Biochemical Pharmacology, Vol. 43, No. 11, pp. 2453–2469, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

IDENTIFICATION OF HUMAN LIVER ALDEHYDE DEHYDROGENASES THAT CATALYZE THE OXIDATION OF ALDOPHOSPHAMIDE AND RETINALDEHYDE*

PATRICIA A. DOCKHAM, MI-OCK LEE and NORMAN E. SLADEK†
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

(*Received* 8 *October* 1991; *accepted* 18 *February* 1992)

Abstract—Biotransformation of the biologically and pharmacologically important aldehydes, retinaldehyde and aldophosphamide, is mediated, in part, by NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of the aldehydes to their respective acids, retinoic acid and carboxyphosphamide. Not known at the onset of this investigation was which of the several known human aldehyde dehydrogenases (ALDHs) catalyze these reactions. Thus, human liver aldehyde dehydrogenases were chromatographically resolved and the ability of each to catalyze the oxidation of retinaldehyde and aldophosphamide was assessed. Only one, namely ALDH-1, catalyzed the oxidation of retinaldehyde; the K_m value was 0.3 μM. Three, namely ALDH-1, ALDH-2 and succinic semialdehyde dehydrogenase, catalyzed the oxidation of aldophosphamide; K_m values were 52, 1193, and 560 μM, respectively. ALDH-4, ALDH-5 and betaine aldehyde dehydrogenase did not catalyze the oxidation of either aldophosphamide or retinaldehyde. ALDH-1 and succinic semialdehyde dehydrogenase accounted for 64 and 30%, respectively, of the total hepatic aldehyde dehydrogenase-catalyzed aldophosphamide (160 μM) oxidation. ALDH-1-catalyzed oxidation of aldophosphamide was noncompetitively inhibited by chloral hydrate; the K_i value was 13 μM. ALDH-2- and succinic semialdehyde dehydrogenase-catalyzed oxidation of aldophosphamide was relatively insensitive to inhibition by chloral hydrate. These observations strongly suggest an important *in vivo* role for ALDH-1 in the catalysis of retinaldehyde and aldophosphamide biotransformation. Succinic semialdehyde dehydrogenase-catalyzed biotransformation of aldophosphamide may also be of some *in vivo* importance.

NAD(P)-linked aldehyde dehydrogenases (AHD; ALDH‡) catalyze the biotransformation of a wide variety of biologically and pharmacologically important aldehydes [1, 2]. For example, acetaldehyde, a toxic intermediate generated during ethanol metabolism, and aldophosphamide, the penultimate aldehyde metabolite of the anticancer drug cyclophosphamide, are detoxified by aldehyde dehydrogenase-catalyzed oxidation to the corresponding carboxylic acids [2–4]. Further, aldehyde dehydrogenases catalyze the oxidation (bioactivation) of retinaldehyde, a vitamin A metabolite and cleavage product of β-carotene, to retinoic acid, a potent modulator of cell growth and differentiation [5–8].

Mouse liver contains eleven aldehyde dehydrogenases (AHDs 1–3, 5, 7–13), plus an additional NAD-dependent enzyme, xanthine oxidase (dehydrogenase form), that catalyze the NAD(P)-dependent oxidation of aldophosphamide, acetaldehyde, benzaldehyde, and/or octanal to their respective carboxylic acids [9]. At least two additional aldehyde dehydrogenases (AHDs 4 and 6) are not found in liver but are present in other mouse tissues [10]. AHD-4 and nine of the hepatic enzymes catalyze the oxidation of aldophosphamide to carboxyphosphamide [9]. Xanthine oxidase (dehydrogenase form) and two hepatic aldehyde dehydrogenases catalyze the oxidation of retinaldehyde to retinoic acid [8]. AHD-2, the major cytosolic aldehyde dehydrogenase, catalyzes the bulk of the total hepatic oxidation of both aldophosphamide and retinaldehyde. Apparent homologs of some of the mouse enzymes have been found in human livers (Table 1).

Five aldehyde dehydrogenases, viz. ALDHs 1–5, that catalyze the oxidation of acetaldehyde and benzaldehyde to their corresponding acids have been identified in human livers [2, 4, 12]. Also present in this tissue are at least two relatively substrate-specific "aldehyde dehydrogenases", viz. betaine aldehyde dehydrogenase (BADH) [13] and γ-aminobutyraldehyde dehydrogenase [14]. Finally, yet another

* Descriptions of parts of this investigation have appeared in abstract form [Dockham *et al.*, *Pharmacologist* **32**: 156, 1990; Lee *et al.*, *Pharmacologist* **32**: 156, 1990] and in the proceedings of a workshop [Sladek *et al.*, In: *Advances in Experimental Medicine and Biology* (Eds. Weiner H, Wermuth B and Crabb DW), Vol. 284, pp. 97–104. Plenum Press, New York, 1990].

† Corresponding author: Dr. Norman E. Sladek, University of Minnesota, Department of Pharmacology, 3-249 Millard Hall, 435 Delaware Street S.E., Minneapolis, MN 55455. Tel. (612) 625-0691; FAX (612) 625-8408.

‡ Abbreviations: AHD, mouse aldehyde dehydrogenase; ALDH, human aldehyde dehydrogenase; MES, 2-[*N*-morpholino]-ethanesulfonic acid; pI, isoelectric point; SDS, sodium dodecyl sulfate; BADH, betaine aldehyde dehydrogenase; and SSDH, succinic semialdehyde dehydrogenase.

Table 1. Human homologs of mouse aldehyde dehydrogenases*

Mouse	Human†	Human‡	Enzyme type§
AHD-1	ALDH-4	ALDH-IV	Glutamic-γ-semialdehyde dehydrogenase
AHD-2	ALDH-1	ALDH-II	Low K_m cytosolic
AHD-3	ALDH-5	ALDH-V	Microsomal
AHD-4	ALDH-3‖	ALDH-III‖	High K_m cytosolic
AHD-5	ALDH-2	ALDH-I	Low K_m mitochondrial
AHD-9	BADH	BADH	Betaine aldehyde dehydrogenase
AHD-12	SSDH	SSDH	Succinic semialdehyde dehydrogenase

* According to Manthey *et al.* [9] and references cited therein.
† Nomenclature used in this manuscript.
‡ Nomenclature used by Manthey *et al.* [9].
§ K_m refers to the K_m obtained when acetaldehyde is the substrate.
‖ ALDH-3 may be the human homolog of AHD-4 [11] but this remains to be established.

relatively substrate-specific "aldehyde dehydrogenase", viz. succinic semialdehyde dehydrogenase (SSDH), while heretofore not reported in human liver, has been identified in human brain [15] and in mouse liver [9].

Not known is which of the human aldehyde dehydrogenases catalyze the oxidation of aldophosphamide and retinaldehyde to their corresponding acids. The present investigation was designed to identify these enzymes and to determine the relative contribution of each to the oxidation of aldophosphamide and retinaldehyde in human liver samples obtained from transplant donors.

MATERIALS AND METHODS

Materials

4-Hydroperoxycyclophosphamide was provided by Dr. Jorge Pöhl, Asta-Werke AG, Bielefeld, FRG. Acetaldehyde, benzaldehyde, octanal, all-*trans*-retinaldehyde (95%), all-*trans*-retinoic acid, tetraphenylethylene, dimethyl sulfoxide (99+%), and methyl sulfide (99+%) were purchased from the Aldrich Chemical Co., Milwaukee, WI. As judged by HPLC/spectrophotometry [8], all-*trans*-retinaldehyde was retinoic acid-free but *cis*-retinaldehyde (<1% of total) was sometimes present. NAD^+, $NADP^+$, NADH, glutathione (reduced form), pyrazole, pyridoxal HCl, betaine aldehyde, succinic semialdehyde, γ-aminobutyraldehyde diethylacetal (90%), DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone, xanthine, bovine serum albumin (crystallized and lyophilized), Lubrol®, Reactive Blue 2-Sepharose CL-6B, and silica gel (70–220 mesh) were purchased from the Sigma Chemical Co., St. Louis, MO. YM-30 ultrafiltration membranes were purchased from Amicon Division, W. R. Grace & Co., Danvers, MA. DEAE-Sephacel, 5′-AMP-Sepharose, Polybuffer Exchanger 94, Polybuffer 74, PD-10 columns, Ampholine PAGplates® (pH 3.5 to 9.5) and an isoelectric point marker kit were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ. EDTA and HPLC-grade solvents were obtained from Fisher Scientific, Ltd., Los Angeles, CA, and the latter were filtered through 0.45 μm Durapore filters (Millipore, Bedford, MA) prior to use. The Bio-Rad Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad Laboratories, Richmond, CA. Chloral hydrate was obtained from the University of Minnesota Hospital Pharmacy, Minneapolis, MN.

Aldophosphamide was generated in aqueous solution by the chemical reduction of 4-hydroperoxycyclophosphamide as described previously [9] except that purer (99+% vs 98%) methyl sulfide was used as the reducing agent. Glutamic-γ-semialdehyde was prepared from DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone as described by Mezl and Knox [16]. γ-Aminobutyraldehyde was prepared fresh daily from γ-aminobutyraldehyde diethylacetal and was partially purified by passage through a 1.0 × 6.5 cm silica gel column according to the procedure of Ambroziak and Pietruszko [17]. It was held at pH 6.5 until just before use.

Human liver samples were obtained from three Caucasian donors through the Liver Tissue Procurement and Distribution System, University of Minnesota, Minneapolis, MN. Donors A and B died as a result of injuries sustained in motor vehicle accidents. An intracerebral aneurysm lead to the death of Donor C. Age and sex of each donor are given in Table 2. All of the donors had been maintained by a life-support system until organ removal. The supplier certified all livers as nonpathological. Liver samples obtained from Donors A and B were kept at 0–4° and were delivered to us within 12 hr of donation. The former, and subcellular fractions thereof, were assayed immediately for aldehyde dehydrogenase activity, that is, without ever being frozen. A portion of the latter was homogenized immediately and submitted to centrifugation to obtain 105,000 *g* soluble and particulate fractions which were then assayed immediately for aldehyde dehydrogenase activity, that is, without ever being frozen; the remaining portion was frozen at −70° and then stored at this temperature until used. The liver sample obtained from Donor C was frozen at −70° within 4 hr of donation and was stored at this temperature until used.

Preparation of hepatic fractions

Human liver 105,000 *g* soluble, and solubilized

particulate, fractions were prepared as previously described for mouse liver [9] except that 0.3% Lubrol®, rather than 0.3% deoxycholate, was used to solubilize 105,000 *g* particulate fractions. Lubrol®-treated whole homogenates were prepared as follows. Liver was cut into 2-mm slices and homogenized in ice-cold homogenization medium (1.15% KCl solution containing 0.3% Lubrol® and 1.0 mM EDTA, pH 7.4) with a Dounce homogenizer. Homogenates (20%) were centrifuged at 105,000 *g* and 4° for 60 min. The supernatants were assayed for protein content and aldehyde dehydrogenase activity, and were submitted to anion exchange chromatography or were frozen at −70° and thawed immediately prior to chromatography or electrophoresis.

Chromatographic resolution and semipurification of human hepatic aldehyde dehydrogenases

DEAE-Sephacel, 5'-AMP-Sepharose, and chromatofocusing column chromatography were performed at 4° as described previously [9]. Dye resin affinity column chromatography with Reactive Blue 2-Sepharose CL-6B was performed at 4° utilizing a 20 cm/hr linear flow rate and a 1.5 × 8 cm column. Protein concentrations of samples loaded onto columns never exceeded 20 mg/mL and typically were much less. Elution of aldehyde dehydrogenase activity off these columns was monitored using the spectrophotometric aldehyde dehydrogenase assay described previously [9]. The NaCl concentration of selected eluate fractions was determined with an Atago refractometer. The pH of selected eluate fractions was determined with a Corning glass pH electrode. Where indicated, the volume of pooled eluate fractions was reduced using an Amicon ultrafiltration stirred-cell apparatus fitted with a YM-30 membrane and pressurized under nitrogen. Transfer of enzyme fractions from one salt/buffer solution to another was accomplished with Pharmacia PD-10 gel filtration columns. Additional details are presented in the figure legends and in Results.

Protein determination

Elution of protein from chromatography columns was routinely monitored at 280 nm with an ISCO UA-5 absorbance monitor. The protein content of crude tissue fractions and selected column pools was determined by the Coomassie Brilliant Blue dye binding assay [18] using commercially available Bio-Rad Protein Assay Reagent and bovine serum albumin as the standard.

Isoelectric focusing

Isoelectric focusing was carried out as described previously [9] except that commercially available Ampholine PAGplates® (pH 3.5 to 9.5) were used. Substrates used to visualize aldehyde dehydrogenases were acetaldehyde (4 mM), glutamic-γ-semialdehyde (200 μM), octanal (100 μM), betaine aldehyde (50 μM), and succinic semialdehyde (100 μM).

Assays for aldehyde dehydrogenase activity

Except that a Beckman DU-70 recording spectrophotometer was used to monitor the appearance of NAD(P)H at 340 nm, spectrophotometric and HPLC/spectrophotometric assays were used as described previously [8, 9] to quantify aldehyde dehydrogenase activity at 37°. The reaction mixture (1 mL, pH 8.2) contained 4 mM NAD, 32 mM tetrasodium pyrophosphate, 0.1 mM pyrazole, 5 mM glutathione, 1 mM EDTA, the substrate of interest, and crude fraction or (semi)purified aldehyde dehydrogenase. Rates expressed per g liver are per g wet weight liver. The freezing, storage and thawing conditions were such that the wet weight of liver that had been frozen did not differ from that obtained before freezing.

Kinetic analysis

Prior to kinetic analysis (as well as to isoelectric focusing), (semi)purified enzymes were first separated (when necessary) from polybuffer and then were transferred, with the aid of PD-10 columns, into 20 mM triethanolamine buffer, pH 7.4, containing 1.0 mM dithiothreitol, and 0.1 mM EDTA; subsequently, these preparations were adjusted to 25% glycerol. Typically, 100 μL of these preparations was used in the aldehyde dehydrogenase assay reaction mixture. K_m, and where indicated, V_{max}, values for the catalysis of retinaldehyde (5 μM), acetaldehyde (10 μM), octanal (10 μM), betaine aldehyde (10 μM), and succinic semialdehyde (10 μM) oxidation by ALDH-1, ALDH-2, ALDH-5, BADH, and SSDH, respectively, were determined by the integrated Michaelis method of analysis of single enzyme progress curves [19]. Double-reciprocal plots of initial rates versus substrate concentrations were used to estimate all other K_m and V_{max} values. Four to ten substrate concentrations were used to generate each pair of K_m and V_{max} values. Wilkinson weighted linear regression analysis [20] was used to fit lines to the double-reciprocal plot values. Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight line functions.

RESULTS

Aldehyde dehydrogenase activity was measured in Lubrol®-solubilized whole homogenates prepared from liver samples obtained from each of three donors (Table 2). The mean aldehyde dehydrogenase activities were 7.1, 2.1 and 0.3 μmol/min/g liver when acetaldehyde (4 mM), aldophosphamide (160 μM) and retinaldehyde (25 μM), respectively, were the substrates. A fourth sample, obtained from yet another donor and held at 0–4° for more than 36 hr after surgical removal, showed approximately 30% of the enzyme activities observed in Donors A, B and C (data not presented). As judged by isoelectric focusing patterns, all donors exhibited the normal Caucasian phenotype [2] with regard to NAD-dependent acetaldehyde-metabolizing enzymes (data not shown). Approximately 80% of the total enzyme-catalyzed oxidation of retinaldehyde to retinoic acid in each sample was NAD dependent (data not shown). Pyridoxal (1 mM), a known inhibitor of aldehyde oxidase, inhibited 44% of

Table 2. Aldehyde dehydrogenase activity in Lubrol®-solubilized whole homogenates obtained from human livers*

Donor	Age (years)	Sex	Aldehyde dehydrogenase activity (μmol/min/g liver)		
			Acetaldehyde	Aldophosphamide	Retinaldehyde
A	2.5	Male	6.4	2.4	0.3
B	22	Male	7.5	1.8	0.3
C	51	Female	7.4	2.2	0.3

* Donor tissue was held at 0–4° for less than 12 hr and assayed for enzyme activity (Donor A), or was stored at −70° for several weeks (Donors B and C), prior to assay. Livers were homogenized in the presence of 0.3% Lubrol® and the 105,000 *g* supernatant fractions prepared from these homogenates were assayed for NAD-dependent aldehyde dehydrogenase activity as described in Materials and Methods. The spectrophotometric assay was used to determine enzyme activity when acetaldehyde (4 mM) and aldophosphamide (160 μM) were the substrates. The HPLC/spectrophotometric assay was used to determine enzyme activity when retinaldehyde (25 μM) was the substrate. Values are means of two determinations.

Table 3. Aldehyde dehydrogenase activity in soluble, and solubilized particulate, fractions obtained from human livers*

Substrate	Aldehyde dehydrogenase activity (μmol/min/g liver)					
	Donor A		Donor B		Donor C	
	Soluble	Particulate	Soluble	Particulate	Soluble	Particulate
Acetaldehyde	3.7	2.6	4.6	4.1	6.1	1.1
Aldophosphamide	2.6	0.8	2.7	0.5	2.5	0.2
Retinaldehyde	0.4	0.0	0.5	0.0	ND†	ND

* Soluble, and solubilized particulate, fractions were prepared from donor livers that had been held at 0–4° for less than 12 hr (Donors A and B), or that had been stored at −70° for several weeks (Donor C), prior to cell fractionation and assay. The spectrophotometric assay was used to determine enzyme activity when acetaldehyde (4 mM) and aldophosphamide (160 μM) were the substrates. The HPLC/spectrophotometric assay was used to determine enzyme activity when retinaldehyde (25 μM) was the substrate. Values are from single, or means of duplicate, determinations.

† ND: not determined.

the retinaldehyde oxidation catalyzed by NAD-independent enzymes present in a 105,000 *g* soluble fraction prepared from Donor B liver that had never been frozen.

Aldehyde dehydrogenase activity was also measured in 105,000 *g* soluble, and solubilized particulate, fractions prepared from liver samples that had never been frozen (Donors A and B) and a sample that had been frozen (Donor C) (Table 3). In agreement with the experience of others [21], freezing apparently affected the distribution of aldehyde dehydrogenase activity between the soluble and solubilized particulate fractions. Thus, for example, about 45% of total aldehyde dehydrogenase-catalyzed acetaldehyde oxidation occurred in the particulate fraction when the liver sample had never been frozen (Donors A and B), but only about 15% did so when it had (Donor C). Isoelectric focusing analysis showed an increased presence of particulate enzymes in the soluble fraction when previously frozen liver was the source of these fractions as compared to that observed when liver samples that had never been frozen were the source (data not shown). It should also be noted that enzyme activity in the whole homogenate prepared from a liver sample that had been frozen previously (Table 2, Donor B) was substantially less than was the sum of the activity in the subcellular fractions obtained from a fresh liver sample from the same donor (Table 3, Donor B). This observation could be interpreted in several ways. One is that freezing causes some loss of enzyme activity.

In subsequent experiments, individual human liver aldehyde dehydrogenases were resolved chromatographically so that each could be examined for its ability to catalyze the oxidation of aldophosphamide and retinaldehyde. Frozen liver from Donor C was used as the starting material because of limited availability of fresh tissue. Subcellular fractionation into soluble and solubilized particulate fractions was not effected prior to column chromatography in the first part of this undertaking because preliminary isoelectric focusing experiments revealed that large amounts of enzymes known to localize in the particulate fraction were present in the soluble fraction prepared from frozen liver, *vide*

supra. Thus, liver tissue was homogenized directly in a 1.15% KCl solution containing 0.3% Lubrol® and 1 mM EDTA, pH 7.4; the homogenate was then submitted to successive ion exchange, affinity, and chromatofocusing column chromatography as described below. The substrates used to monitor the chromatographic separation included acetaldehyde, benzaldehyde, octanal, aldophosphamide, retinaldehyde, succinic semialdehyde, glutamic-γ-semialdehyde, γ-aminobutyraldehyde, and/or betaine aldehyde.

Chromatographic separation of human hepatic aldehyde dehydrogenases

DEAE-Sephacel chromatography of a Lubrol®-solubilized whole homogenate. A 105,000 *g* supernatant fraction prepared from a Lubrol®-treated liver whole homogenate was first submitted to DEAE-Sephacel column chromatography (Fig. 1A). Based on aldehyde dehydrogenase-catalyzed oxidation of the substrates used to monitor enzyme activity, the eluant was divided into seven pools.

Pools 1 and 2 each showed the highest aldehyde dehydrogenase activity with glutamic-γ-semialdehyde as the substrate (data not shown). Smaller amounts of activity were observed with succinic semialdehyde and aldophosphamide as substrates. No activity was detected in either pool with retinaldehyde as the substrate. Thirteen percent of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in Pools 1 and 2.

The enzyme(s) in Pool 3 showed a marked preference for succinic semialdehyde as the substrate; to a lesser extent, the oxidation of aldophosphamide and glutamic-γ-semialdehyde, but not that of retinaldehyde, was catalyzed by this pool. Approximately 17% of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in Pool 3.

Two closely spaced peaks of enzyme activity eluted next (Pools 4 and 5). The enzymes in each pool showed a marked preference for acetaldehyde as the substrate. These pools and the eluate in the gap between Pools 3 and 4 were the only ones that catalyzed the NAD-dependent oxidation of retinaldehyde; moreover, approximately 70% of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in these fractions. In the case of both aldphosphamide and retinaldehyde, the vast majority of the enzyme activity was present in Pool 4. Clearly, however, some was also present in Pool 5 although it could not be ascertained at this point whether this activity was due to the same enzyme(s) present in Pool 4 or to another enzyme. In the case of retinaldehyde, the former was suspected because there was no observable shoulder to the enzyme activity peak. In the case of aldophosphamide, the latter was thought to be the case because there was a clearly observable shoulder present.

Next to elute was a peak of enzyme activity for which betaine aldehyde was the preferred substrate (Pool 6). The enzyme(s) in this pool did not catalyze the oxidation of aldophosphamide or retinaldehyde.

A final peak of enzyme activity (acetaldehyde, octanal, benzaldehyde) was collected into Pool 7; the enzyme(s) in this pool did not catalyze the oxidation of either aldophosphamide or retinaldehyde.

Three "aldehyde dehydrogenases" found in human liver samples by others, viz. γ-aminobutyraldehyde dehydrogenase [14], ALDH-3 [22], and xanthine oxidase (dehydrogenase form) [23, 24], were not detected in this liver preparation. γ-Aminobutyraldehyde dehydrogenase-catalyzed oxidation of γ-aminobutyraldehyde (100 μM) was not observed when DEAE pools were assayed for this activity. ALDH-3 was not present as judged by the fact that no ALDH activity with a preference for benzaldehyde as substrate and NADP as cofactor was observed during chromatography on DEAE-Sephacel. Further, when the DEAE pools were submitted to isoelectric focusing, no ALDH activity (NADP/benzaldehyde) was observed in the pH range (5.9 to 6.4) where ALDH-3 is reported to focus [22]. Others have also observed that ALDH-3 is not expressed in all human liver specimens [25]. NAD-dependent xanthine oxidase-catalyzed oxidation of xanthine (50 μM) was not observed when the Lubrol®-solubilized whole homogenate was assayed for this activity. Xanthine oxidase activity has been observed previously to be lower in humans and carnivores than in rodents and herbivores [24], and to be lost during storage or upon freezing [12].

DEAE Pool 1, 2 and 3 ALDH activity: Further resolution and purification, and enzyme identification. Qualitatively similar profiles of enzyme activity were observed when DEAE Pools 1, 2, and 3 were subjected to affinity chromatography on Reactive Blue 2-Sepharose (Fig. 1, B, C, and D). The relative peak sizes differed but in all three cases an initial peak of enzyme activity with a marked preference for glutamic-γ-semialdehyde as the substrate eluted when a 25 mM sodium phosphate buffer, pH 7.4, was passed through the column, and a second peak of enzyme activity with a marked preference for succinic semialdehyde as the substrate eluted when this buffer was supplemented with 0.7 M NaCl and 1 mM NAD. Hence, these enzymes were identified as glutamic-γ-semialdehyde dehydrogenase, aka ALDH-4, and SSDH, respectively. Several additional observations supported these conclusions.

Upon isoelectric focusing, the enzyme identified as ALDH-4 resolved into two major bands with pI values of 6.7 and 6.9 (Table 4) which stained preferentially with glutamic-γ-semialdehyde as substrate. These pI values are characteristic of ALDH-4 [26]. Some additional lower pI bands were also observed; others have also observed these lower pI bands [26]. They noted that the presence and intensity of these bands varied with time, storage conditions, and repeated freezing and thawing. Finally, the K_m value of 238 μM for enzyme-catalyzed oxidation of glutamic-γ-semialdehyde (Table 4) is similar to the previously reported value of 170 μM for ALDH-4-catalyzed oxidation of this substrate [26].

The enzyme identified as SSDH was further purified with the aid of a 5′-AMP-Sepharose column (experiment not shown). No enzyme activity eluted when the column was washed with a 25 mM MES

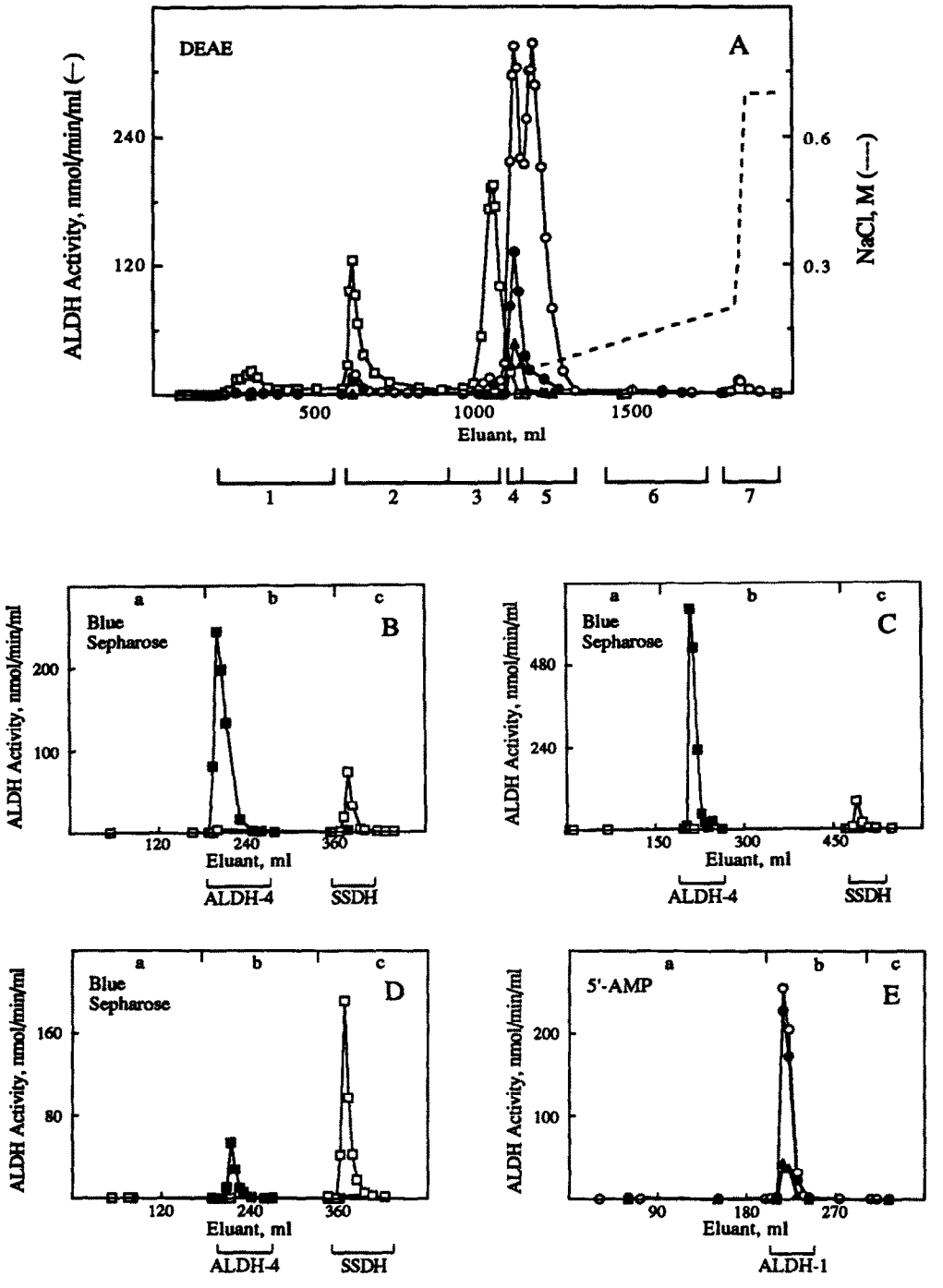

Fig. 1. Chromatographic separation of human liver aldehyde dehydrogenases. (A) A 105,000*g* supernatant fraction obtained from a Lubrol®-treated whole homogenate prepared from a liver sample (6.7 g) originally obtained from Donor C and then frozen, was transferred, with the aid of Pharmacia PD-10 columns, from homogenization medium into a 20 mM imidazole buffer, pH 7.4 (buffer A). The latter preparation (110 mL) was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The loaded column was eluted with 300 mL of buffer A, then with 470 mL of a 20 mM imidazole buffer, pH 6.8 (buffer B), and then with a 0 to 0.2 M NaCl gradient (900 mL) generated in buffer B. Elution was completed with 100 mL of buffer B adjusted to 0.7 M NaCl. Buffers A and B also contained 0.3% Lubrol®. (B) DEAE Pool 1 ALDH activity (400 mL) was concentrated 12-fold and then 28 mL of the concentrated pool was transferred into a 25 mM MES buffer, pH 6.6 (buffer C). This preparation was diluted to 95 mL with buffer C and then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 1 enzyme in buffer C followed by buffer C; (*b*) 25 mM sodium phosphate buffer, pH 7.4 (buffer D); and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (C) DEAE Pool 2 ALDH activity

Table 4. Isoelectric point and K_m values that were obtained and used to identify human liver aldehyde dehydrogenases

ALDH	Substrate	pI*	K_m† (μM)
1	Acetaldehyde	5.2	483
2	Acetaldehyde	4.9	<0.1
4	Glutamic-γ-semialdehyde‡	6.7, 6.9	238
5	Octanal	6.0–6.8	<0.1
BADH	Betaine aldehyde	6.5	0.3
SSDH	Succinic semialdehyde	6.0–7.5	<0.1

* Isoelectric point values were determined for each (semi)purified aldehyde dehydrogenase as described in Materials and Methods and in the legend to Fig. 2. Values are either from one determination or are the means of two to three determinations, each made on a separate gel.

† Aldehyde dehydrogenase activity was measured as described in Materials and Methods using the spectrophotometric assay. Double-reciprocal plots of initial rates (duplicate determinations) versus six to seven substrate concentrations were used to generate the kinetic constants for ALDHs 1 and 4; values are from single experiments. Integrated Michaelis equation analysis of single enzyme progress curves was used to generate the kinetic constants for ALDHs 2 and 5, and for succinic semialdehyde dehydrogenase and betaine aldehyde dehydrogenase; values are the means of three determinations.

‡ In aqueous solution, glutamic-γ-semialdehyde exists in equilibrium with its tautomer, DL-Δ^1-pyrroline-5-carboxylate. No attempt was made to differentiate between the tautomers, i.e. glutamic-γ-semialdehyde concentration was considered to be the total of glutamic-γ-semialdehyde and DL-Δ^1-pyrroline-5-carboxylate concentrations.

buffer, pH 6.6, or with the same buffer supplemented with 0.2 mM NAD. A single peak of enzyme activity (succinic semialdehyde) eluted when the column was subsequently washed with a 25 mM sodium phosphate buffer, pH 7.4, containing 0.7 M NaCl and 1 mM NAD. When visualized by either Coomassie Blue (protein) staining (data not shown) or by enzyme activity staining with succinic semialdehyde as substrate (Fig. 2), the enzyme thus obtained focused as several bands in the pH range 6.0 to 7.5. No bands appeared when glutamic-γ-semialdehyde was used as substrate in the enzyme activity stain. Multiple banding in the pH range of 6.3 to 7.2 has been reported for human brain SSDH [15]. Moreover, despite different assay conditions, the K_m value of <0.1 μM for enzyme-catalyzed oxidation of succinic semialdehyde (Table 4) is grossly similar to the previously reported values of 0.7 and 2 μM for human brain SSDH-catalyzed oxidation of this substrate [15].

SSDH catalyzed the oxidation of aldophosphamide, but ALDH-4 did not (Table 5). Neither catalyzed the oxidation of retinaldehyde (Table 5).

DEAE Pool 4 ALDH activity: Further purification,

Fig. 1. *Continued*
(325 mL) was concentrated 11-fold and 15 mL of the concentrated preparation was then transferred into buffer C. This preparation was diluted to 65 mL with buffer C and then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 2 enzyme in buffer C followed by buffer C; (*b*) buffer D; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (D) DEAE Pool 3 ALDH activity (100 mL) was concentrated 5-fold and then transferred into buffer C. This preparation was diluted to 90 mL with buffer C and 80 mL of the diluted preparation was then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 3 enzyme in buffer C followed by buffer C; (*b*) buffer D; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (E) DEAE Pool 4 ALDH activity (30 mL) was concentrated 3-fold and then transferred into buffer C. This preparation was diluted to 50 mL with buffer C and then loaded onto a 5′-AMP-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 4 enzyme in buffer C followed by buffer C; (*b*) 125 mM sodium phosphate buffer, pH 7.4 (buffer E) containing 0.7 mM NAD; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. In all panels, all buffers referred to also contained 1 mM dithiothreitol and 0.1 mM EDTA. Eluate was collected in fractions of 5–10 mL. Substrates used to monitor ALDH activity included 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲), 4 mM acetaldehyde (○), 100 μM succinic semialdehyde (□) and 200 μM glutamic-γ-semialdehyde (■). Not all of the data obtained with these substrates is shown, nor is any of the data obtained when benzaldehyde (4 mM), octanal (100 μM), γ-aminobutyraldehyde (200 μM), or betaine aldehyde (50 μM) was used as the substrate.

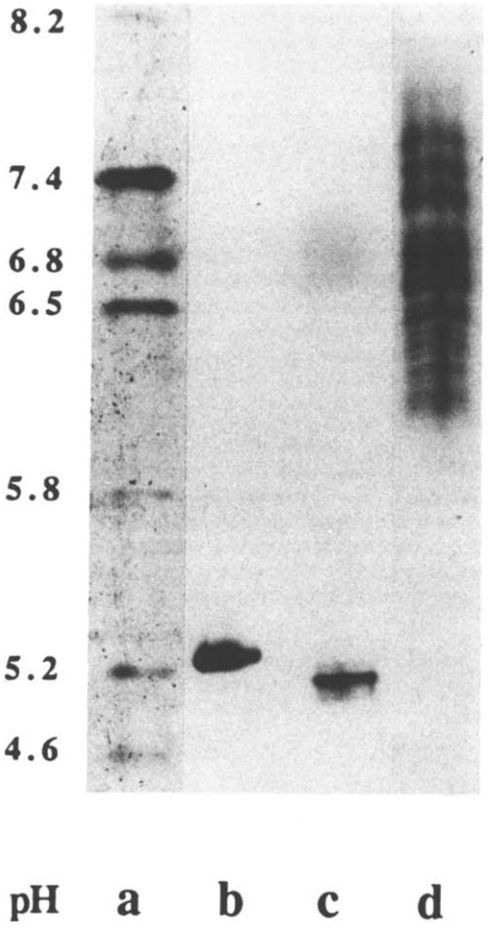

Fig. 2. Isoelectric focusing of relevant hepatic aldehyde dehydrogenases. ALDHs 1 and 2 (eluted from Polybuffer Exchanger columns), succinic semialdehyde dehydrogenase (eluted from a 5′-AMP-Sepharose column), and pI standards were subjected to isoelectric focusing. The amount of each enzyme loaded onto the gel was sufficient to generate 5–10 nmol NADH/min as determined by the spectrophotometric aldehyde dehydrogenase assay and the substrates with which the enzyme was ultimately stained. The nitroblue tetrazolium-based enzyme activity stain was used to visualize the aldehyde dehydrogenases present in lanes b, c and d. The aldehydes used as substrates were acetaldehyde (4 mM) for ALDHs 1 and 2, and succinic semialdehyde (100 μM) for SSDH. Coomassie Brilliant Blue was used to stain lane a. *Lane a*, pI standards; *lane b*, ALDH-1; *lane c*, ALDH-2; and *lane d*, SSDH. Additional details are presented in Materials and Methods.

and enzyme identification. DEAE Pool 4 ALDH activity was loaded onto a 5′-AMP-Sepharose column to which all of the aldehyde dehydrogenase activity bound (Fig. 1E). A single peak of enzyme activity eluted when a 125 mM sodium phosphate buffer, pH 7.4, supplemented with 0.7 mM NAD was subsequently passed through the column.

Several observations supported the identification of this enzyme as ALDH-1. After further purification by chromatofocusing chromatography (Fig. 3A), the enzyme was subjected to isoelectric focusing; a single band with a pI value of 5.2 was observed upon Coomassie Blue (protein) staining (data not shown) as well as upon enzyme activity staining with acetaldehyde as the substrate (Fig. 2 and Table 4). A pI value of 5.2 is characteristic of ALDH-1 [22] but is also not unlike that reported for γ-aminobutyraldehyde dehydrogenase [14]. However, γ-aminobutyraldehyde dehydrogenase focuses as a doublet; pI values are 5.3 and 5.45 [14]. Moreover, ALDH-1 is known to bind to 5′-AMP-Sepharose [21]. The enzyme in question bound to the 5′-AMP-Sepharose column; γ-aminobutyraldehyde dehydrogenase would not be expected to bind to 5′-AMP-Sepharose under the experimental conditions used [14]. Finally, the K_m value of 483 μM for enzyme-catalyzed oxidation of acetaldehyde (Table 4) is very similar to the previously reported value of 348 μM for ALDH-1 [27].

ALDH-1 catalyzed the oxidation of both aldophosphamide and retinaldehyde (Table 5).

DEAE Pool 5 ALDH activity: Further resolution and purification, and enzyme identification. Significant amounts of ALDH-1 were present in DEAE Pool 5. Preliminary experiments showed that resolution of ALDH-1 and the major enzyme in this pool by 5′-AMP-Sepharose and chromatofocusing chromatography was minimal. It was also noted in preliminary experiments with liver samples that had never been frozen, that the major aldehyde dehydrogenase present in DEAE Pool 5 was present in the 105,000 *g* pellet (particulate) fraction. ALDH-1 is a soluble enzyme. Therefore, in order to facilitate the purification of the major DEAE Pool 5 enzyme, it was decided to start with a preparation, viz. a Lubrol®-solubilized hepatic 105,000 *g* particulate fraction, that was virtually free of ALDH-1. The preparation was first submitted to DEAE-Sephacel column chromatography (Fig. 4A). A very minor peak of enzyme activity (acetaldehyde) eluted off the column first; it was closely followed by a major peak of enzyme activity (acetaldehyde). The last approximately two-thirds of the latter was collected into DEAE Pool 5′. DEAE Pool 5′ ALDH activity was then subjected to 5′-AMP-Sepharose affinity chromatography (Fig. 4B). Based on aldehyde dehydrogenase-catalyzed oxidation of the substrates used to monitor enzyme activity, the eluant was divided into three pools. The enzymes in all three pools showed a marked preference for acetaldehyde as substrate. As judged by isoelectric focusing, two aldehyde dehydrogenases were present in each pool, viz. a major band at pH 4.9 and a very minor band at pH 5.2 (data not shown). Pool 9 was further purified by chromatography on a Reactive Blue 2-Sepharose column (Fig. 4C). A trace of enzyme activity (acetaldehyde) eluted when a 25 mM MES buffer, pH 6.6, was passed through the column. A single peak of aldehyde dehydrogenase activity (acetaldehyde) eluted when the column was washed with a 125 mM phosphate buffer, pH 8.0, supplemented with 0.7 mM NAD.

Several observations supported the identification of this enzyme as ALDH-2. After further purification by chromatofocusing column chromatography (Fig. 3B), the enzyme was subjected to isoelectric focusing; a single band with a pI value of 4.9 was observed

Table 5. Data used to conclude that some human aldehyde dehydrogenases catalyze the oxidation of aldophosphamide and/or retinaldehyde whereas others do not

ALDH	Reference substrate*	Index value†	
		Aldophosphamide	Retinaldehyde
1	Acetaldehyde	64.0	9.8
2	Acetaldehyde	2.5	0.3[b]
4	Glutamic-γ-semialdehyde‡	0.04[a]	0.01[b]
5	Octanal	0.2[a]	0.04[b]
BADH	Betaine aldehyde	1.6[a]	0.06[b]
SSDH	Succinic semialdehyde	15.6	0.09[b]

* The indicated reference substrate for each enzyme was chosen as such because it was, or was one of, the preferred substrate(s) for that enzyme.

† Index value = (the observed, or smallest detectable, rate of aldophosphamide or retinaldehyde oxidation) (100)/(rate of oxidation of reference substrate). Values of 0.06(a) and 0.03(b) nmol/min were used to calculate the index values for aldophosphamide and retinaldehyde oxidation, respectively, when there was no measurable rate observed because each was considered to be the smallest detectable rate. Thus, they were used to calculate the upper limits of the relative rate of enzyme-catalyzed aldophosphamide and retinaldehyde oxidation that might be occurring but not be detected. The spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide (160 μM), acetaldehyde (4 mM), glutamic-γ-semialdehyde (200 μM), octanal (100 μM), betaine aldehyde (50 μM) and succinic semialdehyde (100 μM). The HPLC/spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of retinaldehyde (25 μM).

‡ Glutamic-γ-semialdehyde concentration was calculated as described in Table 4.

upon Coomassie Blue (protein) staining (data not shown), as well as upon enzyme activity staining using acetaldehyde as the substrate (Fig. 2 and Table 4). This value is characteristic of ALDH-2 [22]. Despite the difference in assay consitions, the low K_m value of <0.1 μM for enzyme-catalyzed oxidation of acetaldehyde (Table 4) is grossly similar to the previously reported values of <1 μM [28] and 2.4 and 3 μM [21], for ALDH-2-catalyzed oxidation of this substrate.

ALDH-2 catalyzed the oxidation of aldophosphamide but not that of retinaldehyde (Table 5).

DEAE Pool 6 ALDH activity: Further resolution and purification, and enzyme identification. 5'-AMP-Sepharose column chromatography was used to further resolve and purify DEAE Pool 6 ALDH activity (data not shown). An initial, small peak of enzyme activity with a marked preference for acetaldehyde eluted when a 25 mM sodium phosphate buffer, pH 7.4, was passed through the column. A second peak of enzyme activity with a marked preference for betaine aldehyde eluted when a 125 mM sodium phosphate buffer, pH 7.4, was subsequently passed through the column.

On the basis of substrate preference and isoelectric focusing point, viz. 4.9, the first enzyme to elute was identified as ALDH-2.

Several observations supported the identification of the second enzyme to elute as BADH. It focused as a single band at a pI value of 6.5, which stained most intensely when betaine aldehyde was used as the substrate (Table 4). Additionally, the K_m value of 0.3 μM for enzyme-catalyzed oxidation of betaine aldehyde (Table 4) is grossly similar to the previously reported value of 10 μM for oxidation of this substrate by BADH [13].

BADH did not catalyze the oxidation of aldophosphamide or retinaldehyde (Table 5).

DEAE Pool 7 ALDH activity: Enzyme identification. The aldehyde dehydrogenase present in DEAE Pool 7 showed a marked preference for aromatic and long chain aliphatic aldehydes. It was identified as ALDH-5 on the basis of several criteria. First, it focused as a diffuse smear extending from pH 5.5 to 7.5 with most of the enzyme activity appearing between pH 6.0 and 6.8 (Table 4). These values are consistent with those previously reported for ALDH-5 [12]. Second, the K_m value for oxidation of acetaldehyde by the enzyme in DEAE Pool 7 was in the millimolar range (data not shown) while the K_m value of <0.1 μM for the oxidation of octanal by this enzyme was much lower (Table 4). These values are consistent with the reports that ALDH-5 shows a preference for long chain aldehydes as substrates [12, 13].

ALDH-5 did not catalyze the oxidation of either aldophosphamide or retinaldehyde (Table 5).

Michaelis–Menten kinetics and proportional importance of individual hepatic aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde

During the chromatographic separation of the hepatic aldehyde dehydrogenases, careful records were kept regarding the fraction of the total hepatic aldehyde dehydrogenase activity (160 μM aldophosphamide, 25 μM retinaldehyde) that partitioned with each aldehyde dehydrogenase; these values are presented in Table 6 under "%

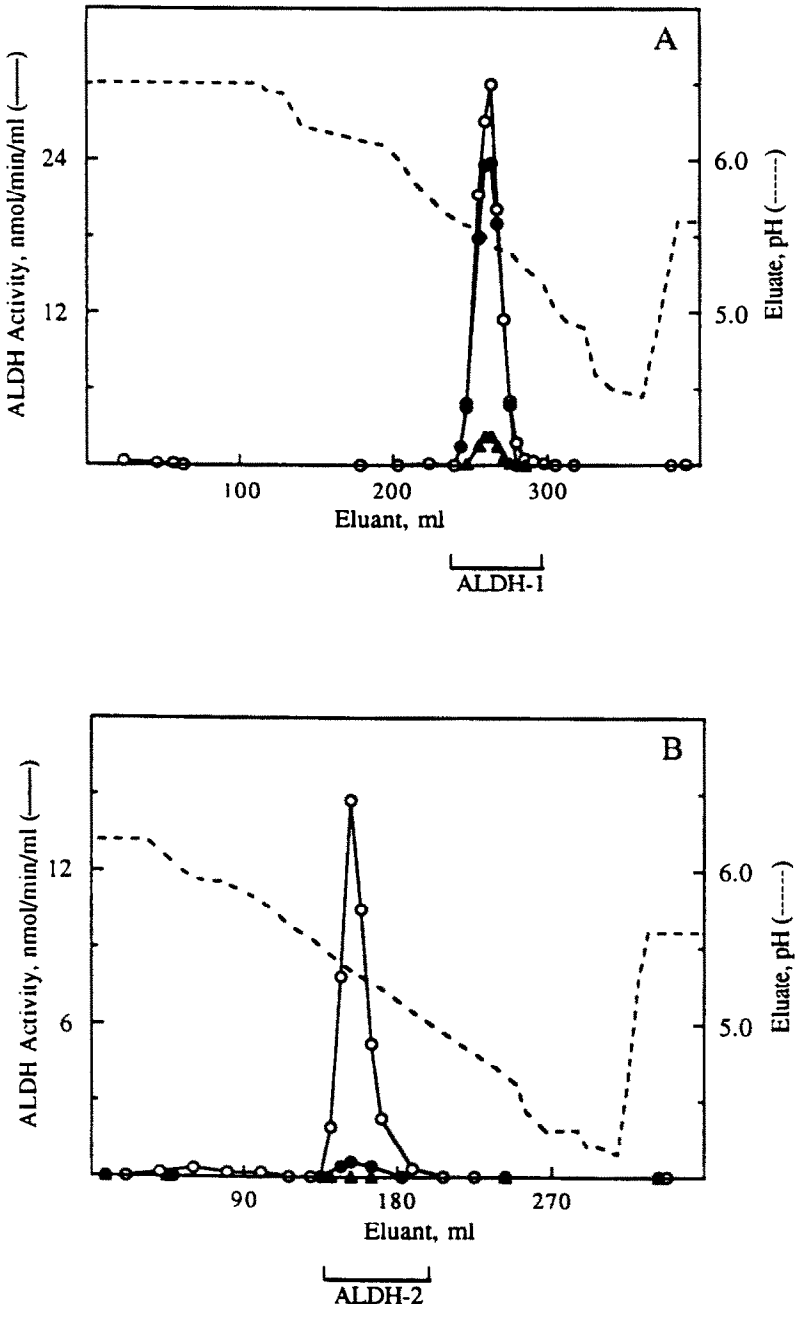

Fig. 3. Chromatofocusing of ALDH-1 and ALDH-2. (A) ALDH-1 recovered from the 5′-AMP-Sepharose column (Fig. 1E) was concentrated 4-fold and transferred, with the aid of PD-10 columns, into a 25 mM histidine buffer, pH 6.5 (buffer F). This preparation (18 mL) was diluted to 100 mL with buffer F and was loaded onto a column of Polybuffer Exchanger 94 that had been equilibrated with buffer F. The column was developed with 250 mL of 10% Polybuffer 74, pH 4.5, followed by 50 mL of this same buffer containing 0.7 M NaCl. The elution maximum was at pH 5.5. (B) ALDH-2 recovered from the Reactive Blue 2-Sepharose column (Fig. 4C) was concentrated 8-fold and transferred into a 25 mM histidine buffer, pH 6.2 (buffer G). This preparation (13 mL) was loaded onto a column of Polybuffer Exchanger 94 that had been equilibrated with buffer G. The column was developed with 300 mL of 10% Polybuffer 74, pH 4.0, followed by 50 mL of this same buffer containing 0.7 M NaCl. The elution maximum was at pH 5.3. In both panels, all buffers referred to also contained 1 mM dithiothreitol, 0.1 mM EDTA, and 20% glycerol. Eluate was collected in fractions of 2–8 mL. Substrates used to monitor ALDH activity were 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲) and 4 mM acetaldehyde (○).

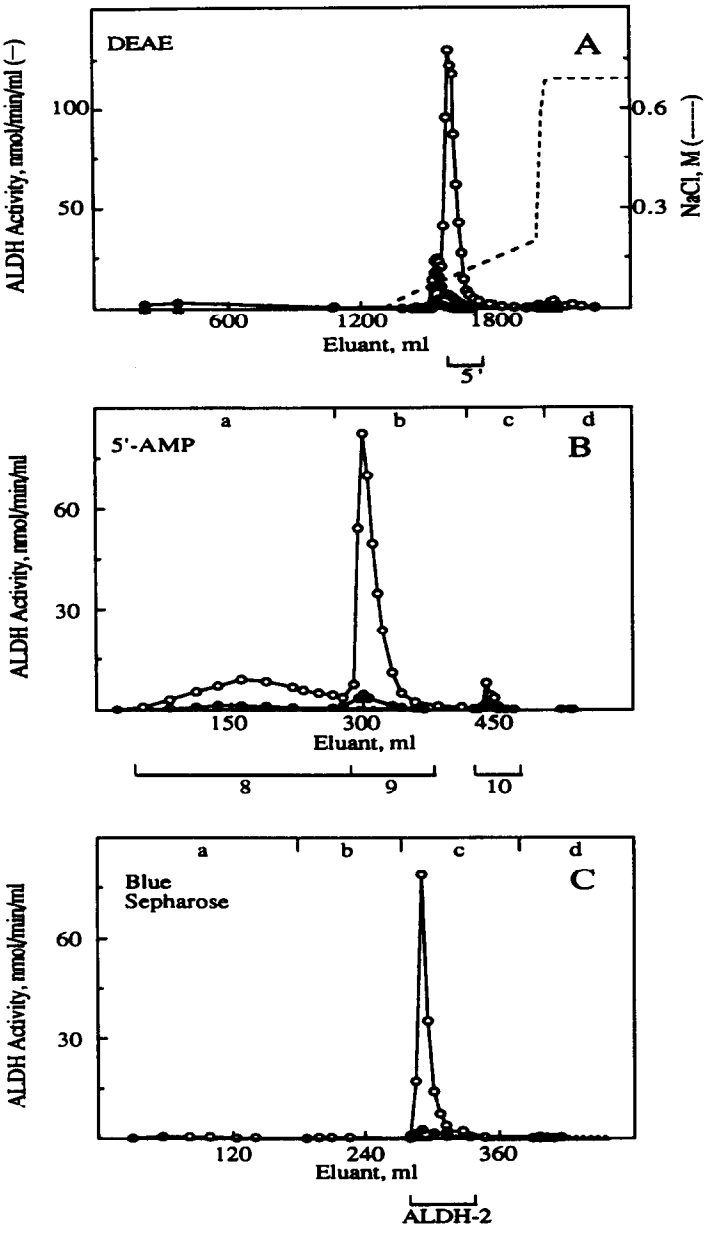

Fig. 4. Chromatographic purification of human liver ALDH-2. (A) A Lubrol®-solubilized 105,000 *g* particulate fraction prepared from a liver sample (15.5 g) originally obtained from Donor C and then frozen, was transferred, with the aid of PD-10 columns, into a 20 mM imidazole buffer, pH 7.4 (buffer A). The latter preparation (340 mL) was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The loaded column was eluted with 550 mL of buffer A, then with 450 mL of a 20 mM imidazole buffer, pH 6.8 (buffer B), and then with a 0–0.2 M NaCl gradient (800 mL) generated in buffer B. Elution was completed with 300 mL of buffer B adjusted to 0.7 M NaCl. (B) DEAE Pool 5′ ALDH activity was concentrated 7-fold and then transferred into a 25 mM MES buffer, pH 6.6 (buffer C). The latter preparation was diluted to 140 mL with buffer C and was then loaded onto a 5′-AMP-Sepharose column equilibrated with the same buffer. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 5′ enzyme in buffer C followed by buffer C; (*b*) 125 mM sodium phosphate buffer, pH 8.0 (buffer H); (*c*) buffer H containing 0.7 mM NAD; and (*d*) 25 mM sodium phosphate buffer, pH 7.4 (buffer D) containing 1 mM NAD and 0.7 M NaCl. (C) Pool 9 from the 5′-AMP column was concentrated 19-fold and then transferred into buffer C. The latter preparation was diluted to 50 mL and was then loaded onto a Reactive Blue 2-Sepharose column equilibrated with the same buffer. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) 5′-AMP Pool 2 enzyme in buffer C followed by buffer C; (*b*) buffer H; (*c*) buffer H containing 0.7 mM NAD; and (*d*) buffer D containing 1 mM NAD and 0.7 M NaCl. In all panels, all buffers referred to also contained 1 mM dithiothreitol and 0.1 mM EDTA. Eluate was collected in fractions of 5–10 mL. Substrates used to monitor ALDH activity were 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲) and 4 mM acetaldehyde (○).

Table 6. Human liver aldehyde dehydrogenases: Kinetics of, and relative contribution to, the catalytic oxidation of aldophosphamide and retinaldehyde*

	Aldophosphamide			Retinaldehyde		
ALDH	K_m (μM)	V_{max}† (μmol/min/mg protein)	% Contribution‡	K_m (μM)	V_{max}† (μmol/min/mg protein)	% Contribution§
1	52 ± 3	4.8 ± 0.1	64	0.3 ± 0.1	0.5 ± 0.1	100
2	1193 ± 110	0.4 ± 0.0	6	NS	0	0
4	NS‖	0	0	NS	0	0
5	NS	0	0	NS	0	0
SSDH	560 ± 48	0.9 ± 0.1	30	NS	0	0
BADH	NS	0	0	NS	0	0

* Double-reciprocal plots of initial rates versus substrate concentrations were constructed as described in Materials and Methods and in the legend to Fig. 5 to estimate the K_m (±SEM) and V_{max} (±SEM) values for enzyme-catalyzed oxidation of aldophosphamide. Values and error estimates are from single experiments (duplicate individual initial rate measurements made at each of four to ten substrate concentrations in each case). The integrated Michaelis method of analysis of single enzyme progress curves (N = 4) was used as described in Materials and Methods to generate the K_m (±SEM) and V_{max} (±SEM) values for ALDH-1-catalyzed oxidation of retinaldehyde.

† ALDHs 1 and 2, purified through chromatofocusing column chromatography, and SSDH, purified through 5′-AMP-Sepharose column chromatography, were used to determine V_{max} values for aldophosphamide and, where indicated, for retinaldehyde, oxidation.

‡ Percentage of total recovered hepatic aldehyde dehydrogenase activity measurable with 160 μM aldophosphamide that co-purifies with the indicated aldehyde dehydrogenase during a typical purification.

§ Percentage of total recovered hepatic aldehyde dehydrogenase activity measurable with 25 μM retinaldehyde that co-purifies with the indicated aldehyde dehydrogenase during a typical purification.

‖ NS: not a substrate.

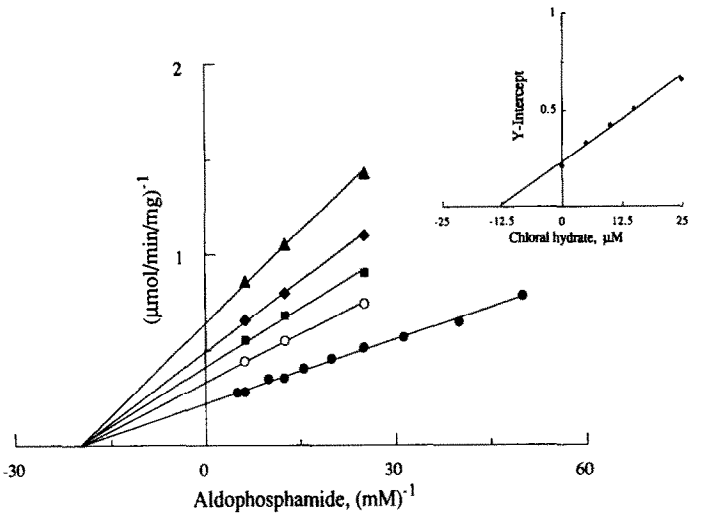

Fig. 5. Inhibition of ALDH-1-catalyzed oxidation of aldophosphamide by chloral hydrate: Lineweaver–Burk kinetic analysis. Initial rates of ALDH-1 catalyzed aldophosphamide oxidation were measured as described in Materials and Methods. The concentrations of chloral hydrate were 0 (●), 5 (○), 10 (■), 15(◆) and 25 (▲) μM. ALDH-1 purified through chromatofocusing chromatography was used in this experiment. V_{max} and K_m values were calculated from the y- and x-intercepts, respectively, of the fitted lines and were 4.8 μmol/min/mg and 52 μM, respectively. Data points represent the means of duplicate determinations. The K_i value was calculated from the plot of the y-intercept values against the chloral hydrate concentrations (inset) and was 13 μM.

Contribution". Three enzymes, viz. ALDH-1, ALDH-2, and SSDH, catalyzed the oxidation of aldophosphamide; ALDH-1 was by far the most important, accounting for 64% of the total when the aldophosphamide concentration was 160 μM. Given the K_m values obtained, ALDH-1 would account for an even greater percentage of the total when the aldophosphamide concentration was pharmacological. K_m values for the catalysis of aldophosphamide by ALDH-1, ALDH-2, and SSDH were 52, 1193 and 560 μM, respectively (Table 6). The Lineweaver-Burk plot used to generate the K_m and V_{max} values for ALDH-1-catalyzed oxidation of aldophosphamide is shown in Fig. 5. ALDH-1 accounted for all of the recovered NAD-dependent enzyme-catalyzed oxidation of retinaldehyde. The K_m value for ALDH-1-catalyzed oxidation of retinaldehyde was 0.3 μM (Table 6).

Sensitivity of ALDH-1-, ALDH-2- and SSDH-catalyzed oxidation of aldophosphamide to inhibition by chloral hydrate

Detoxification of aldophosphamide by human pluripotent hematopoietic progenitor cells appears to be catalyzed by an aldehyde dehydrogenase relatively insensitive to inhibition by chloral hydrate [29]. Therefore, the sensitivity of the three human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide, to inhibition by chloral hydrate was determined. ALDH-1-catalyzed oxidation of aldophosphamide was highly sensitive to inhibition by chloral hydrate, whereas ALDH-2- and SSDH-catalyzed oxidation of this substrate was less sensitive (Table 7). The K_i value for chloral hydrate inhibition of ALDH-1-catalyzed oxidation of aldophosphamide was 13 μM; inhibition was noncompetitive (Fig. 5).

Table 7. Inhibition of aldehyde dehydrogenase-catalyzed aldophosphamide oxidation by chloral hydrate

	Inhibition by chloral hydrate† (%)	
Enzyme*	0.1 mM	1.0 mM
ALDH-1	82	97
ALDH-2	24	51
SSDH	6	16

* ALDHs 1 and 2 were the chromatofocused preparations. SSDH was semipurified from DEAE Peak 3 by passage though Blue Sepharose and 5′-AMP affinity columns. For additional details, see Materials and Methods and the text of Results.

† Initial rates of aldehyde dehydrogenase-catalyzed aldophosphamide (160 μM) oxidation were measured in the presence and absence of chloral hydrate as described in Materials and Methods. Control rates (no chloral hydrate) were 6.1, 0.13 and 2.0 nmol/min for ALDH-1, ALDH-2 and SSDH, respectively.

DISCUSSION

Six aldehyde dehydrogenases were resolved from human liver. One, viz. ALDH-1, accounted for all

of the NAD-dependent enzyme-catalyzed oxidation of retinaldehyde in this organ. Three aldehyde dehydrogenases, viz. ALDH-1, ALDH-2, and SSDH, catalyzed the oxidation of aldophosphamide. ALDH-1 was, by far, the most efficient in this regard. It is not the hepatic aldehyde dehydrogenase that is most important in catalyzing the oxidation of acetaldehyde. That distinction goes to ALDH-2 [30–32]. ALDH-4, ALDH-5, and BADH did not catalyze the oxidation of either aldophosphamide or retinaldehyde.

These observations closely parallel those made in mouse liver. Thus, *in vitro*, AHD-2, the mouse homolog of ALDH-1, catalyzes the bulk of the oxidation of both aldophosphamide and retinaldehyde in mouse liver; AHD-5 and AHD-12, the mouse homologs of ALDH-2 and SSDH, respectively, catalyze the oxidation of aldophosphamide but not that of retinaldehyde, and AHD-3, the mouse homolog of ALDH-5, does not catalyze the oxidation of aldophosphamide or retinaldehyde [8, 9].

There are some differences. Thus, mouse AHDs-1 and -9, unlike their human counterparts, ALDH-4 and BADH, respectively, contribute to the overall hepatic oxidation of aldophosphamide [9]. Further, human homologs of mouse AHDs -7, -8, -10, -11 and -13 were not found. AHD-7 catalyzes the oxidation of retinaldehyde [8], but not that of aldophosphamide [9]. AHDs -8, -10, -11, and -13 catalyze the oxidation of aldophosphamide [9], but not that of retinaldehyde [8]. Failure to find homologs of these enzymes in the human liver sample examined may be due to species differences and/or to loss of activity upon storage and freezing of the human liver sample.

It was not possible to determine whether xanthine oxidase (dehydrogenase form), ALDH-3, or γ-aminobutyraldehyde dehydrogenase catalyzed the oxidation of either retinaldehyde or aldophosphamide because these enzymes were not found in the liver sample used in our investigation. The dehydrogenase form of mouse xanthine oxidase catalyzes the oxidation of retinaldehyde but not that of aldophosphamide [8, 9]. Perhaps the same is true of the human homolog. AHD-4, an aldehyde dehydrogenase found in mouse stomach but not in mouse liver [10], catalyzes, albeit relatively poorly, the oxidation of aldophosphamide but not that of retinaldehyde [8, 9]. ALDH-3 may be the human homolog of this enzyme [11]. In that event, it may catalyze the oxidation of aldophosphamide but not that of retinaldehyde.

Oxazaphosphorines such as cyclophosphamide are detoxified when aldophosphamide is oxidized to carboxyphosphamide [3]. Depending on the type of neoplasm, the oxazaphosphorines often exhibit a relatively favorable therapeutic index [3]. At least in part, this is because certain critical and rapidly renewing normal cell populations, e.g. pluripotent hematopoietic, and intestinal epithelial, progenitor cells contain an aldehyde dehydrogenase which catalyzes the relevant detoxification whereas some tumor cell populations lack such an enzyme or contain much less of it [33–39]. The identity of the relevant aldehyde dehydrogenases in any given normal tissue is, at present, largely uncertain. Whereas the expression of aldehyde dehydrogenase activity of some sort is nearly ubiquitous in normal tissues [40], the expression of any particular aldehyde dehydrogenase is more restricted. Thus, while high levels of ALDH-1 and ALDH-2 are found in liver and kidney, much lower levels are found in intestine, brain, stomach, lung, spleen, testis, lymphocytes, scalp skin, hair roots, cornea and placenta [2, 12, 22, 41, 42]. ALDH-1 is apparently not present in heart [12, 22] and is the only aldehyde dehydrogenase present in erythrocytes [43, 44]. The tissue distribution of SSDH is less clear; to date it has been identified in brain [15] and liver.

ALDH-1 would be expected to confer protection against oxazaphosphorines on any cell in which it is expressed. Conversely, cells which lack ALDH-1, e.g. those of the heart [12, 22], might be expected to be at increased risk to the toxic actions of oxazaphosphorines. Perhaps related is the severe cardiotoxicity observed when high doses of cyclophosphamide are given [45]. Individuals who express a relatively enzymatically nonfunctional variant of ALDH-1 [46, 47] might be at increased risk to the untoward systemic actions of cyclophosphamide and other oxazaphosphorines.

As judged by the kinetic values obtained, ALDH-2, even when present in large amounts, is highly unlikely to confer significant protection on any tissue. Consequently, the large (up to 50%) fraction of Orientals that express an enzymatically nonfunctional variant of this aldehyde dehydrogenase [48] are unlikely to be at increased risk to the untoward systemic actions of the oxazaphosphorines.

SSDH may be an important contributor to detoxification of the oxazaphosphorines in tissues which do not express ALDH-1.

It has been demonstrated that aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide contributes significantly to the relative insensitivity of murine intestinal crypt cells [49] and murine and human hematopoietic progenitor cells to the oxazaphosphorines [33–37]. The aldehyde dehydrogenase present in murine intestinal crypt cells is kinetically and immunologically identical to the major liver cytosolic aldehyde dehydrogenase, viz. AHD-2 [49]. Uncertain is the identity of the operative aldehyde dehydrogenase(s) in hematopoietic progenitor cells. The experiments of Kastan and coworkers [39] suggest that in humans it is ALDH-1 because polyclonal antibodies that they raised to a cytoplasmic (most probably cytosolic) aldehyde dehydrogenase (most probably ALDH-1) purified from Hep G2 hepatoma recognized a protein (presumably ALDH-1) present in hematopoietic progenitor cells (identified as such by flow cytometric assay of cell surface lineage- and maturation-specific antigens). The polyclonal antibodies used to identify the hematopoietic progenitor cell protein also recognized an approximately 55 kDa protein in a cytosolic extract of human liver [39]. Both ALDH-1 and ALDH-2 are tetramers made up of approximately 55 kDa subunits [21]. The specificity of the polyclonal antibodies used to identify the proteins (presumably aldehyde dehydrogenases) in these experiments was not reported. Inconsistent

with the notion that the operative enzyme is ALDH-1 is the observation that even high concentrations of chloral hydrate, a potent inhibitor of human ALDH-1 and mouse AHD-2, did not, or only minimally, potentiate(d) the cytotoxic action of the oxazaphosphorines against multipotent hematopoietic progenitor cells whereas other known inhibitors of these enzymes did [29]. The operative enzyme could be SSDH. It is relatively insensitive to inhibition by chloral hydrate. However, SSDH is a particulate enzyme and, in experiments conducted by yet another laboratory [15], antisera raised to ALDH-1 did not recognize brain SSDH; further, the subunit size of brain SSDH is approximately 62 kDa.

Expression of AHD-2, the mouse homolog of ALDH-1, is a demonstrated mechanism of acquired resistance to the oxazaphosphorines in two mouse leukemia lines [50–52], and increased expression of an unidentified aldehyde dehydrogenase appears to be the underlying basis for the acquired resistance to oxazaphosphorines observed in a rat leukemia [53]. Elevated aldehyde dehydrogenase expression may be a clinically important mechanism for acquired resistant to the oxazaphosphorines in human tumors. In cases of intrinsic and/or acquired tumor cell resistance to the oxazaphosphorines where aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide is the causative event, therapeutic intervention strategies based on selective sensitization of the resistant tumor cell population may be possible if the operative tumor enzyme differs from that present in critical normal cell populations since aldehyde dehydrogenases often differ in their relative sensitivity to various inhibitors, e.g. chloral hydrate [54–57]. On the other hand, given that aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide serves to protect certain critical normal cell populations against the cytotoxic action of oxazaphosphorines, *vide supra*, and that a number of frequently used drugs, e.g. certain cephalosporins, are known to inhibit at least some of the aldehyde dehydrogenases [58, 59], the potential for clinically adverse drug interactions is apparent. Further, given that ALDH-1 activity in erythrocytes and liver is reduced in chronic alcoholics [42, 60–62], and that aldehyde dehydrogenase activity is apparently depressed in humans given certain oral contraceptives [63], these populations may be at increased risk of oxazaphosphorine-induced systemic toxicities. Also, induction of aldehyde dehydrogenase activity is a demonstrated phenomenon in rodents [64, 65].

Retinoids, a family of natural and synthetic vitamin A analogs, are recognized as important regulators of the growth and differentiation of normal and transformed cells [66]. Since retinoic acid is the most potent of the naturally occurring retinoids in many of the systems used to assay retinoids for such activity [66–68], retinol is the major retinoid in the circulation [69], and oxidation of retinol to retinoic acid proceeds via the intermediate, retinaldehyde [7, 70, 71], enzyme-catalyzed oxidation of retinaldehyde to retinoic acid can be viewed as a bioactivation and most likely occurs in target cells. Thus, the rate and extent of retinoid-mediated differentiation of cells could very well be influenced by the rate at which cells convert retinol to retinoic acid. Supporting this notion is the observation that conversion of retinaldehyde to retinoic acid in keratinocytes undergoing differentiation occurs at a greater rate keratinocytes that are not [7].

Aldehyde dehydrogenases, aldehyde oxidase and xanthine oxidase have all been reported to catalyze the oxidation of retinaldehyde to retinoic acid [5, 6, 8, 72–77]. Available evidence suggests that, at least in some tissues, retinaldehyde oxidation is catalyzed primarily by one or more of the cytosolic, NAD-dependent, disulfiram-sensitive, substrate nonspecific, aldehyde dehydrogenases [5–8, 73, 76].

Of the six human hepatic aldehyde dehydrogenases examined in the present investigation, only ALDH-1 catalyzed the oxidation of retinaldehyde to retinoic acid. In addition, a retinaldehyde dehydrogenase apparently distinct from ALDH-1 has been identified in human keratinocytes [7]. The low K_m value for ALDH-1-catalyzed oxidation of retinaldehyde to retinoic acid is consistent with a physiological role for ALDH-1 in the *in vivo* generation of retinoic acid in those tissues where it is present.

Many neoplastic cell populations apparently lack ALDH-1 or, in species other than humans, its homolog [36, 38, 51]. This may preclude their ability to generate retinoic acid from retinaldehyde and, thus, to respond to retinol and β-carotene, another precursor of retinaldehyde [70, 71, 77, 78]. Further, relevant genetic polymorphism, and modulation of ALDH-1 activity, may have profound effects on retinoic acid-dependent processes.

Acknowledgements—This work was supported by USPHS Grant CA 21737. We gratefully acknowledge receipt of human liver specimens from the Liver Tissue Procurement and Distribution System which is supported by NIH Contract N01-DK-6-2274. The authors gratefully acknowledge the technical assistance provided by Mr. Matthew Hedge.

REFERENCES

1. Sladek NE, Manthey CL, Maki PA, Zhang Z and Landkamer GJ, Xenobiotic oxidation catalyzed by aldehyde dehydrogenases. *Drug Metab Rev* **20**: 697–720, 1989.
2. Goedde HW and Agarwal DP, Pharmacogenetics of aldehyde dehydrogenase (ALDH). *Pharmacol Ther* **45**: 345–371, 1990.
3. Sladek NE, Metabolism of oxazaphosphorines. *Pharmacol Ther* **37**: 301–355, 1988.
4. Yoshida A, Isozymes of human alcohol dehydrogenase and aldehyde dehydrogenase. In: *Isozymes: Structure, Function, and Use in Biology and Medicine* (Eds. Ogita Z-I and Markert CL), pp. 327–340. Wiley-Liss, New York, 1990.
5. Connor MJ and Smit MH, Terminal-group oxidation of retinol by mouse epidermis. Inhibition *in vitro* and *in vivo*. *Biochem J* **244**: 489–492, 1987.
6. Leo MA, Kim C-I, Lowe N and Lieber CS, Increased hepatic retinal dehydrogenase activity after phenobarbital and ethanol administration. *Biochem Pharmacol* **38**: 97–103, 1989.
7. Siegenthaler G, Saurat J-H and Ponec M, Retinol and retinal metabolism. Relationship to the state of differentiation of cultured human keratinocytes. *Biochem J* **268**: 371–378, 1990.
8. Lee M-O, Manthey CL and Sladek NE, Identification of mouse liver aldehyde dehydrogenases that catalyze

the oxidation of retinaldehyde to retinoic acid. *Biochem Pharmacol* **42**: 1279–1285, 1991.
9. Manthey CL, Landkamer GJ and Sladek NE, Identification of the mouse aldehyde dehydrogenases important in aldophosphamide detoxification. *Cancer Res* **50**: 4991–5002, 1990.
10. Rout UK and Holmes RS, Isoelectric focusing studies of aldehyde dehydrogenases from mouse tissues: Variant phenotypes of liver, stomach and testis isozymes. *Comp Biochem Physiol* **81B**: 647–651, 1985.
11. Mather PB and Holmes RS, Biochemical genetics of aldehyde dehydrogenase isozymes in the mouse: Evidence for stomach- and testis-specific isozymes. *Biochem Genet* **22**: 981–995, 1984.
12. Duley JA, Harris O and Holmes RS, Analysis of human alcohol- and aldehyde-metabolizing isozymes by electrophoresis and isoelectric focusing. *Alcoholism Clin Exp Res* **9**: 263–271, 1985.
13. Koivula T, Subcellular distribution and characterization of human liver aldehyde dehydrogenase fractions. *Life Sci* **16**: 1563–1570, 1975.
14. Kurys G, Ambroziak W and Pietruszko R, Human aldehyde dehydrogenase: Purification and characterization of a third isozyme with low K_m for γ-aminobutyraldehyde. *J Biol Chem* **264**: 4715–4721, 1989.
15. Ryzlak MT and Pietruszko R, Human brain "high K_m" aldehyde dehydrogenase: Purification, characterization, and identification as NAD^+-dependent succinic semialdehyde dehydrogenase. *Arch Biochem Biophys* **266**: 386–396, 1988.
16. Mezl VA and Knox WE, Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal Biochem* **74**: 430–440, 1976.
17. Ambroziak W and Pietruszko R, Human aldehyde dehydrogenase: Metabolism of putrescine and histamine. *Alcoholism Clin Exp Res* **11**: 528–532, 1987.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
19. Dixon M and Webb EC, *Enzymes*, 3rd Edn, p. 175. Academic Press, New York, 1979.
20. Wilkinson GN, Statistical estimations in enzyme kinetics. *Biochem J* **80**: 324–332, 1961.
21. Greenfield NJ and Pietruszko R, Two aldehyde dehydrogenases from human liver. Isolation via affinity chromatography and characterization of the isozymes. *Biochim Biophys Acta* **483**: 35–45, 1977.
22. Harada S, Agarwal DP and Goedde HW, Electrophoretic and biochemical studies of human aldehyde dehydrogenase isozymes in various tissues. *Life Sci* **26**: 1773–1780, 1980.
23. Della Corte E, Gozzetti G, Novello F and Stirpe F, Properties of the xanthine oxidase from human liver. *Biochim Biophys Acta* **191**: 164–166, 1969.
24. Krenitsky TA, Tuttle JV, Cattau EL and Wang P, A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. *Comp Biochem Physiol* **49B**: 687–703, 1974.
25. Forte-McRobbie CM and Pietruszko R, Aldehyde dehydrogenase content and composition of human liver. *Alcohol* **2**: 375–381, 1985.
26. Forte-McRobbie CM and Pietruszko R, Purification and characterization of human liver "high K_m" aldehyde dehydrogenase and its identification as glutamic semialdehyde dehydrogenase. *J Biol Chem* **261**: 2154–2163, 1986.
27. Henehan GT and Tipton KF, The effects of assay temperature on the complex kinetics of acetaldehyde oxidation by aldehyde dehydrogenase from human erythrocytes. *Biochem Pharmacol* **42**: 979–984, 1991.
28. Henehan GT, Ward K, Kennedy NP, Weir DG and Tipton KF, Subcellular distribution of aldehyde dehydrogenase activities in human liver. *Alcohol* **2**: 107–110, 1985.
29. Maki P and Sladek NE, Cytotoxic activity of mafosfamide against tumor and hematopoietic stem cells in the presence and absence of chloral hydrate. *Proc Am Assoc Cancer Res* **27**: 391, 1986.
30. Weiner H, Aldehyde dehydrogenase: Mechanism of action and possible physiological roles. In: *Biochemistry and Pharmacology of Ethanol* (Eds. Majchrowicz E and Nobel EP), Vol. 1, pp. 107–144. Plenum Press, New York, 1979.
31. Goedde HW, Agarwal DP and Harada S, The role of alcohol dehydrogenase and aldehyde dehydrogenase isozymes in alcohol metabolism, alcohol sensitivity, and alcoholism. In: *Isozymes: Current Topics in Biological and Medical Research* (Eds. Rattazzi M, Scandalios JG and Whitt GS), Vol. 8, pp. 175–193. Alan R. Liss, New York, 1983.
32. Pietruszko R, Aldehyde dehydrogenase isozymes. In: *Isozymes: Current Topics in Biological and Medical Research* (Eds. Rattazzi M, Scandalios JG and Whitt GS), Vol. 8, pp. 195–217. Alan R. Liss, New York, 1983.
33. Kohn FR and Sladek NE, Aldehyde dehydrogenase activity as the basis for the relative insensitivity of murine pluripotent hematopoietic stem cells to oxazaphosphorines. *Biochem Pharmacol* **34**: 3465–3471, 1985.
34. Kohn FR, Landkamer GJ and Sladek NE, Effect of the aldehyde dehydrogenase inhibitor, cyanamide, on the *ex vivo* sensitivity of murine multipotent and committed hematopoietic progenitor cells to mafosfamide (ASTA Z 7557). *Immunopharmacol Immunotoxicol* **9**: 163–176, 1987.
35. Kohn FR and Sladek NE, Effects of aldehyde dehydrogenase inhibitors on the *ex vivo* sensitivity of murine late spleen colony-forming cells (day-12 CFU-S) and hematopoietic repopulating cells to mafosfamide (ASTA Z 7557). *Biochem Pharmacol* **36**: 2805–2811, 1987.
36. Kohn FR, Landkamer GJ, Manthey CL, Ramsay NKC and Sladek NE, Effect of aldehyde dehydrogenase inhibitors on the *ex vivo* sensitivity of human multipotent and committed hematopoietic progenitor cells and malignant blood cells to oxazaphosphorines. *Cancer Res* **47**: 3180–3185, 1987.
37. Sahovic EA, Colvin M, Hilton J and Ogawa M, Role for aldehyde dehydrogenase in survival of progenitors for murine blast cell colonies after treatment with 4-hydroperoxycyclophosphamide *in vitro*. *Cancer Res* **48**: 1223–1226, 1988.
38. Manthey CL and Sladek NE, Aldehyde dehydrogenase-catalyzed bioinactivation of cyclophosphamide. In: *Progress in Clinical and Biological Research* (Eds. Weiner H and Flynn TG), Vol. 290, pp. 49–63. Alan R. Liss, New York, 1989.
39. Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI and Hilton J, Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* **75**: 1947–1950, 1990.
40. Dietrich RA, Tissue and subcellular distribution of mammalian aldehyde-oxidizing capacity. *Biochem Pharmacol* **15**: 1911–1922, 1966.
41. Helander A and Tottmar O, Cellular distribution and properties of human blood aldehyde dehydrogenase. *Alcoholism Clin Exp Res* **10**: 71–76, 1986.
42. Dyck LE, Isoenzymes of aldehyde dehydrogenase in human lymphocytes. *Alcoholism Clin Exp Res* **14**: 534–538, 1990.
43. Inoue K, Nishimukai H and Yamasawa K, Purification and partial characterization of aldehyde dehydrogenase

from human erythrocytes. *Biochim Biophys Acta* **569**: 117–123, 1979.
44. Agarwal DP, Tobar-Rojas L, Harada S and Goedde HW, Comparative study of erythrocyte aldehyde dehydrogenase in alcoholics and control subjects. *Pharmacol Biochem Behav* **18**: 89–95, 1983.
45. Santos GW, Sensenbrenner LL, Burke PJ, Mullins GM, Bias WB, Tutschka PJ and Slavin RE, The use of cyclophosphamide for clinical marrow transplantation. *Transplant Proc* **4**: 559–564, 1972.
46. Yoshida A, Wang G and Dave V, A possible structural variant of human cytosolic aldehyde dehydrogenase with diminished enzyme activity. *Ann Hum Genet* **35**: 1115–1116, 1983.
47. Yoshida A, Dave V, Ward RJ and Peters TJ, Cytosolic aldehyde dehydrogenase (ALDH-1) variants found in alcohol flushers. *Ann Hum Genet* **53**: 1–7, 1989.
48. Goedde HW, Harada S and Agarwal DP, Racial differences in alcohol sensitivity: A new hypothesis. *Hum Genet* **51**: 331–334, 1979.
49. Russo JE, Hilton J and Colvin OM, The role of aldehyde dehydrogenase isozymes in cellular resistance to the alkylating agent cyclophosphamide. In: *Progress in Clinical and Biological Research* (Eds. Weiner H and Flynn TG), Vol. 290, pp. 65–79. Alan R. Liss, New York, 1989.
50. Hilton J, Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210-leukemia. *Cancer Res* **44**: 5156–5160, 1984.
51. Russo JE and Hilton J, Characterization of cytosolic aldehyde dehydrogenase from cyclophosphamide resistant L1210 cells. *Cancer Res* **48**: 2963–2968, 1988.
52. Sladek NE and Landkamer GJ, Restoration of sensitivity to oxazaphosphorines by inhibitors of aldehyde dehydrogenase activity in cultured oxaphosphorine-resistant L1210 and cross-linking agent-resistant P388 cell lines. *Cancer Res* **45**: 1549–1555, 1985.
53. Koelling TM, Yeager AM, Hilton J, Haynie DT and Wiley JM, Development and characterization of a cyclophosphamide-resistant subline of acute myeloid leukemia in the Lewis × Brown Norway hybrid rat. *Blood* **76**: 1209–1213, 1990.
54. Crow KE, Kitson TM, MacGibbon AKH and Batt RD, Intracellular localisation and properties of aldehyde dehydrogenases from sheep liver. *Biochim Biophys Acta* **350**: 121–128, 1974.
55. Sidhu RS and Blair AH, Human liver aldehyde dehydrogenase. Kinetics of aldehyde oxidation. *J Biol Chem* **250**: 7899–7904, 1975.
56. Koivula T and Koivusalo M, Different forms of rat liver aldehyde dehydrogenase and their subcellular distribution. *Biochim Biophys Acta* **397**: 9–23, 1975.
57. Weiner H, Freytag S, Fox JM and Hu JHJ, Reversible inhibitors of aldehyde dehydrogenase. In: *Progress in Clinical and Biological Research* (Eds. Weiner H and Wermuth B), Vol. 114, pp. 91–102. Alan R. Liss, New York, 1982.
58. Brien JF, Tam GS, Cameron RJ, Steenaart NAE and Loomis CW, A comparative study of the inhibition of hepatic aldehyde dehydrogenases in the rat by methyltetrazolethiol, calcium carbimide, and disulfiram. *Can J Physiol Pharmacol* **63**: 438–443, 1985.
59. Brien JF and Loomis CW, Aldehyde dehydrogenase inhibitors as alcohol-sensitizing drugs: A pharmacological perspective. *Trends Pharmacol Sci* **6**: 477–480, 1985.
60. Jenkins WJ and Peters TJ, Selectively reduced hepatic acetaldehyde dehydrogenase in alcoholics. *Lancet* **1**: 628–629, 1980.
61. Thomas M, Halsall S and Peters TJ, Role of hepatic acetaldehyde dehydrogenase in alcoholism: Demonstration of persistent reduction of cytosolic activity in abstaining patients. *Lancet* **2**: 1057–1059, 1982.
62. Lin C-C, Potter JJ and Mezey E, Erythrocyte aldehyde dehydrogenase activity in alcoholism. *Alcoholism Clin Exp Res* **8**: 539–541, 1984.
63. Jeavons CM and Zeiner AR, Effects of elevated female sex steroids on ethanol and acetaldehyde metabolism in humans. *Alcoholism Clin Exp Res* **8**: 352–358, 1984.
64. Deitrich RA, Bludeau P, Stock T and Roper M, Induction of different rat liver supernatant aldehyde dehydrogenases by phenobarbital and tetrachlorodibenzo-*p*-dioxin. *J Biol Chem* **252**: 6169–6176, 1977.
65. Deitrich RA, Bludeau P, Roper M and Schmuck J, Induction of aldehyde dehydrogenases. *Biochem Pharmacol* **27**: 2343–2347, 1978.
66. Lotan R, Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim Biophys Acta* **605**: 33–91, 1980.
67. Breitman TR, Selonick SE and Collins SJ, Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci USA* **5**: 2936–2940, 1980.
68. Williams JB and Napoli JL, Metabolism of retinoic acid and retinol during differentiation of F9 embryonal carcinoma cells. *Proc Natl Acad Sci USA* **82**: 4658–4662, 1985.
69. Wolf G, Multiple functions of vitamin A. *Physiol Rev* **64**: 873–937, 1984.
70. Olson JA, The metabolism of vitamin A. *Pharmacol Rev* **19**: 559–596, 1967.
71. Allen JG and Bloxham DP, The pharmacology and pharmacokinetics of the retinoids. *Pharmacol Ther* **40**: 1–27, 1989.
72. Futterman S, Enzymatic oxidation of vitamin A aldehyde to vitamin A acid. *J Biol Chem* **237**: 677–680, 1962.
73. Elder TD and Topper YJ, The oxidation of retinene (vitamin A_1 aldehyde) to vitamin A acid by mammalian steroid-sensitive aldehyde dehydrogenase. *Biochim Biophys Acta* **64**: 430–437, 1962.
74. Lakshmanan MR, Vaidyanathan CS and Cama HR, Oxidation of vitamin A_1 aldehyde and vitamin A_2 aldehyde to the corresponding acids by aldehyde oxidase from different species. *Biochem J* **90**: 569–573, 1964.
75. Moffa DJ, Lotspeich FJ and Krause RF, Preparation and properties of retinal-oxidizing enzyme from rat intestinal mucosa. *J Biol Chem* **245**: 439–447, 1970.
76. Napoli JL and Race KR, The biosynthesis of retinoic acid from retinol by rat tissues *in vitro*. *Arch Biochem Biophys* **255**: 95–101, 1987.
77. Olson JA, Provitamin A function of carotenoids: The conversion of β-carotene into vitamin A. *J Nutr* **119**: 105–108, 1989.
78. Lakshman MR, Mychkovsky I and Attlesey M, Enzymatic conversion of all-*trans*-β-carotene to retinal by a cytosolic enzyme from rabbit and rat intestinal mucosa. *Proc Natl Acad Sci USA* **86**: 9124–9128, 1989.