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# Methanol Potentiation of Carbon Tetrachloride-Induced Hepatotoxicity (40624)

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It has been established that aliphatic alcohols are capable of potentiating $CCl_4$ hepatotoxicity (1–5). The degree of potentiation has been shown to vary considerably from one aliphatic alcohol to another. Traiger and Plaa (5) showed this variability in mice, using serum glutamic-pyruvate transaminase (SGPT) as an index of toxicity. Pretreatment with methanol, ethanol, isopropanol, *n*-propanol, *n*-butanol, and 2-methyl-2-pentanol showed that only isopropyl, ethyl, and methyl alcohols were significant potentiators in mice. Of the three, isopropanol showed the greatest potentiation, approximately, 7-fold greater than methanol and 20-fold greater than ethanol.

Studies of alcohol potentiation of $CCl_4$ hepatotoxicity have primarily investigated the effects of ethanol; however, additional studies have been concerned with isopropanol. Little information is available concerning methanol potentiation of $CCl_4$ hepatotoxicity. To date, methanol potentiation of $CCl_4$-induced hepatotoxicity has not been investigated *in vivo* in the rat. In addition, the only comparative potentiation study with methanol and other alcohols has been in mice. Similarly, few studies have investigated the mechanism by which alcohols potentiate $CCl_4$-induced hepatotoxicity. Attempts to uncover the mechanism of ethanol and isopropanol potentiation have been made, but the specific site of action is yet unknown (6). The toxicity of $CCl_4$ is dependent on its metabolism and probably involves the resultant trichloromethyl free radical which causes lipid peroxidation and/or covalent binding to cellular macromolecules.

Oral pretreatment of rats with ethanol, methanol, and isopropanol has been found to alter hepatic microsomal metabolism of foreign compounds (7). However, all three alcohols do not cause the same alterations. Ethanol, methanol, and isopropanol all increased aniline hydroxylation, but only isopropanol increased aminopyrine demethylation. Ethanol and methanol inhibited aminopyrine demethylation.

The time course of potentiation of $CCl_4$ hepatotoxicity has been studied with ethanol and isopropanol in rats (5). Maximum potentiation of toxicity occurred when rats were pretreated 18–24 hr prior to $CCl_4$ administration. A similar time-course study has not yet been performed with methanol.

The purpose of the present investigation was to examine the time course of methanol potentiation of $CCl_4$-induced hepatotoxicity in rats. Additionally, the relative magnitude of methanol potentiation in rats was compared to that observed with ethanol and isopropanol. In an attempt to determine the mechanism of methanol potentiation, the effect of methanol pretreatment on $CCl_4$-induced lipid peroxidation and on the irreversible binding of $^{14}CCl_4$ to hepatic microsomal protein and extracted lipids was also investigated.

*Materials and methods. General.* Male, Sprague–Dawley rats, weighing 200–250 g, were used throughout the study. Before and during the experiment, the animals were maintained on an *ad libitum* diet of commercial chow and water.

Analytical grades of methanol, isopropanol, and ethanol were orally administered at doses approximately equal to ½ of the respective $LD_{50}$ (7.0, 2.5, and 6.0 ml/kg, respectively). These values were derived from similar studies by Cornish and Adefuin (4) and Traiger and Plaa (5). Analytical grade carbon tetrachloride was injected intraperitoneally, dissolved in a corn oil vehicle. Orally administered saline (10 ml/kg) was used as control for alcohol pretreatments. Corn oil (10 ml/kg i.p.) was used as control for $CCl_4$.

*Methanol potentiation time-course study.* Methanol was given orally at various times prior to $CCl_4$ injection (0.1 ml/kg i.p.). Animals were anesthetized with ether 24 hr later



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and blood samples were obtained from the dorsal aorta with a syringe rinsed in heparin. Alanine aminotransferase (ALT or SGPT) was measured in plasma by the method of Reitman and Frankel (8).

*Comparative hepatotoxicity study.* Methanol, isopropanol, and ethanol were given orally 24 hr prior to injection of various doses of $CCl_4$. Twenty-four hours after $CCl_4$ injection, rats were anesthetized with ether and blood was taken for ALT activity measurement as described above. Two pieces of liver were then obtained, each weighing approximately 1 g. One section was then used to determine hepatic triglyceride levels by the method of Van Handel and Zilversmit (9), as modified by Butler *et al.* (10). The other section of liver was used to measure hepatic glucose-6-phosphatase activity by the method described by Klaassen and Plaa (11) except a 2.5% liver homogenate was used and samples were incubated for 30 min.

*Microsomal fraction isolation.* Liver from saline- or methanol-pretreated rats was homogenized in 4 vol/g liver of Tris–KCl–EDTA buffer (20 m*M*–1.15%–3.0 m*M*), pH 7.4. The homogenate was centrifuged at 10,000*g* for 30 min in a Sorvall Model RC2-B centrifuge at 4°. The supernatant was removed and centrifuged at 100,000*g* for 65 min at 4° in a Beckman Model L ultracentrifuge yielding a microsomal-rich pellet.

*Irreversible binding of $^{14}CCl_4$ to microsomal protein and lipid.* The microsomal fraction obtained as previously described was resuspended in 4 vol of the same Tris–KCl–EDTA buffer, resulting in a protein concentration of 2–4 mg/ml. The homogenate was incubated under nitrogen at 37° in a total of 8 ml of incubation mixture containing a NADPH regenerating system consisting of the following reagents final concentrations: 2.5 m*M* nicotinamide, isocitrate dehydrogenase (0.02 units/ml), 0.10 m*M* NADP, 20 m*M* DL-isocitrate, and 5.0 m*M* $MgCl_2$. This system is similar to that described by Glende *et al.* (12). Prior to sealing the reaction flasks with airtight caps, the flask and its contents were flushed with nitrogen for 4 min. Radioactive carbon tetrachloride ($^{14}CCl_4$), specific activity 4.7 mCi/mmole (New England Nuclear, Boston, Mass.), was diluted in absolute ethanol and nonradioactive $CCl_4$ such that when 27 $\mu$l of the $CCl_4$–ethanol mixture was injected into the reaction flask, a total of 830 nmole of both radioactive and nonradioactive $CCl_4$ was introduced. Final specific activity of $CCl_4$ was 1.5 mCi/mmole.

Irreversible binding of $^{14}C$ to protein was determined by drawing 1 ml of the incubation mixture into a syringe after 6 min of incubation and immediately injecting the sample into 1 ml of 10% trichloracetic acid. The samples were placed into a boiling water bath for 10 min. After centrifugation at 3000*g* for 15 min and removal of the supernatant, the pellet was resuspended in 5 ml of methanol: ether (3:1), heated to 60° for 10 min, and then centrifuged as before. The resulting supernatant was then removed by aspiration. This washing procedure was repeated six times to assure that any unbound radiolabeled $CCl_4$ was removed. After the final wash, the remaining protein was dissolved in 1 ml of 1 *N* NaOH and the radioactivity determined by liquid scintillation counting. This procedure is similar to that described by Sipes *et al.* (13).

Irreversible binding of $^{14}CCl_4$ to hepatic microsomal lipids after 6 min incubation was determined by removing 1 ml of incubation mixture and injecting it into 9 ml of chloroform:methanol (2:1). The mixture was shaken for 5 min and then centrifuged at 3000*g* for 10 min. The resulting supernatant was then transferred to a graduated tube and a final volume of 10 ml was obtained by adding the chloroform:methanol (2:1). Two milliliters of distilled water was added and after thorough mixing and centrifugation at 3000 *g* for 10 min, the upper phase was aspirated off and discarded. The remaining lower phase was washed twice with 2 ml of upper phase obtained by mixing 25 ml of distilled water and 95 ml of chloroform:methanol (2:1). The washed lower phase was then transferred to a scintillation vial and evaporated under nitrogen at 40°. This method is similar to that described by Castro and Diaz Gomez (14). Radioactivity was determined by liquid scintillation counting. Protein concentrations of microsomal homogenates were determined by the method of Lowry *et al.* (15). The results are expressed as nanomoles $^{14}CCl_4$ irreversibly bound to microsomal lipid or protein per milligram of microsomal protein.

*$CCl_4$-Induced lipid peroxidation.* Rats were

pretreated with either methanol or saline 24 hr prior to $CCl_4$ injection. Exactly 30 min after $CCl_4$ administration, the animals were sacrificed and microsomes were obtained as described above. The determination of lipid peroxidation by measuring diene conjugation has been described by Klaassen and Plaa (11). In this study, however, 1 g of liver was homogenized in 3 vol of buffer. Results are expressed as OD units (243 nm), from extracted microsomal lipids obtained from 1 g of liver.

*Statistics.* Statistical analysis was performed using analysis of variance and Student's *t* test when analysis of variance demonstrated statistical differences (16). The level of statistical significance was chosen as $P < 0.05$.

*Results.* Figure 1 shows alanine aminotransferase activity (ALT) in plasma of rats pretreated with methanol at various times prior to $CCl_4$ injection (0.1 ml/kg). Administration of either methanol or 0.1 ml/kg $CCl_4$ did not produce a significant increase in plasma ALT activity. However, a significant increase in ALT was observed 24 hr following 1.0 ml/kg $CCl_4$ (insert Fig. 1). The potentiation appeared to be maximal when $CCl_4$ was given 48 hr after methanol. However, maximum variability also occurred at this time. It should be noted that when rats were pretreated with methanol, 0.1 ml/kg $CCl_4$ produced a greater elevation of ALT than when a dose of $CCl_4$ 10 times higher was given to control rats.

The effect of pretreatment with methanol, isopropanol, and ethanol on $CCl_4$ hepatotoxicity assessed by plasma ALT, hepatic accumulation of triglycerides, and hepatic glucose-6-phosphatase activity is shown in Fig. 2. The top panel in the figure depicts the plasma ALT in rats pretreated with the alcohols 24 hr before receiving various doses of $CCl_4$. In saline-pretreated rats, ALT values were not significantly altered at any dose of $CCl_4$ administered. Similarly, treatment with

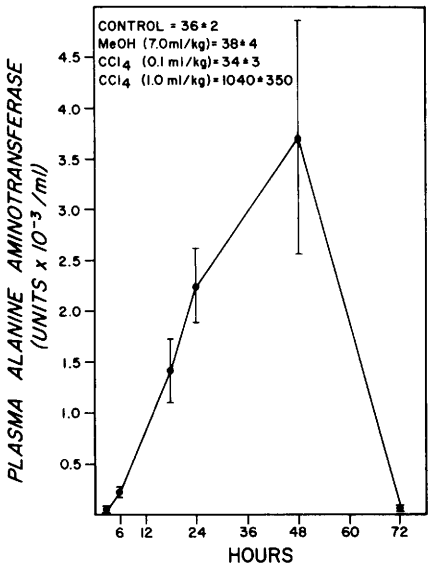


FIG. 1. Plasma ALT in rats pretreated with methanol (7.0 ml/kg p.o.) 24 hr after receiving $CCl_4$ (0.1 ml/kg i.p.). The abscissa indicates the time interval between methanol pretreatment and $CCl_4$ administration. The insert indicates the plasma ALT in control rats, and in rats 24 hr after the described treatments.

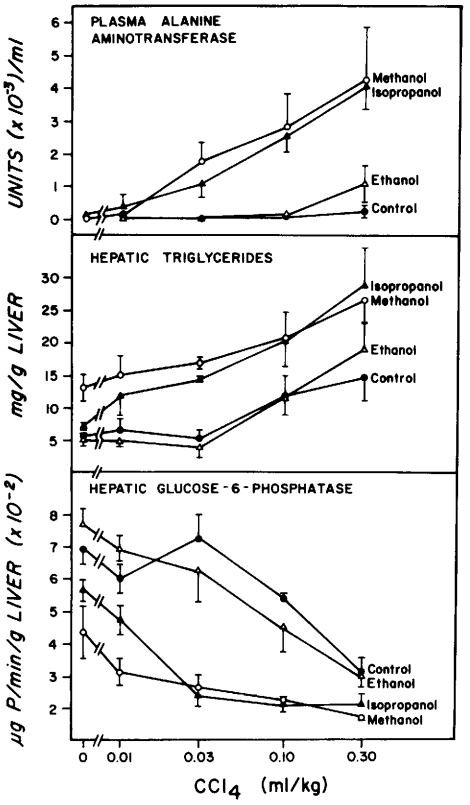


FIG. 2. Assessment of $CCl_4$ hepatotoxicity using plasma ALT, hepatic triglycerides, and hepatic glucose-6-phosphatase in rats pretreated with methanol (7.0 ml/kg p.o.), isopropanol (2.5 ml/kg p.o.), and ethanol (6.0 ml/kg p.o.). Assessment was made 24 hr after the indicated doses of $CCl_4$.

any one of the three alcohols alone did not affect plasma ALT activity. Ethanol pretreatment produced an increase of ALT with the highest dose of $CCl_4$, but this was not statistically significant. In contrast to ethanol, both methanol and isopropanol markedly potentiated the $CCl_4$-induced increase in plasma ALT. Significant increases were observed with $CCl_4$ doses as low as 0.03 ml/kg with both alcohols. This marked potentiation by both methanol and isopropanol was further demonstrated when two of four animals pretreated with either alcohol died as a result of a subsequent 1.0 ml/kg dose of $CCl_4$ while all control rats tested at this dose survived. At all doses of $CCl_4$ used, methanol and isopropanol potentiated the increase in plasma ALT to a similar extent.

The middle panel of Fig. 2 shows the results of a similar study using hepatic accumulation of triglycerides as an index of toxicity. Control rats (those rats not pretreated with alcohols) showed increasing triglyceride levels with increasing doses of $CCl_4$; however, this increase was significant only at the highest dose of $CCl_4$ used. Ethanol did not significantly potentiate the $CCl_4$-induced increase in hepatic triglycerides. Isopropanol pretreatment substantially potentiated $CCl_4$ hepatotoxicity with doses of $CCl_4$ as low as 0.03 ml/kg. Pretreatment with methanol also potentiated $CCl_4$ hepatotoxicity as indicated by increasing the levels of hepatic triglycerides to approximately the same extent as did isopropanol. However, methanol itself produced a significant increase in hepatic triglycerides whereas isopropanol did not (Fig. 2).

The effect of pretreatment with these alcohols on the $CCl_4$ dose related decrease in hepatic glucose-6-phosphatase activity (G6P) is shown in the bottom panel of Fig. 2. $CCl_4$ itself (control) at the two higher doses (0.10 and 0.30 ml/kg) significantly reduced G6P activity. Ethanol tended to potentiate this effect but it was not statistically significant. Both methanol and isopropanol potentiated the decrease in G6P and the degree of potentiation once again appears to be similar. The potentiation with isopropanol was statistically significant with $CCl_4$ doses as low as 0.03 ml/kg. Methanol administration alone caused a significant decrease in G6P activity whereas isopropanol did not.

To determine whether the hepatotoxicity of methanol (reduced G6P activity and increased triglycerides) was present at the time of $CCl_4$ administration, the three parameters just discussed were measured in rats 24 hr after they received methanol. The following results were obtained: Plasma ALT activity was 36 ± 6 units/ml; hepatic triglyceride levels were 5.6 ± 0.1 mg/g liver; and hepatic glucose-6-phosphatase activity was 648 ± 58 μg P/min/g liver. These values are the same as control values.

The effect of methanol pretreatment on $CCl_4$-induced lipid peroxidation *in vivo* is shown in Table I. Pretreatment with methanol, treatment with $CCl_4$ (0.10 ml/kg), or both methanol pretreatment and $CCl_4$ (0.10 ml/kg) did not produce a significant increase in the amount of conjugated dienes. Only injection of $CCl_4$ at 10 times the previously used dose (1.0 ml/kg) produced an increase in the presence of conjugated dienes. Thus, methanol pretreatment did not significantly potentiate the amount of $CCl_4$-induced lipid peroxidation at doses that markedly potentiated the toxicity.

The effect of pretreatment with methanol on the amount of $^{14}C$ irreversibly bound to microsomal protein and lipid after incubation with $^{14}CCl_4$ is shown in Table II. Pretreatment with methanol significantly increased the amount of binding of $^{14}C$ to both microsomal protein and microsomal lipid.

*Discussion.* The ability of methanol to potentiate $CCl_4$-induced hepatotoxicity in the rat has been established in this study. These findings confirm similar findings in mice (5). However, quantitative considerations

TABLE I. EFFECT OF METHANOL ON $CCl_4$-INDUCED LIPID PEROXIDATION

| Treatment[a] | Conjugated dienes[b] |
|---|---|
| Saline–Corn oil | 0.213 ± 0.028 |
| Methanol–Corn oil | 0.235 ± 0.048 |
| Saline–$CCl_4$ (0.10 ml/kg) | 0.258 ± 0.052 |
| Methanol–$CCl_4$ (0.10 ml/kg) | 0.273 ± 0.016 |
| Saline–$CCl_4$ (1.0 ml/kg) | 0.365 ± 0.041* |

[a] Six rats were given saline or methanol (7.0 ml/kg) 24 hr prior to $CCl_4$ (i.p.) or corn oil.

[b] Expressed as OD units (243 λ) ± SE of a lipid extract obtained from microsomes from 1 g of liver 30 min after $CCl_4$ or corn oil injection.

* Significantly different from saline–corn oil ($P < 0.05$).

TABLE II. EFFECT OF METHANOL ON THE IRREVERSIBLE BINDING OF $^{14}CCl_4$ TO MICROSOMAL PROTEIN AND LIPID *IN VITRO*[a]

| Pretreatment | nmole $CCl_4$ bound to protein / microsomal protein (mg) | nmole $CCl_4$ bound to lipid / microsomal protein (mg) |
|---|---|---|
| Saline | 0.33 ± 0.07 | 2.36 ± 0.65 |
| Methanol | 0.57 ± 0.06* | 4.08 ± 0.69* |

[a] Microsomes were obtained from rats pretreated with either saline or methanol 24 hr earlier as described in the methods section. Values represent mean ± SE.

* Significantly different from saline pretreatment ($P < 0.05$).

strongly suggests a significant species difference in susceptibility to potentiating agents. Mice were found to be clearly more sensitive to isopropanol than to methanol. The present study demonstrates that the rat is equally sensitive to methanol and isopropanol as potentiators of $CCl_4$ hepatotoxicity. Studies have shown that the potentiation of $CCl_4$ hepatoxicity by isopropanol is caused by acetone, a metabolite. Whether methanol or a metabolite of methanol is responsible for the potentiation is uncertain. It is unlikely that the potentiation by methanol is due to a decrease in food consumption because even 48-hr fasting only slightly enhances $CCl_4$ hepatotoxicity (22).

Ethanol has been shown to potentiate $CCl_4$ hepatotoxicity in both rats and mice (5, 17). In the present study ethanol did not significantly potentiate $CCl_4$ hepatotoxicity. One possible reason for the discrepancy is that in our study a standard time interval of 24 hr between alcohol and $CCl_4$ was used and the time course for ethanol potentiation performed by Traiger and Plaa (5) showed that ethanol significantly potentiated only when given less than 24 hr before $CCl_4$. Additionally, Maling *et al.* (22) showed that repeated administration of ethanol was required to produce maximum effect.

Methanol treatment alone caused significant alteration in hepatic triglyceride levels and glucose-6-phosphatase activity but not plasma ALT activity (Fig. 2). This suggests that methanol itself has some deleterious effects on the liver. Thus, one might suspect that the enhancement of $CCl_4$ hepatotoxicity is merely due to the additive effects of methanol and $CCl_4$ on the liver. However, this is unlikely since the effect of methanol plus $CCl_4$ on plasma ALT is greater than additive, indicative of a potentiation phenomenon (Fig. 1).

The mechanism of $CCl_4$ hepatotoxicity involves the formation of a reactive metabolite, probably trichloromethyl free radical, which is capable of covalently binding to cellular macromolecules (18) and reacting with polyenoic fatty acids causing lipid peroxidation (19). Sipes *et al.* (13), using various halogenated methane derivatives, found that the potency of the hepatotoxin was directly related to the degree of irreversible binding to microsomal protein. Pretreatment of rats with isopropanol was found to increase the irreversible binding of $CCl_4$ to liver lipids and proteins *in vivo* (20) and *in vitro* (21). In the present study methanol pretreatment significantly increased the amount of $^{14}C$ irreversibly bound *in vitro* after incubation with $^{14}CCl_4$. This suggests that the two alcohols are potentiating the hepatotoxicity by increasing the irreversible binding of $CCl_4$. An increase in $CCl_4$ covalent binding may be the result of an increased metabolism of $CCl_4$. Powis (7) has demonstrated that methanol, isopropanol, and ethanol can cause alterations in hepatic microsomal metabolism of foreign compounds in the rat.

Methanol pretreatment did not significantly increase $CCl_4$-induced lipid peroxidation. Similar studies with isopropanol produced conflicting results. Maling *et al.* (22) showed that pretreatment with isopropanol increased $CCl_4$-induced lipid peroxidation. Lindstrom and Anders (23) showed that isopropanol pretreatment produced only a transient increase in $CCl_4$-induced lipid peroxidation. They, therefore, concluded that lipid peroxidation may not be associated with the increase in $CCl_4$ toxicity observed after acute administration of ethanol or isopropanol. Results from the present study would agree in that it appears that covalent binding is more closely associated with the increased $CCl_4$ hepatotoxicity caused by methanol than is lipid peroxidation.

*Summary.* The potentiation of $CCl_4$-induced hepatotoxicity resulting from oral administration of methanol has been investigated in the rat. Results from the study demonstrate that methanol potentiates $CCl_4$

hepatotoxicity to the same extent as that observed with isopropanol. It appears that the mechanism by which these two alcohols potentiate is similar in that they both increase covalent binding of $CCl_4$ to microsomal proteins and lipids. However, the precise mechanism of potentiation remains as yet undetermined.


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