

Pyrazoles as inhibitors of alcohol oxidation and as important tools in alcohol research: An approach to therapy against methanol poisoning

(methylpyrazole metabolism and toxicity/alcohol-methylpyrazole interaction)

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ABSTRACT 4-Methylpyrazole, in a dose producing inhibition of alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1), was given alone or together with ethanol (10%) as sole drinking fluid to growing rats for up to 38 weeks. Their weight curves remained normal. Electron microscopy of liver, kidney, and heart revealed no changes related to treatment. Hematologic analysis showed normal values for blood and bone marrow. Several clinical chemical parameters showed no impairment of liver or kidney function, except for an enhancement of the microsomal drug-metabolizing activity after concurrent administration of 4-methylpyrazole and ethanol. A study on rats receiving 4-methylpyrazole and ethanol indicated a mutual interaction of the two compounds or the metabolites, leading to increased concentration in the blood of the compounds and reduced formation of 4-hydroxymethylpyrazole, the primary metabolite of 4-methylpyrazole. In monkeys, elimination of 4-methylpyrazole followed a linear course. 4-Hydroxymethylpyrazole accumulated to a level of at most 10% of that of 4-methylpyrazole. Concurrent administration of methanol inhibited the elimination of 4-methylpyrazole about 25%, and 4-methylpyrazole produced a profound inhibition of the oxidation of methanol. 4-Methylpyrazole, at a level in the plasma of more than 10 μM, prevented accumulation of the toxic metabolite formic acid in methanol-poisoned monkeys, and repeated injections of 4-methylpyrazole abolished methanol toxicity in monkeys receiving lethal doses of methanol. The present investigation indicates that 4-methylpyrazole, with its low toxicity and strong inhibition of alcohol oxidation, is a valuable tool for experimental studies of alcohol metabolism and its effects. It illustrates the usefulness of the monkey as a model to study 4-methylpyrazole activity and toxicity in light of its possible use for treating methanol poisoning in human beings.

Alcoholism is a disease limited to human beings. It is therefore important to study the metabolism of ethanol in humans in order to provide a better understanding of the biochemical aspects of alcoholism. In 1963, Theorell and Yonetani (1) reported that pyrazole inhibits the action of horse liver alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) (ADH) *in vitro* by forming a complex with ADH and NAD⁺. In 1969 Li and Theorell (2) reported the kinetics of inhibition of human ADH by pyrazole and some substituted derivatives. They found that, as with the horse enzyme, these compounds form inactive ternary complexes by coupling with ADH and its coenzyme NAD⁺. In 1970 Blomstrand and Theorell (3) reported the use of 4-methylpyrazole (4-MP) in human beings in studies on ethanol oxidation. They emphasized that this type

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of inhibitor should be used as a valuable tool for elucidation of the effect of ethanol on different biochemical variables. It has also been suggested that 4-MP might be used in the treatment of methanol poisoning in humans (4).

4-MP has been used in some investigations in humans according to this early suggestion (5-7). A wider use of 4-MP (8) in clinical alcohol research has been hindered by the pronounced toxicity of its parent compound pyrazole (9). 4-MP is a more specific and much stronger inhibitor of ADH than pyrazole; for example, it does not (10), in contrast to pyrazole (11), inhibit catalase *in vivo*. It is thus important to have a detailed knowledge of the pharmacological and toxicological effects of 4-MP alone or in combination with ethanol (12).

In the present study these effects have been extensively investigated in rats in a 38-week-long feeding experiment. Another part of the present work concerns the effects of 4-MP in the monkey, which is the only species (13) that is susceptible to methanol toxicity in the same manner as humans. This approach to treatment of methanol poisoning in humans with a specific inhibitor of ADH may significantly improve the therapeutic results. A full report of these investigations will be published elsewhere.

MATERIALS AND METHODS

Unlabeled 4-MP was obtained from AB Labkemi (Gothenburg, Sweden) and was pure as shown by gas/liquid chromatography. Unlabeled 4-hydroxymethylpyrazole (4-OH-MP) and 4-carboxypyrazole were gifts from Astra-Ewos (Södertälje, Sweden). All other reagents were of the highest available purity. Details of the synthesis and purification of 4-methyl-[6-²H₃]pyrazole have been given (14). 4-Methyl-[¹⁵N₂]pyrazole was synthesized from 2-methyl-1,1,3,3-tetraethoxypropane and [¹⁵N₂]hydrazine sulfate by a method described for the synthesis of 4-MP (15). 4-Hydroxymethyl-[6-²H₂]pyrazole (4-OH-[6-²H₂]MP) was synthesized from 4-carboxypyrazole after reduction with lithium aluminum deuteride. After derivatization with *t*-butyl-dimethylchlorosilane imidazole reagent, the material had a mass spectrum consistent with *t*-butyl-dimethylsilyl ether of 4-OH-MP with two atoms of deuterium in the molecule.

Assays. 4-MP in plasma was determined by a mass fragmentographic assay basically identical to that described (14), except that 4-[¹⁵N₂]MP was used as the internal standard in the monkey experiments. 4-OH-MP was assayed as follows. To serum samples (0.1 ml), 1 ng of 4-OH-[6-²H₂]MP was added. The mixture was freeze-dried and extracted with ethanol. The

Abbreviations: ADH, alcohol dehydrogenase; 4-MP, 4-methylpyrazole; 4-OH-MP, 4-hydroxymethylpyrazole.

ethanol phase was evaporated under nitrogen and reacted with 50 μ l of *t*-butyl-dimethylchlorosilane imidazole reagent. The derivative was extracted with hexane after addition of normal saline and the hexane phase was concentrated under nitrogen prior to injection. Standard curves were prepared with unlabeled 4-OH-MP, 0.05–3.0 μ g/ml. Samples were analyzed by gas chromatography/mass spectrometry with an LKB 2091 instrument operated in multiple ion detection (MID) mode. A 1.5% SE 30 column was used at a temperature of 150°C. The flow rate of helium was 15 ml/min. The flash heater and ion source temperatures were 275°C.

Formate analyses were made according to Makar *et al.* (16). Ethanol and methanol were determined by gas chromatography after 50 μ l of plasma was mixed with 50 μ l of acetonitrile (2 mg/ml) solution as an internal standard. The rat blood was analyzed for several hematological and clinical chemical variables. Standard analytical procedures were used, and several of these techniques have been standardized against mass fragmentographic methods (17).

Rat Experiments. Male Sprague–Dawley rats were used. On arrival, the rats were allowed a week for acclimatization before the start of the experiment. They were given food and drinking fluid *ad lib*. The food was Astra-Ewos Mouse and Rat Food New R3 (Astra-Ewos).

Three series of experiments were carried out. In series I, 48 rats were divided into four groups of 12 and given drinking fluid as follows: group C, tap water; group E, 1.7 M ethanol; group EM, 1.7 M ethanol plus 1.3 mM 4-MP; and group M, 1.3 mM 4-MP. When the experiment commenced, the rats weighed about 200 g. The amount of food consumed was registered; the rats were weighed once a week and observed daily for clinical signs. The experiment lasted for 12 weeks. At different times during the experiment, blood was obtained from the tip of the tail for hematological and chemical examinations.

In series II, 64 male rats were divided into four groups of 16 and treated with the same type of drinking fluid and food as in series I. The rats were weighed once a week for 16 weeks and the experiment lasted for 38 weeks. At different times during the experiment, the concentration of ethanol and 4-MP was determined in the serum.

After week 22, six animals from each group were killed by a sharp blow on the neck. The livers were immediately removed; microsomes were prepared from the livers and mitochondria from the adrenals. The activity of aniline hydroxylase,

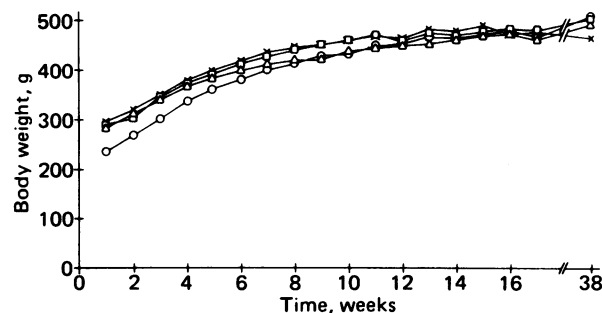


FIG. 1. Weight curves from the different groups: X, control; Δ , ethanol; O, ethanol + 4-MP; and \square , 4-MP. In each group 12 rats were weighed once a week for 16 weeks.

aminopyrine *N*-demethylase, and *p*-nitroanisole *O*-demethylase was determined (18).

After 38 weeks, one animal from each group was anesthetized with ether, the abdomen was opened, and the animal was killed by exsanguination. The liver was perfused with 2% glutaraldehyde and samples for electron microscopy were taken through graded alcohols and then embedded in Epon (19).

In an acute interaction study of 4-MP and ethanol (series III), 15 rats (90–110 g) were randomly divided into three groups. The rats in group 1 were given 4 g of ethanol per kg, those in group 2, 50 mg of 4-MP per kg, and those in group 3, 4 g of ethanol and 50 mg of 4-MP per kg, administered by gastric intubation. After 4 hr, the rats were anesthetized with ether and blood was collected from the aorta. Serum was obtained and frozen for analysis of ethanol, 4-MP, and 4-OH-MP.

Monkey Experiments. Young cynomolgus monkeys (*Macaca fascicularis*), weighing between 1.7 and 3 kg, were used. On the day prior to the experiment, the monkeys were isolated in individual cages and control urine collection was begun. On the morning of the experiment, control blood samples were drawn, and 4-MP was administered intramuscularly as a 2–10% solution (increasing concentration for larger doses), neutralized with NaOH. Methanol was administered as a 20% solution orally via a nasogastric tube. At various times throughout the experiments, lasting 2–4 days, blood samples (2 ml) were obtained by venipuncture from the femoral vein into heparinized syringes. The monkeys were allowed free movement within their cages and free access to food and water.

Table 1. Results of hematological and clinical chemical investigations of series I after 12 weeks of treatment

Variable	Group			
	C	E	EM	M
Fe, μ mol/liter	30 \pm 2	30 \pm 2*	31 \pm 2*	28 \pm 2*
HCRT, %	52 \pm 1	52 \pm 1*	50 \pm 1*	52 \pm 1*
Hb, g/liter	148 \pm 2	158 \pm 3†	152 \pm 4*	154 \pm 2†
WBC, 10 ⁹ /liter	16 \pm 3	15 \pm 1*	14 \pm 0*	16 \pm 1*
PC, 10 ⁹ /liter	1154 \pm 39	1053 \pm 55*	946 \pm 82†	1159 \pm 45*
ASAT, μ kat/liter	1.7 \pm 0.1	1.9 \pm 0.1*	1.6 \pm 0.1*	1.5 \pm 0.1†
ALAT, μ kat/liter	1.4 \pm 0	1.0 \pm 0.1*	1.5 \pm 0.1*	1.3 \pm 0.1*
Albumin, g/liter	41 \pm 1	42 \pm 0*	41 \pm 1*	42 \pm 1*
Bilirubin, μ mol/liter	3.2 \pm 0.1	3.4 \pm 0.2*	2.5 \pm 0.2†	2.8 \pm 0.2*
Urea, μ mol/liter	9.3 \pm 0.6	8.6 \pm 0.7*	9.4 \pm 0.9*	8.9 \pm 0.5*
Uric acid, μ mol/liter	124 \pm 8	151 \pm 13*	98 \pm 11*	121 \pm 13*
Creatinine, μ mol/liter	70 \pm 0	70 \pm 0*	70 \pm 0*	70 \pm 0*
Osmolality, mosM/kg	290 \pm 13	316 \pm 4†	336 \pm 5†	302 \pm 4*

Values are mean \pm SEM ($n = 6$). Variables: HCRT, hematocrit; Hb, hemoglobin; WBC, white cell count; PC, platelet count; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

* $P > 0.05$, † $P \leq 0.05$: significance of difference between different ethanol groups and control group as estimated by Student's *t*-test for unpaired data.

Table 2. Concentration of ethanol (EtOH) and 4-MP in groups E, EM, and M of series II at different time intervals during 38 weeks of treatment

Week	E,	EM		M,
	mM EtOH	mM EtOH	μ M 4-MP	μ M 4-MP
6	1.3	13.8*	31	0*
8	3.3	16.7*	8	0*
9	7.0	15.8*	13	0*
12	10.4	19.6†	36	3†
38	5.7	14.1	25	11†

In each group six rats were investigated. Values shown are mean ($n = 5$).

* $P \leq 0.05$, † $P > 0.05$: significance of difference between groups E and EM and groups EM and M as estimated by Student's *t*-test for unpaired data.

RESULTS

Rat experiments

The rats showed no obvious clinical symptoms during the long-term administration of 4-MP and ethanol. There were no signs of intoxication and their activity seemed unimpaired. The mean daily doses of 4-MP and ethanol were calculated for the last 3 weeks in series I. This gave an average daily intake of 0.14 mmol of 4-MP and 0.18 mol of ethanol per kg. The mean values for the weight gain in the different groups are illustrated in Fig. 1. During the last 3 weeks there was no significant difference among the different groups.

Hematocrit value and serum iron were within reference values of the control group. Hemoglobin was somewhat increased in groups E and M, and erythrocytes showed a slightly decreased concentration in group EM. Altogether, the hematological variables investigated indicated no damage of the bone marrow (Table 1). With regard to blood chemistry, aspartate and alanine aminotransferases and albumin all showed normal values. Bilirubin showed a slight decrease in group EM. The clinical chemical variables investigated, together with the electron microscopy, revealed no damage of liver function. Creatinine concentration was normal in all groups. This, together with normal urea concentration, normal electrolytes,

Table 3. Concentration of 4-MP, 4-OH-MP, and ethanol (EtOH) in serum of rats treated with 4-MP and ethanol (series III)

Compound administered	4-OH-MP, μ M	4-MP, μ M	EtOH, mM
Ethanol	—	—	28 \pm 1
4-MP	27 \pm 3	273 \pm 12	—
4-MP + ethanol	11 \pm 1	309 \pm 30	52 \pm 4

The rats were treated orally with 4-MP (50 mg/kg) or ethanol (4 g/kg) or both, and serum was collected 4 hr after the administration. Group mean value \pm SEM ($n = 5$).

and normal cell organelles (seen by electron microscopy) indicated that there was no damage of kidney function in any of the groups investigated.

The concentrations of ethanol and 4-MP in the long-term experiments at different time intervals are given in Table 2. 4-MP partially inhibited the oxidation of ethanol, leading to a significantly higher blood ethanol concentration than in the rats that drank only ethanol. There was also, however, a higher concentration of 4-MP in the blood of rats drinking both ethanol and 4-MP than in the blood of rats drinking only 4-MP in the tap water.

In Table 3 the results from an acute interaction experiment are given. Four hours after administration of 4-MP and ethanol to a group of rats, the concentration of 4-MP in serum was higher and the concentration of 4-OH-MP only about half the corresponding value in the rats receiving only 4-MP. The concentration of ethanol in serum of the rats receiving both ethanol and 4-MP was about twice that of the rats receiving only ethanol.

Monkey experiments

Metabolism of 4-MP. The data in Fig. 2 show that there was a linear disappearance of 4-MP from the blood after a single dose (50 mg/kg, intramuscularly) to two monkeys. 4-OH-MP accumulated to a level of about 11 μ M (only about 10% of the 4-MP level at the time) and then declined.

Effect of 4-MP on Methanol Metabolism. In contrast to the small interaction of methanol with 4-MP metabolism (see Fig. 3), 4-MP is capable of marked inhibition of methanol metab-

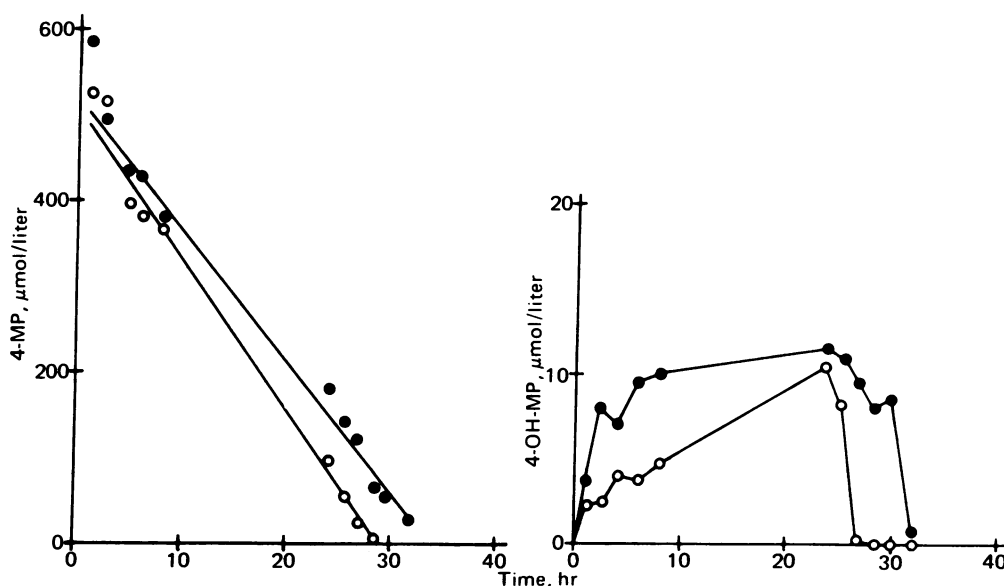


FIG. 2. 4-MP elimination and 4-OH-MP accumulation in the plasma of monkeys O (O) and N (●) after a single dose of 4-MP. 4-MP, in a dose of 50 mg/kg, was administered intramuscularly to each monkey.

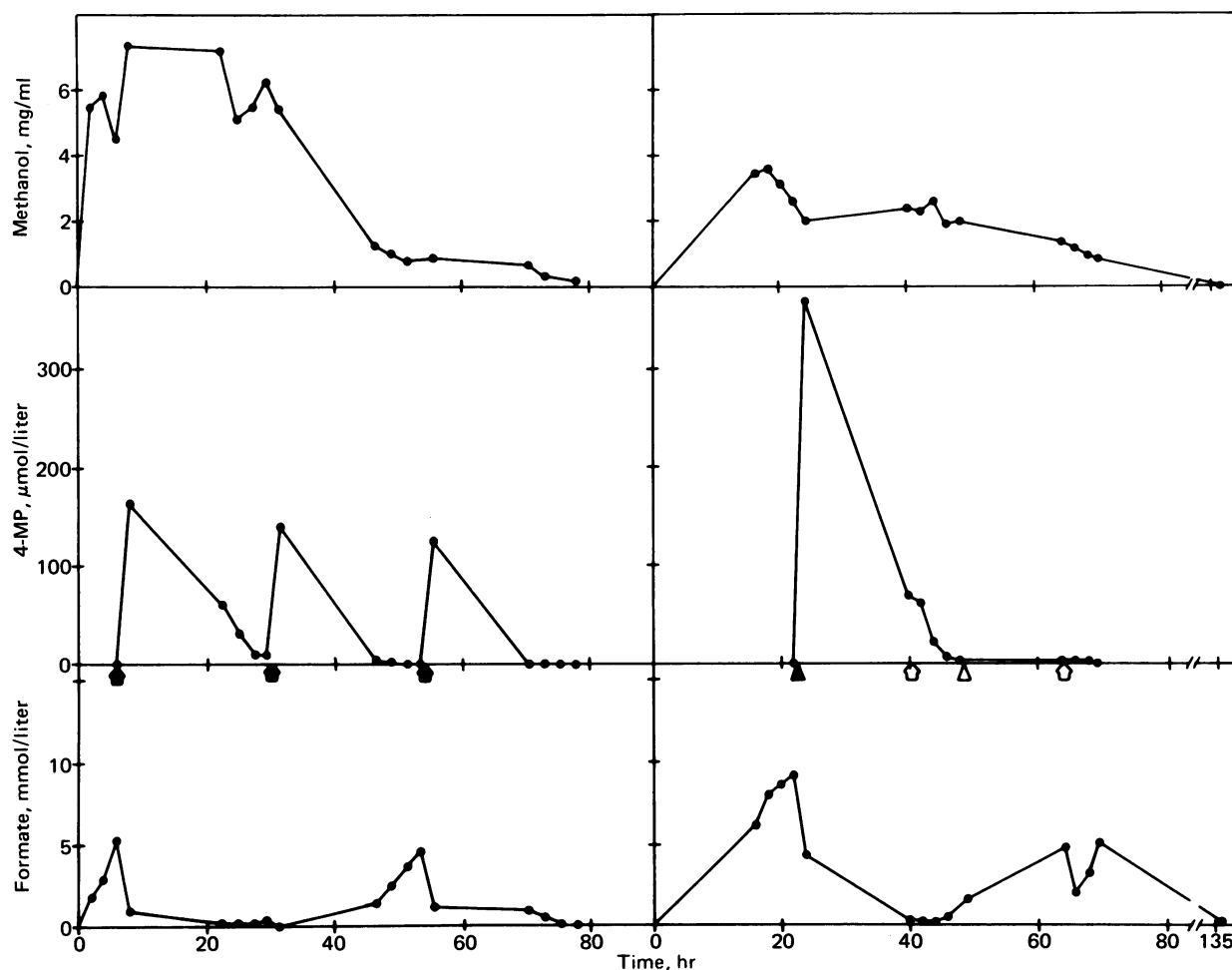


FIG. 3. Effects of 4-MP plasma level on the accumulation of formate in the blood of methanol-poisoned monkeys. Methanol was administered via nasogastric intubation to each monkey in a dose of 3 g/kg. (Left) Data from monkey C; (Right) data from monkey H. 4-MP was administered intramuscularly to monkeys C and H at the times indicated. Doses of 4-MP administered: solid arrows, 20 mg/kg; open arrows, 2.5 mg/kg; solid triangle, 15 mg/kg; and open triangle, 7.5 mg/kg.

olism. A dose of 50 mg/kg was shown to inhibit $^{14}\text{CO}_2$ production from ^{14}C methanol in the monkey by 75% (20). Monkeys C and H were two of a series of monkeys in which the effectiveness of 4-MP in reversing methanol toxicity was tested. The monkeys received 3 or 4 g of methanol per kg, which are potentially lethal doses for the cynomolgus monkey, and were allowed to develop signs of methanol toxicity. We observed an initial slight inebriation, then a latent period of no obvious distress, followed by vomiting, weakness, cold extremities, and general behavioral distress. Within a few hours after 4-MP injection, the monkeys showed recovery from the toxic syndrome; they were alert, strong, competitive, and hungry. Doses of 15 and 20 mg of 4-MP per kg in monkeys C and H were sufficient to rapidly decrease the formate levels to control and were effective for approximately 20 hr. After this initial dose in monkey H, 4-MP was administered twice a day, 2.5 mg/kg in the early morning and 7.5 mg/kg in the late afternoon (Fig. 3).

DISCUSSION

The use of 4-MP in extensive investigations of ethanol metabolism in humans or in treatment of methanol or ethylene glycol poisoning in humans is hindered by the toxicity of pyrazole. It is, however, necessary to stress the difference between these two compounds in their toxic potential. Pyrazole (9, 11) is highly toxic, inducing weight loss, liver necrosis, and cytotoxicity in other organs of rats and mice, especially when administered together

with ethanol (9, 21, 22). Toxicity was observed at doses necessary to inhibit ethanol metabolism *in vivo*. Toxicity has also been observed upon coadministration of pyrazole (100 mg/kg) and methanol in monkeys (23).

In contrast to pyrazole, 4-MP is a relatively nontoxic compound in rats and mice, with no evidence of toxic effects on variables of clinical chemistry or of gross and microscopic pathology at a dose of 100 mg/kg daily for 4 weeks (8) or 12 mg/kg daily for 12 weeks in combination with ethanol (12). In the present study, several clinical chemical variables indicated that neither 4-MP alone nor in combination with ethanol produced any liver damage (Table 1). Electron microscopy of the livers from the different groups after 38 weeks did not reveal any lesions on the cell organelles. Electron microscopy was also carried out on hearts and kidneys from rats in the different groups after 38 weeks. No pathological lesions were revealed on the different cell organelles from these organs. The serum creatinine and electrolytes from rats of the different groups were all in the normal range. The osmolality of the serum was increased in groups E and EM, but this is due to the increased concentration of ethanol in these groups. Kidney function can thus be considered normal in the present investigation.

Chronic administration of ethanol together with an effective inhibitor of ADH, such as 4-MP, should create a metabolic situation in which the metabolic effects produced by influences on the NADH/NAD ratio within cytosol and mitochondria are

less pronounced, albeit the effects of ethanol *per se* should be more pronounced through inhibition of the ADH system.

To further elucidate the mechanism of interaction between ethanol and 4-MP, we carried out an acute interaction study. From the results in Table 3 it is apparent that there was a diminished concentration of 4-OH-MP in the serum of rats receiving both ethanol and 4-MP. Qualitative studies of the metabolism of 4-MP in rats (24) have shown that the major urinary metabolites after administration of tritium-labeled 4-MP were 4-OH-MP, 4-carboxypyrazole, 4-MP-*N*-glucosiduronic acid, and unchanged 4-MP. It is thus possible, as earlier suggested, that ethanol interferes with different steps in the metabolism of 4-MP (25, 26). Ethanol inhibits the metabolism of several drugs, but mutual interactions, as in the present case, are not very common (27).

Although 4-OH-MP has been shown to be an inhibitor of both human and rat liver ADH *in vitro* (28), the relatively high K_i compared to that of 4-MP seems to preclude a significant contribution of 4-OH-MP to the inhibition of ethanol metabolism in these rats.

Even though the present study has shown that the chronic toxicity of 4-MP is minimal, the metabolic interactions observed in combination with ethanol must be taken into consideration in experimental investigations on the effects of ethanol.

The studies in monkeys indicated that a blood level of 4-MP of 9 μ M, but not 6 μ M, was sufficient to inhibit the rate of methanol metabolism so that formic acid did not accumulate in the blood. These levels correlated very well with the K_i of 9 μ M determined for 4-MP in methanol oxidation by monkey liver ADH *in vitro* (10). Because formate is the toxic agent in the acidosis and ocular toxicity of methanol poisoning in monkeys (20), the diminution of formate concentrations by these levels of 4-MP implies that methanol poisoning was averted by the inhibition of ADH activity. In some of these studies, methanol in a dose of 3 or 4 g/kg, considered lethal for monkeys (20), was given and 4-MP administration was delayed to allow methanol toxicity to develop. Previously (10, 20) 4-MP was used to counteract methanol toxicity in doses of 50 mg/kg, but this study indicates that a smaller dose would be sufficient and would avoid high peak blood levels of 4-MP and 4-OH-MP. The prevention of death and the lack of toxicity during the 2-week follow up confirmed earlier reports of the effectiveness of 4-MP in combating methanol poisoning (20, 29). The present results were, however, obtained with lower doses of 4-MP.

Methanol poisoning in humans can be a severe syndrome leading to blindness or death (30). The frequency of toxicity may increase in the near future if the use of methanol as an energy source becomes widespread (31). Thus, the need for refined treatment techniques may increase. Ethanol has become established as a basis for treatment of methanol poisoning (32) since it inhibits methanol oxidation as a competing substrate for ADH. However, the effective dose of ethanol is high, creating the possibility of interaction with methanol in producing central nervous system depression. Also, ethanol is metabolized rapidly, so its levels have to be carefully maintained. 4-MP would offer the attractive alternative of a competitive inhibitor of ADH with a long duration of action and little central nervous system depression at doses that the present study has shown to be successful in preventing the accumulation of formate and, hence, methanol toxicity in monkeys.

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