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FORMATION OF 22-KETO-CHOLESTEROL FROM 22S-HYDROXY-CHOLESTEROL BY A NADPH AND O_2 DEPENDENT, CO-INSENSITIVE ENZYME IN BOVINE ADRENAL CORTEX MITOCHONDRIA

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SUMMARY

(22S)-5-Cholestene-3β,22-diol is a good precursor for pregnenolone formation by the cholesterol side-chain cleavage enzyme system of adrenal cortex mitochondria. We demonstrated the presence in adrenal cortex mitochondria of another enzyme system, capable to convert (22S)-5-cholestene-3β,22-diol into 3β-hydroxy-5-cholestene-22-one. The enzyme is NADPH and O_2 dependent, but its activity is not significantly affected by carbon monoxide. The enzyme therefore should be classified as a CO-insensitive oxygenase.

INTRODUCTION

During investigations on the mechanism of the conversion of cholesterol† into pregnenolone (cholesterol side-chain cleavage) (22S)-5-cholestene-3β,22-diol (22S), has been tested as a possible substrate for the side-chain cleaving enzyme system [1, 2]. 22S was reported to be converted into pregnenolone by acetone powder preparations of bovine adrenal cortex mitochondria. Compared to other sterols as (22R)-5-cholestene-3β,22-diol, (20S)-5-cholestene-3β,20-diol, (20R,22R)-5-cholestene-3β,20,22-triol and (20R,22S)-5-cholestene-3β,20,22-triol, however, the conversion rate of 22S was low [1].

During studies on the conversion of hydroxylated sterols by freeze-damaged bovine adrenal cortex mitochondria, we found, however, that 22S disappeared rapidly from the incubation medium.

In this paper we will describe the identification of 3β-hydroxy-5-cholesten-22-one (22-keto) as a major conversion product of 22S. In order to elucidate the reaction mechanism, we studied the formation of 22-keto under several experimental conditions.

MATERIALS AND METHODS

Bovine adrenal glands were obtained from the local slaughterhouse. After removal of fat and medulla, the cortex was minced in a medium of 0.25 M sucrose, 10 mM Hepes and 0.5 mM EDTA at pH 7.35. The minced tissue was treated in the same medium using a Parr cell disruption bomb (N_2 pressure 900 psi for 15 min) [3]. From the homogenate a mitochondrial suspension was prepared by standard centrifugation procedures [4]. The protein concentration was 23 mg/ml, estimated by the biuret method. The suspension was stored in liquid N_2. 22S and 22-keto were obtained from Ikapharm (Makor). No significant impurities were detectable by t.l.c. and g.l.c. Cyanoketone® was a generous gift from Sterling-Winthrop. Incubations were done at 37°C and pH 7.40 in a Hepes buffer as described by Kraaipoel *et al.*[4]. In all experiments 200 μg 22S was incubated with 1 ml mitochondrial suspension. Total volume was 10 ml. The amounts of 22-keto were estimated 30 min after addition of the various cofactors. Atmospheric conditions (Table 1) were obtained by bubbling the relevant gas mixture through the incubation medium. O_2 tension was monitored with a Clark oxygen electrode. Additional details about the incubation conditions are given in Table 1. Mitochondria used in control experiments were boiled for 5 min.

Gas chromatography (g.l.c.)

Aliquots of 0.5 ml from the incubation mixture were added to 2 ml of a methanol–chloroform (2:1, v/v) mixture at 0°C. 10 μg epicholesterol were added as an internal standard and the solution was left for 1 h at 4°C with occasional shaking. The layers were separated after addition of 0.5 ml double-distilled water and 1 ml chloroform. The organic layer was evaporated under nitrogen. Trimethylsilyl (TMS)

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† The following trivial names will be used throughout this article: 5-Cholesten-3β-ol (cholesterol); 3β-hydroxy-5-pregnen-20-one (pregnenolone); 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one (cyanoketone®) and 5-cholesten-3α-ol (epicholesterol).

Table 1. Products formed from 22S in several experimental conditions, expressed as % of the amounts formed in expt. 1

Expt. no.	Atmosphere	Cofactors added	Pregnenolone	22-keto
1	Air	NADPH gen. system	100	100
2	100% N_2	NADPH gen. system	≤1	≤1
3	98% CO, 2% O_2	NADPH gen. system	5 (2–8)	60 (50–70)
4	98% N_2, 2% O_2	NADPH gen. system	60 (50–70)	60 (50–70)
5	Air	NADP	≤1	≤1
6	Air	NAD	≤1	≤1

ether derivatives were prepared by heating the residue for 1 h at 100°C in 20 μl trimethylsilyl-imidazol (Pierce) to which 2 mg pyridine-HCl had been added. After that time, 1 μl was injected into a HP 402 gas-chromatograph, equipped with a 2 m column, packed with 3% SP 2250 on 80–100 mesh Chromosorb W/HP. Evaluation of peak areas was done by a HP 3352 computer system.

Thin layer chromatography (t.l.c.)

Extracts were prepared as described above. Substrates and products were separated on acetone-precleaned Merck Silicagel plates in the system *n*-pentane–diethylether–acetic acid (20:80:2.5, by vol.).

Gaschromatography–mass spectrometry (GC–MS)

GC–MS analysis was done with a Varian-Mat 112 model coupled with a Varian model 1400 Gaschromatograph (capillary column, length 25 m, I.D. 0.2 mm, OV I). Ion source 250°C, 70 eV.

RESULTS

During incubation of 22S in air, in the presence of a NADPH generating system, significant amounts of two products were formed. The relative retention time (RRT) of the first one was 0.68 (epicholesterol = 1), equal to the RRT of standard pregnenolone. The other compound had a RRT = 2.33. The time course of their formation is shown in Fig. 1.

Thin layer chromatography analysis of 2 ml samples, drawn at the same time showed a decrease of 22S (R_F = 0.63) and the appearance of two substances with R_F's of 0.59 resp. 0.73. The R_F value of standard pregnenolone was 0.59. Gas–liquid chromatography analysis of the compound with R_F = 0.73, isolated from the thin layer plate, yielded a single peak with a RRT = 2.33. The properties of this compound suggested that it was 22-keto. An authentic sample of 22-keto under the same analytical conditions had a RRT = 2.33 and a R_F = 0.73. The mass spectra of the compound and of standard 22-keto were virtually identical (Fig. 2). The amounts of 22-keto and pregnenolone, formed under various incubation conditions are given in Table 1. Results are expressed as the percentage of 22-keto and pregnenolone formed in air in the presence of a NADPH generating system (expt. 1).

The amounts of 22-keto and pregnenolone formed in expt. 3 and 4 show some experimental variation. The range is indicated by the figures between parentheses.

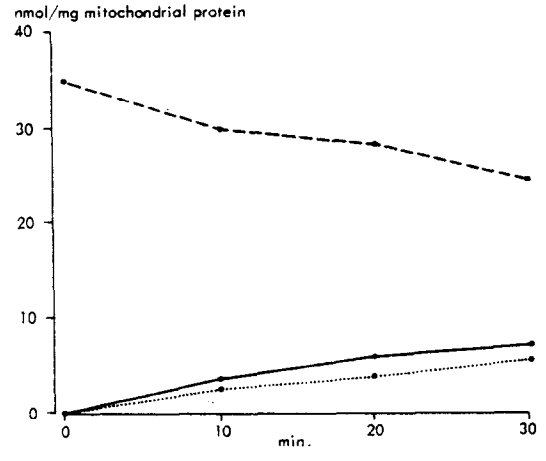


Fig. 1. The conversion of 22S (– – –) into pregnenolone (——) and 22 keto (· · · · ·) by bovine adrenal mitochondria.

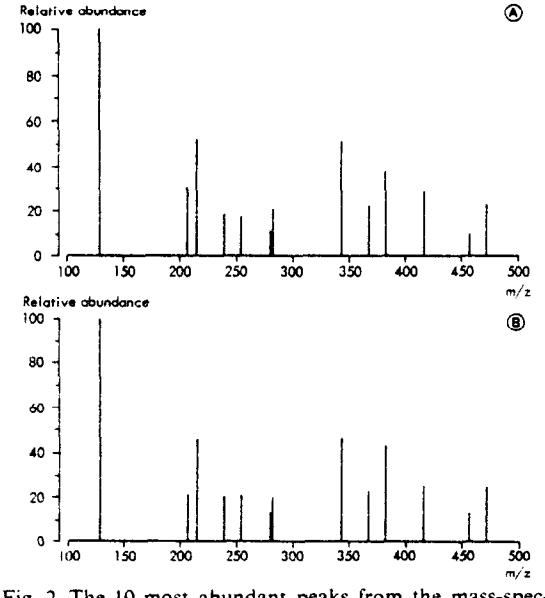


Fig. 2. The 10 most abundant peaks from the mass-spectrum of authentic 22-keto (A), and the 10 most abundant peaks from the mass-spectrum of 22-keto originating from 22S (B). The peaks at *m/z* 129 are put at 100%.

In an atmosphere of 98% CO/2% O_2 (expt. 3), we found that the activity of the side-chain cleaving enzyme system (as estimated by the amount of pregnenolone formed) was almost completely inhibited. Under the same conditions the formation of 22-keto from 22S was inhibited for 40%. Expt. 4 demonstrates the latter effect was not due to the presence of CO, but to the low O_2 concentration: an incubation atmosphere of 98% N_2/2% O_2 had the same inhibitory effect on the formation of 22-keto. Pregnenolone formation too was found to be inhibited for 40%.

No detectable amounts of 22-keto were formed when (22*R*)-5-cholestene-3β,22-diol was tested under the same conditions.

In all control experiments, using boiled mitochondria, no formation of 22-keto or pregnenolone was observed.

DISCUSSION

The combined evidence of t.l.c. and GC–MS demonstrates that in the adrenal an enzyme is present, which is able to convert 22S into 22-keto. This enzyme, although catalyzing the conversion of a hydroxyl group into a keto group can not be classified as a NAD(P) dependent dehydrogenase (expt. 5 and 6).

The dependency on both NADPH and O_2 (expt. 1 and 2) suggests the enzyme is an oxygenase, catalyzing the overall reaction:

$$R_1CHOHR_2 + NADPH + O_2 + H^+ \rightarrow R_1COR_2 + NADP^+ + 2H_2O$$

Its catalytic action is probably comparable to that of a hydroxylase, as can be seen by rewriting the reaction equation as follows:

$$R_1CHOHR_2 + NADPH + O_2 + H^+ \rightarrow R_1C(OH)_2R_2 + NADP^+ + H_2O$$

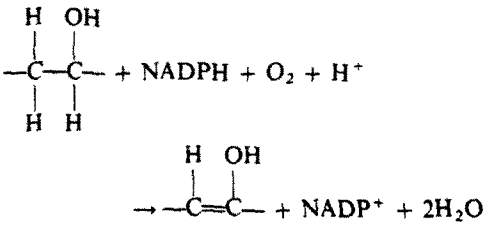
followed by dehydration of the gem-diol to a keto function [6].

An enzyme with comparable activity known to be present in adrenal cortex mitochondria is 18-hydroxysteroid-dehydrogenase [5]. Similar reactions are also involved in C_4 and C_{19} steroid demethylations [7, 8].

Other well-described oxygenases, present in adrenal cortex mitochondria, are steroid 11β-hydroxylase, 18-hydroxylase, 21-hydroxylase and the cholesterol side-chain cleaving enzyme system. All these enzymes contain cytochrome P450. Their activity is inhibited by carbon monoxide [9–12].

Although some CO-sensitive enzymes require quite high CO/O_2 ratios for 50% inhibition [11], on base of the results it is unlikely that the conversion 22S → 22-keto is catalyzed by any of the CO-sensitive enzymes mentioned. The catalyzing enzyme therefore appears to be a CO-insensitive oxygenase.

Our experimental results could also be explained by assuming that 22S is converted by a desaturase, according to the equation:



followed by enol-keto rearrangement to 22-keto. In the sterol field Kraaipoel *et al.*[4], postulated the existence in bovine adrenal cortex mitochondria of a NADPH and O_2 dependent cholesterol 20–22 desaturase, catalyzing the first step of the side-chain cleavage of cholesterol. Such a desaturase might be CO-insensitive, comparable to several fatty acid desaturases found in liver microsomes [13]. Therefore involvement of a desaturase in the conversion of 22S → 22-keto could also explain our experimental results.

The biological significance of the reaction is not yet clear. At present 22S is not known to be formed in the adrenal. It is quite possible, however, that under the right circumstances and in the presence of the right substrate, a 22S hydroxyl group can be introduced in the sterol side-chain.

In this respect it is interesting to note that the adrenal cortex possesses an active 25-hydroxylase, which normally does not participate in steroidogenesis [15]. In metabolic disorders like congenital lipoid adrenal hyperplasia or the 3β-hydroxysteroid dehydrogenase deficiency [16–19] such normally "minor pathways" probably gain importance.

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