In Vivo Metabolism of 15α-Hydroxyprogesterone in Man*

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ABSTRACT: Labeled 15α-hydroxyprogesterone was prepared by the microbiological hydroxylation of [7-^{3}H]progesterone or [4-^{14}C]progesterone. Nonlabeled 15α-hydroxyprogesterone was administered orally to normal male subjects with or without the simultaneous intravenous injection of the labeled steroid, and the urinary conjugates were hydrolyzed with Glusulase. The following six urinary metabolites were isolated and identified in these studies: 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15β,20β-triol, and 5β-pregnane-3α,15α,20β-triol. When a trace amount of [4-^{14}C]15α-hydroxyprogesterone was administered to a subject in the third trimester of pregnancy and to a nonpregnant subject in the luteal phase of the menstrual cycle, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from the sulfate fraction and 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, and 5α-pregnane-3α,15α,20β-triol were isolated from the glucosiduronate fraction of the urine of both subjects.

The specific activities of the metabolites isolated from the urine of the pregnant subject were from 25 to 45 times lower than the specific activity of the injected 15α-hydroxyprogesterone but the same metabolites isolated from the urine of the nonpregnant subject had specific activities which were the same as that of the injected steroid. These results demonstrate that 15α-hydroxyprogesterone and its metabolites are normally excreted in the urine of the pregnant subject but not in the urine of the nonpregnant subject. The studies reported also demonstrate that the 15α-hydroxyl group partially inhibits the reduction of the Δ^4-3-ketone and the C-20 ketone, leads to an increased ratio of 5α:5β-reduced urinary metabolites, and directs the reduction of the C-20 ketone toward the 20β-alcohol.

In a previous communication (Giannopoulos and Solomon, 1967) it was shown that 15α-hydroxyprogesterone is a constituent of human late-pregnancy urine. Because the 16α-hydroxyl group on the steroid nucleus alters the *in vivo* metabolism of C-19 steroids (YoungLai and Solomon, 1967) and modifies to a lesser extent the metabolism of steroids in the C-21 series (Fukushima *et al.*, 1961, 1967; Hirschmann *et al.*, 1961; Calvin and Lieberman, 1962; Ruse and Solomon, 1966; Romanoff *et al.*, 1967), it was therefore of some interest to investigate the metabolic fate of 15α-hydroxyprogesterone[1] administered to humans. We chose to study the metabolism of 15α-hydroxyprogesterone in the pregnant subject during the third trimester because of the findings that the largest amounts of this steroid are excreted in the urine during late pregnancy (Giannopoulos *et al.*, 1969). As a comparative study, we also investigated the *in vivo* metabolism of 15α-hydroxyprogesterone in a nonpregnant subject during the luteal phase of the menstrual cycle.

In the initial stages of the studies to be reported it was necessary to obtain sufficient amounts of the urinary metabolites of 15α-hydroxyprogesterone for their identification and to serve as carrier steroids in subsequent work, because such metabolites had not hitherto been described. In order to accomplish this, two studies were performed in which large amounts of nonlabeled steroid were fed by mouth to normal male volunteers and the urinary metabolites were isolated and identified. In one of these studies the labeled steroid was also injected intravenously.

Materials and Methods

Only those procedures which have not been previously described (Ruse and Solomon, 1966; YoungLai and Solomon, 1967) will be given.

Chromatography. Solvent systems used for chromatography are shown in Table I. All the chromatograms were developed at room temperature.

Standardization of Labeled Acetic Anhydride. One solution of [1-^{14}C]acetic anhydride and two solutions of [^{3}H]acetic anhydride, 20% (v/v) in benzene, were standardized by the acetylation of deoxycorticosterone, chromatography of the acetate on an alumina column, and crystallization to constant specific activity. The [1-^{14}C]acetic anhydride and solutions 1 and 2 of [^{3}H]acetic anhydride had specific activities of 2.30×10^4, 3.26×10^7, and 3.68×10^8 dpm per mg of deoxycorticosterone acetate, respectively.

Formation of Derivatives. A derivative of 15α-acetoxypro-

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[1] The following trivial names and abbreviations are used: progesterone, pregn-4-ene-3,20-dione; 20α-dihydroprogesterone, 20α-hydroxypregn-4-en-3-one; 20α-dihydroprogesterone acetate, 20α-acetoxypregn-4-en-3-one; 20β-dihydroprogesterone, 20β-hydroxypregn-4-en-3-one; 20β-dihydroprogesterone acetate, 20β-acetoxypregn-4-en-3-one; 15α-hydroxyprogesterone, 15α-hydroxypregn-4-ene-3-20-dione; 15α-acetoxyprogesterone, 15α-acetoxypregn-4-ene-3,20-dione; 15α,20β-diacetoxyprogesterone, 15α,20β-diacetoxypregn-4-en-3-one; 15α-hydroxypregnenolone, 3β,15α-dihydroxypregn-5-en-20-one; 16α-hydroxyprogesterone 16α-hydroxypregn-4-ene-3,20-dione; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 15α-hydroxydeoxycorticosterone, 15α,21-hydroxypregn-4-ene-3,20-dione; 15β-hydroxycorticosterone, 15β,21-dihydroxypregn-4-ene-3,20-dione; β-DPNH, dihydro-β-diphosphopyridine nucleotide disodium salt; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

TABLE I: Solvent Systems Used in Chromatography.

System	Type of Chromatography	Solvent Mixture
A	Ppc[a]	Toluene–propylene glycol
B	Ppc	Skellysolve B–methanol–water (10:9:1)
C	Ppc	2,2,4-Trimethylpentane–toluene–methanol–water (5:5:7:3)
D	Ccc[b]	*n*-Heptane–ethyl acetate–methanol–water (10:10:13:7)
E	Ccc	*n*-Hexane–ethyl acetate–methanol–water (10:10:13:7)
F	Ppc	Benzene–methanol–water (20:11:9)
G	Ppc	Toluene–ethyl acetate–methanol–water (9:1:6:4)
H	Tlc[c]	Chloroform–ethanol (9:1)
J	Tlc	Benzene–ethanol (1:1)
K	Tlc	Benzene–ethyl acetate (2:1)

[a] Ppc, paper partition chromatography. [b] Ccc, Celite column chromatography. [c] Tlc, thin-layer chromatography.

TABLE II: Proof of Radiochemical Purity of [4-^{14}C]15α-Hydroxyprogesterone Prepared Microbiologically.

Crystn	Specific Activities (dpm/mg)	
	Crystals	ML[a]
1	2250	2220
2	2180	2140
3	2200	2240
Calcd[b]	2330	

[a] ML, mother liquors. [b] An aliquot of the material eluted from paper containing 1.12×10^5 dpm was mixed with 48.0 mg of carrier 15α-hydroxyprogesterone prior to crystallization.

gesterone was prepared by reduction of the steroid with $NaBH_4$ as described by Norymberski and Woods (1955) and subsequent oxidation of the allylic alcohol at C-3 with DDQ as described by Burn *et al.* (1960). The infrared spectrum (CS_2) of the crystalline product obtained indicated a retention of the acetate group (1738 cm^{-1}), the presence of the Δ^4-3-ketone (1680 cm^{-1}), and of a hydroxyl group (3600 cm^{-1}), and the loss of the 20-ketone. This product had a melting point of 192–193° and absorbed ultraviolet light. The infrared spectrum of this derivative was identical with the spectrum of the product obtained after reduction of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase as described below. In addition, optical rotation studies, described in the Experimental Section, demonstrated that $NaBH_4$ reduction of 15α-acetoxyprogesterone favors the formation of the 20β-alcohol. These results permit the assignment of the structure 15α-acetoxy-20β-hydroxypregn-4-en-3-one to the product obtained by $NaBH_4$ reduction and subsequent DDQ oxidation of 15α-acetoxyprogesterone.

Reduction of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase was performed as follows. An aliquot of 15α-acetoxyprogesterone (3.4 mg) was dissolved in 0.4 ml of ethanol and the solution was added slowly to 20 ml of 0.1 M Tris-HCl and 0.0027 M EDTA buffer (pH 7.6). Following the addition of 194 mg of β-DPNH (Sigma) and 0.2 ml (1.0 mg) of 20β-hydroxysteroid dehydrogenase, Type 11 (Sigma, lot 97B-0490, prepared from *Streptomyces hydrogenans*, and stored at 0–5° as a suspension in 2.2 M ammonium sulfate, 5 mg/ml), the mixture was incubated at 25° in a stoppered flask for 2 hr. The reaction was stopped by the addition of 50 ml of distilled water and the steroids were extracted with ethyl acetate to give a residue weighing 2.8 mg. It was chromatographed on thin-layer chromatography using system K and two ultraviolet-positive bands were observed at average distances of 4.8 and 6.3 cm. The more polar band was eluted to give a residue (2.3 mg) which was crystallized from acetone–Skellysolve B to yield 1.5 mg of coarse needles, mp 192–193°. Its infrared spectrum (CS_2) indicated the retention of the acetate group, the presence of the Δ^4-3-ketone, the loss of the 20-ketone, and the appearance of a hydroxyl band at 3590 cm^{-1}. This spectrum was identical with the spectrum of the material obtained following reduction of 15α-acetoxyprogesterone with $NaBH_4$ and oxidation of the resulting product with the DDQ reagent as described above. The material obtained after incubation of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase was therefore assigned the structure of 15α-acetoxy-20β-hydroxypregn-4-en-3-one.

Nuclear Magnetic Resonance Spectroscopy. The nuclear magnetic resonance spectra were run on a Varian HR-100 spectrometer using deuteriochloroform ($CDCl_3$) as the solvent and tetramethylsilane as an internal standard. A glass microcell (NMR Specialties) of approximately 50-μl volume was used in these determinations. Chemical shift measurements were obtained from individual side-band measurements, using tetramethylsilane as the reference standard. In some instances a Varian HR-100 spectrometer was used, and since the instrument was field-frequency locked, the spectra were displayed on precalibrated charts. Observed chemical shifts were compared with shifts calculated for likely structures from the additive substituent effects given in Zürcher's Tables (Zürcher, 1963).

Mass Spectra. Mass spectra were run by Dr. Alan Duffield, Department of Chemistry, Stanford University. These spectra were determined using an Atlas CH-4 mass spectrometer run at 70 eV using a direct inlet system with the ion source at 180°.

Hydrolysis of Urinary Conjugates. Steroid conjugates were hydrolyzed by the modification of the solvolytic procedure of Jacobsohn and Lieberman (1962) and then with β-glucuronidase as previously described (YoungLai and Solomon, 1967). In some instances steroid conjugates were hydrolyzed by Glusulase as described by Ruse and Solomon (1966).

[7-^{3}H]15α-Hydroxyprogesterone. The preparation, purification, radiochemical purity, and the determination of the specific activity of [7-^{3}H]15α-hydroxyprogesterone have been described (Giannopoulos and Solomon, 1967). The specific activity of this material was 1.74×10^7 dpm/mg.

[4-^{14}C]15α-Hydroxyprogesterone. The microorganism used in this preparation was a strain of penicillium (ATCC 11598)

TABLE III: Determination of the Specific Activity of [4-^{14}C]15α-Hydroxyprogesterone.

	Specific Activities (dpm of ^{3}H/mg)							
	15α-Acetoxyprogesterone[a]				15α-Acetoxy-20β-hydroxypregn-4-en-3-one			
Crystn	Crystals	^{3}H/^{14}C	ML	^{3}H/^{14}C	Crystals	^{3}H/^{14}C	ML	^{3}H/^{14}C
1	1940	1.30	7600	4.90	1720	1.13	1750	1.14
2	1680	1.14	2580	1.70	1690	1.13	1710	1.13
3	1700	1.13	1730	1.14				
Calcd[b]					1710			

[a] An aliquot of [4-^{14}C]15α-hydroxyprogesterone containing 1.20×10^{5} dpm was acetylated with [^{3}H]acetic anhydride, solution 1, and the product was mixed with 65.0 mg of carrier 15α-acetoxyprogesterone. The mixture was chromatographed on a 7-g alumina column prior to crystallization. [b] A portion of the third crystals (32.5 mg) was reduced with $NaBH_4$ and oxidized with DDQ, and the product was chromatographed on a 4-g alumina column to yield 21.0 mg of material containing 3.59×10^{4} dpm of ^{3}H. The calculated specific activity was determined from these values.

supplied by Dr. P. A. Diassi of the Squibb Institute for Medical Research, who also suggested the method of incubation. The details of this procedure have been described recently (Stern *et al.*, 1968). Following incubation of 0.2 mCi of [4-^{14}C]progesterone (specific activity 37.2 mCi/mmole, New England Nuclear Corp.) with penicillium, a methylene chloride extract was prepared. The extract contained 4.07×10^{8} dpm and it was chromatographed on paper in system A for 8 hr. The area corresponding in mobility to 15α-hydroxyprogesterone was eluted to yield 2.8 mg of residue containing 1.05×10^{8} dpm. Chromatography of this residue on paper in system C for 5 hr gave one band of radioactive material with the mobility of 15α-hydroxyprogesterone which on elution afforded a residue containing 9.00×10^{7} dpm. An aliquot of this residue containing 1.12×10^{5} dpm was mixed with carrier 15α-hydroxyprogesterone and the mixture was crystallized to constant specific activity as shown in Table II. The determined and calculated specific activities were in good agreement, indicating a high degree of radiochemical purity and the yield of labeled 15α-hydroxyprogesterone was found to be 20%.

Another aliquot of [4-^{14}C]15α-hydroxyprogesterone containing 1.20×10^{5} dpm was acetylated with [^{3}H]acetic anhydride, solution 1. The product was mixed with 65.0 mg of carrier 15α-acetoxyprogesterone, and the mixture was chromatographed on a 7-g alumina column. Elution with benzene–Skellysolve B (4:1) yielded material which was crystallized from acetone–Skellysolve B, ether–Skellysolve B, and acetone, and the specific activities measured are shown in Table III. A portion of the last crystals (32.5 mg) was reduced with 7.5 mg of $NaBH_4$ in 90 ml of methanol. The products were dissolved in 2.5 ml of dioxane and oxidized with 27.5 mg of DDQ. The product obtained was chromatographed on a 4-g alumina column and the material eluted was crystallized twice from acetone–Skellysolve B and the specific activities were determined as shown in Table III. The specific activity of [4-^{14}C]15α-hydroxyprogesterone was found to be 3.66×10^{8} dpm/mg calculated from the specific activity of the acetic anhydride and the ^{3}H:^{14}C ratio of the second derivative.

Preparation of Nonlabeled 15α-Hydroxyprogesterone. When these studies were started it was necessary to prepare large amounts of 15α-hydroxyprogesterone. This was accomplished by incubating 40 g of progesterone with penicillium, using the conditions described above for the 15α hydroxylation of [4-^{14}C]progesterone, with the exception that the reaction was carried out in a large fermentor (Ayerst Laboratories, Montreal) and a concentration of 400 mg of substrate/l. of incubation medium was employed. The methylene chloride extract of the incubation media weighed 35 g and when crystallized directly from acetone–hexane yielded 11.2 g of crystalline material, mp 208–222°. This material was chromatographed on thin-layer plates (860 plates of 1-mm thickness) using system H. The ultraviolet-positive band corresponding in mobility to 15α-hydroxyprogesterone was eluted from the plates to yield 8.12 g of material which was crystallized twice from acetone to give 6.45 g of coarse needles: mp 230–232°, authentic 15α-hydroxyprogesterone, mp 231–232°, mmp 231–232°. The infrared spectrum (KBr) of the product was identical with that of 15α-hydroxyprogesterone.

Experimental Section and Results

In all studies to be described the labeled 15α-hydroxyprogesterone 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 steroids.

Metabolism of [7-^{3}H]15α-Hydroxyprogesterone by the Normal Male. A total of 2.3×10^{7} dpm of [7-^{3}H]15α-hydroxyprogesterone was injected intravenously in a normal 28-year-old male, and on the same day 800 mg of 15α-hydroxyprogesterone, in 80-mg doses contained in gelatin capsules, was ingested over a period of 10 hr. Urine was collected for 6 days and the steroid conjugates in the individual urines were hydrolyzed with Glusulase. A neutral extract was prepared from each day's urine, and these contained a total of 70.5% of the injected dose. Almost all of the radioactive material excreted (99.7%) was present in the urine collected during the first 4 days. As a result, only these extracts were used in the isolation studies.

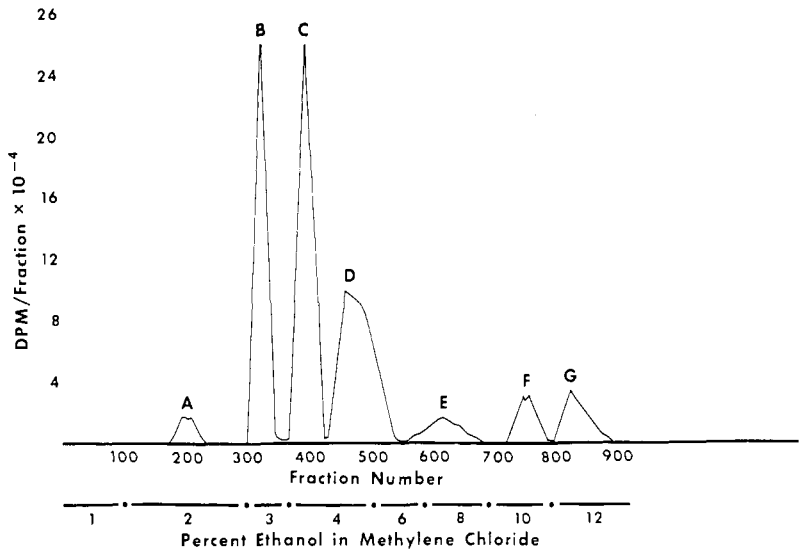

FIGURE 1: Silica gel column chromatography of the neutral extract obtained following Glusulase hydrolysis of the urinary conjugates after the intravenous injection of [7-^{3}H]15α-hydroxyprogesterone and the oral administration of 800 mg of carrier steroid to a normal male.

The combined neutral extract (1.12 g and 1.62 $\times$ 10^7 dpm) was chromatographed on a 150-g silica gel column using increasing concentrations of ethanol in methylene chloride, as previously described (Ruse and Solomon, 1966). The effluent from the column was collected in 10-ml fractions at the rate of 30–40 ml/hr and seven major pools of radioactive material were eluted as shown in Figure 1. Owing to the small weight of material present in pools A, E, and G after further chromatography, the metabolites eluted in these fractions could not be identified. The residue from pool B (80 mg and 3.94 $\times$ 10^6 dpm) was chromatographed on a 100-g Celite column using system D and two major pools of radioactive material, BI and BII, were eluted in the 4th and 5–7 hold-back volumes, respectively. BI (14.1 mg and 4.28 $\times$ 10^5 dpm) was chromatographed on paper in system A for 12 hr and then on a 1-g silica gel column from which 3.3 mg of material containing 2.99 $\times$ 10^5 dpm was eluted with 3% ethanol in methylene chloride. Crystallization of this material from acetone–methanol gave 0.5 mg of coarse needles. The infrared spectrum (KBr) of the crystals showed a strong hydroxyl band at 3450 cm^{-1} and a band at 1695 cm^{-1} indicating the possible presence of a 20-ketone. Because of the small amount isolated the identity of this unknown could not be established.

Residue BII weighed 53.6 mg and contained 3.93 $\times$ 10^6 dpm. Chromatography of a small aliquot of BII on paper in system A for 10 hr indicated that it contained a single band of radioactive material identical in mobility to 15α-hydroxyprogesterone. Therefore the remainder of BII (50.9 mg) was chromatographed on a 5-g alumina column and 43.3 mg of crystalline material containing 3.62 $\times$ 10^6 dpm was eluted with 1% ethanol in benzene. Crystallization of this material from acetone gave 27.4 mg of coarse needles: mp 230–232°, specific activity 1.08 $\times$ 10^5 dpm/mg. The infrared spectrum (KBr) of the crystals was identical with that of authentic 15α-hydroxyprogesterone.

The material present in pool C (Figure 1, 81 mg and 4.52 $\times$ 10^6 dpm) was chromatographed on a 100-g Celite column using system E and a single peak containing 56 mg of material and 4.48 $\times$ 10^6 dpm was eluted in the 4th hold-back volume. This material was further purified by chromatography on paper in system A for 24 hr, and on a 6-g silica gel column from which 51.4 mg of an oily residue containing 4.45 $\times$ 10^6 dpm was eluted with 4% ethanol in methylene chloride. It was crystallized with great difficulty from acetone–ether–Skellysolve B to yield 14.0 mg of fine plates: mp 175–177°, specific activity 1.42 $\times$ 10^5 dpm/mg. An aliquot (8.3 mg) was acetylated with [^{14}C]acetic anhydride and the product was chromatographed on alumina and 10.2 mg of oily residue was eluted from the column with benzene–Skellysolve B (3:2). Crystallization of the acetate from acetone–Skellysolve B afforded 6.5 mg of coarse plates, mp 142–144°. The specific activities of the crystals and the mother liquors were both 4.08 $\times$ 10^4 dpm of ^{14}C/mg (^{3}H/^{14}C = 1.98). These results indicated that the material in pool C had two acylable hydroxyl groups. Its infrared spectrum taken in KBr (Figure 2a) showed a hydroxyl band at 3350 cm^{-1}, a carbonyl band at 1705 cm^{-1} corresponding to a 20-ketone, and a band at 1002 cm^{-1} which is compatible with the 3α–5α stereochemistry in the A ring (Cole *et al.*, 1952). The spectrum (CS_2) of the acetate indicated the absence of a hydroxyl band, the presence of a 20-ketone at 1710, and a band at 1018 cm^{-1} indicative of 3α-acetoxy with an axial conformation (Jones and Herling, 1956). The mass spectrum gave a mass number of 334, which was consistent with the structural formula $C_{21}H_{34}O_3$. Table IV shows the results obtained from nuclear magnetic resonance analysis of the alcohol and its diacetate.

The chemical shifts of the angular methyls (C-18 and C-19) indicate that the compound is of the 5α rather than the 5β series of steroids, and agree well with values calculated from Zürcher's Tables (Zürcher, 1963) for 3α,15α-dihydroxy-5α-pregnan-20-one and its diacetate (Table IV). These same methyl signals differed sufficiently from those calculated for the corresponding 3α,15β- and 3β,15β-diols and their diacetates which confirmed that the substituent at 15 remained in the α configuration. However, the differences between a 3α,15α and 3β,15α substituent were not so great as to be conclusive, although agreement with the values calculated for the 3α isomer was somewhat better than for the 3β. Fortunately this difference is easily distinguished by the spin–spin splitting patterns of the proton at the C-3 position. In the 5α system, a substituent in the 3α position is accompanied by an equatorial proton, while a 3β substituent has

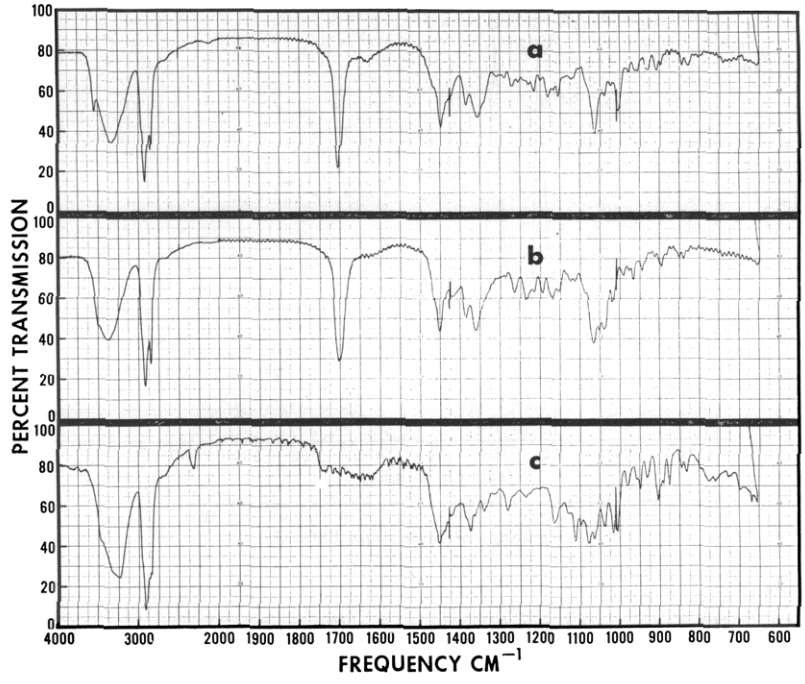

FIGURE 2: Infrared spectra (KBr): (a) metabolite isolated from pool C, (b) metabolite isolated from pool D, and (c) metabolite isolated from pool F.

an axial proton at C-3. In the absence of further substitution at positions 2 and 4, an axial proton at C-3 has two axial–axial coupling interactions (large) and two axial equatorial couplings (small to medium). A similarly placed equatorial proton has two equatorial–axial and two equatorial–equatorial couplings (all small to medium). Thus the axial proton may be distinguished by its broader coupling pattern.

The nuclear magnetic resonance spectrum of C had two proton signals (C-3-H and C-15-H) which were superimposed in the region 3.9–4.2 ppm in the diol and 4.8–5.1 ppm in the diacetate. The appearance was one of a sharper signal atop a much broader one. Identification of the sharper signal as that of C-3-H and thus assignment of this proton as equatorial was accomplished by comparing the low-field region of the nmr of compound C with that of D (*vide infra*) in which two very broad signals occurred. (The fact that D belongs to the 5β series will have little or no effect upon the splitting pattern of its 15α-H.) From the nuclear magnetic resonance results combined with the information obtained from the infrared spectra, the number of acylable hydroxyl groups and from the mass spectrum, the structure 3α,15α-dihydroxy-5α-pregnan-20-one can be assigned to the metabolite isolated from pool C.

The residue from pool D (Figure 1) weighed 117 mg and contained 3.94×10^6 dpm. Chromatography of this residue on a 100-g Celite column in system E yielded 60.3 mg of material containing 3.62×10^6 dpm which was eluted in the 5th and 6th hold-back volume. This material was further purified by chromatography on paper in system A for 24 hr and on a 6-g silica gel column from which 43.0 mg of crystalline residue containing 3.33×10^6 dpm was eluted with 4% ethanol in methylene chloride. Crystallization of this material from acetone–methanol–Skellysolve B afforded 23.0 mg of coarse plates: mp 208–210°, specific activity 7.90×10^4 dpm/mg. An aliquot (9.3 mg) of the crystals was acetylated with [^{14}C]acetic anhydride and the product was chromatographed on an alumina column and elution with benzene–Skellysolve B (3:2) yielded 12 mg of colorless oil. Crystallization of this material from acetone–Skellysolve B afforded 10.9 mg of fine needles, mp 164–166.5°. The specific activities of the crystals and the mother liquors were both 3.89×10^4 dpm of ^{14}C/mg (^{3}H/^{14}C = 1.65). These results indicated the presence of two acylable hydroxyl groups in the unknown in pool D. The infrared spectrum of this material (Figure 2b) showed the presence of a hydroxyl band at 3375, a carbonyl band at 1705 corresponding to a 20-ketone, and a band at 1038 cm^{-1} corresponding to an equatorial 3α-hydroxyl (*Cole et al.*, 1952). The infrared spectrum (CS_2) of the acetate had no hydroxyl band but had a 1710-cm^{-1} band, indicating the presence of a 20-ketone and a band at 1029 cm^{-1} corresponding to an equatorial 3α-acetoxy group (Jones and Herling, 1956). The mass spectrum indicated that the unknown in pool D had a molecular weight of 334, which was consistent with the structural formula $C_{21}H_{34}O_3$.

Table IV shows the results obtained from nuclear magnetic resonance analysis of unknown D and its diacetate. The chemical shifts of the angular methyls identify this compound as being in the 5β series, and agree well with values calculated from Zürcher's tables (Zürcher, 1963) for 3α,15α-dihydroxy-5β-pregnan-20-one and its diacetate. The calculated values for the 3α,15β- and 3α,15β-diols and their diacetates were sufficiently different as to indicate that no change had occurred at the 15 position. The calculated values did not distinguish unambiguously between 3α and 3β. However, two very broad multiplets at approximately 3.63 and 4.02 ppm in the diol which shifted together into the region 4.5 to 5.0 ppm in the diacetate identified the C-3 proton as axial (very broad coupling pattern described above). The nuclear magnetic resonance data therefore indicated that the unknown D was 3α,15α-dihydroxy-5β-pregnan-20-one. This conclusion was reinforced by the data obtained from the infrared spectra, the number of acylable hydroxyl groups, and from the mass spectrum. Confirmatory evidence for the assignment of this

TABLE IV: Nuclear Magnetic Resonance Data for Metabolites in Pools C, D, and F.[a]

Metabolite	Chemical Shifts					Confirmation of C-3 H
	C-19 CH_3	C-18 CH_3	C-21 CH_3	CH_3COO	C-3 H	
C	0.79	0.63	2.10		3.9–4.2	Equatorial
C-diacetate	0.79	0.68	2.10	1.98, 2.03	4.8–5.1	Equatorial
D	0.93	0.64	2.10		3.6–4.1	Axial
D-diacetate	0.94	0.67	2.10	1.98, 2.02	4.5–4.9	Axial
F-triacetate	0.80	0.72	1.21[b]	1.98, 1.99, 2.04	4.95	
3α,15α-Dihydroxy-5α-pregnan-20-one	0.792[c]	0.650[c]				
3α,15α-Diacetoxy-5α-pregnan-20-one	0.809[c]	0.693[c]				
3α,15α-Dihydroxy-5β-pregnan-20-one	0.933[c]	0.650[c]				
3α,15α-Diacetoxy-5β-pregnan-20-one	0.942[c]	0.684[c]				
5α-Pregnane 3α,15α,20α-triacetate	0.809[c]	0.743[c]				
5α-Pregnane 3β,15α,20α-triacetate	0.834[c]	0.734[c]				
5α-Pregnane 3α,15α,20β-triacetate	0.809[c]	0.703[c]				
5α-Pregnane 3β,15α,20β-triacetate	0.834[c]	0.674[c]				

[a] The spectra were measured with a Varian HR-100 spectrometer. Peak positions are expressed in parts per million (ppm) relative to a tetramethylsilane internal standard. The audio-side-band method of calibration was used. [b] Doublet, $J = 6$ Hz. [c] These values were calculated for the compounds designated using Zürcher's tables (Zürcher, 1963).

structure was derived from the specific stereochemical reduction of 15α-hydroxyprogesterone with *Clostridium paraputrificum* (Schubert *et al.*, 1965) through the courtesy of Dr. Josef Schlegel, Deutsche Akademie der Wissenschaften zu Berlin, Jena, Germany. The infrared spectrum (KBr) of the reduced product sent by Dr. Schlegel was identical with that of 3α,15α-dihydroxy-5β-pregnan-20-one except for a shoulder at 1670 cm^{-1} indicating the presence of a small amount of unreacted substrate in the sample.

The residue obtained from pool F (29.0 mg and 6.60×10^5 dpm) was purified by chromatography on paper in system A for 4 days and then on a 2-g silica gel column from which 9.0 mg of oily material containing 6.15×10^5 dpm was eluted with 10% ethanol in methylene chloride. Crystallization of this material from acetone–Skellysolve B yielded 1.6 mg of small plates: mp 211–214°, specific activity 1.96×10^5 dpm/mg. An aliquot (0.95 mg) was acetylated with [^{14}C]-acetic anhydride and the product was chromatographed on alumina. Elution from the column with benzene–Skellysolve B (1:1) yielded 1.2 mg of colorless oil which had a specific activity of 5.52×10^4 dpm of ^{14}C/mg ($^3H/^{14}C = 3.1$). These results indicated the presence of three acylable hydroxyl groups in the unknown F. The infrared spectrum of F (Figure 2c) showed a hydroxyl band at 3250 cm^{-1} and no carbonyl bands indicating that both the Δ^4-3-keto and the 20-keto groups of 15α-hydroxyprogesterone had been reduced during the course of metabolism. The infrared spectrum (CS_2) of the triacetate of F had no hydroxyl band, and a broad acetate band was observed at 1240 cm^{-1}. The mass spectrum indicated that the unknown in pool F had a molecular weight of 336, which was consistent with the structural formula $C_{21}H_{36}O_3$.

Table IV shows the results obtained from nuclear magnetic resonance analysis of the triacetate of metabolite F. Instead of a methyl ketone at C-21 (2.12 ppm) the spectrum showed a doublet at 1.20 ppm ($J = 7$ Hz) due to a secondary methyl group formed by reduction of the C-20 ketone to an alcohol. The positions of the angular methyls corresponded to those of a 5α-steroid, but were similar enough to the calculated values for both 5α-pregnane-3α,15α,20α-triol and the 20β-triol triacetates that it was not possible to determine whether the reduction of the keto group gave the 20α- or 20β-alcohol. The observed values were in somewhat better agreement for the 3α- rather than 3β-acetate, though this difference was also small. In this case the signals of the three protons adjacent to the acetate groups (C-3, C-15, and C-20) overlapped badly and it was not possible to identify the splitting pattern of the proton at C-3.

Following reduction of an aliquot of the diacetate of metabolite C, 3α,15α-diacetoxy-5α-pregnan-20-one, with $NaBH_4$ and acetylation of the product, a triacetate was obtained whose infrared spectrum (CS_2) was identical with that of F–triacetate. Since it is known that $NaBH_4$ reduction

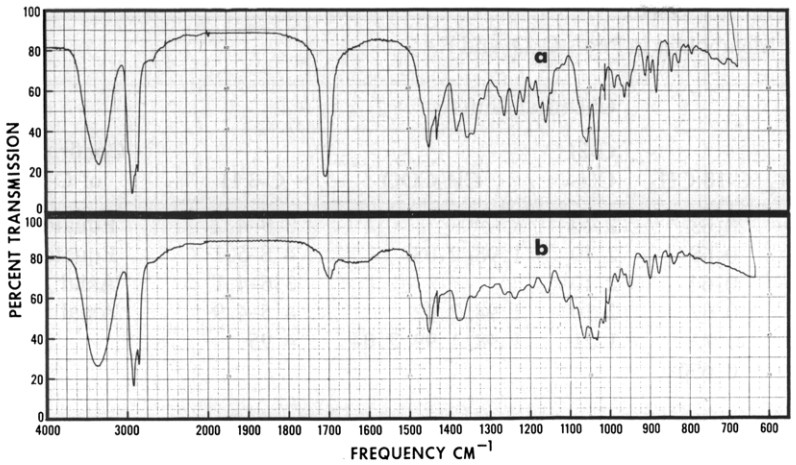

FIGURE 3: Infrared spectra (KBr): (a) metabolite isolated from pool II and (b) metabolite isolated from pool VI.

of a C-20 ketone favors the formation of the 20β-alcohol (Norymberski and Woods, 1955) the unknown F most probably was 5α-pregnane-3α,15α,20β-triol.

To confirm this it was necessary to study the possible effect of the 15α-hydroxyl group on the $NaBH_4$ reduction of 20-ketosteroids. Fieser and Fieser (1948) summarized the molecular rotation, MD, data for 19 pairs of C-20 epimers in the pregnane series and pointed out that the shift in MD on acetylation is positive for 20β-ols and negative for 20α-ols. An aliquot (198 mg) of 15α-acetoxyprogesterone was reduced with $NaBH_4$, the product was oxidized with DDQ, and the material thus obtained was chromatographed on an alumina column. Elution with 0.5% ethanol in benzene yielded a product which was crystallized from acetone–Skellysolve B to give 115 mg of crystals. An aliquot of this material (50 mg) was acetylated and the product was crystallized from methanol–ether. Aliquots of the 20-alcohol and its acetate (30 and 31 mg, respectively) were used for optical rotation studies. The optical rotation of 20α-dihydroprogesterone, 20β-dihydroprogesterone, and their respective acetates were also measured. Table V shows the molecular rotation difference, ΔMD, obtained after acetylation of 20α-dihydroprogesterone and 20β-dihydroprogesterone. The same correlation reported by Fieser and Fieser (1948) was observed here in that the ΔMD of the 20α epimer was slightly negative (−6°) while the ΔMD of the 20β epimer was positive (+212°). As shown in Table V the ΔMD due to the introduction of a 20-acetoxy in 15α-acetoxy-20-hydroxypregn-4-en-3-one was +179. These results provide confirmatory evidence that $NaBH_4$ reduction of 20-ketosteroids, which have a 15α-hydroxy, results in the formation of 20β-alcohols. Further confirmation was obtained by the reduction of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase as described in the Methods section. The infrared spectra (CS_2) of the product obtained after reduction of 15α-acetoxyprogesterone with 20β-hydroxysteroid dehydrogenase in the presence of β-DPNH was identical with the spectrum of the compound obtained after $NaBH_4$ reduction followed by DDQ oxidation. Since reduction of 3α,15α-diacetoxy-5α-pregnan-20-one with $NaBH_4$ and acetylation of the product yielded material having an infrared spectrum identical with the F-triacetate, the structure 5α-pregnane-3α,15α,20β-triol could be assigned to unknown F.

TABLE V: Optical Rotation Data.

Steroid	Molecular Rotation (MD)[a]	ΔMD
20α-Dihydroprogesterone	−306	
20α-Dihydroprogesterone acetate	−312	−6
20β-Dihydroprogesterone	−363	
20β-Dihydroprogesterone acetate	−151	+212
Product obtained from the $NaBH_4$ reduction followed by DDQ oxidation of 15α-acetoxyprogesterone	−242	
15α,20β-Diacetoxyprogesterone[b]	−63	+179

[a] Optical rotations were obtained with a Rudolph polarimeter using 1% chloroform solutions. [b] The diacetate was obtained by reduction of 15α-acetoxyprogesterone with $NaBH_4$, oxidation of this product with DDQ reagent, and subsequent acetylation.

In order to secure large amounts of nonlabeled metabolites of 15α-hydroxyprogesterone for use as carriers in the investigations to be described the following study was performed. Each of four normal male volunteers was given 800 mg of 15α-hydroxyprogesterone by mouth, in 80-mg doses contained in gelatin capsules, over a period of 10 hr. A total of 3.25 g of 15α-hydroxyprogesterone was administered to the four subjects. The urine collected from each subject for 4 days was pooled and the urinary conjugates were hydrolyzed with Glusulase. The neutral extract obtained after hydrolysis (3.5 g) was chromatographed on a 250-g silica gel column using increasing concentrations of ethanol in methylene chloride and the effluent from the column was collected in 20-ml fractions at a rate of 50–60 ml/hr. Since no radioactive steroid was administered in this study, the fractions obtained from the column were pooled on the basis of visual examination of the tubes after evaporation of the solvent. Six pools containing crystalline material were obtained as shown in Table VI.

From pools I, III, and IV, crystalline 15α-hydroxyprogesterone (190 mg), 3α,15α-dihydroxy-5α-pregnan-20-one (110 mg), and 3α,15α-dihydroxy-5β-pregnan-20-one (320 mg), re-

TABLE VI: Combined Fractions from the Initial Silica Gel Column of the Neutral Extract of Urine Following the Ingestion of 15α-Hydroxyprogesterone.

Pool	Fraction	% Ethanol in Methylene Chloride	Weight (mg)	Metabolite Isolated
I	174–230	3	280	15α-Hydroxyprogesterone
II	281–320	3	110	3β,15α-Dihydroxy-5β-pregnan-20-one
III	321–440	4	230	3α,15α-Dihydroxy-5α-pregnan-20-one
IV	441–530	4–6	460	3α,15α-Dihydroxy-5β-pregnan-20-one
V	786–820	10	42	5α-Pregnane-3α,15α,20β-triol
VI	881–995	12	110	5β-Pregnane-3α,15α,20β-triol

TABLE VII: Nuclear Magnetic Resonance Data for Urinary Metabolites II and VI[a] Following Ingestion of 15α-Hydroxyprogesterone by Normal Males.

Metabolite	Chemical Shifts					Confirmation of C-3 H
	C-19 CH_3	C-18 CH_3	C-21 C CH_3	CH_3COO	C-3 H	
II	0.99	0.64	2.12		3.9–4.2	Equatorial
II-diacetate	0.98	0.68	2.12	2.00, 2.05	4.8–5.2	Equatorial
VI-acetate	0.93	0.71	1.21[b]	1.99, 2.00, 2.02	4.7–5.0	
3β,15α-Dihydroxy-5β-pregnan-20-one	0.975[c]	0.650[c]				
3β,15α-Diacetoxy-5β-pregnan-20-one	0.975[c]	0.684[c]				
5β-Pregnane 3α,15β-20α-triacetate	0.942[c]	0.734[c]				
5β-Pregnane 3α,15α,20β-triacetate	0.942[c]	0.684[c]				

[a] The spectra were measured with a Varian HR-100 spectrometer and were displayed on precalibrated charts. Peak positions are expressed in parts per million (ppm) relative to a tetramethylsilane internal standard. [b] Doublet, $J = 6$ Hz. [c] These values were calculated for the compounds designated using Zürcher's tables (Zürcher, 1963).

spectively, were isolated and identified by the methods previously described. From pool II a residue was obtained (110 mg) which was chromatographed on 7 thin-layer chromatography plates using system H and a product migrating as a single band giving a positive phosphomolybdic acid reaction was obtained at an R_F of 0.45 (II). The material eluted weighed 95 mg and it was then chromatographed on a 10-g alumina column. Elution with 1.5% ethanol in benzene yielded 83 mg of crystalline residue which on crystallization from acetone–Skellysolve B afforded 45 mg of coarse plates, mp 192–195°. An aliquot (2.0 mg) of II was acetylated with [^{14}C]acetic anhydride and the product was chromatographed on an alumina column from which 2.1 mg of colorless oil was eluted with benzene–Skellysolve B (3:2). Crystallization of this material from acetone–Skellysolve B afforded 1.6 mg of fine needles, mp 152–155°. The specific activities of the crystals and the mother liquors were both 3.80×10^4 dpm/mg. From this specific activity it was possible to calculate that the unknown II had two acylable hydroxyl groups. The infrared spectrum (KBr) of the unknown II (Figure 3a) showed a hydroxyl band at 3350 cm^{-1}, a carbonyl band at 1710 cm^{-1} indicating the presence of a 20-ketone, and a band at 1032 cm^{-1} corresponding to an axial 3β-hydroxyl (Cole *et al.*, 1952). The infrared spectrum of the acetate (CS_2) indicated the absence of a hydroxyl band, a band at 1710 cm^{-1} corresponding to a 20-ketone, and a band at 1022 cm^{-1} compatible with the presence of an axial 3β-acetoxy (Jones and Herling, 1956). The mass spectrum indicated that 11 had a molecular weight of 334, which was consistent with the structural formula $C_{21}H_{34}O_3$. Table VII shows the results obtained from the nuclear magnetic resonance analysis of unknown II and its diacetate. The chemical shifts of the angular methyls (C-18, C-19) of the diol II and its diacetate corresponded to those of the 5β-steroid series. The presence of a three proton singlet at 2.12 ppm indicated that the 17-acyl group was still intact. The protons at C-3 and C-15 overlapped badly in the diol, but separated enough to be discernible in the diacetate. The signal of the 3 proton

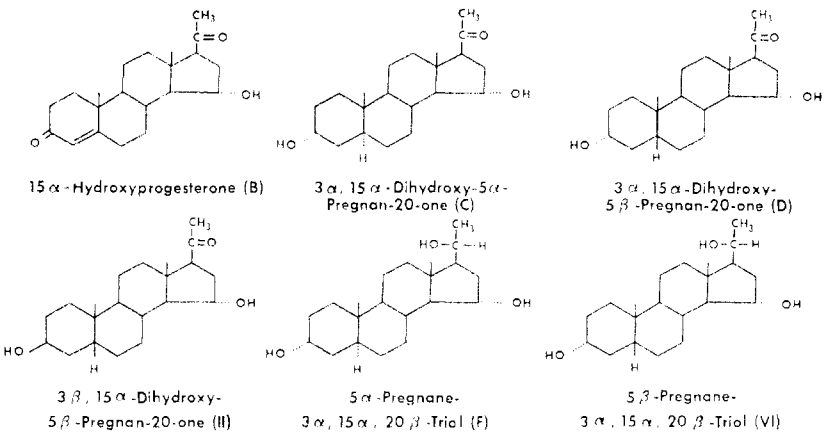

FIGURE 4: Structural formulas of the urinary metabolites of 15α-hydroxyprogesterone.

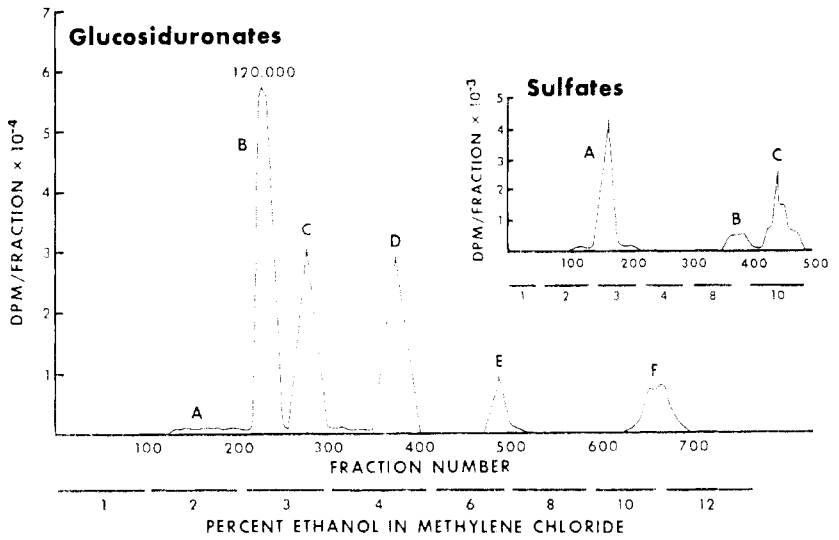

FIGURE 5: Silica gel column chromatography of the neutral extracts obtained following solvolysis (sulfates) and β-glucuronidase hydrolysis (glucosiduronates) of the urinary conjugates after the intravenous injection of [4-^{14}C]15α-hydroxyprogesterone into a normal subject in the 38th week of pregnancy.

appeared at 5.09 ppm, and at half-height had a width of 9 to 10 Hz while that of the 15 proton appeared at 4.89 ppm and was widest at 20 Hz, thus only partly overlapping the 3-proton signal. This permitted assignment of C-3-H as equatorial by comparison with the spectrum of D-diacetate (previous study) in which the only difference is at C-3. These results permit the assignment of the structure 3β,15α-dihydroxy-5β-pregnan-20-one to the metabolite isolated from pool II.

From pool VI a residue was obtained which weighed 110 mg. It was purified by chromatography on eight thin-layer chromatography plates using system J, and then on a 5-g silica gel column to yield 18.5 mg of oily material (VI) which could not be crystallized. Acetylation of an aliquot (12.0 mg) of VI and chromatography on an alumina column yielded 8.0 mg of a crystalline residue. Crystallization of this residue from acetone–Skellysolve B afforded 4.5 mg of small plates, mp 206–208°. The infrared spectrum of VI (Figure 3b) showed a hydroxyl band at 3350 cm^{-1} and no carbonyl bands indicating that both the Δ^4-3-keto and the 20-keto groups of the administered 15α-hydroxyprogesterone had been reduced. The infrared spectrum (CS_2) of the acetate of VI had no hydroxyl band, and a strong acetate band was observed at 1240 cm^{-1}. The mass spectrum of VI indicated that it had a molecular weight of 336, which was consistent with the structure $C_{21}H_{36}O_3$.

The results obtained from nuclear magnetic resonance analysis of VI-acetate are shown in Table VII. The positions of the angular methyl groups placed this compound in the 5β series along with II and its diacetate. The presence of a doublet at 1.21 ppm (J = 6 Hz) and an additional proton in the low-field region of the spectrum (along with signals of H-3 and H-15) indicated that the carbonyl had been reduced to an alcohol and that the three methyl signals in the vicinity of 2 ppm were all acetate methyls. The calculated values of the angular methyls for both the 20α- and 20β-acetates were quite close to the observed ones, thus it was not possible to distinguish between these two structures by nuclear magnetic resonance. Similarly, the differences calculated for the 3α- and 3β-acetates were too small to differentiate between the two structures on that basis alone. With the three overlapping proton signals in the same region, it is difficult to ascertain whether the C-3-H is axial or equatorial, but the data appear more consistent with its being axial and with an equatorial

TABLE VIII: Purification of Metabolites Isolated from Urine Following the Intravenous Injection of [4-^{14}C]15α-Hydroxyprogesterone in a Subject in the 38th Week of Pregnancy.

Radioactive Material Eluted from Silica Gel Column	dpm of ^{14}C Eluted	Wt (mg)	Sequence of Chromatography	Procedure Used for Proof of Radiochemical Purity	Metabolite Identified
			Sulfates		
A	8.70×10^4	35.6	Pc – A[a] → Pc – G → Sg[b] → acetylation[c]	Rid.[d]	3β,15α-Dihydroxy-5β-pregnan-20-one
			Glucosiduronates		
B	1.36×10^6	30.0	Pc – A → PC – B → Sg → acetylation	Rid.	15α-Hydroxyprogesterone
C	3.07×10^5	60.0	Pc – A → Pc – G → Sg → acetylation	Rid.	3α,15α-Dihydroxy-5α-pregnan-20-one
D	6.81×10^5	64.0	Pc – A → Pc – G → Sg → acetylation	Rid.	3α,15β-Dihydroxy-5β-pregnan-20-one
F	1.55×10^5	21.0	Pc – A		
FI[e]	4.20×10^4	4.5			5α-Pregnane-3α,15α,20β-triol
FII	1.05×10^5	5.2	Pc – G → Sg → acetylation	Rid.	

[a] Pc – A, paper chromatography using system A. [b] Sg, silica gel chromatography. [c] Acetylation with [^{3}H]acetic anhydride solution 1. [d] Rid., reverse isotope dilution. [e] The material in this fraction was not identified.

acetate. However, when an aliquot of 3α,15α-diacetoxy-5β-pregnan-20-one was reduced with $NaBH_4$ and the product acetylated, the resulting triacetate had an infrared spectrum almost identical with that of VI-triacetate. The only difference between the two spectra was an extra band at 1145 cm^{-1} in the spectrum of VI-triacetate, which might be due to the presence of impurities in this compound since only small amounts of VI-triacetate were available and the material could only be crystallized once. Because it was established that $NaBH_4$ reduction of 15α-hydroxylated 20 ketosteroids resulted in the formation of 20β-alcohols, metabolite VI was assigned the structure of 5β-pregnane-3α,15α,20β-triol.

In the two studies described above, it was possible to isolate and identify the following urinary metabolites: 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,-20β-triol, 3β,15α-dihydroxy-5β-pregnan-20-one, and 5β-pregnane-3α,15α,20β-triol. The structure of these metabolites are shown in Figure 4.

Metabolism of [4-^{14}C]15α-Hydroxyprogesterone by the Pregnant Female. A total of 13.4 μg of [4-^{14}C]15α-hydroxyprogesterone containing 4.90×10^6 dpm (specific activity 3.66×10^5 dpm/μg) was injected intravenously in a normal volunteer in the 38th weeks of pregnancy. Urine was collected for 6 days and the conjugates present in each day's urine were first hydrolyzed by sovolysis and then with β-glucuronidase. It was found that 6.8% of the injected radioactivity was excreted in the sulfate fraction and 66.1% in the glucosiduronate fraction of the urine. As was observed in the first study, practically all of this radioactivity was found in the first 4 day's urine and the extracts from this period were used for the isolation studies.

The sulfate fraction (450 mg and 3.36×10^5 dpm) was chromatographed on a 50-g silica gel column and three major pools of radioactivity were obtained as shown in Figure 5. Metabolites eluted in pools B and C from this column have not as yet been identified. The glucosiduronate fraction (978 mg and 3.24×10^6 dpm) was chromatographed on a 100-g silica gel column and the radioactive metabolites eluted are shown in Figure 5. Metabolites eluted in pools A and E from this column have not as yet been identified. The procedures used for the purification and identification of the metabolites isolated from the sulfate and glucosiduronate fractions of the urine are outlined in Table VIII. Each pool obtained from the initial silica gel column was purified by chromatography on paper in two systems and the material eluted from the second paper chromatogram was further purified by chromatography on a silica gel column, and then the metabolite was acetylated with [^{3}H]acetic anhydride, solution 1. Following acetylation, the appropriate carrier acetate, obtained in the previous study, was added and the mixture was crystallized to constant specific activity and constant $^3H/^{14}C$ ratio. In all cases except one (metabolite FII, glucosiduronate fraction) a suitable derivative was formed and it was crystallized to constant specific activity and $^3H/^{14}C$ ratio.

A derivative of 15α-acetoxyprogesterone, 15α-acetoxy-20β-hydroxy-pregn-4-en-3-one, was prepared as previously described, and derivatives of 3β,15α-diacetoxy-5β-pregnan-20-one, 3α,15α-diacetoxy-5α-pregnan-20-one, and 3α,15α-diacetoxy-5β-pregnan-20-one were prepared by reduction with $NaBH_4$. A second derivative of 5α-pregnane-3α,15α,20β-triol was not prepared. Using these methods, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from pool A of the sulfate fraction, and 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one,

TABLE IX: Specific Activity and Percentage Recovery of Urinary Metabolites from Injected [4-^{14}C]15α-Hydroxyprogesterone in a Pregnant and a Nonpregnant Subject.[a]

Metabolite Isolated	Pregnant Subject			Nonpregnant Subject		
	Specific Activity[b] (dpm/μg)		Minimal % of Injected Radioactivity in Urinary Product[c]	Specific Activity[d] (dpm/μg)		Minimal % of Injected Radioactivity in Urinary Product
	S[e]	G[f]		S	G	
15α-Hydroxyprogesterone	–[h]	1.56×10^4	18.7	–	3.31×10^5	5.8
3α,15α-Dihydroxy-5α-pregnan-20-one	–	7.90×10^3	2.9	–	3.45×10^5	7.1
3α,15α-Dihydroxy-5β-pregnan-20-one	–	9.70×10^3	7.5	–	3.52×10^5	7.4
3β,15α-Dihydroxy-5β-pregnan-20-one	8.66×10^8	–	1.0	3.06×10^5	–	0.4
5α-Pregnane-3α,15α,20β-triol	–	8.68×10^3	1.8	–	3.35×10^5	2.3

[a] The specific activity of the injected [4-^{14}C]15α-hydroxyprogesterone was 3.66×10^5 dpm/μg. [b] These specific activities were calculated from the specific activity of [^{3}H]acetic anhydride, solution 1, and the ^{3}H/^{14}C ratios obtained following crystallization of the final derivative. The specific activities and ^{3}H/^{14}C ratios agreed within at least 4%. [c] These values were computed from the final specific activity of the crystals and the weight of carrier added. [d] These specific activities were calculated from the specific activity of [^{3}H]acetic anhydride, solution 2, and the ^{3}H/^{14}C ratios obtained following crystallization of the final derivative. The agreement of specific activities and ^{3}H/^{14}C ratios was within 7% for 15α-hydroxyprogesterone and 4% or better for the other metabolites. [e] S, sulfate. [f] G, glucosiduronate. [h] The dashes indicate the metabolite was not isolated.

and 5α-pregnane-3α,15α,20β-triol were isolated from pools B, C, D, and F, respectively, of the glucosiduronate fraction. The specific activities of the isolated metabolites are shown in Table IX. These specific activities were calculated using the specific activity of the [^{3}H]acetic anhydride and the final ^{3}H/^{14}C ratios obtained following crystallization of the derivative.

Metabolism of [4-^{14}C]15α-Hydroxyprogesterone by the Nonpregnant Female. To a normal 23-year-old subject in the luteal phase of the menstrual cycle was administered intravenously 10.2 μg of [4-^{14}C]15α-hydroxyprogesterone containing 3.72×10^6 dpm (specific activity 3.66×10^5 dpm/μg). 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 4.8% of the injected radioactivity was excreted in the sulfate fraction and 57.6% in the glucosiduronate fraction of the urine. Since virtually all of the radioactivity was found in the first 4 day's urine, the extracts from this period were used for the isolation studies described.

The sulfate fraction (248 mg and 1.78×10^5 dpm) was chromatographed on a 50-g silica gel column and four major pools of radioactivity, pools A–D, were obtained as described in the previous study. Metabolites eluted in pools B, C, and D have not as yet been identified. The glucosiduronate fraction (339 mg and 2.14×10^6 dpm) was chromatographed on a 60-g silica gel column and six major pools of radioactivity, pools A–F, were obtained. Metabolites in pools A and E have not as yet been identified. Purification of the metabolites and their identification and proof of radiochemical purity were achieved by procedures identical with those described for the metabolites isolated from pregnancy urine except that [^{3}H]acetic anhydride solution 2 was used for acetylation of the residues following the last chromatographic separation. Using these methods, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from pool A of the sulfate fraction, and 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, and 5α-pregnane-3α,-15α,20β-triol were isolated from pools B, C, D, and F, respectively, of the glucosiduronate fraction. The specific activities of the isolated metabolites are shown in Table IX.

Discussion

In order to study the metabolism of a tracer amount of labeled 15α-hydroxyprogesterone administered to a pregnant female, it was first necessary to obtain sizeable amounts of the urinary metabolites of this steroids, and to establish their identity. This was achieved in the first two studies in which five urinary metabolites of 15α-hydroxyprogesterone, namely, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, 3β,15α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,15α,20β-triol, and 5β-pregnane-3α,15α,20β-triol were isolated and identified.

The last two studies dealt with the metabolism of tracer doses of [4-^{14}C]15α-hydroxyprogesterone injected intravenously into a pregnant subject in the 38th week of gestation and in a nonpregnant subject in the luteal phase of the menstrual cycle. In both studies virtually all of the radioactivity excreted was found in the first 4 day's urine. Most of the radioactive conjugates were hydrolyzed with β-glucuronidase while only a small percentage of the excreted radioactive

metabolites was extracted following solvolysis. It is possible that some of the metabolites present in the glucosiduronate fraction were originally excreted as double conjugates with both sulfuric and glucuronic acids. As is evident from Table IX, 3β,15α-dihydroxy-5β-pregnan-20-one was isolated from the sulfate fraction in both studies, and 15α-hydroxyprogesterone, 3α,15α-dihydroxy-5α-pregnan-20-one, 3α,15α-dihydroxy-5β-pregnan-20-one, and 5α-pregnane-3α,15α,20β-triol were isolated from the glucosiduronate fraction of the urine.

The specific activities of the metabolites isolated from the urine of the pregnant subject (Table IX) were all from 25 to 45 times lower than the specific activity of the injected 15α-hydroxyprogesterone demonstrating that all of these steroids are normal constituents of human late pregnancy urine. However, the specific activities of the metabolites isolated from the urine of the nonpregnant subject were almost identical with the specific activity of the injected 15α-hydroxyprogesterone, demonstrating that these steroids are not normally excreted in the urine of such subjects. In parallel studies we have found (Giannopoulos *et al.*, 1969) that 15α-hydroxyprogesterone is not present in the urine during the luteal phase of the normal menstrual cycle, but is excreted during the second and third trimesters of pregnancy. It was also shown (Giannopoulos *et al.*, 1969) that the conversion of progesterone to 15α-hydroxyprogesterone occurs during late pregnancy in the fetoplacental unit but not in the maternal circulation. Therefore, the results obtained in the pregnant and nonpregnant female are in agreement with these findings.

The specific activities of all the metabolites isolated from the urine of the pregnant subject were very similar with the exception of 15α-hydroxyprogesterone whose specific activity was significantly higher (Table IX). These results suggest that the metabolites of 15α-hydroxyprogesterone may in part be derived from precursors other than 15α-hydroxyprogesterone, such as 15α-hydroxypregnenolone which has not as yet been isolated. It must be borne in mind that [4-^{14}C]15α-hydroxyprogesterone was injected into the maternal circulation and that during late pregnancy it is synthesized in the fetoplacental unit (Giannopoulos *et al.*, 1969). It is therefore possible that 15α-hydroxyprogesterone is metabolized in the fetoplacental unit and that the metabolites cross the placenta and enter into the maternal compartment. If that is true, differences in the relative proportion of the fetal products and the metabolites of [4-^{14}C]15α-hydroxyprogesterone in the maternal compartment may explain the observed differences in the specific activities of the urinary metabolites.

Assuming that urinary 15α-hydroxyprogesterone is a unique metabolite of endogenous 15α-hydroxyprogesterone, the urinary production rate of this steroid in the maternal compartment may be calculated using the expression for a single compartment model (Pearlman *et al.*, 1954). Using this simple model the production rate was calculated to be 79 μg/day. However, the calculated production rate may not reflect the true value and should be considered as a maximal value because of the possibility that other steroids, such as 15α-hydroxypregnenolone (and its sulfate), may serve as important precursors of 15α-hydroxyprogesterone.

It is important to note that the major urinary metabolites of 15α-hydroxyprogesterone are the two dihydroxy ketones, namely 3α,15α-dihydroxy-5α-pregnan-20-one and 3α,15α-dihydroxy-5β-pregnan-20-one, while very small amounts of metabolites reduced at C-20 were isolated. In addition, a large proportion of the injected 15α-hydroxyprogesterone was excreted unchanged in the urine. These results suggest that the 15α-hydroxyl group inhibits the reduction of the 20-ketone and the Δ^4-3-keto group of the steriod nucleus. As shown in Table IX, some of the reduced metabolites of 15α-hydroxyprogesterone are 5α-steroids. The overall ratio of 5α- to 5β-reduced metabolites was found to be approximately 0.55. The generalization has been made that Δ^4-3-keto C_{21} steroids are primarily reduced *in vivo* in man to the 5β form (Dorfman, 1954). That the 15α-hydroxyl group in the C_{21} steroids influence the reduction in the A and B rings in favor of the 5α-metabolites was demonstrated by Ruse and Solomon (1966) who found that the 5α-urinary metabolites of labeled 16α-hydroxyprogesterone injected into a pregnant subject were present in amounts greater than would be expected for steroids of the C_{21} series. It seems, therefore, that the effect of the 15α-hydroxyl group on the reduction of the Δ^4-3-keto group of C_{21} steroids is similar to the effect of the 16α-hydroxyl constituent. Both hydroxyl groups favor an increased 5α/5β ratio of the reduced urinary metabolites but the 15α-hydroxy group has a greater inhibitory effect on the reduction of the Δ^4-3-keto. In addition, reduction of the 20 ketone of 16α-hydroxyprogesterone favors the 20α-alcohols since only 20α-triols of 16α-hydroxyprogesterone were isolated (Ruse and Solomon, 1966). On the other hand, the only C_{20}-reduced metabolites of 15α-hydroxyprogesterone isolated in these studies were 5α-pregnane-3α,15α,20β-triol and 5β-pregnane-3α, 15α,20β-triol, indicating that the 15α-hydroxyl groups directs the reduction at C-20 toward the 20β-alcohols.

It is of interest to note that 15α hydroxylation of neutral steroids is not confined to the human fetoplacental unit. Schneider (1965) found that liver slices of the American bullfrog (*Rana catesbiana*) was capable of converting deoxycorticosterone into a number of hydroxylated products, among which were 15α- and 15β-hydroxydeoxycorticosterone. Recently, it has been found that the feces of conventional rats contain 3α,15α-dihydroxy-15α-pregnan-20-one as the predominant C-21 steroid (Gustafsson, 1968a,b) and that this metabolite is also present in the feces of the germ-free rat. In addition 3α,11β,15α,21-tetrahydroxy-5α-pregnan-20-one and 3α,15α,-21-trihydroxy-5α-pregnane-11,20-dione have been isolated as the major C-21 steroids from the feces of the germ-free rat (Gustafsson and Sjövall, 1968), and in trace amounts from the urine of the germ-free rat (Gustafsson, 1968a,b). Very recently 15α,20β-dihydroxypregn-4-en-3-one (10 μg/beetle) was isolated from the secretion of the prothorax scent gland of the water beetle (*Platambus macilatus*) by Schildknecht *et al.* (1969). As a result of these findings it can be concluded that 15α hydroxylation of neutral steroids may be a common metabolic process in a large number of species.

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Isolation of 15α-Hydroxyandrostenedione and 15α-Hydroxytestosterone from Human Pregnancy Urine*

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ABSTRACT: Five pools of urine were obtained from normal subjects in the third trimester of pregnancy. Purified [4-^{14}C]-15α-hydroxyandrostenedione or [4-^{14}C]15α-hydroxytestosterone was added to the urine as a recovery marker and the steroids were isolated and identified. The amount of 15α-hydroxyandrostenedione excreted in the urine ranged from 4.4 to 5.2 μg per day and that of 15α-hydroxytestosterone from 0.48 to 0.64 μg per day, measured by the isotope derivative procedure using [^{3}H]acetic anhydride. These findings demonstrate that 15α-hydroxyandrostenedione and 15α-hydroxytestosterone are normal excretory products in the third trimester of human pregnancy.

The formation of 15α-hydroxyestrogens from phenolic precursors in the pregnant female and by the newborn has been well documented (Schwers *et al.*, 1965; Hagen *et al.*, 1965; Gurpide *et al.*, 1966). When previable human fetuses were perfused with labeled androstenedione and testosterone, labeled 15α-hydroxyestradiol[1] was detected in the fetal liver (Mancuso *et al.*, 1968), thereby demonstrating that neutral C_{19} steroids can serve as precursors of 15α-hydroxyestrogens. In these latter studies C_{19} 15α-hydroxysteroids could not be detected in the fetal tissues and the authors therefore concluded that 15α hydroxylation occurred after aromatization. With the finding that 15α-hydroxyandrostenedione can be aromatized by human placental tissue (Stern *et al.*, 1968) it appeared likely that 15α-hydroxyestrogens may be formed from 15α-hydroxy neutral precursors in an analogous manner to the

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[1] The following trivial names and abbreviations are used: androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-en-3-one; dehydroisoandrosterone sulfate, 17-oxoandrost-5-en-3β-yl sulfate; 15α-hydroxyandrostenedione, 15α-hydroxyandrost-4-ene-3,17-dione; 15α-hydroxytestosterone, 15α,17β-dihydroxyandrost-4-en-3-one; 15α-hydroxyprogesterone, 15α-hydroxypregn-4-ene-3,20-dione; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; 15α-hydroxyestradiol, estra-1,3,5(10)-triene-3,15α,17β-triol; 15α-hydroxyestriol, estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol; DDQ, 1,2-dichloro-5,6-dicyanobenzoquinone.