Conjugated and Free Estriol in *Corpus Luteum* of Human Pregnancy*

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Free estriol as well as the sulfate and glucosiduronate were found in the *corpora lutea* of pregnant women in the first trimester. The amounts found were 5, 5 and 6 μg/g, respectively from tissue incubated in homologous plasma. The data strongly suggest that estriol is formed in large quantities by luteal tissue of pregnant women.

Studies of estrogens in the human ovary have been mainly concerned with biosynthetic pathways in this organ (Dorfman, 1963; Huang and Pearlman, 1963). It appeared important to clarify the composition of the major products before examining the metabolic pathways in their formation. As a result, our group has been engaged in isolation and identification of estrogens from human ovaries. We have postulated that conjugates may be the major estrogen forms of the ovary. Enzymatic hydrolysis with β-glucuronidase and Mylase-P resulted in complete cleavage of conjugates in blood, and the liberated free estrogen could then be detected in small quantities by gas chromatography (Touchstone *et al.*, 1963).

This report describes the use of this method and results of isolation and quantitation of estriol conjugates and free estriol from *corpora lutea* of human pregnancy.

Experimental Procedure

Tissues from the following patients were analyzed: two women in the first trimester of pregnancy, two normal menstruating women, two patients with Stein-Leventhal syndrome and polycystic ovaries, and one girl with an ovarian tumor (dysgerminoma).

Ovaries were processed as soon as possible after surgical removal. *Corpora leutea* when present were cut out from the rest of the ovary, and preparation and analysis of the dissected tissue portions were conducted separately. The tissues were sliced into sections not exceeding 0.5 × 0.3 cm in size and were placed in Erlenmeyer flasks containing the patient's own plasma. Streptomycin and penicillin were added as preservative. The plasma-tissue ratio never exceeded 1.0 g/5 ml of plasma.

Incubation.—This was started within 2.5 hours after excision of the organ. All flasks with incubates were placed in an Eberbach water-bath shaker and incubated for 24 hours under 95% O_2–5% CO_2 at an agitation rate of 60 complete oscillations per minute.

This procedure was modified from that used in this laboratory for adrenal tissue (Cooper *et al.*, 1958; Varon *et al.*, 1963). The advantage of this method has been the increase by 20-fold in quantity of steroid isolated when compared with simple tissue extraction, with little or no artifact formation (Cooper *et al.*, 1958). Evaluation of results of this procedure with ovary is in progress; no artifacts have been noted.

Extraction of Free Estriol.—This procedure is similar to that described elsewhere for blood (Touchstone *et al.*, 1963). Tissue and plasma were homogenized in a Virtis apparatus. Proteins were precipitated with four volumes of acetone. After centrifugation and washing of the precipitate with acetone, the acetone was removed *in vacuo* in a water bath. Residual water was then extracted with ethyl ether and the aqueous phase remaining after this extraction was subjected to enzymatic hydrolysis. Ether was evaporated and fat was removed from the residue by a 1:1 *n*-heptane–70% methanol partition. After evaporation of the methanol the remaining water was extracted with ether, followed by phenol separation with 1 N sodium hydroxide. Following neutralization of the alkali with HCl, the phenolic fraction was extracted with ether which was washed with bicarbonate and water and evaporated. Separation of an estriol fraction from this extract was then achieved by column chromatography on alumina, as described previously (Touchstone *et al.*, 1963).

Enzymatic Hydrolysis.—The sequential hydrolytic procedure is given in detail elsewhere (Touchstone *et al.*, 1963). Water from which free estriol had been removed by ether extraction was first incubated with a phenol sulfatase, Mylase-P (Nutritional Biochemicals Corp.), for 24 hours at 50° with maleate buffer at *p*H 6.0. After extraction with ether, the *p*H of the residual water was readjusted to 6.8 with sodium hydroxide and the solution was then incubated for 24 hours at 37° with β-glucuronidase (Sigma). After extraction with ether, phenols were separated in the manner described for free estriol.

Justification of features in the β-glucuronidase and Mylase P hydrolytic procedures is discussed in detail in the previous paper (Touchstone *et al.*, 1963).

Chromatography.—Estriol fractions were separated on alumina micro columns with a modification of the technique of Eberlein *et al.* (1958) and Touchstone *et al.* (1963). Recovery from the column averaged 95%.

Gas chromatography was performed on a Glowall Corporation "Chromolab" apparatus Model A110. The conditions were as follows: glass coil column 6 ft × 4 mm; 5% QF-1 on Gas Chrom Z (100–140 mesh); argon pressure, 23 psi; temperatures: column 230°, detector 250°, injection port 260°; chart speed 20 in./hour. About 10% or less of the estriol fractions separated by column chromatography and dissolved in acetone was injected for analysis. Sensitivity for estriol with this technique was 0.2 μg. Gas chromatography served as a probe to set the direction for further confirmatory analyses (unpublished experiments).

Estriol fractions from the alumina column were subjected to paper chromatography on Whatman No. 1 paper strips using the isopropyl ether–ethylene glycol system with development for 36 hours (Touchstone *et al.*, 1962). Zones were located by treatment with the ferric chloride–potassium ferricyanide reagent.

Colorimetry.—The Bachman reagent (Bachman, 1939) phosphoric acid containing 2% sodium *p*-phenol

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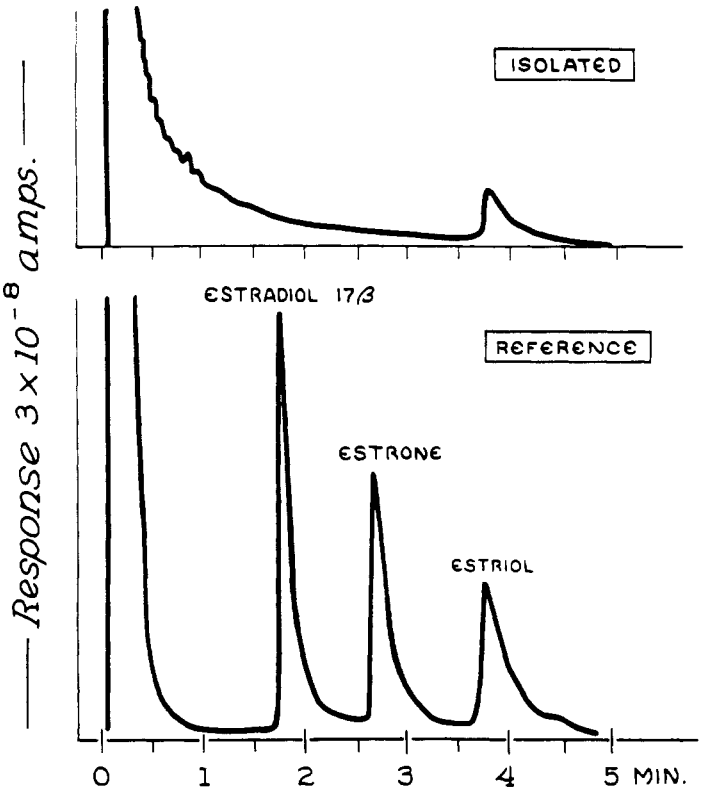

FIG. 1.—Gas chromatography of estriol isolated from human *corpora lutea* incubates. Conditions are given in the text. The amounts of estrone, estradiol 17-β, and estriol were 1, 1, and 2 μg, respectively, in the reference chromatogram.

sulfonate was used for colorimetry with fractions from the column (Touchstone *et al.*, 1963). The formation of a violet-pink color with an absorption maximum at 545 mμ and extinction coefficient ($\Sigma^{1\%}_{1mm}$) of about 600 at 545 mμ is specific for estriol and 16-epiestriol. The latter steroid was largely removed under the conditions used for development of the alumina column and paper chromatography. In the present work no correction for background has been necessary.

Infrared Absorption.—Infrared absorption spectra (Fig. 2) were obtained with a Perkin-Elmer Model 421 spectrometer from the isolated free steroid incorporated in a 1.5-mm KBr pellet. The estriol zone from the paper chromatograms was further purified by alumina-column chromatography prior to incorporation into the KBr.

RESULTS

Free estriol, estriol sulfate, and estriol glucosiduronate after enzymatic hydrolysis were indicated by gas chromatography of extracts of *corpora lutea* of pregnancy after the three sequential steps (Fig. 1). The identity of this steroid was confirmed by paper chromatography and colorimetry with the Bachman reagent, in addition to the fact that the steroid appeared in the estriol fraction after alumina-column chromatography. On gas chromatography the isolated estriol showed a retention time the same as that of the reference (Fig. 1). The infrared spectra of the isolated steroid corresponded to that of reference estriol (Fig. 2).

In Table I the concentrations of free estriol, estriol sulfate, and glucosiduronate are listed. The relative proportion of these materials was about 1:1:1 in the *corpus luteum* of pregnancy. It should be noted that in the remaining (23.5 g) ovarian tissue of the pregnant women, in *corpus luteum* of menstruation, and in all other ovarian tissues, no estriol was detected at a level of sensitivity of 0.2 μg/g (Table I).

DISCUSSION

These results indicate that estriol sulfate, estriol glucosiduronate, and free estriol are relatively abundant in *corpus luteum* of human pregnancy. The fact that these estrogens could not be detected in other portions of ovarian tissue of pregnancy, in *corpora lutea* of menstruation, or in other human ovaries, strongly suggests that luteal tissue of pregnancy may produce estriol. Since about 70% of the estriol was present as a sulfate or glucosiduronate these experiments tend to confirm our postulate that conjugates may be the major estrogen products of the ovary. Because of insufficient material it was not possible to investigate the possible presence of a sulfoglucosiduronate diconjugate which was indicated as a major estriol component in plasma of pregnant women (Touchstone *et al.*, 1963).

The total amount of estriol in the plasma of pregnant women in the last trimester averages 10 μg/100 ml (Touchstone *et al.*, 1963). Since this is approximately 0.5 μg/5 ml plasma and considering that earlier in pregnancy estrogen levels are lower, it appears that the estriol found came from the metabolic processes in the *corpus luteum*. The nature of the precursors and whether they were present in the plasma or in the tissue is a matter of conjecture. Since the ovarian tissues without *corpus luteum* showed no estriol whether incubated or not it appears that the *corpus luteum* is a primary source of the estriol isolated in this study.

Formation of estrogens by human *corpus luteum* of menstruation *in vitro* has been reported recently by Huang and Pearlman (1963) who noted the production of radioactive estrone and 17-β-estradiol after tissue incubation with Δ^4-androstene-3,17-dione-7-H_3. Biological data presented by Greep *et al.*, (1942) has suggested that *corpus luteum* may form estrogens. The isolation of estriol from *corpus luteum* of human preg-

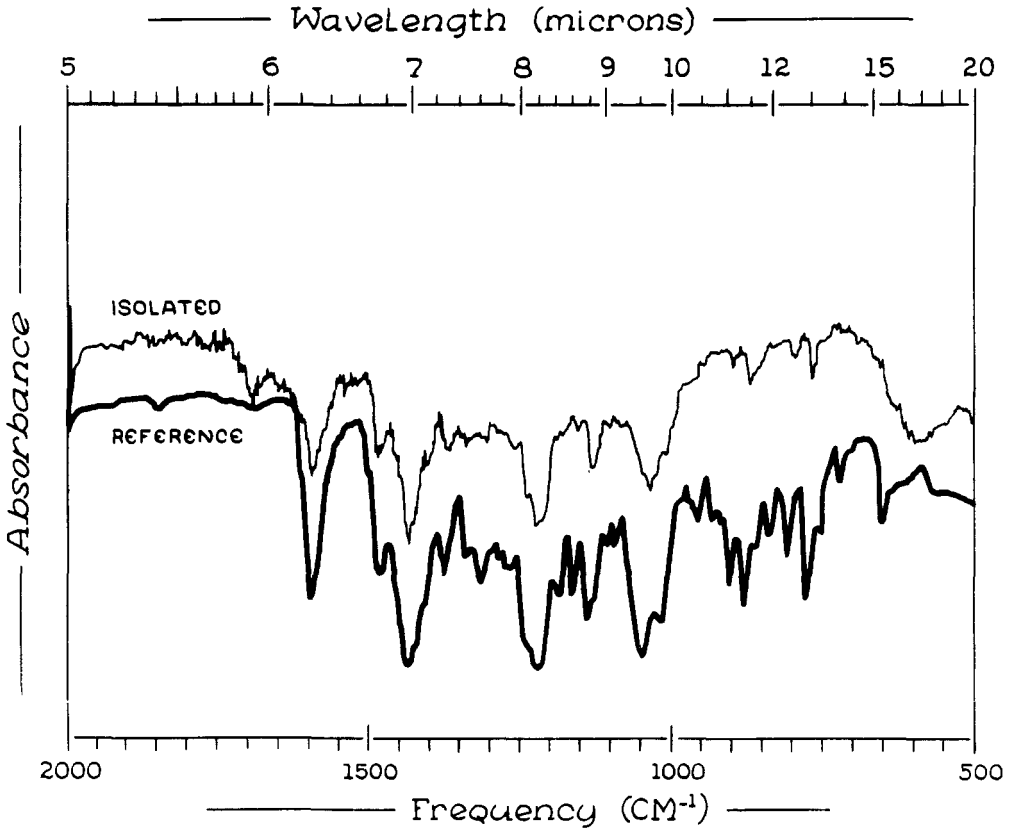

FIG. 2.—Infrared spectra of isolated and reference estriol in 1.5-mm KBr pellets.

nancy provides additional data supporting this suggestion.

Most of the marked elevation of estrogens in blood and urine during pregnancy has been attributed to production of these hormones by the placenta. Diczfalusy (1960) has summarized the strong evidence in favor of production of these steroids by the placenta. Recently Frandsen and Stakemann (1963) have suggested that the fetal adrenal must first produce steroid precursors which are then metabolized into estrogens by the placenta. The results of the present study indicate that production of estriol by the *corpus luteum* of pregnancy may account for at least a part of the increased estrogens found in pregnant women.

These experiments emphasize the value of gas chromatography in determinations of this type. Since estrogens are present in ovaries in low concentrations, most workers have been forced to use radioactive techniques, and isolation and identification of estrogens in tissue has been difficult. With analysis by gas chromatography as described in this study, 0.5 μg of estriol may be detected specifically and, at present, 0.1 μg may be detected. For other estrogens the sensitivity is even greater. As a result, gas chromatography analysis may serve as an initial probe to determine what is present in a small portion of material.

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TABLE I

ESTRIOL AND CONJUGATES OF OVARIAN INCUBATES

Condition and Tissue	No of Patients	Wt. of Tissue	Estriol (μg/g)		
			Free	Sulfate	Glucosiduronate
Pregnant					
(A) *Corpus luteum*	2	7.0	5[a]	5	6
(B) No *corpus luteum*	2	23.5	0[b]	0	0
Menstruating "normal"					
(A) *Corpus luteum*	1	2.0	0	0	0
	1	0.5	0	0	0
(B) No *corpus luteum*	1	8.0	0	0	0
		8.0	0	0	0
		3.5	0	0	0
	1	7.0	0	0	0
Stein-Leventhal	1	7.0	0	0	0
	1	15.0	0	0	0
Tumor (dysgerminoma)	1	34.0	0	0	0
		63.0[d]	0	0	0

[a] Average of two experiments. [b] Detectable at sensitivity 0.5 μg. [c] Different tissue portions from same patient. [d] Not incubated tumor tissue.

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The Conversion of Cholesterol and 20α-Hydroxycholesterol to Steroids by Acetone Powder of Particles from Bovine *Corpus Luteum**

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Acetone-dried powder of "mitochondria" from bovine *corpus luteum* was shown to convert cholesterol and 20α-hydroxycholesterol to pregnenolone and progesterone. Reduced TPN was necessary for this conversion. Interstitial-cell-stimulating hormone *in vitro* was without demonstrable effect on the reaction. Percentage conversion of 20α-hydroxycholesterol to pregnenolone was considerably greater than that of cholesterol to pregnenolone. Evidence is also presented that 20α-hydroxycholesterol is an intermediate in the conversion of cholesterol to pregnenolone by acetone powder of *corpus luteum*.

Present evidence indicates that cholesterol is converted to steroids in the adrenal cortex by way of 20α-hydroxycholesterol which in turn undergoes side-chain cleavage to yield Δ^5-pregnenolone. Halkerston and co-workers (1961) have demonstrated the conversion of cholesterol-4-C^{14} to labeled products which behaved like pregnenolone-C^{14} and progesterone-C^{14} by means of acetone-dried powder of "mitochondria"[1] from bovine adrenal cortex. The conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} by acetone-dried powder of mitochondria from bovine adrenal has also been reported by Constantopoulos and Tchen (1961) and the conversion of 20α-hydroxycholesterol to pregnenolone has been demonstrated by Chanduri *et al.* (1962). These studies also demonstrated that side-chain cleavage of cholesterol requires reduced TPN[2] when intact mitochondria were used, although Constantopoulos and Tchen (1961) reported that soluble preparations derived from mitochondria could use either reduced DPN or reduced TPN. In homogenate of bovine *corpus luteum*, Tamaoki and Pincus (1961) reported that cholesterol is converted to progesterone and that 20α-hydroxycholesterol undergoes side-chain cleavage to yield isocaproic acid. ICSH was without effect on the side-chain cleavage of either cholesterol or 20α-hydroxycholesterol.

It has been shown that ICSH increases the production of progesterone by slices of bovine *corpus luteum in vitro* (Mason *et al.*, 1962). Earlier studies by Stone and Hechter (1954) suggested that ACTH stimulates steroid biosynthesis in the adrenal by promoting the conversion of cholesterol to pregnenolone. Moreover, ICSH causes a fall in the ascorbic acid content of *corpus luteum* in a manner analogous to the well-known adrenal ascorbic acid depletion produced by ACTH (Parlow, 1961). These observations suggest the possibility that the two trophic hormones may stimulate steroid biosynthesis in their respective target organs by means of a mechanism common to both. It was therefore considered important to demonstrate that "mitochondria" from *corpus luteum* convert cholesterol to pregnenolone and that 20α-hydroxycholesterol is an intermediate in this reaction, before detailed studies of the mechanism of action of ICSH upon luteal tissue were undertaken.

The present experiments were designed to demonstrate the capacity of acetone-dried powder of "mitochondria" from bovine *corpus luteum* to convert cholesterol and 20α-hydroxycholesterol to steroids and to characterize the products of this reaction.

Experimental Procedure

Preparation of Tissue.—Bovine ovaries were obtained fresh from a slaughterhouse. *Corpora lutea* were shelled out, stripped of fibrous tissue, and weighed. The tissue was finely chopped and homogenized in two volumes of sucrose (0.44 M) in an all-glass Potter-

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[1] The term "mitochondria" was used by Halkerston *et al.* (1961) to describe the pellet prepared in their experiments. However, these workers did not offer evidence for the exact nature of this pellet. The present authors use the term "mitochondria" in this paper to describe a pellet prepared from *corpus luteum* according to the instructions of Halkerston *et al.* (1961).

[2] The following abbreviations are used: DPN, diphosphopyridine nucleotide; ICSH, interstitial-cell-stimulating hormone; TPN, triphosphopyridine nucleotide; ACTH, adrenocorticotropic hormone; PPO, 2,5-diphenyloxazole; POPOP, 1,4-2-(5-phenyloxazolyl)-benzene.