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Human Liver π-Alcohol Dehydrogenase: Kinetic and Molecular Properties†

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ABSTRACT: A new and distinctive form of human liver alcohol dehydrogenase, π-ADH, has recently been purified to homogeneity [Bosron, W. F., Li, T.-K., Lange, L. G., Dafeldecker, W. P., & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 85–91]. Its general characteristics, i.e., molecular weight of 78 000, dimeric structure, and zinc content, 4 g-atoms per mol, are all similar to those of other mammalian alcohol dehydrogenases. However, its amino acid composition differs from that of both horse liver ADH and previous preparations of human liver ADH containing a mixture of isoenzymes. The kinetics of π-ADH follow an ordered bibi mechanism with cofactor adding first to form a binary enzyme complex. In contrast to the other molecular forms, π-ADH is less stable in vitro, exhibits a more limited substrate specificity, has higher K_M values for ethanol and acetaldehyde, both approximately 30 mM at pH 7.5, and is much less sensitive to inhibition by pyrazole and 4-methylpyrazole, with K_I values of 30 and 2 mM, respectively. Hence, differentiation of ADH-independent ethanol oxidizing pathways in man cannot be based solely upon the lack of inhibition of alcohol oxidation by pyrazole or 4-methylpyrazole, the inhibitors most commonly employed for such purposes. Significantly, π-ADH exhibits markedly lower K_I values toward other pyrazole derivatives, 4-bromo-, 4-nitro-, or 4-pentylpyrazole ranging from 4 to 27 μM. Hence, these pyrazole derivatives may be suitable for quantitative inhibitor studies of all molecular forms of human liver ADH, including π-ADH.

Alcohol dehydrogenase (ADH)[1] is the principal enzyme responsible for the oxidation of ethanol in liver. Human livers contain multiple molecular enzyme forms (Von Wartburg et al., 1964; Smith et al., 1971; Schenker et al., 1971; Pietruszko et al., 1972), whose number and amount vary, seemingly dependent upon genetic background (Smith et al., 1971), state of health of the individual, and time of storage of the tissue after removal (Azevedo et al., 1974; Li & Magnes, 1975). Since the various pharmacologic, addictive, and pathologic consequences of ethanol consumption must relate directly to the biochemical properties of ethanol and/or its metabolic byproducts, the delineation of those ADH isoenzymes principally responsible for ethanol oxidation is a major objective

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[1] Abbreviations used: ADH, alcohol dehydrogenase; CapGapp, 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole; $NaDodSO_4$, sodium dodecyl sulfate.

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in alcoholism research. However, such detailed biochemical studies have become possible only recently, owing to the development of procedures for the rapid and specific separation and purification of ADH isoenzymes based upon affinity chromatography (Lange & Vallee, 1976; Bosron et al., 1977).

In this regard, the recent discovery of a previously unrecognized form of human liver ADH, π-ADH, has been particularly provocative since some of its catalytic properties differ strikingly from those of the other forms (Li et al., 1977). These unique catalytic features include a more limited substrate specificity, a high K_M for ethanol and insensitivity to inhibition by 4-methylpyrazole. In order to characterize π-ADH further, its stability, amino acid and metal composition, steady-state kinetic properties, and inhibition by pyrazole analogues have been studied.

Experimental Procedures

NAD^+ (grade III) and NADH (grade III) were obtained from Sigma Chemical Co., St. Louis, MO; pyrazole and 3-methylpyrazole were from Aldrich Chemical Co., Milwaukee, WI; and 4-bromopyrazole was from Research Plus Laboratories, Denville, NJ. 4-Methylpyrazole was a gift from Astra Chemical Co., Södertälje, Sweden. 4-Iodo-, 4-pentyl-, 4-cyano-, 4-methoxy-, and 4-nitropyrazole derivatives were gifts of Dr. Corwin Hansch, Pomona College, Claremont, CA. All other chemicals were reagent grade and deionized, distilled water was used throughout.

Purification of π-ADH. Human liver samples were obtained at autopsy within 12 h postmortem from individuals succumbing to sudden traumatic death and were stored at −55 °C. Those specimens exhibiting greater than 20% residual enzymatic activity when assayed with 33 mM ethanol and 2.4 mM NAD^+ at pH 10.5 in the presence of 33 μM 4-methylpyrazole were utilized for enzyme preparations. π-ADH was purified by chromatography on DEAE-cellulose, CapGapp-Sepharose, and agarose-AMP as described previously (Li et al., 1977). Enzyme prepared in this manner demonstrated a single band on $NaDodSO_4$ gel electrophoresis and one or two "anodic bands" on starch gel electrophoresis (Li & Magnes, 1975). π-ADH in 5 mM NaP_i, pH 7.5, was stabilized by addition of 10 mM ethanol and storage at 4 °C. Enzyme was gel filtered on Bio-Gel P-6 (Bio-Rad, Richmond, CA) in 5 mM NaP_i, pH 7.5, immediately before use.

Enzymatic Assays. ADH activity was determined in 0.1 M NaP_i, pH 7.5 at 25 °C, by measuring the production or utilization of NADH in the presence of alcohol or aldehyde substrates. The spectrophotometric assay was performed by use of a Cary 118C or Gilford 240 spectrophotometer. Activities are expressed as μmol of NADH utilized or produced per min based on an A_{340} of 6.22 mM^{-1} cm^{-1}. Fluorometric assay was performed with an Aminco-Bowman J4-8203H spectrophotofluorometer. The fluorometer was calibrated with NADH solutions by excitation at 340 nm and emission at 455 nm. All kinetic data were obtained in duplicate or triplicate, and the steady-state kinetic constants were evaluated both graphically and by computer analysis (DEC system 10) with the statistical programs described by Cleland (1976). With the exception of K_{ib} and K_{ip}, the standard errors of the fitted constants were less than 12% of the values themselves. Protein concentration was determined by the method of Lowry et al. (1951) with horse liver ADH as standard, A_{280} = 0.45 mg^{-1} cm^2 (Drum et al., 1969).

Ultracentrifugation. The molecular weight of π-ADH was determined by the meniscus depletion sedimentation equilibrium method of Yphantis (1964) in a Spinco Model E ultracentrifuge at 24 630 rpm and 23.3 °C. Enzyme, 0.36 mg/mL, was dialyzed against 10 mM Tris-Cl and 0.1 M NaCl at pH 7.5. The molecular weight was calculated from the slope of the log $(Y_{(r)} - Y_0)$ vs. r^2 plot assuming a partial specific volume of 0.743 mL/g (Lange et al., 1976).

Metal Analyses. π-ADH was dialyzed for 36 h against multiple changes of 5 mM NaP_i buffer, pH 7.5, that had been rendered metal-free by extraction with 0.01% dithizone in CCl_4 (Thiers, 1957). Quantitative zinc analysis was performed by atomic absorption spectrometry (Fuwa et al., 1964).

Amino acid analyses were performed in duplicate using a Durrum 500 amino acid analyzer. π-ADH, 50–100 μg, was hydrolyzed in 1 mL of 6 N HCl containing 5 μL of redistilled phenol for 24, 48, and 72 h at 110 °C. The final values for Ser and Thr were extrapolated to zero time, while those for Val and Ile were determined after 72 h of hydrolysis. Cysteine was determined after performic acid oxidation (Moore, 1963). Tryptophan content was determined by magnetic circular dichroism (Holmquist & Vallee, 1973). Integral values for amino acids were calculated assuming 355 residues per 39 000 subunit molecular weight.

Table I: Amino Acid Composition of π-ADH

amino acid	residues/ subunit	amino acid	residues/ subunit
Asp	31	Ile	25
Thr	23	Leu	30
Ser	21	Tyr	8
Glu	26	Phe	17
Pro	22	His	7
Gly	37	Lys	28
Ala	32	Arg	10
Val	28	Trp	3
Met	6	Cys	14
		total	368

Results

Molecular Properties of π-ADH. Purified π-ADH exhibits a single band on $NaDodSO_4$ gel electrophoresis with mobility identical with that of horse liver ADH (Bosron et al., 1977). Thus, the subunit molecular weight of π-ADH is approximately 40 000. The molecular weight of π-ADH determined in the ultracentrifuge under nondissociating conditions is 78 000, consistent with the dimeric structure suggested for all mammalian alcohol dehydrogenases thus far examined (Brändén et al., 1975).

The average amino acid composition (Table I) of four different preparations of π-ADH is nearly identical. Based upon 368 amino acids, the subunit molecular weight is calculated to be approximately 39 000. π-ADH contains 3.7 ± 0.2 g-atoms of zinc per mol of ADH.

Previous studies have indicated that π-ADH is much less stable both in vivo and in vitro than other molecular forms of human liver ADH (Li & Magnes, 1975; Bosron et al., 1977). Storage of the purified enzyme in 5 mM NaP_i, pH 7.5 at 4 °C, results in 50% loss of activity within 1 day (Figure 1). Addition of 10 mM NAD^+ or glycerol only partially protects π-ADH against inactivation. However, π-ADH activity can be stabilized effectively for up to 2 weeks by storage at 4 °C in buffer containing 10 mM ethanol.

Kinetic Properties of π-ADH. Since π-ADH has been shown to differ significantly from other molecular forms of ADH with respect to their K_M values for ethanol, the steady-state kinetic constants for all substrates were evaluated at pH 7.5. Analysis of π-ADH activity upon variation of both NAD^+ and ethanol concentrations yields an intersecting linear double-reciprocal plot (Figure 2A). Inhibition of NAD^+

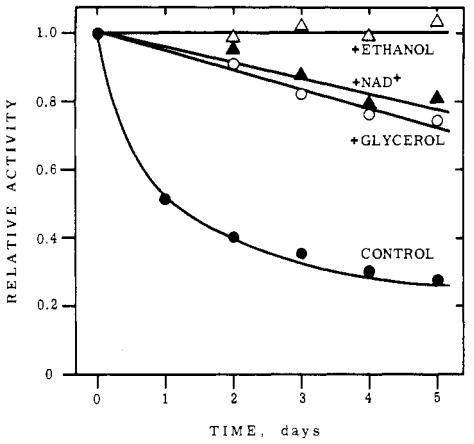

FIGURE 1: Stability of π-alcohol dehydrogenase. π-ADH activity was determined in 0.1 M glycine–NaOH buffer at pH 10.0 with 2.4 mM NAD^+ and 33 mM ethanol after incubation in 5 mM NaP_i buffer at pH 7.5 (●) plus 10 mM ethanol (△), 10 mM NAD^+ (▲), or 10 mM glycerol (○).

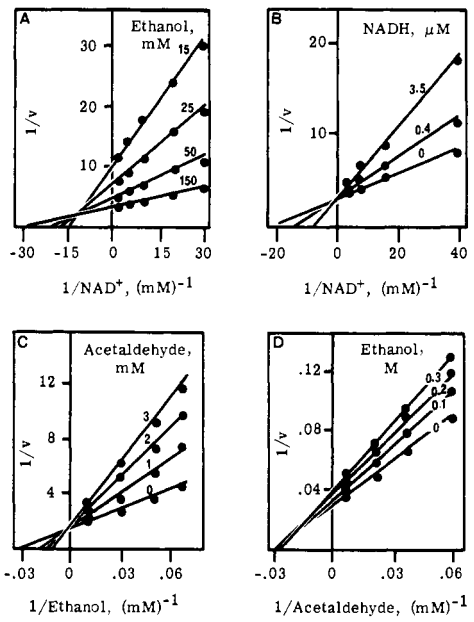

FIGURE 2: Alternate substrate and product inhibition kinetics of π-ADH. Enzyme activity was determined in 0.1 M NaP_i buffer, pH 7.5 at 25 °C. Each figure is a Lineweaver–Burk plot of the primary data and V is expressed as μmol min^{-1} (mg of protein)$^{-1}$. The lines are calculated from least-squares fits of the data to the SEQUEN, COMP, or NONCOMP programs as described by Cleland (1976). Ethanol concentration in panel B is 100 mM; NAD^+ in panel C is 2.4 mM; and NADH in panel D is 0.2 mM.

reduction by NADH with ethanol held constant at 100 mM results in an intersecting pattern characteristic of competitive inhibition (Figure 2B). Both the inhibition of ethanol oxidation by acetaldehyde with 2.4 mM NAD^+ as the constant substrate and the inhibition of acetaldehyde reduction by ethanol with 0.2 mM NADH as the constant substrate result in linear noncompetitive patterns (Figures 2C and 2D). These data are consistent with previous results for horse liver ADH (Wratten & Cleland, 1963; Dworschack & Plapp, 1977) and suggest an ordered bibi mechanism.

Based on this mechanism, data from the alternate substrate and product inhibition studies were utilized to calculate the kinetic constants shown in Table II. However, certain kinetic constants vary substantially for π-ADH prepared from different livers. Thus, the value of the Michaelis constant for ethanol, K_b, is 34 ± 12 mM, ranging from 18 to 48 mM, and

Table II: Kinetic Constants for π-ADH[a]

	mM
K_a (NAD^+)	0.014
K_b (ethanol)	34
K_{ia} (NAD^+)	0.086
K_{ib} (ethanol)	800
K_p (acetaldehyde)	30
K_q (NADH)	0.016
K_{ip} (acetaldehyde)	9
K_{iq} (NADH)	0.0019
	μmol min^{-1} mg^{-1}
V_1/E_t (ethanol oxidizing)	0.50
V_2/E_t (acetaldehyde reducing)	21

[a] Values for K_b, K_p, V_1/E_t, and V_2/E_t were obtained with 2.4 mM NAD^+ or 0.2 mM NADH as cofactor and are the averages of six preparations. K_{ia} and K_a are calculated from data in Figure 2A, K_{iq} from Figure 2B, and approximate values for K_{ib} and K_{ip} are extrapolated from data in Figures 2C and 2D. An apparent value for K_q was obtained with 200 mM acetaldehyde as substrate.

Table III: Inhibition of π-ADH by Pyrazole Analogues

inhibitor	% inhibition[a] 1.0 mM	0.1 mM	K_I[b] (mM)
pyrazole	0	0	30
3-methylpyrazole	3	0	
4-methoxypyrazole	28	3	6.3
4-methylpyrazole	31	1	2.0
4-iodopyrazole	49	14	
4-propylpyrazole	77	29	
4-cyanopyrazole	78	33	
4-nitropyrazole	88	41	0.027
4-bromopyrazole	90	69	0.010
4-pentylpyrazole	98	87	0.0043

[a] Activity was determined with 100 mM ethanol and 2.4 mM NAD^+ in 0.1 M NaP_i, pH 7.5 at 25 °C. [b] K_I values were calculated from the effect of inhibitor on the slope of the primary reciprocal plot. Inhibition was competitive with respect to acetaldehyde as the varied substrate for pyrazole and its 4-methoxy and 4-methyl derivatives (Figure 3A). Inhibition was competitive with respect to ethanol as the varied substrate for 4-bromo-, 4-pentyl-, and 4-nitropyrazole (Figure 3B).

K_p for acetaldehyde is 30 ± 5 mM, varying from 24 to 37 mM for six different preparations. The maximal velocities, V_1/E_t and V_2/E_t, are 0.50 ± 0.09 and 21 ± 4 μmol min^{-1} mg^{-1}, ranging from 0.38 to 0.65 and 18 to 28 μmol min^{-1} mg^{-1}, respectively. An equilibrium constant is calculated from the Haldane relationship

$$K_{eq} = V_1 K_p K_{iq}[H^+]/V_2 K_b K_{ia}$$

to be 14.7×10^{-12} M. This is similar to values determined with horse and yeast ADH by Theorell & Bonnischen (1951) and Racker (1950) to be 8.6 and 11.5×10^{-12} M, respectively.

The effect of other pyrazole analogues on π-ADH activity was determined since π-ADH has been shown to be uniquely insensitive to inhibition by 4-methylpyrazole (Li et al., 1977). Pyrazole and those derivatives containing small neutral substituents such as 4-methoxy- or 4-methypyrazole are relatively ineffective as inhibitors (Table III). The K_I values for pyrazole and 4-methoxy- and 4-methylpyrazole are 30, 6.3, and 2.0 mM, respectively. Moreover, they inhibit π-ADH competitively with respect to acetaldehyde (Figure 3A). Those derivatives containing electronegative or apolar substituents inhibit π-ADH more effectively. The K_I values for 4-nitro-, 4-bromo-, and 4-pentylpyrazole are 27, 10, and 4.3 μM, respectively. In these cases, inhibition is competitive when ethanol is varied (Figure 3B).

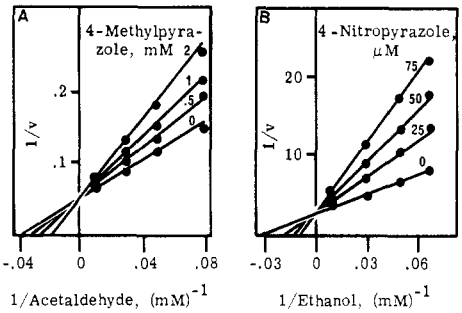

FIGURE 3: Inhibition of π-ADH by 4-methyl- and 4-nitropyrazole. Assay conditions and methods for data analysis are described in Figure 2. NADH concentration in panel A is 0.2 mM and NAD^+ in panel B is 2.4 mM.

Discussion

Certain human livers contain a unique form of ADH, π-ADH, that has a high K_M for ethanol and is relatively insensitive to inhibition by 4-methylpyrazole (Li et al., 1977). The lack of earlier recognition of π-ADH must be attributed to its lability both in vivo (Li & Magnes, 1975) and in vitro (Figure 1). Both the time and conditions for storage of postmortem specimens critically determine the π-ADH content of liver. Rapid affinity chromatography procedures for the purification of large quantities of π-ADH (Bosron et al., 1977) as well as conditions for stabilization of π-ADH activity (Figure 1) now allow the detailed examination of its molecular and catalytic properties.

Ultracentrifugation indicates that π-ADH is a dimer of 78 000 molecular weight. $NaDodSO_4$ gel electrophoresis indicates identical molecular weight subunits of approximately 40 000 (Bosron et al., 1977). π-ADH is a zinc metalloenzyme containing approximately 4 g-atoms of zinc per mol of protein. Thus, the molecular properties of π-ADH closely resemble those of previous preparations of both horse (Drum et al., 1969) and human liver ADH (Lange et al., 1976). However, amino acid analysis of π-ADH (Table I) demonstrates some differences in amino acid composition as compared with the other liver alcohol dehydrogenases. π-ADH contains significantly fewer Val, Lys, and Ser but more Leu, Asp, and Tyr residues than either horse ADH (Jörnvall, 1970) or a preparation of human ADH containing an undefined mixture of isozymes (Lange et al., 1976).

The NAD^+-dependent alcohol dehydrogenases from rat, horse, and human liver have been shown to exhibit broad substrate specificity (Sund & Theorell, 1963; Li, 1977). Many of these substrates, such as glycerol, retinol, and steroid alcohols, are physiologically important but others are oxidized by ADH, ultimately to toxic products. Thus, the toxicity of methanol and ethylene glycol is the result of their conversion to formate and oxalate, respectively (Von Wartburg et al., 1964). Interestingly, π-ADH exhibits a more limited substrate specificity than do the other human liver molecular forms. Neither methanol, glycerol, nor ethylene glycol is oxidized even at concentrations up to 100 mM.

The steady-state kinetic properties of π-ADH examined by means of alternate substrate and product inhibition suggest that the enzyme follows an ordered bibi mechanism with cofactor adding first to the enzyme. Thus, the intersecting pattern obtained when both NAD^+ and ethanol are varied (Figure 2A), the competitive inhibition of NAD^+ reduction by NADH (Figure 2B), and the mixed-noncompetitive inhibition patterns when ethanol and acetaldehyde are alternately substrate or inhibitor (Figure 2C and 2D) are similar to data reported for horse liver ADH (Wratten & Cleland, 1963; Dworschack & Plapp, 1977). Similar mechanistic conclusions have been drawn from recent kinetic studies with what appears to be the $\beta\beta$ isoenzyme of human liver ADH (Dubied et al., 1977).

A direct comparison between kinetic constants for π-ADH at pH 7.5 (Table II) and the $\beta\beta$ isoenzyme at pH 8.0 indicates that the Michaelis constants for cofactors (K_a for NAD^+ and K_q for NADH) as well as the dissociation constants for the E·NAD^+ (K_{ia}) and E·NADH (K_{iq}) complexes are similar. However, the Michaelis constants for ethanol (K_b) and acetaldehyde (K_p) are approximately 20 times greater with π-ADH (Table II) than those reported for the $\beta\beta$ isoenzyme, 1.8 and 1.5 mM, respectively (Dubied et al., 1977). This suggests that the contribution of π-ADH to alcohol metabolism in vivo should become very important at ethanol concentrations that are saturating for the $\beta\beta$ isoenzyme and presumably the other ADH molecular forms as well (Li & Theorell, 1969; Pietruszko et al., 1972; Lange et al., 1976). These two ADH forms additionally differ in their maximal velocities. The $\beta\beta$ form appears more active in oxidizing alcohol than π-ADH. However, the contribution of these and each of the other molecular forms of ADH to ethanol oxidation rate must also depend upon the relative content of the individual forms in liver. Thus, a comprehensive analysis of both catalytic constants and relative content is required before meaningful correlations of individual differences in alcohol elimination rates with isoenzyme patterns can be made. Similar considerations pertain to the elucidation of the enzymatic basis for the phenomenon of increased ethanol elimination rates induced by chronic ethanol ingestion in some alcoholic individuals (Misra et al., 1971).

π-ADH is much less sensitive to inhibition by 4-methylpyrazole than are the other molecular forms of ADH. This difference in pyrazole inhibition has constituted the basis for separation of π-ADH from the pyrazole-sensitive forms on CapGapp-Sepharose affinity columns (Bosron et al., 1977). However, both the mode and degree of inhibition of π-ADH by 3- or 4-substituted pyrazole derivatives depend markedly upon the chemical structure of the substituent (Table III). Previous studies demonstrated that pyrazole and its 3- or 4-substituted analogues inhibit horse and other molecular forms of human ADH competitively with respect to ethanol with K_I values ranging from 10^{-4} to 10^{-8} M (Li & Theorell, 1969; Theorell et al., 1969). Accordingly, it has been proposed that inhibition proceeds by formation of a dead-end ternary complex with enzyme and NAD^+ (Theorell & Yonetani, 1963). For π-ADH, however, inhibition by 4-methylpyrazole is competitive with respect to acetaldehyde (Figure 3A), mixed-noncompetitive with respect to ethanol, and uncompetitive with respect to NAD^+ (unpublished observations). Such patterns are inconsistent with the formation of simple dead-end ternary complexes with either E·NAD^+ or E·NADH alone and suggest a more complex mechanism analogous perhaps to that proposed for the inhibition of pigeon liver malic enzyme by keto- or hydroxymalonate (Schimerlik & Cleland, 1977).

The large decrease in inhibition constant observed when a *n*-pentyl group is substituted at the 4-position of pyrazole (Table III) is consistent with a greater affinity of the enzyme for apolar compounds. Such a substrate binding site has been suggested previously on the basis of a direct relationship between the apolar character of alcohols and their K_M values for horse and human ADH (Hansch et al., 1972; Lange et al., 1976). However, the increased inhibition of π-ADH by 4-bromo- and 4-nitropyrazole requires an alternate explanation.

Introduction of a 4-nitro group onto pyrazole lowers the pK_a from 14 to 9.7 (Janssen et al. 1973). Theorell & Yonetani (1963) found that formation of the E·NAD^+·pyrazole complex is accompanied by release of a proton presumably from pyrazole. They postulated that the pyrazole anion forms a stable covalent complex with NAD^+ at the 4-pyridinium position. Based upon this mechanism, both 4-nitro- and 4-bromopyrazole would be expected to exhibit lower K_I values than pyrazole.

Pyrazole and 4-methylpyrazole have been utilized frequently to differentiate ADH-catalyzed ethanol oxidation from that catalyzed by catalase or a microsomal ethanol oxidizing system both in vivo and in vitro. Such studies in experimental animals and man indicate that a significant fraction of total alcohol oxidizing activity is insensitive to inhibition by pyrazole or 4-methylpyrazole (Teschke et al., 1976; Lieber, 1977; Salaspuro et al., 1978). Since both catalase and the microsomal ethanol oxidizing system are relatively insensitive to inhibition by pyrazole compounds, it has been inferred that this residual oxidation represents contributions by these enzyme systems. The identification of π-ADH as a biochemically distinct form of human liver ADH with K_I of 30 mM for pyrazole, and of 2 mM for 4-methylpyrazole, both as much as 1000 times that of the other molecular forms, indicates that evidence for ethanol oxidizing pathways alternate to ADH in humans cannot be based exclusively on the effects of these compounds. However, other pyrazole analogues such as 4-pentyl-, 4-bromo-, or 4-nitropyrazole exhibit lower K_I values ranging from 4 to 27 μM. These compounds might be more suitable to differentiate ADH from non-ADH ethanol oxidizing capacity in man either in vitro or in vivo.

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