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STEROID 11β-HYDROXYLASE ACTIVITY IN THE MICROSOMAL FRACTION OF HUMAN ADRENALS*

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SUMMARY

The conversion of 11-deoxycortisol and 11-deoxycorticosterone (DOC) to their 11β-hydroxylated metabolites was measured in the 20,000 and 105,000 *g* fractions of human adrenals. The adrenals were obtained from kidney transplantation donors.

The results indicate the presence of relatively high 11β-hydroxylase activity in the microsomal fraction which cannot be attributed to enzyme contamination. In addition there would seem to be further metabolism of cortisol probably by an 17α-hydroxysteroid side chain cleavage enzyme.

INTRODUCTION

The enzymes involved in the adrenocortical biosynthesis are generally considered to be located in the mitochondria and in the endoplasmatic reticulum of adrenocortical cells. Sweat and Hayano [1–3] found the 11β-hydroxylase activity of bovine adrenal glands to occur in the mitochondria. Sweat [1], centrifuging beef adrenal homogenate between 2000 and 19,000 *g*, found essentially all the activity in the granules while only traces were found in the supernatant. It was not examined whether these activity traces were a result of membrane disruption or enzyme presence in particles other than the mitochondria. Hayano *et al.* [2, 3] found the activity in the whole homogenate, to present a supernatant fraction obtained at 1500 *g* and a washed fraction obtained at 5000 *g*. As it appears from this work the activity of the supernatant obtained at 5000 *g* centrifugation has not been determined. Findings which demonstrated the 3β-hydroxy steroid dehydrogenase, 5 ene → 4 ene—isomerase activities to occur in the microsomal and in the mitochondrial fractions [4–9] have encouraged us to examine the presence of 11β-hydroxylase activity in these two cell fractions from human adrenocortical tissue.

Since preliminary electron microscopy examinations showed us that human adrenals obtained from autopsies contained membrane disrupted mitochondria, we had no other choice than to use fresh material which did not undergo freezing and thawing procedures. The only source for these glands were kidney transplantations which have the disadvantage of being very scarce.

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EXPERIMENTAL

Abbreviations:

(1) Androstendione
= 4-Androsten-3,17-dione
(2) Corticosterone
= 11β,21-Dihydroxy-4-pregnene-3,20-dione
(3) Cortisol
= 11β,17α,21-Trihydroxy-4-pregnene-3,20-dione
(4) Cortisone
= 17α,21-Dihydroxy-4-pregnene-3,11,20-trione
(5) 11-Deoxycorticosterone (DOC)
= 21-Hydroxy-4-pregnene-3,20-dione
(6) 11-Deoxycortisol
= 17α,21-Dihydroxy-4-pregnene-3,20-dione
(7) 11-Hydroxyandrostendione
= 11β-Hydroxy-4-androstene-3,17-dione

Materials. The following radioactive substrates were used:

(1) [1,2^{3}H]-deoxycortisol (New England Nuclear Corp., Boston, MA.) was diluted with unlabelled deoxycortisol (Sigma, St. Louis, MO) each corresponding reaction vial in the two experiments contained 5 μg of unlabelled substrate while the activity was 400,000 d.p.m. in the first and 760,000 d.p.m. in the second.

(2) [1,2^{3}H]-DOC (NEN) was diluted with unlabelled DOC, each corresponding reaction vial contained 4·77 μg of unlabelled substrate while the activity was 400,000 d.p.m. in the first experiment (DOC was not used in the second experiment).

The labelled compounds were purified by thin-layer chromatography (HF 254, Merck), using a solvent system of chloroform–methanol (94:6 v/v) [10]. The unlabelled compounds were purified by repeated crystallization using methanol as solvent.

Enzyme preparation. Two separate experiments were carried out, in each of them the adrenal gland

was obtained from kidney donors, both of whom died from head injuries and were 38 and 20 years old respectively. The glands (one in each experiment) were chilled in crushed ice and brought to the laboratory immediately after surgery. The cortex tissue was scraped off with a scalpel and the resultant pulp was homogenized in a Teflon homogenizer with a solution of 0·25 M sucrose, 0·077 M NaCl and 0·05 M sodium phosphate buffer (pH 7·4) to a final concentration of 20% (w/v) [11]. The homogenate was centrifuged at 700 *g* for 15 min in a cooled centrifuge (Sorvall RC2-B). The obtained supernatant was centrifuged up to 20,000 *g* (same centrifuge) for 1 h and the sediment was used as the mitochondrial fraction. The 20,000 *g* supernatant was centrifuged up to 105,000 *g* (Beckman, Spinco L 50) for 1 h and the sediment, after two washings, was used as the microsomal fraction. The washings were carried out with buffer following a centrifugation at 105,000 *g* for 15 min. In the second experiment the 11β-hydroxylase activity of the washing solutions was measured with those of the other fractions (Table 2). The mitochondrial and microsomal sediments were reconstituted to their original concentrations with buffer solutions and samples were taken out for Lowry protein determination (2). The following protein concentrations were measured: (1) first experiment—mitochondria 6·3 mg/ml, supernatant—105,000 *g* 12·8 mg/ml, microsomes 1·1 mg/ml. (2) second experiment mitochondria 6·3 mg/ml, supernatant—105,000 *g* 8·2 mg/ml, microsomes 1·0 mg/ml. In order to see whether apparent microsomal activity could be due to mitochondrial disruption, samples from supernatant 700 *g*, mitochondrial and microsomal fractions were examined by electron microscopy examinations. This procedure was carried out as follows: the respective samples were fixed as pellets in glutaraldehyde, postfixed in osmium tetroxide and imbedded in Epon. Thin sections were examined with an EM 9 S 2. A total of 5 to 10 blocs of the supernatant, the mitochondrial fraction and microsomal fraction were examined and photographed.

In the second experiment the condition of the mitochondrial fraction was examined in addition to the electron microscopy, with a marker enzyme method (glutamate dehydrogenase) [13] (Table 3).

Assay. The incubations were carried out in a shaking bath at 25 °C for 2 h. All the other details including composition of the NADPH generating system, extraction and chromatography are the same as those described in our previous work [14].

Table 1. The 11β-hydroxylase conversion rates in various cell fractions of the human adrenal cortex obtained at kidney transplantation. Sup- = Supernatant. t.l.c. zones = The t.l.c. plates were separated, after chromatography, to three zones: (a) product (b) precursor (c) general, which was the remainder of the plate. Radioactivity % = Per cent of radioactivity calculated from the sum of the plate. Specific activity = Per cent of product radioactivity per reaction vial protein amount (mg) per min. The results of two experiments are described

Incubation	Precursor	t.l.c. zones	Radioactivity %	Mean %	Mean S.A. %/mg/min
Mitochondria (I)	deoxycortisol	product	96·1	96·1	$9{\cdot}08 \times 10^{-2}$
		precursor	3·9		
		general	0·0		
Mitochondria (II)	deoxycortisol	product	96·1		
		precursor	3·9		
		general	0·0		
Supernatant-105,000 *g* (I)	deoxycortisol	product	21·4	18·7	$0{\cdot}83 \times 10^{-2}$
		precursor	74·6		
		general	4·0		
Supernatant 105,000 *g* (II)	deoxycortisol	product	16·0		
		precursor	83·2		
		general	0·7		
Microsomes (I)	deoxycortisol	product	20·1	19·1	$10{\cdot}00 \times 10^{-2}$
		precursor	48·3		
		general	31·6		
Microsomes (II)	deoxycrotisol	product	18·2		
		precursor	53·7		
		general	28·1		
Mitochondria (I)	DOC	product	73·5	72·7	$6{\cdot}90 \times 10^{-2}$
		precursor	13·3		
		general	13·0		
Mitochondria (II)	DOC	product	71·9		
		precursor	14·7		
		general	13·4		
Microsomes (I)	DOC	product	27·2	27·9	$14{\cdot}50 \times 10^{-2}$
		precursor	65·6		
		general	7·2		
Microsomes (II)	DOC	product	28·6		
		precursor	67·2		
		general	4·3		

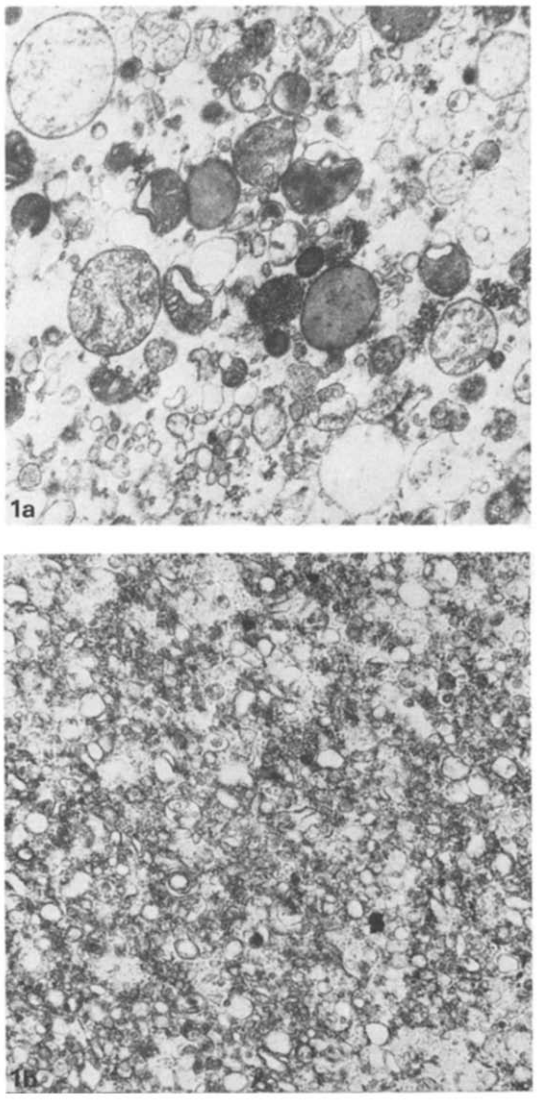

Fig. 1. Electron microscopic aspect of centrifugation fractions in the first experiment. (a) *Mitochondrial fraction* with intact mitochondria, except of some already recognizable ballooning, however without rupture of mitochondrial membrane; (b) *Microsomal fraction* without mitochondrial structure. Both photographs 24,000 ×.

RESULTS

Table 1 shows the results obtained from the first experiment. The following findings are noteworthy: (a) The S.A. values obtained in the incubation of the microsomal fraction with DOC and deoxycortisol were found to be higher than those of the mitochondria, even after the microsomes were washed twice. (b) A zone containing relatively large amounts of radioactivity was found on thin layer chromatograms of the microsomal products derived from deoxycortisol suggesting the production of an additional metabolite. (c) In the present experiment the 11β-hydroxylation of deoxycortisol is higher than that of DOC. This is in contrast to the findings typical to adrenals obtained from autopsies [14].

The mitochondrial fraction of this experiment showed intact mitochondria (Fig. 1a). The microsomal fraction showed vesicular structures from smooth and rough endoplasmatic reticulum, free ribosomes and microbodies. No structures were found which could originate from mitochondria (Fig. 1b). These findings and the relatively high specific activity values obtained in the microsomal fraction support the assumption that the microsomal 11β-hydroxylase activity is due to a distinct microsomal enzyme rather than to a contamination from mitochondrial enzymes.

The results obtained in the second experiment are summarized in Table 2. Attention should be paid to the following results: (a) Washing of the microsomal fraction did not cause loss of 11β-hydroxylase activity. (b) The t.l.c. general fraction of the microsomal incubation reveals relatively high radioactivity counts (75%), probably indicating the production of another metabolite. Thus the true specific activity value might conceivably be considerably higher than that actually obtained.

Table 3 shows the activity of the mitochondrial marker enzyme–glutamate dehydrogenase. These results, more than the electron microscopic examination, show clearly the presence of a relatively intact mitochondria. This is the most convincing finding which proves that the microsomal 11β-hydroxylase activity is not due to mitochondrial enzyme contamination.

In order to detect the expected metabolites (other than cortisol) which could have been produced in the incubation of the microsomal fraction with deoxycortisol, samples from the t.l.c. general fraction were run again with the following steroids (50 μg) added: cortisone, 11β-hydroxyandrostendione and androstendione. After development of the chromatogram, the plates were illuminated with U.V. light, the steroids were scraped off and the remainder of the plates (20 × 20 cm.) was divided into zones with a width of 2 cm. each. All silica gel zones were extracted with ethanol and their radioactivity was measured. No significant radioactivity was found in the corresponding zones of cortisone. Table 4 shows the separation of two plates run in different solution systems. Obviously, most of the radioactivity remains in the zone corresponding to 11β-hydroxyandrostendione and to a lesser extent to androstendione.

DISCUSSION

The experiments described in the present paper (Tables 1, 2) showed 11β-hydroxylase activity in the microsomal fraction of human adrenal tissue. These findings are in disagreement with those of Sweat [1] and Hayano [2, 3] who characterized the 11β-hydroxylase in bovine adrenal as a mitochondrial enzyme.

The following results prove that the microsomal 11β-hydroxylase activity was not a result of enzyme contamination due to mitochondrial membrane disruption: (a) Absence of 11β-hydroxylase activity in

Table 2. The 11β-hydroxylase conversion rates in various cell fractions of the human adrenal cortex obtained of kidney transplantation (second experiment). Washing solution = The buffer solution with which the microsome fraction was washed. Other abbreviations see Table 1. The results of two experiments are described

Incubation	Precursor	t.l.c. zones	Radioactivity %	Mean %	Mean S.A. %/mg/min
Mitochondria (I)	deoxycortisol	product	94·2	93·4	$8{\cdot}83 \times 10^{-2}$
		precursor	2·1		
		general	4·5		
Mitochondria (II)	deoxycortisol	product	92·7		
		precursor	1·7		
		general	5·6		
Supernatant-105,000 *g* (I)	deoxycortisol	product	17·6	15·9	$1{\cdot}16 \times 10^{-2}$
		precursor	77·6		
		general	4·9		
Supernatant 105,000 *g* (II)	deoxycortisol	product	14·2		
		precursor	78·0		
		general	7·8		
First washing solution (I)	deoxycortisol	product	0·7	1·1	
		precursor	91·1		
		general	8·2		
First washing solution (II)	deoxycortisol	product	1·5		
		precursor	97·6		
		general	0·9		
Second washing solution (I)	deoxycortisol	product	0·4	0·2	
		precursor	96·1		
		general	3·4		
Second washing solution (II)	deoxycortisol	product	0·0		
		precursor	97·7		
		general	2·3		
Microsomes (I)	deoxycortisol	product	10·1	10·7	$6{\cdot}33 \times 10^{-2}$
		precursor	13·5		
		general	76·5		
Microsomes (II)	deoxycortisol	product	11·2		
		precursor	15·2		
		general	73·6		

the washing buffer of the microsomal fraction (Table 2), showed that the microsomal 11β-hydroxylase is not removable. (b) The results shown in Fig. 1a reveal that the mitochondria isolated from adrenals removed from kidney donors, remained intact in the course of the preparation. (c) Fig. 1b reveals no presence of mitochondrial particles in the microsomal fraction. (d) The most significant results are those obtained after measuring the activity of the mitochondrial marker-enzyme–glutamate dehydrogenase (Table 3). Comparing the activity of the last enzyme with that of the microsomal 11β-hydroxylase (Tables 1, 2) show clearly that the source of 11β-hydroxylase activity could not be the mitochondria. All these results, together with the relatively high S.A. values obtained with the microsomal fractions, prove the genuineness of the microsomal enzyme.

Table 3. Activity of glutamate dehydrogenase in the various cell fraction preparations obtained in the second experiment

Cellular fractions	Glutamate dehydrogenase activity mU/ml
Mitochondria	220·2
Supernatant 105,000 *g*	15·2
First washing	0·0
Microsomes	3·0

In all experiments carried out with autospy adrenals, the conversion of DOC to corticosterone was higher than that of the deoxycortisol to cortisol [14–16]. The studies with fresh adrenals show that with mitochondria, inverse results are obtained (Table 1). Similar findings are reported by Sauer [17] who isolated mitochondria from adrenal glands surgically removed in patients with Cushing's disease. These contradictory results suggest that the 11β-hydroxylase was probably damaged in the autopsy adrenals. Since the damage is not equal for both hydroxylations, this might imply a difference between the two 11β-hydroxylations.

The results obtained by incubating deoxycortisol with the microsomal fraction revealed another interesting aspect regarding the relatively high radioactive values found on the remainder of the t.l.c. plates after scraping off the product and precursor spots (general t.l.c. zones Tables 1, 2). These results can be attributed to the following reasons: (a) Presence of an enzyme which catalyzes the conversion of deoxycortisol to another metabolite than cortisol. (b) Presence of an enzyme which catalyzes the further metabolism of cortisol. (c) Presence of an enzyme which catalyzes

Table 4. The t.l.c. of the extract of the t.l.c. general zone of the incubation of the microsomal fraction with deoxycortisol. Each plate was loaded with 10% of the extract total amount. The various zones have a width of 2 cm. except of those which contained the steroids (androstendione and 11β-hydroxyandrostendione). Radioactivity % = The per cent of radioactivity of each zone from the whole plate. Be. = Benzene, Chl. = Chloroform

Be: EtOH 90:10 Chromat. system t.l.c. zones	Radioactivity %	Chl.: MeOH 94:6 Chromat. system t.l.c. zones	Radioactivity %
1	17·7	1	11·1
2	2·7	2	1·7
3	23·0	3	1·8
4	31·5	4	18·5
(11β-hydroxy-		5	41·9
androstendione)		(11β-hydroxy-	
5	23·4	androstendione)	
(androstendione)		6	23·6
6	1·4	(androstendione)	
7	0·3	7	1·2
8	0·0	8	0·1

the metabolism of either deoxycortisol and cortisol.

Further t.l.c. chromatographies of the general zone extracts showed no presence of cortisone. However, most of the radioactivity was found to be present in the locations of 11β-hydroxyandrostendione and androstendione (Table 4). These last findings agree with those found by Hudson *et al.* [18, 19] and Axelrod *et al.* [20], and are also supported by the findings that show no conversion of DOC to products other than corticosterone in the microsomes. The lack of 17-hydroxyl group suits this 17-desmolase inactivity. It is probably true that the 11β-hydroxylase specific activity values, obtained by the microsomes, would have been higher if the 17-desmolase activity were taken into account.

The high radioactivity counts found in the t.l.c. general zone of the microsomal deoxycortisol 11β-hydroxylation might explain the results of Sweat [1] who claimed that only traces of 11β-hydroxylase activity were found in the supernatant of 2000 to 19,000 *g* centrifugation of bovine adrenal homogenate. He measured only the presence of cortisol and might have missed other metabolites.

At present nothing can be said concerning the affinity of the microsomal 11β-hydroxylase to DOC and deoxycortisol. Such an experiment might lead to a better understanding of the rare type of congenital adrenal hyperplasia with 11β-hydroxylase deficiency where only one of the two 11β-hydroxylations pathways is blocked, either deoxycortisol to cortisol [21] or DOC to corticosterone [22].

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