EXTRACTION AND IDENTIFICATION OF ESTROGENS AND PROGESTINS IN DOG URINE

by

DONALD MACK SPENCER

A. B., William Jewell College, 1954

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1956

Table of Contents

INTRODUCTION

The knowledge of estrogen and progesterone levels throughout the reproductive cycle of the bitch is only theoretical, and is based on extension of knowledge obtained by work on human levels. Determination of actual hormone levels in the bitch could possibly give a definite means for determining the exact time of ovulation and in abnormal conditions such as prolonged cycles or abortion, provide a basis for proper treatment. The work reported here represents attempts to find a method of hormone determination that could be used routinely in the laboratory without special equipment and excess time.

The ovary is generally considered the principal source of estrogens; however, significant amounts of estrogen have been extracted from the placenta, adrenal cortices, testes, and tissues of certain plants. Approximately ten natural estrogens have been obtained from animal sources, the principal ones being estradiol, estriol, and estrone. Estradiol is the most potent of the estrogens and is considered to be the hormone actually secreted from the ovary. Estriol is the least potent and is regarded as the degradation product, which is excreted in human urine as a water-soluble conjugate, estriol glucuronidate. Estrone is considered as an intermediate product and is also excreted as a glucuronidate.

Progesterone, a steroid closely related to natural estrogens, is produced primarily by the corpus luteum; however, considerable quantities have been found in the placenta and adrenal

glands. Several metabolites of progesterone have been isolated from human pregnancy urine, pregnanediol being the principal one. Pregnanediol is excreted by the kidneys of pregnant women, as an inactive, water-soluble conjugate sodium pregnanediol glucuronidate. Since each molecule of pregnanediol in the urine represents onemolecule of progesterone leaving the system, the prevalence of progesterone during various stages of the reproductive cycle can be measured by pregnanediol determination (21, 23).

Methods have been developed (3, 5, 6, 7, 14, 18, 20, 22) for quantitative determination of estrogen and progesterone in human urine. Methods used with human urine have been modified as necessary and applied to dog urine, in attempts to measure estrogen and progesterone levels.

REVIEW OF LITERATURE

Technics for extraction and identification of progestins are not numerous, and for the most part, each new worker takes the existing procedures and modifies them to suit his needs. The standard procedure may be divided into four steps: I. Collection and preservation, II. hydrolysis, III. extraction, and IV. identification.

Preservation of Urine

Venning et al. (22) and Bucher and Ceschickter (6) found that preservation of the urine was very important in determination of sodium pregnanediol glucuronidate. Venning et al. (22) reported that neither tricresol, chloroform, nor toluene gave

satisfactory preservation at temperatures of 18 to 34°C.; however, sodium cyanide (0.5 percent) prevented hydrolysis for four days at 20°C. Bucher (6) found that free pregnanediol was not present with sterile catheter specimens. Bisset et al. (5) found that temporary preservation was obtained when urine was voided into a bottle containing 3 gm. of a two-to-one (w/w) mixture of finely powdered thymol and salicylic acid (Dingemanse, 10).

Preservation of urine was not so important for estrogen or pregnanediol determinations, but was regularly employed because of theoretical possibilities of bacterial destruction of the desired substances.

Hydrolysis

Most methods of extraction and identification require that the water-soluble, inactive, sodium pregnanediol glucuronidate be broken down into a water-soluble substance; free pregnanediol. This is done by either of two methods, acid hydrolysis and enzymatic hydrolysis.

Acid Hydrolysis. Acid hydrolysis consists of refluxing a quantity of urine in the presence of a water-insoluble solvent and hydrochloric acid. Sommerville et al. (18) hydrolyzed human urine by mixing 100 ml. of urine and 50 ml. sulfur-free toluene in a 500-ml. ground-glass jointed, round-bottomed flask, connected to a reflux condenser. The mixture was brought to a boil, 10 ml. of analytical reagent concentrated hydrochloric acid added, and the boiling continued for exactly 10 minutes. The hydrolyzed mixture was cooled in a water bath to room temperature before

separation was attempted. Jensen (14) modified this method by using one-fortieth of a 24-hour urine sample and an equal volume of toluene. A volume of concentrated hydrochloric acid equal to one-tenth the volume of urine was added through the condenser to the boilding mixture, and boiling continued for 10 minutes. The flask was cooled in ice water, care being taken not to shake the mixture, to avoid violent bubbling and loss of the mixture.

Astwood and Jones (1) used 50-500 ml. of urine in a 2-l. round-bottomed flask with toluene, added up to 100 ml. concentrated hydrochloric acid, depending upon the quantity of urine, and continued the boiling 15 minutes.

Enzymatic Hydrolysis. Fishman (Talbot et al., 20) used an enzyme extract from beef spleen for hydrolysis of sodium pregnanediol glucuronidate, but Talbot et al. (20) was unable to get consistent results with this method. Talbot successfully used an enzyme extract from the liver of rats. In preparation of this extract, six fresh rat livers were ground in a mortar to a paste. Ten volumes of acetone were stirred into the mixture. After allowing 15 minutes for solution, the fluid was removed by filtration in a Buchner funnel. The residue was washed three times with small quantities of anhydrous ether and dried on the filter paper. The residue containing the desired enzyme was sifted through a tea strainer and stored in a dark stoppered bottle in a refrigerator. An aliquot of a 24-hour urine specimen expected to contain 6-9 mg. of pregnanediol was extracted according to Venning et al. (22). The residue was dissolved in 15 ml. of distilled water buffered with 10 ml. of 0.1 N sodium acetate

to pH 5, then incubated with 400 mg. of the powdered rat liver for four hours at 37°C.

Extraction and Purification

Pregnanediol. The methods for extraction and purification of pregnanediol are very similar; however, various workers have developed modifications.

Sommerville et al. (18) proposed a method that will be considered the basic procedure in this report. Following hydrolysis, the cooled mixture was transferred to a 250-ml. separatory funnel and allowed to stand until the layers separated. The lower, aqueous layer was removed and discarded, the toluene and emulsion layers transferred to a Buchner funnel, and filtered through Whatman No. 1 filter paper with gentle suction, to break down the emulsion. The filtrate was transferred to a separatory funnel and the remaining aqueous layer removed. The toluene layer was washed twice with 20-ml. lots of water (to remove undesired substances and neutralize the toluene), transferred to a round-bottomed flask, placed in a water bath, and evaporated to dryness under reduced pressure. By this procedure, pregnanediol and a few other steroids were obtained from the hydrolyzed mixture.

In order to purify pregnanediol the residue was dissolved in ethanol, transferred to a large test tube, and the ethanol reduced in volume to 5 ml. in a warm water bath under a gentle stream of air. With the test tube in the water bath, 20 ml. of 0.1 N sodium hydroxide was added. It was then incubated for two hours at 37°C., allowing time for crystallization of the pregnane-

diol. The pregnanediol crystals were collected by filtering through a sintered glass funnel with pores of 20-30 microns in diameter. They were then redissolved in a test tube with hot ethanol, undesirable coloring matter was removed by addition of 2-4 mg. of "Norite" charcoal which in turn was removed by filtering through Whatman No. 1 filter paper. This procedure was repeated if the solution was not completely clear. The clear alcoholic solution was then evaporated to dryness in a water bath under a gentle stream of air.

Jensen (14) modified the Sommerville method considerably. After hydrolysis was completed, the mixture was shaken vigorously in a separatory funnel for at least one minute to free any pregnanediol still present in the urinary phase, then allowed to separate and the urinary phase discarded. The toluene was washed twice with 20-ml. lots of 4 N sodium hydroxide and three times with 20 ml. of water, then dried with a small amount of sodium sulfate. The purified toluene solution was filtered through a small pore filter paper into a round-bottomed flask and evaporated to dryness under reduced pressure at 80-90°C. The residue was dissolved in 0.5 ml. absolute ethanol with gentle warming. Three ml. of a three per cent solution of hydrogen peroxide and 0.25 ml. of a two per cent sodium bicarbonate solution were added and the mixture heated at 100°C. for four minutes. Pregnanediol crystals formed withon one hour at room temperature. The precipitate was collected in a glass filter, having pores of 15-40 microns in diameter, and washed three times with 10-ml. lots of water without suction. The filter was then placed on a vacuum flask and the

precipitate eluted with 10 ml. of ethanol and the ethanolic solution evaporated to dryness under a stream of air.

Talbot et al. (20) also applied modifications to the method of extraction and purification. Following hydrolysis the mixture was cooled, filtered through Whatman No. 1 filter paper, the residue washed three times with cold water and free pregnanediol extracted four times with 5-ml. lots of hot 95 per cent ethanol. The ethanolic solution was then evaporated to dryness in a boiling water bath, the residue redissolved in 10 ml. of hot ethanol, and the pregnanediol precipitated from solution by slow addition of 40 ml. of water.

Sodium Pregnanediol Glucuronidate. Venning et al. (22) collected and measured a 24-hour specimen of human urine and took an aliquot expected to contain 10-15 mg. of sodium pregnanediol glucuronidate. She extracted four times with a total volume of n-butanol equal to one-third the volume of urine. The urine and n-butanol were mixed by a gentle rotary motion, allowing about one minute for each extraction. If an emulsion formed, it was broken down by centrifuging. The urine layer was then discarded, the clear n-butanol evaporated to dryness under reduced pressure at 40-49°C.

The residue was dissolved in 60 ml. of 0.1 N sodium hydroxide and the alkaline solution extracted four times with n-butanol (20, 20, 10, 10 ml.). The combined n-butanol fractions were allowed to stand ten minutes and then transferred to a separatory funnel. Any precipitate was washed with small amounts of n-butanol and added to the separatory funnel. The n-butanol was washed

once with 3 ml. of 0.1 N sodium hydroxide and twice with 5 ml. of water; after each washing the layers were allowed to completely separate and the aqueous layer discarded. The n-butanol was evaporated to dryness under reduced pressure and the residue dissolved in exactly 5 ml. of water and approximately 10 ml. of acetone. The mixture was transferred quantitatively to a 125-ml. conical flask and the volume made up to 100 ml. with acetone and allowed to stand over night at 5-10°C. during which a precipitate was formed. The supernatant was removed, the remainder of acetone and precipitate centrifuged, and the acetone layer removed. The precipitate was then dissolved in water-acetone, the amount of solvent varying with the approximate amount of precipitate (10 mg., 10 ml.; 5-10 mg., 6 ml.; 5 mg., 4 ml.). As the mixture cooled some of the impurities precipitated and were then removed by filtration. The sodium pregnanediol glucuronidate remained in solution. The filter and centrifuge tube were washed with acetone and the volume made up to 100 ml. with acetone. The pregnanediol glucuronidate precipitated from the solution after a few hours of refrigeration, and was recovered by centrifugation or filtration. The precipitate was dissolved in hot ethanol and a small amount of water added to insure complete solution of sodium pregnanediol glucuronidate. This mixture was filtered, if necessary, then evaporated to dryness in a water bath, and used directly for gravimetric determination.

Modifications of Venning's et al. (22) technic were made by Bucher and Geschickter (6) and Bisset et al. (5). Bucher determined sodium pregnanediol glucuronidate by the same method as

Venning, but considered this inadequate because of the probability of some free pregnanediol in the urine sample. The free pregnanediol remained in solution and was discarded by Venning in the first acetone wash, from which the sodium pregnanediol glucuronidate was precipitated. Bucher retained the clear aqueous acetone decanted from the centrifuge tube, placed it in a 125-ml. Erlenmeyer flask, and evaporated to dryness of a steam bath. The residue was redissolved by adding 10 ml. of acetone and a few drops of 0.1 N sodium hydroxide to the flask and gently warming. Forty ml. of 0.1 N sodium hydroxide was added dropwise, the solution cooled, and placed in a refrigerator over night. The free pregnanediol, containing estrogens, was then separated by filtration with suction in a funnel, the precipitate washed with 5 ml. of water and a small quantity of hexane, transferred with hot acetone to the original flask and evaporated to dryness. The free pregnanediol was further purified by reprecipitation with sodium hydroxide. The precipitate was redissolved in 5 ml. of ethanol and reprecipitated with two volumes of water, which should yield pure crystals of pregnanediol.

Bisset et al. (5) used a highly modified Venning procedure. He mixed 6 gm. of zinc acetate with 300 ml. of urine and filtered. The filtrate was extracted successively with 100, 50, 50 ml. lots of n-butanol. The extracts were combined and washed with 100 ml. of 0.2 N sodium hydroxide and two lots of 20 ml. of sodium chloride solution. The n-butanol was then evaporated to dryness and the residue redissolved with warm water or cold sodium hydroxide to remove gums. The solution was placed in a centrifuge tube,

treated with 0.5 M barium chloride, 1 ml. of 0.05 M sodium acid phosphate, shaken, and allowed to stand 10 minutes at 0°C. Acetone was then used to wash the sides of the tube and the tube drained by inversion on filter paper. Ten milliliters of saturated barium phosphate was then added with a pipette, centrifuged five minutes and drained as before. The precipitate was then measured colorimetrically.

Estrogens

Hydrolysis. Brown (7) hydrolyzed human urine for estrogen extraction by boiling it with 15 per cent volume concentrated hydrochloric acid for one hour and then cooled. Bauld (3) used the same method.

Smith et al. (17) acidified a liter aliquot of a 24-hour urine sample with concentrated sulfuric acid, using congo red as an indicator, extracted four times with 0.125-volume butanol, the butanol extracts pooled, allowed to clarify in a separatory funnel, and distilled to near dryness under reduced pressure at a temperature of less than 100°C. Thirty milliliters of 95 per cent ethanol, 250 ml. of distilled water, and 50 ml. of sulfuric acid were added, and the solution hydrolyzed 10 minutes at 100°C.

Extraction and Purification. Brown (7) extracted the steroids following hydrolysis with di-ethyl ether. The ether extract was washed with concentrated sodium carbonate, to remove the ether-soluble acid fraction at a pH of 10.5. The colored products and some estriol were then removed by washing the ether extract with sodium hydroxide. The sodium hydroxide was then

partially neutralized by adding saturated sodium bicarbonate to the aqueous layer, and by shaking the concentrated carbonate solution with the other ether the estriol was extracted back to the ether layer. The aqueous layer was then discarded and the ether layer washed again with sodium bicarbonate to neutralize any alkali still present. The ether was then washed with a small amount of water to remove the bicarbonate, evaporated to dryness and the residue dissolved in ethanol. A mixture of (1/1) ether and benzene was introduced and extracted with water. To the water extract (containing estriol) was added sodium hydroxide and boric acid, as a buffer; then the estriol was methylated with dimethyl sulfate at 37°C. for 30 minutes and hydrogen peroxide added to remove a portion of urinary contamination. The estriol-methyl ether was then extracted with benzene, the benzene extract washed with water, and chromatographed on an alumina column. The hormones from both columns were eluted and measured by the Kober method. Bauld (3) used a similar method but did not methylate the estrogens and chromatographed on Celite columns.

Smith et al. (17) developed one of the most accurate, quantitative separation methods yet devised. He added 100 ml. of redistilled ether to the hydrolyzed mixture, transferred the entire volume to a separatory funnel, and extracted four successive times with 100 ml. of ether, each time rinsing the hydrolysis flask. The ether extract was washed with 20-ml. lot quantities of 9 per cent sodium bicarbonate until the aqueous phase was almost clear; then washed with three 20-ml. lots of distilled water. The aqueous layer was discarded and the ether layer evap-

orated to dryness at a temperature less than 100°C. Six milliliters of ether and 108 ml. of carbon tetrachloride were added, the solution transferred to a separatory funnel and extracted four times with 50 ml. of 1 N aqueous potassium hydroxide. The ether phase, containing the neutral steroids, was washed twice with 5-ml. lots of water, and the water was added to the potassium hydroxide phase. The potassium hydroxide phase containing the estrogenic phenols was acidified with 6 N sulfuric acid, congo red used as an indicator, and the estrogens removed by extracting five times with 100-ml. lots of ether. The resulting ether extract was washed twice with 30-ml. lots of sodium bicarbonate (9 per cent), twice with 30-ml. lots of distilled water, and as many times as necessary to clear the solution with 20 ml. of 2.5 per cent sodium carbonate. The sodium carbonate extracts were backwashed with ether, the backwash ether added to the ether phase. The ether phase was washed twice with 20 ml. of distilled water and evaporated to dryness. The residue was dissolved in 10 ml. of 95 per cent ethanol and 40 ml. of distilled water, and steam distilled until 500 ml. of distillate was obtained, a volume of about 50 ml. being maintained. This was evaporated to dryness and the residue redissolved in 10 ml. of 95 per cent ethanol. One milliliter of 85 per cent phosphoric acid was added, heated in a boiling water bath for 30 minutes, then allowed to cool. Fluorescence was measured with a Coleman Model 12 photofluorometer with a lamp filter transmitting at a wave length of 436 millimicrons and a photo cell filter transmitting 525 millimicrons. The fluorescence of the unknown sample was compared with the fluo-

rescence of a known standard of crystalline estrone.

Identification

Identification of estrogens and progesterone purified as above may be accomplished by chemicals that produce colored compounds, by paper chromatography, or by bioassay. Since bioassay has been a standard though complicated technic, it will not be considered here.

Sommerville et al. (18) measured the amount of pregnanediol by adding 10 ml. of concentrated sulfuric acid to the dry residue in a test tube, and maintaining the test tube in a 25°C. water bath for 20 minutes with occasional shaking. The intensity of the yellow color produced was measured in a Spekker photoelectric absorptiometer, using spectrum violet number 601 light filter. The absorptiometer readings were interpreted by reference to a calibration curve made with known amounts of pregnanediol and the final amount expressed in milligrams of pregnanediol extracted per 24 hours.

Jensen (14) added 5 ml. of sulfuric acid to the residue, allowed it to stand one hour at room temperature, transferred the acid solution to a 10-ml. cuvette and measured the extinction at 415 and 530 millimicrons determined spectrophotometrically.

Bisset et al. (5) dissolved the precipitate in 2 N hydrochloric acid, heated, and poured the solution into a separatory funnel with 14 ml. di-ethyl ether. The grey color produced, which was stable away from bright sunlight, was measured on a colorimeter.

Chromatographic. Heftmann (12) described a promising method of identification of estrogens and progesterone by paper chromatography. Following hydrolysis and extraction of the hormones, the dried residue was dissolved in as small amount of ethanol as possible. A freshly prepared, saturated, aqueous solution of p-nitrobenzeneazodimethoxyaniline (fast black K salt) was filtered. A volume of the ethanolic mixture, two volumes fast black K salt, and one volume of 20 per cent sodium carbonate were added to a test tube and placed in a boiling water bath for 10 minutes. This coupled the fast black K salt with the hormones. Heftmann (12) stated the coupling could also be done by allowing the mixture to stand over night at room temperature. To extract the coupled hormones, benzene was added and the tube shaken vigorously. The contents of the tube were drawn into a pipette, allowed to separate, the aqueous phase expelled, and the benzene solution applied to the chromatogram.

The combination of solvents found most suitable for separation of the dyes was 200 ml. toluene, 100 ml, petroleum ether (boiling range 35-60°C.), and 30 ml. ethanol with 70 ml. of water. This was shaken in a separatory funnel, allowed to stand over night, and the water layer drawn off. The remainder of the mixture was placed in a chromatographic chamber and the atmosphere allowed to become saturated. Strips of chromatographic paper were suspended from the lid of the chamber with the other end in the solvent mixture. After one and one-half hours the strips were removed, dried, and the spots circled with a pencil. The Rf values for the estrogens and progesterone were found to be:

estrone 0.95, estradiol 0.81, estriol 0.07, and progesterone 0.75. The color given by the estrogens was pink, yellow for progesterone (12).

Using chromatographic technics similar to Heftmann (12), without the benefit of following colored spots, others have developed technics of identifying specific estrogens or progestins.

Fuming sulfuric acid (Axelrod, 2) was pipetted lengthwise onto a glass plate and the chromatograms immersed in the acid with the aid of a glass rod (Axelrod, 2). After one minute the strip was observed under an ultra-violet lamp for fluorescence.

Chromatograms, containing most organic compounds, when placed in potassium permanganate in dilute sulfuric acid will usually give a yellow or white color at the position of the organic compound and may be used to identify spots, if Rf values are calibrated simultaneously (Jensen, 14).

Antimony trichloride in chloroform was proposed by Bloch et al. (4) in determination of pregnanediol. A solution of antimony trichloride saturated at room temperature in chloroform was prepared. About 0.5 cm. of the chloroform solution was added to a flat-bottomed dish and the chromatogram quickly drawn through the solution. The reagent had to be used immediately, since it decomposed rapidly by absorption of water from the atmosphere. Immediately after dipping, the chromatograms were heated in an oven at 90°C. for four minutes. The colors produced by antimony trichloride tend to fade in a few days. With antimony trichloride the allopregnanediols gave a pink color in both white and ultra-violet light; pregnane-3(a), 20(a)-diol gave less intense

pink; pregnane-(a), 17(a), 20(a)-triol and 5 pregnene-3(b)-ol-20-one gave a bluish shade in white light and red shade in ultra-violet light; 17(a)-Hydroxyprogesterone, and allopregne-e-3(b)-ol-20-one produced colors only in ultra-violet light.

Bloch et al. (4) also used zinc chloride in glacial acetic acid. Twenty grams of zinc chloride in 30 ml. of glacial acetic acid was prepared by gentle warming on a hot plate, the zinc chloride having been previously dried in vacuo over phosphoric acid anhydride. The reagent had to be freshly prepared immediately before use, then poured into a flat dish, and the chromatogram drawn through the solution. The chromatograms had to be kept in a dry place because the zinc chloride was very hydroscopic and the chromatograms would disintegrate in the open. The colors produced with zinc chloride were faint brown in white light and blue fluorescence in ultra-violet light from preg-3(a), 20(a)-diol, and pregnane-3(a), 17(a), 20(a)-triol; pink in white and ultra-violet light for 5-pregne-3(b)-ol-20-one; while 17(a)-hydroxyprogesterone and allopregnane-e(b)-ol-20-one gave color only in ultra-violet light.

A freshly diluted (one volume reagent and five volumes water) solution of Folin and Cocalteu's reagent was sprayed onto the chromatograms and placed immediately into a glass tank containing a layer of ammonia solution (specific gravity 0.880) in a method proposed by Mitchell (16) for determination of estrogens. The spots reached maximum intensity within five minutes, the paper was removed and dried at room temperature, giving a permanent-color chromatogram.

Gravimetric. Venning et al. (22) used a gravimetric method for determining the amount of sodium pregnanediol glucuronidate. Bucher (6) employed this method for sodium pregnanediol glucuronidate and pregnanediol, giving total pregnanediol excretion. The final evaporation was made in a weighing bottle and the weight calculated; Bucher combined the weights and calculated for free pregnanediol.

EXPERIMENTAL METHODS AND RESULTS

Dog urine used in the following experiments was collected by placing a dog of desired reproductive condition in a metabolism cage equipped with a drain tray. The outlet was connected by rubber and glass tubing to a liter collection bottle, which would regularly hold the urine voided in a 24-hour period. Because of the septic conditions prevailing during collection, 5-10 ml. of toluene was placed in the collection bottle to inhibit bacterial growth that might be destructive to pregnanediol. Since free pregnanediol is soluble in toluene and insoluble in water, it was assumed that any pregnanediol liberated during collection or storage would pass into the toluene phase, giving less chance that the water-insoluble substance would adhere to the collection bottle and be lost.

Work was started in September, 1955, using the method proposed by Heftmann (12) for determination of urinary estrogens. Although this method was not claimed to be quantitative, it seemed reasonable that by using constant procedures, the size and inten-

sity of the colored spots on the chromatogram could be used to determine the relative levels of estrogens.

Urine from six pregnant and four non-pregnant bitches was collected and extracted and identifications attempted according to Heftmann (Exp. I and II). As checks the K salt was fused with known pure estrogens dissolved in ethanol (Exp. III); with extracts from human male urine, and with distilled water (Exp. IV) in the same way as the test samples were prepared. These check preparations were chromatographed in the same way as, and in many cases, in the same tank with test samples.

Experiment I E. Urine collected from pregnant bitches was shaken, to get a homogeneous mixture, a 10-ml. aliquot removed, and placed in a large test tube. Three milliliters of concentrated hydrochloric acid was added, the test tube placed in a boiling water bath, hydrolyzed 10 minutes, and cooled. Ten milliliters of ethylene dichloride was added to the hydrolyzed urine, the mixture shaken five minutes, centrifuged and the top aqueous layer discarded. An excess of sodium hydroxide (6 pellets) added, the mixture shaken, and filtered through Whatman No. 1 filter paper. The ethylene dichloride layer was then drawn off, evaporated to dryness and the residue dissolved in 2 ml. of ethanol. Two milligrams of freshly prepared, saturated, aqueous solution of the K salt, 1 ml. of the ethanolic solution, and 1 ml. of 20 per cent sodium carbonate were added to a test tube and placed in a boiling water bath for 20 minutes. A glass bulb was placed over the mouth of the test tube to prevent excess evaporation. Two milligrams of benzene were added and the tube shaken

vigorously. The benzene solution was then placed on Whatman No. 1 chromatographic strips and the strips placed in a chromatographic tank, for one and one-half hours (Pl.I:A). The mobile phase used was 200 ml. benzene, 100 ml. petroleum ether, and 100 ml. 30 per cent ethanol; this mixture was allowed to equalize over night and the aqueous layer discarded. Benzene was found to work equally as well in the solvent mixture as toluene suggested by Heftmann (12).

Four distinct colored spots were found on the chromatograms, a yellow spot having an Rf of 0.96 and three pink spots with Rf's of approximately 0.1, 0.7, and 0.9. These latter spots were approximately in the same position as spots described by Heftmann for estrone, estradiol, and estriol (0.95, 0.81, 0.07) and therefore assumed to be the estrogen hormones and the yellow spot at Rf 0.96 was assumed to be progesterone.

Experiment II E. Non-pregnant urine was treated in the same manner as the pregnant urine. Spots exactly the same color and appearing at the same position on the chromatogram as observed in Experiment I were found repeatedly in non-pregnant urine.

Experiment III E. Quantities of pure estradiol, estrone, estriol, and progesterone were dissolved in a volume of ethanol. The ethanolic solution was then fused with the K salt and chromatographed in the same manner as in Experiments I and II.

The same spots occurred on the chromatograms as in Experiments I and II, showing no variation caused by the presence of pure hormones.

Experiment IV E. Separate tests were made using triple dis-

tilled water and human male urine in the same manner as the urine in Experiments I and II. In repeated tests the same results were obtained as in Experiments I and II.

Discussion of Results Using Heftmann's Method

Although Heftmann (12) reported the presence of only a yellow spot produced by the K salt in blank tests, the yellow spot plus a graded series of pink to purple spots was present each time in the laboratory. Checks with triple distilled water and human male urine gave the same results as the dog urine extracts. As identical spots were obtained repeatedly from triple distilled water, human male urine, pregnant dog urine, and preparations of pure hormones; the technic proposed by Heftmann was considered to be of no value for determination of estrogens or progesterone.

Following continuous failure in identifying the questioned material by Heftmann's technic, further attempts were made in paper separations.

Experiment V E. In attempts to identify the hormones on chromatograms, ethanolic solutions containing estradiol, estrone, and pregnanediol were prepared and applied to filter paper strips. Another series of strips was prepared for sensitivity tests with graded amounts of each hormone, varying from 0.002 mg. to 0.2 mg. Both series of strips were tested with Folin-Ciocalteus reagent (16), fuming sulfuric acid (2), antimony trichloride (4), potassium permanganate in dilute sulfuric acid (Lederer and Lederer, 15) (0.5 N permanganate in 0.3 N acid).

Three identification methods not found in the literature were

also tried. (A) A series of permanganate solutions was made by diluting the stock solution with equal volumes of water, resulting in dilutions of 1/1, 1/2, 1/4, 1/8, and 1/16. Varying amounts of the hormones were placed first in the highest dilution, then transferred to increasingly greater concentrations until a reaction was visible, to determine if the larger quantities of hormone could be identified in more dilute permanganate solutions. (B) Strips were sprayed with N sodium hydroxide and allowed to dry; another series of strips was sprayed with concentrated phosphoric acid and dried. The strips were then observed for fluorescence under long (3600-3650 Angstrom units) and short (2500 Angstrom units) ultra-violet light. (C) Strips with known quantities of estrone, estradiol, and pregnanediol were placed in a chromatographic tank, the mobile phase (benzene-ether 2/1 saturated with water) allowed to advance to about 300 mm., air dried and observed for fluorescence under long and short ultra-violet light.

Folin-Ciocalteus reagent gave a blue color in the presence of two micrograms of each hormone, but failed to give a method for quantitative measurement (Mitchell and Davis, 16).

Fuming sulfuric acid yielded a pink color with estrone, orange with estradiol, and brown with pregnanediol in quantities exceeding two micrograms, but failed to be useful for quantitative measurement (Axelrod, 2).

Antimony trichloride produced a pink color with estrone and estradiol and a light green color with pregnanediol (Bloch et al., 4). An attempt was made to measure the width of bands with dif-

ferent quantities of hormones; this failed because the hormones were soluble in chloroform, which caused the bands to spread. The antimony trichloride was then sprayed on the strips but, due to the hygroscopic activity of antimony trichloride, color production was inhibited.

The permanganate solution gave a brown color with all three hormones, but offered no means of quantitative measurement (Lederer and Lederer, 15). Color was produced in all dilutions of the permanganate except 1/16. The quantity of hormone present exceeding a minimum concentration of 0.002 mg. per sq. cm. made no difference in the various dilutions.

The complete strips fluoresced after being sprayed with sodium hydroxide or phosphoric acid, again giving no means for identification and measurement of estrogens or pregnanediol. When placed under ultra-violet light without being sprayed none of the hormone fluoresced.

Known amounts of estradiol, estrone, progesterone, and pregnanediol dissolved in ethanol were chromatographed in efforts to determine the Rf values for the pure hormones. The mobile phase was varied from that suggested by Heftmann (12). Benzene-ether (2/1), benzene-ether (2/1) saturated with water, benzene-ethylene dichloride (1/1), benzene-ether (1/1), benzene-ether (1/2), benzene-ether (3/1). Benzene, petroleum ether, ethylene dichloride, and ethanol were used as single solvents for the mobile phase.

None of the chemical tests gave consistent identification, and no quantitative results could be interpreted. When the chromatographic strips were subjected to ultra-violet light, negative

results were again obtained except with progesterone. Chromatographic strips containing known amounts of progesterone placed under short ultra-violet light (2500 Angstrom units), a purple fluorescence was observed. Different concentrations of chromatographed progesterone could be measured in direct proportion with the width of the fluorescent band produced (10 micrograms = 5 mm., 20 micrograms = 1 mm.).

Experiment VI E. The method proposed by Brown (7) for extraction and purification of estrogens was then explored, using urine from both pregnant and non-pregnant dog urine. Eighty-five milliliters of urine and 15 ml. of concentrated hydrochloric acid were hydrolyzed by boiling for one hour, cooled, and extracted with di-ethyl ether. The ether was washed with 20 ml. of concentrated sodium carbonate solution, then 20 ml. of 1 N sodium hydroxide was added and shaken with the ether. Twenty milliliters of concentrated sodium bicarbonate was added, shaken, and separated. The aqueous layer was discarded. The ether containing the estrogens, if any, was then washed with 5 ml. of water, evaporated to dryness, and the residue dissolved in 5 ml. of ethanol. The ethanolic solution was applied to chromatograms and chromatographed using benzene-ether (2/1), saturated with water, for the mobile phase.

The chromatographed strips were subjected to all the tests listed in Experiment V E, with consistent negative results.

Pregnanediol

Because of the inconclusiveness of chemical tests for estro-

gens on chromatograms, work was concentrated on a method for quantitative determination of pregnanediol. Sommerville's technic (18) for extraction was found to be workable and was followed closely.

Experiment I P. As a check on the technic, a series of tests was made using 1-mg. samples of pure pregnanediol dissolved in ethanol mixed with 100 ml. of water and 50 ml. toluene, then hydrolyzed by boiling 10 minutes with 10 ml. concentrated hydrochloric acid (PLX.C.). The mixture was cooled, transferred to a separatory funnel, shaken, and the aqueous layer discarded. The toluene and emulsion layers were filtered with gentle suction through Whatman No. 1 filter in a Buchner funnel, and transferred back to the separatory funnel by quantitative washings of the filter and flask with toluene. The remaining aqueous layer was discarded. The toluene layer was washed twice with 20-ml. lots of N sodium hydroxide, twice with 20-ml. lots of water, then transferred to a round-bottomed, ground-glass jointed flask and evaporated to dryness under reduced pressure in an 80-90°C. water bath (P.I.B.). The residue was then washed quantitatively from the flask with hot ethanol to a large test tube. The ethanolic solution was evaporated to 5 ml. in a warm water bath (75°C.) and 20 ml. of 0.1 N sodium hydroxide added from a buret over a three-minute period with occasional stirring. The test tube was then transferred with the water bath to an incubator and incubated for two hours at 37°C., during which time a flocculent precipitate formed if pregnanediol was present. Contents of the test tube were then filtered through a sintered glass funnel and the residue

eluted from the funnel into a test tube by three washings with 5-ml. lots of hot ethanol. Two to four milligrams of "Norite" charcoal were added, the test tube placed in a warm water bath for two minutes, and the contents filtered through Whatman No. 1 filter paper. The ethanolic solution was evaporated to dryness, the residue dissolved in 10 ml. of concentrated sulfuric acid. The resulting yellow color was compared with the color produced by 0.5, 1.0, and 1.5 mg. of pregnanediol in sulfuric acid.

A yellow color of the same visual intensity as that produced with 1 mg. pregnanediol in 10 ml. sulfuric acid was produced by the final residue in 10 ml. sulfuric acid, denoting recovery of nearly 100 per cent.

Experiment II P. Human pregnancy urine, collected from three females 60 to 120 days pregnant, was subjected to the same extraction, purification, and colorimetric determination as the sample in Experiment I.

A dark yellow color was produced when 10 ml. concentrated sulfuric acid was added to the test tube containing a residue from the final evaporation. A dark brown color was produced upon addition of 10 ml. of a 3 per cent solution of antimony pentachloride in ethylene dichloride (10) to the test tube containing the final residue, indicating a recovery of approximately 1.5 mg. pregnanediol from each 100-ml. sample.

Experiment III P. Castrate dog urine collected from a hysterectomized bitch was treated in the same manner as other urine samples.

No color was produced when concentrated sulfuric acid was

added to the residue following regular hydrolysis, extraction, and purification technics.

Experiment IV P. Eight extractions were made with urine collected from pregnant bitches in early, midterm, and late pregnancy. Volumes of urine ranging from 100 to 750 ml. with 10 per cent (v/v) hydrochloric acid and 50 ml. toluene were hydrolyzed, extracted and purified as before.

No visible color change occurred when concentrated sulfuric acid was added to the residue after final evaporation of the ethanolic solution. Antimony pentachloride likewise gave negative results on all samples except one: a light brown color was obtained from a hydrolyzed 48-hour (750-ml.) sample of urine from a three-week-pregnant bitch. This shows a negligible quantity of pregnanediol in the dog urine tested.

Experiment V P. Stools obtained from a bitch in early pregnancy and one from a bitch in late pregnancy were macerated separately in water, and strained through a tea strainer. The aqueous mixture was placed in a liter round-bottomed flask, layered with toluene, and refluxed with hydrochloric acid as described in Experiment I P. No attempt was made at this time to observe quantitative technics. The mixture was cooled to room temperature, shaken and filtered through a Buchner funnel with suction. The filtrate was transferred to a separatory funnel, the aqueous layer discarded, and the toluene washed with sodium hydroxide and water. The procedure was then carried to completion in the same manner as the previous experiments.

A dark brown color was produced by addition of 10 ml. of an-

timony pentachloride solution to the final evaporation tube. Color was given immediately after the addition of antimony pentachloride at room temperature indicating a high concentration of pregnanediol at least equivalent to 1 mg.

Experiment VI P. Two other methods for determining the relative amounts of pregnanediol were carried out, using known quantities of pure pregnanediol. Five-tenths, 1, 2, and 4 mg. amounts were dissolved in 5 ml. of ethanol and precipitated in test tubes with 20 ml. of 0.1 N sodium hydroxide. The test tubes containing the precipitated pregnanediol were shaken and the turbidity in each compared. The precipitates were then transferred to calibrated centrifuge tubes, centrifuged, the amount of precipitate in each measured, and compared with the other tubes.

Turbidity of the tube containing 1 mg. obsdrved without instruments was approximately twice that of the tube containing 0.5 mg. and one-half that produced by the tube containing 2 mg. The tube containing 4 mg. appeared the same as the tube containing 2 mg. of pregnanediol. The amount of precipitate after centrifuging the tubes containing 0.5, 1, 2, and 4 mg. did not give results that could be used for quantitative measurement.

Summary of Results

Because of the absence of notable differences between tests and controls, it appears that the method for colored paper chromatographic separation of estrogens, proposed by Heftmann (12) cannot be applied to estrogens. Colorimetric methods for deter-

mination and measurement of estrogens on chromatograms, proposed by various workers, gave colors for identification but not for measurement of estrogens or pregnanediol.

Results using the Sommerville (18) technic for determination of urinary pregnanediol in human pregnancy urine proved to be impractical with dog urine because of the small amount of pregnanediol in the pregnant bitch was obtained only by using 48-hour samples of urine from a bitch that should, if the level in dogs is similar to that of women, have been at the highest stage of pregnanediol excretion in the reproductive cycle.

The modified Sommerville technic, applied to pregnant dog feces, gave positive results from 25-gram stools collected from bitches in early and late pregnancy. However, the technic has not yet been standardized for quantitative determination. The color produced by sulfuric is non-specific; the antimony pentachloride reaction seemed to be more specific. Since the antimony pentachloride solution yields quantitative colorimetric results with diethylstilbesterol (Dingemanse, 10), it is probable that a colorimetric curve can be produced using graded quantities of pregnanediol dissolved in uniform volumes of the antimony pentachloride solution.

DISCUSSION

At the onset of this project a review of literature led to the belief that a method already established need only to be learned and applied to dog urine. This has not been the case,

at least with estrogens. Communications with Hansel of Cornell University,[1] who is working on a method for blood or urinary estrogen measurement by chromatography using Smith's method (17) applied to cattle, and Sykes[2] of the U. S. D. A. in Beltsville, Maryland, working with blood estrogens in cattle, have reported results similar to those obtained in this laboratory. They report that by addition of estrogens to blood or urine, quantitative extractions have been made, but no results have been obtained in extraction of estrogens naturally occurring in blood or urine.

Work with pregnanediol excretion reviewed in the literature dealt only with human urine. When the method described by Sommerville (18) was used with human pregnancy urine, positive results were obtained, but when applied to pregnant dog urine, results were negative. It was then assumed that pregnanediol was excreted through some other body excrement, so fecal extractions were attempted. A substance was recovered giving upon precipitation a mass visually the same as that derived from similar treatment of pure pregnanediol and of extracts of residues obtained from human pregnancy urine. The results obtained from fecal extractions appear to demonstrate that pregnanediol is excreted through the feces in measurable quantities in the pregnant bitch.

Hydrochloric acid (1/10 by volume) was used for hydrolysis of urine samples in the laboratory as suggested by Sommerville (18). Ten minutes was the time used for hydrolysis; other work-

[1]Correspondence March 19, 1956.
[2]Correspondence March 13, 1956.

ers have suggested different concentrations of acid and have varied the time for hydrolysis without marked effects on the extract; however, it was felt that by using a constant concentration and time, the amount of hydrolysis would be constant and recoveries comparable. In tests where sodium pregnanediol glucuronidate was given orally to male patients, the various workers report between 70 and 80 per cent of the pregnanediol was recovered as free pregnanediol after hydrolysis, extraction, and purification of urine samples. Toluene was used as suggested by Sommerville (18). Other workers (22, 6, 4) used n-butanol, but no reason for this was given. Talbot (20) used water for precipitation of pregnanediol; however, satisfactory results were obtained in this laboratory using 0.1 N sodium hydroxide as proposed by Sommerville (18). The Sommerville method (18) was chosen in this laboratory because it offered the most rapid method for measurement of pregnanediol to be found in the literature.

SUMMARY

I. A review of the literature is given covering (a) hydrolysis, (b) extraction, and (c) identification of estrogens and progestins.

II. Since none of the described systems contains all the necessary procedures for giving a reasonably rapid determination of the desired hormones, modifications were attempted.

III. Attempts to determine quantities of estrogen excretions in dog urine failed possibly through lack of identification

and possibly because estrogens, like pregnanediol, may not be excreted in dog urine.

IV. Positive results have been obtained in extracting pregnanediol from dog feces; however, this procedure is not yet quantitative.

EXPLANATION OF PLATE I

Fig. A. Chromatographic tank with chromatograms in position.

Fig. B. Apparatus for evaporation under reduced pressure.

Fig. C. Reflux apparatus for hydrolysis.

PLATE 1

ACKNOWLEDGMENTS

The author would like to acknowledge Dr. H. T. Gier of the Zoology Department for his constant patience and supervision, which made this paper possible.

Acknowledgments are also made at this time to the Haver-Glover and Upjohn laboratories for supplying pure crystalline hormones for this project.

LITERATURE CITED

1. Astwood, E. B., and G. E. Seegar Jones.
   A simple method for the quantitative determination of pregnanediol in human urine. J. Biol. Chem. 137:397. 1941.

2. Axelrod, L. R.
   The quantitative separation of estrogens by paper partition chromatography. J. Biol. Chem. 201:59. 1953.

3. Bauld, W. S.
   Separation of oestrogens in urinary extracts by partition chromatography. Biochem. J. 59:294-99. 1955.

4. Bloch, H. S., B. Zimmerman, and S. L. Cohen.
   Paper chromatography of pregnanediol and related compounds. J. Clin. Endocrinol. and Metabolism. 13:1206-12. 1953.

5. Bisset, N. G., B. W. L. Brooksbank, and G. A. D. Haslewood.
   The estimation of a fraction resembling pregnanediol glucuronide in human urine. Biochem. J. 42:366. 1948.

6. Bucher, N., and C. F. Geschickter.
   Corpus luteum studies. Endocrinology. 27:727. 1940.

7. Brown, J. B.
   A new method for the determination of oestrogens in urine and its application to a study of the oestrogen excretion in the menstrual cycle. Biochem. J. 56:426. 1954.

8. Bush, I. E.
   Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. Biochem. J. 50:370-78. 1952.

9. Cheng, E. W., and B. Wise.
   Determination of small amounts of diethyl-stilbesterol in feeds. Association of Official Agricultural Chemists. 38:146-150. 1955.

10. Dingemanse, E.
    A colour reaction of diethyl-stilbesterol. Nature. 145:825. 1940.

11. Emmens, C. W.
    Hormone assay. New York: Academic Press, 1950.

12. Heftmann, E.
    Identification of estrogens by paper chromatography. Science. 111:571-72. 1950.

13. Heusghem, C.
Partition chromatography of natural estrogens. Nature. 171-42. 1953.

14. Jensen, C. C.
Quantitative determination of urinary pregnanediol and allopregnanediol for clinical use. Acta Endocrinol. 18:281-87. 1955.

15. Lederer, E., and M. Lederer.
Chromatography. Amsterdam-Houston-London-New York: Elsevier, 1954.

16. Mitchell, F. L., and R. E. Davies.
Isolation and estimation of steroid oestrogens in placental tissues. Biochem. J. 54:690. 1954.

17. Smith, E. P., W. M. Dickson, and R. E. Erb.
Fluorimetric estimation of estrogens in bovine urine. Journal of Dairy Science. 39:162-170. 1956.

18. Sommerville, I. F., G. F. Marrian, and R. J. Keller.
A rapid determination of urinary pregnanediol. Lancet ii, 89. 1948.

19. Stover, R. F., and J. P. Pratt.
Pregnanediol excretion. Endocrinology. 21:711. 1939.

20. Talbot, N. B., J. Ryan, and J. K. Wolfe.
Enzymatic hydrolysis of urinary sodium pregnanediol glucuronidate to free pregnanediol. J. Biol. Chem. 151:607-613. 1943.

21. Turner, C. Donnell.
General endocrinology. Philadelphia: Saunders, 1955.

22. Venning, E. H., P. G. Weil, and J. S. L. Browne.
Excretion of sodium pregnanediol glucuronidate in the adrenogenital syndrome. J. Biol. Chem. 128:cvii. 1938.

23. Wolstenholme, G. E. W.
Ciba foundation colloquia on endocrinology. New York: Blakiston, 1952.

EXTRACTION AND IDENTIFICATION OF ESTROGENS AND PROGESTINS IN DOG URINE

by

DONALD MACK SPENCER

A. B., William Jewell College, 1954

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Zoology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1956

Knowledge of estrogen and progesterone levels throughout the reproductive cycle of the bitch is only theoretical, based on information obtained by work on human levels. By obtaining the actual, or at least relative levels of these hormones in the bitch, possibly a definite means for determining the exact time of ovulation could be established. Abnormal conditions such as prolonged cycles and threatened abortion could be studied and treated.

Estrogens are secreted mainly by the follicle as estradiol, which undergoes metabolism to form estrone and estriol, the latter being the main excretion product, being excreted as a conjugated substance. Progesterone is secreted mainly by the corpus luteum; this substance undergoes metabolism, forming several inactive metabolites, the main one being pregnanediol. Pregnanediol is excreted as a conjugate, sodium pregnanediol glucuronidate in urine.

An attempt has been made to find a method that could be used in daily laboratory routine without special equipment. The method proposed by Heftmann for paper chromatography was investigated, with no success. A modified method proposed by Brown was then attempted but when no satisfactory method for measurement of estrogens was found, this method was abandoned.

Attention was then focused on a method for determination of pregnanediol. The method proposed by Sommerville was selected because of its rapidity, specificity, and quantitative results. Trial determinations were made with pure pregnanediol in ethanol. Human pregnancy urine was used to test the workability of the

procedure. Samples of urine collected from bitches in various stages of pregnancy were then hydrolyzed, extracted, and purified according to Sommerville. The color produced by the action of either concentrated sulfuric acid or antimony pentachloride in chloroform upon the final residue obtained by extraction was compared with the color produced by the same solutions with pure pregnanediol crystals. Color was produced only when a 48-hour urine sample from a three-week-pregnant bitch was used, showing that negligible amounts of pregnanediol are excreted in the urine of pregnant bitches.

Stools from bitches in early and late pregnancy were treated according to Sommerville, using modifications as necessary. Color, comparable to that produced when pure pregnanediol was treated with sulfuric acid or antimony pentachloride, was observed with each sample.

The results of these experiments show that pregnanediol is not excreted in measurable quantities in dog urine. However, a substance was extracted from feces, using the same procedure, that yielded the same colorimetric results as pure pregnanediol. This indicates that pregnanediol or some similar substance is excreted through the feces of the dog in high enough concentrations that measurement can be made.