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3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN BOVINE ADRENAL CORTEX MITOCHONDRIA: CONVERSION OF OXYGENATED 3β-HYDROXY-STEROLS

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SUMMARY

Adrenal cortex 3β-hydroxysteroid dehydrogenase (3β-HSD) is able to convert many C_{19} and C_{21} 3β-OH-5-ene steroids into products with a 3-keto-4-ene structure. In the present investigation we describe the conversion of a number of C_{27} and C_{24} 3β-5-ene sterols by adrenal mitochondrial 3β-HSD. Among these substrates were (20S)-5-cholestene-3β,20-diol, (22R)-5-cholestene-3β,22-diol and (20R,22R)-5-cholestene-3β,20,22-triol, compounds occurring as intermediates in the cholesterol side-chain cleavage reaction. Cholesterol itself was not converted to a measurable extent.

INTRODUCTION

The sequence cholesterol† → pregnenolone → progesterone is the main pathway of adrenal progesterone biosynthesis.

In the conversion of cholesterol to pregnenolone (Cholesterol side-chain cleavage or CSCC) several side-chain hydroxylated sterols such as (20S)-5-cholestene-3β,20-diol, (22R)-5-cholestene-3β,22-diol and (20R,22R)-5-cholestene-3β,20,22-triol have been proposed as intermediates [1, 2].

In the step following CSCC, the 3β-OH-5-ene structure as present in pregnenolone is converted into the 3-keto-4-ene structure of progesterone. This reaction is catalyzed by a 3β-OH-steroid dehydrogenase 5 → 4-ene-isomerase (3β-HSD) enzyme system. While CSCC activity is located in the mitochondria, adrenal 3β-HSD is found in both mitochondria and microsomes [3].

As to the substrate specificity of adrenal 3β-HSD it is known that most of the common C_{21} and C_{19} steroids like pregnenolone, 17α-OH-pregnenolone and dehydroepiandrosterone can be converted into their 3-keto-4-ene analogues [4]. NAD is the preferred cofactor [5]. Little information is available, however, concerning the formation of 3-keto-sterols with e.g. 27 C atoms. In fact, apart from cholesterol very few sterols have been tested as a substrate for adrenal 3β-HSD.

Because unusual adrenal metabolites with a C_{19} or C_{21} structure are known to be produced in various disorders such as the 21, 17α or 11β-hydroxylase defects, we have investigated if unusual metabolites with a C_{27} structure could also be produced from cholesterol or other C_{27} sterols.

In the present study cholesterol, the 3 hydroxylated sterols (20S)-5-cholestene-3β,20-diol, (22R)-5-cholestene-3β,22-diol and (20R,22R)-5-cholestene-3β,20,22-triol and several other C_{27} compounds were tested as substrates for 3β-HSD of bovine adrenal cortex mitochondrial preparations.

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) [6]. From the homogenate a mitochondrial suspension was prepared by standard centrifugation procedures [7]. The protein concentration was 25 mg/ml, estimated by the biuret method. The suspension was stored in liquid N_2.

Incubation conditions

Incubations were done at 37°C in a medium containing 154 mM KCl, 11.5 mM NaCl, 50 mM nicotinamide, 20 mM Hepes (pH 7.40), 5 mM $CaCl_2$, 4 mM sodium azide, 1% bovine serum albumine (w/v), 5 μM

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† The following trivial names will be used throughout this article: 5-cholesten-3β-ol(cholesterol); 5-cholesten-3α-ol(epicholesterol); 5α-cholestan-3β-ol(cholestanol); 4-cholesten-3-one(cholestenone); 3β-hydroxy-5-pregnen-20-one(pregnenolone); 3β,17α-dihydroxy-5-pregnen-20-one(17α-OH-pregnenolone); 4-pregnene-3,20-dione(progesterone) and 3β-hydroxy-5-androsten-17-one(dehydroepiandrosterone).

Table 1. Relative retention times (RRT) and R_F values of 3β-OH-5-ene sterols and their 3-keto-4-ene analogues: the latter were prepared as described in the section "Reference compounds"

	g.l.c. RRT; silylated sterols (Epicholesterol = 1)			t.l.c. R_F; free sterols	
	3β-OH-5-ene	3-keto-4-ene		3β-OH-5-ene	3-keto-4-ene
Cholesterol	1.28	2.23	2.35	0.57	0.70
20S	1.75	2.21	3.11	0.54	0.68
22R	1.66	2.07	2.99	0.47	0.60
22S	1.57	2.05	2.87	0.47	0.62
25-OH	2.37		4.03	0.41	0.57
22-keto	2.40	2.67	3.08	0.58	0.70
17,20	3.03	*		0.41	0.52
20R,22R	2.25	3.06	4.20	0.36	0.49
20R,22-keto	*	*		0.46	0.69
3-OH-cholenic	2.09	2.66	3.88	0.22	0.30

* No accurate RRT measurement possible.

antimycin A and 1 ml mitochondrial suspension, in a final volume of 10 ml. In all incubations 200 μg sterol dissolved in 100 μl ethanol was added as a substrate.

At $t = 0'$, 2 mg NAD was added to start the reaction. Both at $t = 0'$ and at $t = 30'$, 0.5 and 3.5 ml aliquots were drawn from the medium for g.l.c. and t.l.c.

The following sterols (with abbreviation and source) were used as substrates: 5-cholesten-3β-ol {cholesterol} Sigma; (20S)-5-cholestene-3β,20-diol {20S} Sigma; (22R)-5-cholestene-3β,22-diol {22R} Ikapharm; (22S)-5-cholestene-3β,22-diol {22S} Ikapharm; 5-cholestene-3β,25-diol {25-OH} Steraloids; 3β-hydroxy-5-cholesten-22-one {22-keto} Sigma; (17S,20S)-5-cholestene-3β,17,20-triol {17,20} Steraloids; (20R,22R)-5-cholestene-3β,20,22-triol {20R,22R} Biosynthesis [8]; (20R)-3β,20-dihydroxy-5-cholesten-22-one {20R,22-keto} Own synthesis [9]; 3β-hydroxy-5-cholen-24-oic acid {3-OH-cholenic} Schwartz–Mann.

Using the g.l.c. and t.l.c. methods mentioned below, all sterols yielded 1 peak resp. 1 spot. Retention times relative to the internal standard epicholesterol and R_f values are given in Table 1.

Gas–liquid chromatography (g.l.c.)

Aliquots of 0.5 ml from the incubations 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) either derivatives were prepared by heating the residue in 20 μl trimethylsilyl-imidazol (Pierce) containing 2 mg pyridine–HCl for 1 h at 100°C. Retention time and peak area relative to the internal standard were measured on a HP 402 gaschromatograph. The column (length 2 m) was packed with 3% SP 2250 on 80–100 mesh Chromosorb W/HP. Evaluation of peak areas was done by a HP 3352 computer system. Most 3-keto-4-ene compounds when treated as described yielded two peaks, probably as a result of enolisation of the 3-keto group.

Because methoximation, followed by silylation, also yielded two peaks, the simplest method (silylation only) was used.

Thin layer chromatography (t.l.c.)

Extracts were prepared from 3.5 ml aliquots as described above. Substrates and products were separated on Merck silicagel plates (with F 254) in the solvent system chloroform–methanol (95:5, v/v).

Reference compounds

All 3β-5-ene sterols used in the present study were converted into their 3-keto-4-ene analogues by oxidation with cholesterol oxidase using the following procedure: 100 μg sterol dissolved in 50 μl ethanol was added to 3.5 ml 1 M phosphate buffer, containing 1 mM EDTA, at pH 7.40. After addition of 0.5 U cholesterol oxidase (Boehringer) and 25 U catalase (Boehringer) the mixture was left standing overnight at room temperature. The mixture was extracted with methanol-chloroform and separated by t.l.c. as described. The conversion of the substrate was not always complete, but in all cases besides the parent sterol a spot could be detected with higher R_F, showing fluorescence at 254 nm. These spots, after elution with acetone, were used as reference 3-keto-4-ene sterols.

RESULTS

With the exception of cholesterol all substances investigated were to a measurable extent converted into a less polar metabolite (Table 2). In all cases the R_F values of these metabolites were identical with the R_F's of the appropriate 3-keto-4-ene reference compounds, prepared as described in the Methods-section.

Table 2. The conversion of 3β-OH-5-ene sterols into their 3-keto-4-ene analogues, measured by g.l.c. and t.l.c.

Substrate	% substrate disappeared at $t = 30'$. g.l.c.	3-keto-4-ene formation g.l.c.	t.l.c. (a)
Cholesterol	n.s.	n.s.	n.s.
20S	40	(c)	yes
22R	n.s.	(d)	yes
22S	40	(c)	yes
25-OH	15	(d)	yes
22-keto	45	(d)	yes
17,20	70	(d)	yes
20R,22R	35	(d)	yes
20R,22-keto	(b)	(b)	yes
3-OH-cholenic	65	(c)	yes

n.s. Not significant. (a) Detection limit for all compounds $\leq 1\ \mu g$. (b) No quantitative g.l.c. measurements due to inadequate derivatization. (c) Well-defined peaks; but no reliable quantitative g.l.c. measurement due to unsatisfactory derivatization. (d) No quantitative g.l.c. measurements due to unsatisfactory derivatization and/or poorly defined peaks.

The various metabolites all showed fluorescence at 254 nm, characteristic for 3-keto-4-ene compounds. Based on these results, the detected less polar compounds were assumed to have a 3-keto-4-ene structure. This conclusion was also confirmed by mass-spectrometric evidence. As can be inferred from the chemical structure of the used substrates, in most of them other hydroxy → keto conversions are incompatible with an intact sterol structure. Only substrates containing a 22-hydroxy group (22R; 22S and 20R,22R) could, in addition to 3β-OH oxidation, have undergone 22-OH oxidation as well. The R_F values of the 22R and 22S metabolites, however, were clearly different from each other and from the R_F of the 22-keto metabolite. The R_F of the 20R,22R metabolite also was different from the R_F of the metabolite of 20R,22-keto.

Gas-liquid chromatography measurement showed for most of the substrates a decrease with time (Table 2) with the exception of 22R and cholesterol. In view of the difficulties met with gaschromatographic determination of the 3-keto-4-ene sterols, we did not attempt to obtain a quantitative estimation of the latter compounds. However, neither on g.l.c. nor on t.l.c. did we detect significant amounts of other metabolites than the 3-keto-4-ene sterols arising from the 3β-5-ene substrates.

DISCUSSION

Our results show that adrenal mitochondrial 3β-HSD is able to convert (besides the already mentioned C_{19} and C_{21} compounds) 3β-OH-5-ene C_{27} sterols with widely different structures into 3-keto-4-ene compounds. The rate of conversion of cholesterol is very poor. From more than 200 μg cholesterol (endogenous plus 200 μg added), less than 10 μg cholestenone was formed. All other compounds tested, however, proved to be better substrates; some were metabolized with a high reaction rate. Three of the compounds tested by us as substrates (20S; 22R and 20R,22R), are commonly accepted as CSCC intermediates. All three were found to be converted more efficiently into their 3-keto-4-ene analogues than cholesterol itself.

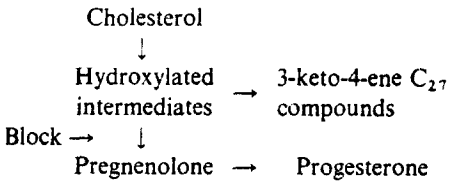
Due to experimental uncertainties, no significant change of 22R could be demonstrated by g.l.c. By t.l.c., however, the formation of its 3-keto-4-ene metabolite in amounts $> 10\ \mu g$ could clearly be demonstrated. The decrease of 20S and 20R,22R was much more rapid (40%, resp. 35%). In the case of 20R,22R the presence of the 3-keto-4-ene metabolite could be detected unequivocally by t.l.c., but the very high retention time of this metabolite made detection by g.l.c. virtually impossible.

Both the formation of hydroxylated sterols from cholesterol and their conversion into pregnenolone are dependent on the presence of NADPH inside the adrenal cortex mitochondria. This cofactor can be generated inside mitochondria from NADP by oxidation of e.g. malate [10] or isocitrate [11]. 3β-HSD activity, as already stated in the introduction, is NAD dependent. The *in vivo* effect of ACTH on the intramitochondrial concentrations of these nucleotides is insignificant [12]. The intramitochondrial concentrations of NADPH and NAD are coupled by the action of the enzyme transhydrogenase, catalyzing the reaction:

$$NADPH + NAD \rightleftarrows NADP + NADH$$

It has been reported that in the presence of compounds as ATP or citric acid cycle intermediates this equilibrium is shifted far to the left [13]. A mitochondrial milieu, which would facilitate the production of side-chain hydroxylated sterols from cholesterol therefore also appears capable to convert these sterols into their 3-keto-4-ene analogues. Usually this conversion is of little importance *in vivo*, probably because of the rapid conversion of the hydroxylated sterols involved, into pregnenolone.

If, however, the later steps in SCC are blocked by a metabolic defect, these compounds, after the formation of high intramitochondrial concentrations, could be converted by adrenal mitochondrial 3β-HSD into 3-keto-4-ene compounds according to the scheme:



In the clinical condition, known as congenital lipoid adrenal hyperplasia or CLAH there is, besides deficient production of gluco- and mineralocorticoids, virtually no androgen production [14].

Free and esterified cholesterol is found accumulated in the adrenals [15]. CLAH is believed to arise from a metabolic defect in the CSCC [16, 17]. In spite of adequate steroid replacement therapy, patients often died at young age. This observation has led some investigators to assume that in CLAH, there is production of toxic metabolites [18, 24]. This hypothesis has parallels in the various conditions, known collectively as the adrenogenital syndrome. When a metabolic pathway leading to gluco- and mineralocorticoids following CSCC (e.g. 21-hydroxylation) is blocked by an enzyme defect, there is elevated production of substances with androgenic activity. These androgens can be considered as toxic metabolites.

Another parallel can be drawn between this model of CLAH and the metabolic disorder cerebrotendinous xanthomatosis (CTX). In CTX, cholestanol accumulates in many parts of the body [21]. This disease is due to a defect in bile-acid biosynthesis [22, 23]. Under these circumstances cholesterol is first metabolized into cholestenone by a liver 3β-HSD. The cholestenone on its turn is converted by liver 3β,5α-reductase into cholestanol.

The finding that most of the 3β-5-ene sterols investigated can be converted into 3-keto-4-ene analogues, suggests that the latter compounds might play a role in CTX, CLAH and comparable diseases. In this respect, it is interesting to note that investigations into the toxicity of cholestenone have shown that feeding of this substance to rats causes systemic toxic effects. In addition adrenal hyperplasia [19] and -dysfunction can be observed [20].

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