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Inhibition of microsomal oxidation of ethanol by pyrazole and 4-methylpyrazole *in vitro*

Increased effectiveness after induction by pyrazole and 4-methylpyrazole

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Pyrazole and 4-methylpyrazole, which are inhibitors of alcohol dehydrogenase, were also found to be effective inhibitors of the oxidation of ethanol by liver microsomes (microsomal fractions) *in vitro*. Ethanol oxidation by microsomes from rats previously treated for 2 or 3 days with either pyrazole or 4-methylpyrazole appeared to be especially sensitive to inhibition *in vitro* by pyrazole or 4-methylpyrazole. The kinetics of inhibition by pyrazole or 4-methylpyrazole in all microsomal preparations were mixed, as the K_m for ethanol was elevated while V_{max} was lowered. However, K_i values for pyrazole (about 0.35 mM) and especially 4-methylpyrazole (about 0.03–0.10 mM) were much lower than those found with the saline controls (about 0.7–1.1 mM). In contrast, K_i values for dimethyl sulphoxide as an inhibitor of microsomal ethanol oxidation were similar in all microsomal preparations. Pyrazole and 4-methylpyrazole reacted with microsomes to produce type II spectral changes whose magnitude increased after treatment with either pyrazole or 4-methylpyrazole. Thus the increased inhibitory effectiveness of pyrazole and 4-methylpyrazole appears to be associated with increased interactions with the cytochrome *P*-450 isoenzyme(s) induced by these compounds. These isoenzymes have properties similar to those of the isoenzyme induced by chronic ethanol treatment. Therefore, caution is needed in the use of pyrazole or 4-methylpyrazole to assess pathways of ethanol metabolism, especially after chronic ethanol treatment, since these agents, besides inhibiting alcohol dehydrogenase, are also effective inhibitors of microsomal ethanol oxidation.

INTRODUCTION

Pyrazole and 4-methylpyrazole are potent inhibitors of alcohol dehydrogenase and of ethanol metabolism (Goldberg & Rydberg, 1969; Li & Theorell, 1969; Reynier, 1969). However, these agents also interact with microsomes (microsomal fractions), and some of these interactions are very similar to those found after chronic ethanol treatment (Evarts *et al.*, 1982; Feierman & Cederbaum, 1985*a*; Krikun & Cederbaum, 1984; Tu *et al.*, 1981). *In vitro*, ethanol has been known to affect the metabolism of a wide variety of drugs by the mixed-function oxidase system. This inhibition by ethanol has been attributed to competition for metabolism by cytochrome *P*-450 (Rubin *et al.*, 1970; Rubin & Lieber, 1971), or to hydrophobic interactions and displacement of drug substrates from cytochrome *P*-450 (Cinti *et al.*, 1973), or, in intact cells, to interference with the availability of the NADPH cofactor (Dicker & Cederbaum, 1983; Reinke *et al.*, 1980; Thurman & Kauffman, 1980).

The interactions of pyrazole and 4-methylpyrazole with liver microsomes, especially after induction with various inducers, have not been as well characterized. *In vitro*, pyrazole was shown to inhibit the metabolism of ethanol and aminopyrine (Cederbaum & Berl, 1982; Lieber *et al.*, 1970) and to bind with control microsomes to produce a type II spectral change (Rubin *et al.*, 1971). *In vitro*, 4-methylpyrazole also inhibited the metabolism of ethanol and aminopyrine by control microsomes (Cederbaum & Berl, 1982) and could bind to microsomes to produce a type II spectral change (Feierman & Cederbaum, 1985*b*). The present work was carried out to evaluate the effects of pyrazole and 4-methylpyrazole on oxidation of ethanol by microsomes from control rats as well as rats treated with the inducers, pyrazole or 4-methylpyrazole, to characterize the kinetics of inhibition by these agents, and to attempt to correlate their inhibitory effectiveness with the efficiency of binding to cytochrome *P*-450 in the microsomes.

For comparative purposes, the effects of dimethyl sulphoxide (DMSO) on microsomal oxidation of ethanol were also studied. Morgan *et al.* (1982) showed that DMSO interacted with microsomes or cytochrome *P*-450 from ethanol-treated animals, but not controls, to produce a modified type II spectral change. DMSO was shown to be a very effective inhibitor of microsomal ethanol oxidation by liver microsomes isolated from imidazole-treated rabbits as compared with other inducers (Kaul & Novack, 1984). In rabbits, imidazole and ethanol appear to induce the same cytochrome *P*-450 isoenzyme (Koop *et al.*, 1985). In microsomes from pyrazole-treated (Krikun & Cederbaum, 1984) or 4-methylpyrazole-treated (Feierman & Cederbaum, 1985*b*) rats, but not from controls, DMSO produced a modified type II spectral change. The above results suggest that DMSO may have a special interaction with the cytochrome *P*-450 induced by alcohol or alcohol-like

Abbreviation used: DMSO, dimethyl sulphoxide.

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inducers (imidazole, pyrazoles), and could therefore be a more effective inhibitor of cytochrome *P*-450-catalysed reactions in these preparations.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing about 150 g were injected intraperitoneally with saline (0.9% NaCl) for 2 or 3 days, or pyrazole (200 mg/kg body wt.) for 2 days, or 4-methylpyrazole (200 mg/kg body wt.) for 3 days. The rats were starved overnight and killed 24 h after the last injection. Liver microsomes were prepared as previously described (Cederbaum & Berl, 1982). Experiments were carried out with either fresh microsomes or microsomes that had been stored at −70 °C. Identical results were obtained with freshly prepared or frozen preparations.

The oxidation of ethanol was assayed at 37 °C in a reaction system consisting of 100 mM-potassium phosphate, pH 7.4, 10 mM-$MgCl_2$, 0.4 mM-$NADP^+$, 1 mM-NaN_3 (to inhibit catalase), ethanol (present at a final concn. of 55 mM in most experiments) and about 2 mg of microsomal protein in a final volume of 1 ml. Reactions were initiated by the addition of a mixture containing 10 mM-glucose 6-phosphate plus 0.7 unit of glucose-6-phosphate dehydrogenase and were terminated after 5 min by the addition of 0.3 ml of 1 M-HCl. The production of acetaldehyde was determined by a headspace g.l.c. procedure (Cederbaum & Cohen, 1984). All values were corrected for zero-time controls, which contained HCl added before the NADPH-generating system.

EDTA was not present in the reaction system, in order to minimize the production of hydroxyl radicals (Feierman & Cederbaum, 1983). This was necessary since ethanol is oxidized by hydroxyl radicals, and agents such as DMSO (Anbar & Neta, 1967) and pyrazole or 4-methylpyrazole (Cederbaum & Berl, 1982) are potent hydroxyl-radical scavenging agents. All buffers and the water used to prepare solutions were passed through Chelex-100 resins to remove metals such as iron and thereby minimize the production of hydroxyl radicals.

Binding-spectrum experiments with either pyrazole or 4-methylpyrazole as the substrate were carried out with a Perkin–Elmer model 554 dual-beam spectrophotometer. Microsomal protein (2 mg) was placed into 6 ml of 100 mM-phosphate buffer, pH 7.4, and the sample was divided into two 3 ml portions. A baseline correction was performed to compensate for any differences between the two cuvettes before the addition of various concentrations of pyrazole or 4-methylpyrazole.

All values are means ± S.E.M. Statistical analyses were performed by Student's *t* test (two-tailed). *P* values were calculated relative to the appropriate saline control. The content of cytochrome *P*-450 was determined by the method of Omura & Sato (1964).

RESULTS

Inhibition of the microsomal oxidation of ethanol by pyrazole, 4-methylpyrazole and DMSO

At a substrate concentration of 55 mM, ethanol was oxidized at a rate of 6–7 nmol/min per mg of protein by control microsomes and, as described previously (Krikun & Cederbaum, 1984; Feierman & Cederbaum, 1985*a*), this rate was increased about 2-fold after treating rats with pyrazole or 4-methylpyrazole. A dose–response curve for pyrazole inhibition of microsomal ethanol oxidation is shown in Fig. 1(*a*). Microsomes from rats treated with pyrazole appear to be especially sensitive to inhibition by pyrazole, as there was an initial rapid decrease in activity such that most of the increase in ethanol oxidation produced by the pyrazole treatment was blocked by low concentrations of the inhibitor. Micro-

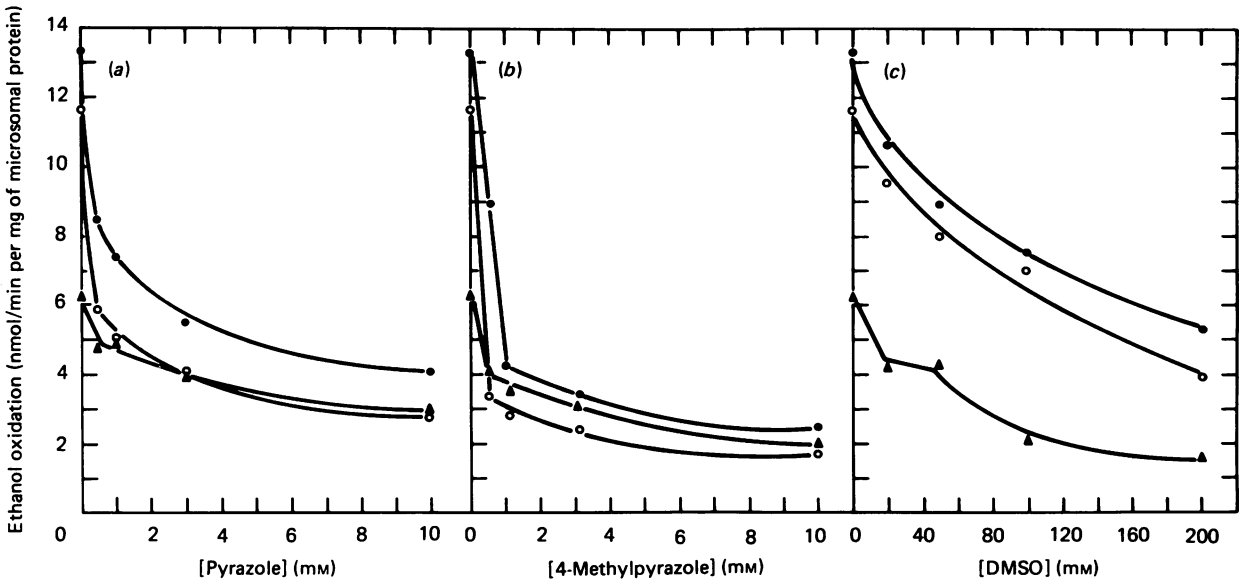

Fig. 1. Inhibition of the microsomal oxidation of ethanol by addition of pyrazole (*a*), methylpyrazole (*b*) or DMSO (*c*) *in vitro*

The oxidation of 55 mM-ethanol by microsomes from rats treated with saline (▲), pyrazole (○) or 4-methylpyrazole (●) was assayed as described in the Materials and methods section, in the presence of the indicated concentrations of pyrazole, 4-methylpyrazole or DMSO. Results are from three to four experiments. For ease of presentation, only mean values are presented, but differences between experiments did not exceed 10%.

somes from rats treated with 4-methylpyrazole also appear to be very sensitive to inhibition by pyrazole, as there was an initial rapid decrease in ethanol oxidation at low concentrations of pyrazole, followed by a less-pronounced decrease at higher concentrations of pyrazole. This less-pronounced decrease resembles the pattern observed with microsomes from saline-treated rats (Fig. 1*a*).

Fig. 1(*b*) shows similar types of experiments with 4-methylpyrazole added as the inhibitor *in vitro* of ethanol oxidation. In all three types of microsomal preparations, but especially in those from rats treated with pyrazole or 4-methylpyrazole, there was an initial rapid inhibition of ethanol oxidation by low concentrations of 4-methylpyrazole, followed by a less sensitive phase. The increase in ethanol oxidation produced by prior treatment with pyrazole or 4-methylpyrazole was completely lost in the presence of low concentrations of 4-methylpyrazole, analogous to results with pyrazole as the inhibitor *in vitro* (Figs. 1*a* and 1*b*).

Experiments were also carried out with DMSO, since this agent was shown to be an especially effective inhibitor of ethanol oxidation by liver microsomes from imidazole-treated rabbits (Kaul & Novack, 1984). Much higher concentrations of DMSO, as compared with pyrazole and 4-methylpyrazole, were required to inhibit ethanol oxidation (Fig. 1*c*). In contrast with results with pyrazole or 4-methylpyrazole, the inhibition by DMSO was the same in all three microsomal preparations, i.e. prior treatment with pyrazole or 4-methylpyrazole did not induce a population of cytochrome *P*-450 isoenzymes with ethanol-oxidizing activity that was especially sensitive to inhibition by DMSO. Indeed, at all concentrations of DMSO tested, the rate of ethanol oxidation by microsomes from rats treated with either pyrazole or 4-methylpyrazole remained higher than the rates for saline controls (Fig. 1*c*).

Kinetics of inhibition of microsomal oxidation of ethanol

To evaluate the kinetics of inhibition by pyrazole, 4-methylpyrazole and DMSO, the effect of these agents on the oxidation of various concentrations of ethanol was studied in microsomes isolated from rats treated with saline, pyrazole or 4-methylpyrazole. In all experiments, DMSO was used at a concentration of 50 mM. However, in view of the increased effectiveness of pyrazole and 4-methylpyrazole as inhibitors of microsomal ethanol oxidation after treatment with pyrazole or 4-methylpyrazole, lower concentrations of pyrazole (0.25 and 0.50 mM) and 4-methylpyrazole (0.15 and 0.50 mM) were utilized in experiments with microsomes isolated from rats treated with these agents than with the saline controls (1 mM- and 3 mM- pyrazole or 4-methylpyrazole). Results are summarized in Table 1. The rate of oxidation of ethanol increased over the ethanol concentration range of 11–110 mM in all three microsomal preparations (saline, pyrazole- or 4-methylpyrazole-treated). In the absence of any inhibitors, the rate of microsomal oxidation of ethanol was higher in the pyrazole- or 4-methylpyrazole-treated rats than in saline controls at all four concentrations of ethanol employed (Table 1, no additions). The presence of pyrazole, 4-methylpyrazole or DMSO caused an inhibition of ethanol oxidation at all ethanol substrate concentrations studied, in all three microsomal preparations. Pyrazole and 4-methylpyrazole, but not DMSO, were more effective inhibitors of ethanol oxidation in microsomes isolated from rats treated with pyrazole or 4-methylpyrazole than from saline controls (also note different concentration of pyrazole and 4-methylpyrazole utilized, Table 1).

To determine the kinetics of inhibition, the data shown in Table 1 were plotted as Lineweaver–Burk plots. The reciprocal plots in the absence or presence of inhibitors were linear over the ethanol concentration range of

Table 1. Effect of pyrazole, 4-methylpyrazole and DMSO on the microsomal oxidation of various concentrations of ethanol

Experiments were carried out in the presence of the indicated concentrations of pyrazole, 4-methylpyrazole and DMSO added to microsomes isolated from rats treated with saline, pyrazole or 4-methylpyrazole. Results are from three experiments.

Treatment	Addition	Concn. of ethanol (mM) . . .	Rate of oxidation of ethanol (nmol/min per mg) 11	27.5	55	110
Saline	—		3.69±0.11	5.29±1.08	7.24±0.63	8.96±0.42
	1 mM-pyrazole		2.07±0.37	3.30±0.29	4.61±0.71	6.03±0.77
	3 mM-pyrazole		1.37±0.12	2.20±0.28	3.73±0.82	5.28±0.80
	1 mM-4-methylpyrazole		1.57±0.17	2.68±0.35	3.80±0.54	5.26±0.53
	3 mM-4-methylpyrazole		1.06±0.14	2.12±0.24	3.34±0.84	4.83±0.84
	50 mM-DMSO		1.56±0.43	2.82±0.37	3.87±0.59	5.30±0.35
Pyrazole	—		6.16±0.70	8.46±0.12	11.47±0.31	13.41±0.64
	0.25 mM-pyrazole		3.51±0.43	5.12±0.24	7.83±0.25	8.86±0.33
	0.50 mM-pyrazole		2.88±0.08	4.06±0.21	5.84±0.29	6.90±0.31
	0.15 mM-4-methylpyrazole		1.73±0.26	2.82±0.24	3.97±0.42	4.97±0.72
	0.50 mM-4-methylpyrazole		1.11±0.24	2.08±0.27	3.62±0.40	4.54±0.38
	50 mM-DMSO		2.44±0.45	4.98±0.39	7.10±0.95	8.37±0.72
4-Methylpyrazole	—		8.36±1.13	10.37±0.63	13.84±0.38	18.21±0.92
	0.25 mM-pyrazole		5.64±0.36	8.98±0.89	11.14±0.64	15.00±1.38
	0.50 mM-pyrazole		4.55±0.29	6.68±0.62	9.34±1.32	12.61±0.73
	0.15 mM-4-methylpyrazole		3.41±0.53	4.45±0.88	6.60±1.26	8.00±1.13
	0.50 mM-4-methylpyrazole		2.40±0.43	4.24±0.45	6.17±0.93	8.28±1.27
	50 mM-DMSO		4.57±0.17	7.41±0.73	10.08±0.88	13.44±1.00

Table 2. Kinetic constants for the inhibition of microsomal oxidation of ethanol by pyrazole, 4-methylpyrazole and DMSO

The kinetic constants were derived from linear regressions of Lineweaver–Burk plots of the data shown in Table 1. The K_i values for pyrazole as an inhibitor were significantly ($P < 0.05$) lower in microsomes from pyrazole-treated (about 0.32 mM average) or 4-methylpyrazole-treated (about 0.37 mM average) rats as compared with saline controls (about 1.1 mM average). The K_i values for 4-methylpyrazole as an inhibitor were also significantly ($P < 0.01$) lower in microsomes from pyrazole-treated (about 0.03 mM average) or 4-methylpyrazole-treated (about 0.10 mM) rats as compared with saline controls (about 0.70 mM average). The K_i values for DMSO as an inhibitor were not significantly different for the various preparations ($P > 0.05$).

Treatment	Addition	K_m (mM)	$V_{max.}$ (nmol/min per mg)	K_i (mM)
Saline	—	18	9.6	—
	1 mM-pyrazole	26	6.8	0.97
	3 mM-pyrazole	40	6.2	1.22
	1 mM-4-methylpyrazole	33	6.2	0.56
	3 mM-4-methylpyrazole	66	7.4	0.80
	50 mM-DMSO	36	6.6	27.1
Pyrazole	—	15	14.3	—
	0.25 mM-pyrazole	21	10.1	0.25
	0.50 mM-pyrazole	26	7.5	0.40
	0.15 mM-4-methylpyrazole	27	5.9	0.04
	0.50 mM-4-methylpyrazole	58	6.9	0.02
	50 mM-DMSO	46	12.8	20.7
4-Methylpyrazole	—	12	17.3	—
	0.25 mM-pyrazole	21	16.3	0.36
	0.50 mM-pyrazole	25	13.2	0.39
	0.15 mM-4-methylpyrazole	16	8.2	0.08
	0.50 mM-4-methylpyrazole	37	10.4	0.12
	50 mM-DMSO	26	15.2	34.5

11–110 mM (results not shown). Kinetic values are summarized in Table 2. In the absence of inhibitors, treatment with pyrazole or 4-methylpyrazole resulted in an increase in $V_{max.}$ values per mg of protein; the 4-methylpyrazole treatment also produced a small decrease in the K_m value for ethanol (Table 2). The kinetics of inhibition by pyrazole and 4-methylpyrazole were complex, as the inhibitors increased K_m values for ethanol while lowering the $V_{max.}$ values in all three microsomal preparations. The kinetics of inhibition by DMSO, although mixed, appeared to have a large competitive component (Table 2). Since all three inhibitors appeared to show a mixed type of inhibition, K_i values were estimated from the equation (Segal, 1975):

$$K_i = \frac{\frac{[I]}{\alpha}\left(\frac{K_m^*}{K_m} - 1\right)}{1 - \frac{K_m^*}{K_m}}$$

where [I] is inhibitor concentration and K_m^* is the apparent K_m in the presence of inhibitor. The values for α were estimated from the Lineweaver–Burk plots by graphically solving for the abscissa co-ordinate at the point of intersection of the control rate, and the rate in the presence of the inhibitor; this co-ordinate value is equal to $-1/\alpha K_m$ (Segal, 1975). K_i values are summarized in the last column of Table 2. With microsomes from saline-treated rats, pyrazole had a K_i of about 1.1 mM; this value was decreased about 3-fold after treatment with pyrazole or 4-methylpyrazole. K_i values for 4-methylpyrazole were about 0.7, 0.03 and 0.1 mM in microsomes from saline, pyrazole- and 4-methylpyrazole-treated rats respectively. Thus 4-methylpyrazole was a better inhibitor of microsomal ethanol oxidation than pyrazole was, and was especially effective after treatment with pyrazole and 4-methylpyrazole. By contrast, the K_i values for DMSO appeared to be similar with all three microsomal preparations (Table 2).

The possibility that 10 mM-pyrazole, in the presence of NADPH, may act as a suicide inhibitor of cytochrome *P*-450 was suggested (Craft, 1985). However, under the conditions of our experiments, the combination of pyrazole plus NADPH was no more effective than NADPH alone in causing loss of the cytochrome *P*-450 binding spectrum (about 10% loss of the CO-binding spectrum was found after a 5 min incubation period with microsomes from pyrazole-treated rats). No cytochrome *P*-420 was observed, despite this 10% loss of cytochrome *P*-450. In other experiments, neither 1 mM-pyrazole nor 1 mM-4-methylpyrazole had any effect on the activity of NADPH–cytochrome *P*-450 reductase (results not shown).

Binding of pyrazole and 4-methylpyrazole to rat liver microsomes

Pyrazole *in vitro* was shown to react with microsomes from control rats to produce a type II spectral change, with a peak at 430 nm and a trough at 394 nm (Rubin *et al.*, 1971). A typical spectrum for pyrazole interacting with saline microsomes is shown in Fig. 2(*a*), and confirms the previous results of Rubin *et al.* (1971). The magnitude of this spectral change increased with microsomes isolated from rats treated with either pyrazole or 4-methylpyrazole (Fig. 2*a*). In a similar fashion, 4-methylpyrazole also interacted with micro-

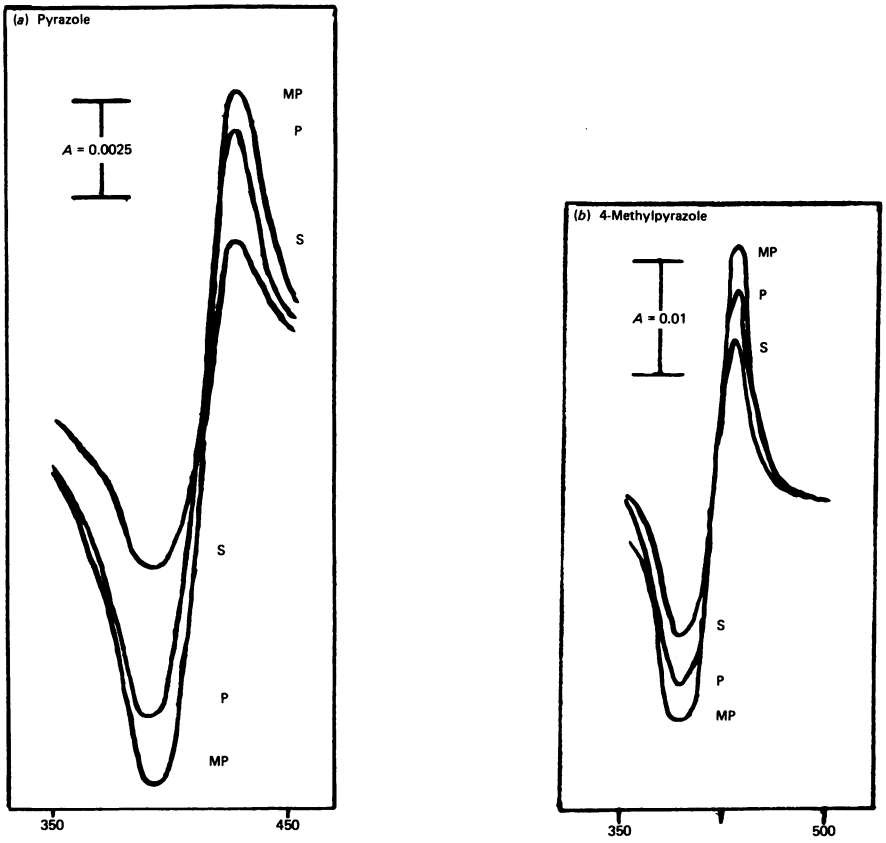

Fig. 2. Spectra associated with the binding of (*a*) pyrazole or (*b*) 4-methylpyrazole to microsomes from rats treated with saline (S), pyrazole (P) or 4-methylpyrazole (MP)

Spectral studies were carried out as described in the Materials and methods section, with either 2.67 mM-pyrazole or 2.67 mM-4-methylpyrazole as substrate. Results from a typical spectrum are shown.

somes from saline controls to produce a type II spectrum with a peak at 429 nm and a trough at 392 nm (Fig. 2*b*). The magnitude of the spectral change associated with 4-methylpyrazole interacting with cytochrome *P*-450 also increased after rats were treated with either pyrazole or 4-methylpyrazole (Fig. 2*b*). The wavelengths for peak height and trough were identical with those found for the saline controls.

Various concentrations of pyrazole or 4-methylpyrazole were added to microsomes from the saline-, pyrazole- or 4-methylpyrazole-treated rats, and the magnitude of the binding spectrum, as reflected by the change in absorbance (429−392 nm), was determined. With all three preparations of microsomes, the magnitude of the spectral change increased as the concentration of pyrazole or 4-methylpyrazole was increased over the range of 0.33–2.67 mM (results not shown). A Hanes–Wolfe plot of these data was linear with both pyrazole

Table 3. Kinetic constants for the binding of pyrazole and 4-methylpyrazole to microsomes

The kinetic constants were derived from Hanes–Wolfe plots of experiments in which the concentration of pyrazole or 4-methylpyrazole was varied over the range 0.33–2.67 mM, and the change in absorbance (ΔA) determined as peak minus trough (429–392 nm). Microsomes from saline-treated controls or from rats treated with pyrazole or 4-methylpyrazole were utilized. Results are from four to seven experiments. [a]$P < 0.01$; [b]$P < 0.001$; [c]$P < 0.005$.

Substrate	Kinetic parameter	Treatment		
		Saline	Pyrazole	4-Methylpyrazole
Pyrazole	K_s (mM)	0.320±0.024	0.202±0.023[a]	0.333±0.031
	V_s (ΔA/mg of protein)	0.0083±0.0004	0.0169±0.0015[b]	0.0179±0.001[b]
	V_s (ΔA/nmol of cytochrome *P*-450)	0.0123±0.004	0.0244±0.002[b]	0.0099±0.002
4-Methylpyrazole	K_s (mM)	0.319±0.019	0.116±0.009[b]	0.223±0.009[c]
	V_s (ΔA/mg of protein)	0.0165±0.0011	0.0244±0.0004[b]	0.0310±0.001[b]
	V_s (ΔA/nmol of cytochrome *P*-450)	0.0219±0.0015	0.0372±0.002[b]	0.0187±0.0005

and 4-methylpyrazole as substrates (results not shown), and kinetic constants were calculated from linear-regression analyses. Spectral dissociation constants (K_s) and maximal spectral changes (V_s) from the $A_{429}-A_{392}$ were calculated from these plots and are summarized in Table 3. The K_s values for pyrazole and 4-methylpyrazole binding to microsomes from saline-treated rats were the same, 0.32 mM, whereas maximal binding values per mg of protein or per nmol of cytochrome *P*-450 were 2-fold higher with 4-methylpyrazole as the substrate than with pyrazole. Treatment with pyrazole caused a lowering of the K_s values for both pyrazole and 4-methylpyrazole, as well as an almost 2-fold increase in maximal binding values for both substrates (Table 3). Treatment with 4-methylpyrazole lowered the K_s value for 4-methylpyrazole, but not for pyrazole as the substrate. Maximal binding values for both substrates were doubled when expressed as ΔA per mg of microsomal protein, but no such changes were observed when the spectral changes were expressed per nmol of cytochrome *P*-450 (Table 3). The content of cytochrome *P*-450 was about 0.70 nmol/mg of microsomal protein for the saline- and the pyrazole-treated rats, and about 1.7 nmol/mg of microsomal protein for the 4-methylpyrazole-treated rats.

DISCUSSION

Results in the present paper demonstrate that *in vitro* pyrazole and 4-methylpyrazole can inhibit the microsomal oxidation of ethanol, and that the inhibitory effectiveness of these agents is increased in rats treated with pyrazole and 4-methylpyrazole. Pyrazole treatment appears to induce a cytochrome *P*-450 isoenzyme with properties similar to the isoenzyme induced by chronic ethanol treatment (Evarts *et al.*, 1982; Koop *et al.*, 1985; Krikun & Cederbaum, 1984; Tu *et al.*, 1981). Similar results have been suggested for 4-methylpyrazole treatment (Feierman & Cederbaum, 1985*a*). The increased sensitivity of microsomal ethanol oxidation to inhibition by pyrazole or 4-methylpyrazole probably reflects the induction of an alcohol-preferring cytochrome *P*-450 isoenzyme by the pyrazole or 4-methylpyrazole treatment. Indeed, the increased rate of ethanol oxidation produced by the pyrazole or 4-methylpyrazole treatments is especially sensitive to inhibition by these compounds *in vitro* (Figs. 1*a* and 1*b*), suggesting that the cytochrome *P*-450 isoenzyme(s) responsible for the increase in microsomal ethanol oxidation reflect the isoenzyme(s) which are sensitive to pyrazole and 4-methylpyrazole.

Pyrazole and 4-methylpyrazole produce type II binding spectra with the microsomes, and the magnitude of the spectral changes increased after treatment with pyrazole or 4-methylpyrazole. This increased interaction of pyrazole and 4-methylpyrazole with cytochrome *P*-450 after treatment with these agents may explain, in part, the increased inhibitory effectiveness towards ethanol oxidation. For example, the K_i for pyrazole in inhibiting microsomal oxidation of ethanol is lowered about 3-fold after pyrazole or 4-methylpyrazole treatment (Table 2). Associated with this 3-fold decrease in K_i is a 2-fold increase in values for the maximal spectral change (V_s) associated with pyrazole binding after pyrazole or 4-methylpyrazole treatment (Table 3).

The kinetics associated with 4-methylpyrazole inhibition of microsomal ethanol oxidation appear somewhat unusual, in that as the concentration of 4-methylpyrazole is raised there is an increase in K_m for ethanol, but $V_{max.}$ does not decrease any further (Table 2). One interpretation for these results could reflect several populations of cytochrome *P*-450 isoenzymes in the microsomal preparations with which 4-methylpyrazole interacts, e.g. lower concentrations appear to interact with one population of cytochrome *P*-450 isoenzymes and inhibit ethanol metabolism via a mixed type of inhibition, whereas higher concentrations may react with another population of cytochrome *P*-450 isoenzymes and inhibit ethanol metabolism via a competitive type of mechanism. That 4-methylpyrazole may interact with several populations of cytochrome *P*-450 can be suggested from the increase in total cytochrome *P*-450 content observed after treatment with 4-methylpyrazole, but not pyrazole (Feierman & Cederbaum, 1985*a*), and the cytochrome *P*-450 profiles obtained after SDS/polyacrylamide-gel electrophoresis (Krikun *et al.*, 1986).

The potential role of potent oxidants such as the hydroxyl radical in the microsomal pathway of ethanol oxidation has been described (Cederbaum *et al.*, 1978; Cederbaum & Dicker, 1983; Ingelman-Sundberg & Johansson, 1984). Since ethanol, DMSO, pyrazole and 4-methylpyrazole are all potent scavengers of the hydroxyl radical, efforts were made to minimize the contribution of this radical in order to avoid complexities in interpreting the results. Under the reaction conditions as described in the Materials and methods section, we estimate that about 90% of the ethanol is oxidized by a cytochrome-*P*-450-dependent pathway that is independent of a role for hydroxyl radicals (Feierman & Cederbaum, 1983; Feierman *et al.*, 1985).

Pyrazole and 4-methylpyrazole are often utilized to assess the contribution of alcohol dehydrogenase towards the overall metabolism of ethanol. This assessment, however, will be complicated, in view of the inhibition by these agents of microsomal oxidation of ethanol. In particular, if the increased sensitivity to inhibition by pyrazole or 4-methylpyrazole after treatment with these agents could be extended to an increased microsomal sensitivity to these agents after chronic ethanol treatment (in view of induction of similar isoenzymes of cytochrome *P*-450), extreme caution would be required in the use of pyrazole or 4-methylpyrazole to assess the role of alcohol dehydrogenase-dependent and -independent (e.g. microsomal) pathways in contributing towards the metabolic tolerance associated with chronic ethanol consumption. Preliminary experiments have shown that ethanol oxidation by microsomes isolated from rats chronically fed on the Lieber–DeCarli (1982) ethanol diet is indeed more sensitive to inhibition by pyrazole or 4-methylpyrazole than is that by microsomes from pair-fed controls.

DMSO was shown to interact with microsomes or cytochrome *P*-450 from ethanol-treated rats or rabbits to produce a modified type II spectral change (Morgan *et al.*, 1981, 1982). This suggested the possibility of a special interaction between DMSO and the alcohol-inducible cytochrome *P*-450 isoenzyme. DMSO was a very effective inhibitor of ethanol oxidation by microsomes isolated from imidazole-treated rabbits (Kaul & Novack, 1984), and this appears to reflect induction of the same cytochrome *P*-450 isoenzyme by imidazole and ethanol (Koop *et al.*, 1985). However, in rats (unlike rabbits), imidazole treatment does not appear to induce

an alcohol-preferring isoenzyme of cytochrome *P*-450 (Reinke *et al.*, 1985). Since pyrazole and 4-methylpyrazole appear to be 'alcohol-like' inducers in rats, it was considered that, analogous to results with imidazole, DMSO would be an especially effective inhibitor of microsomal ethanol oxidation after treatment with pyrazole or 4-methylpyrazole. This proved not to be the case, as the K_i for inhibition by DMSO was similar in all three microsomal preparations. Thus, although DMSO produces a binding spectrum with microsomes from pyrazole-treated (Krikun & Cederbaum, 1984) or 4-methylpyrazole-treated (Feierman & Cederbaum, 1985*a*) rats (but not with controls), the significance of this interaction is unclear, since its does not result in enhanced inhibitory effectiveness by DMSO towards ethanol oxidation.

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