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

G. Giannopoulos and S. Solomon

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

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

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

formation of estriol. It was, therefore, important to demonstrate the formation of C_{19} 15α-hydroxysteroids during human pregnancy and a search for 15α-hydroxyandrostenedione and 15α-hydroxytestosterone in late pregnancy was initiated. In this paper data for the isolation and identification of 15α-hydroxyandrostenedione and 15α-hydroxytestosterone from human late-preganacy urine will be presented.

Materials and Methods

Techniques of counting, chromatography, infrared analysis, and enzymatic hydrolysis of urinary steroid conjugates with Glusulase have been described (Ruse and Solomon, 1966). Solvent systems used in chromatography are shown in Table I. Tritium-labeled acetic anhydride, 20% (v/v) in benzene, was standardized as previously described (Giannopoulos *et al.*, 1970) and two solutions (solutions 1 and 2) were used in these studies. The specific activities of solutions 1 and 2 were 3.67×10^8 and 3.26×10^7 dpm per mg of deoxycorticosterone acetate, respectively.

Formation of Derivatives. A derivative of 15α-acetoxyandrostenedione was prepared by reduction of the steroid with sodium borohydride and subsequent oxidation of the product with the DDQ reagent as described by Stern *et al.* (1968).

A derivative of 15α-hydroxytestosterone diacetate was prepared by reduction of the steroid with $NaBH_4$. The product obtained was chromatographed on thin-layer chromatographic plates using system L. On spraying a 0.5-cm portion of the plates with phosphomolybdic acid, one major spot was observed and the material was eluted and crystallized. The infrared spectrum (KBr) of the crystalline material showed the retention of the acetate bands, the absence of the α,β-unsaturated ketone and the presence of a C=C band at 1610 cm^{-1}. Since it is known (Norymberski and Woods, 1955) that $NaBH_4$ reduction of a Δ^4-3-ketosteroid gives mainly the 3β-alcohol, this derivative most probably was 15α,17β-diacetoxyandrost-4-en-3β-ol.

Preparation of [4-^{14}C]15α-Hydroxyandrostenedione and [4-^{14}C]15α-Hydroxytestosterone. The preparation of [4-^{14}C]-15α-hydroxyandrostenedione by the microbiological hydroxylation of labeled androstenedione and the proof of its purity have been described previously (Stern *et al.*, 1968). An aliquot of [4-^{14}C]15α-hydroxyandrostenedione containing 2.42×10^5 dpm was acetylated with [^{3}H]acetic anhydride (solution 1) and the product was mixed with 65 mg of carrier 15α-acetoxyandrostenedione. The mixture was chromatographed on a 7-g alumina column from which 63 mg of oily material was eluted with benzene–Skellysolve B (1:1). This material was difficult to crystallize and it was therefore reduced with $NaBH_4$ and then oxidized with DDQ, and the product (59.0 mg) was chromatographed on a 6-g alumina column. Elution with 0.5% ethanol in benzene yielded 41.0 mg of a residue which was crystallized to constant specific activity and a $^3H:^{14}C$ ratio as shown in Table II. From the final $^3H:^{14}C$ ratio the specific activity of [4-^{14}C]15α-hydroxyandrostenedione was calculated to be 4.26×10^8 dpm/mg.

[4-^{14}C]15α-Hydroxytestosterone was prepared by reduction of an aliquot (1.47×10^7 dpm) of [4-^{14}C]15α-hydroxyandrostenedione with $NaBH_4$ and subsequent oxidation of the product with the DDQ reagent as previously described (Stern *et al.*, 1968). A product containing 8.80×10^6 dpm was obtained and it was judged to be at least 99% pure by the use of the isotope dilution technique on an aliquot. The calculated specific activity of [4-^{14}C]15α-hydroxytestosterone, based on the specific activity of [4-^{14}C]15α-hydroxyandrostenedione, and the increase in molecular weight, was 4.23×10^8 dpm/mg.

TABLE I: Solvent Systems Used in Chromatography.

System	Type of Chromatography	Solvent Mixtures
A	Ppc[a]	Benzene–cyclohexane (1:1)–propylene glycol
B	Ppc	Toluene–ethyl acetate–methanol–water (9:1:6:4)
C	Ppc	Benzene–methanol–water (20:11:9)
D	Ppc	2,2,4-Trimethylpentane–toluene–methanol–water (5:5:7:3)
E	Ppc	2,2,4-Trimethylpentane–methanol–water (10:9:1)
F	Ppc	Skellysolve B–methanol–water (10:9:1)
G	Tlc[b]	Benzene–ethanol (1:1)
H	Tlc	Benzene–ethanol (9:1)
J	Tlc	Benzene–ethyl acetate (2:1)
K	Tlc	Benzene–diethyl ether (1:1)
L	Tlc	Ethyl acetate–*n*-hexane (1:1)

[a] Ppc = paper partition chromatography. [b] Tlc = thin-layer chromatography.

Experimental Section and Results

To a 62-day collection of late-pregnancy urine (approximately 80 l.), 6.93×10^5 dpm of [4-^{14}C]15α-hydroxytestosterone (specific activity 4.23×10^8 dpm/mg) was added. The urine was concentrated at a reduced pressure to one-tenth of the original volume and the urinary conjugates were hydrolyzed with Glusulase. Steroids were extracted from the urine with ethyl acetate and a neutral extract was obtained by washing the organic phase with 1 N NaOH and then with water until neutral. The neutral extract (21 g) was chromatographed on a 1.3-kg silica gel column which was developed with methylene chloride and then with increasing concentrations of ethanol in methylene chloride. The effluent was collected in 15–20-ml fractions at the rate of six fractions per hour. A single band of radioactive material was eluted with 6% ethanol in methylene chloride (fractions 5230–5720) to yield a residue which weighed 400 mg and contained 3.14×10^5 dpm. This material was purified by chromatography on a 40-g alumina column and then on three thin-layer chromatography plates using system G. An ultraviolet-absorbing band with the mobility of 15α-hydroxytestosterone was observed on the plates and on elution afforded 47 mg of residue which was further purified by chromatography on paper using systems B and C. The material eluted from the last paper was further chromatographed on a small alumina column to yield a residue which weighed 1.5 mg and contained 1.30×10^5 dpm. It was acetylated with [^{3}H]acetic anhydride (solution 2), and the product was chromatographed on a thin-layer chromatography plate in

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

	15α-Acetoxy-17β-hydroxyandrost-4-en-3-one[a]			15α-Hydroxytestosterone Diacetate[b]		
	Crystals			Crystals		
Crystzn	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C
1	4570	1.55	18.0	2900	1.06	1.05
2	3320	1.10	2.80	2860	1.06	1.06
3	3230	1.06	1.05			

[a] An aliquot of [4-^{14}C]15α-hydroxyandrostenedione was acetylated with [^{3}H]acetic anhydride solution 1, and the product was mixed with 65.0 mg of carrier 15α-acetoxyandrostenedione. The mixture was reduced with $NaBH_4$, oxidized with DDQ, and then chromatographed on an alumina column prior to crystallization. [b] The third crystals and mother liquors were combined and acetylated with nonlabeled acetic anhydride.

TABLE III: Determination of the Specific Activity of 15α-Hydroxytestosterone Isolated from the 62-Day Urine Pool.

	15α-Hydroxytestosterone Diacetate[a]			15α,17β-Diacetoxyandrost-4-en-3β-ol[b]		
	Crystals			Crystals		
Crystzn	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C
1	16,800	7.80	19.20	9300	4.74	4.80
2	10,500	4.89	5.50	9300	4.76	4.72
3	10,400	4.79	4.82			

[a] The urinary 15α-hydroxytestosterone was acetylated with [^{3}H]acetic anhydride (solution 2) and the diacetate was purified by thin-layer chromatography and paper chromatography. Then it was mixed with 32.7 mg of carrier 15α-hydroxytestosterone diacetate and the mixture was crystallized. [b] The third crystals and mother liquors were combined and the mixture was reduced with $NaBH_4$. The product was chromatographed on a thin-layer chromatography plate prior to crystallization.

system J. An ultraviolet-absorbing band with the mobility of 15α-hydroxytestosterone diacetate was observed on the plate, which was eluted, and the residue was chromatographed on paper in system F. One ultraviolet-absorbing band was observed with a mobility slower than that of 15α-hydroxytestosterone diacetate.

The material on the paper corresponding to the mobility of 15α-hydroxytestosterone diacetate was not ultraviolet positive at this stage and it was eluted (9.5×10^5 dpm of ^{3}H and 8.6×10^4 dpm of ^{14}C), mixed with 32.7 mg of carrier 15α-hydroxytestosterone diacetate, and crystallized to constant specific activity and an ^{3}H:^{14}C ratio as shown in Table III. The third crystals and mother liquors were combined and the mixture was reduced with $NaBH_4$. Following chromatography of the product on two thin-layer chromatographic plates, using system L, the material eluted was crystallized to constant specific activity and an ^{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]15α-hydroxytestosterone added to the urine, and the specific activity of the [^{3}H]acetic anhydride, the amount of 15α-hydroxytestosterone excreted in the urine was calculated to be 0.64 μg/day.

At this stage, it was considered that 15α-hydroxyandrostenedione may be excreted in the urine in larger amounts than 15α-hydroxytestosterone and it was therefore decided to examine the fractions eluted from the initial silica gel column for the presence of this steroid. It was known that 15α-hydroxyandrostenedione when chromatographed on silica gel can be eluted from the column with 3% ethanol in methylene chloride. Therefore, fractions 2845–3860, containing material eluted with 3 and 4% ethanol in methylene chloride from the initial silica gel column of the neutral extract of the 62-day urine collection were combined to give a residue weighing 2 g. To this residue 1.92×10^6 dpm of [4-^{14}C]15α-hydroxyandrostenedione was added and the mixture was chromatographed on a 200-g silica gel column. A single band of radioactive material was eluted with 3% ethanol in methylene chloride (fractions 280–335) which gave a residue weighing 800 mg and containing 1.47×10^6 dpm. In order to reduce the mass, it was further chromatographed on a 80-g alumina column and elution with 1% ethanol in benzene afforded 200 mg of residue containing 1.21×10^6 dpm. This residue was then chromatographed on ten thin-layer chromatography plates using system H. Two ultraviolet-positive bands were observed on the plates, the less polar band having the mobility of 15α-hydroxyandro-

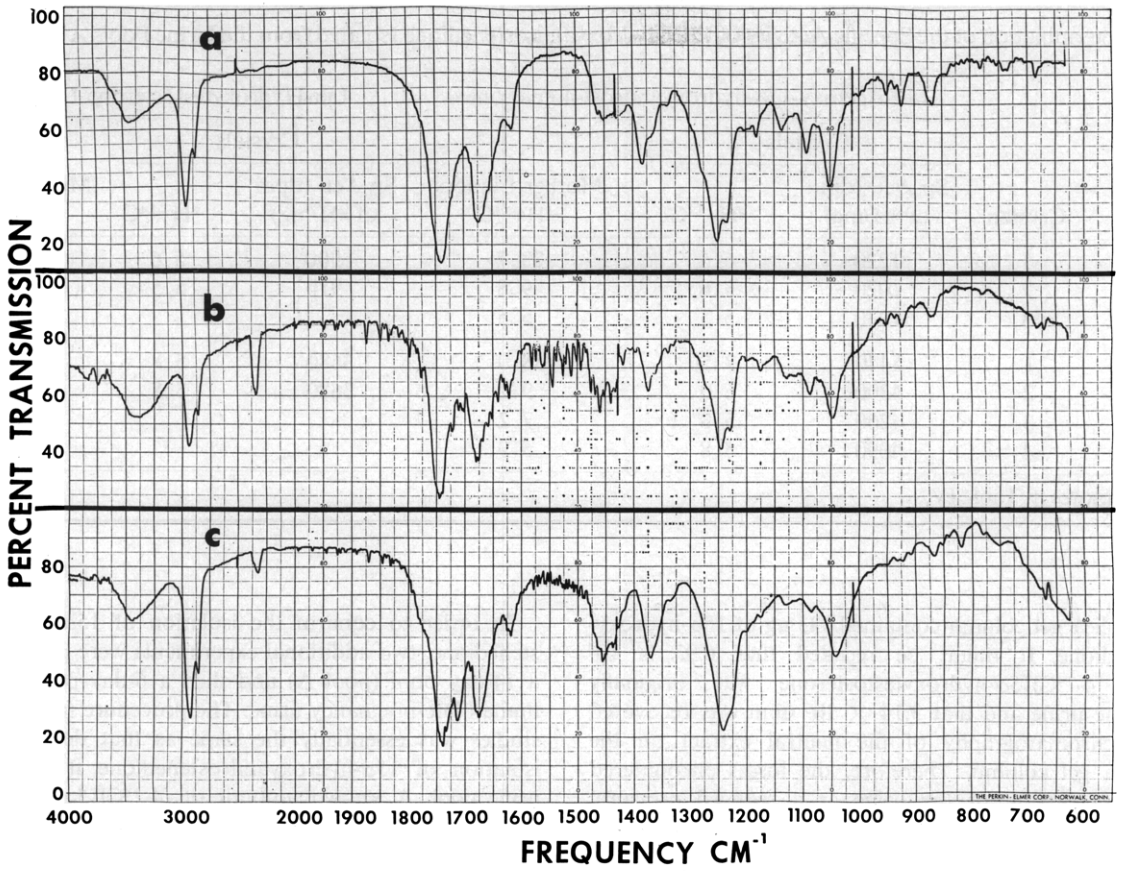

FIGURE 1: Infrared spectra (KBr) of authentic and isolated 15α-acetoxyandrostenedione. (a) Authentic 15α-acetoxyandrostenedione (240 μg), (b) authentic 15α-acetoxyandrostenedione (10 μg), and (c) acetylation product of isolated steroid (estimated weight 10 μg).

TABLE IV: Determinations of the Specific Activity of 15α-Hydroxyandrostenedione Isolated from the 62-day Urine Pool.

	15α-Acetoxy-17β-hydroxyandrost-4-en-3-one[a]			15α-Hydroxytestosterone Diacetate[b]		
	Crystals			Crystals		
Crystzn	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C	Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C
1	16,000	4.90	23.90	8,400	2.85	3.10
2	9,600	2.95	5.32	8,400	2.79	2.81
3	9,500	2.80	2.90			

[a] An aliquot of the urinary 15α-hydroxyandrostenedione (1.97×10^5 dpm) was acetylated with [^{3}H]acetic anhydride (solution 2). Then 15α-acetoxy-17β-hydroxyandrost-4-en-3-one was formed as a derivative and it was chromatographed on an alumina column prior to crystallization. [b] The third crystals and mother liquors were combined and acetylated with nonlabeled acetic anhydride. The 15α-hydroxytestosterone diacetate formed was then crystallized.

stenedione. This material was therefore eluted and further purified by chromatography on paper in system B and then on a small alumina column. The residue obtained weighed 2.1 mg and contained 5.90×10^5 dpm. A one-third aliquot of the residue (0.7 mg and 1.97×10^5 dpm) was acetylated with [^{3}H]acetic anhydride (solution 2) and the product was mixed with 60 mg of carrier 15α-acetoxyandrostenedione. The mixture was then reduced with $NaBH_4$, oxidized with DDQ, and finally chromatographed on an alumina column. The resulting 15α-acetoxy-17β-hydroxyandrost-4-en-3-one was crystallized to a constant specific activity and a ^{3}H:^{14}C ratio as shown in Table IV. The third crystals and mother liquors were then combined, the mixture was acetylated, and the product was crystallized to constant specific activity and a ^{3}H:^{14}C ratio as shown in Table IV. From this final ^{3}H:^{14}C ratio it was calculated that fractions 2845–3860 eluted from

TABLE V: Determination of the Specific Activity of 15α-Hydroxyandrostenedione Isolated from the Urine Pools A and B.

Urine Pool	Crystzn	15α-Acetoxy-17β-hydroxyandrost-4-en-3-one[a] Crystals Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{3}H:^{14}C	15α-Hydroxytestosterone Diacetate[a] Crystals Sp Act. (dpm of ^{3}H/mg)	^{3}H:^{14}C	Mother Liquors ^{4}H:^{14}C
A	1	1220	6.6	23.7	900	5.6	5.7
	2	1020	5.6	9.8	910	5.5	5.6
	3	1030	5.5	5.5			
B	1	5440	7.50	38.0	3680	5.8	6.0
	2	4300	6.0	9.2	3680	5.8	5.8
	3	4200	5.8	7.2			
	4	4090	5.8	5.9			

[a] See footnotes in Table IV.

the initial silica gel column contained 133 μg of 15α-hydroxyandrostenedione. It might be recalled that 6.93×10^8 dpm of [4-^{14}C]15α-hydroxytestosterone was originally added in the urine and that 3.14×10^5 dpm was recovered following chromatography of the neutral extract on the initial silica gel column. Therefore, the losses encountered during concentration of the urine at a reduced pressure, hydrolysis, and extraction of the urinary steroids, and chromatography on the silica gel column, were approximately 55%. On the basis of these results it was calculated that 296 μg of 15α-hydroxyandrostenedione was present in the urine pool, corresponding to an excretion of 4.8 μg/day.

The remaining two-thirds of the residue used for the isolation of 15α-hydroxyandrostenedione (1.4 mg and 3.9×10^5 dpm) was acetylated with nonlabeled acetic anhydride and the product was chromatographed on paper in system F for 20 hr. An ultraviolet-absorbing band with the mobility of 15α-acetoxyandrostenedione was observed and it was eluted to yield a residue containing 2.90×10^5 dpm. This residue was rechromatographed on paper in system E for 36 hr and again an ultraviolet-positive band with the mobility of 15α-acetoxyandrostenedione was observed. This material was eluted and further chromatographed on a thin-layer chromatography plate in system K and the ultraviolet-positive area was eluted with a minimum volume (3 ml) of ethyl acetate to yield a residue containing 1.50×10^5 dpm. On the basis of the amount of radioactivity recovered and the results obtained from the isotope dilution studies, it was calculated that this residue contained approximately 10 μg of 15α-acetoxyandrostenedione. The infrared spectrum (KBr) of this material is shown in Figure 1. The infrared spectra of 240 and 10 μg of standard 15α-acetoxyandrostenedione are also in this figure (spectra a and b, respectively). It can be seen that as little as 10 μg of standard 15α-acetoxyandrostenedione gives essentially the same spectrum as that obtained with 240 μg of this steroid. The spectrum of the acetate of the urinary 15α-hydroxyandrostenedione (spectrum c) is almost identical with that of authentic 15α-acetoxyandrostenedione (spectrum b); the only differences noted were an exaggeration of the band at 1715 cm^{-1} seen in spectrum b and an extra band at 820 cm^{-1}.

In order to confirm the isolation of 15α-hydroxyandrostenedione, two additional experiments were performed using a 14-day pool (pool A) and then a 6-day pool (pool B) of human late-pregnancy urine. The procedure followed in these studies was essentially the same as that described for the isolation of 15α-hydroxyandrostenedione from the 62-day urine pool. A known amount of [4-^{14}C]15α-hydroxyandrostenedione was added to each urine pool (5.40×10^5 dpm was added to pool A and 1.86×10^5 dpm was added to pool B). The urinary conjugates were hydrolyzed with Glusulase and a neutral extract was prepared and chromatographed on a silica gel column as previously described. The following procedures were followed for the purification and the determination of the specific activity of the urinary 15α-hydroxyandrostenedione: (a) chromatography on alumina; (b) thin-layer chromatography in system H; (c) paper chromatography in systems A and B; (d) alumina column; (e) acetylation with [^{3}H]acetic anhydride, solution 2; (f) addition of carrier 15α-acetoxyandrostenedione, formation of 15α-acetoxy-17β-hydroxyandrost-4-en-3-one and crystallization to constant specific activity and ^{3}H:^{14}C ratio; and (g) formation of 15α-hydroxytestosterone diacetate and crystallization to constant specific activity and ^{3}H:^{14}C ratio. The data establishing the specific activity of 15α-hydroxyandrostenedione isolated from the urine pools A and B are shown in Table V. From the final ^{3}H:^{14}C ratios the amount of 15α-hydroxyandrostenedione excreted in the urine pool A was calculated to be 5.2 μg/day and 4.4 μg/day for urine pool B.

A further experiment was also performed to confirm the isolation of 15α-hydroxytestosterone from human pregnancy urine. To a 5-day urine pool obtained from a normal subject in week 32 of gestation was added 2.76×10^5 dpm of purified [4-^{14}C]15α-hydroxytestosterone. The urinary conjugates were hydrolyzed with Glusulase and a neutral extract was prepared and chromatographed on a silica gel column as previously described. The following procedures were employed for the purification and the determination of the specific activity of the urinary 15α-hydroxytestosterone: (a) chromatography on an alumina column; (b) thin-layer chromatography in system G; (c) paper chromatography in systems

TABLE VI: Determination of the Specific Activity of 15α-Hydroxytestosterone Isolated from the 5-Day Urine Pool.

	15α-Hydroxytestosterone Diacetate[a]			15α,17β-Diacetoxyandrost-4-en-3β-ol[a]		
	Crystals			Crystals		
Crystzn	Sp Act. (dpm of ^3H/mg)	$^3H:^{14}C$	Mother Liquors $^3H:^{14}C$	Sp Act. (dpm of ^3H/mg)	$^3H:^{14}C$	Mother Liquors $^3H:^{14}C$
1	3800	19.2	50.80	1550	0.89	0.90
2	1750	0.87	4.60	1550	0.88	0.88
3	1740	0.88	0.90			

[a] See footnotes in Table III.

B and C; (d) acetylation with [^3H]acetic anhydride, solution 2; (e) addition of carrier 15α-hydroxytestosterone diacetate and crystallization to constant specific activity and $^3H:^{14}C$ ratio; (f) formation of 15α,17β-diacetoxyandrost-4-en-3-β-ol and crystallization to constant specific activity and $^3H:^{14}C$ ratio. The data shown in Table VI establish the constancy of the specific activity of urinary 15α-hydroxytestosterone. From these data the excretion of 15α-hydroxytestosterone in urine was calculated as 0.48 μg/day.

Discussion

The results reported in this paper demonstrate that 15α-hydroxyandrostenedione and 15α-hydroxytestosterone are excreted in human late-pregnancy urine. The identity of these two steroids and their quantitation in urine was established by the use of isotope derivative methods described. Further proof of identity of the urinary 15α-hydroxyandrostenedione was achieved by obtaining an infrared spectrum of its acetate which compared well with that of the authentic standard. The amount of 15α-hydroxyandrostenedione excreted in late-pregnancy urine ranged from 4.4 to 5.2 μg per day and that of 15α-hydroxytestosterone from 0.48 to 0.64 μg per day.

These are the first neutral 15α-hydroxylated C_{19} steroids isolated from human sources. In previous communications we have reported the isolation of 15α-hydroxyprogesterone (Giannopoulos and Solomon, 1967) and its metabolites (Giannopoulos *et al.*, 1970) from human late-pregnancy urine. The first indication that neutral C_{19} 15α-hydroxysteroids can be formed by mammalian tissues came from the work of Neher and Wettstein (1960) who presented chromatographic evidence for the presence of 15α-hydroxytestosterone in extracts of bull testes. Recently, 5α-androstane-3α,15α,17β-triol and 3α,15α-dihydroxy-5α-androstan-17-one have been isolated from the feces of the germ-free rat (Gustafsson and Sjövall, 1968) and 5α-androstane-3α,15α,17β-triol has also been isolated from the feces of the conventional rat (Gustafsson, 1968). The presence of 15α-hydroxyestrogens in human late-pregnancy urine is also well documented (Knuppen *et al.*, 1965; Lisboa *et al.*, 1967).

The finding that 15α-hydroxyandrostenedione and 15α-hydroxytestosterone are normal constituents of human late-pregnancy urine fits well with their role as important precursors of the urinary 15α-hydroxyestrogens. Recent reports support this possibility. Stern *et al.* (1968) have shown a 30–40% conversion of labeled 15α-hydroxyandrostenedione into 15α-hydroxyestradiol following incubation of the precursor with a 10,000*g* supernatant fraction of human placental tissue and Solomon *et al.* (1969) have shown that 15α-hydroxyestradiol is the major product following perfusion of human placenta *in situ* with labeled 15α-hydroxyandrostenedione. Following the intravenous administration of various labeled C_{19} steroids to subjects in the third trimester of pregnancy and the isolation of labeled 15α-hydroxyestrogens from the urine it was found that 15α-hydroxyandrostenedione was the best precursor of urinary 15α-hydroxyestradiol (YoungLai and Solomon, 1968). In the latter studies it was also shown that 15α-hydroxyandrostenedione was almost as good a precursor of urinary 15α-hydroxyestriol as was dehydroisoandrosterone sulfate which was the best precursor of 15α-hydroxyestriol among the labeled steroids injected. In a more recent study (YoungLai *et al.*, 1969) in which labeled substrates were introduced into the fetus during transfusion *in utero* for erythroblastosis fetalis, it was found that 15α-hydroxyandrostenedione was a better precursor than dehydroisoandrosterone sulfate or urinary 15α-hydroxyestradiol and 15α-hydroxyestriol.

The studies discussed above give good support to the concept that 15α-hydroxyandrostenedione and possibly 15α-hydroxytestosterone can serve as efficient substrates of 15α-hydroxyestrogens during late human pregnancy. In view of these findings it is possible that larger amounts of 15α-hydroxyandrostenedione and 15α-hydroxytestosterone are made during late pregnancy despite the very small amounts of these steroids isolated from urine. Different types of studies have to be devised to measure the urinary production rates of these steroids during pregnancy.

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Refereces

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Kinetics of Carboxypeptidase A. II. Inhibitors of the Hydrolysis of Oligopeptides*

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ABSTRACT: The carboxypeptidase-catalyzed hydrolyses of benzoylglycylglycyl-L-phenylalanine and carbobenzoxyglycylglycyl-L-phenylalanine do not display the substrate activation characteristic of their dipeptide analogs, benzoylglycyl-L-phenylalanine and carbobenzoxyglycyl-L-phenylalanine. Moreover, a known activator of the hydrolysis of the dipeptides, carbobenzoxyglycine, competitively inhibits the hydrolyses of these tripeptides with a K_i of 0.027 M.

As with dipeptides, acetylation of carboxypeptidase A (Anson) reduces activity toward the tri- and tetrapeptides to less than 3% of the control. The relative order of the Michaelis constant, K_m, can now be established as Co = Zn = Ni > Mn for the respective metallocarboxypeptidases. K_m is likely to be a measure of the binding affinity of the tripeptide since no relationship is found between the values of K_m and k_{cat} when one particular metallocarboxypeptidase is examined on its action on a series of tripeptides or when a series of metallocarboxypeptidases act on one tripeptide. The order of k_{cat} is the same as for the dipeptides, *i.e.*, Co > Zn = Ni > Mn. These kinetic studies reveal significant differences in the hydrolysis of these peptides and esters. Indole-3-acetate, phenylacetate, and β-phenylpropionate are all noncompetitive inhibitors of tripeptide hydrolyses. This class of compounds has long been known to be competitive inhibitors of the hydrolysis of esters. These studies are consistent with the proposed multiple loci model (Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. C., Auld, D. S., and Sokolvsky, M. (1968), *Biochemistry 7*, 3547).

The kinetics of hydrolyses of CbzGly-L-Phe and BzGly-L-Phe catalyzed by a series of metallocarboxypeptidases are characterized by substrate activation. The range of substrate concentration over which the activation occurs varies slightly for the different metalloenzymes. Similarly, all the various metallocarboxypeptidases exhibit substrate inhibition when employing hippuryl-β-DL-phenyllactic acid, the substrate which commonly serves for esterase activity measurements. Since the Lineweaver–Burk plots are multiphasic, it would seem likely that the extrapolated parameters "V_{max}" and "K_m" might be composite terms. Thus, it was of interest to note that there was apparent ordering of the constants for the substrates, regardless of the metal present, indicating that the course of the kinetic complications may be due, in part, to multiple interactions of the small synthetic dipeptide and depsipeptide substrates used for the kinetic analysis (Davies *et al.*, 1968a,b). A model proposing binding of substrates at multiple loci is consistent with the data obtained thus far (Vallee, 1967; Vallee *et al.*, 1968, 1969). The model suggests that an increase in the length of the substrate and/or bulk of the blocking group should place more constraints on its manner of binding, and the enzymatic hydrolysis of such substrates should approach Michaelis–Menten kinetics. This hypothesis has been tested by investigating the kinetics of hydrol-

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