Biochemical Pharmacology, Vol. 60, pp. 1629–1637, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00469-X

Betaine Aldehyde, Betaine, and Choline Levels in Rat Livers during Ethanol Metabolism

Ming Kai Chern, Douglas A. Gage† and Regina Pietruszko*‡*

*Center of Alcohol Studies and Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, Piscataway, NJ; and †Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.

ABSTRACT. Betaine aldehyde levels were determined in rat livers following 4 weeks of ethanol feeding, employing the Lieber–De Carli liquid diet. The results showed that the levels of betaine aldehyde are unaffected by alcohol feeding to rats. These levels in both experimental and control animals were found to be quite low, 5.5 nmol/g liver. Betaine aldehyde levels have not been determined previously in mammalian liver because of methodological difficulties. This investigation employed fast atom bombardment–mass spectroscopy to determine the levels of betaine aldehyde, betaine, and choline. The decrease in betaine levels following ethanol administration confirmed the results of other investigators. Choline levels determined during this investigation were lower than previously reported. The reason for starting this investigation was the fact that the enzyme that catalyzes betaine aldehyde dehydrogenation to betaine, which is distributed in both mitochondria and the cytoplasm, was found to also metabolize acetaldehyde with K_m and V_{max} values lower than those for betaine aldehyde. Thus, it appeared likely that the metabolism of acetaldehyde during ethanol metabolism might inhibit betaine aldehyde conversion to betaine and thereby result in decreased betaine levels (Barak *et al.*, *Alcohol* 13: 395–398, 1996). The fact that betaine aldehyde levels in alcohol-fed animals were similar to those in controls demonstrates that competition between acetaldehyde and betaine aldehyde for the same enzyme does not occur. This complete lack of competition suggests that betaine aldehyde dehydrogenase in the mitochondrial matrix may totally metabolize betaine aldehyde to betaine without any involvement of cytoplasmic betaine aldehyde dehydrogenase. BIOCHEM PHARMACOL **60**;11:1629–1637, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. betaine aldehyde; alcohol-fed; rat livers

Chronic ethanol ingestion leads to the deposition of neutral lipids in the liver. Although the precise reason is not yet clear, it has been speculated that five major mechanisms are involved: increased availability of precursors for lipid synthesis, increased biosynthesis of lipid, decreased lipid breakdown, decreased secretion of lipid from liver, and enhanced uptake of lipid from circulation [1]. A deficiency in dietary lipotropic factors such as choline and methionine can also produce a fatty liver in growing rats [2] and, to a lesser extent, in baboons [3]. In rat liver, the effects of ethanol intake also include decreased betaine and increased N^5-methyltetrahydrofolate levels, impaired methionine synthetase, and enhanced betaine:homocysteine methyltransferase activities, resulting in an increased utilization of betaine [4]. Supplementation of betaine in the diet has been shown to prevent the ethanol-induced increase in fat accumulation [5].

Aldehyde dehydrogenases (EC 1.2.1.3) in the presence of NAD catalyze irreversible dehydrogenation of aldehydes to the corresponding acids. The enzymes have broad aldehyde substrate specificity, which includes a large variety of aldehydes with different structures. The substrates usually include acetaldehyde, even though in some cases it is utilized at a high K_m value. More recently, it was shown that substrates such as betaine aldehyde, γ-aminobutyraldehyde, and retinaldehyde are also metabolized by aldehyde dehydrogenase, rather than by specific enzymes previously believed to perform this function [6–10]. The enzyme investigated in this study catalyzes dehydrogenation of aliphatic and aromatic aldehydes, amino aldehydes, and betaine aldehyde. Its broad substrate specificity and low K_m for acetaldehyde were observed when it was first purified to homogeneity from human liver cytoplasm [7]. Since that time, the enzyme has been cloned and sequenced [11, 12] and investigated by x-ray crystallography [13]. The enzyme is coded for by the *aldh9* gene, but most of its properties are described in the literature as human E3 enzyme (a third human aldehyde dehydrogenase with low K_m for acetaldehyde, capable of metabolizing ethanol-derived acetaldehyde at low concentrations). Rothschild and Guzman Barron [14], who first partially purified this enzyme from rat liver, reported that the enzyme was inhibited by acetaldehyde. Therefore, it became interesting to investigate the interaction of betaine biosynthesis with acetaldehyde metabolism, since enzyme properties suggest that hepatic

‡ Corresponding author: Dr. Regina Pietruszko, Center of Alcohol Studies, 607 Allison Road, Piscataway, NJ 08854-8001. Tel. (732) 445-3643; FAX (732) 445-3500; E-mail: pietrusz@rci.rutgers.edu

Received 9 March 2000; accepted 9 May 2000.

betaine aldehyde and acetaldehyde may compete for the same enzyme in an ethanol-metabolizing subject. If such competition occurs, the accumulation of hepatic betaine aldehyde due to the inhibition of its metabolism by acetaldehyde is predicted. This inhibition also may contribute to the now well established reduced betaine levels [4] and to hepatic lipid accumulation induced by ethanol ingestion. The measurement of enzyme constants and of the hepatic levels of betaine aldehyde, betaine, and choline in ethanol-fed rats in comparison with those from the control group, employing FAB–MS* methodology, which was developed originally for analysis of quaternary ammonium compounds in plants, is presented in this paper. Betaine aldehyde levels have not been determined previously in mammalian liver due to methodological difficulties; this investigation is, therefore, the first report on betaine aldehyde levels in mammalian liver.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats were obtained from Harlan Sprague–Dawley. Lieber–DeCarli liquid diet was purchased from Dyets Inc. A Partisil 5 silica column was obtained from Supelco, and AG 50W-X8 (200–400 mesh, hydrogen form) from Bio-Rad. [*Methyl*-^{14}C]choline chloride (389 μCi/mg) was obtained from Amersham. Deuterium-labeled choline chloride (trimethyl-d_9) was purchased from Cambridge Isotope Laboratories. Deuterium-labeled glycine betaine (trimethyl-d_9) and betaine aldehyde (methyl-d_3) chloride were gifts from Dr. Andrew D. Hanson, University of Florida at Gainsville. Yeast alcohol dehydrogenase was obtained from Boehringer Mannheim. Richter drinking tubes were made by the machine shop of the Rutgers Chemistry Department. Microreaction vessels and PTFE/silicone septa were purchased from Supelco. All other chemicals were reagent grade.

Preparation and Analysis of [methyl-^{14}C]Betaine and of [methyl-^{14}C]Betaine Aldehyde

Two procedures were used to prepare [*methyl*-^{14}C]betaine:

(A) CHEMICAL PROCEDURE. Prior to reaction, [*methyl*-^{14}C]choline (2 μL, 0.4 μCi, 7.3 nmol) was freeze-dried to remove residual ethanol. Aqueous solution (200 μL) containing 1 mg $KMnO_4$, 2 μL H_2SO_4 and [*methyl*-^{14}C]choline was heated in a boiling water bath for 1 hr and 40 min. The reaction mixture was extracted with 2 vol. of chloroform and 1 vol. of methanol.

(B) ENZYMIC PROCEDURE. Tris–HCl buffer (0.1 mL, 0.1 M, pH 8.0), containing 5 U of choline oxidase (*Alcaligenes* species, Sigma; 1 unit converting 1 μmol choline to betaine per min at pH 8.0 at 37°), 1000 U of catalase (bovine liver, Sigma; 1 unit converting 1 μmol H_2O_2 to H_2O at pH 7.0 at 25°), and 3.6 μL [*methyl*-^{14}C]choline (0.72 μCi, 13.1 nmol), was incubated at 37° for 24 hr. The enzyme was inactivated by the addition of 1/10 vol. of 1.2 M HCl, and then the reaction mixture was extracted with 2 vol. of chloroform and 1 vol. of methanol. Aliquots of the upper phase were taken for analysis.

[*Methyl*-^{14}C]betaine aldehyde was prepared as previously described [15], with some modifications in quantity and timing. The reaction medium (100 μL) contained glycine (40 mM, pH 8.5), [*methyl*-^{14}C]choline (0.715 μCi, 13 nmol), and rat liver mitochondria. Rat mitochondrial protein (60 μg) was added three times in succession, with each addition followed by a 12-hr incubation at 37°. The reaction was terminated by adding 1/10 vol. of 1.2 M HCl. The reaction products were extracted with 1 vol. of methanol and 2 vol. of chloroform. The upper phase was collected for further analysis and separation. One volume of the upper methanol–water phase was mixed with 2 vol. of methanol and analyzed on a Partisil 5 silica column (4.6 x 250 mm). After sample application, the column was eluted and washed according to Zhang *et al.* [15]. Aliquots of 1.5-mL fractions were counted in a liquid scintillation counter (1219 RACKBETA, LKB) in 7 mL Ecoscint (National Diagnostics). Each peak was collected and lyophilized for further experiments. Sodium borohydride reduction, used for identification of betaine aldehyde, was carried out according to Zhang *et al.* [15].

For TLC of unlabeled betaine aldehyde, [*methyl*-^{14}C]choline, and the putative [*methyl*-^{14}C]betaine aldehyde, 4 × 8 cm TLC plates (SIL G, Alltech Associates) were used. The plates were developed with a solvent mixture containing chloroform, methanol, and 0.1 M HCl (65:30:4, by vol.) [15]. The unlabeled aldehyde was visualized by spraying with 2,4-dinitrophenylhydrazine reagent. The radioactive spot was determined by counting the radioactivity of thirty-two 2-mm-wide slices of each radioactive compound-containing lane that covered from the origin to the solvent front.

Rat Feeding

Fourteen male Sprague–Dawley rats, 145–185 g, were housed individually in a temperature and light-controlled room (18–22°; 12-hr light–dark cycle). Rats were paired by their body weight. The ethanol-containing Lieber–DeCarli [16] liquid diet was given *ad lib.* to one member of the animal pair, while its control was given an isocaloric diet in the amount consumed by the ethanol-fed rat the previous day on a day-to-day basis. The control diet was divided into three equal parts and given every 8 hr in order for the control rats to consume at a rate similar to that of the ethanol rats. The drinking tubes were always found empty before the control diet was refilled. Rats were kept on this regimen for 4 weeks before decapitation. Livers were freeze-clamped at −77° in a dry ice–acetone system and then stored at −70°.

* *Abbreviation*: FAB–MS, fast atom bombardment–mass spectroscopy.

Blood Ethanol Analysis and Hepatic Triglyceride Levels

Blood taken by heparinized capillary tubes was deproteinized in 10% $HClO_4$, and ethanol was measured using yeast alcohol dehydrogenase in the presence of semicarbazide [17]. Total lipids were extracted from the liver by the chloroform–methanol procedure of Folch *et al.* [18]. Saponification and quantitation of triglycerides in the extract were done as directed by Sigma, employing the Sigma diagnostic kit No. 320-UV and triolein standard.

Preparation of Rat Liver Mitochondria and Isoelectric Focusing

Livers from Sprague–Dawley rats were used. Mitochondria were isolated as described by Hogeboom [19]. The mitochondrial pellet was washed three times by resuspending in 9 mL of 0.25 M sucrose to 1 g of mitochondrial pellet. Isoelectric focusing was done using rat liver mitochondrial matrix and cytoplasm, employing a Pharmacia Multiphore II Apparatus and isoelectric focusing standards for the pH range of 3–10. The gels were developed with 1 mM betaine aldehyde in 0.1 M sodium phosphate, pH 7.4, in the presence of NAD (20 mg/30 mL), nitroblue tetrazolium (20 mg/30 mL), and phenazine methosulfate (2 mg/30 mL).

Purification of Enzymes and Calculation of Kinetic Effects

The rat and human enzymes were purified from human and rat livers as previously described [7, 20]. Kinetic constants were determined (see footnote to Table 1) and calculated by using the statistical method of Cleland [22]. The effect of acetaldehyde on the rate ($V_{a'}$) of betaine aldehyde metabolism via betaine aldehyde dehydrogenase was calculated by using the following equation [23]:

$$V_{a'} = \frac{V_a}{1 + \frac{K_a}{a}\left(1 + \frac{b}{K_b}\right)}$$

where V_a is the maximal velocity with betaine aldehyde, K_a and K_b are Michaelis constants, and a and b are concentrations of betaine aldehyde and acetaldehyde, respectively. Protein was determined by the microbiuret procedure [24].

Hepatic Betaine and Betaine Aldehyde Levels

Rat livers were cut on dry ice and weighed. Each 0.1 g of liver received 0.6 nmol d_3-betaine aldehyde and 50 nmol d_9-glycine betaine as FAB–MS internal standards and then was homogenized in 0.9 mL of 1 N HCl on ice. The homogenate was centrifuged at 18,000 *g* for 60 min. To trace the compounds during chromatography, 2000 dpm equivalents of [*methyl*-^{14}C]glycine betaine and [*methyl*-^{14}C]betaine aldehyde were added to each 2-mL supernatant, which was then applied to a 1.3-mL AG 50W-X8 (H^+) column (55 × 5.5 mm). The column was eluted with 8 mL of 2.5 N HCl. The radioactive fractions were collected and freeze-dried. For choline and betaine aldehyde, the radioactive fractions (all fractions 1.3 mL) occurred from fractions 4 to 7 and peaked around fraction 5, while the radioactivity for betaine ranged from fractions 3 to 6 and peaked around fraction 4. When the supernatant of rat liver homogenate was fractionated, turbidity of the same appearance as that of the loaded sample constantly occurred in fractions 2 and 3 and mostly gathered in fraction 2. Thus, these two fractions were not included for the FAB–MS experiments to keep the resulting mass spectrum as simple as possible. Betaine and betaine aldehyde were derivatized to form dibutanol acetal derivative for FAB–MS analysis as done by Rhodes *et al.* [25].

Hepatic Choline Levels

Each 0.2 g of rat liver received an internal standard of 0.25 nmol d_9-choline chloride and then was homogenized in 1.8 mL of 1 N HCl on ice and centrifuged at 18,000 *g* for 60 min. Two milliliters of supernatant, after receiving 2000 dpm [*methyl*-^{14}C]choline chloride, was applied to an AG 50W-X8 (H^+) column (55 × 5.5 mm), which was eluted with 8 mL of 2.5 N HCl. The radioactive fractions were collected, freeze-dried, and analyzed by FAB–MS.

Mass Spectrometry

A 1-μL aliquot of an aqueous sample solution was mixed on the tip of a metal probe with *ca.* 1 μL glycerol. The probe was then introduced via an airlock into the ion source of a JEOL HX110 double-focusing mass spectrometer (JEOL USA). There, the matrix–sample droplet was bombarded by a high energy (6 KeV) beam of xenon atoms. The acceleration voltage was 10 kV, and the instrument was scanned from *m/z* 50 to 500 in 20 sec.

RESULTS

Metabolism of Choline by Rat Liver Mitochondria

[*Methyl*-^{14}C]choline when applied to silica HPLC produced a radioactive peak at a retention time of 38 min; [*methyl*-^{14}C]betaine, prepared chemically or enzymically, appeared at 27 min. The unlabeled betaine aldehyde was eluted at 23 min from the HPLC column. This was confirmed further by sodium borohydride reduction of radioactive betaine aldehyde, eluting at 23 min, which upon reduction was converted to choline (identified by its elution from the HPLC column at 38 min). The identity of radioactive betaine aldehyde was also confirmed by TLC versus unlabeled betaine aldehyde. When an attempt was made to prepare [*methyl*-^{14}C]betaine aldehyde by incubation of [*methyl*-^{14}C]choline with rat liver mitochondria, three radioactive peaks were visualized upon HPLC separation, at retention times of 23, 27, and 38 min, respectively. These peaks

varied in quantity, depending on the length of incubation time. When the incubation period was brief, only 23- and 38-min peaks were visible. The size of the 27-min peak became larger if the incubation time lasted longer (Fig. 1). Thus, it appeared that the metabolism of choline proceeded all the way to betaine in mitochondria. This was unexpected because our previous experiments [7, 26] indicated that betaine aldehyde dehydrogenase is a cytoplasmic enzyme.

Assay and Isoelectric Focusing of Rat Liver Cytoplasm and of Mitochondrial Matrix

When mitochondrial betaine aldehyde dehydrogenase was assayed, it was found to constitute only 5% of total betaine aldehyde dehydrogenase activity; 95% was in the cytoplasm. An attempt was made to visualize mitochondrial betaine aldehyde dehydrogenase by isoelectric focusing of rat liver mitochondrial matrix. The results are shown in Fig. 2, where rat cytoplasmic and mitochondrial betaine aldehyde dehydrogenases are compared in male rats. The mitochondrial enzyme appeared to have the same pI as the more cathodal component of the cytoplasmic enzyme.

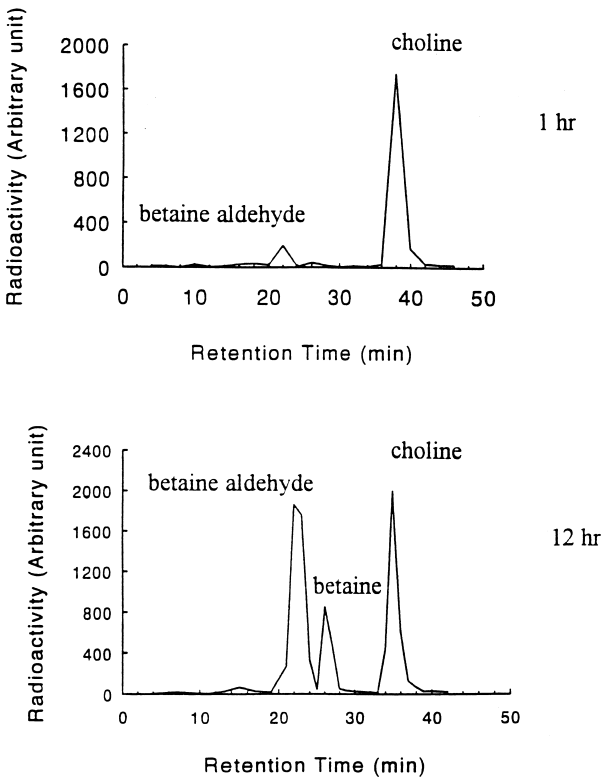

FIG. 1. Incubation of rat liver mitochondria with [*methyl*-^{14}C]choline: formation of betaine aldehyde and betaine. Upper panel: 1-hr incubation; lower panel: 12-hr incubation. The reaction medium (100 μL) contained glycine (40 mM, pH 8.5), [*methyl*-^{14}C]choline (0.715 μCi, 13 nmol), and rat liver mitochondria (60 μg protein).

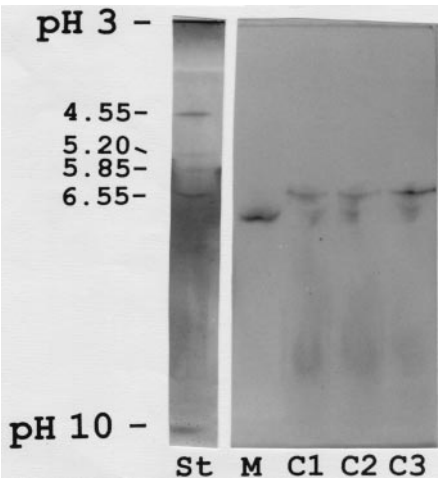

FIG. 2. Isoelectric focusing of betaine aldehyde dehydrogenase from rat liver cytoplasm and mitochondrial matrix. The staining solution used was: 0.1 M Tris–HCl buffer, pH 8.5, containing NAD (20 mg/30 mL), nitroblue tetrazolium (20 mg/30 mL), phenazine methosulfate (2 mg/30 mL), and 1 mM betaine aldehyde. St = pI standards visualized with Coomassie Brilliant Blue (pI values of standards are shown in the left-hand column); M = mitochondrial matrix; C1, C2, and C3 = cytoplasmic fractions.

Kinetic Constants for Betaine Aldehyde and Acetaldehyde

Kinetic constants for human and rat liver mitochondrial betaine aldehyde dehydrogenases are shown in Table 1. Both human and rat enzymes were active with betaine aldehyde and acetaldehyde; in both cases, the K_m and V_{max} values for acetaldehyde were considerably lower than those for betaine aldehyde. And, in both cases, acetaldehyde was a worse substrate than betaine aldehyde, as reflected by the V_{max}/K_m ratios differing by almost an order of magnitude (Table 1). In fact, the kinetic constant for the rat enzyme could not be determined by a direct procedure; it was determined by inhibition kinetics, measuring the effect of

TABLE 1. Kinetic properties of purified betaine aldehyde dehydrogenase from human and rat livers with betaine aldehyde and acetaldehyde as substrates

Varied substrate	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m
Human enzyme			
Betaine aldehyde	260 ± 45.8	7.9 ± 0.38	0.03
Acetaldehyde	52 ± 8.7	0.23 ± 0.002	0.004
Rat enzyme			
Betaine aldehyde	118 ± 9.5	3.4 ± 0.13	0.029
Acetaldehyde	14 ± 0.8	0.064	0.004

Kinetic constants (K_m and V_{max}) were measured in 0.1 M sodium phosphate buffer, pH 7.4, containing 500 μM NAD (constant substrate) and 1 mM EDTA at 25°. Values (mean ± SEM, N = 3) were determined by the Lineweaver-Burk procedure [21], except for acetaldehyde with the rat enzyme where inhibition kinetics versus varied betaine aldehyde was used to obtain the K_m while velocity was determined at 126 μM acetaldehyde and adjusted to the V_{max} mathematically. At pH 9.0 in 0.1 M sodium pyrophosphate buffer, the K_m for the human enzyme was 380 μM and the V_{max} was 14.5 μmol/min/mg.

TABLE 2. Calculations of the effect of 100 μM acetaldehyde on betaine adlehyde metabolism by human and rat enzymes

Betaine aldehyde (μM)	Reaction rate at acetaldehyde concentration		Activity with betaine aldehyde (%)
	0	100 μM	
Human enzyme			
100	2.19	0.92	42
50	1.27	0.49	39
5	0.149	0.05	34
Rat enzyme			
100	1.56	0.32	21
50	1.01	0.15	14.9
5.5*	0.151	0.0194	12.8

The rates of the enzyme-catalyzed reaction are expressed as μmol NADH formed/min/mg enzyme at 25° at which measurements were made. Kinetic constants (Table 1) were used for the calculation, employing the equation described in Materials and Methods.

*Average concentration of betaine aldehyde in rat liver (see Table 4).

acetaldehyde on betaine aldehyde metabolism [27]. Kinetic constants of the rat and human enzymes (Table 1) then were employed for theoretical calculations to see what effect acetaldehyde would have on betaine aldehyde dehydrogenase activity (Table 2). The concentration of acetaldehyde used was that reported to be present in the liver during alcohol metabolism [28]. It can be seen (Table 2) that acetaldehyde is a good inhibitor of betaine aldehyde dehydrogenase activity.

Characterization of Rats

There were no significant differences in the initial and final body weights between the control and the ethanol-fed groups (Table 3), as may be expected by a pairwise isocaloric regimen. However, the ethanol group gained more liver weight than the control group. The ethanol diet also produced a significant increase in both blood ethanol and liver triglyceride levels (Table 3), indicating the occurrence of ethanol absorption and fat accumulation in the liver, respectively. No significant increase in the liver protein level was observed, suggesting that hepatomegaly

TABLE 3. Characterization of ethanol-fed rats

Characterization	Controls	Ethanol-fed
Initial body weight (g)	167.8 ± 4.5	162.2 ± 4.0*
Final body weight (f)	225.8 ± 13.6	209.0 ± 14.7*
Liver weight (g/100 g body weight)	3.2 ± 0.08	4.2 ± 0.2*
Blood ethanol† (mg/mL blood)	0.010 ± 0.005	1.76 ± 0.29‡
Triglyceride (μmol/g liver)	8.79 ± 1.07	29.44 ± 3.55‡
Protein (mg/g liver)	96.8 ± 4.1	104.1 ± 4.0*

Values are means ± SEM for seven male controls and seven male alcohol-fed rats. Statistical comparison performed using Student's *t*-test: $P < 0.05$ was considered significant.

*Not significant.

†Blood samples were taken pairwise occasionally, with a total of 18 samples per group.

‡$P < 0.001$.

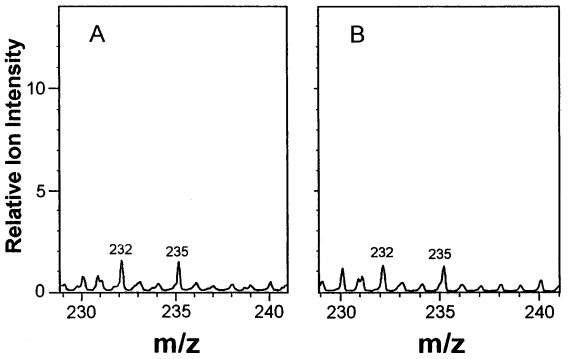

FIG. 3. FAB–MS spectra for betaine aldehyde fractions of the control rat liver (A) and the ethanol-fed rat liver (B). The matrix was an aqueous solution of 50% (v/v) glycerol. Note the relative sizes of the peaks at *m/z* 235 (M^+ for d_3-betaine aldehyde di-*n*-butylacetal) and *m/z* 232 (M^+ for the endogenous betaine aldehyde di-*n*-butylacetal).

was not produced by the overall increase of protein synthesis.

Betaine Aldehyde, Betaine, and Choline Levels

Radiolabeled choline, betaine aldehyde, and betaine were employed to trace endogenous compounds of the same kind during AG 50W-X8 (H^+) chromatography. When individual radiolabeled compounds were tested, 90, 90, and 75% recovery were found for choline, betaine aldehyde, and betaine, respectively. Endogenous betaine aldehyde was quantified according to the relative abundance of the ions at *m/z* 232 and 235 (the M^+ ions of d_0- and d_3-betaine aldehyde di-*n*-butylacetal, respectively, Fig. 3). The endogenous glycine betaine was quantified according to the relative abundance of the ions at *m/z* 174 and 183 (representing M^+ for the *n*-butyl esters of d_0- and d_9-glycine betaine, respectively, Fig. 4). As shown in Table 4, whereas there was a significant decrease in betaine levels in the ethanol group, no significant difference in betaine aldehyde levels was found between the two groups. The average betaine levels were 0.77 nmol/g liver for the ethanol group and 1.45 nmol/g liver for the control group. The average betaine aldehyde level was 5.5 nmol/g liver for both groups. Since betaine aldehyde levels were not altered during ethanol ingestion, hepatic choline was also measured to see whether a feedback had occurred at the choline levels. The endogenous (d_0) choline was quantified according to the relative abundance of ions at *m/z* 104 (M^+ of d_0-choline) and *m/z* 113 (M^+ of d_9-choline) (Fig. 5). The choline level was found to average 0.23 nmol/g liver, without a significant difference between the two groups (Table 4).

DISCUSSION

When the kinetic properties of human aldehyde dehydrogenases E1, E2, and E3 were investigated [7, 29], it was apparent that all enzymes were of broad substrate specificity, which included acetaldehyde as a substrate at low

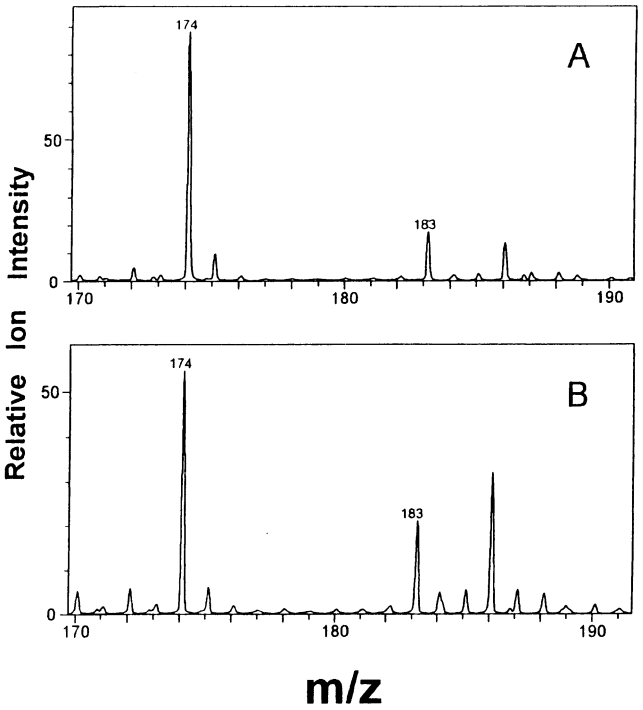

FIG. 4. FAB–MS spectra for the betaine fractions of the control rat liver (A) and the ethanol-fed rat liver (B). The matrix was an aqueous solution of 50% (v/v) glycerol. Note the relative sizes of the peaks at *m/z* 183 (M^+ for d_9-betaine *n*-butyl ester) and *m/z* 174 (M^+ for the endogenous betaine *n*-butyl ester).

micromolar concentrations. With the E3 isozyme, both the K_m and the V_{max} values for acetaldehyde were considerably lower than those for betaine aldehyde, suggesting that acetaldehyde may inhibit betaine aldehyde metabolism. In fact, such inhibition was readily demonstrated in our laboratory [27] and by other investigators [14]. Theoretical considerations (Table 2) for three concentrations of betaine aldehyde being metabolized in the absence and presence of 100 μM acetaldehyde (the level in rat liver during ethanol perfusion [28]) showed that betaine aldehyde metabolism would be inhibited by 100 μM acetaldehyde generated in the liver during ethanol metabolism and would contribute to the decrease in the hepatic betaine levels during alcohol ingestion [4, 5]. For this reason rats were subjected to a pair-feeding technique employing the liquid diet of Lieber *et al.* [16]. Three indicators for alcohol metabolism were used in this investigation: blood ethanol

TABLE 4. Betaine aldehyde, betaine, and choline levels in liver

Group	Betaine aldehyde (nmol/g liver)	Betaine (nmol/g liver)	Choline (nmol/g liver)
Control	5.37 ± 0.46	1.45 ± 0.2	0.256 ± 0.106
Ethanol	5.71 ± 0.48*	0.77 ± 0.12†	0.212 ± 0.065*

Values are means ± SEM for seven rats per group. Statistical comparison was performed using Student's *t*-test; $P < 0.05$ was considered significant.

*Not significant.

†$P < 0.05$.

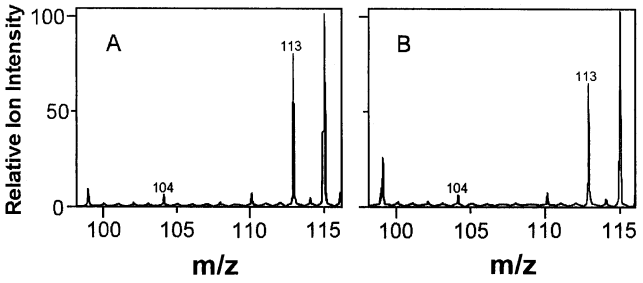

FIG. 5. FAB–MS spectra for the choline fractions of the control rat liver (A) and the ethanol-fed rat liver (B). The matrix was an aqueous solution of 50% (v/v) glycerol. Note the relative sizes of the peaks at *m/z* 113 (M^+ for d_9-choline) and *m/z* 104 (M^+ for the endogenous choline).

levels, liver weights, and hepatic triglyceride levels. All three were higher in the ethanol group than in the control (Table 3), showing that the rats of the ethanol group had consumed alcohol from the alcohol–liquid diet to the extent of clinical relevance. The pair-feeding technique employed in this investigation should eliminate the possibility of nutritional difference between the two groups. This technique makes possible the observation of changes in several tissue–organ systems, among which betaine and methionine metabolism in the liver has been studied systematically by Barak *et al.* [30].

The betaine aldehyde concentration in mammalian liver has not been determined previously. Hise and Mansbach [31] attempted to measure the endogenous betaine aldehyde in liver homogenate, but failed to detect it within the detection limit of their method, 25 nmol/g liver. FAB–MS methodology has been developed for determining quaternary ammonium metabolites in higher plants [25]. This method takes advantage of the permanent charge on the quaternary ammonium moiety of these compounds. The zwitterionic acid can be esterified to create a net positive species that simply requires transfer to the gas phase for mass analysis. The ionization efficiency, and therefore the sensitivity, can be affected by the hydrophobic aliphatic chain portions of the solute. When the length of the carbon chain of the ester group was increased, signal intensity per nanomole of betaine ester was also increased markedly [25]. Both the *n*-butyl and *n*-propyl esters of glycine betaine appeared highly suitable for quantitative application, with the lower limits of detection at *ca.* 0.05 nmol/μL glycerol matrix. In the same reaction mixture where the acid was esterified, betaine aldehyde was converted to di-alcohol acetal derivatives, which also exhibited strong FAB–MS signals. The lower limit of detection above glycerol background for the di-*n*-butylacetal derivative of betaine aldehyde reached 5 pmol/μL glycerol matrix [25]. As shown in Table 4, no significant accumulation of hepatic betaine aldehyde was observed in the ethanol group, arguing against the prediction based on the kinetic properties of betaine aldehyde dehydrogenase, which predicts 58–79% inhibition of betaine aldehyde dehydrogenase metabolism in the

presence of 100 μM acetaldehyde. The level of betaine aldehyde in both groups was 5.5 nmol/g liver.

It is important to note that the *n*-butyl ester of valine gives a molecular ion of identical mass to that of betaine. The preliminary purification of betaine on an AG 50W-X8 (H^+) column does not guarantee the removal of valine. To remove valine and other amino acids, an additional ion exchange column (Dowex-1-OH^-) is necessary [25]. However, this step may decrease the overall yield of compounds of interest. It is a matter of compromise between the cleanness of the sample and the yield of the compounds to be quantitated. It has been found that chronic ethanol feeding does not cause changes in the concentrations of amino acids when ethanol-treated and control rats are compared [32]. The valine concentration has been found to be *ca.* 200 nmol/g liver in both ethanol-treated and control groups of rats [33]. Therefore, the hepatic betaine level was estimated by subtracting the valine concentration. The hepatic betaine level decreased to approximately half of that in the control group, confirming previous results [5], which employed the spectrophotometric method. The mean values of the betaine levels found for the control and ethanol groups were comparable to those obtained using a spectrometric assay (1.18 and 0.50 μmol/g liver for control and ethanol groups, respectively; see Fig. 2 in Ref. 5). This result favors an enhanced consumption rather than insufficient synthesis of betaine as the cause of reduced hepatic betaine levels.

Unlike betaine aldehyde, whose metabolism is restricted to the choline–betaine pathway with betaine aldehyde as an intermediate, choline serves as a precursor of three metabolic pathways: phosphorylation, acetylation (in cholinergic neurons), and oxidation (choline → betaine). The phosphorylation rate has been found to become higher upon ethanol exposure [34], whereas the uptake of choline does not appear to change in the ethanol-perfused liver [35]. However, the rate of change of oxidation in the choline–betaine pathway has not been studied. A measurement of choline levels in the ethanol and control groups, thus, was carried out. Although no significant difference in choline levels was found between the two groups, the concentrations obtained (*ca.* 0.23 μM, Table 4) were too low to be tenable in comparison to those measured by other procedures (60 μM [36]; *ca.* 88 μM [31]). Since an underestimate of the d_9-choline standard would result in an underestimate of the endogenous choline levels, the concentration of the d_9-choline standard was checked by FAB–MS. When the d_9-choline standard used for FAB–MS was quantified against a known amount of unlabeled choline, the concentration of the standard was found to be correct (data not shown). Therefore, a possible problem with the d_9-choline standard may be ruled out. A comparison of various procedures showed that the components of solvent used for extraction, the length of extraction time, and multiple steps of extraction may be essential for obtaining free choline levels from the tissue. Thus, it is possible that the extraction method employed here resulted in choline levels lower than those determined by other investigators.

The effects of ethanol metabolism on the metabolism of serotonin, norepinephrine, and dopamine [37–39] have been observed in the liver. Similar to betaine, the levels of the corresponding acids of the above-mentioned monoamine metabolites are reduced in the ethanol-treated groups. This result is probably due to an enhancement of the reductive pathway via alcohol dehydrogenase, since the corresponding alcohols were increased and the aldehydes were decreased [40]. However, the same mechanism cannot apply to betaine aldehyde, because choline is not a substrate for alcohol dehydrogenase. In mammalian liver, choline is converted to betaine aldehyde by choline dehydrogenase, which is present in the inner mitochondrial membrane [15]. Although *in vitro* assays of choline dehydrogenase have employed phenazine methosulfate or dichloroindophenol as electron acceptors [41], its endogenous cofactor has not been identified fully. Whether the reaction catalyzed by choline dehydrogenase is reversible in the presence of the reduced cofactor remains an open question. In the liver, aldehyde reductase (EC 1.1.1.2) is responsible for reducing a broad range of aldehydes, including isocorticosteroids, aromatic aldehydes, sugar aldehydes, and aliphatic aldehydes. Hence, liver homogenate was also tested for aldehyde reductase activity. No decrease of NADPH (500 μM) or NADH (500 μM) absorbance was detected in the presence of added betaine aldehyde (1 mM) (data not shown), indicating that aldehyde reductase may not play a role in betaine aldehyde reduction.

Although the overall hepatic concentration of acetaldehyde was found to be 60–250 μM in ethanol-perfused rat livers [28], subcellular compartmentations and spatial organizations need to be taken into account in the estimation of the acetaldehyde concentration that is actually experienced by betaine aldehyde dehydrogenase. Interestingly, immunohistochemical studies on liver biopsies from alcoholic patients have shown that the acetaldehyde–protein adducts occurred in rough endoplasmic reticulum, in some peroxisomes, and in the cytosol of the hepatocyte, but none were detected in mitochondria [42]. These three locations coincided with those where acetaldehyde is produced from ethanol. The fact that mitochondria are the principal site of acetaldehyde oxidation [43, 44] may also help minimize the mitochondrial level of acetaldehyde if the mitochondrial influx rate of acetaldehyde is lower than the mitochondrial oxidation rate of acetaldehyde. Thus, it appears likely that *in vivo* dehydrogenation of betaine aldehyde, which is produced from choline by choline dehydrogenase in mitochondria, may be carried out exclusively in mitochondria where some of betaine aldehyde dehydrogenase is localized [27, 45].

References

1. Lieber CS, Ethanol and lipid disorders, including fatty liver, hyperlipemia, and atherosclerosis. In: *Medical and Nutritional*

Complications of Alcoholism: Mechanism and Management (Ed. Lieber CS), pp. 91–126. Plenum Press, New York, 1992.
2. Best CH, Hartroft WS, Lucas CC and Ridout JH, Liver damage produced by feeding alcohol or sugar and its prevention by choline. *Br Med J* **2:** 1001–1006, 1949.
3. Hoffbauer FW and Zaki FG, Choline deficiency in baboon and rat compared. *Arch Pathol* **79:** 364–369, 1965.
4. Barak AJ, Beckenhauer HC, Tuma DJ and Donohue TM Jr, Adaptive increase in betaine-homocysteine methyltransferase activity maintaining hepatic S-adenosylmethionine level in ethanol treated rats. *IRCS Med Sci* **12:** 866–867, 1984.
5. Barak AJ, Beckenhauer HC, Junnila M and Tuma DJ, Dietary betaine promotes generation of hepatic *S*-adenosylmethionine and protects the liver from ethanol-induced fatty infiltration. *Alcohol Clin Exp Res* **17:** 552–555, 1993.
6. Chern M-K and Pietruszko R, Human aldehyde dehydrogenase E3 isozyme is a betaine aldehyde dehydrogenase. *Biochem Biophys Res Commun* **213:** 561–568, 1995.
7. Kurys GL, Ambroziak W and Pietruszko R, Human aldehyde dehydrogenase. Purification and characterization of a third isozyme with low K_m for γ-aminobutyraldehyde. *J Biol Chem* **264:** 4715–4721, 1989.
8. Lee M-O, Manthey CL and Sladek NE, Identification of mouse liver aldehyde dehydrogenases that catalyze the oxidation of retinaldehyde to retinoic acid. *Biochem Pharmacol* **42:** 1279–1285, 1991.
9. Yoshida A, Hsu LC and Dave V, Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase. *Enzyme* **46:** 239–244, 1992.
10. Ambroziak W, Izaguirre G and Pietruszko R, Metabolism of retinaldehyde and other aldehydes in soluble extracts of human liver and kidney. *J Biol Chem* **274:** 33366–33373, 1999.
11. Kurys G, Shah PC, Kikonyogo A, Reed D, Ambroziak W and Pietruszko R, Human aldehyde dehydrogenase. cDNA cloning and primary structure of the enzyme that catalyzes dehydrogenation of 4-aminobutyraldehyde. *Eur J Biochem* **218:** 311–320, 1993.
12. Lin SW, Chen JC, Hsu LC, Hsieh C-L and Yoshida A, Human γ-aminobutyraldehyde dehydrogenase (ALDH9), cDNA sequence, genomic organization, polymorphism, chromosomal localization, and tissue expression. *Genomics* **34:** 376–380, 1996.
13. Johansson K, El-Ahmad M, Ramaswamy S, Hjelmqvist L, Jornvall H and Eklund H, Structure of betaine aldehyde dehydrogenase at 2.1 Å resolution. *Protein Sci* **7:** 2106–2117, 1998.
14. Rothschild HA and Guzman Barron ES, The oxidation of betaine aldehyde by betaine aldehyde dehydrogenase. *J Biol Chem* **209:** 511–523, 1954.
15. Zhang J, Blusztain JK and Zeisel SH, Measurement of the formation of betaine aldehyde and betaine in rat liver mitochondria by high pressure liquid chromatography-radioenzymatic assay. *Biochim Biophys Acta* **1117:** 333–339, 1992.
16. Lieber CS, Jones DP and De Carli LM, Liquid diet technique of ethanol administration: 1989 Update. *Alcohol Alcohol* **24:** 197–211, 1989.
17. Jones D, Gerber LP and Drell W, A rapid enzymatic method for estimation of ethanol in body fluids. *Clin Chem* **16:** 402–407, 1970.
18. Folch J, Lees M and Stanley GHS, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226:** 497–509, 1957.
19. Hogeboom GH, Fractionation of cell components of animal tissues. *Methods Enzymol* **1:** 16–19, 1955.
20. Hempel JD, Reed DM and Pietruszko R, Human aldehyde dehydrogenase: Improved purification procedure and comparison of homogeneous isozymes E1 and E2. *Alcohol Clin Exp Res* **6:** 417–425, 1982.
21. Lineweaver H and Burk D, The determination of enzyme dissociation constants. *J Am Chem Soc* **56:** 658–667, 1934.
22. Cleland WW, Statistical analysis of enzyme kinetic data. *Methods Enzymol* **63:** 103–138, 1979 .
23. Dixon M and Webb EC, *The Enzymes*, pp. 72–73. Academic Press, New York, 1979.
24. Goa J, A microbiuret method for protein determination. Determination of total protein in cerebrospinal fluid. *Scand J Clin Invest* **5:** 218–222, 1953.
25. Rhodes D, Rich PJ, Myers AC, Reuter CC and Jamieson G, Determination of betaines by fast atom bombardment mass spectrometry: Identification of glycine-betaine deficient genotypes of *Zea mays*. *Plant Physiol* **84:** 781–788, 1987.
26. Ambroziak W, Kurys G and Pietruszko R, Aldehyde dehydrogenase (EC 1.2.1.3) comparison of subcellular localization of the third isozyme that dehydrogenates 4-aminobutyraldehyde in rat, guinea pig and human liver. *Comp Biochem Physiol B* **100:** 321–327, 1991.
27. Chern M-K and Pietruszko R, Evidence for mitochondrial localization of betaine aldehyde dehydrogenase in rat liver: Purification, characterization and comparison with human cytoplasmic E3 isozyme. *Biochem Cell Biol* **77:** 179–187, 1999.
28. Eriksson CJP, Regulation of acetaldehyde metabolism during ethanol oxidation in perfused rat liver. In: *Biological Aspects of Ethanol* (Ed. Gross MM), pp. 225–236. Plenum Press, New York, 1977.
29. Greenfield NJ and Pietruszko R, Two aldehyde dehydrogenases from human liver. Isolation via affinity chromatography and characterization of the isozymes. *Biochim Biophys Acta* **483:** 35–45, 1977.
30. Barak AJ, Beckenhauer HC and Tuma DJ, Betaine, ethanol, and the liver: A review. *Alcohol* **13:** 395–398, 1996.
31. Hise MK and Mansbach CM II, Determination of intracellular choline levels by an enzymatic assay. *Anal Biochem* **135:** 78–82, 1983.
32. Morland J, Flengsrud R, Prydz H and Svendsen L, Hepatic amino acid levels in rats after long-term ethanol feeding. *Biochem Pharmacol* **28:** 423–427, 1979.
33. Botiglieri T, The effect of storage of rat tissues and human plasma amino acid levels determined by HPLC. *Biomed Chromatogr* **2:** 195–196, 1987.
34. Roberti R, Binaglia L, Michal G, Brunetti M and Porcellati G, The effect of acute ethanol ingestion on *in vitro* metabolism of choline and ethanolamine derivatives in rat liver. *Biochem Pharmacol* **23:** 3289–3298, 1974.
35. Tuma DJ, Barak AJ and Beckenhauer HC, Study on the effect of ethanol on physiological levels of choline in an isolated perfused liver system. *Biochim Biophys Acta* **239:** 81–83, 1971.
36. Haubrich DR and Gerber NH, Choline dehydrogenase: Assay, properties and inhibitors. *Biochem Pharmacol* **30:** 2993–3000, 1981.
37. Davis VE, Brown H, Huff JA and Cashaw JL, The alteration of serotonin metabolism to 5-hydroxytryptophol by ethanol ingestion in man. *J Lab Clin Med* **69:** 132–140, 1967.
38. Davis VE, Brown H, Huff JA and Cashaw J, Ethanol-induced alteration in norepinephrine metabolism in man. *J Lab Clin Med* **69:** 787–799, 1967.
39. Davis VE, Walsh MJ and Yamanaka Y, Augmentation of alkaloid formation from dopamine by alcohol and acetaldehyde *in vitro*. *J Pharmacol Exp Ther* **174:** 401–412, 1970.
40. Eccleston D, Reading WH and Richie IM, 5-Hydroxytryptamine metabolism in brain and liver slices and the effects of alcohol. *J Neurochem* **16:** 274–276, 1969.
41. Rendina G and Singer PT, Studies on choline dehydrogenase. I. Extraction in soluble form, assay, and some properties of the enzyme. *J Biol Chem* **234:** 1605–1610, 1959.

42. Paradis V, Scoazek J-Y, Kolinger M, Holstege A, Moreau A, Feldman G and Bedossa P, Cellular and subcellular localization of acetaldehyde-protein adducts in liver biopsies from alcoholic patients. *J Histochem Cytochem* **44:** 1051–1057, 1996.
43. Havre P, Margolis JM and Abrams MA, Subcellular site of acetaldehyde oxidation in monkey liver. *Biochem Pharmacol* **25:** 2757–2759, 1976.
44. Cao QN, Tu G-C and Weiner H, Mitochondria as the primary site of acetaldehyde metabolism in beef and pig liver slices. *Alcohol Clin Exp Res* **12:** 720–724, 1988.
45. Dragolovich J and Pierce SK, Characterization of partially purified betaine aldehyde dehydrogenase from horseshoe crab (*Limulus polyphenus*) cardiac mitochondria. *J Exp Zool* **270:** 417–425, 1994.