The Isolation and Origin of Urinary 16α-Hydroxyprogesterone*

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ABSTRACT: Labeled 16α-hydroxyprogesterone was isolated from the urine of a pregnant female and a normal male following the intravenous administration of [7-^{3}H]16α-hydroxyprogesterone to both subjects. From the urine of the pregnant subject, weighable amounts of 16α-hydroxyprogesterone were isolated. When [4-^{14}C]-progesterone and [7-^{3}H]16α-hydroxyprogesterone were administered to a pregnant subject, the urinary 16α-hydroxyprogesterone did not contain any ^{14}C, indicating that peripheral 16α-hydroxylation did not occur. Following the administration of [7-^{3}H]16α-hydroxypregnenolone and [4-^{14}C]16α-hydroxyprogesterone (^{3}H/^{14}C = 4.9), the urinary 16α-hydroxyprogesterone had both labels and the ^{3}H/^{14}C was higher (6.9) than the injected ratio. This demonstrated that administered 16α-hydroxypregnenolone was a precursor of urinary 16α-hydroxyprogesterone but was not oxidized exclusively to the free circulating form of the latter.

We have reported (Ruse and Solomon, 1966) that 16α-hydroxyprogesterone[1] is metabolized to urinary 16α-hydroxy steroids by human subjects. A pregnant subject given labeled 16α-hydroxyprogesterone excreted labeled 3α,16α-dihydroxy-5α-pregnan-20-one, 3α,16α-dihydroxy-5β-pregnan-20-one, and 5β-pregnane-3α,16α-20α-triol. Isopregnanolone, a known metabolite of 16α-hydroxyprogesterone (Calvin and Lieberman, 1962), was also isolated. The conclusion that 16α-hydroxyprogesterone is a normal precursor of these urinary steroids depends on the demonstration that it is actually produced *in vivo*. This paper is a report of the isolation of 16α-hydroxyprogesterone from the urine of a pregnant female and a normal male. We have also demonstrated that 16α-hydroxypregnenolone is a possible precursor of urinary 16α-hydroxyprogesterone but that the latter may not be derived from circulating progesterone.

Methods

Techniques of counting, solvent preparation, chromatography, and enzymatic hydrolysis of urinary steroid conjugates have been described (Ruse and Solomon, 1966). In one experiment, steroid sulfates were cleaved by the solvolytic procedure of Burstein and Lieberman (1958) and glucosiduronidates by the use of β-glucuronidase (Baylove Chemicals, Musselburgh, Scotland). Solvent systems used for chromatography are shown in Table I.

TABLE I: Solvent Systems Used in Chromatography.

System	Solvents
B	*n*-Heptane–ethyl acetate–methanol–water (50:50:65:35)
E	*n*-Heptane–propylene glycol
F	Skellysolve C–propylene glycol
G	Toluene–propylene glycol
J	Ethyl acetate–*n*-hexane (2:1)

The preparation of [7-^{3}H]16α-hydroxyprogesterone has been described (Ruse and Solomon, 1966). The [4-^{14}C]16α-hydroxyprogesterone used in these experiments was prepared in the same way. A portion of the product was mixed with carrier 16α-hydroxyprogesterone and crystallized three times from acetone–Skellysolve B. The specific activities of crystals and mother liquors (Table II) reflect a satisfactory degree of purity. The specific activity of the product was 2.1 × 10^7 cpm/mg.

Labeled 16α-hydroxypregnenolone was prepared by the incubation of 1 mc of [7-^{3}H]pregnenolone (New England Nuclear Corp, Boston, Mass) and 7.1 mg of

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[1] The following trivial names are used: progesterone, pregn-4-ene-3,20-dione; pregnenolone, 3β-hydroxypregn-5-en-20-one; isopregnanolone, 3α-hydroxy-5β,17α-pregnan-20-one; 16α-hydroxyprogesterone, 16α-hydroxypregn-4-ene-3,20-dione; 16α-hydroxypregnenolone, 3β,16α-dihydroxypregn-5-en-20-one; 16-dehydropregnanolone, 3α-hydroxy-5β-pregnan-16-en-20-one; desoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; testosterone, 17β-hydroxyandrost-4-en-3-one; androstenedione, androst-4-ene-3,17-dione; testosterone glucuronide, 3-ketoandrost-4-ene-17β-yl-β-D-glucopyranosiduronic acid; 16α-hydroxyprogesterone glucuronide, 3,20-diketopregn-4-ene-16α-yl-β-D-glucopyranosiduronic acid; 16α-hydroxypregnenolone sulfate, 16α-hydroxy-20-diketopregn-5-ene-3β-yl sulfate; DDQ, dichlorodicyanobenzoquinone; hbv, holdback volume.

TABLE II: Radiochemical Purity of Injected Steroids.

	Specific Activity (cpm/mg)					
	[4-^{14}C]16α-Hydroxyprogesterone[a]		[7-^{3}H]16α-Hydroxypregnenolone[b]		[4-^{14}C]Progesterone ($\times 10^{-3}$)	
Crystallization	X11s[c]	M_L[c]	X11s	M_L	X11s	M_L
1	3020	2970	29500	29500	3650	3450
2	3060	3030	30400	29700		
3	3060	3020	30200	29000		
Calcd	2960		28700		3560	

[a] 1.56×10^5 cpm mixed with 52.7 mg of carrier. [b] 1.45×10^6 cpm mixed with 50.4 mg of carrier. [c] X11s, crystals; M_L, mother liquors.

pregnenolone with a strain of *Streptomyces roseochromogenus* (ATCC 3347) supplied by Dr. C. Vezina, Ayerst Laboratories, Montreal. The procedure used for the incubation was the same as the one described previously for the synthesis of labeled 16α-hydroxyprogesterone (Ruse and Solomon, 1966). The final extract was chromatographed on thin layers of silica gel in system J and on paper in system G. A portion of the product was mixed with carrier and crystallized from methanol–ethyl acetate, acetone–methanol, and methanol. The specific activities of crystals and mother liquors (Table II) indicate a satisfactory degree of radiochemical homogeneity. The specific activity of the product was 1.28×10^6 cpm/mg.

[4-^{14}C]Progesterone (10 μc) (New England Nuclear Corp.) was purified by chromatography on paper in system E. The eluate was mixed with 5.2 mg of progesterone and chromatographed on a small alumina column. Elution with *n*-hexane–benzene (2:8) and benzene yielded 4.5 mg of crystalline material containing 1.6×10^7 cpm. It was crystallized from ether–Skellysolve B and the specific activities are shown in Table II. The crystals were used for the *in vivo* studies.

Standardization of [1-^{14}C]Acetic Anhydride. Solutions of [1-^{14}C]acetic anhydride, 10% (v/v) in benzene, were standardized by acetylation of desoxycorticosterone, and crystallization of the product to constant specific activity. Solutions 1 and 2 had specific activities of 3.2×10^5 and 3.2×10^4 cpm/mg of desoxycorticosterone, respectively.

Formation of Acetates. Steroids were dissolved in two parts of pyridine and one part of acetic anhydride and left at room temperature for 18 hr. Steroids acetylated with labeled acetic anhydride were dissolved in two parts of pyridine and three parts of 10% acetic anhydride in benzene and incubated at 37° for 16–18 hr. Solvents were removed under nitrogen or by addition of the reaction mixture to acidified ice water and subsequent formation of a neutral extract.

Derivative Formation. A derivative of 16α-hydroxyprogesterone acetate was prepared by reduction of the 20-ketone with $NaBH_4$ as described by Norymberski and Wood (1955) and subsequent oxidation of the allylic alcohol at C_3 with dichlorodicyanobenzoquinone (DDQ) as described by Burn *et al.* (1960).

In a trial preparation, a solution of 30.5 mg of 16α-hydroxyprogesterone acetate in 6 ml of methanol was cooled at 0° and treated with 4.9 mg of $NaBH_4$. After 1 hr the reaction was stopped with a few drops of glacial acetic acid and the methanol evaporated. The residue was dissolved in ethyl acetate which was then washed with 0.1 N NaOH and water, dried over Na_2SO_4, and evaporated *in vacuo*. The products were dissolved in 2 ml of freshly distilled dioxane together with 22.2 mg of DDQ. The solution was left at room temperature for 24 hr and then filtered and evaporated to dryness. The products were dissolved in ethyl acetate, washed with 0.5 N NaOH and water, dried over Na_2SO_4, and the solution evaporated *in vacuo*. The residue, 32.6 mg, was chromatographed on a small silica gel column. Elution with 3% ethanol in benzene gave 22.6 mg of yellow oil, which on crystallization from ethanol–ether–Skellysolve B yielded 13.2 mg of small coarse needles, mp 173–177°. An infrared spectrum (KBr) showed major bands at 1730, 1665, 1615, 1245, and 1085 cm^{-1}. There was no absorption at 1700 cm^{-1} where the 20-ketone of 16α-hydroxyprogesterone acetate is observed. The spectrum indicates the retention of the acetate group and the Δ^4-3 ketone and disappearance of the 20-ketone. *Anal.* Calcd for $C_{23}H_{34}O_4$: C, 73.76; H, 9.09. Found: C, 73.80; H, 9.06. We were not able to compare the product to an authenticated compound but the structure 16α,20β-dihydroxypregn-4-en-3-one 16-acetate may be assigned with some confidence.

Experimental Section and Results

Four subjects have been studied. A pregnant subject and a normal male subject were given [7-^{3}H]16α-hydroxyprogesterone intravenously. A second pregnant subject was given [4-^{14}C]progesterone and [7-^{3}H]16α-hydroxyprogesterone, and a third pregnant subject [7-^{3}H]16α-hydroxypregnenolone and [4-^{14}C]16α-hydroxyprogesterone. Urine was collected from each subject for 4–9 days after the injections.

Experiment 1. A normal subject in the 34th week of

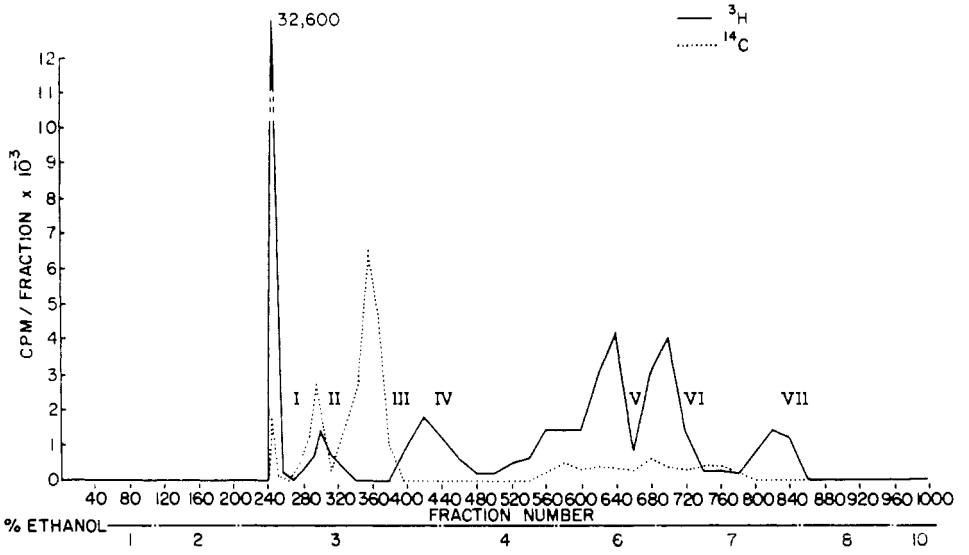

FIGURE 1: Chromatography on a 240-g silica gel column of the neutral extract of urine after the administration of [7-^{3}H]16α-hydroxyprogesterone and [4-^{14}C]progesterone to a pregnant female. The solvent used for elution was methylene chloride with increasing concentrations of ethanol as shown on the abscissa.

TABLE III: Radiochemical Purity of 16α-Hydroxyprogesterone Isolated in Experiment 1.

	Specific Activity (cpm of ^{3}H/mg)							
	16α-Hydroxyprogesterone Acetate				16α,20β-Dihydroxypregn-4-en-3-one 16-Acetate[b]			
Crystallization	X11s[a]	^{3}H/^{14}C	M_L[a]	^{3}H/^{14}C	X11s	^{3}H/^{14}C	M_L	^{3}H/^{14}C
1	430	1.7	440	1.2	410	1.7	390	1.8
2	420	1.7	420	1.7	410	1.7	390	1.7
Calcd[c]	410				420			

[a] X11s, crystals; M_L, mother liquors. [b] After the second crystallization of the acetate, 38 mg was reduced with $NaBH_4$ and the allylic alcohol at C-3 was oxidized with dichlorodicyanobenzoquinone. [c] A total of 2.9 mg containing 4.3×10^4 cpm of ^{3}H was acetylated with [1-^{14}C]acetic anhydride. The product was mixed with 100 mg of carrier 16α-hydroxyprogesterone acetate and the mixture was chromatographed on a silica gel column. The calculated specific activity was determined on the product eluted prior to crystallization.

pregnancy was given [7-^{3}H]16α-hydroxyprogesterone, 4.6×10^6 cpm, by intravenous injection and urine was collected for 4 days. The hydrolysis of urinary steroid conjugates, extraction, and preliminary fractionation on a silica gel column have been described (Ruse and Solomon, 1966). The neutral extract, 1.65 g and 2.34 $\times 10^6$ cpm, was chromatographed on a 225-g silica gel column, using methylene chloride with increasing concentrations of ethanol. The effluent was collected in 10-ml fractions, at the rate of 30–40 ml/hr. The residue, eluted by 3 and 3.5% ethanol, in tubes 368–444 (Figure 1, peak V, Ruse and Solomon, 1966), weighed 38 mg and contained 1.5×10^5 cpm. It was chromatographed on a 70-g Celite column in system B and two peaks of radioactivity were found in the effluent. The residue, eluted in the 7th and 8th holdback volume (hbv), 4.5 mg and 1.0×10^5 cpm, was chromatographed on a small silica gel column and then filtered through alumina, leaving 2.3 mg of faintly yellow oil, containing 6.8×10^4 cpm. Crystallization from acetone–*n*-hexane and acetone–*n*-heptane gave 0.5 mg of small plates, mp 218–221°, mmp 218–221°, authentic 16α-hydroxyprogesterone mp 218–221°. An infrared spectrum (KBr) was identical with that of standard 16α-hydroxyprogesterone.

The steroid used for infrared spectroscopy was recovered, added to the remaining crystals and mother liquors, and acetylated with [1-^{14}C]acetic anhydride, solution 2. The product was mixed with 100 mg of carrier 16α-hydroxyprogesterone acetate, chromatographed on a 40-g silica gel column, crystallized from ether and ether–Skellysolve B, and the specific

TABLE IV: Radiochemical Purity of 16α-Hydroxyprogesterone Isolated in Experiment 2.

	Specific Activities (cpm of ^3H/mg)							
	16α-Hydroxyprogesterone Acetate				16α,20β-Dihydroxypregn-4-en-3-one 16-Acetate[b]			
Crystallization	X11s[a]	$^3H/^{14}C$	M_L[a]	$^3H/^{14}C$	X11s	$^3H/^{14}C$	M_L	$^3H/^{14}C$
1	390	2.7	620	0.1	400	4.9	350	4.6
2	400	3.9	420	0.4	390	4.6	380	4.6
3	390	4.5	400	2.2				
4	390	4.7	320	4.3				
Calcd[c]	480				390			

[a] X11s, crystals; M_L, mother liquors. [b] The fourth crystals and mother liquors were used in the formation of this derivative. [c] The 16α-hydroxyprogesterone eluted from the alumina column weighed 0.9 mg and contained 4.8×10^4 cpm ^3H. It was acetylated with [1-^{14}C]acetic anhydride and to the acetate was added 100 mg of carrier 16α-hydroxyprogesterone acetate prior to crystallization. The calculated specific activity was determined from these values.

activities measured as shown in Table III. A portion of the last crystals, 38 mg, was reduced with 6.1 mg of $NaBH_4$ in 8.0 ml of methanol. The products were dissolved in 2.0 ml of dioxane and oxidized with 28.2 mg of DDQ. The final product was chromatographed on a 5-g silica gel column and crystallized twice from methanol–ether–Skellysolve B, and the specific activities were determined as shown in Table III. The specific activity of the urinary 16α-hydroxyprogesterone was 6.2×10^4 cpm/mg, calculated from the specific activity of the acetic anhydride and the $^3H/^{14}C$ ratio of the second derivative.

Experiment 2. A 38-year-old normal male was given an intravenous injection of [7-^{3}H]16α-hydroxyprogesterone containing 4.6×10^6 cpm. Urine was collected for 4 days and extracted with ethyl acetate to remove free steroids. Steroid sulfates were cleaved by the solvolytic method of Burstein and Lieberman (1958) and a neutral ethyl acetate extract obtained. The glucosiduronidates in the residual aqueous phase were hydrolyzed with 15 mg of β-glucuronidase powder/100 ml of urine and the liberated steroids were extracted with ethyl acetate. The unconjugated fraction contained less than 1% of the injected radioactivity.

The sulfate fraction weighed 1.57 g and contained 9.8×10^4 cpm. It was first chromatographed on a large silica gel column, then on an alumina column, and finally on paper in system F. Two radioactive compounds were found, the most polar of which had chromatographic mobilities comparable to those of isopregnanolone. The compounds were not further investigated. There was no evidence for the presence of 16α-hydroxyprogesterone in the sulfate fraction.

The fraction containing steroids excreted as glucosiduronidates weighed 430 mg and contained 1.45×10^6 cpm. It was chromatographed on a 225-g silica gel column, in the manner described under expt 1, and eight distinct peaks of radioactivity were found. The residue, eluted by 4% ethanol in methylene chloride, weighed 24.7 mg and contained 1.2×10^5 cpm. It was chromatographed on a 30-g Celite column in system B and two peaks of radioactivity were eluted, the first in the 3rd hbv and the second peak in the 5th and 6th hbv. The residues corresponding to the two peaks were pooled together, in error, giving 15.0 mg of material containing 1.1×10^5 cpm. This residue was chromatographed on paper in system G and then on a 2-g alumina column. Elution of the latter with 2% ethanol in benzene yielded 0.9 mg of yellow oil containing 4.8×10^4 cpm. This oil was acetylated with [1-^{14}C]acetic anhydride, solution 1, the product was mixed with 100 mg of 16α-hydroxyprogesterone acetate, and the mixture was crystallized from ether–methanol, ether, ether–Skellysolve B, and methanol–ether–Skellysolve B. The specific activities obtained following crystallization are shown in Table IV.

The final crystals and mother liquors were pooled, reduced with $NaBH_4$, and oxidized with DDQ as described above. The product was crystallized twice from methanol–ether–Skellysolve B (Table IV). The calculated specific activity of the urinary 16α-hydroxyprogesterone was 1.67×10^6 cpm/mg.

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Experiment 3. A normal subject in the 26th week of pregnancy was given [7-^{3}H]16α-hydroxyprogesterone, 2.85×10^6 cpm, and [4-^{14}C]progesterone, 1.85×10^6 cpm, by intravenous injection. Urine was collected for 4 days, treated with Glusulase, and extracted with ethyl acetate. The neutral extract weighed 1.07 g and contained 6.4×10^5 cpm of tritium (uncorrected for quenching) and 1.7×10^5 cpm of ^{14}C. It was chromatographed on 240 g of silica gel and the radioactivity eluted is shown in Figure 1. The residue eluted by 3 and 4% ethanol, in tubes 390–485 (peak IV), weighed 29.3 mg and contained 7.7×10^4 cpm of tritium and 5.5×10^3 cpm of ^{14}C. It was chromatographed on a 30-g Celite column in system B. Two major peaks of tritium were found and the residue eluted in the 6th and 7th hbv was chromatographed on a small alumina column. Elution with 2% ethanol in benzene gave 1.2 mg of colorless oil, containing 2.3×10^4 cpm of tritium. Pro-

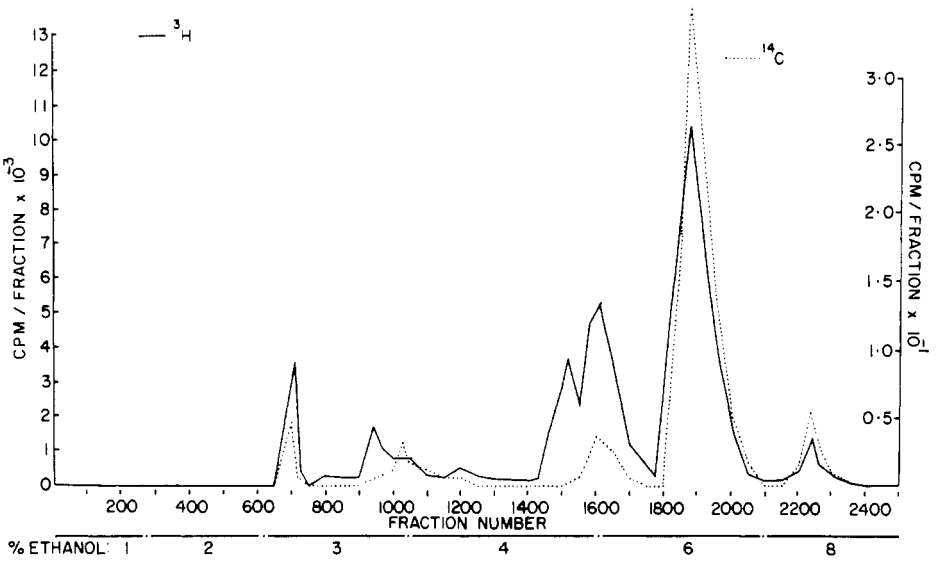

FIGURE 2: Silica gel chromatography of the neutral extract of urine following the administration of [7-^{3}H]16α-hydroxypregnenolone and [4-^{14}C]16α-hydroxyprogesterone to a pregnant female. A 1000-g silica gel column was used and it was eluted with methylene chloride containing increasing amounts of ethanol as shown on the abscissa.

longed counting revealed no evidence of the presence of ^{14}C. The residue was mixed with 89.0 mg of 16α-hydroxyprogesterone and crystallized from acetone methanol, acetone–methanol–Skellysolve B and twice from ethanol–Skellysolve B. The last crystals were acetylated and the product crystallized twice from ethanol–Skellysolve B and from ether–ethanol. The specific activities of crystals and mother liquors are shown in Table V.

Experiment 4. The subject, a normal female in the 32nd week of pregnancy, was given an intravenous injection containing [7-^{3}H]16α-hydroxypregnenolone, 5.31 $\times$ 10^{6} cpm, and [4-^{14}C]16α-hydroxyprogesterone, 1.08 $\times$ 10^{6} cpm. Urine was collected for 9 days. Steroid conjugates were hydrolyzed with Glusulase and a neutral ethyl acetate extract obtained. The extract, which weighed 3.91 g and contained 2.76 $\times$ 10^{6} cpm of tritium and 5.3 $\times$ 10^{5} cpm of ^{14}C, was chromatographed on a 1000-g silica gel column and the radioactive material eluted is shown in Figure 2. The residue of tubes 990–1300 was further fractionated on a 200-g Celite column in system B as is shown in Figure 3. The contents of fractions 120–164 (6th and 7th hbv), from the Celite column, were chromatographed on paper in system G, mixed with 18.0 mg of 16α-hydroxyprogesterone, and chromatographed on a 5-g alumina column. Elution with 1 and 1.5% ethanol in benzene gave 19.1 mg of oil, containing 1.3 $\times$ 10^{4} cpm of tritium and 1.9 $\times$ 10^{3} cpm of ^{14}C. The residue was crystallized twice from ethanol–Skellysolve B and once from acetone. The last crystals and mother liquors were acetylated and the product crystallized from ethyl acetate–Skellysolve B and from ethanol–Skellysolve B. The specific activities of crystals and mother liquors are shown in Table VI. The ^{3}H/^{14}C ratio of the isolated 16α-hydroxyprogesterone was higher than that of the injected compounds.

TABLE V: Radiochemical Purity of 16α-Hydroxyprogesterone Isolated in Experiment 3.

Crystallization	Specific Activity (cpm ^{3}H/mg)			
	16α-Hydroxyprogesterone[a]		16α-Hydroxyprogesterone Acetate[b]	
	X11s[c]	M_L[c]	X11s	M_L
1	220	420	190	160
2	210	250	180	180
3	210	230	180	180
4	210	220		
Calcd	250		180	

[a] The isolated 16α-hydroxyprogesterone was mixed with 89 mg of carrier prior to crystallization. [b] The fourth batch of crystals were used to form the acetate. [c] X11s, crystals; M_L, mother liquors.

Discussion

In expt 1 we isolated crystalline 16α-hydroxyprogesterone from the urine of a pregnant subject. Losses in isolation are not known but the recovery of 0.5 mg of crystalline steroid suggests that relatively large amounts of 16α-hydroxyprogesterone were excreted when compared to the excretion in urine of Δ^{4}-3-keto steroids. The finding supports previous conclusions (Ruse and Solomon, 1966) that 16α-hydroxyprogesterone may be an

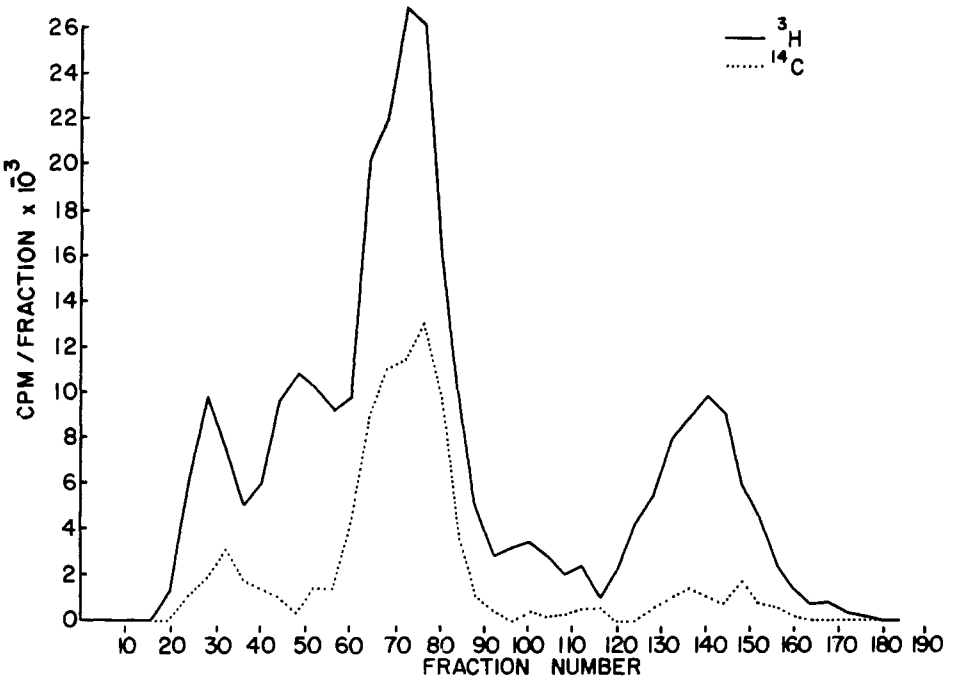

FIGURE 3: Celite partition chromatography of the residue from fractions 990 to 1300, Figure 2. The residue was chromatographed on a 70-g column using system B and fractions 120 to 164 were taken for further purification.

TABLE VI: Radiochemical Purity of 16α-Hydroxyprogesterone Isolated from Experiment 4.

	Specific Activity (cpm of ^3H/mg)							
	16α-Hydroxyprogesterone				16α-Hydroxyprogesterone Acetate[b]			
Crystallization	X11s[a]	$^3H/^{14}C$	M_L	$^3H/^{14}C$	X11s	$^3H/^{14}C$	M_L	$^3H/^{14}C$
1	750	6.7	310	6.9	640	6.6	520	7.1
2	780	6.6	590	7.0	640	6.9	610	6.9
3	770	6.6	790	7.1				
Calcd[c]	670				690			

[a] X11s, crystals; M_L, mother liquors. [b] The third crystals and mother liquors were combined prior to formation of the acetate. [c] The 16α-hydroxyprogesterone isolated from the Celite partition column was chromatographed on paper in system G and then mixed with 18.0 mg of carrier and the mixture chromatographed on an alumina column. A total of 19.1 mg was eluted containing 1.3×10^4 cpm of ^3H and 1.9×10^3 cpm of ^{14}C. These values were used to obtain the calculated specific activity.

endogenous precursor of the urinary 16α-hydroxylated steroids described.

From the urine of a normal male we were also able to isolate 16α-hydroxyprogesterone but in much smaller quantities (expt 2). At the stage of acetylation, only 4.8 $\times 10^4$ cpm of tritium remained. If we assume that all this radioactivity was in the form of 16α-hydroxyprogesterone, then we may calculate that a maximum of 30 μg was recovered. In this study, 16α-hydroxyprogesterone was found only in the neutral extract of urine following β-glucuronidase hydrolysis.

It is apparent from the results of expt 3 that the progesterone injected into the maternal circulation was not converted to 16α-hydroxyprogesterone by the maternal liver or by fetal tissues. With the present experimental design we would not expect to detect ^{14}C with a $^3H/^{14}C$ ratio of more than 100, so that it is possible that a minute conversion could not have been detected. Fotherby *et al.* (1957) have reported measurable conversions of parenteral administered dehydroisoandrosterone to 16α-hydroxydehydroisoandrosterone by an adrenalectomized, oophorectomized subject. It is possible that a more sensitive experiment might demonstrate the conversion of small amounts of progesterone

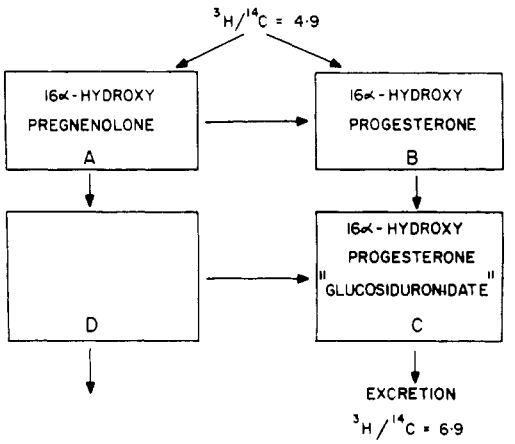

FIGURE 4: An outline of the routes of metabolism of [7-^{3}H]16α-hydroxypregnenolone and [4-^{14}C]16α-hydroxyprogesterone. Chemical compartments are designated by A–D. Compartment D may represent a chemical species such as 16α-hydroxypregnenolone sulfate. The two substrates were injected with a $^3H/^{14}C$ ratio of 4.9 and 16α-hydroyprogesterone was isolated having a $^3H/^{14}C$ ratio of 6.9.

to 16α-hydroxyprogesterone. The results of expt 4 demonstrate conclusively that administered 16α-hydroxypregnenolone was converted to urinary 16α-hydroxyprogesterone.

From the comparison of the $^3H/^{14}C$ ratio in the urinary 16α-hydroxyprogesterone (6.9) and that of the injected mixture (4.9), it is apparent that the injected 16α-hydroxypregnenolone did not contribute to urinary 16α-hydroxyprogesterone by exclusive transformation to free circulating 16α-hydroxyprogesterone. If the latter were true, the $^3H/^{14}C$ ratio of the urinary compound could not exceed that of the injected mixture. Figure 4 shows a model which includes a second route to urinary 16α-hydroxyprogesterone. We assume the latter compound was excreted as the glucosiduronidate as it was in the study of the male subject. Compartment D may represent an unknown chemical species, such as 16α-hydroxypregnenolone sulfate, which reaches the liver and is partially converted by hydrolysis, oxidation, and conjugation to 16α-hydroxyprogesterone glucosiduronidate. Korenman and Lipsett (1964) have proposed a similar scheme for the conversion of androstenedione to testosterone glucosiduronidate in liver. Fukushima *et al.* (1961) have suggested that 16α-hydroxypregnenolone may be a precursor of urinary 16α-hydroxyprogesterone and its metabolites. Recently we succeeded in isolating 16α-hydroxypregnenolone (J. L. Ruse and S. Solomon, unpublished results) from urine in expt 4 and this metabolite has been found in the urine of the normal newborn (Reynolds, 1963). Thus it is likely that in late pregnancy, 16α-hydroxypregnenolone is an endogenous precursor of urinary 16α-hydroxyprogesterone.

It is possible to calculate the production rates of 16α-hydroxyprogesterone in the subjects of expt 1 and 2. However, if 16α-hydroxypregnenolone does in fact contribute to urinary 16α-hydroxyprogesterone, the calculations are invalid.

The site of production of 16α-hydroxyprogesterone in the intact subject is not known. In the intact male it may be elaborated by the testes or the adrenals. A number of adult and fetal tissues are known to possess 16α-hydroxylase activity (Viscelli *et al.*, 1965; Villee *et al.*, 1961; Villee *et al.*, 1957; Ward and Grant, 1963; Villee, 1964; Warren and Salhanick, 1961; Little *et al.*, 1963; Acevedo *et al.*, 1963; Bloch, 1964). In pregnancy, the probable site of formation of 16α-hydroxyprogesterone is the feto-placental unit. In addition to the adrenals the kidney and residue of the perfused previable human fetus can convert progesterone to 16α-hydroxyprogesterone (C. E. Bird, N. Wiqvist, E. Diczfalusy, and S. Solomon, submitted for publication, 1966). It is also possible that 16α-hydroxypregnenolone is produced in the feto-placental unit from pregnenolone formed in the placenta and it could then be converted to 16α-hydroxyprogesterone in the placenta itself or in the maternal circulation.

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The Biosynthesis of Chlorobium Chlorophylls-660. The Isolation and Purification of Porphyrins from *Chlorobium thiosulfatophilum*-660*

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ABSTRACT: Concentrated suspensions of *Chlorobium thiosulfatophilum*-660, when incubated with the tetrapyrrole precursors glycine and 2-ketoglutarate or δ-aminolevulinic acid, have produced coproporphyrin III, uroporphyrins I and III, traces of metalloporphyrins, porphyrins with 2, 3, 5, 6, and 7 carboxyl groups, and tetracarboxylic porphyrins with phyllo type spectra.

We have also studied the effect of ethionine on such incubations. A new and useful type of chromatography has been developed, whereby porphyrins are eluted from powdered polyethylene columns according to the number of carboxyl groups, the most highly carboxylated being removed first. This polyethylene chromatography has been used in conjunction with countercurrent distribution for separation and purification of the excreted porphyrins. No *meso*-alkylation of the tetrapyrrole nucleus has occurred before the coproporphyrinogen III stage during the biosynthesis of chlorobium chlorophyll-660 by this organism.

Although a great deal of work has appeared concerning the biosynthesis of chlorophyll *a* (Bogorad, 1960; Granick and Mauzerall, 1961) and bacteriochlorophyll (Lascelles, 1961, 1964), relatively little has appeared on the biosynthesis of the chlorobium chlorophylls. Recently, structures have been assigned to the chlorophylls of the green sulfur bacterium, *Chlorobium thiosulfatophilum* (Holt *et al.*, 1962, 1963; Mathewson *et al.*, 1963a,b). A unique feature of the chlorophylls of the 660 series is that in addition to the γ-substituent involving the cyclopentenone ring E, there is a *meso*-alkyl (methyl or ethyl) substituent on the tetrapyrrole nucleus.[1] We have been interested in determining the point in the biosynthetic sequence of production of the chlorobium chlorophylls-660 at which the *meso*-alkyl substituent is attached to the macrocycle.

Porphyrins have been detected in the regular growth medium of *C. thiosulfatophilum* (Lascelles and Cooper, 1955; Lascelles, 1955; Erokhin and Krasnovskii, 1963; Uspenskaya and Kondrat'eva, 1964). Also, although *C. thiosulfatophilum* cannot grow on media containing organic compounds without the normal inorganic nutrients being present (Larsen, 1953; Moshentseva and Kondrat'eva, 1962) the biomass and porphyrin excretion is increased if precursors of tetrapyrroles are included along with the regular growth medium (Uspenskaya, 1965a,b).

We have also observed porphyrin excretion by *C. thiosulfatophilum*-660 when the organism was cultured under normal growth conditions. However, much larger quantities of porphyrins were excreted if the bacteria were first grown under normal conditions and then concentrated and incubated with the tetrapyrrole precursors. Following work done with purple bacteria by Lascelles (1955, 1956) and Gibson *et al.* (1962), working with *Rhodopseudomonas spheroides*, and by Cooper (1963), working with *R. capsulata*, we have identified the main porphyrins excreted by *C. thiosulfatophilum*-660 under similar conditions as coproporphyrin III and uroporphyrins I and III. In addition we have detected traces of metalloporphyrins, porphyrins with 2, 3, 5, 6, and 7 carboxyl groups, and tetracarboxylic porphyrins with phyllo type spectra (for explanation of spectral types see Falk, 1963); however, protoporphyrin was not specifically identified as the dicarboxylic

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[1] Holt *et al.* (1962, 1963) have assigned the *meso*-alkyl substituents to the δ-position. However, on the basis of the exchange properties of the pheophorbide (Mathewson *et al.*, 1963a) and the chlorophyll (Mathewson *et al.*, 1963b), we have assigned the *meso*-alkyl substituents to either the α- or β-position.