ON THE INHIBITORY POWER OF SOME FURTHER PYRAZOLE DERIVATIVES OF HORSE LIVER ALCOHOL DEHYDROGENASE

by

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SUMMARY

We found in 1969 (1) that the inhibitory power of pyrazole on LADH was greatly increased by methyl substitution in the 4-position. In this paper we have studied the effect of increasing the size of this side chain. The inhbitory power was found to increase by a factor of two for each methyl group added in a normal side chain. Some other side chains were tested. Already the 4-butyl and 4-pentyl pyrazoles were so active that for the calculation of their true inhibition constants these had to be corrected for the concentration of the enzyme (0.0005 μN). To our knowledge this never happened before.

Pyrazole and some of its derivatives have been more or less known since long time (2) (1893, Buchner et al.). A large number of pyrazoles substituted by 4-methyl- and further up to 4-hexadecyl side chains, and a few others (4-phenyl-, 4-phenyl-3-methyl- and 4-benzyl-) were synthesized and patented by Karmas in 1960 (3). They were stated to have anticonvulsive properties for the treatment of epilepsy.

Theorell and Yonetanin (4) in 1963 found that pyrazole inhibits LADH by forming a firm ternary complex with LADH and NAD^+.

We further found (4) by pH-titration and spectrophotometry that the inhibitory action of pyrazole depends upon the formation of a bridge between a zinc atom in ADH and N-2 in pyrazole, whereas the N-1 in pyrazole was concluded to be bound under liberation of H^+ to C-4 in the nicotinamide ring of NAD^+. This has recently been confirmed by the X-ray crystallographical results of Brändén et al. (5) showing the presence of a zinc atom in the expected position in the ADH molecule, being bound to the protein by three bonds, and having one bond free for binding to N-2 in the pyrazole after liberation of a water molecule from Zn. Pyrazole in this way blocks the binding site for alcohol (competitive inhibition). This theory requires that substitution of the H-atom in  should abolish its inhibitory power. This was found to be true.

Already in 1969 Theorell et al. (1) found that substitutions in positions 3 and 5 of pyrazole practically abolish the inhibitory power. On the contrary

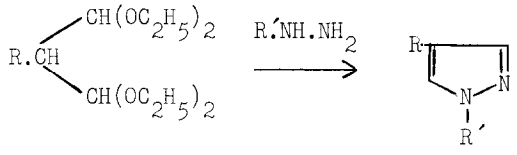
substitutions in position 4 of pyrazole are found to increase the inhibition. Of the 31 compounds tested 12 were pyrazole derivatives. Of these 12 two had $K_I > 125$ μM-∞.

It was especially interesting to notice that the pyrazoles substituted only in the 4-position (4-methyl-, 4-Br- and 4-I-) were at least 1000 times stronger than any of the other pyrazole compounds. Of course there is an obvious possibility that the very low activities found in some compounds could be due to minimal impurities.

After our discoveries of ADH inhibition by pyrazole (4) in 1963, and substituted pyrazoles (1) in 1969 an intense interest in the possible usefulness for combating the ill-effects of alcohol abuse by pyrazoles aroused (see the papers by Blomstrand and Theorell, 1970, (6), Blomstrand and Öhman (7), Blomstrand and Kager (8), Blomstrand et al. (9) and many others). At first sight it would seem harmful to retard the alcohol combustion and thus prolong intoxication. However, after it was found that the harmful effects of ethanol are caused by its combustion, seriously disturbing both fat and carbohydrate metabolism, it seemed logical to slow down ethanol combustion by partial inhibition of LADH activity by pyrazoles. Fortunately full normalization is obtained already by partial inhibition, which seems to open up the possibility of combating alcohol damages.

These results made it obviously interesting to investigate further derivatives of pyrazoles substituted in the 4-position.

Most of the 4-alkyl- and aryl-substituted pyrazoles used in this work are known from the literature and have been prepared by different methods. We prepared them in good yields by reacting an alkyl-substituted malondialdehyde tetraethyl acetal with hydrazine or substituted hydrazine (10).



The pyrazoles were usually isolated and characterized as salts which have not been reported previously in the literature.

Experimental

Melting points were determined with calibrated Anschütz thermometers and are uncorrected. IR and NMR spectra were in accordance with the proposed structures.

4-Propylpyrazole hydrochloride. Propylmalondialdehyde tetraethyl acetal (29.0 g) was added dropwise with stirring to a solution of hydrazine sulphate (12.2 g) and 2 N HCl (5 ml) in water (20 ml) at 45-50°. After 1 h

the temperature was raised to 100° for 20 min. After cooling 40% NaOH (80 ml) was added and the mixture was thoroughly extracted with ether. The ethereal solution was dried ($MgSO_4$) and fractionated under reduced pressure affording 4-propylpyrazole (7.9 g, 65%), b.p. 100-101° (1.5 mm). The base was converted to the hydrochloride, m.p. 125-126° (from ethanol-ether).

Anal. Calcd for $C_6H_{10}N_2$.HCl: C 49.15; H 7.56; N 19.1. Found: C 48.8; H 7.75; N 19.1.

The following compounds were prepared similarly:

4-Ethylpyrazole hydrochloride, m.p. 123.5-125° (from ethanol-ether), yield 64%.

Anal. Calcd for $C_5H_8N_2$.HCl: C 45.3; H 6.84; N 21.1 Found: C 45.1; H 6.75; N 21.2.

4-Isopropylpyrazole oxalate, m.p. 164-165° dec. (from ethanol-ether), yield 59%.

Anal. Calcd for $C_6H_{10}N_2 \cdot 1/2(COOH)_2$: C 54.1; H 7.15; N 18.05. Found: C 54.3; H 7.23; N 17.9.

4-Butylpyrazole hydrochloride, m.p. 122.5-124° (from ethanol-ether), yield 50%.

Anal. Calcd for $C_7H_{12}N_2$.HCl: C 52.3; H 8.16; N 17.4.Found: C 52.3; H 8.20; N 17.3.

4-Pentylpyrazole hydrochloride, m.p. 127-128.5° (from ethanol-ether), yield 70%.

Anal.Calcd for $C_8H_{14}N_2$.HCl: C 55.0; H 8.66; N 16.0. Found: C 55.3; H 8.65; N 15.9.

4-Phenylpyrazole, m.p. 231.5-232.5° (from ethanol), (10) 230-231°, yield 56%.

4-Ethyl-1-phenylpyrazole, b.p. 107-108° (1.5 mm), (10) 114-115° (2 mm), yield 68%.

The inhibitory power of the new pyrazole derivatives was tested in the fluorimeter designed in 1954 by Theorell and Nygaard (11) by observing the change of fluorescence of the coenzyme on oxidation and reduction.

The experiments were performed in 1 cm cuvettes containing 3 ml phosphate buffer, pH 7.0, 23,5°C, 500 μM NAD^+, 0.0005 μN horse-LADH, inhibitor and ethanol in different concentrations.

RESULTS

If the inverse of the reaction velocity, 1/v, is plotted on the ordinate, the inverse of the ethanol concentration, 1/S, on the abscissa, straight lines with different slopes for each I , all intercepting on the ordinate, where 1/v for $S = \infty$, are obtained. This was to be expected from the well-known fact that the pyrazoles are competitive with the substrate, ethanol.

Table 1

Compound	$K_{EO,I}$, μM
pyrazole	0.22
4-methylpyrazole	0.013
4-ethyl- "	0.007
4-propyl- "	0.004
4-butyl- "	0.0018
4-pentyl- "	0.0008
4-isopropylpyrazole	0.008
4-phenyl- "	0.1
1-phenyl-4-ethylpyrazole	100
4-iodopyrazole	0.02
4-bromo- "	0.02
4-trifluoromethylpyrazole x)	0.08

x) Kindly provided by Prof. S. Gronowitz, Lund.

Since NAD^+ was added in manyfold excess over the dissociation constant (K_{EO}, pH 7 = 150 μM) the inhibition constant, "K_I", becomes closely equal to

$$K_{[EO,I]} = \frac{[EO][I]}{[EO,I]}$$

However, this formula is valid only under the assumption that I is $\gg$EO,I. Interestingly enough we found 4-butyl- and 4-pentylpyrazole to be so strong inhibitors that the enzyme concentration, 0.0005 μN, could not be neglected since it binds so much of the inhibitor that this must be taken into account. Therefore a modified formula had to be used.

$$K_{[EO,\ I]} = \frac{[EO]\ [I\text{-}EO]}{[EO]}$$

In the case of 4-butylpyrazole this correction of $K_{EO,I}$ amounted to -10%, for 4-pentylpyrazole -25%. To our knowledge this is the first time that so strong inhibitors have been found that the enzyme concentration has to be

taken into account, because it is of the same order of magnitude as the inhibitor. The results are given in Table 1.

In the case of 4-methylpyrazole the value found in this series of tests differs from the one given in 1969 (1), $K_{EO,I} = 0,08$ μM. We now find $K_{EO,I} = 0,013$ μM.

Discussion

The importance of substitutions in position 4 is very obvious. Further introductions of alkyl groups are clearly of great interest. Substitutions in position 1 abolish the activity. This fits with our observation (4) that pyrazole inhibits by its 1- and 2-nitrogens forming a bridge between zinc in the enzyme and NAD^+ and explains why 1-phenyl-4-ethylpyrazole is inactive. N-2-substitution is not so easy since N-2 is ternary.

Of course we are aware of the fact that the usefulness of our inhibitors depends not only on their inhibition constants, but also on the speed of elimination with or without chemical modifications. The work along these lines is being continued.

Acknowledgements

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References

1. Theorell, H., Yonetani, T., and Sjöberg, B. (1969) Acta Chem. Scand. 23, 255-260.
2. Buchner, E., and Fritsch, M. (1893) Liebigs Ann. 273, 256-266.
3. Karmas, G. (1960) U.S. Pat. 2.931.814, 5 April.
4. Theorell, H., and Yonetani, Y. (1963) Biochem. Z. 338, 537-553.
5. Brändén, C.-I., to be published.
6. Blomstrand, R., and Theorell, H. (1970) Life Sciences, 9, 631-640.
7. Blomstrand, R., and Öhman, G. (1973) ibid. 13, 107-112.
8. Blomstrand, R., and Kager, L. (1973) ibid. 13, 113-123.
9. Blomstrand, R., Kager, L., and Lantto, O. (1973) ibid. 13, 1131-1141.
10. Protopopova, T.V., Klimko, V.T., and Skoldinov, A.P. (1959) Khim. Nauka i Prom. 4, 805-806.
11. Theorell, H., and Nygaard, A.P., (1954) Acta Chem. Scand. 8, 877-888.