

**THE PRODUCTION RATE OF ESTRADIOL IN THE
STEIN-LEVENTHAL SYNDROME**

by

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This thesis submitted by Ruben A. Puebla, M.D. has been examined and approved by an appointed committee of the Faculty of the Graduate Division of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Date

50145

This volume is dedicated

to

Robert B. Greenblatt, M.D., Professor and Chairman of the
Department of Endocrinology and Virendra B. Mehta, Ph.D.,
D.Phil., Associate Research Professor of Endocrinology in
appreciation for their skillful guidance and their cheerful
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INTRODUCTION

Extensive studies have been carried out on the biosynthesis of steroids in the polycystic ovary. These studies suggest that there are enzymatic abnormalities in such ovaries resulting in impaired estrogen synthesis and excessive production of androgens.

The studies of in vitro biosynthesis of steroids in the polycystic ovaries have, without doubt, provided valuable information concerning the various enzymatic activities of the ovary, however, it is difficult to interpret the results in terms of ovarian function under physiological conditions. Similarly, analysis of the steroid content of ovarian tissue has also been of limited value because it does not give any indication of the rate of secretion. Certainly, the estrogen level in the ovarian venous blood of patients with Stein-Leventhal syndrome, as compared with that of normal women, constitutes the best procedure in determining whether the estrogen secretion is decreased or normal in this syndrome. The necessity of surgical procedures and technical complexity make this method impractical and realizable only in special conditions.

During the last few years, the use of isotopically labeled steroids in the measurement of the secretion rate of steroid hormones in the human has proven extremely valuable (Pearlman, 1957; Peterson, 1959; Curpide et al. 1962a, 1963; Vande Wiele et al. 1963).

Isotope dilution principle has been applied to the measurement of secretion rate of estrogen in normal and pregnant women (Carpide et al. 1962a, 1963; Morse et al. 1963). The production rate of estrogens in patients with Stein-Leventhal syndrome has thus far not been determined.

In the present study, the production rate of estradiol has been determined in five patients with the Stein-Leventhal syndrome, one patient with secondary amenorrhea of hypothalamic origin, and one patient with Sheehan's syndrome. The significance of the results obtained is discussed.

REVIEW OF THE RELATED LITERATURE

The presence of estrogenic and androgenic activities in the follicular fluid of hog ovaries was demonstrated by Allen & Doisy (1923) and by Champy & Kritch (1925). During the last few years there have been numerous publications of the *in vitro* biosynthesis of steroids in the normal and polycystic ovary by incubation with various steroid precursors (O'Donnell & McCaig, 1939; Sweat et al. 1960; Axelrod & Goldzieher, 1961, 1962; Forchielli et al. 1961; Kase et al. 1961; Mahesh & Greenblatt, 1961; Lanthier & Sandor, 1962; Leon et al. 1962; Zander et al. 1962; Chapdelaine et al. 1963). Based on the results of these investigations, it is generally accepted that the major pathway of the biosynthesis of estrogens in the normal ovary is the conversion of cholesterol into estrogens via Δ^5 -pregnenolone-progesterone-17 α -OH progesterone and Δ^4 -androstenedione. Recently, Ryan & Smith (1961) reported a minor pathway in the synthesis of estrogen from cholesterol via Δ^5 -pregnenolone-17 α -OH- Δ^5 -pregnenolone-dehydroepiandrosterone (DHA)- Δ^4 -androstenedione.

The isolation of large quantities of 17 α -OH progesterone and Δ^4 -androstenedione from the ovarian tissue of normal subjects after *in vivo* stimulation with human-pituitary follicle-stimulating hormone (HP-FSH) has been shown by Mahesh & Greenblatt (1964). Furthermore, the absence of significant amounts of these steroids in the urine and/or ovarian venous plasma

under similar conditions, in contrast with a marked increase in estrogen excretion, indicates that these steroids were converted into estrogen prior to being secreted by the ovary. On the other hand, large quantities of either Δ^4 -androstenedione, 17 α -OH-progesterone or 17 α -hydroxy- Δ^5 -pregnenolone and DHA have been isolated from the ovarian tissue of patients with Stein-Leventhal syndrome (Mahesh & Greenblatt, 1962; Starks et al. 1962; Baulieu et al. 1963). High levels of Δ^4 -androstenedione in the follicular fluid have been observed by Short & London (1961) and Mahajan et al. (1963) in patients with polycystic ovaries.

During control periods, Mahesh & Greenblatt (1964) found the urinary excretion of estrogen within normal limits in Stein-Leventhal patients. When the ovaries were stimulated with H.P.-FSH, three of five patients showed a marked rise in androgen output rather than estrogen. These findings are suggestive of abnormal steroid biosynthesis.

In vitro studies have demonstrated two types of enzymatic abnormalities concerning the biosynthesis of steroid hormones in Stein-Leventhal patients.

(1) Abnormal aromatization of Δ^4 -androstenedione to produce estrogens. The presence of this enzymatic defect in polycystic ovaries was confirmed by the finding of high levels of Δ^4 -androstenedione in the ovarian venous blood and follicular fluid (Short & London, 1961; Short, 1962; Mahajan et al. 1963; Mahesh & Greenblatt, 1964). Moreover, Short (1962) has reported that the follicular fluid from polycystic ovaries contains very small

amounts of estrogens as compared to the follicular fluid obtained from normal ovaries.

(2) Abnormalities in 3 β -O1-dehydrogenase have been shown in *in vitro* incubation of ovarian slices with 7-³H DHA. Polycystic ovaries with decreased 3 β -O1-dehydrogenase activity showed poor conversion of DHA to Δ^4 -androstenedione and estrogens. This abnormality was confirmed by Mahesh & Greenblatt (1964) by (a) isolation of DHA in ovaries of untreated patients with Stein-Leventhal syndrome (b) presence of large quantities of DHA and 17 α -OH- Δ^5 -pregnenolone in the polycystic ovaries after stimulation with HP-FSH and (c) by the presence of DHA in ovarian venous blood of these patients.

Dokunov & Dashev (1963) concluded that the enzymatic abnormalities in the Stein-Leventhal syndrome is localized in cells that produce steroid hormones. The same conclusions were made by Leventhal (1962) after functional and morphological studies of the adrenal and ovaries of two patients with polycystic ovaries.

Normal urinary estrogen levels in patients with polycystic ovaries have been reported by several investigators. This finding does not rule out defective ovarian biosynthesis of estrogens. It has been explained by the presence of an incomplete block, the participation of a larger number of follicles in estrogen production and the presence of increased concentration of substrate present in ovarian tissue. The level of estrogen in the ovarian venous blood of polycystic ovaries has been found

to be comparable to normal ovaries under control conditions.

Mahech & Greenblatt (1964) found that estrogen/androgen ratio in ovarian venous blood of normal patients varied from 1.4 to more than 4.9, and a ratio of 0.028 to 0.15 was found in patients with Stein-Leventhal syndrome. Furthermore, after stimulation with H.P.-FSH the ratio increased to more than 5.1 in normal patients and was 0.058 and 0.098 in two patients with Stein-Leventhal syndrome. This abnormal ratio in polycystic ovaries indicates the presence of enzymatic abnormalities even though significant levels of estrogens are present.

The in vivo conversion of androgens to estrogen has been demonstrated in normal women by Nathanson et al. (1952) and in castrated and adrenalectomized women by West et al. (1956). It has been suggested that the augmented androgen synthesis and secretion by the polycystic ovaries may be another factor contributing to the presence of estrogen in the urine of these patients.

MATERIALS AND METHODS

PURIFICATION OF 6,7-³H ESTRADIOL

Estradiol -6,7-³H was obtained from New England Nuclear Corporation and purified by using extraction and paper chromatography according to the method described by Mahesh (1964). The material was transferred to a 250 ml round-bottom flask with methanol and evaporated to dryness. It was then taken up in toluene (100 ml) and the toluene transferred to a separatory funnel. The flask was washed with 25 ml N. sodium hydroxide and the sodium hydroxide solution transferred to the same separatory funnel and mixed with toluene. After that, the sodium hydroxide layer was separated; the sodium hydroxide wash of the flask and the toluene was done three more times (using in each one, 25 ml N. sodium hydroxide). The combined sodium hydroxide mixture was acidified to pH of 9±0.5 with 12 per cent sulfuric acid and extracted four times with 0.25 volumes of ether. The combined ether extract was washed once with 0.05 volume water and evaporated to dryness in a round-bottom flask. The dry extract was then dissolved in ethyl acetate: methanol mixture (2:1) and applied over the entire width of a Whatman 3MM paper strip (6" x 22"). Two strips (2" wide) with 50μg of estradiol were prepared to serve as standards. The chromatograms were run in a system consisting of ligroin: benzene: methanol: water (67:33:80:20) for 3.5 hours after a preliminary equilibration for 2 hours.

The estradiol zone was located on standard strip with the Folin-Ciocalteu reagent and the zone marked on the unknown strips. The radioactivity was also located in the estradiol zone by scanning through a Batzel Atomic 4 II paper scanner. The estradiol zone was eluted from paper with ethyl acetate: methanol. The extract was dried and dissolved in 10 ml ethanol and aliquots counted in a Packard Liquid Scintillation Counter. It was then diluted with ethanol to give a final concentration of 3.0 μ c per ml.

INJECTION OF RADIOACTIVE ESTRADIOL AND URINE COLLECTION

3.0 μ c of radioactive $6,7-\text{H}$ estradiol was transferred to counting vials, evaporated to dryness and dissolved in 1 ml of ethanol; the volume was further made up to 10 ml with acetile saline solution and injected i.v. to each patient. The syringe is flushed several times with blood to complete the transfer of radioactivity and the empty vial counted for residual radioactivity. The difference gave the amount of radioactive injected. Following the injection of radioactive estradiol, twenty-four-hour urine specimens were collected for three days in stoppered bottles containing 100 ml toluene, and refrigerated.

DETERMINATION OF PRODUCTION RATES

The basic steps involved in the determination of production rates were: a) hydrolysis of estrogen conjugates by treatment of urine with concentrated hydrochloric acid. b) extraction of the liberated steroids with ether. c) partition of the extract between NaOH and toluene, to separate the phenolic fraction. d) preliminary purification of the phenolic fraction by using

gradient elution liquid partition chromatography system. e) preparation of samples for counting. f) further purification of various estrogen fractions by paper chromatography. g) estimation of free estrogens by fluorimetry of Ittrich extract of Kober chromogens and by measurement of radioactivity. h) calculation of production rate.

a) Acid hydrolysis: The advantages and disadvantages of acid hydrolysis as compared to enzymatic hydrolysis have been discussed by Brown & Blair (1958) and Preedy & Aitken (1961). Acid hydrolysis has been found suitable for the estimation of estrone, estradiol, and estriol. Acid hydrolysis was carried out by the method of Preedy & Aitken (1961). A 25 per cent aliquot of the 72 hr. urine specimen was brought to boiling under reflux and concentrated hydrochloric acid (15 ml acid per 85 ml urine) added. The mixture was refluxed for 45 minutes and cooled rapidly.

b) Preliminary ether extraction: Extraction was carried out by a procedure similar to Engel et al. (1950). The hydrolyzed urine was put in a separatory funnel and extracted four times with 0.25 volumes freshly distilled ether. The combined ether extract was washed twice with 0.05 volumes saturated sodium bicarbonate and once with 0.05 volumes water and evaporated to dryness in a round-bottom flask.

c) Partition of extract between toluene and sodium hydroxide: For the separation of the phenolic fraction from other impurities, the following procedure was followed: Toluene (100 ml)

and N sodium hydroxide (25 ml) were added to the extract, the mixture transferred to a separatory funnel, and the sodium hydroxide layer separated. The flask containing the original extract and the toluene were washed three more times with 25 ml N sodium hydroxide. The combined sodium hydroxide mixture was acidified to pH of 9±0.5 with 12 per cent sulfuric acid and extracted four times with 0.25 volumes of ether. The combined ether extract was washed once with 0.05 volume water and evaporated to dryness in a round-bottom flask.

d) Preliminary purification by column chromatography: The dry extract was dissolved in a small amount of 2:1 ethyl acetate-methanol mixture and transferred to a stoppered tube and evaporated to dryness. A glass column 96 cm long and 1.6 cm in diameter was used. A small piece of washed cotton was packed at the bottom of the column. The column was then packed with a mixture of 10 gm of celite (Johns-Manville celite 545 acid washed) and 3 ml water (section C, figure 1). The material was packed with a plunger in segments of 1.5 to 2 cm in height. The column was then packed with a mixture of 30 gm celite and 9 ml 90 per cent ethylene glycol in water (section B, figure 1). The extract was dissolved in 0.9 ml of 90 per cent ethylene glycol in water, mixed with 3 gm celite and then packed in the column (Section A). Iso-octane (2,2,4-trimethylpentane) was carefully introduced and allowed to flow by gravity until solvent emerged from the column. The volume of solvent required to fill the column, hold back volume (HBV), was measured directly or by

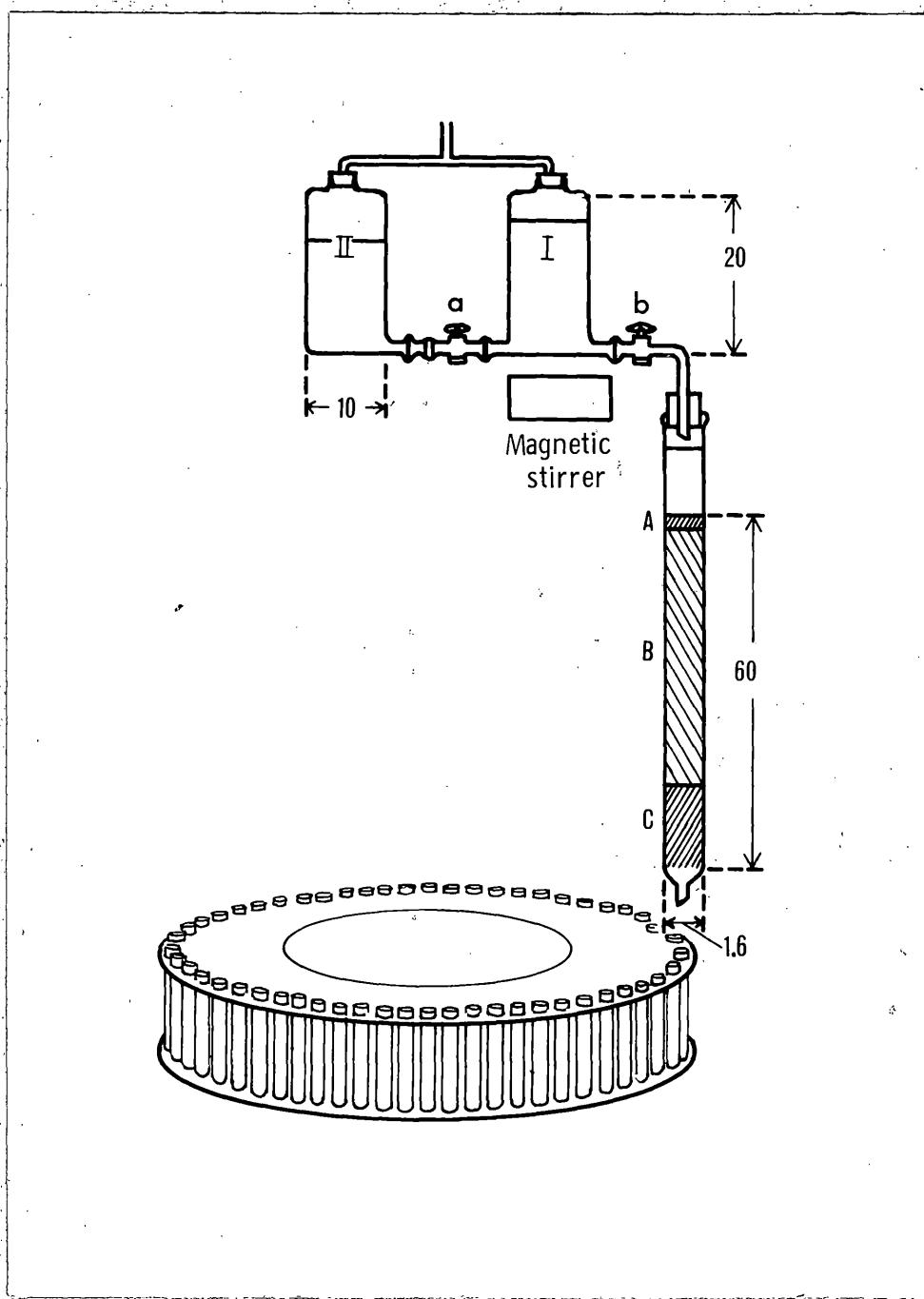


Fig. 1. Apparatus for gradient elution partition chromatography of urinary estrogens.
Dimensions are given in centimeters.

adding a measured amount of solvent and determining the length of column that it occupied. Gradients of increasing solvent polarity were produced by a system of two cylinders (10 cm x 20 cm) as shown in figure 1. Cylinder I constitutes the mixing chamber and is filled with two outlets having size 18/9 standard ball joints; cylinder II is of identical size but with only one outlet at the bottom. Both cylinders are provided with 24/40 standard taper joints at the top for application of pressure to the system. A tube connects the two cylinders and is fitted with 18/9 socket joint, a teflon stopcock and a coarse sintered glass plug which minimizes spurious flow of solvent between the two cylinders which were placed to the same level. A delivery tube is also fitted with a socket joint and passes into the column through a rubber stopper. The entire apparatus is shown in figure 1. Gradient cylinder I was filled with 900 ml of iso-octane and cylinder II with 697.5 ml of ethyl acetate. The magnetic stirrer was started and stopcocks "a" and "b" cautiously opened and hydrostatic equilibrium between cylinders I and II was adjusted. The solvent was allowed to flow through the column at a rate of 60 to 100 ml per hour until both cylinders were empty. Finally, 200 ml of ethyl acetate was passed through the column. About 150 fractions of 10 ml each were collected in a fraction collector and aliquots of 200λ from each tube were taken in order to identify the radioactivity of the estrogenic fractions.

e) Preparation of samples for counting: All samples were pre-

pared for liquid scintillation counting in the following manner: An aliquot of the sample was transferred to counting vials and the solvent evaporated. The sample was then dissolved in 20 ml toluene containing 0.4 per cent 2,5-diphenyloxazole (PPO) and .01 per cent 1,4-Bis-2 (5-phenyloxazolyl) benzene (POPOP). (LSP-2 and LSP-7, Tracerlab). The various estrogen fractions were then pooled and transferred to flasks and each tube was washed 3 times with ethyl acetate: methanol mixture 2:1. The flasks were then evaporated to dryness under vacuum at 45°C by using a flash evaporator (Laboratory Glass and Instrument Corp., Model FE-2). The residue was then transferred in a similar way to a test tube using ethyl acetate: methanol mixture 2:1 and evaporated to dryness in a Rotatory Evapomix.

f) Paper Chromatography: Whatmann 3 MM chromatographic paper strips (2" x 22½") were washed in a soxhlet type extraction apparatus for 48 hours with a mixture of ethyl acetate and ethanol (1:1). The dry estrogen fractions were dissolved in 500 λ of ethyl acetate: methanol mixture 2:1 and aliquots of 200 λ or 300 λ applied to the entire width of the paper. One paper strip was used for each estrogen fractions. Two standard strips each containing 50 µg quantities of estrone, estradiol, epiestradiol and 2-methoxy-estrone were prepared for the separation of estrone and estradiol. The standard strips and the experimental strips were run in a system consisting of ligroin: benzene: methanol: water (67:33:80:20) for 2½ to 3 hours after a preliminary equilibration of 2 hours. A small amount of Sudan IV

was used to mark the solvent front. The paper strips were then dried at room temperature. The standard strips were developed with Folin-Ciocalteu reagent (alkaline-phenol reagent). The estrone, estradiol zones on the unknown chromatograms were marked by comparison with the standards. The radioactive compounds were then eluted from paper using ethyl acetate: methanol mixture 2:1. The solution was evaporated to dryness. Each estrogenic fraction was re-dissolved in 200 λ or 300 λ of ethyl acetate: methanol 2:1. One aliquot of 50 λ was then taken of each fraction and its radioactivity measured, using a Tracerlab Dual Channel Liquid Scintillation Counter and from 100 λ to 250 λ were used for estimation by fluorescence.

g) Fluorescence Reaction: The Kober reaction was carried out by heating the dry extract containing 20 mg of hydroquinone with 1.1 ml of 65 per cent sulfuric acid on boiling water bath for 40 minutes. After cooling in an ice bath, 1.5 ml of ice cold water is added to each tube and the tube shaken after further cooling in the ice bath. Ice cold chloroform solution (3 ml) containing 2 per cent p-nitrophenol (w/v) and 1 per cent ethanol (v/v) is added to each tube and the tubes shaken. The aqueous layer is removed by suction and the organic layer transferred to pre-cooled cuvettes (10 mm x 75 mm). The cuvettes are centrifuged at 2000 rpm for 5 minutes at 0°C and the fluorescence read in a Turner Fluorometer (Model 110) using a combination of Wratten 2 A and 61 as primary filters, and Wratten 23 A as secondary filter with a light standard of 535 m μ . A blank tube

and a set of 5 standard tubes containing .05, 0.1, 0.25, 0.5 and 1 μ g respectively of estrone or estradiol were prepared as described above, in order to determine the amount of estrogen present in the unknown fractions. Quantities as low as 0.02 μ g can be detected by this fluorescence reaction.

h) Formula for calculation of secretion rate: The secretory rate of the endogenously produced hormone is calculated from the cumulative specific activity (sa) of a urinary metabolite according to the formula employed by Curpide et al. (1962a, 1962b).

$$\text{Production Rate} = \frac{\text{Radioactivity injected}}{\text{Cumulative sa of urinary metabolite} \times \text{time}}$$

which is valid when the tracer is injected into the same compartment into which the hormone is secreted.

The numerator of this formula (radioactivity injected) is calculated as follows: The quantity of 3.03 μ c is converted into counts per minute. This is done by multiplying the disintegration per minute (dpm) in 3.03 μ c ($3.03 \times 2,220,000$) with the efficiency of the Scintillation Counter during the counting. The efficiency is determined for each run using a known Tritium standard. The difference between these counts and the residual counts in the empty vial after transfer of radioactivity to the syringe gives the counts of radioactivity injected.

The specific activity of the urinary metabolite is the counts per μ g of the steroid isolated from urine. The time of collection was 3 days. From these values, the production rates of estradiol in μ g per day is calculated using the above men-

tioned formula for production rate determination.

RESULTS

Separation of Urinary Estrogen Fractions by Column and Paper

Chromatography:

For column chromatography the solvent system used (ethyl acetate: iso-octane) with gradients of increasing polarity was convenient for adequate separation of the different estrogenic fractions. The HBV (volume of the mobile phase retained by the column) was approximately equal to 100 ml. The mobile phase was run at rate 1 HBV/hour and fractions of 0.1 HBV were collected.

The tritium counts of the various fractions collected are shown in the figure 2. The chemical structure of each estrogenic metabolite producing different solubility and absorptions to the column chromatography system influence their rate of appearance through the column. For example, the extra carbon atom in the 2-methoxy estrone molecule is responsible by its early appearance in tubes 6 to 10 (0.6 to 1 HBV); presence of keto group in estrone molecule is responsible for its emergence earlier than estradiol. Peak of estrone was found between tubes 38-50 (3.8 to 4.2 HBV) and estradiol fraction emerged between tubes 45-50 (4.5 to 5.0 HBV); moderate amounts of radioactivity corresponding to epiestriol was found between tubes 58-63 (5.8 to 6.3 HBV) and estriol peak emerged between tubes 100-120. Extensive work on the paper chromatography of estrogens has been carried out in the past and has been reviewed

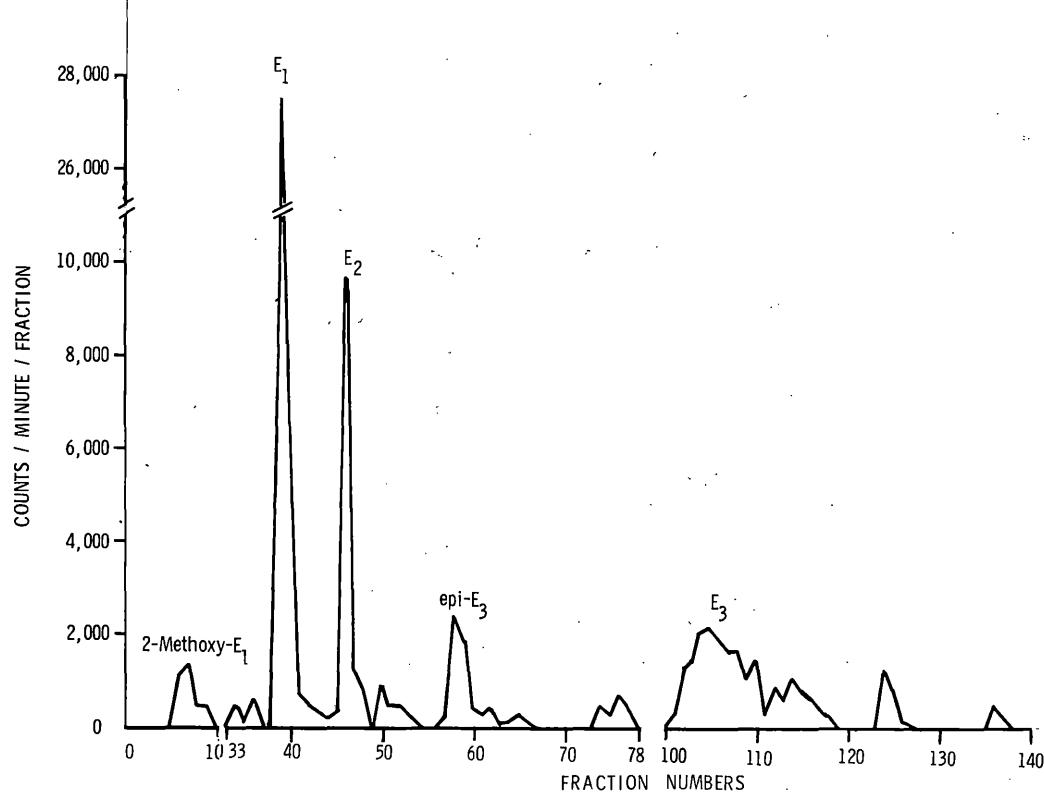


Fig. 2. Results of gradient elution chromatography of a phenolic extract from 4,700 ml. of urine of a patient with Stein-Leventhal syndrome. Estradiol-6,7-³H (3.03 μ c) was administered intravenously and urine was collected for 3 days. Fraction numbers are plotted against their tritium content. The column hold back volume was 100 ml. and fractions of 10 ml. were collected. The following abbreviations have been used: 2-methoxy E₁ for 2-methoxy estrone, E₁ for estrone, E₂ for estradiol, epi E₃ for epiestriol, and E₃ for estriol.

in detail by Oakey (1962). The separation of the different estrogenic fractions by paper chromatography is shown in the figure 3. The estrone, estradiol, epiestradiol, and 2-methoxy estrone zones are adequately separated by this procedure.

Establishment of Radiochemical Purity:

In order to measure the production rate of one hormone by an endocrine gland, using the isotope dilution principle, the determination of the specific activity (counts per $\mu\text{g}.$) of one of its metabolites is indispensable. In the present study, the amount of estrogen produced by the ovary after i.v. administration of 6,7- ^3H estradiol was determined by measurement of urinary specific activity of estradiol and estrone in all of the patients. The cumulative specific activity of urinary estradiol was also determined in two of the patients with Stein-Leventhal syndrome.

For the determination of production rates, it is essential that a correct specific activity be obtained and radiochemical purity established. The establishment of radiochemical purity of urinary estrone and estradiol was studied in the following manner:

Acid hydrolysis was carried out on one of the urine specimens and a phenolic extract was prepared as described previously. The extract was then subjected to paper chromatographic separation and the estrone and estradiol fractions eluted from paper. Aliquots of these fractions were run on a partition column described by Preedy and Aitken (1961) and radioactive as well as fluorimetric

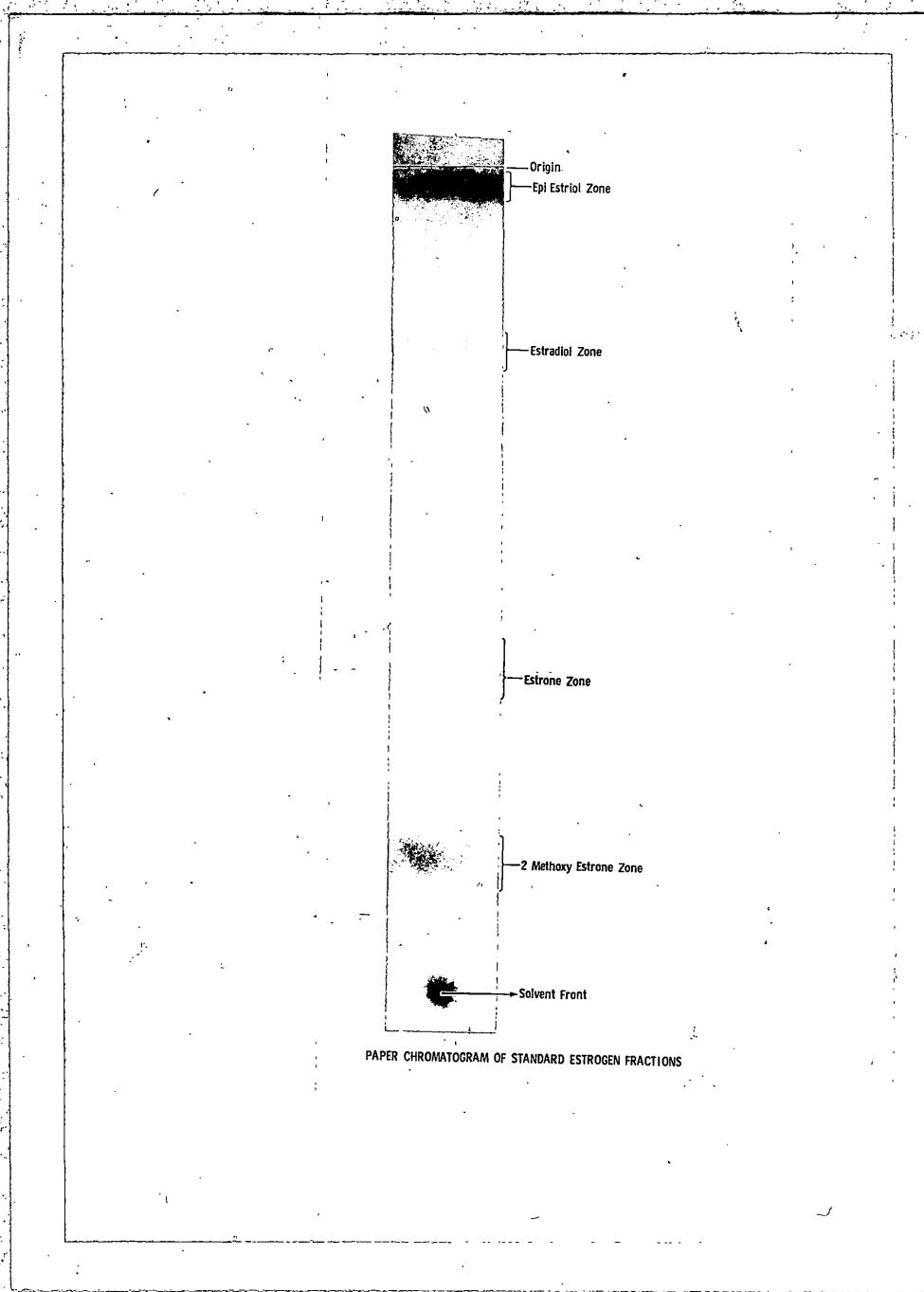


Fig. 3. Separation of estrone, estradiol, epiestradiol and 2-methoxy estrone fractions by paper chromatography in a system of ligroin: benzene: methanol: water (67:33:80:20) for 2½ to 3 hours. The strips were developed with Folin-Cicalteu reagent.

determination done on each tube. The results are shown in the figure 4. By these figures it can be seen the radioactivity and fluorescence from estrone and estradiol appears as symmetrical gaussian elution curves, and there is a good agreement in terms of counts per unit of fluorescence in each tube. It is important to point out that the counts per unit of fluorescence obtained directly on the paper extract agrees very well with those obtained after purification, using the column described by Preedy and Aitken (1961). It is thus demonstrated that after paper chromatography the estrone and estradiol fractions are pure enough to be estimated by fluorimetry of the Ittrich extract of Kober Chromogens, for the determination of specific activities. Furthermore, it has been amply demonstrated by Gurpide et al. (1962a, 1962b, 1963) that after the injection of tritiated estradiol to normally ovulating women, the specific activities found of the estrone, estradiol, and estriol fractions are identical. In the present investigation, the production rate is calculated from two or three metabolites of estradiol and in almost all cases there is a good agreement between the results from urinary estrone and estradiol and this further confirms the validity of the present experiments.

In the present investigation, the production rate of estradiol was determined in five patients with the Stein-Leventhal syndrome, one patient with secondary amenorrhea probably of hypothalamic origin, and a patient with Sheehan's syndrome. In one of the patients with the Stein-Leventhal syndrome (E.G.) the pro-

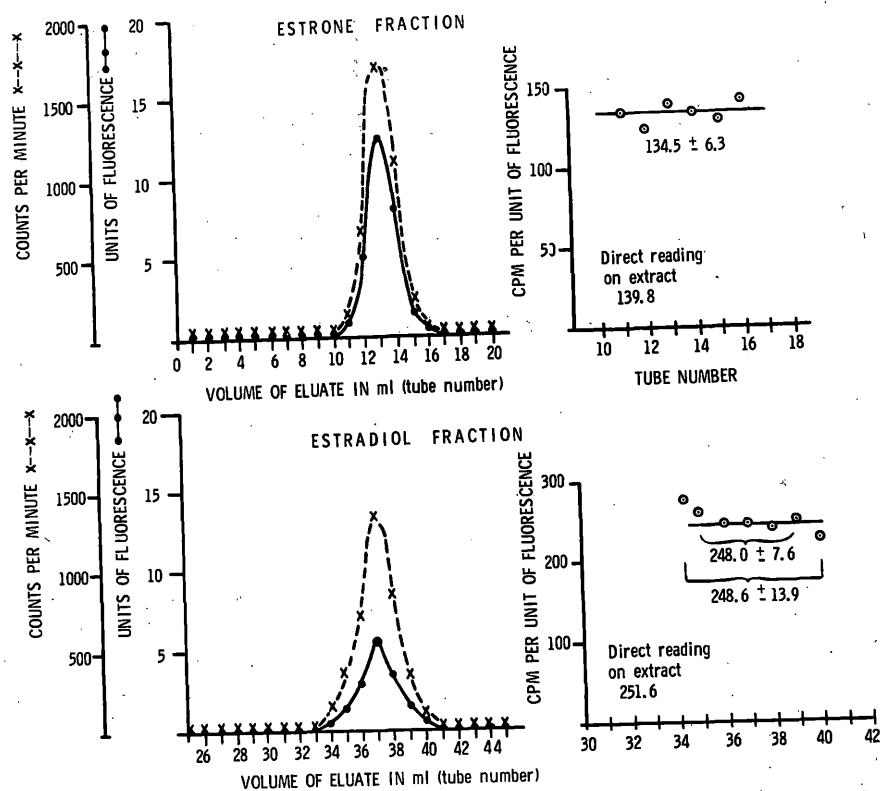


Fig. 4. Aliquots of estrone and estradiol fractions of a urine extract after paper chromatography, were rechromatographed on a celite column. (Freedy and Aitken). The figure shows perfect agreement of symmetrical gaussians elution curves as determined by Iuttich fluorescence and radioactivity in the two fractions. The counts per unit of fluorescence show good agreement from tube to tube and also with direct determinations carried on the paper extract.

duction rate studies were carried out before and after wedge resection of the polycystic ovaries.

The total radioactivity injected in the form of 6,7-³H estradiol in each patient, the fluorescence and radioactivity of estrone, estradiol and estriol isolated from the urine are presented in table I. The specific activity of each fraction (the counts per microgram of the estrogen) was then calculated. The production rate of estradiol was calculated on the basis of the specific activity of estrone and estradiol using the expression:

$$\text{Production Rate} = \frac{\text{Radioactivity injected}}{\text{Cumulative sa of urinary metabolite} \times \text{time}}$$

In two cases the production rate was also calculated by using the specific activity of estriol. The production rates of estradiol thus calculated for the seven patients are shown in table II.

TABLE I

Patient	Diagnosis	Total Radioactivity injected (cpm)	Total radioactivity found in "phenolic extracts" (cpm)			Quantity of compound by Fluorescence (ug)		
			Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol
1-S.C.	S.L.S.	1,027,598	910	490	1,671	.420	.215	.755
2-E.C.	S.L.S.	1,112,645	1,178	485	2,230	.915	.385	1.721
3-J.G.	S.L.S.	1,014,582	3,849	766	-	.875	.139	-
4A-E.G.*	S.L.S.	1,223,872	5,643	4,730	-	.407	.397	-
4B-E.G.**	S.L.S.	1,013,402	7,161	1,518	-	1.100	.232	-
5-V.W.	S.L.S.	1,209,073	5,966	1,713	-	.473	.133	-
6-E.M.	Sheehan's Syndrome	1,023,957	13,791	7,940	-	.225	.046	-
7-M.W.	Hypothalamic Amenorrhea	1,007,293	1,214	1,156	-	.275	.116	-

*PR estimated before wedge resection of polycystic ovaries

**PR estimated five months after wedge resection of polycystic ovaries

Fluorescence and radioactivity of various estrogen fractions isolated from the urine of seven patients after the injection of 6,7-³H estradiol.

TABLE II

Patient	Diagnosis	SA (cpm/ μ g.)			Calculated PR of E_2 (μ g/day) from the SA of		
		Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol
1-S.G.	S.L.S.	2,166.66	2,279.00	2,213.24	158.09	150.29	154.76
2-E.G.	S.L.S.	1,287.43	1,259.74	1,295.75	288.07	294.41	286.23
3-J.C.	S.L.S.	4,399.37	5,510.79	-	76.87	61.36	-
4A-E.G.*	S.L.S.	13,864.00	14,340.00	-	29.42	28.40	-
4B-E.G.**	S.L.S.	6,510.00	6,544.60	-	51.89	51.00	-
5-V.W.	S.L.S.	12,613.00	12,381.00	-	31.90	31.28	-
6-E.M.	Sheehan's Syndrome	61,296.00	172,620.00	-	5.56	1.97	-
7-M.W.	Hypothalamic Amenorrhea	4,414.54	9,971.12	-	76.05	33.67	-

*PR estimated before wedge resection of polycystic ovaries

**PR estimated five months after wedge resection of polycystic ovaries

Cumulative specific activities of urinary E_1 , E_2 , and E_3 and calculated production rates of E_2

DISCUSSION

The production rate of estradiol has been determined in five patients with the Stein-Leventhal syndrome, one patient with secondary amenorrhea probably of hypothalamic origin and in a patient with Sheehan's syndrome. In all cases the production rate has been calculated from the specific activities of estrone and estradiol and with the exception of one case, (case 7) which is under further study, there was a good agreement between the results obtained. In two cases, the production rate was also calculated from the specific activities of the estriol fraction and once again good agreement was obtained with the results from estrone and estradiol estimations.

The production rates of estradiol varied from 29.4 to 288 µg per 24 hours in five patients with the Stein-Leventhal syndrome as calculated from the specific activity of urinary estrone. Similar calculations from urinary estradiol gave values varying from 26.4 to 294.4 µg per 24 hours (Table II). Gurpide et al. (1962a) reported the secretion rate of estradiol to be 200 and 500 µg at the time of ovulation in two normally menstruating females. In a subsequent study they reported secretion rates of 50 to 250 µg in various phases of the menstrual cycle (Gurpide et al. 1963). The results in the present investigation indicate that patients with the Stein-Leventhal syndrome may secrete moderately low to elevated levels of estrogens. In

one of the patients (E.G.) the production rate was measured before and after wedge resection of the polycystic ovary and the values were almost twice those found before wedge resection. This shows that a change was produced by wedge resection. There seems to be some measure of disagreement on the production rate of estradiol in a patient with secondary amenorrhea of hypothalamic origin as calculated from urinary estrone and estradiol. This is being further investigated. It is also of interest to note that, as expected, a patient with Sheehan's syndrome has almost negligible production rate of estradiol. Although such a phenomenon has been suggested by clinical manifestations, this is the first actual demonstration of the very low estrogen production rate.

Mahesh and Greenblatt (1964) have demonstrated the presence of two types of enzymatic abnormalities in polycystic ovaries. They are a) failure of conversion of Δ^4 -androstenedione to estrogens and b) failure of the ovarian 3β -O_H-dehydrogenase. Both of these enzyme defects would appear to interfere with estrogen synthesis (figure 5). However, the patients with Stein-Leventhal syndrome consistently show a) moderate to good estrogen secretion as judged by breast development, vaginal smears and endometrial biopsies; b) normal urinary estrogen levels; c) the level of estrogens in the ovarian venous blood of polycystic ovaries has been found to be slightly lower or comparable to normal ovaries under control conditions. The presence of estrogens in the patients with the Stein-Leventhal

MAJOR PATHWAYS OF BIOSYNTHESIS OF STEROIDS IN THE OVARY

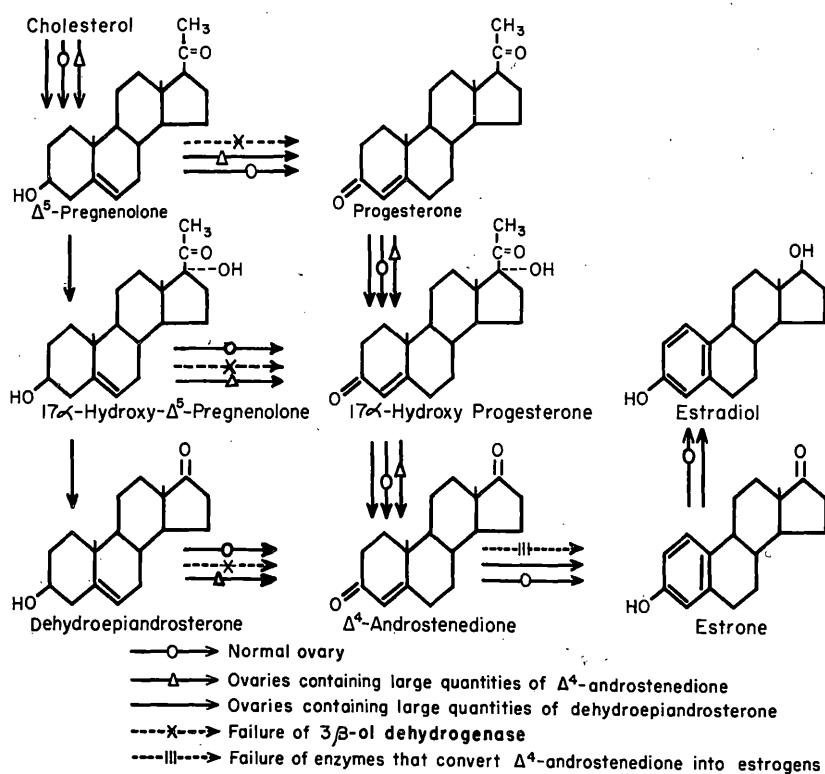


Fig. 5. Major pathways of biosynthesis of steroids in the normal ovary and enzymatic abnormalities present in the Stein-Leventhal Syndrome.

syndrome, however, does not rule out the presence of enzymatic abnormalities. The manifestations of enzymatic abnormalities is readily shown by the very low estrogen to androgen ratio in ovarian venous blood from polycystic ovaries as compared to that obtained from normal ovaries (Mahesh & Greenblatt, 1964).

In the present investigation the isotope dilution principle for the measurement of production rate of estrogens by the ovary has been shown to be of value in the evaluation of estrogen secretion of a patient. There has been a considerable amount of controversy about the ability of the polycystic ovary to produce estrogens. Axelrod & Goldzieher (1962) have reported on the basis of in vitro studies, that there is a complete absence of synthesis of estrogens by the polycystic ovary. Short & London (1961) and Short (1962) were also unable to isolate estrogens from follicular fluid from polycystic ovaries. The results obtained in this study show that patients with the Stein-Leventhal syndrome can secrete moderately low to elevated levels of estrogens and thereby confirm the findings of Mahesh & Greenblatt (1964) that polycystic ovaries can secrete physiological levels of estrogens along with excessive amounts of androgens.

SUMMARY

Estradiol 6,7-³H (3.03 μ c) was injected i.v. to five patients with Stein-Leventhal syndrome, one patient with secondary amenorrhea of hypothalamic origin, and one patient with Sheehan's syndrome. Twenty-four hours urine collection was carried out for three days following the injection of labeled estradiol. The urine was hydrolyzed using hot acid hydrolysis and extracted. Preliminary purification of the phenolic fractions was done by using partition column chromatography. The crude estrogen fractions were further purified by paper chromatography to constant specific activity. Fluorometry on Ittrich extract of Kober chromogens was then used for the determination of the amount of estrogenic fraction excreted in the urine. The estradiol production rate of five patients with Stein-Leventhal syndrome varied from 28.4 μ g to 288 μ g/24 hours. These results support earlier observations of estrogen levels in urine and estrogen content of ovarian venous blood indicating that polycystic ovaries can secrete significant amounts of estradiol.

The clinical findings such as involution of mammary glands and genital organs seen in patients with Sheehan's syndrome is suggestive of the lack of estrogens secretion by the ovaries. This was confirmed by estradiol production rate studies in one such patient.

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