ABSTRACT

Thomas Theodore Powell, Jr. THE DETECTION AND QUANTITATION OF ESTRADIOL-17β FROM OVARIAN TISSUE OF THE ALBINO RAT USING THIN LAYER AND GAS LIQUID CHROMATOGRAPHY. (Under the direction of Everett C. Simpson and Takeru Ito) Department of Biology, 1972.

Forty-seven albino rats of the Holtzman strain were divided into three groups based on the stage of the estrous cycle. The estrous cycle stage was determined by vaginal lavage. The first group of fourteen rats were taken in proestrus, the second group of thirteen were taken in estrus and the third group of twenty were used as prepuberal controls.

The ovaries of rats within a specific stage of the estrous cycle were surgically removed, weighed to the nearest tenth of a milligram and pooled in preparation for extraction procedures. A homogenate was prepared from the freshly excised ovaries prior to extraction.

The ovarian tissue homogenate was first extracted with diethyl ether. After evaporation to dryness under nitrogen, the resultant residue was dissolved in toluene. The toluene

was extracted with NaOH and the pooled alkaline extract was neutralized with HCl. The neutralized extract was extracted again with diethyl ether. The diethyl ether was washed twice with ten volumes of deionized water to remove salts. The ether extract was taken to dryness under nitrogen.

As a prior purification step, the extract was subjected to TLC on an alumina coated chromagram. The extract was spotted against an estradiol-17β standard. The extract location was ascertained from the R_f value of the estradiol-17β standard.

Prior to GLC, a estradiol diacetate derivative was prepared by using an acetylation reagent.

Separation and detection of estradiol diacetate was carried out by GLC using a flame ionization detector. The identity of the estradiol in the ovarian extract was ascertained from the retention time of standard estradiol diacetate. These standards were injected into the column prior to the ovarian extract sample. The retention time of the estradiol diacetate standards and the estradiol diacetate from the ovarian extract was twenty-two minutes under the conditions used.

Quantitation was carried out by performing TLC on a series of estradiol-17β standards of known concentration. After elution from the plate, diacetate derivatives were prepared from the estradiol-17β standards. The estradiol diacetate standards were injected into the column. From these, a standard curve of ng of estradiol vs. peak area in cm^2 was made. The quantity of estradiol in the ovarian extract was reported as ng/mg.

An average of 4.62 ng/mg of estradiol-17β was found in proestrus ovaries, but none was demonstrated to be present in estrus or prepuberal ovaries.

Estradiol-17β may have been present in estrus tissue, but in concentrations beneath the detection capability of the instrument used.

THE DETECTION AND QUANTITATION OF ESTRADIOL-17β FROM OVARIAN TISSUE OF THE ALBINO RAT USING THIN LAYER AND GAS LIQUID CHROMATOGRAPHY

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CHAPTER I

INTRODUCTION

The main active estrogen secreted by the ovary is estradiol-17β. Estradiol-17β is synthesized in the follicular phase by the follicle and its surrounding cells. The ovarian thecal cells, a layer of cells surrounding the granulosa cells, are responsible for at least a portion of estradiol biosynthesis. The resulting estradiol-17β is stored within the follicular fluid (Sawin, 1969).

It is postulated that two peaks in estrogen secretion exist. One occurs during the estrus portion of the estrous cycle under the influence of FSH, and the other occurs during the luteal phase (Young, 1961; Turner, 1966). Since estradiol secretion reaches a peak during estrus, it can be postulated that titers of estradiol increase within the ovary during the proestrus phase. A sensitive analytical procedure for separation, identification and quantitation of estradiol-17β from ovarian tissue would be necessary in order to test this hypothesis.

The quantitative analysis of steroid hormones in animal tissues, particularly progestins and androgens, using gas liquid chromatography has met with considerable success. Less

success has been achieved with estrogens in biological extracts. This is primarily due to the minute amounts of estrogens being produced. It has been found that prior purification with thin layer or column chromatography is necessary for optimum results with gas liquid chromatography.

The development of gas liquid chromatographic procedures for the identification and quantitation of estradiol-17β in ovarian tissue extracts would be of great value in subsequent steroid hormone studies.

CHAPTER II

REVIEW OF LITERATURE

Present work is confined to procedural techniques for extraction and quantitation of estradiol-17β. It is felt that the review of literature should deal extensively with techniques.

EXTRACTION PROCEDURE

Removal of estrogens from plasma or tissue follows the same general procedure. A solvent-phenol extraction is generally used. This procedure entails the extraction of the estrogens into a relatively non-polar solvent such as diethyl ether or ethyl acetate. This solvent would also remove lipids, cholesterol and other non-polar compounds. The second phase of the procedure entails the extraction of the estradiol into a very alkaline solution such as NaOH or KOH. This phase is selective to any acidic compound including those compounds containing free hydroxyl groups. Essentially, the hydrogen ion is removed from the free hydroxyl groups at the high pH of the alkaline solution thus increasing the polarity of the compound. Acidification to neutrality results in a gain of the hydrogen ion to reform the unionized hydroxyl groups. The estrogens can then be extracted from the neutralized extract into a non-polar solvent.

Touchstone, Varon and Murawec (1963) extracted estriol from the corpus luteum of human ovarian tissue by first extracting into ether, then into 1N NaOH and then back into ether after neutralization of the NaOH with 8N HCl. Attal (1969) employed an almost identical extraction procedure using sheep testicular homogenate. He extracted both estradiol-17β and estriol from this tissue.

Menini and Norymberski (1966) obtained estradiol-17β from human full term placenta by extracting the homogenate first with ethanol and then with methanol. The extract, from which the methanol was removed, was taken into ether. After the extraction of the ether with 1N NaOH, the alkaline extract was neutralized and extracted with ether prior to chromatography.

Attal and Eik-Nes (1968) extracted estradiol from aliquots of human blood plasma using a procedure identical with that Attal (1969) used for extracting estrogens from fetal sheep testicular tissue. Exley (1966) also extracted free estradiol-17β from blood plasma by extracting first into ethyl acetate and then into 2N NaOH. This alkaline extract was neutralized with 6N HCl and re-extracted into ether prior to chromatography.

THIN LAYER CHROMATOGRAPHY

TLC is adsorption chromatography performed on open layers of adsorbent supported on an inert support. A variety of materials including silica gel or alumina may be used as adsorbents. The prepared plates may be activated by heating in an oven for one hour at 100°C. Samples may be spotted on the surface of the plate with micropipettes. After the solvent has evaporated, the plates are placed in a vertical glass tank containing an appropriate solvent system. Separation is produced by the solvent rising through components of the sample spots from the origin depending on the degree of adsorption of the components on the adsorbent (Conn and Stumpf, 1966).

Ismailov and Shraiber (1938) first described the use of thin layers of adsorbent on glass plates for the separation of galenicels. Since 1938, TLC has been used extensively in the separation and purification of biologically active compounds.

Lisboa and Diczfalusy (1962) studied extensively separation and characterization of estrogens by thin layer chromatography. These workers reported changes in R_f values of the estrogens with various solvent systems and tank saturation conditions. They found that in some experiments, using

tissue extracts, it became evident that certain impurities were more efficiently separated from the estrogens when incomplete rather than complete saturation of the tanks was employed.

Struck (1961) reported the separation and quantitative determination of estrone and estradiol-17β. The solvent mixture used was a benzene : ethanol (9:1 v/v). Stahl (1965) reported detection of estradiol, estriol and estrone on TLC plates using U.V. light at 245 nm.

Wotiz and Chattoraj (1964) reported on the use of TLC and GLC procedures in conjunction with each other. They reported that GLC separated and quantitated the eluates from TLC quite rapidly. These workers employed a silica gel thin layer with benzene : ethyl acetate (1:1 v/v) and petroleum ether: dichloromethane : ethanol (10:9:1 v/v) as solvent systems. Van der Molen and Groen (1966) verified the work of Wotiz *et al.* by using TLC prior to GLC. Excellent results were obtained on GLC if the extract was purified on TLC prior to injection into the column.

Brooks (1966) studied the fractionation of urinary steroids using two dimensional chromatography prior to GLC.

He employed silica gel plates using chloroform:ethanol (12:1 v/v) as one solvent system and toluene:ethyl acetate (9:1 v/v) as the other solvent system.

Attal and Eik-Nes (1968) used TLC and subsequent GLC in the purification and quantitation of estradiol-17β in blood plasma. A silica gel thin layer was utilized along with a solvent system of chloroform:ethyl acetate (5:1 v/v). Similar procedures were carried out by Kliman (1965) in the separation of testosterone from human plasma. He used silica gel plates and benzene:ether (2:1 v/v) as a solvent system in one direction and hexane:ethyl acetate (1:1 v/v) in the other.

Attal (1969) used TLC to purify estrogens from sheep fetal testicular tissue extracts prior to GLC. He performed TLC on alumina layers using benzene:methanol (96:4 v/v) and benzene:ethyl acetate (90:10 v/v) as solvent systems.

GAS LIQUID CHROMATOGRAPHY

In this procedure, an inert "carrier gas" such as nitrogen, is used to sweep the vaporized diacetate derivative of estradiol-17β at a high temperature through a long heated capillary tube containing a liquid stationary phase supported on an inert solid. Various compounds in a mixture partition

themselves between the gas phase and the liquid phase, depending on their individual gas-liquid partition coefficients. The separated compounds in a mixture containing estradiol diacetate may be detected by a flame ionization detector. When organic material is burned in a hydrogen flame, electrons and ions are produced. The negative ions and electrons move in a high voltage field to an anode and produce a very small current, which is changed to a measurable signal by appropriate circuitry. The electric current is directly proportional to the amount of material burned (Lehninger, 1970; Conn and Stumpf, 1966).

Gas chromatography was first proposed by Martin and Synge (1941) but their idea was not explored until some years later when James and Martin (1952) showed that the technique was feasible. Since then, it has been applied to detecting a wide range of natural body constituents, including long chain alcohols, wax esters, sugars, amino acids, blood gases, fatty acids and steroids.

The separation of estrogens by GLC was first described by Vanden Heuvel, Sweely and Horning (1960). Wotiz and Martin (1961) first investigated methods of estimation of estrogens.

The difficulty in estimating estrogens in tissue extracts occurs because of the minute quantities usually present in biological tissues and blood plasma. Thayer, Jordan, and Doisey (1930) found that the ratio of estradiol to follicular fluid in the hog ovary was about 1 : 15,000,000. This was substantiated in regard to blood plasma by the work of Attal and Eik-Nes (1968). They found only 0.283 µg of estradiol-17β per 10 ml. of plasma from pregnant women. Attal (1969) found that extracts of sheep fetal testis contained only 1.6 ng per 83 mg of tissue.

Ideally an assay procedure should be specific, reproducible, sensitive and rapid enough to allow multiple analysis. Using GLC, Kuksis (1966) found that overall recoveries of estrogens added to placenta homogenates were of the order of 80%. As little as 1.0 µg of steroids could be examined. Wotiz and Martin (1961), working with the acetate derivatives of estradiol, estriol and estrone on GLC, found that the approximate lower limit of detection of these steroids was 0.15 µg. They found that as little as 0.09 µg of estrone and estradiol acetates could be detected. Wotiz and Chattoraj (1964) found that the GLC was sensitive to as little as 0.2 µg of estrogen in urine specimens collected over a 24 hour period.

Wotiz and Martin (1962), also working with estrogens in human pregnancy urine, used GLC for separation, detection and quantitation. The phenolic extraction was carried out subsequent to hydrolysis of the urine extract. No purification procedures such as thin layer or column chromatography were carried out before GLC. However, these workers reported excellent separation of the acetate derivatives of estradiol, estriol and estrone. It was reported that 81 µg of estradiol-17β per 1,000 ml of urine was separated and quantitated by this method. A retention time of 11.0 minutes was reported for estradiol. The conditions for this separation were as follows. The nitrogen flow rate was maintained at 60 ml per minute with a column temperature of 228°C. The injection port temperature was maintained at 250°C. A 4-foot, 1/8 i.d. stainless steel column was utilized. The column was packed with 3% SE-30 on 80-100 mesh, Diatoport S. Aldercreutz et al. (1966) separated estradiol and estriol from urine samples by GLC with prior purification of the extract using sephadex chromatography. After extraction of the sephadex eluant, further purification was carried out by TLC. After these extensive purification procedures, the sensitivity of the method was found to be about 2 µg in a urine specimen collected over a 24 hour period. The sensitivity was the same for both estriol and estradiol.

Attal and Eik-Nes (1968) determined the concentration of free estradiol-17β in blood plasma using GLC. Using 10 ml aliquots of blood plasma, an ether extraction was followed by a sodium hydroxide extraction. Purification prior to GLC was carried out by TLC. The mean value of estradiol-17B detected was 0.239 µg per 10 ml of blood plasma.

Attal (1969) used GLC to determine the levels of estrone and estradiol-17β in the testis of fetal sheep. He extracted the testicular homogenate using a solvent-phenol type of extraction procedure. After prior purification by TLC, GLC procedures were employed. They reported that 1.6 ng of estradiol-17β per 83 mg of testicular tissue were detected by this method.

McKern and Norstrand (1964) studied regulation of estrogen biosynthesis in human placenta and ovary. Their tissue extraction procedure consisted of ethyl acetate extraction followed by a water wash of the extract. These workers did not use TLC or column chromatography prior to GLC because they felt that some of the advantages of GLC were lost if extensive pre-purification of the extracts was carried out.

Touchstone, Varon and Murawec (1963) extracted and quantitated free estriol in corpus luteum of human pregnancy.

The free estriol was separated from the ovarian tissue by ethyl acetate extraction, subjected to subsequent sodium hydroxide extraction, and then a final ether extraction separated the estriol for purification on an alumina column. After purification on the alumina column, GLC was carried out. Sensitivity for the estriol was found to be 0.2 µg for the procedure.

The TLC and GLC procedures used in this study were done to identify and quantitate estradiol-17β in ovarian tissue extracts of the white rat. The ovarian tissue was taken at three distinct periods of the reproductive cycle. These periods were proestrus, estrus and prepuberal.

CHAPTER III

MATERIALS AND METHODS

ANIMALS

Both mature and prepuberal female albino rats of the Holtzmann strain were used in this study. These animals were subjected to daily light periods of fourteen hours and dark periods of ten hours in order to maintain the normal photoperiodic estrous cycle. They were fed Purina Lab Chow. ad. lib.

EXPERIMENTAL PROCEDURES

VAGINAL LAVAGE

The vaginal lavage technique was employed to ascertain the various stages of the estrous cycle (Appendix A). Approximately 0.25 cc of water was introduced into the vagina with a medicine dropper and aspirated. Sloughed cells were placed on a microscope slide with the medicine dropper and the identity of these cells, as seen under the microscope, served as a criterion for determining the specific stage of the estrous cycle.

The animals were divided into three groups based on the stage of the estrous cycle. The first group were taken in

the proestrus stage, the second in the estrus stage and the third group were utilized as pre-puberal controls. A total of 47 animals was used.

SURGICAL REMOVAL OF OVARIES

After sacrificing the rats by cervical dislocation, an incision was made from the pubic bone to the base of the sternum. The ovaries were quickly removed and trimmed of excess fat. The follicles of proestrus ovaries were swollen and distended. Ovaries of a specific estrus stage were pooled prior to extraction procedures.

TREATMENT OF OVARIAN TISSUE PRIOR TO EXTRACTION PROCEDURE

After surgical removal, four to eight ovaries were weighed to the nearest tenth of a milligram. They were placed in a small Duall ground glass homogenizer along with approximately 1.0 ml of distilled water and homogenized until a creamy homogenate was obtained.

EXTRACTION PROCEDURE

The ovarian tissue homogenate was placed in a 250 ml. separatory funnel. The homogenate was extracted six times with 4.0 ml of diethyl ether. The ether was evaporated to dryness under nitrogen. The resulted residue was dissolved in 3.0 ml of toluene. The toluene was extracted four times

with 2.0 ml of 1N NaOH and the pooled alkaline extract was neutralized with 8N HCl. The neutralized extract was extracted four times with 5.0 ml of diethyl ether. The ether was washed twice with ten volumes of deionized water to remove salts. The ether was evaporated to dryness under nitrogen in preparation for TLC.

THIN LAYER CHROMATOGRAPHY

A 10 µl Hamilton syringe was used to spot approximately 10 µl of the extract dissolved in ether on an Eastman Chromagram coated with an alumina thin layer. A 150 µg estradiol-17β standard from Sigma Chemical Company was spotted 12.0 cm abreast of the extract. The TLC plate was placed in an Eastman Chromagram Chamber plate set and dipped into troughs containing a chloroform:ethyl acetate (5:1 v/v) mixture. The solvent front was allowed to run within 2.0 to 4.0 cm from the top edge of the plate before removal from the chamber. Running time ranged from 1.5 to 2 hours. The standard was located under U.V. light at 254 nm. A large area corresponding to the R_f value of the estradiol standard was cut from the plate. The standard was not removed from the plate. The cut area was eluted with acetone, which was evaporated to dryness under nitrogen.

DERIVATIVE PREPARATION

Estradiol diacetate derivatives were prepared prior to injection into a GLC column. Acetylation reagent was prepared by mixing 10.0 ml of acetic anhydride with 5.0 ml of pyridine (Fisher Analytical Grade). This reagent (2.0 ml) was added to the dried estradiol sample eluted from the TLC plates. This mixture was allowed to stand overnight. Prior to injection, the acetylation mixture was heated in a water bath for one hour at 60-65°C. Excess acetylation reagent was removed under nitrogen.

GAS LIQUID CHROMATOGRAPHY

GLC was carried out on a Bendix Chroma-lab Series 2100. A 1/8 inch i.d., six foot stainless steel column was used. The column was packed with 3% OV-17 coated on Chromosorb WHP (80-100 mesh) from Chemical Research Services, Inc. A flame ionization detector was used for detection.

The detector temperature was maintained at 260°C, the injection port at 265°C, and the oven at 245°C. Nitrogen gas was used as the mobile phase and the flow rate was maintained at 47 ml/min at 60 p.s.i. Oxygen and hydrogen flow rates were adjusted until maximum peak height was obtained. The hydrogen flow rate reading on a Brooks tube R-2-15-AAA

(CO) rotometer was 60. The oxygen flow rate reading on a Brooks tube 2-65-A rotometer was 1.6. Tank pressure was 35 p.s.i. The electrometer attenuation was X 10.

CALCULATION OF RETENTION TIME

The identity of estradiol diacetate in the ovarian extract was ascertained from the retention time of standard estradiol diacetate. These standards were injected into the column prior to the ovarian extract sample. The retention time of the estradiol diacetate standard and the estradiol diacetate from the ovarian extract was twenty-two minutes under the conditions used (Figure 1 and 2).

QUANTITATION PROCEDURE

In order to quantitate the estradiol found in ovarian extracts, a series of estradiol-17β standards of known concentration were subjected to TLC. After TLC, the estradiol standards were eluted from the TLC plate and acetylated. The prepared diacetate derivatives of estradiol-17β were injected into the column (Figure 3). From these standards, a standard curve of ng of estradiol standard vs. peak area in cm^2 was made. The quantity of estradiol in the ovarian extract in ng/mg of ovarian tissue was calculated from the standard curve.

CHAPTER IV

RESULTS AND DISCUSSION

The use of GLC in conjunction with TLC is predicated on two basic needs. After extraction of tissue, the components of the extract may be separated by means of TLC as a prior purification step. GLC allows further separation of the components of the TLC eluant as well as their detection.

The method of estrogen analysis in rat ovaries investigated includes solvent extraction, purification by TLC and final analysis of acetylated derivatives by GLC. Peaks obtained from ovarian extracts which showed the retention times similar to those exhibited by acetylated estradiol standards were identified as estradiol.

A periovarian fat sample was extracted, and subjected to TLC and GLC to verify that the sample peak indeed came from ovarian tissue. No estrogens were detected in the periovarian fat.

The weight of a single group of pooled proestrus ovaries averaged 199.2 with a standard deviation of $\pm$27.5 mg (Table 3). The average weight of each group of estrus ovaries did not vary significantly from that of each group of proestrus

ovaries (Table 2). Ovarian tissue was pooled because it was thought that the amount of estrogens would be too small to be detected from the tissue of one animal. The number of ovaries used in each experimental run was not constant.

Estradiol-17β was not detected during estrus or in prepuberal rats by the method used, which could give a discernable peak with the amounts of estradiol as small as 250 ng (Table 1 and 2).

The lowest amount of estradiol found in proestrus ovarian tissue was 320 ng per three animals (Table 3). This level was well above the minimum level of the estradiol detected by a similar column used by other investigators. The levels of estradiol in proestrus ovarian tissue ranged from 1.4 ng/mg of tissue to 8.1 ng/mg of tissue with an average of 4.62 $\pm$2.13 ng/mg tissue (Table 3). While there was no measurable amount of estradiol-17β in the ovarian tissue of rats in estrus, this is not taken to imply that no estradiol was present. It may simply reflect that the level of this estrogen is below the capacity of this instrument to detect.

Visual examination of data showed a significant difference in levels of estradiol obtained between proestrus and

estrus tissue. This significance is due to the fact that the detectable quantities of estradiol was found only during proestrus.

During the proestrus phase, under the influence of FSH, estradiol synthesis is heightened and is thought to come from either the membrana granulosa or the theca interna. Estradiol collects in the follicle and is elaborated as the onset of estrus ensues (Sawin, 1969; Turner, 1966). Considering that estradiol collects in the follicle during proestrus, somewhat higher levels of estradiol were expected in proestrus tissue. The variation in quantity of estradiol in the ovarian tissue of the proestrus group (Table 3) may have been the result of the time within the proestrus portion of the cycle when the tissue was procured. These higher levels of estradiol may have been detected when the follicular thecal or granulosa layer were at the height of estradiol synthesis. The lower levels may be accounted for in either removal of the ovaries in early proestrus, and before maximum estradiol synthesis, or late enough in proestrus that the peak of estradiol synthesis was passed.

Estrus is the period of sexual receptivity in the rat. Under the influence of FSH, the ovarian follicles grow

rapidly. This phase of the cycle shows increased estrogen secretion. Estrogen secreted causes hyperemia and hyperplasia of the rat uterus (Turner, 1966). Since estrogen leaves the follicle to exert its action upon the uterus to prepare it for implantation, it is not surprising that no levels of estradiol-17β were found in the ovarian tissue during this period (Table 2).

Although qualitative analysis of estradiol could be done quite well with this procedure, accurate quantitation was more difficult. No procedure was employed to determine possible losses during the extraction and TLC procedures. Experimental procedures outlined in literature reviewed called for the use of tritiated estradiol standards to be used during extraction and TLC to estimate losses. These procedures called for special modifications on the gas liquid chromatograph employed. There were none available for this work. However, great care was taken during extraction and TLC separation to reduce losses to a minimum.

A rather long retention time of twenty-two minutes was noted for estradiol-17β. Estrogen acetates are known for their long retention times. Coupled with this fact was the

presence of a tightly packed column and a consequently slowed gas flow rate. Figure 1 shows a GLC tracing of an estradiol diacetate standard.

Figure 2 shows a peak of a diacetate derivative obtained from a proestrus ovarian extract. The retention times are the same for both peaks. The peak appearing at a retention time of five minutes in Figure 2 is that of an acetate derivative of cholesterol.

Further work should be done in this area. If the sensitivity of the instrument used could be increased, there is a possibility that estradiol-17β could be detected during estrus. Further pooling of estrus ovarian tissue is necessary along with possible increased sensitivity adjustments of the instrument used.

SUMMARY

Estradiol-17β was detected and quantitated from proestrus ovarian tissue of the white rat. No estradiol was detected in estrus ovaries nor in prepuberal ovaries.

A total of 47 animals was used in this work. The animals were divided into three groups based on the stage of the estrous cycle. The first group were taken in the proestrus stage, the second in the estrus stage and the third were used as prepuberal controls.

The stage of the estrous cycle was determined by vaginal lavage. The ovaries of rats within the specific stage of the estrous cycle were surgically removed, weighed, and pooled in preparation for extraction procedures.

After extraction from the ovarian tissue by a phenolic extraction procedure, the estradiol was separated from extraneous compounds in the extract by TLC. The location of the estradiol-17β on the TLC plate was determined from its R_f value.

Prior to GLC, it was necessary to prepare a diacetate derivative of estradiol-17β. Separation and detection of

the estradiol diacetate was carried out by GLC using a flame ionization detector.

Quantitation of estradiol was carried out by injecting a series of estradiol diacetate standards onto the column. From these standards, a standard curve was constructed. From the standard curve, the quantity of estradiol in the extract was ascertained.

No estradiol was demonstrated to be present in estrus ovarian tissue. This was not taken to mean that no estradiol was present in these ovaries. The level of estradiol in estrus ovarian tissue may have been below the detection capacity of the instrument used.

Higher estrogen levels were found in proestrus ovarian tissue. This was not surprising, since it is thought that there is an accumulation of estradiol in the follicle prior to the onset of estrus.

APPENDIX A

Different stages in the rat estrous cycle and corresponding cell types in the vaginal lavage.

Stage of Cycle	Cell Type
Diestrus	Lavage dominated by leucocytes
Proestrus	Nucleated epithelial cells
Estrus	Large masses of cornified epithelial cells with degenerate nuclei
Metestrus	Many leucocytes with a few cornified epithelial cells

APPENDIX B

LIST OF ABBREVIATIONS

FSH	follicle stimulating hormone
GLC	gas liquid chromatography
R_f	the ratio of the distance traveled by the compounds to the distance traveled by the solvent front on a TLC plate
TLC	thin layer chromatography
UV	ultra violet

APPENDIX C

Table 1. The quantity of estradiol-17β present in pre-puberal ovaries as detected by GLC.

Experiment Number	Number of Animals Used in Each Experiment	Pooled Weight of Ovaries in Each Experiment (mg)	Total E-17β in Each Experiment (ng)	Levels of E-17β in Each Experiment (ng/mg of ovaries)
1	4	120	0	0
2	4	122	0	0
3	4	119	0	0
4	4	121	0	0
5	4	123	0	0
Average		121	0	0
Standard Deviation		±1.41		

APPENDIX D

Table 2. The quantity of estradiol-17β present in estrus ovaries as detected by GLC.

Experiment Number	Number of Animals Used in Each Experiment	Pooled Weight of Ovaries in Each Experiment (mg)	Total E-17β in Each Experiment (ng)	Levels of E-17β in Each Experiment (ng/mg of ovaries)
1	2	195	0	0
2	3	217	0	0
3	2	175	0	0
4	3	220	0	0
5	3	225	0	0
Average		206.4	0	0
Standard Deviation		$\pm$18.4		

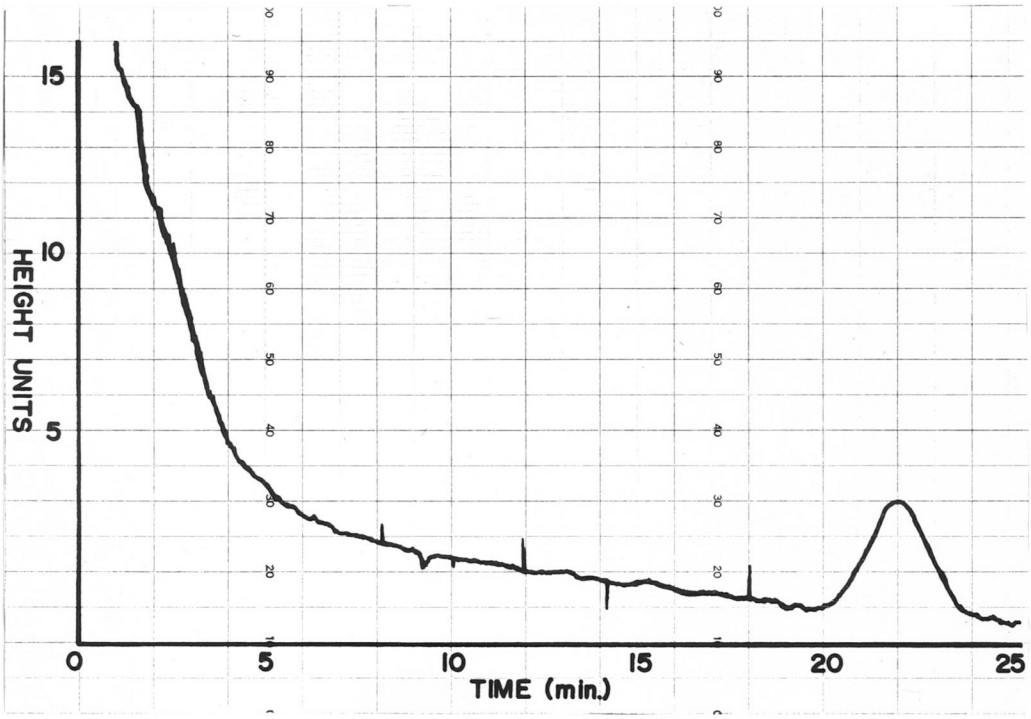
APPENDIX E

Table 3. The quantity of estradiol-17β present in proestrus ovaries as detected by GLC.

Experiment Number	Number of Animals Used in Each Experiment	Pooled Weight of Ovaries in Each Experiment (mg)	Total E-17β in Each Experiment (ng)	Levels of E-17β in Each Experiment (ng/mg of ovaries)
1	3	219	1,000	4.6
2	3	190	920	4.8
3	2	150	610	4.1
4	3	210	1,730	8.1
5	3	227	320	1.4
Average		199.2	916	4.62
Standard Deviation		±27.5	±477	±2.13

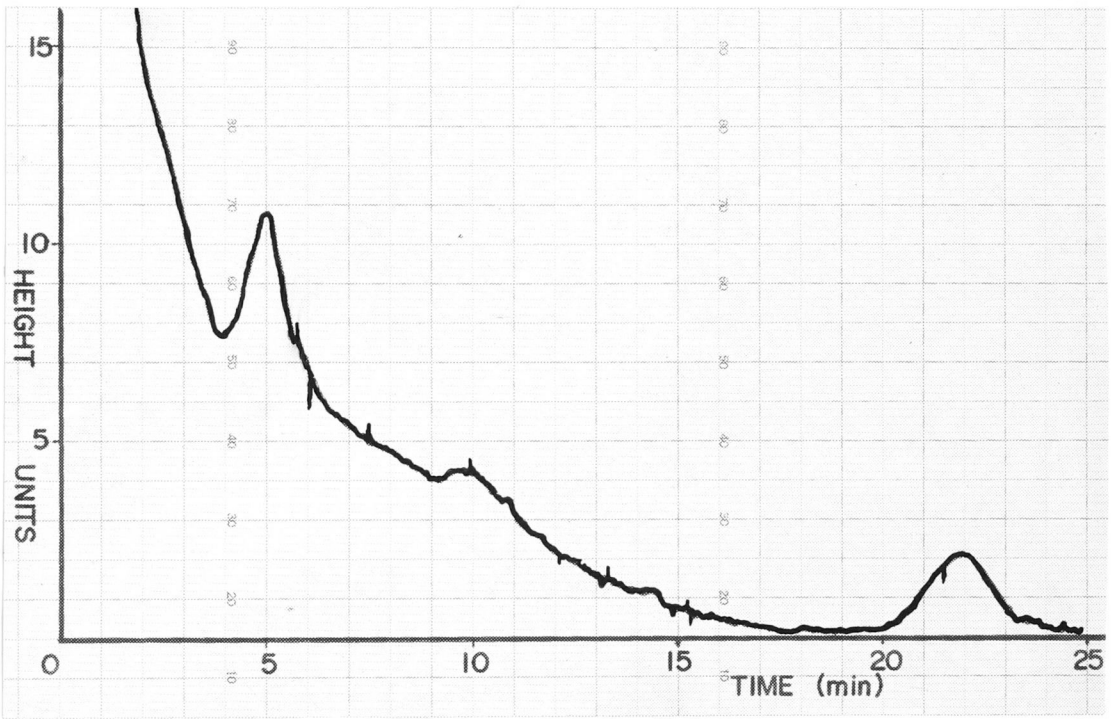
APPENDIX F

Figure 1. A GLC tracing of an estradiol diacetate standard. Details are in the text.



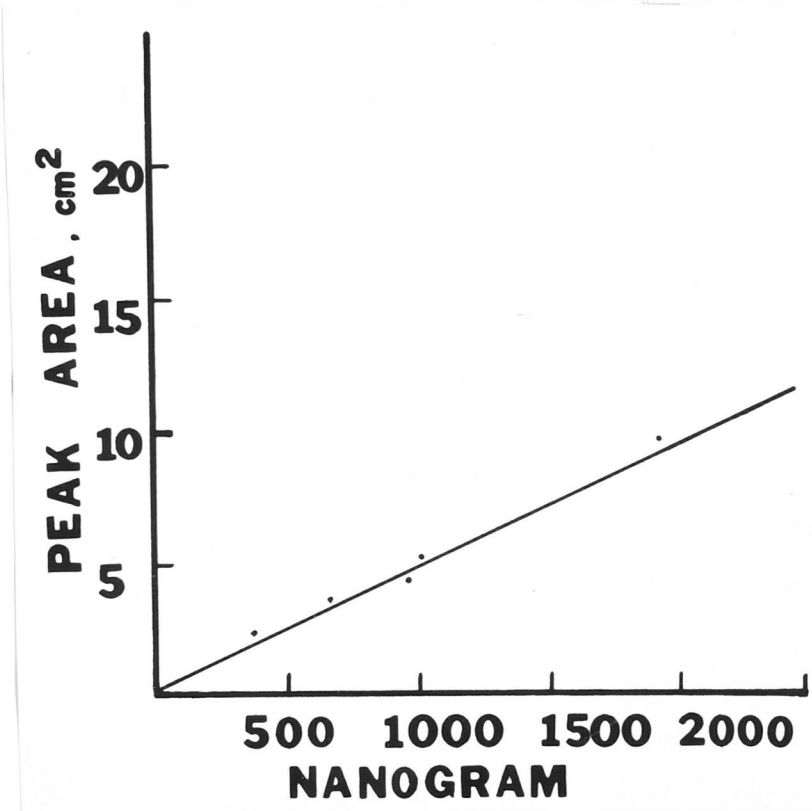
APPENDIX G

Figure 2. The best GLC tracing of estradiol diacetate from an ovarian extract. Details are in the text.



APPENDIX H

Figure 3. The standard curve from which the quantity of estradiol-17β in the ovarian extract was ascertained.



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