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5β-ANDROSTANE-3α,17β-DIOL: AN ENDOGENOUS SUBSTRATE FOR RABBIT LIVER 3-HYDROXYHEXOBARBITAL DEHYDROGENASE

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Abstract—Dehydrogenation of 5β-androstane-3α,17β-diol to 5β-androstan-3α-ol-17-one was found to be catalysed by rabbit liver 3-hydroxyhexobarbital dehydrogenase. Rabbit liver cytosol contained several enzyme activities for the dehydrogenation of 5β-androstane-3α,17β-diol. One of the activities was not separable from 3-hydroxyhexobarbital dehydrogenase in the course of purification and on polyacrylamide gel disc electrophoresis. The activity of 3-hydroxyhexobarbital dehydrogenase was inhibited competitively by 5β-androstane-3α,17β-diol. Results of a mixed substrate method, thermal inactivation and inhibition by *p*-chloromercuribenzoate also supported the interpretation that a single enzyme was responsible for the dehydrogenation of 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. It was shown that, in the rabbit liver, 3-hydroxyhexobarbital dehydrogenase was separate from testosterone 17β-dehydrogenase (NADP) (EC 1.1.1.64) by TEAE-cellulose column chromatography, although both enzymes were found to be identical in the case of guinea-pig liver.

3-Hydroxyhexobarbital dehydrogenase which catalyses the reversible reaction of 3-hydroxyhexobarbital to 3-oxohexobarbital is present in rabbit liver cytosol and it has been shown that the enzyme is different from classical alcohol dehydrogenase (EC 1.1.1.1) [1–4].

Recently, we indicated that 3-hydroxyhexobarbital dehydrogenase from rabbit liver cytosol was able to catalyse the dehydrogenation of alicyclic alcohols (e.g. 3-hydroxyhexobarbital, 1-indanol) and acyclic secondary alcohols (e.g. β-ionol, styrylmethylcarbinol), but the enzyme metabolized testosterone very poorly [5]. On the other hand, Kageura and Toki [6, 7] demonstrated that the guinea-pig liver enzyme catalyses the dehydrogenation of a variety of androgens having a 17β-hydroxyl group, and that the enzyme is identical with testosterone 17β-dehydrogenase (NADP) (EC 1.1.1.64).

In the present experiment, to investigate the endogenous substrates of rabbit liver 3-hydroxyhexobarbital dehydrogenase, various hydroxysteroids were tested. Among these compounds, only 5β-androstane-3α,17β-diol gave appreciable activity. A difference of the enzyme from testosterone 17β-dehydrogenase was also confirmed.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: NAD(H) and NADP(H) (Oriental Yeast Co., Ltd., Tokyo, Japan); Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden); triethylaminoethyl (TEAE)-cellulose (Serva-Entwicklungslabor, Heidelberg, Germany); hydroxylapatite and acrylamide (Seikagaku Kogyo Co., Ltd., Tokyo, Japan); 5α-androstan-3α-ol-17-one, testosterone and oestradiol-17β (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan); 4-androsten-6β,17β-diol-3-one (The Upjohn Co., Kalamazoo, MI, U.S.A.). All of other steroids were from Sigma Chemical Co., St. Louis, MO, U.S.A. or Steraloids, Inc., Pawling, NY, U.S.A. α-3-Hydroxyhexobarbital was prepared by the method of Takenoshita and Toki [5].

Preparation and purification of 3-hydroxyhexobarbital dehydrogenase were described in the previous paper [5]. The 105,000 *g* supernatant fluid of rabbit liver homogenate was used as the source of the enzyme. The fractionation with ammonium sulphate was followed by Sephadex G-100 gel filtration, TEAE-cellulose column chromatography and hydroxylapatite column chromatography. Sodium phosphate buffer, pH 8.0, containing 0.05% (w/v) 2-mercaptoethanol was used as a elution buffer on column chromatography.

Enzyme activities were measured spectrophotometrically by the change of the absorbance at 340 nm at 25°. For the determination of the enzyme activity, α-3-hydroxyhexobarbital (1 mM), testosterone (0.1 mM) or 5β-androstane-3α,17β-diol (25 or 50 μM) was used as substrate. The reaction mixture contained 0.1 ml of substrate, 1.5 μmoles of NAD (or 0.6 μmole of NADP), a suitable quantity of enzyme solution, and 0.1 M glycine buffer, pH 9.5 (or 10.5 for testosterone), to make a total volume of 1.5 ml. A unit of activity is defined as the amount of enzyme which forms 1 μmole of NAD(P)H per min at 25°. Protein concentration was determined by the method of Lowry *et al.* [8] after all the proteins were precipitated by the method of Folin and Wu [9]. Bovine serum albumin was used as standard.

Polyacrylamide gel disc electrophoresis was performed as described by Davis [10]. Location of

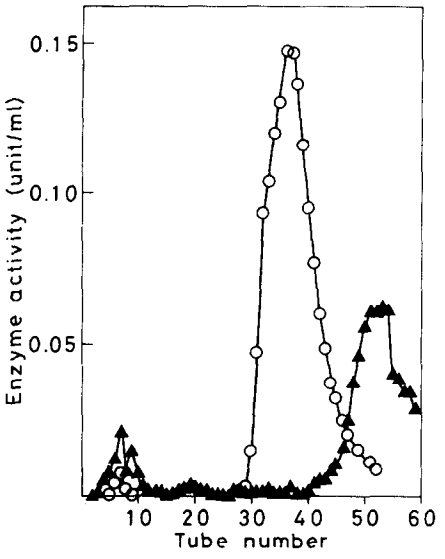

Fig. 1. TEAE-cellulose column chromatography of 3-hydroxyhexobarbital dehydrogenase and testosterone 17β-dehydrogenase. Sephadex G-100 fraction was applied to a TEAE-cellulose column (1.5 × 23 cm). The column was eluted with a linear gradient of 300 ml each of the elution buffer, from 5 mM to 50 mM, and 10 ml fractions were collected. ○, activity for 3-hydroxyhexobarbital; ▲, activity for testosterone.

protein bands and demonstration of enzyme activities on polyacrylamide gel were described in the previous paper [5].

RESULTS

Separation of 3-hydroxyhexobarbital dehydrogenase from testosterone 17β-dehydrogenase. The two enzyme activities for 3-hydroxyhexobarbital and testosterone were not separable at the purification steps of ammonium sulphate fractionation and Sephadex G-100 gel filtration. However, 3-hydroxyhexobarbital dehydrogenase was separated distinctly from testosterone 17β-dehydrogenase by TEAE-cellulose column chromatography (Fig. 1). Accordingly, in contrast with guinea-pig liver, 3-hydroxyhexobarbital and testosterone are metabolized by the different enzymes and testosterone does not function as a physiological substrate for rabbit liver 3-hydroxyhexobarbital dehydrogenase.

Substrate specificity. In order to find the endogenous substrate for the rabbit liver enzyme, a wide variety of steroids possessing hydroxyl group(s) at 3(α or β), 6(β), 7(α), 11(β), 12(α), 17(α or β), 20(β) and/or 21 position of the steroid nucleus were tested (Table 1). Among these compounds, only 5β-androstane-3α,17β-diol showed a comparable activity to α-3-hydroxyhexobarbital. 5β-Androstane-3β,17β-diol, 5β-androstan-17β-ol-3-one, 5β-androstan-17β-ol, 5α-androstane-3α,17β-diol, and 4-androstene-3β,17β-diol were also metabolized, but the relative rate was less than 40 per cent of that of α-3-hydroxyhexobarbital. The other hydroxysteroids including testosterone were not metabolized or exhibited merely a negligible activity. In general, 5β-androstanes gave higher activities than corresponding 5α-isomers.

Table 1. Relative rates of the dehydrogenation of steroids by 3-hydroxyhexobarbital dehydrogenase*

Substrate	Relative rate with NAD	with NADP
α-3-Hydroxyhexobarbital	100	39
5β-Androstane-3α,17β-diol†	70	120
5β-Androstane-3β,17β-diol‡	18	18
5β-Androstan-17β-ol-3-one‡	17	17
5β-Androstan-17β-ol§	24	21
5α-Androstane-3α,17β-diol†	18	38
5α-Androstan-17β-ol-3-one‡	8	5
5α-Androstan-17β-ol§	7	12
Testosterone‡	3	6
4-Androstene-3β,17β-diol‡	12	33

* Relative rate was expressed by per cent activity of α-3-hydroxyhexobarbital at the same concentration. Assay system consisted of 0.1 ml of methanolic solution of each steroid, 1.5 μmoles of NAD (or 0.6 μmole of NADP), 0.1 ml of enzyme solution and 0.1 M glycine buffer, pH 9.5 (or 10.5 for NADP) to make a final volume of 1.5 ml. The following steroids were found to be inactive as substrate for 3-hydrocyhexobarbital dehydrogenase: 5β-androstan-3α-ol-17-one, 5β-androstan-3β-ol-17-one, 5β-androstane-3α,11β,17β-triol, 5β-pregnane-3α,11β,20β-triol, 5β-pregnan-3α,17α,20β,21-tetrol-11-one, 5β-pregnan-3α,11β,17α,21-tetrol-20-one, 5β-pregnan-3α-ol-20-one, 5β-pregnane-3α,20β-diol, 5β-pregnane-3α,11β,17α, 20β,21-pentol, cholic acid, desoxycholic acid, androsterone, 5α-androstan-3β-ol-17-one, 5α-androstane-3α,11β,17β-triol, 5α-pregnan-3α,11β,17α,21-tetrol-20-one, epitestosterone, 19-nortestosterone, 4-androsten-6β,17β-diol-3-one, 4-androstene-11β-ol-3,17-dione, 5-androsten-3β-ol-17-one, 5-androstene-3β,17β-diol, 4-pregnen-20β-ol-3-one, corticosterone, cortisol and oestradiol-17β.

† 0.05 mM.

‡ 0.1 mM.

§ 0.01 mM.

The metabolite of 5β-androstane-3α,17β-diol was identified to be 5β-androstan-3α-ol-17-one (R_f 0.44) by t.l.c. on silica gel HF_{254} (E. Merck A.-G., Darmstadt, Germany) with benzene/acetone (4:1, v/v) as solvent. 5β-Androstan-3α-ol-17-one gave a red colour after spraying with H_2SO_4–methanol (1:1, v/v) followed by heating moderately. The reversibility of the reaction was confirmed by production of 5β-androstane-3α,17β-diol (R_f 0.25, purple colour) from 5β-androstan-3α-ol-17-one.

Elution patterns of the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. Figure 2A shows that one of the dehydrogenation activities for 5β-androstane-3α, 17β-diol coincided with the 3-hydroxyhexobarbital dehydrogenase activity on Sephadex G-100 gel filtration. The active fraction (tubes 49–55) was applied to a TEAE-cellulose column. The enzyme activity for 5β-androstane-3α,17β-diol was separated into several peaks, and one of them (tube 34) coincided with that of the main activity of 3-hydroxyhexobarbital dehydrogenase (Fig. 2B). The results of polyacrylamide gel disc electrophoresis of the active fraction (tubes 30-37) showed that the main protein bands concerned with the dehydrogenation of both 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. The fraction was then applied to a hydroxylapatite column. The elution patterns of the

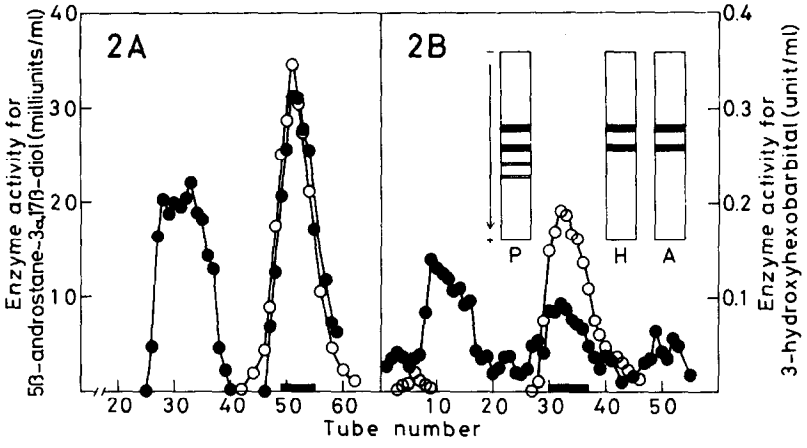

Fig. 2A. Sephadex G-100 gel filtration of the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. Ammonium sulphate fraction (6 ml) was applied to a Sephadex G-100 column (2.5 × 90 cm). The column was eluted with 5 mM elution buffer and 6 ml fractions were collected. ○, activity for 3-hydroxyhexobarbital; ●, activity for 5β-androstane-3α,17β-diol.

Fig. 2B. TEAE-cellulose column chromatography and polyacrylamide gel disc electrophoresis of the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. Sephadex G-100 fraction (tubes 49–55) was applied to a TEAE-cellulose column (1.5 × 23 cm). Elution was carried out as described in Fig. 1. ○, activity for 3-hydroxyhexobarbital; ●, activity for 5β-androstane-3α,17β-diol; P, protein; H, activity for 3-hydroxyhexobarbital; A, activity for 5β-androstane-3α,17β-diol.

dehydrogenation activities for the two substrates gave good agreement (Fig. 3). These results indicated that the rabbit liver cytosol contained several enzyme activities for the dehydrogenation of 5β-androstane-3α,17β-diol; however, only one of them was able to oxidize 3-hydroxyhexobarbital.

Mixed substrate method. As shown in Table 2, the dehydrogenation rate of a mixture of 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol was less than the sum of the rates of dehydrogenation of each substrate added separately. These data indicated that a single enzyme is responsible for the oxidation of the two substrates [11].

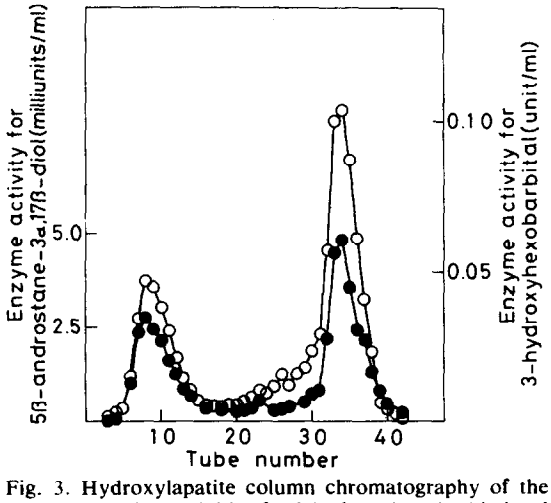

Fig. 3. Hydroxylapatite column chromatography of the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol. TEAE fraction (tubes 30–37) was applied to a hydroxylapatite column (1.5 × 10 cm). The column was eluted with a linear gradient of 150 ml each of elution buffer, from 20 mM to 100 mM and 6 ml fractions were collected. ○, activity for 3-hydroxyhexobarbital; ●, activity for 5β-androstane-3α,17β-diol.

Effects of thermal treatment and p-*chloromercuribenzoate.* Table 3 shows that the enzyme activity for 5β-androstane-3α,17β-diol was lost by thermal treatment to the same degree as that for 3-hydroxyhexobarbital at each temperature tested. The ratios of the enzyme activity for 3-hydroxyhexobarbital to that for 5β-androstane-3α,17β-diol gave the almost constant values of 1.3–1.4, at every temperature examined.

The enzyme activities for the two substrates were inhibited by *p*-chloromercuribenzoate noncompetitively. The K_i values calculated from Lineweaver–Burk [12] plots were very similar for the two substrates: 3.5 μM for 3-hydroxyhexobarbital and 3.2 μM for 5β-androstane-3α,17β-diol.

Inhibition of 3-hydroxyhexobarbital dehydrogenase by 5β-androstane-3α,17β-diol. 3-Hydroxyhexobarbital dehydrogenase was inhibited by 5β-androstane-3α,17β-diol competitively (Fig. 4). This result indicated that both compounds bound to the same site of the enzyme. The K_i value for 5β-androstane-3α,17β-diol was 23 μM.

Other properties. As shown in Table 4, the K_m value for 5β-androstane-3α,17β-diol was about one-sixth of that of 3-hydroxyhexobarbital. Therefore, the enzyme has higher affinity for the former compound than the latter one. The K_m values for NAD and NADP with 5β-androstane-3α,17β-diol were one-tenth of those with 3-hydroxyhexobarbital. The reason the K_m values for pyridine nucleotides differ greatly between the two substrates is unknown. The lower K_m values for the steroid substrate may have some physiological significance. Maximal velocities for 5β-androstane-3α,17β-diol were one-eighteenth with NAD and one-third with NADP in comparison with those for 3-hydroxyhexobarbital. In contrast to 3-hydroxyhexobarbital, 5β-androstane-3α,17β-diol gave a higher activity with NADP than NAD.

Table 2. Mixed substrate method*

Substrate	Concentration	NAD(P)H formed with NAD	NAD(P)H formed with NADP
	mM	nmoles/ml/20 min	
α-3-Hydroxyhexobarbital	0.1	59.0	28.1
5β-Androstane-3α,17β-diol	0.025	10.1	15.9
Combined		55.1	28.9

* Assay system consisted of 0.1 ml of enzyme solution, 3 μmoles of NAD (or 0.3 μmole of NADP) and 0.2 ml of methanolic solution of substrate or 0.2 ml of methanolic solution of two substrates in 0.1 M glycine buffer, pH 9.5 (or 10.5 for NADP).

Table 3. Effect of thermal treatment on the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol*

Temperature	Activity for 3-hydroxyhexobarbital NADH formed (A)		Activity for 5β-androstane-3α,17β-diol NADH formed (B)		A/B
	nmoles/ml	%	nmoles/ml	%	
2°	9.4	100	6.8	100	1.39
40°	8.8	94	6.4	95	1.37
45°	7.4	79	5.4	80	1.37
48°	2.9	31	2.1	31	1.38
52°	0.6	7	0.5	7	1.33
55°	0	0	0	0	

* Enzyme solutions were heated at each temperature for 5 min and immediately cooled in ice. Aliquots of the enzyme solution were utilized for measurements of the dehydrogenation activities for 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol simultaneously.

DISCUSSION

It was presumed that the oxidation of 3-hydroxyhexobarbital would be catalysed by alcohol dehydrogenase (EC 1.1.1.1), because of wide substrate specificity of the enzyme including cyclic alcohols [13]. However, Toki and Tsukamoto [3, 4] established that, during purification, 3-hydroxyhexobarbital dehydrogenase obtained from rabbit liver cytosol was separated from alcohol dehydrogenase, and that horse liver alcohol dehydrogenase was unable to oxidize 3-hydroxyhexobarbital.

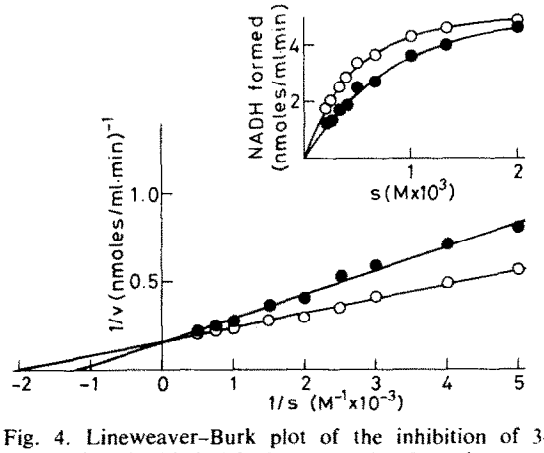

Fig. 4. Lineweaver–Burk plot of the inhibition of 3-hydroxyhexobarbital dehydrogenase by 5β-androstane-3α,17β-diol. The enzyme solution (0.1 ml) in glycine buffer, pH 9.5 (1.1 ml) was preincubated with 5β-androstane-3α,17β-diol (22.5 nmoles in 0.1 ml of methanol) or methanol (0.1 ml) for 3 min at 25°, then at intervals of 1 min NAD (3 μmoles) and 3-hydroxyhexobarbital were added to start the reaction. ○, activity without 5β-androstane-3α,17β-diol; ●, activity with 5β-androstane-3α,17β-diol.

Later, 3-hydroxyhexobarbital dehydrogenase was purified to a homogeneous protein from rabbit liver and guinea-pig liver [5, 7]. Both enzymes showed a marked difference in substrate specificity [5, 7]. The rabbit liver enzyme oxidized a wide variety of foreign alcoholic compounds (e.g. styrylmethylcarbinol, β-ionol, 1-indanol and 1-tetralol), while the substrate for the guinea-pig liver enzyme was on the other hand restricted to C_{19}-17β-hydroxysteroids. Kageura and Toki [6] demonstrated that guinea-pig liver 3-hydroxyhexobarbital dehydrogenase is identical with testosterone 17β-dehydrogenase (NADP) (EC 1.1.1.64).

The present study revealed that, in the case of rabbit liver, among various steroids, only 5β-androstane-3α,17β-diol exhibited the relatively high activity. This compound reacted at the same site of the enzyme as 3-hydroxyhexobarbital, and the enzyme attacked the 17β-hydroxyl group of 5β-androstane-3α,17β-diol. It was also shown that testosterone 17β-dehydrogenase was separated from 3-hydroxyhexobarbital dehydrogenase. Thus, the characteristic features of 3-hydroxyhexobarbital dehydrogenase are very different between two species.

5β-Androstane-3α,17β-diol is biotransformed from testosterone and this compound is inactive as an androgenic or anabolic agent [14–16]. Granick

Table 4. Comparison of the properties of 3-hydroxyhexobarbital dehydrogenase when 3-hydroxyhexobarbital and 5β-androstane-3α,17β-diol were used as substrates

	5β-Androstane-3α,17β-diol		3-Hydroxyhexobarbital	
	NAD	NADP	NAD	NADP
Optimum pH	9.5–10.2	9.8–10.5	9.5	10.5
K_m(μM) for				
Substrate	77	67	530	400
Cofactor	190	2.2	1900	22
V_{max} (μmoles/min/mg protein)	0.6	1.0	10.6	3.0

and Kappas [17] reported that steroid metabolites of 5β-H type including 5β-androstane-3α,17β-diol strongly stimulate porphyrin biosynthesis in chick embryo liver cells. However, the physiological role of 5β-androstane-3α,17β-diol is still unknown.

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