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HUMAN LIVER ALCOHOL DEHYDROGENASE—INHIBITION OF METHANOL ACTIVITY BY PYRAZOLE, 4-METHYLPYRAZOLE, 4-HYDROXYMETHYLPYRAZOLE AND 4-CARBOXYPYRAZOLE*

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Abstract—With human liver alcohol dehydrogenase of high purity at pH 7·0 and 500 μM NAD the K_m for methanol is 7·0 mM (ten times greater than the K_m for ethanol) and the turnover number 1·4/active site/min (about one-tenth of the turnover with ethanol in the same conditions). From secondary kinetic plots it can be calculated that at saturating concentrations of both substrates, namely methanol and NAD, these constants do not change appreciably: the K_m for methanol is somewhat lower (5·2 mM) and the turnover number slightly higher (1·7/active site/min). The difference in turnover numbers with methanol and ethanol as substrates suggests that the kinetic mechanism for methanol is different from that for ethanol dehydrogenation. The dissociation constant between human alcohol dehydrogenase and NAD, determined kinetically with methanol as substrate, is 127 μM. The K_i values for pyrazole, 4-methylpyrazole and 4-hydroxymethylpyrazole are 0·54, 0·09 and 6·6 μM respectively; 4-carboxypyrazole (100 μM) at 3 mM methanol does not inhibit human ADH. The inhibitory effect of 4-methylpyrazole is therefore not likely to be enhanced by a possible metabolic conversion to 4-hydroxymethylpyrazole and 4-carboxypyrazole.

Pyrazole and some of its derivatives are potent inhibitors of purified horse liver alcohol dehydrogenase (ADH) [1], human liver ADH [2] and rat ADH [3] with ethanol as substrate. These inhibitors form slowly dissociating ternary complexes with ADH and NAD [1, 2] and act as competitive inhibitors with respect to ethanol. Yeast ADH is also inhibited by pyrazole [3] but to a lesser extent than the corresponding mammalian liver enzymes. Pyrazole is also known to inhibit ethanol [4, 5] and methanol [6] metabolism *in vivo* in the rat. The low K_i values of ADH with pyrazole (10^{-6} M) and the even lower values with some of substituted pyrazoles (10^{-7} M), together with the effectiveness of these inhibitors *in vivo*, suggest the possibility of their therapeutic use after intake of methanol to prevent the formation of the more toxic products of methanol metabolism. Current studies in animals have shown that pyrazole, in the dosage required for effective inhibition of ethanol metabolism, is itself toxic and produces undesirable side effects [7]. Substitution in position 4 by alkyl groups [8] yields compounds of lower toxicity, and in the case of the 4-methyl compound increases its effectiveness as an ADH inhibitor [1–3]. Some studies with 4-methylpyrazole *in vivo* have been carried out in rats and humans [9]. A recent study [10] has demonstrated that in mice 4-methylpyrazole is metabolized to 4-hydroxymethylpyrazole and 4-carboxypyrazole. In the present study the effect of pyrazole, 4-methylpyrazole and its metabolites on human liver ADH of high purity, employing methanol as substrate, has been investigated.

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MATERIALS AND METHODS

Since ADH from human liver, even at saturating concentrations of the respective substrates, is only about one-tenth as active with methanol as with ethanol [11–13], extreme precautions have to be taken to avoid contamination with higher alcohols and aldehydes which, due to their higher affinity for the enzyme, can appreciably alter kinetic data.

Water used in all experiments was distilled from alkaline permanganate and used fresh, as it was subject to aldehyde contamination from the surrounding air. Na_2HPO_4 and NaH_2PO_4 (E. Merck AG, Germany) were used as purchased. Buffers were kept in tightly stoppered flasks, but were usable with these precautions for no longer than 1 week. Just before kinetic determinations, the reactants were tested for aldehyde contamination employing yeast alcohol dehydrogenase (Boehringer und Soehne GmbH, Germany) and NADH (Sigma Chemical Co., U.S.A.). NAD (Grade III, Sigma) was purified on a diethylaminoethyl cellulose column (DE-11, W. Balston Ltd., England) according to the procedure described by Dalziel [14] employing buffers in water distilled from alkaline permanganate. NAD solutions were stored frozen at −18° and were suitably diluted before use. Methanol (E. Merck AG, Germany) was purified by fractional distillation using a Podbielniak fractionating column until it contained no traces of formaldehyde (tested by the chromotropic acid method [15]). Pyrazole, 4-methylpyrazole, 4-hydroxymethylpyrazole and 4-carboxypyrazole were a gift from Astra Chemical Co. (Sweden) and were used without further purification. Carboxymethyl cellulose (CM-11 and CM-32) was purchased from W.H. Balston Ltd., England.

Sephadex G-100 was obtained from Pharmacia, Sweden. Human livers, obtained 24 hr after death, were either extracted immediately or kept frozen at −18°. Normality of the enzyme was determined by titration with standardized NADH in the presence of 0·1 M isobutyramide in phosphate buffer, $\mu = 0{\cdot}1$, pH 7·0 [16], using a recording fluorometer at the excitation wavelength of 330 nm and emission wavelength of 410 nm. Protein concentration was measured at 280 nm as described by Mourad and Woronick [17] employing their extinction coefficient (0·61 A/mg/ml of solution in 1-cm cuvettes). Concentrations of NAD and NADH were determined by employing yeast ADH and ethanol or acetaldehyde [18] and calculated from the extinction coefficient for 1 mM NADH (6·22 A at 340 nm in 1-cm cuvettes). Kinetic constants were determined from double-reciprocal plots. Measurements of initial rates of enzyme activity were made with a recording fluorometer [19] modified to yield enchanced stability and sensitivity [20]. Fluorescence-free glass cuvettes were used. The rates of enzymic reaction are expressed in turnover numbers × active site^{-1} × min^{-1} unless stated otherwise; these measurements were made in phosphate buffer, $\mu = 0{\cdot}15$, pH 7·4 at 23·5°. The enzyme concentration was 0·4 μN throughout.

RESULTS

Preparation of the enzyme. The enzyme was first prepared according to the method of Mourad and Woronick [17], including their crystallization procedure. This preparation, however, contained an appreciable amount of an inert protein impurity which could be readily visualized by electrophoresis on starch gels. The enzyme was therefore purified further by chromatography on Sephadex G-100 and carboxymethyl cellulose (CM-32). After this additional purification, no inert protein could be detected in the purified ADH. The enzyme consisted of four electrophoretically distinct components (bands 1, 2, 3 and 4 [20] present in approximately equal amounts; bands 5, 6 and 7 were not present). From the normality data, the published mol. wt, 80,000 [21], and the assumption of two catalytic sites per molecule, the protein concentration was calculated and found to be in excellent agreement with that obtained from direct measurement at 280 nm. In glycine buffer, 0·062 M, pH 10·0, at 500 μM NAD the enzyme had a turnover number with ethanol (V) of 90 × active site^{-1} × min^{-1}; the turnover number for methanol was 8 × active site^{-1} × min^{-1}. At pH 7·0 in 0·1 M phosphate buffer at 500 μM NAD, the turnover number for ethanol (V) was 16·7 × active site^{-1} × min^{-1} and the turnover number for methanol was 1·4 × active site^{-1} × min^{-1}.

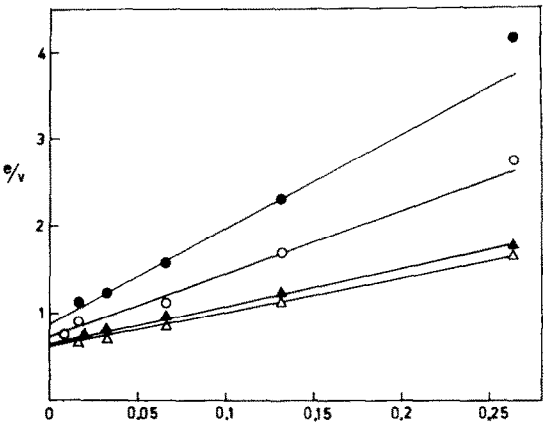

Fig. 1. Lineweaver–Burk plots. Variation of the reciprocal of the initial rate of methanol dehydrogenation with the reciprocal of methanol concentration at several constant concentrations of NAD (μM): ●, 50; ○, 100; ▲, 250; △, 500, at pH 7·4 and 23·5°. e = Concentration of ADH active sites, μN; v = NADH formed, μM/min.

Kinetics in the absence of inhibitors. For determination of kinetic constants in the absence of inhibitors, the reaction was started by addition of the enzyme and followed by continuous recording.

At low concentrations of methanol (up to 31 mM), the reaction progress curves (fluorescence increase × time) appeared normal (concave downward), but at high methanol concentrations (above 61 mM) concave (upward) curves were obtained independently of whether methanol or the enzyme was added last. No explanation for this effect is known. If the enzyme exhibits a hysterectic property [22], it has not been observed with substrates other than methanol; normal curves are produced with ethanol as substrate. If the rate were measured about 1 min after addition of the enzyme, the reciprocals fell on the line of the Lineweaver–Burk plots obtained by measurement of the initial rates at lower concentrations of methanol; these values were, therefore, used.

The primary double-reciprocal plots at varying concentrations of methanol and four different concentrations of NAD (Fig. 1) show that the mechanism of methanol dehydrogenation is sequential. The calculated Dalziel [23] constants derived from these plots are listed in Table 1. Thus the K_m (NAD) at the saturating concentrations of methanol is 24 μM. The K_m (methanol) at saturating concentrations of NAD is 5·2 mM and the turnover number at saturating concentrations of both substrates is 0·028 × active site^{-1} × sec^{-1}. At 500 μM NAD, used in inhibition experiments, the K_m for methanol was 7·0 mM and the turnover number was 0·023 × active site^{-1} sec^{-1}. The corresponding set of constants for the reaction

Table 1. Kinetic constants derived from secondary plots of slopes and intercepts of Fig. 1

Intercept of intercept plot	ϕ'_0	35·4 sec
Slope of intercept plot	ϕ'_1	846 × sec × μM
Intercept of slope plot	ϕ'_2	184,200 × sec × μM
Slope of slope plot	ϕ'_{12}	23,202,000 × sec × μM
K_m NAD at saturating methanol	ϕ'_1/ϕ'_0	24 μM
K_m methanol at saturating NAD	ϕ'_2/ϕ'_0	5,200 μM
V at saturating methanol and NAD	$1/\phi'_0$	0·028 × active site^{-1} × sec^{-1}

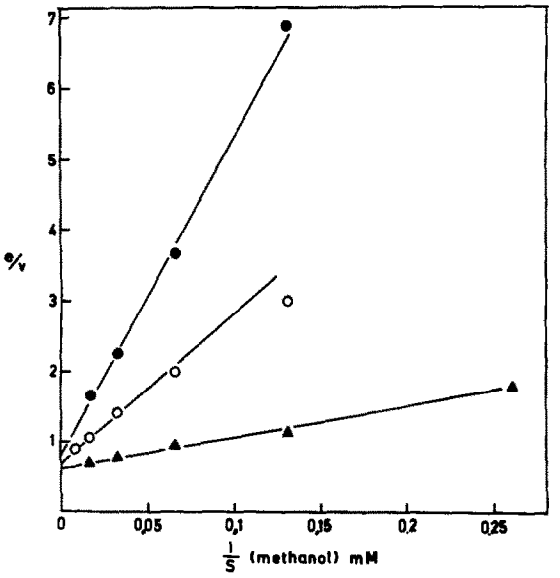

Fig. 2. Variation of the reciprocal of the initial rate of methanol dehydrogenation with the reciprocal of methanol concentration in the presence of pyrazole (μM): △, 0·0; ○, 1·0; ●, 5·0, at pH 7·4 and 23·5° with 500 μM NAD. e = Concentration of ADH active sites, μN; v = NADH formed, μM/min.

of NADH with formaldehyde to form NAD and methanol is not available, mainly because of difficulty in obtaining formaldehyde of sufficient purity.

Kinetics in the presence of inhibitors. In the presence of pyrazoles, inhibition could not be readily demonstrated if the enzyme was added last; the initial rate of the reaction was indistinguishable from that in the absence of pyrazole, and only after about 4 min was the rate observed to be less than that in the control. This effect was previously observed with horse liver ADH [1] and human liver ADH [2] with ethanol as substrate and results from the slow rate of formation of ternary complexes of pyrazoles with ADH and NAD. For the measurement of the rate in the presence of inhibitors, the reaction was started by addition of methanol. In this case, concave (upward) curves were produced, with the rate gradually increasing and giving a true measurement of the extent of inhibition after 4 min of recording.

Inhibition by pyrazole. In Fig. 2, pyrazole inhibition of methanol dehydrogenation is presented at pyrazole concentrations of 1 and 5 μM. The K_i for pyrazole, calculated from three separate determinations (0·66, 0·56, 0·40 μM) is 0·54 μM. The intersection of the slopes at the Y-axis indicates that pyrazole is competitive with respect to methanol, as with ethanol and horse [1] and human liver ADH [2]. However, with methanol as substrate, the K_i value is about five times less than that determined with ethanol [2].

Inhibition by 4-methylpyrazole. Figure 3 shows inhibition of human liver ADH in the presence of methanol by 4-methylpyrazole; although the slopes do not intersect at the Y-axis, the inhibition may still be competitive because of the great inhibition exercised by 4-methylpyrazole (K_i = 0·09 μM), five times as effective as pyrazole under the same conditions (Fig. 3). At 500 μM NAD, the K_m for methanol is 7·0–8·0 mM or about 80,000 times greater than the K_i, so that the inhibition by 4-methylpyrazole cannot be fully reversed. A similar phenomenon had been observed by Li and Theorell [2] when ethanol was employed as substrate with the strongly binding 4-iodopyrazole; the K_m for ethanol established by Li and Theorell [2] was 0·45 mM, substantially less than that found for methanol here.

Since the K_i value is about four times less than the enzyme concentration (enzyme, 0·4 μM; K_i, 0·09 μM), depletion of the free inhibitor may occur, invalidating the Michaelis–Menton derivation. However, replot of data according to the procedure of Henderson [24], specifically divised for such cases, has shown that the inhibition by 4-methylpyrazole is competitive. Employing the Henderson procedure, deviations from linearity were observed with 4-methylpyrazole.

Inhibition by 4-hydroxymethylprazole. Inhibition of human liver ADH by 4-hydroxymethylpyrazole is presented in Fig. 4; the K_i is 6·6 μM and the inhibition is competitive with respect to methanol. 4-Hydroxymethylpyrazole is therefore a much less potent inhibitor of the enzyme than either pyrazole itself or 4-methylpyrazole. It should be noted that 4-hydroxymethylpyrazole is itself a substrate for human liver ADH; NADH was produced from NAD and 4-hydroxymethylpyrazole in excess of that in the control containing NAD and enzyme. From the preliminary data, the K_m for 4-hydroxymethylpyrazole is approximately 200 μM and V is 0·9 × active site^{-1} × min^{-1}, approximately 50 per cent of the V for methanol.

4-Carboxypyrazole at concentrations of 25 and 100 μM with 500 μM NAD at methanol concentrations ranging between 3·0 and 30 μM did not inhibit human liver ADH.

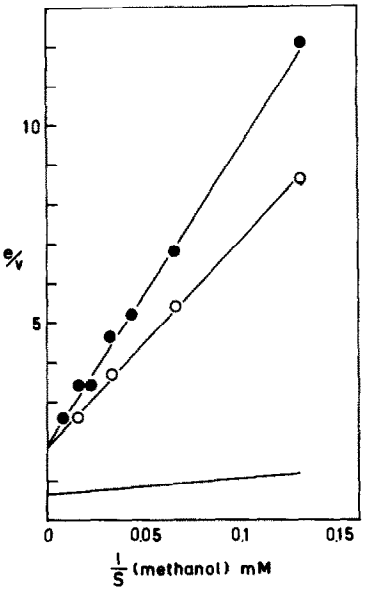

Fig. 3. Variation of the reciprocal of the initial rate of methanol dehydrogenation with the reciprocal of methanol concentration in the presence of 4-methylpyrazole (μM): ——, 0·0; ○, 1·0; ●, 1·5, at pH 7·4 and 23·5° with 500 μM NAD. e = Concentration of ADH active sites, μN; v = NADH formed, μM/min.

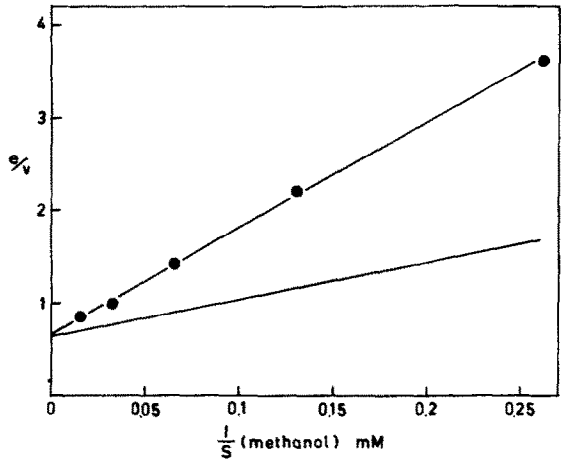

Fig. 4. Variation of the reciprocal of the initial rate of methanol dehydrogenation with the reciprocal of methanol concentration in the presence of 4-hydroxymethylpyrazole (μM): ——, 0·0; ●, 10·0, at pH 7·4 and 23·5° with 500 μM NAD. e = Concentration of ADH active sites, μN; v = NADH formed, μM/min.

DISCUSSION

The mechanism of the reaction of human ADH with NAD and methanol appears to be sequential (Fig. 1) and is probably also ordered, the enzyme combining first with NAD and then with methanol to form the reactive ternary complex. In the case of horse liver ADH, the interconversion of the ternary enzyme–NAD–methanol complex to the enzyme–NADH–formaldehyde complex is the rate-limiting step [25] rather than the dissociation of ADH-bound NADH, which is the rate-limiting step during ethanol oxidation [26]. Inasmuch as the V for methanol with human ADH is less than one-tenth of that with ethanol, some step slower than the dissociation of ADH-bound NADH must occur and this step, in all probability, is also interconversion of the ternary complex. It should be noted that V values for horse and human ADH are similar [12]. Based on these considerations, the reciprocal of the Dalziel constant $1/\phi'_0$ 0·28 × sec^{-1}) probably represents the rate constant for the interconversion of enzyme–NAD–methanol to enzyme–NADH–formaldehyde. If the mechanism is ordered, in that the enzyme combines with NAD before methanol, the ratio of ϕ'_{12}/ϕ'_2 represents a dissociation constant of the enzyme with NAD (the value so calculated, 127 μM, is almost that of horse ADH with NAD, 160 μM [19]). At pH 7·4, at saturating concentrations of NAD the K_m for methanol is 5 mM. This value, ten times greater than that reported for ethanol [2], confirms that methanol is not a good substrate for human ADH. At saturating concentrations of methanol, the K_m for NAD is 24 μM and that determined at 5 mM ethanol by Li and Theorell [2] is 22 μM. K_m values for NAD are therefore similar when determined with either substrate.

Because of the low activity of human ADH with methanol, relatively large concentrations of the enzyme were used (0·4 μM). These concentrations are of an order of magnitude similar to the K_i values for pyrazole and 4-methylpyrazole. Since these two inhibitors are very potent, it was not possible to use inhibitor concentrations greater than 10–15 times the K_i values. As a consequence, it was suspected that depletion of the free inhibitor, due to binding with the enzyme, could not have been negligible. However, application of the equation described by Henderson [24] to the data obtained with pyrazole gave linear replots of slopes and the K_i value calculated was 0·45 μM as compared with 0·54 μM derived from the Michaelis–Menton equation. Both methods also showed that the inhibition with pyrazole is competitive. The K_i value for pyrazole, determined kinetically with methanol as substrate, is five times less than that previously reported [2] determined with ethanol as substrate and also directly. The reason for this discrepancy is not known, but the isozyme composition of enzyme preparations may have been different.

With methylpyrazole, however, although application of the Henderson equation still showed that the inhibition was competitive, the replot of slopes was not linear. The latter did not make it possible to recheck the numerical values of K_i for 4-methylpyrazole. The K_i value with 4-methylpyrazole is less than that with pyrazole and identical with that previously determined with ethanol as substrate [2]. 4-Hydroxymethylprazole, an established mouse metabolite of 4-methylpyrazole, is a much less effective inhibitor of methanol dehydrogenation. 4-Carboxypyrazole could not be demonstrated to inhibit human liver ADH. Inasmuch as 4-hydroxymethylpyrazole is 70 times less effective than 4-methylpyrazole as an inhibitor of human liver ADH, the inhibitory effect of 4-methylpyrazole in man is not likely to be enhanced by a possible metabolic conversion to 4-carboxypyrazole, if the metabolic pathway in man is similar to that in the mouse [10].

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