In Vitro Metabolism of 15α-Hydroxyprogesterone by Rabbit Liver†

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ABSTRACT: A large-scale incubation of 15α-[7-^{3}H]hydroxyprogesterone (500 mg) with a rabbit liver (640 g) homogenate was performed using phosphate buffer fortified with nicotinamide (70 mM) and DPN^+ (2.5 mM) at 37° in air for 2 hr. The following seven metabolites were isolated and identified by infrared and mass spectroscopy, melting points, and partial synthesis: 15-ketoprogesterone, 15β-hydroxyprogesterone, 20α-hydroxypregn-4-ene-3,15-dione, 15α,20α-dihydroxypregn-4-en-3-one, 15β,20α-dihydroxypregn-4-en-3-one, 15α-hydroxydeoxycorticosterone, and 3α,15α-dihydroxy-5β-pregnan-20-one. Only one metabolite, 3α,15α-dihydroxy-5β-pregnan-20-one has been isolated previously. The results demonstrate that the metabolism of 15α-hydroxyprogesterone is different from that of progesterone under the same conditions and *in vivo*. This study also demonstrates that the 15α-hydroxyl group inhibits the reduction of the Δ^4-3-ketone and the 20-ketone, and contrary to the *in vivo* findings in man, directs the reduction of the C-20 ketone toward the 20α alcohol rather than 20β alcohol. Two of the metabolites isolated, 15-ketoprogesterone and 15β-hydroxyprogesterone are of special interest because they possess antimineralocorticoid activity. The results also show that along with the other metabolites, rabbit liver was capable of 21-hydroxylation. To our knowledge this is the first example of a 21-hydroxylase in liver.

Shortly after the isolation of 15α-hydroxyprogesterone (Giannopoulos and Solomon, 1967) a study of its *in vivo* metabolism was undertaken (Giannopoulos *et al.*, 1970) and it was demonstrated that the presence of the 15α-hydroxyl group inhibited the reduction of the Δ^4-3-ketone and the C-20 ketone and led to an increased ratio of 5α/5β reduced products. In addition it was also shown that the 15α-hydroxy group directed the reduction of the 20 ketone to the 20β alcohol. In a search for an *in vitro* system for studying the metabolism of 15α-hydroxyprogesterone, our attention was directed to the rabbit liver preparation described by Taylor (1955) because in these preparations, progesterone was reduced predominantly to metabolites having a 5β configuration and a 20α alcohol. The aim of our study was to determine whether the metabolites previously isolated from human pregnancy urine (Giannopoulos *et al.*, 1970) were products of hepatic metabolism and whether the rabbit liver preparation would serve as a suitable system for the large-scale preparation of these steriods. In addition we wanted to test the hypothesis that during its course of metabolism 15α-hydroxyprogesterone would give rise to products having a greater antimineralocorticoid activity (Tweit and Kagawa, 1964) than the substrate.

Materials and Methods

All solvents were distilled prior to use and were peroxide free. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 3002 and 4322). Samples to be counted were first dissolved in 2 ml of absolute methanol to which was added 13 ml of toluene–phosphor solution containing 0.4% of Omnifluor (New England Nuclear) in toluene. The counts of quenched samples were corrected by the use of an external standard (Hayes *et al.*, 1964).

Melting points were determined on a Kofler block and were corrected. Infrared spectra (KBr) were obtained with a Model 221 Perkin-Elmer infrared spectrometer. Mass spectra were measured on a LKB Model 9000 run at 12 or 70 eV using a direct inlet system with the ion sources at 290°.

Chromatography. The separation and purification of steroids was accomplished by using the solvent systems described in Table I. Celite partition columns were prepared by first mixing the stationary phase with Celite (0.5 ml/g) in a plastic bag until homogeneous, and this material was then dry packed as described by Kelly *et al.* (1962). The columns used had a length to width ratio of approximately 40. For example, 120 g of Celite was packed in a column having a length of 90 cm and a width of 2.4 cm. Paper chromatography was done using Whatman No. 2 or 3MM papers (15 cm wide and 58 cm long). Steroids were eluted from paper with methanol. Thin-layer chromatography was performed on plates of silica gel in the usual manner. Elution of steroids from thin-layer plates was done with acetone.

Experimental Section and Results

Synthesis of 15α-[7-^{3}H]Hydroxyprogesterone. The [7-^{3}H]-progesterone (specific activity 20 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass., and its purity was greater than 96% when checked by the isotope dilution technique. This material, after dilution with pro-

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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; 15α-hydroxyprogesterone, 15α-hydroxypregn-4-ene-3,20-dione; 15α-hydroxydeoxycorticosterone, 15α,21-dihydroxypregn-4-ene-3,20-dione; 15β-hydroxyprogesterone, 15β-hydroxypregn-4-ene-3,20-dione; 15-ketoprogesterone, pregn-4-ene-3,15,20-trione; DPN^+, β-diphosphopyridine nucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propandiol.

TABLE I: Solvent Systems Used in Chromatography.

System	Type of Chromatography	Solvent Mixtures
A	Tlc[a]	Benzene–ethanol (9:1)
B	Tlc	Chloroform–methanol (85:15)
C	Tlc	Chloroform–ethanol (85:15)
D	Tlc	Benzene–ethanol (93:7)
E	Tlc	Benzene–ethanol (95:5)
F	Tlc	Benzene–methanol (9:1)
G	Tlc	Benzene–ethanol (85:15)
H	Tlc	Benzene–ethanol (80:20)
I	Tlc	Hexane–ethanol (70:30)
J	Tlc	Chloroform–ethanol (9:1)
K	Paper[b]	2,2,4-Trimethylpentane–*tert*-butyl alcohol–methanol–water (10:2:7:1)
L	Paper or Celite	Skellysolve C–benzene–methanol–water (10:5:8:2)
M	Paper	Benzene–methanol–water (2:1:1)
N	Celite[c]	Skellysolve C–methanol–water (100:75:25)
O	Celite	Toluene–propylene glycol
P	Celite	Benzene–methanol–water (2:1:1)
Q	Celite	Toluene–ethyl acetate–methanol–water (9:1:6:4)

[a] Tlc, thin-layer chromatography. [b] Paper, paper partition chromatography. [c] Celite, Celite partition chromatography.

TABLE II: Proof of Radiochemical Purity of 15α-[7-^{3}H]-Hydroxyprogesterone.

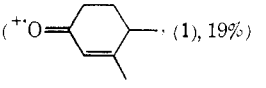
Crystallization	Specific Activities (dpm/mg) of Crystals: 15α-Hydroxyprogesterone	15α-Acetoxyprogesterone
1	3500	1520
2	3620	1570
3	3500	
Calculated	3260[a]	1560[b]

[a] A total of 8.8×10^4 dpm of material was mixed with 27 mg of carrier 15α-hydroxyprogesterone prior to crystallization. The calculated specific activity is based on these values. [b] A total of 9.94 mg of the third crystals containing 3.5×10^4 dpm was mixed with 9.95 mg of carrier 15α-hydroxyprogesterone and the mixture was then acetylated. The calculated specific activity has been corrected for changes in molecular weights and for the addition of more carrier.

gesterone, was converted microbiologically to 15α-[^{3}H]hydroxyprogesterone as described below.

A total of 2.46×10^9 dpm of [^{3}H]progesterone (20.2 mg) was incubated with a strain of *Penicillium* ATCC 11598 using two types of media. Medium I consisted of 30 g of corn steep liquors, 10 g of brown sugar, 6 g of $NaNO_3$, 1 mg of $ZnSO_4$, 1.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 5 g of $CaCO_3$, 2 g of lard oil, all in 1 l. of distilled water. Medium II consisted of 6 g of corn steep liquors, 3 g of $NH_4H_2PO_4$, 2.5 g of $CaCO_3$, and 2.2 g of soybean oil, all in 1 l. of distilled water. The pH of medium II was adjusted to 7. A total of 50 ml of medium I was inoculated with the *Penicillium* growth from a 2-week-old glucose agar slant under aseptic conditions and it was incubated for 3 days at 25°. Then 20% of this suspension was transferred to 100 ml of medium II to which was added a solution of [^{3}H]progesterone in alcohol. This mixture was incubated for 3 days at 25°, the medium was then filtered, and the filtrate was extracted five times with 200 ml of dichloromethane. This extract was washed with water (three times with 10 ml), dried over Na_2SO_4, and evaporated to dryness. The residue weighed 120 mg and contained 2.28×10^9 dpm. It was purified by chromatography on a 10-g alumina (5% H_2O) column using benzene as the initial solvent followed by increasing concentrations of ethanol in benzene. A peak of radioactive material was eluted with 0.5% ethanol in benzene which when pooled yielded a residue which weighed 13 mg and contained 893×10^6 dpm. This residue was purified by thin-layer chromatography on 6 plates in system A and an ultraviolet-absorbing material, corresponding in mobility to 15α-hydroxyprogesterone, was eluted and the residue crystallized twice, from ethanol, methanol, and hexane mixtures, to yield 3.0 mg of crystals containing 345×10^6 dpm. The infrared spectrum of this material was identical with that of authentic 15α-hydroxyprogesterone. Radiochemical purity of this material was checked by isotope dilution and it was found to be over 98%, as shown in Table II. This material was used as a substrate after further dilution with carrier 15α-hydroxyprogesterone.

Synthesis of 15β-Hydroxyprogesterone. The organism used in this preparation was a strain of *Bacillus megaterium* No. NRRLB-938, obtained from ARS U. S. Department of Agriculture, Peoria, Ill. The bacterium was first grown in 50 ml of medium in a 250-ml flask. The medium consisted of 50 g of commerical dextrose (cerelose), 20 g of Edamine, and 5.0 g of corn steep liquors, all in 1 l. of water. The pH of the medium was adjusted to 6.5. The flask was incubated for 48 hr at 28° and then a 10% transfer was made to a set of two flasks each containing 60 ml of the same medium. A solution of 8 mg of progesterone in 1 ml of ethanol was added to each flask and the flasks were incubated for 72 hr at 28°. Following incubation, an ethyl acetate extract was prepared. The extract was purified by chromatography on tlc in system A and ultraviolet-absorbing material, slightly less polar than 15α-hydroxyprogesterone, was eluted. It was rechromatographed on tlc in system B and the eluted material was evaporated to give a residue which was crystallized from ethanol–hexane mixtures to yield 3.8 mg of white crystalline material, mp 203–206° (lit. (Fried *et al.*, 1955) mp 204–205°). The mass spectrum indicated that the compound has a molecular weight of 330, which was consistent with the structural formula $C_{21}H_{30}O_3$, *m/e* 330 (M^+, 4%), 315 (M − 15, 1.3%), 312 (M − 18, 26%), 270 (M − 60, 6%), 269 (M − 61, 10%), 124



The infrared spectrum of this material was identical with that of 15β-hydroxyprogesterone synthesized chemically as described by Ramm and Caspi (1969) (Figure 1B,C).

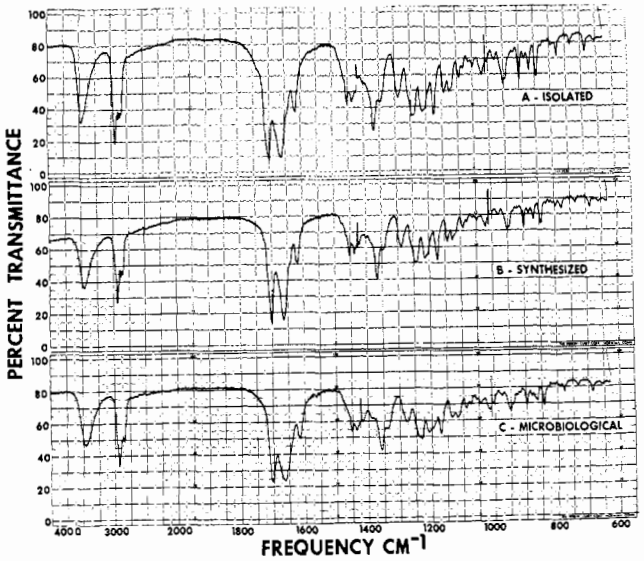


FIGURE 1: Infrared spectra (KBr) of 15β-hydroxyprogesterone: (A) isolated from pools VI and VII; (B) synthesized chemically; (C) synthesized microbiologically.

Synthesis of 15β,20α-Dihydroxypregn-4-en-3-one. This compound was prepared by microbiological 15β-hydroxylation of 20α-hydroxypregn-4-en-3-one as described above for the synthesis of 15β-hydroxyprogesterone. A total of 15 mg of 20α-hydroxypregn-4-en-3-one was incubated and the product obtained was purified by chromatography on tlc in system C. A major ultraviolet-absorbing material, having R_F 0.5, was eluted and crystallized twice from ethanol–hexane mixtures to yield 3 mg of a white crystalline material, mp 218–220°. The mass spectrum of this material indicated that the compound has a molecular weight of 332 which was consistent with the structural formula $C_{21}H_{32}O_3$, *m/e* 332 (M^+, 12%), 314 (M − 18, 34%), 296 (M − 18 + 18, 52%), 290 (M − 42, 15%), 124 (**1**, 100%). The infrared spectrum is shown in Figure 2A. As there was no authentic sample available for comparison, the structure 15β,20α-dihydroxypregn-4-en-3-one was assigned to the above material for the following reasons. Firstly, other isomers, 15β,20β-dihydroxypregn-4-en-3-one, 15α,20α-dihydroxypregn-4-en-3-one, and 15α,20β-dihydroxypregn-4-en-3-one have all been prepared in this laboratory by specific methods and the infrared spectra of these steroids did not match with the spectrum of the above product. Secondly, it is known that *Bacillus megaterium* hydroxylates steroids in the 15β configuration (Fried *et al.*, 1955). Thirdly, the fragmentation pattern obtained during mass spectral analysis is compatible with the structure of 15β,20α-dihydroxypregn-4-en-3-one.

Synthesis of 15α,20α-Dihydroxypregn-4-en-3-one. This compound was prepared by microbiological 15α-hydroxylation of 20α-dihydroprogesterone using the same procedure employed in the preparation of 15α-hydroxyprogesterone. A total of 140 mg of 20α-dihydroprogesterone was incubated and the product obtained after extraction was purified by tlc using system C. An ultraviolet-absorbing material having an R_F of 0.4 was eluted and the residue was crystallized twice from ethanol–hexane and acetone–hexane mixtures to yield 17 mg of product, mp 161–161.5°. The mass spectrum of this material indicated it has a molecular weight of 332, which was consistent with the structural formula $C_{21}H_{32}O_3$, *m/e* 332 (M^+, 77%), 314 (M − 18, 13%), 290 (M − 42, 25%), 272 (M − 60, 20%), 124 (**1**, 100%). The infrared spectrum of this material is shown in Figure 3A. As there is no authentic sample available for direct comparison, we had to rely on the physical characteristics of the product to deduce the structure. It is known that *Penicillium* ATCC 11598 hydroxylates steroids at the 15α position. The infrared and mass spectra of this material were different from those of the other three possible isomers namely 15α,20β-dihydroxypregn-4-en-3-one, 15β,-20α-dihydroxypregn-4-en-3-one, and 15β,20β-dihydroxypregn-4-en-3-one, hence the structure 15α,20α-dihydroxypregn-4-en-3-one can be assigned to this material.

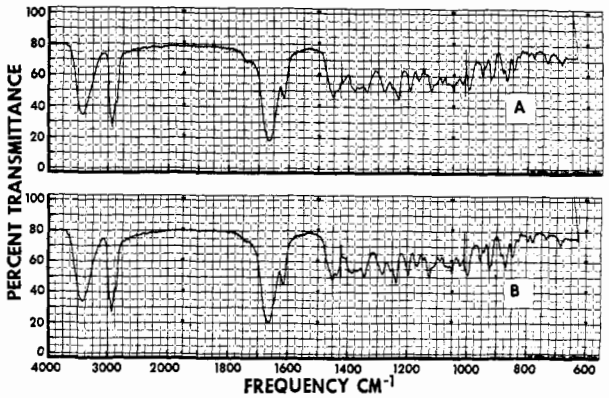


FIGURE 2: Infrared spectra (KBr) of 15β,20α-dihydroxypregn-4-en-3-one: (A) synthesized microbiologically; (B) isolated from pool XIII.

Synthesis of 15-Ketoprogesterone. A total of 50 mg of 15α-hydroxyprogesterone in 5 ml of glacial acetic acid was treated with a solution of CrO_3 (25 mg) in acetic acid (5 ml). The reaction mixture was stirred for 1 hr at room temperature. Ethanol (2 ml) was added and the mixture stirred for a further 10 min, then 20 ml of water was added, and the mixture was extracted with ethyl acetate. The organic phase was washed with $NaHCO_3$ and water until neutral and evaporated to dryness. The residue was purified by chromatography on a 2-g silica gel column. Elution with 2.5% ethanol in methylene dichloride yielded a residue weighing 45 mg which on crystallization from acetone–hexane yielded 36 mg of crystals, mp 155–157° (lit. mp 157–160°). The mass spectrum of this material gave a molecular weight of 328, consistent with the formula $C_{21}H_{28}O_3$, *m/e* 328 (M^+, 100%), 313 (M − 15, 15%), 286 (M − 42, 53%). The infrared spectrum of this material, Figure 4A, indicates the presence of a five-membered ring ketone (C-15 C=O), an α,β unsaturated ketone (C-3 C=O),

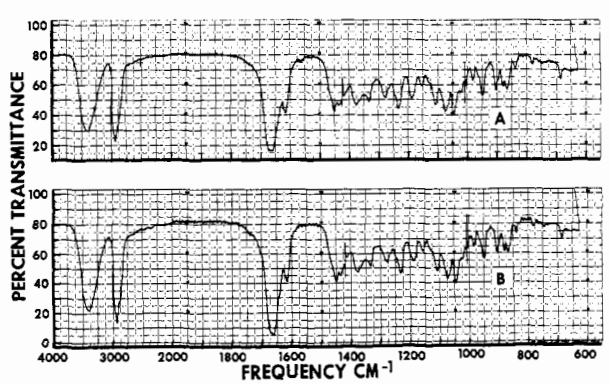


FIGURE 3: Infrared spectra (KBr) of 15α,20α-dihydroxypregn-4-en-3-one: (A) synthesized microbiologically; (B) isolated from pool XIV A.

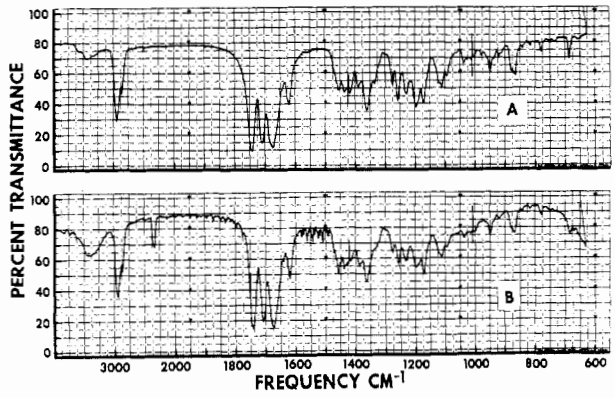


FIGURE 4: Infrared spectra (KBr) of 15-ketoprogesterone: (A) synthesized chemically; (B) isolated from pool IV E.

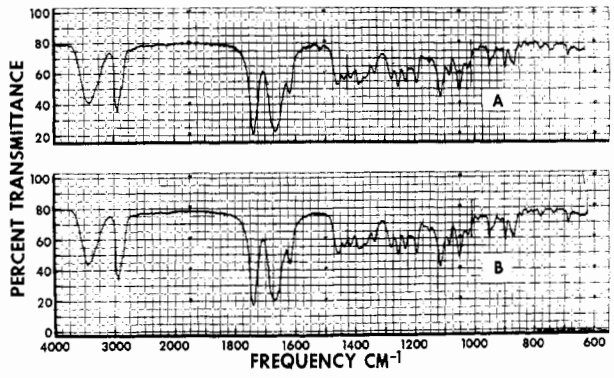


FIGURE 5: Infrared spectra (KBr) of 20α-hydroxypregn-4-ene-3,-15-dione: (A) synthesized microbiologically; (B) isolated from pool VIII E.

and a C-20 ketone. Both the mass and infrared data are consistent with the structure of 15-ketoprogesterone.

Synthesis of 20α-Hydroxypregn-4-ene-3,15-dione. It was possible to synthesize 20α-hydroxypregn-4-ene-3,15-dione by the microbiological reduction of 15-ketoprogesterone with *Rhodotula longissima*, NRRL NO7-2243 obtained from USDA, Peoria, Ill., using the following conditions. The incubation medium consisted of 3 g of yeast, 3 g of malt extract, 5 g of peptone, 10 g of glucose, all in 1 l. of distilled water. This medium (50 ml) was sterilized and inoculated with a lyophilized preparation of *Rhodotula longissima* and the mixture incubated for 48 hr at 25°. Then 12 ml of this suspension was transferred to 120 ml of fresh medium and 15 mg of 15-ketoprogesterone in 1 ml of ethanol was added. This mixture was then incubated for 3 days at 25° on a shaker. The medium was extracted with ethyl acetate and the organic phase washed with water, dried over Na_2SO_4, and evaporated to dryness under vacuum. The oily residue was partitioned between 80% methanol and hexane and the methanol phase evaporated to dryness. The residue obtained weighed 26 mg and was purified by chromatography on two thin-layer plates using system D. Two ultraviolet-absorbing zones were observed, the less polar one having the mobility of 15-ketoprogesterone. The more polar zone was eluted and the residue after two crystallizations from ethanol–hexane mixtures yielded 4.3 mg of crystals, mp 155–157°. The mass spectrum indicated that the molecular weight was 330, which is consistent with the formula $C_{21}H_{30}O_3$, *m/e* 330 (M^+, 100%), 315 (M − 15, 12%), 312 (M − 18, 8%), 288 (M − 42, 52%), 124 (**1**, 88%). The infrared spectrum is shown in Figure 5A. As there is no authentic sample available for direct comparison, we synthesized the other possible isomer, 20β-dihydropregn-4-ene-3,15-dione, by enzymatic reduction of 15-ketoprogesterone with 20β-hydroxysteroid dehydrogenase. The 20β isomer obtained has mp 196–198° and an infrared spectrum which is distinct from that of the 20α isomer. After taking into consideration the fragmentation pattern obtained during mass spectral analysis, the infrared spectrum, and the fact that *Rhodatula logissima* reduces the C-20 carbonyl to the 20α alcohol, one can therefore assign the structure 20α-dihydropregn-4-ene-3,15-dione to this material with some confidence.

Purification of 15α-Hydroxydeoxycorticosterone. Crude 15α-hydroxydeoxycorticosterone, prepared by microbiological hydroxylation with a strain of *Penicillium* ATCC 11598, was obtained through the courtesy of Dr. C. Vézina and Dr. S. N. Sehgal, Ayerst Laboratories, Montreal, and was purified by chromatography on a silica gel column. Elution with 4.5% ethanol in benzene yielded a residue which was crystallized from acetone–ethanol–hexane mixtures. The crystals had mp 202–205° and an infrared spectrum identical both with that of a pure sample of 15α-hydroxydeoxycorticosterone (AY20551 supplied through the courtesy of Dr. M. Givner, Ayerst Laboratories, Montreal) and with that reported by Meystre *et al.* (1955). The mass spectrum indicated a molecular weight of 346 which agrees with the formula $C_{21}H_{30}O_4$, *m/e* 346 (M^+, 5%), 315 (M − 31, 100%), 287 (M − 31 + 28, 31%), 269 (M − 31 + 28 + 18, 74%), 251 (M − 31 + 28 + 36, 10%), 227 (M − 31 + 28 + 18 + 42, 20%), 124 (—, 18%). The infrared spectrum of 15α-hydroxydeoxycorticosterone is given in Figure 6A. The structure of this compound was confirmed by converting it to 15α-hydroxyandrost-4-en-3-one, 17β-carboxylic acid by oxidation with periodic acid or sodium bismuthate. The periodic acid oxidation was done according to a method described by Zaffaroni and Burton (1951) as follows. A total of 8.8 mg of 15α-hydroxydeoxycorticosterone was dissolved in 2 ml of methanol and to this solution was added 1 ml of 0.03 M periodic acid reagent (68.4 mg of H_5IO_6 in 10 ml of 0.2 N H_2SO_4), and the mixture was allowed to stand in the dark at room temperature for 15 hr. The reaction mixture was then diluted with 50 ml of water and extracted with ethyl acetate (three times with 150 ml). The organic phase was washed with water until neutral and then evaporated to dryness. The residue (14.5 mg) was purified by thin-layer chromatography using system H and a major ultraviolet-absorbing zone (R_F 0.5) was observed. This material was eluted and the residue obtained was crystallized from an ethanol–acetone mixture to yield 3.2 mg of crystals. The mass spectrum of this material gave a molecular weight of 332, which is consistent with the structural formula $C_{20}H_{28}O_4$, *m/e* 332 (M^+, 95%), 317 (M − 15, 9%), 314 (M − 18, 11%), 290 (M − 42, 100%), 272 (M − 60, 39%), 124 (**1**, 89%). The infrared spectrum of 15α-hydroxyandrost-4-en-3-one, 17β-carboxylic acid is given in Figure 7A and is essentially identical with the one reported by Meystre *et al.* (1955).

The sodium bismuthate oxidation was done according to the method described by Brooks and Norymberski (1953) as follows. A total of 8.3 mg of 15α-hydroxydeoxycorticosterone was dissolved in 5 ml of glacial acetic acid and to this was added 5 ml of water and 150 mg of $NaBiO_3$. The mixture was stirred for 30 min and was then diluted with 50 ml of water and extracted with ethyl acetate (three times with 150 ml). The organic phase was washed with water and finally evaporated to dryness. The residue obtained was purified as described above. The infrared spectrum of the crystals ob-

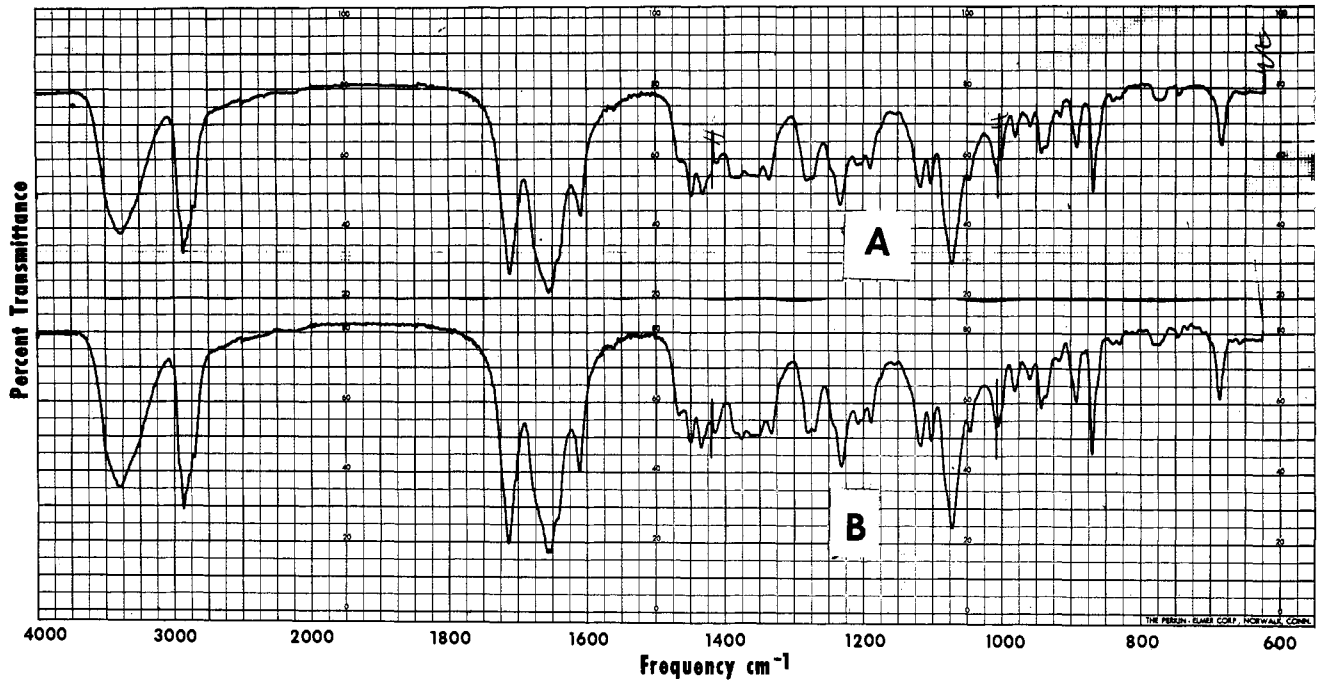


FIGURE 6: Infrared spectra of 15α-hydroxydeoxycorticosterone: (A) authentic; (B) isolated from pool XI.

tained is given in Figure 7B and is identical with that of the product obtained by periodic acid oxidation (Figure 7A).

Incubation. Male and female rabbits weighing 9–12 lb were killed by a blow on the neck. The livers were rapidly removed, washed in cold 0.15 M KCl, and then blotted on filter paper and weighed. A total of 640 g of liver was chopped finely with scissors and homogenized in 1000 ml of 0.15 M KCl containing 29 g of nicotinamide in a Waring blender. The resulting suspension was filtered through a gauze, and the filtrate diluted to 2400 ml with 0.1 M phosphate buffer, pH 7.4. To this homogenate was added 8 g of DPN^+ and 502 mg of 15α-[^{3}H]-hydroxyprogesterone (2.2×10^8 dpm) dissolved in a 50-ml propylene glycol–ethanol mixture (1:1). The final incubation medium contained approximately 70 mM nicotinamide, 2.5 mM DPN^+, with a tissue to steroid ratio of 1200:1. This mixture was then equally divided into 18 500-ml beakers and incubated for 2 hr at 37° in air on a rotary shaker. At the end of the incubation period a neutral extract was prepared by extraction with ethyl acetate. The neutral extract obtained was defatted by dissolving it in 70% methanol and precipitating the fats at −20°. The supernatant fluid was evaporated to dryness and a residue was obtained which weighed 2.95 g and contained 1.8×10^8 dpm. This residue was chromatographed on a 300-g silica gel column using increasing concentrations of ethanol in methylene dichloride. The effluent from the column was collected in 10-ml fractions at the rate of 60 ml/hr and was divided into 16 pools as shown in Figure 8. The weight and radioactivity in each pool is described in Table III. Pools IV, VI, VII, VIII, XI, XII, XIII, and XIV have been processed to date and six new metabolites have been isolated and identified from them.

Pool IV. The residue from pool IV (400 mg and 6.4×10^6 dpm) was chromatographed on a 120-g Celite column using system N. Five pools of radioactive materials were obtained. The major pool of radioactive material (3.7×10^6 dpm and 30 mg) IV E was eluted in 15–19 holdback volumes and it was chromatographed sequentially in systems F and G. In both these systems the material had the mobility of 15-ketoprogesterone. Most of the material eluted from the system was lost accidentally and only 8.15×10^5 dpm (3.8 mg) was recovered. This residue was further chromatographed on tlc using the system H, and the ultraviolet-absorbing zone corresponding in mobility to 15-ketoprogesterone was eluted. An aliquot of this eluate (150 μg) was taken for infrared analysis and the spectrum was identical with that of authentic 15-ketoprogesterone (Figure 4B). Attempts to crystallize this small quantity were not successful. At this stage the identity of the material was confirmed by the isotope dilution tech-

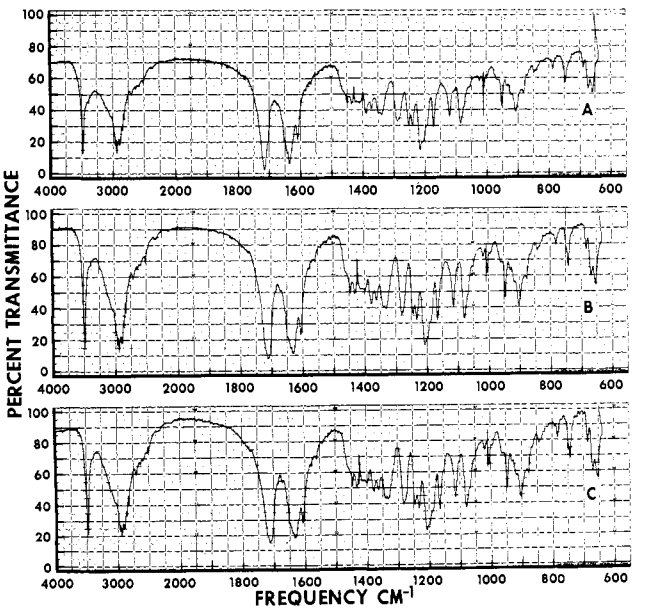


FIGURE 7: Infrared spectra of 15α-hydroxyandrost-4-en-3-one,-17β-carboxylic acid: (A) authentic 15α-hydroxydeoxycorticosterone oxidized with periodic acid; (B) oxidized with sodium bismuthate; (C) isolated 15α-hydroxydeoxycorticosterone oxidized with periodic acid.

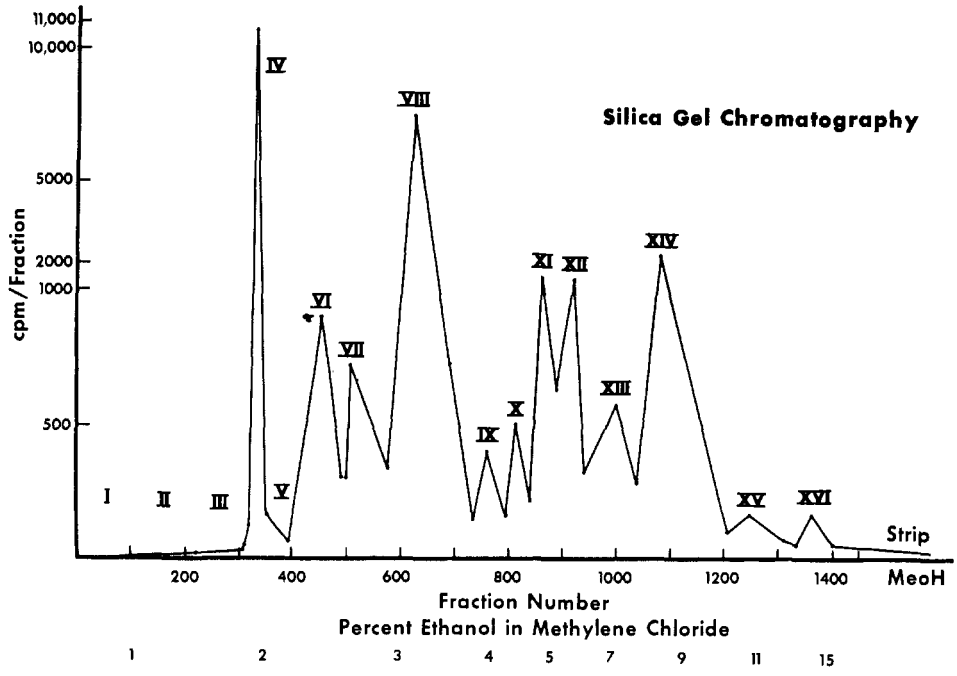


FIGURE 8: Silica gel column chromatography of the neutral extract.

nique. An aliquot containing 7.2×10^3 dpm was mixed with 30.6 mg of carrier 15-ketoprogesterone and the mixture was crystallized to constant specific activity (235, 234, 231 dpm per mg in the crystals with a calculated value of 235 dpm/mg). This then confirms the identification of the major radioactive material in pool IV as 15-ketoprogesterone. The radioactive metabolites present in the remaining pools have not yet been identified.

Pools VI and VII. The material present in pool VI (122 mg and 6.7×10^6 dpm) was chromatographed on four papers (Whatman No. 3MM) using system L for 8 hr and on tlc in system C. A major ultraviolet-absorbing material having the mobility of 15β-hydroxyprogesterone was observed in both of these systems. The material was eluted and crystallization from ethanol–hexane mixtures yielded 6 mg of crystals (specific activity 3.8×10^5 dpm/mg), mp 202–204°. The infrared spectrum of this material was identical with that of authentic 15β-hydroxyprogesterone (Figure 1A). The mass spectrum gave the molecular weight as 330, *m/e* 330 (M^+, 4%), 315 (M − 15, 1%), 312 (M − 18, 39%), 270 (M − 60, 5%), 269 (M − 61, 8%), 124 (**1**, 13%). The mass spectrum is essentially identical with that of authentic 15β-hydroxyprogesterone.

The residue from pool VII weighed 211 mg and contained 3.4×10^6 dpm. Sequential chromatography of this material in systems L (paper) and C yielded 6.3 mg of crystalline residue (2.3×10^6 dpm), mp 202°. The infrared spectrum of this material was identical with that of authentic 15β-hydroxyprogesterone and the material isolated from pool VI. Thus 15β-hydroxyprogesterone was isolated and identified from pools VI and VII.

Pool VIII. The residue from pool VIII (988 mg and 98.5×10^6 dpm) was chromatographed on a 140-g Celite column using system L. The radioactive material eluted from the column in the various fractions was pooled as shown in Figure 9. Owing to the small mass of material in pools A, B, C, and G, the metabolites eluted in these fractions have not been further investigated. From pool D (holdback volumes 8–9) a residue was obtained which weighed 25 mg and contained 1.4×10^6 dpm. It was identified as 15β-hydroxyprogesterone using the method described above. The material from pool E (holdback volumes 10–11) weighed 28 mg and contained 5.4×10^6 dpm. It was chromatographed on tlc in the system E and a major ultraviolet-absorbing material migrating slightly ahead of 15α-hydroxyprogesterone was observed. This material was eluted and the residue was crystallized from acetone–hexane to yield 4.5 mg of crystals, mp 152–153°, specific activity 3.7×10^5 dpm/mg. The infrared spectrum of this material was identical with that of authentic 20α-hydroxypregn-4-ene-3,15-dione (Figure 5B). The mass spectrum gave a molecular weight of 330, *m/e* 330 (M^+, 100%), 315 (M − 15, 10%), 312 (M − 18, 7%), 288 (M − 42, 54%), 124 (**1**, 63%).

TABLE III: Distribution of Radioactive Material in Different Fractions.

Pool No.	Wt of Crude Residue (g)	Dpm × 10^6
I	0.03	0
II	0.03	0.26
III	0.09	0
IV	0.40	6.4
V	0.10	1.5
VI	0.12	6.7
VII	0.21	3.4
VIII	0.99	98.5
IX	0.40	4.5
X	0.17	2.4
XI	0.31	12.4
XII	0.37	18.7
XIII	0.15	8.6
XIV	0.15	19.2
XV	0.37	2.1
XVI	1.22	1.3
XVII		2.5

This mass spectrum was identical with that obtained with authentic 20α-hydroxypregn-4-ene-3,15-dione.

The material from pool F (Figure 9, holdback volumes 13–17) weighed 217 mg and contained 90 × 10^6 dpm and it was crystallized twice directly from acetone–ethanol–hexane mixtures to yield 80 mg of crystals, mp 231–232° (authentic 15α-hydroxyprogesterone, mp 231–232°). The infrared spectrum of this material was identical with that of 15α-hydroxyprogesterone. The specific activity of the isolated 15α-hydroxyprogesterone was 3.8 × 10^5 dpm/mg. Three steroids have been identified from pool VIII, namely 15β-hydroxyprogesterone, 15α-hydroxyprogesterone, and 20α-hydroxypregn-4-ene-3,-15-dione.

Pool XI. The residue from pool XI (310 mg and 12.4 × 10^6 dpm) was chromatographed on a 120-g Celite column using system O. A broad peak of radioactive material was eluted in holdback volumes 19–22. This material contained some propylene glycol and was therefore partitioned between ethyl acetate and water. The organic phase was dried over Na_2SO_4 and then evaporated to dryness. A residue weighing 17.2 mg which contained 6.6 × 10^6 dpm was obtained, which on crystallization from acetone–ethanol–hexane mixtures afforded 13 mg of crystals, mp 200–202° (authentic 15α-hydroxydeoxycorticosterone, mp 202–204°), specific activity 3.5 × 10^5 dpm/mg. The infrared spectrum of this material was identical with that of authentic 15α-hydroxydeoxycorticosterone (Figure 6B). The mass spectrum gave a molecular weight of 346, *m/e* 346 (M^+, 5%), 315 (M − 31, 100%), 287 (M − 31 + 28, 44%), 269 (M − 31 + 28 + 18, 72%), 251 (M − 31 + 28 + 18 + 18, 12%), 227 (M − 31 + 28 + 18 + 42, 23%), 124 (**1**, 22%). This mass spectrum was identical with that obtained with authentic 15α-hydroxydeoxycorticosterone. The structure of the isolated 15α-hydroxydeoxycorticosterone was confirmed by its oxidation to 15α-hydroxyandrost-4-en-3-one,17β-carboxylic acid with periodic acid. A total of 5 mg (specific activity 3.5 × 10^5 dpm/mg) was oxidized and purified as described above in the Experimental Section. The crystals obtained had a specific activity of 3.6 × 10^5 dpm/mg and an infrared spectrum (Figure 7C) identical with that of authentic 15α-hydroxyandrost-4-en-3-one,17β-carboxylic acid (Figure 7A,B).

Pool XII. The residue from pool XII (370 mg and 18.7 × 10^6 dpm) was chromatographed on a 150-g Celite column using system P. Two major peaks of radioactive material were obtained in holdback volumes 1–2 (A) and 3–4 (B). Peak XII A weighed 35 mg and contained 7 × 10^6 dpm. It was purified by sequential chromatography in systems L (paper), M, and H. The major zone was detected with phosphomolybdic acid. The material eluted from system H was crystallized to yield 3 mg of crystals, mp 210–212°, specific activity 3.7 × 10^5 dpm/mg. The infrared spectrum of this material was identical with that of authentic 3α,15α-dihydroxy-5β-pregnan-20-one (mp 208–210°, Giannopoulos *et al.*, 1970). The mass spectrum gave a molecular weight of 334 which is compatible with the structural formula $C_{21}H_{34}O_3$, *m/e* 334 (M^+, 21%), 316 (M − 18, 22%), 298 (M − 18 + 18, 36%), 283 (M − 18 + 18 + 15, 17%), 273 (M − 61, 17%).

The material in peak XII B weighed 10 mg and contained 2.3 × 10^6 dpm and was chromatographed on tlc in system G. A major ultraviolet-absorbing material having the mobility of 15α-hydroxydeoxycorticosterone was eluted and crystallized to yield 3.5 mg of crystals, mp 198.5–200°, specific activity 3.9 × 10^5 dpm/mg. The infrared spectrum of this material was identical with that of 15α-hydroxydeoxycorticosterone.

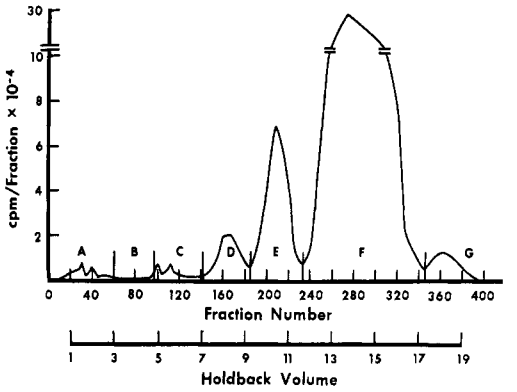


FIGURE 9: Celite partition chromatography of the residue from pool VIII of Figure 8 using System L.

Pool XIII. The residue from pool XIII (150 mg and 8.6 × 10^6 dpm) was chromatographed on a 120-g Celite column using system O. A major peak of radioactive material was eluted in holdback volumes 19–22 (2.6 × 10^6 dpm). This material was chromatographed sequentially in systems M and C and an ultraviolet-absorbing material was noted in both the systems. Crystallization of the residue eluted from the last chromatogram gave 4 mg of crystals, mp 218–220°, specific activity 3.7 × 10^5 dpm/mg. The infrared spectrum (Figure 2B) of this material was identical with that of 15β,20α-dihydroxypregn-4-en-3-one and the mass spectrum gave a molecular weight of 332, *m/e* 332 (M^+, 11%), 314 (M − 18, 43%), 296 (M − 18 + 18, 71%), 290 (M − 42, 21%), 124 (**1**, 100%). This spectrum was identical with that obtained with authentic 15β,20α-dihydroxypregn-4-en-3-one. From pool XIII it was possible to isolate and identify 15β,20α-dihydroxypregn-4-en-3-one. The remaining radioactive materials eluted from the Celite column have not been processed.

Pool XIV. The residue from pool XIV (150 mg and 19.2 × 10^6 dpm) was chromatographed on a 120-g Celite column using system P. Two major peaks of radioactive material were eluted in holdback volumes 3–5 (A) and 7–10 (B). The material in A weighed 25 mg and contained 5.04 × 10^6 dpm and was chromatographed on a 2-g silica gel column. Elution of the column with 9% ethanol in methylene dichloride gave a peak of radioactive material (4.3 × 10^6 dpm) which was then chromatographed sequentially in systems H, I, C, K, and C. A single ultraviolet-absorbing zone was obtained in all of the above systems having the mobility of 15α,20α-dihydroxypregn-4-en-3-one. Attempts to crystallize this oily material (9.2 mg) were unsuccessful. Its infrared spectrum was identical with that of 15α,20α-dihydroxypregn-4-en-3-one (Figure 3B).

The material in peak B (45 mg and 10.7 × 10^6 dpm) was chromatographed on a 3-g silica gel column. Elution with 9% ethanol in methylene dichloride gave a peak of radioactive material and the residue obtained was an oil weighing 39 mg and contained 9.4 × 10^6 dpm. This residue was chromatographed on a 50-g Celite column using system Q. A single peak of radioactive material was obtained in holdback volumes 6–8. This material (9.1 × 10^6 dpm and 30 mg) was chromatographed sequentially in systems L (paper) and J, and a single ultraviolet-absorbing compound was eluted (R_F 0.5 in system J). The residue was crystallized from ethanol–hexane mixtures to yield 8 mg of crystals, mp 182–183°. The infrared spectrum of this material showed characteristic bands at 3425 (OH), 1705 (C-20, C=O), 1680 (C-3, C=O), 1620 (Δ^4 C=C) cm^{-1}, and other bands at 1053, 1030, 876, 725, and 707 cm^{-1}.

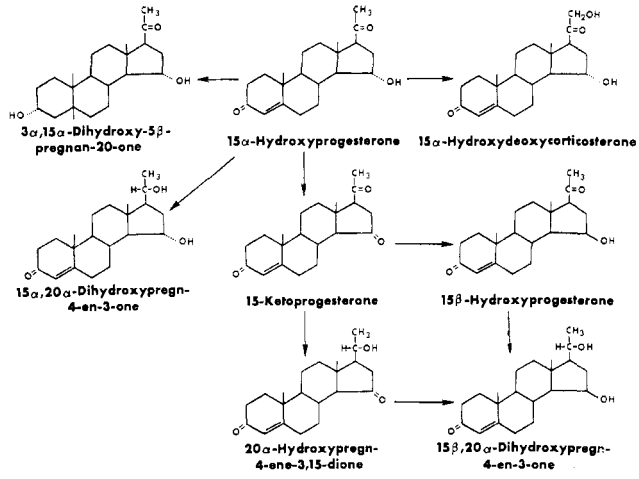


FIGURE 10: Pathways of 15α-hydroxyprogesterone metabolism in rabbit liver.

The mass spectrum indicated a molecular weight of 346. This material has not as yet been identified.

TABLE IV: *In Vivo* and *in Vitro* Metabolism of 15α-Hydroxyprogesterone.

Metabolites Isolated from Human Urine	Metabolites Isolated from Rabbit Liver Homogenates
3α,15α-Dihydroxy-5α-pregnan-20-one	Pregn-4-ene-3,15-20-trione (1.3)[a]
3α,15α-Dihydroxy-5β-pregnan-20-one	20α-Hydroxypregn-4-ene-3,15-dione (1.6)
3β,15α-Dihydroxy-5β-pregnan-20-one	15β-Hydroxyprogesterone (1.8)
5α-Pregnane-3α,15α,20β-triol	15α,20α-Dihydroxypregn-4-en-3-one (1.0)
5β-Pregnane-3α,15α,20β-triol	15β,20α-Dihydroxypregn-4-en-3-one (1.0)
	15α,21-Dihydroxypregn-4-ene-3,20-dione (3.3)
	3α,15α-Dihydroxy-5β-pregnan-20-one (0.4)

[a] Minimum per cent formed.

Discussion

In this study seven metabolites were isolated and identified by physiochemical means and comparison to standards prepared by partial synthesis. Out of these seven (Table IV) only one metabolite, 3α,15α-dihydroxy-5β-pregnan-20-one has been previously isolated from human pregnancy urine (Giannopoulos *et al.*, 1970). Approximately 50% of the 15α-hydroxyprogesterone remained unchanged and the minimal per cent conversions to the various metabolites are given in Table IV.

The formation of 15-ketoprogesterone and 15β-hydroxyprogesterone from 15α-hydroxyprogesterone by a liver homogenate is of interest because of the finding of Tweit and Kagawa (1964) who showed that the presence of a 15-keto and 15β-hydroxy group on the progesterone molecule produced a compound with antimineralocorticoid properties. Therefore it is conceivable that during the course of metabolism, 15α-hydroxyprogesterone is converted to compounds having antialdosterone activity. Whether this occurs *in vivo* in the rabbit remains to be demonstrated.

The conversion of 15α-hydroxyprogesterone to 15α-hydroxydeoxycorticosterone demonstrates that rabbit liver is capable of hydroxylation at position 21. To our knowledge this is the first demonstration of the presence of a 21-hydroxylase in liver. The presence of 21-hydroxylase has previously been demonstrated only in the adrenal (Hayano and Dorfman, 1952) and the testis (Dominguez *et al.*, 1960). Helmreich and Huseby (1962) demonstrated that a synthetic steroid such as 6α-methyl-17α-acetoxypregn-4-ene-3,20-dione (Medroxyprogesterone) when administered, in doses ranging from 40 to 200 mg per day to postmenopausal women with metastatic or recurrent breast cancer, can be metabolized to 6α-methyl-17α-acetoxy-6β,21-dihydroxypregn-4-ene-3,20-dione. These authors indicated that both C-21 and C-6 hydroxylations were not dependant upon either adrenal or gonadal functions or to be influenced appreciably by the presence of bacteria in the gut. Furthermore, these authors have suggested that the 17α-acetoxy group in some way facilitates 21-hydroxylation by nonendocrine tissues. It is possible that these hydroxylations may have occurred in the liver or in other peripheral tissues. The adrenal 21-hydroxylase is a microsomal enzyme (Ryan and Engel, 1957) while preliminary results with rabbit liver 21-hydroxylase indicate that the major portion of the enzyme activity resides in the mitochondria (B. R. Bhavnani, K. N. Shah, and S. Solomon, 1971, unpublished data). In the study described in this paper, the substrate concentration was 0.425 mM, which is rather high and the question arose as to whether the 21-hydroxylation occurred due to the high substrate concentration. This point was resolved by repeating the above experiment at low substrate concentration (12–60 μM) and again 15α-hydroxydeoxycorticosterone was isolated (B. R. Bhavnani, K. N. Shah, and S. Solomon, 1971, unpublished data), indicating that this activity was not artifactually induced by a high substrate concentration.

A scheme representing the reduction and oxidation of 15α-hydroxyprogesterone by rabbit liver is shown in Figure 10, and a summary of the metabolites obtained in this study as compared with the *in vivo* metabolism of 15α-hydroxyprogesterone in man is shown in Table IV. The metabolism of 15α-hydroxyprogesterone in the two systems is completely different in that only a single metabolite, 3α,15α-dihydroxy-5β-pregnan-20-one was isolated and identified from both studies. It is interesting to note that 15α-hydroxyl group does indeed inhibit the reduction of a Δ^4-3-ketone but more so in the *in vitro* study. Significant differences between the *in vivo* and *in vitro* studies are that the reduction of the C-20 ketone is toward 20α in the *in vitro* study and not toward 20β as found in the *in vivo* study. The observation *in vivo* of an increased 5α:5β ratio in the reduced metabolites does not hold in the *in vitro* situation as only a single 5β-reduced product was isolated. The *in vitro* metabolism of 15α-hydroxyprogesterone (present study) and progesterone (Taylor, 1955) by rabbit liver is also significantly different. All of the progesterone metabolites isolated were ring A reduced products while in the case of 15α-hydroxyprogesterone only one ring A reduced product was isolated, even though the conditions of the incubation were essentially the same in both studies. In view of these results, the inhibition of the Δ^4-3-ketone reduction can be attributed to the presence of a 15α-hydroxy group.

There are two main similarities in the metabolism of progesterone and 15α-hydroxyprogesterone in rabbit liver, in that the C-20 reduction is toward 20α and that this reduction in both is not very extensive.

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References

Brooks, C. J. W., and Norymberski, J. K. (1953), *Biochem. J. 55*, 371.
Dominguez, O. V., Acevedo, H. F., Huseby, R. A., and Samuels, L. T. (1960), *J. Biol. Chem. 235*, 2608.
Fried, J., Thoma, R. W., Pearlman, D., Etterz, J., and Borman, A. (1955), *Rec. Prog. Horm. Res. 11*, 158.
Giannopoulos, G., Durham, L. J., and Solomon, S. (1970), *Biochemistry 9*, 584.
Giannopoulos, G., and Solomon, S. (1967), *Biochemistry 6*, 1226.
Hayano, M., and Dorfman, R. I. (1952), *Arch. Biochem. Biophys. 36*, 237.
Hayes, F. N., Schrodt, A. G., and Gibbs, J. A. (1964), 9th Symposium on Advances in Tracer Methodology, San Francisco, Calif.
Helmreich, M. L., and Huseby, R. A. (1962), *J. Clin. Endocrin. Metab. 22*, 1018.
Kelly, W. G., Bandi, L., and Lieberman, S. (1962), *Biochemistry 1*, 172.
Meystre, Ch., Vischer, E., and Wettstein, A. (1955), *Helv. Chim. Acta 38*, 381.
Ramm, P. J., and Caspi, E. (1969), *J. Biol. Chem. 244*, 6065.
Ryan, K. J., and Engel, L. L. (1957), *J. Biol. Chem. 225*, 103.
Taylor, W. (1955), *Biochem. J. 60*, 380.
Tweit, R. C., and Kagawa, C. M. (1964), *J. Med. Chem. 7*, 524.
Zaffaroni, A., and Burton, R. B. (1951), *J. Biol. Chem. 193*, 749.

Gentamicin Resistance in Strains of *Pseudomonas aeruginosa* Mediated by Enzymatic N-Acetylation of the Deoxystreptamine Moiety*

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ABSTRACT: Strains of *Pseudomonas aeruginosa* which are gentamicin resistant but tobramycin sensitive have been found to inactivate gentamicin by enzymatic acetylation. Physical and chemical characterization of the purified acetylgentamicin has revealed that the 3-amino group of the 2-deoxystreptamine moiety is acetylated in the antibiotic. *In vitro* studies with the partially purified enzyme have shown that the gentamicin C antibiotics and sisomicin are all excellent substrates, whereas closely related antibiotics such as tobramycin, kanamycins A, B, and C, and gentamicin A are either poor substrates or not acetylated.

The amino glycoside antibiotics, which are effective agents against gram-negative infections, can be enzymatically inactivated by many resistant strains isolated in clinical situations. A number of different enzymes have been identified in R-factor-containing strains which can modify these antibiotics by phosphorylation, acetylation, or adenylylation (Davies *et al.*, 1971). In Pseudomonads, neomycin–kanamycin resistance *via* phosphorylation has been studied by Umezawa *et al.* (1968) but other amino glycoside resistance mechanisms have not been identified. The antibiotic resistance patterns of bacterial strains provide a convenient way of recognizing the mechanism of resistance, since different enzymes have different amino glycoside substrate specificities. We recently obtained several clinical isolates of *Pseudomonas aeruginosa* which showed a resistance pattern different from those already known, since they were gentamicin resistant but tobramycin (nebramycin factor 6) sensitive. All other *Pseudomonas* strains which we have studied are sensitive to both antibiotics. Several strains of *Klebsiella* and *Escherichia coli* have been shown to be resistant to both antibiotics as a result of enzymatic adenylylation (Benveniste and Davies, 1971b). Analysis of extracts of the resistant *P. aeruginosa* strains has revealed a new acetylating enzyme with high specificity for the gentamicin C antibiotics.

Physical and chemical studies of the inactivated antibiotic have shown that acetylation occurred on the 3-amino group of the 2-deoxystreptamine ring. This is the first report of enzymatic modification of this cyclitol moiety in amino glycoside antibiotics.

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