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ALDEHYDE REDUCTASE FROM RAT LIVER IS A 3α-HYDROXYSTEROID DEHYDROGENASE

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Abstract—Rat liver was previously shown to contain [V. G. Erwin and R. A. Deitrich, *Biochem. Pharmacol.* **21**, 2915 (1972)] two aldehyde reductases (EC 1.1.1.2) in addition to alcohol dehydrogenase (EC 1.1.1.1). The present paper demonstrates that one of these aldehyde reductases also catalyzes reversible reduction of 3-ketosteroids of A/B *cis* configuration. The product of reduction appears to be 3α-alcohol, rather than 3β-alcohol, the product of reduction of the same 3-ketone by rat liver alcohol dehydrogenase. This difference in the steric course of reduction may be useful for distinguishing aldehyde reductase from alcohol dehydrogenase. Since 3α-alcohols of A/B *cis* configuration are universally distributed in the bile of vertebrates, the steroid activity of aldehyde reductase may be connected with its physiological function.

At physiological pH values, reduction of biological aldehydes and ketones to alcohols can be easily accomplished by alcohol dehydrogenases (EC 1.1.1.1) which are universally distributed in animal tissues [1, 2]. In addition to alcohol dehydrogenase, mammalian tissues also contain a group of cytoplasmic enzymes with somewhat narrower substrate specificity, called aldehyde reductases (EC 1.1.1.2) distributed in brain, liver, kidney, lung, heart and other tissues [3–5]. These enzymes were named aldehyde reductases on the assumption that they catalyzed an irreversible reaction [3]. Further studies, however, have shown that aldehyde reductases, like alcohol dehydrogenase, catalyze reversible alcohol–aldehyde interconversion [4, 5]. Unlike alcohol dehydrogenase, the majority of aldehyde reductases are specific for NADPH as coenzyme, but some can also utilize NADH. Ethanol and acetaldehyde are generally not substrates. Aldehyde reductases can be further distinguished from alcohol dehydrogenases by the fact that they are not inhibited by pyrazole or isobutyramide but are susceptible to inhibitors such as barbiturates [4].

In 1972 Erwin and Deitrich [5] published a separation profile of enzymes catalyzing aldehyde reduction from livers of Sprague–Dawley rats. Employing a calcium phosphate gel–cellulose column, the enzymes catalyzing aldehyde reduction were eluted in three peaks numbered I, II and III in the order of elution. Enzyme activities were followed with *m*-nitrobenzaldehyde and propionaldehyde as substrates with NADH and NADPH as coenzymes. Peak III, active with *m*-nitrobenzaldehyde and propionaldehyde as substrates and NADH or NADPH as coenzymes, was identified as the classical alcohol dehydrogenase (EC 1.1.1.1). The enzymes eluted in peaks I and II were inactive with propionaldehyde and were identified as aldehyde reductases (EC 1.1.1.2). Peak I enzyme was specific for NADPH while peak II could utilize both NADH and NADPH.

From the data of Ris and von Wartburg [6] it appears that aldehyde reductases, like alcohol dehydrogenase, utilize longer chain and aromatic aldehydes more effectively than the corresponding short-chain compounds as substrates. Alcohol dehydrogenase (EC 1.1.1.1) from rat liver can also catalyze reduction of cyclohexanone (the simplest steroid analog) and of steroidal 3-ketones of A/B *cis* configuration [7–9]. Since alddehyde reductases seem to resemble alcohol dehydrogenases in substrate specificity [6], it appeared possible that steroids might also function as substrates for aldehyde reductases. The experiments described in this paper show that aldehyde reductase (EC 1.1.1.2) isolated by Erwin and Deitrich in peak II can catalyze reduction of cyclohexanone and is also a steroid dehydrogenase specific for the 3-keto group of steroids of A/B *cis* configuration.

MATERIALS AND METHODS

3-Keto-4-androsten-17β-ol (testosterone), 4-pregnene-3,20-dione (progesterone), 1,3,5(10)-estatriene-3-ol-20-one (estrone), 3β-hydroxy-5β-androstan-17-one (etiocholan-3β-ol-17-one), 3-keto-5β-androstan-17β-ol (5β-dihydrotestosterone), 5-pregnene-3β-ol-20-one (pregnenolone) and 3β-hydroxy-5α-androstan-17-one (epiandrosterone) were obtained from Sigma Chemical Co. St. Louis, Mo. 3α-Hydroxy-5β-androstan-17-one (etiocholanolone), 3α-hydroxy-5α-androstan-17-one (androsterone) and 3-keto-5α-androstan-17β-ol (5α-dihydrotestosterone) were obtained from Steraloids, Pawling, N.Y.

Sprague–Dawley rats were used. The livers were removed immediately after decapitation and homogenized in 0.1 M sodium phosphate, pH 7.4. The procedure of extraction, centrifugation and ammonium sulfate fractionation was described by Erwin and Deitrich [5]. Calcium phosphate gel–cellulose columns were prepared as described by Massey [10]. Whatman cellulose powder (microgranular form CC31) was washed three times with each: 0.1 M NaOH, 0.1 M HCl and deionized water. Cellulose (20 g dry weight) was suspended in 200 ml of distilled water and mixed with 100 ml of a suspension of calcium phosphate gel

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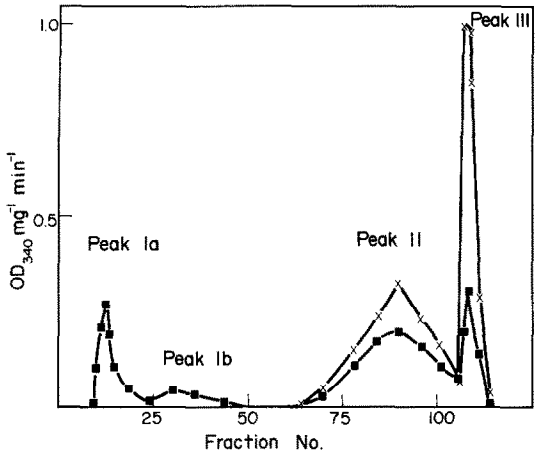

Fig. 1. Separation of enzymes reducing *m*-nitrobenzaldehyde with NADH and NADPH as coenzymes on a calcium phosphate–cellulose column. Key: (■——■) activity with *m*-nitrobenzaldehyde (1.3 mM) as substrate with NADPH (170 μM) as coenzyme; and (×——×) activity with 3-keto-5β-androstan-17β-ol (114 μM) as substrate with NADH (170 μM) as coenzyme. Fractions 1–24 eluted with 0.01 M phosphate, pH 6.0. Fractions 25–60 eluted with 0.1 M phosphate, pH 6.0. Fractions 61–106 eluted with 0.1 M phosphate, pH 6.7. Fractions 107–120 eluted with 0.1 M phosphate, pH 6.7, containing 0.5 M sodium chloride.

(Sigma Chemical Co. St. Louis, Mo.) diluted to 30 mg of solids/ml. A column (2.5 × 30 cm) of calcium phosphate gel–cellulose was prepared and washed successively with 600 ml of 0.1 M sodium phosphate, pH 6.0, and 0.01 M sodium phosphate, pH 6.0. All buffers contained 0.05 mM ethylene diamine tetraacetate and dithiothreitol (30 mg/liter). Dialyzed 40–70% saturated ammonium sulfate fraction (5 ml) containing 150–180 mg protein was placed on the column and eluted in the manner described by Erwin and Deitrich [5]. Column eluates were analyzed with NADH (170 μM) and NADPH (170 μM) as coenzymes and with acetaldehyde (1.2 mM), 3-keto-5β-androstan-17β-ol (114 μM), added to 3 ml volume in 10 μl dioxan, and *m*-nitrobenzaldehyde (1.3 mM) as substrates in 0.1 M phosphate buffer, pH 7.0. The eluted enzymes were concentrated in vacuum dialysis concentrators (Schleicher & Schuell, Inc., Keene, N.H.). Coenzyme oxidation was followed at 340 nm in a Beckman DB-GT spectrophotometer. Protein concentrations were determined by employing the method of Lowry *et al.* [11] using bovine serum albumin (Fraction V, Sigma Chemical Co.) as a primary standard.

RESULTS

Identity of enzymes in peaks I, II and III. The elution profile was the same as that obtained by Erwin and Deitrich [5] in that *m*-nitrobenzaldehyde reducing enzymes have been eluted off the calcium phosphate–cellulose column in three major peaks as shown in Fig. 1. The enzyme peaks (separated reproducibly in three experiments) could be easily distinguished by employing different substrates with NADH and NADPH as coenzymes.

In addition to being active with all substrates and coenzymes used here (see Table 1), the enzyme isolated in peak III was inhibited by pyrazole and isobutyramide, the inhibitors of alcohol dehydrogenase. Peak III, therefore, in accordance with previous observation [5], is alcohol dehydrogenase (EC 1.1.1.1).

Peaks I and II showed narrower substrate and coenzyme specificity (Table 1) and were not inhibited by pyrazole or isobutyramide at concentrations of 30 mM. Peaks I and II are, therefore, aldehyde reductases (EC 1.1.1.2).

Coenzyme and substrate specificity of peak I enzyme. In accordance with the previous report [5], the enzyme eluted in peak I has been found to be specific for NADPH as coenzyme; of the substrates used, only *m*-nitrobenzaldehyde and cyclohexanone were reduced. Activity of aldehyde reductases (EC 1.1.1.2) with cyclohexanone as substrate was never reported previously. During this investigation, it was also observed that front and tail portions of peak I were eluted in phosphate buffers of different ionic strengths: peak Ia in 0.01 M phosphate, pH 6.0, and peak Ib in 0.1 M phosphate, pH 6.0 (see Fig. 1); it

Table 1. Ratios of activity with acetaldehyde, cyclohexanone and 3-keto-5β-androstan-17β-ol with NADH and NADPH as coenzymes to the activity with *m*-nitrobenzaldehyde with NADPH as coenzyme*

		N-NADPH	N-NADH	A-NADPH	A-NADH	S-NADPH	S-NADH	C-NADPH	C-NADH
Before chromatography		1.0	2.3	0.5	4.4	0.6	3.1	†	†
Peak I (EC 1.1.1.2)	Ia	1.0	‡	‡	‡	‡	‡	0.01	‡
	Ib	1.0	‡	‡	‡	‡	‡	0.5	‡
Peak II (EC 1.1.1.2)		1.0	0.8	‡	‡	0.7	1.6	0.9	0.6
Peak III (EC 1.1.1.1)		1.0	5.5	1.4	11.2	0.6	3.1	†	11.4

* N = *m*-nitrobenzaldehyde (1.3 mM); A = acetaldehyde (1.2 mM); S = 3-keto-5β-androstan-17β-ol (114 μM); C = cyclohexanone (12.8 mM) in phosphate buffer 0.1 M, pH 7.0, with NADPH (170 μM) or NADH (170 μM) as coenzymes. All measurements were made in a Beckman DB-GT recording spectrophotometer at 25°C. The reaction was started by addition of enzyme. The specific activities with *m*-nitrobenzaldehyde as substrate and NADPH as coenzyme in μmoles NADPH oxidized/mg of protein/min were: 0.08 for peak Ia; 0.06 for peak Ib; 0.16 for peak II; and 0.08 for peak III.

† Not determined.

‡ Tests showed no activity.

appeared possible that the enzymes present in peaks Ia and Ib could be further distinguished. By employing cyclohexanone as substrate it could be demonstrated that aldehyde reductases eluted in peaks Ia and Ib were different. The ratio of cyclohexanone to *m*-nitrobenzaldehyde activity has been found to be 0.01 for peak Ia and 0.5 for peak Ib enzyme (Table 1).

The column fractions from peaks Ia and Ib were pooled and concentrated by vacuum dialysis. With NADPH as coenzyme, 3-keto-5β-androstan-17β-ol was inactive. Both peaks showed slight activity with testosterone and progesterone as substrates. The activity was, however, less than 5 per cent of that with *m*-nitrobenzaldehyde as substrate.

Steroid activity of aldehyde reductase eluted in peak II. Like peak I enzyme, aldehyde reductase eluted in peak II utilizes cyclohexanone as substrate (Table 1), the activity being of the same magnitude as that with *m*-nitrobenzaldehyde.

From the results presented in Fig. 1 and Table 1 it is evident that the activity of peak II enzyme with 3-keto-5β-androstan-17β-ol as substrate is also of the same magnitude as that with *m*-nitrobenzaldehyde. The ratio of steroid to *m*-nitrobenzaldehyde activity in various peak II fractions was constant, suggesting that the steroid and *m*-nitrobenzaldehyde activities of this peak are associated with the same enzyme. However, this does not eliminate the possibility that a contaminating steroid dehydrogenase is eluted in the same protein peak. To resolve this, comparison of reaction rates with *m*-nitrobenzaldehyde and 3-keto-5β-androstan-17β-ol as substrates separately and in a mixture was attempted after concentration of peak II enzyme by vacuum dialysis (Table 2). With NADPH as coenzyme the rate with the mixture of these two substrates was equal to the rate of either substrate used singly, suggesting that the same enzyme was catalyzing steroid and *m*-nitrobenzaldehyde reduction. With NADH as coenzyme the rate with the mixture of both substrates was greater than with *m*-nitrobenzaldehyde alone but less than with steroid alone, again suggesting that the same enzyme was catalyzing both reactions. When NADH and NADPH were used singly and in a mixture with 3-keto-5β-adrostan-17β-ol as substrate, the results obtained with the mixture were not additive, suggesting that peak II probably contains a single enzyme which can function with either coenzyme (Table 2).

Pentobarbital (4 mM) and chlorpromazine (0.2 mM) inhibited activity with 3-keto-5β-androstan-17β-ol with NADH as coenzyme 40 and 22 per cent respectively. *m*-Nitrobenzyl alcohol (1 mM) inhibited reduction of 3-keto-5β-androstan-17β-ol by 35 per cent.

Reversibility and steric course of reduction of 3-keto-5β-androstan-17β-ol. Since reduction of 3-keto-5β-androstan-17β-ol can only occur at the ketone group in position 3, the product of the reduction has to be either the corresponding 3α-alcohol or 3β-alcohol. The product of reduction of 3-keto-5β-androstan-17β-ol was not characterized directly by isolation. Instead, 3α-hydroxy-5β-androstan-17-one (50 μM) and 3β-hydroxy-5β-androstan-17-one (50 μM) were tested as substrates with NAD (500 μM) or NADP (500 μM) as coenzymes. Only 3α-alcohol was found to produce an increase of $O.D._{340}$ with both coenzymes. It appears, therefore, that 3α-alcohol rather than the corresponding 3β-alcohol is the product of the reduction of 3-keto-5β-androstan-17β-ol by aldehyde reductase isolated in peak II (Fig. 1).

Substrate specificity of peak II enzyme. The results presented in Table 3 show that the enzyme eluted in peak II is specific for steroids of the A/B *cis* configuration. A steroid ketone of A/B *trans* configuration, 3-keto-5α-androstan-17β-ol, is reduced at only *ca.* 5 per cent of the rate of reduction of 3-keto-5β-androstan-17β-ol (Table 3) with both coenzymes. When steroidal 3-alcohols were tested with NAD and NADP as coenzymes, it was observed that the A/B *trans* alcohol, 3α-hydroxy-5α-androstan-17-one, was oxidized at only 10 per cent of the rate obtained with the corresponding A/B *cis* compound, 3α-hydroxy-5β-androstan-17-one. Only negligible reaction rates were observed with testosterone and progesterone with NADPH as coenzyme. Estrone, 5-pregnene-20-one and 17-ketosteroids were inactive with NADH as coenzyme. These results indicate rather narrow substrate specificity, essentially confined to 3-ketosteroids of A/B *cis* configuration.

DISCUSSION

Although aldehyde reductases differ from alcohol dehydrogenases in molecular weight [6] and suspectibility to inhibition with pyrazole and isobutyramide [3, 4], there appears to be a considerable overlap in their substrate specificity. Aldehydes of longer chain length and aromatic aldehydes are better substrates for both enzymes than the corresponding short-chain

Table 2. Comparison of reaction rates with 3-keto-5β-androstan-17β-ol and *m*-nitrobenzaldehyde separately and in a 1:1 mixture in the presence of peak II enzyme.*

Coenzyme	*m*-Nitrobenzaldehyde (1.3 mM)	3-Keto-5β-androstan-17β-ol (114 μM)	*m*-Nitrobenzaldehyde (1.3 mM) and 3-keto-5β-androstan-17β-ol (114 μM)
NADPH (170 μM)	0.335	0.319	0.312
NADH (170 μM)	0.112	0.455	0.318
NADPH (85 μM)	†	0.293	†
NADH (85 μM)	†	0.334	†
NADPH (85 μM) and NADH (85 μM)	†	0.323	†

* The reaction was carried out in 0.1 M phosphate buffer, pH 7.0, in the presence of 10 μl dioxan/3 ml of cuvette volume. The rates of enzyme-catalyzed reactions are expressed as E/mg/min.

† Not determined.

Table 3. Steroid substrate specificity of the enzyme isolated from rat liver in peak II

Substrate	Coenzyme			
	NADH	NADPH	NAD	NADP
3-Keto-5β-androstan-17β-ol (5β-dihydrotestosterone)	100	100	*	*
3-Keto-5α-androstan-17β-ol (5α-dihydrotestosterone)	6	4	*	*
3-Keto-4-androsten-17β-ol (testosterone)	*	0.05	*	*
4-Pregnene-3,20-dione (progesterone)	*	0.2	*	*
1.3.5(10)Estratriene-3-ol-20-one (estrone)	*	†	†	†
5-Pregnene-3β-ol-20-one (pregnenolone)	*	†	†	†
3β-Hydroxy-5β-androstan-17-one (etiocholan-3β-ol-17-one)	*	†	*	*
3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	†	†	*	*
3α-Hydroxy-5β-androstan-17-one (etiocholanolone)	*	†	100	100
3α-Hydroxy-5α-androstan-17-one (androsterone)	†	†	†	10

* No activity.
† Not determined.

aldehydes (compare Refs. 2 and 6). Cyclohexanone, a classical alcohol dehydrogenase substrate [7], and 3-keto-5β-androstan-17β-ol can now be added to this common substrate list.

Activity with cyclohexanone of peak Ib and peak II enzymes is of similar magnitude to that with *m*-nitrobenzaldehyde (see Table 1), while that of peak Ia is low and may be uncertain, since only $O.D._{340}$ was measured and no products were isolated. Because of considerable difference in activity with cyclohexanone it is concluded that peaks Ia and Ib represent distinct enzymes; rat liver, therefore, contains three aldehyde reductases.

Steroid activity of peak II enzyme appears to be associated with the enzyme sites catalyzing *m*-nitrobenzaldehyde reduction. The rate of coenzyme oxidation in the presence of a mixture of *m*-nitrobenzaldehyde and 3-keto-5β-androstan-17β-ol was considerably less than the sum of rates with these substrates separately (see Table 2). Furthermore, steroid activity was inhibited by pentobarbital, chlorpromazine and *m*-nitrobenzyl alcohol. Therefore, aldehyde reductase (EC 1.1.1.2) isolated from rat liver in peak II is also a steroid reductase. In its activity with steroids it resembles alcohol dehydrogenase from rat liver and isozyme SS from the horse [9, 12]. Aldehyde reductase, isolated in peak II, like alcohol dehydrogenase is specific for steroids of A/B *cis* configuration. Steroids of A/B *trans* configuration are interconverted at rates less than 10 per cent of those with A/B *cis* compounds (see Table 3).

The steric course of reduction of 3-keto-5β-androstan-17β-ol appears, however, to be distinct from that established for alcohol dehydrogenase [9, 13]. Steroid-active alcohol dehydrogenases catalyze reduction of 3-ketosteroids of the A/B *cis* configuration to the corresponding 3β-alcohols. From our results it appears that the 3α-alcohol and not the 3β-alcohol is the product of reduction of 3-keto-5β-androstan-17β-ol by the aldehyde reductase isolated from rat liver in peak II.

Since aldehyde reductases and alcohol dehydrogenases appear to have many substrates and inhibitors in common [1, 2, 6], steric course of reduction of 3-keto-5β-androstan-17β-ol can serve as additional means of distinguishing between these enzymes.

Activity with cyclohexanone and steroids as substrates appears to be a common property for three groups of enzymes: alcohol dehydrogenases (EC 1.1.1.1), steroid dehydrogenases (EC 1.1.1.50 and EC 1.1.1.51) [14] and aldehyde reductases (EC 1.1.1.2). Alcohol dehydrogenase from horse liver also resembles steroid dehydrogenases with regard to stereospecificity with certain bicyclic compounds [15]. In view of these considerations, it appears likely that although none of the steroids tested appeared to be good substrates for peak Ia and Ib enzymes, peak I aldehyde reductases may also be steroid dehydrogenases whose substrate has not yet been identified.

The physiological significance of the steroid activity of alcohol dehydrogenase (EC 1.1.1.1) is uncertain. 3β-Hydroxysteroids of A/B *cis* configuration do not occur naturally [16] thereby suggesting that the physiological role of this enzyme is not connected with its steroid activity. 3α-Hydroxysteroids of A/B *cis* configuration, however, occur naturally and are normal metabolites. Cholic acid, which has 3α-hydroxy A/B *cis* structure, is the major bile acid component of most vertebrates. It is conceivable, therefore, that aldehyde reductase (EC 1.1.1.2), isolated here, plays some role in steroid metabolism.

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