The *in Vivo* Metabolism of 16α-Hydroxydehydroisoandrosterone in Man*

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ABSTRACT: Labeled 16α-hydroxydehydroisoandrosterone was prepared by the microbiological hydroxylation of [7-^{3}H]dehydroisoandrosterone. The labeled steroid was administered intravenously to a normal male who also ingested 600 mg of nonlabeled steroid and to a woman in the third trimester of pregnancy. From the "sulfate" fraction of the male urine the following metabolites were isolated and identified: 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3β,16α,17β-triol, androst-5-ene-3α,16α,17β-triol, and androstanetriol. From the "glucosiduronate" fraction the following metabolites were isolated and identified: 16α-hydroxyandrostenedione, 16α-hydroxydehydroisoandrosterone, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3α,16α,-17β-triol, and 16α-acetoxy-3α-hydroxyandrost-5-en-17-one. Three of these metabolites, namely, 16α-hydroxydehydroisoandrosterone, androst-5-ene-3β,16α,17β-triol, and androstanetriol have been previously isolated from human urine. With the exception of androstanetriol, androst-5-ene-3α,16α,17β-triol, and 16α-acetoxy-3α-hydroxyandrost-5-en-17-one, all of the above steroids were isolated and identified as metabolites of [7-^{3}H]16α-hydroxydehydroisoandrosterone in late pregnancy urine. Labeled estriol was also isolated from both the "sulfate" and "glucosiduronate" fractions of the pregnancy urine and it had a low specific activity which was of the same order of magnitude as 16α-hydroxydehydroisoandrosterone and androst-5-ene-3β,16α,17β-triol isolated from the "sulfate" fraction. The specific activities of these metabolites were much lower than those of the other metabolites isolated and indicated that a portion of the injected steroid had entered the feto–placental unit where it was diluted prior to being metabolized and excreted as urinary products.

The most abundant single urinary metabolite of the injected 16α-hydroxydehydroisoandrosterone was 3α,-16α-dihydroxyandrost-5-en-17-one. This was added proof that the presence of a 16α-hydroxy group on the steroid nucleus partially inhibits the Δ^4-isomerase enzyme.

The formation of estriol from 16α-hydroxylated C_{19} precursors by the human placenta *in vitro* was demonstrated by Ryan (1959). Subsequently it was shown that umbilical cord blood contains large amounts of 16α-hydroxydehydroisoandrosterone (Magendantz and Ryan, 1964; Colas *et al.*, 1964; Easterling *et al.*, 1966). Thus it seems likely that in man estriol is formed by the placental aromatization of 16α-hydroxylated precursors synthesized in the fetus. The fetal liver seems to be the site of 16α-hydroxylation of C_{19} β,γ-unsaturated steroids. Slaunwhite *et al.* (1965) reported the *in vitro* formation of 16α-hydroxydehydroisoandrosterone[1] from dehydroisoandrosterone by human fetal liver, while Reynolds (1966) showed that the liver of the newborn can form 16α-hydroxypregnenolone from pregnenolone, and Heinrichs *et al.* (1966) demonstrated that liver microsomes from the newborn anencephalic infant are capable of 16α-hydroxylating dehydroisoandrosterone. When [4-^{14}C]pregnenolone was injected into the umbilical vein at the time of therapeutic abortion, at 13 weeks of gestation, labeled 16α-hydroxypregnenolone, was isolated only from the fetal liver (M. Iwamiya, F. J. Tweedie, and S. Solomon, in preparation). In an analogous study in which labeled dehydro-

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[1] The following trivial names and abbreviations are used: dehydroisoandrosterone, 3β-hydroxyandrost-5-en-17-one; pregnenolone, 3β-hydroxypregn-5-en-20-one; pregnanediol, 5β-pregnane-3α,20α-diol; androstanetriol, 5α-androstane-3α,16α-17β-triol; etiocholanetriol, 5β-androstane-3α,16α,17β-triol; 16α-hydroxydehydroisoandrosterone, 3β,16α-dihydroxyandrost-5-en-17-one; 16β-hydroxydehydroisoandrosterone, 3β,16β-dihydroxyandrost-5-en-17-one; 16α-hydroxyandrostenedione, 16α-hydroxyandrost-4-ene-3,17-dione; 16α-hydroxyandrosterone, 3α,16α-dihydroxy-5α-androstan-17-one; 16α-hydroxyetiocholanolone, 3α,-16α-dihydroxy-5β-androstan-17-one; 16α-hydroxypregnenolone, 3β,16α-dihydroxypregn-5-en-20-one; 16α-hydroxyprogesterone, 16α-hydroxypregn-4-ene-3,20-dione; 11-dehydrocorticosterone, 21-hydroxypregn-4-ene-3,11,20-trione; dehydroisoandrosterone sulfate, 17-oxo-androst-5-ene-3β-yl-sulfate; estriol, estra-1,3,5-(10)-triene-3,16α,17β-triol; DDQ, dichlorodicyanobenzoquinone.

isoandrosterone sulfate and dehydroisoandrosterone were perfused in the previable human fetus, androst-5-ene-3β,16α,17β-triol was isolated from the aqueous extract of the liver (Bolté *et al.*, 1966).

The presence of 16α-hydroxylated C_{19} steroids in human urine has been demonstrated by a number of investigators (Hirschmann, 1943; Marrian and Butler, 1944; Lieberman *et al.*, 1953; Fotherby *et al.*, 1957; Fotherby, 1958; Okada *et al.*, 1959; Bongiovanni, 1962, 1966; Reynolds, 1963). The investigations reported in this paper describe the urinary metabolites of labeled 16α-hydroxydehydroisoandrosterone in a normal male fed large amounts of carrier steroid, and in a pregnant female given the labeled steroid.

Materials and Methods

Only those procedures which have not been described previously (Ruse and Solomon, 1966a) will be given in this paper.

Counting. Samples insoluble in toluene were first dissolved in 2 ml of methanol before the addition of 10 ml of phosphor solution. Two models of the Packard Tri-Carb liquid scintillation spectrometer were used in these studies. Efficiency of counting in the Model 3002 and Model 4322 was approximately 40% for ^3H and 91% for ^{14}C but when methanol was present the efficiencies were about 20% for ^3H and 85% for ^{14}C. At double-labeled settings the efficiencies were approximately 31% for ^3H and 63% for ^{14}C. The counts of quenched samples were corrected by the use of an internal standard. ^3H- and ^{14}C-labeled *n*-hexadecane (Amersham) were used to standardize the liquid scintillation spectrometers.

Chromatography. Solvent systems used for chromatography are shown in Table I. Metabolites eluted from paper chromatograms were percolated through small silica gel columns for further purification. The material was applied to the column in methylene chloride and was eluted with increasing concentrations of ethanol in methylene chloride. In some instances small alumina columns were employed for the same purpose but here the material was applied in mixtures of Skellysolve B and benzene and eluted with an increasing concentration of benzene in Skellysolve B. Whatman AC 81 acetylated papers were used in reversed-phase chromatographic systems.

Standardization of [1-^{14}C]Acetic Anhydride. Two solutions of [1-^{14}C]acetic anhydride, 10% (v/v) in benzene, were standardized by the acetylation of 16α-hydroxydehydroisoandrosterone and crystallization of the diacetate to constant specific activity. Solutions 1 and 2 had specific activities of 1.1×10^5 and 1.1×10^4 dpm/mg of 3β,16α-diacetoxyandrost-5-en-17-one, respectively.

Formation of Derivatives. A derivative of 16α-acetoxyandrostenedione was prepared by reduction of the steroid with sodium borohydride and subsequent oxidation of the allylic alcohol at C-3 with DDQ as described by Ruse and Solomon (1966b). The infrared spectrum (CS_2) of the crystalline product obtained indicated the retention of the acetate and the presence of a Δ^4-3-ketone, the presence of a hydroxyl group, and the loss of the 17-ketone. The product had a melting point of 175–177° and was ultraviolet positive. *Anal.* Calcd for $C_{21}H_{30}O_4$: C, 72.8; H, 8.73. Found: C, 72.7; H, 8.23. We were not able to compare the derivative to an authenticated standard but the structure 16α-acetoxy-17β-hydroxyandrost-4-en-3-one (16α-acetoxytestosterone) may be assigned with some confidence.

TABLE I: Solvent Systems Used in Chromatography.

System	Solvents
A	Ligroin B–methanol–water (100:90:10)
B	*n*-Hexane–ethyl acetate–methanol–water (50:50:65:35)
C	Benzene–cyclohexane–methanol–water (1:2:3:3)
D	2,2,4-Trimethylpentane–toluene–methanol–water (25:25:35:15)
E	Toluene–ethyl acetate–methanol–water (95:5:65:35)
F	2,2,4-Trimethylpentane–*t*-butyl alcohol–methanol–water (500:200:75:225)
G	2,2,4-Trimethylpentane–ethyl acetate–methanol–water (2:1:1:1)
H	Benzene–ethyl acetate–cyclohexane–methanol–water (8:1:1:8:2)
J	Benzene–cyclohexane–propylene glycol
K	Methylene chloride–ethylene glycol
L	Toluene–propylene glycol
M	Cyclohexane–methanol–water (100:88:12)
N	2,2,4-Trimethylpentane–methanol–water (100:90:10)
O	Benzene–methanol (75:25)
P	Ethyl acetate–*n*-hexane (2:1)

Hydrolysis of Urinary Conjugates. Steroid conjugates were hydrolyzed first by a modification of the solvolytic procedure of Jacobsohn and Lieberman (1962) and then with β-glucuronidase (Baylove Chemicals, Musselburgh, Scotland), 600 Fishman units/ml at 37° for 5 days. Solvolysis of steroid conjugates was performed at 37° for 16 hr. Purified tetrahydrofuran was used to extract the conjugates from urine to which was added 20% (w/v) of NaCl. To the solution of the conjugates in tetrahydrofuran was then added perchloric acid (0.09 ml of 70% perchloric acid/100 ml of solution). After cleavage of the conjugates the unconjugated steroids were extracted with ethyl acetate rather than with benzene. The "sulfates" and "glucosiduronates" thus obtained were processed separately.

Preparation of [7-^3H]16α-Hydroxydehydroisoandrosterone. Labeled 16α-hydroxydehydroisoandrosterone was prepared by the incubation of 1 mc of [7-^3H]-dehydroisoandrosterone (New England Nuclear Corp., Boston, Mass.) and 6.76 mg of carrier dehydroisoan-

drosterone with a strain of *Streptomyces roseochromogenes* (ATCC 3347) supplied by Dr. C. Vezina, Ayerst Laboratories, Montreal. The procedure used for the incubation was the same as the one previously described, for the synthesis of [7-^{3}H]16α-hydroxyprogesterone (Ruse and Solomon, 1966a). After chromatography of the final extract by thin layer chromatography (tlc) in system P, and on paper in system D, the residue was mixed with 8.5 mg of carrier 16α-hydroxydehydroisoandrosterone and crystallized from acetone–ether. The crystals and mother liquor had specific activities of 5.0×10^7 and 4.8×10^7 dpm/mg, respectively. These crystals were used in the *in vivo* studies to be reported below. An aliquot of the crystalline product containing 3.1×10^5 dpm was mixed with 30.3 mg of carrier steroid and the mixture was crystallized as the diol and as the diacetate as shown in Table II.

TABLE II: Proof of Radiochemical Purity of [7-^{3}H]16α-Hydroxydehydroisoandrosterone.

	Specific Activity of Crystals (dpm/mg)	
Crystzn	16α-Hydroxydehydroisoandrosterone	3β,16α-Diacetoxyandrost-5-en-17-one
1	9,870	8,120
2	10,110	8,140
Calculated[a]	10,260	

[a] An aliquot containing 3.1×10^5 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone was mixed with 30.3 mg of carrier. The diacetate was prepared from the second crystals and mother liquor of the diol.

The specific activities of the mother liquors were 11,030 and 10,540 dpm/mg for the diol and 8100 and 8160 dpm/mg for the diacetate.

Identity and Radiochemical Purity of Isolated Metabolites (isotope ratio procedure, irp). In the second study to be reported, minute quantities of some of the metabolites were isolated. These metabolites were acetylated with [^{14}C]acetic anhydride and the acetates were chromatographed on paper or on columns. When the peak of radioactivity was located in the column eluates or on paper, aliquots were taken from the middle of the peak and from both the proximal and distal ends. The ^{3}H:^{14}C ratio was determined for the three aliquots and the remaining material was rechromatographed in additional systems until two successive chromatographic procedures gave the same ^{3}H:^{14}C ratio. When the ^{3}H:^{14}C ratio was constant, radiochemical purity of the metabolite was achieved. To this radiochemically pure product was then added a known amount of ^{3}H- or ^{3}H- and ^{14}C-labeled acetate, prepared from the purified metabolites previously isolated in the first study, and the mixture was rechromatographed. When the final ^{3}H:^{14}C ratio was constant and corresponded to the predicted ratio when the labeled acetate was added, then the identity of the metabolite was established. The specific activity of each metabolite thus identified was determined from the ^{3}H:^{14}C ratio obtained after acetylation but prior to the addition of authentic labeled acetate which was previously shown by chromatography to be radiochemically pure. This technique was developed because of the unavailability of large amounts of carrier for reverse isotope dilution analyses and will be referred to as the irp.

Radiochemical purity of a metabolite was established when the specific activities in the crystals and mother liquors agreed within 10% in the unaltered steroid and in a derivative. When the irp was employed the final and penultimate ^{3}H:^{14}C ratios were in close agreement and the specific activities calculated from these were within 5%.

Experimental Section and Results

The metabolism of 16α-hydroxydehydroisoandrosterone has been studied in a normal male and a normal pregnant female. The first study was designed to provide urinary metabolites in amounts sufficient for their identification and for reference standards. In both studies the labeled 16α-hydroxydehydroisoandrosterone was dissolved in 0.5 ml of absolute ethanol and this solution was diluted with 10 ml of isotonic saline prior to intravenous injection. There were insignificant amounts of radioactivity left in the syringe, needle, and vial used in the preparation and administration of the labeled steroid.

Metabolism of [7-^{3}H]16α-Hydroxydehydroisoandrosterone by the Normal Male. The subject, a 24-year-old male, was given 2.3×10^7 dpm of [7-^{3}H]16α-hydroxydehydroisoandrosterone. On the same day 600 mg of 16α-hydroxydehydroisoandrosterone was administered orally, in 50-mg doses contained in gelatin capsules, over a period of 12 hr. Urine was collected for 5 days after the injection of the labeled steroid. Steroid conjugates contained in the individual urines were hydrolyzed first by solvolysis and then with β-glucuronidase. Neutral extracts from both hydrolytic procedures were prepared by washing the organic phase with 5% $NaHCO_3$ and then with water. In such an extraction scheme, the urinary estrogens remained in the organic phase. The urinary sulfates and glucosiduronates contained 13.8 and 56.1% of the injected dose, respectively, but almost all of this radioactivity (>96%) was present in the urine collected during the first 48 hr. As a result, only the extracts of the first 2 days' urine were used in the isolation studies.

The sulfate fraction (406 mg and 3.1×10^6 dpm) was chromatographed on a 100-g silica gel column using increasing concentrations of ethanol in methylene chloride. The effluent from the column was collected

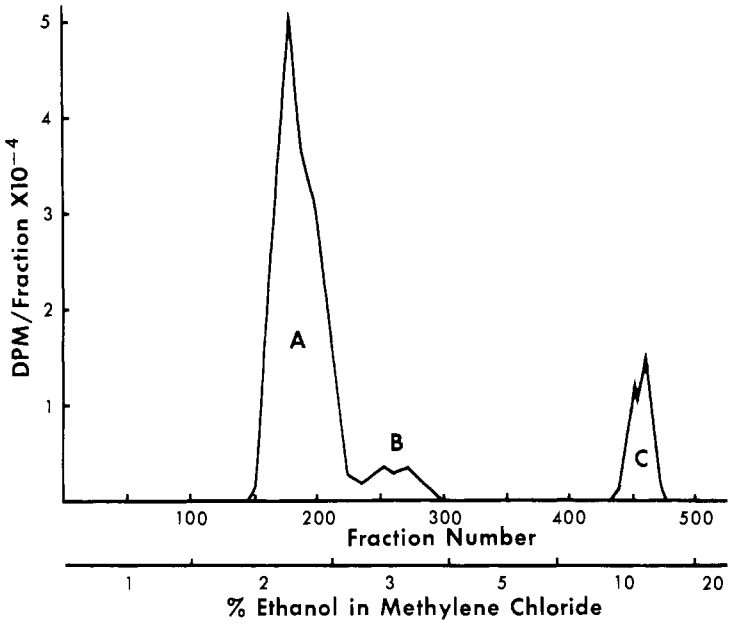

FIGURE 1: Silica gel chromatogram of the extract resulting from the solvolysis of the urinary conjugates following the intravenous administration of [7-^{3}H]16α-hydroxydehydroisoandrosterone as well as the ingestion of nonlabeled steroid.

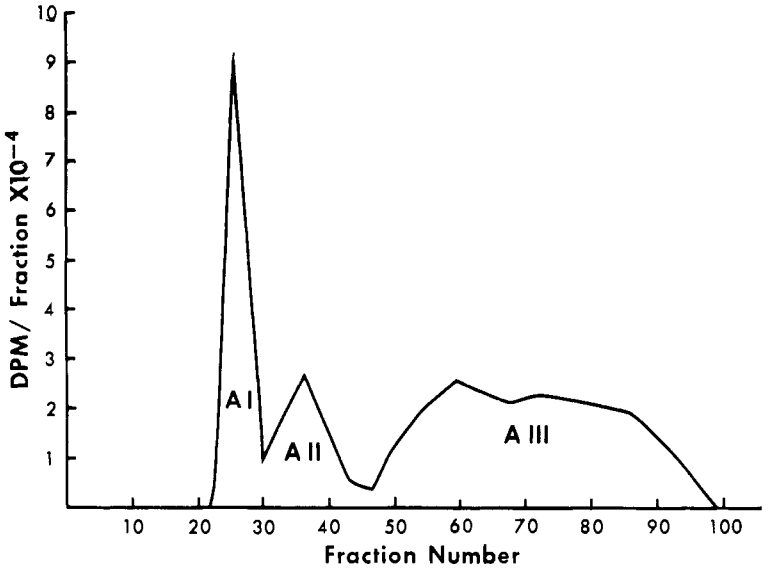

FIGURE 2: Celite partition chromatography of the residue from pool A of Figure 1 using system C.

in 10-ml fractions at the rate of 30–40 ml/hr. Three major pools of radioactivity were found as shown in Figure 1. The residue from pool A, which weighed 117 mg and contained 2.2×10^6 dpm was chromatographed on an 84-g Celite column in system C. Three major pools of radioactivity were found in the fractions collected as shown in Figure 2: AI (10.2 mg and 5.0×10^5 dpm), AII (12.3 mg and 2.9×10^5 dpm), and AIII (79.6 mg and 1.4×10^6 dpm).

AI was first chromatographed on paper in system L for 18 hr and then on a 1-g silica gel column from which 7.3 mg of crystals containing 3.2×10^5 dpm were obtained. Crystallization from acetone–Skellysolve B afforded 2.9 mg of fine needles, mp 181–183°, sp act. 4.9×10^4 dpm/mg. An aliquot of the mother liquor (1 mg) was acetylated and the infrared spectrum (CS_2) of the acetate was identical with that of authentic 3α,16α-diacetoxy-5α-androstan-17-one. Another aliquot

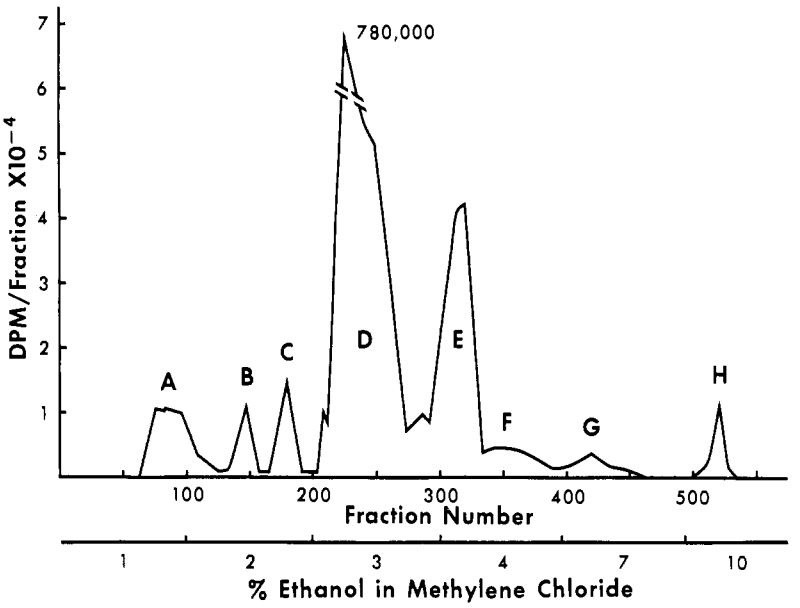

FIGURE 3: Silica gel chromatogram of the extract resulting from the β-glucuronidase hydrolysis of the urinary conjugates following the intravenous administration of [7-^{3}H]16α-hydroxydehydroisoandrosterone as well as the ingestion of nonlabeled steroid.

of the mother liquor (2.9 mg) was reduced with $NaBH_4$. The product was percolated through a 1-g silica gel column and crystallized from methanol–ether to yield 0.9 mg of needles, mp 253–257°, mmp 255–258°, androstanetriol standard mp 255–258°, lit. (Lieberman *et al.*, 1953) mp 254–256°, sp act. 5.0 × 10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of androstanetriol. It was not possible to obtain a sample of 16α-hydroxyandrosterone for direct comparison but the infrared spectrum (KBr) of AI was identical with that of 16α-hydroxyandrosterone in the files of Dr. T. F. Gallagher.

Residue AII was purified by chromatography on paper in system E and then by passage through a 1-g silica gel column. The material eluted was crystallized with great difficulty from methanol–ethyl acetate to yield 3.3 mg of fine plates, mp 127–130°, sp act. 5.3 × 10^4 dpm/mg. It gave a positive reaction with the blue tetrazolium reagent, a pink color when treated with 77% sulfuric acid in ethanol, a negative Zimmerman reaction, and was ultraviolet negative. The product obtained after reduction of AII with $NaBH_4$ had the same infrared spectrum (KBr) as that of CIII isolated from peak C of Figure 1.

The residue from AIII was chromatographed on a 70-g Celite column using system C. One radioactive peak was obtained in the third and fourth holdback volume and the residue weighed 71.4 mg and contained 1.1 × 10^6 dpm. It was crystallized from acetone–Skellysolve B to yield 40.1 mg of fine needles, mp 175–178°, mmp 177–179°, standard 16α-hydroxydehydroisoandrosterone mp 177–179°, lit. (Fotherby *et al.*, 1957) mp 177–181°, sp act. 1.7 × 10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of authentic 16α-hydroxydehydroisoandrosterone.

The material of pool B of Figure 1 (1.63 × 10^5 dpm and 16.6 mg) was purified by chromatography on paper in system E and on a 1-g silica gel column to yield 4.0 mg of a yellow oil which contained 9.4 × 10^4 dpm. This oil was acetylated and the product was percolated through a 1-g alumina column to yield 1.9 mg of colorless oil which contained 3.3 × 10^4 dpm. Crystallization from acetone–Skellysolve B afforded 1.1 mg of coarse plates, mp 188–190°, 3α,16α-diacetoxy-5β-androstan-17-one standard mp 185–188°, sp act. 1.9 × 10^4 dpm/mg. The infrared spectrum of the crystalline substance (CS_2) was identical with that of authentic 3α,16α-diacetoxy-5β-androstan-17-one. It was not possible to obtain a sample of the unacetylated compound for direct comparison.

From pool C of Figure 1 a residue (31.1 mg and 3.14 × 10^5 dpm) was obtained which was crystallized directly from methanol to yield 4.7 mg of large needles, mp 258–262°. The infrared spectrum (KBr) of the crystals was identical with that of androst-5-ene-3β,-16α,17β-triol (Hirschmann's triol). The crystals were chromatographed on thin layer plates in system P and the material eluted from the plates was crystallized from methanol to yield 4.5 mg of needles, mp 266–270°, mmp 265–267°, standard Hirschmann's triol mp 264–266°, lit. (Hirschmann, 1943) mp 265–270°, sp act. 1.8 × 10^4 dpm/mg.

The mother liquor obtained after crystallization of the residue from pool C was chromatographed on paper in system K for 37 hr and it was resolved into radioactive peaks CI, CII, and CIII with mobilities of 13.4, 21.3, and 31.2 cm, respectively. After elution the material from CI was percolated through a 1-g

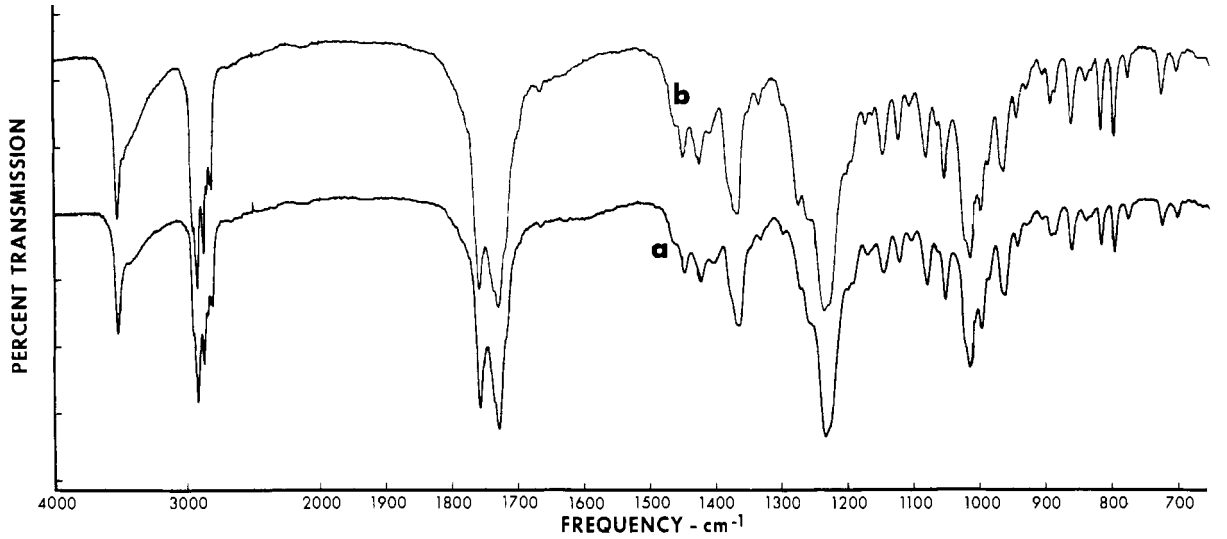

FIGURE 4: Infrared spectra (KBr) of (a) the metabolite isolated from pool A of Figure 3, and (b) D II B, the monoacetate synthesized from 3α,16α-dihydroxyandrost-5-en-17-one.

silica gel column and the product obtained was crystallized from methanol to yield 0.8 mg of fine needles which were identical with Hirschmann's triol present in the crystals. The eluate of CII was purified by passage through a 1-g silica gel column to give 2.2 mg of a residue which contained 8.0×10^4 dpm. Crystallization from methanol afforded 1.0 mg of small plates, mp 255–257°, androstanetriol mmp 253–256°, sp act. 5.0×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of authentic androstanetriol.

Further purification of the residue from CIII was achieved by chromatography on a 1-g silica gel column and the material eluted was crystallized from methanol to give 0.7 mg of fine cubes, mp 270–273°, sp act. 5.0×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of the $NaBH_4$ reduction product of the unknown AII and did not correspond to any known C_{19} triol.

The "glucosiduronate" fraction from the first 2 days' urine weighed 570 mg and contained 1.4×10^7 dpm. It was chromatographed on a 150-g silica gel column as previously described for the "sulfate" fraction and the radioactivity in the fractions eluted from the column is shown in Figure 3. Owing to the small weight of material in pools B, F, and G, the metabolites eluted in these fractions could not be identified.

From pool A a residue (29.1 mg and 5.4×10^5 dpm) was obtained which was purified by chromatography on paper in system A for 24 hr, then on a 2-g silica gel column to yield 6.0 mg of crystalline material containing 2.5×10^5 dpm. Crystallization from acetone–*n*-hexane yielded 4.4 mg of small plates, mp 195–196°, sp act. 5.0×10^4 dpm/mg. The infrared spectrum (KBr) of this metabolite is shown in Figure 4. This unknown was Zimmerman negative, ultraviolet negative, and gave a slight color with 77% sulfuric acid in ethanol and a positive reaction with the blue tetrazolium reagent after 5 min. A high-resolution mass spectrum analysis (courtesy of Dr. C. Djerassi, Stanford University) of this unknown, indicated that it had a molecular weight of 346 which would correspond to the monoacetate of a steroid such as 16α-hydroxydehydroisoandrosterone. This is in keeping with the strong band observed in the infrared region at 1235 cm^{-1} (Figure 4). These deductions are strengthened by the existence of a peak in the mass spectrum at *m/e* 286 which corresponds to the loss of acetic acid. The 100-Mcycle nuclear magnetic resonance (nmr) spectrum (courtesy of Dr. C. Djerassi) of this unknown, had a peak of 4.08 ppm which corresponds to a 3β proton. When compared to the spectra in Figure 5 the absorption peaks due to the 3β and 6 proton of DII were also present in the unknown but there was an upfield shift in the 16β proton corresponding to that of DII A, which was identified as 3α,16α-diacetoxyandrost-5-en-17-one (see below). An examination of the infrared spectrum of the unknown (Figure 4) revealed two carbonyl absorption bands at 1730 and 1757 cm^{-1}, the latter being indicative of a 16α-acetoxy-17-keto group. From the foregoing data the structure of 16α-acetoxy-3α-hydroxyandrost-5-en-17-one was assigned to the unknown in pool A. Because this unknown had the same infrared spectrum as that of DII B (Figure 4) the structure of the latter was also established.

Pool C gave a residue which weighed 12.9 mg and contained 2.3×10^5 dpm. It was chromatographed on paper in system J for 6 hr and one main radioactive peak was observed with the mobility of 16α-hydroxyandrostenedione. An aliquot of the material eluted from the paper had an infrared spectrum (KBr) which was similar to that of 16α-hydroxyandrostenedione. The residue (2.4×10^4 dpm and 0.8 mg) was chromatographed in system D for 4 hr and the eluate from

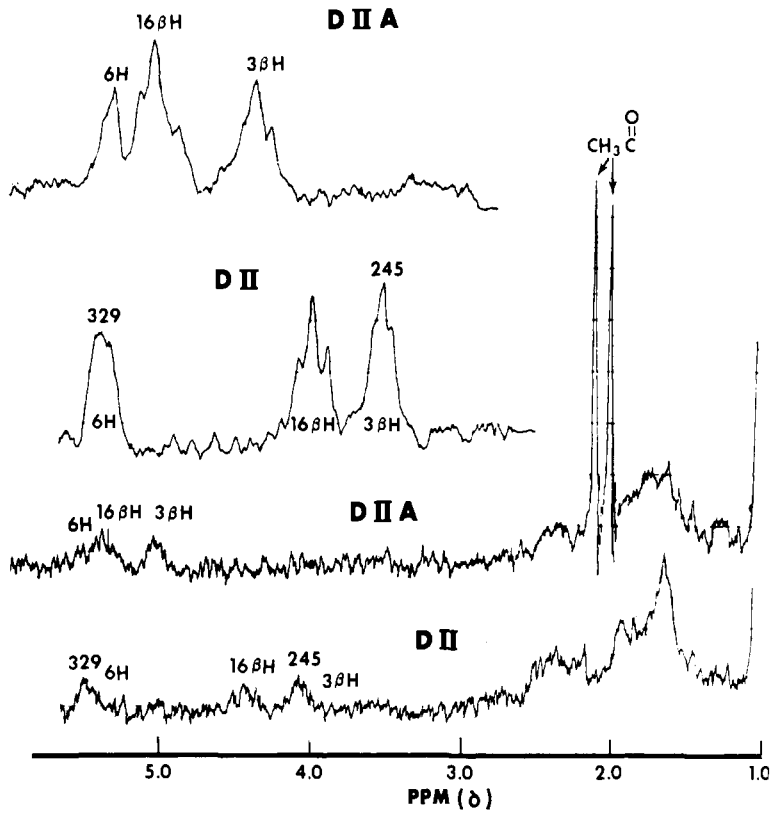

FIGURE 5: Nmr spectra of the unknown D II and its diacetate, D II A in $CDCl_3$. The enlarged top scans represent computer spectra at four-thirds scale of the bottom scans.

the paper was percolated through a small silica gel column to yield 0.2 mg of material containing 1.8×10^4 dpm. This purified material was mixed with 14.9 mg of authentic carrier 16α-hydroxyandrostenedione and the mixture was crystallized to constant specific activity and again after the formation of 16α-acetoxyandrostenedione.

The residue from pool D weighed 230 mg and contained 1.1×10^7 dpm. Chromatography of this residue on a Celite column in system C yielded three main radioactive peaks: DI in the third holdback volume, DII in the fourth and fifth holdback volume, and DIII in the sixth holdback volume. The residue from DI contained 1.9×10^6 dpm and weighed 46.0 mg and was rechromatographed on a Celite column in system C. One radioactive peak (1.8×10^6 dpm and 43 mg) was obtained in the third and fourth holdback volume. Crystallization of this material from acetone yielded 26.9 mg of coarse needles, mp 185–189°. Its infrared spectrum (KBr) was identical with that of 16α-hydroxyandrosterone isolated from the sulfate fraction.

Residue DII (150 mg and 7.6×10^6 dpm) was chromatographed on a Celite column in system C to yield 129 mg of material and 6.9×10^6 dpm. This material was crystallized with great difficulty from *n*-hexane–ether–acetone, to yield 56 mg of slightly yellow fine plates, mp 125–129°, sp act. 5.3×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of the unknown AII from the sulfate fraction and gave the same color reactions previously described for that unknown. The mass spectrum (courtesy of Dr. C. Djerassi) indicated that DII had a molecular weight of 304, which was consistent with the structural formula $C_{19}H_{28}O_3$. An aliquot (10.8) of DII was acetylated with [^{14}C]acetic anhydride, solution 2, and the product was chromatographed on alumina to yield two residues, DII A and DII B, eluted with 60% benzene in Skellysolve B and 2% ether in benzene, respectively. Crystallization of DII A from *n*-hexane afforded 2.8 mg of coarse plates (mp 156–159°) and the specific activities of the crystals and the mother liquors were both 1.1×10^4 dpm of ^{14}C/mg (^{3}H:^{14}C = 2.2). These results indicated the presence of two acetylable hydroxyl groups in the unknown DII. After crystallization of DII B from acetone–Skellysolve B, 2.0 mg of plates was obtained with a mp of 192–193° and a specific activity of 1.1×10^4 dpm of ^{14}C/mg (^{3}H:^{14}C = 4.4). Samples of 10 mg of DII A and DII were sent for nmr analysis to Dr. J. Fishman of the Institute for Steroid Research, Montefiore Hospital, New York, N. Y. The spectra shown in Figure 5 revealed the presence of an equatorial 3β-proton peak at 4.08 ppm in the unacetylated compound and 5.03 ppm in the diacetate. When compared to 16α-hydroxydehydroisoandrosterone there was no change in the C-6 proton, the 16β proton, and C_{18} and C_{19} methyl

TABLE III: Purification of Metabolites Isolated from Pregnancy Urine.

Material Eluted from Silica Gel Columns	Dpm of ^3H Eluted	Wt (mg)	Sequence of Chromatography	Procedure for Proof of Radiochemical Purity	Metabolite Identified
			Sulfates		
A	1.7×10^5	31.8	PC-D[a]		
AI	3.9×10^4	12.2	SG[b] → acetylation	rid[d]	16α-Hydroxydehydroisoandrosterone
AII	4.6×10^4	8.8	SG → acetylation	irp[e]	16α-Hydroxyandrosterone?
B	1.4×10^5	36.1	PC-E → SG	rid	Estriol
C	2.3×10^5	25.3	PC-H → tlc-O[c]	rid	Hirschmann's triol
			Glucosiduronates		
C	6.3×10^5	26.3	PC-J → PC-A → PC-D → acetylation	rid	16α-Hydroxyandrostenedione
D	3.3×10^6	98.7	Celite column C		
DI	8.6×10^5	16.4	PC-D → PC-A → acetylation	irp	16α-Hydroxyandrosterone
DII	1.5×10^6	14.9	PC-D → PC-A → acetylation	irp	3α,16α-Dihydroxyandrost-5-en-17-one
DIII	4.1×10^5	16.1	PC-E → PC-A → acetylation	rid	16α-Hydroxydehydroisoandrosterone
F	9.4×10^5	18.7	PC-D → SG → acetylation	irp	16α-Hydroxyetiocholanolone
H	9.8×10^5	55.1	Celite Column-F	Crystallization	Estriol

[a] PC-D, paper chromatography using system D. [b] SG, silica gel chromatography. [c] tlc-O, thin layer chromatography using system O. [d] rid, reverse isotope dilution. [e] irp, isotope ratio procedure as described in Materials and Methods.

group absorptions. These data indicated that the unknowns AII and DII were 3α,16α-dihydroxyandrost-5-en-17-one. The reduced unknown, CIII, from the sulfate fraction, was therefore compatible with the structure of androst-5-ene-3α,16α,17β-triol. The monoacetate DII B had the same infrared spectrum (KBr) as that of 16α-acetoxy-3α-hydroxyandrost-5-en-17-one (Figure 4).

Confirmatory evidence for the structure of DII was furnished by chemical conversion to a known steroid. DII (10 mg) was acetylated and the diacetate was chromatographed on an alumina column to yield 11.2 mg of an oily residue. Reduction with $NaBH_4$ and chromatography of the product on alumina yielded 9.8 mg of colorless oil which was reduced by catalytic hydrogenation over platinum oxide as described by Fukushima *et al.* (1961). The product was chromatographed on an alumina column to yield 6.7 mg of yellow oil which was saponified in 0.4 M methanolic KOH and chromatographed on a small silica gel column. Elution with 5% ethanol in methylene chloride gave 4.2 mg of crystalline material which was crystallized from methanol to give 1.9 mg of coarse granules, mp 274–276°, mmp 275–277°, standard etiocholanetriol mp 275–278°, lit. (Lieberman *et al.*, 1953) mp 276–278°. The infrared spectrum (KBr) was identical with that of etiocholanetriol. Identity of the unknown with etiocholanetriol established the presence of a 3α-hydroxy-5-ene group because it has been shown by Lewis and Shoppee (1955) and Fukushima *et al.* (1961) that a 3α-acetoxy-5-ene group gives mainly the 5β configuration after catalytic hydrogenation (confirmed in our laboratory with the model steroid, 3α-acetoxyandrost-5-en-17-one).

Residue DIII (8.0 mg and 5.1×10^5 dpm) was purified by chromatography on thin layer plates in system P to yield 5.5 mg of crystalline material containing 3.8×10^5 dpm. Crystallization of this material from acetone–Skellysolve B afforded 2.0 mg of fine needles, mp 177–180°, with authentic 16α-hydroxydehydroisoandrosterone mp 177–179°, sp act. 8.4×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of

16α-hydroxydehydroisoandrosterone.

The residue of pool E (1.3×10^6 dpm and 63.6 mg) was chromatographed sequentially on a Celite column in system B, paper in system D, and 6-g silica gel column to yield 30.3 mg of colorless oil containing 7.5×10^5 dpm. Crystallization from acetone–Skellysolve B afforded 15.8 mg of fine needles, mp 157–160°, sp act. 2.4×10^4 dpm/mg. Acetylation of an aliquot (1.7 mg) and crystallization of the acetate gave 0.8 mg of plates, mp 181–185°, sp act. 1.8×10^4 dpm/mg. Its infrared spectrum (CS_2) was identical with that of 3α,16α-diacetoxy-5β-androstan-17-one.

From pool H a residue was obtained (3.0×10^5 dpm and 38.7 mg) which was chromatographed on a Celite column in system H to yield 11.1 mg of material containing 2.1×10^5 dpm. Recrystallization from methanol afforded 2.3 mg of fine needles, mp 269–271°, sp act. 5.3×10^4 dpm/mg. Its infrared spectrum (KBr) was identical with that of androst-5-ene-3α,16α,17β-triol isolated from the sulfate fraction.

Metabolism of [7-^{3}H]16α-Hydroxydehydroisoandrosterone by the Pregnant Female. A total of 1.5×10^7 dpm in 310 μg of [7-^{3}H]16α-hydroxydehydroisoandrosterone was injected intravenously to a 23-year-old subject in the 36th week of pregnancy. Urine was collected for 5 days and the conjugates present in each day's urine were first hydrolyzed by solvolysis and then with β-glucuronidase. A total of 10.5% of the injected radioactivity was excreted in the sulfate and 64.8% in the glucosiduronate fraction. As in the first study most of this radioactivity was found in the first 2 days' urine and the extracts from this period were used for the isolation studies.

The sulfate fraction (660 mg and 1.6×10^6 dpm) was chromatographed on a silica gel column as previously described and three major pools of radioactivity, pools A–C, were obtained. The chromatographic procedures used to isolate the metabolites and the methods employed to establish radiochemical purity are outlined in Table III. Pool A resolved into two fractions, AI and AII. From AI it was possible to isolate 16α-hydroxydehydroisoandrosterone and its radiochemical purity was demonstrated by the reverse isotope dilution technique as shown in Table IV, which will serve as an example for other metabolites thus purified. Except for the first mother liquor, which had a specific activity of 710 dpm/mg, the specific activities and ^{3}H:^{14}C ratios of all the other mother liquors were the same as those of the crystals. From the final ^{3}H:^{14}C ratio, the specific activity of the 16α-hydroxydehydroisoandrosterone was calculated to be 4.1×10^4 dpm/mg. Chromatographic evidence was obtained for the presence of 16α-hydroxyandrosterone in AII but the lack of sufficient radioactivity prevented its identification.

Estriol was isolated from pool B and Hirschmann's triol from pool C. These metabolites were isolated in crystalline form and were identified by their melting point, mixture melting point, and infrared spectra. Then aliquots of the crystals were mixed with carrier and radiochemical purity established by reverse isotope dilution.

The glucosiduronate fraction (600 mg and 1.0×10^7 dpm) was chromatographed on a silica gel column as previously described and the radioactivity eluted is shown in Figure 6. Metabolites eluted in pools A, B, G, and J have not as yet been identified. As shown in Table III, the identity and radiochemical purity of the material in pool C was established by reverse isotope dilution and this metabolite was found to be 16α-hydroxyandrostenedione. Pool D resolved into three major fractions (DI, DII, and DIII) after chromatography on a Celite column. Two consecutive paper chromatographic separations served to purify DI, at which stage 2.3 mg of residue was obtained containing 3.4×10^5 dpm. It was acetylated with [1-^{14}C]acetic anhydride, solution 1, and the product was subjected to a number of chromatographic separations in the irp as exemplified in Table V. From these data it can be seen that DI was identical with 16α-hydroxyandrosterone. This same type of procedure was used to prove the radiochemical purity and identity of the 3α,16α-dihydroxyandrost-5-en-17-one and 16α-hydroxyetiocholanolone isolated from fractions DII and F, respectively (Table III). From DIII, 16α-hydroxydehydroisoandrosterone was isolated and radiochemical purity was established by reverse isotope dilution analysis, while 15 mg of estriol was isolated in crystalline form from pool H and identified by melting point, mixture melting point, and infrared analysis. Radiochemical purity of the estriol was also established by

TABLE IV: Proof of Radiochemical Purity of 16α-Hydroxydehydroisoandrosterone Isolated from the Sulfate Fraction of Pregnancy Urine.

	Specific Activity (dpm of ^{3}H/mg)			
	3β,16α-Diacetoxyandrost-5-en-17-one		3β,16α-Diacetoxyandrost-5-en-17β-ol[a]	
Crystzn	Crystals	^{3}H:^{14}C	Crystals	^{3}H:^{14}C
1	1270	2.8	1260	2.9
2	1280	2.8	1200	2.9
3	1280	2.9		
Calculated[b]	1050			

[a] The third crystals and mother liquor of the diacetate were reduced with sodium borohydride and the product was chromatographed on a small alumina column prior to crystallization. [b] After chromatography on silica gel 2.5×10^4 dpm contained in 1.7 mg of oily material was acetylated with [1-^{14}C]acetic anhydride solution 2 and the product was mixed with 20.3 mg of carrier 3β,16α-diacetoxyandrost-5-en-17-one. The mixture was chromatographed on an alumina column to yield 20 mg of crystalline material which contained 2.1×10^4 dpm of ^{3}H. The calculated specific activity is based on these values.

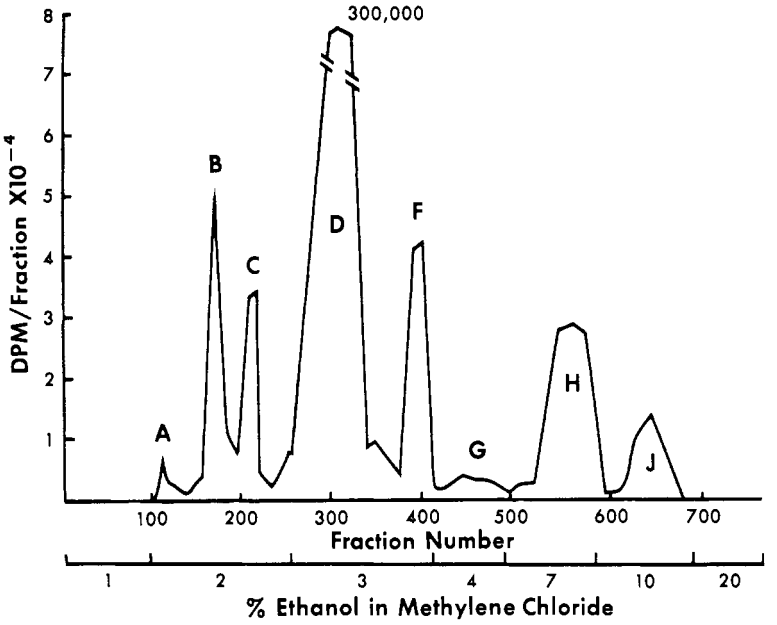

FIGURE 6: Silica gel chromatogram of the extract resulting from the β-glucuronidase hydrolysis of the urinary conjugates following the intravenous administration of [7-^{3}H]16α-hydroxydehydroisoandrosterone to a subject in the third trimester of pregnancy.

TABLE V: Proof of Radiochemical Purity of 16α-Hydroxyandrosterone Isolated from the Glucosiduronate Fraction of Pregnancy Urine.

	Chromatographic System	Support	R_F or HBV[b]	^{3}H:^{14}C after Chromatography[a]			
				Proximal	Middle	Distal	Av
1	A	Paper	0.83	9.5	11.5	9.9	10.3
2	N	Celite	4	10.5	12.0	12.2	11.6
3	Benzene	Silica gel	—	—	12.2	11.9	12.1
4	M	Celite	3	12.3	12.1	11.8	12.1
5	N	Celite	3–4	16.4	17.0	15.0	16.1
6	A	Paper	0.82	16.4	16.1	—	16.3

[a] After purification the residue from DI weighed 2.3 mg and contained 3.4 × 10^5 dpm. This was acetylated with [1-^{14}C]acetic anhydride, solution 1, and the product was chromatographed in several systems. After the fourth chromatographic procedure the residue eluted from the column had 1.2 × 10^5 dpm of ^{3}H and 1.0 × 10^4 dpm of ^{14}C. This residue was mixed with 3α,16α-diacetoxy-5α-androstan-17-one containing 4.2 × 10^4 dpm of ^{3}H to give a calculated ^{3}H:^{14}C ratio of 16.2 and the mixture was rechromatographed in systems 5 and 6. [b] HBV, holdback volume.

the determination of its specific activity after crystallization of the alcohol and the triacetate.

In Table VI are shown the specific activities of the urinary metabolites isolated from pregnancy urine. The values shown were computed when radiochemical purity was achieved using reverse isotope dilution analysis with the formation of a derivative or with the irp as exemplified in Table V. In addition, the per cent conversion of the injected radioactivity to each metabolite was computed on the basis of weight of carrier used and the final specific activity. In some instances this value was calculated from the amount of radioactivity in the metabolite prior to acetylation with [^{14}C]acetic anhydride and subsequent analysis by reverse isotope dilution or by the isotope ratio procedure. All these values for both studies are minimal because no correction was made for losses incurred during isolation.

Discussion

Large amounts of 16α-hydroxydehydroisoandro-

TABLE VI: Urinary Metabolites of 16α-Hydroxydehydroisoandrosterone.

Metabolite Isolated	Specific Activity[a] (dpm/μg) Urine of Pregnant Female		% Conversion of Injected Radioactivity[b]	
	Sulfate	Glucosiduronate	Pregnant Female	Normal Male
16α-Hydroxydehydroisoandrosterone	41	390	2.9	3.5
16α-Hydroxyandrosterone	250[c]	1710	5.9	5.7
16α-Hydroxyetiocholanolone	*d*	1190	6.0	1.6
16α-Hydroxyandrostenedione	—	1840	1.9	0.1
3α,16α-Dihydroxyandrost-5-en-17-one	—	3060	9.5	12.7
Hirschmann's triol	68	—	0.9	0.3
Androst-5-ene-3α,16α,17β-triol	—	—	—	0.5
Androstanetriol	—	—	—	0.2
Estriol	27	44	6.0	—

[a] These final values were computed after radiochemical purity was established. [b] These values were computed from the final specific activity and weight of the crystals where radiochemical purity was established or from the amount of radioactivity obtained prior to acetylation with [1-^{14}C]acetic anhydride in the isotope ratio procedure. [c] Radiochemical purity of this metabolite could not be fully established because of the small amount of radioactivity isolated. [d] The dashes indicate that the metabolite was looked for but was not detected.

sterone sulfate are elaborated by the human fetus in late pregnancy as is evidenced by its high plasma titer in the umbilical vein and artery at term (Magendantz and Ryan, 1964; Colas *et al.*, 1964; Easterling *et al.*, 1966). The high levels of 16α-hydroxydehydroisoandrosterone sulfate found in the umbilical vein indicate that not all of the steroid reaching the placenta is hydrolyzed and then aromatized to form estriol. It, therefore, appeared reasonable to us that some of the 16α-hydroxydehydroisoandrosterone sulfate which was not aromatized would be transported to the maternal circulation where it would be metabolized and excreted as urinary products. With this as our working hypothesis we began to study the metabolism of labeled 16α-hydroxydehydroisoandrosterone in late human pregnancy. Our first task was to determine the nature of the urinary metabolites of this steroid and to isolate enough of the metabolites for use in further investigations.

In both the studies reported virtually all of the radioactivity excreted was found in the first 2 days' urine. Most of the radioactive conjugates were hydrolyzed with β-glucuronidase. It is possible that some of the metabolites present in the glucosiduronate fraction were originally excreted in the urine conjugated to both sulfuric and glucuronic acids because solvolysis was used first and was followed by β-glucuronidase hydrolysis.

Three of the nine neutral metabolites isolated in the first study have been found previously in human urine. These are, 16α-hydroxydehydroisoandrosterone, Hirschmann's triol, and androstanetriol. A number of investigators have isolated 16α-hydroxydehydroisoandrosterone from normal male urine (Fotherby *et al.*, 1957; Bongiovanni, 1966), from the urine of newborn infants (Reynolds, 1963), from the urine of a patient with an adrenal tumor (Okada *et al.*, 1959) and from the urine of subjects with congenital adrenal hyperplasia with a metabolic block in the 3β-hydroxy steroid dehydrogenase enzyme (Bongiovanni, 1962). Hirschmann's triol was first isolated from the urine of a boy with adrenocortical carcinoma (Hirschmann, 1943) and subsequently from normal male urine (Marrian and Butler, 1944; Fotherby, 1958; Bongiovanni, 1966). Androstanetriol was isolated by Lieberman *et al.* (1953) from a large pool of urine obtained from patients with unspecified illnesses. The other six metabolites have not hitherto been isolated from human urine. These are, 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 16α-hydroxyandrostenedione, 3α,16α-dihydroxyandrost-5-en-17-one, androst-5-ene-3α,16α,17β-triol, and 16α-acetoxy-3α-hydroxyandrost-5-en-17-one.

When authentic standards were available for comparison the urinary metabolites were identified by their melting point, mixture melting point, and infrared spectra. The nmr spectra of DII and its diacetate (Figure 5) indicated the presence of an equatorial 3β proton, while all the other absorption peaks were the same as those found in 16α-hydroxydehydroisoandrosterone. In addition, the evidence from the color reactions and the fact that the unknown had two acylable hydroxyl groups, indicated that it was 3α,16α-dihydroxyandrost-5-en-17-one. Confirmatory evidence for this structure was obtained by chemical conversion of the unknown to etiocholanetriol. As can be seen from the per cent conversion of the injected radioactivity to urinary metabolites shown in Table VI, the most abundant single urinary metabolite isolated in these studies had a 3α-hydroxy-5-ene grouping. This would indicate that the presence of a 16α-hydroxyl group interferes in part with the Δ^4-isomerase enzyme which would normally play a role in the conversion of a β,γ-unsaturated alcohol to an α,β-unsaturated ketone. The studies of Fukushima *et al.* (1962) suggested that a 3-keto-5-ene may be an intermediate in the formation of a 3α-hydroxy-5-ene metabolite when

they demonstrated the conversion of androst-5-ene-3,17-dione but not of dehydroisoandrosterone to urinary 3α-hydroxyandrost-5-en-17-one. Previously, Fukushima *et al.* (1961) had isolated large amounts of pregn-5-ene-3α,16α,20α-triol from the urine of a patient with adrenocortical carcinoma. Although large amounts of dehydroisoandrosterone were isolated from the urine of this patient, 3α-hydroxyandrost-5-en-17-one could not be detected. The isolation of 3α-hydroxy-5-ene steroids in these studies is added proof that the 16α-hydroxyl group in a steroid partially inhibits the Δ^4-isomerase enzyme and represents the first report of the isolation of a urinary 3α-hydroxy-5-ene C_{19} steroid.

Although 11-dehydrocorticosterone acetate has been isolated from human plasma (Weichselbaum and Margraf, 1960), urinary steroid acetates (Lieberman *et al.*, 1948) have been considered as artifacts encountered during the course of isolation. Recently, Schubert and Wehrberger (1965) reported the isolation of 3β-acetoxyandrost-5-ene-7,17-dione from the unconjugated fraction of a large pool of human urine. In our study we have isolated 16α-acetoxy-3α-hydroxyandrost-5-en-17-one from the glucosiduronate fraction of urine under conditions where transesterification seems unlikely. It is possible that this urinary acetate was isolated because we used nothing more basic than 5% $NaHCO_3$ to prepare our neutral extracts in order to avoid degrading the α-ketols expected as urinary metabolites. Further studies are planned to determine whether this acetate was formed artifactually during the course of isolation. Although the presence of this acetate was also indicated in the glucosiduronate fraction of the pregnancy urine (pool A, Figure 6), repeated chromatography of the material did not afford sufficient product for proper identification.

In view of the unavailability of sufficient amounts of nonradioactive standards for use as carrier steroid in reverse isotope dilution analysis, another method was devised in order to identify the steroids isolated. This method, herein called the irp, involved the use of labeled acetates of the metabolites isolated from the urine of the normal male. With the use of this type of procedure, the identity and specific activities of a number of metabolites were determined and their radiochemical purity was demonstrated.

With the exception of androst-5-ene-3α,16α,17β-triol, 16α-acetoxy-3α-hydroxyandrost-5-en-17-one, and androstanetriol, all of the metabolites isolated from the urine of the normal male could also be found in the urine of the pregnant female. Estriol was also isolated from the sulfate and the glucosiduronate fractions. As shown in Table VI, the specific activities of urinary 16α-hydroxydehydroisoandrosterone from the sulfate fraction and urinary estriol from both fractions, were similar. The low specific activity of urinary 16α-hydroxydehydroisoandrosterone in the sulfate fraction indicated that a part of the injected steroid was transported to the placenta and possibly to the fetus, where it mixed with a large pool of endogenously formed steroid prior to being converted to estriol. Some of this diluted 16α-hydroxydehydroisoandrosterone (sulfate) was then transported to the maternal circulation along with the estriol formed from it. Large amounts of 16α-hydroxydehydroisoandrosterone do not seem to enter the maternal circulation from the placenta and fetus because if this were so, then the specific activities of 16α-hydroxyandrosterone, 16α-hydroxyetiocholanolone, 16α-hydroxyandrostenedione, and 3α,16α-dihydroxyandrost-5-en-17-one would approach that of estriol instead of being considerably higher. Thus it appears that these urinary metabolites with high specific activities probably reflect metabolism occurring in the maternal circulation.

It is of interest to note that when [7-^{3}H]16α-hydroxydehydroisoandrosterone was administered to the pregnant subject, the majority of the urinary metabolites were isolated from the glucosiduronate fraction. Metabolites such as 16α-hydroxyandrostenedione, however, are probably excreted in the urine conjugated with glucuronic acid, a situation analogous to that found for 16α-hydroxyprogesterone in pregnancy urine (Ruse and Solomon, 1966b).

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Adrenocorticotrophin-Induced Changes in the Steroidogenic Activity of Adrenal Cell-Free Preparations*

Robert V. Farese

ABSTRACT: After incubation of rat adrenal quarters *in vitro* with adrenocorticotrophic hormone (ACTH), the steroidogenic activity of subsequently derived homogenates is enhanced. This *induced* effect of ACTH in the homogenates occurs despite maximal quantities of a reduced triphosphopyridine nucleotide (TPNH)-generating system and does not appear to be *directly* due to ACTH or 3′,5′-adenosine monophosphate (3′,5′-AMP), and the inductive process (in the intact tissue) is blocked by puromycin. The effect appears to be largely owing to a factor(s) recovered in the 60,000*g* supernatant which interacts with adrenal mitochondria and enhances total corticosterone production, conversion of [^{14}C]cholesterol to corticosterone, and cholesterol side-chain cleavage (relative to control supernatant). The ACTH-induced supernatant factor is present in the macromolecular fraction excluded by Sephadex G-25, and the factor seems to be $(NH_4)_2SO_4$ precipitable, heat labile, and trypsin labile. Cyclic AMP induces comparable changes in the cholesterol side-chain-cleaving activity of rat adrenal supernatant, as does ACTH in the human adrenal cortex. The control rat adrenal supernatant inhibited cholesterol side-chain cleavage.

The factor(s) responsible for inhibition was present in the fraction excluded by Sephadex G-25, and appeared to be $(NH_4)_2SO_4$ precipitable and heat labile. This factor was observed in adrenal tissue from the rat, dog, rabbit, pig, and man, but not in extraadrenal rat tissues. It is unknown whether ACTH stimulates steroidogenesis in the cell-free system by counteracting the control inhibitory factor, or *via* a stimulatory factor which functions independently of the inhibitor.

The biological events that occur between the initial interaction of ACTH[1] with adrenal tissue and its subsequent effect on steroidogenesis have remained obscure. This ignorance is largely due to the ineffectiveness of ACTH in cell-free systems. Although studies with inhibitors of protein synthesis in more intact systems have suggested a role for protein synthesis during the steroidogenic action of ACTH (Ferguson, 1963; Farese, 1964; Garren *et al.*, 1965), the correct interpretation of these studies is doubtful since it is not certain that the inhibitors were operative *via* effects on protein synthesis. In the present investiga-

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[1] Abbreviations used: ACTH, adrenocorticotrophic hormone; KRB, Krebs–Ringer bicarbonate buffer; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; SN, supernatant fluid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.