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On the Effects of Some Heterocyclic Compounds on the Enzymic Activity of Liver Alcohol Dehydrogenase

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During the course of kinetic investigations of horse liver alcohol dehydrogenase [1] it was found that the LADH-catalyzed oxidation of ethanol by NAD^+ is either inhibited or stimulated by imidazole depending on the concentration of the substrate used. However, since imidazole concentrations above millimolar were required *in vitro* for obtaining substantial effects it was decided to try to find more potent substances with similar mode of action. It seemed possible that compounds that increase the rate of oxidation of ethanol *in vivo* might be of practical interest. Therefore, a number of heterocyclic compounds related to imidazole were synthesized and tested for their effects on the enzymic activity of horse LADH. The results of this survey study will be described in this paper.

EXPERIMENTAL

Materials. A preparation of three times recrystallized LADH** was made from fresh horse liver according to a modification of Dalziel's method.[2] The purity of the enzyme preparation was estimated to be nearly 100 % on the basis of a molecular weight of 84 000 per two coenzyme-binding sites. The enzyme concentration was expressed as N, the normality of the coenzyme-binding site per liter, which was determined spectrophotometrically by titrating LADH (20 to 50 μN) with NAD^+ *** in the presence of 1 mM pyrazole.[3] The enzyme was dialyzed against sodium phosphate buffer, pH 7.0, ionic strength 0.1 at 4°C for several days prior to the assay and kinetic experiments. NAD^+ and NADH † were purchased from Pabst Laboratories. The concentration of the coenzyme was spectrophotometrically determined on the basis of the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ at 340 mμ in a reduced state.[4]

The compounds used in the activity tests (listed in Table 1) were either commercially available or prepared according to known methods.

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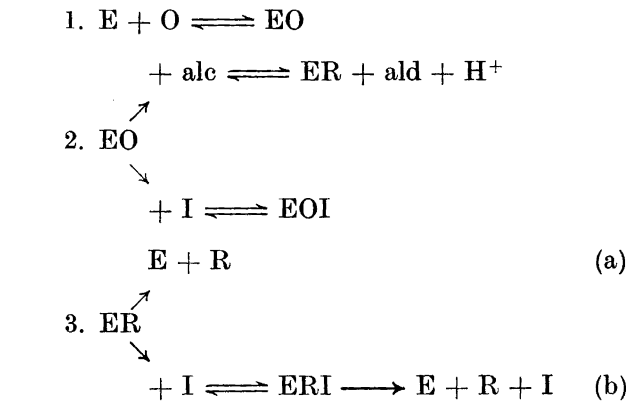
** LADH, liver alcohol dehydrogenase; *** NAD^+, oxidized nicotiniumamide dinucleotide; † NAD, reduced nicotiniumamide dinucleotide.

Methods. The effects of these heterocyclic compounds on the kinetics of the LADH reaction were studied fluorimetrically [5] by observing the rate of change of the fluorescence of the coenzyme on oxidation and reduction.[6] The initial rates thus measured were plotted according to Lineweaver and Burk [7] to calculate K_I values. The effects of these compounds were mostly examined in the direction of oxidation of ethanol by NAD^+. When the concentration of NAD^+ or ethanol was varied, the concentration of its reaction partner (ethanol or NAD^+, respectively) was kept constant at an optimal value: 6.2 mM ethanol for varied concentrations of NAD^+ from 20 to 500 μM and 500 μM NAD^+ for varied concentrations of ethanol from 100 μM to 6.2 mM. The experiments were performed in sodium phosphate buffer, pH 7.0, ionic strength 0.1 at 23.5°C.

RESULTS AND DISCUSSION

The heterocyclic compounds tested and their effects on the enzymic activity of LADH are listed in Table 1. The effects of these compounds were expressed in terms of the type of inhibition, as defined in Fig. 1, and K_I values. In order to understand the relations between the different types of inhibition it may be helpful to recall that the inhibitors (I) here in question operate by forming ternary complexes with the binary enzyme coenzyme complexes, LADH·NAD^+ (EO), or LADH·NADH (ER).[1] In the ternary complexes (EOI) the inhibitor occupies the binding site of alcohol, in ERI that of aldehydes. Some inhibitors like pyrazoles [3] or fatty acids [8] only give EOI complexes, others like fatty acid amides only ERI complexes, whereas imidazole gives both types. Therefore, pyrazole gives competitive inhibition (C) with ethanol, amides with aldehyde.

A general scheme for the action of inhibitors in the "Theorell-Chance" mechanism of LADH may be pictured:



Inhibition type CIS, as with imidazole, occurs when both EOI and ERI are formed, and the liberation of E for another reaction cycle is faster *via* ERI (b) than *via* ER (a). Type C will occur if ERI is not formed, or if the overall velocity in (b) is=(a). This is a very special case which would require that the binding of I to ER would not influence the stability of the complex.

Types M, N and U-N and U will appear, in this order, the greater the stability—and thus the lower the decomposition velocity—of ERI compared with EOI.

Table 1.

		Inhibitor behaviour toward				
		NAD+			Ethanol	
No.	Name	Source	Type	K_I	Type	K_I
1.	Imidazole	a	CIS	3.6 mM	CIS	7.6 mM
2.	α-4(5)-Imidazoleisobutyronitril	b	CIS	—	CIS	7.7 mM
3.	4(5)-*tert*-Butylimidazole	b	M	47 μM	C	280 μM
4.	β-4(5)-Imidazoleisobutylamine	b	CIS	6.5 mM	CIS	4.5 mM
5.	*N*,*N*-Dimethyl-β-4(5)-imidazoleisobutylamine	b	CIS	5.7 mM	CIS	>10 mM
6.	Ethyl-α-4(5)-imidazoleisobutyrate	b	M	270 μM	C	280 μM
7.	Pyridine	a	C	900 μM	C	2.1 mM
8.	2-Methyl-pyridine (α-picoline)	a	M	*ca.* 1 mM	M	2.5 mM
9.	3-Methyl-pyridine (β-picoline)	a	C	180 μM	C	390 μM
10.	4-Methyl-pyridine (γ-picoline)	a	M	260 μM	C	350 μM
11.	2,6-Dimethyl-pyridine (lutidine)	a	C, CIS	2.9 mM	C	7.5 mM
12.	Pyrazine	a	CIS	25 mM	—	∞
13.	2-Methyl-pyrazine	a	CIS	4 mM	CIS	20 mM
14.	2,5-Dimethylpyrazine (ketine)	c	C,CIS	4 mM	CIS	7.8 mM
15.	1,2,4-Triazole	a	U	—	C	410 μM
16.	3-Amino-1,2,4-triazole	a	CIS	14 mM	CIS	14 mM
17.	4-Amino-1,2,4-triazole	a	CIS	36 mM	CIS	69 mM
18.	1,2,3,5-Tetrazole	a	U—N	—	C	3.1 mM
19.	Pyrazole	a	U	—	C	0.2 μM
20.	1-Methylpyrazole	e	M	6.8 mM	N	17 mM
21.	3-Methylpyrazole	d	U—N	—	C	125 μM
22.	4-Methylpyrazole	f	U	—	C	0.08 μM
23.	3,5-Dimethylpyrazole	a	C	1.8 mM	C	7.1 mM
24.	3,4-Dimethyl-5-pyrazolone	d	U—N	100 μM	C	?
25.	4-Bromo-pyrazole	g	U	—	C	0.02 μM
26.	3,4-Dibromopyrazole	g	U	—	C	20 μM
27.	3,4,5-Tribromo-pyrazole	g	U	—		20 μM
28.	4-Iodopyrazole	h	U	—	C	0.02 μM
29.	3,4-Diiodopyrazole	h	U	—	C	30 μM
30.	3,4,5-Triiodopyrazole	h	U	—	C	10 μM
31.	Benzpyrazole	a	U	—	C	14 μM

[a] Fluka AG, and purified. [b] Jönsson, Å. *Acta Chem. Scand.* **8** (1954) 1389. [c] EGA-Chemie KG, and purified. [d] Aldrich Chemical Company, and purified. [e] Finar, L. and Lord, G. H. *J. Chem. Soc.* **1957** 3314. [f] Pino, P. and Ercoli, R. *Gazz. Chim. Ital.* **81** (1951) 757. [g] Hüttel, R., Wagner, H. and Jochum, P. *Ann.* **593** (1955) 179. [h] Morgan, G. T. and Ackerman, I. *J. Chem. Soc.* **123** (1923) 1308.

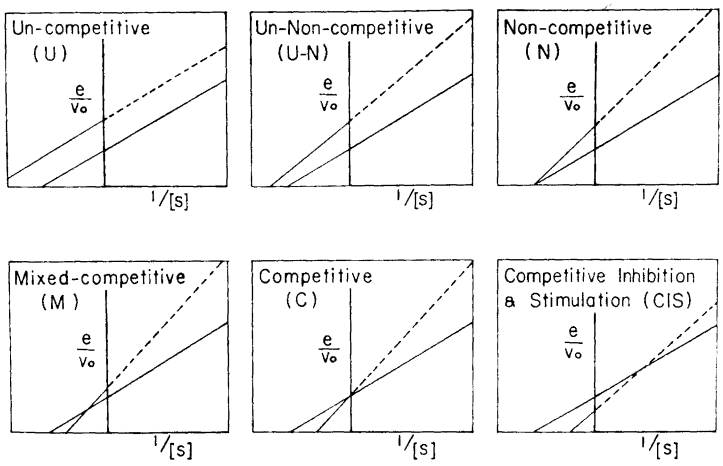


Fig. 1. Types of inhibition in the Lineweaver and Burk plot. Whole-drawn and dotted lines are obtained in the absence and presence of a fixed concentration of an inhibitor, respectively.

Type U appears if only ERI, but not EOI is formed, for instance with isobutyramide.

The inhibition constant, K_I, here refers to the inhibition caused by the formation of the complex EOI, and is taken as the concentration of inhibitor which doubles the *slope* of the Lineweaver-Burk plot compared with the slope without inhibitor. Under our conditions, high concentration of NAD^+ and varied ethanol concentration, it is easily seen that when [alc]→zero the whole system approaches true equilibrium instead of steady state, and $K_I = K_{EO,I}$, the *dissociation constant* operating between EO and I, and this will not depend on the type of inhibition. The difference between the types can be described as a parallel displacement of the lines with inhibitor present, caused by the formation of ERI complexes, which do not change the *slope* of the line which is a function of the formation of EOI. The enzymic activity of LADH was affected immediately on the addition of the majority of these compounds except for pyrazole and some of its derivatives (see below). As reported previously [1] imidazole exhibits either inhibition or stimulation of Type CIS (*cf.* Fig. 1) toward both NAD^+ (K_I=3.6 mM) and ethanol (K_I= 7.6 mM). Several other compounds also exhibited the inhibition of Type CIS. However, none of these inhibitors of Type CIS was found to be substantially more potent than imidazole. Therefore, our initial aim to discover an effective stimulator of the ethanol oxidation has not been thus far attained. No systematic correlation between the inhibition of Type CIS and the chemical structure of the inhibitor has yet been found.

During the course of this survey, made in 1963, a series of extremely potent inhibitors of LADH which compete with ethanol were discovered. Pyrazole, an isomer of imidazole, was found to be a potent ethanol-competitive inhibitor of LADH (K_I of 0.1 to 0.2 μM). It was shown that the inhibition of LADH by

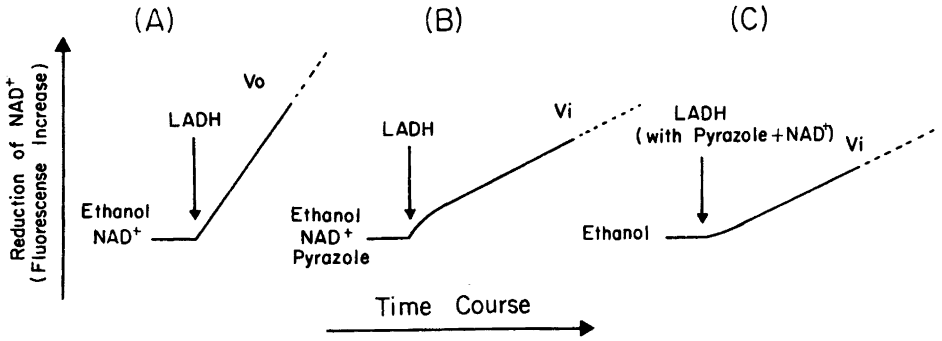


Fig. 2. Kinetics of the inhibition by pyrazole of the LADH-catalyzed oxidation of ethanol in the presence of NAD^+. The reaction was monitored fluorimetrically by measuring the increase in the fluorescence of NADH formed. The reaction without pyrazole was initiated by the addition of the enzyme to the reaction mixture (A). The reaction in the presence of pyrazole was initiated either by the addition of the enzyme to the reaction mixture (B), or by the addition of the enzyme preincubated with pyrazole and NAD^+ to ethanol (C). Since the ternary complex of LADH with NAD^+ and pyrazole is formed (B) and dissociated (C) rather slowly, the steady state rates of the LADH reaction in the presence of pyrazole were obtained only after short periods of incubation (~30 sec) (*cf.* B and C).

pyrazole was caused by the formation of an inactive ternary complex of LADH with NAD^+ and pyrazole (EOPy).[3] The rates of formation and dissociation of this ternary complex seem to be slow under the conditions of kinetic measurements of initial steady state rates. If ethanol, NAD^+, and pyrazole are added first to the buffer solution in the cuvette and the reaction started by adding LADH the reaction velocity is uninhibited at $t=0$. Inhibition occurs during an induction period, whereafter a steady state is reached (Fig. 2 (B)). Under these conditions the formation of the ternary complex LADH-NAD^+-pyrazole (EOPy) is slow because the steady state of the "Theorell-Chance" mechanism is established very rapidly, leaving only small concentrations of EO for Py to react with. Conversely, if preformed EPyO is added to buffer+ethanol the reaction velocity at $t=0$ is near zero, because the concentration of EO is very low in accordance with a low dissociation constant of EPyO. After an induction period the same steady state velocity is reached as in the first case (see Fig. 2 (C)). Under our conditions the induction periods were of the order of magnitude of 20—60 sec depending on the concentration of the reactants. The formation of the ternary complex EOPy is characterized by the appearance of a specific absorption at 290 mμ.[3] Several derivatives of pyrazole were also found to inhibit LADH. It appears that the substitution of the hydrogen at either position 1 or position 3 (*cf.* 3-methyl pyrazole and 3-iodopyrazole) practically abolish the inhibitory potency. Substitution of the hydrogen atom at position 4 on the contrary enhances the inhibitory effect of pyrazole. For example, 4-iodo-pyrazole has a K_I value of 0.03 μM. Since it contains a relatively heavy atom of iodine and has such a small K_I value, 4-iodopyrazole might be useful in X-ray crystallography of its ternary complex with NAD^+ and LADH in order to label the active site of the enzyme.

The results are summarized in Table 1. It is seen that the inhibition varies within wide limits, even for closely related compounds, for instance for (28) 4-iodo-pyrazole, K_I *vs.* ethanol is=0.03 μM (29), 3,4-diiodopyrazole K_I *vs.* ethanol=30 μM, a factor of 1000. In such cases the value 30 μM is to be regarded as a minimum. The true value may be=∞, since the observed value may result from an analytically scarcely observable contamination with 0.1 % of 4-iodopyrazole.

In the group of imidazole and its derivatives (1—6) 3 and 6 are considerably stronger than imidazole as inhibitors. However, 3 and 6 do not show the CIS-type of effect on the reaction velocity. Since the stimulation of velocity in the CIS-type depends more upon the formation of *destabilized* complexes of the type E-imidazole-NADH,[1] the CIS-type can therefore scarcely be expected to occur in stabilized complexes with *lower* dissociation constants, and in general lower dissociation velocities than the ternary imidazole complexes. Compounds 2, 4, and 5 in agreement herewith having about the same inhibition constants as imidazole also show the CIS-type of behaviour.

Furthermore, only the weakest inhibitors, 11—14, 16—17 in the pyridine, pyrazine, and triazole series with K_I's equal to or higher than those of imidazole give the CIS-type. Lower K_I's invariably give inhibition types between competitive (C) and uncompetitive (U).

The pyrazole and its derivatives are of particular interest. The very strong inhibitory power of pyrazole is enhanced by a factor of 5—10 by substitution with iodine, bromine, or methyl in the 4-position. Substitution in the positions 1 or 3 abolish the inhibition.

In the formation of the EPyO complex one proton is released into the solution as we found already in 1963.[3] It was then assumed that in the formation of EPyO the N(2) of the pyrazole was bound to Zn^{2+} in the enzyme, the N(1) of the pyrazole to the C(4) of the pyridine ring with loss of the imino-H as a proton, and neutralization of the resulting negative charge of the pyrazole by the positive charge of the pyridine ring in NAD^+. The loss of the pyrazole inhibitory action on methylation of N(1) is in agreement herewith, but the loss of activity upon substitution of the hydrogen atom in C(3) of the pyrazole may open up other explanations in addition to the possibility that C(3)-substitution causes steric hindrance for the formation of the ternary complex.

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