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PRESENCE OF CYTOSOLIC ALDEHYDE DEHYDROGENASE ISOZYMES IN ADULT AND FETAL RAT LIVER*

QING-NA CAO,† GUANG-CHOU TU† and HENRY WEINER‡
Department of Biochemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

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Abstract—A colony of Wistar-strain rats bred at Purdue University was composed of animals with two different isozyme patterns of liver cytosolic aldehyde dehydrogenase (EC 1.2.1.3, ALDH) as determined by isoelectric focusing. One cytosolic isozyme pattern had a major activity band with a pI = 5.8 and a minor activity band at pI = 6.2. The other pattern contained three major isozymes with pI values of 5.3, 5.4 and 5.6 along with the pI 6.2 isozyme and a trace of the 5.8 one. The 5.8 and 6.2 isozymes were recognized by antibodies produced against horse and beef liver cytosolic ALDH, whereas the set of three (5.3–5.6) were not. The cytosolic isozymes were inhibited by low levels of disulfiram and had K_m values for acetaldehyde in the 100 μM range, properties typical for cytosolic ALDHs. All animals contained the same isozymes of liver mitochondrial ALDH. These were a major activity with a pI ≈ 5.2 and minor activities associated with isozymes of pI = 6.4 and 6.6. These isozymes were recognized by antibodies produced against pure horse and beef liver mitochondrial ALDHs. Both cytosolic and mitochondrial ALDHs were found in fetal liver as early as day 15 of gestation. The total activity for mitochondrial ALDH increased between day 15 and day 21 whereas that for cytosolic ALDHs remained relatively constant during development. It appeared that both cytosolic and mitochondrial ALDH were present by at least the third trimester and could afford the fetus some protection against the toxic action of endogenous or exogenous aldehydes.

Liver aldehyde dehydrogenase (EC 1.2.1.3, ALDH§) is found in all major subcellular organelles. One role of the enzyme is to remove aldehydes which, in general, are toxic compounds. Substrate specificities of the cytosolic and mitochondrial isozymes are broad so that it is not possible by K_m values alone to suggest their natural substrates [1]. It had been shown that the metabolism of acetaldehyde, derived from ethanol, and of 3,4-dihydroxyphenylacetaldehyde, derived from dopamine, occurs primarily in mitochondria [2, 3]. Thus far, the potential endogenous or exogenous substrates for cytosolic isozymes have not been identified from metabolic studies.

Various isozymes of cytosolic ALDH are inducible by different xenobiotics. Phenobarbital [4], tetrachlorodibenzo-*p*-dioxin [5] and 2-acetylaminofluorine [6] all cause the induction of the isoenzymes in rodents. Similar experiments have not been done in non-rodents, but the constitutive cytosolic isozymes from human [7], horse [8] and sheep [9] have been purified and studied extensively. Cytosolic ALDHs from beef [10], dog [11], and mouse [12] have also been characterized. There is some question as to whether cytosolic isoenzymes exist to any degree in some rat strains [13].

Using Wistar rats, we previously reported that it was possible not only to detect the presence of cytosolic ALDH but also to show that there was a genetic component to the ALDH isozyme patterns [14]. Three distinct major isozyme patterns have been detected [15]. Some animals possess a triplet pattern with the pI values of *ca.* 6.2, 6.0, and 5.8, whereas others have only one isozyme at either 6.2 or 5.8. A correlation between voluntary alcohol consumption and isozyme patterns has also been found in these animals [15]. More recently we showed that antibodies prepared against horse and beef liver cytosolic ALDH could cross-react with rat cytosolic ALDH isozymes but they do not recognize the isozymes obtained from mitochondria [16]. Hence it appears, at least in the Wistar rat, that there is a gene which codes for a non-inducible cytosolic ALDH.

If both mitochondrial and cytosolic isozymes are constitutive, one might expect to find them in fetal liver. It is known that human and rat fetal liver can oxidize acetaldehyde [17]. Recently it was reported that by day 15 of gestation mitochondrial, but not cytosolic, ALDH could be found in fetal livers of Wistar rats [18]. Insamuch as the Wistar rat colony we originally used was destroyed and a new one begun, we wanted to determine if indeed cytosolic

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† Permanent address: Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, The People's Republic of China.

‡ Recipient of a Research Scientist Award, AA 00028, from the National Institute on Alcohol Abuse and Alcoholism. To whom correspondence should be addressed.

§ Abbreviations: ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; αHC, αBC and αHM, antibodies prepared in rabbit against horse and beef liver cytosolic and horse liver mitochondrial aldehyde dehydrogenase respectively; IEF, isoelectric focusing; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

ALDHs were present in the adult, as we found previously, and if the liver isozymes from the two organelles were detectable in the developing fetus.

METHODS

Materials. Acetaldehyde, propionaldehyde and *p*-nitrobenzaldehyde were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Agarose IEF and Pharmalyte pH 5–8 were from Pharmacia (Piscataway, NJ). Pyrazole, pyruvate, NAD, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylterazolium bromide (MTT), Coomassie Brilliant Blue R, bovine carbonic anhydrase, β-lactoglobulin and disulfiram were purchased from the Sigma Chemical Co. (St. Louis, MO). Phenazine methosulfate was supplied by Calbiochem (San Diego, CA), *d*-sorbitol was from Fisher (Fairlawn, NJ) and ribonuclease was a product of Boehringer (Indianapolis, IN). Goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate was obtained from Bio-Rad (Richmond, CA). Nitrocellulose was from Schleicher & Schuell (Woburn, MA). Other chemicals available from commercial sources were of reagent grade.

Distilled aldehyde-free water was used to prepare solutions. Buffers were from sodium salts.

Animals. Wistar rats originally from Harlan Sprague–Dawley were obtained from the animal facility of the Biochemistry Department at Purdue University. To study fetal ALDH isozymes, a male and famale pair were housed separately. If a vaginal plug was found in the morning, it was assumed that the female conceived the day before, which was labeled day 1 of gestation. On different days the females were killed with CO_2 and the pups were removed quickly.

Homogenization and subcellular fractionation. The homogenization and subcellular fractionation of rat livers were performed as described by Tank *et al.* [3]. Alcohol dehydrogenase, glutamate dehydrogenase and glucose-6-phosphatase were used as the marker enzymes of cytosolic, mitchondrial and microsomal fractions, respectively, to verify the extent of contamination of each other. Less than 6.1% of the cytosolic marker, ADH, was found in mitochondria and less than 6.1% of the mitochondrial marked glutamic dehydrogenase was found in the cytosolic fraction. This is similar to what we previously found [3].

Enzyme assays. The cytosolic ALDH activity was assayed at 25° in 100 mM phosphate buffer, pH 7,4, containing 0.5 mM NAD, 10 mM pyrazole and 1 mM acetaldhyde, by measuring NADH formation with an Aminco Fluoro-Microphotometer. The mitochondrial and microsomal ALDH activity assays employed 10 μM and 10 mM acetaldhyde respectively, as the substrate. ADH, glutamate dehydrogenase and glucose-6-phosphatase activities were assayed according to Tank *et al.* [3].

Determination of K_m *values.* Michaelis constants for ALDH isozymes were calculated from Line Weaver–Burk plots.

Disulfiram inhibition. Various concentrations of disulfiram were incubated with ALDH isozyme in 0.1 M phosphate buffer, pH 7.4, containing 0.5 mM NAD at 25° for 10 min. The activity remaining was determined by the addition of the substrate.

Antigenetic property. Double diffusion and immunoblotting (Western blotting) were carried out as described by McMichael *et al.* [16].

Isoelectric focusing (IEF). Analytic isoelectric focusing was performed in a 1% agarose gel slab using Pharmalyte pH 5–8. The focusing was carried out in the Pharmacia model 3000 flatbed apparatus at 10° for 90 min according to the instructions of the manufacturer. Cytosolic and mitochondrial fractions (15 μl) were loaded separately on the gel. At the completion of focusing, the gel was cut into two pieces. The piece of gel containing pI standards was stained for protein and the other containing isozyme samples was stained for ALDH activity. Column isoelectric focusing was performed as described [3].

Activity staining. Following isoelectric focusing, the isozymes were stained in 0.1 M pyrophosphate buffer (aldehyde-free), pH 9.0. The composition of staining mixture was described by Holmes [19] but did not contain agar.

Protein staining. The proteins were stained with Coomassie Brilliant Blue R according to Pharmacia's instruction.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A 10% polyacrylamide gel was prepared in a slab-gel mould and overlayed with a 4% polyacrylamide stacking gel. Electrophoresis was carried out vertically at room temperature according to a manual from Hoefer Scientific Instruments.

Determination of protein concentration. The concentration of protein was determined using Bio-Rad protein determination reagent as described in the instruction sheets of the manufacturer, using crystalline bovine serum albumin as the standard.

RESULTS

Aldehyde dehydrogenase activity identified in rat liver cytosol. When livers from ten animals were pooled and subjected to subcellular fractionation, the cytosolic fraction was found to contain many isozymes of ALDH in contrast to the mitochondrial fraction (Fig. 1B). When individual animals were analyzed, rather than using pooled livers, it was found that two distinct isozyme patterns emerged (Fig. 1C). Some animals had a major band with pI = 5.8, whereas others had a major triplet of isozymes with pIs of 5.3, 5.4, and 5.6. The pattern with the dominant form at pI 5.8 will be called the A-pattern and the other the B-pattern. Nineteen animals were analyzed separately, and the A-pattern appeared in eight, while the B-pattern was in the remaining eleven. There was no sex preference to the patterns. In Table 1 are summarized the pI values for the major and minor isozymes found for each pattern. Occasionally a band of activity was found corresponding to a cytosolic enzyme with a pI value between 6.9 and 7.0. Lindahl and Evces [20] reported that a tumor specific and some xenobiotic inducible isozymes have pI values in this range. No attempts were made to characterize or search for the origin of this activity.

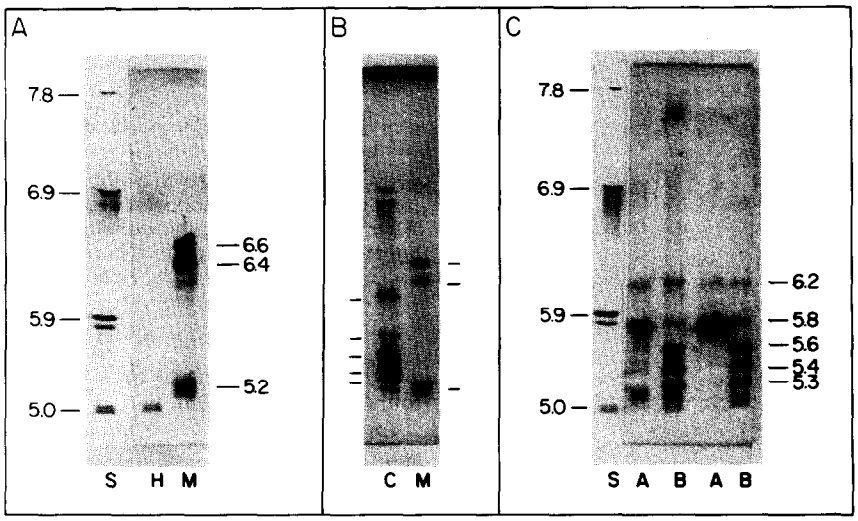

Fig. 1. Agarose-gel IEF electrophoretograms of rat liver ALDH isozymes. Samples (25 μl) were focused at 4° for 90 min and stained either for ALDH activity with propionaldehyde as substrate or for proteins. Key: (panel A) mitochondrial isozymes; (panel B) a comparison of cytosolic isozymes with mitochondrial ones; (panel C) two distinct IEF patterns of cytosolic isozymes; S, pI standard proteins; H, horse liver mitochondrial ALDH; M, rat liver mitochondrial homogenate; C, rat liver cytosolic fraction; A, cytosolic fraction of A-pattern rat liver; and B, cytosolic fraction of B-pattern rat liver.

Liver ALDHs were shown to possess broad substrate specificity in that they oxidize aliphatic, aromatic and aldehydes derived from biogenic amines [3]. It was found that the isozyme forms isolated in the study all possessed activity towards *p*-nitrobenzaldehyde (data not shown). Only the enzyme with a pI of 6.9 (see Fig. 1B) showed activity against succinate semi-aldehyde. It was suggested recently that a human enzyme that oxidizes acetaldehyde is really a succinate semi-aldehyde dehydrogenase [21].

Rat liver mitochondrial ALDH. In Fig. 1A is presented an electrophoretogram showing that the major activity of mitochondrial ALDH was found at pH 5.2. Additional bands of catalytic activity were found at pH 6.4 and 6.6. This was similar to what we previously reported from both electrophoretograms and isolation of mitochondrial isozymes [3]. The isozyme forms were recognized by antibodies prepared against horse or beef mitochondrial ALDH using either double diffusion or Western blotting. They did not cross-react with antibodies prepared against horse or beef cytosolic isozyme.

Rat liver microsomal ALDH. No attempts were made to solubilize the enzyme. If crude microsomes were subjected to IEF and then stained for ALDH activity, no definite band of activity could be found. A smear of activity was always found between pH 5 and 6. This is in contrast to what occurred with the mitochondrial and cytosolic ALDHs. Hence, we conclude that no contamination by microsomes occurred in the analysis of cytosolic ALDH to be presented.

Isolation of cytosolic ALDHs by column isoelectric focusing. Livers were first phenotyped with respect to their isozyme patterns. Separately, livers containing A or B isozyme patterns were subjected to column IEF to separate and partially characterize the individual isozymes.

Livers with the A-isozyme pattern yielded a major and a minor activity peak (Fig. 2). Aliquots from each were subjected to gel isoelectric focusing. It was found that the minor peak (II) contained an active ALDH band with pI = 6.2, whereas the major peak (I) contained the 5.8 isozyme.

Table 1. Properties of isozymes of rat liver cytosolic ALDH

Isozyme pattern	pI	K_m* (μM)	Disulfiram concentrations for 50% inhibition (μM)	Antibody recognition	
				αHC and αBC	αHM
A-pattern	6.2	500	0.1	+	+†
	5.8	170	20	+	+
B-pattern	6.2	500	0.1	+	+
	5.8	170	20	+	+
	Triplet‡	170	5	−	−

* K_m for acetaldehyde.
† It has been shown that αHM will recognize cytosolic ALDHs [16].
‡ Cluster of isozymes with pIs of 5.3, 5.4 and 5.6.

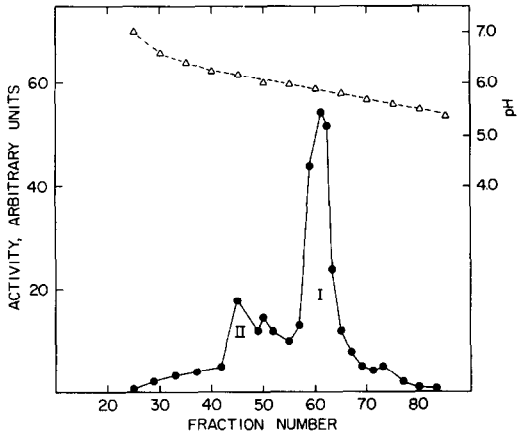

Fig. 2. Separation of A-pattern isozymes by column IEF. Cytosol from 2 g of A-pattern rat liver was subjected to column IEF. The catalytic activity in each fraction was measured by monitoring the fluorescence of NADH formation using propionaldehyde as a substrate (●), and the pH of each fraction was determined (△). Fractions containing the separated isozymes were subjected to agarose-gel IEF and were found to focus as a single band.

The same separation technique was used with livers containing the B-isozyme pattern. Two nearly equal peaks of catalytic activity were obtained. A sharp band was found at pH 6.2, while the other (broader) band was found between pH 5.8 and 5.4. An aliquot from the fractions isolated at pH 6.2 was subjected to gel IEF and only one activity band was noted. The broad peak was divided into three sections, as indicated in Fig. 3. Each portion contained components of the triplet, but the ratio of the individual isozymes appeared to change across the peak (Fig. 3). It was also possible to detect some

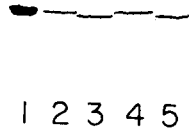

Fig. 4. Western blotting of SDS–PAGE. Fractions obtained from column IEF (Fig. 2) were subjected to SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose. Lane 1: purified beef liver cytosolic ALDH; 2 and 4: pI 6.2 fractions; 3 and 5: pI 5.8 fraction.

activity corresponding to the pI 5.8 isozyme as was found in the A-pattern.

Immunological identification of the cytosolic isozymes. It has been established that antibodies against horse or beef cytosolic ALDH (αHC or αBC) will not recognize a mitochondrial isozyme [16]. The active fractions obtained from the A-pattern (Fig. 2) were subjected to SDS–PAGE followed by Western blotting analysis using antibody prepared against beef liver cytosolic ALDH. Both the minor component at pH 6.2 and the major one at pH 5.8 were recognized by the antisera. The two rat isozymes had nearly the same subunit molecular weights as pure beef ALDH, 55 kD. As shown in Fig. 4, the pI 5.8 isozyme was perhaps 1 kD smaller than the 6.2 one. It was not possible to obtain unequivocal data when the B-isozyme pattern was analyzed. The minor component with pI = 6.2 was positively recognized by the antibody, but the family of isozymes found near pH 5.4 did not yield positive results using Western blotting or double diffusion. They also were not recognized by antibodies prepared against mitochondrial isozymes. Hence it is not possible to state that these isozyme forms were cytosolic in origin based upon their antigenetic properties.

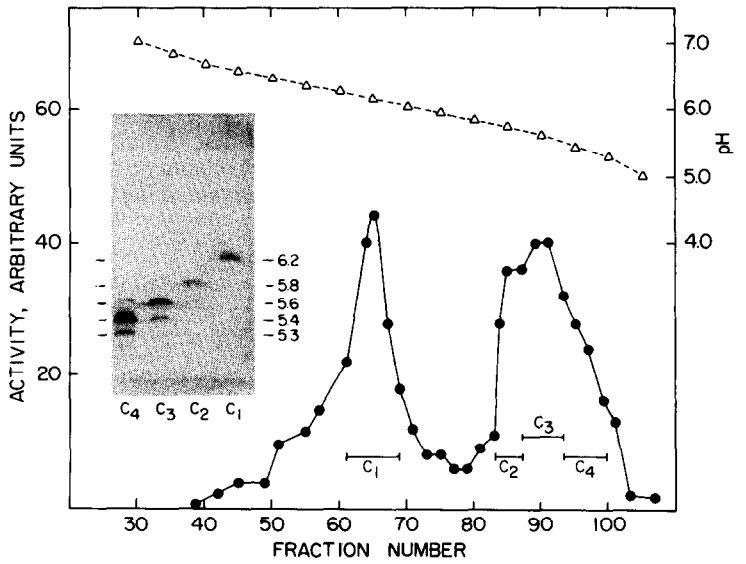

Fig. 3. Separation of B-pattern isozymes by column IEF. The experiment was carried out as in Fig. 2. Insert: agarose-gel IEF of the fractions C_1 to C_4.

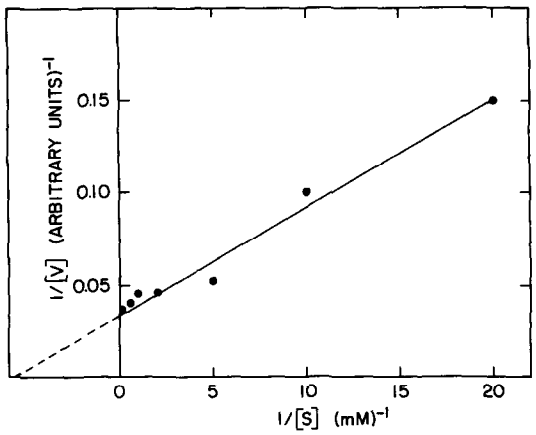

Fig. 5. Double-reciprocal plot of B-pattern isozymes (pI 5.3–5.6) using acetaldehyde as the varied substrate (S) and NAD as the fixed substrate (500 μM).

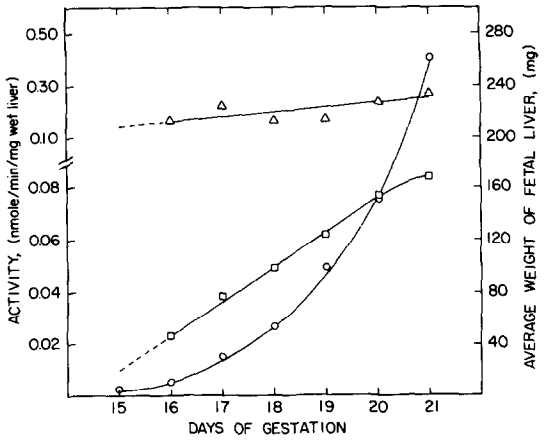

Fig. 7. Changes in fetal liver weight (○) and activity of both cytosolic (△) and mitochondrial (□) ALDH during development.

K_m *values towards acetaldehyde.* It has been shown that K_m values towards acetaldehyde with mitochondrial ALDHs are in the submicromolar range, whereas with cytosolic ALDHs they are typically in the >100 μM range [1]. The K_m values of rat liver cytosolic ALDHs were determined with the isozymes separated by column IEF (Figs. 2 and 3). The values for the K_m are presented in Table 1. Though isozymes from the B-pattern clustered at pI ~5.4 were not separated from each other, the double-reciprocal plot, shown in Fig. 5, was linear. This suggests that all three isozymes had essentially the same K_m value. The major band from the A-pattern (pI = 5.8) had virtually the same K_m as did the B-cluster of isozymes. The common pI = 6.2 isozyme had the highest K_m value (500 μM).

Inhibition by disulfiram. Cytosolic ALDHs have been shown to be very susceptible to *in vitro* inhibition by disulfiram, whereas isozymes isolated from mitochondria are relatively insensitive to inhibition [7]. All of the rat isozymes isolated by column isoelectrofocusing were inhibited by relatively low concentrations of disulfiram. The concentrations of disulfiram necessary to cause 50% inhibition are presented in Table 1. It was found that the concentrations of disulfiram employed failed to inhibit mitochondrial ALDHs.

ALDH activity in livers of newborn pups and in fetuses. Lindahl [22] originally showed that the isozyme pattern in newborn Wistar rat pups was virtually identical to that of the adult but also reported that no cytosolic isozyme exists [13]. Sanchis and Guerri [18] reported that they could find no high K_m cytosolic ALDH activity on day 21 but could on the day of birth (day 22). In contrast, it was found after IEF that livers from newborn or 21st day of gestation pups possessed isozyme patterns virtually identical to those found in the adult. The results showed that, at least for the Wistar strain used in these experiments, cytosolic and mitochondrial enzymes were present before birth as well as in the newly born animal.

Livers from animals isolated at different days post-conception were pooled and mitochondria separated from cytosol. It was possible to detect ALDH activity corresponding to cytosolic isozymes as early as day 15 of gestation (Fig. 6). Mitochondrial isozyme activity was also detected as early as day 15 by both activity

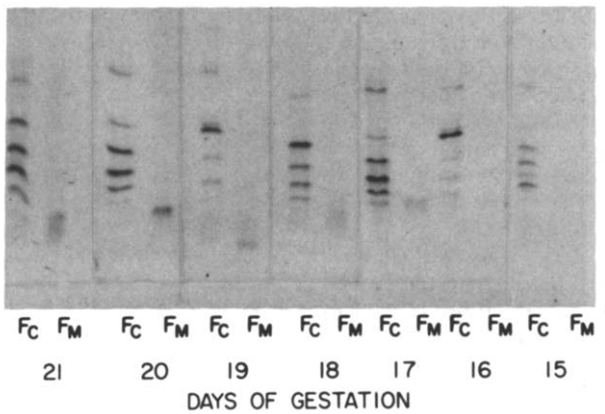

Fig. 6. Agarose-gel IEF patterns of rat liver ALDH isozymes from different gestation days. F_C and F_M are cytosolic fraction and mitochondrial homogenate from fetal livers respectively.

staining (Fig. 6) and by Western blotting (data not shown). No attempts were made to analyze livers from earlier times as they were too small to fractionate and analyze.

Changes in activity of ALDH during development. To assess the change in activity of ALDHs in the developing fetus one pregnant animal was killed on each day of gestation. The livers from the fetuses were pooled and mitochondria then were separated from cytosol. Average fetal liver weight increased dramatically during development (Fig. 7). The activity of the mitochondrial ALDH per mg of wet fetal liver also increased. In contrast, the activity of the cytosolic enzymes per mg of wet fetal liver remained relatively constant during development. On day 21 and in the newborn, the cytosolic activity was *ca.* three times greater than the mitochondrial activity, whereas in the adult the ratio was *ca.* two [2]. It is of potential interest to have found that the activity of mitochondrial but not cytosolic ALDH increased during development. The physiological significance of this cannot be explained at present.

DISCUSSION

It has been shown by Lindahl and Evces [13] that very little cytosolic ALDH exists in some rat strains. It is also well established though that xenobiotics can cause the induction of various isozymes of ALDH in cytosol [4–6, 20]. The data presented in this study show that our colony of Wistar-strain rats possess ALDHs that can be isolated from the cytosol fraction which were neither mitochondrial nor microsomal in origin.

Our colony appears to be composed of animals with two different liver aldehyde dehydrogenase phenotypes. One set of livers contain a major isozyme with a pI of 5.8 and a minor one of pI 6.2. Both isozymes were susceptible to inhibition by disulfiram and cross-reacted with antibodies produced against horse or beef liver cytosolic ALDHs.

The second isozyme pattern found was more complex. Three isozymes with pIs, determined by gel isoelectric focusing, of 5.3, 5.4 and 5.6 were found. These livers also possessed the pI 6.2 isozyme found in the other liver phenotype. Trace amounts of the pI 5.8 form were also identified. The three isozymes found between pH 5.3 and 5.6 appeared to have similar properties with respect to their K_m for acetaldehyde (170 μM) and inhibition by disulfiram. These forms, though, were not recognized by the antibodies prepared against horse and beef cytosolic ALDH. Since they are not present in mitochondria and have some properties of typical cytosolic ALDHs, it appears that these forms probably represent cytosolic ALDHs. The origin of this heterogeneity was not investigated further.

The isozyme patterns of cytosolic ALDH were somewhat different from those reported to be present in a different colony of Wistar rats previously bred in our department. In that colony it was found that the rats possessed cytosolic isozymes with pIs of 6.2, 6.0 and 5.8 [15]. Breeding studies revealed that those isozymes were the result of only two gene products which coded for subunits of the 6.2 and 5.8 isozymes [14].

An early study by Pikkarainen [17] showed that human fetal livers possess aldehyde oxidizing capabilities as early as the third month. He found, though, little aldehyde oxidizing activity in fetal rat livers. This latter result was similar to what was reported recently by Sanchis and Guerri [18] who also relied on spectrophotometric activity assays to determine aldehyde oxidizing capabilities. The data presented here clearly show that ALDH activity is detectable on isoelectric focusing gels as well as by fluorometric assays as early as day 15 of gestation. Both mitochondrial and cytosolic ALDH isozymes could be detected. Thus, it appears that both isozyme types are constitutive to the liver and do not require induction by xenobiotics.

Inasmuch as ALDH is involved in the metabolism of endogenous and exogenous aldehydes it is not surprising that it is present at an early stage of development. Lad *et al.* [23] reported that alcohol dehydrogenase was not detectable until day 18 of gestation, whereas Sanchis and Guerri [18] reported that no detectable activity of that enzyme was found until day 21. A radioimmunoassay for the enzyme was employed in the former study while the latter depended on *in vitro* enzyme activity assays. It was somewhat surprising to have found that ALDH may have been expressed earlier in development than was ADH. The results of this study suggest that the developing rat fetus has the capacity to metabolize ethanol and aldehydes by at least the third trimester.

The natural substrates for ALDHs are not known. It was of interest to note that the activity of cytosolic enzymes remained relatively constant during development while that of the mitochondrial isozyme continually increased. This observation supports the notion that the isozymes are involved in different metabolic pathways. The genes coding for them could be under the influence of different hormonal regulation.

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