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INHIBITION OF 11β-HYDROXYLATION OF DEOXYCORTICOSTERONE IN MITOCHONDRIA OF BOVINE ADRENAL CORTEX

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SUMMARY

The kinetics of inhibition of 11β-hydroxylation of deoxycorticosterone in bovine mitochondria was determined for dehydroepiandrosterone (DHA) and metyrapone. Both compounds inhibited competitively the 11β-hydroxylase. The Michaelis–Menten constant of the reaction using intact mitochondria was evaluated at $K_m = 102$ μmol. The K_i for DHA was found to be 72×10^{-5} mol and that for metyrapone $K_i = 28 \times 10^{-5}$ mol.

INTRODUCTION

SHARMA *et al.*[1] reported on the inhibitory effects of certain steroids and pharmacological inhibitors on 11β-hydroxylation of 11-deoxycorticosterone *in vitro*. Androstenedione was found to be a competitive inhibitor. DHA, DHA-sulfate and testosterone were also found to be inhibitory, but whether their action is competitive or noncompetitive has not been determined. As for the inhibition by metyrapone (SU 4885), it 'cannot be relieved by increasing the substrate concentration'. The competitive nature of 11β-hydroxylase inhibition by metyrapone has been demonstrated by Dominguez and Samuels[2].

Metyrapone and DHA are both compounds of considerable physiological interest. Metyrapone is known to prevent 11β-hydroxylation in human and animal adrenal glands[3, 4]. In man, this compound is widely used to prevent cortisol and corticosterone synthesis and thus to determine whether the hypothalamo-hypophyseal-adrenal system is functioning normally[5, 6]. The biological role of the large concentration of DHA-sulfate present in the circulation is not known. Various non-significant effects have been ascribed to DHA, e.g. being slightly androgenic, anabolic[7] and estrogenic[8]. DHA is a potent inhibitor of glucose-6-phosphate dehydrogenase[9, 10] and of cholesterol biosynthesis [11]. Perfusion of dog adrenal gland with a solution of 5% glucose saturated with DHA causes a decreased conversion of DOC to corticosterone[12].

The above findings led us to study the type of inhibition by metyrapone and by DHA on 11β-hydroxylation in isolated mitochondria of bovine adrenal cortex.

MATERIALS AND METHODS

Separation of adrenal cortex mitochondria was done according to Rosenthal and Narasimhulu[13]. Freshly excised adrenals were collected in crushed ice at the local slaughter house and processed within 2 hr. Adhering fat was trimmed, the glands bisected and the medullary tissue excised. Cortical tissue was scraped off the capsule, the scrapings collected in a beaker, rinsed with 0·25 M sucrose

and homogenized in 0·25 M sucrose by means of a Potter–Elvehjem type homogenizer. Approximately 4 parts of 0·25 M sucrose per 1 part of cortex tissue were used. The homogenate was diluted with 0·25 M sucrose 1 : 1, v/v and centrifuged for 10 min at $900 \times g$. The resulting supernatant was centrifuged for 10 min at $9000 \times g$. The sediment was resuspended in 0·25 M sucrose and centrifuged again for 10 min at $9000 \times g$. The resulting sediment was taken as "mitochondria" for the enzyme assay. Protein concentration of the mitochondrial suspension was adjusted with 0·25 M sucrose to 30 mg protein per ml.

The incubation mixture consisted of 240 mM sucrose; 20 mM KCl; 15 mM triethanolamine at pH 7·2; 10 mM potassium phosphate buffer, pH 7·2 and 5 mM $MgCl_2$.

Hydroxylation assay

The mitochondrial suspension was diluted with the incubation mixture to a concentration of 2 mg protein per ml. To 5 ml of reaction mixture 33 μl of a 1 M solution of equal concentration of succinate and malate were added as hydrogen donors for the generation of NADPH. The appropriate quantity of DOC dissolved in methanol was placed on the bottom of a 25 ml Erlenmeyer flask and the alcohol evaporated in a stream of dry air. One drop of propyleneglycol was added to dissolve the steroid in the aqueous solution. The incubation mixture was preheated to 25°C before its addition to the Erlenmeyer flasks and incubation was done at 25°C for 20 min in a Dubnoff metabolic shaker. At the end of the incubation two 1-ml aliquots from the flasks were transferred to test tubes containing 1 ml of a 0·5% solution of $HgCl_2$ to stop further reaction, followed by the addition of 10 ml of dichloromethane.

Extraction of corticosterone

The contents of the tubes were thoroughly mixed, the phases separated by centrifugation at $1000 \times g$ and the organic layer removed with an aspirator. The aqueous layer was extracted 3 times more with dichloromethane and the combined organic solvent evaporated with a flash evaporator. The resulting lipid extract was separated by thin-layer chromatography (t.l.c.) in a system of benzene : acetone (2 : 1, v/v). The plates were examined for U.V.-absorbing steroids under an U.V. lamp with a peak emission at 253 nm. The silica gel with the corticosterone was scraped off, packed in small columns and the steroid eluted with methanol.

Corticosterone was estimated fluorimetrically [14]. Protein was determined by the method of Lowry [15].

Chemicals

Analytical reagent grade methanol, ethanol, acetone and dichloromethane were redistilled through a 50 cm Vigreux column. Metyrapone (SU 4885, 1,2-bis (3-pyridyl)-2-methyl-1-propanone) was a gift of Prof. F. G. Sulman (Hebrew University of Jerusalem). Steroids were obtained from Ikapharm, Ramath-Gan, Israel. Other reagents were commercial preparations, used without further purification.

RESULTS

The time course of the conversion of DOC to corticosterone by the action

of the 11β-hydroxylase in the absence of inhibitor, followed over a range of several substrate concentrations, is presented as a Lineweaver and Burk plot in Fig. 1. Resulting values of the slope and intercept of the reciprocal function were statistically analyzed and found to be linear with acceptable deviations, the regression coefficient of the line being $r = 0{\cdot}96$. The resulting K_m was evaluated at 102 μmol.

To determine whether the inhibition of the tested compounds is competitive or not, the affinity of the enzyme for inhibitors was evaluated by the method of Dixon[16] as shown in Fig. 2. The differences in V_{max} in the presence and absence of inhibitor prove that DHA (as well as metyrapone) inhibit competitively the steroid 11β-hydroxylase. The competitive nature of 11β-hydroxylase inhibition by metyrapone has already been established by Dominguez and Samuels[2]. Therefore, we determined the K_1 for this compound by using a single substrate concentration for plotting 1/v against metyrapone and use of the intersection with a horizontal line at the height of $1/V_{max}$.

The level of DHA-sulfate in plasma and probably also in the adrenal gland

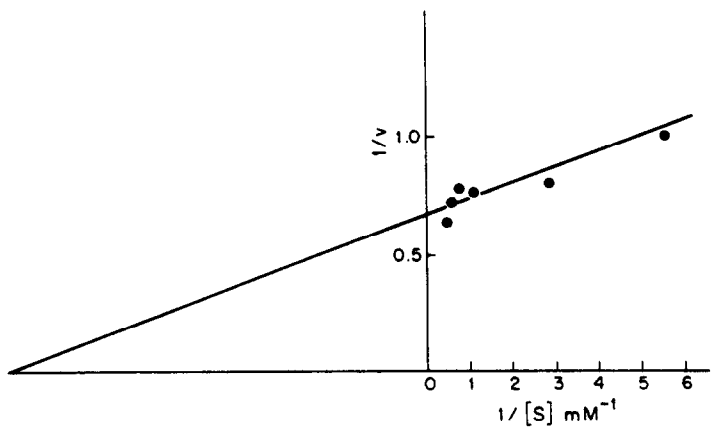

Fig. 1. Lineweaver and Burk plot for the determination of the K_m of 11β-hydroxylation of DOC to corticosterone. The substrate (DOC) was incubated with 2·0 mg of mitochondria for 20 min. v represents μ moles of corticosterone formed per liter per min. K_m = 102 μmol.

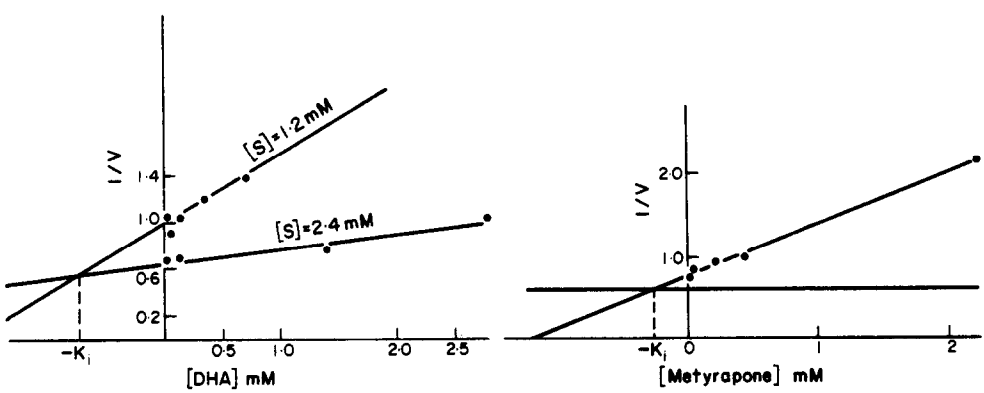

Fig. 2. Determination of K_i by the method of Dixon[16]. The substrate (DOC) concentrations. 2·4 and 1·2 mmoles per liter for DHA and 2·4 mmol per liter for metyrapone. were incubated with 2·0 mg of mitochondria for 20 min. v represents μ moles of corticosterone formed per liter per min. $K_{i\,DHA} = 72 \times 10^{-5}$ mol (to the left); $K_{i\,metyrapone} = 28 \times 10^{-5}$ mole (to the right).

is by far greater than that of free DHA. In a preliminary experiment the effect of DHA-sulfate on 11β-hydroxylation was examined. From Table 1 it seems that DHA-sulfate exerts a considerable inhibitory effect on 11β-hydroxylation.

Table 1. Inhibitory effect of DHA-sulfate on 11β-hydroxylation in bovine adrenal cortex mitochondria. The substrate (DOC) was incubated with 2·0 mg of mitochondria for 20 min

Substrate (mmol per liter)	Inhibitor (mmol per liter)	Corticosterone isolated (μmol per liter per min)
2·4	-	1·80
2·4	0·94	1·06
1·2	-	1·52
0·4	-	1·38

DISCUSSION

The Michaelis–Menten constant of 11β-hydroxylation of DOC to corticosterone was estimated by Sharma *et al.*[1] and by Rosenthal and Narasimhulu[13] with a purified enzyme preparation obtained from sonicated bovine mitochondria. Values reported were 8·5 and 20 μmol respectively. The value of 102 μmol found in the present paper with intact mitochondria seems therefore reasonable.

Metyrapone and DHA inhibit 11β-hydroxylation of DOC to corticosterone at different sites. Metyrapone enters the adrenal cortex and binds to a site(s) on cytochrome P-450, thereby diminishing its ability to react with DOC and thus affecting the rate of steroid 11β-hydroxylation. Addition of DOC to adrenal mitochondria produced a spectral change which is thought to represent an interaction between DOC and cytochrome P-450[17]. DHA, on the other hand, affects the rate at which NADPH is generated in adrenal tissue. It is assumed that DHA may thereby affect the biosynthesis of steroids in the adrenal gland[10]. Therefore, it is remarkable that both metyrapone and DHA inhibit competitively the same reaction, and that the K_i of their inhibition is similar.

In contrast to the finding of Sharma *et al.*[1] who worked with sonicated mitochondria, the results of Dominguez and Samuels[2] working with whole rat adrenal homogenate, as well as our results, show that the inhibition of metyrapone can be relieved by increasing the substrate concentration. This finding seems to be of importance, since otherwise it could not be recommended to use a non-competitive inhibitor whose reduction product (SU 5236) is also strongly inhibitory[17], for diagnostical purposes. This discrepancy might be due to the different preparations used as the source of the hydroxylase.

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