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EFFECT OF PYRAZOLE *IN VIVO* ON ALDEHYDE METABOLISM IN RAT LIVER AND BRAIN*

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Abstract—The effect of administration of pyrazole *in vivo* on aldehyde reduction and oxidation has been studied in rat liver and brain. It was found that while pyrazole is capable of complete inhibition of NADH dependent reduction of a number of aldehydes in liver, it is only partially effective as an inhibitor of NADPH dependent aldehyde reduction and is ineffective as an inhibitor of NAD dependent aldehyde oxidations in liver. No effect of pyrazole on oxidation or reduction of aldehydes in brain was found.

Pyrazole has a rapid onset of action *in vivo* and has an effective half life for inhibition of NADH dependent aldehyde reduction in rat liver of about 76 hr following a dose of 360 mg $\times$ kg^{-1} intraperitoneally.

PYRAZOLE is known to be a potent inhibitor of alcohol dehydrogenase (EC 1.1.1.1) *in vitro*[1,2] and also has been found to inhibit ethanol metabolism in the intact animal[3,4] and in perfused liver.[5] Since the K_i value is very low with liver alcohol dehydrogenase,[1] it should be possible to demonstrate inhibition of the enzyme by direct assay after administration of pyrazole to the animal. It is known that there are various enzymes with "alcohol dehydrogenase" activity present in various tissues, including brain,[6] and liver[7] as well as other tissues.[8] Some of these enzymes may be isozymes of the well-known alcohol dehydrogenase or they may be entirely different enzymes with different substrate and co-factor specificity. This paper describes the effect of administered pyrazole on the activity of liver alcohol dehydrogenase as well as on other enzymes involved in alcohol and aldehyde metabolism.

METHODS

Enzyme preparation

Alcohol dehydrogenase. Male Sprague–Dawley rats, 150–300 g, were used throughout. Livers were removed, homogenized in 0·25 M sucrose to make a 10% suspension and the homogenate centrifuged at 147,000 *g* for 1 hr. The supernatant solution (cytosol) was used directly as the source of alcohol dehydrogenase enzyme.

Aldehyde dehydrogenase. For preparation of aldehyde dehydrogenase, the livers were removed, weighed, and homogenized with a loose fitting teflon pestle in sufficient

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0·25 M sucrose to make a 20% homogenate. This was centrifuged at 600 *g* for 10 min and the supernatant decanted and saved. The pellet was rehomogenized in 0·25 M sucrose with a tight fitting teflon pestle and centrifuged again at 600 *g* for 10 min. The final combined 600 *g* supernatant fraction was diluted to approximately 10 per cent (g/ml) with respect to the original liver wet weight. This fraction was then subjected to centrifugation at 10,000 *g* for 10 min to isolate mitochondria. The mitochondria were washed twice with 0·25 M sucrose by centrifugation and diluted to a volume equal to the original wet weight of the liver. A 5-ml aliquot was removed and diluted with an equal volume of cold water. This preparation was sonically treated with the microprobe for the Branson Model 125 sonicator at a setting of 2 for a total of 3 min. During the three sonication periods of 1 min each, the suspension was immersed in an ice-salt water mixture at −7°. At least 1 min elapsed between each sonication period during which time the suspension was placed in an ice bath at 0°. The temperature did not rise above 4°. The sonicated mitochondrial preparation was centrifuged at 110,000 *g* for 30 min and the mitochondrial supernatant used for determination of aldehyde dehydrogenase.

The cytosol was centrifuged at 122,000 *g* for 1 hr. An aliquot was placed on 2 × 10 cm columns of Sephadex G 25 (medium) equilibrated with 0·25 M sucrose. The red protein was followed visually. The recovery of protein was 99·4%. Recovery of aldehyde dehydrogenase as measured by indoleacetic acid production was complete.[9]

Brain enzymes. Brains were removed and homogenized in 0·32 M sucrose. The whole homogenate was used to determine the rate of oxidation of indoleacetaldehyde as previously described.[9] Brain NADPH-dependent aldehyde reduction was determined in the 122,000 *g* supernatant as described by Tabakoff and Erwin.[6]

Enzyme Assays

Liver alcohol dehydrogenase was determined by following the rate of NADH (0·33 mM) oxidation spectrophotometrically in the presence of 0·33 mM aldehyde at 25°. The buffer was 0·1 M phosphate, pH 7·4. Final volume was 3 ml. The reaction was linear with protein concentration from 1·0 mg to 1·5 mg protein per 3 ml. The rate was determined during the first minute of the reaction. This assay was chosen since aldehyde reduction is more rapid than alcohol oxidation and it can be carried out at physiological pH. Further discussion of the assay is presented below. Several experiments were carried out using acetaldehyde instead of propionaldehyde. Essentially identical results were obtained with either substrate; however, acetaldehyde boils at less than 20° at this altitude (5280 ft) and thus is difficult to work with. Therefore, propionaldehyde was routinely used.

Mitochondrial aldehyde dehydrogenase was determined at pH 9·6 in 0·015 M pyrophosphate buffer containing 0·33 mM each NAD and aldehyde by following the rate of NADH formation in a Gilford 2000 spectrophotometer at 340 mμ, 25°, final volume, 3 ml. Blank cuvettes contained no aldehyde. The concentration of the aldehydes was determined enzymatically.[9] NADH oxidase and alcohol dehydrogenase were absent in this preparation. Aldehyde dehydrogenase in the cytosol was determined similarly except that 33·3 μM. (final concentration) pyrazole was added to completely inhibit alcohol dehydrogenase.

Pyrazole was dissolved in water and injected intraperitoneally. Doses are presented in each figure and table.

RESULTS

The time course of inhibition of propionaldehyde reduction is shown in Fig. 1. Inhibition observed in the assay *in vitro* is complete within 1 hr of intraperitoneal injection. The effective half time for recovery of activity after a dose of 360 mg of pyrazole per kg body weight is approximately 76 hr. 3-Methylpyrazole, given in the same dose, was found not to be an inhibitor as expected from earlier studies.[4]

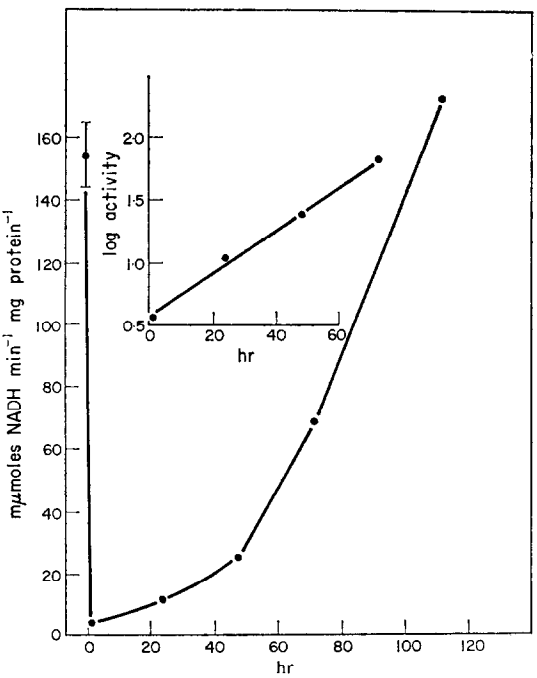

FIG. 1. Time course of inhibition of propionaldehyde reduction by NADH in rat liver after pyrazole administration. Assay of aldehyde reduction was carried out as described in the text at the times indicated following i.p. injection of 360 mg (5·29 m-moles) kg^{-1} of pyrazole. Two animals were employed for each point except for the control animals (zero time) where 22 animals are represented ± the standard error of the mean in brackets. "Hours" is the time following injection of the drug. The second point is 1 hr after injection of pyrazole.

A dose–response curve for inhibition of propionaldehyde reduction after administration of pyrazole *in vivo* is presented in Fig. 2. Since there is a dilution factor of 300 from the whole liver to the assay cuvette, smaller doses probably are completely inhibitory *in vivo*.

Table 1 illustrates the usefulness of pyrazole in determining the pathways of alcohol oxidation and aldehyde reduction. It is seen that either propionaldehyde or indoleacetaldehyde reduction by NADH is almost completely inhibited by administration of pyrazole either *in vivo* or *in vitro*. Reduction of these aldehydes by NADPH, however, is much less effectively inhibited with 33·3 μM pyrazole. This difference narrows at 10 mM pyrazole.

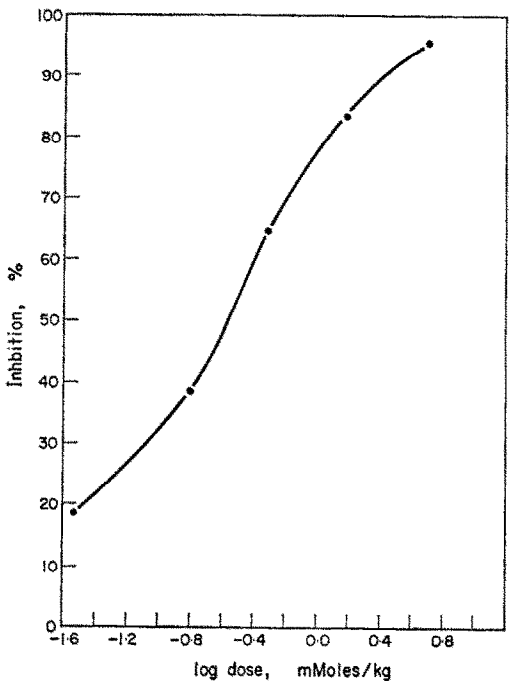

FIG. 2. Dose–response curve for inhibition of propionaldehyde reduction by NADH in rat liver after pyrazole administration. Assay of aldehyde reduction was carried out as described in the text 1 hr after injection of pyrazole i.p. into rats.

TABLE 1. INHIBITION OF ALDEHYDE REDUCTION BY PYRAZOLE*

Substrate 0·33 mM	Co-enzyme 0·33 mM	NAD(P)H oxidized (nmoles) $\times$ mg^{-1} $\times$ min^{-1}	Inhibition by pyrazole (%) *In vivo*	*In vitro* 33·3 μM	*In vitro* 10 mM
Propionaldehyde	NADH	156·1	95·9	97·3	100·0
Same	NADPH	31·6	48·6	63·8	100·0
Indoleacetaldehyde	NADH	44·4	79·7	96·5	100·0
Same	NADPH	35·7	27·2	35·9	67·6
p-Nitrobenzaldehyde	NADH	35·1		24·1	19·1
Same	NADPH	51·8		16·4	38·5
p-Carboxybenzaldehyde	NADH	50·1		92·8	97·4
Same	NADPH	12·3		6·5	55·0
Glycolaldehyde	NADH	10·0	91·1	81·8	94·3
Same	NADPH	0·6			
Glyoxylic acid	NADH	72·8	−9·5†	−4·2†	
Same	NADPH	4·2			

* Aldehyde and co-enzyme concentration was 0·33 mM in all cases. Pyrazole (360 mg/kg) was given i.p. 1 hr before sacrifice for inhibition *in vivo*. Pyrazole was present in the experiment *in vitro* at a concentration of 33·3 μM or 10 mM. The results are from at least 2 duplicate experiments in each case. Rat liver supernatant (147,000 *g*, 1 hr) was used in each case.

† Stimulation.

Reduction of *p*-nitrobenzaldehyde by either NADH or NADPH is not inhibited greatly by pyrazole indicating that more than 1 NADH dependent enzyme is present. Glycolaldehyde reduction by NADH also is inhibited by pyrazole. However, glycolaldehyde is not reduced by NADPH. Reduction of glyoxylic acid by NADH is not inhibited by pyrazole. Presumably this reduction is catalyzed by lactic acid dehydrogenase.[10,11]

Table 2 demonstrates that pyrazole given to the animal has virtually no effect on NAD-dependent aldehyde oxidation in rat liver. Pyrazole at a concentration of 10 mM *in vitro* had no effect on either the supernatant or the mitochondrial enzyme activity.

TABLE 2. EFFECT OF PYRAZOLE ADMINISTRATION ON ALDEHYDE DEHYDROGENASE OF RAT LIVER

Treatment	nmoles NADH × mg protein^{-1} ± S.E.M. Supernatant	*n*	Mitochondria	*n*
Control	14·4 ± 2·2	5	161·5 ± 13·2	5
Pyrazole	13·5 ± 1·3	10	143·7 ± 3·7	10

* Aldehyde dehydrogenase was determined as described in the text with propionaldehyde as substrate. The values given are for 5 min of reaction. Pyrazole was given i.p. in a dose of 360 mg/kg and the livers taken from 15 min to 10 hr later. Since there was no detectable effect on the activity of aldehyde dehydrogenase regardless of when the livers were taken, the data for all times have been combined.

The effect of pyrazole administration on brain aldehyde oxidation and reduction is presented in Table 3.

TABLE 3. EFFECT OF ADMINISTRATION OF PYRAZOLE ON RAT BRAIN ALDEHYDE REDUCTION AND OXIDATION*

Substrate	nmoles product × mg^{-1} × min^{-1} Control ± S.E.M.	Pyrazole ± S.E.M.	Reaction
p-Nitrobenzaldehyde†	3·14 ± 0·21	3·06 ± 0·53	Aldehyde reduction
p-Carboxybenzaldehyde†	1·88 ± 0·14	2·11 ± 0·39	Aldehyde reduction
Indoleacetaldehyde‡	2·75 ± 1·13	2·55 ± 0·43	Aldehyde oxidation

* The determinations were carried out as described in the text 1 hr after i.p. injection of 360 mg/kg of pyrazole; $n = 4$ in all cases.
† Reduction of aldehyde by NADPH.
‡ Oxidation of aldehyde by NAD.

DISCUSSION

The assay chosen for "alcohol dehydrogenase" was that of aldehyde reduction rather than alcohol oxidation. This procedure has several advantages: (1) it is a more sensitive assay since the rate of aldehyde reduction is much faster than alcohol oxidation; (2) the reaction is easily carried out near physiological pH, whereas alcohol oxidation is usually determined at high pH; and (3) it circumvents the necessity of adding alcohols which are known to be competitive with pyrazole. This last factor is especially important following administration *in vivo* of the inhibitor since the

inhibition observed in the assay would be a function not only of the amount of inhibitor present but also of the amount of alcohol added. Since pyrazole is noncompetitive with the aldehyde and the reduced nucleotide,[12] inhibition is observed even at saturating concentrations of both. Pyrazole does not appreciably bind to the enzyme unless NAD is present.[1] There must be therefore endogenous NAD in the enzyme preparation or one cycle of the enzymatic process is required to generate a molecule of NAD.

It is clear that pyrazole *in vivo* or *in vitro* is a potent inhibitor of NADH-linked aldehyde reduction. It is also apparent that NADPH reduction of aldehydes is relatively immune to this inhibition. The question of whether or not liver alcohol dehydrogenase is capable of reaction with NADPH is not settled. We observed that commercial preparations of the enzyme do function with NADPH instead of NADH as coenzyme, although at very much slower rates, and that the process was not sensitive to pyrazole inhibition. However, since "pure" liver alcohol dehydrogenase is known to contain several distinct catalytic activities,[7,13] it is possible that there is another form of this enzyme, present in small quantities, which is responsible for the observed reaction.

The time course of inhibition of aldehyde reduction after giving pyrazole yields a half time of approximately 76 hr. It is not known whether the observed inhibition is due to pyrazole itself, a metabolite or both. The determination of the effective half time for recovery of aldehyde reduction is probably more useful than a direct determination of the half life of pyrazole itself in the body since the two may not be equal because of the very tight binding of the inhibitor to the enzyme–NAD complex.

There is no marked effect of pyrazole on aldehyde dehydrogenase of liver. The slight inhibition observed in the mitochondria is of doubtful significance ($0{\cdot}1 < P < 0{\cdot}2$) (Table 2) especially since addition of 10 mM pyrazole *in vitro* has no effect. In the case of the enzyme in the cytosol, one can only be sure that administration of pyrazole *in vivo* causes no more inhibition than that imposed by 33·3 μM pyrazole required in the assay mixture. Addition of 10 mM pyrazole did not cause any inhibition. It seems unlikely that administration of pyrazole *in vivo* is bringing about inhibition of aldehyde oxidation in the assay system used here for the supernatant enzyme.

As might have been expected from the results with the liver enzymes, pyrazole administration has no effect on NADPH-dependent aldehyde reduction in the brain, nor upon indoleacetaldehyde oxidation. Pyrazole inhibition is thus demonstrated not to extend to those metabolic events.

It is well established that there exist pathways of aldehyde reduction besides that catalyzed by liver alcohol dehydrogenase.[6] It is possible that these pathways are responsible for the reduction of endogenous aldehydes such as those arising from norepinephrine and serotonin. Pyrazole and similar compounds promise to be useful tools in investigations of the metabolism of these aldehydes since it is possible to virtually completely remove the NADH-linked reductions of aldehydes while leaving relatively untouched the NADPH-dependent reduction pathways as well as the NAD-linked oxidative step.

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