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IDENTIFICATION OF MOUSE LIVER ALDEHYDE DEHYDROGENASES THAT CATALYZE THE OXIDATION OF RETINALDEHYDE TO RETINOIC ACID*

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Abstract—NAD(P)-linked aldehyde dehydrogenases catalyze the oxidation of a wide variety of aldehydes. Thirteen of these enzymes have been identified in mouse tissues; eleven are found in the liver. Some are substrate-nonspecific; others are relatively substrate-specific. The present investigation sought to determine which of these enzymes are operative in catalyzing the oxidation of retinaldehyde to retinoic acid, a metabolite of vitamin A that promotes the differentiation of epithelial and other cells. Spectrophotometric and HPLC assays were used for this purpose. Enzyme-catalyzed oxidation of retinaldehyde (25 μM) was restricted to the cytosol (105,000 *g* supernatant fraction) and occurred at a rate of 211 nmol/min/g liver; oxidation of acetaldehyde (4 mM) by this fraction proceeds about ten times faster. At least 90% of this activity was NAD dependent. Of the approximately 10% that was apparently NAD independent, two-thirds was inhibited by 1 mM pyridoxal, a known inhibitor of aldehyde oxidase. Of the six cytosolic aldehyde dehydrogenases, only two, viz. AHD-2 and AHD-7, catalyzed the oxidation of retinaldehyde to retinoic acid. An additional NAD-dependent enzyme, viz. xanthine oxidase (dehydrogenase form), also catalyzed the reaction. Catalysis by AHD-2 accounted for more than 90% of the total NAD-dependent activity. K_m values were 0.7, 0.6 and 0.9 μM, respectively, for the AHD-2-, AHD-7- and xanthine oxidase (dehydrogenase form)-catalyzed reaction. AHD-4, an aldehyde dehydrogenase found in the cytosol of mouse stomach epithelium and cornea, did not catalyze the reaction.

Retinoids, a family of natural and synthetic vitamin A analogues, are recognized as important regulators of the growth and differentiation of normal and transformed cells [1]. In many of the systems used to assay retinoids for such activity, retinoic acid is the most potent of the naturally occurring retinoids [1–3]. *In vivo*, the source of retinoic acid is retinaldehyde; oxidation of retinol and metabolic cleavage of β-carotene give rise to this intermediate [4–7].

Enzyme-catalyzed retinaldehyde oxidation to retinoic acid has been observed in intact cells as well as the cytosol of many tissues/species [4, 8–22]. NAD-dependent dehydrogenase(s), aldehyde oxidase (EC 1.2.3.1), and xanthine oxidase (EC 1.2.3.2) have been reported to participate in the catalysis of this reaction [5, 9, 12, 13, 19, 23–25]. Available evidence suggests that, at least in some tissues, retinaldehyde oxidation is catalyzed primarily by one or more of the cytosolic, NAD-linked, disulfiram-sensitive, substrate nonspecific, aldehyde dehydrogenases (EC 1.2.1.3) [12, 13, 19, 21, 24].

Thirteen aldehyde dehydrogenases have been identified in mouse tissues [26]. All but one, viz. AHD-8, exhibit a rather broad substrate specificity though some markedly favor specific aldehydes as substrates. Some appear to be relatively tissue-specific whereas others are relatively broadly distributed. Most are present in the mitochondria, endoplasmic reticulum or cytosol of liver. Thus, eleven of these enzymes have been found in DBA/2 mouse liver; six of them and XOX(D)‡ are found in the cytosol (105,000 *g* supernatant fraction). Not known is which of these catalyze the oxidation of retinaldehyde to retinoic acid. The present investigation was designed to identify these enzymes and to determine the relative contribution of each of the relevant enzymes to the oxidation of retinaldehyde to retinoic acid in mouse liver.

MATERIALS AND METHODS

Materials. All-*trans*-retinaldehyde, all-*trans*-retinoic acid, acetaldehyde, benzaldehyde, octanal, dimethyl sulfoxide (> 99%) and tetraphenylethylene were purchased from the Aldrich Chemical Co., Milwaukee, WI. As judged by HPLC/spectrophotometry, *vide infra*, all-*trans*-retinaldehyde was retinoic acid-free but *cis*-retinaldehyde (< 1% of total) was sometimes present. Betaine aldehyde, NAD^+, $NADP^+$, NADH, pyrazole, glutathione, pyridoxal HCl, Lubrol® and bovine serum albumin (crystallized and lyophilized) were purchased from the Sigma Chemical Co., St. Louis, MO. Aldophosphamide was prepared as described previously [27, 28]. HPLC-grade solvents were

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‡ Abbreviation: XOX(D), xanthine oxidase (dehydrogenase form).

obtained from Fisher Scientific Ltd., Los Angeles, CA, and were filtered through 0.45 μm Durapore filters, Millipore, Bedford, MA, prior to use. DEAE-Sephacel, 5′-AMP-Sepharose and PD-10 columns were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ. The Ultrasphere-ODS HPLC column and the Adsorbosphere-ODS guard column were obtained from Beckman, Fullerton, CA, and Alltech Associates, Inc., Deerfield, IL, respectively. Female DBA/2 mice, 8- to 10-weeks-old, were purchased from the Harland Sprague Dawley Co., Indianapolis, IN, and were maintained on standard laboratory diet. Mouse liver 105,000 *g* soluble, and solubilized particulate, fractions were prepared as previously described [28] except that 0.3% Lubrol®, rather than 0.3% deoxycholate was used to solubilize 105,000 *g* particulate fractions. With the exception of AHD-7, the semipurified aldehyde dehydrogenases and the XOX(D) used in the initial experiments (see Table 2) were those prepared previously [28]. They had been stored for up to 19 months at −70° in a 20 mM triethanolamine buffer, pH 7.4, containing 1 mM dithiothreitol, 0.1 mM EDTA, and 25% glycerol. Fresh AHD-7, AHD-2 and XOX(D) were prepared as described previously [28].

Chromatographic resolution of hepatic soluble fraction aldehyde dehydrogenases. DEAE-Sephacel and 5′-AMP-Sepharose column chromatography was used sequentially as described previously [28]. Elution of aldehyde dehydrogenase activity off these columns was followed using the spectrophotometric aldehyde dehydrogenase assay described below. Several substrates, viz. retinaldehyde (5 μM), acetaldehyde (4 mM), benzaldehyde (4 mM) and octanal (100 μM), were used for this purpose. A refractometer was used to estimate the NaCl concentration of selected eluate fractions. Elution of protein from chromatography columns was routinely monitored at 280 nm. Protein content was also determined by the Coomassie brilliant blue protein dye binding assay [29]; a commercially available (Bio-Rad) reagent was used. Bovine serum albumin served as the standard.

Spectrophotometric assay for aldehyde dehydrogenase activity. Except when retinaldehyde was the substrate, enzyme activity was always quantified at 37° by monitoring the appearance of NADH at 340 nm with a Gilford Response spectrophotometer. 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, semipurified XOX(D) or a semipurified aldehyde dehydrogenase. Retinaldehyde, octanal and benzaldehyde were added in 5 μL of nitrogen-bubbled dimethyl sulfoxide, 2 μL of methanol, and 50 μL of a water:methanol mixture (9:1), respectively. Acetaldehyde and betaine aldehyde (25 μM) were added in 50 μL of water. Aldophosphamide was added in a volume of 50 μL as described previously [27, 28]. The reaction was initiated by addition of substrate. Rates were measured (and were linear) over the first 3 min of assay except when the integrated Michaelis–Menten method of analysis of single enzyme progress curves [30] was used to determine K_m values. In some cases, enzyme-catalyzed oxidation of retinaldehyde to retinoic acid was also quantified at 37° by monitoring at 340 nm with a Gilford Response spectrophotometer. However, in addition to NADH, retinaldehyde and retinoic acid absorb at this wavelength. This was taken into account when calculating the rate of enzyme-catalyzed retinaldehyde oxidation; known molar absorption coefficients (E_{340}) of retinoic acid, retinaldehyde and NADH were used for this purpose [24]. Where possible, all manipulations were done under dim light when retinaldehyde was used as a substrate.

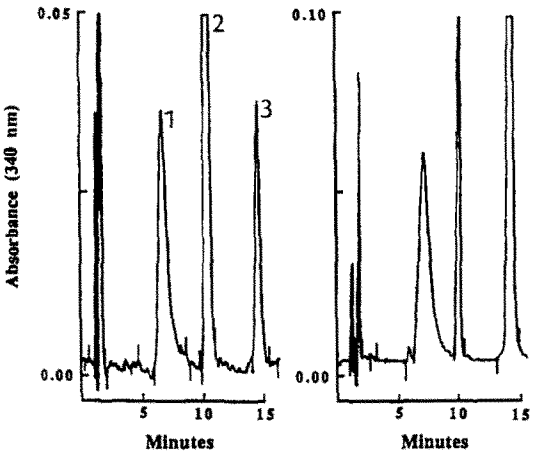

Fig. 1. HPLC chromatograms of all-*trans*-retinaldehyde, all-*trans*-retinoic acid and tetraphenylethylene. Left: A solution of all-*trans*-retinaldehyde and all-*trans*-retinoic acid in dimethyl sulfoxide was added to a solution of butanol/acetonitrile (1:1) containing tetraphenylethylene and the resultant mixture was injected into an ultrasphere-ODS HPLC column. Peak 1, 100 pmol of all-*trans*-retinoic acid; peak 2, 1 nmol of tetraphenylethylene; and peak 3, 100 pmol of all-*trans*-retinaldehyde. Right: All-*trans*-retinaldehyde (25 μM) and semipurified AHD-2 were incubated for 5 min. The reaction mixture was then extracted and 20 μL of the extract was injected into an ultrasphere-ODS HPLC column. Details are as described in Materials and Methods.

HPLC/Spectrophotometric assay for retinoids. This assay [17] was used to confirm and sometimes to quantify aldehyde dehydrogenase-catalyzed oxidation of retinaldehyde (25 μM) to retinoic acid. All-*trans*-retinaldehyde was added to the reaction mixture in 25 μL of nitrogen-bubbled dimethyl sulfoxide; otherwise, the reaction mixture was as described above and was incubated in foil-wrapped disposable borosilicate glass tubes at 37°. After 5 min of incubation, the reaction was stopped by placing the reaction mixture into an ice-water bath. Four hundred microliters of a butanol/acetonitrile (1:1) mixture containing 50 μM tetraphenylethylene (internal standard) was added to the reaction mixture. After vortexing for 60 sec, 300 μL of a saturated dipotassium monohydrogen phosphate in water solution was added and the mixture was vortexed for 30 sec [31]. It was then centrifuged at 500 *g* for 10 min. Twenty microliters of the resultant

Table 1. Oxidation of retinaldehyde catalyzed by enzymes in the soluble and solubilized particulate fractions of mouse liver

Subcellular fraction	Enzyme activity (nmol/min/g liver)		
	Retinaldehyde (25 μM)		
	+NAD (4 mM)	−NAD	Acetaldehyde (4 mM)
Soluble	211 ± 7†	15 ± 2†	2468‡
Particulate	0	0	2399‡

Soluble and solubilized particulate fractions were prepared from pooled livers of three, six and ten mice and were assayed for aldehyde dehydrogenase activity. The HPLC/spectrophotometric assay was used to quantify the rate of retinaldehyde oxidation; the spectrophotometric assay was used to quantify the rate of acetaldehyde oxidation.

† Values are means ± SEM of three determinations, each made with fractions obtained from different preparations.

‡ Values are the means of duplicate measurements made with one of the preparations (pooled livers of three mice).

organic phase (extraction efficiency approached 100%) was injected directly into an ultrasphere-ODS HPLC column (4.6 mm i.d. × 25 cm) fitted with a guard column. Elution was with acetonitrile/water (4:1) containing 1% ammonium acetate, at a flow rate of 2 mL/min. A Beckman model 322 programmable liquid chromatographic system with a Beckmann model 160 UV detector was used to monitor the column eluate at 340 nm. Representative chromatograms are presented in Fig. 1. Retinoic acid and retinaldehyde concentrations were estimated by determining the peak areas of these retinoids and comparing them to that of tetraphenylethylene, the internal standard. A Hewlett–Packard-3390A integrator was used to determine peak areas. The lower limit of detection was 7.5 pmol for both retinoic acid and retinaldehyde. All experiments were done in duplicate and under dim light.

RESULTS

Initial experiments indicated that enzyme-catalyzed retinaldehyde oxidation by mouse liver was localized in the soluble fraction of this organ (Table 1). Retinaldehyde oxidation occurred at the rate of 211 nmol/min/g liver when NAD was present in the incubation mixture; omission of NAD reduced activity by more than 90%. The pyridine nucleotide-dependent reaction was not supported by NADP. Acetaldehyde oxidation catalyzed by the soluble fraction proceeded about ten times faster than retinaldehyde oxidation. Pyridoxal (1 mM), a known inhibitor of aldehyde oxidase, inhibited approximately two-thirds of the NAD-independent, enzyme-catalyzed, retinaldehyde oxidation, suggesting that aldehyde oxidase was involved in the catalysis of this part of the reaction.

In keeping with the foregoing observations, none of the five individual aldehyde dehydrogenases present in the solubilized particulate fraction of mouse liver was able to catalyze the oxidation of retinaldehyde to retinoic acid (Table 2). Of the six aldehyde dehydrogenases present in the soluble fraction of mouse liver, only two, viz. AHD-2 and AHD-7, catalyzed the reaction. XOX(D) also catalyzed it. An aldehyde dehydrogenase present in the soluble fraction of mouse stomach but not mouse liver, viz. AHD-4, was unable to catalyze the oxidation of retinaldehyde.

The relative contribution of AHD-2, AHD-7 and XOX(D) to total mouse hepatic NAD-dependent enzyme-catalyzed oxidation of retinaldehyde to retinoic acid was determined in the next series of experiments. DEAE-Sephacel and 5′-AMP-Sepharose column chromatography was used sequentially to separate and semipurify the enzymes of interest present in the soluble fraction of freshly obtained mouse liver (Fig. 2). The relative contribution of each enzyme to NAD-dependent enzyme-catalyzed oxidation of retinaldehyde is presented in Table 3. It is obvious that AHD-2 is by far the most prominent in this regard. K_m values were also determined; all were less than 1 μM.

DISCUSSION

We found that mouse hepatic particulate fractions did not catalyze the oxidation of retinaldehyde to retinoic acid and that NAD was required for the bulk (> 90%) of retinaldehyde oxidation catalyzed by mouse liver cytosol. NAD-dependent enzyme-catalyzed retinaldehyde oxidation in the cytosol of various tissues/cells obtained from several mammalian species has been reported by others. In close agreement with our observations, 90–95% of the enzyme activity of interest was NAD dependent in rat liver [19]. Still others report values of 76% (rat liver) [12], 70% (rat kidney) [12], 57% (rat kidney) [15], 51% (rat intestinal mucosa) [14], 21% (deer mouse liver) [19], 14% (mouse epidermis) [10], and 82% (human keratinocytes) [21]. Some of these differences are probably due to differences in experimental conditions. Others are undoubtedly

Table 2. Data used to conclude that some mouse aldehyde dehydrogenases catalyze the oxidation of retinaldehyde whereas others do not

Subcellular fraction	AHD-	Index value*
Liver soluble	2	11.28
	7	1.28
	8	0.44†
	9	0.03†
	10	0.45†
	11	0.14†
	XOX(D)	5.01
Liver particulate	1a	0.15†
	1b	0.23†
	3	0.09†
	5	0.33†
	12a	0.13†
	12b	0.17†
	12c	0.68†
	13	0.15†
Stomach soluble	4	0.06†

* Index value = (0.03 nmol/min or observed rate of retinaldehyde oxidation) (100)/(rate of oxidation with reference substrate). Reference substrates were 4 mM benzaldehyde (XOX(D)), 160 μM aldophosphamide (AHD-8), 25 μM betaine aldehyde (AHD-9), and 100 μM octanal (all other enzymes). The indicated reference substrate for each enzyme was chosen as such because it was one of the preferred substrates for that enzyme [28]. The value of 0.03 nmol/min was used (†) to calculate the index value when there was no measurable rate observed because it was considered to be the smallest detectable rate. Thus, it was used to calculate the upper limit of the relative rate of enzyme-catalyzed retinaldehyde oxidation that, while not detectable, might be occurring. The spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde, aldophosphamide, betaine aldehyde and octanal. The HPLC/spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of retinaldehyde (25 μM).

due to tissues/species differences in the relative content of relevant aldehyde dehydrogenases, aldehyde oxidase, xanthine oxidase, and perhaps additional, as yet unidentified, enzymes.

The six aldehyde dehydrogenases present in mouse liver cytosol and mouse liver XOX(D) were examined for their capacity to catalyze the oxidation of retinaldehyde to retinoic acid. Only three of these enzymes, viz. AHD-2, AHD-7, and XOX(D), were found to be capable of doing so. As judged by K_m values (Table 3), retinaldehyde is a good substrate for each of these enzymes. Of the three, AHD-2 is clearly the most important in mouse liver since it accounted for 95% of the total dehydrogenase-catalyzed retinaldehyde oxidation to retinoic acid in this tissue. AHD-2 is also of primary importance when benzaldehyde or aldophosphamide is the substrate [26]. It is not of primary importance when acetaldehyde is the substrate; AHD-5 is [26]. AHD-2 is present in a number of mouse tissues, e.g. liver, lung and testes [32]. A homologue of AHD-2 is the only aldehyde dehydrogenase found in human red blood cells [33]. However, AHD-2 is apparently not expressed in mouse kidney, stomach, ovary, heart and brain [32]. Whether it is expressed in mouse intestine is not clear [32, 34]. AHD-7 is expressed in mouse liver and kidney but apparently not in mouse stomach, intestine, testes, ovary, heart, lung and brain [32].

In agreement with the findings of others [23, 25], the present study shows that XOX(D) catalyzes NAD-dependent retinaldehyde oxidation and suggests that aldehyde oxidase may catalyze the NAD-independent reaction. Xanthine oxidase and aldehyde oxidase are metalloflavoproteins. They exhibit overlapping substrate specificities [35] and coexist in several species including humans [36–38]. Xanthine oxidase is present in many mammalian tissues; the distribution of aldehyde oxidase is more limited [32, 39]. They are probably relatively important catalysts of retinaldehyde oxidation in certain tissues, viz. those in which the aldehyde dehydrogenases are not present or are expressed at a low level. For example, AHD-4, AHD-8 and low levels of a particulate fraction aldehyde dehydrogenase, viz. AHD-5, are the only aldehyde dehydrogenases found in the stomach [28]; these enzymes were not capable of catalyzing the oxidation of retinaldehyde to retinoic acid (Table 2). However, xanthine oxidase activity is not found in this tissue [32], and the presence of aldehyde oxidase activity is questionable [32, 39]. On the other hand, xanthine oxidase and/or aldehyde oxidase activity is present in certain other tissues that lack AHD-2, e.g. kidney, ovary, heart and brain [32].

Since species homology of certain aldehyde dehydrogenases has been noted [28], the observations reported herein may apply to human hepatic aldehyde dehydrogenase-catalyzed oxidation of retinaldehyde to retinoic acid. If the mouse is a relevant model, ALDH-1 (also called ALDH-II or E1 [40]), the purported human homologue of mouse AHD-2, is likely to be the primary mediator of NAD-dependent enzyme-catalyzed oxidation of retinaldehyde to retinoic acid in human liver.

Given that retinoid-mediated differentiation of cells in culture is generally more sensitive to retinoic acid than to retinol, the major retinoid in the circulation [1], and thus, that oxidation of retinol to retinoic acid is likely to occur in target cells, the rate and extent of retinoid-mediated differentiation of cells could very well be directly influenced by the rate at which such cells convert retinol to retinoic acid. An important determinant in such a scenario could be, at least in some cells, one or more of the enzymes that convert retinaldehyde to retinoic acid. Included would be the relevant aldehyde dehydrogenases identified in the present study. Clearly, understanding the metabolism of the retinoids is potentially important for the foregoing reasons and perhaps to better understand the etiology of retinoid-related diseases. It should be remembered that although XOX(D), AHD-7 and, especially, AHD-2 are able to convert retinaldehyde to retinoic acid *in vitro*, it is uncertain how important they and/or other enzymes that catalyze this reaction are in this regard *in vivo*. Moreover, other factors, including tissue uptake or efflux of retinoids, cellular

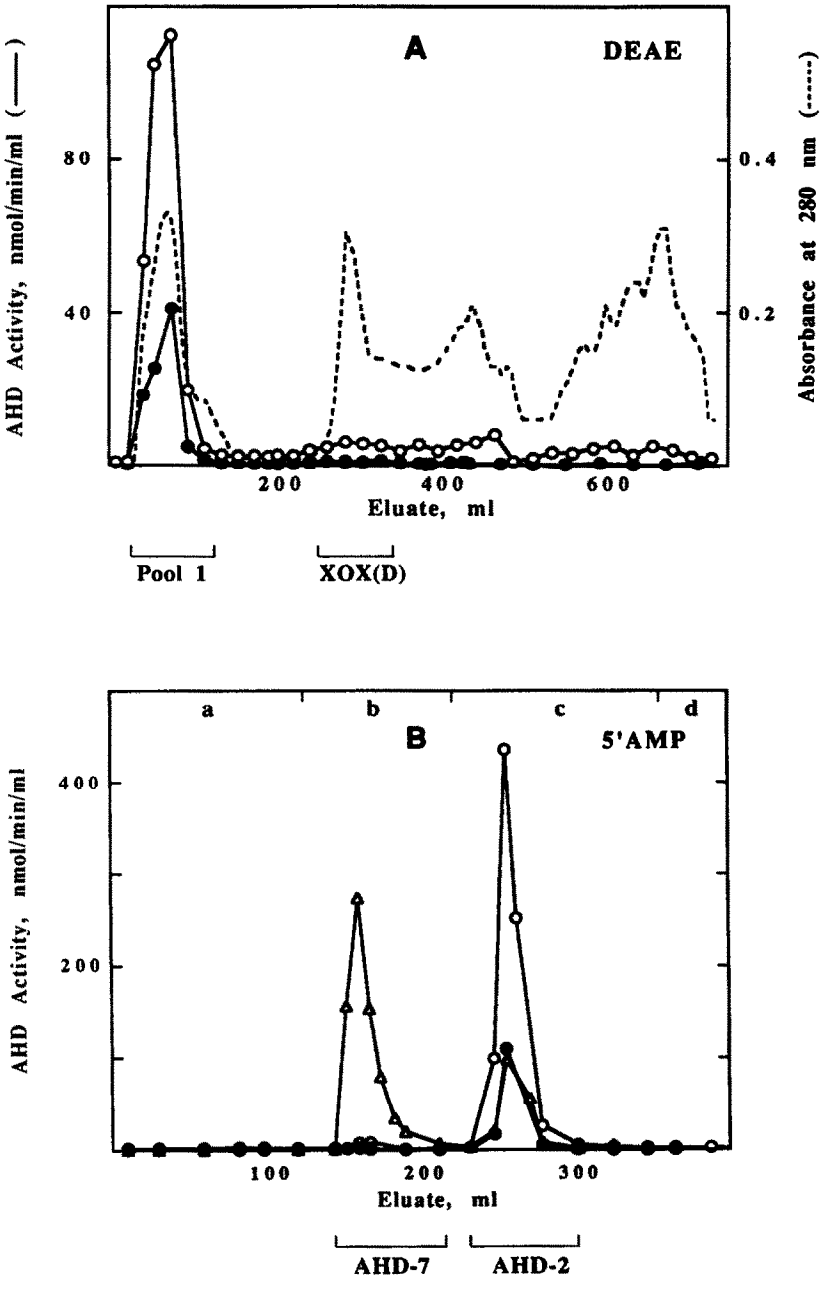

Fig. 2. Chromatographic separation of mouse liver 105,000 *g* soluble fraction aldehyde dehydrogenases. (A) A 105,000 *g* soluble fraction prepared from pooled livers of five mice (5.2 g liver) was transferred, with the aid of Pharmacia PD-10 columns, from homogenization medium into a 20 mM imidazole buffer, pH 7.2 (buffer A). The latter preparation (30 mL) was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The loaded column was eluted with 100 mL of buffer A and then with a 0.0 to 0.2 M NaCl gradient (350 mL) generated in buffer A. Elution was completed with 200 mL of buffer A adjusted to 0.7 M NaCl. (B) DEAE-Pool 1 aldehyde dehydrogenase activity (110 mL) was loaded directly onto a 5′-AMP-Sepharose column equilibrated with buffer A. The loaded column was developed as delineated at the top of the panel: (a) DEAE-Pool 1 enzyme in elution buffer A; (b) 50 mM sodium phosphate buffer pH 7.4; (c) 50 mM sodium phosphate buffer, pH 7.4, containing 0.5 mM NAD; and (d) buffer A containing 0.5 mM NAD and 0.7 M NaCl. All of the buffers referred to above also contained 1.0 mM dithiothreitol and 0.1 mM EDTA. Eluant was collected in fractions of 6–10 mL. Substrates used to monitor aldehyde dehydrogenase activity were 5 μM retinaldehyde (●), 4 mM acetaldehyde (○), and 4 mM benzaldehyde (△). The spectrophotometric assay was used to monitor enzyme activity. The crude soluble fraction contained aldehyde dehydrogenase activities of 258 and 1,817 nmol/min/g liver when 5 μM retinaldehyde and 4 mM acetaldehyde, respectively, were used as substrates. Not shown is the further purification of XOX(D) by passage of the pool demarcated as containing this enzyme in panel A through a 5′-AMP-Sepharose column as described previously [28].

Table 3. AHD-2-, AHD-7-, and XOX(D)-catalyzed oxidation of retinaldehyde

Enzyme	Percent contribution*	K_m† (μM)
AHD-2	94.9	0.7
AHD-7	0.9	0.6
XOX(D)	4.2	0.9

* Relative contribution to total hepatic NAD-dependent enzyme-catalyzed oxidation of 25 μM retinaldehyde. Fractions off 5'-AMP-Sepharose columns containing the enzyme of interest were pooled in each case (Fig. 2), and the HPLC/spectrophotometric assay was used to quantify enzyme activity.

† Values are the means of two experiments utilizing the spectrophotometric assay. The initial retinaldehyde concentration was 5 μM. Lines were fitted to data points using least squares regression analysis.

retinol and retinoic acid binding proteins (CRBP and CRABP), and specific nuclear receptors are also likely to affect retinoid-promoted cellular differentiation.

Establishing the importance, or lack thereof, of these enzymes in retinoid metabolism, and thus biological action, is particularly important because aldehyde dehydrogenases are sensitive to a wide variety of physiological, pharmacological and environmental inhibitors/suppressors/inducers [40–43]. For example, rat cytosolic aldehyde dehydrogenase activity is inducible 30- and 100-fold by phenobarbital and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, respectively [41, 42]; human hepatic and red blood cell aldehyde dehydrogenase activity, specifically ALDH-1, is less than usual in alcoholics [40, 44, 45]; and indirect evidence suggests that a decrease in human aldehyde dehydrogenase activity is elicited by oral contraceptives [46]. Moreover, so-called "alcohol deterrents" such as disulfiram are thought to be of clinical value specifically because they inhibit aldehyde dehydrogenases. Directly relevant, phenobarbital or chronic ethanol administration to rats results in increased hepatic "retinaldehyde dehydrogenase" activity [19].

Of potential relevance also are the observations that neoplastic tissues expressed much lower levels of aldehyde dehydrogenase activity than did a variety of normal tissues [39], that human multipotent hematopoietic progenitor cells contain aldehyde dehydrogenase activity but that it is lost or diminished upon transformation of these cells to malignant cells [47], and that "retinaldehyde oxidase" activity is less in *N*-methyl-*N*-nitrosourea-induced rat mammary carcinomas as compared to that in normal mammary gland [48]. A characteristic of malignant cells is that they fail to fully differentiate. The hypothesis could be formulated that, at least in some cases, the absence/diminished activity of the enzymes that activate retinoids, failure to differentiate, and malignant transformation are related.

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