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Synthesis of 14α-Hydroxyprogesterone and Its Isolation from Human Late-Pregnancy Urine*

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ABSTRACT: Nonlabeled and high specific activity [4-^{14}C]-14α-hydroxyprogesterone was synthesized from 14α-hydroxydeoxycorticosterone, prepared by microbiological hydroxylation of deoxycorticosterone. Purified [4-^{14}C]-14α-hydroxyprogesterone of known specific activity was added as a recovery marker to a large pool of late-pregnancy urine (82 l.) and the steroid was isolated and identified. The 14α-hydroxyprogesterone excreted in the urine was quantitated by the isotope derivative procedure by first reducing it to 14α,20β-dihydroxypregn-4-en-3-one with 20β-hydroxysteroid dehydrogenase followed by acetylation with [^{3}H]acetic anhydride.

From the ^{3}H/^{14}C ratio of the purified derivative the excretion of 14α-hydroxyprogesterone was found to be 0.52 μg/day. Identification of the isolated 14α-hydroxyprogesterone was confirmed by infrared analysis.

The microbiological hydroxylation at C_{15} and C_{16} of the steroid nucleus has been described and the subject has been extensively reviewed (Dorfman and Ungar, 1965). In recent years these hydroxylations have also been demonstrated to occur in mammalian tissues. A number of neutral 16α- and 15α-hydroxy steroids have been isolated from human urine, (Hirschmann and Hirschmann, 1945, 1950; Lieberman *et al.*, 1953; Fotherby *et al.*, 1957; Neher *et al.*, 1959; Hirschmann *et al.*, 1961; Fukushima *et al.*, 1961; Bongiovanni, 1962; Reynolds, 1965, 1966; Ruse and Solomon, 1966a,b; YoungLai and Solomon, 1967; Giannopoulos and Solomon, 1967). These findings prompted us to initiate a study to determine whether 14α-hydroxylated steroids could also be formed by mammalian tissues. We therefore set out to synthesize labeled 14α-hydroxyprogesterone[1] in order to use it as an aid in its isolation.

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[1] The following trivial names have been used: progesterone = pregn-4-ene-3,20-dione; pregnanediol = 5β-pregnane-3α,20α-diol; deoxycorticosterone = 21-hydroxypregn-4-ene-3,20-dione; deoxycorticosterone acetate = 21-acetoxypregn-4-ene-3,20-dione; 14α-hydroxyprogesterone = 14α-hydroxypregn-4-ene-3,20-dione; testosterone = 17β-hydroxyandrost-4-en-3-one; 14α-hydroxytestosterone = 14α,17β-dihydroxyandrost-4-en-3-one; estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; 14α-hydroxyestrone = 3,14α-dihydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol = estra-1,3,5(10)-triene-3,17β-diol; 14α-hydroxyestradiol = estra-1,3,5(10)-triene-3,14α,17β-triol.

TABLE I: Solvent Systems Used in Chromatography.

System	Solvents
A	Benzene–cyclohexane–methanol–water (1:2:3:3, v/v)
B	Benzene–methanol (9:1, v/v)
C	Benzene–methanol–water (10:5:5, v/v)
D	Skellysolve C–benzene–methanol–water (10:5:8:2, v/v)
E	*n*-Hexane–ethyl acetate–methanol–water (18:2:7:3, v/v)
F	Isooctane–*t*-butyl alcohol–methanol–water (50:10:35:5, v/v)
G	Benzene–ethanol (9:1, v/v)

While these studies were in progress it was demonstrated that bovine (Knuppen *et al.*, 1967) and porcine adrenal preparations (Loke and Gan, 1968) are capable of converting estrone into 14α-hydroxyestrone. This paper describes the synthesis of nonlabeled and ^{14}C-labeled 14α-hydroxyprogesterone and its isolation from human late-pregnancy urine.

Methods

Techniques of counting, solvent preparation, and hydrolysis of urinary conjugates have been described previously (Ruse and Solomon, 1966a,b; YoungLai and Solomon, 1967). Infrared spectra (KBr) were obtained using a Perkin-Elmer infrared spectrometer (Model 221). Melting points were determined on a Kofler block and are corrected, and solvent systems used for chromatography are given in Table I. Paper chromatography in systems A and C were performed on strips of paper 110 cm in length in tanks 120 cm high, fitted with a solvent saturating device described by Kimball *et al.* (1966). Steroids were eluted from paper with methanol. Thin-layer chromatography was performed using silica gel G in the usual manner. Steroids were eluted from thin-layer plates with acetone.

Preparation of 14α-Hydroxyprogesterone. Microbiological hydroxylation of progesterone to yield 14α-hydroxyprogesterone with *Mucor griseo-cyanus* (ATCC 1207) according to the method described by Singh *et al.* (1967) was unsuccessful. A method was therefore devised whereby 14α-hydroxydeoxycorticosterone (II), obtained through the courtesy of Dr. C. Vézina, Ayerst Laboratories, Montreal, was degraded chemically to 14α-hydroxyprogesterone (VI) as shown in Figure 1. The methods used for the formation of the tosylate (III), chloride (IV), and iodide (V), and the conversion of iodide (V) into 14α-hydroxyprogesterone (VI) were similar to those described by Reichstein and Fuchs (1940) for the conversion of corticosterone into progesterone. In one such experiment 2 g of 14α-hydroxydeoxycorticosterone (II) was dissolved in 15 ml of a (1:9) mixture of pyridine and chloroform (alcohol free), then 2.3 g of *p*-toluenesulfonyl chloride was added and the solution was kept in a desiccator at room temperature for 24 hr. The reaction mixture was then poured into a beaker containing 1000 ml of ether and a

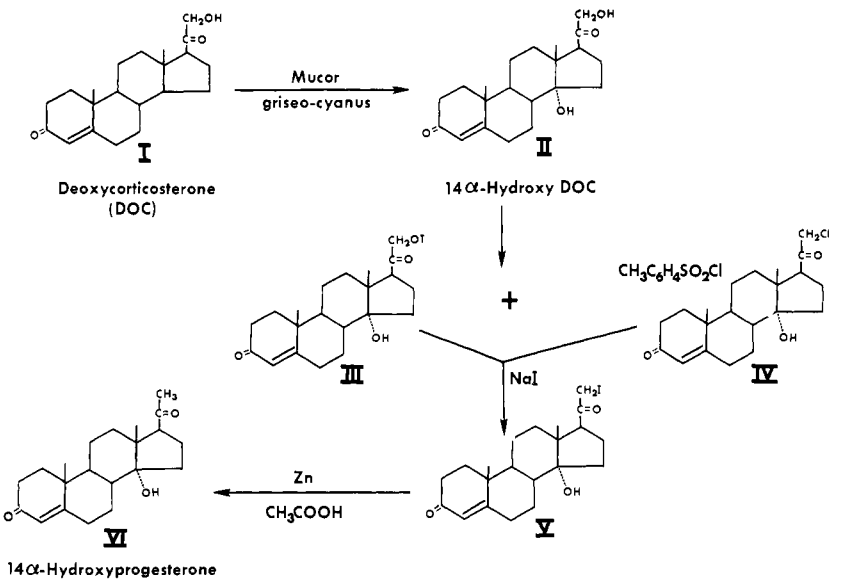

FIGURE 1: Synthesis of 14α-hydroxyprogesterone.

yellow oily precipitate separated out which was discarded. The ether phase was decanted into a separatory funnel and was washed first with 0.1 N HCl, then with 10% Na_2CO_3, and finally with water until neutral, dried over Na_2SO_4, and evaporated to dryness *in vacuo*. The residue in the flask (mixture of III and IV) was treated with 2 g of NaI in 30 ml of anhydrous acetone at 60° for 5 min. The mixture was then filtered and evaporated to dryness and yielded a greenish residue of the iodide V. This residue was dissolved in 10 ml of glacial acetic acid, 700 mg of zinc dust was added, and the mixture was stirred until a colorless solution was obtained (15–20 min), which was then filtered. The filtrate was transferred to a separatory funnel containing 1 l. of ethyl acetate and the organic phase was then washed with 10% Na_2CO_3, with water until neutral, dried over Na_2SO_4, and evaporated *in vacuo* to yield a residue (VI) weighing 1.05 g. This residue was chromatographed on a 100-g Celite partition column using system A and the material eluted between the second and third holdback volumes was pooled. The residue from this pool weighed 495 mg and was further purified by chromatography on a 20-g alumina column. Elution with benzene yielded a residue (357 mg) which after crystallization from ethanol–hexane gave 305 mg of granular crystals, mp 193–197° (the melting point of authentic 14α-hydroxyprogesterone is 193–196°, supplied through the courtesy of Dr. D. H. Peterson, The Upjohn Co., Kalamazoo, Mich.) whose infrared spectrum was identical with that of authentic 14α-hydroxyprogesterone.

14α,20β-Dihydroxypregn-4-en-3-one. This steroid was prepared by reduction of the 14α-hydroxyprogesterone with $NaBH_4$ as described by Norymberski and Wood (1955) and subsequent oxidation of the allylic alcohol at C_3 with dichlorodicyanobenzoquinone as described by Burn *et al.* (1960). The reduced product was purified by thin-layer chromatography in system B, and then crystallized from ethanol–hexane to give granular crystals, mp 181–182°. An infrared spectrum (KBr) showed the retention of the Δ^4-3-ketone and disappearance of the 20-ketone. We were not able to compare the product with an authentic sample, but the structure 14α,20β-dihydroxypregn-4-en-3-one may be assigned to it because the same compound was obtained by the enzymatic reduction of the 20-ketone to 20β-hydroxy with 20β-hydroxysteroid dehydrogenase (Sigma type II).

The enzymatic reduction was done according to the method described by Henning and Zander (1962). In a trial preparation 25 mg of 14α-hydroxyprogesterone was dissolved in a few drops of ethanol and to this solution was added 1 ml of a 2.2 M ammonium sulfate suspension of 20β-hydroxysteroid dehydrogenase (5 mg), 100 ml of Sorensen buffer (97 ml of 0.15 M KH_2PO_4 and 3 ml of 0.067 M Na_2HPO_4, pH 5.5) containing 2.7×10^{-3} M EDTA, and 10 ml of 0.1 M Tris buffer (pH 8.1) which also contained 250 mg of NADH. The mixture was incubated for 2 hr at 37° in air. At the end of the incubation the mixture was extracted three times with twice the volume of ethyl acetate and the organic phase was washed with water until neutral, dried over Na_2SO_4, and evaporated to dryness. The residue was chromatographed on a silica gel plate in system B and two ultraviolet-positive materials were observed; the more polar material corresponding in mobility to the 14α,20β-dihydroxypregn-4-en-3-one was eluted. Two crystallizations of this product from ethanol–hexane yielded 8.8 mg of crystals (mp 181–182°) whose infrared spectrum (KBr) was identical with that of 14α,20β-dihydroxypregn-4-en-3-one synthesized above.

20β-Acetoxy-14α-hydroxypregn-4-en-3-one. This compound was prepared in the usual manner by dissolving 14α,20β-dihydroxypregn-4-en-3-one in two parts of pyridine and one part of acetic anhydride. The product after crystallization had a melting point of 258–259°.

20β-Acetoxy-14α-hydroxypregn-4-ene-3-thiosemicarbazone. This derivative was prepared by dissolving 5 mg of 20β-acetoxy-14α-hydroxyprogesterone in 0.2 ml of glacial acetic acid, then 8 mg of thiosemicarbazide was added, and the mixture was gently boiled until a clear solution was obtained. The reaction was allowed to proceed for 3 hr at 70° after which the acetic acid was removed under nitrogen, 2 ml of a 10% Na_2CO_3 solution was added, and the mixture was extracted three times with methylene chloride. The organic phase was washed with water until neutral and evaporated to dryness. The residue was crystallized twice from methylene chloride–ethanol to yield 6.4 mg of crystals having a melting point of 196–198° and its infrared spectrum showed characteristic bands at 3225, 1575 (NH), 1720 (acetate), 1625 (Δ^4), 1365 (C=S), 1245 cm^{-1} (acetate), and other major bands at 965, 930, 870, 850, 835, and 780 cm^{-1}. The spectrum indicated the retention of the acetate, the Δ^4 group, and the disappearance of the 3-ketone.

[4-^{14}C]-14α-Hydroxyprogesterone. The procedure described above for the synthesis of nonlabeled 14α-hydroxyprogesterone was also used for the preparation of the labeled steroid. Since the starting material [4-^{14}C]-14α-hydroxydeoxycorticosterone was not available it was prepared by the microbiological hydroxylation of [4-^{14}C]deoxycorticosterone, 212×10^6 dpm, specific activity 35.0 mCi/mmole (New England Nuclear Corp., Boston, Mass.). Carrier deoxycorticosterone (3.8 mg) was added prior to hydroxylation and the procedure used was the same as the one described by Singh *et al.* (1967). At the end of the incubation the medium was extracted with ethyl acetate, and the dried extract was chromatographed on paper in systems A and C.

The radioactive material corresponding in mobility to 14α-hydroxydeoxycorticosterone in the above two systems accounted for only 2.3% (4.6×10^6 dpm) of the original radioactivity and it was next converted to 14α-hydroxyprogesterone by the procedure described above (Figure 1). The final reaction product was chromatographed sequentially in systems A, D, and E. In all three systems the radioactive material had the mobility of 14α-hydroxyprogesterone, and a total of 1.1×10^6 dpm was recovered from the last chromatogram. Radiochemical purity at this stage was determined by isotope dilution (4.45×10^4 dpm and 29.3 mg of

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

Crystallization	Specific Activities (dpm/mg)	
	Crystals	Mother Liquors
1	2920	
2	2880	2900
3	2870	2830
Calculated[a]	2920	

[a] A total of 4.1 $\times$ 10^4 dpm of material from the last chromatogram was mixed with 14 mg of carrier 14α-hydroxyprogesterone prior to crystallization. The calculated specific activity is based on these values.

carrier 14α-hydroxyprogesterone) and was found to be only 80%. Further purification was achieved by acetylating the remaining product with pyridine and acetic anhydride. The acetylated mixture was chromatographed on paper in system D and two areas of radioactive material were observed, the more polar of the two corresponded in mobility to 14α-hydroxyprogesterone. This polar material was further purified by paper chromatography in system F, and a single area of radioactive material corresponding in mobility to 14α-hydroxyprogesterone was observed and was eluted. Radiochemical purity at this stage was over 98% as shown in Table II and this material was used as a recovery tracer in the isolation study to be described.

Determination of the Specific Activity of [4-^{14}C]-14α-Hydroxyprogesterone. An aliquot (5.3 $\times$ 10^4 dpm) of the purified 14α-hydroxyprogesterone was reduced enzymatically with the 20β-hydroxy steroid dehydrogenase as described earlier. The reduced product was purified by paper chromatography in system D and then acetylated with tritiated acetic anhydride (specific activity 3.25 $\times$ 10^4 dpm/μg of deoxycorticosterone acetate). The acetylated product was mixed with carrier 20β-acetoxy-14α-hydroxypregn-4-en-3-one, and the mixture was chromatographed on a small alumina column. The material eluted with benzene was crystallized to constant ^{3}H/^{14}C ratio as shown in Table III. From the final ^{3}H/^{14}C ratio, the specific activity of the [4-^{14}C]-14α-hydroxyprogesterone was calculated to be 3.05 $\times$ 10^4 dpm/μg.

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

Crystallization	Sp. Act. (dpm of ^{14}C/mg)	^{3}H/^{14}C
1	1700	2.2
2	1710	1.2
3	1630	1.3
4	1600	1.2

[a] An aliquot, 5.3 $\times$ 10^4 dpm of [4-^{14}C]-14α-hydroxyprogesterone, was reduced enzymatically to 14α,20β-dihydroxypregn-4-en-3-one which was then acetylated with [^{3}H]acetic anhydride and the product was mixed with 10.2 mg of carrier 20β-acetoxy-14α-hydroxypregn-4-en-3-one. The mixture was purified (as described in the text) and was crystallized from ethanol–benzene, methanol–Skellysolve B, acetone, and acetone–Skellysolve C mixtures.

Experimental Section and Results

A 62-day urine pool (82 l.) was collected from normal pregnant women in the third trimester of pregnancy. The urine was collected by a large number of females and frozen prior to processing during a 2-week period. To this urine pool was added 5.1 $\times$ 10^5 dpm of [4-^{14}C]-14α-hydroxyprogesterone (16.9 μg) and the urine was then concentrated to 9200 ml *in vacuo* in a flash evaporator (courtesy of Ayerst Laboratories, Montreal). The urinary steroid conjugates were hydrolyzed with 200 ml of Glusulase (Endo Laboratories, Garden City, N. Y.), at pH 5.2 and 37° for 5 days, and a neutral extract was prepared which weighed 21 g. This extract was purified by chromatography on a 1.3-kg silica gel column using methylene chloride and increasing concentrations of ethanol in methylene chloride. The effluent was collected in 15-ml fractions at a rate of eight fractions per hour. A peak of radioactive material was eluted with 2% ethanol in methylene chloride (fractions 1981–2845) which when pooled yielded a residue weighing 2.6 g which contained 2.9 $\times$ 10^5 dpm. This residue had large amounts of pregnanediol as this steroid is also eluted with 2% ethanol in methylene chloride. In order to separate the pregnanediol, the residue was chromatographed on a 75-g alumina column. Elution with benzene yielded a peak of radioactive material weighing 400 mg which contained 2.55 $\times$ 10^5 dpm. This residue probably still contained some pregnanediol and related steroids and in order to separate these from 14α-hydroxyprogesterone, it was acetylated with pyridine and acetic anhydride in the usual manner. The acetylated material was chromatographed on a 10-g alumina column and elution with Skellysolve B and increasing concentrations of benzene removed all the steroid acetates leaving behind 14α-hydroxyprogesterone which was then eluted from the column with benzene. The benzene eluate contained 2.3 $\times$ 10^5 dpm and weighed 86 mg. This residue was chromatographed sequentially on Whatman No. 3MM papers in the systems D, F, and A. In all three systems a single area of radioactive material was observed with the same mobility as 14α-hydroxyprogesterone, and on elution from the last chromatogram a residue was obtained which weighed 2.8 mg and contained 1.7 $\times$ 10^5 dpm.

The specific activity of this material was determined by first reducing two-fifths (6.8 $\times$ 10^4 dpm) of it with 20β-hydroxysteroid dehydrogenase as described earlier; the reduced product was purified by paper chromatography in system D. A single area of radioactive material having the mobility of 14α,20β-dihydroxy-

TABLE IV: Proof of Radiochemical Purity of 14α-Hydroxyprogesterone Isolated from Late-Pregnancy Urine.

	20β-Acetoxy-14α-hydroxypregn-4-en-3-one		20β-Acetoxy-14α-hydroxypregn-4-ene-3-semicarbazone	
Crystallization	Sp. Act. (dpm of ^{14}C/mg)	$^{3}H/^{14}C$[b]	Sp. Act. (dpm of ^{14}C/mg)	$^{3}H/^{14}C$[b]
1	3090	3.7	2740	3.2
2	2870	3.4	2890	3.3
3	2860	3.5	2790	3.2
Calculated[a]			2860	

[a] The third crystals (specific activity 2860 dpm/mg) were converted into the thiosemicarbazone and the specific activities have been corrected for changes in molecular weight. [b] The $^{3}H/^{14}C$ used in the calculation of the specific activity of the isolated 14α-hydroxyprogesterone was 3.45. It was the average value of the second and third crystals and their mother liquors.

pregn-4-en-3-one was observed. This material was eluted and yielded a product containing 5.6×10^4 dpm which was acetylated with [^{3}H]acetic anhydride. The acetate was mixed with 12.6 mg of carrier 20β-acetoxy-14α-hydroxypregn-4-en-3-one and the mixture was chromatographed on an alumina column. Elution with 1% ethanol in benzene yielded a residue which weighed 12.6 mg and contained 4.7×10^4 dpm of ^{14}C. This residue was crystallized to constant specific activity and $^{3}H/^{14}C$ ratio as shown in Table IV. The third crystals (3.8 mg) were treated with thiosemicarbazide as described earlier and the product formed was crystallized to constant specific activity and $^{3}H/^{14}C$ ratio as shown in Table IV. From the final $^{3}H/^{14}C$ ratio and the specific activity of acetic anhydride the specific activity of urinary 14α-hydroxyprogesterone was calculated to be 1.06×10^4 dpm/μg of ^{14}C. From this specific activity and the amount of 14α-hydroxyprogesterone added to the urine it was calculated that the amount of 14α-hydroxyprogesterone excreted was 0.52 μg/day. The over-all recovery of labeled 14α-hydroxyprogesterone prior to acetylation was 33%.

The remaining 14α-hydroxyprogesterone (1.0×10^5 dpm) was also converted to 20β-acetoxy-14α-hydroxypregn-4-en-3-one as described above and it was further purified by paper chromatography in systems F and D followed by thin-layer chromatography in system G. A single ultraviolet-absorbing material having the mobility of 20β-acetoxy-14α-hydroxypregn-4-en-3-one was observed. This material was eluted from the thin-layer chromatographic plate and it contained 3.8×10^4 dpm of ^{14}C and 1.33×10^5 dpm of ^{3}H ($^{3}H/^{14}C$ = 3.5). This ratio is the same as that obtained after crystallization (Table IV). From this ratio the amount of 20β-acetoxy-14α-hydroxypregn-4-en-3-one present in the sample was calculated to be 4 μg. The infrared spectrum (KBr) of this material was almost identical with that of authentic 20β-acetoxy-14α-hydroxypregn-4-en-3-one (4 μg) as shown in Figure 2. However a small amount of impurity is still present in the isolated material which exhibited absorption bands in the region of Δ^4-3-ketone, thus giving rise to shoulders in the band between 1700 and 1750 cm^{-1}, and the band at 1440 cm^{-1} observed in the standard was obscured. The fingerprint region was identical with that of the standard. Further purification of this material was not possible due to insufficient amounts. It is noteworthy that in the 4 μg present at this stage, 1.4 μg was accounted for by the amount of tracer added to the original urine pool and that trials with a 2-μg sample did not give a recognizable infrared spectrum.

Discussion

Attempts to synthesize 14α-hydroxyprogesterone by direct microbiological hydroxylation of progesterone with *Mucor griseo-cyanus* according to a method described by Singh *et al.* (1967) was unsuccessful. The procedure described by Eppstein *et al.* (1958) for microbiological hydroxylation of progesterone was not attempted as these authors reported that three samples of 14α-hydroxyprogesterone obtained from three different microbiological experiments differed from each other significantly and contained contaminants. It appears therefore that microbiological 14α-hydroxylation of progesterone is not a suitable way of preparing this steroid. As a result nonlabeled 14α-hydroxyprogesterone was synthesized by chemical degradation of 14α-hydroxydeoxycorticosterone.

A similar method was used for the preparation of high specific activity ^{14}C-labeled 14α-hydroxyprogesterone; however, in this case the starting material was [4-^{14}C]deoxycorticosterone which was first converted to [4-^{14}C]-14α-hydroxydeoxycorticosterone by microbiological hydroxylation with *Mucor griseo-cyanus*, and then to [4-^{14}C]-14α-hydroxyprogesterone by the same route as described in the preparation of the nonlabeled steroid. Radiochemical purity of [4-^{14}C]-14α-hydroxyprogesterone was achieved after extensive purification and it was shown to be at least 98% pure.

The presence of 14α-hydroxyprogesterone in late-pregnancy urine was established from the results of the double-isotope derivative study and were confirmed by the isolation of sufficient amounts for a micro infrared spectrum. In this investigation, trace amounts of ^{14}C-labeled 14α-hydroxyprogesterone of known specific activity was added to the urine to facilitate the isolation

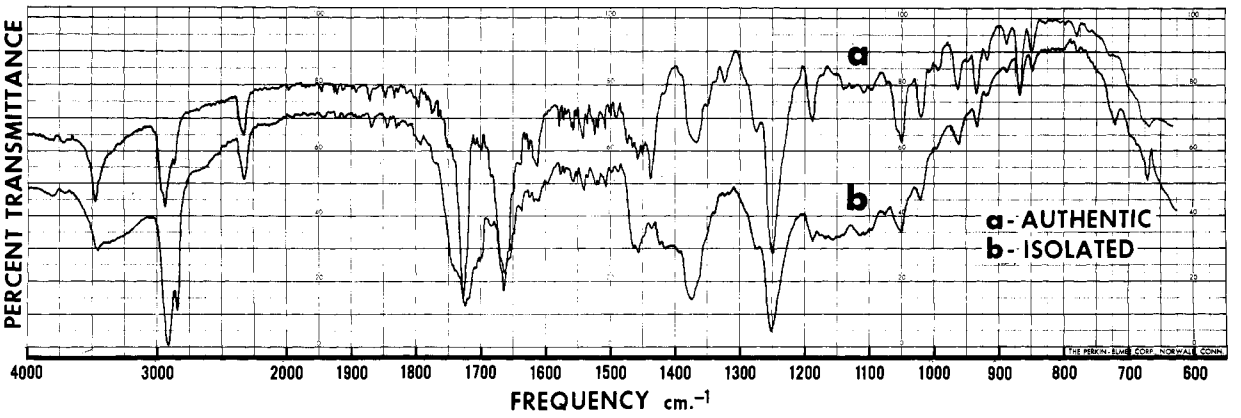

FIGURE 2: Infrared spectra of 20β-acetoxy-14α-hydroxypregn-4-en-3-one.

of small amounts of this steroid. The urine was processed, and two-fifths of the purified material was utilized for quantitation of the isolated 14α-hydroxyprogesterone by the double-isotope derivative technique, as described in the text. The final $^{3}H/^{14}C$ ratio, after crystallization (Table IV), was 3.45 and the specific activity with respect to ^{14}C of the isolated product was calculated to be 1.06×10^{4} dpm/μg (specific activity of material added to urine was 3.05×10^{4} dpm/μg). A threefold reduction in specific activity had occurred and thus the amount excreted was calculated to be 0.52 μg/day. No information regarding the nature of the conjugates of 14α-hydroxyprogesterone is available at the present time and since the tracer was added to the urine in the nonconjugated form, the calculated value of 0.52 μg/day must be considered as minimal. To confirm the isolation, the remaining three-fifths of the purified material was also converted into 20β-acetoxy-14α-hydroxypregn-4-en-3-one. The product after purification by chromatography had a $^{3}H/^{14}C$ ratio of 3.5 which is the same as the one obtained in the first determination. The infrared spectrum of this isolated material was almost the same as that of authentic 20β-acetoxy-14α-hydroxypregn-4-en-3-one. This then confirmed the results obtained from the isotope derivative study, that a small amount of 14α-hydroxyprogesterone is present in human late-pregnancy urine, and it can therefore be concluded that 14α hydroxylation of neutral steroids is a pathway of steroid metabolism during late pregnancy. This observation was strengthened by our recent finding that very small amounts of 14α-hydroxytestosterone are excreted in human late-pregnancy urine (YoungLai *et al.*, 1969).

The possibility that progesterone is 14α-hydroxylated in late-pregnancy urine during storage and processing of the urine is remote. There is very little progesterone in pregnancy urine, microbial 14α hydroxylation of progesterone does not occur readily, and the urines were frozen from the time of collection until they were processed.

While this work was in progress, Knuppen *et al.* (1967) and Loke and Gan (1968) demonstrated the *in vitro* formation of 14α-hydroxyestrone from estrone with adrenal preparations. The biological activity of 14α-hydroxyprogesterone is not known and therefore the physiological significance of the formation of this steroid during late pregnancy will have to await further investigations. It is possible that *in vivo* 14α-hydroxyprogesterone is uniquely elaborated in the human fetus and may serve as a precursor for the 14α-hydroxylated neutral steroids such as 14α-hydroxytestosterone, which in turn can be converted into the 14α-hydroxylated estrogens. That this pathway may be operative is suggested by the finding of Knuppen *et al.* (1967) who were able to demonstrate that 14α-hydroxyandrost-4-ene-3,17-dione can be aromatized by human placental microsomes to yield 14α-hydroxyestrone. It is interesting to note that the rate of aromatization of 14α-hydroxyandrost-4-ene-3,17-dione equaled that of testosterone to 17β-estradiol. However, these authors (Knuppen *et al.*, 1967) failed to isolate 14α-hydroxyestrone and 14α-hydroxyestradiol from human pregnancy urine. In view of our results, it is quite possible that 14α-hydroxylated estrogens are also present in very minute amounts and it would be necessary to work with large volumes of human pregnancy urine and very sensitive methods of detection before one can draw any definite conclusions regarding the presence or absence of 14α-hydroxylated estrogens.

It is of interest to note that while 14α-hydroxytestosterone and 14α-hydroxyprogesterone are the first two naturally occurring 14α-hydroxylated neutral steroids isolated from a mammalian source a 14α-hydroxysterol such as ecdysone (Butenandt and Karlson, 1954) has been isolated from insects and 14β-hydroxy steroids present in cardiac glycosides and toad poisons have been previously isolated from plants and amphibians (Fieser and Fieser, 1959).

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Studies on the Binding of Soluble Antigens to a Unique Ribonucleoprotein Fraction of *Macrophage Cells**

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ABSTRACT: The response of macrophages to an immunogenic synthetic copolymer of L-glutamic acid, L-alanine, and L-tyrosine has been studied. This synthetic antigen is taken up by rat macrophages *in vitro* and is complexed exclusively to a distinct ribonucleoprotein of the macrophage. This ribonucleoprotein, which has previously been shown to exist only in macrophages, is separable from the rest of the cell's ribonucleic acid by virtue of a unique density in cesium sulfate.

Glu-Ala-Tyr can be partially released from the ribonucleoprotein of the macrophage by solutions of high ionic strength. The relation of these findings to those of other investigators is discussed.

A low molecular weight RNP[1] fraction of macrophages has been shown to be associated with particulate antigens to which the macrophage cells are experimentally exposed (Gottlieb, 1968a; Bishop, 1968). The RNP fraction derived from cells exposed to T2 bacteriophage specifically stimulates the production of neutralizing antibody against this bacterial virus. This ribonucleoprotein is characterized by an effective buoyant density of 1.588 g/cm^3 in cesium sulfate solution. Other physical properties are to be reported subsequently (Gottlieb and Straus, 1969).

Roelants and Goodman (1968) have studied the association of poly-γ-D-glutamic acid with RNA from macrophage cells. These workers reported that poly-γ-D-glutamic acid was associated with the 4–5S fraction of RNA in a strong and possibly covalent linkage and that they could not detect a specific association of the polypeptide with the light-density RNP fraction of macrophage RNA.

In an attempt to clarify the relation between antigen and RNP of the macrophage, we report here additional

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[1] RNP = ribonucleoprotein.