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ENZYMOLOGY AND SUBCELLULAR LOCALIZATION OF ALDEHYDE OXIDATION IN RAT LIVER

OXIDATION OF 3,4-DIHYDROXYPHENYLACETALDEHYDE DERIVED FROM DOPAMINE TO 3,4-DIHYDROXYPHENYLACETIC ACID*

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Abstract—In the presence of ethanol, the metabolism of dopamine in rat liver slices is altered such that the major product is 3,4-dihydroxyphenylethanol, not 3,4-dihydroxyphenylacetic acid (DOPAC). It has been proposed that this metabolic alteration is due to the inhibition of the oxidation of 3,4-dihydroxyphenylacetaldehyde (DOPAL) by acetaldehyde, the first metabolite of ethanol. The oxidation of DOPAL in rat liver slices, however, is not inhibited dramatically by relatively high concentrations of acetaldehyde [A. W. Tank and H. Weiner, *Biochem. Pharmac.* **28**, 3139 (1979)]. Thus, it is possible that acetaldehyde and DOPAL are oxidized by different isozymes of aldehyde dehydrogenase (ALDH) present in different subcellular compartments. Acetaldehyde is oxidized by isozymes of ALDH that are found in the matrix space of the mitochondria of rat liver. The subcellular site of the oxidation of most other aldehydes is not known. Mitochondrial, microsomal and cytosol fractions of the rat liver were isolated by differential centrifugation, and the isozymes of ALDH present in the cytosol and mitochondrial fractions were separated by column isoelectric focusing. Five isozymes of ALDH were isolated from the cytosol, and three isozymes were isolated from the mitochondria. The K_m values for acetaldehyde, *p*-nitrobenzaldehyde and DOPAL for each of the isolated isozymes were determined and were found to range from approximately 1 μM to 1 mM. Each subcellular fraction was incubated with [ethylamine-2-^{14}C]dopamine to determine its ability to oxidize DOPAL. Partially purified monoamine oxidase was used to generate DOPAL for those incubations which did not contain mitochondria. Intact mitochondria were capable of oxidizing virtually all the DOPAL to DOPAC in the presence or absence of added pyridine nucleotide coenzymes. Cytosol and microsomal fractions were capable of oxidizing the aldehyde, but not to the same extent as the intact mitochondria. ALDH activity present in the mitochondrial matrix space was inhibited by the addition of rotenone. This treatment inhibited formation of DOPAC by 80 per cent in isolated intact mitochondria in the absence of added pyridine nucleotides. Inclusion of rotenone caused the inhibition of DOPAC formation by *ca.* 50 per cent when intact mitochondria, microsomes and cytosol were incubated together with dopamine. These results suggest that an isozyme of ALDH present in the mitochondrial matrix space is primarily responsible for the oxidation of DOPAL in rat liver, though nonmitochondrial enzymes can contribute to the oxidation.

Dopamine is oxidatively deaminated by monoamine oxidase (monoamine:O_2 oxidoreductase, EC 1.4.3.4, MAO‖) to its corresponding aldehyde derivative 3,4-dihydroxyphenylacetaldehyde (DOPAL). This aldehyde can then be either oxidized to its corresponding acid by an NAD-dependent aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3, ALDH) or reduced to its corresponding alcohol by an NAD-dependent alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1, ADH) or an NADP-dependent aldehyde reductase (alcohol:NADP oxidoreductase, EC 1.1.1.2, ALRed). In rat liver the two reducing enzymes are found in the cytosol, whereas multimolecular forms of ALDH are found in cytosol, mitochondria and microsomes. The different forms of rat liver ALDH have been described by a number of workers [1–10]. Shum and Blair [3], Deitrich *et al.* [6], and Koivula and Koivusalo [5] have all demonstrated the presence of at least two isoenzymes in rat liver cytosol. Other workers [1, 4, 5] have reported the presence of at least two aldehyde dehydrogenases in rat liver mitochondria. It has been shown that one of these mitochondrial enzymes is located on the outer membrane or in the space between the membranes, while the other enzyme is found in the matrix space [7–9]. Rat microsomal

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‖ Abbreviations: MAO, monoamine oxidase; ALDH, aldehyde dehydrogenase; DOPET, 3,4-dihydroxyphenylethanol; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPAC, 3,4-dihydroxyphenylacetic acid; THP, tetrahydropapaveroline; and HVA, homovanillic acid.

ALDH has been described by Tottmar *et al.* [1] and Koivula and Koivusalo [5]. Using isoelectric focusing it has been shown that a number of isozymes are found in rat liver cytosol, and at least three are found in mitochondria [10–12].

In rat liver and brain, dopamine is primarily metabolized to its acid derivative, 3,4-dihydroxyphenylacetic acid (DOPAC) [13, 14]. However, in the presence of ethanol the metabolism of dopamine in the liver is altered such that 3,4-dihydroxyphenylethanol (DOPET) is the major metabolite [14]. This alteration in biogenic amine metabolism also occurs in man during ethanol ingestion [15, 16]. It has been proposed that acetaldehyde produced during ethanol metabolism prevents oxidation of DOPAL since both aldehydes are oxidized by ALDH to acids [14, 17]. The proposed inhibition of DOPAL oxidation may then allow the biogenic aldehyde to be reduced to DOPET, presumably by the cytosolic aldehyde reductase. It has been shown that acetaldehyde is an inhibitor of the oxidation of 5-hydroxyindoleacetaldehyde in rat liver homogenates [17]. Davis *et al.* [14] demonstrated a similar inhibition of the oxidation of DOPAL by acetaldehyde in rat liver homogenates. However, both studies employed relatively high concentrations of acetaldehyde (1–4 mM) and, in their use of homogenates, disregarded the possible cellular compartmentation of aldehyde oxidation. We have recently reported the results of similar studies using rat liver slices, a model system which maintains the cellular compartmentation of the various isozymes of ALDH [18]. Under these conditions low levels of acetaldehyde (50 μM) do not inhibit DOPAL oxidation. Higher concentrations of acetaldehyde (0.2–1.0 mM) only slightly inhibit DOPAL oxidation when compared to the inhibition produced by ethanol. These results were unexpected since the K_m of ALDH for acetaldehyde is in the micromolar range [7–9], suggesting that acetaldehyde should act as a good competitive inhibitor of DOPAL oxidation. Because isozymes of the enzyme exist in different cellular compartments, it is possible that acetaldehyde and DOPAL are oxidized in different subcellular regions. Alternatively, these two aldehydes may be oxidized by different isozymes in the same subcellular compartment.

Though originally it was suggested that the cytosol is the major site of oxidation [19] of acetaldehyde, most of the evidence supports the thesis that acetaldehyde is oxidized primarily by an isozyme(s) of ALDH present in the mitochondrial matrix of rat liver [2, 20–22]. In a preliminary report we have shown that DOPAL also appears to be oxidized by an ALDH present in rat liver mitochondria [23]. In the experiments reported here, we investigated further the subcellular site of DOPAL oxidation in the rat liver and partially characterized the isozymes of ALDH isolated from each subcellular compartment.

MATERIALS AND METHODS

Materials. [Ethylamine-2-^{14}C]dopamine-hydrochloride, from the Amersham-Searle Co. (Arlington Heights, IL), was dissolved in distilled water with sufficient nonlabeled dopamine to prepare a solution of the desired specific activity. The solution was adjusted to pH 5 and stored at −20°. Unlabeled dopamine, and the available metabolites derived from dopamine that were used for chromatography standards, were from the Sigma Chemical Co. (St. Louis, MO). Tetrahydropapaveroline (THP) was a gift from Dr. A. Collins, School of Pharmacy, University of Colorado, Boulder, CO. The methods used to synthesize DOPAL have been described elsewhere [18]; they involved either the pinacol–pinacolone rearrangement of epinephrine as described by Robbins [24], or the enzymatic oxidative deamination of dopamine by rat liver MAO. DOPET was available at times from the Regis Chemical Co. (Morton Grove, IL) and was also synthesized by the reduction of DOPAL using sodium borohydride [18]. Unless otherwise specified, all chemicals were of the highest purity available and were used without further purification.

Animals. Adult male rats weighing between 350 and 400 g were employed. All of the rats were of an original Wistar strain and were bred in the facilities of the Biochemistry Department at Purdue University.

Partial purification of monamine oxidase. Beef liver MAO was partially purified by the method of Nara *et al.* [25]. The enzyme was precipitated after the alumina C_γ step by adding ammonium sulfate to 45% saturation. Rat liver MAO was prepared by isolating the outer membrane of the mitochondria as described by Greenawalt [26]. Before use, an aliquot of either enzyme preparation was dialyzed three times against 2 liters of 10 mM sodium phosphate buffer, pH 7.4, and assayed according to the procedure of Tabakoff and Alivisatos [27]. ALDH and aldehyde reductase activities were undetectable in the beef liver preparation. Barely detectable ALDH activity and no aldehyde reductase activity were present in the rat liver preparation.

Subcellular fractionation of rat liver. Rats were killed by cervical dislocation, and the livers were rapidly removed, placed on ice, and minced with a tissue grinder. A 20% (w/v) suspension of the liver in 0.25 M sucrose–10 mM Tris–HCl buffer, pH 7.4 (referred to as isolation medium) was homogenized at 340 rpm on a Potter–Elvehjem homogenizer. Centrifugation of the homogenate at 900 *g* for 15 min was performed, and the pellet which represented the nuclear fraction was discarded. The mitochondria were isolated by centrifugation at 9,000 *g* for 15 min. The pellet was washed twice by rehomogenization in 10 ml isolation medium, followed by centrifugation at 9,000 *g* for 10 min. This pellet was designated the mitochondrial pellet. Centrifugation at 12,000 *g* for 15 min was performed on the supernatant fraction of the first 9,000 *g* centrifugation. The pellet was designated the lysosomal pellet. The supernatant fraction was submitted to centrifugation at 104,000 *g* for 60 min. The final pellet was rinsed twice with isolation medium and designated the microsomal pellet. An aliquot of the final supernatant fraction was passed through a Sephadex G-25 column (1.2 × 38 cm) that had been equilibrated previously with isolation medium. The fractions containing 90–95 per cent of the ALDH, alcohol dehydrogenase and aldehyde reductase activities that

were placed on the column were pooled and designated the cytosol fraction.

Alcohol dehydrogenase and glutamate dehydrogenase (L-glutamine:NAD oxidoreductase, EC 1.4.1.3) were used as enzyme markers for the cytosol and mitochondrial fractions respectively. Only 5 per cent of the total alcohol dehydrogenase activity present in the original homogenate was found in the mitochondrial fraction, indicating minimal contamination of the mitochondrial fraction with cytosol enzymes. Likewise, only 5 per cent of the total glutamate dehydrogenase activity present in the original homogenate was found in the cytosol fraction, indicating minimal contamination of the cytosol with mitochondrial enzymes.

Enzyme assays. The mitochondrial pellet was resuspended in 10 ml of 10 mM sodium phosphate buffer, pH 7.4, kept in an ice bath, and sonicated for 30 sec at 90 W of power with a Branson Sonifier cell disruptor. This suspension was used for the mitochondrial assays. All other fractions were resuspended in 100 mM sodium phosphate buffer, pH 7.4, and used without further treatment unless otherwise specified.

All assays of dehydrogenase enzymes were performed by following NADH production on an Aminco filter fluoromicrophotometer at 25° using a Corning 7-60 filter for excitation and a Wratten 2A filter for emission. The fluorometer was calibrated before each experiment by determining the magnitude of fluorescence obtained with known quantities of NADH. The assay mixtures contained 0.5 mM NAD and 2 μM rotenone and were run at 25° in 100 mM phosphate buffer, pH 7.4, in a total volume of 1.0 ml. In the assays containing cytosol fractions, 10 mM pyrazole and 20 mM isobutyramide were added to inhibit alcohol dehydrogenase activity (except when alcohol dehydrogenase itself was assayed). Aliquots of the liver fraction, NAD and rotenone were first mixed together, and the increase in NADH, if any, due to endogenous substrates, was recorded. When this activity diminished, substrate was added to initiate the reaction, and the rate of NADH production was monitored.

ALDH activity was measured using 10 mM acetaldehyde as the substrate. Alcohol dehydrogenase activity was measured using 16 mM ethanol as substrate. Fractions were sonicated at 90 W with a Branson Sonifier cell disrupter for 30 sec before assaying glutamate dehydrogenase using 20 mM sodium glutamate as substrate. Aldehyde reductase activity was measured by following the decrease in the fluorescence of NADPH in an assay mixture containing 0.2 mM NADPH, 0.2 mM *p*-nitrobenzaldehyde and an aliquot of enzyme. Protein was determined by the method of Lowry *et al.* [28].

Isoelectric focusing studies. Column isoelectric focusing was performed in an LKB electric focusing apparatus following the directions supplied by the manufacturer. Cytosol and mitochondrial fractions were isolated as described above from 1 g of rat liver. The isolated mitochondria were lysed as described above. The cytosol and mitochondrial fractions were subjected separately to isoelectric focusing in a pH 5–8 gradient prepared from LKB ampholines. It took 36–48 hr to complete the focusings. After that time the column was drained and fractions were collected. The pH and the ALDH and aldehyde reductase activities in each fraction were determined. Kinetic analysis was performed on the isolated isozymes of ALDH and aldehyde reductase. These isozyme activities were stable at 4° for 1–2 weeks. No attempt was made to purify further any of the isolated isozymes.

Integrity of isolated mitochondria. The two indices used to verify the integrity of the isolated mitochondria were: (1) the coupling of the electron transport system to oxidative phosphorylation, and (2) the impermeability of the inner mitochondrial membrane to NAD. The first verification tested the ability of the isolated mitochondria to synthesize ATP from ADP during electron transfer. This test was performed with the use of an oxygen electrode. First, the consumption of oxygen by the mitochondrial suspension was determined in the absence of added substrates (Fig. 1). The addition of malate and pyruvate to the mitochondrial suspension produced an increase in oxygen consumption. A dramatic increase in oxygen utilization occurred upon the

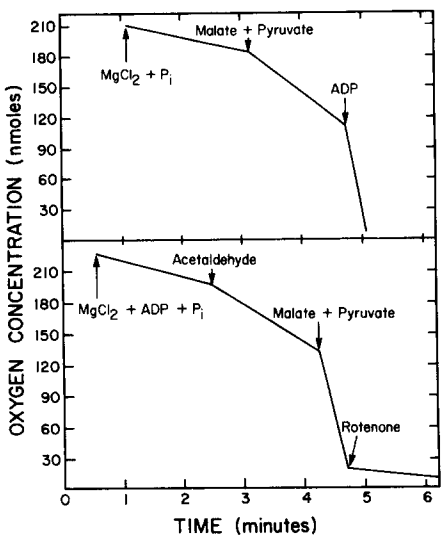

Fig. 1. Oxygen utilization by intact mitochondria. Experiments were performed with a Gilson Medical Electronics Oxygraph, model KM, which was equipped with a Clark oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH). The instrument was calibrated with a potassium chloride solution (0.1 M KCl possesses an oxygen tension of 290 nmoles/ml at 25°). The assay mixtures contained 2–3 mg mitochondrial protein, 10 mM $MgCl_2$ 0.25 M sucrose, and 10 mM sodium phosphate, pH 7.4, in a total volume of 1.6 ml. Additions of 32 μmoles sodium malate, 32 μmoles sodium pyruvate, 32 μmoles sodium phosphate, 1.6 μmoles ADP, 160 nmoles acetaldehyde, and 3.2 nmoles rotenone were made as shown in the figure. Assays were run at 25°, and the consumption of oxygen by the mitochondria after the addition of different compounds was monitored. The top panel shows that ADP stimulated the rate of electron transport. The bottom panel shows that the electron transport system was not rate-limiting during acetaldehyde oxidation, for the inclusion of malate/pyruvate caused a greater rate of O_2 utilization.

addition of ADP to the suspension (top panel). This result demonstrated the coupling of the electron transport system to oxidative phosphorylation (energy state 3). As shown in Fig. 1 (bottom panel), the increased oxygen consumption by the mitochondria during the oxidation of acetaldehyde was demonstrated along with the complete inhibition of this oxidation by rotenone. The addition of pyruvate and malate caused an increased rate of oxygen utilization over that produced by acetaldehyde alone. This implies that the rate of acetaldehyde oxidation under these conditions was not limited by the rate of the electron transport system.

The second test of mitochondrial integrity was performed to determine whether there was damage to the mitochondrial inner membrane during its isolation, permitting exogenously supplied NAD to diffuse freely into the matrix space. This test was based on two facts: (1) in intact mitochondria the inner mitochondrial membrane is impermeable to exogenously supplied NAD, whereas the outer membrane is permeable to this cofactor [29]; and (2) ALDH activity present in the mitochondrial matrix has a low K_m (1 μM) for acetaldehyde, whereas the activity present in the interstitial space of the mitochondria has a high K_m (1 mM) for acetaldehyde [8]. If the endogenous NAD pool of the matrix were first depleted, no oxidation of low concentrations (10 μM) of acetaldehyde should be observed in the presence of exogenously supplied NAD, providing the mitochondrial membranes were intact. Mitochondrial suspensions were incubated at 37° for 30 min in isolation medium containing 1 mM ADP, 10 mM $MgCl_2$ and 10 mM sodium phosphate, pH 7.4. Aliquots of this suspension were removed every 5 min to determine the ALDH activity in the intact mitochondrial matrix by the fluorometric assay described below. The aliquot was added to a cuvette containing rotenone (2 μM) and acetaldehyde (10μM) in a total volume of 1.0 ml isolation medium. This suspension was allowed to incubate at room temperature for 1–2 min, to deplete the endogenous stores of NAD in the mitochondrial matrix. The change in fluorescence in this assay solution due to new NADH formation was then monitored upon the addition of 0.5 μmole NAD and 10 nmoles acetaldehyde to the NAD-depleted mitochondria. An increase in fluorescence upon the addition of these reagents to the suspension indicated damage to the mitochondrial matrix membrane. Fluorescence was monitored on an Aminco filter fluoromicrophotometer at 25° using a Corning 7–60 filter for excitation and a Wratten 2A filter for emission. Using this assay we found that mitochondrial matrix membranes were undamaged in mitochondrial suspensions incubated for up to 20 min at 37°. However, when incubated for longer than 20 min, mitochondrial suspension produced significant increases in fluorescence upon addition of NAD and acetaldehyde, indicating that damage to the matrix membrane ultimately occurred. This assay was run for each mitochondrial preparation employed in these studies.

Incubations of subcellular fractions with dopamine. Incubations of dopamine with the various subcellular liver fractions were carried out in the isolation medium in a total volume of 0.70 ml. Additions of cofactors and subcellular fractions were made as specified in the table legends. [Ethylamine-2-^{14}C]dopamine (2 μmoles, 0.2 μCi) was added to all assay mixtures to initiate the reaction. The reactions were terminated after 15 min at 37° by the addition of two drops of 0.2 N HCl, and the cellular debris was removed after centrifugation at 40,000 g for 10 min. The supernatant fraction was analyzed for dopamine metabolites by two-dimensional paper chromatography–electrophoresis system as described previously [18, 30]. The metabolites were visualized by spraying the paper with 50% aqueous phenol reagent followed by 20% Na_2CO_3, cut out, and placed in scintillation vials. The radioactivity was determined with a Beckman LS-100C liquid scintillation counter. The scintillation mixture consisted of 700 ml toluene, 300 ml Triton X-100, 4 g diphenyloxazole and 100 mg 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene. The recovery of radioactivity in the supernatant fraction was about 85 per cent and the radioactivity in the R_f values representing the metabolites presented in the tables comprised greater than 90 per cent of the recovered radioactivity.

RESULTS

Isoenzymes of ALDH from various liver subcellular fractions. A previous study from this laboratory showed that all the isozymes of ALDH present in rat liver were found in the pH range of 5–8 when analyzed by gel isoelectric focusing [10]. When the liver was subjected to subcellular fractionation, and the mitochondrial and cytosolic fractions subjected to column isoelectric focusing, three major isozymes were obtained from the mitochondrial fraction, while five isozymes were obtained from the cytosolic fraction (Figs. 2 and 3). The isoelectric points of the

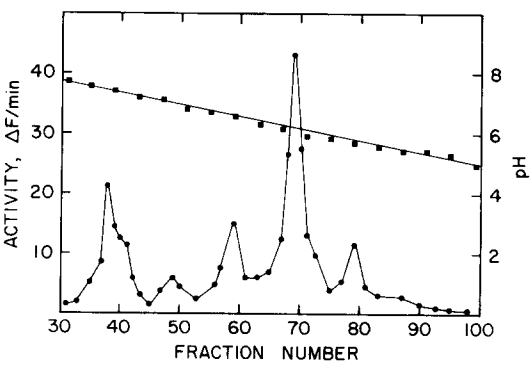

Fig. 2. Isozyme pattern of aldehyde dehydrogenase obtained from rat liver cytosol. Cytosol was subjected to column isoelectric focusing as described in Materials and Methods. The catalytic activity in each fraction was measured by monitoring the fluorescence of NADH formation (●) using *p*-nitrobenzaldehyde as a substrate, and the pH of each fraction was determined (■). Fractions containing the separated isoenzymes were subjected to isoelectric focusing in polyacrylamide gels [11] and were found to focus as a single band. Though many rats from our colony contain all three isozymes at pI = 5.8, 6.05 and 6.15, some animals have just the 5.8, while others just the 6.15 enzyme forms [11].

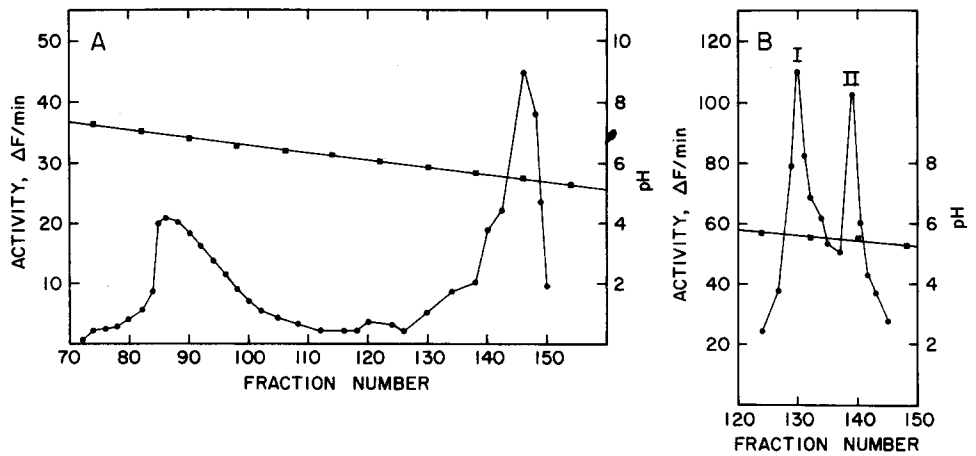

Fig. 3. Isozyme pattern of aldehyde dehydrogenase obtained from rat liver mitochondria. Isolated lysed mitochondrial proteins were subjected to column isoelectric focusing as described in Materials and Methods. Catalytic activity (●) and pH (■) were determined as indicated in Fig. 2. Panel A: Representative pattern of enzyme activity. Panel B: Fractions 140–150 were pooled and focused using a narrow pH gradient. Two separate enzyme activities were obtained. These each focused as a single band on polyacrylamide gel isoelectric focusing and proved to be differentially inhibited by disulfiram [31], an *in vivo* (and *in vitro*) inhibitor of aldehyde dehydrogenase.

mitochondrial enzymes were different from those of the cytosol enzymes; hence, it could be concluded that the cytosol isozymes were not artifacts due to leakage of enzyme activity from the mitochondria. The microsomal fraction was not subjected to isoelectric focusing.

Kinetic properties of isozymes of ALDH. The Michaelis constants of the various isozymes of ALDH were determined using *p*-nitrobenzaldehyde, acetaldehyde and DOPAL as substrates. No attempt was made to determine K_m for NAD, but it was found that the concentration employed was saturating with respect to coenzyme for all the isozymes. Results of these experiments are presented in Table 1. In general, all enzyme forms possessed a low K_m for *p*-nitrobenzaldehyde except for the cytosol, pI = 6.6, and the microsomal enzyme. The various enzyme forms found in the mitochondrial fraction possessed widely different values K_m for acetaldehyde and DOPAL, ranging from 10^{-7} to 10^{-3} M. Siew *et al.* [8] showed that the enzyme form associated with the outer membrane of the mitochondria had a high K_m for acetaldehyde and used NADP as a cofactor. The mitochondrial isozyme with pI = 6.9 possessed a high K_m for both acetaldehyde and DOPAL and also used NADP as a coenzyme. Presumably then, this form (pI 6.9) is from the outer mitochondrial membrane. The five cytosol enzyme forms also possessed widely differing K_m values for DOPAL and acetaldehyde (range μM to mM). The crude suspension of the microsomal pellet was used to determine the Michaelis constants for this enzyme form. The enzyme possessed a high K_m for both acetaldehyde and DOPAL.

Table 1. K_m for isozymes of rat liver aldehyde dehydrogenase and aldehyde reductase separated by isoelectric focusing from different subcellular organelles

Liver fraction	pI*	Apparent K_m (mM)			% Inhibition by ADP†
		p-Nitrobenzaldehyde	Acetaldehyde	DOPAL	
Aldehyde dehydrogenase					
Cytosol	7.4	2.4×10^{-3}	13	2.5×10^{-1}	58
	6.6	1.3×10^{-1}	9.4×10^{-1}	2.2	16
	6.15	5.7×10^{-4}	1.0×10^{-2}	2.8	—
	6.05	3.1×10^{-4}	2.2×10^{-3}	5.1×10^{-3}	—
	5.8	7.0×10^{-4}	4.5×10^{-3}	5.1×10^{-2}	—
Mitochondrial	6.9	9.0×10^{-2}	1.3	1.8×10^{-1}	—
	5.4	$<10^{-3}$	$<10^{-3}$	1.7×10^{-3}	14
	5.6	$<10^{-3}$	1.3×10^{-4}	3.0×10^{-4}	2
Microsomes	‡	5.7×10^{-1}	4.0	4.5×10^{-1}	—
Aldehyde reductase					
Cytosol	6.9	1.7×10^{-3}		9.0×10^{-2}	
	6.5	2.7×10^{-2}		1.7×10^{-1}	
	6.0	5.0×10^{-2}			

* pI ± 0.1 except for entry indicated 6.15 or 6.05.

† *p*-Nitrobenzaldehyde (0.1 mM) substrate; [ADP] = 1 mM; dash means essentially no inhibition was found.

‡ Not subjected to isoelectric focusing.

For the metabolic experiments to be discussed, it was necessary to employ 1 mM ADP in the incubations containing mitochondria to maintain coupling of the electron transport system to oxidative phosphorylation, assuring maximal reoxidation of NADH to NAD; hence, the effect of ADP on the isolated isozymes was determined (Table 1). ADP (1 mM) did not dramatically affect any of the isolated mitochondrial isozymes or the microsomal enzyme. However, ADP (1 mM) was found to inhibit two cytosol forms of ALDH, particularly the pI 7.4 isozyme. Only a slight increase in inhibition was observed if 2 mM ADP was employed, and no inhibition was found if 0.1 mM ADP was employed. The mode of inhibition by ADP was not investigated. It was found, though, that AMP or ATP neither inhibited the activity of ALDH nor prevented the ADP inhibition from occurring.

Isozymes of rat liver aldehyde reductase. The activity profile for aldehyde reductase obtained by column isoelectric focusing of the cytosolic fraction of rat liver is presented in Fig. 4. No isozymes were found in the mitochondrial fraction, though, as discussed below, enzymatic activity of aldehyde reductase may be associated with the mitochondria.

Kinetic constants of aldehyde reductase. The isozymes of aldehyde reductase all possessed low K_m values for *p*-nitrobenzaldehyde. The K_m values for two of the cytosol enzymes were approximately 0.1 mM with respect to DOPAL (Table 1). The third form (pI 6.0) exhibited no activity when DOPAL was used as a substrate. No activity could be detected with any of the isozymes when acetaldehyde was used as a substrate.

Oxidation of DOPAL in incubation media containing intact mitochondria. Incubations of dopamine and isolated mitochondria were performed without exogenously supplied pyridine nucleotide cofactors to determine the oxidative capacity of the matrix ALDH with respect to the generated DOPAL (Table 2, line 1). Eighty per cent of the deaminated dopamine was converted to DOPAC. Only small quantities of DOPAL and THP, the condensation product of the reaction between dopamine and DOPAL, accumulated, indicating that the matrix enzymes possessing a low K_m for DOPAL were capable of oxidizing almost all of the DOPAL produced by

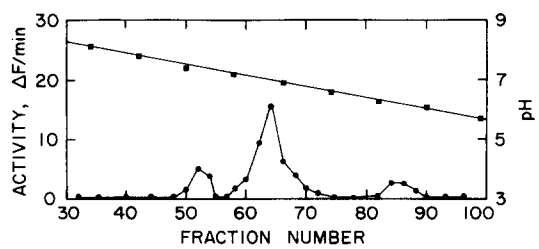

Fig. 4. Isozyme pattern of aldehyde reductase obtained from rat liver cytosol. Cytosol was subjected to column isoelectric focusing as described in Materials and Methods. The catalytic activity in each fraction was measured by monitoring the fluorescence decrease (●) of NADPH using *p*-nitrobenzaldehyde as a substrate, and the pH of each fraction was determined (■). No activity was found when assaying the mitochondrial fraction.

Table 2. Percentages of the deaminated products formed from the metabolism of dopamine in incubations of rat liver mitochondria*

Additions			Per cent of deaminated products						
	Pyridine nucleotide coenzymes	Rotenone	THP	DOPAL	DOPET	DOPAC	HVA	Unknown	Per cent deamination
1	−	−(6)	4.6 ± 0.9[a, b]	9.3 ± 2.9[c]	2.2 ± 0.5[e, f]	80.0 ± 2.6[g]	1.5 ± 1.6	2.2 ± 0.6	14.3 ± 2.3
2	−	+(6)	10.5 ± 1.9[a]	53.7 ± 7.4[c, d]	8.5 ± 2.4[e]	22.9 ± 5.7[g, h]	2.9 ± 1.1	3.7 ± 1.5	14.7 ± 2.3
3	+	−(6)	4.5 ± 1.1	8.0 ± 2.0[d]	1.9 ± 0.9	82.7 ± 4.2[h]	0.8 ± 0.7	1.9 ± 0.6	13.6 ± 2.6
4	+	+(5)	8.9 ± 1.5[b]	36.9 ± 4.1[c, d]	7.0 ± 3.5[f]	42.0 ± 6.8[g, h]	1.7 ± 1.4	3.2 ± 1.1	13.8 ± 2.9

* The experiments were performed as described in Materials and Methods. All incubations contained 2.0 mg mitochondrial protein, 1.0 mM ADP, 10 mM $MgCl_2$, and 10 mM sodium phosphate, pH 7.4. Rotenone (3 μM) and pyridine nucleotide coenzymes (0.5 mM NAD, 0.5 mM NADPH, 1 μM NADH, and 5 μm NADP) were supplied as designated in the table. The percentages were calculated as described elsewhere [18] and are presented as the means ± S.D. obtained from experiments with the number of rats shown in parentheses. Statistical significance was determined by Student's *t*-test; the statistically compared values are designated with the same small letter superscript, and the level of significance is $P < 0.01$.

MAO, even at the high dopamine concentration employed. The inclusion of rotenone, which prevents the reoxidation of NADH in the mitochondrial matrix by inhibiting NADH dehydrogenase of the electron transport system, in incubations of mitochondria and dopamine caused an 80 per cent inhibition of DOPAC formation, a 5-fold increase in DOPAL level, and a 2-fold increase in THP (line 2). The residual DOPAC produced during rotenone inhibition (20 per cent of the products) was due either to incomplete depletion of the matrix NAD pool or to oxidation of DOPAL by the high K_m outer membrane enzyme.

The results obtained from incubations containing added pyridine nucleotide cofactors were the same as those obtained from incubations performed in the absence of the added cofactors (line 1 vs line 3). A measure of the outer membrane ALDH activity could be obtained by inhibiting the matrix enzyme activity with rotenone and adding pyridine nucleotide cofactors to the incubation mixture (line 4). The outer membrane enzyme was capable of oxidizing DOPAL; however, the formation of DOPAC was less than that seen when the matrix enzymes were not inhibited. This result supports the thesis that the outer membrane isozyme has a higher K_m for DOPAL than the matrix isozymes.

Oxidation of DOPAL in incubation media containing cytosol and microsomal fractions. In the previous experiments, 1 mM ADP was added to the incubation media to maintain energy state 3 of the mitochondria. This concentration of ADP did not significantly affect the activity of the mitochondrial isozymes of ALDH, but it inhibited one of the cytosol isozymes of ALDH dramatically (Table 1). Hence, incubations with crude cytosol were run in the absence and presence of added ADP. For these incubations MAO partially purified from beef liver or rat liver mitochondria was used to generate DOPAL from dopamine. When either MAO preparation was incubated with dopamine, the major metabolite that accumulated was DOPAL (lines 1 and 3 of Table 3). Negligible quantities of DOPAC were formed. Though aldehyde reductase activity was not detected in either the beef liver or rat liver MAO preparation, significant quantities of radioactivity which co-chromatograph and co-electrophores with DOPET were observed in incubations containing dopamine and either MAO preparation (lines 1 and 3, Table 3).

In incubations of the cytosol fraction containing 1 mM ADP and either preparation of MAO (only the beef liver MAO data are shown in Table 3), little of the generated DOPAL was converted to either DOPAC or DOPET, even though both NAD and NADPH were supplied to the incubation medium. Using the values obtained with the MAO preparation alone for DOPET and DOPAC as blank values, DOPET and DOPAC accounted for *ca.* 10 and 5 per cent of the products formed, respectively, whereas DOPAL built up to 50 per cent of the products (line 2, Table 3). In the absence of ADP, cytosol ALDH activity was no longer inhibited, and DOPAC formation was enhanced. DOPAC levels reached 38 per cent of the products, while DOPAL levels decreased to 33 per cent of the products.

Table 3. Percentages of the deaminated products formed from the metabolism of dopamine in incubations of rat liver cytosol and microsomes with partially purified MAO*

Liver fractions	MAO preparation	[ADP] (mM)	Per cent of deaminated products						Per cent deamination
			THP	DOPAL	DOPET	DOPAC	HVA	Unknown	
1	Beef liver (4)	1	12.4 ± 1.6	59.2 ± 4.4	21.6 ± 2.9	2.3 ± 0.6	2.8 ± 1.1	1.8 ± 1.0	18.2 ± 5.9
2 Cytosol	Beef liver (2)	1	5.5	50.4	30.6	6.5	3.9	3.1	17.5
3	Rat liver (3)	0	13.9 ± 0.7	60.0 ± 2.0	12.4 ± 0.6	6.5 ± 2.1[a, b]	2.3 ± 0.6	4.9 ± 1.0	22.8 ± 1.7
4 Cytosol	Rat liver (3)	0	11.5 ± 0.2	33.1 ± 1.8	10.0 ± 0.4	38.4 ± 1.5[a]	2.3 ± 0.4	4.7 ± 0.3	17.5 ± 3.9
5 Microsomes	Rat liver (3)	0	13.3 ± 0.6	47.3 ± 3.6	10.4 ± 1.0	22.6 ± 2.4[b]	2.1 ± 0.6	4.3 ± 0.4	21.7 ± 0.8
6 Cytosol + microsomes	Rat liver (3)	0	9.0 ± 1.4	28.2 ± 2.5	8.9 ± 3.4	49.4 ± 5.7	1.7 ± 0.9	2.7 ± 0.1	10.3 ± 2.7

* The experiments were performed as described in Materials and Methods. All incubations contained 10 mM $MgCl_2$, 10 mM sodium phosphate, pH 7.4, and pyridine nucleotide coenzymes at the concentrations described in the legend of Table 2. The incubations contained 2.5 mg and 3.0 mg of microsomal and cytosol protein, respectively, plus added rat or beef liver MAO. The percentages were calculated as described elsewhere [18] and are presented as the means ± S.D. obtained from experiments with the number of rats shown in parentheses. Statistical significance was determined by Student's *t*-test, and the statistically compared values are designated as described in Table 2 with $P < 0.01$.

DOPAC was formed in incubations of dopamine with the microsomal fraction, but most of the deaminated products remained as the aldehyde. Incubation of dopamine with both microsomal and cytosolic fractions together increased the percentage of DOPAC formed when compared to the incubation of either fraction alone. However, subcellular fractions combined did not oxidize DOPAL to the same extent as the mitochondrial matrix.

Oxidation of DOPAL in incubation media containing mitochondria, cytosol and microsomal subcellular fractions. Incubations containing mitochondria plus the other subcellular liver fractions without added pyridine nucleotide cofactors yielded results which were nearly identical to those seen in incubations containing mitochondria alone without added cofactors (compare lines 1 and 2 of Table 2 with lines 1 and 2 in Table 4). In the absence of rotenone, DOPAC production predominated and only small amounts of DOPAL were formed. Since no cofactors were supplied, this production of DOPAC must be attributed to the action of the mitochondrial matrix ALDH. The addition of rotenone confirmed this conclusion, since DOPAC formation was inhibited by 70–80 per cent of its presence.

In the presence of added coenzymes the distribution of metabolites obtained from the reconstituted system (line 3, Table 4) was very similar to that obtained with intact liver slice incubations [18, 30]. DOPAC levels constituted 50–60 per cent of the products, and DOPAL levels reached approximately 25 per cent of the products. Very little DOPET or THP was formed. Adding rotenone to the reconstituted system in the presence of coenzymes led to an inhibition of oxidation as noted by the decrease in DOPAC formation and the doubling of DOPAL levels (line 3 vs line 4, Table 4). These results again emphasize the importance of the mitochondrial matrix isozyme(s) in the oxidation of DOPAL.

DISCUSSION

In man aldehydes derived from the biogenic amines, as well as acetaldehyde derived from ethanol, are predominantly oxidized to acids by non-specific ALDHs [13]. It has been well documented that ethanol alters the *in vivo* as well as the *in vitro* metabolism of the biogenic amines such that the oxidation of the biogenic aldehydes to their respective acids is inhibited, while the reduction to their respective alcohols is enhanced [13–16]. Two major explanations for this ethanol-induced alteration have been presented. The first is that during the metabolism of ethanol a large decrease in the cellular NAD/NADH ratio occurs, producing a more favorable environment for the reduction of the biogenic aldehydes in the cytosol [13, 31]. The second reason is that acetaldehyde acts as a competitive inhibitor of biogenic aldehyde oxidation [14, 17, 32]. When this inhibition occurs, the biogenic aldehyde levels increase and are then capable of being reduced by aldehyde reductase. We have presented evidence suggesting that neither of the above hypotheses is valid, and have proposed an alternative hypothesis originally suggested by von Wartburg [33] to explain the alteration of biogenic aldehyde metabolism in the presence of ethanol [18, 30]. It was proposed that, during the metabolism of ethanol, liver alcohol dehydrogenase is converted from the E–NAD oxidizing complex into an E–NADH reducing complex. This complex, which is only formed in liver during the metabolism of an alcohol, is then capable of reducing the biogenic aldehyde faster than it can be oxidized by ALDH.

Though this mechanism can explain how a biogenic aldehyde or even chloral hydrate [34] is reduced in the presence of ethanol, we are not able to explain why the oxidation of DOPAL in rat liver slices is not dramatically inhibited in the presence of acetaldehyde [18]. We observed only a 25 per cent inhibition of DOPAL oxidation in liver slices in the presence of 1 mM acetaldehyde. One possible explanation for this lack of inhibition is that different isozymes of ALDH may be responsible for the oxidation of the two aldehydes.

The isozymes of ALDH possessing low K_m values for DOPAL also possess relatively low K_m values for acetaldehyde. Thus, we cannot postulate that a particular isozyme with a low K_m for DOPAL and a high K_m for acetaldehyde is primarily responsible for DOPAL oxidation.

Table 4. Percentages of the deaminated products formed from the metabolism of dopamine in incubations of a mixture of liver microsomes, cytosol and mitochondria*

Pyridine nucleotide coenzymes		Rotenone	Per cent of deaminated products						Per cent deamination
			THP	DOPAL	DOPET	DOPAC	HVA	Unknown	
1	−	−	4.1 ± 0.4	14.2 ± 5.0[a]	3.1 ± 2.2	73.8 ± 1.3[b]	1.9 ± 1.7	2.7 ± 2.2	10.7 ± 3.0
2	−	+	9.4 ± 0.8	58.9 ± 7.7[a]	10.0 ± 6.3	17.8 ± 1.2[b]	0.9 ± 1.1	2.6 ± 1.2	12.5 ± 0.1
3	+	−	5.1 ± 1.6	23.9 ± 8.9	6.9 ± 4.4	57.9 ± 6.9[c]	3.1 ± 3.2	2.8 ± 2.8	9.2 ± 3.2
4	+	+	5.2 ± 0.8	45.5 ± 9.7	12.3 ± 9.5	32.0 ± 13.9[c]	2.5 ± 2.5	2.2 ± 0.9	11.6 ± 2.6

* The experiments were performed as described in Materials and Methods. All incubations contained 1.0 mM ADP, 10 mM $MgCl_2$ and 10 mM sodium phosphate, pH 7.4. Rotenone (2 μM) and pyridine nucleotide coenzymes at the concentrations described in the legend of Table 2 were added as designated in the table. The incubations contained 3.0 mg, 2.0 mg, and 2.5 mg of cytosolic, mitochondrial, and microsomal proteins respectively. The percentages were calculated as described elsewhere [18] and are presented as the means ± S.D. obtained from experiments with three rats. Statistical significance was determined by Student's *t*-test, and the statistically compared values are designated as described in Table 2. Key: (a) $P < 0.01$; (b) $P < 0.01$; and (c) $P < 0.05$.

An alternative explanation for this lack of inhibition is that the isozymes of ALDH involved in DOPAL and acetaldehyde oxidation are located in different subcellular compartments. It has been established that acetaldehyde oxidation in rat primarily occurs in the matrix space of the liver mitochondria [20–22]. The data presented in this study lead us to suggest that the mitochondrial matrix also is the primary site of DOPAL oxidation.

Eighty per cent of the products formed in incubations containing intact mitochondria and dopamine was DOPAC. Only a small accumulation of DOPAL occurred (10 per cent of the products or *ca.* 0.05 mM). The use of rotenone helped establish the fact that the oxidation of DOPAL predominantly occurs in the mitochondrial matrix, since DOPAC was inhibited by 80 per cent in the presence of rotenone.

The matrix membrane was impermeable to exogenously supplied pyridine nucleotide coenzymes, where the outer membrane is permeable to these cofactors [29]; hence, the enzyme located in the non-matrix region of the mitochondria is exposed to these added cofactors. This enzyme can oxidize DOPAL to a limited degree, as observed in the incubations containing intact mitochondria, rotenone and NAD. However, a large accumulation of DOPAL occurs, suggesting that the non-matrix regions possess an enzyme with a high K_m for DOPAL. This result agrees with the K_m data presented in Table 1. The pI 6.9 enzyme, which is presumably located in or on the outer membrane of the mitochondria, has a K_m of 0.18 mM for DOPAL; the two matrix enzymes have low K_m values for DOPAL. Thus, under physiological conditions when DOPAL levels are presumably low, it can be assumed that the non-matrix enzyme of the mitochondria is not contributing significantly to the oxidation of DOPAL.

The cytosol and microsomes contain ALDH activity (Table 1), and these subcellular fractions are capable of oxidizing DOPAL, but not at a rate comparable to that of the mitochondria (Table 2). A final concentration of 1 mM ADP is supplied routinely to incubations of intact mitochondria to ensure that the electron transport system is operating near its maximal capacity. This concentration of ADP does not significantly affect the mitochondrial activity of ALDH; however, the pI 7.4 cytosolic isozyme is inhibited by 60 per cent in the presence of ADP. When the cytosol fraction is incubated with dopamine plus ADP, only 6 per cent of the deaminated products are recovered as DOPAC, and a concomitant build-up of the aldehyde intermediate is observed. When ADP is omitted from the incubation mixture, the inhibition is relieved, as revealed by the increased formation of DOPAC (38 per cent). This result suggests that the bulk of DOPAL oxidation in the cytosol is due to the action of the pI 7.4 isozyme of ALDH, since this isozyme is the only one appreciably inhibited by ADP. We cannot explain why the pI 6.05 and pI 5.8 isozymes, which are not inhibited by ADP, do not contribute significantly to the oxidation of DOPAL when the cytosol fraction is incubated with dopamine and ADP. Even in the absence of ADP, the cytosol and microsomal fractions together do not oxidize DOPAL to the same extent as the mitochondrial fraction.

Incubations of mitochondria with the other subcellular fractions support the concept that the cytosol and microsomal enzymes play a relatively minor role in the oxidation of low levels of DOPAL. Even when the cytosol, microsomal and mitochondrial fractions are incubated together with dopamine and added cofactors, the metabolic distribution obtained is similar to that seen in incubations of dopamine with liver slices [18]. The addition of rotenone to this reconstituted system produces a 45 per cent inhibition of DOPAC formation and a concomitant rise in DOPAL accumulation. Since the oxidation in cytosol is inhibited by physiological levels of ADP, it appears likely that the mitochondria are the site of DOPAL oxidation. The accumulated evidence supports the hypothesis that DOPAC is predominantly oxidized by a low K_m ALDH in the mitochondrial matrix space, though other enzyme forms can contribute to oxidation.

Our data support the concept that DOPAL, as well as acetaldehyde, is oxidized in the mitochondrial matrix space. Why, then, does not acetaldehyde inhibit DOPAL oxidation to a greater extent in liver slices? A possibility is that the cytosol and the outer membrane mitochondrial enzymes are capable of performing the oxidation when the mitochondrial matrix enzymes are being utilized for acetaldehyde oxidation. This could occur under the conditions employed because the concentration of DOPAL could rise to as high as 0.1–0.2 mM after the 30-min incubation [18]. This concentration approaches the K_m of the mitochondrial outer membrane enzymes and is approximately the same as seen in the incubations with cytosol in the absence of ADP. However, only if the cellular ADP concentration is low in the liver slices will the cytosol enzymes become important in DOPAL oxidation, since much of this activity is inhibited by physiological concentrations of ADP.

An alternative explanation for the lack of inhibition is that DOPAL and acetaldehyde are oxidized by different isozymes of ALDH in the mitochondrial matrix. Both matrix isozymes possess low K_m values for DOPAL and acetaldehyde; however, our previous results show that disulfiram, a known inhibitor of ALDH [35, 36], inhibits the pI 5.4 isozyme to a greater degree than it inhibits the pI 5.6 isozyme [31]. These results are intriguing, for it is known that disulfiram inhibits acetaldehyde oxidation to a large degree [35, 36], but that it has relatively little effect on DOPAL oxidation in rat liver [12]. These observations lead us to suggest that the pI 5.4 isozyme, which is inhibited by *ca.* 60 per cent after disulfiram treatment, may be primarily involved in acetaldehyde oxidation. The pI 5.4 enzyme, which is inhibited by *ca.* 25 per cent by disulfiram, may be primarily involved in DOPAL oxidation.

Three different forms of aldehyde reductase were isolated from the cytosol. Two of these isozymes have relatively high K_m values for DOPAL, and the third isozyme does not reduce DOPAL at all. At low concentrations of DOPAL, these enzymes would probably play a very small role in the disposition of

DOPAL. The data presented in Table 3 support this thesis. The formation of DOPET in incubations containing cytosol and dopamine is minimal, unless DOPAL levels are raised substantially. The difference in the K_m values of the oxidizing and reducing enzymes must contribute to the fact that *in vivo* dopamine is primarily metabolized to DOPAC and not DOPET.

An unexpected result is observed when dopamine is incubated with the combination of mitochondrial, microsomal and cytosolic fractions. An increase in DOPAL concentration is found compared to when incubations are performed with just mitochondria alone. This increase is not statistically significant, due to the large variation in the data; it was, however, observed in every individual experiment. The accumulation of DOPAL was seen only in the presence of the cytosol fraction; it does not occur when mitochondria and microsomes are incubated with dopamine (data not shown). Thus, it appears that the cytosol contains some system capable of binding DOPAL, especially when pyridine nucleotide cofactors are present.

Aldehydes derived from the biogenic amines have been shown to bind tenaciously to cellular material [37, 38]. It has been shown that sulfhydryl-containing compounds prevent the binding [37]. While studying the binding of *p*-hydroxyphenylacetaldehyde to bovine serum albumin, it was observed that the binding is amine-catalyzed.* Why pyridine nucleotide coenzymes enhance the degree of binding in the present study is not known. Since in the presence of ADP the cytosol is not converting the aldehyde to acid, the enhanced level of DOPAL may be due to the binding of the substrate and coenzyme to ALDH, which is inactivated by ADP. Alternatively, the aldehyde could possibly bind to an alcohol dehydrogenase NAD complex, forming an abortive ternary complex.

The data presented in this study indicate that the mitochondrial matrix is the major site of DOPAL oxidation. Since the matrix is also the site of acetaldehyde oxidation, we still cannot explain unequivocally why acetaldehyde does not inhibit the oxidation of DOPAL in liver slices [18]. One possible explanation is that the specificity of enzymes *in vivo* differs from that measured *in vitro*. Conceivably, cofactors such as metals which change the catalytic properties of mitochondrial ALDH [39] or nucleotides may alter the specificity of particular isozymes towards different substrates.

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