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A STUDY OF STEROID 11β-HYDROXYLATION BY ADRENAL MITOCHONDRIA OF MARSUPIALS— PART I. A COMPARISON OF 11β-HYDROXYLASE ACTIVITY AND SPECIFICITY FOR DIFFERENT STEROID SUBSTRATES BY POSSUM (*TRICHOSURUS VULPECULA*), KANGAROO (*MACROPUS MAJOR*) AND BEEF*

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SUMMARY

A kinetic approach was used to investigate the properties of adrenal mitochondrial 11β-hydroxylation in two marsupials, possum (*Trichosurus vulpecula*) and kangaroo (*Macropus major*), as compared to beef under similar experimental conditions. For possum, kangaroo and beef, the K_M values of 11β-hydroxylation of 11-deoxycortisol (S) were 86, 714 and 38 μM respectively and for 17α-OH-progesterone (17OHP) 147, 294 and 118 μM respectively. The V values for S were in the respective species, 36, 32 and 2 270 pmol/mg mitochondrial protein/min and for 17OHP 59, 101 and 11 pmol/mg/min. The K_M values for 11β-hydroxylation of progesterone and androstenedione by kangaroo mitochondria were 769 and 87 μM and the V values were 6.3 and 39 pmol/mg/min respectively. With possum and kangaroo simultaneous additions of S and 17OHP acting as alternate inhibitors, showed a mutual competitive inhibition of 11β-hydroxylation in the range of 24–52%, indicating a single 11β-hydroxylase enzyme system. No evidence of competitive inhibition was found with beef, indicating two, substrate-specific 11β-hydroxylases. The findings are discussed in terms of possible evolutionary processes in adrenal steroidogenesis.

INTRODUCTION

In marsupials the presence in adrenal venous blood of a number of unusual steroids, in relatively large amounts [1–4], suggests that the sequence of steroid hydroxylations may be different from that of eutherian species. The mechanism of 11β-hydroxylation is of special interest, since most marsupial species secrete 21-deoxycortisol in relatively large amounts [4]. This product has not been isolated from blood of eutherian species, but in humans it was isolated from the peripheral blood of a patient with adrenogenital syndrome [5].

The accumulation of 21-deoxycortisol could be the result of a deficiency in the 21-hydroxylating mechanisms [6], inability to 21-hydroxylate a previously 11β-hydroxylated substrate [7, 8] or a different 11β-hydroxylating system [6, 9, 10]. In marsupials the first possibility is unlikely, since their major secretory product, cortisol, is produced in adequate amounts [4, 11]. The second possibility has been tested *in vivo* and *in vitro* and it was found that 21-deoxycortisone or 21-deoxycortisol was readily 21-hydroxylated by the adrenal tissue of both eutherian and marsupial species [12]. The possibility that the 11β-hydroxylating enzyme system of marsupials could differ from that of eutherian species remained to be tested.

The cellular location of the 11β-hydroxylase is in the mitochondrial fraction of the adrenal cortex and it requires NADPH and oxygen as cofactors [13]. Addition of Krebs-cycle intermediates to intact mitochondria will generate NADPH formation. The degree of 11β-hydroxylation is influenced by the type of intermediate and depends on the species of animal employed [14–18]. These differences in enzyme responses substantiate the view that several mechanisms exist for the generation of intra-mitochondrial NADPH [16, 19]. However, when sufficient Ca^{2+} is added to swell mitochondria, exogenously supplied NADPH can permeate and ensure optimum hydroxylation [20]. Thus in beef 11β-hydroxylation of 11-deoxycortisol is the same in the presence of malate as in the presence of NADPH and Ca^{2+} [16, 21].

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The following abbreviations are used: progesterone (PROG), 11β-OH-progesterone (11OHP), 17-OH-progesterone (17OHP), 11-deoxycortisol (S), 21-deoxycortisol (21-DF), cortisol (F), androstenedione (AND), 11β-OH-androstenedione (11OHAND).

Factors such as these must be taken into consideration when enzyme activities of different species are compared.

A further complexity of the 11β-hydroxylase system points to the fact that in some species multiple 11β-hydroxylases exist, specific for particular steroid substrates. Kinetic studies with intact beef mitochondria revealed three different 11β-hydroxylases specific for 11-deoxycortisol, 11-deoxycorticosterone and androstenedione [21, 22] and indirect evidence with human adrenal tissue resulted in similar conclusions [9, 10, 23]. More specific studies remain to be carried out to provide clarification, whether substrate specificity of 11β-hydroxylase extends to other eutherian species. It is now well established that the 11β-hydroxylase system consists of several components, involving NADPH, flavoprotein, a non-heme iron protein and a cytochrome P-450 [20]. It seems justified to suspect that substrate specificity could occur at any level of this multicomponent system [24], although dissociation of each of the individual components is as yet not feasible.

The aim of the present investigation was to study, by means of a kinetic approach, the properties of the adrenal mitochondria 11β-hydroxylase system in two representative marsupials (possum and kangaroo), as compared to beef under similar experimental conditions. The view was taken that this information would help to clarify the process by which 21-deoxycortisol is formed in marsupials.

MATERIALS AND METHODS

Animals. Five male and three female brush-tailed possums (*Trichosurus vulpecula*) and two female kangaroos (*Macropus major*) were used. Four adrenal glands from beef were used. Adrenal glands from marsupials were removed under ether anaesthesia. Bovine adrenals were obtained from the slaughter-house within 20 min of death, put in ice cold buffer and processed immediately.

Substrates. Radioactive steroids were obtained from New England Nuclear and purified by repeated chromatography. The following labelled steroids were used; [4-^{14}C]-progesterone (S.A. 53 mCi/mmol), [7(n)-^{3}H]-17α-OH-progesterone (S.A. 11 Ci/mmol), [1,2-^{3}H]-11-deoxycortisol (S.A. 44.1 Ci/mmol), [4-^{14}C]-androstenedione (S.A. 60 mCi/mmol). Crystalline forms of the same steroid were obtained from Sigma and Ikapharm.

Radioactivity measurement. Radioactivity was detected on paper with a Nuclear Chicago 4π scanner and on t.l.c. by autoradiography using Kodak X-ray film [25]. Radioactivity from eluates was estimated by liquid scintillation using 0.05 g POPOP and 4 g PPO per litre toluene and counted in a Searle Analytic Inc. Isocap/300 liquid scintillation counter. The channels ratio was used for quench correction.

Media and buffers. The buffers were prepared basically as described by Tsang and Peron[26]. The incubation medium contained: Tris buffer 15 mM, 15 mM NaCl, 154 mM KCl, 72 mM sucrose, 5 mM $CaCl_2$ and 5 mM $MgCl_2$ at pH 7.4. Solution A contained: Tris buffer 15 mM, pH 7.4, 0.25 M sucrose, 1 mM EDTA. Solution B contained: 0.2 mM sucrose, 2 mM EDTA. Solution C contained: 0.25 M sucrose. Linear sucrose gradients at concentrations extending from 0.8–1.8 M, containing 1 mM EDTA, were prepared in MSE nitrocellulose tubes by means of an LKB Ultrograd model 11300 Gradient maker.

Tissue preparation. All procedures were carried out on ice. The glands were cleared of fat and for bovine and kangaroo adrenals the cortex which was separated from the medulla was used for mitochondrial preparation. For possums, whole adrenal glands were used, and a correction was made for medullary protein (see Calculation of results). Each experiment consisted of pooled glands from 3 or 4 possums.

Preparation of mitochondrial fraction. Mitochondrial fractions were prepared essentially as described by McMurray and Dawson[27] and Pollock and Nunn[28]. The tissue was cut into small pieces with scissors, homogenized in solution A and made up to 10% (w/v). The suspension was centrifuged at 900 g for 15 min. The supernatant was removed and centrifuged at 6,300 g for 15 min. The mitochondrial pellet was washed three times by resuspending in medium B between each spin. After the last wash the pellet was resuspended in a small vol. of solution B and layered on top of the sucrose gradients. Isopycnic centrifugation was carried out in a MSE superspeed 65 MK 11 ultracentrifuge with a swing out rotor at 110,000 g for 2 h. The mitochondrial band which was visible at sucrose concentrations of 1.3–1.4 M was removed with a syringe, diluted to a concentration of approximately 0.8 M with solution C, and centrifuged at 12,500 g for 20 min. The pellet was resuspended with an appropriate vol. of incubation medium to give a protein concentration of approximately 4 mg/ml. The suspension was added to incubation vessels, and aliquots were reserved for protein estimation and enzyme assays.

Incubation procedure. Incubation flasks contained; the steroid substrate in amounts as indicated in Results, dissolved in a few drops of propylene glycol, the incubation medium, the cofactors, consisting of 0.4 mM ATP, 0.4 mM NAD, 0.1 mM NADP, 2.5 mM glucose-6-phosphate, 5 i.u. glucose-6-phosphate dehydrogenase, supplemented with 10 mM fumarate [14]; and the mitochondrial suspension, equivalent from 0.3 to 0.9 mg protein. The final incubation vol. was 3 ml. Control incubations containing all constituents except mitochondria were included. Incubations were carried out at 37° in a Dubnoff metabolic shaker for periods of 30–60 mins.

Analytical. Incubates were extracted with methylene chloride and the extract washed with 0.1 M NaOH and distilled water. The organic phase was evaporated and the residue subjected to sequential paper chromatography in the appropriate chroma-

tography systems. The following systems were employed: T/75, L/80, LB 53/80 and LT 32/80 [29]; t.l.c. silica gel 'G' (Stahl); benzene–ethyl acetate (1:1, v/v). Eluates of conversion products were subjected to acetylation with pyridine–acetic anhydride and to oxidation with 2% chromic trioxide. The derivatives formed were rechromatographed in appropriate systems in the presence of reference standards. Aliquots were removed for counting, for spectrophotometry and for crystallization.

The isolated conversion products were finally identified by crystallization to constant S.A. To each of the isolated conversion products 10 mg of crystalline carrier was added and three or four successive crystallizations were made in two different solvent mixtures; ethyl acetate–*n*-hexane, acetone–*n*-hexane. The specific activities (d.p.m./mg) of the last two successive crystallizations did not differ by more than ±3%. In experiments where the products were insufficient fractions from a number of incubations were pooled and used for crystallizations.

The quantitative estimation of products was made by the absorption maximum at 240 nm, in methanol with a Zeiss PMQ quartz prism spectrophotometer, employing Allen's correction [30].

Assays. Mitochondrial protein was estimated by the method of Lowry[31] using bovine serum albumin standards.

In order to determine the extent of mitochondrial disruption, succinic dehydrogenase (SDH), which is located on the inner membrane of the mitochondrion [32], was assayed. The SDH activity was determined by the method of Wilson *et al.*[33]. From each experiment two determinations were made; (a) with intact mitochondria (separated on sucrose gradients), (b) with disrupted mitochondria. Disruption was carried out by sonication on ice at 30 s bursts with a Raythéon 10 Kc, 90 Watt sonic oscillator. The formazan produced was expressed as μmol formazan/mg protein/min. The results obtained with disrupted mitochondria were taken to be 100% and that of intact mitochondria were expressed as a fraction of this value.

Histology. Possum adrenals were fixed in 10% formol saline, cut into slices (10 μ thick) and stained with haemotoxylin and eosin. The sections were viewed under a light microscope fitted with an eye piece graticule, by which it was possible to estimate the surface area of cortex and medulla. The ratio of the two surface areas was calculated. Assuming that the relative densities of the two areas were equal, the ratios were expressed in terms of adrenal gland weight. On average 1/3 of the total adrenal weight consisted of medullary tissue.

Calculation of results. In beef and kangaroo experiments adrenal cortical tissue was used for incubations and the 11β-hydroxylase activity was expressed in terms of unit product/mg mitochondrial protein/min. In possum where whole adrenal glands were used corrections were made for the presence of medullary protein as determined by histological examinations of adrenal sections. This assumes that the mitochondrial protein per unit wt. of adrenal tissue was the same in both cortex and medulla.

The amount of each conversion product was determined from its radioactive content as well as the result of U.V. determination at 240 nm. The results of both methods were in good agreement. Losses were accounted for by the recovery of total radioactivity from conversion products and the non-metabolized substrate. The recovered radioactivity expressed as a percentage of the initially added substrate was found to be in the range of 70–85%.

Lineweaver–Burk plots [34] were constructed over a range of concentrations for the 11β-hydroxylation of each substrate. Plotting $1/s$ against $1/v$, where s is the substrate concentration and v is the reaction velocity (expressed as unit product/mg protein/min), a straight line is obtained if the reaction obeys Michaelis–Menten kinetics. The kinetic parameters, K_M (affinity) and V (maximum velocity) were obtained from the intercepts of the straight line with the horizontal and with the vertical axis respectively. As a further check, values of K_M and V were also determined by the graphical method of De Miguel Merino[35].

The inhibitor constant, K_i, was calculated from the Michaelis–Menten equation for competitive inhibition [36]. In two experiments K_i was also determined by the method of Dixon[37]. In this method the labelled substrate is kept at a constant concentration whereas the concentration of the inhibitor changes.

RESULTS

Preliminary studies

From the results of the SDH assay the mitochondrial preparation was found to consist of approximately 90–95% intact mitochondria. In all species 11β-hydroxylation was optimal in the presence of Mg^{2+} and Ca^{2+} ions and the NADPH generating system and fumarate as cofactors. Similar findings were reported in rats by Guerra *et al.*[14]. In the presence of fumarate alone and in the absence of Ca^{2+} and NADPH, 11β-hydroxylation was minimal. The formation of cortisol (F) and 21-deoxycortisol (21-DF) from 11-deoxycortisol (S) and 17α-OH-progesterone (17OHP) respectively, proceeded linearly over a 90 min incubation period. There was also a linear relationship between product formation and amount of enzyme over a range of 0.3–0.9 mg of mitochondrial protein in a total of 3 ml of incubation medium. Standard experimental conditions were arranged for each incubation in such a way that the reaction velocity was linear with respect to time and amount of enzyme used. In each experiment the amount of product formed was below 10% of added substrate.

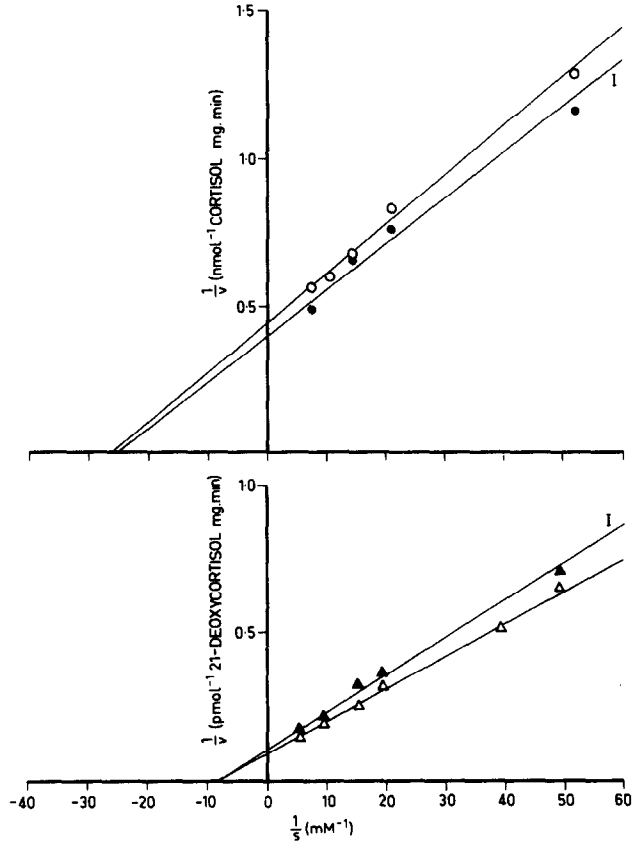

Fig. 1. Lineweaver-Burk plots for 11β-hydroxylation of S and 17OHP by adrenal mitochondria from beef. (○) S, (● I) S and 133 μM 17OHP; (△) 17OHP, (▲ I) 17OHP and 150 μM S. Each point represents the arithmetic mean of two experiments. Values of K_M and V are listed in Table 1. For experimental details see text.

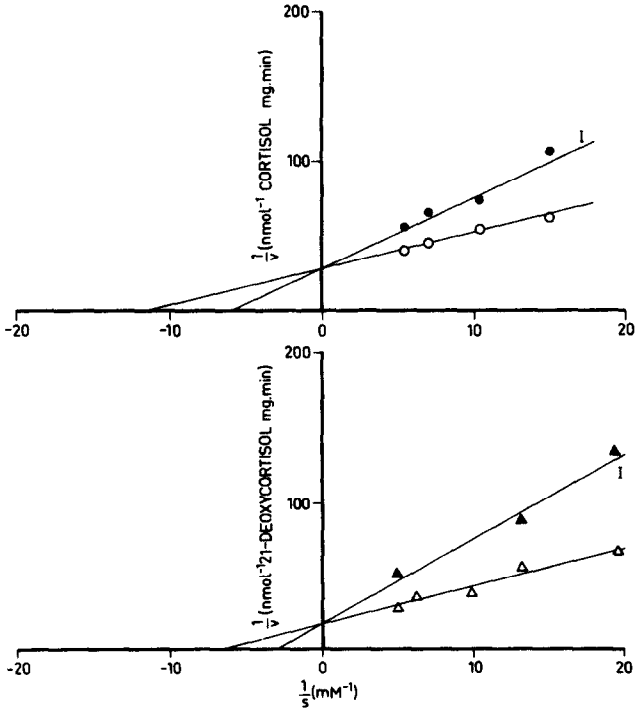

Fig. 2. Lineweaver-Burk plots for 11β-hydroxylation of S and 17OHP by adrenal mitochondria from possum. (○) S, (● I) S and 182 μM 17OHP; (△) 17OHP, (▲ I) 17OHP and 200 μM S. Each point represents the arithmetic mean of 2 experiments for S and 3 experiments for 17OHP. Values of K_M and V are listed in Table 1. For experimental details see text.

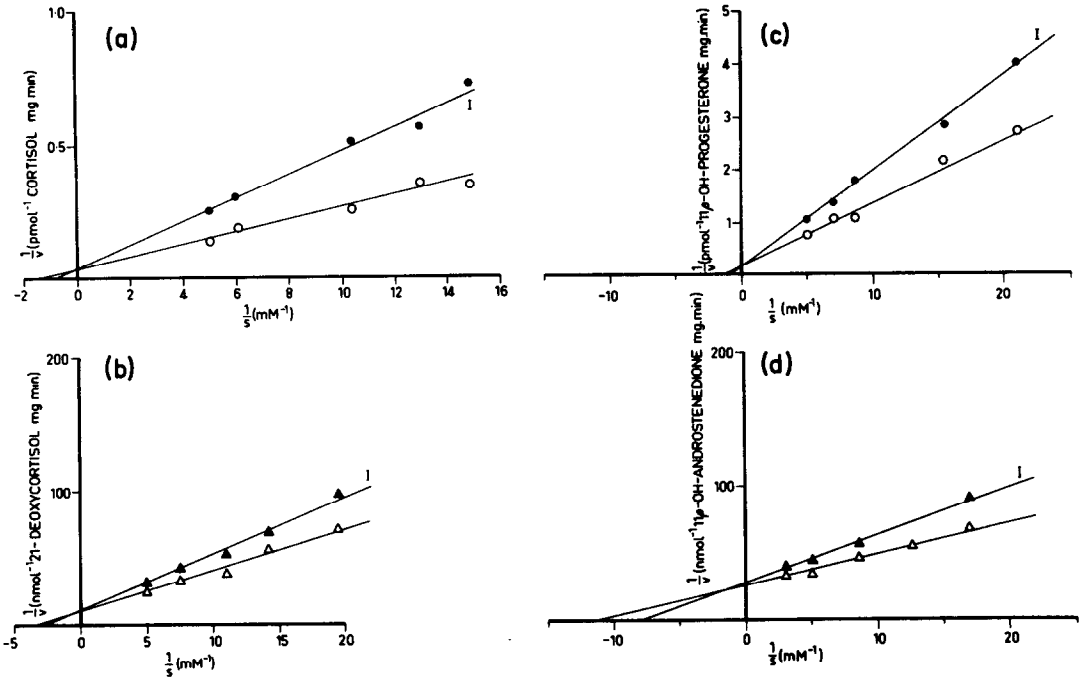

Fig. 3. Lineweaver–Burk plots for 11β-hydroxylation of different steroid substrates by adrenal mitochondria from kangaroo. (a) (○) S. (● I) S and 200 μM 17OHP, (b) (△) 17OHP, (▲ I) 17OHP and 200 μM S, (c) (○) progesterone, (● I) progesterone and 200 μM S, (d) (△) androstenedione, (▲ I) androstenedione and 333 μM S. Each point represents the arithmetic mean of 2 experiments for S and 17OHP. Data for progesterone and androstenedione substrates are from 1 experiment. Values of K_M and V are listed in Table 1. For experimental details see text.

Kinetic studies

· *Determination of* K_M *and* V *values.* The conversion of S and 17OHP substrates to 11β-hydroxylated products, namely F and 21-DF, by bovine, possum and kangaroo adrenal mitochondria, and of progesterone and androstenedione to 11β-OH-progesterone and 11β-OH-androstenedione by kangaroo mitochondria, are presented as Lineweaver–Burk plots (Figs 1–3). Each point represents the arithmetic mean of two or three experiments. The same figures contain also results of inhibition studies, denoted by I, which will be described separately.

Each incubation flask contained 0.29–0.86 mg mitochondrial protein and 2–6 μCi [^{3}H]-labelled or 2 μCi [^{14}C]-labelled substrate. Except for androstenedione, which ranged from 20–333 μM, all the other unlabelled substrates were at concentrations ranging from 20–200 μM, as indicated in Figs. 1–3. The incubation conditions were as described in Materials and Methods.

The K_M and V values, representing enzyme affinity and activity respectively, for each substrate are presented in Table 1. A comparison of the K_M values for S in the three species indicated that the affinity of the 11β-hydroxylase with possum and beef mito-

Table 1. K_M and V values for 11β-hydroxylation of different steroid substrates by adrenal mitochondria from beef, possum and kangaroo

Substrate	K_M (μM)	V (pmol/mg/min)	$K_{M/V}$	$K_{M/V}$ Relative to 11-deoxycortisol
Beef				
11-deoxycortisol	38	2270	0.02	—
17α-OH-progesterone	118	11	11	1:635
Possum				
11-deoxycortisol	86	36	2.4	—
17α-OH-progesterone	147	59	2.5	1:1.04
Kangaroo				
11-deoxycortisol	714	32	22	—
17α-OH-progesterone	294	101	2.9	1:0.13
progesterone	769	6	122	1:5.53
androstenedione	87	39	2.3	1:0.10

chondria were similar, whereas the affinity with kangaroo was approximately 1/10 that of the other species. On the other hand the affinities for 17OHP were of a similar order of magnitude in all 3 species. Of the two additional substrates investigated with kangaroo mitochondria, androstenedione had the highest affinity for the 11β-hydroxylase, while that of progesterone was similar to that of S (Table 1). A comparison of V values for S and 17OHP showed in marsupials, activities of a similar order of magnitude for both substrates, whereas in beef the activity for S was about 200 times greater than that for 17OHP (Table 1). When the $K_{M/V}$ values (which may be broadly regarded as an inverse measure of enzyme efficiency) for S are compared with those of 17OHP within each species, it is evident that with beef the efficiency for S was approximately 600 times higher than that for 17OHP, with kangaroo it was 8 times lower, whereas with possum the efficiencies for both substrates were similar. With kangaroo the enzyme had the lowest efficiency for the progesterone substrate whereas the highest efficiencies were for 17OHP and androstenedione (Table 1). For each substrate the $K_{M/V}$ ratios relative to S are also listed in Table 1 (last column).

Inhibition studies. To determine if single or multiple enzymes are responsible for the 11β-hydroxylation of S and 17OHP, simultaneous incubations were carried out with the substrate alone (Figs 1–3, open symbols) and in the presence of another substrate acting as inhibitor (Figs 1–3, closed symbols, plots designated I). For each pair of substrates two series of incubations were carried out, in which one and then the other substrate acted as the inhibitor. The inhibitor was unlabelled and kept at a constant concentration, whereas the substrate carried the radioactive label and varied in amount over a range of four or five different concentrations, one of the concentrations being equimolar with that of the inhibitor. The position of the intercept of the two lines was indicative of the type of inhibition which occurred [38].

With beef the plots for the 11β-hydroxylation of S and 17OHP alone and in the presence of each other were the same (Fig. 1), indicating that there was no inhibitory effect. The results suggest that two hydroxylases, one specific for 11β-hydroxylation of S, the other for 17OHP, were operating independently. With possum and kangaroo, the reciprocal plots for the 11β-hydroxylation of S in the presence of constant amounts of 17OHP, and vice versa (Figs 2 and 3a, b) show that each substrate competitively inhibited the hydroxylation of the other one, indicating that a single enzyme was involved. The 11β-hydroxylation of two additional steroids, progesterone and androstenedione, was competitively inhibited in the presence of constant amount of S (Fig. 3c, d). It would there-

Table 2. Competitive inhibition studies of 11β-hydroxylation of different steroid substrates in the presence of a second steroid by adrenal mitochondria of beef, possum and kangaroo

Substrate (μM)		Product estimated	Observed rate of product formation (pmol/mg/min)	Calculated rate of product formation (pmol/mg/min) No. of Enzymes		Inhibition of estimated product (%)	K_i (μM)
				One	Two		
Beef							
S	17OHP						
19.2	—	F	781		760		
19.2	133	F	869	432		0	—
—	20.2	21-DF	1.5		1.6		
105	20.2	21-DF	1.4	0.48		8.5	—
Possum							
S	17OHP						
67.1	—	F	15.9		15.6		
67.1	182	F	9.4	9.2		41	133
—	51	21-DF	15.2		15.1		
200	51	21-DF	7.5	5.6		50	93
Kangaroo							
S	17OHP						
67.1	—	F	2.9		2.8		
67.1	200	F	1.4	1.7		52	286
—	51.3	21-DF	14.5		15.0		
200	51.3	21-DF	10.2	12.1		30	698
S	Prog						
—	47.6	11OHPROG	0.37		0.36		
200	47.6	11OHPROG	0.25	0.29		32	701
S	And						
—	58.1	11OHAND	14.7		15.4		
333	58.1	11OHAND	11.1	12.1		24	705

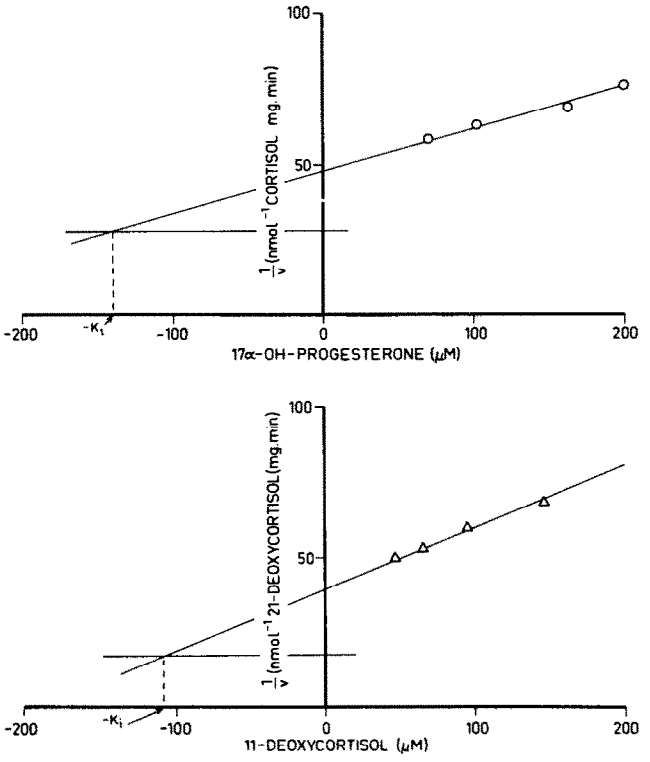

Fig. 4. Determination of K_i by the method of Dixon[37]. The substrates, S and 17OHP at a concentration of 100 μM each, were incubated with adrenal mitochondria from possum. (○) S, (△) 17OHP. The K_i values for S and 17OHP were 108 and 140 μM respectively. For experimental details see text.

fore appear that with kangaroo, S, 17OHP, progesterone and androstenedione are all hydroxylated by the same single enzyme system.

The summarized results of the competitive inhibition studies from the three species are shown in Table 2. Each figure represents the arithmetic mean of two or three experiments, with a standard deviation range as shown in the caption of Table 2. The experimentally obtained rates for product formation were compared with the calculated values obtained from the equations of Dixon and Webb[38] by which it is possible to predict if a one or a two enzyme system is operating. As seen in Table 2, in beef the results fit a two enzyme system, whereas in possum and kangaroo single enzyme systems are indicated for all substrates. The percentage inhibition was calculated from values obtained at the lowest substrate, and highest inhibitor concentrations. This combination showed maximal inhibition under the incubation conditions employed. The percentage inhibition of the estimated product in the mixture was negligible in beef, whereas in the two marsupials it ranged from 24–52%. With possum the inhibition of hydroxylation of 17OHP by S was higher than that of S by 17OHP, while in the kangaroo the reverse was the case (Table 2). The 11β-hydroxylation of progesterone and androstenedione in the presence of S was inhibited by 32.4% and 24.4% respectively.

The K_i values for each substrate for possum and kangaroo experiments were calculated from the Michaelis–Menten equation and they are listed in Table 2 (last column). As a further check the K_i values from two possum experiments were also determined from Dixon plots (Fig. 4). They were for S 108 and for 17OHP 170 μM, which is in good agreement with the above listed values (Table 2).

DISCUSSION

The 11β-hydroxylation of different steroid substrates has been extensively investigated with adrenal mitochondria from numerous eutherian species. This is a first report of a kinetic study of 11β-hydroxylation by adrenal mitochondria of marsupials.

In agreement with the findings in eutherians, 11β-hydroxylation by marsupial mitochondria requires the presence of reducing equivalents in the form of NADPH. Of the Krebs-cycle intermediates tested, fumarate had a low efficiency with both beef and possum mitochondria, while malate which is very effective in beef [16, 21], was almost completely ineffective with possum mitochondria (authors' unpublished observations). To maintain uniform conditions, essential for comparative studies, exogenous NADPH was supplied in the presence of Ca^{2+} in all experiments. Under these conditions, high yields of 11β-hydroxylated end-products were obtained with mitochondria from both marsupials, and with beef the K_M and V values for S were of a similar order of magnitude to those reported by other workers [16, 21, 39]. Since

21-DF is not a constituent of adrenal venous blood of beef, it was one of our interests to establish the kinetic properties of 11β-hydroxylase for 17OHP in this species and compare it with that of marsupials. We confirmed the observations of Sharma *et al.*[40] that 17OHP was a very poor substrate for 11β-hydroxylation by beef adrenal mitochondria. The efficiency $K_{M/V}$) for 17OHP, which was only about 1/600 that for S, was due mainly to the relatively low activity of the enzyme for this substrate (see *V*, Table 1). Identical experiments carried out with possum and kangaroo mitochondria revealed that the efficiency of 11β-hydroxylation for 17OHP was in both species of a similar order of magnitude, being approximately four times higher than that for beef. On the other hand the enzyme efficiency for S of possum was 150 times, and of kangaroo 1,300 times less than that of beef (Table 1). These differences in enzyme efficiencies could in themselves serve to explain the reason for the absence of 21-DF in beef, and its presence in adrenal venous effluent of marsupials.

The findings that the 11β-hydroxylase activity for S was much lower in marsupials than in beef, prompted us to determine whether this pattern of activity also applies, relative to other eutherians. The reported rates for 11β-hydroxylation of S (pmol/mg/min), at substrate concentrations ranging from 90–170 μM were; for human, 2,240 [41], for beef 2,690 and 8,858 [16, 21], for guinea pig, 1,570 [42] and of DOC for rat, 3,500 and 8,522 [18, 43]. Some of the listed values were obtained indirectly, by calculations made from relevant data available from the respective publications. The validity of these comparisons should be considered with some reservations due to differences in the experimental conditions, especially with regard to addition of cofactors e.g. the presence of Ca^{2+} in unphysiological concentrations [16, 18, 21, 43]. However, it is apparent that the rate of 11β-hydroxylation of S in possum and kangaroo is 1/200–1/1000 that of eutherian species (Figs 1–3), under incubation conditions (exogenous NADPH and 5 mM Ca^{2+}) which are assumed to yield optimal rates of 11β-hydroxylation in most species [16, 20, 21]. Furthermore, with kangaroo mitochondria the 11β-hydroxylase efficiency was higher for 17OHP, than for the S substrate, whereas with possum it was the same for both (Table 1). Consequently these data suggest that the biosynthesis of F in kangaroo could proceed via 17OHP → 21-DF → F, in preference to the S → F pathway, whereas in possum both pathways could be equally effective. That possum adrenal tissue can very efficiently 21-hydroxylate the 21-DF substrate was reported in a previous publication [12].

Additional information of the properties of 11β-hydroxylase were drawn from the competitive inhibition studies which give an indication of enzyme substrate specificity. Our results with beef mitochondria indicated two separate enzyme systems, one specific for S, the other for 17OHP. Reports by other workers with beef have shown that substrate specific 11β-hydroxylases exist also for DOC and androstenedione [21, 22]. In contrast, in marsupials the significant competitive inhibition of one substrate by the other, indicated, that only a single enzyme system was operating. The non-specificity of the 11β-hydroxylase extended to four tested substrates, namely; S, 17OHP, progesterone and androstenedione. The results obtained from inhibition studies were verified when compared with the calculated, theoretically expected values for a one and a two enzyme system (Table 2). The fact that the *V* values for all substrates were of a similar order of magnitude substantiated further the concept of a single enzyme system. In addition the ratio of K_i (inhibitor) to K_M (substrate), was comparable with the ratio K_M (inhibitor) to K_M (substrate), implicating that the same enzyme sites were involved.

Enzyme substrate specificity may be of importance with regard to evolutionary processes in adrenal steroidogenesis. Multiple enzyme forms within a single organism possessing analogous biological activities (iso-enzymes) are widespread [44]. Current knowledge indicates that the main advantage of substrate specific enzymes lie in the ability of regulating cell metabolism with more precision. Hence the substrate specific enzymes with the different efficiencies in beef, may be the result of a higher evolution, which could allow a greater and more rapid response to enzyme regulators and act as a safeguard to the organism if one of the enzyme systems becomes deficient.

Finally it is of interest to consider the relationship between mitochondrial 11β-hydroxylation by marsupials and by humans with diseases where 21-DF is formed. Reports indicate, that human mitochondria possess multiple substrate specific 11β-hydroxylases [9, 10, 23], and a defect in a particular 11β-hydroxylase could result in an over, or an undersecretion of the respective hydroxylated product, without necessarily affecting the formation of other products [9, 10]. The experimental data suggest that in marsupials the formation of 21-DF is due to a non-substrate-specific 11β-hydroxylase with an equal efficiency for 17OHP and S, while in the adrenogenital syndrome, the presence of 21-DF could be the result of either a deficiency in the 21-hydroxylase [6], or a defect in the specific 11β-hydroxylase system.

In conclusion, the results obtained in the present study offer an explanation for the secretion of 21-DF in marsupials and its absence in eutherian species. A single 11β-hydroxylase for hydroxylation of 21-deoxy, 21-hydroxy and C-19 steroids, was found in marsupials, whereas separate 11β-hydroxylases of grossly different efficiencies, specific for S and 17OHP, were found in beef. It seems likely that the adaptive changes in eutherians occurred with respect to an increase in the rate of formation of F from S, making the 21-DF to F pathway redundant. The presumable evolution of the multi-enzyme system would have physiological advantages with regard to individual re-

sponses to enzyme regulators and a finer control of biosynthetic pathways.

The adjoining second part of this communication provides further evidence in support of a single 11β-hydroxylase system in the possum.

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