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DIMETHYLAMINOETHANOL—AN IMPROBABLE SUBSTRATE *IN VIVO* FOR ALCOHOL DEHYDROGENASE IN THE RAT*

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Abstract—Dimethylaminoethanol (DMAE) was studied as a possible substrate for liver alcohol dehydrogenase (ADH) *in vivo* and *in vitro*. Although kinetic studies showed that DMAE compared favorably as a substrate with ethanol (EtOH) for horse and rat liver ADH at pH 9·5, experiments with fasted male Sprague–Dawley rats indicated that the rate of elimination of $^{14}CO_2$ from [1-^{14}C]ethanol (1·08 to 16·3 m-moles/kg i.p.) was not altered significantly by the injection (i.p.) of DMAE (at molar ratios DMAE/EtOH of 0·61 to 10·3); nor was the metabolism of [1, 2-$^{14}C_2$]DMAE (2 and 5 m-moles/kg) affected by concurrent administration of ethanol (21·7 and 43·4 m-moles/kg), as shown by the unaltered urinary excretion pattern of radioactive compounds; the greater part of injected DMAE is eliminated unchanged. These results were supported by the finding that while both horse and rat liver ADH gave K_m and V_{max} values for ethanol at pH 7·0 comparable to values obtained at pH 9·5, no activity at pH 7·0 was observed with DMAE as substrate. DMAE thus appears an unlikely substrate *in vivo* for rat and horse liver ADH.

The abundance of liver alcohol dehydrogenase (ADH; alcohol: NAD oxidoreductase, EC 1.1.1.1) in animals with little contact with substantial quantities of ethanol and the relatively low substrate specificity of this enzyme, which reacts with a variety of primary and secondary alcohols, aldehydes and ketones, raise the possibility of physiological substrates other than ethanol for liver ADH [1, 2].

Recently Winer [3] reported that 2-dimethylaminoethanol (DMAE) compares favorably with ethanol as a substrate for horse liver ADH *in vitro*; since this substance is a precursor of the phospholipid, phosphatidyl choline, the possible involvement of DMAE in ethanol oxidation *in vivo* in the rat was studied by us.

Our results indicate that DMAE is, indeed, an effective substrate for horse liver ADH and, as we show here, for rat liver ADH at pH 9·5. However, it has no significant effect on the oxidation of ethanol in the rat nor is it a substrate for horse or rat liver ADH at physiological pH *in vitro*. The fact that the major part of the substantial quantities of DMAE injected into rats in our experiments is not metabolized is not, of course, inconsistent with the fact that far lesser amounts of DMAE may be involved in DMAE's role as a physiological substrate in other non-ADH reactions.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (Charles River, CD), weighing 200–250 g were used. The animals were maintained on Purina Laboratory Chow and were fasted overnight (16 hr) before use. [1-^{14}C]ethanol (specific activity, 12·3 mCi/m-mole) and [1,2-$^{14}C_2$]DMAE (specific activity, 3·64 mCi/m-mole; as the hydrochioride) were obtained from New England Nuclear and diluted appropriately with cold ethanol or DMAE immediately prior to use. Unlabeled DMAE was purchased from Matheson, Coleman & Bell; *N*-methylaminoethanol (MMAE; practical grade, Eastman) was distilled under nitrogen prior to use. Horse liver ADH, NAD and NADH were from Boehringer Mannheim Corp.; the ADH, a crystalline suspension consisting largely of the isoenzyme EE [4], was exhaustively dialyzed against 0·05 M sodium phosphate buffer, pH 7·5. Rat liver ADH, prepared according to the method of Markovic *et al.* [5], was kindly supplied by Miss F. Chen and Dr. R. Pietruszko of this Center.

Determination of the rate of ethanol oxidation in vivo. The rate of ethanol oxidation was estimated from the rate of radioactive CO_2 production after the intraperitoneal injection of [1-^{14}C]ethanol, approximately 1 μCi/kg as a 1 or 10 per cent (w/v) solution in isotonic saline, at doses shown in Table 1. Ethanol was given

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Table 1. Ethanol oxidation in the rat with or without 2-dimethylaminoethanol*

[1-^{14}C]ethanol (m-moles/kg i. p.)	DMAE (m-moles/kg i. p.)	Ratio of DMAE to ethanol	Ethanol oxidation (m-moles/kg/hr ± S.D.) Control	With DMAE
1·08	9·95	9·95	0·59 (1)†	0·64 (1)
2·17	2·24–22·4	1·03–10·3	1·09 ± 0·09 (8)	1·00 ± 0·08 (8)
16·30	9·95	0·61	3·40 (1)	3·33 (1)

* 2-Dimethylaminoethanol as a 10% (w/v) solution with pH adjusted to 7·4 was given intraperitoneally 15 min prior to the administration of [1-^{14}C]ethanol, approximately 1 μCi/kg as a 1% or 10% (w/v) solution in saline. The highest dose of DMAE borders on the lethal, exceeding the reported LD_{50} in Sprague–Dawley rats [12].

† Number of experiments.

15 min after the intraperitoneal injection of DMAE (see Table 1). The rat was placed in a glass respiratory chamber (model 350-C, Aerospace Industries, Inc.) through which CO_2-free air at 1 liter/min was drawn. Expired CO_2 was collected in approximately 40 ml of an ethanolamine–methanol mixture (1 : 4, v/v) for successive 15-min periods over 2–3 hr; the mixture absorbed CO_2 completely. After adding methanol to the absorbent mixture to a final volume of 50 ml, the radioactivity of a 5-ml aliquot, made up to 15 ml with toluene scintillation solution containing 42 ml Liquifluor (Intertechnique) per liter of toluene, was measured in an Intertechnique liquid scintillation spectrometer (model SL30). The counting efficiency of each sample was determined by an external standards ratio technique which had been checked with an added [^{14}C]toluene internal standard; all calculations were based upon the computed radioactivity as dis./min. The rate of ethanol metabolism was computed from the maximal rate of radioactive CO_2 elimination, calculated from the maximal slope of the best fitting polynomial of the data. The slope than allowed calculations of the minimum time for elimination of all radioactivity: dividing dose by time gave the rate of ethanol disposition as m-moles/kg/hr. Although the rate so obtained is somewhat less than the value for ethanol disappearance estimated from analysis of blood ethanol, the values obtained give parallel measures of the rates of oxidation and allow comparisons in the absence and presence of injected DMAE.

Urinary analysis of DMAE. Rats were given intraperitoneally 2 or 5 m-moles/kg of [1,2-$^{14}C_2$]DMAE (molar solution, pH adjusted to 7·4; 5 μCi/kg) with or without 21·7 or 43·3 m-moles/kg of ethanol (10% solution, w/v in saline). When both agents were administered, ethanol was given 5 min before the injection of DMAE.

A metabolic chamber was used for urine collection. One ml of the sample was diluted with 14 ml Triton X-100–toluene–Liquifluor mixture (65 : 31 : 4, v/v) and counted for total radioactivity. DMAE and possible metabolites were analyzed by paper and gas–liquid chromatography.

For paper chromatography, Whatman No. 4 filter paper and a solvent system consisting of methanol–diethyl ether–HCl–water (10 : 10 : 1 : 3, v/v) were employed. With this solvent system, an R_f value of 0·5 was observed for DMAE. After development, the chromatogram was divided into 1-cm segments and the radioactivity of the segments was directly counted in the toluene–Liquifluor solution. In some cases, the paper strips were eluted with 50% ethanol in an elution chamber; the eluates were then counted. Direct counting gave 80 per cent recovery; essentially 100 per cent recovery was obtained by the elution technique.

For gas–liquid chromatography, a 5 ft × $\frac{1}{8}$ inch (o.d.) stainless steel column, filled with a 60/80 Gas Chrom Q support coated with 30 per cent by weight of a 16% Amine 220 and 84% Apiezon L mixture, was used at 90° with a hydrogen flame ionization detector; the carrier gas was nitrogen. Under our conditions, the DMAE peak appeared in about 65 sec. Urine diluted with water was injected into the chromatograph; in the urine of the fasted rat, at the attenuation and dilution employed, no peak other than that of DMAE was detected.

Determination of the rate of ethanol oxidation by rat liver homogenate. The method used was a modification of Lundquist *et al.* [6]. Rat liver was homogenized with 5 vol. of 0·1 M phosphate buffer, pH 7·1. Nicotinamide solution (1 M) was added to the buffer solution immediately before the homogenization to give a final concentration in the suspension of 0·04 M. NAD (20 mg/ml) was then added to a final concentration of 0·2 mM. One ml of the suspension was pipetted into each test tube and ethanol was added to give a final concentration of 9 mM. The sealed tubes were then shaken mechanically in a water bath at 23°. Tubes were removed at 15-min intervals and deproteinized with 1 ml of 5·1% perchloric acid. The supernatant solution was used for the determination of ethanol by the yeast ADH method [7]. In experiments where the effects of DMAE (10 mM) and pyrazole (1 mM) were examined, these substances were added 10 min prior to the addition of ethanol. Although there is some inhibition of yeast ADH by pyrazole [8, 9], under our experimental conditions, neither pyrazole nor DMAE interfered with the ethanol determination.

Measurement of enzyme activities. The initial velocities of the oxidation of substrates (ethanol, DMAE or MMAE) were determined at constant NAD concentration (0·5 mM) and various substrate concentrations in 0·1 M phosphate buffer, pH 7·0, or in 0·1 M glycine–NaOH buffer, pH 9·5, by monitoring the appearance of NADH on a Beckman DBGT recording spectrophotometer at 25°. Concentration of enzyme was expressed as the normality, N (NADH-binding sites/liter), and was determined fluorometrically by titration with NADH in the presence of 0·1 M isobutyramide, according to the method of Winer and Theorell [10]. The concentration of NADH was calculated from its extinction coefficient of 6·22 mM^{-1} cm^{-1} at 340 nm [11].

The lines of Figs. 1–4 were computed by the method of least squares utilizing all experimental values, except those for DMAE at substrate inhibitory concentrations (see Fig. 2).

RESULTS AND DISCUSSION

The effects of DMAE on ethanol oxidation in rats are presented in Table 1. At doses of DMAE in the range of 2·24 to 22·4 m-moles/kg, the rates of ethanol oxidation for the control vs. the DMAE-treated rats were found to be 0·59 vs. 0·64, 1·09 vs. 1·00 and 3·40 vs. 3·33 m-moles/kg/hr at ethanol levels of 1·08, 2·17 and 16·3 m-moles/kg respectively. If DMAE is indeed a substrate of ADH, one would expect its competition with the oxidation of ethanol to result in lower ethanol oxidation rates. We observed no significant differences between DMAE-treated and control rats when the ratio of DMAE to ethanol was varied from 0·61 to 10·3, making DMAE an unlikely substrate *in vivo* for liver ADH.

Furthermore, in the first 6 hr after intraperitoneally administered [1,2-$^{14}C_2$]DMAE (2 and 5 m-moles/kg), less than 1·5 per cent appeared as $^{14}CO_2$ in expired air and about 60 per cent of the radioactivity appeared in the urine, essentially all as unchanged DMAE. These values were determined by gas–liquid and paper chromatographic analyses, irrespective of the presence or absence of ethanol (21·7 and 43·4 m-moles/kg). The radioactivity which remained in the body after 6 hr was excreted thereafter in the urine at a much slower pace, and at 72 hr a total of 75 per cent was recovered, again virtually all as unchanged DMAE.

Because the ineffectiveness of DMAE on ethanol metabolism and the ineffectiveness of ethanol on DMAE metabolism may result from DMAE not being transported to the liver cytosol, experiments were performed with rat liver homogenates. The results of these experiments are presented in Fig. 1. After 60 min of incubation, the ethanol concentration in both control and DMAE-treated homogenates dropped to 6·6 mM. As in the case *in vivo*, the presence of DMAE did not alter the rate of ethanol disappearance, which remained at 15 μmoles/g of liver/hr, far greater than the inhibited rate seen in the presence of pyrazole, a strong competitive inhibitor of ADH.

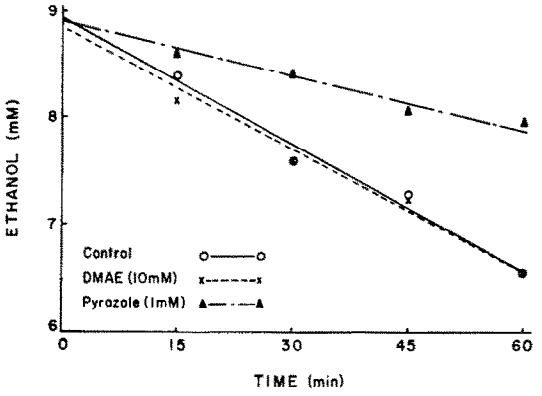

Fig. 1. Ethanol metabolism in rat liver homogenates with or without DMAE and pyrazole at 23°. The reaction mixture contained 1 ml liver homogenate (equivalent to 0·2 g liver), 10 μl each of ethanol (900 mM) and DMAE (1 M) or pyrazole (100 mM). In control experiments, 10 μl of 0·1 M phosphate buffer, pH 7·1, was substituted for DMAE or pyrazole. Each point shown is the mean of duplicate determinations.

Since these experiments *in vivo* and *in vitro* did not appear to agree with Winer's report [3] that DMAE was a substrate of ADH, we re-examined Winer's results with both horse liver ADH and rat liver ADH in 0·1 M phosphate buffer, pH 7·0, and in 0·1 M glycine–NaOH buffer, pH 9·5. The results are presented in Figs. 2 and 3 and Table 2.

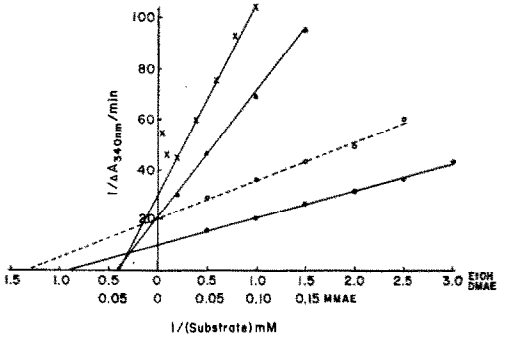

Fig. 2. Kinetics of oxidation catalyzed by horse liver alcohol dehydrogenase. The reaction mixture contained 2 ml of 0·75 mM NAD in buffer plus 1 ml buffer with varying amounts of substrates (if necessary, adjusted to proper pH). The total volume of 3 ml was equilibrated at 25°, then 10 μl enzyme (14·4 μN) was added to initiate the reaction. ——, 0·1 M glycine–NaOH buffer, pH 9·5; – – –, 0·1 M phosphate buffer, pH 7·0; (○) ethanol, concentration varying from 0·4 to 2·0 mM; (●) ethanol, concentration varying from 0·33 to 2·0 mM; (X) DMAE, concentration varying from 1·0 to 20·0 mM; (▲) MMAE, concentration varying from 6·67 to 50·0 mM.

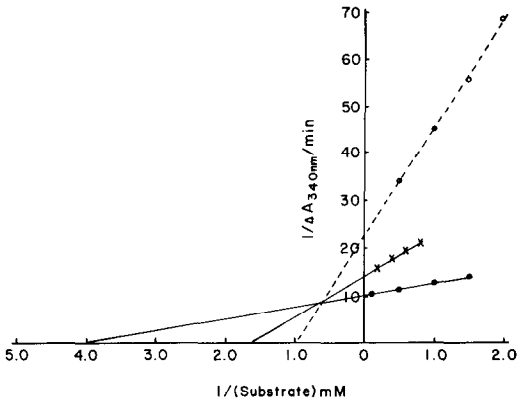

Fig. 3. Kinetics of oxidation catalyzed by rat liver alcohol dehydrogenase. The reaction mixture contained 2 ml of 0·75 mM NAD in buffer plus 1 ml buffer with varying amounts of substrate (if necessary, adjusted to proper pH). The total volume of 3 ml was equilibrated at 25°, then 10 μl enzyme (66·5 μN) was added to initiate the reaction. ---, 0·1 M glycine NaOH buffer, pH 9·5; ----, 0·1 M phosphate buffer, pH 7·0; (○) ethanol, concentration varying from 0·5 to 2·0 mM; (●) ethanol, concentration varying from 0·67 to 10·0 mM; (X) DMAE, concentration varying from 1·25 to 5·0 mM.

K_m and V_{max} values of horse liver ADH for ethanol, 0·76 mM and 327 moles/liter/min/mole of ADH at pH 7·0, and 1·09 mM and 665 moles/liter/min/mole of ADH at pH 9·5, are in good agreement with those of Winer [3] and others [13, 14].

Ethanol was also used as a substrate for the kinetic studies of rat liver ADH, and a K_m value of 1·02 mM at pH 7·0 was obtained, higher than the reported value of 0·5 mM [15]. At pH 9·5, the K_m value was found to be 0·24 mM, which is much lower than reported values of 2·13 mM (pH 10) [5] and 40 mM (pH 8·8) [16]. These discrepancies cannot be explained; however, the instability of the enzyme, varying purities of the preparation and the possible existence of isoenzymes might be contributing factors.

K_m and V_{max} values of DMAE and MMAE at pH 9·5 for horse liver ADH were found to be 2·6 and 24·0 mM, and 226 and 323 moles/liter/min/mole of ADH respectively. Substrate inhibition occurred when the DMAE concentration was above 5 mM. Although these observation differ somewhat from Winer's [K_m = 1·2 and 13·0 mM; V_m = 135 and 160 moles/l/min/mole of ADH and substrate (DMAE) inhibition, > 10 mM] [3], they are of the same magnitude. For rat liver ADH, at pH 9·5, the K_m and V_{max} values of DMAE were 0·59 mM and 101 moles/l/min/mole of ADH respectively. However, at pH 7·0, there is no activity with either horse or rat liver ADH toward DMAE as substrate, or for horse liver ADH toward MMAE. Kinetic values of DMAE or MMAE as substrates of ADH at pH 7·0 have not been reported heretofore.

These extreme differences in activity of DMAE (and MMAE) at pH 7·0 and pH 9·5 may result from the ionization characteristics of DMAE and the hydrophobic substrate binding site of ADH [17, 18]. With pK_a values for DMAE of 9·15 [19] or 9·31 [20] and for MMAE of 9·77 [21], it is reasonable to assume that at pH 7·0 they are almost completely protonated, carrying positive charges, while at pH 9·5 they probably exist in the uncharged state. Since the substrate binding site is hydrophobic, a charged substrate will have difficulty entering the binding site, and no activity will be observed at pH 7·0. Contrariwise, at pH 9·5, uncharged DMAE or MMAE will be able to enter the hydrophobic binding site and reaction can proceed. Although we did not use a non-phosphate buffer at pH 7·0 to exclude the possible inhibitory action of phosphate, the agreement of the results *in vitro* (using a phosphate buffer) with the measures *in vivo* and the non-inhibition of ethanol oxidation in phosphate buffer at pH 7·0 give little support for such an alternative.

The product formed in the liver ADH-catalyzed reaction of DMAE at pH 9·5 was not studied, but it is assumed to be the aldehyde of DMAE. Double reciprocal plots of the initial rates versus DMAE concentrations were made at two NAD concentrations (0·1 and 0·5 mM) and are shown in Fig. 4; since the two

Table 2. Michaelis constants (K_m) and maximal velocities (V_{max}) of liver alcohol dehydrogenase for different substrates at pH 7·0 and 9·5*

Enzyme	Substrate	K_m (mM) pH 7·0	K_m (mM) pH 9·5	V_{max} (moles/l./min/mole ADH) pH 7·0	V_{max} (moles/l./min/mole ADH) pH 9·5
Horse liver ADH	Ethanol	0·76	1·1	327	665
	DMAE	ND†	2·6	0	226
	MMAE	ND	24·0	0	323
Rat liver ADH	Ethanol	1·02	0·24	64	140
	DMAE	ND	0·59	0	101

* K_m and V_{max} were determined by double reciprocal plots of the rate of NADH increase vs. substrate concentration, with a minimum of three determinations at each of four to seven concentrations (Figs. 2 and 3).

† ND = not determinable.

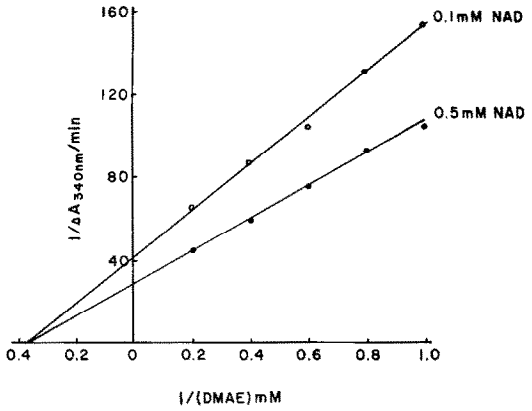

Fig. 4. Kinetics of oxidation catalyzed by horse liver alcohol dehydrogenase at pH 9·5 with DMAE at two concentrations of NAD. The reaction mixture contained 2 ml of 0·75 or 0·15 mM NAD in 0·1 M glycine–NaOH buffer, pH 9·5, and 1 ml of the same buffer with varying amounts of substrate; if necessary, the pH was adjusted to 9·5. The total volume of 3 ml was equilibrated at 25°, then 10 μl enzyme (14·4 μN) was added to initiate the reaction. The concentration of DMAE was varied from 1·0 to 5·0 mM.

lines have a common intersection on the substrate axis with different slopes, the reaction appears to follow a random mechanism [22]. The reaction does not seem to follow the Theorell–Chance mechanism, a compulsory mechanism involved in the reversible oxidation of primary, secondary and cyclic alcohols [18, 23, 24]. However, more detailed kinetic experiments with submaximal concentrations of the constant reaction partner need to be carried out and subjected to the Dalziel [25] tests, which distinguish the Theorell–Chance mechanism from other alternatives.

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