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INDUCTION OF MICROSOMAL DIMETHYLNITROSAMINE DEMETHYLASE BY PYRAZOLE

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Abstract—Pyrazole, a potent inhibitor of alcohol dehydrogenase, was found to be a potent inducer of the activity of low K_m dimethylnitrosamine demethylase (DMN-d). One injection of pyrazole (200 mg/kg body wt) to weanling Wistar rats changed the microsomal DMN demethylase activity by 1.7, 1.9 and 2.5 times the control values at 6, 12 and 24 hr after the injection respectively. Pyrazole administration reduced arylhydrocarbon hydroxylase (AHH) activity. When animals were injected with pyrazole (200 mg/kg body wt) for 1, 2, 3 or 4 consecutive days, the values for DMN-d activity were 277, 297, 306 and 319% of the control values. The corresponding values for AHH were 91, 67, 57 and 45% for 1, 2, 3 and 4 injections respectively. Pyrazole-induced DMN-d activity was NADPH dependent and was inhibited by CO; *n*-butanol gave a 50% inhibition at a concentration of 2×10^{-3} M. The corresponding value for metyrapone was 1×10^{-2} M. Cytochrome P-450 was slightly increased by pyrazole and its CO-complex gave an absorption maximum around 451 nm. When the microsomal proteins were separated using sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis, a large increase in a band at about 51,000 daltons was found in the liver microsomes of pyrazole-treated animals.

The first step in the metabolism of dimethylnitrosamine (DMN) is enzymatic hydroxylation of the methyl group. The hydroxymethyl derivative of DMN undergoes nonenzymatic rearrangement to a methylating intermediate with simultaneous liberation of formaldehyde, which is further oxidized to formic acid and CO_2. The formation of the hydroxymethyl derivative of DMN is thought to be mediated via a microsomal mixed-function oxidase system [1, 2]. However, the general microsomal enzyme inducers phenobarbital and 3-methylcholanthrene are either without effect or else decrease the activity of the enzyme involved in the metabolism of DMN [3]. This enzyme, dimethylnitrosamine demethylase I (DMN-d) [4], is induced by indole and tryptophan, which are, however, fairly weak inducers [5, 6]. Both of these compounds contain the heterocyclic pyrrole ring. On the other hand, pyrazole (1,2-diazole), a potent inhibitor of alcohol dehydrogenase, inhibits the formation of CO_2 from DMN when given 30 min to 1 hr before the administration of ^{14}C-labeled DMN [7]. Several pyrazole derivatives are clinically important because of their antipyretic, analgesic and anti-inflammatory properties. In the present paper we present data showing that pyrazole is a potent inducer of low-substrate level DMN-d in the liver and possibly gives rise to a new specific cytochrome P-450 species that is involved in the metabolism of DMN.

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MATERIALS AND METHODS

Chemicals. Pyrazole and DMN were purchased from the Aldrich Chemical Co. (Milwaukee, WI), and NADP, NADPH and cytochrome *c* from the Sigma Chemical Co. (St. Louis, MO); metyrapone [2-methyl-1,2-bis-(3-pyridyl)-1-propanone] was a gift from the Ciba Pharmaceutical Co. (Summit, NJ); *n*-butanol was purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ), sodium dithionite from the Fisher Scientific Co. (Fairlawn, NJ), and carbon monoxide gas from the Matheson Co., Inc. (East Rutherford, NJ). Chemicals for electrophoresis were purchased from the Bio-Rad Laboratories (Richmond, CA).

Wistar weanling rats were obtained from the Charles River Breeding Laboratories. They were housed five to a cage and received water and food (Wayne Lab Blox) *ad lib.* The animals were killed by decapitation. Perfused livers were homogenized in a buffer containing 10 mM EDTA and 150 mM KCl (pH 7.25) and were centrifuged at 9000 *g* for 15 min. The supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and the centrifugation at 105,000 *g* was repeated. The incubation medium for DMN demethylation contained the following components: NADPH-generating system consisting of 0.4 mM monosodium NADP, 4 mM monosodium glucose-6-phosphate, 4 units/ml of glucose-6-phosphate dehydrogenase, 2 mM $MgCl_2$, 7.5 mM semicarbazide hydrochloride, and 2 mg microsomal protein/ml in 0.1 M potassium phosphate buffer. Substrate concentration was 4×10^{-3} M. The incubation time was 30 min. DMN-d

activity was determined as described earlier [5]. AHH activity was determined according to Wiebel and Gelboin [8]. Protein was determined by the method of Lowry *et al.* [9]. For the spectral studies, microsomes were suspended in 0.25 M potassium phosphate buffer (pH 7.25) containing 30% glycerol. Cytochrome P-450 was determined by the method of Omura and Sato [10] using 91 $mM^{-1} \cdot cm^{-1}$ as the molecular extinction coefficient. NADPH–cytochrome *c* reductase was measured according to Phillips and Langdon [11]. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed according to Laemmli [12]. The concentration of polyacrylamide in the stacking gel was 3% and in the separating gel, 7.5%. Samples containing 20 μg of solubilized microsomal protein were submitted to electrophoresis on polyacrylamide gels (1.5 mm thick). Albumin, catalase, glutamate dehydrogenase, and ovalbumin were used as protein standards. The subunit molecular weights of these proteins are 68K, 58K, 53K and 43K correspondingly. The gels were stained with Coomassie Blue R250.

RESULTS

In the first experiment with pyrazole, weanling male Wistar rats were given one i.p. injection of pyrazole (200 mg/kg body wt), and the animals were killed 6, 12 and 24 hr after the administration of pyrazole. Within 6 hr the activity of DMN-d was 170% of the control values and at 24 hr the enzyme activity was 250% of the control value (Fig. 1).

In the second experiment, rats received pyrazole (200 mg/kg body wt) as 1 to 4 injections on consecutive days. On day 5 of the experiment, 24 hr after the last injection, the animals were killed and the livers were removed. The activities of DMN-d, AHH and cytochrome P-450 were determined. An increase of about 200% in DMN-d activity was obtained when the animals received 1 to 2 injections of pyrazole. There was a slight rise in the cytochrome P-450 concentration, whereas the activity of AHH decreased and after the fourth pyrazole injection it was less than 50% of the control value (Fig. 2).

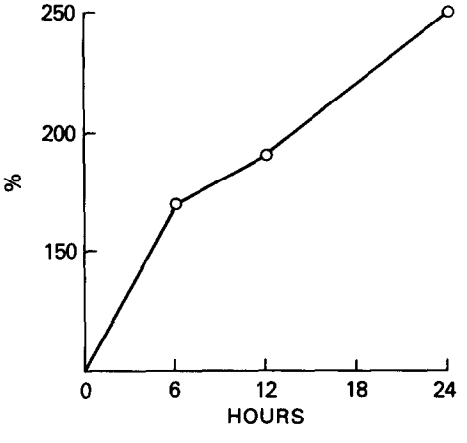

Fig. 1. Effect of one injection of pyrazole on the activity of DMN-d. Animals were killed 6, 12 and 24 hr after the administration of pyrazole. Three treated and three control animals were killed at each time interval. The values are expressed as a percentage of the values obtained for control animals.

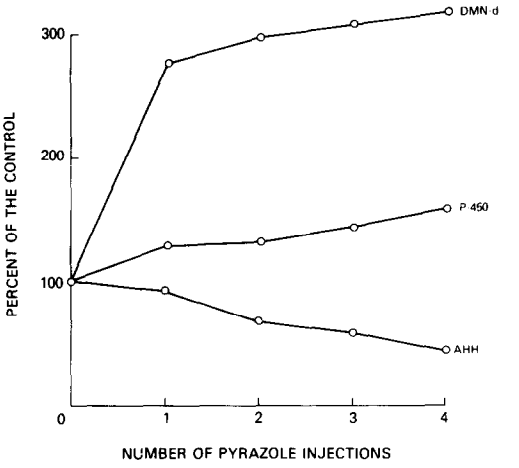

Fig. 2. Effect of pyrazole administration on the microsomal DMN-d, AHH and cytochrome P-450 levels. The animals received pyrazole (200 mg/kg body wt) as 1 to 4 injections on consecutive days. Each point is the average value of three animals. The values for DMN-d activity were 139 (control) and 385, 413, 426 and 443 nmoles $\cdot hr^{-1} \cdot$ (mg microsomal protein)$^{-1}$ for 1, 2, 3 and 4 injections of pyrazole, respectively; for AHH, 180 (control) and 163, 121, 102 and 81 pmoles $\cdot min^{-1} \cdot$ (mg microsomal protein)$^{-1}$; and for cytochrome P-450, 0.533 (control) and 0.688, 0.693, 0.750 and 0.844 nmoles $\cdot$ (mg microsomal protein)$^{-1}$.

Table 1 shows the effect of pyrazole in liver DMN-d, cytochrome P-450 and cytochrome *c*-reductase of the male and female Wistar rats. There were no significant differences between the sexes in the activity of DMN-d or in the concentration of cytochrome *c* reductase. A slight increase of cytochrome P-450 concentration was present after pyrazole administration.

To gain further evidence of the involvement of the microsomal mixed-function oxidase system in the metabolism of DMN, the requirement for NADPH and the effect of CO treatment of microsomes on the activity of DMN-d were studied. As shown in Table 2, without the presence of NADPH-generating system the enzyme activity was practically nonexistent. Flushing the flask with CO for 2 min before incubation in dim light caused 63% inhibition of the enzyme activity. When microsomes were boiled for 5 min before their use for incubation, 94% of the enzyme activity was destroyed.

The formation of formaldehyde was linear with increasing concentration of microsomal protein when control microsomes were used. A slight deviation from linearity was observed at 2 mg/ml protein concentration, when pyrazole microsomes were used. The formation of formaldehyde showed a linear increase with increasing incubation time when either control of pyrazole microsomes was used.

Metyrapone, a frequently used microsomal enzyme inhibitor, which is specific for phenobarbital inducible forms of P-450, increased the activity of DMN-d at low concentrations, when control microsomes were used, but produced inhibition at a higher

Table 1. Effect of pyrazole treatment on the activity of dimethylnitrosamine demethylase, and on the concentrations of cytochrome P-450 and NADPH–cytochrome *c*-reductase*

	Number of animals	Microsomal protein (mg/g liver)	DMN-d[nmoles·hr^{-1}·(mg microsomal protein)$^{-1}$]	P-450 (nmoles/mg microsomal protein)	NADPH–cytochrome *c*-reductase (nmoles/mg microsomal protein)
Male (control)	4	22 ± 1.3	110 ± 4.6	0.617 ± 0.031	100 ± 2.7
Male (pyrazole)	4	24 ± 1.1	400 ± 4.4†	0.792 ± 0.041‡	86 ± 7.3
Female (control)	4	23 ± 1.1	102 ± 5.8	0.564 ± 0.028	91 ± 3.8
Female (pyrazole)	4	26 ± 0.9	392 ± 9.0†	0.717 ± 0.015†	76 ± 5.4

* Weanling Wistar rats were given 3 injections of pyrazole (200 mg/kg body wt) and killed 24 hr after the last injection. Values are means ± S.E.
† Significant at 1% level.
‡ Significant at 5% level.

concentration (4×10^{-2} M). When pyrazole-induced microsomes were used, only the inhibitory effect was observed (Fig. 3A). On the contrary, *n*-butanol inhibited both the control and pyrazole-induced microsomes. It gave 50% inhibition of DMN-d activity at a concentration of 2×20^{-3} M (Fig. 3B).

Figure 4 shows the electrophoretograms of control microsomes and of microsomes induced by pyrazole, 3-methylcholanthrene, and phenobarbital. Pyrazole-induced microsomes showed a heavy band at the area of 51,000 daltons molecular weight.

DISCUSSION

The present experiment showed that pyrazole is a potent inducer of DMN-d. Earlier experiments showed a substantial inhibition in CO_2 formation and labeling of the macromolecules when ^{14}C-labeled DMN was administered shortly after animals had been treated with pyrazole [7, 13]. It is possible that the initial effect of pyrazole is inhibition of enzyme activity. However, in our experiment the activity of DMN-d was above the control value 6 hr after the administration of pyrazole and was still increasing 24 hr later. Possibly pyrazole may have a biphasic effect on microsomal enzyme systems, similar to that of piperonyl butoxide [14, 15]. This compound initially inhibited and then induced microsomal enzyme activities by 24 hr after treatment.

The precise mechanism of the induction of microsomal enzymes by xenobiotics has not been fully elucidated. The most potent inducers generally are lipophilic compounds, for induction is greater with compounds which remain in the body. The half-life for pyrazole, a water-soluble compound, is about 14 hr [16]. In a preliminary study we found that imidazole (1,3-diazole) was a good inducer for both DMN-d and AHH, whereas pyrazole decreased the activity of AHH. This indicates that pyrazole was a more specific inducer of DMN-d. The heavy band

Table 2. Requirement of NADPH for the microsomal DMN demethylase activity

Treatment	Number of animals	HCHO [nmoles·hr^{-1}·(mg microsomal protein)$^{-1}$] + NADPH	− NADPH
Control	6	79 ± 4.6	6 ± 1.7
Pyrazole	6	368 ± 23.2	8 ± 2.2

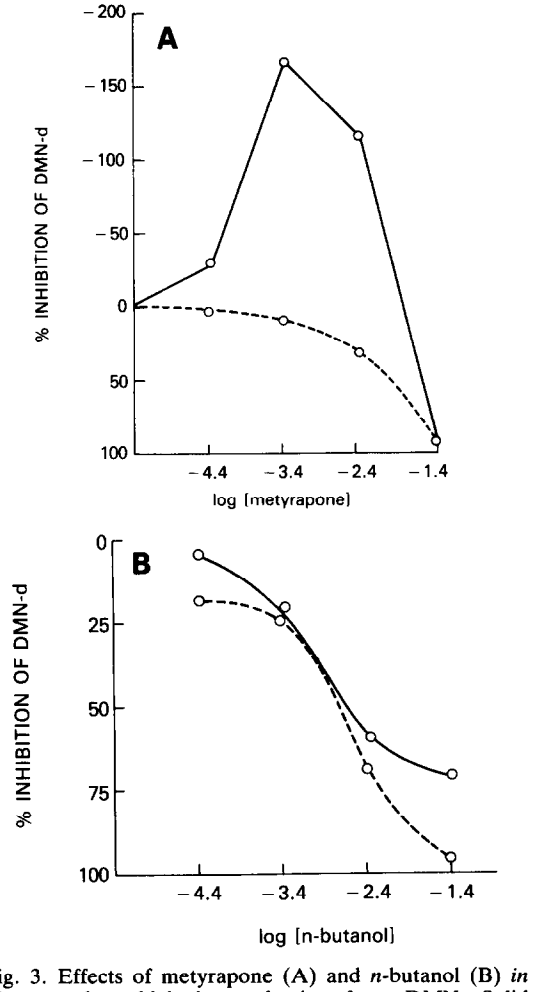

Fig. 3. Effects of metyrapone (A) and *n*-butanol (B) *in vitro* on formaldehyde production from DMN. Solid line = control microsomes; broken line = pyrazole microsomes.

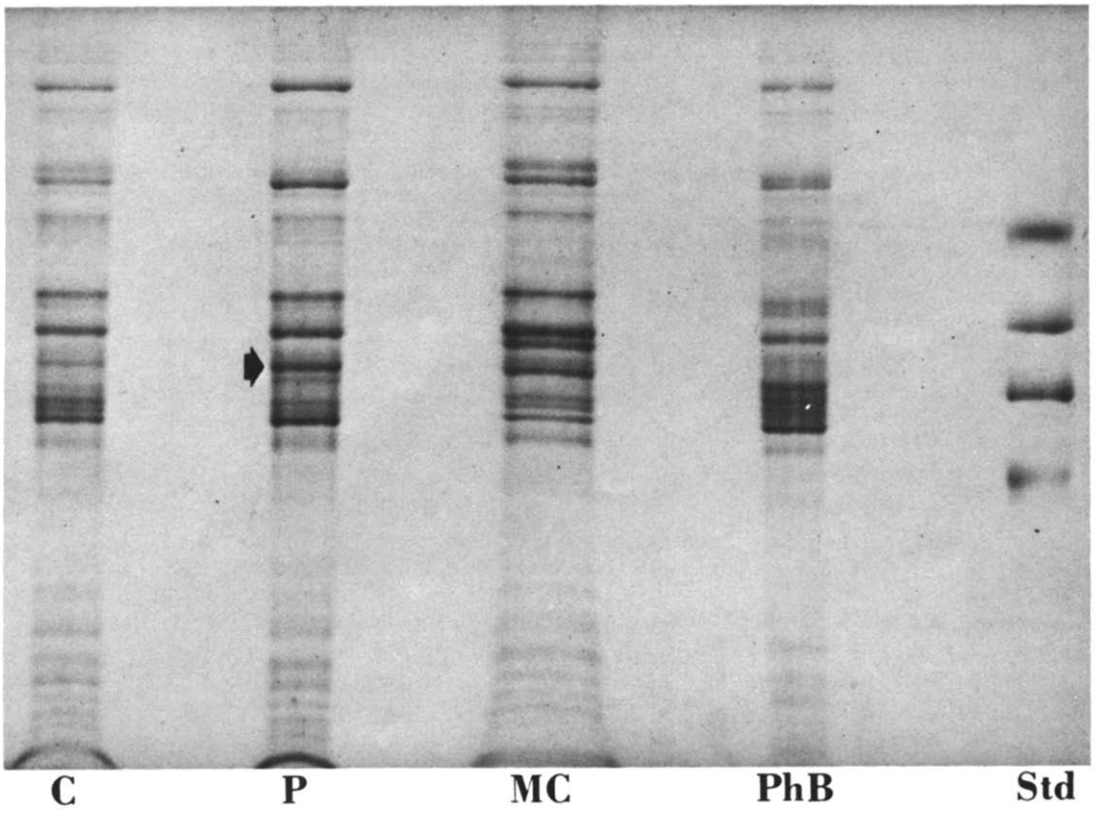

Fig. 4. Electrophoretograms of control (C) microsomes and of microsomes induced by pyrazole (P), 3-methylcholanthrene (MC) and phenobarbital (PhB). The heavy band induced by pyrazole is indicated by an arrow. The standards used were: albumin, catalase, glutamate dehydrogenase and ovalbumin.

at the area of 51,000 molecular weight may represent a specific cytochrome P-450 species involved in the metabolism of DMN. However, further information as to the nature of this protein will require its isolation for assay in a reconstituted enzyme system and a comparison of its specific activity with that of the other proteins of the cytochrome P-450 family. Using highly purified cytochrome P-450 fractions from phenobarbital-induced rabbit and rat liver, Guengerich [17] showed that a single P-450 was most active. However, the concentration of DMN in these experiments was 200 mM. Inhibition of the pyrazole-induced enzyme activity by CO and its dependence on NADPH indicates that cytochrome P-450 is involved in the metabolism of DMN, corroborating the earlier experiments [18, 19]. In addition, the lack of bacterial mutagenicity when a CO-treated microsomal preparation was used has been regarded as evidence that cytochrome P-450 is involved in the metabolism of DMN [1]. On the other hand, the longer storage stability of DMN-d as compared to the other cytochrome P-450-dependent mixed-function oxidases suggests that cytochrome P-450 is not involved in the metabolism of DMN [20]. However, it is possible that the different species of cytochrome P-450 have different stabilities. Our finding that metyrapone increases the activity of DMN-d agrees with that of Lake *et al.* [20]. Metyrapone also enhances the microsomal metabolism of acetanilide and trichloroethylene [21], but the mechanism of this action by metyrapone is not known, although a shift in the electron flow has been suggested [22].

There have been conflicting reports as to the consequences of oxidative demethylation of DMN on its mutagenicity, carcinogenicity and toxicity in animals. Partly due to the lack of an efficient inducer of the demethylase enzyme system, only inhibitors of DMN-d have been used previously for experiments *in vivo* and for mutagenicity testing. However, in a recent study Garro *et al.* [23] showed that chronic ethanol ingestion increased hepatic microsomal DMN-d activity and that the increased activity resulted in an increase in hepatic microsomal activation of DMN to a mutagen. The significant action of pyrazole in animals affords new possibilities to pinpoint the specifics of DMN activation in the living organism and in *in vitro* experiments as well.

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