The *in Vivo* Metabolism of 16α-Hydroxyprogesterone*

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ABSTRACT: Labeled 16α-hydroxyprogesterone was prepared by the microbiological hydroxylation of [4-^{14}C]-progesterone or [7-^{3}H]progesterone. From the urine of a normal male who was injected with labeled 16α-hydroxyprogesterone and then given 1.5 g of the steroid *per os*, the following labeled metabolites were isolated and identified: 3α-hydroxy-5β,17α-pregnan-20-one, 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, 5α-pregnane-3α,16α,-20α-triol, and 5β-pregnane-3α,16α,20α-triol.

When a trace amount of [7-^{3}H]16α-hydroxyprogesterone was administered to a subject in the third trimester of pregnancy, four of these metabolites were isolated from the urine but labeled 5α-pregnane-3α,16α,20α-triol could not be found.

A small number of neutral C-21, 16α-hydroxy steroids have been isolated from human urine (Hirschmann and Hirschmann, 1945; Hirschmann and Hirschmann, 1950; Lieberman *et al.*, 1953; Neher *et al.*, 1959; Hirschmann *et al.*, 1961; Fukushima *et al.*, 1961; Bongiovanni, 1962). For the most part, the precursors of these steroids have been a matter of speculation. It has been reported that 16α-hydroxyprogesterone[1] is present in human umbilical blood (Zander *et al.*, 1962) and that the human fetal adrenal (Villee *et al.*, 1961), the human adult adrenal (Villee *et al.*, 1962; Ward and Grant, 1963; Villee, 1964), the ovary (Warren and Salhanick, 1961), and the placenta (Little *et al.*, 1963) are capable of introducing a 16α-hydroxyl group on C-21 steroids. This paper describes the synthesis of ^{3}H- and ^{14}C-labeled 16α-hydroxyprogesterone and presents evidence that 16α-hydroxyprogesterone can serve as a precursor of urinary 16α-hydroxylated steroids.

Methods

Counting. Aliquots of samples to be counted were dried under nitrogen in 5-dram vials (Wheaton Glass Co.) and dissolved in 5 ml of toluene containing 0.3% (w/v) of 2,5-diphenyloxazole and 0.01% of 1,4-bis-2 (5-phenyloxazolyl)benzene. The samples were counted in one of two models of the Packard Tri-Carb liquid scintillation spectrometer. Efficiency of counting by the Model 314-X was about 18% for tritium and 60% for ^{14}C, when counted separately. Corresponding efficiencies of the Model 3002 were 40% and 92%, respectively. The ^{3}H and ^{14}C content of doubly labeled compounds were determined by the discriminator ratio method of Okita *et al.* (1957) as modified by Ulick (1961). The counts of quenched samples were corrected by the use of an internal standard.

Solvents. Solvents were distilled prior to use, with the exception of Skellysolve B and C (Skelly Oil Co., Kansas City, Mo.).

Chromatography. Solvent systems used for chromatography are shown in Table I. Celite was prepared as described previously (Watanabe *et al.*, 1963) and covered with mobile phase prior to packing into columns. Holdback volumes of Celite columns were de-

TABLE I: Solvent Systems Used in Chromatography.

System	Solvents
A	*n*-Hexane–methanol–water (100:90:10)
B	*n*-Heptane–ethyl acetate–methanol–water (50:50:65:35)
C	*n*-Hexane–ethyl acetate–methanol–water (50:50:65:35)
D	Benzene–cyclohexane–methanol–water (1:2:3:3)
E	*n*-Heptane–propylene glycol
F	Skellysolve C–propylene glycol
G	Toluene–propylene glycol
H	Ethyl acetate–*n*-hexane (7:3)
J	Ethyl acetate–*n*-hexane (2:1)
K	Chloroform–methanol–water (90:10:1)

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[1] The following trivial names and abbreviations are used: progesterone, pregn-4-ene-3,20-dione; pregnenolone, 3β-hydroxypregn-5-en-20-one; 16α-hydroxyprogesterone, 16α-hydroxypregn-4-ene-3,20-dione; 16α-hydroxypregnenolone, 3β,16α-dihydroxypregn-5-en-20-one; pregnanolone, 3α-hydroxy-5β-pregnan-20-one; isopregnanolone, 3α-hydroxy-5β,17α-pregnan-20-one; 16-dehydropregnanolone, 3α-hydroxy-5β-pregn-16-en-20-one; tlc, thin layer chromatography.

termined with Sudan IV, as described by Johnson (1959), and averaged 1.2 ml/g of support. The effluent from the columns was collected at the rate of 0.2 to 0.4 ml/g of Celite/hr. Thin layer chromatograms (tlc) were made with Silica gel G (Merck), spread in layers 20 × 20 cm and 1 mm thick. Steroids were located on papers and thin layers by their absorption of ultraviolet light or by their reaction with phosphomolybdic acid (Kritchevsky and Kirk, 1952). Radioactivity on paper chromatograms was located with a scanner (Vanguard, Model 880). Alumina (Harshaw Scientific) was prepared according to the procedure described by Solomon *et al.* (1960) and deactivated by the addition of 5% (v/w) of water. Silica gel (Davison Chemical, 100–200 mesh) was used as purchased. Migration rates and elution volumes of various steroids are shown in Table II. All the chromatograms were developed at room temperature.

TABLE II: Rates of Migration and Elution Volumes of Steroids in the Chromatographic Systems Used.

Paper Systems			
Steroid	System	Time (hr)	(cm)[a]
Isopregnanolone	E	12	20
3α,16α-Dihydroxy-5α-pregnan-20-one	G	35	35
5α-Pregnane-3α,16α,20α-triol	G	40	16
3α,16α-Dihydroxy-5β-pregnan-20-one	G	50	25
5β-Pregnane-3α,16α,20α-triol	G	50	17
16α-Hydroxyprogesterone	G	10	16

Celite Systems		
	System	Elution Volume (hbv)[a]
Isopregnanolone	A	4, 5
3α,16α-Dihydroxy-5α-pregnan-20-one	C	5
5α-Pregnane-3α,16α-20α-triol	D	6
3α,16α-Dihydroxy-5β-pregnan-20-one	D	7, 8
5β-Pregnane-3α,16α,20α-triol	D	8, 9
16α-Hydroxyprogesterone	B	7, 8

[a] These represent the average values from multiple runs at room temperature. The hbv refers to holdback volumes.

Infrared spectra were determined with the use of a Model 221 Perkin-Elmer spectrometer. Samples were examined using the KBr disk technique or in CS_2 solutions. Melting points were determined on a Kopfer block (H. O. Post Scientific, Long Island, N. Y.) and were corrected.

Steroid conjugates were hydrolyzed with Glusulase (100,000 units of β-glucuronidase and 50,000 units of sulphatase/ml, Endo Laboratories, Garden City, N. Y.). Neutral extracts of urine were prepared by extraction with 1.5–2 volumes of ethyl acetate. The organic phase was washed with NaOH and with water until neutral, dried over Na_2SO_4, and evaporated *in vacuo*. Steroid conjugates were extracted from urine using the method described by Edwards *et al.* (1953).

Preparation of Labeled 16α-Hydroxyprogesterone. ^{14}C- and tritium-labeled 16α-hydroxyprogesterone were prepared by incubation of labeled progesterone with hydroxylating microorganisms.

[4-^{14}C]16α-Hydroxyprogesterone. The incubation of [4-^{14}C]progesterone with the microorganism and extraction with ethyl acetate were done by the CIBA Co. of Basel, Switzerland, through the courtesy of Dr. R. Neher. Progesterone, 180 mg, was mixed with 80 μc of [4-^{14}C]progesterone (The Radiochemical Centre, Amersham, England) and incubated with an hydroxylating organism (CIBA-A-7894). The neutral ethyl acetate extract prepared after incubation weighed 258 mg and contained 1.2×10^8 cpm. This extract was chromatographed on Celite in system B and on paper in system G and then on a 35-g silica gel column starting with benzene and continuing with increasing concentrations of ethyl acetate in benzene. The product eluted with benzene–ethyl acetate (1:4) was crystallized twice from ether–acetone and once from acetone–Skellysolve B, to yield 7.1 mg of coarse needles, mp 215–220° (standard mp 217–220°, obtained from Dr. S. Bernstein, Lederle Laboratories, Pearl River, N. Y.). An infrared spectrum (KBr) was identical with that of authentic 16α-hydroxyprogesterone. The specific activity of the third batch of crystals was 3.7×10^5 cpm/mg. These crystals were used in the first *in vivo* study.

[7-^{3}H]16α-Hydroxyprogesterone. The organism used in this preparation was a strain of *S. Roseochromogenus* (SC-1624), supplied by Dr. J. Fried of the Squibb Institute for Medical Research, who also suggested the method of incubation. A 2-week growth on Gould's agar was transferred to 50 ml of liquid medium in a 250-ml flask. The medium consisted of 30 g of glucose, 20 g of soybean meal, 2.2 g of hydrogenated soybean oil, and 2.5 g of $CaCO_3$, all in 1 l. of water. The flask was incubated for 72 hr at 25° and then a 10% transfer was made to a second flask containing the same medium. [7-^{3}H]Progesterone (0.5 mc weighing 0.016 mg, New England Nuclear Corp.) was diluted with 7.8 mg of progesterone carrier. The labeled steroid was dissolved in 0.25 ml of dimethylformamide and was added to the second flask and incubated for 48 hr at 25°. The calculated specific activity of the substrate was 2.8×10^7 cpm/mg.

Following incubation a methylene chloride extract was prepared. The extract was purified by tlc in system H. An ultraviolet absorbing material, 3.5 cm from the origin, was eluted and chromatographed on paper in

system G, yielding a residue which contained 1.4×10^6 cpm. A portion of this residue was mixed with carrier 16α-hydroxyprogesterone and crystallized from acetone–Skellysolve B, acetone–ether, and acetone–Skellysolve B. The specific activities of the crystals and mother liquors are shown in Table III. The determined and calculated specific activities were in good agreement, indicating a satisfactory degree of radiochemical purity.

TABLE III: Proof of Radiochemical Purity of [7-^{3}H]16α-Hydroxyprogesterone Prepared Microbiologically.

	Specific Activities (cpm/mg)	
	[7-^{3}H]16α-Hydroxyprogesterone[a]	
Crystallization	Crystals	M_L[b]
1	1970	2060
2	2040	2090
3	2010	1990
Calcd	2010	

[a] An aliquot containing 1.2×10^5 cpm was mixed with 59.7 mg of carrier 16α-hydroxyprogesterone prior to crystallization. The carrier was obtained from Dr. Seymour Bernstein, Lederle Laboratories, and had a mp of 217–220°. [b] M_L, mother liquors.

Preparation of 3α-Hydroxy-5β,17α-pregnan-20-one (Isopregnanolone). Isopregnanolone was prepared by isomerization of pregnanolone. A solution of 201 mg of pregnanolone in 30 ml of 5% KOH in methanol (w/v) was refluxed for 5 hr. A neutral ethyl acetate extract was prepared and chromatographed on a 20-g alumina column. Elution with *n*-hexane–methylene chloride (1:1) yielded 151 mg of starting material. The residue eluted with methylene chloride was crystallized from ether–*n*-heptane, giving 31 mg of fine needles, mp 147–149°. An infrared spectrum (CS_2) was identical with that of an authentic sample of isopregnanolone provided by Dr. David Fukushima. The yield of isopregnanolone was 15%.

Experimental Section and Results

The metabolism of 16α-hydroxyprogesterone has been studied in two volunteers. The first experiment was designed to provide urinary metabolites in amounts sufficient for identification. The subject was given an oral load of 16α-hydroxyprogesterone as well as an intravenous injection of labeled steroid. The subject of the second study, a patient in the third trimester of pregnancy, was given the labeled steroid alone. In both experiments the labeled 16α-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 steroid.

Metabolism of [4-^{14}C]16α-Hydroxyprogesterone by the Normal Male. The subject, a 36-year-old normal male, was given 1.3×10^6 cpm of [4-^{14}C]16α-hydroxyprogesterone in 2 mg by intravenous injection. Several weeks later, 1.5 g of 16α-hydroxyprogesterone was administered orally, in 100-mg doses, over a period of 48 hr. Urine was collected for 4 days after the injection of the labeled steroid (days 1–4) and for 3 days after the first oral dose of carrier steroid (days 5–7).

The individual urines were extracted by the procedure of Edwards *et al.* (1953), using a total of 1.5 volumes of ether–ethanol (3:1) for each day's urine. The extracts of days 1–4 were each dissolved in 160 ml of 0.5 N acetate buffer (pH 5.2) and the steroid conjugates were hydrolyzed by the addition of 2 ml of Glusulase and incubation at 37° for 60–80 hr. The extracts of days 5–7 were pooled and processed in a similar manner, using 500 ml of buffer, 6 ml of Glusulase, and an 18-hr incubation. Following incubation, a neutral ethyl acetate extract of each hydrolysate was prepared.

The combined neutral extract of the 7-days' urine weighed 2.7 g and contained 3.9×10^5 cpm. It was chromatographed on a 300-g silica gel column, using the solvent series *n*-hexane, benzene, ether, and ethyl acetate. No useful resolution was achieved with this procedure and fractions containing ^{14}C were pooled, yielding a residue which weighed 1.7 g and contained all the radioactivity applied to the column. This residue was then chromatographed on a 100-g silica gel column, using methylene chloride and increasing concentrations of ethanol in methylene chloride. The residue eluted by 2 and 4% ethanol (pool A) weighed 750 mg and contained 1.1×10^5 cpm. A second residue (pool B), eluted by 8 and 10% ethanol, weighed 420 mg and contained 1.8×10^5 cpm. The residue of pool A was purified by tlc, using system J. Elut on and counting of the residue of 1-cm strips of trial chromatograms showed that the bulk of the radioactivity was in an area between a line 6 cm from the origin and a distinctive band of red pigment at about 13 cm. The extract was chromatographed on 30 thin layer plates. Elution of the area described yielded an oily purple residue weighing 330 mg and containing 6.7×10^4 cpm. This residue was chromatographed on a 130-g Celite column in system A. A plot of radioactivity *vs.* fraction number is shown in Figure 1.

The residue of tubes 55–100, which weighed 43 mg and contained 4.2×10^4 cpm, was chromatographed on paper in system E. Elution of the area opposite standard isopregnanolone yielded a residue which weighed 24.4 mg and contained 2.1×10^4 cpm. This residue was further purified by chromatography on a 1-g silica gel column and crystallized from ether–Skellysolve B, giving 7.3 mg of fine needles: mp 147–149°, standard isopregnanolone mp 147–149°, mmp 147–148°. An infrared spectrum was identical with that of authentic isopregnanolone. The specific activity of the metabolite was 1.4×10^3 cpm/mg.

The residue of pool B from the silica gel column was

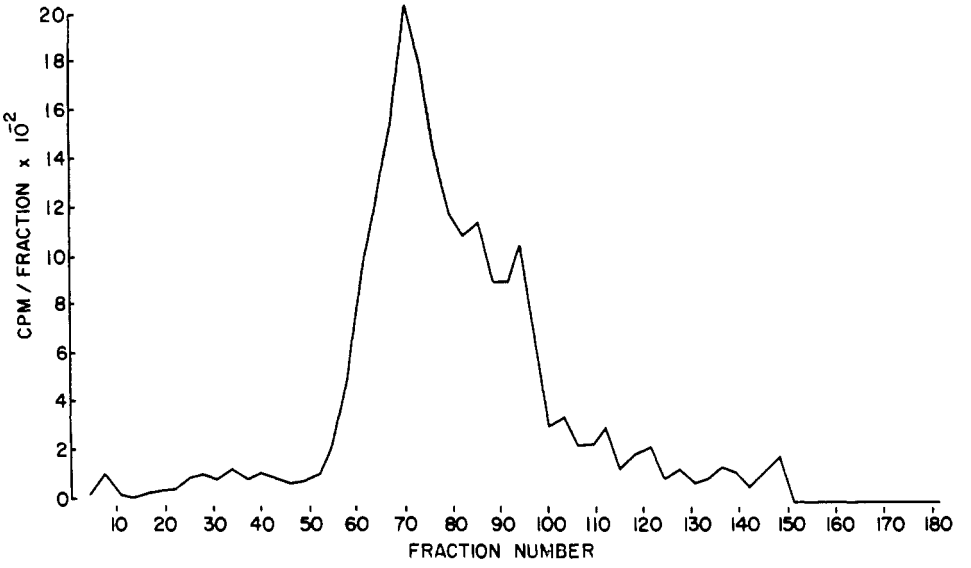

FIGURE 1: Counts per minutes per fraction times 10^{-2} *vs.* fraction number following chromatography of Fraction A on a Celite partition column.

purified by tlc in system K. Elution of the area between the standards cortisol and 16α-hydroxyprogesterone, yielded a residue which weighed 158 mg and contained 1.17×10^5 cpm. It was chromatographed on a 160-g Celite column in system D. Two major peaks of radioactivity were found in the fractions collected as shown in Figure 2. The first peak of radioactive material, B I, weighed 31.1 mg and contained 2.6×10^4 cpm. The residue from the tubes containing the second peak, B II, weighed 36.1 mg and contained 7.0×10^4 cpm.

The residue of B I was chromatographed on paper in system G. Two areas of radioactivity were found at average distances of 16 (B III) and 23 cm (B IV). The first, B III, yielded a residue which weighed 14.4 mg and contained 8.2×10^3 cpm. The residue from the second area, B IV, weighed 21.4 mg and contained 1.1×10^4 cpm.

The residue of B III was percolated through a 1-g silica gel column and after crystallization from acetone–Skellysolve B, 4.6 mg of fine needles were obtained: mp 230–232°, specific activity 1.3×10^3 cpm/mg. The infrared spectrum of this crystalline material indicated that it did not contain carbonyl groups. The crystals (3 mg) were acetylated in 0.2 ml of pyridine and 0.1 ml of acetic anhydride at room temperature overnight. The infrared spectrum (CS_2) of the product was identical with that of authentic 5α-pregnane-3α,16α,20α-triol triacetate, generously donated by Dr. Hans Hirschmann. We were not able to crystallize the triacetate. The melting point of the free steroid agreed well with the reported value, 230–232° (Hirschmann *et al.*, 1961). It was not possible to obtain a sample of the free compound for direct comparison.

The residue of B IV was percolated through a small silica gel column and afforded 9.2 mg of colorless oil. The infrared spectrum (KBr) of this material was identical with that of authentic 3α,16α-dihydroxy-5α-pregnan-20-one, obtained from Dr. R. Neher. Crystallization from acetone–Skellysolve B gave 4.6 mg of fine needles: mp 209–211°, mmp 208–210°, standard mp 210–211°. Its specific activity was 1.1×10^3 cpm/mg.

Fraction B II, from the Celite column, was chromatographed on paper in system G. Two areas of radioactivity were found at average distances of 15 (B V) and 25 cm (B VI). The eluate of the area B V contained 13.8 mg of yellow oil, and the eluate of the area B VI contained 19.1 mg of colorless oil.

Residue B V was percolated through silica gel and crystallized from acetone–Skellysolve B, yielding 3.7 mg of fine needles. The infrared spectrum of the crystals was identical with that of authentic 5β-pregnane-3α,16α,20α-triol, provided by Dr. Hans Hirschmann. Recrystallization from acetone–methanol and acetone gave 0.5 mg of fine needles: mp 224–226°, mmp 227–228°, standard mp 227–228°. The specific activity of this metabolite was 2.4×10^3 cpm/mg.

The residue from fraction B VI was crystallized from acetone–Skellysolve B to give 9.4 mg of fine needles: mp 211–212°, specific activity 2.8×10^3 cpm/mg. The crystals (3 mg) were acetylated in pyridine and acetic anhydride (2:1) at room temperature overnight. The infrared spectrum (CS_2) of the product was identical with that of 3α,16α-dihydroxy-5β-pregnan-20-one diacetate provided by Dr Hans Hirschmann. The diacetate was crystallized from acetone–methanol to yield 1.9 mg of small plates: mp 129–130°, mmp 129–130°, standard mp 129–130°.

Metabolism of [7-^{3}H]16α-Hydroxyprogesterone by the Pregnant Female. A normal 23-year-old subject in the 34th week of pregnancy was given [7-^{3}H]16α-hydroxyprogesterone by intravenous injection. The injected steroid weighed 165 μg and contained 3.60

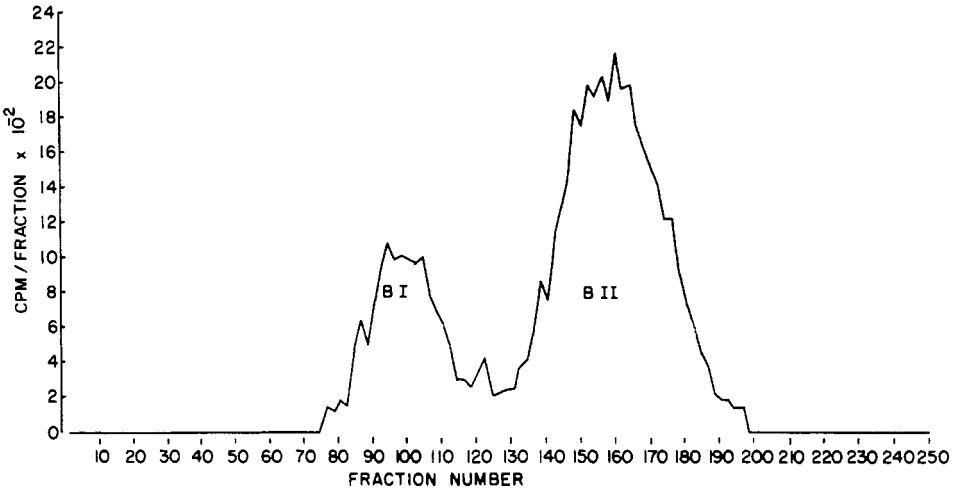

FIGURE 2: Counts per minute per fraction $\times 10^{-2}$ *vs.* fraction number following chromatography of Fraction B on a Celite partition column.

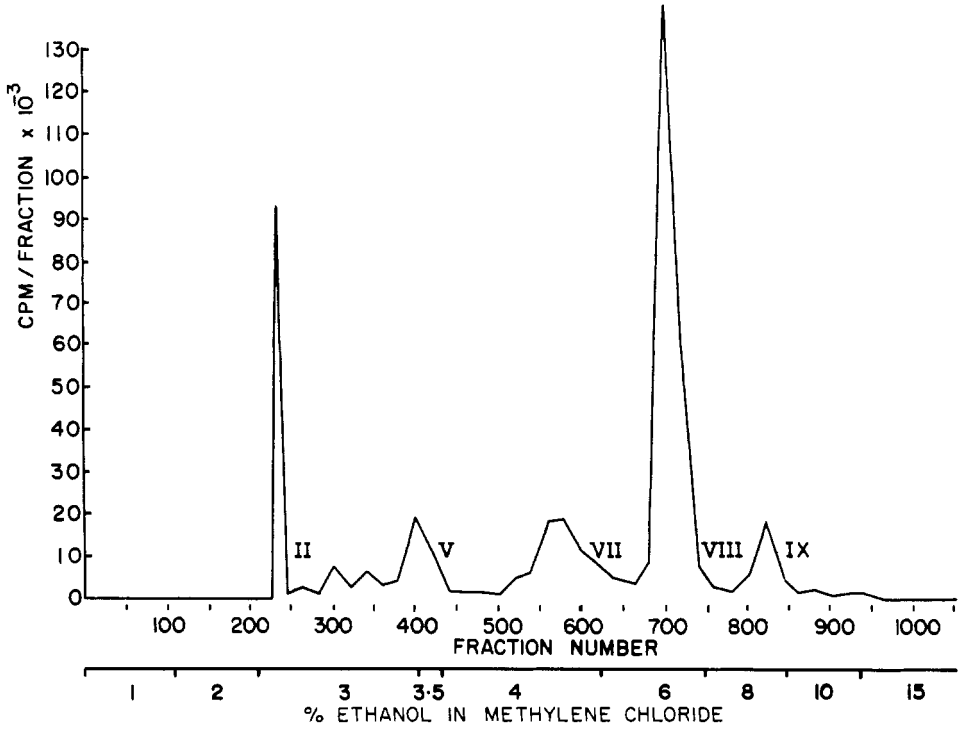

FIGURE 3: Silica gel chromatography of neutral extract from pregnancy urine after administration of [7-^{3}H]16α-hydroxyprogesterone.

$\times 10^6$ cpm. Urine was collected for 4 days, adjusted to pH 5.2, acetate buffer was added, and the mixture was incubated with Glusulase, 0.5% (v/v) for 4 days at 37°. A neutral ethyl acetate extract was prepared which weighed 1.63 g and contained 2.3×10^6 cpm. It was chromatographed on a 225-g silica gel column, using increasing concentrations of ethanol in methylene chloride. The effluent from the column was collected in 10-ml fractions at the rate of 30–40 ml/hr. Five major peaks of radioactivity were found as shown in Figure 3.

ISOLATION OF ISOPREGNANOLONE. The residue of peak II (Figure 3) was chromatographed on a 30-g Celite column in system A. The residue of fractions 16–25 weighed 14.4 mg and contained 7.2×10^4 cpm. This material was chromatographed on a 2-g alumina column, starting with *n*-hexane–methylene chloride (1:1). Elution with *n*-hexane–methylene chloride (1:1,

4:6, 3:7) gave 6.1 mg of crystalline material having an infrared spectrum identical with that of pregnanolone. The isolated pregnanolone was devoid of radioactivity. Elution with *n*-hexane–methylene chloride (3:7) and methylene chloride yielded 1.9 mg of yellow oil containing 6.3×10^4 cpm. A portion of this material, 0.5 mg, was acetylated and the acetate was mixed with 20.1 mg of isopregnanolone acetate. The mixture was percolated through a small alumina column and the material, 17.0 mg and 7.5×10^3 cpm, was crystallized from acetone–*n*-heptane, ether–*n*-heptane, and acetone–*n*-heptane. The specific activities of the crystals and mother liquors are shown in Table IV.

TABLE IV: Radiochemical Purity of Isopregnanolone Isolated from the Urine of a Pregnant Subject.

Crystallization	Specific Activity (cpm/mg) Crystals	Mother Liquors
1	410	400
2	370	360
3	360	360
Calcd[a]	440	

[a] After chromatography through a small alumina column 17.0 mg of isopregnanolone acetate containing 7.5×10^3 cpm was crystallized.

ISOLATION OF 3α,16α-DIHYDROXY-5α-PREGNAN-20-ONE. The residue of peak VII (Figure 3) of the original silica gel column weighed 97.4 mg and contained 2.7×10^5 cpm. It was chromatographed on an 80-g Celite column in system C and a single peak of radioactivity was eluted. The residue containing the radioactivity (41.7 mg and 2.7×10^5 cpm) was chromatographed on paper in system G for 25 hr. Two areas of radioactivity were located on the chromatograms at an average distance of 18 and 30 cm, respectively. The less polar material was eluted and it weighed 24.3 mg and contained 1.3×10^5 cpm. This material was percolated through a small silica gel column yielding 17 mg of colorless oil. An infrared spectrum of this oil (KBr) matched that of the final crystalline material. Crystallization from acetone–Skellysolve B gave only 6.0 mg of fine needles, and attempts at recrystallization were unsuccessful. Accordingly, the residue of the mother liquors, which weighed 11.0 mg, was crystallized twice from acetone–Skellysolve B, yielding 1.5 mg of small plates: mp 226–228°, mmp 224–227°, standard mp 209–211°, 227–229° (Neher *et al.*, 1959). Its infrared spectrum (KBr) was identical with that of 3α,16α-dihydroxy-5α-pregnan-20-one. The specific activity of this metabolite was 5.6×10^3 cpm/mg.

ISOLATION OF 3α,16α-DIHYDROXY-5β-PREGNAN-20-ONE. The residue of peak VIII (Figure 3) from the original silica gel column weighed 119 mg and contained 8.10×10^5 cpm (uncorrected for quenching). It was chromatographed on a 100-g Celite column in system D from which a single peak of radioactivity was eluted. The corresponding residue weighed 40 mg and contained 9.3×10^5 cpm. It was chromatographed on paper in system G and then percolated through a small alumina column, yielding 11.8 mg of colorless oil. The infrared spectrum (KBr) of this material was identical with that of 3α,16α-dihydroxy-5β-pregnan-20-one isolated in the previous experiment. Crystallization from acetone–Skellysolve B and methanol–acetone–Skellysolve B afforded 2.4 mg of fine needles: mp 210–211°, mmp 210–212°, standard mp 210–212°. The specific activity of this metabolite was 6.8×10^4 cpm/mg.

ISOLATION OF 5β-PREGNANE-3α,16α,20α-TRIOL. The residue of peak IX (Figure 3), which weighed 98 mg and contained 1.2×10^5 cpm, was chromatographed on a 120-g Celite column in system D. A single peak of radioactivity was found and the residue weighed 5.7 mg and contained 1.1×10^5 cpm. This residue was chromatographed on paper in system G and was percolated through a small silica gel column, giving 2.0 mg of colorless oil. An infrared spectrum of this material was identical with that of 5β-pregnane-3α,16α,20α-triol. Crystallization from acetone–Skellysolve B gave 0.7 mg of fine needles: mp 225–226°, mmp 224–226°, standard mp 227–228°. The specific activity of the steroid was 4.2×10^4 cpm/mg.

Discussion

Isopregnanolone is a known metabolite of 16α-hydroxyprogesterone, as demonstrated by Calvin and Lieberman (1962). The same authors found that 16-dehydropregnanolone is derived from 16α-hydroxyprogesterone and that a Δ^{16} compound may be an intermediate in the formation of isopregnanolone. The other four metabolites reported in this paper have been isolated from human urine although their precursor(s) were not known. Neher *et al.* (1959) found the two dihydroxy ketones, 3α,16α-dihydroxy-5α-pregnan-20-one and 3α,16α-dihydroxy-5β-pregnan-20-one, in the urine of a patient with congenital adrenal hyperplasia accompanied by salt loss. Hirschmann *et al.* (1961) isolated the two triols, 5α- and 5β-pregnane-3α,16α,20α-triol, from pregnancy urine and Fukushima *et al.* (1961) found the 5α-triol in the urine of a patient with adrenocortical carcinoma. Hirschmann *et al.* (1961) has commented on the apparent preponderance of 5α-metabolites in the urinary 16α-hydroxylated, C-21 steroids. The urinary metabolites of C-21, Δ^4-3-keto steroids are predominantly of the 5β configuration (Dorfman and Unger, 1965). In our studies, as in those of Hirschmann *et al.* (1961), recoveries of steroids were not quantitative; nevertheless it would seem that the urinary 5α-metabolites were present in amounts greater than would be expected, suggesting that the 16α-hydroxyl group influences the reduction in the A and B rings.

The distribution of radioactivity in the metabolites of injected labeled 16α-hydroxyprogesterone was not influenced by the oral administration of nonlabeled

steroid because the latter was given to the subject several weeks after the injection. It should be noted that the injected labeled steroid could not be considered as a tracer dose. In contrast to the results obtained in the feeding experiment, the specific activity of the isolated 3α,16α-dihydroxy-5α-pregnan-20-one was considerably lower than that of other metabolites isolated from the urine of the pregnant subject. This latter observation raises the possibility of the existence of precursors of the neutral 16α-hydroxypregnanes in pregnancy urine other than 16α-hydroxyprogesterone. We failed to find 5α-pregnane-3α,16α,20α-triol among the labeled metabolites in the urine of the pregnant subject. The specific activities of the metabolites isolated from the urine of the male subject were of the same order of magnitude, indicating that the ingested steroid and the injected labeled steroid were converted to the various urinary metabolites in about the same proportions.

The isolated metabolites of 16α-hydroxyprogesterone were identified by their melting points, mixture melting points, and infrared spectra, either as the free steroid or as the acetate. Due to the limited amount of carrier steroids available, rigorous demonstration of radiochemical homogeneity was not possible. The evidence presented demonstrates that administered 16α-hydroxyprogesterone is a precursor of urinary 3α,16α-dihydroxy-5α-pregnan-20-one and its 5β epimer and of 5α- and 5β-pregnane-3α,16α,20α-triol. Finally, when a tracer dose of [7-^{3}H]16α-hydroxyprogesterone was administered to a subject in the third trimester of pregnancy, weighable amounts of the urinary metabolites were isolated but labeled 5α-pregnane-3α,16α,20α-triol was not found.

Added in Proof

Since this paper was submitted we have succeeded in isolating labeled 5α-pregnene-3α,16α,20α-triol from pregnancy urine following the intravenous administration of [^{14}C]16α-hydroxyprogesterone and [^{3}H]16α-hydroxypregnenolone. The metabolite contained both labels.

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