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DIFFERENCE IN 11β-HYDROXYLATION OF DEOXYCORTISOL AND DEOXYCORTICOSTERONE BY HUMAN ADRENALS*

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SUMMARY

Human adrenals obtained up to 12 h after death, were homogenized and used as a 11β-hydroxylase preparation. Two kinds of incubations, each containing 11-deoxycorticosterone (DOC) and 11-deoxycortisol (compound S) as substrates, were performed.

One incubation was carried out at various temperatures (25–38°C) and the other using a heat inhibition method. The results indicate a difference in the hydroxylation of 11-deoxycortisol to cortisol (compound F) and 11-deoxycorticosterone to corticosterone (compound B).

INTRODUCTION

Zachmann *et al.* [1] described a young female subject withcongenital adrenal hyperplasia due to 11β-hydroxylase deficiency in which the secretion of compound F (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) was low and that of compound S (17α,21-dihydroxy-4-pregnene-3,20-dione) was high, while the secretion rates of DOC (21-hydroxy-4-pregnene-3,20-dione) and compound B (11β,21-dihydroxy-4-pregnene-3,20-dione) were normal.

This is in contrast to other cases of 11β-hydroxylase deficiency, where the secretion of DOC was high and that of compound B low [2, 3]. Sharma *et al.* [4], using bovine adrenals found that the substrates, compound S and DOC, undergo 11β-hydroxylation at the same active sites of the enzyme. They concluded that one 11β-hydroxylase is responsible for the two conversions. Tomkins *et al.* [5, 6], however, working with calf adrenals, pointed out that at least one of the components involved in the hydroxylation of DOC was different from those required for hydroxylation of compound S.

We have tried to confirm this by means of an Arrhenius plot method [7], but failed to find any difference between the conversions in the temperature range from 7–30°C. Only in the temperature range from 30–37°C, there remained uncertainty. In the present work, the conversion of DOC and compound S to their respective 11β-hydroxylated metabolites was studied under the following conditions: 1. Various incubation temperatures (25–38°C); 2. Heat inhibition incubation [8–11].

EXPERIMENTAL

Materials. The following radioactive substrates were used: 1. [1·2^{3}H]-compound-S (New England Nuclear Corp., Boston, Mass.) was diluted with unlabelled compound S (Sigma, St. Louis, U.S.A.), each reaction vial contained 5 μg of unlabelled substrate plus an activity of 1,525,000 d.p.m. 2. [1·2^{3}H]-DOC (NEN) was diluted with unlabelled DOC, each reaction vial contained 4·77 μg of unlabelled substrate plus an activity of 1,525,000 d.p.m. The labelled compounds were purified by thin layer chromatography (HF 254, Merck), using a solvent system of 94:6 chloroform–methanol [14]. The unlabelled compounds were purified by repeated crystallization using methanol as a solvent.

Enzyme preparation. Human adrenal glands were frozen after autopsy and stored for about two months. The glands were thawed and the cortex tissue scraped off with a scalpel. The resultant pulp was homogenized in a Teflon homogenizer with a mixture of equal volumes of 0·154 M sodium chloride and 0·1 M sodium phosphate buffer (pH 7·4). The final concentration of the homogenate was 20% (w/v) [12]. The homogenate was centrifuged at 700 *g* for 15 min in a Sorvall RC2-B refrigerated centrifuge, and the supernatant was used as an enzyme solution.

Assay

Incubation. The incubations were carried out in a shaking bath and performed as follows:

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1. Each incubation flask contained substrate dissolved in propylene glycol (0·1 ml).

2. In addition, each flask received 0·1 ml of a water solution containing 1·0 μmol $NADP^+$ (Sigma), 6·0 μmol glucose-6-phosphate (Sigma), and 1·4 μmol $MgCl_2$ (Merck, Darmstadt, West Germany).

3. The incubations were started by addition of 1·4 ml of adrenal cortex homogenate and 1·0 Korenberg unit of glucose-6-phosphate dehydrogenase (Sigma) dissolved in 0·05 ml of water. The incubation flasks were open and the incubation lasted 2 h.

4. Incubations were carried out at (a) various temperatures between 25–38 C; (b) at 25 C, after treating the enzyme solution by heat (39 C) for various periods of time as a method of inactivation.

Extraction and chromatography procedure

The incubations were stopped and extracted with distilled chloroform (3 × 5 ml) (Fluka, Switzerland). DOC and compound B, or compounds S and F, 50 μg of each were added to the respective extracts in order to detect subsequently the steroids in U.V. light. The chloroform was dried in a vacuum rotary evaporator and the residues were dissolved in ethanol, purified according to the method of Peterson [13]. The extract was then applied to silica gel HF 254 (Merck) thin layer plates. The plates were developed in chloroform–methanol (94:6, v/v) [14] the methanol being purified according to the method of Bush [15]. After chromatography the plates were viewed in U.V. light at 254 nm. The product and substrate spots and the remainder of the plate (for recovery calculations) were scraped off and extracted three times with ethanol (final volume 5·0 ml). Samples of 0·05 ml were transferred into scintillation vials and the radioactivity was read in a liquid scintillation spectrometer (Packard, model 3003).

Heat inactivation

Before the incubations were started, the enzyme solution (pH 7·4) was inactivated by heat according to the method of Dahlqvist [8–10]. A preliminary experiment was performed with a sample from step 4a (see assay section), which was maintained at 38·5°C for 75 min before incubation at 30 C. The results from this incubation were compared with those from incubation at 30°C without previous heat treatment. Based on the preliminary experiment a second series of inactivations was performed, in which the incubation and inactivation temperatures were constant (25 and 39 C respectively), but the inactivation periods varied (5, 10, 20, 40 and 60 min). After inactivation, the samples were stored in crushed ice until the incubation was started, samples not treated by heat were used as "zero" inactivation samples.

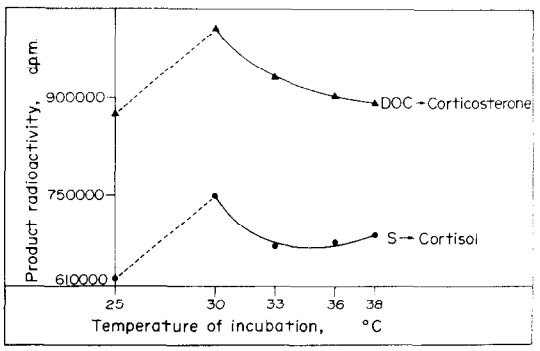

Fig. 1. Human adrenals 11β-hydroxylase. Incubations at various temperatures. Effect of incubation at various temperatures on the conversion of DOC to compound B and compound S to compound F, performed by human adrenal cortex homogenate. The incubations were carried out at temperatures from 25 to 38°C for 2 h, the flasks were open to air and contained 5 μg unlabelled compound S plus an activity of 1,525,000 d.p.m. or 4·77 μg unlabelled DOC plus an activity of 1,525,000 d.p.m. respectively. The product radioactivity results are a mean of two experiments.

RESULTS

Table 1 reveals a large difference between the activities of the adrenal homogenate treated by heat before the incubation on one hand, and those which were kept cool on the other hand. From this, it was concluded that heat inactivation at 39 C is most suitable for experiment 4b (see assay section). Figure 1 shows the change of product radioactivity of both conversions (compound S to compound F and DOC to compound B) at the temperature range of 25 to 38 C. Each

Table 1. The inhibiting influence of heat on the 11β-hydroxylation of compound S to compound F and DOC to compound B, at 38·5°C in human adrenal tissue

	Precursor	Precursor activity (d.p.m.)	Product activity (d.p.m.)	Conversion (%)
Control	Compound S	1,525,000	750,220	49
Heat treated	Compound S	1,525,000	83,470	5
Control	DOC	1,525,000	1,006,000	66
Heat treated	DOC	1,525,000	141,500	9

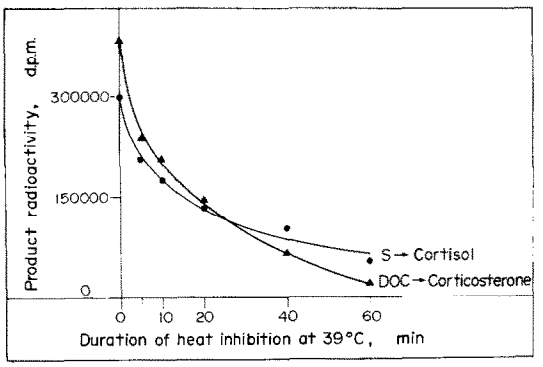

Fig. 2. Human adrenal 11β-hydroxylase. Inhibition by heat. Effect of heat inhibition on the 11β-hydroxylation of DOC to compound B and of compound S to compound F, performed by human adrenal cortex homogenate. The heat inactivation was performed by bringing the enzyme solution to a temperature of 39°C, samples were taken out at the intervals 0, 5, 10, 20, 40 and 60 min and stored in crushed ice until the start of the incubation. The incubations were carried out at 25°C for 2 h, the flasks were open to air and contained 5 μg unlabelled compound 2 plus an activity of 1,525,000 d.p.m. or 4·77 μg unlabelled DOC plus an activity of 1,525,000 d.p.m. respectively. The product radioactivity results are a mean of two experiments.

graph point is the mean of two experiments. With both conversions, the activity increases between 25 and 30°C and decreases at higher temperatures. The activity of the conversion DOC to compound B continues to decrease moderately, at least, up to 38°C, while the compound S to compound F activity ceases to decrease at 33°C and increases moderately, at least, up to 38°C. Figure 2 reveals the influence of inactivation temperature on the compound F and compound B production. It appears that the heat inactivation is not equal in both conversions. This is also demonstrated by the production ratio (Table 2) of compound B to compound F, which changes from 1·3 at temperature untreated experiments to 0·40 at 1 h treatment.

Table 2. The compound B to compound F production ratio at various inhibition times

Inhibition time (min)	Ratio compound B/compound F
0	1·3
5	1·2
10	1·2
20	1·0
40	0·6
60	0·4

The ratio terms were calculated from the heat inhibition experiment data (Fig. 2).

DISCUSSION

From our studies, it appears that the human 11β-hydroxylase system is heat sensitive. Its activity diminished above 30°C (Fig. 1) when the damage was irreversible (Table 1, Fig. 2) in spite of the fact that, *in vivo*, the temperature is higher. The heat sensitivity of the enzyme might also be due to the increased activity at temperatures above 30°C of the homogenate proteases. The heat sensitivity might explain why human adrenals obtained at autopsies failed to be suitable as a source of 11β-hydroxylase in previous works [15–20], since usually these experiments were carried out at 37°C. The decreasing extent of both conversions at temperatures above 30°C (Fig. 1) could possibly be explained by damage of a common component. The increment of compound S to compound F conversion and the reduction of DOC to compound B at temperatures above 33°C could be explained by a difference between the components replacing the damaged factor. Those which participate in the compound S to compound F conversion might be more stable than those which participate in the DOC to compound B conversion. This last hypothesis appears to be possible from the results on Fig. 2 and Table 2, which show that at approximately 20 min of heat inactivation, the conversion from compound S to compound F becomes faster than that of DOC to compound B.

Figure 2 reveals the typical system heat inactivated curve [8] (not following first order kinetics) caused by a mixture of several enzymes with different heat sensitivities. From this curve, it may be concluded that the almost parallel decrease up to 10 min inactivation is probably caused by a common heat damage of the sensitive components. The later slope up to 60 min inactivation, and the change of compound B to compound F production ratio (Table 2), are caused by different heat sensitive components participating in each conversion. In conclusion we have presented evidence that the adrenal 11β-hydroxylation of compound S to compound F differs, at least, in some respect from that of DOC to compound B.

Generally both hydroxylations are blocked in congenital adrenal hyperplasia with 11β-hydroxylase deficiency. This is, however, not in contradiction with the assumption of different enzyme systems, because some components might be in common for both conversions. Our previously reported unusual case [1] of 11β-hydroxylase deficiency, where only the conversion of compound S to compound F is inhibited, is in favour of the assumption of more than one enzyme system. Here there could be a defect of a component that plays a role in only one of these reactions.

Previous studies have demonstrated that steroid 21-hydroxylation is "substrate specific" [21, 22] and that

the 21-hydroxylation of progesterone and 17-hydroxyprogesterone is probably performed by two systems. Basch *et al.* [23] and Cowan *et al.* [24] have shown that the activity of rat adrenal 3β-hydroxy steroid dehydrogenase is found in mitochondria as well as in microsomal fractions and that there is a distinct difference in the K_m for NADP in both fractions. The work of Tomkins *et al.* [5, 6], Sweat *et al.* [25] and our own results ([7] and the present) describe 11β-hydroxylase of compound S and of DOC as two systems with at least one different component. However, the competition of the two substrates at one active enzyme site [4], shows that the two systems have some factors in common.

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