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STUDIES OF 11β-HYDROXYLATION BY BEEF ADRENAL MITOCHONDRIA

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SUMMARY

The 11β-hydroxylations of androstenedione (Δ^4A), 11-deoxycortisol (S) and deoxycorticosterone (DOC) were studied using mitochondria from calf or heifer adrenal tissue. Standard assay conditions were: non-radioactive androstenedione (30.0 μM) 11-deoxycortisol (24.5 μM) or deoxycorticosterone (26.0 μM) plus 6.0×10^4 d.p.m. ^{14}C-steroid, 0.2 mM NADPH, 1.0 mM Mg^{2+}, 1.0 mM Ca^{2+} and the mitochondrial fraction equivalent to 20 mg of adrenal tissue in a final vol. of 3 ml of 0.1 M HEPES buffer. pH 7.4. Incubations were performed at 37°C for 4 min. Product formation under these conditions was identical to product formation measured when the NADPH and Ca^{2+} were replaced with 10 mM malate. The 11β-hydroxylation of Δ^4A showed a requirement for NADPH and oxygen, indicating that the enzyme involved is a mixed-function oxidase. The K_m values for calf adrenal mitochondria were 3.8, 8.5 and 8.0 μM for Δ^4A, S and DOC, respectively. For heifer adrenal mitochondria, the K_m values were 12 and 15 μM respectively, for Δ^4A and S. Competition studies in which equal amounts of two substrates were incubated simultaneously, revealed that Δ^4A, S and DOC did not compete for the same enzymatic site, but were hydroxylated to the same degree in the presence or absence of each of the other two precursors. The 11β-hydroxylations of S and DOC were stimulated by Mg^{2+} at a concentration of 1.0 mM, while the 11β-hydroxylation of Δ^4A was inhibited by this concentration of Mg^{2+}. In experiments in which the mitochondria were preheated at 50°C for 6 min, the 11β-hydroxylation of Δ^4A, under standard assay conditions, was 96% of the unheated value, while the 11β-hydroxylation of S and DOC was 77 and 59%, respectively, of the unheated values. These studies indicate that there are three substrate specific 11β-hydroxylases in beef adrenal mitochondria.

INTRODUCTION

The pathway involving pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenedione is considered to be the major route of synthesis of 11β-hydroxyandrostenedione (11βOHA) in the human adrenal gland [1–3]. The enzyme system mediating 11β-hydroxylation has been studied extensively and has been considered to consist of an NADPH specific flavoprotein, a non-heme iron protein and cytochrome P450 [4–11]. The 11β-hydroxylation of deoxycorticosterone and 11-deoxycortisol has been reported in some detail [4–6, 11], however, relatively little has been written about the mechanism of the 11β-hydroxylation of androstenedione.

There has been some controversy as to whether a single enzyme hydroxylates androstenedione, 11-deoxycortisol and deoxycorticosterone, or whether there are three separate, substrate specific enzymes [12–18]. Evidence presented by Sharma *et al.* [12–13], Eckstein and Klein [14] and Colby and Brownie [15] suggests that there is a single 11β-hydroxylase in beef and rat adrenal tissues. However, *in vitro* [16, 17] and *in vivo* [18] evidence has been presented suggesting that there is more than one 11β-hydroxylase in both human and beef adrenal tissues.

The purpose of this paper is to report studies of the 11β-hydroxylation of androstenedione by beef adrenal mitochondria and to present evidence that three separate enzymes specific for the 11β-hydroxylation of deoxycorticosterone, 11-deoxycortisol and androstenedione can be demonstrated in this tissue.

MATERIALS AND METHODS

Radioactive steroids and chemicals. The radioactive steroids used ([4-^{14}C]-androstenedione-58.8 mCi/mmol; [4-^{14}C]-11-deoxycortisol-40.2 mCi/mmol; [4-^{14}C]-deoxycorticosterone-54.3 mCi/mmol; [1,2^{3}H]-11βOHA-55 Ci/mmol; [1,2-^{3}H]-cortisol 55 Ci/mmol; [1,2-^{3}H]-corticosterone-50 Ci/mmol) were obtained from the New England Nuclear Corporation, Boston, Mass. Each steroid was purified by paper chromatography prior to use and the purity was checked by recrystallization with authentic standards. Non radioactive steroids were obtained from Ikapharm, Ramat-Gan, Israel and Mann Research Laboratories, New York, N.Y. NADPH was obtained from B.D.H. Laboratories, Toronto, Canada, HEPES buffer (N-2-hydroxyethylpiperazine-N^1-2-ethane sulphonic acid-p*K* 7.55) was obtained from B.D.H. Laboratories. It was adjusted to the desired pH with NaOH.

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The following abbreviations and trivial names are used: pregnenolone: 3β-hydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone: 3β, 17α-dihydroxy-5-pregnen-20-one; androstenedione (Δ^4A): 4-androstane-3, 17-dione; 11β-hydroxyandrostenedione (11βOHA): 11β-hydroxy-4-androstane-3, 17-dione; 11-deoxycortisol (S): 17α,21-dihydroxy-4-pregnen-3,20-dione; 11β-hydroxyandrosterone: 3α,11β-dihydroxy-5α-androstan-17-one.

MES buffer (2-(N-morpholino)-ethanesulphonic acid-p*K* 6.1) was obtained from Calbiochem, Los Angeles, Cal. It was adjusted to the desired pH with NaOH. (Tris(hydroxymethyl)-aminomethane-p*K* 8.3) was adjusted to the desired pH with hydrochloric acid. $MgSO_4$ was added to the buffers to a final Mg^{2+} ion concentration of 1.0 mM. Malate was obtained from Calbiochem, Los Angeles, Cal.

Preparation of mitochondrial fraction. Beef adrenal tissue was obtained from the abattoir immediately after slaughter and kept in ice-cold buffer until used. All preparations were carried out at 2–4 C. The results reported in this paper were obtained from calf adrenal tissue unless stated otherwise. It was found that results were more consistent with this tissue than with other types of beef adrenal tissue.

After being dissected free of fat, portions of the glands from several animals were ground in a Waring Blender for 5–10 s in 0.25 M sucrose buffered in 0.1 M HEPES buffer at pH 7.4. The tissue: buffer ratio was one g tissue to 5 ml buffer. The mince was homogenized with a motor-driven glass–Teflon Potter–Elvejhem homogenizer and the homogenate was centrifuged at 2000 rev./min for 15 min. The supernatant was decanted and centrifuged at 9000 *g* for 30 min. The mitochondrial pellet obtained was resuspended in 0.25 M sucrose in buffer and both the resuspended pellet and the 9000 *g* supernatant were centrifuged at 9000 *g* for a further 30 min. The resulting pellets were combined and suspended in 0.1 M HEPES buffer to a concentration equivalent to 200 mg of original adrenal tissue/ml. This corresponded to a protein concentration of 4–6 mg/ml. The 9000 *g* supernatant was further centrifuged at 105,000 *g* for 65 min. The resultant microsomal pellet was suspended in 0.25 M sucrose in buffer and recentrifuged at 105,000 *g* for 65 min along with the initial high speed supernatant. The pellets obtained were combined and suspended in 0.1 M HEPES buffer to a concentration equivalent to 200 mg of original adrenal tissue/ml. This corresponded to a protein concentration of 0.4 to 0.7 mg/ml. The protein concentration in the 105,000 *g* supernatant was 5–7 mg/ml and that of the whole homogenate was 10–12 mg/ml. After establishing that there was only minimal 11*β*-hydroxylase activity in the microsomal fraction or the 105,000 *g* supernatant fraction, the 9000 *g* supernatant was routinely discarded.

Protein determination. Protein determinations were performed by the method of Lowry *et al.* [19] using BSA as the standard.

Incubation procedure. The reactions studied were the 11*β*-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone. Each substrate was incubated separately, or, in those experiments in which competition studies were performed, approximately equal amounts of two substrates were incubated simultaneously. To ensure that the steroids would be soluble in the aqueous incubation mixture, they were first dissolved in 3 drops of propylene glycol in the incubation flasks. The rest of the incubation mixture was then added to each flask.

Standard assay conditions were: non-radioactive androstenedione (30.0 μM), 11-deoxycortisol (24.5 μM) or deoxycorticosterone (26.0 μM) plus 6.0×10^4 d.p.m. ^{14}C-steroid, 0.2 mM NADPH, 1.0 mM Mg^{2+}, 1.0 mM Ca^{2+} and the mitochondrial fraction equivalent to 20 mg of original adrenal tissue, in a total vol. of 3 ml HEPES buffer, pH 7.4. The quantities of steroid substrates used were found to be sufficient to saturate the enzymes studied. Incubations were performed in a metabolic shaker for 4 min at 37 C in room air. Any incubation conditions which differed from the standard assay will be described in the Results Section.

Product isolation and quantitation. Incubations were terminated by the addition of 12 ml of methylene chloride. Known amounts of ^{3}H-labelled steroids were added to monitor losses during product isolation. The unconjugated steroids from the incubation mixtures were extracted with methylene chloride and the residues of these extracts, after evaporation, were chromatographed on paper. The separation of substrates and products was effected by the systems outlined in Table 1.

The regions corresponding to the 11*β*OHA, cortisol or corticosterone standards were eluted with methanol. In pilot experiments, radiochemical homogeneity of the steroids in the eluates was determined by recrystallization with known amounts of authentic standards. The recrystallization data is shown in Table 2. The procedure followed for recrystallization,

Table 1. Paper chromatography systems used in separating substrates and products

Substrate or product	System
Androstenedione + 11*β*OHA	Isooctane–Toluene–Methanol–Water (3:2:3:1 by vol.)
11-Deoxycortisol + Cortisol	Benzene–Methanol–Water (2:1:1 by vol.)
Deoxycorticosterone + Corticosterone	Isooctane–Toluene–Methanol–Water (3:2:3:1 by vol.)
Androstenedione + 11*β*OHA + 11-Deoxycortisol + Cortisol	Isooctane–Toluene–Methanol–Water + (3:2:3:1 by vol.) Benzene–Methanol–Water (2:1:1 by vol.)
Androstenedione + 11*β*OHA + Deoxycorticosterone + Corticosterone	Isooctane–Toluene–Methanol–Water (3:2:3:1 by vol.)
11-Deoxycortisol + Cortisol + Deoxycorticosterone + Corticosterone	Toluene–Propylene Glycol + Benzene Methanol–Water (2:1:1 by vol.)

derivative formation and recrystallization of the derivatives were identical to those previously reported [3]. Product formation was expressed in nmol/min/mg protein, from the radioactivity eluted from the paper, and corrected for losses, and the specific activity of the substrate.

The data reported in this paper are the results of single experiments performed with the adrenal tissue obtained from 4–6 different animals. The data were confirmed by obtaining similar results on at least one additional occasion.

Anaerobic experiments. In order to study the oxygen requirement of the 11β-hydroxylation of androstenedione, certain experiments were performed in a nitrogen atmosphere. The incubation flasks containing substrate, cofactors and buffer were stoppered with cotton wool and oxygen-free nitrogen was blown into the flask for 10 min prior to addition of the enzyme. The incubations were then allowed to proceed in a nitrogen atmosphere. Product formation was determined as in the standard assay.

Determination of radioactivity. Radioactivity was determined in a Philips Liquid Scintillation Analyser. Samples to be counted were dissolved in 2 ml of methanol and 10 ml of toluene phosphor containing 5.0 g PPO (2,5-diphenyloxazole) and 0.1 g POPOP (1,4-bis-2, (5-phenyloxazolyl) benzene)/l of toluene. The efficiency of counting was approx. 24% of ^{3}H and 50% for ^{14}C when both isotopes were counted simultaneously. A minimum of 5000 counts were collected for each sample. The d.p.m. in each sample were calculated with the use of an external standard.

RESULTS

11β-Hydroxylase activity in subcellular fractions of calf adrenal tissue. The results obtained when the whole homogenate and subcellular fractions equivalent to 100 mg of adrenal tissue were incubated with

Table 2. Recrystallization data showing specific activity of crystals (C) and mother liquors (ML) of isolated products and derivatives following incubation of substrates with calf adrenal mitochondria, in experiments on the Effect of Substrate Concentration on 11βHydroxylation (2a) and Competition Studies (2b)

Compound	S.A. (d.p.m./mg) ^{14}C	^{3}H	Derivative	S.A. (d.p.m./mg) ^{14}C	^{3}H
(2a)		Substrate—11-deoxycortisol			
Cortisol	C 445	200	11βOHA	C 530	240
	ML 425	210		ML 515	225
	C 455	205			
	ML 435	200			
		Substrate—Deoxycorticosterone			
Corticosterone	C 485	165	Corticosteroneacetate	C 470	145
	ML 495	150		ML 450	150
	C 505	155			
	ML 515	165			
		Substrate—Androstenedione			
11βOHA	C 325	185	11β-hydroxytestosterone	C 315	180
	ML 350	170		ML 325	180
	C 310	185			
	ML 300	180			
(2b)		Substrate—Androstenedione, 11-deoxycortisol			
11βOHA	C 315	175	11β-hydroxytestosterone	C 310	190
	ML 290	180		ML 285	175
	C 305	180			
	ML 285	170			
Cortisol	C 460	205	11β-hydroxy-Androstenedione	C 535	225
	ML 420	220		ML 505	245
	C 470	215			
	ML 440	200			
		Substrate—Androstenedione, Deoxycorticosterone			
11βOHA	C 330	160	11β-hydroxytestosterone	C 300	160
	ML 315	155		ML 310	160
	C 325	165			
	ML 305	155			
Corticosterone	C 465	140	Corticosteroneacetate	C 445	140
	ML 485	150		ML 435	150
	C 480	155			
	ML 450	170			
		Substrate—Deoxycorticosterone, 11-deoxycortisol			
Corticosterone	C 505	180	Corticosteroneacetate	C 470	140
	ML 455	165		ML 485	155
	C 500	175			
	ML 465	170			
Cortisol	C 475	215	11βOHA	C 505	235
	ML 430	195		ML 535	250
	C 465	195			
	ML 495	210			

6.2×10^4 d.p.m. ^{14}C-androstenedione for 10 min are shown in Table 3. This pilot experiment, with only radioactive androstenedione as substrate, was performed, in the absence of added Ca^{2+}, before the requirements for 11β-hydroxylation were determined, in order to demonstrate that the 11β-hydroxylation of androstenedione occurred in the mitochondrial fraction. It can be seen that the activity associated with the mitochondria was 60% of the activity of the whole homogenate when expressed as total d.p.m. 11βOHA formed. When expressed as d.p.m./mg protein, the activity of the mitochondria exceeded the activity of the whole homogenate. The activity of each of the other fractions was less than 2% of the total mitochondrial activity.

Control incubations. Control incubations, in which conditions were identical to the standard assay, except that no mitochondrial protein was added to the incubation flasks, were carried out with each of the three substrates. Eluates of the regions of the chromatograms corresponding to the 11βOHA, cortisol and corticosterone standards contained no radioactivity.

Effect of protein concentration and time. Figure 1 illustrates the effect of protein concentration and time on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone by calf adrenal mitochondria. In each case, the formation of product was proportional to time for at least 9 min and the rate of product formation was proportional up to a protein concentration of at least 0.8 mg/3 ml under standard assay conditions. This weight of protein corresponded to about 40 mg of original adrenal tissue. All subsequent experiments reported were performed under conditions of linearity with respect to time and protein concentration.

Effect of electron donor on 11β-hydroxylation. The effect of electron donor on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone by calf adrenal mitochondria is shown in Table 4. It can be seen that the formation of 11βOHA, cortisol and corticosterone is the same in the presence

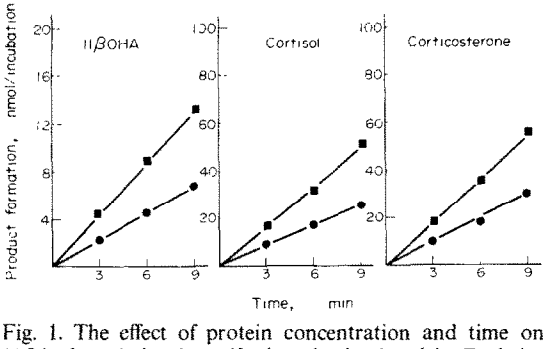

Fig. 1. The effect of protein concentration and time on 11β-hydroxylation by calf adrenal mitochondria. Each incubation mixture contained: [^{14}C]-steroid (androstenedione, 30 μM, 11-deoxycortisol, 33 μM or deoxycorticosterone, 31 μM), NADPH, Mg^{2+} and Ca^{2+} as in the standard assay, and 0.4 or 0.8 of mitochondrial protein equivalent to 20 (●) and 40 (■) mg, respectively, of adrenal tissue, in a final vol. of 3 ml HEPES buffer pH 7.4. Products were assayed at 3, 6 and 9 min.

Table 3. The 11β-hydroxylation of androstenedione by subcellular fractions of beef adrenal tissue

Fraction	Product formation d.p.m. 11βOHA formed/100 mg adrenal tissue	d.p.m. 11βOHA formed/mg protein
Whole homogenate	19,000	2111
Mitchondria	12,000	3000
Microsomes	200	500
Supernatant	140	24

Each incubation flask contained: 6.2×10^4 d.p.m. ^{14}C-adrostenedione, 0.2 mM NADPH, 1.0 mM Mg^{2+} and the equivalent of 100 mg adrenal tissue in a total volume of 3 ml of 0.1 M HEPES buffer, pH 7.4. The time of incubation was 10 min.

of 0.2 mM NADPH and 1.0 mM Ca^{2+} as in the presence of 10 mM malate. In the experiments reported in this paper, NADPH was used as the source of reducing equivalents, in the presence of 1.0 mM Ca^{2+}.

Effect of pH on the 11β-hydroxylation of androstenedione and 11-deoxycortisol. The effect of pH on the 11β-hydroxylation of androstenedione and 11-deoxycortisol was studied over the range from 5.0 to 9.0 in a series of different buffers. The pH optimum for both substrates lay between 7.0 and 7.5. The pH optimum for the 11β-hydroxylation of deoxycorticosterone has been shown to be close to 7.4 [12] and these studies were not repeated in this series of experiments.

Requirements for the assay of the 11β-hydroxylation of androstenedione. Table 5 lists the requirements for assaying the 11β-hydroxylation of androstenedione. The enzymatic activity demonstrated an absolute requirement for NADPH. In the absence of exogenous NADPH, there was virtually no activity. The enzyme also revealed a requirement for oxygen. Incubations in a nitrogen atmosphere markedly reduced enzymatic activity to 10% of the activity found under standard conditions. The presence of Ca^{2+} ions at a concentration of 1.0 mM greatly enhanced substrate conversion to 8 times the conversion seen in the absence

Table 4. A comparison of the source of electrons on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone by calf adrenal mitochondria

Substrate (μM)	Length of incubation (min)	Product formation (nmol/mg protein) (a) NADPH+ Ca^{2+}	(b) Malate
Androstenedione	3	6.15	6.15
(30)	6	12.56	13.21
	9	19.19	18.22
11-Deoxycortisol	3	25.11	25.60
(33)	6	48.79	47.73
	9	72.67	73.79
Deoxy-	3	27.00	25.11
corticosterone (31)	6	49.74	48.22
	9	86.14	88.00

Each incubation flask contained the substrate, Mg^{2+} (1.0 mM) and mitochondrial protein (equivalent to 20 mg adrenal tissue) as in the standard assay, plus: (a) NADPH (0.2 mM) and Ca^{2+} (1.0 mM), (b) Malate (10 mM).

Table 5. Requirements for the 11β-hydroxylation of androstenedione by calf adrenal mitochondria

Omission	11β-Hydroxylase activity nmol/min/mg protein	%
None	2.20	100
Ca^{2+}	0.26	12
Mg^{2+}	2.73	124
Mg^{2+} + Ca^{2+}	0.66	30
Ca^{2+} + Mg^{2+} (2 mM EDTA added)	0.73	33
NADPH	0.07	3
NADPH + Ca^{2+}	0.07	3
NADPH + Mg^{2+}	0.09	4
NADPH, Mg^{2+}, Ca^{2+} (2 mM EDTA added)	0.07	3
None, but incubated in N_2 Atmosphere	0.22	10
None, but TRITON X-100 (0.1% v/v) added	0	0
Mitochondrial Protein	0	0

The complete incubation mixture consisted of androstenedione, NADPH, Mg^{2+}, Ca^{2+} and mitochondria as in the standard assay.

of added Ca^{2+} ions. This enhancement of enzyme activity by the addition of exogenous Ca^{2+} ions was consistently seen in other experiments. The omission of Mg^{2+} from the incubation mixture also enhanced substrate conversion. The addition of EDTA at a concentration of 2 mM had no further effect on enzymatic activity measured in the absence of Ca^{2+} and Mg^{2+}. Triton X-100 at a concentration of 0.1% (v/v) completely abolished 11β-hydroxylase activity.

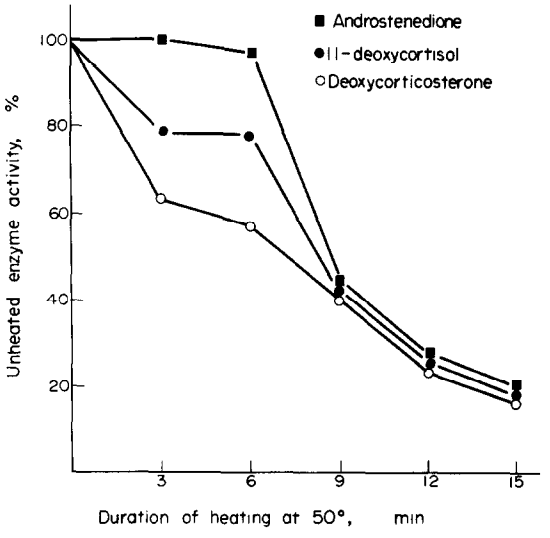

Fig. 2. The effect of preheating calf adrenal mitochondria on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone. The mitochondria were preheated at 50°C for varying periods of time prior to incubation with the substrate. Incubations were then carried out under standard assay conditions. Activity, as percent of unheated enzyme activity, is plotted against duration of preheating at 50°C. Unheated enzyme activity was 2.05, 9.26 and 12.0 nmol/min/mg protein, respectively, for the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone.

The effects of Mg^{2+} and Ca^{2+} on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone. Because Mg^{2+} ions had been shown to enhance the 11β-hydroxylation of deoxycorticosterone by beef adrenal mitochondria [12], the effects of Mg^{2+} on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone were compared. As can be seen in Table 6, the effect of Mg^{2+} on the 11β-hydroxylation of 11-deoxycortisol and deoxycorticosterone was similar, but opposite to the effect on the 11β-hydroxylation of androstenedione. The omission of exogenous Mg^{2+} ions reduced the 11β-hydroxylation of the former substrates by about 2/3, but stimulated the formation of 11βOHA from androstenedione. The omission of exogenous Ca^{2+} reduced the 11β-hydroxylation of all three substrates.

Effect of preheating mitochondria at 50°C on enzyme activity. The effect of pre-heating the mitochondria at 50°C for varying periods of time prior to assay of the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone under standard conditions is shown in Fig. 2. It can be seen that the longer the mitochondria were exposed to 50°C, the greater the diminution of activity. However, the reduction in activity differed for the three substrates. The 11β-hydroxylation of androstenedione was the least sensitive, with the activity after 6 min at 50°C still 96% of the unheated value. The activities for the 11β-hydroxylation of 11-deoxycortisol and deoxycorticosterone after the same length of heating were 77 and 59%, respectively, of the unheated values. Heating for a longer period resulted in a precipitous fall in the hydroxylation of all three substrates.

Effect of substrate concentration on 11β-hydroxylation. The effect of substrate concentration on 11β-hydroxylation by calf adrenal mitochondria is shown in Fig. 3. When the reciprocal of velocity was plotted against the reciprocal of substrate concentration, a straight line was obtained for each substrate. This finding indicates that 11β-hydroxylase activity obeys Michaelis–Menten kinetics for all three substrates. The K_m values were found to be 3.8 μM 8.5 μM, and 8.0 μM respectively, for androstenedione, 11-deoxycortisol and deoxycorticosterone. The respective maximum velocities were 2.10, 9.36, and 12.54 nmol/min/mg protein. Similar studies using heifer adrenal mitochondria revealed that the calculated K_m values for this tissue were 12 μM and 15 μM for androstenedione and 11-deoxycortisol, respectively.

Competition studies. The 11β-hydroxylation of each substrate was compared under standard assay conditions in the presence and absence of approximately equal amounts of each of the other two substrates.

These studies were performed, with calf adrenal mitochondria, at 5 substrate concentrations. In Table 7 are shown the results of this comparison at 2 substrate concentrations. Results were similar for the other 3 concentrations.

The predicted velocities for an incubation in the presence of two substrates, each of which is hydroxylated by a different 11β-hydroxylase, is the same as

Table 6. Comparison of the effects of Mg^{2+}, Ca^{2+} and EDTA on the 11β-hydroxylation of androstenedione, 11-deoxycortisol and deoxycorticosterone

Omission	11β-Hydroxylase activity (nmol/min/mg protein) Androstenedione	11-Deoxycortisol	Deoxycorticosterone
None	2.15	8.15	9.10
Ca^{2+}	0.19	0.65	1.37
Mg^{2+}	3.23	2.36	3.19
Mg^{2+}, Ca^{2+}	0.88	0.33	0.46
Mg^{2+}, Ca^{2+} with 2 mM EDTA added	1.03	0.25	0.27

The complete incubation mixture contained the substrate, NADPH, Mg^{2+}, Ca^{2+} and mitochondria as in the standard assay.

the velocities obtained in the presence of each substrate alone. The calculated velocities for an incubation in the presence of two substrates, each of which is competing for the same enzyme, are given by:

$$v_1 = \frac{V_1\left[\frac{A_1}{K_1}\right]}{1 + \frac{A_1}{K_1} + \frac{A_2}{K_2}}$$

and

$$v_2 = \frac{V_2\left[\frac{A_2}{K_2}\right]}{1 + \frac{A_1}{K_1} + \frac{A_2}{K_2}}$$

where v_1 and v_2 are the velocities in the presence of two substrates, A_1 and A_2 are the concentrations of the two substrates, V_1 and V_2 are the maximum velocities and K_1 and K_2 are the Michaelis constants [20]. From the experimental and predicted values shown in Table 7, it can be seen that the substrates did not compete for the same active centre. This indicates that, under the assay conditions employed, three 11β-hydroxylases were demonstrated in calf adrenal tissue. Under similar assay conditions, heifer adrenal tissue was shown to contain at least two separate 11β-hydroxylases, one for androstenedione and one for 11-deoxycortisol. The 11β-hydroxylation of deoxycorticosterone was not studied in heifer tissue.

DISCUSSION

The enzymes mediating the 11β-hydroxylation of 11-deoxycortisol and deoxycorticosterone in adrenal cortical tissue have been shown to consist of three components, an NADPH specific flavoprotein, a non-heme iron protein and cytochrome P-450 [11]. Relatively little has been reported about the mechanism of the 11β-hydroxylation of androstenedione. The results reported in this paper confirm that beef adrenal mitochondria can 11β-hydroxylate androstenedione. Under the assay conditions employed, the rate of synthesis of 11βOHA, cortisol and corticosterone was proportional to protein concentration and constant with time. This linearity permitted a meaningful comparison of enzyme activity for each substrate.

The rate of formation of 11βOHA, cortisol and corticosterone was the same in the presence of 0.2 mM NADPH and 1.0 mM Ca^{2+} as in the presence of 10 mM malate. The rates of formation of cortisol and corticosterone were close to those reported by Harding *et al.* (about 4.0 nmol/min/mg protein for cortisol) [21] and Cooper *et al.* (13.6 nmol/min/mg protein for corticosterone) [22], who used malate to support 11β-hydroxylation by steer whole mitochondria and reconstituted mitochondrial 11β-hydroxylase components, respectively. Interestingly, Harding *et al.* [21] also showed that the 11β-hydroxylation of 11-deoxycortisol by whole adrenal mitochondria was the same in the presence of NADPH and Ca^{2+} as in the presence of malate. The effect of the added Ca^{2+} is probably due to mitochondrial swelling and free entry of the exogenous NADPH into the mitochondria [21, 23]. In the experiments reported in the present paper, some synthesis of 11βOHA occurred even in the absence of added Ca^{2+}. In the absence of exogenous NADPH, there was minimal activity, suggesting that intramitochondrial NADPH was insufficient to support the 11β-hydroxylation measured during these incubations. Harding *et al.*[21] observed similar results during the 11β-hydroxylation of 11-deoxycortisol by steer adrenal mitochondria.

The data reported in the present paper indicate that calf and heifer adrenal tissue contain more than one 11β-hydroxylase. This conclusion was reached because of the results of the competition studies, in which the substrates were hydroxylated to the same extent in the presence or absence of each of the other two substrates, because of the different sensitivities to heat, and because of the different responses to exogenous magnesium ions. The data is at variance with evidence supplied by Sharma *et al.*[12, 13] which indicated that a single enzyme hydroxylated all three substrates. In separate experiments, these investigators showed that 11-deoxycortisol and deoxycorticosterone competed for the same 11β-hydroxylase [12] and that androstenedione competitively inhibited the 11β-hydroxylation of deoxycorticosterone [13]. The reason for the difference in results between those reported by Sharma *et al.* and the present paper is not known, but may be due, at least in part, to the different enzyme preparations. The experiments in the present paper were performed with fresh whole mitochondria, while those in the paper of Sharma *et al.* were performed with sonicated mitochondria

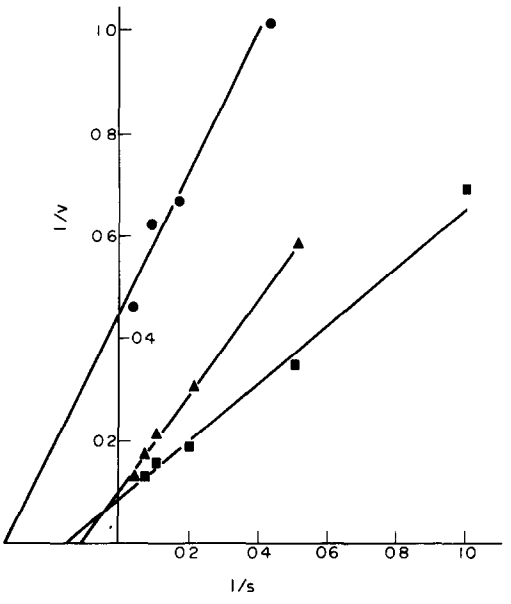

Fig. 3. The effect of substrate concentration on 11β-hydroxylation by calf adrenal mitochondria. Each incubation flask contained [^{14}C]-androstenedione (●)—2.33, 5.83, 11.7 or 35.0 μM, [^{14}C]-11-deoxycortisol (▲)—1.92, 4.80, 9.61, 14.4 or 28.8 μM, or [^{14}C]-deoxycorticosterone (■)—1.00, 2.00, 5.05, 10.1 or 15.2 μM, NADPH, Mg^{2+}, Ca^{2+} and HEPES buffer in a final vol. of 3 ml, as in the standard assay. Incubation time was 4 min.

obtained from beef adrenal tissue which had been frozen and thawed before homogenization. The rates of formation of cortisol and corticosterone, reported by Sharma *et al.*, were about 0.10 [12] and 0.10 to 0.20 [12, 13] nmol/min/mg protein, respectively.

The much lower rate of formation reported by Sharma *et al.* in comparison with those reported in the present paper and by Harding *et al.*[21] and Cooper *et al.*[22] suggests that some factor in Sharma's preparation, perhaps electron flow, was rate limiting. If electron flow were rate limiting, then it is not surprising that the presence of two substrates for 11β-hydroxylation would result in data which was consistent with an interpretation that the substrates competed for a single enzyme site. An alternative possibility is that the freezing and thawing of the tissue damaged one or more components of the 11β-hydroxylases so that only one 11β-hydroxylase, with markedly reduced activity, remained.

Studies of the 11β-hydroxylation of androstenedione demonstrated a requirement for NADPH and oxygen. These requirements indicate that the enzyme involved is a mixed-function oxidase. The similarity of requirements to those for the 11β-hydroxylations of 11-deoxycortisol and deoxycorticosterone, which have been considered to be mediated by cytochrome P-450 and the cytochrome P-450 reductase system

Table 7. Competition studies of the 11β-hydroxylation of androstenedione (Δ^{4}A), 11-Deoxycortisol (S) and Deoxycorticosterone (DOC) by calf adrenal mitochondria

1	2	3	4		5	6	7		8
Substrate (μM)		Observed Velocity (nmol/min/mg)	Calculated Velocity (nmol/min/mg) one enzyme(s)	two	No. of Enzymes	Observed Velocity (nmol/min/mg)	Calculated Velocity (nmol/min/mg) one enzyme(s)	two	No. of Enzymes
DOC	S		DOC → B				S → F		
15.2	—	7.98				—			
—	14.4	—				5.82			
15.2	14.4	7.08	5.10	7.98	2	5.82	3.48	5.82	2
30.3	—	7.32				—			
—	28.8	—				7.50			
30.3	28.8	7.08	5.76	7.32	2	7.38	3.90	7.50	2
DOC	Δ^{4}A		DOC → B				Δ^{4}A → 11βOHA		
10.1	—	6.84				—			
—	11.7	—				1.62			
10.1	11.7	5.64	2.52	6.84	2	1.68	1.32	1.62	2
30.3	—	7.32				—			
—	35.0	—				2.16			
30.3	35.0	7.14	2.82	7.32	2	1.98	1.50	2.16	2
S	Δ^{4}A		S → F				Δ^{4}A → 11βOHA		
14.4	—	5.82				—			
—	17.5	—				1.62			
14.4	17.5	5.76	1.86	5.82	2	1.68	1.44	1.62	2
28.8	—	7.50				—			
—	35.0	—				2.16			
28.8	35.0	7.08	1.98	7.50	2	2.04	1.50	2.16	2

Each substrate was incubated alone or in the presence of each of the other two substrates, under standard assay conditions. The substrates are listed in columns 1 and 2. Columns 3 and 6 record the observed velocities for each substrate alone and in the presence of the other substrate. In columns 4 and 7 are recorded the calculated velocities for either one or two enzymes. The conclusions as to the number of enzymes present, recorded in columns 5 and 8, were determined by comparing the observed velocities, in columns 3 and 6, when two substrates were incubated simultaneously, with the calculated velocities for one and two enzymes in columns 4 and 7, respectively.

[4–11] suggests that the mechanism of hydroxylation of all three substrates is similar and likely involves cytochrome P-450.

The widespread involvement of cytochrome P-450 as the terminal oxidase for a number of steroid hydroxylations occurring in adrenal mitochondria and microsomes has led to speculation that there may be several different cytochrome P-450's or a number of substrate specific proteins associated with cytochrome P-450 [4–6, 24, 25]. Cooper *et al.*[6] have shown that deoxycorticosterone will not bind to cytochrome P-450 of sonicated steer adrenal microsomes, but will bind to the steer mitochondrial pigment. This suggests that either the cytochrome or a protein associated with the cytochrome is different in each of the subcellular fractions. Jeffcoate *et al.*[26] have isolated two cytochrome P-450 fractions in rat adrenal mitochondria which exhibit substrate specificity, one mediating 11β-hydroxylation, the other cholesterol side-chain cleavage. These observations indicate the presence of substrate specific cytochrome P-450's in adrenal tissue. The data in the present paper demonstrates the presence of substrate specific 11β-hydroxylases. Cytochrome P-450 has been shown to be the site of substrate binding during 11β-hydroxylation [6], but since it was not specifically isolated during the experiments reported in the present paper, it is not possible to conclude that the demonstrated substrate specificity was due to different cytochrome P-450's.

That the demonstration of substrate specific 11β-hydroxylases may have clinical relevance is shown in a report by Zachmann *et al.*[18] who demonstrated that a patient with congenital adrenal hyperplasia had normal deoxycorticosterone, corticosterone and aldosterone secretion rates in the presence of markedly elevated 11-deoxycortisol and low normal cortisol secretion. They postulated a defect in the enzyme hydroxylating 11-deoxycortisol, with normal activity of a deoxycorticosterone specific enzyme. In the same patient, the urinary excretion of 11β-hydroxyandrosterone, the major metabolite of 11βOHA [27], was not detected. This latter finding suggests a total lack of 11β-hydroxylation of androstenedione, although the authors did not comment on this possibility. Klein and co-workers [28], using homogenates of human adrenal tissue, have shown that the 11β-hydroxylations of deoxycorticosterone and 11-deoxycortisol differ in their sensitivities to heat. They have concluded that the 11β-hydroxylases for these two substrates differ in at least one component. In a report of the *in vitro* metabolism of [^{14}C]-progesterone by homogenates of hyperplastic human adrenal tissue, Adadevoh *et al.*[17] reported a lack of synthesis of [^{14}C]-corticosterone despite good conversion to [^{14}C]-deoxycorticosterone. The same tissue synthesized [^{14}C]-cortisol and [^{14}C]-11βOHA in substantial yield. They postulated a defect in the hydroxylase specific for deoxycorticosterone, with normal activity of the enzymes specific for 11-deoxycortisol and androstenedione.

Under the conditions of the assay described, three 11β-hydroxylases, specific for androstenedione, 11-deoxycortisol and deoxycorticosterone, have been demonstrated in calf adrenal tissue. The substrates also differed in response to Mg^{2+} ions and in sensitivity to heat. Two 11β-hydroxylases were demonstrated in heifer adrenal tissue.

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