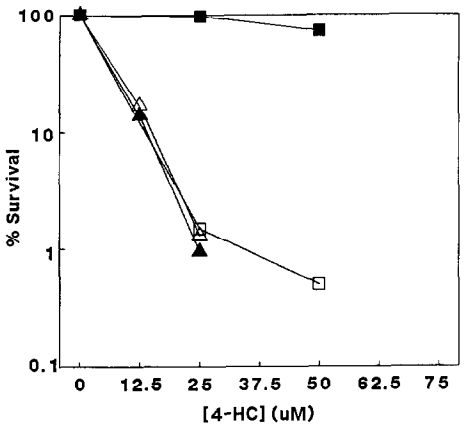

Fig. 2. Effect of DEAB on L1210/0 and L1210/CPA survival. Cells were treated with 50 μM DEAB or vehicle for 5 min prior to exposure to 4-hydroperoxycyclophosphamide (4-HC) for 30 min at 37°. The cells were then washed free of inhibitor and drug and plated in semisolid agar. Survival values are the mean of at least two survival assays. Key: (□) L1210/CPA [+DEAB], (■) L1210/CPA [−DEAB], (△) L1210/0 (+DEAB), and (▲) L1210/0 [−DEAB].

(data not presented) indicate that DEAB was also an effective ALDH inhibitor *in vivo* in mice as well as of the human cytosolic ALDH isozyme in liver, erythrocytes, and the HEPG2 hepatoma cell line.

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Inhibition of the oxidation of the urinary bladder carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine by pyrazole and 4-substituted pyrazoles

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N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BHBN*) is widely used as an experimental urinary bladder carcinogen [1]. The principal urinary metabolite of BHBN in the rat is BCPN, formed by oxidation of the alcoholic group of BHBN to the carboxyl group [1, 2]. BCPN itself is a urinary bladder carcinogen, and it has been suggested that BCPN is the proximate carcinogenic metabolite of BHBN [1]. To study further the biochemical mechanism of the organospecificity of BHBN in cancer induction, it was necessary to find some means of inhibiting the metabolism of BHBN to BCPN. The enzymological aspects of the conversion of BHBN to BCPN have not been studied. The principal enzymes involved in oxidation of alcohols are the NAD^+-dependent alcohol dehydrogenases and aldehyde dehydrogenases, which are relatively specific for NAD^+ as coenzyme but exhibit broad substrate specificity with respect to alcohols and aldehydes [3, 4]. One would expect BHBN to be oxidized by an alcohol dehydrogenase to form the aldehyde intermediate BFPN (Fig. 1). BFPN has not been detected as a urinary metabolite of BHBN, or as a metabolite in isolated hepatocytes, because it is very rapidly

* Abbreviations: BHBN, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine; BFPN, *N*-butyl-*N*-(3-formylpropyl)nitrosamine; BCPN, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

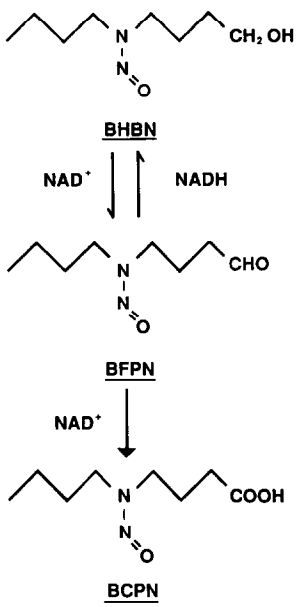

Fig. 1. Oxidation of BHBN to BCPN by rat liver cytosol.

oxidized to BCPN by disulfiram-insensitive isozymes of aldehyde dehydrogenase [2]. We report here the results of studies on the effect of pyrazole and some 4-substituted pyrazoles on the NAD^+-dependent oxidation of BHBN in rat liver cytosol.

Materials and methods

Male Wistar rats (200–250 g) were obtained from Charles River Breeding Laboratories, North Wilmington, MA. NAD^+, NADH, pyrazole, and 4-methylpyrazole were purchased from the Sigma Chemical Co., St Louis, MO. 4-Bromopyrazole was obtained from Research Plus, Inc., Bayonne, NJ. BHBN [5], BFPN [6] and BCPN [7] were synthesized as previously described.

Liver from rats was homogenized in 4 vol. of ice-cold 0.05 M HEPES–0.33 mM dithiothreitol, pH 8.4, and the homogenate was centrifuged at 40,000 rpm (105,000 *g*, for R_{av}) for 60 min at 2–3°. The supernatant (cytosol) was used as the enzyme source. The rate of BHBN-dependent reduction of NAD^+ was measured at 340 nm (23°) by the method used by Lumeng *et al.* [8] to study ethanol oxidation by rat liver cytosol. The reverse reaction was measured by following the oxidation of NADH in the presence of BFPN. The NAD^+ concentration was 2.5 mM and substrate concentrations were 0.025 to 1.5 mM; final assay volume was 3.0 ml. Initial reaction velocities were linear with respect to time (first 5 min) and amount of cytosol added. The rate of NAD^+ reduction was corrected for low blank values found in the absence of added substrate. For study of enzyme inhibition, the pyrazole compounds were preincubated with the cytosol in the presence of NAD^+ for 5 min at 23° prior to addition of substrate. Enzyme activity for BHBN oxidation was expressed as nanomoles of NADH formed per minute per milligram of protein. The concentration of NADH was calculated based on an A_{340} of 6.22 $mM^{-1} \cdot cm^{-1}$. Protein concentration was determined by the procedure of Lowry *et al.* [9] with crystalline bovine serum albumin as standard. Lineweaver–Burk plots were used to calculate the kinetic parameters V_{max}, K_m and K_i. In some experiments with the 4-substituted pyrazoles, K_i values were also determined by plotting $1/V$ vs $[I]$ where the x intercept = $-K_i$.

For analysis of nitrosamines in the 3-ml final assay volume, reaction times were extended to 30 min, and the reaction was terminated by addition of 0.06 ml of 33% acetic acid and 1.3 ml acetonitrile. Following centrifugation, aliquots of the supernatant fraction were analyzed for BHBN, BFPN and BCPN by the HPLC method previously described [2].

Results and discussion

We found that NAD^+ was reduced to NADH by rat liver cytosol in the presence of BHBN, with a mean rate (V_{max}) of 12.4 nmol/min/mg protein. Although the initial oxidation product of BHBN was not directly identified in these studies, it is reasonable to infer that it was BFPN (Fig. 1). The finding that BFPN was reduced to BHBN by NADH and rat liver cytosol at a rate of 12.9 nmol/min/mg protein is consistent with this interpretation. When reaction times were extended from 3–5 min to 30 min, BCPN was found to be the end product of the NAD^+-dependent oxidation of BHBN. It was shown that BFPN can be oxidized to BCPN by a cytosolic isozyme of aldehyde dehydrogenase that is insensitive to disulfiram inhibition [2].

Pyrazole inhibited the NAD^+-dependent oxidation of BHBN and the NADH-dependent reduction of BFPN by rat liver cytosol, but had no effect on the NAD^+-dependent oxidation of BFPN to BCPN. The inhibition of BHBN oxidation by pyrazole was non-competitive (Fig. 2), in contrast to the competitive inhibition reported for the oxidation of simpler primary alcohols by liver alcohol dehydrogenases [3]. As a control, we checked the inhibition of NAD^+-dependent oxidation of *n*-butanol in rat liver cytosol by pyrazole and found the expected competitive type inhibition. V_{max} values found for butanol oxidation were 7.0, 7.0 and 7.2 nmol/min/mg protein for 0, 2.5 and 10 μM pyrazole, respectively, with apparent K_m values for butanol of 8, 21 and 62 μM and a K_i for pyrazole of 1.5 μM.

The inhibitory activities of 4-methylpyrazole and 4-bromopyrazole for the NAD^+-dependent oxidation of BHBN by rat liver cytosol were ten to fifteen times that found for pyrazole (Fig. 3). The type of inhibition found was also non-competitive. The kinetic data for the effect of pyrazole and the 4-substituted derivatives on BHBN oxidation by rat liver cytosol are given in Table 1.

In summary, we have demonstrated the NAD^+-dependent oxidation of BHBN to BFPN in rat liver cytosol with a V_{max} of 12–13 nmol/min/mg protein at 23° and a K_m for BHBN of 0.05 mM. The observed NAD^+-dependent oxidation of BHBN was sensitive to pyrazole inhibition with a K_i of about 5 μM pyrazole. Furthermore, the oxidation of BHBN was ten to fifteen times more sensitive to inhibition by 4-methylpyrazole and 4-bromopyrazole. These data pro-

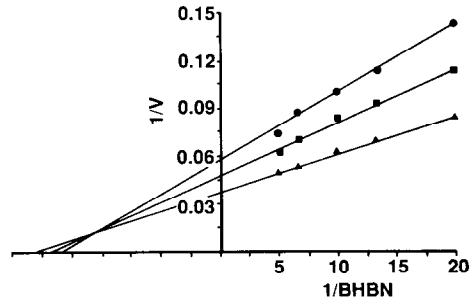

Fig. 2. Non-competitive inhibition of NAD^+-dependent oxidation of BHBN in rat liver cytosol by pyrazole. Key: control, no pyrazole (▲——▲); 1 μM pyrazole (■——■); and 2.5 μM pyrazole (●——●). In this representative experiment, the calculated K_i for pyrazole inhibition was 3.9 μM.

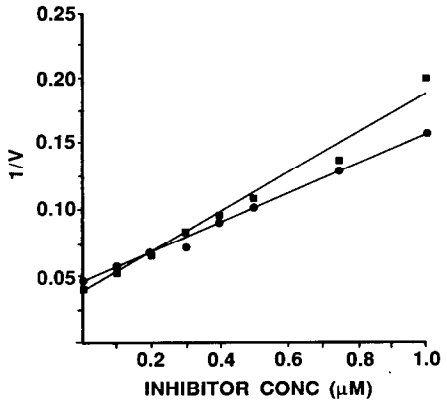

Fig. 3. Representative kinetic plots showing inhibition of NAD^+-dependent oxidation of BHBN in rat liver cytosol by 4-methylpyrazole (●——●) and by 4-bromopyrazole (■——■). The concentration of BHBN was 0.2 mM. In these experiments, for the 4-methylpyrazole slope = 0.111, r = 0.996, x intercept ($-K_i$) = −0.413, and for the 4-bromopyrazole slope = 0.149, r = 0.981 and the x intercept = −0.261.

vide evidence for a pyrazole-sensitive cytosolic form of alcohol dehydrogenase as an initial step in the oxidation of BHBN in rat liver. The effect of 4-methylpyrazole on the metabolism and carcinogenicity of BHBN in the rat is being studied.

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Table 1. Inhibition of rat liver cytosolic NAD^+-dependent oxidation of BHBN by pyrazole, 4-methylpyrazole and 4-bromopyrazole

Inhibition	K_m (μM)	K_i (μM)
None*	51 ± 3†	
Pyrazole	58 ± 5	4.85 ± 0.66
4-Methylpyrazole	54 ± 8	0.50 ± 0.03‡
4-Bromopyrazole	57 ± 2	0.35 ± 0.03‡§

* The control rate (V_{max}) of BHBN oxidation was 12.4 ± 0.4 nmol/min/mg protein.
† Mean ± SE for eight to nine rats in each group.
‡ $P < 0.001$ compared to pyrazole.
§ $P < 0.001$ compared to 4-methylpyrazole.

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