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$C_{19}O_2$ Steroids and Some of Their Precursors in Blood from Normal Human Adrenals *

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The ready response of the main urinary 11-deoxy-17-ketosteroids to adrenal suppression and adrenal stimulation leaves no doubt that a considerable fraction of urinary androsterone (3α-hydroxy-5α-androstan-17-one), 5β-androsterone (3α-hydroxy-5β-androstan-17-one), and androstenolone (3β-hydroxyandrost-5-en-17-one, dehydroepiandrosterone) must be derived from adrenal precursors. However, when adrenal vein blood was examined for such compounds, no consistent pattern emerged (3–9); therefore the identity of the precursors remained in doubt. The presence of androstenedione (androst-4-ene-3,17-dione), which was tentatively identified by Pincus and Romanoff (3), was not confirmed by Lombardo, McMorris, and Hudson (6), who examined 12 samples of human adrenal blood. In fact, these investigators (6) obtained from only one individual a substance, androstenolone, that could be classed as an efficient precursor of the urinary 11-deoxy-17-ketosteroids. The scope of the investigation widened when Baulieu (8) reported that in two instances, adrenal tumors secreted androstenolone sulfate but no free androstenolone. The purpose of this communication is to document and extend the findings of our preliminary reports (1, 2) and to show that the normal human adrenal consistently secretes androstenolone sulfate, androstenolone, and androstenedione. These three compounds appear to be the principal precursors of the urinary 11-deoxy-17-ketosteroids.

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Preliminary accounts of some of our findings have been given (1, 2).

Methods

Patients. The subjects studied were five women (I.A., M.R., C.B., M.M., and R.R.) and three men (J.J., L.W., and F.B.). Their ages and serial numbers are given in Table III. R.R. had hirsutism but normal ovulatory menses and F.B. had diabetes. There was no other evidence of endocrinologic disease or hepatic dysfunction in any of these subjects. The patients fasted on the morning of the procedure. Secobarbital (100 mg orally) was given at 8 a.m., and 50 mg meperidine hydrochloride was given subcutaneously just before the catheterization,[1] which was usually done between 10 a.m. and noon essentially in the manner performed by Cranston (5). The location of the catheter was ascertained by venogram. In most patients this produced sufficient pain to require additional meperidine. This was unnecessary with L.W. and F.B., who had minimal discomfort. Blood was collected after the iv injection of 100 mg of heparin. The adrenal blood sample (usually about 100 ml) was collected during a measured interval. This was followed by the withdrawal of a similar volume of peripheral blood. Thereafter, corticotropin was administered to R.R. and F.B., and a second sample was taken from the adrenal vein. In the case of R.R. this collection was begun 10 minutes after the completion of an iv infusion (11 minutes) of 25 U of corticotropin and of F.B. 16 minutes after the rapid injection of 40 U of corticotropin.

Radioactivity measurements. Radioactivity was determined by scintillation counting with a two-channel spectrometer.[2] Free steroids were dissolved in 5 ml of scintillator solution (toluene containing 20 mg of 2,5-diphenyloxazole and 0.5 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene), sulfates in 0.3 ml of methanol before 5 ml of the scintillator solution was added. When methanol was present, corrections for the diminished efficiency of counting were determined by internal standards.

In calculating the isotope ratios C^{14}_2/H^3_1, we used the equation, $C^{14}_2/H^3_1 = [(N_2/N_1) - a]/[1 - (1/b)(N_2/N_1)]$. In this expression, the N values are the observed rates of scintillation corrected for background; the subscripts 1

[1] A more detailed description will be given elsewhere by Levy and Katz.

[2] Tricarb, Packard Instrument Co., La Grange, Ill. Samples from Patients 1 to 5 were analyzed on a model 314 AX, the others on a model 314 EX.

TABLE I

*Test for radiochemical purity of androstenolone-7-H³ sulfate**

Fraction	Solvent	Weight	
		mg	*cpm/μmole*
Mother liquor	Methanol	4.8	1,375
Crystals		8.2	1,318
Mother liquor	95% Ethanol	0.8	1,347
Crystals		6.8	1,305
Sample solvolyzed and recrystallized Crystals	Methanol	0.3	1,391

* Nonradioactive androstenolone sulfate (13 mg) was added before recrystallization. Samples were assayed for radioactivity and for steroid content by the Zimmermann reaction.

and 2 refer to the two channels of the spectrometer. The symbol C^{14}_2, therefore, signifies the number of disintegrations of C^{14} that are registered in channel 2 per unit time. The efficiency ratios, *a* and *b*, equal the N_2/N_1 ratios of reference samples that contain only a single isotope, H^3 or C^{14}, respectively. Spectrometer [2] model 314 AX was operated at settings that resulted in efficiency ratios $a \approx 0.2$ and $b \approx 5.1$; model 314 EX gave $a \approx 0.05$ and $b \approx 11.8$.

Color reactions and spectroscopy. The Zimmermann reaction was carried out essentially according to the modification described by Wilson (10). In Cases 1 to 5, the Porter-Silber reaction was done on the neutral water-soluble ketonic plasma fraction as described (9); in the others, on 5-ml samples of plasma by the method of Peterson, Karrer, and Guerra (11). Infrared spectra were measured with a double beam spectrometer [3] with beam condenser on samples (*ca.* 1%) embedded in KBr disks (1.5 mm).

Chromatography. Systems for chromatographic separations were prepared from mixtures of the following solvents in the volume ratios specified: *1*) toluene, isoöctane, *n*-heptane, methanol, and water, 10:5:5:16:4; *2*) isoöctene, methanol, and water, 5:4:1; *3*) benzene, isoöctane, methanol, and water, 10:20:12:3; *4*) ligroin, methanol, and water, 10:9:1; *5*) isopropyl ether, *t*-butanol, and 2.8% aqueous ammonia, 3:2:5; *6*) toluene, *n*-butanol, and 2.8% aqueous ammonia, 1:1:2; and *7*) toluene, isoöctane, methanol, and water, 15:5:16:4. We have previously described solvent systems 1 to 4 (9); Schneider and Lewbart (12) described systems 5 and 6 for the fractionation of steroid sulfates. Eluates from blank chromatograms were prepared for correcting all spectrographic data except the infrared curves.

Materials. The radioactive tracers, androstenedione-4-C^{14}, testosterone-4-C^{14}, 17-hydroxyprogesterone-4-C^{14} (17-hydroxypregn-4-ene-3,20-dione), androstenolone-7-H^3, and 17-hydroxypregnenolone-7-H^3 (3β,17-dihydroxypregn-5-en-20-one) were the same preparations as we described before (9) and were generally used in the same amounts. They were periodically tested for purity by paper chromatography and scanning [4] for radioactivity. Cortexolone-1,2-H^3 (17,21-dihydroxypregn-4-ene-3,20-dione) was a commercial sample that was purified by Girard separation and chromatography on paper in system 1. Androstenediol-4-C^{14} (androst-5-ene-3β,17β-diol) was prepared from commercial testosterone-4-C^{14} by a procedure essentially like the one subsequently published by Gut and Uskokovič (13). The resulting androstenediol diacetate melted at 161.5 to 163° C; [5] the free diol melted at 181 to 183° C and showed a single symmetrical peak when chromatographed in system 7. Androstenolone-7-H^3 sulfate [6] was prepared from the free steroid (1.9 μg, 1.1×10^6 cpm N_1) in 1 ml of chloroform with 50 mg of pyridine-sulfur trioxide according to the method of McKenna and Norymberski (15). The product was converted to the potassium salt by treatment with aqueous potassium hydroxide. The solution was then washed with ether and extracted with *n*-butanol, which was washed with water and taken to dryness *in vacuo*. The product was chromatographed on paper in system 6. The radioactive zone that had the same R_f as a recrystallized unlabeled reference sample (mp, 219 to 221° C, mp in reference 16, 219 to 223° C) was eluted and used as tracer [7,000 cpm N_1 (corrected)/sample]. Its purity was tested by dilution of a sample with cold carrier, recrystallization, and solvolysis (16) followed by recrystallization. The results are shown in Table I. At its last use as tracer the preparation was still in its conjugated state, since only 1% of the radioactivity could be extracted from water with benzene.

The preparations of acetic anhydride were commercial samples that had been diluted with benzene. Unless noted otherwise the one containing H^3 introduced about 250 cpm H^3_1 into 1 mμmole of a monohydroxysteroid; the one with C^{14}, about 180 cpm C^{14}_2 into 1 mμmole. Precise counts were determined by acetylation of reference compounds of known weight that contained the same amounts of steroid tracer as the unknowns. These reference acetates were prepared at the same time and purified in the same manner as the acetates from blood. The amount of steroid in the test sample (s μg) was calculated by the following equation, $s = t\ [(H^3_1/C^{14}_2)_{unk.}\ (C^{14}_2/H^3_1)_{ref.} - 1]$, when t μg of a C^{14}-labeled steroid tracer had been added to the plasma or plasma fraction. The subscripts unk. and ref. relate to the tritiated acetates from blood and from the pure steroid tracer, respectively. When tritiated steroid tracers of negligible weight were used, the same amount of radioactivity was added to the unknown solution containing s μg of steroid and to the unlabeled reference sample (r μg). In this case, the

[3] Perkin-Elmer, model 421, Perkin-Elmer Corp., Norwalk, Conn.

[4] Atomic Accessories, Bellerose, N. Y.

[5] All melting points reported are corrected.

[6] A procedure for the preparation of labeled sulfate with much lower specific activity has been reported previously (14).

unknown (unk.) and reference (ref.) acetates-C^{14} are related by the equation, $s = r\ (C^{14}_2/H^3_1)_{unk.}\ (H^3_1/C^{14}_2)_{ref.}$.

Fractionation procedures. 1) Free ketonic steroids. Our basic procedure for the separation of free ketonic steroids of human plasma has been described in detail before (9) and entails the following steps: *1)* precipitation of proteins by the addition of a mixture (3:1, vol:vol) of ethanol and ethyl ether; *2)* evaporation of the filtrate *in vacuo; 3)* partition of the residue between methylene chloride and water; *4)* removal of lipid impurities by extracting the residue of the organic phase with methanol, by precipitation with water, and by washing of the resulting supernatant solution in dilute methanol with petroleum ether; *5)* Girard separation and removal of acidic compounds; *6)* partition of the neutral ketonic fraction between benzene and water; *7)* partition chromatography on powdered cellulose in system 3 of the fraction soluble in benzene (the efficacy of this step for the separation of androstenedione, androstenolone, 17-hydroxyprogesterone, and 17-hydroxypregnenolone is illustrated in reference 9); *8)* chromatography, if appropriate, on alumina; chromatography on paper for the further purification of these steroids and of testosterone; acetylation and recrystallization with carrier when applicable. Full details for each compound are given in reference 9.

This procedure is an improvement over one initially used (Cases 1 to 3), in which step 7 was omitted. The method was modified further as we gained experience and expanded our objectives. Specifically, in Cases 6 to 8 we omitted the benzene-water partition (step 6) and the chromatography on alumina of step 8 since these procedures were no longer needed. Finally when steroid sulfates were investigated (Cases 6 to 8), a twofold partition between equal volumes (30 to 75 ml) of ethyl acetate and 5% sodium bicarbonate at 4° C [7] was substituted for the distribution between methylene chloride and water. This change became necessary because we found that in the presence of blood lipids, water removed less than 6% of labeled androstenolone sulfate from the organic phase.[8]

2) Conjugated steroids. The combined aqueous phases of the ethyl acetate-bicarbonate distribution removed all tritiated androstenolone sulfate from the organic phases but carried about 4% of the C^{14}-labeled neutral steroids.[9] The aqueous phases were combined and extracted with *n*-butanol, which was then washed with water and taken to dryness *in vacuo* (sulfate fraction).

The sodium bicarbonate fraction and the washings (case F.B., sample A_2) were combined, acidified (pH 1), and extracted with *n*-butanol. The extract was washed with water and taken to dryness *in vacuo*. The residue was dissolved in 0.5 M sodium acetate buffer (6 ml, pH 5.0) and incubated with 10 ml of β-glucuronidase (Ketodase, 50,000 U) at 38° C for 24 hours. The mixture was extracted with ether, which was washed with hydrochloric acid, sodium hydroxide, and water. The residue contained 2% of the C^{14}_2 and 4% of the H^3_1 counts added to the plasma. Labeled androstenolone, androstenedione, and testosterone were added, and the mixture was fractionated by Girard separation and partition column and paper chromatography as described for the free steroids.

The sulfate fraction was applied to a partition column containing 12 g cellulose and 6 ml of the lower phase of system 5 and was eluted with the upper phase in 5-ml portions. Labeled androstenolone sulfate was usually found in fractions 25 to 37. These were combined and chromatographed on paper in system 6. The area containing radioactivity was extracted; the eluate was assayed for tritium and by the Zimmermann reaction and then solvolyzed. The neutral reaction product was chromatographed on paper in system 1. The eluate from the radioactive zone was again assayed for tritium and 17-ketosteroid content. In patient J.J. (2) the isolation of the sulfates was omitted, and the aqueous fraction containing the conjugated steroids and androstenolone-H^3 sulfate was solvolyzed and after the addition of 17-hydroxypregnenolone-H^3 chromatographed on cellulose in system 3 (9). The two fractions containing peaks of radioactivity were chromatographed individually on paper in system 1. Androstenolone was measured both by the Zimmermann reaction and by forming an acetate with acetic-C^{14} anhydride (30 μl) in pyridine (40 μl); after the addition of unlabeled carrier (20 mg) the product was recrystallized until the ratio N_2/N_1 for crystals and mother liquors agreed within 2%. 17-Hydroxypregnenolone was acetylated in the same manner. The prod-

TABLE II

*Recrystallization of adrenal 17-hydroxypregnenolone 3-acetate**

Fraction		Sample A			Sample B		
		N_2	N_1	N_2/N_1	N_2	N_1	N_2/N_1
		cpm	*cpm*		*cpm*	*cpm*	
Mother liquor	n-1				340	1,049	0.324
Crystals	n-1	201	121	1.65			
Mother liquor	n	128	77	1.67	260	804	0.323
Crystals	n	193	115	1.67	116	360	0.321

* Sample A was obtained from the free steroids of J.J, sample B from the fraction that had been solvolyzed with ethyl acetate. Both contained tritiated tracer, were acetylated with acetic-C^{14} anhydride, and recrystallized after the addition of unlabeled carrier from methanol, n = 7 times (A) and n = 5 times (B), respectively. The minimal net count per channel was 17,000 per sample.
N = observed rates of scintillation corrected for background; the subscripts 1 and 2 refer to the two channels of the spectrometer.

[7] This precaution was taken because cleavage of the dihydroxyacetone side chain to 17-ketosteroids has been reported to occur in alkaline solution (17).

[8] After a twofold distribution of the pure tracer between 4 vol of methylene chloride and 1 vol of water, 93% of the radioactivity was in the aqueous phase. Burstein and Dorfman (18) observed the anomalous behavior of this sulfate on distribution in the presence of tissue lipids. In their case, lowering of the ionic strength was found to decrease the amount of sulfate soluble in toluene.

[9] The volume used in the distribution had no discernible effect on this.

TABLE III

*μg steroids per 100 ml plasma**

Subject No.	Sex Age	Sample†	Time‡	17,21-di-OH-20-ones§	Androstenolone		Andro-stene-diol	Andro-stene-dione	Testos-terone	17-OH-pregnenolone		17-OH-proges-terone
					Free	Sulfate‖				Free	Sulfate‖	
			min									
I.A.	F	A		181	19§		0.4	6¶				
1	50	P			0§			0¶				
M.R.	F	A	30	220	+§		0.4	6§				
2	34	P			0§			0¶				
C.B.	F	A	26	504	74§		0.8	12¶				
3	46	P			<1§			0¶				
M.M.	F	A	34	153**	20			4¶				
4	22											
R.R.	F	A_1	7	29	15			0				
5	33	A_2	7	141	100			7				
J.J.	M	A	79	532	102¶	356††		28	1.3¶	123¶	18¶	21¶
6	50	P		20	0¶	77††		0	1.0¶	<1¶	<1¶	0¶
L.W.	M	A	35	31		78						
7	57	P		15		44						
F.B.	M	A_1	29	17	0¶	89		0	1.1	<1¶		
8	45	P		4	0¶	51		0	1.0	<1¶		
		A_2	24	621	137¶	245		37	1.2	70¶		

* Unless noted otherwise tracers were added to plasma.

† A = adrenal; P = peripheral; A_1, A_2 = adrenal before and after corticotropin.

‡ Time for collecting 100 ml of plasma.

§ Determined without tracer.

‖ Weight as micrograms of free steroid.

¶ Tracer added to partially fractionated plasma at a stage at which the recoveries of related steroids were about 80%. No corrections were made for losses of steroids before the addition of tracer.

** Measurement on chromatographically purified cortisol.

†† Tracer added to whole blood.

uct was chromatographed in system 1. The eluate from the radioactive zone was diluted with nonradioactive carrier and recrystallized as shown in Table II.

Nonketonic free steroids. The neutral nonketonic fraction of the free steroids was adsorbed on alumina (1.4 g) and eluted with benzene containing decreasing amounts of petroleum ether (75 to 0%) and benzene with 0.1% of ethanol. The radioactive fractions were combined, chromatographed on paper in system 1 or 7, and acetylated in pyridine with acetic-H^3 anhydride. The product was added to carrier androstenediol diacetate and recrystallized from methanol until the ratio of isotopes became constant.

Tests of procedure. Cortexolone (13 μg, 38×10^3 cpm H^3_1) and androstenedione (2.4 μg, 13×10^3 cpm C^{14}_2) were added to 30 ml of plasma. Androstenedione was isolated by the modified procedure that was used in the last three cases. The dione contained 1.2% of the H^3 and 45% of the C^{14} that had been added to the plasma.

When unlabeled cortexolone (10.3 and 100 μg, respectively) and 1 μg of androstenedione-C^{14} were chromatographed on 1 g acid-washed, nearly neutral alumina, the eluates containing androstenedione-C^{14} were found to contain 1.1 and 4.6 μg, respectively, of unlabeled material. Although in Cases 1 to 3 fractions that still contained some cortexolone were chromatographed on alumina, we estimated that cleavage to androstenedione would have yielded about 0.5 μg (C.B.) or less per 100 ml of plasma.

To test the recovery of androstenolone from blood, a tritiated sample was added with stirring to each of two 30-ml samples of heparinized blood. One was centrifuged immediately, the other after it had stood for 30 minutes at 0° C. The steroid fraction obtained after protein precipitation and methylene chloride-water partition in the usual manner (9) contained 83 and 81%, respectively, of the radioactivity added.

To test whether our measurements of androstenolone sulfate determine an artifact as Oertel (19) has suggested for many procedures, we compared our method with one that has been reported to minimize the alleged artifactual formation of the sulfate from a more complex lipid in peripheral blood that Oertel presumes to be a sulfatide. Tritiated androstenolone sulfate (14×10^3 cpm N_1) was added to 70 ml of plasma from peripheral blood. One-half was fractionated as described for the isolation of the conjugate and was found to contain as the sulfate, 126 μg of androstenolone per 100 ml of plasma. The other half was extracted by method D of Oertel (19) and fractionated (19) on a DEAE-Sephadex (20) column. The first two fractions were solvolyzed and found to contain about 2% of the tritium added to the plasma sample and 19 μg per 100 ml of plasma of a 17-ketosteroid that traveled with the R_f of androstenolone in system 1. Fraction 6 (19) contained the bulk of the tracer. The androstenolone was obtained by solvolysis and assayed in the Zimmermann reaction after chromatography. The measurement after correction for losses of isotope indicated that 100 ml of plasma contained 129 μg of androstenolone as the sulfate. As we obtained consistent and rather high values for the sulfate by both procedures, we did not pursue the matter further.

Results

Our results on eight samples of plasma from apparently normal adrenals are summarized in Table III. As this presentation implies that all

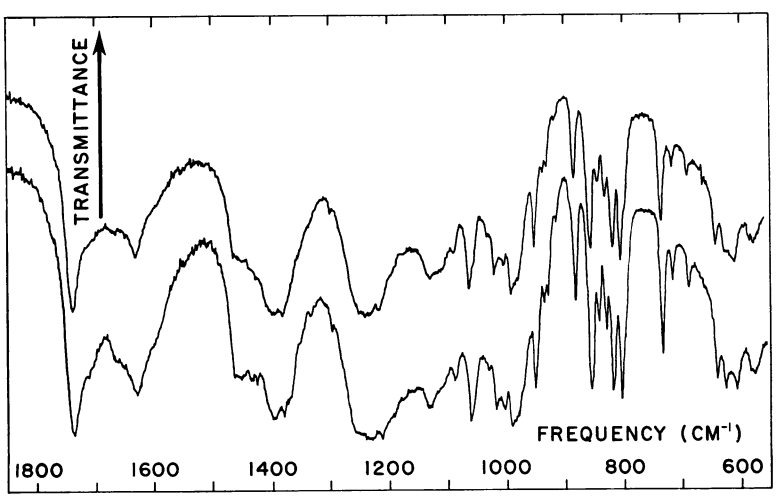

FIG. 1. INFRARED SPECTRA OF SYNTHETIC (UPPER CURVE) AND OF ISOLATED (F.B., SAMPLE A_2) ANDROSTENOLONE SULFATE. Both samples were chromatographed on paper in system 6, eluted with methanol, and embedded in potassium bromide without further purification. The samples presumably represent ammonium salts. A somewhat different reference curve has been published previously (21) for this compound. (The positive and the negative peaks of the upper curve near 667 cm^{-1} are instrumental artifacts.)

TABLE IV

*Recrystallization of adrenal androstenolone acetate**

Fraction	N_2	N_1	N_2/N_1
	cpm	*cpm*	
Mother liquor no. 4	1,219	654	1.87
Mother liquor no. 5	1,091	584	1.87
Crystals	430	232	1.85

* Sample from J.J. containing tritiated tracer was purified, acetylated with acetic-C^{14} anhydride, and recrystallized from methanol after the addition of unlabeled androstenolone acetate.

compounds measured were adequately purified and correctly identified, the following observations are offered as evidence. The conjugated 17-ketosteroid from adrenal vein blood that was found to accompany a tracer of androstenolone sulfate through two solvent partitions and on fractionation in two chromatographic systems gave an infrared spectrum which agreed closely with that of a reference specimen of androstenolone sulfate (Figure 1). The isolated compound was cleaved by a procedure that has been found effective only with sulfates (2). The product was chromatographed and the free steroid with the same R_f as androstenolone measured. In general the estimates were in accord with those made on the sulfates, and no systematic deviations were observed. As the mean of the ratios of the two determinations was 1.02, one may conclude that no further purification had occurred. The free androstenolone was similarly identified. Again a sample that had accompanied the tracer in two chromatographic systems gave an infrared spectrum in good agreement with that of a reference specimen that had been chromatographed in the same manner. Acetylation with acetic-C^{14} anhydride gave a product that could not be separated from the tracer by crystallization (Table IV). Moreover the isotope ratio signified an amount of adrenal androstenolone that agreed within 6% with that determined colorimetrically on the unacetylated steroid (2). In the case of free and of conjugated 17-hydroxypregnenolone, three chromatographic systems (two before, one after acetylation) were used, and again no separation of steroid and tracer occurred during these steps or on recrystallization (Table II). The identity of androstenediol (androst-5-ene-3β,17β-diol) rests on the behavior of a sample that had been purified as described in the experimental section, on countercurrent distribution (Table V). Androstenedione also accompanied the tracer through two chromatographic systems. It gave the same spectrum as the reference compound in the ultraviolet and after the Zimmermann reaction in the visible. Estimates based on these two sets of data were in reasonable agreement (25 and 28 μg, respectively). A sample derivatized with tritiated dinitrophenylhydrazine could not be separated from the corresponding hydrazone of the dione tracer by chromatography on alumina or by recrystallization.

Less detailed characterization was used to establish the presence of 17-hydroxyprogesterone, which has been found repeatedly in blood from normal adrenals (6), and of testosterone, which is a normal constituent of the peripheral plasma of either sex (22). In the case of 17-hydroxyprogesterone, the material that had followed the tracer through two chromatographic separations was found to give the same spectrum as the reference compound in the Zimmermann reaction, and at least fairly consistent estimates by this method (21 μg) and ultraviolet absorption (16 μg) were obtained. Testosterone after the same chromatographic purification could not be separated from the tracer by recrystallization of the acetate.

The plasma fraction that was hydrolyzed with β-glucuronidase was examined in one instance (sample A_2 of F.B.). No androstenolone was

TABLE V

*Countercurrent distribution of androstenediol diacetate**

Tube no.	N_2	N_1	N_2/N_1
	cpm	*cpm*	
7	146	75	1.96
8	276	143	1.93
9	435	227	1.91
10	599	311	1.93
11	735	381	1.93
12	747	386	1.94
13	658	342	1.92
14	483	247	1.96
15	288	149	1.94
16	160	86	1.87

* Sample from adrenal blood of C.B. and 2.4 μg of androstenediol-C^{14} (15,800 cpm N_2) were purified, acetylated with acetic-H^3 anhydride, and recrystallized with 12 mg carrier as described in Methods. The solvents used for the 24-fold distribution in the Craig apparatus were 380 ml 95% ethanol, 20 ml water, and 400 ml petroleum ether. The N_2/N_1 ratio for the acetate of the pure tracer was 2.33.

found, and the amounts of androstenedione (1.1 μg per 100 ml of plasma) and testosterone (0.2) were too small to warrant any conclusions, particularly in view of the imperfect separations of free and conjugated steroids.

Discussion

Adrenal venous blood was sampled through a catheter in the hope that this method would allow examination of glandular secretion under more nearly basal conditions than would be possible by cannulation at surgery. The measured rate of secretion of 17-hydroxycorticoids ranged from 0.03 (F.B) to 1.2 mg per hour per adrenal (C.B.). It is difficult to attribute this large difference merely to variations in basal rates of different individuals. High values seem explicable if the varying degree of pain suffered on the injection of the radiopaque dye caused the release of corticotropin in some patients.[10] Very low secretion rates were attributed at first to incomplete collections of the adrenal secretion and very low concentrations to dilution of the adrenal blood. Numerous samples were discarded on this basis although the X-ray films indicated correct placement of the catheter in the adrenal vein and only minor dilution by collateral circulation. However, the dramatic effect of corticotropin on the 17-hydroxycorticoid secretion of F.B. suggested that incomplete collection and dilution may not always be major factors in such cases and that there may be much greater variations in secretion during short intervals than are apparent from published ranges of the mean secretion rates of cortisol that are measured over several hours (23). In some instances (24) the decline of peripheral 17-hydroxycorticoids after 8 a.m. was at a rate close to that observed after the iv administration of cortisol (25). In such situations the rate of cortisol secretion must be very small. Moreover, Lombardo and associates (6), who obtained their samples at surgery, observed as large a variation in cortisol concentration as we did with the concentrations of the free 17-hydroxycorticoids. Although there may be no need, therefore, to question our sampling method on the basis of our findings, it would seem advisable not to emphasize absolute amounts but to compare the ratios at which the various steroids were found in our samples of adrenal venous blood. Such ratios, of course, would be expected not to be constants characteristic of an individual but to vary with the extent of adrenal stimulation. This view is supported by data obtained on F.B., which indicate that exogenous corticotropin stimulated the secretion of various steroids to a different extent.

Our measurements show quite consistently the secretion of androstenolone sulfate, androstenolone, and androstenedione. Although we failed to find the dione twice and androstenolone once when the 17-hydroxycorticoid values were very low (< 30 μg per 100 ml of plasma), corticotropin caused the secretion of the free steroids in both these patients. These findings agree with those of Short (7), who isolated both free compounds from adrenal blood samples that were obtained at surgery from three women who had received corticotropin. In view of these consistent findings it is difficult to understand why other investigators have failed to obtain similar results. As the methodology varied, it seemed important to show that our results were not caused by artifactual formation of the free steroids during fractionation procedures. Hydrolysis of androstenolone sulfate to the free compound was considered. This possibility seemed very remote because we consistently failed to find androstenolone in peripheral blood [11] although it is known to contain appreciable amounts of the sulfate. The hydrolysis of androstenolone sulfate was definitely eliminated as the source of free androstenolone obtained from subjects J.J. and F.B. because we found that the tritium that had been added as androstenolone-H^3 sulfate to their blood or plasma was removed from the fraction that subsequently yielded the free steroid in large amounts. Numerous conditions exist that can convert steroids with the dihydroxyacetone side chain to 17-keto compounds. Control experiments showed, however, that only neg-

[10] Data obtained on J.J. suggested that his adrenals were stimulated because they produced at least 15 mg per day of C_{19} precursors of 11-deoxy-17-ketosteroids if the observed rates of production were maintained. On the day before catheterization the total urinary 17-ketosteroids amounted to 8 mg.

[11] The sensitivity of the colorimetric method does not exceed 1 μg per 100 ml of plasma. The presence of such amounts of this or other steroids, as were recently announced by Gandy and Peterson (26), is therefore not in conflict with our findings.

ligible amounts could have formed during our procedure. We, therefore, regard androstenolone and androstenedione as genuine secretion products of the human adrenal.

The differences in adrenal and peripheral androstenolone sulfate exceeded those of the free compound, both when stimulated and nonstimulated adrenals were examined. Our series of observations that demonstrate the secretion of the sulfate by the normal adrenal is enlarged by findings of Baulieu (27), who also observed higher levels of androstenolone sulfate in adrenal than in peripheral blood samples of three normal subjects.[12]

Our data do not establish that there is secretion of androstenediol or testosterone [13] by the normal human adrenal but show that such secretion, if it occurs, must be quite small compared to that of the corresponding ketones. Short (7) also failed to find testosterone in his samples by methods he estimates to be capable of detecting 2 μg per 100 ml of plasma. These results could not be foreseen from *in vitro* studies in which the ratio of yields of testosterone and androstenedione from progesterone was 0.5 (30). If our findings are typical of the normal adrenal, its contribution to peripheral testosterone levels may be more through peripheral metabolism of its secretion products (31) than by the secretion of testosterone itself. After the completion of our studies, Burger, Kent, and Kellie (32) reported differences for testosterone between adrenal and peripheral plasma of hirsute women. Other C_{19} steroids were not measured. They found rather large values in two patients (7.76 and 0.85 μg per 100 ml) but differences comparable to those reported in Table III in others.

While this work was in progress Vande Wiele and co-workers (28) considerably refined their method for measuring the *in vivo* production of C_{19} steroids by measurements of the specific activities of selected metabolites in the urine of subjects who had been injected with labeled specimens of the presumed precursors. Since these methods are intended to measure total production, their results (28) are not directly comparable to those reported here for adrenal secretion. These workers deduced the following production rates for a normal woman: androstenolone sulfate, 7.7; androstenolone, 0.7; androstenedione, 3.8; and testosterone, 0.7 mg per day; but more nearly comparable rates of androstenolone and its sulfate for two normal men. Although Vande Wiele and associates took great pains to point out that their calculations are based on the assumption that their model is representative of the physiological situation, others have taken their results as proof that the compounds for which production rates were computed must be actual secretion products (33). Although Vande Wiele and his colleagues' measurements were capable of detecting erroneous assumptions through inconsistent results (28), they evidently have not yet uncovered all differences between the model and the actual situation (34, 35). It would seem that direct examination of the blood from endocrine glands is necessary to prove secretion of a compound and that it, therefore, is an essential step in the development of indirect methods for the estimation of secretion rates.

Our other measurements were done on compounds believed to be the adrenal precursors of the adrenal 17-ketosteroids. They demonstrate for the first time the capacity of the normal human adrenal to secrete 17-hydroxypregnenolone and a conjugate believed to be a sulfate, since it was cleaved by ethyl acetate solvolysis to 17-hydroxypregnenolone. An attempt to isolate the compound directly was unsuccessful, since the tracer 17-hydroxypregnenolone-H^3 3-sulfate was extensively rearranged during chromatography in an alkaline system.[14] The analysis of the presumed sulfate in the blood of J.J. represents a minimal figure, since the tracer was the free steroid that had to be added at a stage in the purification where partial decomposition of the natural compound could have occurred. In this

[12] One of these observations was mentioned in a discussion remark to a paper by Vande Wiele, MacDonald, Gurpide, and Lieberman (28), which was published after submission of our preliminary report (2). We thank Dr. Baulieu for permission to cite his new results.

[13] The specific activity of the acetic anhydride was too low for reliable measurements of the small differences between adrenal and peripheral testosterone that we observed but would have been suitable for measuring the larger amounts of testosterone that were expected from the estimate that normal adrenals secrete about 500 μg of testosterone per day (29).

[14] Calvin and Lieberman (36) recently reported the successful purification of this sulfate in such a system.

case at least, the three 17-ketosteroids appear to be accompanied by the corresponding 17-hydroxy-20-oxopregnenes, and in this series, too, the free Δ^5-unsaturated compound predominated over the α,β-unsaturated ketone. This predominance is in contrast to the situation encountered with the plasma derived from an adrenal tumor that had yielded more 17-hydroxyprogesterone than free 17-hydroxypregnenolone (9). The earlier report discusses this problem more fully and also makes reference to the isolation of 17-hydroxypregnenolone from human urine and from canine adrenal blood.

Calvin and associates (36, 37) have presented evidence for the existence of enzyme systems that can effect the conversions, pregnenolone (3β-hydroxypregn-5-en-20-one) sulfate → 17-hydroxypregnenolone sulfate → androstenolone sulfate. Our probable identification of 17-hydroxypregnenolone sulfate in adrenal vein blood indicates that the substrate of the cleavage reaction occurs in the human adrenal. As was discussed previously (36) such a demonstration is needed if the reaction is to be regarded as an actual rather than merely a potential pathway to androstenolone sulfate.

Summary

Steroids were measured in peripheral and in adrenal venous blood that was obtained by catheterization. The main $C_{19}O_2$ steroids secreted by the adrenal were found to be 3β-hydroxyandrost-5-en-17-one sulfate, 3β-hydroxyandrost-5-en-17-one, and androst-4-ene-3,17-dione. Androst-5-ene-3β,17β-diol was found only in small amounts in adrenal blood. The secretion by the normal adrenal of this steroid and of testosterone, which was not definitely established, must have been very much smaller than the output of the corresponding 17-ketones. 3β,17-Dihydroxypregn-5-en-20-one was identified as a secretion product of the normal human adrenal, and evidence for the presence of its sulfate was obtained.

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