A COMPARISON OF RAT AND HUMAN LIVER FORMALDEHYDE DEHYDROGENASE

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SUMMARY

An NAD- and GSH-dependent formaldehyde dehydrogenase (formaldehyde: NAD^+ oxidoreductase, EC 1.2.1.1) was purified from rat and human liver, and the properties of these enzymes were compared. The GSH requirement of the enzyme obtained from both species could not be replaced by dithiothreitol, CoA or cysteine, and NADP could not substitute for NAD. The pH optimum, and the K_m of formaldehyde and NAD^+, were similar for both rat and human liver formaldehyde dehydrogenase. By employing inhibitors such as folic acid and 1,10-phenanthroline, several qualitative differences between rat and human liver formaldehyde dehydrogenase have been detected.

The molecular weight of purified human formaldehyde dehydrogenase was estimated at 90000. However, the molecular weight of human formaldehyde dehydrogenase appeared to be greater than 250000 when it was determined in preparations which also contained alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) and aldehyde dehydrogenase (aldehyde: NAD^+ oxidoreductase, EC 1.2.1.3). These data suggest that formaldehyde dehydrogenase exists in a complex with other proteins or in a polymeric form until the ultimate steps in purification.

The capacity for NAD-linked formaldehyde oxidation was greater in human liver than in rat liver. The possible implications of this are discussed in regard to the unique susceptibility of man to methanol poisoning.

INTRODUCTION

Methanol poisoning has been characterized in man as a severe metabolic acidosis and blindness which occurs 18–24 h after ingestion[1]. Because of the latency observed prior to the onset of symptoms and because of the reversal of these phenomena by ethanol administration, this syndrome has been thought to be due to me-

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tabolic products of methanol rather than methanol *per se*. Furthermore, this toxicity is not observed in lower species with the possible exception of the monkey[2]. While our knowledge of the first step in the oxidation of methanol in the mammal has increased considerably over the last decade[3] the second step in this process, the disposition of formaldehyde, has received little attention. Consideration of formaldehyde metabolism is important because it may be the toxic metabolite responsible for methanol poisoning[2]. The fact that there is a decided species difference in susceptibility to methanol and the lack of information about formaldehyde utilization led to the studies reported here.

A number of aldehyde dehydrogenases have been reported to employ formaldehyde as substrate but analysis of the properties of these enzymes have usually been performed with substrates other than formaldehyde[4,5]. In 1955, STRITTMATTER AND BALL[6] identified a formaldehyde-specific, NAD-dependent dehydrogenase (formaldehyde:NAD oxidoreductase, EC 1.2.1.1) in beef liver. The enzyme showed a requirement for GSH which was suggested to participate in the reaction rather than acting non-specifically to protect protein sulfhydryl groups[6]. No studies have been performed on human liver or species where methanol metabolism has been examined in detail. In addition, formaldehyde is one of the products formed in the demethylation of drugs by the hepatic microsomal drug hydroxylation system[7]. An understanding of the disposition of formaldehyde would extend our knowledge of the utilization of one-carbon fragments generated by the demethylation of many drugs.

This report documents the presence of an NAD- and GSH-dependent formaldehyde dehydrogenase in rat and human liver, compares certain properties of these enzymes, and demonstrates qualitative differences between them.

MATERIALS AND METHODS

Chemicals

NAD (Grade III), NADP, cytochrome *c* (Type III), DEAE-cellulose, CM-cellulose, Sephadex G-200, bovine serum albumin, yeast alcohol dehydrogenase, calcium phosphate gel, GSH, folic acid, and 1,10-phenanthroline were purchased from Sigma Chemical Company. Pyrazole was purchased from K and K Laboratories, Inc. Ovalbumin and myoglobin were purchased from Nutritional Biochemical Company. Methotrexate was a gift from Lederle Laboratories. All other reagents were employed in the highest purity available.

Source of liver

Rat livers were obtained from male Holtzman rats (250 g). Human livers were obtained at autopsy, not later than 10 h *post mortem*. In all instances the case history and gross pathological examination indicated the livers to be free of disease. Similar results were obtained when liver from either sex was employed and when fresh human liver was examined. Fresh human liver was obtained from a 50-year-old female who had died of a stroke. Artificial ventilation and extracorporeal perfusion had been instituted for 1 h prior to removal of a portion of the liver.

Enzyme assays

One unit of enzyme activity is defined as the amount of enzyme which cata-

lyzes the reduction of 1 μmole of NAD per min under the conditions described. Specific activity is expressed as units per mg protein.

Formaldehyde dehydrogenase. Formaldehyde dehydrogenase activity was determined spectrophotometrically by measuring the rate of reduction of NAD at 340 nm on a Gilford Model 2000 spectrophotometer. All determinations were carried out at 22°. The reaction mixture contained in a final volume of 1 ml: enzyme; NAD, 1 μmole; GSH, 1 μmole; formaldehyde, 0.12 μmole; and phosphate buffer[28], 50 mM, pH 8.0.

Alcohol dehydrogenase. Alcohol dehydrogenase activity was determined spectrophotometrically as described for formaldehyde dehydrogenase. The reaction mixture contained in a final volume of 1 ml: enzyme, ethanol, 100 μmoles; NAD, 2 μmoles; and glycine–NaOH buffer, 37.5 mM, pH 10.0.

Aldehyde dehydrogenase. Aldehyde dehydrogenase activity was determined spectrophotometrically as described for formaldehyde dehydrogenase. The reaction mixture contained in a final volume of 1 ml: enzyme, acetaldehyde (or benzaldehyde when indicated in the text), 10 μmoles; pyrazole, 1 μmole; NAD, 2 μmoles; phosphate buffer[28], 50 mM, pH 8.0.

Purification of rat liver formaldehyde dehydrogenase

Preparation of hepatic cytosol fraction. It was determined in preliminary experiments that greater than 90% of the total activity of formaldehyde dehydrogenase resided in cytosol fractions of rat and human liver homogenates. Rats were decapitated, the livers were rapidly removed and placed in an ice-cold solution of 0.01 M phosphate buffer[28], pH 7.4, containing 0.001 M disodium EDTA and 0.0001 M dithiothreitol (Medium A). All procedures for the preparation of the enzyme were carried out at 0–4°. About 300 ml of a 50% (w/v) homogenate were prepared in Medium A with a Waring blender (21 000 rev./min, 45 sec). The homogenate was centrifuged at 12 500 × g for 20 min. The supernatant fluid containing microsomes and cytosol was diluted with Medium A to 300 ml and centrifuged in a Spinco Model L ultracentrifuge at 78 500 × g for 2 h using a Type 30 rotor. The microsomes were discarded and the cytosol was diluted with Medium A to about 30 mg protein per ml. A portion of the cytosol was dialyzed against 100 times its volume of Medium A and used for analysis of formaldehyde dehydrogenase. The rest was employed for other purification procedures.

Ammonium sulfate fractionation. An NAD-, GSH-dependent formaldehyde dehydrogenase was precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation. Solid $(NH_4)_2SO_4$ was slowly added to obtain the appropriate concentration and the pH was maintained at 7.4 by the addition of small amounts of NH_4OH. The 50–70% $(NH_4)_2SO_4$ precipitate was brought to 100 ml (30 mg protein per ml) in Medium A and dialyzed for 12 h against 10 l of Medium A prior to treatment with an ethanol–chloroform mixture.

Ethanol–chloroform fractionation. Most of the hemoglobin is removed by slowly adding with stirring 2 ml of a mixture of 95% ethanol and chloroform (2:1, by vol.) to each 10 ml of dialyzed enzyme preparation. This mixture was centrifuged at 12 500 × g for 20 min and the resulting supernatant fluid was dialyzed for 12 h against 10 l of Medium A. This preparation (about 20 mg protein per ml) was used for DEAE-cellulose chromatography.

Chromatography on DEAE-cellulose. The enzyme preparation was applied to a column of DEAE-cellulose (5 cm × 45 cm), which had been previously equilibrated with Medium A. Elution was effected with a linear gradient of KCl (0 to 0.5 M) in Medium A. The flow rate was adjusted to 60 ml/h and 20-ml fractions were collected.

Chromatography on hydroxylapatite. Certain fractions from DEAE-cellulose chromatography containing formaldehyde dehydrogenase and small amounts of alcohol dehydrogenase were pooled and applied to a column of hydroxylapatite (2cm × 10 cm) which had been equilibrated previously with Medium A. Stepwise elution with phosphate buffer (0.01–0.15 M), pH 7.4[28], containing 0.001 M EDTA and 0.0001 M dithiothreitol was employed. The flow rate was adjusted to 10 ml/h and 5-ml fractions were collected.

Purification of human liver formaldehyde dehydrogenase

Preparation of hepatic homogenate. Autopsy samples of human liver were obtained, and homogenized as described for rat liver. A hepatic cytosol fraction was prepared (as described for rat liver) from a small portion of the homogenate. The major portion was treated with an ethanol–chloroform mixture.

Ethanol–chloroform fractionation. The homogenate was treated with ethanol–chloroform (2:1 by vol.), in the same manner as that described for the rat liver cytosol. The resulting mixture was centrifuged at 12 500 × g for 20 min and the supernatant was dialyzed for 12 h against 5 l of Medium A. The dialysis procedure was repeated once more, and the preparation (230 ml, 14 mg protein per ml) was then subjected to $(NH_4)_2SO_4$ fractionation.

Ammonium sulfate fractionation. An NAD- and GSH-dependent formaldehyde dehydrogenase was precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation. Solid ammonium sulfate was employed. This step was carried out as described for the rat liver enzyme.

Chromatography on DEAE-cellulose. The enzyme preparation after ammonium sulfate fractionation was applied to a DEAE-cellulose column (2.5 cm × 25 cm) previously equilibrated with Medium A and was eluted with a linear gradient of KCl (0–0.2 M) in Medium A. The flow rate was adjusted to 30 ml/h and 10-ml fractions were collected. Certain fractions containing formaldehyde dehydrogenase activity, along with alcohol dehydrogenase and aldehyde dehydrogenase activity, were pooled and dialyzed for 12 h against 5 l of 0.01 M phosphate buffer, pH 7.1[28], containing 0.001 M EDTA and 0.0001 M dithiothreitol prior to treatment with calcium phosphate gel.

Adsorption on calcium phosphate gel. Calcium phosphate gel was added to the enzyme preparation obtained by DEAE-cellulose chromatography (2 mg dry weight calcium phosphate gel per mg protein). This mixture was stirred for 20 min at 0° and centrifuged at 10000 × g for 10 min. The supernatant, containing formaldehyde dehydrogenase and some alcohol dehydrogenase activity was removed and stored. The pellet, consisting of protein adsorbed on calcium phosphate gel, was suspended in 10 ml of 0.05 M phosphate buffer, pH 7.1[28], containing 0.001 M EDTA and 0.0001 M dithiothreitol and stirred for 20 min prior to centrifugation at 10000 × g for 10 min. This procedure was repeated once more and the three supernatants containing formaldehyde dehydrogenase activity and a small amount of alcohol dehydrogenase activity were combined. This preparation was concentrated to a volume of 35 ml using

a Diaflow Ultrafiltration Cell with a 10000 mol.wt. filter. The concentrated preparation was then dialyzed against 3.5 l of phosphate buffer[28] (0.005 M, pH 7.0) containing 0.001 M EDTA and 0.0001 M dithiothreitol (Medium B) prior to chromatography on CM-cellulose.

Chromatography on CM-cellulose. The enzyme preparation was applied to a CM-cellulose column (2.5 cm × 20 cm) which had been previously equilibrated with Medium B. Elution with 350 ml of Medium B followed. The flow rate was adjusted to 30 ml per h and 5-ml fractions were collected.

Estimation of molecular weight

The molecular weight of rat and human hepatic formaldehyde dehydrogenase was estimated by gel filtration on a Sephadex G-200 column according to the method of ANDREWS[8].

Determination of Michaelis constants

The K_m of formaldehyde and NAD$^+$ for rat and human formaldehyde dehydrogenase was determined by the method of LINEWEAVER AND BURK[9].

Protein determination

Protein was determined by the Biuret[10] method or by ultraviolet absorption[11].

RESULTS

Purification of formaldehyde dehydrogenase from rat liver

The complete procedure for the purification of formaldehyde dehydrogenase from rat liver is summarized in Table I. When EDTA and dithiothreitol were used throughout the purification procedure, enzyme activity could be maintained at 0–5° without measurable loss for at least 3 weeks.

TABLE I

PURIFICATION OF FORMALDEHYDE DEHYDROGENASE FROM RAT LIVER

Procedure	Vol. (ml)	Concn. (units/ml)	Total units	Protein (mg/ml)	Specific activity	Yield (%)	Purification
Soluble fraction	300	0.027	8.1	30.1	0.0009	100	1
50–70% (NH$_4$)$_2$SO$_4$	100	0.067	6.7	30.3	0.0022	83	2.5
Chloroform–ethanol	85	0.070	6.0	19.5	0.0036	74	4
DEAE-cellulose	100	0.032	3.2	3.0	0.0107	39	12
Hydroxylapatite	10	0.024	0.24	0.26	0.0920	3	102

Slight activity was observed in the cytosol of rat liver when GSH was omitted from the reaction mixture. This is probably due to the presence of other aldehyde dehydrogenases which depend on NAD and not on GSH. The protein which precipitated between 0 and 50% (NH$_4$)$_2$SO$_4$ saturation showed almost equal activity with and without GSH in the reaction mixture (Table II). However, the protein which precipitated between 50 and 70% (NH$_4$)$_2$SO$_4$ saturation showed very little activity toward formaldehyde if GSH was omitted from the reaction mixture. Formaldehyde dehydrogenase activity in all fractions was calculated by subtracting

TABLE II

FORMALDEHYDE OXIDATION IN RAT LIVER

	$+GSH$	$-GSH$	Formaldehyde dehydrogenase (units)*
Soluble fraction	0.035	0.008	0.027
0–50% $(NH_4)_2SO_4$	0.019	0.014	0.005
50–70% $(NH_4)_2SO_4$	0.071	0.004	0.067
Chloroform–ethanol treatment of 50–70% $(NH_4)_2SO_4$	0.070	0.000	0.070

* Reaction rate (μmoles NAD reduced per ml per min) in the presence of GSH *minus* the rate when GSH was omitted. Assay conditions were those described in METHODS.

the rate of NAD reduction in the absence of GSH from the rate obtained when GSH was employed in the reaction. After treatment with chloroform–ethanol there was no detectable NAD reduction if GSH was omitted from the reaction mixture (Table II). Furthermore, no NAD reduction was observed in this fraction when acetaldehyde was employed as substrate. These observations indicate that separation of rat hepatic formaldehyde dehydrogenase and aldehyde dehydrogenase occurs after chloroform–ethanol treatment following $(NH_4)_2SO_4$ fractionation. This procedure offers a more effective and rapid method of separating these enzymes than the procedures employed by STRITTMATTER AND BALL[6]. The results obtained after chromatography on DEAE-cellulose are shown in Fig. 1. The majority of the alcohol dehydrogenase activity was eluted prior to formaldehyde dehydrogenase, but an incomplete resolution was observed. The specific activity of formaldehyde dehydrogenase was in-
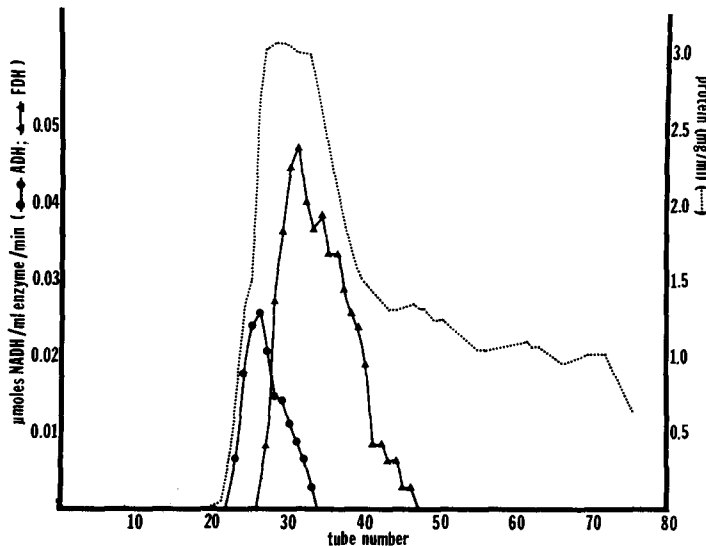

Fig. 1. DEAE-cellulose chromatography (column, 5 cm × 45 cm) of rat liver formaldehyde dehydrogenase. Elution was performed with a linear gradient of KCl (0 to 0.5 M, in 0.01 M phosphate buffer[28], pH 7.4, containing 0.001 M EDTA and 0.0001 M dithiothreitol). The flow rate was adjusted to 60 ml/h and 20-ml fractions were collected. Fractions 28–32 were pooled and chromatographed on a hydroxylapatite column (Fig. 2). ●—●, alcohol dehydrogenase; ▲—▲, formaldehyde dehydrogenase; ......, protein.
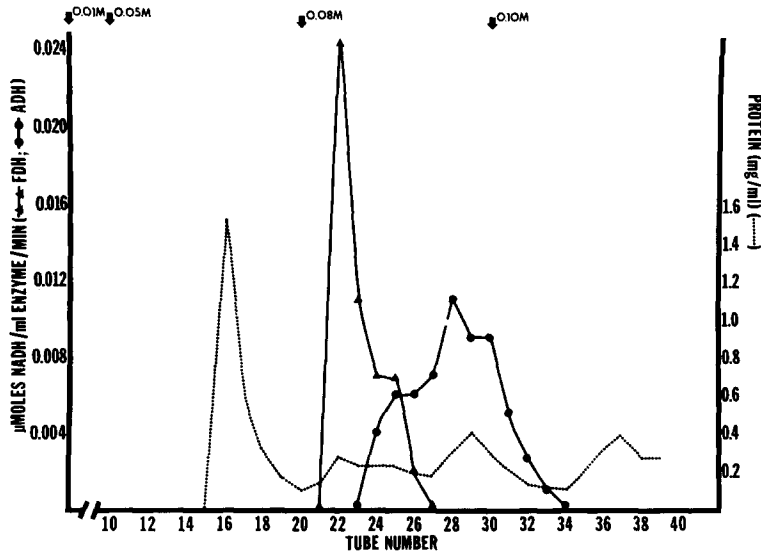
Fig. 2. Hydroxylapatite chromatography (column, 2 cm × 10 cm) of rat liver formaldehyde dehydrogenase. Elution was performed stepwise, with increasing molarity of phosphate buffer (0.01–0.10 M, pH 7.4[28]) containing 0.001 M EDTA and 0.0001 M dithiothreitol). The flow rate was adjusted to 10 ml/h and 5-ml fractions were collected. ●—●, alcohol dehydrogenase; ▲—▲, formaldehyde dehydrogenase; ......, protein.

creased about 12-fold over that of the soluble fraction. Certain fractions (28–32) which contained formaldehyde dehydrogenase activity and a small amount of alcohol dehydrogenase activity were pooled and chromatographed on a hydroxylapatite column (Fig. 2). The specific activity of Fraction 22 represents about a 102-fold purification of formaldehyde dehydrogenase This fraction was free of alcohol dehydrogenase activity.

Purification of formaldehyde dehydrogenase from human liver

The complete procedure for the purification of formaldehyde hydrogenase from human liver is summarized in Table III. The values for human liver cytosol, which had been prepared in the same manner as that of rat liver, are included for compari-

TABLE III

PURIFICATION OF FORMALDEHYDE DEHYDROGENASE FROM HUMAN LIVER

Procedure	Vol. (ml)	Concn. (units/ml)	Total units	Protein (mg/ml)	Specific activity	Yield (%)	Purification
Soluble fraction	400	0.041	16.4	28.8	0.001	100	1
Homogenate after chloroform– ethanol	230	0.042	9.7	13.9	0.003	59	3
50–70% (NH₄)₂SO₄	50	0.185	9.3	29.0	0.006	56	6
DEAE-cellulose	50	0.102	5.1	4.4	0.023	31	23
Calcium phosphate gel	35	0.087	3.0	1.9	0.045	18	45
CM-cellulose	40	0.036	1.4	0.16	0.225	8	225

son. When EDTA and dithiothreitol were used throughout the purification procedure, enzyme activity could be maintained without measurable loss at $0-5°$ for at least 3 weeks.

A considerable amount of formaldehyde oxidation occurred in the cytosol fraction when GSH was omitted from the reaction mixture which is probably due to the presence of relatively non-specific aldehyde dehydrogenases. This is also the case when a human liver homogenate is fractionated with chloroform–ethanol. The protein which precipitated between 0 and 50% $(NH_4)_2SO_4$ saturation showed almost equal activity with or without GSH in the reaction mixture. However, the protein which precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation exhibited very little activity if GSH was omitted from the reaction mixture. These results are shown in Table IV.
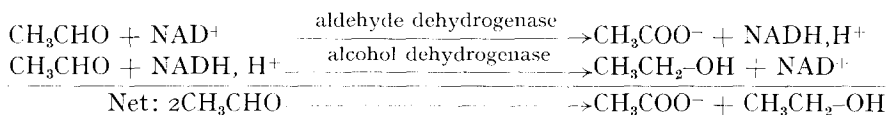
TABLE IV

FORMALDEHYDE OXIDATION IN HUMAN LIVER

	$+GSH$	$-GSH$	Formaldehyde dehydrogenase (units)*
Soluble fraction	0.090	0.051	0.039
Homogenate after chloroform–ethanol	0.181	0.071	0.110
0–50% $(NH_4)_2SO_4$	0.163	0.174	0.000
50–70% $(NH_4)_2SO_4$	0.193	0.013	0.180

* Reaction rate (μmoles NAD reduced per ml per min) in the presence of GSH *minus* the rate when GSH was omitted. Assay conditions were those described in METHODS.

The protein which precipitated between 50 and 70% $(NH_4)_2SO_4$ saturation contained a considerable amount of alcohol dehydrogenase activity and at first appeared to contain only a minimal amount of aldehyde dehydrogenase activity. However, in view of the fact that the equilibrium of the alcohol dehydrogenase reaction lies far in favor of acetaldehyde reduction[12], it seemed possible that the following series of reactions could be occurring:

$$CH_3CHO + NAD^+ \xrightarrow{\text{aldehyde dehydrogenase}} CH_3COO^- + NADH, H^+$$
$$CH_3CHO + NADH, H^+ \xrightarrow{\text{alcohol dehydrogenase}} CH_3CH_2-OH + NAD^+$$
$$\text{Net: } 2CH_3CHO \longrightarrow CH_3COO^- + CH_3CH_2-OH$$

This series of reactions would, therefore, not yield a net increase in NADH and, thus, the aldehyde dehydrogenase reaction would be masked if measurement of NADH formation was used as the assay. A direct means of testing this hypothesis was provided by the alcohol dehydrogenase inhibitor, pyrazole[13,14]. Table V shows that the addition of pyrazole to the 50–70% $(NH_4)_2SO_4$ fraction results in a profound increase in aldehyde dehydrogenase activity. The concentration of pyrazole employed was capable of completely inhibiting the alcohol dehydrogenase reaction when 10 mM ethanol was employed as substrate (Table V). Because of this observation, assays for aldehyde dehydrogenase were carried out routinely in the presence of 1 mM pyrazole. Aldehyde dehydrogenase, separated from alcohol dehydrogenase, was completely active in the absence of pyrazole and, in addition, aldehyde dehydrogenase activity was not inhibited by pyrazole in concentrations up to 5 mM. When the 50–70%

TABLE V

THE EFFECT OF ALCOHOL DEHYDROGENASE ON THE DETERMINATION OF ALDEHYDE DEHYDROGENASE ACTIVITY

The enzyme preparation employed was the 50–70% $(NH_4)_2SO_4$ fraction which was prepared as described in METHODS.

	Aldehyde dehydrogenase*	Alcohol dehydrogenase	
		100 mM ethanol	10 mM ethanol
No pyrazole	0.0003	0.0421	0.0166
Pyrazole (1 mM)	0.0205	0.0213	0

* μmoles of NAD reduced per mg protein per min.

$(NH_4)_2SO_4$ fraction was applied to a CM-cellulose column, formaldehyde dehydrogenase and aldehyde dehydrogenase are both eluted by 0.005 M phosphate buffer[28], pH 7.0, while alcohol dehydrogenase remained on the column.

The results obtained after applying the 50–70% $(NH_4)_2SO_4$ fraction on a DEAE-cellulose column are shown in Fig. 3. This procedure results in a purification of formaldehyde dehydrogenase, alcohol dehydrogenase and aldehyde dehydrogenase but does not provide for effective separation of these activities. Fractions 16–20 were combined and treated with calcium phosphate gel. When the gel was washed with 0.05 M phosphate buffer[28], pH 7.1, a separation of aldehyde dehydrogenase from formaldehyde dehydrogenase and alcohol dehydrogenase was seen. Aldehyde dehydrogenase and most of the alcohol dehydrogenase remained adsorbed to the gel and the final preparation containing formaldehyde dehydrogenase and a small amount of alcohol dehydrogenase activity was chromatographed on a CM-cellulose column (Fig. 4). This resulted in a complete separation of formaldehyde dehydro-
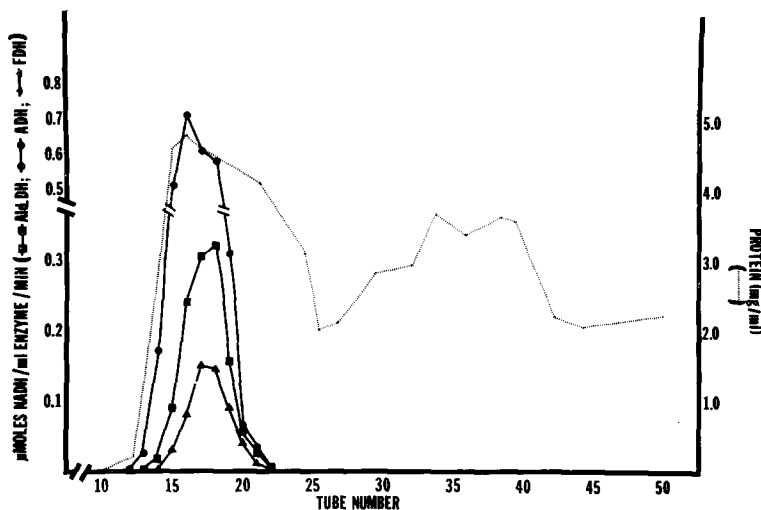

Fig. 3. DEAE-cellulose chromatography (column, 2.5 cm × 25 cm) of human liver formaldehyde dehydrogenase. Elution was performed with a linear gradient of KCl (0–0.2 M in 0.01 M phosphate buffer[28], pH 7.4, containing 0.001 M EDTA and 0.0001 M dithiothreitol). The flow rate was adjusted to 30 ml/h and 10-ml fractions were collected. Fractions 16–20 were pooled and treated with calcium phosphate gel. ●—●, alcohol dehydrogenase; ▲—▲, formaldehyde dehydrogenase; ■—■, aldehyde dehydrogenase; ......, protein.
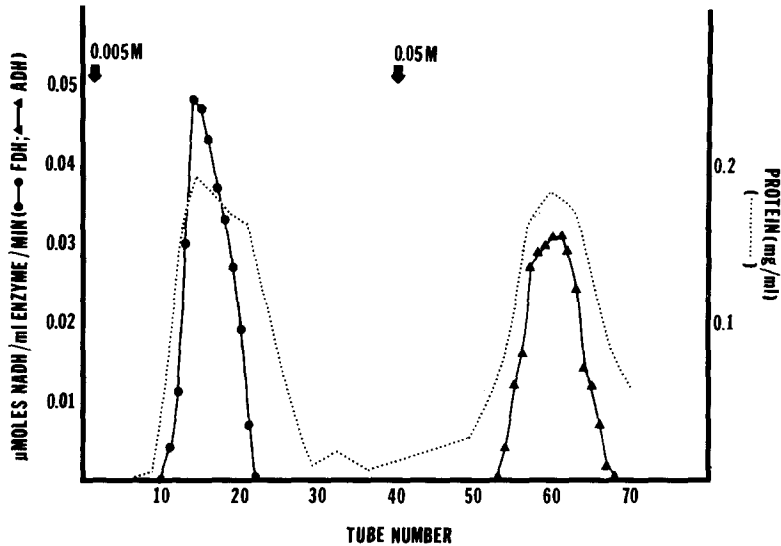
Fig. 4. CM-cellulose-chromatography (column, 2.5 cm × 20 cm) of human liver formaldehyde dehydrogenase. Elution was performed stepwise with 0.005 M and 0.050 M phosphate buffer[28], pH 7.0, containing 0.001 M EDTA and 0.0001 M dithiothreitol. The flow rate was adjusted to 30 ml/h and 5-ml fractions were collected. ▲—▲, alcohol dehydregenase; ●—●, formaldehyde dehydrogenase; ......, protein

genase from alcohol dehydrogenase activity. When Fractions 13–20 (Fig. 4) were pooled, this preparation had a specific activity which was about 225 times that of the hepatic cytosol.

Comparison of NAD-linked formaldehyde oxidation in rat and human liver soluble fractions

A comparison of NAD-linked formaldehyde oxidation in rat and human liver is presented in Table VI. The total amount (units/g liver) of formaldehyde dehydrogenase activity in human liver was found to be about 50% higher than that of rat liver. In addition to this, the capacity for NAD-linked formaldehyde oxidation which is not dependent upon GSH was several fold higher (units/g liver and specific activity) in human liver.

TABLE VI

COMPARISON OF NAD-LINKED FORMALDEHYDE OXIDATION IN RAT AND HUMAN LIVER

These studies were performed using hepatic soluble fractions prepared as described in METHODS.

| | *Formaldehyde dehydrogenase* | | *Non-GSH dependent formaldehyde oxidation* | |
	Units/g liver	Specific activity	Units/g liver	Specific activity
Rat	0.054	0.0009	0.016	0.0003
Human	0.078	0.0010	0.102	0.0013

Properties of rat and human liver formaldehyde dehydrogenase

Purified rat and human liver formaldehyde dehydrogenase preparations were employed for these studies. The preparation represented 102- and 225-fold purification of the rat and human liver enzymes, respectively.

Substrate specificity. Both rat and human liver formaldehyde dehydrogenase were unreactive towards acetaldehyde and benzaldehyde, in concentrations up to 10 mM of substrate. These aldehydes have been reported to be good substrates for human liver aldehyde dehydrogenase[5,15]. The K_m of formaldehyde for rat liver formaldehyde dehydrogenase was found to be $7.1 \cdot 10^{-6}$ M, while the K_m for the human liver enzyme was $8.7 \cdot 10^{-6}$ M. The K_m of formaldehyde for bovine liver formaldehyde dehydrogenase can be calculated to be $5 \cdot 10^{-6}$ M from the data presented by STRITTMATTER AND BALL[6]. The K_m of NAD^+ for rat and human hepatic formaldehyde dehydrogenase was 10^{-5} M and $7 \cdot 10^{-6}$ M, respectively. When NADP, in concentrations up to 5 mM, was substituted for NAD in the assay of the rat and human liver formaldehyde dehydrogenase, no reaction was detected. No reaction was observed when GSH was omitted from reaction mixtures. Furthermore, the GSH requirement of both rat and human liver formaldehyde dehydrogenase could not be replaced by cysteine, dithiothreitol or CoA in concentrations up to 5 mM. This is consistent with the proposal that GSH participates directly in the formaldehyde dehydrogenase reaction, rather than acting non-specifically to protect protein sulfhydryl groups[6].

pH optimum. Both enzymes exhibited broad pH optima. The pH optimum for rat liver formaldehyde dehydrogenase was 7.5–8.4 (Fig. 5) while the maximal activity of human liver formaldehyde dehydrogenase was seen between pH 7.6 and 8.8 (Fig. 5). These data are in contrast to the sharp pH optimum of 8.0 reported by STRITTMATTER AND BALL[6] for bovine liver formaldehyde dehydrogenase.
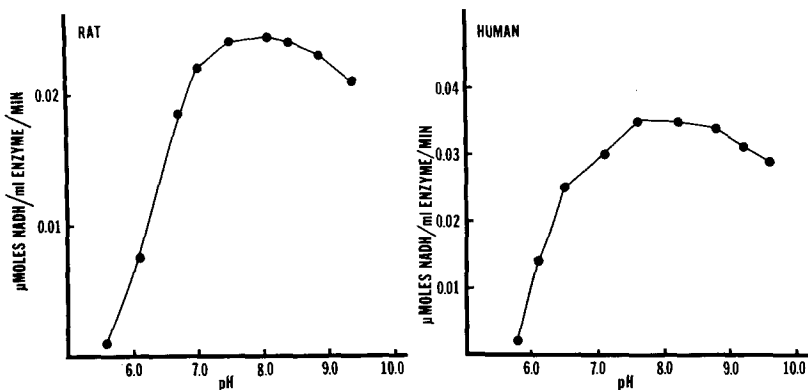

Fig. 5. Effect of pH on the activity of purified rat and human liver formaldehyde dehydrogenase. Reactions were carried out at 22°; reaction mixtures contained in a final volume of 1 ml: enzyme, 0.2 ml; NAD, 1 μmole; GSH, 1 μmole; formaldehyde, 0.12 μmole; and sodium phosphate or sodium pyrophosphate, 25 μmoles, adjusted to the desired pH with 1 M HCl. Sodium phosphate was employed up to pH 7.5 and sodium pyrophosphate was used above this pH value.

Effect of various inhibitors. The well-known inhibitor of alcohol dehydrogenase, 1,10-phenanthroline, was studied for its effects on rat and human liver formaldehyde dehydrogenase. Table VII shows that 1,10-phenanthroline had no effect on rat liver formaldehyde dehydrogenase, but does inhibit human liver formaldehyde dehydrogenase. Since 1,10-phenanthroline is a chelating agent, these data suggest that the human liver enzyme is metal-containing, whereas the rat liver enzyme is not. How-

ever, a direct inhibitory effect of 1,10–phenanthroline on human liver formaldehyde dehydrogenase cannot be ruled out at this time.

Table VII shows that folic acid (10^{-4} M) inhibited rat liver formaldehyde dehydrogenase but had no effect on the human liver enzyme indicating another difference between these enzymes. Methotrexate ($5 \cdot 10^{-4}$ M) had no effect on either enzyme (Table VII).

TABLE VII

EFFECT OF VARIOUS INHIBITORS ON PURIFIED RAT AND HUMAN FORMALDEHYDE DEHYDROGENASE

Rates are expressed as μmoles of NAD reduced per mg protein per min. Assay conditions were those described in METHODS.

	Rat	Human
Formaldehyde	0.12	0.10
Formaldehyde *plus* 1,10-phenanthroline (0.1 mM)	0.12	0.07
Formaldehyde *plus* 1,10-phenanthroline (1.0 mM)	0.12	0
Formaldehyde *plus* folic acid (0.1 mM)	0	0.10
Formaldehyde *plus* methotrexate (0.5 mM)	0.12	0.10

Folic acid and methotrexate have been shown to be inhibitors of a wide variety of dehydrogenases, including malic dehydrogenase, glucose 6-phosphate dehydrogenase and alcohol dehydrogenase[16,17]. Folic acid has been shown to be a zinc chelator[18]. However, the possibility that folic acid and methotrexate inhibit alcohol dehydrogenase by a chelation mechanism was rejected[17] for several reasons, (1) inhibition of dehydrogenase activity was neither prevented nor reversed by the addition of zinc; (2) the inhibition by folic acid was pH independent. In order to chelate folic acid with free zinc, the hydroxyl group (pK_a 8.3) in the number 4 position of folic acid must be ionized. Thus, if folic acid inhibited by chelation, inhibition should have been greater at higher pH values; (3) methotrexate, which has little chelating potentiality, was as effective as folic acid in inhibiting alcohol dehydrogenase. Therefore, the mechanism of dehydrogenase inhibition produced by folic acid and methotrexate remains unclear. However, these results point to a qualitative difference between rat and human liver formaldehyde dehydrogenase.

Effect of pyrazole. Since pyrazole has been shown to be a potent inhibitor of liver alcohol dehydrogenase[13,14] it was important to examine the effect of this compound on rat and human liver formaldehyde dehydrogenase. Fig. 6 shows that pyrazole stimulates the oxidation of formaldehyde by rat liver formaldehyde dehydrogenase. Furthermore, it appears to be capable of substituting for GSH in the formaldehyde dehydrogenase reaction. In contrast to the results obtained with rat liver formaldehyde dehydrogenase, pyrazole had no effect on 5 out of 6 human liver formaldehyde dehydrogenase preparations. At a concentration of 1 mM, pyrazole produced a 20% inhibition of the rate of formaldehyde oxidation by formaldehyde dehydrogenase isolated from one human liver. In addition to indicating a further difference between rat and human liver formaldehyde dehydrogenase, these results may indicate that certain human livers may contain an atypical formaldehyde dehydrogenase. The presence of atypical alcohol dehydrogenase in some human liver is well-documented and the atypical enzyme has been shown to be less sensitive to inhibition by pyrazole than the normal enzyme[19]. Imidazole, an isomer of pyrazole,

in concentrations up to 5 mM, had no effect on rat or human liver formaldehyde dehydrogenase.
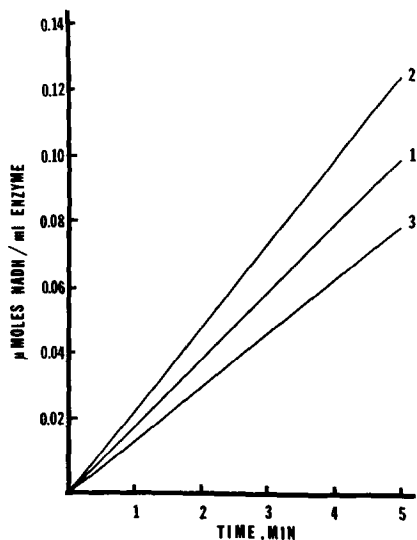

Fig. 6. Effect of pyrazole on purified rat liver formaldehyde dehydrogenase. All reaction mixtures contained 0.2 ml of enzyme (0.4 mg protein per ml) and 0.12 μmole of formaldehyde in a final volume of 1 ml: (1) NAD, 1 μmole; GSH, 1 μmole; (2) NAD, 1 μmole; GSH, 1 μmole; pyrazole, 1 μmole; (3) NAD$^+$, 1 μmole; pyrazole, 1 μmole. When pyrazole and GSH were omitted from reaction mixtures there was no detectable rate of NAD reduction. Curves represent tracings taken from the recorder of a Gilford Model 2000 spectrophotometer.

Estimation of molecular weight.(a) Human liver formaldehyde dehydrogenase. The molecular weight of human liver formaldehyde dehydrogenase was estimated by gel filtration through a Sephadex G-200 column (Fig. 7). The molecular weight of purified human liver formaldehyde dehydrogenase was estimated at 90000. However, when a relatively crude preparation of human liver formaldehyde dehydrogenase which also contained alcohol dehydrogenase and aldehyde dehydrogenase was applied to a Sephadex G-200 column (Fig. 7), formaldehyde dehydrogenase was eluted along with aldehyde dehydrogenase soon after blue dextran, in a volume indicating its molecular weight to be over 250000. Most of the alcohol dehydrogenase was eluted in a volume indicating its molecular weight to be 80000. The molecular weight of purified human liver alcohol dehydrogenase has been reported to be 87000 (ref. 20).

(b) Rat liver formaldehyde dehydrogenase. The molecular weight of rat liver formaldehyde dehydrogenase was estimated at about 110000 by gel filtration through a Sephadex G-200 column (Fig. 7).

DISCUSSION

These studies have shown the presence of specific formaldehyde dehydrogenase requiring NAD and GSH for activity, in rat and human liver. A 102-fold purification of formaldehyde dehydrogenase from rat liver and a 225-fold purification of formaldehyde dehydrogenase from human liver was achieved.
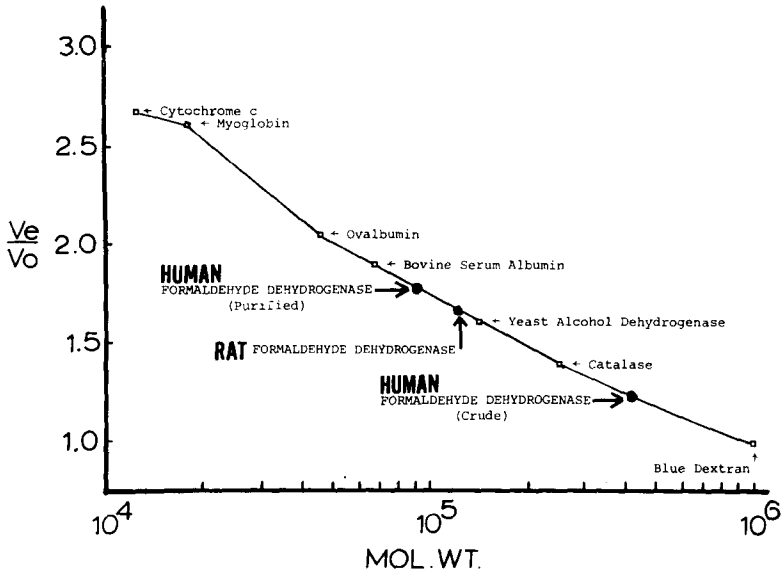
Fig. 7. Estimation of the molecular weight of rat and human liver formaldehyde dehydrogenase. A Sephadex G-200 column (2 cm × 50 cm) was calibrated with six crystalline proteins. The molecular weights of the crystalline proteins were those given by ANDREWS[8]. The elution volume of a 4-fold purified rat liver formaldehyde dehydrogenase preparation (obtained after chloroform–ethanol treatment, Table I) was measured, and the molecular weight was estimated to be 110000. The elution volume of a purified human liver formaldehyde dehydrogenase preparation (obtained after CM-cellulose chromatography, Table III) and a crude human liver formaldehyde dehydrogenase preparation obtained after ammonium sulfate fractionation, Table III were measured. The molecular weights of the enzyme in purified and crude preparations were estimated at 90000 and over 250000, respectively. V_e = elution volume, V_o = void volume (62 ml).

Rat and human hepatic formaldehyde dehydrogenase were found to be similar in several aspects. Both enzymes were unreactive towards acetaldehyde and benzaldehyde, substrates which are known to react favorably with human liver aldehyde dehydrogenase[5,10]. In addition, the GSH requirement of the enzyme obtained from both species could not be replaced by dithiothreitol, CoA or cysteine, and NADP was not capable of replacing NAD. The K_m of formaldehyde was similar for both rat and human liver enzymes; $7.1 \cdot 10^{-6}$ M and $8.7 \cdot 10^{-6}$ M, respectively. The K_m for NAD^+ was 10^{-5} and $7 \cdot 10^{-6}$ M for the rat and human liver enzyme respectively, and the pH optimum of rat hepatic formaldehyde dehydrogenase was between 7.5 and 8.4, while the human liver enzyme has a pH optimum which was slightly broader and higher, 7.6–8.8. This contrasts with the sharp pH optimum of 8.0, that has been reported for beef liver formaldehyde dehydrogenase[6] and to a pH optimum between 9 and 10 reported for human liver aldehyde dehydrogenase.

Several qualitative differences were noted between rat and human liver formaldehyde dehydrogenase. 1,10-Phenanthroline had no effect on the rat liver enzyme, but did inhibit human liver formaldehyde dehydrogenase activity. Since 1,10-phenanthroline is a chelating agent, these data suggest that the enzyme from human liver is metal-containing. However, a rather high concentration (1 mM) of o-phenanthroline was required to inhibit the enzyme and, therefore, the inhibition may not necessarily have been due to chelaton of a metal. Folic acid inhibited rat

liver formaldehyde dehydrogenase, but had no effect on the human liver enzyme, whereas methotrexate had no effect on either enzyme.

Pyrazole, the potent inhibitor of liver alcohol dehydrogenase[13,14] was examined for its effect on formaldehyde dehydrogenase. Contrary to expectations, pyrazole stimulated purified rat liver formaldehyde dehydrogenase activity and it appeared to be capable of substituting for GSH in the reaction. It is possible that formaldehyde reacts with pyrazole to form N-hydroxymethylpyrazole, analogous to the reaction of formaldehyde with amino and imino groups[21], which then serves as a substrate for rat liver formaldehyde dehydrogenase. In contrast to the results obtained with the rat liver enzyme, pyrazole had no effect on 5 out of 6 human liver formaldehyde dehydrogenase preparations. However, in a concentration of 1 mM, pyrazole did produce a 20% inhibition of formaldehyde dehydrogenase purified from one human liver. This suggests the existence of an atypical human liver formaldehyde dehydrogenase in certain individuals. VON WARTBURG AND SCHURCH[19], have reported the existence of atypical human liver alcohol dehydrogenase in 20% of a Swiss and 4% of a London population. The typical and atypical alcohol dehydrogenase enzymes differed in their susceptibility to inhibition by pyrazole. At an ethanol concentration of $1.6 \cdot 10^{-2}$ M, pyrazole ($4 \cdot 10^{-5}$ M) inhibited normal alcohol dehydrogenase by 50% while the atypical enzyme was inhibited by 86%[19]. In the current study, the human liver formaldehyde dehydrogenase preparation which was inhibited by pyrazole was as sensitive to inhibition by 1,10-phenanthroline as was the formaldehyde dehydrogenase preparation which was not inhibited by pyrazole. This is in contrast to studies done on alcohol dehydrogenase[19] where the I_{50} for o-phenanthroline was $6.7 \cdot 10^{-5}$ M for the normal enzyme and $3.3 \cdot 10^{-4}$ M for the atypical enzyme. However, at this point one cannot conclusively state that an atypical human liver formaldehyde dehydrogenase exists.

Pyrazole was shown to be useful in the study of aldehyde dehydrogenase when this enzyme is contaminated by alcohol dehydrogenase. In the presence of alcohol dehydrogenase, aldehyde dehydrogenase activity is masked due to the cycling of pyridine nucleotides. Pyrazole inhibits alcohol dehydrogenase without affecting human hepatic aldehyde dehydrogenase and, thus, provides a means of determining the actual amount of aldehyde dehydrogenase in preparations containing a high level of alcohol dehydrogenase activity.

Formaldehyde dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase are 3 functionally related enzymes. Aldehydes serve as substrates for these enzymes and a product of the alcohol dehydrogenase reaction, acetaldehyde, is a substrate for aldehyde dehydrogenase. It is quite interesting that these human liver dehydrogenases remained together after a variety of purification procedures. Only by employing a procedure involving adsorption of calcium phosphate gel between chromatography on DEAE-cellulose and on CM-cellulose columns was formaldehyde dehydrogenase freed from alcohol dehydrogenase and aldehyde dehydrogenase. The molecular weight of the purified human liver formaldehyde dehydrogenase was estimated at 90000. However, when a relatively crude preparation of human liver formaldehyde dehydrogenase which also contained alcohol dehydrogenase and aldehyde dehydrogenase was applied to a Sephadex G-200 column, formaldehyde dehydrogenase was eluted in a volume indicating its molecular weight to be over 250000. The aldehyde dehydrogenase and about 10% of the alcohol dehydrogenase present in these prepara-

tions were also eluted in the same volume as formaldehyde dehydrogenase. The remainder of the alcohol dehydrogenase was eluted in a volume indicating its molecular weight to be about 80 000. The molecular weight of purified human liver alcohol dehydrogenase has been reported to be 87 000. These data suggest the possibility that formaldehyde dehydrogenase, alcohol dehydrogenase and aldehyde dehydrogenase exist in a multifunctional enzyme complex within the soluble fraction of the human hepatic cell. This would explain the difficulty encountered in isolating these dehydrogenases and the observation that in a purified preparation the molecular weight of formaldehyde dehydrogenase was estimated at 90 000, while in relatively crude preparations in which alcohol dehydrogenase and aldehyde dehydrogenase were also present the molecular weight of formaldehyde dehydrogenase was estimated at over 250 000. The physical association of enzymes located in the cytosol as multifunctional enzyme complexes has been reported to exist in bacteria[22-24]. It is possible that this occurs in mammals, also. However, it is also possible that formaldehyde dehydrogenase dissociates into subunits during the final stages of the purification procedure and that this accounts for the discrepancy in molecular weight between crude and purified preparations.

A low capacity of human liver to oxidize formaldehyde could have at least partially explained the unique susceptibility of man to methanol poisoning. This was not the case. The total amount (units/g liver) of formaldehyde dehydrogenase in human liver was found to be about 50% higher than in rat liver. In addition, the capacity for NAD^+-linked formaldehyde oxidation which is not dependent upon GSH was several fold higher (in terms of units/g liver and specific activity) in human liver as compared to rat liver. While it is possible that there are other pathways for formaldehyde disposition which are operative to a greater extent in rat liver, on the basis of these studies it appears that the conversion of formaldehyde to formate can proceed more readily in human liver than in rat liver. Perhaps, the explanation for the susceptibility of man to methanol poisoning lies in the formate utilization step. Previous studies from this laboratory have shown a minimal capacity for hydrogen peroxide generation in human liver[25]. If formate oxidation relies on a peroxidative mechanism as has been suggested[26,27] the toxicity of methanol may be explained on the basis of a lack of peroxidative oxidation in human liver.

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