least one of the washings should contain chloroform because of the relatively low solubility of estrogens in methanol. The elution procedure, therefore, consisted of one washing with 5 ml chloroform: methanol (1:1, v/v) and a second with 5 ml methanol. The following procedure was used for both washings. About 2 ml of the eluant was added, the tube stoppered and then thoroughly shaken for 10-15 seconds on a Clay-Adams Cyclo-Mixer. The remaining eluant was used to rinse down the tube which was then centrifuged for 15 minutes at 1000 x G. After centrifugation, the eluates were decanted into test tubes.

The tube contents were dried with either a stream of nitrogen in a water bath at 38°C or under vacuum with a Buchler Rotary Evapomix at the same temperature.

To all tubes was added 1.0 ml absolute ethanol. For recovery rate determination purposes, 0.1 ml was removed from each of the estrone, estradiol-17α and estradiol-17β tubes and placed in separate counting vials. To each vial was added 10 ml of aqueous scintillation fluid. Radioactivity was then counted with a Packard Tricarb model 3314 liquid scintillation spectrometer. Window settings were 50 and 600 with a gain of 10. At least two one minute counts were taken, with the average being used in the calculations. In addition to the samples, a blank vial and

vials containing 11,100 dpm each of estrone-16-^{14}C and estradiol-17β-4-^{14}C were counted each time. In computing per cent recovery the background was first subtracted, the resulting value multiplied by 10 to get the total sample count, and this then divided by the standard count and multiplied by 100. The rationale for counting the estradiol-17α tube was to check for contamination by estradiol-17β since the two compounds were not readily separable in the TLC systems used. If counts were detected in the TLC systems used. If counts were detected in the estradiol-17α tube, a correction of the fluorescence value was later made.

The fluorometric procedure was a modification of McAnally and Hausman's (1954) with volumes adjusted to suit the cuvettes used (Mellin, 1964). To each tube was added 0.1 ml absolute ethanol and 0.7 ml 90% (v/v) sulfuric acid. This mixture was then heated in a water bath at 80°C for 20 minutes. After heating, the tubes were cooled to room temperature, 4.3 ml 65% (v/v) sulfuric acid added and the tube adequately shaken. After the bubbles had disappeared, the contents were transferred to matched 12 x 75 mm round Pyrex cuvettes for reading in the fluorometer. A model 110 Turner fluorometer was employed with a primary filter combination of 47B + 2A (the 2A filter closest to the lamp) and a secondary combination of a 2A-12 + 10% neutral density filter (the latter nearest the sample). Filter numbers were Kodak Wratten designations. The 2A filter passes wavelengths longer than 415 mμ and the 47B has its transmission peak at 436 mμ. The 2A-12 filter transmits wavelengths longer than 510 mμ. The 10% neutral density filter was used to reduce the sensitivity of the instrument by a factor of 10 in order to maintain linearity between estrogen concentration and fluorescence (Mellin, 1964). Wherever possible,

and in most cases it was, the aperture setting used was 30 X. A voltage regulator was used to stabilize the input current and minimize any drift of the fluorometer galvanometer.

Standard stock solutions of estrone, estradiol-17β, estriol (Calbiochem) and estradiol-17α and 16-epiestriol (Mann Research Laboratories) were made up to a concentration of 40 µg/ml in chloroform:ethanol (1:1, v/v). The stock solution was diluted to 0.4 µg/ml which served as the working standard. Fluorescence curves for each standard estrogen were obtained by adding appropriate amounts of the working standard to test tubes, drying the tubes and running the fluorescence reaction as described above. Depending upon the estrogen, five to seven points were used for each curve. Quantities of 0.04, 0.08, 0.16, 0.24, 0.32 and 0.40 µg were used for the estrone and estradiol-17α curves. Similar amounts as well as 0.60 and 0.80 µg for estradiol-17β, estriol and 16-epiestriol, and up to 1.0 µg for estriol and 16-epiestriol were used since these steroids exhibit a lower fluorescence. In all cases standard tubes and blanks received the same amount of chloroform:ethanol (1:1, v/v). In addition a blank area from a TLC plate run in the usual systems was eluted as described and small aliquots of this added to each standard and blank tube. The estrogen curves were run at least twice and the best points or average values used. The standard curves used in all of the calculations can be seen in Figure 1, page 8.

Calculation of the quantity of each estrogen was done by converting the fluorometer reading to micrograms of estrogen with the aid of a standard curve. To correct for the counting aliquot removed from the estrone, estradiol-17α and estradiol-17β samples, the microgram quantities determined

for these estrogens were increased by one tenth. The corrected values were then divided by the fractional recovery and by the original plasma volume. This μg/ml value was then multiplied by 100 to convert it to the conventional expression of μg estrogen/100 ml plasma. In instances where radioactivity (ie., estradiol-17β contamination) was detected in the estradiol-17α fraction, correction for fluorometric readings had to be made. By running many fluorometric determinations the average estradiol-17α:estradiol-17β fluorescence ratio was found to be 1.9. The following equation was therefore used to correctly re-distribute the fluorometric values:

2.9 X = initial estradiol-17α fluorometer reading

X = estradiol-17β contamination

1.9 X = actual estradiol-17α value

RESULTS

Efficiency of the extraction procedure was checked by taking a tracer count at every step. Overall efficiency was found to be 90% or better.

Appearance of an oily residue followed the benzene partition during extraction in the majority of cases. The contaminant prolonged spotting time, effected normal chromatographic migration of the estrogens and interferred with fluorometry. The implications of these interferences will be discussed later.

Qualitative Analysis:

Preliminary investigations employing the use of one- and two-dimensional thin-layer chromatography (TLC) gave indications of there being an estriol-like compound, or compounds, and estrogens of the estradiol and estrone type.

Two-dimensional TLC of methyl ether and acetylated derivatives of eluates from the paper chromatographic fractions contributed evidence that estrone, estradiol-17α, estradiol-17β and estriol were present. Indications of another polar estrogen (finally concluded to be 16-epiestriol) were also obtained. Co-chromatography resulted in no other estrogens, thereby supporting the derivative data.

The standard 16-epiestriol used for spotting standard plates underwent a chemical change during the course of the experiment; most likely oxidation of the 16- and 17-hydroxyl group. The new estrogen migrated faster on TLC than did 16-epiestriol, but not as fast as 16-keto-estradiol-17β. On several occasions a spot corresponding exactly with this 16-epiestriol impurity showed up on sample plates. Whether dog plasma

actually contains the 16-epiestriol derivative or whether it was due to oxidation of 16-epiestriol in the sample extract was not determined. However, this "accident" did give further proof of the presence of 16-epiestriol.

Quantitative Analysis:

As mentioned previously, contamination did lead to erroneous results in some cases. Evaluation of fluorometric data was further complicated by low recoveries as determined by the tracer recovery method (the loss of estrogen occurred at some step, or steps following the extraction procedure). Recovery percentages as low as 5-10%, and in a few instances 0%, were obtained for both the labelled estrone and estradiol-17β. Combining all recovery percentages, mean recoveries of 16.4 and 31.6% were arrived at for estrone and estradiol-17β, respectively. In general, the recovery percentage of estradiol-17β-4-^{14}C was used in the calculations of estradiol-17α, estradiol-17β, estriol and 16-epiestriol. In cases where the estradiol-17β-4-^{14}C recovery was below the mean of 31.6%, the mean recovery was used in calculating estriol and 16-epiestriol. This was done because the positions of estriol and 16-epiestriol standards varied little from plate to plate and there was little chance of missing the two estrogens when eluting. In those instances where the percent recovery was zero and fluorometric readings were obtained, mean recoveries were employed in the calculations.

There were frequently sample readings below the blank value. Whether this was solely a cause of differences in amount of gel eluted or due to a previously undetected impurity in the blank area was not resolved. The values were recorded as zero.

The only difficulty experienced with the standard curves (Fig. 3, page 20) was with the estriol curve. Regardless of how many times the standard curve was run, a straight-line curve could not be obtained. Evidently, the 10% neutral density filter was not adequate to maintain a linear relationship between fluorescence and estriol concentration. Finally 9-10 concentrations were run several times, the points plotted and the best straight line drawn.

Estrous Cycle:

The values obtained during the estrous cycle were divided into early estrous cycle (initial slight bleeding of the vulva; ie., early proestrus), middle estrous cycle (full bleeding; ie., late proestrus) and late estrous cycle (cessation of bleeding; ie., estrus). The values for the early estrous cycle are from only one sample and, therefore, must be interpreted with caution.

Mean values for the concentrations of individual estrogens during the early, middle and late estrous cycle have been listed in Table 1, page 21 and graphically represented in Fig. 4, page 22. Table 2, page 23 and Fig. 5, page 24 consist of the combined estrogen concentration means during the estrous cycle.

Pregnancy:

The estrogen levels during pregnancy were observed on a biweekly basis. An average of six samples per biweekly period were examined.

Trends in both individual (Fig. 6, page 25) and combined (Fig. 7, page 27) estrogen levels became apparent. The least prevalent estrogen, estradiol-17α, remained at fairly constant levels throughout pregnancy, ranging from a low of 0.4 to a high of 3.0 μg/100 ml plasma. Likewise,

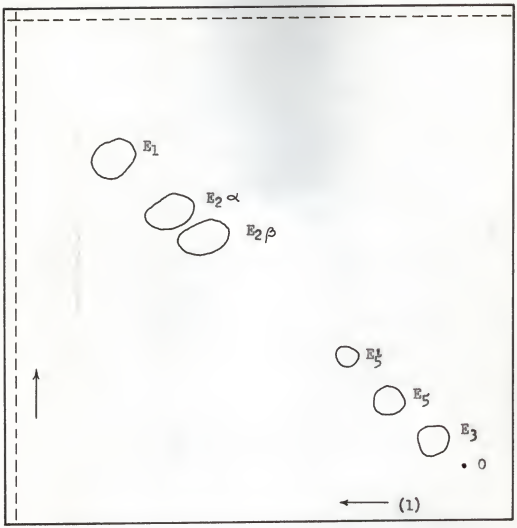

Fig. 2. Two-dimensional TLC of estrogen standards. First direction run in chloroform:ethyl acetate (2:1) and the second in ethyl acetate: n-hexane (1:1). Designations: O, origin; E_1, estrone; $E_{2\alpha}$, estradiol-17α; $E_{2\beta}$, estradiol-17β; E_3, estriol; E_5, 16-epiestriol; and E_4, 16-epiestriol impurity.

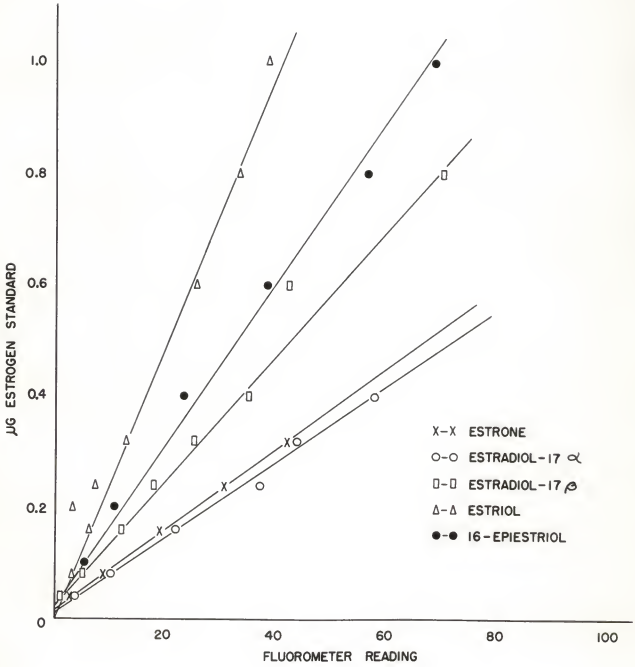

Fig. 3. Sulfuric acid-fluorescence curves for the following standard estrogens: estrone (E_1), estradiol-17α(E_2α), estradiol-17β(E_2β), estriol (E_3) and 16-epiestriol (E_5). Each point represents the means of 2-9 runs.

Table 1. The mean levels and ranges (ng/100 ml) of the individual free estrogens in plasma during the early, middle and late estrous cycle periods. N = the number of samples per period.

Period	Estrone	Estradiol-17α	Estradiol-17β	Estriol	16-Epiestriol	n
Early	38.4	22.5	49.4	26.1	6.6	1
Middle	30.3 (0.0-73.2)	7.6 (0.4-23.3)	7.5 (1.1-20.3)	14.8 (3.2-50.6)	8.9 (0.0-27.5)	6
Late	5.8 (2.0-9.6)	3.2 (0.0-6.3)	5.3 (0.0-10.6)	12.0 (11.5-12.6)	3.3 (0.0-6.6)	2

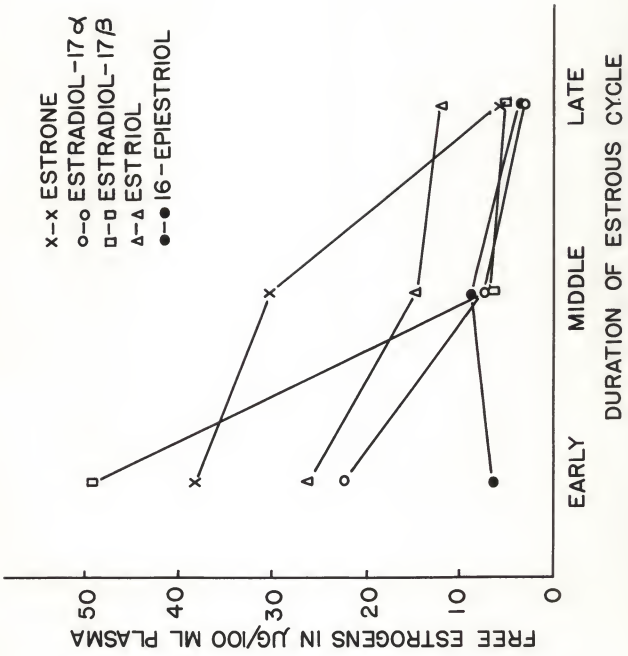

Fig. 4. Individual free estrogen levels in plasma during the estrous cycle. Early estrous cycle represents one sample whereas middle and late estrous cycle are means of six and two samples, respectively.

Table 2. The total free estrogen concentrations (µg/100 ml) in plasma during the early, middle and late estrous cycle periods. N = the number of samples per period.

Period	Mean	Range	n
Early Estrous	143.0	---	1
Middle Estrous	69.2	6.7-128.4	6
Late Estrous	29.6	13.5-45.7	2

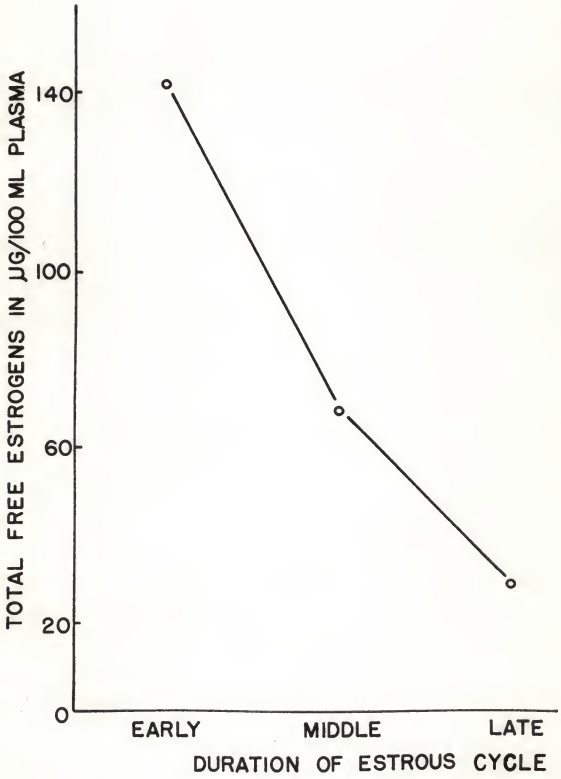

Fig. 5. Combined free estrogen levels in dog plasma during the estrous cycle.

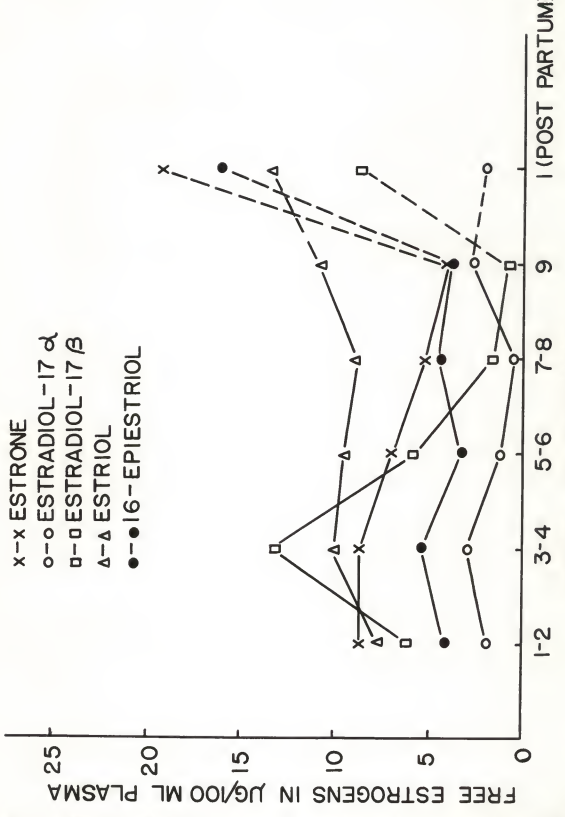

Fig. 6. Biweekly levels of individual free estrogens in dog plasma during pregnancy and including the first week post-partum. Each point represents the mean of 3-7 samples.

Table 3. The means and ranges (µg/100 ml) of the individual free estrogens in plasma during the first 4 biweekly periods, the ninth week of pregnancy and the first week post-partum. The number of samples = n.

Week	Estrone	Estradiol-17α	Estradiol-17β	Estriol	16-Epiestriol	n
1-2	8.6 (0.0-35.4)	1.7 (0.0-7.3)	6.1 (0.0-17.5)	7.6 (3.1-20.9)	4.2 (0.0-14.7)	7
3-4	8.6 (4.0-15.2)	3.0 (0.0-8.3)	13.4 (0.0-35.3)	10.1 (0.2-19.8)	5.4 (1.0-12.4)	6
5-6	6.9 (0.0-21.9)	1.2 (0.0-6.0)	5.7 (0.0-14.2)	9.6 (2.5-25.8)	3.2 (0.0-4.6)	6
7-8	5.2 (0.0-17.6)	0.4 (0.0-2.6)	1.6 (0.0-11.2)	8.8 (0.4-23.4)	4.5 (0.0-13.7)	7
9	4.0 (0.0-9.1)	2.8 (0.0-8.4)	0.7 (0.0-2.2)	10.9 (0.0-29.4)	3.9 (0.0-6.2)	3
1 p.p	19.2 (2.9-36.3)	2.0 (0.6-3.5)	8.8 (0.0-17.0)	13.5 (3.2-26.4)	16.3 (1.8-43.6)	4

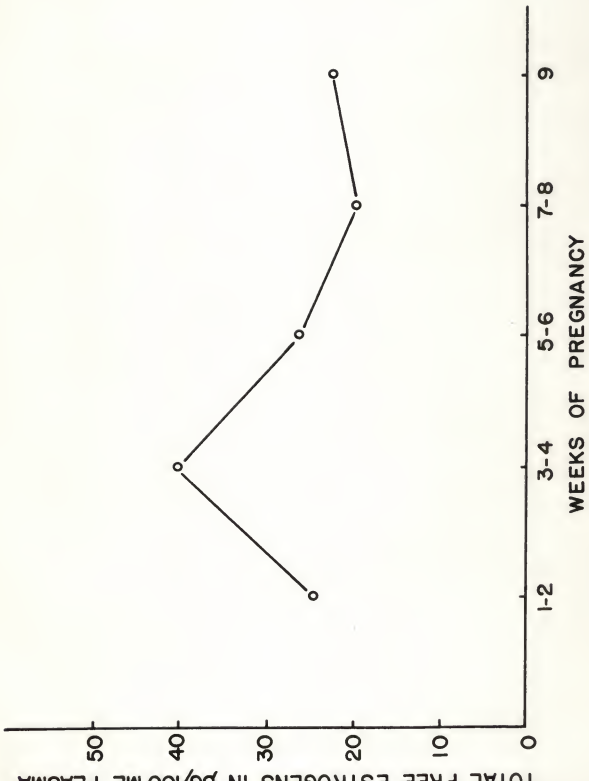

Fig. 7. Biweekly levels of combined free estrogens in dog plasma throughout pregnancy.

Table 4. The means and ranges (µg/100 ml) of the total free estrogens during the first 4 biweekly periods, the ninth week of pregnancy and the first week post-partum. N = the number of samples per period.

Week	Mean	Range	n
1-2	24.4	8.5-49.6	7
3-4	40.5	7.5-67.9	6
5-6	26.4	11.1-50.9	6
7-8	19.6	3.0-48.3	7
9	22.4	0.0-38.4	3
1 p.p	59.8	41.9-87.7	4

16-epiestriol also held fairly constant (3.2 - 5.4 µg/100 ml). In contrast though, both estrone and estradiol-17β showed a continuous decrease in concentration after reaching a peak of 8.6 and 13.4 µg/100 ml, respectively, during the 3-4 week period. The most pronounced decrease was that of estradiol-17β from a peak of 13.4 µg/100 ml at 3-4 weeks to a low of 0.7 µg/100 ml at nine weeks. Peak levels were attained by all of the estrogens at 3-4 weeks except for estriol, but it too was essentially at its peak. The 3-4 week period was again prominent when the individual values were combined. An average free estrogen level of about 23.2 µg/100 ml was maintained throughout pregnancy except for the 3-4 week period when the concentration rose to 40.5 µg/100 ml plasma.

During the first week post-partum there was a substantial increase in every estrogen except estradiol-17α. The means and ranges of four samples during this period can be found in Fig. 6, page 25, Table 3, page 26 and Fig. 7, page 27, Table 4, page 28.

DISCUSSION

The 90% plus efficiency of the extraction procedure obtained in this work was in good agreement with that reported by Veenhuizen et al. (1960).

The oily residue encountered after extraction was finally traced to the silicone stopcock grease (Dow Chemical Co.) on separatory funnels used to partition the benzene and neutralized aqueous fractions.

It was also concluded that stopcock grease contamination caused a distortion in normal distribution of the estrogens on thin-layer plates, thus contributing to the possible error in eluting and decreasing recovery efficiency. The distortion became progressively more pronounced as the front was approached (ie., greatest possible elution error was experienced with estrone and least with estriol). It is also likely that the oily residue hindered completeness of sample transfer from test tubes to thin-layer plates while spotting.

Although the stopcock grease contaminant caused some fluorescence upon exposure to short UV, it was not the sole source, for extracts obtained without stopcock grease also displayed such fluorescence. The presence of non-specific fluorescing materials stemming from both reagents and plasma extracts has been reported by Veldhuis (1953) and Slaunwhite and Sandberg (1950). Veldhuis (1953) suggested that the interferring materials might be lipids, and such might also be the case in the present investigation.

Qualitative Analysis:

The Skellysolve B:benzene:formamide paper chromatographic system of Veenhuizen et al. (1960) proved satisfactory for separating estrone, estradiol-17α, estradiol-17β and estriol. The difficulty encountered in the rechromatography of the estriol fraction was due to a procedural error. An

interesting phenomenon was observed in the apparent division of estriol into two spots on the paper chromatogram as determined by TLC of the acetylated products (ie., acetylation of estriol fractions 'a' and 'c' gave identical products with respect to chromatographic migration). It would seem probable that acetylation resulted in a racemization; the acetylation of the 16α- and β-hydroxyl groups yielded acetate groups spacially-oriented in only one direction.

Results from TLC analysis of unsubstituted extracts, acetylated and methylated derivatives and co-chromatography with standards showed that estrone, estradiol-17α, estradiol-17β and estriol were present as free estrogens in dog plasma. It should be kept in mind that the estrogen samples for TLC were initially separated into discrete fractions with paper chromatography. Identification of 16-epiestriol was based primarially upon inference. The initial one- and two-dimensional TLC analyses indicated an estrogen migrating slightly ahead of estriol, corresponding most closely with 16-epiestriol. Also, the standard 16-epiestriol impurity did exactly match the extra metabolite observed on several plates. This latter observation suggests the possible presence of yet another estrogen metabolite. Whether dog plasma actually contains this 16-epiestriol derivative or whether it was due to oxidation of 16-epiestriol in the sample extract was not determined, however.

The present results indicating the presence of estrone, estradiol-17α, estradiol-17β, estriol and 16-epiestriol in the dog are in good agreement with the findings of others. Early workers (Longwell & McKee, 1942), using the vaginal smear method in ovariectomized rats for the assay, could not detect any estrogens in the bile or urine of control dogs. However, upon

administration of estrone nearly all of the total estrogen detected in the bile extracts was in the non-ketonic fraction. Between 61.8 - 80.9% of the total estrogen in urine extracts was non-ketonic. This suggested that the dog has the enzyme systems necessary for transforming estrogens. As a further note, Paschkis et al. (1947) reported that after intramuscular injections of acetyl-estrone the major portion of estrogenic material was recovered in the bile, a lesser amount in the urine and a yet smaller, but measurable quantity in the feces. The majority of the estrogenic material in the bile was in the "free" form and the ratio of conjugated to free in the feces was small while this ratio was much greater in the urine.

Pearlman et al. (1947) questioned whether the dog was capable of transforming estrone into estriol to any extent. Siegel et al. (1962) also suggested that estriol is not a major metabolite in the dog. However, Siegel et al. (1962) did report finding trace amounts of estriol as well as other 16-oxygenated estrogens in dog urine. In contrast, Longwell and McKee (1942) found appreciable estrogenic activity in the 0.3 M Na_2CO_3-soluble, benzene-insoluble phenolic fraction (ie., that fraction consisting of estriol). Pearlman et al. (1945) also detected estrogenic activity in similar material in dog bile after injection of estradiol-17β. In addition to traces of estriol and other 16-oxygenated estrogens, Siegel et al. (1962) also found estrone, estradiol-17α and estradiol-17β in the urine and blood of the dog.

Quantitative Analysis:

In addition to causing distortions in the estrogen distribution pattern, the extraction contaminants also interferred with the fluorometry.

At times the interference was obvious and the value had to be discarded, but at other times there seemed to be little or no interference. Since it could not easily be discerned whether materials visible under short UV would interfere with fluorometric readings, it was preferable to avoid the contaminants wherever possible. It is also possible that impurities were present which did not show up under short UV, but did fluoresce when heated with sulfuric acid. Experience has shown, however, that if the plate appeared clean under short UV, the chance of there being extraneous fluorescence after acid treatment is slight.

The very low recovery percentages of the radioactively-labelled estrone and estradiol-17β may be attributed to a number of factors. The primary factor was most likely an error in estimating the position of the unknown from the distribution on the standard plate. While the fronts on both standard and unknown plates generally reached the same heights, impurities in the unknown could still cause distortions in estrogen distribution. It was concluded that use of a "cold" standard plate to determine the distribution of the unknowns was not always reliable and that the preferred method would be to use a TLC-plate scanner to accurately locate the radioactively-labelled estrogens. Other likely factors involved were loss during transfer of the sample from test tube to TLC plate, avoidance of short UV-fluorescing areas even when they corresponded to the possible estrogen locations, and loss when scraping gel off the plate. This latter factor has been considered by some investigators (Nandi & Bern, 1963) as being one of the drawbacks of using TLC for quantitative studies. Although in some instances in the present study estrogens were observed to remain on the glass, the technique was quite

effective if care and a sharp razor were used.

It has also been reported that silica gel impurities interfere with some methods of quantitation. Nandi and Bern (1963) have noted this effect in UV specroscopy, Randerath (1962) in fluorometry and Siegel and Dorfman (1963) with the Kober reaction. However, present evidence indicates that TLC can be used in conjunction with fluorometry. While the sample blank gives a considerably higher fluorometric reading than does the reagent blank, the causative factor(s) is not wholly due to the silica gel. Methanol-washed silica gel G was added to a reagent blank with little effect on the degree of fluorescence. It was observed that if the sample was eluted from the silica gel soon after the plate was run, the background fluorescence was higher than if the sample was eluted one to two days after running. The longer the plate remained exposed to the atmosphere before eluting the lower was the background fluorescence. However, it was considered inadvisable to permit exposure of estrogens to the atmosphere for too long a period since under such conditions oxidation can occur. Therefore, plates were kept in a light-tight box for about two to four days before eluting. It would appear, therefore, that one or more of the solvents used in the TLC systems and/or elution were responsible for the extra fluorescence and that with time the contaminant either evaporates or undergoes degradation to a non-fluorescing product. Veldhuis (1953), among others, has also reported background fluorescence from eluting reagents. In most cases during the present investigation, the extra fluorescence could be adequately blanked out with the instrument.

Though the use of mean recovery percentages in calculating the

quantitative data in the instances mentioned previously may not appear particularly desirable, to have used recoveries as low as 5% for estriol and 16-epiestriol would have resulted in values even further from the truth. In these cases and those where fluorometric readings were obtained even though the percent recovery was essentially zero as determined by radioactive counting, the use of mean recoveries seemed justified.

While the method of re-distributing the fluorometric values (see page 15) when radioactivity was detected in the estradiol-17α fraction is not unequivocally correct, it does offer a better approximation of the true values than if no correction were made.

In discussing results of both the estrous cycle and pregnancy it must be kept in mind that data for free estrogens only partially contribute to the overall estrogen status of the animal. Values for the circulating levels of both protein-bound and conjugated fractions are also required in order to more completely establish any conclusions.

Circulating estrogen levels in either the non-pregnant or pregnant subject have only been thoroughly studied in the human (Preedy & Aitken, 1957; Roy & Brown, 1960; Roy & Mackay, 1962; Roy et al., 1965; Smith, 1966) and the white-tailed deer (Eleftheriou et al., 1966; Church & Eleftheriou, 1966). Despite the quantity of data, direct comparison of most such results with this present work is difficult due to the use of different blood components for analysis. Whether or not there is a significant difference in whole blood estrogens as compared to plasma remains to be resolved. The works of Smith (1966) and Eleftheriou et al. (1966), however, do offer results for comparison.

Estrous Cycle:

For the follicular phase of the menstrual cycle in the human, Roy et al. (1965) have reported the following total (free, protein-bound and conjugated) estrogen levels in peripheral whole blood: estrone, 0.020 ± 0.011; estradiol-17β, 0.013 ± 0.008; and estriol, 0.025 ± 0.012 ug/100 ml. Their values for the ovulatory phase were 0.070 ± 0.025; 0.028 ± 0.017; and 0.037 ± 0.023 µg/100 ml, respectively. It, therefore, appears that at least in the case of the human, the general belief that estrogen levels increase as the follicules mature and rupture holds true. If this is also the case in the dog, and from bioassay evidence it seems to be so (Gier, 1960), then the progressive decline in free estrogens from proestrus through estrus (Fig. 3) would indicate a concomittant rise of the estrogens in some other form(s). Whether the other forms are protein-bound and/or conjugated fractions or an ever increasing binding of the estrogens at their tissue receptor sites and subsequent metabolism are possibilities, but ones which require supporting evidence.

If approached from a slightly different angle, the problem of explaining the decline in free estrogen levels becomes even more perplexing. The free form is taken to be the biologically active fraction and since sex-accessory tissue (eg. uterus, vagina) response appears to indicate progressively increasing amounts of active estrogen during the estrous cycle, it would appear logical that the present study should have shown a steady increase in the free estrogen levels. This not being the case, the following is proposed. The tissues in question have been sufficiently predisposed by the initial high level of free estrogen, and quite likely by other hormonal agents such as progesterone and the

gonadotropins, so as to be sensitized to estrogen. Therefore, higher active levels of estrogen would not be required to initiate maximal tissue responses. The requirement of a high concentration of estrogen to change the physiologic condition of the sex-accessory tissues from an anestrus to proestrus state is not outside the realm of possibility since it is apparent that the dog is relatively insensitive to estrogens when compared with the human and the cow.

Assuming the above hypothesis to be true and since the estrogen production by the ovary continually increases, the extra estrogen would have to be directed to protein-bound and conjugated forms with, quite likely, a corresponding increase in excretion rate. With respect to the latter however, Kristoffersen and Velle (1960) found very low levels of estrogen in the urine of the dog. Discussion of the possible implications of renal clearances will follow.

Pregnancy:

It is known that as pregnancy progresses the circulating and urinary estrogen levels increase. This is initially due to increased ovarian activity and later as a function of placental participation. Roy and Mackay (1962) and Roy et al. (1965) have shown the progressive increase of total estrogens in the whole blood of women from the 6-7 week period to the 40th week. They did state, however, that the increase for each estrogen measured was not always continuous. Their values for the 6-7 week period were for estrone, 0.138 ± 0.081; estradiol-17β, 0.050 ± 0.021; and estriol, 0.051 ± 0.023 μg/100 ml and for the 40th week period: 5.44 ± 2.36; 1.19 ± 0.41; and 9.71 ± 5.00 μg/100 ml, respectively. The increase in estrogens during pregnancy also increases the concentration

of plasma proteins.

With the above in mind, the results of the present study, which indicate a fairly constant free estrogen level throughout pregnancy (except during the 3-4 week period), can best be explained in the following manner.

The phenomenon observed is most likely a combination of factors. In order to maintain a constant level of an X while the source is producing more of X than before, the excess X must be diverted to other state or places. In the case of a constant free estrogen level, this diversion could very well be contributed by a combination of: 1) target-tissue requirements continually and gradually increase, thereby affecting an increased rate of metabolism of the estrogens; 2) estrogens, themselves, are known to stimulate an increase in the amount of plasma proteins which in turn could lead to increased protein binding of the estrogens; and 3) an increased excretion rate, particularly renal clearance.

The latter may well be the direct consequence of increased blood estrogen levels. Brown et al. (1964) have studied the renal clearance of estrogens in the human during late pregnancy and concluded that while the urinary/plasma ratios for estrone and estradiol-17β are essentially the same, that for estriol is significantly different when compared to either estrone or estradiol-17β. Furthermore, after examining the relationship between plasma concentration and renal clearance, they suggested the possibility of a tubular secretion maximum for estriol. However, Brown et al. (1964) did not distinguish between the various forms in which estrogens exist in both plasma and urine, but rather arrived at

values for total estrogens.

The work of Brown et al. (1964) may offer an explanation for the presence of low levels of free estradiol-17α in plasma while a major urinary estrogen metabolite in the dog (Siegel et al., 1962). The difference between the free plasma level and urinary total estradiol-17α level is most likely due to another form of estradiol-17α (ie., protein-bound and/or conjugated) and/or an increased conversion of another estrogen (eg., estrone) to estradiol-17α in the liver. Just the reverse may be true for estriol, which is in high concentrations in the plasma while it is not reported to be a major metabolite in the urine (Siegel et al., 1962). Although these metabolic conversions and variances in physico-chemical forms are good possibilities, the possibility of there also being a selective renal tubular secretion mechanism cannot be excluded.

The estrogen-protein relationship has been investigated by various workers (Szego & Roberts, 1946; Bischoff et al., 1954; Antoniades et al., 1957). In a review by Sandberg et al. (1957) on the binding of steroids to human plasma proteins, it was concluded that steroid-protein binding involves free and conjugated steroids, that the binding relationship is weak and reversible, and that of all the steroids examined the estrogens are the most tightly bound. Szego and Roberts (1946) were able to progressively dissociate the estrogen-protein complex by dialysis. Their results indicate a simple equilibrium between the estrogen and protein and, therefore, the degree of binding is logically a function of both the estrogen and protein concentrations. Since both estrogen and plasma protein concentrations increase during pregnancy, it would follow that the amount of estrogen-protein binding would also increase and thereby

aid in maintaining a constant free estrogen level.

During pregnancy the progressive proliferation of the placenta, uterus, mammaries and other tissues involved in reproduction is directly controlled by a balance of various hormones including estrogens. It is well known that hormone ratios change throughout pregnancy, indicating changes in production rates and/or changes in metabolic rates.

As proposed for the estrous cycle (see page 37), the sex-accessory tissues require less estrogen for a given response after having been initially primed by a high dose of estrogen. If an organ (eg., uterus) is taken as a whole, its overall estrogen requirement increases throughout pregnancy since maintenance and proliferation continue. However, on the cellular level the amount of estrogen per unit response is lower than that required initially. These tendencies would cancel to some degree, although the former would probably predominate. The total estrogen level (free as well as protein-bound and conjugated) may increase in proportion to the requirement for free (ie., "active") estrogen. This would mean a continual drawing off of free estrogen and a constant readjustment of the equilibrium in order to maintain a constant free estrogen level. This would also imply a shift from combined estrogens back to free; a subsequent decline in total estsogen if it were not for the increased rate of estrogen production by the placenta.

In this work the 43% increase above the mean of the dog plasma estrogens during the 3-4 week period can best be attributed to the initiation of placental secretory activity. It is during this time that placental development is completed (Gier, 1965) and its estrogen secretion combined with that of the ovary without a concomittant increase in tissue estrogen requirement would result in an increased free estrogen level.

With the cessation of ovarian activity and re-establishment of a relationship between tissue requirement and physicochemical equilibrium, the free estrogen level would be expected to decline to its normal mean value of 23.2 µg/100 ml.

Comparison of the present data with free estrogen levels in white-tailed deer plasma (Eleftheriou et al., 1966) indicates a similarity in that during most of pregnancy free estrogen plasma levels remain fairly constant. Eleftheriou et al. (1966) however, report a significant rise in free estrogen levels from estrus to 6-8 weeks of pregnancy, whereas in the present study no notable difference was found between estrus and the first week of pregnancy. This discrepancy need not necessarily be true since the 6-8 week period of pregnancy in the deer may correspond to the 3-4 week period of the dog. On such a basis the dog would also show a significant rise above the estrus level.

Smith (1966) found relatively constant levels of free estrone and estriol and slightly increasing levels of estradiol-17β in human plasma from 15 weeks before the onset of labor to term. The present study also indicates constant free estrogen levels in the dog during the latter stages of pregnancy, except for estradiol-17β, which shows a progressive decline.

A surprising result was the spectacular rise in free estrogens during the first week post-partum. It is generally assumed that in an animal without a post-partum heat the total estrogen concentration declines after parturition since the major source of the hormone (ie., placenta) is expelled. If this assumption is correct, the results suggest a sudden shift in equilibrium to predominately the free form. However, this rise

in free estrogen would be expected to be transient since the sex-accessory tissues show little evidence of estrogenic activity during anestrus. If the phenomenon is not transient, either the tissues are de-sensitized to estrogen following parturition or the values are incorrect. The latter appears unlikely. Considering the changes in other hormonal concentrations following parturition, a de-sensitization is quite probable.

CONCLUSION

The qualitative and quantitative techniques used in this study appear to be sound. Specificity, sensitivity, speed and simplicity are advantages of TLC plus fluorometry although a drawback is the presence of interferring fluorescent materials. The interferring materials, however, can be reduced to a minimum by using the proper combination of pure reagents. Addition of refinements adopted during the course of this study should prove useful. Final procedural refinements include: 1) use of teflon stopcocks on the separatory funnels in the extraction procedure (overcomes silicone grease contamination and subsequent difficulties in spotting, distortion of normal chromatographic migration, and excess residual fluoroescence) and 2) use of chloroform as the solvent for elution from silica gel rather than methanol (this decreases the amount of silica gel residue and background fluorescence).

SUMMARY

Integration of solvent partitioning plus paper and thin-layer chromatography with sulfuric acid-fluorometry led to the successful identification and quantitation of estrone, estradiol-17α, estradiol-17β, estriol and 16-epiestriol as free estrogens in dog plasma. Sulfuric acid-fluorescence was used to quantitate the estrogens.

The estrous cycle was divided into early, middle, and late phases corresponding approximately to early proestrus, late proestrus and estrus, respectively. Mean values (μg/100 ml plasma) for the early phase were estrone, 38.4; estradiol-17α, 22.5; estradiol-17β, 49.4; estriol, 26.1; and 16-epiestriol, 6.6. Middle phase values in the same order were 30.3, 7.6, 7.5, 14.8 and 8.9 μg/100 ml plasma. Values for the late phase were: 5.8, 3.2, 5.3, 12.0 and 3.3 μg/100 ml plasma, respectively. Combined values averaged 143.0, 69.2 and 29.6 μg/100 ml plasma for the early, middle and late phases, respectively. The progressive decline in total estrogen was attributed to increased tissue sensitivity to estrogen after a priming effect and to a shift in the equilibrium from the free form to protein-bound and conjugated states.

Except for estrone and estradiol-17β individual estrogen levels remained fairly constant throughout pregnancy with estradiol-17 being the least in concentration and estriol the greatest. Both estrone and estradiol-17β decreased after 3-4 weeks. Combined values indicated a constant level averaging 23.2 μg/100 ml plasma throughout pregnancy except for the 3-4 week period when the level rose to 40.5. Mean values for total estrogen in μg/100 ml plasma during biweekly periods were: 1-2 (24.4); 3-4 (40.5); 5-6 (26.4); 7-8 (19.6); and 9 (22.4).

A number of hypotheses have been explored in an attempt to explain the correlations between free estrogen levels and various stages of the estrous cycle and pregnancy.

ACKNOWLEDGEMENTS

I wish to thank the following people without whose aid this study would have been exceedingly difficult, if not impossible: Dr. B. E. Eleftheriou for his financial and moral support; The Kansas Agriculture Experiment Station for my research assistantship; Dr. T. Mellin for advice on the paper chromatographic and fluorometric procedures; Mr. H. Minocha for counting the radioactive samples; and Dr. M. Fox for the plasma samples.

BIBLIOGRAPHY

Aitken, E. H., J. R. K. Preedy, B. Eton and R. V. Short. 1958. Oestrogen and progesterone levels in foetal and maternal plasma at parturition. Lancet. 2:1096.

Allen, E. 1922. The estrous cycle of the mouse. Am. J. Anat. 30:297.

Allen, E. and E. A. Doisy. 1923. An ovarian hormone; preliminary report on its localization, extraction and partial purification and action in test animals. J. Am. Med. Assoc. 81:819.

Antoniades, H. N., J. W. McArthur, R. B. Pennell, F. M. Ingersoll, H. Ulfelder and J. L. Oncley. 1957. Distribution of infused estrone in human plasma. Am. J. Physiol. 189:455.

Astwood, E. B. 1938. A six-hour assay for the quantitative determination of estrogen. Endocrinology. 23:25.

Bates, R. W. and H. Cohen. 1947. Quantitative fluorescent micro-method for the determination of natural estrogens. Fed. Proc. 6:236.

_____. 1950. Experimental basis for selecting optimal conditions for quantitative fluorometry of natural estrogens. Endocrinology. 47:166.

_____. 1950. Fluorescence spectra of natural estrogens and their application to biological extracts. Endocrinology. 47:182.

Bischoff, F., R. D. Stauffer and C. L. Gray. 1954. Physio-chemical state of the circulating steroids. Am. J. Physiol. 177:65.

Brown, C. H., B. D. Saffan, C. M. Howard and J. R. K. Preedy. 1964. The renal clearance of endogenous estrogens in late pregnancy. J. Clin. Invest. 43:295.

Brown, C. H., B. D. Saffan and J. R. K. Preedy. 1960. Renal handling of estrogens in late pregnancy. J. Clin. Invest. 39:975.

Brown, J. B. 1955. A chemical method for the determination of oestriol, oestrone and oestradiol in human urine. Biochem. J. 60:185.

Bush, I. E. 1961. The Chromatography of Steroids. Pergamon Press. pp. 358 and 367.

Butenandt, A. 1929. Über "Progynon", ein kristallisiertes weibliches Sexualhormon. Naturwissenschaften. 17:879.

Cantarow, A., A. E. Rakoff, K. E. Passchkis, L. P. Hansen and A. A. Walkling. 1943. Excretion of exogenous and endogenous estrogen in bile of dogs and humans. Proc. Soc. Exp. Biol. Med. 52:256.

Church, R. L. and B. E. Eleftheriou. 1966. Unpublished data.

Cohen, S. L. and G. F. Marrian. 1934. The application of the Kober test to the quantitative estimation of oestrone and oestriol in human pregnancy urine. Biochem. J. 28:1603.

Diczfalusy, E. 1953. Chorionic gonadotrophin and oestrogens in the human placenta. Acta endocr. (Kbh.). Supp. 12.

Diczfalusy, E. and P. Lindkvist. 1956. Isolation and estimation of free estrogens in human placenta. Acta endocr. (Kbh.). 22:203.

Diczfalusy, E. and A. M. Magnusson. 1958. Tissue concentration of oestrone, oestradiol-17B and oestriol in the human foetus. Acta endocr. (Kbh.). 28:169.

Doisy, E. A., C. D. Veler and S. A. Thayer. 1929. Folliculin from urine of pregnant women. Am. J. Physiol. 90:329.

Dorfman, R. I. and A. S. Dorfman. 1948. Studies on the bioassay of hormones --- the assay of estrogens by a chick oviduct method. Endocrinology. 42:85.

Eleftheriou, B. E., K. W. Boehlke, A. Zolovick and F. Knowlton. 1966. Free plasma estrogens in the deer. Proc. Soc. Exptl. Biol. Med. 121:88.

Engel, L. L., W. R. Slaunwhite, Jr., P. Carter and I. T. Nathanson. 1950. The separation of natural estrogens by counter-current distribution. J. Biol. Chem. 185:255.

Fellner, O. O. 1913. Experimentelle Untersuchungen über die Wirkung von Gewebsextrakten aus der Plazenta und den weiblichen Sexualorganen auf das Genitale. Arch. Gynäk. 100:641.

Finck. 1936. Rec. Med. Vet. 112:628.

Frank, R. T. and J. Rosenbloom. 1915. Physiologically active substances contained in the placenta and corpus luteum. Surg. Gynec. Obstet. 21:649.

Gier, H. T. 1960. Estrous cycle in the bitch: vaginal fluids. Veterinary Scope. V:2.

_____. 1965. Personal communication.

Helm, K. 1931. Tierärzt. Rdsch. 37:671.

Herrmann, E. 1915. Ueber eine wirksame Substanz im Eierstocke und in der Placenta. Mschr. Geburtsh. Gynäk. 41:1.

Iscovesco, H. 1912. Les lipoides de l'ovarire. C. R. Soc. Biol. (Paris). 63:16.

Ittrich, G. 1958. Eine neur Methode zur chemischen Bestimmung der östrogenen Hormone im Harn. Hoppe-Seyl. Z. f. physiol. Chemie. 312:1.

Jailer, J. W. 1948. A fluorometric method for the clinical determination of estrone and estradiol. J. Clin. Endocrinol. 8:564.

Kober, S. 1931. Eine kolorimtrisch Bestimmung des Brunsthormons (Menformon). Biochem. Z. 239:209.

Kristoffersen, J. and W. Velle. 1960. Urinary estrogens of the dog. Nature. 185:253.

Küst. 1931. Die Feststellung der Trächtigkeit der Stute durch den Nachweis des Ovarialhormons im Harn. Duetsche Tierärztl. Wochenschr. 39:33.

Ladany, S. and M. Finkelstein. 1963. Isolation of estrone, estradiol-17B and estriol from female human urine. Steroids. 2:297.

Lesbouyries, et Berthelon. 1936. Le diagnostic de la gestation chez la chienne. Bull. Acad. Vet. France. 9:62.

Lisboa, B. P. and E. Diczfalusy. 1962. Separation and characterization of steroid estrogens by means of TLC. Acta endocr. (Kbh.). 40:60.

Long, J. A. and H. M. Evans. 1922. The estrous cycle in the rat and its associated phenomena. Mem. Univ. Calif. 6:1.

Longwell, B. B. and F. S. McKee. 1942. The excretion of estrogens in the bile and urine after the administration of estrone. J. Biol. Chem. 142:757.

MacCorquodale, D. W., S. A. Thayer and E. A. Doisy. 1935. The crystalline ovarian follicular hormone. Proc. Soc. Exp. Biol. Med. 32:1182.

Manner, F. D., B. D. Saffan, R. A. Wiggins, J. D. Thompson and J. R. K. Preedy. 1963. Interrelationships of estrogen concentrations in the maternal circulation, fetal circulation and maternal urine in late pregnancy. J. Clin. Endocr. Metabol. 23:445.

Marrian, G. F. 1929. The chemistry of oestrin. II. Methods of purification. Biochem. J. 23:1233.

McAnally, J. S. and E. R. Hausman. 1954. The determination of urinary estrogens by fluorescence. J. Lab. Clin. Med. 44:647.

Mellin, T. 1964. Personal communication.

Mitchell, F. L. and R. E. Davies. 1954. The isolation and estimation of the steroid oestrogens in placental tissue. J. Biol. Chem. 56:690.

Nandi, J. and H. A. Bern. 1965. Chromatography of corticosteroids from teleost fishes. Gen. Comp. Endocr. 5:1.

Nilsson, S. A. 1948. Om sambandet mellan dermatoser och sexual-endokrina rubningar hos tik. Skand. Vet.-Tidskr. 38:643.

Paschkis, K. E., A. Cantarow, A. E. Rakoff, L. P. Hansen and A. A. Walkling. 1943. Excretion of androgens, 17-keto-steroids and estrogens in the dog. following administration of androgens. Proc. Soc. Exp. Biol. Med. 53:213.

_____. 1944. Excretion in the dog of androgens and estrogens in the bile following injections of androgens. Proc. Soc. Exp. Biol. Med. 55:127.

Paschkis, K. E. and A. E. Rakoff. 1950. Some aspects of the physiology of estrogenic hormones. Rec. Prog. Hormone Res. 5:115.

Pearlman, W. H., K. E. Paschkis, A. E. Rakoff, A. Cantarow, A. A. Walkling and L. E. Hansen. 1945. A note on the biliary excretion of exogenous estrogen. Endocrinology. 36:284.

Pearlman, W. H., A. E. Rakoff, A. Cantarow, K. E. Paschkis and A. A. Walkling. 1947. The metabolism of estrone in the dog. Fed. Proc. 6:283.

_____. 1948. The metabolic fate of estrone in bile fistula dogs. J. Biol. Chem. 173:175.

Preedy, J. R. K. and E. H. Aitken. 1957. Plasma-estrogen levels in late pregnancy. Lancet. 272:191.

_____. 1961. Determination of estrone, estradiol-17B and estriol in urine and plasma with column partition chromatography. J. Biol. Chem. 236:1300.

Randerath, K. 1964. Thin-Layer Chromatography. Academic Press. p. 120.

Roy, E. J. and J. B. Brown. 1960. A method for the estimation of oestriol, oestrone and oestradiol-17B in the blood of the pregnant woman and of the foetus. J. Endocr. 21:9.

Roy, E. J., R. A. Harkness and M. G. Kerr. 1965. The concentration of oestrogens in the peripheral blood of women during the normal menstrual cycle and in the first trimester of pregnancy. J. Endocr. 31:177.

Roy, E. J. and R. Mackay. 1962. The concentration of oestrogens in blood during pregnancy . J. Obstet. Gynaec. Brit. Cwlth. 69:13.

Sandberg, A. A., W. R. Slaunwhite, Jr. and H. N. Antoniades. 1957. The binding of steroids and steroid conjugates to human plasma proteins. Rec. Prog. Hormone Res. 13:209.

Siegel, E. T. and R. I. Dorfman. 1963. A chemical method for the determination of estrone, 17 -estradiol and 17 -estradiol in canine urine. Steroids. 1:409.

Siegel, E. T., R. I. Dorfman, R. S. Brodey and M. H. F. Friedman. 1962. Conversion of 6,7-H^3-estradiol-17 into estrone and estradiol-17 in the mature male dog. Proc. Soc. Exp. Biol. Med. 111:533.

Slaunwhite, W. R., Jr., L. L. Engel, J. F. Scott and C. L. Ham. 1953. Fluorescence and absorption spectra of estrogens heated in sulfuric acid. J. Biol. Chem. 201:615.

Slaunwhite, W. R., Jr. and A. A. Sandberg. 1959. Phenolic steroids in human subjects. III. Estrogens in plasma of pregnant women. Proc. Soc. Exp. Biol. Med. 101:544.

Smith, O. W. 1966. Free and conjugated estrogens in blood and urine before and during parturition in normal human pregnancy. Acta endocr. (Kbh.). 51(Suppl. 104):3.

Stahl, E. 1958. Thin-layer chromatography. II. Standardization, detection documentation, and application. Chemiker-Ztg. 82:323.

Stiasny, H. 1937. Z. Geburtsh. Gynäk. 116:108.

Stockard, C. R. and G. N. Papanicolau. 1917. The existence of a typical oestrous cycle in the guinea pig with a study of its histological and physiological changes. Am. J. Anat. 22:225.

Szego, C. M. and S. Roberts. 1946. The nature of circulating estrogens. Proc. Soc. Exp. Biol. Med. 61:161.

Veenhuizen, E. L., R. E. Erb and J. Gorski. 1960. Quantitative determination of free estrone, estradiol-17 and estradiol-17 in bovine fetal cotyledons. J. Dairy Science. 43:270.

Veldhuis, A. H. 1953. A chemical method for the determination of estrogens in plasma. J. Biol. Chem. 202:107.

Wintersteiner, O., E. Schwenk and B. Whitman. 1935. Estrogenic dihydroxy compounds in the urine of pregnant mares. Proc. Soc. Exp. Biol. Med. 32:1087.

THE QUALITATIVE AND QUANTITATIVE DETERMINATION OF FREE ESTROGENS IN DOG PLASMA DURING THE ESTROUS CYCLE AND PREGNANCY

by

FRED METZLER, JR.

B. A., University of Maine, 1963.

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1966

The direct determination and measurement of biologically-active molecules, rather than their effects is a major innovation. With the means now at hand, it is possible to obtain data on specific hormonal levels in situ. The availability of such data greatly contributes to the understanding of basic physiological processes from the organismic to the cellular level. For these reasons, a study of the free estrogens in plasma of cycling and pregnant dogs were undertaken.

The procedure consisted of combining solvent extraction, chromatography (paper and thin-layer), radioisotopes and sulfuric acid fluorometry.

Prior to extraction estrone-16-^{14}C and estradiol-17β-4-^{14}C were added to each sample for recovery determination purposes.

Extraction consisted of first extracting with diethyl ether, evaporating the ether fraction and dissolving the residue in toluene. The toluene solution was then treated with 5% NaOH, the NaOH fraction neutralized with 6N H_2SO_4 and extracted with benzene.

The qualitative analysis entailed chromatography of unsubstituted, acetylated and methylated estrogen derivatives. Initial chromatography was on Whatman No. 1 paper (saturated with 50% formamide in methanol) using a Skellysolve B: benzene (1:1, v/v) system saturated with formamide. The eluate from the origin (ie., polar estrogens) was re-run in an ethyl acetate:toluene (15:85, v/v) system saturated with 50% methanol. Separate portions of these fractions were then acetylated or methylated and run 2-dimensionally on silica gel G thin-layer plates in chloroform:ethyl acetate (2:1, v/v) for the first direction and ethyl acetate:n-hexane (1:1, v/v) for the second. Chromatography led to the conclusion that the free estrogens in female dog plasma include estrone, estradiol-17α, estradiol-17β,

estriol and 16-epiestriol.

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 (1:1, v/v) mixture. The eluate was then evaporated, the residue redissolved in 1.0 ml absolute ethanol and a 0.1 ml aliquot taken for purposes of counting radioactivity. The remainder was evaporated and to the residue was added 0.1 ml absolute ethanol and 0.7 ml 90% sulfuric acid. This mixture was heated for 20 minutes at 80°C. After cooling, 4.3 ml 65% sulfuric acid was added. The fluorescence of the mixture was measured with a fluorometer using a primary combination of 47B + 2A and a secondary combination of 2A-12 + 10% neutral density Kodak Wratten filters. After correcting for recovery rate and original sample volume, values for estrogen in µg/100 ml plasma were obtained.

The estrous cycle was divided into early, middle and late phases corresponding approximately to early proestrus, late proestrus and estrus, respectively. Mean values (µg/100 ml plasma) for the early phase were estrone, 38.4; estradiol-17α, 22.5; estradiol-17β, 49.4; estriol, 26.1; and 16-epiestriol, 6.6. Middle phase values in the same order were 30.3, 7.6, 7.5, 14.8 and 8.9 µg/100 ml. Values for the late phase were: 5.8, 3.2, 5.3, 12.0 and 3.3 µg/100 ml, respectively. Combined values averaged 143.0, 69.2 and 29.6 µg/100 ml for the early, middle and late phases, respectively. The progressive decline in total free estrogen was attributed to increased tissue sensitivity to estrogen after a priming effect and to a shift in the equilibrium from the free form to protein-bound and conjugated states.

Except for estrone and estradiol-17β, individual estrogen levels remained fairly constant throughout pregnancy with estradiol-17α being the least in concentration and estriol the greatest. However, both estrone and estradiol-17β decreased after 3-4 weeks. Combined values indicated a constant level averaging 23.2 μg/100 ml throughout pregnancy except for the 3-4 week period when the level rose to 40.5. Mean values for total free estrogen in μg/100 ml plasma during the biweekly periods were: 1-2 (24.4); 3-4 (40.5); 5-6 (26.4); 7-8 (19.6); and 9 (22.4). It is felt by the author that the following hypotheses best explain the maintenance of constant free estrogen levels during pregnancy: 1) continual and gradual increased sex-accessory tissue requirement for active estrogen; 2) increased protein binding; and 3) increased and selective excretion. The 3-4 week period increase was considered due to the initiation of placental secretory activity and an altered free: combined equilibrium. The first week post-partum values showed increases in all free estrogens except estradiol-17α. The combined value was 59.8 μg/100 ml. This rise suggested a shift in equilibrium to the free form, concomitant with a decreased tissue sensitivity.

In addition to contributing estrogen data, the present study stimulated thought on further hypotheses concerning estrogen problems.