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Pyrazole Inhibition and Kinetic Studies of Ethanol and Retinol Oxidation Catalyzed by Rat Liver Alcohol Dehydrogenase*

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The inhibition effect of pyrazole is studied on a ten times purified rat liver alcohol dehydrogenase (LADH) preparation. Ethanol and retinol oxidation are equally inhibited. 4-Iodo, 4-bromo, and 4-methyl-pyrazole are more potent inhibitors. Pyrazole has no effect on several other enzymes. The inhibition constant for pyrazole is higher with yeast ADH than with rat LADH. Yeast ADH is weakly inhibited by 4-halogenated pyrazole derivatives.

The kinetic studies of the oxidation of ethanol and other primary alcohols catalyzed by mammalian liver alcohol dehydrogenase (LADH) *** have been mainly performed with horse LADH.[1] Crystalline horse LADH served Theorell and his associates as a model for an extensive study of the mechanism of pyridine nucleotide-linked hydrogen transfer.[2] Kinetic and physico-chemical properties of purified ADH preparations from human [3] and monkey [4] livers also have been investigated. On the other hand, no kinetic studies on rat LADH are available in the literature. The rat is an experimental animal currently used in biochemical, pharmacological, and toxicological studies. Therefore it seems useful to know the properties of an enzymatic system in this species, and, as far as possible, to control its activity with a specific inhibitor.

This paper is concerned with (a) the preparation of a ten times purified fraction, (b) the kinetics of ethanol and retinol (vitamin A alcohol) oxidation catalyzed by this preparation with NAD^+ as coenzyme, (c) the inhibition effect of pyrazole [5] in the kinetics of rat LADH catalysed ethanol and retinol

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*** The following abbreviations are used: LADH, liver alcohol dehydrogenase, E.C. 1.1.1.1, alcohol: NAD^+-oxidoreductase. NAD^+ and NADH, oxidized and reduced nicotinamide pyridine nucleotide, respectively.

oxidation, (d) the effect of pyrazole on several other zinc-containing enzymes, (e) the inhibition effects of various pyrazole derivatives, some of which are more potent than pyrazole.

MATERIALS AND METHODS

Rat LADH preparation. Wistar male rats of 250 g in weight were used. The thorax was opened under ether anesthesia and the rats were killed by cardiac puncture. About 50 ml of a 0.9 % NaCl solution were perfused through the abdominal aorta in order to eliminate the blood of the liver vessels.[6] Livers which were not used immediately were stored at −18°C. Each preparation started with three livers (about 40 g). All operations were performed at temperature of 4°C. Homogenization was performed with an Ultra-Turrax homogenizer, model TP 18/2, using 3 ml phosphate buffer (0.01 M, pH 7.5) per gram of fresh liver. The homogenate was centrifuged for 60 min at 38 000 *g* in a MSE High-Speed 18 centrifuge. The enzyme was precipitated with ammonium sulfate between 35 and 70 % saturation. The pH was maintained at 7 by addition of ammonia. The precipitate was resuspended in the minimum amount of a tris-phosphate buffer (0.005 M, pH 8.0) and dialyzed overnight with two changes of one liter of this buffer. The solution was then passed through a DEAE-cellulose column, prepared according to Peterson and Sober [7] and equilibrated with the same buffer. The ADH activity was not retained on the column and appeared in the eluate which was dialyzed for 6 h against phosphate buffer (pH 7.2, ionic strength 0.05). The solution was then passed through a CM-cellulose column, prepared according to Peterson and Sober [7] and equilibrated with the same buffer. Phosphate buffers (pH 7.2, 0.05 μ and pH 7.8, 0.05 μ) were used for elution. Active eluates were dialyzed against tris-phosphate buffer (0.005 M, pH 8.0). These solutions were stable at 4°C for several days.

Effect of pH and temperature on rat LADH stability. After ammonium sulfate fractionation (35—70 %), the enzyme preparation was dissolved in 0.01 M phosphate buffer pH 8.0 and dialyzed against the same buffer. Aliquots of the pH 8.0 solution were then dialyzed for 24 h against 0.01 M phosphate buffers of varied pH. The ADH activity was measured at zero time and after 24 h. Other samples of the pH 8.0 solution were exposed at various temperatures for different times.

LADH activity determination. Rat LADH activity was assayed at 23.5°C in the same conditions as for horse LADH:[8] 1.85 ml glycine buffer (0.1 M, pH 10.0), 1.00 ml NAD+ solution (1 mg/ml), 0.15 ml ethanol solution (1 ml 96 % solution to 100 ml with distilled water), 0.10 ml enzyme preparation in a 1 cm cuvette. One unit of activity, U, is the amount of enzyme which catalyzes the transformation of 1 μmole of substrate per 60 sec.[9] Practically, the activity was determined from measurements of the time in seconds necessary to obtain a 0.2 optical density change at 340 mμ in the assay conditions. In these conditions, the unit previously used by Zachman and Olson [6] corresponds to 5.79×10^{-3} U.

Preparation of aqueous retinol solution. Retinol is poorly soluble in water. It was dispersed in water with Tween 80 [6,10] according to the following procedure. An acetone solution was first prepared and an aliquot of this solution mixed with petroleum ether (b.p. 30—60°). The retinol concentration was determined from the absorbancy of this solution at 325 mμ on the basis of an absorbancy index $E^{1\,\%}_{1\,cm} = 1830$.[11] An aliquot of the acetone solution was mixed with an acetone solution of Tween 80. The mixture was evaporated under nitrogen flow at room temperature and the viscous residue was dispersed in distilled water. The solution thus obtained was perfectly clear. In these conditions, an aqueous stock solution containing 0.09 % Tween 80 and 120 μmole/l retinol was easily prepared. For the kinetic studies, an aliquot of this stock solution was added to the buffered reaction mixture. In each incubation medium there was a constant ratio between retinol and Tween 80.

Retinal was dissolved in hexane. The absorbancy of this solution at 370 mμ permitted the calculation of the retinal concentration on the basis of an absorbancy index $E^{1\,\%}_{1\,cm} = 1690$.[12] The aqueous solution was then prepared in the same way as for retinol.

The yellow aqueous solution contained in our conditions 0.18 % Tween 80 and 250 μmole/l retinal.

The retinol and retinal aqueous stock solutions were prepared in weak light and used within the next two hours.

Rate determination of the ethanol and retinol oxidation. The rate of reduction of NAD+ coupled with ethanol oxidation was measured fluorimetrically.[13] With retinol the concentration change of the product (retinal) was spectrophotometrically measured at 400 mμ with the expanded scale of a Cary recording spectrophotometer, model 14. The rate determination of retinal reduction was followed at 400 mμ with the normal scale of the Cary spectrophotometer. With retinol the reaction was started by the addition of the substrate and, with ethanol and retinal, by the addition of ADH. Initial velocities are expressed as micromoles per liter per second and plotted according to Lineweaver and Burk [14] in terms of e/v against 1/[varied partner].[13] All kinetic studies were carried out in phosphate buffer (ionic strength 0.1, pH 7) at 23.5°C.

Other determinations. Yeast ADH activity was determined according to Wallenfels and Sund.[15] The kinetic studies were performed fluorimetrically as for LADH. Carbonic anhydrase activity was determined according to Lindskog [16] at 3°C, carboxypeptidase according to Snoke and Neurath [17] from the measurement of phenylalanine [18] liberated from carbobenzoxy-glycyl-phenylalanine. Lactic and glutamic dehydrogenases were assayed according to Neilands [19] and Strecker,[20] respectively, alkaline phosphatase according to Garen and Levinthal,[21] glyceraldehyde-3-phosphate dehydrogenase according to Velick,[22] α-glycerophosphate dehydrogenase according to Beisenherz *et al.*,[23] glucose-6-phosphate dehydrogenase according to Kornberg and Horecker,[24] malate dehydrogenase according to Ochoa,[25] "isomerase" activity of LADH according to Van Eys,[26] catalase according to Bonnichsen *et al.*,[27] and triose phosphate isomerase according to Racker *et al.*[28] Proteins were determined with the Lowry method.[29]

Reagents. Retinol and retinal were obtained from Hoffman-LaRoche, Basel or from Distillation Products Industry (Eastman Kodak). NAD+ and NADH were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Pyrazole was purchased from Theodor Schuchardt, München. Pyrazole derivatives were gifts from Dr. B. Sjöberg, AB Astra, Södertälje, Sweden. DEAE and CM-cellulose were prepared from Whatman cellulose (CF 11) according to Peterson and Sober.[7] Absorbancy indices of NADH and NAD+ used were $A_{340}=6.22$ ($mM^{-1}\times cm^{-1}$) [30] and $A_{260}=18.0$ ($mM^{-1}\times cm^{-1}$),[31] respectively. Retinol and retinal spectra in phosphate buffer (ionic strength 0.1, pH 7) were recorded with a Cary spectrophotometer, model 14. Yeast ADH, lactate dehydrogenase (rabbit muscle), glutamate dehydrogenase (beef liver), alkaline phosphatase (calf intestine), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), α-glycerophosphate dehydrogenase (rabbit muscle), glucose-6-phosphate dehydrogenase (yeast), malate dehydrogenase (pig heart) and triose phosphate isomerase (rabbit muscle) were commercial enzyme preparations from Boehringer. Carbonic anhydrase was kindly supplied by Dr. B. Strandberg, Uppsala, Sweden, catalase by Miss G. Heimbürger in this laboratory.

RESULTS

Ammonium sulfate fractionation. The ADH activity of rat liver is precipitated between 35 and 70 % of saturation. This fraction was used without further purification for the stability experiments and after further purification by chromatography for the kinetic studies.

Effect of pH and temperature on rat LADH stability. After ammonium sulfate fractionation, the resuspended dialyzed active precipitate (see materials and methods) is not stable at pH below 8. After 24 h at pH 6.0 and 4°C, all of the activity is destroyed. In the same conditions, at pH 7.0, only 60 % of the activity remained (Fig. 1). During heat treatment, at pH 8.0, marked inactivation occurs from 45°C and is more pronounced at 50°C. The previous observations of Zachman and Olson [6] at 53°C are in good accordance with the present results (Fig. 2).

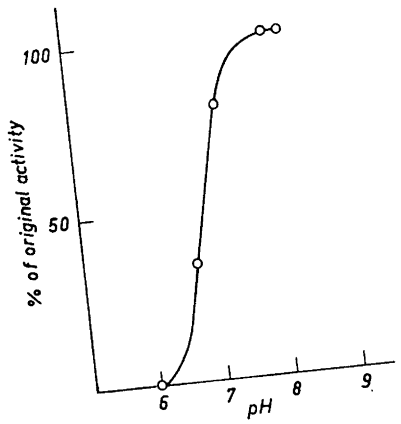

Fig. 1. Effect of pH on rat LADH stability. Conditions: see Materials and Methods.

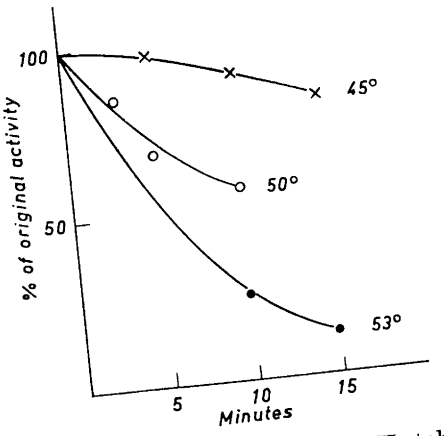

Fig. 2. Effect of heat on rat LADH stability. Conditions: see Materials and Methods.

Rat ADH purification (Table 1). The ammonium sulfate fractionation eliminates some of the inactive proteins. After DEAE-cellulose chromatography, the ADH preparation is about 6 times purified with 80 % yield. In accordance with previous observations,[32] the rat liver ADH activity is not retained at pH 8.0 (tris-phosphate buffer 0.005 M) on the anion exchanger and appears in the first protein fractions. On the subsequent CMC chromatography, at pH 7.2 (phosphate buffer 0.05 μ), the main part of the activity is only slightly retained on the column and appears in the eluates somewhat slower than the total protein peak. The tail fraction is rapidly eluted with phosphate buffer pH 7.8, ionic strength 0.05 (Fig. 3). On Fig. 3 the pH of the eluates were measured at 4°C and thus deviated somewhat from the pH values of the original buffer solutions in which pH was measured at room temperature.

Table 1. Purification procedure of rat LADH.

	Protein (mg)	Activity (mU)	Specific activity (mU/mg)	Time purification	Yield %
First supernatant of whole homogenate	2950	28 300	9.6	—	100
Precipitate (35—70 % Am_2SO_4)	2100	25 700	12.2	1.3	91
After DEAE-chromatography	367	22 800	62.2	6.5	80
After CMC-chromatography	186	14 500	78	8.1	51

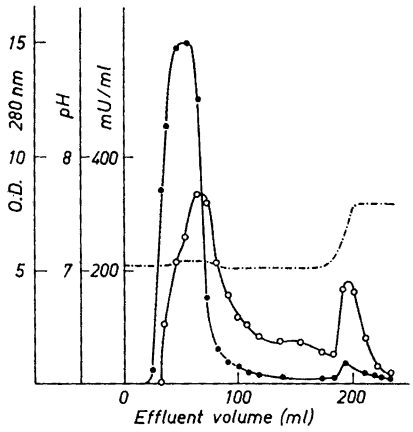

Fig. 3. Carboxymethyl-cellulose chromatography in rat LADH preparation. Column was equilibrated with phosphate buffer (pH 7.2, ionic strength 0.05). (●) optical density at 280 mμ. (○) activity expressed as mU/ml. (—.—) pH of elution fractions.

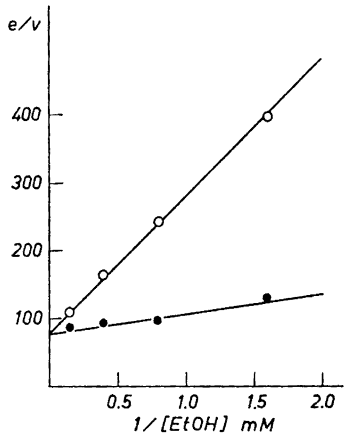

Fig. 4. Ethanol oxidation kinetics with $[NAD^+]=210$ μM. Conditions: phosphate buffer (pH 7.0, ionic strength 0.1), temperature 23.5°C, fluorimetry. (●) control. (○) with [pyrazole]$=25$ μM. $v=$μM NADH sec^{-1} and $e=$U/l.

Kinetics and pyrazole inhibition of ethanol oxidation. The kinetic studies were performed with the first and main fraction (50—110 ml of elution volume on Fig. 3) eluted from the CMC chromatography. Between 6.25 mM and 0.625 mM ethanol concentrations, the reaction rates follow a linear relation in the Lineweaver-Burk plots (Fig. 4). In contrast with horse LADH no induction period was seen. The Michaelis constant K_m is 0.5×10^{-3} M. The V_{max}/e is 13×10^{-3} μM U^{-1} l^{-1} sec^{-1}. These values were obtained from experiments which were carried out with $[NAD^+]=210$ μM.

As for horse LADH, pyrazole is an inhibitor of rat LADH activity. With ethanol as varied substrate the inhibition is competitive (Fig. 4). The inhibition constant K_i is 4.2 μM.

Kinetics and pyrazole inhibition of retinol oxidation. The retinol oxidation or the retinal reduction reactions were followed at 400 mμ. The retinol and retinal spectra in phosphate buffer (pH 7, ionic strength 0.1) with 0.02 % Tween 80 are shown on Fig. 5. The absorption maximum of retinol is observed at 328 mμ and at 380 mμ for retinal. At 400 mμ the molar absorbancy index of retinal is 29 500.

In the 1—2 μM retinol concentration range the reaction rates of the oxidation follow a linear relation in the Lineweaver-Burk plots (Fig. 6). The Michaelis constant K_m is 0.7×10^{-6} M. The V_{max}/e is 1.45×10^{-3} μM U^{-1} l^{-1} sec^{-1}. With $[NADH]=100$ μM rat LADH catalyzes the retinal reduction. In the Lineweaver-Burk plots the reaction rates follow a linear relation between 5 and 25 μM retinal concentrations (Fig. 7).

Pyrazole is a competitive inhibitor of retinol oxidation (Fig. 6). The inhibition constant K_i is 3.6 μM. In the conditions used, with 50 μM pyrazole

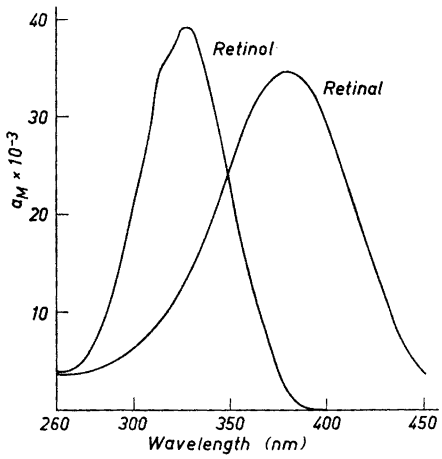

Fig. 5. Retinol and retinal spectra in aqueous solution. Conditions: phosphate buffer (pH 7.0, ionic strength 0.1), 0.02 % Tween 80. Cary spectrophotometer, model 14. Temperature 23.5°C.

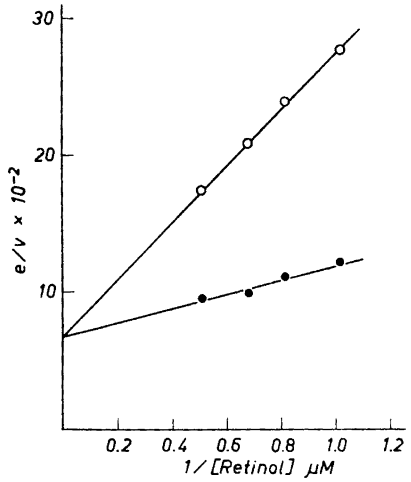

Fig. 6. Retinol oxidation kinetics. Conditions: $[NAD^+]=205$ μM, phosphate buffer (pH 7.0, ionic strength 0.1), temperature 23.5°C. Control (●). With [pyrazole]=10 μM (○). Cary spectrophotometer 10 times expanded scale. $v=\mu M$ retinal sec^{-1} and $e=U/l$.

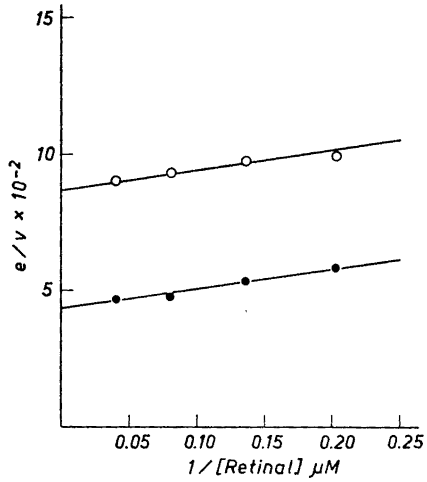

Fig. 7. Retinal reduction kinetics. Conditions: [NADH]=100 μM, phosphate buffer (pH 7.0, ionic strength 0.1), temperature 23.5°C. Control (●). With [pyrazole]=50 μM (○). $v=\mu M$ retinal sec^{-1} and $e=U/l$.

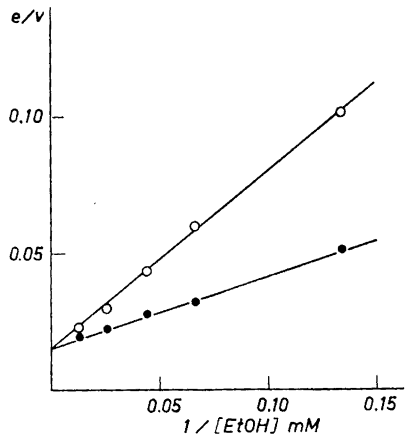

Fig. 8. Kinetics of ethanol oxidation catalyzed by yeast ADH. Conditions: phosphate buffer (pH 7.0, ionic strength 0.1), temperature 23.5°C. Control (●). With [pyrazole]=41.5 μM (○). Concentration of $NAD^+=1070$ μM.

concentration the retinal reduction is 50 % uncompetitively inhibited (Fig. 7). In Table 2 the values of the kinetic inhibition constants obtained with rat LADH are summarized.

Table 2. Kinetic and pyrazole inhibition constants of rat LADH with ethanol and retinol as substrates.

	Rat LADH	
Substrate	Ethanol	Retinol
K_m (mM)	0.50	0.0007
K_i (μM) for pyrazole	4.2	3.6

Table 3. Assay of pyrazole derivatives as inhibitors of rat LADH. Conditions: phosphate buffer (pH 7.0, ionic strength 0.1), temperature 23.5°C. [NAD^+] = 200 μM, [ethanol] = 1.25 mM, [inhibitors] = 25 μM, rat LADH = 0.87 U/l.

Pyrazole derivatives	Relative initial rate (%)	K_i (μM)
None	100	—
Pyrazole	51	4.3
4-Bromo-pyrazole	9	0.8
3,4-Dibromo-pyrazole	68	12
3,4,5-Tribromo-pyrazole	82	> 30
4-Iodo-pyrazole	10	0.6
3,4-Diiodo-pyrazole	98	> 100
3,4,5-Triiodo-pyrazole	75	20
4-Nitro-pyrazole	88	> 30
3,5-Dimethyl-pyrazole	97	> 100
1-Methyl-pyrazole	74	20
3-Methyl-pyrazole	80	> 30
4-Methyl-pyrazole	18	1.2
4-Nitro-3,5-dimethyl-pyrazole	86	> 30
4-Nitro-imidazole	80	> 30
Imidazole	83	> 30

Inhibition effect of pyrazole derivatives. Several substituted derivatives can be prepared from pyrazole. Some of them were tested for their inhibition effect on rat LADH activity. Three derivatives are more potent inhibitors than pyrazole: 4-bromo, 4-iodo and 4-methyl-pyrazole. Substitutions on carbon 3 or 5, or on nitrogen 1 decrease the inhibition property (Table 3). The kinetic studies show that 4-bromo, 4-iodo, and 4-methyl-pyrazole, like pyrazole, are competitive inhibitors with ethanol as substrate. The inhibition constants for 4-iodo, 4-bromo, and 4-methyl-pyrazole are 0.6, 0.8, and 1.2 μM, respectively (Table 4).

Table 4. Pyrazole derivatives inhibition constants of rat LADH and yeast ADH.

		K_i (μM)			
Enzyme	Substrate	Pyrazole	4-Iodo-pyrazole	4-Bromo-pyrazole	4-Methyl-pyrazole
Rat LADH	Ethanol	4.2	0.6	0.8	1.2
Yeast ADH	Ethanol	29	7000	2000	—

Table 5. Pyrazole and zinc-containing enzymes.

Enzymes	Substrate varied	Pyrazole concentration	Inhibition
Horse LADH	Ethanol		Competitive $K_i = 0.2$ μM
	NAD^+		Mixed un-non competitive
	Acetaldehyde		Uncompetitive
	NADH		Uncompetitive
Rat LADH	Ethanol		Competitive $K_i = 4.2$ μM
Yeast ADH	Ethanol		Competitive $K_i = 29$ μM
	NAD^+		Uncompetitive
	Acetaldehyde		Uncompetitive
	NADH		Uncompetitive
Carbonic anhydrase	CO_2 (2—8 mM)	6 mM	0
Lactic dehydrogenase	Lactate (0.1—1 mM) $NAD^+ = 200$ μM	6 mM	0
Glutamic dehydrogenase	Glutamate (1.6—6 mM) $NAD^+ = 140$ μM	3 mM	0
Alkaline phosphatase	*p*-Nitrophenylphosphate (0.16—1.6 mM)	10 mM	0
Carboxypeptidase	Carbobenzoxy-glycyl-L-phenylalanine (2—4 mM)	10 mM	0
Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate (10—215 μM) $NAD^+ = 97$ μM	5 mM	0
α-Glycerophosphate dehydrogenase	Dihydroxyacetone phosphate (12.5—200 μM) NADH = 10 μM	8 mM	0
Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate (40—400 μM) $NADP^+ = 400$ μM	5 mM	0
Malate dehydrogenase	Oxaloacetate (20—200 μM) NADH = 9 μM	4 mM	0

Pyrazole effect on zinc-containing enzymes (Table 5). The pyrazole effect was investigated on other enzyme systems which, like horse LADH, contain zinc in their molecule:[34,35] yeast ADH, carbonic anhydrase, carboxypeptidase, lactate dehydrogenase, glutamate dehydrogenase, alkaline phosphatase, glyceraldehyde-3-phosphate dehydrogenase, α-glycerophosphate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase. Catalase and triose-phosphate isomerase were also tested for their sensitivity to pyrazole. With 4—10 mM concentration range, 100 to 1000 times higher than the active concentration for horse or rat LADH, pyrazole had no inhibition effect on the assayed enzymes. Only yeast ADH was found to be appreciably inhibited. The kinetic studies of the yeast ADH inhibition show that pyrazole is a competitive inhibitor with ethanol as substrate (Fig. 8) and uncompetitive when acetaldehyde, NAD^+ or NADH are substrates. The 4-iodo and 4-bromo derivatives also are competitive inhibitors of yeast ADH with ethanol as substrate. The inhibition constants K_i for pyrazole, 4-iodo, and 4-bromopyrazole are 29 μM, 7 mM, and 2 mM, respectively (Table 4).

DISCUSSION

Rat LADH purification. Liver ADH is a soluble enzyme found in the supernatant after cell disruption and fractionation.[36] Thus the homogenization of the livers with an Ultra-Turrax blender or by the Potter-Elvehjem method gives the same ADH activity in the homogenate.[37] But the stronger homogenization with the Ultra-Turrax blender at high speed (24 000 rpm) probably liberates more proteolytic enzymes [38] which may explain the sensitivity of this rat ADH preparation to acidic pH and heat treatment. With the ammonium sulfate fractionation and DEAE-cellulose chromatography the ADH activity is several times purified with a good yield. However, in the case of rat LADH and in the conditions used, the CMC chromatography is less useful than for horse LADH. The increase of purity and the yield are low and the electrophoretically observed rat LADH fractions [39] could not be separated. Because of the instability of the preparation below pH 7.2, it was not possible to use a lower pH in the chromatography on CMC.

The calculation of enzyme activity per unit of liver weight shows that rat liver contains 6 to 10 times less activity than horse liver. The horse liver contains about 1 g of enzyme per kg of liver. In 1 kg of rat liver there are about 580 to 870 Units. In the assay conditions, 0.182 mg of horse LADH corresponds to one Unit (calculated from the relation $mg/ml = 1.05/(t_{0.2} \times v)$[8]). Thus, one kilogram of rat liver contains an activity equivalent to that of 105 to 158 mg of horse LADH, *viz.* 10 to 6 times less.

Kinetic properties of rat LADH. From the Michaelis constants of rat LADH for ethanol and retinol (Table 2), it appears that rat LADH has a greater affinity for retinol than for ethanol. In the case of horse LADH, it is known that the Michaelis constant decreases when the chain length of primary alcohols increases. In the same way, the longer the chain of primary alcohols, the lower is the concentration at which substrate inhibition occurs.[40] In the retinol concentrations used (1—2 μM) no substrate inhibition was

observed. On the other hand, V_{max} is 10 times higher with ethanol than with retinol. The difference observed between the kinetic coefficients of retinol and ethanol oxidation demonstrates once more the importance of the lipophilic chain attached to the primary alcohol group.

Inhibition effect of pyrazole. Pyrazole is an inhibitor of rat LADH as well as of horse LADH. Theorell and Yonetani [5] were the first to observe the inhibition effect of pyrazole on horse LADH. Pyrazole is a stronger inhibitor of ethanol or retinol oxidation catalyzed by horse LADH (K_i=0.20 and 0.17 μM, respectively) than by rat LADH (K_i=4.2 and 3.6 μM, respectively). But the inhibition is stronger with both mammalian liver ADH's than with yeast ADH (K_i=29 μM). The difference is more striking with the 4-halogenated pyrazole derivatives, 4-bromo and 4-iodo-pyrazole, more potent inhibitors of horse and rat LADH than pyrazole, are much less, if at all, inhibitory on yeast ADH (Table 4). Thus the enzymatic alcohol determination with yeast ADH is possible in blood or tissue extracts containing 4-iodo or 4-bromo-pyrazole.

On the basis of their observations with horse LADH, Theorell and Yonetani [5] described the mechanism of the pyrazole inhibition. A bond formation between pyrazole and zinc of ADH expels one proton and a bond is formed between the pyrazole and the C-4 of the pyridine ring of NAD^+. The binding of pyrazole to both NAD^+ and the zinc of horse LADH may account for its potent inhibition effect on this system and the apparent specificity of this inhibition. Other zinc-containing enzymes and enzymes like catalase and triose-phosphate isomerase are not inhibited by pyrazole in a concentration range up to 10 mM. Among all the tested enzymes, only liver and yeast ADH were inhibited by pyrazole. Since yeast ADH is not present in mammalian tissues it seems reasonable to assume that pyrazole would have a highly specific effect on LADH *in vivo*.

The possibility to inhibit an enzymatic reaction specifically *in vivo* is an interesting point. With pyrazole it is now possible to study the participation of LADH to the metabolism of retinol (vitamin A alcohol), of aliphatic primary alcohols and of various chemical structures with primary or secondary alcohol group attached to an appropriate lipophilic radical. The *in vitro* studies, which were reported in this work, were performed with the LADH of the rat in which it is possible to analyze the effects of the inhibition *in vivo* of LADH after pyrazole administration. Further pharmacological work along this line is actually in progress.

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REFERENCES

1. Sund, H. and Theorell, H. *The Enzymes*, Academic, New York 1963, Vol. 7 pp. 25–83.
2. Theorell, H. *Bull. Soc. Chim. Biol.* **46** (1964) 1533.
3. von Wartburg, J. P., Bethune, J. L. and Vallee, B. L. *Biochemistry* **3** (1964) 1775.

4. Papenberg, J., von Wartburg, J. P. and Aebi, H. *Biochem. Z.* **342** (1965) 95.
5. Theorell, H. and Yonetani, T. *Biochem. Z.* **338** (1963) 537.
6. Zachman, R. D. and Olson, J. A. *J. Biol. Chem.* **236** (1961) 2309.
7. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
8. Dalziel, K. *Acta Chem. Scand.* **11** (1957) 397.
9. Dixon, M. and Webb, E. C. *Enzymes*, Longmans, London 1964.
10. Bliss, A. F. *Arch. Biochem. Biophys.* **31** (1951) 197.
11. Embree, N. D., Ames, S. R., Lehman, R. W. and Harris, P. L. *Methods Biochem. Analy.* **4** (1957) 47.
12. Plack, P. A., Kon, S. K. and Thompson, S. Y. *Biochem. J.* **71** (1959) 467.
13. Theorell, H. and McKinley-McKee, J. S. *Acta Chem. Scand.* **15** (1961) 1797, 1811, 1834.
14. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
15. Wallenfels, K. and Sund, H. *Biochem. Z.* **239** (1957) 17.
16. Lindskog, S. *Biochim. Biophys. Acta* **39** (1960) 218.
17. Snoke, J. E. and Neurath, H. *J. Biol. Chem.* **181** (1949) 789.
18. Moore, S. and Stein, W. H. *J. Biol. Chem.* **179** (1948) 367.
19. Neilands, J. B. *Methods in Enzymology* **1** (1955) 449.
20. Strecker, H. J. *Methods in Enzymology* **2** (1955) 220.
21. Garen, A. and Levinthal, C. *Biochim. Biophys. Acta* **38** (1960) 470.
22. Velick, S. F. *Methods in Enzymology* **1** (1955) 401.
23. Beisenherz, G., Bucher, T. and Garbade, K. H. *Methods in Enzymology* **1** (1955) 391.
24. Kornberg, A. and Horecker, B. L. *Methods in Enzymology* **1** (1955) 323.
25. Ochoa, S. *Methods in Enzymology* **1** (1955) 735.
26. Van Eys, J. *J. Biol. Chem.* **236** (1961) 1531.
27. Bonnichsen, R. K., Chance, B. and Theorell, H. *Acta Chem. Scand.* **1** (1947) 685.
28. Racker, E., Klybas, V. and Schramm, M. *J. Biol. Chem.* **234** (1959) 2510.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
30. Horecker, B. L. and Kornberg, A. *J. Biol. Chem.* **175** (1948) 385.
31. Kronberg, A. and Pricer, W. E. *Biochem. Prep.* **3** (1953) 201.
32. Moore, B. W. and Lee, R. H. *J. Biol. Chem.* **235** (1960) 1359.
33. Theorell, H., Taniguchi, S., Åkeson, Å. and Skursky, L. *Biochem. Biophys. Res. Commun.* **24** (1966) 603.
34. Malmström, B. and Neilands, J. B. *Ann. Rev. Biochem.* **33** (1964) 331.
35. Vallee, B. L. *The Enzymes*, Academic, New York 1960, Vol. 3, p. 255.
36. Nyberg, A., Schubert, J. and Änggard, L. *Acta Chem. Scand.* **7** (1953) 1170.
37. Büttner, H. *Biochem. Z.* **341** (1965) 300.
38. de Duve, C. *Ciba Foundation Symposium*, Churchill, London 1963.
39. Koen, A. L. and Shaw, C. R. *Biochim. Biophys. Acta* **128** (1966) 48.
40. Shore, J. D. and Theorell, H. *Arch. Biochem. Biophys.* **117** (1966) 375.

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