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Human Liver Alcohol Dehydrogenase: Inhibition by Pyrazole and Pyrazole Analogs

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Alcohol dehydrogenase from human liver has been purified 400-fold and the effects of pyrazole and several of its derivatives examined. Pyrazole, 4-bromopyrazole, 4-methylpyrazole, and 4-iodopyrazole inhibit the enzyme by competing with ethanol. The inhibition constants are 2.6, 0.29, 0.21, and 0.12 μM, respectively. Di- and trisubstituted derivatives are less effective inhibitors than the monosubstituted ones. Pyrazole interacts with the enzyme and coenzyme, NAD^+, to form a ternary complex generating a new absorption maximum at 295 mμ. The values of the dissociation constants for pyrazole and iodopyrazole in their ternary complexes are in good agreement with the respective inhibition constants, indicating that ternary complex formation is the basis of inhibition. The data suggest that these compounds have pharmacologic significance in the study of alcohol metabolism.

Alcohol dehydrogenase from human liver has been purified substantially [1] and recently crystallized.[2] Many of its catalytic, physical and chemical properties are similar to those of the enzyme from horse liver which has been studied in considerable detail.[3] Both enzymes have broad substrate specificity. In addition to ethanol and other longer chain aliphatic alcohols, the human enzyme also oxidizes methanol, monohalo derivatives of ethanol, ethylene glycol, and other diols.[4] These reactions have physiologic as well as pharmacologic significance since their products may have profound metabolic and adverse effects in man. Thus it would be of interest to show that human liver alcohol dehydrogenase ** may be specifically and effectively inhibited, preferably by a compound of low toxicity.

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** Abbreviations: LADH-Liver alcohol dehydrogenase; NAD^+-nicotinamide adenine dinucleotide; $K_{EO,I}$-dissociation constant for inhibitor I in a ternary complex consisting of enzyme, NAD^+, and inhibitor.

In their studies of the inhibitory properties of heterocyclic compounds, Theorell and Yonetani found that pyrazole and some of its substituted derivatives are potent inhibitors of horse LADH competing strictly with ethanol.[5,6] This paper reports the kinetics of inhibition of human LADH by some of these compounds and demonstrates that, as with the horse enzyme, they form inactive ternary complexes by coupling with LADH and the coenzyme, NAD^+.

MATERIALS AND METHODS

Preparation of enzyme. A single liver, obtained at autopsy from a healthy man succumbing to an automobile accident was used. The liver weighed 1.8 kg and appeared normal on gross inspection. LADH was extracted from ground liver with water, and purified by ammonium sulfate precipitation, chloroform-ethanol extraction, and chromatography on carboxymethylcellulose and diethylaminoethylcellulose according to Mourad and Woronick.[2] The enzyme was stored as a precipitate in 80 % ammonium sulfate at pH 8, 4°. Enzyme activity remained stable in this way for several months.

Chemicals. Whatman carboxymethylcellulose (CM 1) and diethylaminoethylcellulose (DE 1) were products of W & R Balston Ltd., England, and prepared for use according to Peterson and Sober.[7] Ethanol, 95 % (Extra Finsprit) was used without further purification. β-NAD^+ (grade III) from Sigma Chemical Co., was 99 % pure. Its concentration was determined spectrophotometrically, using $A_{260}=18.0$ ($mM^{-1}\times cm^{-1}$). Pyrazole was a product of Dr. Theodor Schuchardt Co., Munich, Germany. 4-Bromopyrazole, 4-iodopyrazole, 4-methylpyrazole, 3,4-dibromopyrazole, 3-methyl-4-iodopyrazole, and tribromopyrazole were gifts of Dr. B. Sjöberg, Astra AB, Södertälje, Sweden. All other chemicals were reagent grade.

Enzyme activity and protein concentration. Activity was measured at 20° in a Beckman DU spectrophotometer by observing the increase in absorbance at 340 mμ when NAD^+ was reduced by ethanol at pH 10. The assay conditions were those described by Dalziel.[8] A unit of activity was defined as the formation of 1 μmole of NADH per ml in one minute under the conditions of assay. Protein concentration was estimated spectrophotometrically, assuming $A_{280}=0.61$ cm^{-1} for a 1 mg/ml solution of protein in 0.1 μ sodium phosphate buffer, pH 7.0.[2] Specific activity was defined as enzyme units per milligram of protein.

Kinetics. Measurements were made with a recording fluorimeter [9] modified to yield enhanced stability and sensitivity[10]. Fluorescence-free glass cuvettes, 1×1 cm and 4 ml in volume, were employed. The rates of enzymatic reaction were expressed as inches of recorder-pen deflection per minute, where a deflection of 7 inches represented the formation of 2 μM DPNH. All measurements were made in 0.15 μ sodium phosphate buffer, pH 7.4 at 23° and kinetic and inhibition constants were determined from double reciprocal plots.

Spectrophotometry. Absorption spectra and spectral titrations were recorded with a Cary 14 recording spectrophotometer equipped with "0—1.0—2.0" and "0—0.1—0.2" slide wires. All measurements were made with 1 cm quartz cuvettes in 0.15 μ sodium phosphate buffer, pH 7.4, 20°.

RESULTS

Enzyme preparation. The enzyme was purified about 400-fold and recovered in a yield of 32 %. The A_{280}/A_{252} ratio of the protein was 1.48 in 0.1 M Tris buffer, pH 9.0. The specific activity of the preparation was 0.73 units per mg protein. The enzyme was not *atypical* [11] since the activity at pH 10 was higher than that at pH 8.5.

At pH 7.4 and 23°, the K_m for ethanol is 4.5×10^{-4} M, at an NAD^+ concentration of 350 μM. The K_m for NAD^+ is 2.2×10^{-5} M with 5 mM ethanol

under the same conditions. Substrate inhibition is not observed with this particular enzyme preparation until the ethanol concentrations exceeded 0.3 M.

Inhibition by pyrazole and analogs. At pH 7.4, the activity of human LADH is inhibited by pyrazole and its halo- and methyl-substituted derivatives (Table 1). Modification of the pyrazole ring in the 4 position increases the

Table 1. Inhibition of human LADH by pyrazole and derivatives.

Inhibitor	Concentration (μM)	V_i/V_c (%)
None	–	100
Pyrazole	1	93
	10	63
4-Bromopyrazole	1	66
	10	18
3,4-Dibromopyrazole	1	90
	10	53
3,4,5-Tribromopyrazole	10	87
4-Iodopyrazole	1	47
	10	8
4-Methylpyrazole	1	58
	10	14
3-Methyl-4-iodopyrazole	1	96
	10	69

Conditions: [NAD^+], 350 μM; [Ethanol], 2.5 mM; [Enzyme], 8 μg/ml; 0.15 μ sodium phosphate buffer, pH 7.4, 23°.

effectiveness of inhibition markedly. Of the 3 monosubstituted derivatives examined, 4-iodopyrazole is the most potent inhibitor, followed by 4-methylpyrazole and 4-bromopyrazole. However, the di-substituted derivatives are not any more effective than pyrazole itself and tribromopyrazole is less inhibitory than pyrazole.

Time course of inhibition during reaction. Theorell and Yonetani first noted that the inhibition of horse LADH with pyrazole has a short induction period [5] due to the time required for the formation or breakdown of the LADH—NAD^+-pyrazole ternary complex. The same phenomenon is observed with human LADH. Thus when the enzyme reaction is started by the addition of enzyme to a solution of ethanol, NAD^+, and inhibitor, the initial velocity almost equals that of the uninhibited control, but activity progressively decreases. The reaction becomes linear only after about 1 min in the presence of pyrazole and after about 1.5 min in the presence of iodopyrazole (Fig. 1A). In contrast, if the reaction is initiated by the addition of ethanol to a solution of enzyme, NAD^+, and inhibitor, activity is virtually absent immediately, and increases gradually in the first minutes. Zero order rates are attained only after about 1.5 min with pyrazole and after about 2 min with iodopyrazole (Fig. 1B). These circumstances dictate that the enzyme reactions be followed for more than 4 min to measure the steady-state velocities of the inhibited

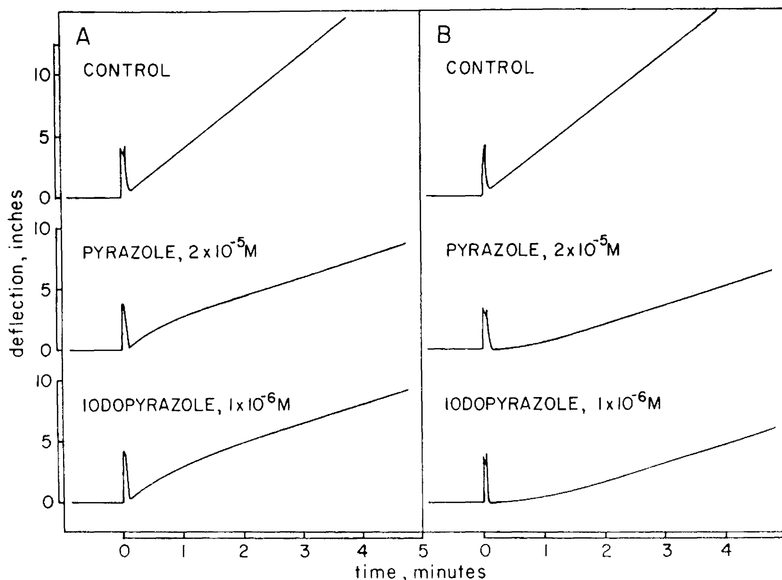

Fig. 1. Inhibition of LADH activity by 2×10^{-5} M pyrazole and 1×10^{-6} M iodopyrazole. The time course of the reaction was followed fluorimetrically at pH 7.4, 23.5°. A. Enzyme was added last to initiate the reaction. B. Ethanol was added last to initiate the reaction. Conditions: enzyme, 4 μg/ml; ethanol 1.25 mM; NAD^+, 350 μM.

reactions. To ensure that the reaction rates of the uninhibited controls also remain constant over this time period, the enzyme concentration employed for the kinetic studies was reduced to 4 μg/ml.

Inhibition kinetics. The effects of pyrazole and the more potent monosubstituted derivatives were examined kinetically at pH 7.4. The inhibition by pyrazole (Fig. 2), 4-bromopyrazole, and 4-methylpyrazole are all competitive with ethanol, regardless of whether enzyme or ethanol is added to initiate the reactions. The inhibition constants, K_I, are 2.6×10^{-6} M, 2.9×10^{-7} M, and 2.1×10^{-7} M, respectively.

Iodopyrazole also competes with ethanol when the enzyme is added to start the reaction (Fig. 3). The K_I is 1.2×10^{-7} M. However, the inhibition by iodopyrazole becomes mixed competitive-noncompetitive when the addition of ethanol is used to start the reaction. Furthermore, over the concentration range of 0.5—7.5 mM ethanol, a deviation from linearity is observed in the reciprocal plot at the higher ethanol concentrations (Fig. 4A). It suggests that the degree of noncompetitive inhibition would decrease in a higher concentration range of substrate. This can be discerned from the ratios of the maximal velocities for the uninhibited (V_{max}) and the inhibited (V_p) reactions, obtained by extrapolation of the reciprocal plots to infinite substrate concentration. When the range of substrate concentration is increased to 5—37.5 mM ethanol (Fig. 4B), V_p/V_{max} is 1.1 as compared with a value of

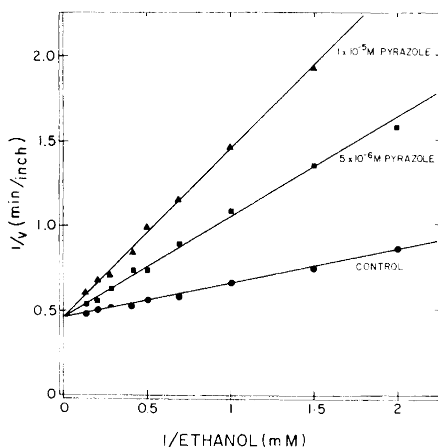

Fig. 2. Double reciprocal plot of the kinetics of LADH inhibition by pyrazole. The steady state rates of LADH in the absence of inhibitor (●), in the presence of 5×10^{-6} M pyrazole (■) and 1×10^{-5} M pyrazole (▲) were measured at varied ethanol concentrations. The reaction rates were identical regardless of whether ethanol or enzyme was added last to initiate the reaction. Conditions: enzyme, 4 μg/ml; NAD^+, 350 μM in 0.15 μ phosphate buffer, pH 7.4, 23.5°.

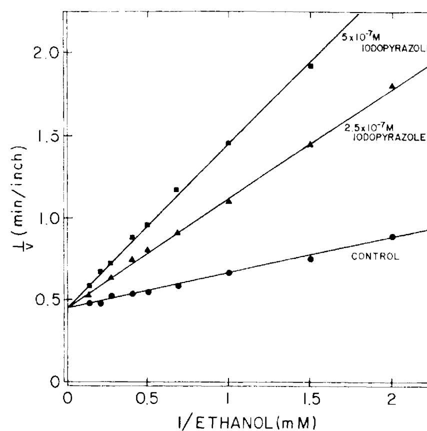

Fig. 3. Double reciprocal plot of the kinetics of LADH inhibition by iodopyrazole, when enzyme was added last to start the reaction. The steady-state rates were measured at varied ethanol concentrations in the absence of inhibitor (●), and in the presence of 2.5×10^{-7} M iodopyrazole (▲) or 5×10^{-7} M iodopyrazole (■). Conditions: enzyme, 4 μg/ml; NAD^+, 350 μM; pH 7.4, 23.5°.

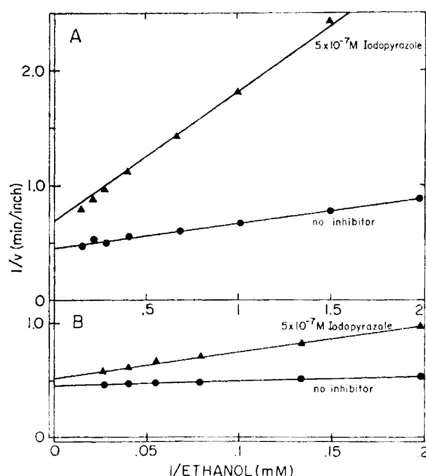

Fig. 4. Double reciprocal plot of the kinetics of LADH inhibition by iodopyrazole when ethanol was added last to start the reaction. The steady state rates were measured at varied ethanol concentrations in the absence of inhibitor (●) and in the presence of 5×10^{-7} M iodopyrazole (▲). Ethanol concentrations of 0.5 – 7.5 mM were employed in A, and of 5 – 37.5 mM in B. Conditions: enzyme 4 μg/ml; NAD^+, 350 μM; pH 7.4, 23.5°.

1.5 at the lower concentration range, demonstrating a striking decrease in the noncompetitive inhibition. Thus it would appear that the noncompetitive nature of the inhibition in these measurements is only a reflection of the relative slowness of dissociation of iodopyrazole from the enzyme in conjunction with the larger uncertainties in determining the points at which steady-state rates are attained when the activities are low. Increasing the ethanol concentration both accelerates the dissociation of the inhibitor and provides higher reaction rates thus allowing more accurate estimation of the steady-state rates. The K_I for iodopyrazole, calculated from the slopes of the reciprocal plots in Fig. 4A and 4B are 1.4×10^{-7} M and 1.0×10^{-7} M, respectively.

The inhibition constants for pyrazole and the 3 monosubstituted analogs are summarized in Table 2. The inhibition by these compounds when NAD^+ concentration is varied is noncompetitive — almost uncompetitive (Fig. 5).

Table 2. Inhibition constants (K_I) and dissociation constants ($K_{EO,I}$) for pyrazole and analogs.

	K_I (μM)	$K_{EO,I}$ (μM)
Pyrazole	2.6	2
Bromopyrazole	0.29	—
Iodopyrazole	0.12	0.1
Methylpyrazole	0.21	—

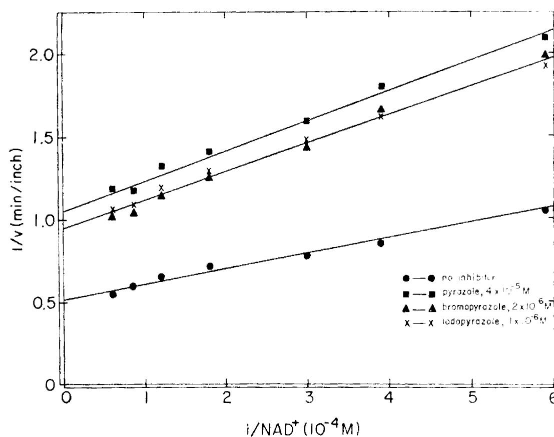

Fig. 5. Double reciprocal plot of the kinetics of LADH inhibition by pyrazole, bromopyrazole, and iodopyrazole. Enzyme was added last to start the reaction. The steady state rates were measured at varied NAD^+ concentrations in the absence of inhibitors (●) and in the presence of 4×10^{-5} M pyrazole (■), 2×10^{-6} M bromopyrazole (▲), or 1×10^{-6} M iodopyrazole (×). Conditions: enzyme, 4 μg/ml; ethanol, 5 mM; pH 7.4, 23.5°.

Ternary complex formation. The kinetic data indicate that these compounds compete with ethanol for binding to the human enzyme. It has been demonstrated for the horse enzyme that this occurs through the formation of an inactive enzyme-NAD^+-inhibitor ternary complex which can be discerned spectrophotometrically.[5] Hence the effect of pyrazole on the spectral properties of human LADH and NAD^+ was examined.

The addition of 1 mM pyrazole to 1.8 mg/ml LADH or 30 μM NAD^+ does not alter the absorption spectrum of the enzyme or that of NAD^+. However, when it is added to a solution containing both enzyme and NAD^+, an increase in absorptivity between 270 mμ and 230 mμ is observed. These changes are most clearly illustrated by comparing the spectrum of NAD^+ with the *difference* spectrum of (LADH+NAD^++pyrazole) *minus* LADH (Fig. 6). A new absorption maximum appears at 295 mμ and the absorptivity of NAD^+ at 260 mμ decreases. These findings are very similar to those observed with the horse enzyme and demonstrate directly the interaction of pyrazole with human LADH and NAD^+. Virtually identical spectral changes are observed with iodopyrazole.

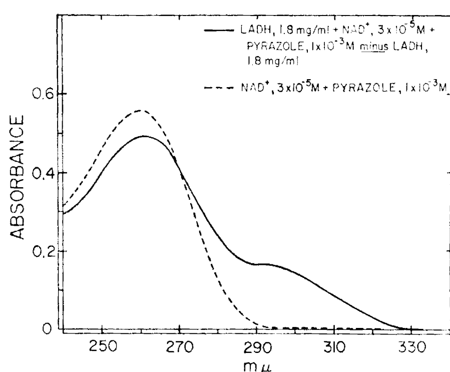

Fig. 6. Absorption spectrum of NAD^+ and pyrazole and difference spectrum in the presence of LADH. The absorption of 3×10^{-5} M NAD^+ and 1×10^{-3} M pyrazole (- - -) and of 1.8 mg/ml LADH, 3×10^{-5} M NAD^+ and 1×10^{-3} M pyrazole *minus* 1.8 mg/ml LADH (—) were recorded from 340 mμ to 240 mμ. The enzyme (1.8 mg/ml) in the absence of pyrazole did not alter the absorption of 3×10^{-5} M NAD^+ since they remain dissociated at these concentrations.

Titration of NAD^+ binding sites and dissociation constants for pyrazole and iodopyrazole. Since pyrazole increases the association constant of NAD^+ for LADH by more than 1500-fold,[5] binding of NAD^+ to human LADH and hence the number of active sites can be measured from the increase in absorptivity at 295 mμ. In the presence of excess pyrazole, 1.4 mg of protein binds 1.7×10^{-5} mmoles of NAD^+ (Fig. 7). Assuming a M.W. of 87 000 for the enzyme,[1] there are 1.06 moles of active sites per mole of protein in this enzyme preparation. More highly purified preparations have been reported to bind at least 2 moles of NADH/mole protein.[1] The number of active sites per molecule of protein in crystalline human LADH[2] has not been determined. Titration of the enzyme with pyrazole in the presence of excess (100 μM) NAD^+, yielded an identical end point, *i.e.* 1.7×10^{-5} mmoles pyrazole/1.4 mg protein, demonstrating that pyrazole and NAD^+ bind to the enzyme in a 1:1 relationship. Thus the ternary complex consists of one molecule each of pyrazole and NAD^+ per active site.

These titrations show, in addition, that the A_{295} of 1×10^{-5} M ternary complex is 0.097, and on this basis, the dissociation constant, $K_{EO,I}$, for pyrazole and iodopyrazole in the ternary complex can be determined.[5] This has been performed by titrating 0.18—0.28 mg/ml of enzyme with pyrazole and iodopyrazole in the presence of excess ($1\times10^{-5}-1\times10^{-4}$ M) NAD^+. The $K_{EO,I}$ for pyrazole is 2×10^{-6} M and that for iodopyrazole 1×10^{-7} M, in good agreement with the K_I values for these compounds (Table 2).

DISCUSSION

Derivatives of pyrazole have wide metabolic and physiologic action; many are effective therapeutic agents. Some have been reported to cause profound alterations in carbohydrate and fat metabolism while others are inhibitors of enzymes. The enzymes known to be inhibited include LADH, catalase, monoamine oxidase, and xanthine oxidase. Depending upon the nature and size of the substituents, still others have diuretic, anabolic, anti-inflammatory, carcinostatic, anticonvulsant, and antimicrobial properties.[12]

Pyrazole itself is an effective inhibitor of liver alcohol dehydrogenase, but some of its simplest derivatives exhibit even greater potency. Among those examined, the compounds which are monosubstituted with nucleophilic groups in the 4 position of the pyrazole ring are the most effective. Substitution in other positions of the ring or in multiple positions results in lesser degrees of enhanced potency or in reduced potency (Table 1). These relationships have now been examined in LADH from 3 species, *viz.* horse,[6] man (this paper), and rat,[13] and have been found to pertain in all. However, the magnitudes of the inhibition constants differ for each of the 3 enzymes. The K_I values for human LADH are 3—8 times greater than the corresponding values for the horse enzyme, but 2—5 times smaller than those for the rat enzyme. Such variation is not unexpected, since the enzymes differ in specific activity and other kinetic parameters.

Pyrazole and its derivatives inhibit human LADH by competing with ethanol but not with NAD^+. As with the horse enzyme, catalytic activity of the human enzyme in the presence of these inhibitors reaches a steady-state rate only 1 or 2 min after the start of the reaction (Fig. 1). During this initial period, formation or breakdown of the inhibitor complex can be observed, depending upon whether or not the enzyme is first exposed to pyrazole and NAD^+. When the enzyme is added to a solution of ethanol, NAD^+, and inhibitor, the progression curve is convex, reflecting formation of the inhibitor complex. When ethanol is added to enzyme, NAD^+, and inhibitor to start the reaction, the curve is concave reflecting dissociation of the complex due to competition by ethanol. However, if the reaction is initiated by the addition of NAD^+ alone, NAD^+ plus ethanol, or NAD^+ plus inhibitor, the progression curves are all convex, indicating that the inhibitor complex which forms is a ternary complex consisting of enzyme-NAD^+-inhibitor. Moreover, its formation has a "compulsory" order: the inhibitor interacts with the enzyme only after the coenzyme has bound. In the case of iodopyrazole, the most potent of the inhibitors, the dissociation of the complex is so slow that unless very

high ethanol concentrations are employed, the inhibition kinetics appear to be mixed competitive-noncompetitive (Fig. 4).

Formation of the ternary complex results in the appearance of an absorption band, centered at 295 mμ (Fig. 6). Titration of the enzyme with NAD^+ in the presence of excess pyrazole (Fig. 7) and with pyrazole in the presence

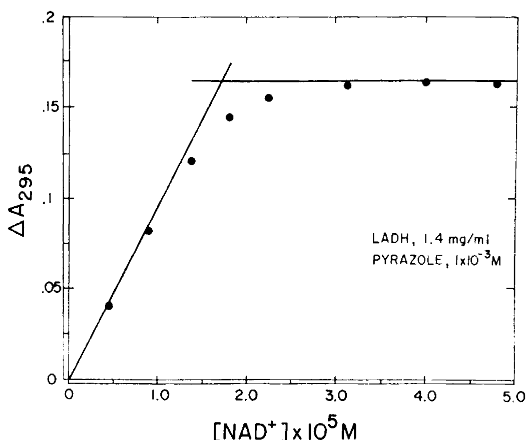

Fig. 7. Spectral titration of 1.4 mg/ml LADH with NAD^+ in the presence of 1×10^{-3} M pyrazole. The increase in absorptivity at 295 mμ was recorded with increasing concentrations of NAD^{+1}at pH 7.4 and 20°.

of excess NAD^+ shows that the inhibitor and coenzyme react with the enzyme in a 1:1 ratio. Thus the ternary complex consists of 1 molecule each of pyrazole and NAD^+ per active enzymatic site. The good agreement between the K_I and $K_{EO,I}$ values for pyrazole and iodopyrazole indicates that formation of the inactive ternary complex accounts fully for the inhibition of the enzyme.

As has been shown by Theorell and Yonetani,[5] titrations of LADH with NAD^+ in the presence of excess pyrazole can be employed to determine the purity of enzyme preparations if the M.W. and the number of active enzymatic sites per molecule of enzyme is known. Thus assuming a M.W. of 87 000 and two active sites per molecule of human LADH [1] the purity of the present preparation is 50 %. Based upon the increase in specific activity during purification and the final specific activity of crystallized enzyme,[2] human LADH at the stage of purification of the preparation used should be about 45 % pure.

The spectral changes accompanying the binding of pyrazole and NAD^+ to human LADH are similar to those observed for the horse enzyme (Fig. 6). The shifts in the spectrum of NADH upon binding to horse LADH and to the human enzyme are also closely similar.[1] These observations suggest that the active enzymatic centers of these two enzymes share major structural features in spite of dissimilarities in their amino acid compositions.[2] However, minor differences in the environment of the active centers must exist to account for the differing kinetic parameters and inhibition constants. Such differences,

however, are not reflected in the spectral properties of the binary and ternary complexes. The formation of the inactive pyrazole ternary complex in horse LADH has been ascribed to the bonding of the negatively charged pyrazole ion with the 4-carbon of the pyridinium ring of NAD^+, at or near the substrate binding site of the enzyme.[5] The zinc atoms at the active centers of LADH are thought to promote the dissociation of H^+ from pyrazole and the subsequent interaction of the pyrazole anion with NAD^+. The inhibition of human LADH probably occurs by a similar mechanism. The effect of pyrazole upon a number of other zinc metalloenzymes and zinc containing NAD^+-dependent dehydrogenases has been examined. With the exception of yeast alcohol dehydrogenase which is only weakly inhibited, they are not inhibited at all by pyrazole.[13] Thus it is probably the unique affinity of pyrazole for the substrate binding sites present in the enzyme-coenzyme binary complexes which governs its specificity and potency as an inhibitor of the liver alcohol dehydrogenases.

The low K_I values of 1 and 2×10^{-7} M for iodopyrazole and methylpyrazole indicate that they should be effective inhibitors of LADH activity *in vivo* and suggest an approach and rational basis for therapy. Profound disturbances in carbohydrate and lipid metabolism occur following heavy ethanol consumption.[14,15] Poisoning by both methanol and ethylene glycol is thought to arise through their oxidation, by LADH to products which are toxic.[16] Whether or not the pyrazole derivatives can serve as clinically useful agents remains to be examined experimentally. It is pertinent that experiments in dogs and rats have already shown that the administration of pyrazole can induce a prolonged and dramatic inhibition of ethanol oxidation.[3] Since iodopyrazole and methylpyrazole have 10—20 times greater inhibitory capacity than does pyrazole (Table 2), much smaller doses would be required. On the basis of inhibition kinetics, approximately 400 mg iodopyrazole or 600 mg methylpyrazole would be expected to cause 90 % inhibition when the ethanol concentration is 20 mM in a 70 kg man, calculated on the assumption that the pyrazole derivatives are freely and equally distributed in the total body water. For comparable concentrations of substrates such as methanol and ethylene glycol, even lesser amounts would suffice, since their K_m values are higher than that for ethanol.[4] Whether or not these compounds themselves have short and long term untoward effects is being studied in experimental animals and in man.*

The activity per mg of the human LADH preparation employed for these studies is 1/2 that reported by Mourad and Woronick [2] at a comparable stage of purification.[2] Wide variations in the activity per unit weight of liver from different individuals has been noted previously.[4] Moreover, heterogeneity has also been encountered in preparations from single organs where one, two, or three electrophoretically different forms have been observed.[4] These observations have suggested that variations in activity and/or molecular forms of the enzyme may be significant in relation to the differences of indi-

* *Addendum to proofs May 7, 1969:* Preliminary results (H.T. in collaboration with Dr. R. Blomstrand) indicate that inhibitory effects of pyrazole derivatives on ethanol oxidation can be obtained in man.

viduals in their ability to metabolize alcohol. Another finding may be pertinent in this regard: the enzyme studied here was not inhibited by ethanol until the concentration exceeded 0.3 M. With other preparations, substrate inhibition was observed at 0.02 M ethanol,[1] a level more comparable with that for the horse enzyme. Since blood alcohol concentrations of 0.02 M or higher are not uncommonly attained in man during acute ingestion, differences in sensitivity to ethanol inhibition may be an additional factor underlying the widely varying tolerances of man to intoxication by alcohol.

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REFERENCES

1. von Wartburg, J. P., Bethune, J. L. and Vallee, B. L. *Biochemistry* **3** (1964) 1775.
2. Mourad, N. and Woronick, C. L. *Arch. Biochem. Biophys.* **121** (1967) 431.
3. Theorell, H. *Harvey Lectures, Ser.* **61** (1967) 17.
4. Blair, A. H. and Vallee, B. L. *Biochemistry* **5** (1966) 2026.
5. Theorell, H. and Yonetani, T. *Biochem. Z.* **338** (1963) 537.
6. Theorell, H., Yonetani, T. and Sjöberg, B. *Acta Chem. Scand.* **23** (1969) 255.
7. Peterson, E. A. and Sober, H. A. *Methods Enzymol.* **5** (1962) 3.
8. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 1706.
9. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1797.
10. Shore, J. D. and Theorell, H. *Arch. Biochem. Biophys.* **117** (1966) 375.
11. von Wartburg, J. P., Papenberg, J. and Aebi, H. *Can. J. Biochem.* **43** (1965) 889.
12. Orth, R. E. *J. Pharm. Sci.* **57** (1968) 537.
13. Reynier, M. *To be published.*
14. Isselbacher, K. J. and Greenberger, N. J. *New Engl. J. Med.* **270** (1964) 351, 402.
15. Freinkel, N., Cohen, A., Arky, R. A. and Foster, A. E. *J. Clin. Endocrinol. Metab.* **25** (1965) 76.
16. Wacker, W. E. C., Haynes, H., Druyan, R., Fisher, W. and Coleman, J. E. *J. Am. Med. Assoc.* **194** (1965) 1231.

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