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Studies on the Mechanism of Methanol Poisoning: Purification and Comparison of Rat and Human Liver 10-Formyltetrahydrofolate Dehydrogenase

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SUMMARY

Methanol poisoning in primates and humans is due to formate accumulation as a result of low rates of formate oxidation. This toxicity is not seen in rats, where formate oxidation rates are high. Formate oxidation *in vivo* is dependent on hepatic tetrahydrofolate levels and on the activity of the enzyme 10-formyltetrahydrofolate (10-formyl-H_4folate) dehydrogenase (EC 1.5.1.6). Because hepatic 10-formyl-H_4folate dehydrogenase activity is lower in human liver than in rat liver, studies were performed investigating the properties of this enzyme in rat and human liver. 10-Formyl-H_4folate dehydrogenase was purified to homogeneity from rat and human liver and was found to possess similar subunit molecular weights on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (96,000). N-Terminal amino acid analysis of the pure proteins showed an identical sequence for the first 16 amino acids. Antibodies raised in rabbits against the rat liver enzyme were inhibitory toward the activity of both rat and human liver enzymes and appeared to recognize only the 10-formyl-H_4folate dehydrogenase in cytosolic preparations of rat and human liver. Immunoblots of pure rat and human liver 10-formyl-H_4folate dehydrogenase showed similar staining intensity. It is concluded that rat and human liver 10-formyl-H_4folate dehydrogenase possess very similar properties and that the activity of the enzyme in human liver is lower than that of rat liver, due to a reduced amount of enzyme protein in human liver. This may be an important factor in regulating formate oxidation in humans and may explain, in part, the accumulation of formate and the mechanism of toxicity of methanol in humans.

The metabolism of formate in mammalian tissues is catalyzed by two systems, a catalase peroxidative oxidation to carbon dioxide (1) and a folate-dependent pathway. The folate-dependent pathway relies upon the conversion of formate to 10-formyl-H_4folate, as catalyzed by 10-formyl-H_4folate synthetase (EC 6.3.4.3) (2), followed by oxidation to carbon dioxide through the mediation of 10-formyl-H_4folate dehydrogenase EC 1.5.1.6) (3). Studies from this and other laboratories have demonstrated that the folate-dependent route is the major one in most mammalian species (4–7) and the exclusive one in primates (8). This is of major significance in understanding the mechanism of methanol poisoning, because it has been shown that only monkeys and humans develop formic acidemia after the administration of methanol (9). Formate accumulation in body fluids and tissues largely accounts for the metabolic acidosis and the sequelae such as blindness that are characteristic of this syndrome (10–13). Rats, mice, and other common laboratory animals do not develop the signs of methanol poisoning (10, 14, 15), because they have rates of formate oxidation that are adequate to deal with the formate generated from methanol oxidation.

Recent studies have shown that there is an excellent correlation between concentrations of hepatic H_4folate and the capacity of an animal to oxidize formate. In rats, where it has been possible to reduce hepatic H_4folate levels by folate-deficient diets or by nitrous oxide treatment (4–6), formate oxidation rates are reduced. After methanol administration, these rats become acidotic, with formic acid accumulation in the blood (6). Recently, studies on the hepatic folate in monkeys and humans showed that hepatic H_4folate was markedly lower in monkeys and that hepatic H_4folate and total folate was also low in humans (7, 16). In monkeys, the administration of 5-formyl-H_4folate (folinic acid) to methanol-treated animals partially reverses the acidosis and formate accumulation and also enhanced formate oxidation (17).

Until recently, no studies have been performed to investigate the proximate catalyst for formate oxidation to CO_2, 10-formyl-H_4folate dehydrogenase, in species either sensitive or insensi-

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ABBREVIATIONS: 10-formyl-H_4folate; 10-formyltetrahydrofolate; H_4folate, tetrahydrofolate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

tive to methanol. We have discovered that 10-formyl-H_4folate dehydrogenase activity in human and monkey liver cytosol was only 26 and 37%, respectively, of that in rat liver (7). Case *et al.* (18) have recently purified 10-formyl-H_4folate dehydrogenase from rat liver and have studied certain properties of the rat liver enzyme. The current studies have focused on purifying 10-formyl-H_4folate dehydrogenase to homogeneity from rat and human liver and raising antibodies to these proteins. In addition, NH_2-terminal amino acid analysis has been performed for each protein.

Materials and Methods

Chemicals. 5-Formyl-H_4folate, hexyl-agarose, red 120-agarose, and NADP were purchased from Sigma Chemical Co. (St. Louis, MO), whereas Tris-acryl-DEAE was purchased from IBF Biotechnics (Savage, MD). Complete and incomplete Freund's adjuvant were obtained from Difco (Detroit, MI). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Cooper Biomedical (Malvern, PA). Electrophoresis-grade reagents for SDS-PAGE, Tween 20, and the protein assay kit were obtained from Bio-Rad (Richmond, CA).

Liver sources. Rat livers were purchased from Pel-Freez Biologicals (Rogers, AR) as harvested from Sprague-Dawley rats and were shipped on dry ice. These were stored at −70°.

Human liver tissue was obtained from the organ donor program at The University of Iowa. Livers were obtained from patients who were determined by established procedures to have cerebral death but in whom cardiopulmonary function had been maintained. Donors were almost exclusively victims of traumatic head injuries, and livers from individuals used for this study had no evidence of acute or chronic disease. The livers were harvested by standard retrieval techniques and were perfused with Collins solution. Livers were transported on ice, and sectioned in 30-g blocks, wrapped in aluminum foil, and then submersed in liquid nitrogen. All livers were stored at −70° until used.

Enzyme preparation. Livers were removed from −70° storage and placed in a cooled glass beaker, into which 3 volumes/weight homogenizing buffer were added. Typical liver amount was 30 g. The homogenizing buffer consisted of 25 mM Tris-HCl with 1 mM EDTA and 0.1 mM dithiothreitol, pH 8.5. This buffer was also used throughout all the following procedures. The liver was sectioned into small pieces using scissors and was homogenized using a Potter-Elvehjem homogenizer. All steps were carried out at 4°. The homogenate was centrifuged at 15,000 × *g* for 20 min and the supernatant of that step was collected after filtration through cotton gauze. The preparation was then recentrifuged at 100,000 × *g* for 1 h and filtered through cotton gauze. Thirty milliliters of preparation were then brought to 200 ml with buffer and applied to a hexyl-agarose column (1.5 cm × 20 cm) that was previously equilibrated with buffer. After 200 ml of buffer were applied, a 0 to 0.4 M NaCl gradient in buffer was used to elute the enzyme. Fractions (5.0 ml) containing activity were pooled (40–50 ml) and brought to 150 ml with buffer. This pool was then applied to a Tris-acryl-DEAE column. After application of the enzyme, the column was washed with 4 column volumes of 100 mM NaCl in buffer. Then the activity was eluted with a linear gradient of 0.1 to 0.4 M NaCl in buffer. The fractions containing activity were pooled and applied to a column of red 120-agarose (7 ml). The column was treated with 25 ml each of 0.5 M, 1 M, and 2 M NaCl in buffer and the enzyme was eluted in 2 M NaCl. Fractions with enzyme activity were pooled, concentrated, and desalted using Centricon concentrators with an exclusion molecular weight of 30,000. Usually, 10 ml of enzyme preparation were centrifuged six times at 4000 × *g* for 20 min, and were resuspended in either buffer or distilled water.

Enzyme assays. 10-Formyl-H_4folate dehydrogenase activity was measured according to the method of Kutzbach and Stokstad (3) or Case *et al.* (18). The assay was carried out at room temperature. 10-Formyl-H_4folate hydrolase was measured using the method described by Case *et al.* (18).

Antibody production to rat liver 10-formyl-H_4folate dehydrogenase. To elicit the production of antibodies, each of two male New Zealand white rabbits was treated with 290 μg of homogeneous rat liver 10-formyl-H_4folate dehydrogenase that was suspended in 5 ml of Freund's complete adjuvant. Animals were treated subcutaneously in multiple sites on the back. After 2 weeks, each rabbit received booster injections of 100 μg of protein in incomplete Freund's adjuvant. Preimmune serum was collected before immunization. Four weeks after the initial injections and at 2-week intervals for 3 months, rabbits were bled and serum was prepared. IgG was prepared from both preimmune and immune sera by means of ammonium sulfate precipitation.

Immunoinhibition studies. Rat and human 10-formyl-H_4folate dehydrogenase, as isolated from hexyl-agarose chromatography, were used for immunoinhibition studies. Various concentrations of preimmune and immune IgG were added to a reaction mixture containing enzyme, 10-formyl-H_4folate, and buffer. The reaction was initiated by the addition of NADP.

Immunoblotting and SDS-PAGE. Samples of rat or human 10-formyl-H_4folate were subjected to SDS-PAGE, using the method of Laemmli (19), in 8% acrylamide gels. The gels were normally run at 10 mA/gel for 14–16 hr. Immunoblotting was conducted by the method of Towbin *et al.* (20). Proteins were transferred to nitrocellulose (BA85; Schleicher and Schuell, Keene, NH), using a Hoefer Scientific Instruments (San Francisco, CA) Transphor Apparatus for 2 hr at 1 A. The electrotransfer buffer contained 190 mM glycine, 20 mM Tris·HCl, 20% methanol, and 0.05% SDS, pH 8.3. Tween 20 was used to block the nonspecific binding of IgG to the nitrocellulose. The secondary antibody was horseradish peroxidase-conjugated IgG produced in goats. Antibody concentrations and incubation times are given in figure legends.

NH_2-Terminal amino acid sequence analysis. Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An Applied Biosystems, Inc. (Foster City, CA) Model 470A gas phase sequencer was employed for the degradations (21), using the standard sequencer cycle 03RPTH. The respective phenylthiohydantoin-amino acid derivatives were identified by reverse phase high performance liquid chromatography analysis in an on-line fashion using an Applied Biosystems Model 120A PTH Analyzer fitted with a Brownlee 2.1-mm i.d. PTH-C18 column. Similarities between the obtained sequence and known sequences were investigated using the computer program FASTP (22). Similarity searches were performed against the National Biomedical Research Foundation protein sequence database (23) and the translated GENBANK DNA database (24).

Results

Purification of rat and human liver 10-formyl-H_4folate dehydrogenase. A typical purification procedure and recovery of 10-formyl-H_4folate dehydrogenase from rat and human liver are shown in Tables 1 and 2. New procedures were employed when it was found that previously published methods (18, 25) yielded enzyme preparations that were not homogeneous, as determined by the silver staining technique on SDS-PAGE. The new methods did allow for the preparation of homogeneous protein. This was considered to be of great im-

TABLE 1
Summary of purification of rat liver 10-formyl-H_4folate dehydrogenase

	Volume	Total Activity	Total Protein	Specific Activity	Recovery
	ml	*units*	*mg*	*units/mg*	%
Cytosol	198	5148	733	7	100
Hexyl-agarose	32	1440	24	60	28
Tris-acryl-DEAE	20	800	3.4	235	16
Red 120-agarose	11	110	0.15	733	2

TABLE 2
Summary of purification of human liver 10-formyl-H_4folate dehydrogenase

	Volume	Total Activity	Total Protein	Specific Activity	Recovery
	ml	*units*	*mg*	*units/mg*	%
Cytosol	198	2574	634	4	100
Hexyl-agarose	16	800	29	28	31
Tris-acryl-DEAE	18	396	3.4	116	15
Red 120-agarose	8	80	0.1	800	3

portance because the raising of antibodies against the enzyme was a major goal of this work. Occasionally, a minor contaminant protein was observed from human liver preparations. Separation of 10-formyl-H_4folate synthetase from 10-formyl-H_4folate dehydrogenase was accomplished by hexyl-agarose chromatography. At this step, 5,10-methylene-H_4folate reductase coeluted with 10-formyl-H_4folate dehydrogenase. These proteins were readily separated by Tris-acryl-DEAE ion-exchange chromatography. The 10-formyl-H_4folate dehydrogenase was purified to apparent homogeneity using red 120-agarose dye ligand chromatography. Although similar purification results were obtained for both rat and human liver, the activity of human liver cytosolic 10-formyl-H_4folate dehydrogenase was always lower, as we have reported previously (7) (Table 2). Recoveries of activity from rat and human liver averaged about 3%. In general, the activity of these proteins was quite labile. Rat liver preparations were used within 1 week and human liver preparations were used within 3 days. Purified rat and human 10-formyl-H_4folate dehydrogenases resolved from red 120-agarose were found to be free of 10-formyl-H_4folate hydrolase activity. Silver stains of SDS-PAGE gels of rat and human liver 10-formyl-H_4folate dehydrogenase are shown in Fig. 1. Both rat and human liver preparations appear to be homogeneous and exhibit the same monomeric molecular weight, 96,000. This is similar to the value of 94,000 recently reported by Case *et al.* (18) for rat liver 10-formyl-H_4folate dehydrogenase.

Kinetic studies on rat and human 10-formyl-H_4folate dehydrogenases were carried out using racemic 10-formyl-H_4folate. A convex relationship was observed when Lineweaver-Burk plots were analyzed. This was also observed by Case *et al.* (18). Kinetic constants for the rat liver enzyme for 10-formyl-H_4folate were found to be very similar to those reported by Case *et al.* (18). The K_1 and K_2 values were 2 and 40 μM, respectively. Values for the human liver enzyme were 0.4 and 20 μM for the K_1 and K_2, respectively.

Immunoinhibition and immunoblotting studies. Immunoinhibition studies were carried out using an IgG preparation that was prepared from sera of rabbits inoculated with purified rat liver 10-formyl-H_4folate dehydrogenase. This preparation directly inhibited partially purified rat and human liver 10-formyl-H_4folate dehydrogenase activity and the inhibition was concentration dependent. Data for the rat liver enzyme are shown in Fig. 2. Similar results were obtained for the human liver enzyme (Fig. 3), although a different antibody preparation was used in studies with the human liver enzyme.

Immunoblotting experiments using purified rat and human 10-formyl-H_4folate dehydrogenase and rabbit anti-rat 10-formyl-H_4folate dehydrogenase IgG were performed. Dot-blots in Fig. 4 show that staining intensity is about the same for a series

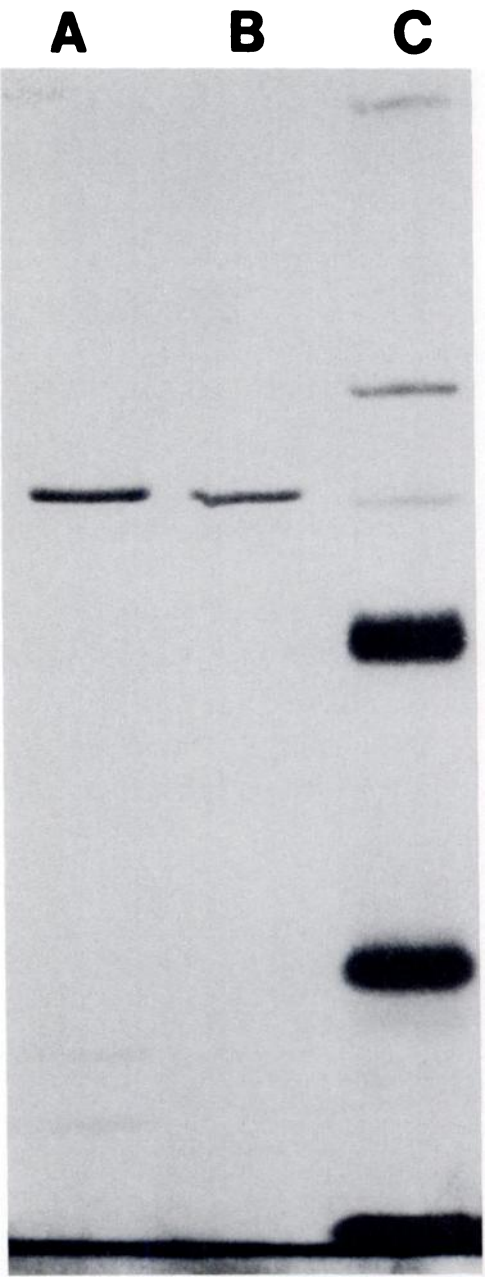

Fig. 1. SDS-PAGE of rat and human liver 10-formyl-H_4folate dehydrogenase obtained from red 120-agarose. *Lanes* show the human enzyme (A) (1 μg of protein), rat enzyme (B) (1 μg of protein), and standards (C) (4 μg), respectively. Commercial molecular weight standards were myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (97,400), bovine serum albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000). The migration was from *top* to *bottom*.

of concentrations of the rat and human liver enzymes (100 to 12.5 ng of protein).

Western blots of purified rat and human liver 10-formyl-H_4folate dehydrogenases and of cytosolic preparations of rat and human liver are shown in Fig. 5. Both rat and human liver 10-formyl-H_4folate dehydrogenase are recognized by rabbit anti-rat 10-formyl-H_4folate dehydrogenase, and only a single stained band is apparent from the cytosolic preparations of rat and human liver. Protein profiles stained by the silver method are also seen in Fig. 5. These data show that rabbit anti-rat 10-formyl-H_4folate dehydrogenase recognizes purified rat and hu-

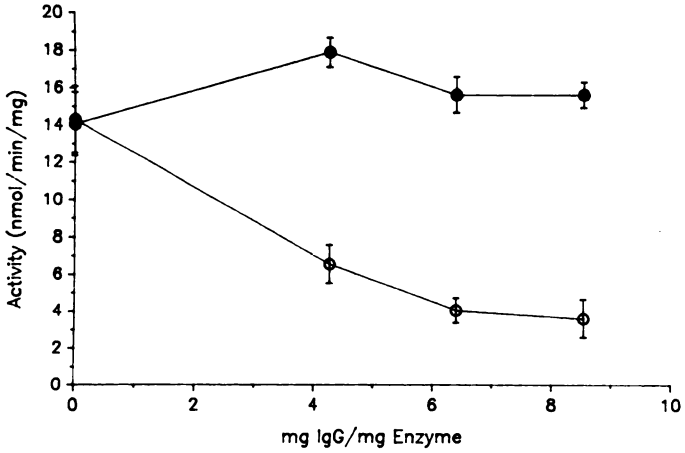

Fig. 2. Immunoinhibition of rat 10-formyl-H_4folate dehydrogenase activity by rabbit anti-rat 10-formyl-H_4folate dehydrogenase IgG. ●, Preimmune IgG; ○, immune IgG. Values are the mean ± standard deviation and assays were performed in triplicate.

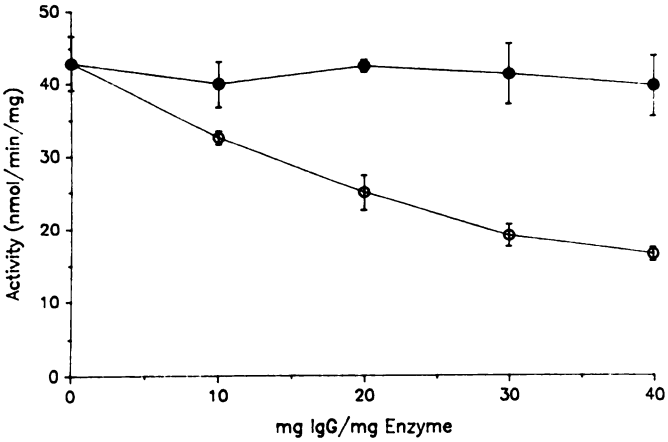

Fig. 3. Immunoinhibition of human 10-formyl-H_4folate dehydrogenase activity by rabbit anti-rat 10-formyl-H_4folate dehydrogenase IgG. ●, Preimmune IgG; ○, immune IgG. Values are mean ± standard deviation and assays were performed in triplicate. The IgG used for these inhibition studies was obtained from a different rabbit than that used in Fig. 2.

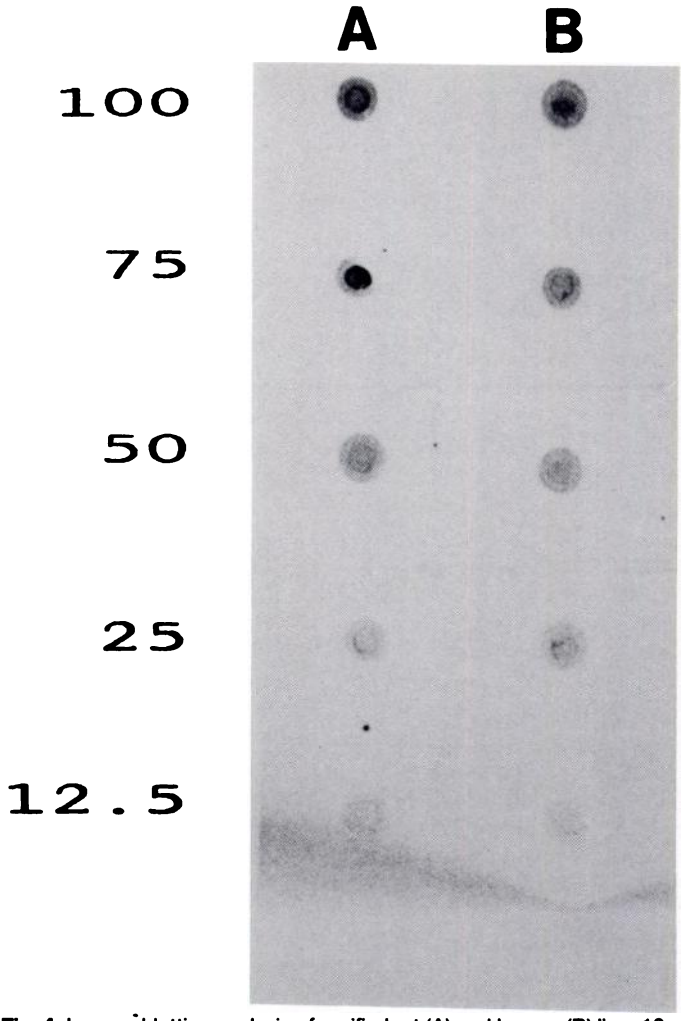

Fig. 4. Immunoblotting analysis of purified rat (A) and human (B) liver 10-formyl-H_4folate dehydrogenase using rabbit anti-rat 10-formyl-H_4folate dehydrogenase IgG. The amount of each enzyme (ng) is indicated at the *left*. The immunoblots were incubated with rabbit antibody (1:250 dilution) overnight, followed by 4 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution), and were developed using 4-chloro-1-naphthol as the peroxidase substrate.

man liver enzymes and that the antibody appears to recognize only the 10-formyl-H_4folate dehydrogenase in both rat and human liver cytosolic fractions.

NH_2-Terminal sequence of rat and human liver 10-formyl-H_4folate dehydrogenases. The sequences of the NH_2-terminus of purified rat and human liver 10-formyl-H_4folate dehydrogenases are shown in Table 3. The sequences appear to be identical for the first 16 amino acids, although a more complete amino acid sequence for the rat liver protein was obtained.

Discussion

The enzyme 10-formyl-H_4folate dehydrogenase is the important catalyst for formate oxidation in both rats and humans and, as such, plays a pivotal role in disposing of formate generated from exogenous methanol exposure. In humans, toxicity can occur from excessive formate levels following methanol ingestion, due to low rates of formate oxidation. Rats are capable of adequately metabolizing formate and usually do not demonstrate adverse effects following methanol administration. The observation that hepatic levels of 10-formyl-H_4folate dehydrogenase in human liver are only about 26% of those observed for rat liver led to the studies reported here. A qualitative comparison of the nature of this enzyme has been made. It would appear from studies presented here that 10-formyl-H_4folate dehydrogenase is quite similar in both rat and human liver. The monomeric molecular weights of the rat and human enzyme are essentially the same (96,000). Also, kinetic studies indicate that the two proteins are similar with respect to their reaction with the folate substrate. The values obtained were similar to those recently reported by Case *et al.* (18). These data suggest that differences in activity observed between the rat and human liver are due to differences in the amount of enzyme present.

The NH_2-terminal amino acid sequence analysis provides further evidence for the similarity of rat and human liver 10-formyl-H_4folate dehydrogenase, where positions 2–16 are identical. Molecular biological work is now in progress to obtain a cDNA with a full length coding sequence for both the rat and human liver enzymes. It is interesting to note that, when the sequence was used in homology searches of the translated

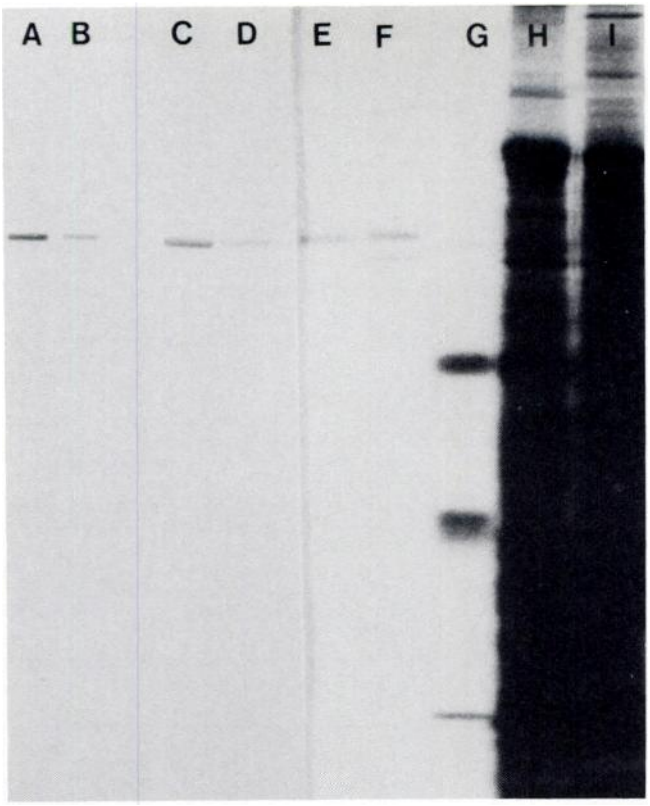

Fig. 5. Western blots of purified rat and human liver 10-formyl-H_4folate dehydrogenases and of rat and human liver cytosolic preparations. *Lanes A* and *B*, approximately 0.5 μg of purified rat and human liver enzyme, respectively. *Lanes C* and *D*, 100 μg of protein from rat and human liver cytosol, respectively. Antibody concentrations and incubation times were identical to those given for Fig. 4. *Lanes E* and *F* are identical to *lanes A* and *B* and *lanes H* and *I* are identical to *lanes C* and *D*, except that they were stained for protein by the silver method. Molecular weight standards are shown in *lane G*.

GENBANK DNA sequence database, no significant homologies were shown. Also, a search against the National Biomedical Research Foundation database showed limited homology (32% identity through 25 amino acids) to the NH_2-terminus of glyceraldehyde-3-phosphate dehydrogenase. Thus, it may be that 10-formyl-H_4folate dehydrogenase possesses a relatively unique NH_2-terminal sequence.

Experiments carried out with antibodies raised against purified rat liver 10-formyl-H_4folate dehydrogenase have proven to be very useful. The antibody preparations appear to be monospecific. Thus, they recognize only a single polypeptide corresponding to the dehydrogenase (96,000 daltons) from both rat and human liver cytosol preparations. Furthermore, the intensity of staining for each purified protein, either rat or human, based on a given amount tested is very similar. This should allow for future studies to estimate the amount of enzyme present in human liver using only small amounts of tissue. Studies carried out so far indicate that the amount of enzyme present in human liver is less than that in rat liver. Indeed, in Fig. 5, less intense staining for the enzyme from human liver cytosolic preparation is consistent with this hypothesis. However, more work is needed to confirm these findings in a larger number of human livers. Variability in susceptibility to methanol poisoning has been reported (10) but whether this is due to environmental factors leading to low folate cosubstrate levels or low enzyme levels in certain individuals is not known. However, the use of a monospecific antibody reagent may help to dissociate environmental and/or genetic factors in an understanding of the variability in responsivity to human methanol poisoning.

TABLE 3

NH_2-terminal sequences of rat and human liver 10-formyl-H_4folate dehydrogenases

Approximately 200 pmol of proteins were applied to the automated sequencer.

No.	Rat		Human	
	Amino acid	Phenylthiohydantoin	Amino acid	Phenylthiohydantoin
		pmol		*pmol*
1	Xxx		Xxx	
2	Lys	31	Lys	27
3	Ile	45	Ile	32
4	Ala	39	Ala	28
5	Val	34	Val	24
6	Ile	35	Ile	22
7	Gly	34	Gly	17
8	Gln	32	Gln	18
9	Ser		Ser	12
10	Leu	24	Leu	14
11	Phe	25	Phe	14
12	Gly	18	Gly	13
13	Gln	24	Gln	11
14	Glu	22	Glu	9
15	Val	14	Val	5
16	Tyr	14	Tyr	9
17	(Cys)			
18	Gln	1		
19	Leu	10		
20	Arg			
21	Lys	8		
22	Glu	7		
23	Gly	8		

It has been pointed by Case *et al.* (18) that the physiological role of 10-formyl-H_4folate dehydrogenase is unknown. Although its physiological function may be unknown, it plays a major role in the disposition of formate generated from one-carbon moieties of environmental chemicals, drugs, and foods. The importance of this enzyme in the toxicology of methanol is now well known. Other one-carbon environmental chemicals also can, in part, be converted to CO_2 through formate by 10-formyl-H_4folate dehydrogenase activity (e.g., methylene chloride). In addition, drugs possessing methyl groups are converted to CO_2 through formate and 10-formyl-H_4folate dehydrogenase. Black *et al.* (26) have shown that depletion of hepatic H_4folate in rats led to a depressed rate of conversion of the methyl group of aminopyrine to CO_2 and that this was due to the reduced rate of formate oxidized to CO_2, with subsequent elevation of urinary formate. In that same study, rats with lowered hepatic H_4folate demonstrated elevated formate excretion even without the administration of drugs. Thus, it is possible that dietary components contribute directly or indirectly to one-carbon moieties that lead to formate and that need to be disposed of through the folate biochemical system and 10-formyl-H_4folate dehydrogenase.

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