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REDUCTASES FOR CARBONYL COMPOUNDS IN HUMAN LIVER

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Abstract—Two aldehyde reductases with mol. wt 78,000 and 32,000 and one carbonyl reductase with mol. wt 31,000 were purified to homogeneity from human liver cytosol. The high molecular weight aldehyde reductase exhibited properties similar to alcohol dehydrogenase; it had a single subunit of mol. wt 41,000 and a pI value of 10 to 10.5, and showed preference for NADH over NADPH as cofactor and sensitivity to SH-reagents, pyrazole, *o*-phenanthroline and isobutyramide. The enzyme reduced aliphatic and aromatic aldehydes, alicyclic ketones and α-diketones and an optimal pH of 6.0, and oxidized various alcohols with NAD as a cofactor at an optimal pH of 8.8. The identity of the enzyme with alcohol dehydrogenase was established by starch gel electrophoresis and co-purification of the two enzymes. The other enzymes were NADPH-dependent and monomeric reductases; the aldehyde reductase reduced aldehydes, hexonates and α-diketones and was sensitive to barbiturates, diphenylhydantoin and valproate, while the carbonyl reductase showed a broad substrate specificity for aldehydes, ketones and quinones and was inhibited by SH-reagent, quercitrin and benzoic acid. The latter enzyme appeared in three multiforms with different charges which occurred in differing ratios in liver specimens. Comparison of kinetic constants for aldehydes among the enzymes indicated that alcohol dehydrogenase is the best reductase with the highest affinity and K_{cat} values. The enzyme also catalyzed oxidation and reduction of aromatic aldehydes in the presence of NAD at physiological pH of 7.2. Tissue distribution of the three enzymes and variation of their specific activities in human livers were examined.

Several xenobiotic carbonyl compounds are reduced to corresponding alcohols *in vivo* [1], and the enzymes responsible for the reduction are thought to be NAD-dependent alcohol dehydrogenase and NADPH-dependent aldo/keto reductases [2, 3]. Aldo/keto reductases have been purified from animal tissues [4–10] and are divided into two groups, aldehyde reductase and ketone reductase [11, 12]; the latter enzyme has recently been referred to as carbonyl reductase [13].

In human tissues, liver contains two aldehyde reductases which differ in molecular weight and inhibitor sensitivity [14, 15]. The low molecular weight aldehyde reductase has been characterized as having properties similar to animal aldehyde reductases [5, 14], whereas the high molecular weight enzyme purified was reported to be composed of two different subunits and to have alkaline pI and dual cofactor specificity for NADH and NADPH similar to alcohol dehydrogenase [14]. However, we have suggested that the human liver high molecular weight aldehyde reductase is alcohol dehydrogenase because of interaction of the enzyme with 4-[3-(*N*-6-aminocaproyl) aminopropyl]pyrazole-immobilized Sepharose which is a specific affinity resin for alcohol dehydrogenase and because of identity of the aldehyde reductase activity with alcohol dehydrogenase activity on isoelectric focusing [15]. In addition to the aldehyde reductases, carbonyl reductase which reduced not only various carbonyl compounds but also potassium ferricyanide and 2,6-dichlorophenol indophenol has been purified from brain [13]. Recently, the enzyme purified from liver has been shown to have substrate specificity slightly different from the brain enzyme, although it shows three heterogeneous forms similar to the brain enzyme [15]. Thus, the aldehyde reductases and carbonyl reductase are involved in reduction of xenobiotic carbonyl compounds, but the relationship between the chemical structure of the substrate and the reactivity of these enzymes and the capacity of the enzymes for reducing carbonyl compounds in a given tissue are unknown. It is necessary from a view of detoxication of carbonyl compounds to compare the substrate specificity of the enzymes from the same tissue and to study the content of the respective enzymes in human tissues. This paper describes the purification of the high-molecular weight aldehyde reductase from human liver in addition to the low-molecular weight aldehyde and carbonyl reductases to identify the enzyme with alcohol dehydrogenase and to compare substrate specificity among the three enzymes. In addition, individual differences in contents of the enzymes in human livers and their distribution in other tissues are studied. A preliminary account of this work has been presented.‡

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‡ T. Nakayama, K. Yashiro, A. Hara and H. Sawada, *J. Pharm. Dyn.* **6**, s-73 (1983).

EXPERIMENTAL PROCEDURES

Materials. Indole-3-acetaldehyde bisulfite, steroids, and proteins used as molecular weight

markers were purchased from the Sigma Chemical Co. 3,3-Tetramethyleneglutaric acid, 1-indanone, 2-indanone and phenylglyoxal were obtained from the Aldrich Chemical Co., and pyridine nucleotides from the Oriental Yeast Co. Prostaglandins (Ono Central Research Institute, Osaka, Japan), metyrapone (Ciba Pharmaceutical Co., Summit, NJ, U.S.A.), befunolol (Kaken Yaku-kako Co. Ltd., Osaka, Japan), oxisuran (Warner-Lambert Research Institute, Morris, NJ, U.S.A.), naloxone (Sankyo Co., Ltd., Tokyo, Japan), and Cibacron Blue F3GA (Ciba–Geigy, Basel, Switzerland) were provided by their manufacturers. DEAE-cellulose (DE 32) was obtained from Whatman Ltd., and Sephadex G-100, Sepharose 4B and pH carrier ampholyte were from Pharmacia Fine Chemicals. Blue-Sepharose and hydroxylapatite were prepared by the methods of Heyns and De Moor [16] and Levin [17] respectively. 4 - [3 - (*N* - 6 - Aminocaproyl)aminopropyl]pyrazole (CapGapp) was synthesized and coupled with Sepharose 4B as described by Lange and Vallee [18]. Human prostatic acid phosphatase was purified as described previously [19]. Indole-3-acetaldehyde was prepared free of $NaHSO_3$ before use by the method of Erwin *et al.* [20]. 4-Nitrobenzaldehyde and 4-nitrobenzyl alcohol were recrystallized from water. Other chemicals of reagent grade were used without further purification.

Extraction of reductase from tissues. Human livers were obtained from autopsies within 24 hr of death caused by traffic accidents, homicides, suicides without drugs and CO-poisonings. Liver, kidney, heart, lung and brain were excised from an autopsy case of a 32-year-old man. The organs were stored at −75° until used.

The organs were thawed, minced and homogenized with 4 vol. of 0.15 M KCl containing 50 mM K_2HPO_4 in a Waring blender for 2 min. The homogenates were centrifuged at 105,000 *g* for 1 hr, and the supernatant fractions were analyzed for enzyme and protein. All steps involving homogenization and centrifugation were carried out at 0–4°

Purification of high and low molecular weight aldehyde reductases. A typical experiment with a human liver (about 500 g) is described. All of the following procedures were performed at 0–4°, and all solutions contained 2 mM EDTA and 1 mM 2-mercaptoethanol.

The human liver was homogenized and centrifuged as described above. The crude extract was fractionated by adding ammonium sulfate with constant stirring and adjusting the pH to 7.5 with 0.2 N NH_4OH. The precipitate collected between 35 and 75% saturation was suspended in 80 ml of 10 mM Tris–HCl buffer, pH 8.0, and then dialyzed against the same buffer (3 liters). After 16 hr of dialysis, the dialyzed solution was centrifuged at 12,000 *g* for 20 min, and the supernatant fraction was filtrated in two batches on a 4.6 × 100 cm Sephadex G-100 column. Elution was carried out with descending conditions (40 ml/hr, 15 ml/fraction). Two peaks of aldehyde reductase activity were found as previously reported [15]. A large peak of enzyme, which was inactive towards D-glucuronate and 4-benzoylpyridine and which was designated as a high molecular weight aldehyde reductase, was eluted first (fractions 33–56). The low molecular weight aldehyde reductase and carbonyl reductase which most efficiently reduced D-glucuronate and 4-benzoylpyridine were recovered in the second peak (fractions 59–76).

The high molecular weight aldehyde reductase fractions were pooled and applied directly to a 2.6 × 40 cm DEAE-cellulose column equilibrated with 10 mM Tris–HCl buffer, pH 8.0. The enzyme was not adsorbed on the column, and the enzyme fractions in the effluent were collected. The fractions were concentrated by ultrafiltrations using an Amicon YM-10 membrane to about 10 ml, and NAD was added to a 0.37 mM concentration and applied to a 1.6 × 20 cm CapGapp–Sepharose column equilibrated with 10 mM Tris–HCl buffer, pH 7.5, containing 0.37 mM NAD. After the column was washed with 100 ml of the equilibration buffer, the enzyme was eluted with the buffer containing 0.5 M ethanol without NAD. The enzyme fractions were pooled and applied directly to a 1.6 × 15 cm hydroxylapatite column equilibrated with 10 mM Tris–HCl buffer, pH 7.5. The enzyme was eluted with 500 ml of a linear gradient from 0 to 0.1 M potassium phosphate, pH 7.5, in the Tris–HCl buffer. Fractions containing enzyme activity were pooled and applied to a 1.6 × 25 cm Blue-Sepharose column which was equilibrated and washed with 2 vol. of 10 mM Tris-HCl buffer, pH 7.5. Elutions were initiated with 100 ml of 50 mM Tris–HCl buffer, pH 8.5, containing 0.3 NaCl. The enzyme active fractions were pooled, concentrated by ultrafiltration, and stored in an ice bath.

The low molecular weight aldehyde reductase and carbonyl reductase were separated from each other by 1.6 × 15 cm Blue-Sepharose column chromatography of the second peak from the Sephadex G-100 column. The column was washed with 100 ml of 10 mM Tris–HCl buffer, pH 7.5, which was also used as an equilibration buffer on the column. Aldehyde reductase was first eluted out by rinsing the column with 50 mM Tris–HCl buffer, pH 8.5, containing 0.3 M NaCl, and then carbonyl reductase was eluted with the Tris–HCl buffer containing 1.0 M NaCl. The two enzyme fractions were separately pooled, concentrated by ultrafiltration, and dialyzed against 10 mM Tris–HCl buffer, pH 8.0.

The dialyzed concentrate of aldehyde reductase fractions was applied to a 1.6 × 15 cm DEAE-cellulose column equilibrated with 10 mM Tris–HCl buffer, pH 8.0. The enzyme was eluted with 600 ml of a linear gradient from 0 to 0.1 M NaCl in the buffer. The enzyme fractions were pooled and applied to a 1.6 × 8 cm hydroxylapatite column equilibrated with 10 mM Tris–HCl buffer, pH 8.0. The enzyme was not adsorbed on the column and appeared in the effluent. The fractions containing enzyme activity were pooled, concentrated by ultrafiltration, and stored in an ice bath. The carbonyl reductase fractions were first focused in an LKB focusing column for 40 hr. The enzyme separated into three peaks; a major peak was at pH 8.3 and minor peaks at pH 7.9 and 8.9. The pI 8.3 enzyme was further purified; the enzyme fraction was passed through a 2.6 × 90 cm Sephadex

G-25 column equilibrated with 10 mM Tris–HCl buffer, pH 7.5, and applied to a 1.6 × 10 cm DEAE-cellulose column equilibrated with the same buffer. The enzyme was eluted out during washing of the column with the equilibration buffer. The enzyme fractions were pooled and applied to a 1.6 × 8 cm hydroxylapatite column which had been equilibrated with the Tris–HCl buffer, pH 7.5. The enzyme was eluted with 300 ml of a linear gradient from 0 to 50 mM potassium phosphate, pH 7.5, in the buffer. Fractions containing enzyme activity were pooled, concentrated by ultrafiltration and stored in an ice bath.

Enzyme assay and protein determination. The reductase activity was monitored spectrophotometrically at 340 nm [8]. The standard assay mixture for the high molecular weight aldehyde reductase contained 80 mM potassium phosphate buffer, pH 6.0, 160 μM NADPH, 3.6 mM pyridine-4-aldehyde and enzyme solution, in a final volume of 2.5 ml. For assay of the activity of the low molecular weight aldehyde reductase and carbonyl reductase, D-glucuronate and 4-benzoylpyridine were used as substrate, respectively, and the concentration of NADPH was 80 μM. In separate experiments with low concentrations of NAD(P)H (less than 10 μM), the oxidation rate of the reduced cofactor was fluorometrically determined at 340 nm (excitation) and 450 nm (emission). The reactions were initiated by the addition of NAD(P)H. The reverse reaction rate of the enzymes was determined by monitoring the formation of NAD(P)H in 2.5 ml of the assay mixture consisting of 80 mM sodium pyrophosphate buffer, pH 8.8, or glycine–NaOH buffer, pH 10.0, 0.32 mM NAD(P), various concentrations of alcohols, and the enzyme solution. One unit of the enzymes was defined as the amount that catalyzed oxidation and reduction of 1 μmole of the cofactors at 25°.

For determining the effect of pH on the activity, we used the following 80 mM buffers with the indicated pH ranges: sodium citrate (pH 4.2 to 6.6), potassium phosphate (pH 5.8 to 7.8), Tris–HCl (pH 7.0 to 8.9) and glycine–NaOH (pH 7.8 to 11.0).

Alcohol dehydrogenase activity was assayed at 25° according to the method of Lange and Vallee [18]. Protein was determined by the method of Lowry *et al.* [21] with bovine serum albumin as a standard.

Product analysis. Concentrations of the oxidized and reduced products of pyridine-4-aldehyde were determined by a high performance liquid chromatograph (Waters Ltd., model 204A). The oxidation rates of NAD(P)H by the high molecular weight aldehyde reductase was first determined fluorometrically in the standard reaction mixture containing various concentrations of NAD(P) at 25°. The reaction was allowed to continue for a total incubation time of 30 min before it was terminated by adding an equal volume of acetonitrile. The mixture was allowed to stand for more than 10 min in an ice bath and centrifuged at 12,000 *g* for 10 min. The supernatant fraction was filtered through a Millipore fluorinert filter (0.5 μm). The filtrate (5–10 μl) was injected into the instrument with a μBondapak C_{18} (0.39 × 30 cm). The analyses of isonicotinic acid and 4-hydroxymethylpyridine were done with solvent A [10% acetonitrile aqueous solution containing 5 mM $(NH_4)_2HPO_4$ and 5 mM tetra-*n*-butylammonium phosphate, pH 7.5] and solvent B [10% acetonitrile aqueous solution containing 50 mM $(NH_4)_2HPO_4$, pH 7.5] as a mobile phase, respectively, to separate the product from other products, cofactors and substrate. The flow rate was 1.0 ml/min. The retention time of isonicotinic acid in solvent A was 6.8 min and that of 4-hydroxymethylpyridine in solvent B was 5.4 min. The concentrations of the products were determined by their peak heights at 254 nm. Each analysis was run twice.

Electrophoresis. Polyacrylamide gel electrophoresis at pH 8.9 or with sodium dodecyl sulfate (SDS) was performed according to the methods of Davis [22] and Weber and Osborn [23] respectively. Urea gel electrophoresis was accomplished with 10% acrylamide gels containing 8 M urea using a buffer system described by Panyim and Chalkley [24]. The gels were stained for protein with Coomassie brilliant blue R250 and for reductase activity as described by Sawada *et al.* [8]. Starch gel electrophoresis was carried out at 4° and a constant voltage of 7 V/cm by the method of Li and Magnes [25]. After 24 hr electrophoresis, the starch gel was cut horizontally into two slices. One slice was sprayed with a mixture consisting of 0.2 M potassium phosphate buffer, pH 7.0, 4.5 mM pyridine-4-aldehyde and 3 mg/ml of NADPH, and the aldehyde reductase activity bands were observed as dark spots under ultraviolet rays of 254 nm. Another slice of the gel was stained for alcohol dehydrogenase activity by spraying it with a mixture consisting of 0.1 M sodium pyrophosphate buffer, pH 8.8, 50 mM ethanol, 3 mg/ml of NAD, 0.3 mg/ml of nitrotetrazolium blue, and 0.08 mM Meldola blue.

Molecular weight determination. The molecular weights of the enzymes denatured in 1% SDS and 1% 2-mercaptoethanol were estimated by SDS-electrophoresis using bovine serum albumin (mol. wt 67,000), ovalbumin (mol. wt 45,000), trypsin (mol. wt 23,000) and myoglobin (mol. wt 17,800) as reference proteins, and those of the native enzymes were determined by Sephadex G-100 filtration in 10 mM Tris–HCl buffer, pH 7.5, containing 0.15 M KCl, 1 mM 2-mercaptoethanol, and 2 mM EDTA. The column (1.5 × 95 cm) was calibrated with Blue Dextran 2000, prostatic acid phosphatase (mol. wt 100,000), bovine serum albumin, ovalbumin and myoglobin.

Isoelectric focusing. Isoelectric focusing was carried out at 4° with the aid of an LKB 8101 electrofocusing column containing a pH gradient of 5–8, 8–10.5 or 5–10.5 in a 0–50% sucrose gradient. After electrofocusing for 40 hr at a maximum electrical output of 4 W, fractions (2.2 ml) were collected from the gradient, analyzed for pH, and assayed for reductase activity. Isoelectric focusing on polyacrylamide gel was performed as described previously [10] and the gels were stained for reductase activity and for protein as described above.

Table 1. Purification of human liver high molecular weight aldehyde reductase

Step	Protein (mg)	Aldehyde reductase* Activity (units)	Aldehyde reductase* Specific activity (units/mg)	Alcohol dehydrogenase† Activity (units)	Alcohol dehydrogenase† Specific activity (units/mg)	Ratio‡
105,000 *g* Supernatant	17650	1955	0.11	2045	0.12	0.96
35–75% Saturated ammonium sulfate	13452	2213	0.17	1660	0.12	1.33
Sephadex G-100	8465	1480	0.18	1771	0.21	0.84
DEAE-cellulose	1198	1237	1.03	1123	0.94	1.10
CapGapp-Sepharose	448	923	2.06	729	1.63	1.27
Hydroxylapatite	164	694	4.23	555	3.38	1.25
Blue-Sepharose	114	591	5.18	485	4.25	1.22

* The reductase activity was assayed in the standard reaction mixture with 3.6 mM pyridine-4-aldehyde and 160 μM NADPH.

† The dehydrogenase activity was assayed in the reaction mixture containing 80 mM pyrophosphate buffer, pH 8.8, 40 mM ethanol and 2.6 mM NAD.

‡ The ratio of aldehyde reductase activity to alcohol dehydrogenase activity.

RESULTS

Purification of high molecular weight aldehyde reductase and its identity with alcohol dehydrogenase. To test the possibility that the high molecular weight aldehyde reductase is alcohol dehydrogenase, we copurified NADPH-dependent high molecular weight aldehyde reductase with alcohol dehydrogenase activity (Table 1). The enzyme was first separated from the low molecular weight reductases on Sephadex G-100 filtration as reported previously [15]. The enzyme appeared as a single activity peak coincident with alcohol dehydrogenase activity, and the ratios of aldehyde reductase activity to alcohol

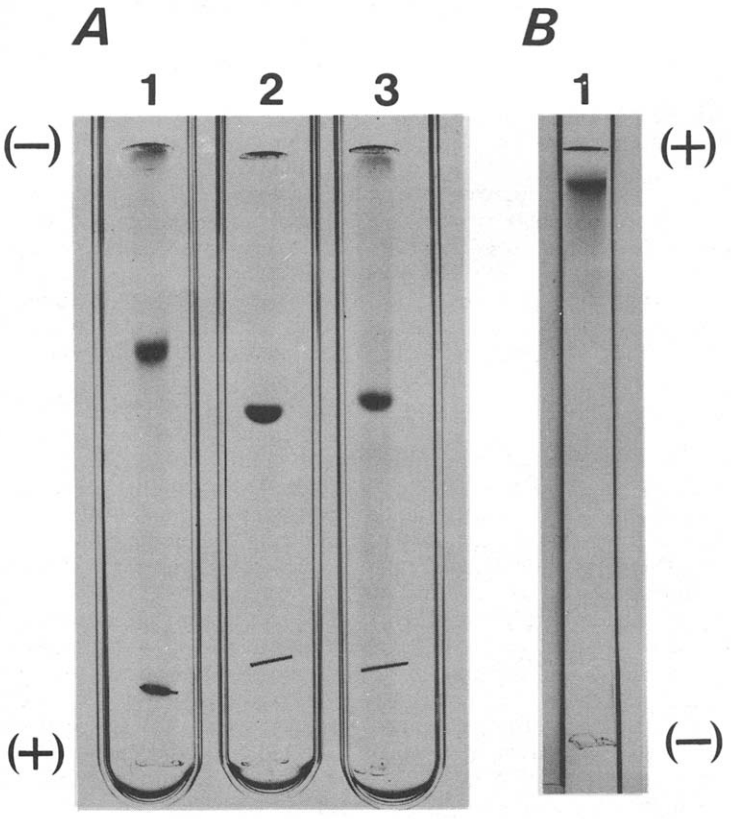

Fig. 1. Polacrylamide gel electrophoresis of the purified reductases. About 10 μg each of the high molecular weight aldehyde reductase (1), carbonyl reductase (2) and low molecular weight aldehyde reductase (3) were run in the presence of SDS (A) and urea (B), and the gels were stained for protein.

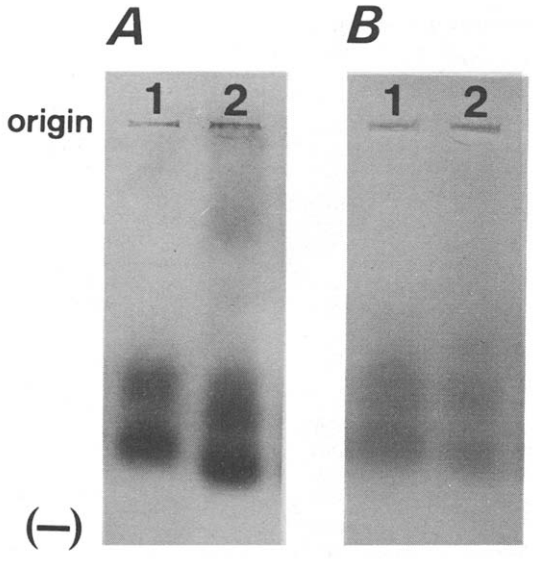

Fig. 2. Starch gel electrophoresis of the high molecular weight aldehyde reductase. The purified enzyme (1) and the enzyme preparation partially purified from DEAE-cellulose column (2) were run and stained for aldehyde reductase activity (A) and alcohol dehydrogenase activity (B).

dehydrogenase activity were constant in all subsequent purification steps. The purified enzyme showed a single protein band on SDS- and urea-gel electrophoresis (Fig. 1), although it did not enter the polyacrylamide gel by electrophoresis without SDS at pH 8.9. When being analyzed on starch gel electrophoresis, the enzyme was stained as two NADPH-dependent aldehyde reductase activity bands whose mobilities also corresponded with alcohol dehydrogenase isozymes stained on the same gel (Fig. 2). Furthermore, the activities of the NADPH-dependent aldehyde reductase and alcohol dehydrogenase were identically and broadly focused at pH ranges from 10 to 10.5.

Purification of low molecular weight aldehyde and carbonyl reductases. The enzymes were purified from the low molecular weight aldehyde reductase fraction on Sephadex G-100 chromatography of the ammonium sulfate fraction of the same human liver cytosol (Table 2). Aldehyde reductase was obtained with a good yield, but the recovery of carbonyl reductase was low. Carbonyl reductase shows three heterogeneous forms on DEAE-cellulose chromatography [15]. In this study we have improved the purification procedure by introducing isoelectric focusing to obtain more clear separation of the three forms, and the major form with pI of 8.3 was further purified. The final preparation of aldehyde reductase and carbonyl reductase gave a single protein band on electrophoresis with SDS (Fig. 1) and on gel isoelectric focusing, in which the pI value of aldehyde reductase was 5.3.

Molecular weight. The molecular weights of the high and low molecular weight aldehyde reductases and carbonyl reductase estimated by Sephadex G-100 filtration were 78,000, 32,000 and 31,000 respectively; values of 41,000, 38,000 and 37,000 respectively, were obtained by SDS-gel electrophoresis.

Catalytic properties. Substrate specificity of the three purified reductases for various carbonyl compounds was compared in Table 3. Although aromatic and aliphatic aldehydes, α-diketones and α-keto-aldehydes were common substrates for all the enzymes, their kinetic constants for the substrates differed significantly. The high molecular weight aldehyde reductase reduced aromatic and aliphatic aldehydes except for 4-carboxybenzaldehyde and

Table 2. Purification of low molecular weight aldehyde reductase and carbonyl reductase

	Low molecular weight aldehyde reductase*			Carbonyl reductase†		
Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Protein (mg)	Activity (units)	Specific activity (units/mg)
105,000 *g* Supernatant	17650	121	0.0069	17650	244	0.014
37–75% Saturated ammonium sulfate	13450	122	0.0091	13450	236	0.018
Sephadex G-100	2726	118	0.043	2726	180	0.066
Blue-Sepharose	185	108	0.58	174	103	0.59
Isoelectric focusing				14	67	4.9
DEAE-cellulose	21	99	4.7	6.8	51	7.4
Hydroxylapatite	16	82	5.2	2.7	34	12.6

* The enzyme activity was assayed with 5 mM sodium D-glucuronate as a substrate.
† The enzyme activity was assayed with 1 mM 4-benzoylpyridine as a substrate.

Table 3. Michaelis and catalytic constants for various substrates of the three reductases

Substrate	Conc (mM)	High molecular weight aldehyde reductase* NADPH K_m (mM)	NADPH k_{cat}† (sec^{-1})	NADH K_m (mM)	NADH k_{cat}† (sec^{-1})	Low molecular weight aldehyde reductase K_m (mM)	Low molecular weight aldehyde reductase k_{cat}† (sec^{-1})	Carbonyl reductase K_m (mM)	Carbonyl reductase k_{cat}† (sec^{-1})
2-Nitrobenaldehyde	1	0.024	8.7	0.021	24.2	—	(0.2)†	2.3	16.1
3-Nitrobenzaldehyde		0.024	5.7	0.022	25.1	4.9	6.1	1.1	1.4
4-Nitrobenzaldehyde		0.021	8.1	0.021	49.2	0.96	7.3	1.2	3.2
4-Carboxybenzaldehyde	10	0.083	3.1	0.14	19.4	0.07	10.7	—	(0.2)
Pyridine-3-aldehyde		0.36	4.2	2.2	39.5	1.2	9.8	15.6	4.9
Pyridine-4-aldehyde		0.036	6.8	0.24	50.0	2.6	10.5	3.7	12.9
Phenylglyoxal		0.85	6.9	1.0	26.2	0.92	10.4	7.0	7.6
Methylglyoxal		1.4	6.7	3.2	29.0	2.0	7.1	49.0	3.8
Indole-3-adetaldehyde		0.033	8.5	0.016	82.5	2.9	5.4	2.2	1.1
Acetaldehyde	10	0.016	10.5	0.037	47.3	—	(0.2)	—	0
n-Butyraldehyde	10	0.022	14.7	0.053	57.3	68.5	3.2	—	(0.1)
Chloral hydrate		4.6	2.4	5.9	8.0	142	10.4	146	0.8
DL-Glyceraldehyde	10	4.0	0.85	7.0	2.8	2.9	1.5	—	(0.1)
D-Glucuronate	10	—	0	—	0	5.1	6.8	—	0
D-Xylose	100	—	(0.1)	—	(0.1)	—	(0.2)	—	0
4-Nitroacetophenone	1	—	0	—	0	—	0	5.6	1.5
4-Benzoylpyridine	1	—	0	—	0	—	0	0.84	1.7
Metyrapone	1	—	0	—	0	—	0	—	(0.1)
Oxisuran	10	—	0	—	0	—	0	—	(6.5)
Befunolol	1	—	0	—	0	—	0	—	(0.2)
Acetone	10	—	(0.1)	—	(0.1)	—	0	—	0
Cyclohexanone	10	1.2	2.9	0.85	4.0	—	(0.1)	—	(0.6)
1-Indanone	1	—	(0.1)	—	(0.1)	—	0	—	(0.1)
2-Indanone	1	—	(0.5)	—	(0.8)	—	0	—	0
Naloxone	2	—	(0.1)	—	(0.1)	—	0	—	0
Prostaglandin E_2	1	—	0	—	0	—	0	—	(0.1)
1,4-Cylohexanedione	10	—	(0.7)	—	(1.3)	—	0	—	(0.2)
1,2-Cyclohexanedione	10	—	(0.5)	—	(0.4)	—	(0.9)	—	(1.3)
2,3-Butanedione		1.1	0.8	0.92	1.8	20.0	9.2	4.1	15.0
Benzoylacetone	1	—	(0.1)	—	(0.1)	—	(0.3)	0.94	0.8
2,5-Toluquinone	0.2	—	(0.6)	—	0	—	0	0.091	11.3
Menadione	0.2	—	0	—	0	—	0	0.063	11.7
Hydrindantin	0.1	—	(0.1)	—	(0.6)	—	(0.3)	0.26	7.4
NADPH§		0.044		—		0.005		0.010	
NADH§		—		0.029		—		—	

* The enzyme activity was assayed with both 160 μM NADPH and NADH.

† The catalytic constants were calculated assuming molecular weights of high and low molecular weight aldehyde reductases and carbonyl reductase of 78,000, 32,000 and 31,000 respectively.

‡ Values in parentheses were calculated with the activities with indicated concentrations of the substrates.

§ The activity was assayed with 3.6 mM pyridine-4-aldehyde as a substrate.

DL-glyceraldehyde at lower K_m values than the other enzymes. While the enzyme gave similar K_m values and catalytic constants (k_{cat}) for all the nitrobenzaldehydes, the low molecular weight aldehyde reductase and carbonyl reductase showed the highest values of k_{cat}/K_m and 4- and 2-nitrobenzaldehyde respectively. α-Ketoaldehydes were efficiently reduced by the two aldehyde reductases; carbonyl reductase reduced α-diketones. D-Glucuronate was reduced by only the low molecular weight aldehyde reductase, which was inactive towards other aldoses such as D-glucose, D-galactose and D-mannose tested as substrate. A clearer difference in substrate specificity was observed with the other ketones and quinones. Alicyclic ketones were slowly reduced by the high molecular weight aldehyde reductase and carbonyl reductase, and aromatic ketones and quinones by carbonyl reductase.

The high molecular weight aldehyde reductase showed higher k_{cat} values for the substrates with NADH as a cofactor than with NADPH, and its K_m value for NADH was also lower than that of NADPH. In contrast, the low molecular weight reductase and carbonyl reductase were specific for NADPH as a cofactor.

The maximal reduction rate of the high molecular weight aldehyde reductase with pyridine-4-aldehyde as a substrate and either NADH or NADPH as a cofactor was observed at pH 5.9 to 6.2, and that of

carbonyl reductase at pH 6.1, while the low molecular weight aldehyde reductase showed a more broad pH optimum of 5.8 to 6.5.

The high molecular weight aldehyde reductase oxidized not only ethanol but also the following alcohols maximally at pH 8.8 in the presence of NAD as a cofactor. The relative oxidation rates of 40 mM ethanol, 4-hydroxymethylpyridine, 2,3-butanediol, cyclohexanol and glycerol (each 10 mM) to NADPH-dependent reductase activity obtained with 3.6 mM pyridine-4-aldehyde under standard conditions were 96, 71, 45, 36, and 9% respectively. However, no NADPH formation was observed with NADP as a cofactor. In contrast, the low molecular weight aldehyde reductase and carbonyl reductase oxidized glycerol and cyclohexanol of the above alcohols, respectively, with NADP as a cofactor at pH 10.0, but the rates were less than 2% of the reductase activity of the respective enzymes.

Inhibition studies. The inhibitor sensitivity of the three enzymes was compared under the same conditions (Table 4). The effects of SH-reagents, barbiturates, diphenylhydantoin, quercitrin, 3,3-tetramethyleneglutaric acid, lithium sulfate, and sodium chloride on the enzymes were essentially the same as reported with high and low molecular weight aldehyde reductases from the same tissue and carbonyl reductase from human brain by other workers [5, 13, 14]. However *o*-phenanthroline and pyrazole, which are known as inhibitors of alcohol dehydrogenase [26], and NAD of the oxidized cofactors greatly inhibited only the high molecular weight aldehyde reductase.

Since the physiological ratios of NAD/NADH and NADP/NADPH in liver cytosol are 100–1000 and 0.02, respectively [27], the effects of concentrations of NAD and NADP on both NADH- and NADPH-dependent activity of the high molecular weight aldehyde reductase were determined by measuring the decrease of fluorescence of the reduced cofactors (Fig. 3A). The NADH-dependent activity was gradually inhibited with increasing concentrations of NAD, while the NADPH-dependent activity showed greater inhibition by NAD. However, when the reaction products were analyzed by high performance liquid chromatography, we found that the reaction mixture with NADH as a cofactor contained two products; one was the reduced product, 4-hydroxymethylpyridine, and the other was the oxidized product, isonicotinic acid. The rate of formation of the two products was increased with elevating ratios of NAD/NADH and the amounts of the two products exceeded that of the oxidized NADH calculated from the decreasing rate of fluorescence of the cofactor, but formation of the two products was inhibited almost completely by the addition of pyrazole (Fig. 3B). In contrast, only 4-hydroxymethylpyridine was formed in the reaction mixture with NADPH as a cofactor and the mole ratio of 4-hydroxymethylpyridine formed to NADPH oxidized was almost 1:1. No formation of the oxidized product was observed even in the reaction mixture containing the ratio of NADP/NADPH of 20. It should be noted that the enzyme also produced 4-hydroxymethylpyridine and isonicotinic acid at rates of 270 and 340 nmoles/min, respectively, when incubated with 2 mM NAD but without the reduced cofactors, which suggests that NAD is a predominant cofactor for the high molecular weight aldehyde reductase to form the two products.

Tissue distribution. The tissue contents of the high and low molecular weight aldehyde reductases and carbonyl reductase in cytosols of several tissues from one man were measured with substrates and cofactors specific for the respective enzymes (Table

Table 4. Comparison of susceptibility to various inhibitors among high and low molecular weight aldehyde reductases and carbonyl reductase*

Inhibitor	Conc (mM)	Inhibition (%)		
		High molecular weight aldehyde reductase	Low molecular weight aldehyde reductase	Carbonyl reductase
$HgCl_2$	0.001	74	28	97
$CuSO_4$	0.01	99	82	78
Quercitrin	0.01	26	82	83
p-Chloromercuribenzoate	0.1	98	45	99
Diphenylhydantoin	0.1	0	89	13
NADP	1	22	58	97
NAD	1	92	1	0
o-Phenanthroline	1	92	31	20
3,3-Tetramethyleneglutarate	1	0	94	62
Valproate	1	11	88	6
Barbital	5	8	88	0
Phenobarbital	5	7	95	8
Pyrazole	10	80	5	6
Isobutyramide	10	35	11	23
Li_2SO_4	100	94	17	1
NaCl	100	76	31	0

* After the enzymes were added to the incubation mixture containing 80 mM potassium phosphate buffer, pH 6.0, 3.6 mM pyridine-4-aldehyde and inhibitors, the reactions were immediately initiated by the addition of 80 μM NADPH.

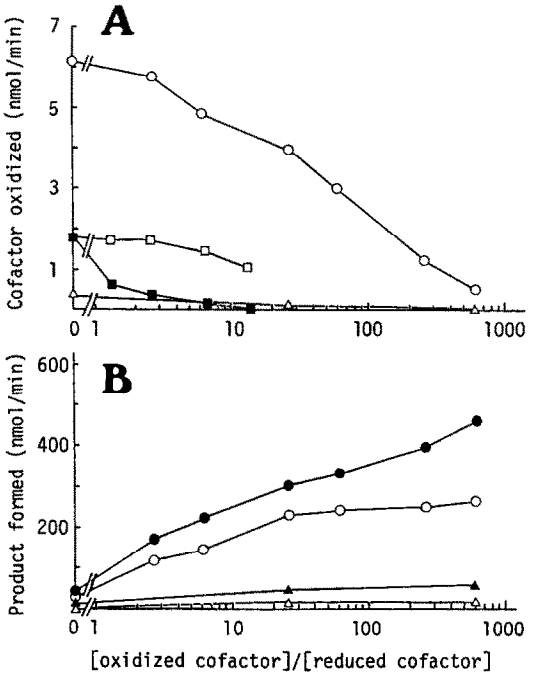

Fig. 3. Effect of oxidized cofactor on pyridine-4-aldehyde reductase activity of the high molecular weight aldehyde reductase and on formation of the oxidized and reduced products. The reaction was initiated by adding 0.1 ml of 12 μg/ml of the purified enzyme diluted with 2% bovine serum albumin in 10 mM potassium phosphate buffer, pH 7.2, to 1.9 ml of the mixture consisting of 100 mM potassium phosphate buffer, pH 7.2, 10 mM pyridine-4-aldehyde and cofactors in the absence (○, ●, □, ■) or presence (△, ▲) of 10 mM pyrazole. The NADH and NADPH concentrations were kept at 4 and 380 μM, respectively, and oxidized cofactor concentrations ranging from 0 to 4.7 mM were added. (A) Oxidation of NADH and NADPH. The rates were assayed fluorometrically in the reaction mixtures containing various ratios of NAD/NADH (○, △), NADP/NADPH (□) or NAD/NADPH (■). (B) product analysis. The reduced product, 4-hydroxymelthylpyridine (□, △), and the oxidized product, isonicotinic acid (●, ▲), in the reaction mixtures containing various ratios of NAD/NADH were determined by high performance liquid chromatography.

Table 5. Tissue distribution of three reductases

Tissue*	Activity (units/g of tissue)		
	High molecular weight aldehyde reductase†	Low molecular weight aldehyde reductase	Carbonyl reductase
Liver	20.1	0.38	0.50
Kidney	0.16	0.43	0.22
Brain	0.07	0.06	0.26
Lung	1.10	0.08	0.06
Heart	<0.01	0.04	0.05

* The tissues were obtained from an autopsy case of a 32-year-old man.

† High moleculat weight aldehyde reductase activity was assayed in the standard reaction mixture except that 160 μM NADH replaced NADPH.

5). The high molecular weight aldehyde reductase was predominantly distributed in liver, but the low molecular weight aldehyde reductase activity was high in liver and kidney and the carbonyl reductase activity in liver, kidney and brain.

Individual difference. The activities of the high and low molecular weight aldehyde reductases and carbonyl reductase in liver cytosols from each of three men and women were measured (Table 6). The specific activity of the low molecular weight aldehyde reductase was constant in all specimens, but those of the other enzymes varied greatly. In addition, carbonyl reductase from four specimens was fractionated by ammonium sulfate, chromatographed on a Sephadex G-100 column and subsequently on a Blue-Sepharose column, and the partially purified carbonyl reductase fractions were analyzed on isoelectric focusing. Although a pI 8.3 peak of carbonyl reductase appeared as a major form in all specimens, the occurrence and contents of the other forms at pH 7.9 and 8.9 differed markedly (Fig. 4). On polyacrylamide gel focusing, these isoelectric patterns were not altered in the presence or absence of 10 mM 2-mercaptoethanol.

Table 6. Specific activities of high and low molecular weight aldehyde reductases and carbonyl reductase in liver specimens

Specimen (age, sex)	Specific activity*		
	High molecular weight aldehyde reductase†	Low molecular weight aldehyde reductase	Carbonyl reductase
1. 32, M	248	4.6	6.2
2. 40, M	224	7.3	8.5
3. 51, M	180	6.5	21.7
4. 35, F	302	5.8	26.4
5. 40, F	358	9.4	14.5
6. 70, F	19.7	4.5	11.1

* The activity of liver cytosols was assayed and is expressed as munits/mg of cytosol protein.

† High molecular weight aldehyde reductase activity was assayed in the standard mixture except that 160 μM NADH replaced NADPH.

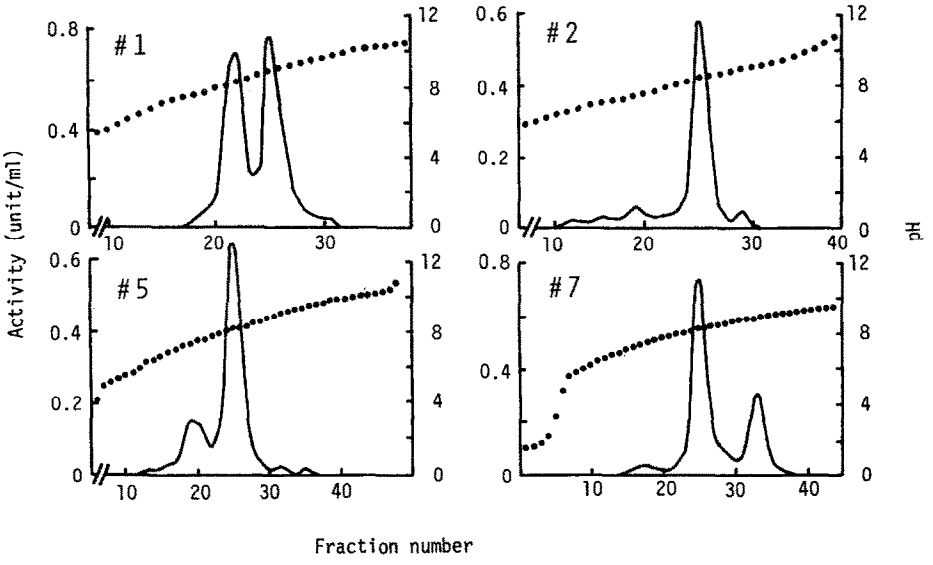

Fig. 4. Isoelectric focusing analyses of multiforms of carbonyl reductase in human liver autopsies. The numbers of the specimens are the same as those indicated in Table 6, except that No. 7 specimen was obtained from an autopsy of a 26-year-old woman. Carbonyl reductases from the livers were partially purified on chromatography with Sephadex G-100 and Blue-Sepharose and focused in pH 5 to 10.5 gradient. The enzyme activity and pH are expressed as solid and dashed lines respectively.

The three forms of the enzyme obtained from the different liver specimens showed almost the same substrate specificity and inhibitor sensitivity (Table 7). These analyses were performed with the enzyme preparations partially purified by Blue-Sepharose chromatography, because the preparations obtained from ammonium sulfate fractionation or Sephadex G-100 filtration gave one major peak at about pH 6.0 and very weak peaks around pH 7.0 to 9.0. It should be noted that the enzyme preparations from the Blue-Sepharose column were also focused around pH 6.0 when incubated with 0.1 mM NADPH for 1 hr at 0° and focused in the presence of 0.1 mM NADPH, but their pI values were not altered by pretreatment with 10 mM 2-mercaptoethanol. These results suggest that pI values of carbonyl reductase

Table 7. Substrate specificity and inhibitor sensitivity of the multiforms of human liver carbonyl reductase

	Conc (mM)	Relative activity* (%) pI 7.9	pI 8.3	pI 8.9
Substrate				
Pyridine-4-aldehyde	10	100	100	100
Pyridine-3-aldehyde	10	30	30	25
4-Nitrobenzaldehyde	1	29	25	21
4-Benzoylpyridine	1	71	74	77
Menadione	0.36	89	92	100
Potassium ferricyanide	1	0	0	0
Cytochrome c	0.05	0	0	0
2,6-Dichlorophenol indophenol	0.05	0	0	0
Inhibitor†				
Quercitrin	0.01	26	17	19
Benzoic acid	1	29	22	31
3,3-Tetramethyleneglutarate	1	39	38	37
o-Phenanthroline	1	86	80	83

* The activities with the substrates are relative to that with 10 mM pyridine-4-aldehyde as a substrate but without inhibitors.

† Effect of inhibitor was measured with 10 mM pyridine-4-aldehyde as a substrate.

were changed by binding to the cofactor as reported with human brain carbonyl reductase [13], and that the enzyme may be present as a complex of enzyme and cofactor in liver cytosol.

DISCUSSION

We have presented evidence here which confirms our previous suggestion that the high molecular weight aldehyde reductase is alcohol dehydrogenase [15]. First, the high molecular weight aldehyde reductase was accompanied by alcohol dehydrogenase activity through the purification process and they were not separated until the enzyme obtained was homogeneous on SDS-gel electrophoresis. As alcohol dehydrogenase shows several isozymes on starch gel electrophoresis [26, 28], the present enzyme preparation exhibited two electrophoretical alcohol dehydrogenase bands whose mobilities were coincident with those of aldehyde reductase activity bands. Second, properties of the enzyme including molecular weight, subunit structure, pI, substrate specificity for aliphatic aldehydes and alicyclic ketones and sensitivity to pyrazole, *o*-phenanthroline and isobutyramide are almost the same as those of alcohol dehydrogenase [2, 26]. Finally, the specific activity of the present enzyme in ethanol oxidation was 4.2 units/mg which is also comparable to the values of alcohol dehydrogenase purified by other workers [2, 26, 28] and its optimal pH of 8.8 in the ethanol oxidation is identical with that of an atypical type of alcohol dehydrogenase which is mainly found in liver of Orientals [28]. Therefore, we have concluded that the enzyme purified as the high molecular weight aldehyde reductase is an alcohol dehydrogenase. Human liver alcohol dehydrogenase has been shown to reduce biogenic aldehydes and aromatic aldehydes as well as aliphatic aldehydes and alicyclic ketones with NADH as a cofactor [2, 29]. In this study the enzyme also reduced various aldehydes and α-diketones with both NADPH and NADH as a cofactor, although the maximal velocity of NADPH-dependent activity was lower than that of NADH-dependent activity. The cofactor and substrate specificity of the present enzyme is similar to that of the high molecular weight aldehyde reductase purified by Petrash and Srivastava [14], who have suggested that the enzyme is different from alcohol dehydrogenase. The present enzyme has the electrophoretic mobility at pH 8.9 or in the presence of urea, molecular weight, optimal pH and inhibitor sensitivity reported for the enzyme purified by Petrash and Srivastava [14], but differs in subunit structure. However, Petrash and Srivastava have not characterized their enzyme with respect to reverse reaction with ethanol and sensitivity to alcohol dehydrogenase inhibitors, which would allow further comparison with the present enzyme. The high molecular weight aldehyde reductase preparations purified from four more liver specimens showed properties similar to alcohol dehydrogenase and gave a single subunit with molecular weight of 40,000, which suggests that at least in Japanese liver there is no high molecular weight aldehyde reductase which is composed of different subunits.

The low molecular weight aldehyde reductase exhibited almost the same physical and enzymatic properties as an aldehyde reductase purified previously [5, 14]. Carbonyl reductase can be also regarded as a multiform of aldehyde reductase with respect to its ability to reduce aldehydes, although it differs from the low molecular weight aldehyde reductase by the other properties. A simple nomenclature of aldehyde reductases has been proposed recently [30]. Judging from the substrate specificity and inhibitor sensitivity for the two enzymes, the low molecular weight aldehyde reductase and carbonyl reductase are identical with the temporary nomenclature of aldehyde reductase 1 (EC 1.1.1.2) and aldehyde reductase 3 (unclassified) respectively.

Comparison of substrate specificity of the purified three enzymes and their amounts in liver seems to indicate that alcohol dehydrogenase is the major enzyme in the reductive metabolism of xenobiotic aldehydes. Although the function as a reductase of alcohol dehydrogenase *in vivo* has been doubtful because of a high ratio of NAD/NADH in liver cytosol [27] and strong inhibition of the enzyme by NAD, it has been reported by several workers [31, 32] that alcohol dehydrogenase is capable of reducing some aldehydes in the presence of ethanol because it is converted into enzyme–NADH complex which can then reduce the aldehydes. Furthermore, Anderson and Dahlquist [33] have reported recently that horse liver alcohol dehydrogenase catalyzes the oxidation and reduction of an aromatic aldehyde, trifluorobenzaldehyde, in an *in vitro* system including the aldehyde, enzyme and NAD; they proposed a mechanism that the aldehyde is first oxidized by the enzyme as aldehyde dehydrogenase and that the produced enzyme–NADH complex then reduces the aldehyde to alcohol [33]. In the reduction of pyridine-4-aldehyde by human liver alcohol dehydrogenase, the apparent oxidation rate of NADH was inhibited by increasing concentrations of NAD as reported in the reductions of 4-nitrobenzaldehyde [31] and chloral hydrate [32] by rat liver alcohol dehydrogenase, but the oxidized and reduced products were observed in the reaction media. Since NAD was an essential cofactor required for the formation of the two products by human liver alcohol dehydrogenase, the enzyme may catalyze both reactions by the same mechanism as that proposed with horse liver alcohol dehydrogenase. We have also observed that almost equal amounts of the oxidized and reduced products of pyridine-4-aldehyde are formed when the aldehyde and NAD were incubated in the homogenate of human liver and that the formation was inhibited to 25% by the addition of 10 mM pyrazole (unpublished data), which suggests the possibility that alcohol dehydrogenase might be involved in the reductive metabolism of xenobiotic aldehydes even in the absence of ethanol.

Aldehyde reductase and carbonyl reductase were NADPH-dependent enzymes and may always operate as reducing enzymes because of a low ratio of NADP/NADPH [27]. Aldehyde reductase showed a higher affinity and k_{cat} values for most of the aldehydes than carbonyl reductase and it reduced

more efficiently some aldehydes such as 4-carboxybenzaldehyde and D-glucuranate than did alcohol dehydrogenase. In contrast to alcohol dehydrogenase, aldehyde reductase activity was detected in all extrahepatic tissues. Therefore, aldehyde reductase may be the important reducing enzyme for aldehydes, especially in the extrahepatic tissues in which alcohol dehydrogenase activity is low or absent. On the other hand, carbonyl reductase reduced quinones and ketones which were not substrates for the other two enzymes. The substrate specificity of liver carbonyl reductase is similar to that of brain carbonyl reductase [13]. The liver enzyme also showed essentially the same molecular weight, pI values, and inhibitor sensitivity as the brain enzyme, but the liver enzyme lacks the ability to reduce potassium ferricyanide and 2,6-dichlorophenol indophenol which are good substrates for the brain enzyme. In addition to the difference in the NADPH-diaphorase activity, the relatively high specific activity of the enzyme in brain may suggest a specific function beside drug metabolism in the tissue.

Specific activities of the high molecular weight aldehyde reductase and carbonyl reductase in human livers varied markedly, while aldehyde reductase activity was relatively constant. The variation of high molecular weight aldehyde reductase activity is consonant with that of alcohol dehydrogenase activity in the tissue autopsies reported by Li [34], who suggested that the variation is due to the health of the individuals before death and the presence of a labile component in the enzyme activity. The same explanation might be possible in carbonyl reductase, since the variation of specific carbonyl reductase activity was not related to other factors such as age and sex. However, the occurrence of multiforms of the enzyme differed in the individuals. The three multiforms with different pI values had almost the same molecular weight, substrate specificity and inhibitor sensitivity, and the properties of the liver enzyme quite resemble those of brain carbonyl reductase, in which heterogeneity has been thought to be due to small structural modification [13]. Further investigation is needed to establish whether the heterogeneity is caused by post-translational and/or post mortem modification or is genetically determined.

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