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EFFECT OF 4-METHYLPYRAZOLE AND PYRAZOLE ON THE INDUCTION OF FATTY LIVER BY A SINGLE DOSE OF ETHANOL

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Abstract—Pyrazole (272 mg/kg), 4-methylpyrazole (4-MP; 200 mg/kg) or saline was injected intraperitoneally into fasted male and female rats. Ten min later, ethanol (4 or 6 g/kg) or an equicaloric dose of sucrose was given by stomach tube. Hepatic triglyceride (TG) levels were measured at 6, 12 or 16 hr after the gavage. With a 4 g/kg dose of ethanol, pyrazole reduced the accumulation of TG at 6 hr in females, but not at 12 and 16 hr. In males, ethanol gave relatively little TG accumulation at 6 hr and pyrazole did not affect this, but at 16 hr the TG levels in the ethanol–pyrazole group had not risen as much as in the ethanol–saline group. In contrast to pyrazole, 4-MP by itself increased liver TG content, and significantly increased the TG accumulation caused by a 4 g/kg dose of ethanol in both males and females at 16 hr. However, 4-MP caused a significantly smaller TG accumulation in females at 6 hr after the ethanol, but not in males. With a larger dose of ethanol (6 g/kg), both pyrazole and 4-MP decreased the accumulation of TG at 16 hr in males. It is concluded that ethanol *per se*, ethanol as a metabolic substrate, and pyrazoles as pharmacological agents with complex actions may all contribute to the development of acute fatty liver. Therefore, pyrazole and 4-MP do not appear to be suitable tools for resolving the controversy about the mechanism of production of alcoholic fatty liver.

THE ADMINISTRATION of a single large dose of ethanol to well nourished rats is known to result in a significant increase in liver triglyceride concentration at times between 6 and 20 hr after the dose.[1] Since this does not occur in the absence of the hypophysis and adrenals,[2] it was first believed that the underlying mechanism was increased mobilization of fatty acids from adipose tissue to the liver, in response to the stress of intoxication. On the other hand, it has also been reported[3,4] that an acute dose of ethanol inhibits the oxidation of fatty acids in the liver even when there is no increase of mobilization. The available evidence has not yet resolved the relative importance of these two processes in the production of acute alcohol-induced fatty liver.[5]

One approach to this problem has been the use of pyrazole derivatives which inhibit the oxidation of ethanol by alcohol dehydrogenase.[6,7] This would be expected to prevent hepatic steatosis, if the latter is an indirect result of the metabolism of ethanol, but not if it is the result of pharmacological actions of ethanol *per se*. The results of such studies have appeared contradictory. Some investigators have reported complete prevention of the acute alcohol-induced fatty liver by pyrazole[8] or 4-methylpyrazole (4-MP),[9] while others found pyrazole to be without effect.[10] Johnson *et al.*[11] suggested that the discrepancy in results might depend on the time after alcohol administration: at 6 hr, pyrazole-treated animals showed less triglyceride (TG) accumulation than

those receiving ethanol alone, but at 16 hr there was no difference between the two groups. Domanski *et al.*[12] attributed the discrepancy to sex difference in the animals used: they found that pyrazole completely prevented the alcohol-induced TG accumulation in male rats, but was very much less effective in females. In contrast, Nordmann *et al.*[13] found the sex of the animals, the dose of pyrazole and the time interval between the administration of ethanol and pyrazole to be unimportant, whereas the dose of alcohol was critical. Pyrazole prevented the TG accumulation when the dose of ethanol was 6 g/kg, but not when it was 4 g/kg. They, therefore, concluded that hepatic TG accumulation at the lower alcohol dose did not depend on ethanol metabolism, while at the higher dose it did.

Unfortunately none of these explanations appears to resolve the discrepancy between the results of Morgan and DiLuzio[8] and Blomstrand and Forsell[9] and those of Bustos *et al.*[10] The explanation offered by Johnson *et al.*[11] is inconsistent with the fact that Morgan and DiLuzio and Blomstrand and Forsell measured TG at 20 hr after the ethanol. That of Domanski *et al.*[12] conflicts with DiLuzio's finding of identical results in males and females (personal communication). The interpretation offered by Nordmann *et al.*[13] is difficult to reconcile with the fact that the ethanol-oxidizing mechanism based on alcohol dehydrogenase (ADH) is saturated at doses much below 4 g/kg so that the increase to 6 g/kg could not affect the rate of ethanol metabolism.

We have, therefore, re-investigated the effect of both pyrazole and 4-MP on TG accumulation in the livers of both male and female rats at 6, 12 or 16 hr after ethanol.

MATERIALS AND METHODS

Female and male Wistar rats, obtained from High Oak Farms, were used in all experiments. In each experiment, the animals were distributed into four groups matched on the basis of sex and body weight. After a 16- to 17-hr fast, during which tap water was available at all times, one group (ES) received an intraperitoneal injection of saline, followed 10 min later by gavage with ethanol (4 or 6 g/kg) given as a 25% v/v solution in water. Controls (GS) received the injection of saline, followed by intubation with glucose solution, equal in volume and caloric value to the ethanol solution given to the ES group. The two remaining groups (EMP and GMP) received the same doses of ethanol and glucose, respectively, 10 min after the intraperitoneal injection of 4-MP (200 mg/kg, as a 1% solution in saline). The latter dose is the same as that used by Blomstrand and Forsell.[9] In other experiments, the 4-MP was replaced by pyrazole (272 mg/kg, as a 1·7% solution in saline); the groups are then designated EP and GP.

At 6, 12 or 16 hr after the administration of ethanol or glucose, the animals were decapitated. The abdomen was opened and the livers were rapidly removed, blotted on filter paper and homogenized in phosphate buffer, 0·066 M, pH 7·0. Triglycerides were extracted[14] and measured colorimetrically.[15] In one such experiment with 4-methylpyrazole, capillary blood samples of 0·05 ml were taken from the tip of the tail at 2, 4, 8, 12, 16 and 20 hr after intubation, from each rat receiving ethanol, for measurement of ethanol by gas–liquid chromatography.[16]

RESULTS

In the control rats, there was an almost linear fall of blood alcohol level between

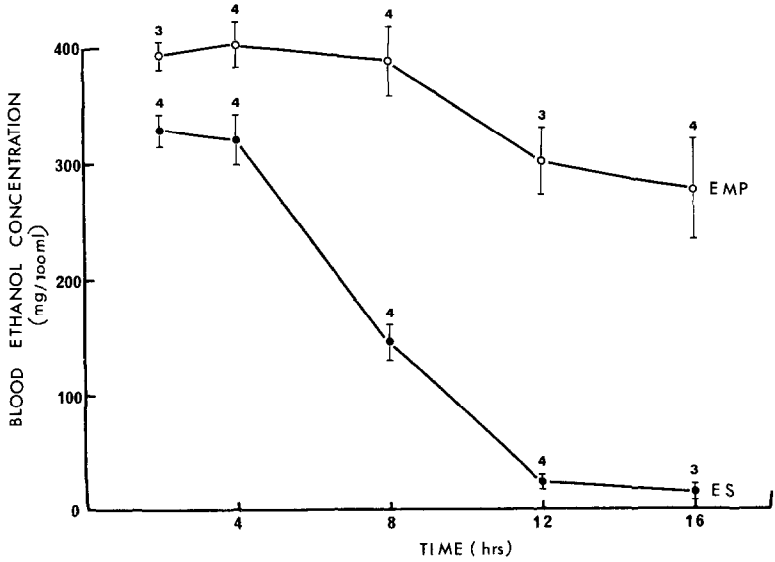

FIG. 1. Mean blood ethanol concentrations in the ethanol + 4-methylpyrazole (EMP; ○) and ethanol + saline (ES; ●) groups. Vertical lines indicate standard errors. Number of animals for each point shown on top of the vertical lines.

2 and 12 hr, at a mean hourly rate of about 30·5 mg/100 ml (Fig. 1). The 200 mg/kg dose of 4-MP inhibited ethanol metabolism almost completely for the first 8 hr; at later times the blood ethanol level declined at a mean hourly rate of approximately 11 mg/100 ml, or approximately one-third the control rate. In earlier work,[10] we found that pyrazole, in the same dose used here, inhibited alcohol disappearance virtually completely over a period of 16 hr. The difference between the duration of effect of pyrazole and 4-MP is consistent with the shorter half-life of 4-MP in com-

TABLE 1. HEPATIC TG CONCENTRATIONS IN RATS AFTER ADMINISTRATION OF ETHANOL (4 g/kg) IN THE PRESENCE AND ABSENCE OF 4-METHYLPYRAZOLE AND PYRAZOLE*

	Time (hr)	Hepatic TG (mg/g liver)			
4-Methyl-pyrazole experiments		EMP	ES	GMP	GS
Females	6	24·90 ± 1·40(15)	28·62 ± 2·19(15)	14·38 ± 1·06(15)	11·80 ± 0·75(15)
	12	29·66 ± 2·58(8)	25·60 ± 1·25(8)	14·58 ± 1·47(8)	10·63 ± 0·45(8)
	16	32·42 ± 2·95(17)	20·79 ± 1·86(17)	12·92 ± 0·63(17)	10·71 ± 1·02(12)
Males	6	11·65 ± 0·78(11)	10·04 ± 0·89(12)	7·80 ± 0·71(12)	6·89 ± 0·65(12)
	16	18·11 ± 1·52(31)	12·49 ± 0·63(32)	9·92 ± 0·49(32)	8·49 ± 0·25(31)
Pyrazole experiments		EP	ES	GP	GS
Females	6	22·05 ± 1·31(8)	30·68 ± 2·46(8)	11·21 ± 0·54(8)	9·51 ± 0·79(8)
	12	28·61 ± 1·34(8)	32·38 ± 2·16(8)	12·36 ± 0·72(8)	11·54 ± 1·36(8)
	16	35·20 ± 4·15(7)	34·00 ± 2·38(8)	15·30 ± 1·9(8)	12·83 ± 1·17(8)
Males	6	11·69 ± 0·48(7)	10·18 ± 0·58(8)	7·80 ± 0·44(8)	7·58 ± 0·45(8)
	16	12·11 ± 0·40(17)	15·00 ± 1·03(17)	9·52 ± 0·23(17)	9·19 ± 0·26(17)

* Values are expressed as mean ± S. E. M., with number of animals per group in parentheses. Doses as described in text.

parison to pyrazole.[17] The degree of inhibition produced by pyrazole is also consistent with the dose–response curve for pyrazole obtained by Deitrich *et al.*[18]

The hepatic TG concentrations at different times after administration of ethanol (4 g/kg) or isocaloric glucose in male and female rats treated with pyrazole and 4-MP are presented in Table 1. The results were subjected to analysis of variance for a factorial design involving proportionate subclasses, using logarithms of the TG levels to correct for non-homogeneity of variances among the subclasses. In each case, the ES, EP and EMP groups showed highly significant increases in the liver TG concentration, when compared with the corresponding GS, GP and GMP controls, at all times tested. These observations confirm the well known fact that ethanol by itself, in this dosage, increases hepatic TG content.

An unexpected finding was that 4-MP by itself increases TG content also. The values for the GMP group exceeded those of the GS group significantly in the females at all times tested ($P < 0.025$, 0.02 and 0.005 at 6, 12 and 16 hr, respectively) and in the males at 16 hr ($P < 0.005$). The value appeared to be greater in the males at 6 hr also, but because of the smaller value of N, the difference was not statistically significant. In contrast, pyrazole by itself did not increase TG concentrations; the values for the GP group did not differ significantly from those of the GS group in either sex, at any of the times tested.

The combination of ethanol with pyrazole or 4-MP produced variable results, depending on the sex and the time studied. With 4-MP, in the female rats, the alcohol effect was reduced at 6 hr, i.e. the value of ES–GS was greater than that of EMP–GMP ($P < 0.005$). By 12 hr, there was no longer a difference between the alcohol effects with and without 4-MP. By 16 hr, the value of EMP–GMP was actually significantly greater than that of ES–GS ($P < 0.05$). The TG level in the female EMP group at 16 hr was also significantly greater than that of the ES group ($P < 0.001$). In the males, the alcohol effect at 6 hr did not differ, regardless of the presence or absence of 4-MP. But at 16 hr the value of EMP–GMP was clearly greater than that of ES–GS, i.e. the alcohol effect was increased in the presence of 4-MP ($P < 0.005$).

With pyrazole, the alcohol effect in the females was diminished at 6 hr ($P < 0.001$); by 12 hr the difference was no longer significant and by 16 hr the value of ES–GS was essentially the same as that of EP–GP. In males, the situation was exactly the reverse, pyrazole apparently not influencing the ethanol effect at 6 hr, but reducing it at 16 hr ($P < 0.01$).

The hepatic TG concentrations at 16 hr after administration of a larger dose of alcohol (6 g/kg) or isocaloric glucose, in male rats receiving 4-MP or pyrazole, are shown in Table 2. Again, pyrazole by itself did not increase TG concentration, but the value is higher in the GMP than in the GS group ($P < 0.05$). With the larger dose of alcohol, both 4-MP and pyrazole reduced the alcohol effect ($P < 0.001$ and $P < 0.02$, respectively), i.e. the difference between ES and GS was greater than the corresponding difference between the other two groups. It is noteworthy that the effect of pyrazole in this respect was greater, and that of 4-MP even reversed, when compared with their effects in the experiments with the 4 g/kg dose of ethanol. This finding is consistent with the observations of Nordmann *et al.*[13]

DISCUSSION

Despite the fact that pyrazole and 4-MP are both highly effective inhibitors of

TABLE 2. HEPATIC TG CONCENTRATIONS AT 16 HR IN MALE RATS AFTER ADMINISTRATION OF ETHANOL (6 g/kg) IN THE PRESENCE AND ABSENCE OF 4-METHYLPYRAZOLE AND PYRAZOLE*

4-Methylpyrazole experiment		Pyrazole experiment	
Group	Hepatic TG (mg/g liver)	Group	Hepatic TG (mg/g liver)
EMP	12·58 ± 0·98(4)	EP	12·35 ± 1·51(3)
ES	18·30 ± 1·14(5)	ES	19·55 ± 1·48(4)
GMP	10·77 ± 0·77(3)	GP	8·39 ± 0·50(4)
GS	8·13 ± 0·81(5)	GS	8·47 ± 0·58(4)

* Results are expressed as mean ± S. E. M., with number of animals per group in parentheses; 4-methylpyrazole and pyrazole doses as described in text.

alcohol dehydrogenase, they cannot be considered fully equivalent, because the present findings show clearly that 4-MP by itself tends to raise hepatic triglyceride levels. Further, the duration of action of 4-MP is considerably shorter than that of pyrazole, as shown by the fact that some fall in blood alcohol concentration began at 8 hr in the present study (Fig. 1) while a dose of pyrazole, which was smaller than that of 4-MP relative to their respective K_i values, virtually prevented alcohol disappearance for at least 16 hr in our earlier study.[10] This finding is in satisfactory agreement with the known difference in the rate of metabolism of pyrazole and 4-MP.

These facts help to reconcile many of the apparently contradictory or conflicting findings in the literature. The reduction of alcohol metabolism by 4-MP was virtually complete during the first 8 hr (Fig. 1), and during this time the ethanol effect on TG accumulation was reduced in the female rats (Table 1, 6 hr). By 12 hr, the inhibitory effect of the 4-MP was wearing off, and some ethanol metabolism was occurring; this is consistent with increased TG accumulation in both male and female rats at the later times, superimposed upon the previous direct effect of 4-MP. The lack of an inhibitory effect of 4-MP on TG accumulation in the males at 6 hr may merely reflect a slower process of lipid turnover in the males, with a considerably smaller ethanol effect even in the saline controls at that time, so that any change produced by the 4-MP was not significant.

In contrast, the inhibitory effect of pyrazole on ethanol oxidation continued throughout the 16-hr period, as shown in previous work.[10] The reduction by pyrazole of the ethanol-induced TG accumulation was, therefore, evident in the females at 6 hr, and in the males at 16 hr, consistent with the postulated slower lipid turnover in the males. The fact that the TG level in the EP group of females at 16 hr did not exceed that in the ES group presumably reflects a "ceiling" effect on TG levels, together with the absence of a direct effect of pyrazole comparable to that of 4-MP. All of the above interpretation is consistent with the observed time differences in the course of hepatic TG accumulation in males and females described by Johnson *et al.*,[11] and with the contribution of ethanol metabolism to the acute fatty liver as proposed by Morgan and DiLuzio,[8] Blomstrand and Forsell[9] and Johnson *et al.*[11]

In contrast, the greater effect of pyrazole on TG accumulation produced by a high dose of alcohol (6 g/kg) than with the smaller dose (4 g/kg) is not explainable on the basis of inhibition of ethanol metabolism. It is well known that the rate of oxidation of ethanol by ADH is essentially zero order at all blood ethanol concentrations above

approximately 25 mg/100 ml.[5] On the other hand, pyrazole has been shown to have a definite CNS depressant effect of its own, which is synergistic with the actions of ethanol.[19–21] The animals which received pyrazole plus the high dose of alcohol were profoundly depressed. It seems highly probable that in such a condition there was a marked reduction in blood flow through peripheral adipose tissue and through the viscera, which could well have contributed significantly to the reduced TG accumulation in the liver.

The data of Blomstrand and Forsell,[9] in studies with 4-MP and ethanol doses ranging from 4·5 to 8·0 g/kg, point clearly to an alcohol dose-dependent effect of 4-MP on TG accumulation, although the authors themselves failed to mention this point. Further, Morgan and DiLuzio[8] also used a 6 g/kg dose of ethanol rather than the 4 g/kg used in our work. Nordmann *et al.*[13] called attention to the difference in pyrazole effects at ethanol doses of 4 vs 6 g/kg, and showed that the ethanol-induced decrease of NAD/NADH ratio was inhibited by pyrazole at both alcohol doses. They were, therefore, not justified in concluding that the TG accumulation was due to ethanol *per se* at the lower dose, but to the metabolism of ethanol at the higher dose.

The present findings suggest that the TG accumulation due to ethanol, and the effect of pyrazole derivatives upon it, are the resultant of at least three separate actions: (1) an accumulation of TG due to some action of ethanol *per se*, as distinct from the consequences of ethanol metabolism, which is represented by that portion of the TG accumulation that was resistant to the effects of pyrazole or 4-MP, especially at 6 hr when the metabolism of ethanol was blocked almost completely; (2) accumulation of TG as a consequence of disturbances in intermediary metabolism produced by the metabolism of ethanol itself, equivalent to that portion of the TG accumulation which was sensitive to pyrazole or 4-MP, especially in the female rats; and (3) accumulation of TG due to the influence of 4-MP itself, in both sexes. Because of this complexity of actions, the pyrazole derivatives are probably not good pharmacological tools for resolving problems concerning the mechanism of hepatic metabolic effects produced by alcohol, either acutely or chronically.

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