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ADRENOCORTICAL FACTORS IN HYPERTENSION—II THE SIGNIFICANCE OF 16-OXYGENATED C-19 STEROIDS*

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SUMMARY

One of the proposed steroid abnormalities in low renin hypertension, increased production of 16β-hydroxydehydroepiandrosterone, has been evaluated and not confirmed. Double isotopic and radioimmunoassay methods were developed and their specificity evaluated. Two isomeric 16-oxygenated C-19 steroids were also measured, the 16α-hydroxy epimer and the 16-keto,17β-hydroxy derivative, and no significant elevations were observed in hypertensive patients. Plasma levels of 16-keto-5-androstene-3β,17β-diol were also measured and found to be elevated normally in late pregnancy.

The proposal that the syndromes of mineralocorticoid excess and low renin essential hypertension share a common mechanism is based on certain similarities between the two disorders, and the ability of agents which inhibit or antagonize adrenocortical secretion to lower blood pressure in the low renin hypertensive group. However, the beneficial effect of the removal or neutralization of the adrenocortical contribution need only constitute evidence for volume dependency of blood pressure in the low renin group. Evidence for the existence of an adrenocortical factor in low renin hypertension must rest on the isolation of an active substance which reproduces the effect when readministered. This criterion has not yet been met.

INTRODUCTION

The hypertensive syndromes in man, summarized in Table 1, in which adrenocortical hormones have a clearly demonstrated etiologic role, together represent only a small fraction of the hypertension problem. Several lines of indirect evidence have suggested the participation of the adrenal cortex in a larger fraction of the hypertensive population. The incidence of adrenocortical hyperplasia in hypertension at autopsy of 1% [1] is appreciable in view of the incidence of hypertension. Low plasma renin activity is found in primary aldosteronism [2, 3] and in approximately 20–30% of patients with benign essential hypertension [4–7] without evidence of overproduction of known mineralocorticoids. The ability of adrenal inhibitors or antagonists to lower blood pressure in low renin hypertensives has been cited by Woods *et al.* [8] and Spark and Melby [9] as evidence for the secretion of unknown adrenal factors.

Two proposed steroid abnormalities in low renin hypertension are increased production of 18-hydroxy-11-deoxycorticosterone and of 16β-hydroxydehydroepiandrosterone. Interest in 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) appears initially to have been prompted by its structural resemblance to aldosterone and 18-hydroxycorticosterone. 18-Hydroxy-11-deoxycorticosterone was first identified in adrenal incubates by Kahnt *et al.* [10], synthesized by Pappo [11], and identified as a major corticosteroid in the rat adrenal by Birmingham and Ward [12] and by Péron [13]. In that species, 18-OH-DOC arises from the fasciculata zone of the adrenal cortex [14, 15] and is ACTH-dependent [16]. 18-OH-DOC is not an important precursor of aldosterone [17] since it arises in a zone which lacks the terminal oxidase mechanism for aldosterone synthesis. Most bioassays of 18-OH-DOC indicate a sodium-retaining activity of approximately one-fifth that of 11-deoxycorticosterone [18]. Its activity as a glucocorticoid is also very low [19]. If DOC is considered to have 4% of the mineralocorticoid activity of aldosterone in man [20], then one would require a 100-fold increase of 18-OH-DOC level in order to produce an elevation of one aldosterone equivalent. Thus, the

Table 1. Adrenocortical hypertensive syndromes

Mineralocorticoid excess
Aldosterone
Primary aldosteronism due to adenoma
Idiopathic hyperaldosteronism due to bilateral hyperplasia
Dexamethasone-suppressible hyperaldosteronism
DOC (11-deoxycorticosterone)
17α-hydroxylase defect
11β-hydroxylase defect
Ectopic ACTH syndrome
Glucocorticoid excess
Cushing's syndrome (cortisol excess)
High dosage steroid therapy

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reported 2–3-fold elevation in 18-OH-DOC excretion in hypertensive patients [21] is unlikely to have biologic significance. In addition, 18-OH-DOC has not been shown to be uniquely increased in experimental hypertension. It is not elevated in the spontaneously hypertensive rat [22] and its elevation in adrenal regeneration hypertension occurs only after the hypertension has been established [23]. It does not appear to be specifically increased in salt-susceptible hypertension since the 18-OH-DOC/corticosterone ratio in the salt-sensitive animal is the same as that found in the control rats. It is the resistant rats that have an abnormally low ratio [24].

In a recent summary of the role of 18-OH-DOC in experimental and human hypertension, we found no evidence for a specific etiologic role [25, 26]. 18-OH-DOC was not increased in a small series of consecutive low renin spironolactone-responsive essential hypertensives. Its secretion responded normally to ACTH rather than to the renin-angiotensin system. 18-OH-DOC was markedly elevated in 17α-hydroxylase defect and was slightly elevated in primary aldosteronism due to an adenoma, but not in idiopathic or dexamethasone-suppressible hyperaldosteronism. On the basis of a large body of evidence showing parallelism between 11β- and 18-hydroxylase functions of the fasciculata zone, including impairment of 18-hydroxylase in the 11β-hydroxylase form of congenital adrenal hyperplasia, we proposed* both enzymic functions were functionally related and might involve the same enzyme protein and catalytic site. According to this view, the secretion of 18-OH-DOC would have no special significance of its own but would be an obligatory consequence of the secretion of fasciculata zone corticosterone. A testable corollary of this hypothesis would be that the 18-OH-DOC/corticosterone secretory ratio should remain relatively constant despite variations in the absolute secretory rate.

16β-Hydroxydehydroepiandrosterone was encountered in the course of a search for the identity of a sodium-retaining factor which was found to be increased in the urine of low renin hypertensives [27], but is now considered not to be etiologically related to the hypertension since the suppression of its production by dexamethasone does not lead to lowering of blood pressure.† In addition, its biological activity has not been confirmed [28, 29]. We have investigated the excretion of 16β-hydroxydehydroepiandrosterone and related 16-oxygenated-C-19 steroids in low renin hypertension as a possible indicator of a metabolic abnormality in this group apart from the question of its biological activity. Previous estimates of the excretion of 16β-OH-DHEA and related steroids in urine have used gas chromatographic techniques [30]. In order to provide more certain quantitation and avoidance of problems of isomerization we have developed double isotopic and confirmatory radioimmunoassay methods for this purpose.

EXPERIMENTAL

Sources of steroids

16α-OH-DHEA and 3β,17β-dihydroxy-5-androstene-16-one were purchased from Steraloids, Wilton, New Hampshire, and their purity verified by gas chromatography on XE-60 and OV-1 as the TMS and MO-TMS derivatives [30]. Before 16β-OH-DHEA became available, its preparation from the diacetate derivative (Steraloids) was attempted, but all efforts at acid, alkaline or enzymatic hydrolysis resulted in considerable isomerization to the 16-keto-17β-hydroxy derivative. The free ketol, 16β-OH-DHEA, was generously provided by the Searle Co.

Preparation of tritium-labeled steroids

Each of the three 16-oxygenated C-19 steroids was labeled by catalytic exchange. Under typical conditions, 2.0 mg steroid in dimethylformamide was treated with 10 Ci tritiated water in the presence of a rhodium on alumina catalyst at 50° for 16 h (performed by the New England Nuclear Corp.). Methanol-exchangeable hydrogen isotope was removed, and the product chromatographed in the toluene–propylene glycol and toluene–formamide systems at least four times or until the labeled product was seen to migrate as a single narrow band by radioactive scanning.

Chromatography

Non-volatile stationary phases were applied by dipping the paper in a 25% solution of glycol or formamide. In some instances the formamide concentration was increased to 50% to retard the migration rate. Silica gel thin layer plates were from Quantum Industries (Q6F) and alumina from the Brinkmann Instrument Co. (Alox). The latter were neutralized to pH 4.5 by dipping in 10% acetic acid in ethanol for 5 min and drying in a forced-air oven at 60° for 20 min.

Patients

Urine specimens from patients seen at the Hypertension Center of New York Hospital-Cornell Medical Center were kindly provided by Dr. John H. Laragh, whose Laboratory also measured plasma renin activity and urinary sodium excretion and classified the hypertensive patients according to their published criteria [31]. The 24-h specimens were frozen until analyzed. Urine specimens from pregnant subjects were obtained at the Mount Sinai Hospital Prenatal Clinic, with the kind assistance of Dr. Erlio Gurpide.

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† See Discussion by Dr. Grant Liddle.

Measurement of 16-oxygenated C-19 steroids by double isotopic methods

Hydrolysis and extraction of urinary steroid sulfates. Initially, urine specimens were subjected to a sequential procedure in which free, glucuronide, pH 1-hydrolyzable and solvolyzable steroid fractions were prepared. Subsequently it was found that direct chemical solvolysis of urine gave the same results without prior removal or hydrolysis of other conjugates.

A known amount of one or all three [^{3}H]-labeled steroids was added to 100 ml of urine to permit correction for recovery. The pH was adjusted to 1.0 with 2 ml or more of 18 N sulfuric acid. Ammonium sulfate (50 g per 100 ml urine) was added and the urine was extracted with three 100-ml portions of ethyl acetate. The extract was dried over sodium sulfate, filtered and incubated at 37° for 18 h. After neutralization of the extract with ammonium hydroxide, it was washed with 5% sodium carbonate, water, dried and evaporated in preparation for chromatography. The recovery of labeled dehydroisoandrosterone sulfate added to urine under these solvolysis conditions was 90–95%.

Isolation, purification and measurement. The solvolyzed extract was chromatographed according to the sequence of Table 2. The steroid or its diacetate derivative were located after each chromatogram by radioactive scanning. Radiochemical purity was always achieved by this purification scheme, as indicated by agreement within 10% between the ^{3}H/^{14}C ratios in the last two steps. Excretory rates were calculated in the usual manner from the c.p.m. [^{3}H] internal standard, the urinary aliquot, the specific activities of the acetylating reagent and the ^{3}H/^{14}C ratio of the purified urinary steroid.

Measurement of 16-oxygenated-C-19 steroids by radioimmunoassay

The antiserum (S-37 No. 2) prepared by Buster and Abraham [32] for the radioimmunoassay or 16α-OH-DHEA in plasma was found to cross-react with the 16β-OH and the 16-keto-17β-OH derivatives (Fig. 1). Randomly-labeled [^{3}H]3β,17β-dihydroxy-5-androstene-16-one (8.6 Ci/mmol) was used as the tracer in all assays.

Plasma. [^{3}H]-16-ketoandrostenediol (10,000 c.p.m., 700 pg) was added to 10 ml plasma and the mixture equilibrated at room temperature for 1 h. The amount of unlabeled steroid added with the tracer did not exceed the minimum endogenous level. Extraction was carried out with methylene chloride (20 ml) and the extract was washed twice with 5 ml 0.1 N sodium hydroxide, once with water and evaporated under a stream of nitrogen.

Each extract was chromatographed on a 2 × 50 cm strip of unwashed Whatman No. 1 paper in the toluene–propylene glycol system for approx. 24 h or until the F_5 dye of Neher had run at least 3/4 of the distance to the edge. The dried chromatogram was scanned in a Vanguard Model 930 scanner to locate the tracer, and the radioactive zone cut up and eluted by shaking gently on a Dubnoff shaker for 15 min with 1.7 ml 0.05 M phosphate buffer, pH 8.0, in a counting vial.

Non-specific blanks were removed from the eluate by filtration through a small glass wool column as suggested by Dr. P. Vecsei.‡ Unwashed Pyrex brand glass wool was gently packed into a Pasteur pipette and washed once with 0.05 M phosphate buffer, pH 8.0. The paper chromatogram eluate was passed through the column and aliquots taken for radioimmunoassay and calculation of recovery.

Urine. An aliquot of the toluene–propylene glycol eluate from the double isotopic method for solvolyzed C-16 oxygenated C-19 steroids was taken for radioimmunoassay.

Radioimmunoassay. The standard curve was prepared by evaporating a methanolic solution of steroid

‡ Personal communication.

Table 2. Chromatographic isolation and purification of 16-oxygenated C-19 steroids

Chromatogram No.	
(1) Toluene–propylene glycol 24 h. Mobility relative to F_5 dye:	
16β-OH-DHEA	0.33
16α-OH-DHEA	0.56
16-keto-androstenediol	0.71
Acetylate with ^{14}C-acetic anhydride to form the diacetate.	
(2) Heptane–formamide to the edge. (Mobility of diacetates relative to progesterone 1.7 ± 0.2)	
(3) Repeat No. 2	
(4) Silica gel thin layer: dichloroethane–methanol (99:1 v/v)	
(5) Alumina (acid washed) thin layer: toluene	

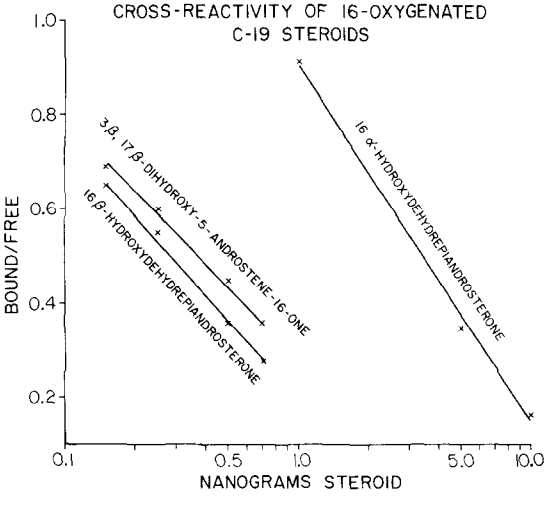

Fig. 1.

into the assay tubes to cover the range from 100–1000 pg.

Abraham antibody S-37 No. 2 was diluted approx. 1:9000 to give a bound/free ratio of 1.0 for the tracer alone. Each tube contained the diluted antibody, approximately 3500 c.p.m. of tracer, and 5 μl human gamma globulin in a total volume of 0.2 ml 0.05 M phosphate buffer, pH 8.0. All tubes were mixed in a vortex mixer and placed in an orbital shaker operating at low speed in a cold room at 9° overnight. To separate bound and free steroid, 0.1 ml 5% bovine gamma globulin and 0.7 ml polyethylene glycol (6000–7500 mol wt) 30 g/100 ml buffer was added. The tubes were mixed and centrifuged at 2500 rev./min for 30 min and either the bound or free steroid or both were measured. The supernatant (free) was decanted into 10 ml of the phosphor and the precipitate (bound) dispersed in the same total volume of phosphor. Either the free fraction alone was counted and the bound calculated by subtraction from the total or the bound fraction was determined experimentally. There was no significant difference between bound/free ratios determined by the two methods.

Validity of the radioimmunoassay. The B/F value for the tracer in 9 determinations was 1.04 $\pm$ 0.058. The Δ B/F for this standard deviation was equivalent to a sensitivity of 30 pg. The practical sensitivity was approximately twice this value and was limited by the relatively low S.A. of the tracer. In the tube containing tracer alone, 3500 c.p.m. represented 250 pg of carrier steroid. The coefficients of variation were 7.0% within assays and 10.0% between assays. The mean overall recovery in 30 plasma assays was 19 $\pm$ 5.9%.

RESULTS

Validity of the methods

Purity of the steroid and stability of the tritium label. Table 3 illustrates the use of carrier dilution to verify the radiochemical homogeneity and stability of two of the C-16-oxygenated-C-19 steroids. The doubly-labeled diacetate derivatives were purified to constant $^3H/^{14}C$ ratio using the same sequence of chromatograms as in the analytical determination. Chromatography in the toluene–propylene glycol system did not significantly change the S.A., indicating that radiochemical purity had already been achieved and that there was no loss of tritium during this step. Although the calculated specific activity was subject to errors in counting and weighing it too showed satisfactory agreement with the two values obtained by the isotope derivative technique.

Additional evidence for the stability of the labeled steroid in the analytic procedure was obtained in a recovery experiment summarized in Table 4, where the analysis of a urine pool before and after the addition of 100 μg 3β,17β-dihydroxy-5-androstene-16-one showed quantitative recovery.

Specific activity of exchange-labeled steroids. For the determination of specific activity, radioimmunoassay was used to determine the pg of steroid for amounts of tracer over the range of 1000–5000 c.p.m. Total c.p.m. were plotted against pg, the best straight line drawn through the points, and the S.A. calculated from the slope. Specific activities were at least 5 Ci/mmol and for the 16-keto-androstenediol it was 8.6 Ci/mmol. This value was about one fifth that of the commercially available [1,2-^{3}H]-labeled steroids, and was adequate for the measurement of urinary excretory rates but limited the sensitivity of the plasma radioimmunoassay.

Chromatographic stability. 16β-Hydroxy-DHIA, when chromatographed on silica gel thin layer plates was partly converted to its 16-keto isomer, 16-keto-5-androstenediol. When each of the three labeled 16-oxygenated-C-19 steroids were chromatographed and rechromatographed on the toluene–propylene glycol system, each appeared as a separate diacetate zone. There was no evidence of interconversion.

Stability toward solvolysis. Each labeled 16-oxygenated-C-19 steroid was added to an aliquot of urine and the solvolysis procedure carried out as described and the extract chromatographed on the toluene–propylene glycol system. Radioactive scanning showed

Table 3. Demonstration of radiochemical purity of titriated steroids by carrier dilution

	S.A. c.p.m./μg		
		Found for diacetate: Effect of chromatography*	
Steroid	Calculated	before	after
16β-hydroxydehydroisoandrosterone 5.35×10^6 c.p.m. + 100 μg	53500	49800	47500
3β,17β-dihydroxy-5-androstene-16-one 7.0×10^6 c.p.m. + 70 μg	10000	11000	9900

After addition of carrier steroid, an aliquot of the mixture was reacted with ^{14}C-acetic anhydride and purified chromatographically as described under Method. Another aliquot of the mixture was chromatographed on the toluene–propylene glycol system before formation, purification and measurement of the doubly labeled diacetate.

* Free steroid on toluene–propylene glycol system.

Table 4. Recovery of 3β,17β-dihydroxy-5-androstene-16-one by double isotopic method

Experiment	Other	3β,17β-dihydroxy-5-androstene-16-one added μg	3β,17β-dihydroxy-5-androstene-16-one recovered μg
1	100 ml urine pool A		4
2	100 ml urine pool A	100	98
3	+ 250 μg 16α-OH-DHEA	50	52
4	+ 250 μg 16α-OH-DHEA	50	48

Recovery of steroid added to urine and of steroid in the presence of a known excess of 16α-OH-DHEA.

that in each experiment the radioactive component migrated as a single peak with the same mobility as the authentic steroid. Thus there was no decomposition or interconversion of the two 16-hydroxy epimers of dehydroepiandrosterone or of the 16-keto derivative under conditions of solvolysis and paper chromatography.

Specificity of the isotopic method. The recovery experiments of Table 3 cited above provided evidence as well for the specificity of the method. The separation of the three C-16-oxygenated steroids was largely achieved by the toluene–propylene glycol chromatogram step, in which the 16β-hydroxy derivative migrated at a much slower rate than both its 16α-epimer and the 16-keto-17β-hydroxy derivative. Although the latter two were also clearly separated from each other when the F_5 dye was allowed to run to the edge of the paper, it was felt desirable to verify that the more abundant urinary steroid, 16α-hydroxydehydroepiandrosterone, did not interfere in determinations of the 16-ketone.

A mixture of known amounts of labeled and unlabeled 16-keto derivative was analyzed as described under Experimental, beginning with the toluene–propylene glycol chromatogram step and proceeding to acetylation with labeled acetic anhydride, and purification to constant isotope ratio. Another aliquot of the same mixture was enriched with a 5-fold excess of 16α-hydroxydehydroepiandrosterone and analysis carried out as described above. The calculated content of 16-keto derivative in the two analyses showed no significant difference and therefore no interference from the 16α derivative in the determination of the 16-ketone. The demonstration of the stability of all three steroids toward solvolysis and chromatography was cited above.

Comparison of double isotopic and radioimmunoassay methods

A comparison of urinary excretion of 16-keto-5-ene-androstenediol by double isotopic and radioimmunoassay methods is shown in Table 5. There was, in all instances, a satisfactory agreement between the two methods. Hypertensives excreted normal amounts of the steroids and patients during late pregnancy excreted increased amounts, as has been previously shown for 16α-hydroxydehydroepiandrosterone [33].

16-Oxygenated C-19 steroids in hypertension

Table 6 shows a group of 21 hypertensive subjects classified according to plasma renin activity relative to urinary sodium excretion [31]. These data are also shown in Fig. 2 along with the normal range in the shaded area based on the data of Jänne and Vihko for their gas chromatographic method [30]. Most determinations were in the normal range but a few determinations of the 16α- and 16β-hydroxydehydroepiandrosterone appeared to be slightly above this range but not strikingly elevated. All determined values of 16-keto-5-ene-androstenediol in hypertensive patients were normal in contrast to the marked elevations seen in late pregnancy.

Plasma levels by radioimmunoassay

The mean concentration of 3β,17β-dihydroxy-5-androstene-16-one in four normal plasma pools was 14.6 ± 5.5 ng/100 ml. This value was approx. one sixth that of 16α-OH-DHEA [33] and is in keeping with the relatively greater urinary excretory rate of the 16α-hydroxy derivative. Specificity of the method

Table 5. Comparison of double isotope and radioimmunoassay measurement of urinary 16-oxygenated C-19 steroids

Subjects	16-keto-5-ene-androstene-3β,17β-diol, μg/day double isotope	16-keto-5-ene-androstene-3β,17β-diol, μg/day radioimmunoassay
Hypertensives		
L.B.	36	32
M.K.	40	34
P.B.	54	52
D.C.	114	132
D.C.	163	180
Pregnancy, 3rd trimester		
Ve	332	360
Ma	680	990
Ca	920	960
Wi	1006	1220

Table 6. Excretory rates of 16-oxygenated C-19 steroids in hypertensive patients

Patient	Age	Sex	Urinary steroids, μg/day 16β-OH-DHEA	16-keto-5-An-diol	16α-OH-DHEA	^{U}Na m-equiv./day	PRA
Low PRA							
K.B.*	53	F	11.1	36.8	14.2	38	1.5
N.G.	36	M	11.4	56		84	0.8
E.W.	50	F	12	11	86	41	1.8
A.P.	25	F	14.5	100		48	0.3
C.T.	53	F	67	93	183	69	1.1
M.M.	58	M	71	187	478	66	1.1
M.C.	58	M	196	183	499	128	0.3
L.F.	48	M	209	217	2060	67	0.4
T.W.	56	M		55		85	1.8
Borderline low PRA							
G.L.	65	F	10.2	33	59	48	2.2
B.K.	49	M	14.5	123	99.4	34	3.0
C.M.	54	F	28	64	103	70	1.8
J.E.	35	M	151	136	1273	172	1.5
Normal PRA							
M.A.	55	M		39		158	2.1
H.R.	57	F		40		31	4.6
M.K.	46	M		40		93	2.9
L.B.	45	M	36	51		46	4.0
P.B.	50	F		54		53	7.2
Labile hypertensives							
R.A.	59	F	28.7	71.4	495	76	1.3
D.C.	33	M	120	195		65	3.3
A.D.	15	F		89		107	1.1

* Black. All other patients are white.

was shown by the failure of a large excess of 16α-OH-DHEA to interfere with the determination (Table 7).

Plasma levels of 16α-OH-DHEA are known to be increased in pregnancy [33]. Table 7 shows that 16-keto-androstenediol was also increased in the third trimester. Thus the increase in 16-hydroxylation by the fetal-placental unit is also associated with increased levels of the 16-keto derivative in the maternal circulation.

The sensitivity of the radioimmunoassay could probably be improved, even without increasing the specific activity of the tracer, by decreasing the amount of tracer in the assay tubes and the amount of internal standard. We used a relatively large amount of internal standard to permit localization on the paper chromatogram by direct radioactive scanning in order to provide greater analytic certainty in these initial observations, but localization could probably be achieved as well by the use of reference tracer strips on either side of the plasma extract.

DISCUSSION

One of the proposed steroid abnormalities in low renin hypertension [27], increased production of 16β-hydroxydehydroepiandrosterone, has been evaluated in this report and not confirmed. Methods have been devised which provide an accurate measure of each of the three isomeric 16-oxygenated C-19 steroids, avoiding problems of interconversion. Determinations were confirmed in some instances by both

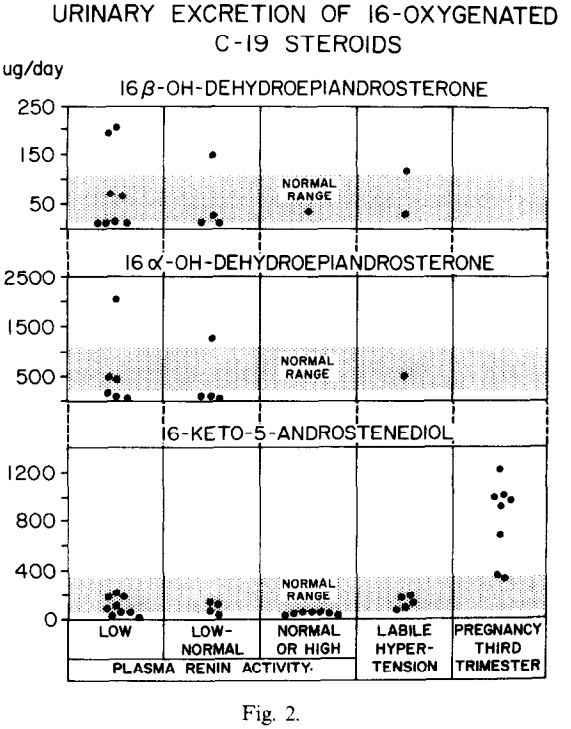

Fig. 2.

Table 7. Radioimmunoassay of 3β,17β-dihydroxy-5-androstene-16-one in plasma

Subject		Plasma level ng/100 ml
Normal No. 1		13, 21
Normal No. 2		7, 7
Normal No. 3		15
Normal No. 4		21, 18
Normal No. 4 + 7 ng/ml 16α-OH-DHEA		19, 15
Pregnancy	Month gestation	
F.M.	5	13
L.W.	8	53
C.R.	8	120

double isotopic and radioimmunoassay techniques. Although the immunoassay was of limited sensitivity when applied to plasma, it did demonstrate that the 16-ketone derivative was present in the circulation and was not an artifact of extraction. It also demonstrated elevated values in normal late pregnancy.

We previously failed to confirm increased production of 18-OH-DOC in low renin hypertension but found small increases in its production in aldosterone-producing adenomas and suggested that small increases in 18-OH-DOC in low renin hypertension might indicate the presence of a histological variant of Conn's syndrome [25, 26]. The subsequent proposal, that a 16α-hydroxy metabolite of 18-OH-DOC might augment the mineralocorticoid activity of aldosterone [34] has been recently subjected to testing at the level of the kidney cystosol mineralocorticoid receptor. Fuller and coworkers [35] found that 16α,18-dihydroxy-DOC did not exert a co-operative or allosteric effect on the binding of aldosterone to the receptor.

The suggestion of adrenocortical involvement in low renin hypertension is based on certain similarities between these patients and those with syndromes of mineralocorticoid excess [8, 9]. Both have low plasma renin activity, volume-sensitive hypertension, and respond to inhibition or blockade of the adrenocortical secretion. An analogy between the two syndromes, however, does not necessarily establish a common mechanism. Since the blood pressure is especially volume sensitive in the low renin group, particularly if the group is actually defined operationally in terms of the sensitivity of blood pressure to vol. depletion, any measure which acts on one of the components of the homeostatic chain which normally maintain intravascular vol. such as the adrenal cortex, the kidney, or sodium balance, would be expected to affect blood pressure. Thus, reduction of blood pressure in low renin hypertensives by steroid antagonists or inhibitors does not necessarily constitute evidence for the operation of another adrenal factor, since these agents need only act on the normal components of sodium homeostasis to exert a beneficial effect. Proof of the existence of an adrenocortical steroid mechanism in low renin hypertension must rest on the demonstration of the presence of a biologically active substance and the administration of that substance to produce the effect. This criterion has not yet been met.

An important difference between low renin hypertension and mineralocorticoid excess is that the former group generally have a normal serum potassium. One question that can be raised is whether hypokalemia is an obligatory manifestation of mineralocorticoid excess or whether a steroid can cause hypertension without exhibiting hypokalemia. The normal renal tubular coupling of sodium reabsorption to potassium excretion by mineralocorticoids would seem to require that a sufficient degree of sodium retention by a mineralocorticoid mechanism would be accompanied by kaluresis. Dissociation between sodium-retaining and kaluretic effects can occur under certain circumstances [26, 35] but at the present state of knowledge the absence of hypokalemia argues against a state of mineralocorticoid excess.

Another question concerns the possibility of whether a steroid which is neither a potent mineralocorticoid nor glucocorticoid itself can exert a hypertensive effect. Preliminary reports suggest that very high doses of 18-OH-DOC may be hypertensive in the sensitized dog [36]. An interesting mechanism uncovered by the Melbourne group of investigators in studies of the mechanism of ACTH-induced hypertension in sheep, is that a steroid having very little activity itself, 17α,20α-dihydroxy-4-pregnene-2-one, is capable of causing hypertension when administered along with the other normal adrenocortical secretory products [37]. The factors regulating blood pressure can be considered in terms of mechanisms affecting either plasma volume or vasoconstriction. Mineralocorticoids act predominantly to increase plasma volume whereas glucocorticoids may have an effect on vascular reactivity and vasoconstriction [38]. Other possible steroidal mechanisms are an effect on cardiac output by way of myocardial receptors or an effect on the synthesis of renin substrate [39]. Thus it may be theoretically possible but unlikely for an unknown steroid to have a potent effect on blood pressure and yet not be recognizable either as a potent mineralocorticoid or glucocorticoid.

Since the oversecretion of an etiologically important active substance of adrenocortical origin has not yet been demonstrated in low renin essential hypertension, it would seem more promising to direct attention to the smaller subgroup of hypertensive patients who exhibit hypokalemia in the face of subnormal production of known mineralocorticoids. The patients that have been described have a spironolactone-correctable syndrome of apparent mineralocorticoid excess but with low levels of both aldosterone and 11-deoxycorticosterone [40–42]. Their response to mineralocorticoid antagonists distinguishes them from the hypokalemic hypertensive

patients described by Liddle and coworkers [43]. A search for an unknown biologically active steroid in such patients is in progress [42].

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DISCUSSION

Liddle. I wonder if you have measured 16β-hydroxy-DHEA only in urine or if you've also measured it in blood with your radioimmunoassay system or with the double isotope derivative method?

Ulick. We've measured only 16-keto-5-androstenediol in plasma by radioimmunoassay, not the 16β-hydroxy-DHEA derivative. The sensitivity was borderline and we haven't made many observations. I can only say that the steroid is present in plasma. It does circulate and its formation is not artifactual. The levels in normals and in hypertensives were less than in 3rd trimester pregnancy.

Liddle. Perhaps you would permit me to supplement your observations by referring to work by Dr. Sekihara in our laboratory. During the past year, he has used a method for measuring plasma 16β hydroxy DHEA and has found that it apparently is regulated largely by ACTH but also to some extent apparently by gonadotrophins. It does rise during pregnancy and it is not significantly different in various groups of hypertensive patients. This, I might say, is essentially in confirmation of your observation.

Adlercreutz. I can tell you that we measured 16β-hydroxy-DHA in urine in normal individuals by capillary gas chromatography and we have at present the normal range, which goes up to almost 300 micrograms per day.

Ulick. Thank you for your comments. That would put our values in hypertensives within the normal range.

Pasqualini. If I make a good calculation you show that 20% of the 18-hydroxy-DOC production is converted to its tetrahydro derivative. Do you have any idea what the

remaining metabolites are. Also do you have some data on the conjugates of 18-hydroxy-DOC or the tetrahydro derivative?

Ulick. We really don't know much about other metabolites because we just looked at the major tetrahydro derivative. We have not examined other radioactive components following the injection of labeled 18-hydroxy-DOC. The conjugate we measure is glucuronidase-hydrolyzable and cleaved by periodic acid.

Fraser. I read with interest your recent paper on the interaction of 18-hydroxy-11-deoxycorticosterone with alcohol solvents. There is some evidence that 18 hydroxycorticosteroids can form dimers in solution. Have you examined this phenomenon?

Ulick. Dr. Pappo of G. D. Searle first called attention to the phenomenon of dimerization of 18-OH-DOC. He noted it in the course of working with milligram amounts. We have his reference sample of 18-hydroxy-DOC dimer and have found it different from the less polar form of 18-hydroxy-DOC that is formed in dilute alcoholic solution (Roy, A. K., Ramirez, L. C., and Ulick, S.: Structure and mechanism of formation of the two forms of 18-hydroxy-11-deoxycorticosterone. *J. steroid Biochem.* **7** (1976) 81–87). However, the intermediate in both reactions is probably the carbonium ion at C-20. In dilute solution there is greater probability for the steroid to react with the solvent but if you have a lot of molecules together in more concentrated solution, the tendency will be for it to react with itself. The mechanism of both reactions is probably the same.

Crabbé. May I ask you to expand a little bit on your conclusion that the enzyme system carrying out 11β and 18 hydroxylation is one and the same? Does one observe a reduction in 18-hydroxy-DOC for instance when metapyrone is being given?

Ulick. The answer to your question is yes. Dr. Birmingham has made that observation. That is one of the points of similarity between the 11β- and 18-hydroxylases, that both share the same inhibitors and cofactors. Our experimental evidence consists only of the observation of the absence of 18-hydroxylation in patients with the 11β-hydroxylase defect, but as we mentioned one can assemble an impressive amount of evidence showing that the two activities are parallel. The purest preparations of mitochondrial fasciculata zone 11β-hydroxylase contain proportional amounts of 18-hydroxylase. The hypothesis of two hydroxylating functions for a single enzyme protein has not been proposed before for mammalian systems, although there is evidence for it that I cited in microbial systems—of a single catalytic site of diminished specificity. A fortunate thing about this hypothesis is that it can be tested. A corollary would be that the 18-hydroxy-DOC/corticosterone ratio should be constant despite wide ranges in the absolute rate of secretion of either. Suppression of stimulation of 18-hydroxy-DOC should be associated with a proportional change in fasciculata zone corticosterone. One can also obtain evidence *in vitro* from Lineweaver–Burk plots of both hydroxylating activities. It would be an extraordinary coincidence for the two enzymic activities to show the same intercept and the same Michaelis constant if the catalytic site were not the same.

Birmingham. I'm always for a unifying hypothesis. However, I can think of three examples where corticosterone dissociates from 18-hydroxydeoxycorticosterone: When you study the effect of the alcoholic derivative of metapyrone you get the disconcerting observation that you stimulate 18-hydroxy-DOC formation by the rat adrenal whereas you have no effect on the production of corticosterone (*Steroids* **13** (1969) 57–465). I don't see that that necessarily negates your theory because there may be various conditions under which the same enzyme will have different requirements for different substrates. Other instances in which corticosterone dissociates from 18-OH-DOC is in the freshly regenerating rat adrenal, where we found 18-hydroxy-DOC formation recovers before corticosterone formation. The third evidence is work with cyclic nucleotides. My student Wah-Tung Hum has found that when you incubate cyclic GMP as opposed to cyclic AMP you find that the ratio of 18-OH-DOC to corticosterone is consistently increased compared to the ratio obtained in the presence of cyclic AMP (*Proc. Con. Fed. Biol. Soc.* **19** (1976) 470). As I said this does not necessarily oppose your theory, but it's something one has to consider.