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SUBCELLULAR DISTRIBUTION AND PROPERTIES OF RABBIT LIVER ALDEHYDE DEHYDROGENASES

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Abstract—In rabbit liver, both NAD^+- and $NADP^+$-dependent aldehyde dehydrogenases were identified. The activities were distributed among at least three major groups of isozymes identifiable by gel electrophoresis. These isozymes also differed in their substrate and coenzyme preferences, subcelluar distributions, and/or responses to effectors. The NAD^+-dependent aldehyde dehydrogenase activity was distributed among the mitochondrial, microsomal, and cytosolic fractions. The $NADP^+$-dependent aldehyde dehydrogenase activity was largely microsomal, with little true cytosolic $NADP^+$-dependent activity demonstrable. Aliphatic aldehydes were oxidized equally well by aldehyde dehydrogenases in all three fractions. Aromatic aldehydes, however, were preferentially oxidized by microsomal aldehyde dehydrogenases. Disulfiram significantly inhibited mitochondrial (45 per cent) and cytosolic (93 per cent) NAD^+-dependent aldehyde dehydrogenase, but it did not cause significant inhibition of microsomal NAD^+-dependent activity. Disulfiram inhibited the $NADP^+$-dependent aldehyde dehydrogenase activity (>71 per cent) in all subcellular fractions. Diethylstilbestrol activated both NAD^+- and $NADP^+$-dependent aldehyde dehydrogenases in mitochondria and cytosol. Microsomal aldehyde dehydrogenases were not affected by diethylstilbestrol.

Evidence for multiple molecular forms of aldehyde dehydrogenase (aldehyde: $NAD(P)^+$ oxidoreductase, EC 1.2.1.3 and EC 1.2.1.5) in mammalian liver is steadily increasing. At least three isozymes of aldehyde dehydrogenase are demonstrable in normal rat liver mitochondrial, microsomal, and/or cytosolic fractions [1–3]. In several other species, a mitochondrial and a cytosolic isozyme are identifiable [4–7]. For the rat, it is generally accepted that one or more of the mitochondrial isozymes are responsible for the oxidation of acetaldehyde during ethanol metabolism. For other species, including man, it is not yet clear which isozymes are involved in ethanol metabolism. Moreover, the roles of the microsomal and cytosolic aldehyde dehydrogenases have remained largely undefined.

In addition to a role in ethanol metabolism, it is becoming increasingly apparent that aldehyde dehydrogenases may play an important role in the metabolism of other potentially harmful xenobiotics [8]. Phenobarbital [9, 10], 2-acetylaminofluorene, dimethylaminoazobenzene, ethionine [11], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polychlorinated biphenyls, and mirex [12–14] induce a variety of aldehyde dehydrogenase isozymes not detectable in normal rat or mouse liver. A common characteristic of these inducible isozymes is that they are almost exclusively cytosolic. Moreover, these inducible activities possess very broad substrate specificities and, except for the phenobarbital-inducible isozyme, utilize $NADP^+$ as efficiently as NAD^+ as coenzyme [11, 12].

Among the mammalian liver aldehyde dehydrogenases, few reports are concerned with rabbit liver activities. Maxwell and Topper [15] and Duncan [16, 17] have purified and characterized a steroid-sensitive aldehyde dehydrogenase from the soluble fraction of rabbit liver. Raison *et al.* [18] reported two aldehyde dehydrogenases in rabbit liver. Both are NAD^+-dependent and differ in their abilities to oxidize aliphatic and aromatic aldehydes.

Reports of inducible aldehyde dehydrogenase isozymes and our observations of significant $NAD(P)^+$-dependent aldehyde dehydrogenase activity capable of oxidizing both aliphatic and aromatic substrates not only in rat liver [3], but also in mouse and Mongolian gerbil liver [19], have prompted us to reexamine the liver aldehyde dehydrogenase activities of other mammalian species. This study was undertaken to establish the normal rabbit liver aldehyde dehydrogenase phenotype and provide a partial characterization of the activities observed.

MATERIALS AND METHODS

Aldehydes and pyrazole were from the Aldrich Chemical Co., Inc. (Milwaukee, WI). NAD^+, $NADP^+$, EDTA, 2-mercaptoethanol, rotenone, 2,2-dinitrophenyl-5,5′- diphenyl- 3,3′- (3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (NBT), *N*-methylphenazonium methosulfate (PMS), tetraethylthiuram disulfide (disulfiram), sodium *p*-hydroxymercuribenzoate and diethylstilbestrol were from the Sigma Chemical Co. (St. Louis, MO). Reagents for electrophoresis and isoelectric focusing were from Eastman Organic Chemicals (Rochester, NY), except for isoelectric focusing ampholytes, which were from LKB Instruments, Inc. All other chemicals were of reagent grade.

Tissue preparation. Adult male albino rabbits were obtained from a local supplier. Animals were killed by suffocation by sublimation of solid CO_2 in a large

container. Livers were removed and rinsed free of blood. A portion of the liver was placed in ice-cold 60 mM sodium phosphate buffer (pH 8.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (hereafter referred to as buffer). The remaining liver was quick-frozen, as described [20], and stored at −70° for later use. Whole liver was homogenized in buffer, made to 1.0% with Triton X-100 and centrifuged as described [3]. The resulting supernatant fraction was the enzyme source for whole liver assays.

Fresh or quick-frozen livers were fractionated as described [3], using 0.25 M sucrose buffered to pH 8.5. Subcellular fractions were made to 1.0% with Triton X-100 and centrifuged as described. No significant changes in enzyme distributions or activities were caused by the use of quick-frozen liver [3].

Enzyme assays. Aldehyde dehydrogenase activity was determined spectrophotometrically at 23° by monitoring the A_{340} of NADH or NADPH during the oxidation of aldehydes in a modification of the previously described assay [20]. The reaction mixture contained: 1.0 ml of buffer, 1.0 ml of 7.5 mM NAD or NADP, 0.25 ml of 75 mM propionaldehyde or acetaldehyde or a saturated benzaldehyde solution, 10–100 μl of enzyme, and water to 3.0 ml. Pyrazole (0.5 mM final concentration) and rotenone (2 μM final concentration) were included to inhibit alcohol dehydrogenase and NADH oxidase respectively. Usually, enzyme was added last and the increase in A_{340} recorded at 1-min intervals for 5 min. All appropriate controls for substrate- and enzyme-independent changes in A_{340} were made. Occasionally, tetraethylthiuram disulfide (disulfiram, 100 μM final concentration), *p*-hydroxymercuribenzoate (13 μM final concentration) or diethylstilbestrol (20 μM final concentration) was included to study their effects on aldehyde dehydrogenase. Disulfiram and diethylstilbestrol were prepared as stock solutions in methanol, which was without effect on aldehyde dehydrogenase. The influence of the effectors was determined as described [3, 16], by mixing 10 μl of the effector stock solution with 2.74 ml of assay mixture containing sample, but no substrate. After a 5-min preincubation, the reaction was initiated by the addition of 0.25 ml of substrate solution. When effectors were to be included, 2-mercaptoethanol was omitted from the fractionation and assay buffers.

Marker enzyme assays for the various subcellular fractions were performed as described [3]. All enzyme activities are expressed as milliunits/mg of protein (1 milliunit = 1 nmole of NAD(P)H produced/min). Protein concentration was determined by the method of Lowry *et al.* [21], using bovine serum albumin as standard.

Miscellaneous methods. Polyacrylamide gel electrophoresis and analytical isoelectric focusing in gel slabs were performed as described [19]. Gels were stained for aldehyde dehydrogenase as described [11]. Control gels were stained without added substrate to test for activity due to endogenous substrates or without coenzyme to test for aldehyde oxidase [22].

RESULTS

In rabbit liver, both NAD^+- and $NADP^+$ dependent aldehyde dehydrogenases are identifiable (Fig. 1; Table 1). NAD^+ was the preferred coenzyme with all the aliphatic and most aromatic aldehyde substrates tested, except for 4-chloro- and 4-nitroben-

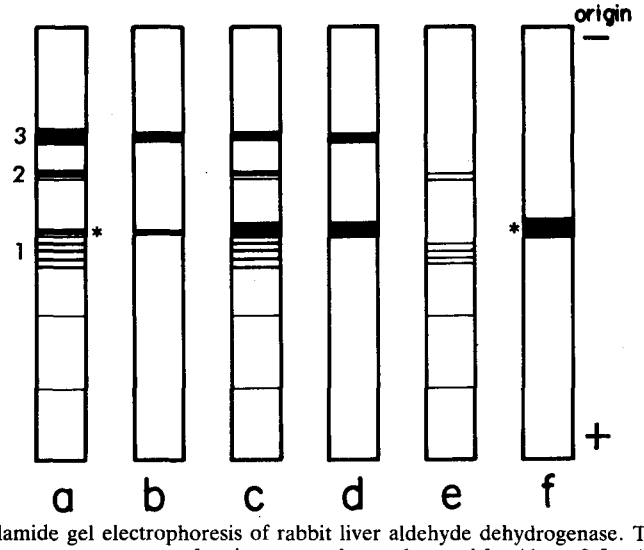

Fig. 1. Polyacrylamide gel electrophoresis of rabbit liver aldehyde dehydrogenase. Ten μl samples of rabbit liver homogenate supernatant fractions were electrophoresed for 4 hr at 2.5 mA/gel through 7% acrylamide gels. Gels were stained [20] as follows: (a–d) for aldehyde dehydrogenase with (a) propionaldehyde, NAD; (b) propionaldehyde, NADP; (c) benzaldehyde, NAD; (d) benzaldehyde, NADP; (e) without added aldehyde, NAD, to test for activity due to endogenous substrates; and (f) benzaldehyde, without coenzyme, to test for aldehyde oxidase [22]. Major groups of aldehyde dehydrogenase isozymes are denoted as 1, 2 and 3 in order of decreasing mobility toward the anode. Isozyme groups 1 and 2 represent mitochondrial aldehyde dehydrogenases identified by electrophoresis of mitochondrial fractions as described above for whole tissue. Isozyme 3 represents a microsomal aldehyde dehydrogenase as determined by electrophoresis of microsomal fractions. The asterisk (*) signifies aldehyde oxidase activity.

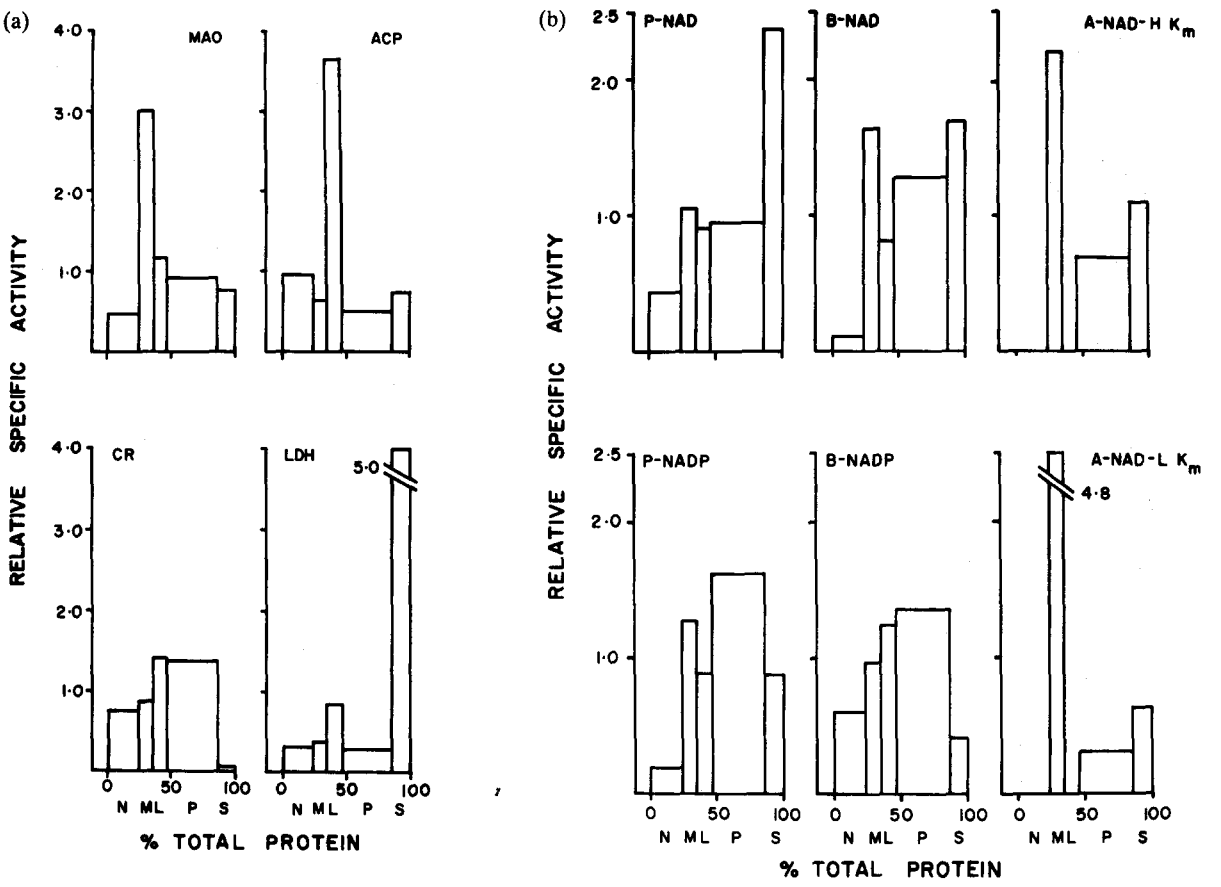

Fig. 2. Marker enzyme-activity distributions (a) and aldehyde dehydrogenase activity (b) in rabbit liver subcellular fractions. (Panel a) Key: MAO, monoamine oxidase; ACP, acid phosphatase; CR, NADPH-cytochrome *c* reductase; and LDH, lactate dehydrogenase. Subcellular fractions are: N, nuclear; M, mitochondrial; L, lysosomal; P, microsomal; and S, cytosolic. Relative specific activity is the ratio of percentage of activity to the percentage of protein in each fraction. (Panel b) Key: P, propionaldehyde; B, benzaldehyde; and A, acetaldehyde. High K_m activity was measured with 5 mM acetaldehyde; low K_m activity was measured with 5 μM acetaldehyde. The fractions are as in panel a.

Table 1. Activity of rabbit liver aldehyde dehydrogenase

Substrate, coenzyme	Specific activity (mU/mg protein ± S.E.M.)
Propionaldehyde, NAD^+	26.9 ± 1.7 (8)*
Propionaldehyde, $NADP^+$	11.1 ± 0.6 (8)
Benzaldehyde, NAD^+	7.5 ± 0.4 (8)
Benzaldehyde, $NADP^+$	6.8 ± 0.4 (8)

* Number of livers assayed.

zaldehyde where $NADP^+$ was the preferred coenzyme (data not shown).

Gel electrophoresis resolved several isozymes of rabbit liver aldehyde dehydrogenase (Fig. 1). In addition, an aldehyde oxidase isozyme was also resolved by electrophoresis (Fig. 1). Gel isoelectric focusing resolved a single major and two minor NAD^+-dependent aldehyde dehydrogenases. The isoelectric point (pI) of the major isozyme was near pH 5.8; the pIs of the minor forms, near pH 5.5.

Table 2. Aldehyde dehydrogenase activity in rabbit liver subcellular fractions

Substrate, coenzyme	Total activity (mU/g liver ± S.E.M.) N*	M	L	P	S	Sum N–S
Propionaldehyde, NAD (8)†	11.8 ± 2.8	44.7 ± 7.8	10.4 ± 1.4	49.7 ± 16.4	41.9 ± 16.4	158.5
Benzaldehyde, NADP (8)	9.1 ± 0.8	18.6 ± 5.0	8.4 ± 1.4	46.0 ± 15.3	4.6 ± 1.5	86.7
Acetaldehyde, NAD (5 mM) (2)	ND‡	33.4	ND	42.6	37.6	ND
Acetaldehyde, NAD (5 μM) (2)	ND	22.2	ND	6.0	6.8	ND

* Abbreviations: N, nuclear; M, mitochondrial; L, lysosomal; P, microsomal; and S, cytosolic.
† Number of livers assayed.
‡ Not determined.

Table 3. Influence of various effectors on rabbit liver aldehyde dehydrogenase

Substrate, coenzyme	Control activity (mU/mg protein ± S.E.M.)	% Inhibition		% Activation by diethylstibesterol
		Disulfiram	*p*-Hydroxymercuribenzoate	
Mitochondria				
Propionaldehyde, NAD^+	69.1 ± 17.8 (5)*	44.9†	67.8†	122.4‡
Benzaldehyde, $NADP^+$	15.0 ± 2.9 (5)	70.9†	95.7†	131.8
Microsomes				
Propionaldehyde, NAD^+	75.5 ± 23.9 (5)	28.0	66.3†	0.0
Benzaldehyde, $NADP^+$	22.9 ± 4.4 (5)	76.0†	100.0†	0.0
Cytosol				
Propionaldehyde, NAD^+	43.9 ± 6.1 (5)	93.2†	88.6†	142.9
Benzaldehyde, $NADP^+$	3.4 ± 0.7 (5)	79.4†	100.0†	63.0

* Number of livers assayed for the effects of disulfir m or *p*-hydroxymercuribenzoate.
† Activity in the presence of disulfiram (100 μM) or *p*-hydroxymercuribenzoate (13 μM) was significantly less than its corresponding control at, at least, the $P < 0.05$ level by a paired *t*-test.
‡ Average per cent activation by diethylstilbesterol (20 μM) compared to corresponding controls for two determinations.

Rabbit liver aldehyde dehydrogenase was localized primarily in mitochondria, microsomes, and the cytosol (Fig. 2; Tables 2 and 3). Based on the relative specific activities (Fig. 2b) and total activities (Table 2), clear compartmentalization of the aldehyde dehydrogenases was demonstrable. Using 5 μM acetaldehyde as substrate, the mitochondrial fraction possessed the vast majority of the NAD^+-dependent aldehyde dehydrogenase activity. No significant $NADP^+$-dependent activity was detectable in any subcellular fraction, using 5 μM acetaldehyde. Using aldehyde concentrations in the mM range, significant aldehyde dehydrogenase activities were demonstrable in the mitochondrial, microsomal, and cytosolic fractions (Fig. 2b; Tables 2 and 3). With NAD^+, microsomes appeared to preferentially oxidize aromatic aldehydes, aliphatic aldehydes being oxidized largely, but not exclusively, by mitochondria and/or cytosol (Table 2). With $NADP^+$ as coenzyme, microsomes were the major source of aldehyde dehydrogenase using either aliphatic or aromatic aldehydes (Table 2). With little $NADP^+$-dependent aldehyde dehydrogenase was present in the cytosol (Fig. 2b; Tables 2 and 3).

Gel electrophoresis of aldehyde dehydrogenases from subcellular fractions also indicated a clear compartmentalization (Fig. 1). Mitochondrial aldehyde dehydrogenase activity was resolved into the anodally migrating NAD^+-dependent series of isozymes (1 in Fig. 1), and an intermediate-mobility NAD^+-dependent isozyme (2 in Fig. 1). Microsomal aldehyde dehydrogenase migrated as a single $NAD(P)^+$-dependent slow-mobility isozyme (3 in Fig. 1). Cytosolic aldehyde dehydrogenase consisted exclusively of the single intermediate-mobility NAD^+-dependent isozyme (2 in Fig. 1). Gel isoelectric focusing of aldehyde dehydrogenase from subcellular fractions indicated that the major isozyme (pI 5.8) identifiable in whole tissue preparations corresponded to isozyme 2 of mitochondria and cytosol. Isozyme 3 was not resolved by isoelectric focusing, and isozyme(s) 1 of mitochondria resolved as the minor isozymes at pI 5.5.

Disulfiram significantly inhibited the NAD^+-dependent aldehyde dehydrogenase of rabbit liver mitochondria and cytosol (Table 3). The cytosolic NAD^+-dependent activity was particularly disulfiram-sensitive (> 90 per cent inhibition). Microsomal NAD^+-dependent aldehyde dehydrogenase activity was only slightly reduced by disulfiram. Disulfiram significantly inhibited the $NADP^+$-dependent aldehyde dehydrogenase activity of all subcellular fractions (Table 3). With either NAD^+ or $NADP^+$, *p*-hydroxymercuribenzoate significantly inhibited rabbit liver aldehyde dehydrogenase in all subcellular fractions, inhibiting $NADP^+$-dependent activity more than 95 per cent (Table 3). Diethylstilbestrol activated both NAD^+- and $NADP^+$-dependent aldehyde dehydrogenases in mitochondria and cytosol (Table 3). Microsomal aldehyde dehydrogenases were not affected by diethylstilbestrol.

DISCUSSION

Rabbit liver was shown to possess both NAD^+- and $NADPH^+$-dependent aldehyde dehydrogen-

ases. The activities were distributed among at least three major groups of isozymes. These isozymes differed in their substrate and coenzyme preferences, subcellular distribution, and response to effectors. They were identifiable by gel electrophoresis and/or gel isoelectric focusing. The substrate and coenzyme specificities of rabbit liver aldehyde dehydrogenase were similar to those of the rat [3, 23, 24], Mongolian gerbil [19], and mouse [25]. The subcellular distribution of rabbit liver aldehyde dehydrogenase was also similar to that of the rat [2, 3], except that rabbit liver possessed significantly more true cytosolic activity.

As with rat liver [3, 23, 26], acetaldehyde was oxidized by rabbit liver mitochondrial, microsomal, and cytosolic aldehyde dehydrogenases. Two distinct acetaldehyde oxidizing isozymes were detectable in mitochondria (micromolar and millimolar K_m forms). Only the millimolar K_m isozyme was identifiable in microsomal and cytosolic fractions. Distinct NAD^+ and $NAD(P)^+$-dependent aldehyde dehydrogenases capable of oxidizing both aliphatic and aromatic aldehydes were identifiable in both mitochondria and microsomes of rabbit liver. Rabbit liver cytosolic aldehyde dehydrogenase consisted almost exclusively of NAD^+-dependent activity, with little, if any, true cytosolic $NADP^+$-dependent activity demonstrable.

Gel electrophoresis resolved several isozymes of rabbit liver aldehyde dehydrogenase and clearly demonstrated the association of different isozymes with various subcellular components. Since the first report of Robbins [27], electrophoresis has only infrequently been used as a tool for characterizing mammalian liver aldehyde dehydrogenases in whole tissues or subcellular fractions. Recently, Holmes [28] has resolved several isozymes of mouse liver aldehyde dehydrogenase. Harada *et al.* [29] have resolved three isozymes of human liver aldehyde dehydrogenase. We have routinely used gel electrophoresis and isoelectric focusing in the characterization of various mammalian liver aldehyde dehydrogenases [3, 19, 20]. Only for rabbit liver, however, was the resolution of the various aldehyde dehydrogenase isozymes sufficient to associate the isozymes observed with clearly defined subcellular fractions.

The various effectors examined in this study also provide evidence for multiple forms of rabbit liver aldehyde dehydrogenase. As with rat liver [2, 3, 10, 24], both disulfiram-sensitive and disulfiram-insensitive NAD^+-dependent aldehyde dehydrogenases were present in rabbit liver. The differential disulfiram-inhibition of NAD^+- and $NADP^+$-dependent isozymes, especially in the microsomal fraction, indicates the presence of two $NAD(P)^+$-dependent aldehyde dehydrogenases in rabbit liver, as in the rat [3]. Rabbit liver cytosolic NAD^+-dependent aldehyde dehydrogenase was particularly disulfiram-sensitive. This appears to be a common characteristic of mammalian liver cytosolic aldehyde dehydrogenase, including the inducible isozymes [3, 10, 30–32].

Diethylstilbestrol activates a cytosolic aldehyde dehydrogenase purified from rabbit liver [15, 16]. The phenobarbital-inducible isozyme, purified from rat liver, is also activated by diethylstilbestrol [31]. By examining subcellular fractions and using both NAD^+ and $NADP^+$ as coenzyme, we showed that, in addition to the cytosolic activity, rabbit liver mitochondrial NAD^+- and $NADP^+$-dependent aldehyde dehydrogenases were activated by diethylstilbestrol. However, the microsomal isozymes, both NAD^+- and $NADP^+$-dependent, were unaffected by diethylstilbestrol.

The results reported here indicate that rabbit liver aldehyde dehydrogenase exists as a variety of isozymes that are readily distinguishable by a number of properties. The relative ease with which the various isozymes can be identified in whole tissue and subcellular preparations makes rabbit liver aldehyde dehydrogenase a particularly useful system for studying the regulatory events that control the expression of these isozymes.

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