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DIHYDRODIOL DEHYDROGENASES IN GUINEA PIG LIVER

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Abstract—Four major and four minor dihydrodiol dehydrogenases, with similar apparent molecular weights of 28,000 to 34,000 but with different charges, were purified from male guinea pig liver cytosol. One of the minor enzymes catalyzed only the oxidation of benzene dihydrodiol with a high K_m value of 5.0 mM and was identified immunologically with aldehyde reductase. The other enzymes oxidized xenobiotic alicyclic alcohols and 17β-hydroxysteroids as well as benzene dihydrodiol. These enzymes exhibited higher affinity for 17β-hydroxysteroids than for alicyclic alcohols and benzene dihydrodiol, and immunologically cross-reacted with testosterone 17β-dehydrogenase purified from the same source. Four major enzymes and one minor with K_m values for benzene dihydrodiol of about 0.2 mM, possessed specificity for 5β-androstane—17β-hydroxysteroids and dual cofactor requirement, whereas the other two minor enzymes with high K_m values of over 5 mM showed apparent NADP and 5α-androstane specificity. The dihydrodiol dehydrogenase activity was localized in the cytosol of liver. The results indicate that the hepatic oxidation of dihydrodiols in the guinea pig is mediated by cytosolic testosterone 17β-dehydrogenase isozymes and aldehyde reductase. Testosterone 17β-dehydrogenase immunologically identical to the liver enzymes was detected only in kidney, whereas aldehyde reductase was detected in all tissues of the guinea pig.

A cytosolic NADPH-dependent dihydrodiol dehydrogenase (*trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase, EC 1.3.1.20) from rat liver has been shown to decrease the mutagenic activity of benzo[*a*]pyrene [1] and the ultimate reactive dihydrodiol epoxide derivative of benz[*a*]anthracene [2] in the Ames test, and thus may play a role in the metabolic inactivation of dihydrodiol epoxides derived from polycyclic hydrocarbons. The rat liver enzyme catalyzes not only the oxidation of 3α-hydroxysteroids but also the reduction of 3-keto-steroids and aromatic carbonyl compounds [3, 4], and has been subsequently identified with 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) [5, 6]. On the other hand, four soluble dihydrodiol dehydrogenases have been purified from mouse liver, three of which exhibit 17β-hydroxysteroid dehydrogenase activity [7]. In addition, more than four forms of dihydrodiol dehydrogenase have been detected in rabbit liver cytosol and most of the rabbit enzymes do not show 3α-hydroxysteroid dehydrogenase activity [5]. These findings suggest that a variety of enzymes, which play differing physiological roles, act as dihydrodiol dehydrogenase in different species.

Recently, we reported that cytosolic aldehyde reductase (EC 1.1.1.2) and two isozymes of testosterone 17β-dehydrogenase (NADP) (EC 1.1.1.64) from guinea pig liver can catalyze the oxidation of benzene dihydrodiol [8]. However, the benzene dihydrodiol dehydrogenase activity in the liver cytosol is not precipitated completely by antisera against testosterone 17β-dehydrogenase and aldehyde reductase [8]. In the present study, we purified eight dihydrodiol dehydrogenases from guinea pig liver cytosol, and characterized their chemical, enzymatic and immunological properties to elucidate whether only testosterone 17β-dehydrogenase and aldehyde reductase are responsible for the oxidation of dihydrodiols. In addition, the distribution of the enzymes in intracellular fractions of liver and in other tissues was studied.

EXPERIMENTAL PROCEDURES

Materials. Benzene dihydrodiol was synthesized by the method of Platt and Oesch [9]. Reduced and oxidized cofactors were obtained from the Oriental Yeast Co.; steroids, phenylmethylsufonyl fluoride, pepstatin A and soybean trypsin inhibitor were from the Sigma Chemical Co.; 1-acenaphthenol, 1-indanol, cyclohex-2-en-1-ol and 1,2,3,4-tetrahydro-1-naphthol were from the Aldrich Chemical Co.; Sephadex G-100, DEAE-Sephacel, CM-Sepharose, pI markers and standard proteins for molecular weight determination were purchased from Pharmacia Fine Chemicals; peroxidase conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories; and carrier ampholytes (Ampholine) were from LKB Produkter AB. Blue-Sepharose [10] and hydroxyapatite [11] were prepared as described.

Preparation of subcellular fractions. Liver, kidney, lung, heart, brain, testis and adrenal of male albino guinea pigs (300–400 g body weight) of the Hartley strain were exised. The organs were homogenized in 4 vol. of 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 600 *g* for 10 min at 4°, and the supernatant fraction was centrifuged at 10,000 *g* for 10 min at 4° to obtain the mitochondrial fraction. The microsomal and cytosolic fractions were separated by centrifugation of the resulting

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supernatant fraction at 105,000 *g* for 1 hr at 4°. The proteins of particulate fractions were suspended at a concentration of 20 mg of protein/ml in the sucrose solution and then solubilized by being stirred in the presence of 1% Triton X-100 and 0.5 M KCl at 4° for 1 hr. The solubilized fractions and cytosol were analyzed for enzyme and protein.

Enzyme purification. A typical purification of dihydrodiol dehydrogenases starting from twenty-one guinea pig livers (300 g) is described. All procedures were performed at 0–4°, and all buffers contained 0.25 M sucrose, 5 mM 2-mercaptoethanol and 0.5 mM EDTA unless otherwise noted.

Seven fresh livers were homogenized with 3 vol. of 0.25 M sucrose containing 20 mM 6-aminohexanoic acid, 5 mM EDTA, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 mg/liter pepstatin A and 1 mg/liter soybean trypsin inhibitor, using a Waring blender. The homogenate was centrifuged for 1 hr at 105,000 *g*. The supernatant was fractionated by the addition of solid $(NH_4)_2SO_4$. The pH of the enzyme solution was adjusted to 7.5 by titration with 0.2 N NH_4OH. The 0.35 to 0.75 $(NH_4)_2SO_4$ precipitate was suspended in 30 ml of 10 mM Tris–HCl, pH 8.5, plus 0.15 M KCl and the above proteinase inhibitors and then dialyzed overnight against 20 vol. of the same buffer. The dialyzed solution was passed through a 4.6 × 100 cm Sephadex G-100 column in 10 mM Tris–HCl, pH 8.5, containing 0.15 M KCl. The enzyme activity appeared as a single peak. The enzyme fractions were combined with those similarly purified from two batches of seven fresh livers and concentrated to 50 ml through an Amicon membrane YM-10. The concentrate was dialyzed overnight against 2 liters of 10 mM Tris–HCl, pH 8.5, and applied to a 2.5 × 40 cm DEAE-Sephacel column that had been equilibrated with the same buffer. When the column was washed with this buffer, four enzyme peaks (D1–D4) appeared from the void volume, and three further enzyme peaks (D5–D7) were subsequently eluted with 20 mM and 40 mM Tris–HCl, pH 8.0, and a linear 0–0.06 M NaCl gradient (see Fig. 1A).

The fractions of D1–D4 were separately pooled and adsorbed on four 1.5 × 9 cm Blue-Sepharose columns that were equilibrated with 10 mM Tris–HCl, pH 8.5. After the columns were washed with this buffer containing 0.2 M NaCl, the enzymes were eluted with the buffer containing 0.5 M NaCl. These enzyme fractions were separately concentrated by ultrafiltration, dialyzed against 10 mM potassium phosphate, pH 7.0, and then applied to four CM-Sepharose columns (each 1.2 × 9 cm), that were equilibrated with the same buffer. Linear 0–120 mM NaCl gradients in this buffer were used to elute the enzymes; the main peaks of D1, D2, D3 and D4 appeared at NaCl concentrations of 100, 70, 50 and 40 mM respectively. The enzyme fractions were separately pooled, concentrated by ultrafiltration, and stored in an ice bath.

The enzyme fractions of D5–D7 from the DEAE-Sephacel column were separately concentrated by ultrafiltration, dialyzed against 10 mM Tris–HCl, pH 8.0, and then applied to three 1.5 × 9 cm Blue-Sepharose columns which were equilibrated with the same buffer. After the columns were washed with the buffer containing 0.1 M NaCl, D5 was eluted with 0.5 M NaCl, and D6 and D7 with 0.3 M NaCl in the same buffer. The enzyme fractions were separately concentrated by ultrafiltration, dialyzed against 10 mM Tris–HCl, pH 8.0, and then applied to three 1.2 × 5 cm hydroxyapatite columns that were equilibrated with the same buffer. While D7 was eluted out during washing of the column with the buffer, D5 and D6 were retained on the column and eluted with a linear 0–0.1 M potassium phosphate, pH 8.0, in the buffer. At this stage, D7 was purified to homogeneity. The other enzyme preparations were separately dialyzed against 10 mM potassium phosphate, pH 6.0, and purified with two CM-Sepharose columns (each 1.2 × 9 cm) equilibrated with the same buffer. After the enzyme preparations were applied to the column, D5 was eluted as a single peak in a linear 0–0.1 M NaCl gradient in this buffer, whereas D6 resolved into two peaks (D6a and D6b, see Fig. 1B). These enzyme active fractions were separately concentrated by ultrafiltration to protein concentrations of more than 1 mg/ml and stored at 4°.

Major isozymes of testosterone 17β-dehydrogenase and aldehyde reductase, which had been called aldehyde-ketone reductases 2 and 3, respectively, were separately purified from guinea pig liver as described previously [12, 13].

Enzyme assay. Dihydrodiol dehydrogenase activity was assayed fluorometrically by recording NADPH formation at 25° as described [8]. The standard reaction mixture consisted of 50 mM glycine-NaOH, pH 10.5, 1.8 mM benzene dihydrodiol, 0.25 mM NADP and enzyme in a total volume of 2.0 ml. When 1-acenaphthenol was used as a substrate, the dehydrogenase activity was determined spectrophotometrically at 340 nm. Reductase activity was also assayed fluorometrically in 2.0 ml of 80 mM potassium phosphate, pH 6.0, containing various concentrations of carbonyl compounds, 80 μM NADPH and enzyme. One unit of enzyme is defined as the amount that catalyzes reduction or oxidation of 1 μmole NADPH per min. For the study of pH dependence of the dehydrogenase activity of the enzyme, 0.1 M buffers composed of sodium phosphate (pH 6.5 to 8.0 and 11.0 to 11.8), Tris–HCl (pH 8.3 to 9.0) and glycine–NaOH (pH 9.0 to 11.0) were used. Protein concentration was determined by the method of Lowry *et al.* [14] with bovine serum albumin as the standard.

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed at pH 10.0 [15]. Sodium dodecyl sulfate (SDS*)- polyacrylamide slab gel electrophoresis was carried out according to the method of Laemmli [16]. For determining the molecular weights of the enzymes, the enzymes in 1% SDS and 1% 2-mercaptoethanol were heated for 5 min at 100° and then subjected to electrophoresis in 12.5% (w/v) polyacrylamide gel. The gels were stained with Coomassie brilliant blue R-250.

Isoelectric focusing. Isoelectric focusing on 5% (w/v) polyacrylamide disc gel containing 2% (w/v)

* Abbreviations: SDS, sodium dodecyl sulfate; testosterone, 17β-hydroxyandrost-4-en-3-one; and 5α- or 5β-dihydrotestosterone, 5α- or 5β-androstan-17β-ol-3-one.

Ampholine (pH 5–8 or pH 7–11) was performed at 4° as described [17]. Enzyme activity in the gel was detected by incubating at 25° in a mixture consisting of 50 mM Tris–HCl, pH 9.0, 9.5 μM Meldola blue, 0.18 mg/ml nitro blue tetrazolium, 0.6 mM NADP and 4 mM benzene dihydrodiol. The pH gradient formed in the gel was assessed by focusing pI markers simultaneously.

Molecular weight determination. The molecular weight of the native enzyme was estimated by high performance liquid chromatography with a TSKgel G3000SW (0.75 × 60 cm) in 10 mM potassium phosphate, pH 7.0, containing 0.15 M KCl, 5 mM 2-mercaptoethanol and 0.5 mM EDTA. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen A and cytochrome *c*.

Immunochemical methods. Antibodies against testosterone 17β-dehydrogenase and aldehyde reductase from guinea pig liver were raised in young female rabbits as described previously [13]. Immunological identity of the enzymes was analyzed by the double-diffusion techniques of Ouchterlony [18] using 1.2% agarose in 20 mM Tris–HCl, pH 7.5, containing 0.14 M NaCl. Tissue distribution of the enzymes was analyzed by immuno-blot assay with the two antibodies as the first antibody. Proteins in the cytosols from the tissues were preincubated at 100% ammonium sulfate saturation and dialyzed overnight against 20 vol. of the Tris-buffered saline before immuno-blot assay. The proteins were run on SDS–polyacrylamide gels as described above. They were electrophoretically transferred onto nitrocellulose membrane and reacted with rabbit antiserum against aldehyde reductase or testosterone 17β-dehydrogenase (1:500 dilution) in the Tris-buffered saline containing 1% (w/v) gelatin. Peroxidase conjugated goat anti-rabbit IgG (1:2,000 dilution) in the same saline was used as the second antibody, and the reaction product was stained with 4-chloro-1-naphthol.

RESULTS

Purification and purity. The soluble dihydrodiol dehydrogenase activity from guinea pig liver resolved into seven peaks (D1–D7) by DEAE-Sephacel chromatography (Fig. 1A). The four major enzymes (D1–D4), which eluted at or near the void volume of the column, were subsequently purified by chromatography on Blue-Sepharose and CM-Sepharose to remove the other overlapping enzymes (Table 1). On the other hand, the purification of the minor enzymes of D5–D7 was achieved by employing hydroxyapatite chromatography in addition to the purification procedures for D1–D4 (Table 2). D6 further resolved into two activity peaks (D6a and D6b) on CM-Sepharose chromatography, but the other enzymes appeared as a single activity peak.

The purity of the eight dihydrodiol dehydrogenase preparations was evaluated by polyacrylamide electrophoresis. All the enzymes exhibited a single protein band on disc gel electrophoresis at pH 10.0. SDS-Slab electrophoresis also resulted in a single band of the enzymes, except that D6b showed two protein bands (Fig. 2). The mobilities of D1 through D6a were the same and also identical to that of testosterone 17β-dehydrogenase which was separately purified from guinea pig liver. D7 gave a single band at the same mobility as that of aldehyde reductase from guinea pig liver.

Catalytic properties. The maximum NADP-dependent oxidation rates of benzene dihydrodiol catalyzed by D1–D5 were observed at identical pH ranges from 10.4 to 10.6. When NAD was used as the cofactor, 35–45% of the NADP-dependent activities of the five enzymes were obtained at an optimal pH of 10.0. D6a and D6b showed the same pH optimum of 10.0 to 10.2 and D7 exhibited a broad pH optimum from 8.5 to 9.5, but the NAD-dependent dehydrogenase activities of these enzymes were less than 3% of their NADP-dependent activities.

Table 1. Purification of four major forms of dihydrodiol dehydrogenase from guinea pig liver cytosol

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
105,000 *g*					
Supernatant	18,900	168	0.009	1	100
$(NH_4)_2SO_4$ fraction	13,800	177	0.013	1.4	105
Sephadex G-100	3,940	175	0.044	4.9	104
DEAE-Sephacel					
D1	206	53.9	0.262	29	32
D2	56.5	50.1	0.886	98	30
D3	181	77.9	0.431	48	46
D4	173	29.2	0.169	19	17
Blue-Sepharose					
D1	15.5	26.1	1.69	188	16
D2	7.6	28.4	3.73	414	17
D3	17.0	64.2	3.80	422	38
D4	23.0	17.9	0.775	86	11
CM-Sepharose					
D1	3.3	18.9	5.68	631	11
D2	3.1	10.2	3.29	366	6
D3	6.3	43.7	6.87	763	26
D4	3.1	9.9	3.22	358	6

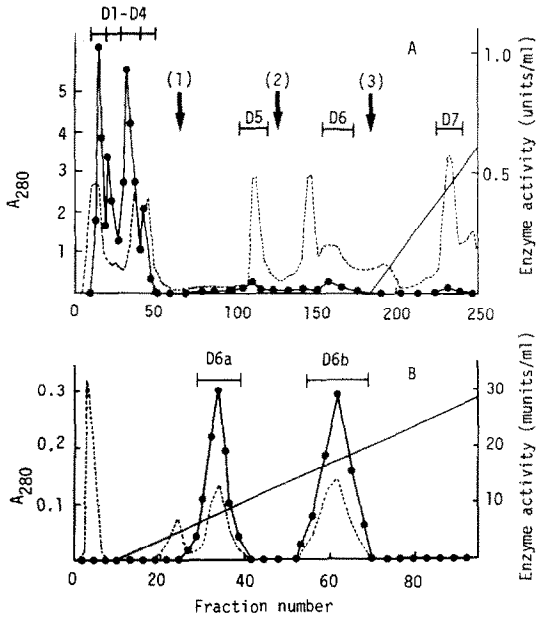

Fig. 1. Resolution of multiple forms of guinea pig liver dihydrodiol dehydrogenase by column chromatography on DEAE-Sephacel (A) and CM-Sepharose (B). The DEAE-Sephacel column was washed with the equilibration buffer described in the text, and then eluted with 20 mM Tris–HCl, pH 8.0 (1); 40 mM Tris–HCl, pH 8.0 (2); and a linear 0–0.06 M NaCl gradient (——, 3) at the positions indicated with arrows. The CM-Sepharose column was eluted with a linear 0–0.12 M NaCl gradient. The fractions were assayed for activity (●) and protein (---).

Tables 3 and 4 compare substrate specificities of the dihydrodiol dehydrogenases for various alcohols. D1 through D5 showed similar reactivities and affinities for alicyclic alcohols and 17β-hydroxysteroids, in which the enzymes oxidized 5β-dihydrotestosterone more actively than testosterone and 5α-dihydrotestosterone, whereas D6a and D6b showed high activity for testosterone and 5α-dihydrotestosterone. D6a and D6b were further differentiated by affinity for the steroidal substrates. The K_m values of D1–D6b for 17β-hydroxysteroids were lower than those for benzene dihydrodiol and 1-acenaphthenol, which suggests that the best substrates for D1 through D6b are 17β-hydroxysteroids. D7 was distinct from the other enzymes in its inability to oxidize these xenobiotic and steroidal monoalcohols.

All the dihydrodiol dehydrogenases reduced various carbonyl compounds (Table 5), except that D5 was not studied because of its small amount. D7 showed high activities for aromatic aldehydes and glucuronate, and the other enzymes reduced aromatic aldehydes and ketones and 17-ketosteroids at different rates. D1–D4 showed essentially similar substrate specificities for the compounds although their reductase activities were much lower than their dihydrodiol dehydrogenase activities, D6a exhibited high reductase activity for all the carbonyl compounds tested, and D6b efficiently reduced only aromatic aldehydes and ketones.

Comparison of other properties. An identical molecular weight of 34,000 was estimated for D1–D6a by SDS-electrophoresis; the values of the two subunits of D6b were 26,5000 and 14,500, and that of D7 (37,000) was slightly higher. Similar molecular weights of 28,000–30,000 were obtained for D1–D6b by gel permeation chromatography and that of D7 was 35,000. D7 was an acidic protein with a pI value of 6.0, whereas the other enzymes were basic proteins with different pI values: 9.2, 8.9, 8.7, 8.3, 7.8 and 8.0 for D1, D2, D3, D4, D6a and D6b, respectively; D5 further resolved into three enzyme forms with pI values of 8.5, 8.7 and 8.8 by gel-focusing analysis. The pI values of D6a and D7 were identical to those of testosterone 17β-dehydrogenase and aldehyde reductase respectively. These results suggest that the basicity of D1–D6b may cause the low molecular weights by gel permeation chromatography and that the enzymes, except D6b, are monomers.

Table 2. Purification of four minor forms of dihydrodiol dehydrogenase from guinea pig liver cytosol

Step*	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
DEAE-Sephacel					
D5	221	9.6	0.043	5	5.7
D6	169	8.2	0.049	5	4.9
D7	212	2.1	0.010	1	1.3
Blue-Sepharose					
D5	7.0	2.8	0.400	44	1.7
D6	20	4.3	0.215	24	2.6
D7	24	1.1	0.045	5	0.7
Hydroxyapatite					
D5	4.9	1.8	0.367	41	1.1
D6	8.0	2.5	0.312	35	1.5
D7	6.1	1.1	0.180	20	0.7
CM-Sepharose					
D5	0.3	0.7	2.33	259	0.4
D6a	3.0	1.2	0.400	44	0.7
D6b	2.9	0.9	0.322	36	0.5

* The purification results of the preceding steps are the same as described in Table 1.

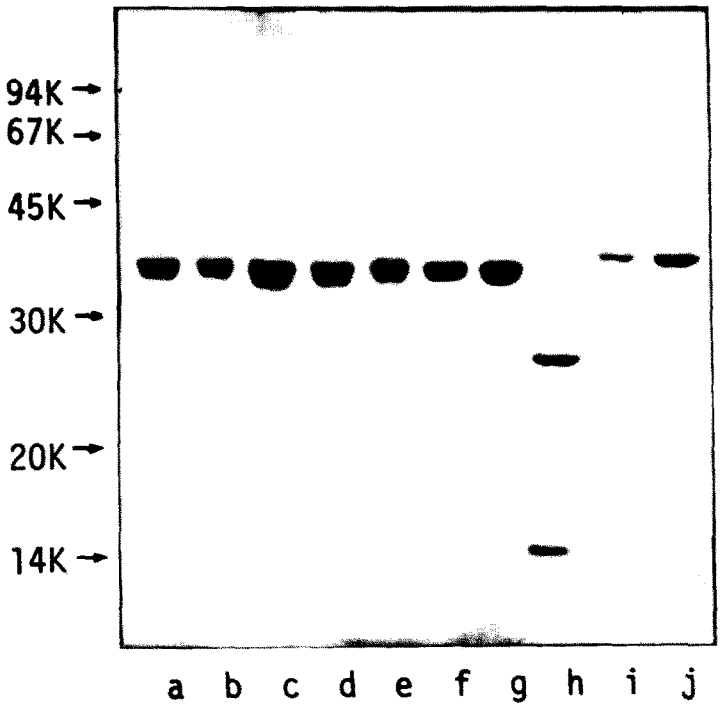

Fig. 2. Slab gel electrophoresis of the purified dihydrodiol dehydrogenases. The samples (approximately 10 μg each) were run on a 12.5% polyacrylamide gel containing 0.1% SDS. The samples were D1 (a); D2 (b); D3 (c); D4 (d); D5 (e); D6a (f); testosterone 17β-dehydrogenase (g); D6b (h); D7 (i); and aldehyde reductase (j). The arrows indicate the positions of standard proteins: phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (45K), carbonic anhydrase (30K), soybean trypsin inhibitor (20K), and α-lactalbumin (14.4K).

Since D1 through D6b catalyzed the oxidation of 17β-hydroxysteroids and reduction of 17-ketosteroids, immunoreactivity of the purified dihydrodiol dehydrogenases to the antisera against testosterone 17β-dehydrogenase and aldehyde reductase was examined to identify the enzymes with testosterone 17β-dehydrogenases (Fig. 3). The antiserum against testosterone 17β-dehydrogenase reacted to the dihydrodiol dehydrogenases, except D7, and formed two precipitin lines: one fused line was observed among D6a, D6b and the enzyme antigen, testosterone 17β-dehydrogenase, and another fused line among D1 through D5. The precipitin lines gave spur formation. These results indicate that the group of D6a and D6b and that of D1–D5 are immunologically identical within the respective groups of the enzyme and that the two groups have some common antigenic sites. On the other

Table 3. Substrate specificity of guinea pig liver dihydrodiol dehydrogenases for alicyclic and steroidal alcohols

Substrate	Conc (mM)	Relative activity* (%) D1	D2	D3	D4	D5	D6a	D6b	D7
Benzene dihydrodiol	1.8	100	100	100	100	100	100	100	100
1-Acenaphthenol	0.5	169	231	281	205	229	364	135	0
Cyclohex-2-en-1-ol	1.0	137	182	190	295	269	114	108	0
Tetralol†	1.0	65	93	88	76	80	296	87	0
1-Indanol	1.0	78	79	80	58	55	242	96	0
Cyclohexanol	1.0	23	36	33	61	44	77	87	0
Testosterone	0.05	15	21	16	24	19	563	412	0
19-Nortestosterone	0.05	10	17	13	25	19	163	117	0
5α-Dihydrotestosterone	0.05	5	8	6	8	8	730	466	0
5β-Dihydrotestosterone	0.05	69	78	72	114	94	44	112	0

* Values are relative to the benzene dihydrodiol dehydrogenase activities of the respective enzymes with specific activities described in Tables 1 and 2, and are an average of three determinations. 5α-Androstan-3α-ol-17-one, 5β-androstan-3β-ol-17-one and 17α-epitestosterone were not oxidized by the enzymes.

† 1,2,3,4-Tetrahydro-1-naphthol.

Table 4. Michaelis constants of guinea pig liver dihydrodiol dehydrogenases for representative alcohols

Substrate	Apparent K_m value* (μM)							
	D1	D2	D3	D4	D5	D6a	D6b	D7
Benzene dihydrodiol	200	190	260	290	210	7200	5200	5000
1-Acenaphthenol	230	200	280	240	—†	120	220	—
Testosterone	12	8	9	10	9	48	123	—
5α-Dihydrotestosterone	10	9	8	7	—	44	155	—
5β-Dihydrotestosterone	7	10	6	6	5	35	14	—

* Values are an average of two determinations.
† Not determined.

hand, the antiserum against aldehyde reductase formed a precipitin line only with D7.

Distribution in hepatic subcellular fractions and in other organs. About 90% of benzene dihydrodiol dehydrogenase activity in liver homogenate was recovered in the cytosol (specific activity of 16.0 ± 4.9 munits/mg protein) and less than 5% of the activity in the microsomal and mitochondrial fractions. The valid but low dihydrodiol dehydrogenase activity was also detected in cytosols from kidney, testis, adrenal, spleen, heart, lung and brain. The respective specific activities were 4.1 ± 0.5, 2.9 ± 0.7, 1.9 ± 0.4, 1.8 ± 0.6, 1.6 ± 0.2, 1.2 ± 0.3 and 0.9 ± 0.2 munits/mg protein (N = 3). When immunoperoxidase staining of nitrocellulose membrane was performed, on which proteins from the cytosols were blotted and reacted with the antiserum against aldehyde reductase or testosterone 17β-dehydrogenase, the antigenic proteins with molecular weight of 37,000 against anti-(aldehyde reductase) serum were detected in the cytosols of all organs, whereas those with molecular weight of 34,000 against anti-(testosterone 17β-dehydrogenase) serum were found only in kidney cytosol.

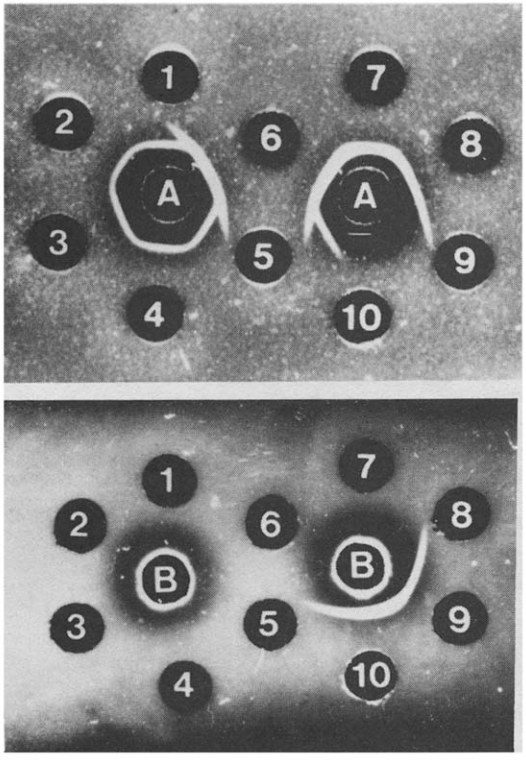

Fig. 3. Double-immunodiffusion tests of dihydrodiol dehydrogenases to antisera against testosterone 17β-dehydrogenase and aldehyde reductase. Center wells: (A) anti-(testosterone 17β-dehydrogenase) serum; (B) anti-(aldehyde reductase) serum. Peripheral wells: (1) D1; (2) D2; (3) D3; (4) D4; (5) D5; (6) testosterone 17β-dehydrogenase; (7) D6a; (8) D6b; (9) D7; and (10) aldehyde reductase.

DISCUSSION

The main object of this study was the identification of the enzymes that act as dihydrodiol dehydrogenase in guinea pig liver. The enzyme activity resolved into eight multiforms during the present purification. Of the multiforms, D7 was identified immunologically with aldehyde reductase which is consistent with our earlier finding [8]. The other seven forms of dihydrodiol dehydrogenase oxidized alicyclic alcohols and 17β-hydroxysteroids, showing a much higher affinity for 17β-hydroxysteroids than for the other alcohols and benzene dihydrodiol, and reduced xenobiotic carbonyl compounds and 17-ketosteroids. The substrate specificity of the enzyme forms resembles that of two testosterone 17β-dehydrogenase isozymes [8, 12, 19], and the immunological cross-reactivity of D1–D6b with testosterone 17β-dehydrogenase clearly indicates that the enzymes are all testosterone 17β-dehydrogenases. Based on the amount contained in the liver and pI values of the multiforms, D6a and D3 are the major and minor isozymes of testosterone 17β-dehydrogenase, respectively, described in our previous papers [8, 12, 13]. It has been shown that, in guinea pig liver cytosol, there are several forms of testosterone 17β-dehydrogenase which can be divided into two groups of the isozymes: the first group of enzymes exhibits higher activity for 5β-androstanes than for testosterone and 5α-androstanes; the second group shows a preference for testosterone and 5α-androstanes [20]. Judging from the difference in substrate specificity for 17β-hydroxysteroids of the present enzymes, D1 through D5 correspond to the first group of testosterone 17β-dehydrogenase, and D6a and D6b to the second group. The dimeric structure of D6b has been also observed in multiforms of the second group of testosterone 17β-dehydrogenase isozymes [20]. The immunological partial identity between the two isozymes has been reported [13]. Therefore, we concluded that dihydrodiol dehydro-

Table 5. Substrate specificity of guinea pig liver dihydrodiol dehydrogenases for various carbonyl compounds

Substrate*	Specific activity (units/mg)						
	D1	D2	D3	D4	D6a	D6b	D7
D-Glucuronate	ND†	ND	ND	ND	ND	ND	5.16
n-Butyraldehyde	0.21	0.14	0.20	0.12	0.24	0.06	0.61
Pyridine-3-aldehyde	0.44	0.30	0.53	0.13	0.81	0.13	9.41
4-Nitrobenzaldehyde	0.38	0.29	0.35	0.22	1.20	0.68	8.62
4-Benzoylpyridine	0.30	0.10	0.20	0.18	0.57	0.21	ND
4-Nitroacetophenone	0.12	0.08	0.18	0.16	1.64	0.25	ND
5α-Androstan-3α-ol-17-one	0.01	0.02	0.05	0.01	0.13	0.05	ND
5β-Androstan-3α-ol-17-one	0.78	0.46	0.65	0.42	1.94	0.08	ND
5β-Androstan-3β-ol-17-one	0.30	0.13	0.33	0.17	0.15	0.01	ND
5β-Androstane-3,17-dione	0.51	0.33	0.50	0.30	0.46	0.06	ND

* The concentration of the substrates was 1 mM except that the steroids were 0.05 mM concentration.

† Not detected.

genases in guinea pig liver cytosol are all testosterone 17β-dehydrogenase isozymes and aldehyde reductase, and that there is no enzyme distinct from the two enzyme species in there. The incomplete precipitation of benzene dihydrodiol dehydrogenase activity by the antisera against testosterone 17β-dehydrogenase and aldehyde reductase [8] may be due to weak binding of the antibody to D1–D5 which showed greater dihydrodiol dehydrogenase activity than D6a and D6b.

The number of multiforms (D1–D5) of the present enzyme, which corresponds to the first group of testosterone 17β-dehydrogenase isozymes, is larger than that reported by other workers [20]. Since the five proteinase inhibitors were added in the homogenization buffer, and since the multiplicity of the enzyme was observed in three different purifications from four guinea pig livers, the heterogeneity of the present enzyme may not be an artifact of the purification, nor due to a few aberrant livers. The occurrence of the multiforms of the second group of testosterone 17β-dehydrogenase isozymes has been reported to be dependent on time of initiation and duration of the extraction of the liver [20]. Since fresh livers were processed in this study, the number of dihydrodiol dehydrogenases, which correspond to this group of testosterone 17β-dehydrogenase isozymes, may be smaller than that reported previously [20].

The two isozymes of testosterone 17β-dehydrogenase, which were purified as aldehyde-ketone reductases in our previous studies [12, 13, 19], may be identical to one of D1–D5 and to D6a from their similarities in molecular weight, isoelectric point and substrate specificity for carbonyl compounds. In addition, D6a is, probably, the same enzyme as 3-hydroxyhexobarbital dehydrogenase from guinea pig liver which is a monomer with molecular weight and substrate specificity for alicyclic alcohols and 17β-hydroxysteroids similar to D6a [21]. Billings *et al.* [22] have purified indanol dehydrogenase from bovine liver and shown that the enzyme oxidizes alicyclic alcohols but not hydroxysteroids. Although testosterone derivatives with a 17-hydroxy group have not been tested as substrates for the bovine enzyme, D1 through D5 resemble the bovine enzyme in molecular weight, dual cofactor requirement, optimal pH and substrate specificity for alicyclic alcohols.

D1 through D4 were the major dihydrodiol dehydrogenases in guinea pig liver cytosol. The K_m values of D1–D4 for benzene dihydrodiol are lower than not only those of D6a, D6b and D7 but also those of dihydrodiol dehydrogenases from rat [3] and mouse [7] liver. The dehydrogenase activities of the four enzymes were much higher than their reductase activities and the enzymes utilized both NADPH and NAD as cofactors. Since the ratios of NAD/NADH and NADP/NADPH are 1164 and 0.014, respectively, in mammalian liver cytosol [23], D1–D4 may be more important in the oxidative metabolism of dihydrodiols than D6a, D6b and D7 which were essentially NADP-dependent.

Testosterone 17β-dehydrogenase immunologically identical to liver enzyme was found only in kidney of the extrahepatic organs which contained aldehyde reductase. This suggests that aldehyde reductase is involved in the metabolism of dihydrodiols in the organs that lack testosterone 17β-dehydrogenase. However, the distribution of benzene dihydrodiol dehydrogenase activity in guinea pig organs is not consistent with that of aldehyde reductase activity. Testis has higher dihydrodiol dehydrogenase activity than heart, lung and brain, whereas aldehyde reductase contents of the tissues are almost the same [24]. A dihydrodiol dehydrogenase chemically and immunologically distinct from the hepatic enzymes has been identified in testicular cytosol*, and guinea pig lung carbonyl reductase also oxidizes benzene dihydrodiol at a low rate [25]. It is likely that dihydrodiol dehydrogenase different from hepatic aldehyde reductase and testosterone 17β-dehydrogenase is present in some extrahepatic tissues.

The identification of most guinea pig liver dihydrodiol dehydrogenases with multiple forms of two testosterone 17β-dehydrogenase isozymes differs from the reports that rat liver dihydrodiol dehydrogenase is identified with 3α-hydroxysteroid dehydrogenase

* Unpublished work.

[5, 6], but is in agreement with the observation that mouse liver dihydrodiol dehydrogenase exhibits 17β-hydroxy steroid dehydrogenase [7]. These data, collectively, suggest that some hydroxysteroid dehydrogenases can act as dihydrodiol dehydrogenases, although the type of hydroxysteroid dehydrogenase differs depending on the animal species.

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