Enhanced Hepatic Microsomal Activity by Pretreatment of Rats with Acetone or Isopropanol (36996)

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It is generally accepted that carbon tetrachloride (CCl_4) must be converted to an active intermediate by the liver microsomal system to produce its hepatotoxic effects (1, 2, 3). This active metabolite can then react with tissue macromolecules and alter the integrity of the cell (2, 4).

Cornish and Adefuin (5) have shown that the acute inhalation toxicity of CCl_4 was potentiated by several aliphatic alcohols. Recently, Traiger and Plaa (6) reported that of a series of alcohols, isopropanol was the most effective in potentiating the elevated levels of serum glutamic-pyruvic transaminase and liver triglycerides and the decreased liver glucose-6-phosphatase activity caused by CCl_4. This potentiation did not occur immediately, but reached a peak when isopropanol was administered 16 to 18 hr before CCl_4. Since the administration of acetone also potentiates the toxicity caused by CCl_4, it is possible that the potentiating effects of isopropanol may be mediated by this metabolite (7).

Recently, this laboratory (8, 9) reported that $^{14}CCl_4$ is covalently bound to protein in an enzyme preparation containing liver microsomes and a NADPH-generating system. The purpose of the present study was to compare the effects of isopropanol and acetone pretreatment on the conversion of CCl_4 to its active intermediate by liver microsomes with other indices of the drug metabolizing system—namely cytochrome P-450 levels and cytochrome *c* reductase activity. The effects of pretreatment with isopropanol or acetone on the metabolism of two other hepatotoxins, chloroform ($CHCl_3$) and dimethylnitrosamine (DMN), were also studied.

Methods. Male Sprague-Dawley rats (180–235 g) received orally 2.5 ml/kg of acetone or isopropanol as a 25% solution in water and were sacrificed at specified time intervals. Livers were homogenized in cold 0.02 *M* Tris-1.15% KCl buffer (pH 7.4) and the microsomal fraction was obtained by differential centrifugation (10). The isolated microsomes were then resuspended in Tris-KCl and adjusted to desired protein concentration as measured by the method of Gornall *et al.* (11).

The amounts of cytochrome P-450 were determined as described by Omura and Sato (12) and the NADPH-cytochrome *c* reductase activity was measured according to Phillips and Langdon (13).

The formation of an active intermediate was studied by following the covalent binding of the intermediate to microsomal protein. The covalent binding of CCl_4 or $CHCl_3$ to protein was determined by a method recently developed in our laboratory (9) which consists of incubating ^{14}C-labeled CCl_4 or $CHCl_3$ with rat liver microsomes and a NADPH-generating system. The final incubation mixture (1 ml) consisted of rat liver microsomes (2 mg protein), dialyzed 105,000*g* liver soluble fractions (3 mg), 0.124 m*M* NADH, 2 m*M* nicotinamide, 0.2 m*M* NADP, 2 m*M* glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase and 1 m*M* of $^{14}CCl_4$ or $^{14}CHCl_3$ (sp act 1 μCi/μmole). The mixture was incubated with shaking in a closed vial for 10 min at 37° and then 1 ml of 10% TCA was added to stop the enzymatic reaction and precipitate protein. The sample was then centrifuged, and the resulting pellet resuspended in 5 ml of methanol:ether (3:1), heated at 60° for 10 min and then centrifuged. The washing procedure with the metha-

nol:ether mixture was repeated 10 times to remove radioactivity that was not covalently bound to protein. After aspiration of the final wash the protein pellet was dissolved in 1 ml of 1.0 *N* NaOH. Aliquots of the resulting solution were then removed for determination of covalently bound radioactivity and of protein by the method of Lowry (14). When denatured microsomes were used there was no covalent binding of $^{14}CCl_4$ or $^{14}CHCl_3$. After pronase hydrolysis of the protein containing covalently bound $^{14}CCl_4$, amino acid analysis indicated the presence of one amino acid peak containing 80% of the radioactivity.

The metabolism of ethylmorphine and dimethylnitrosamine (DMN) was determined in a 3.0-ml incubation mixture consisting of 5 m*M* $MgCl_2$, 12 m*M* glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 0.33 m*M* NADP, 50 m*M* Tris-KCl buffer (pH 7.4), 10 mg of microsomal protein and 5 m*M* ethylmorphine or 10 m*M* DMN. The mixture was incubated at 37° for 10 min with shaking, after which the reactions were terminated with 1 ml 8.9% $ZnSO_4$. The degree of N-demethylation was estimated by measuring the amount of formaldehyde formed according to the method of Nash (15).

Student's *t* test was used to evaluate the significance of differences between treated animals and the corresponding controls.

Results. Figure 1 summarizes the temporal effects of the oral pretreatment of rats with acetone and isopropanol on various indices related to the metabolic activity of liver microsomes. The covalent binding of $^{14}CCl_4$ to protein was increased 3 to 4 times when microsomes were isolated 16 to 24 hr after pretreatment with either acetone or isopropanol. A similar 3 to 4-fold increase in the microsomal N-demethylation of DMN was observed at 16 and 24 hr after acetone or isopropanol pretreatment. Since there were no significant changes in either of these enhanced activities at 1, 2, 4 or 40 hr it appears that they reach their maximum level between 4 and 16 hr after pretreatment, remain high through 24 hr, and then decrease with time. No apparent changes occurred in the microsomal content of cytochrome P-450 or cytochrome *c* reductase when

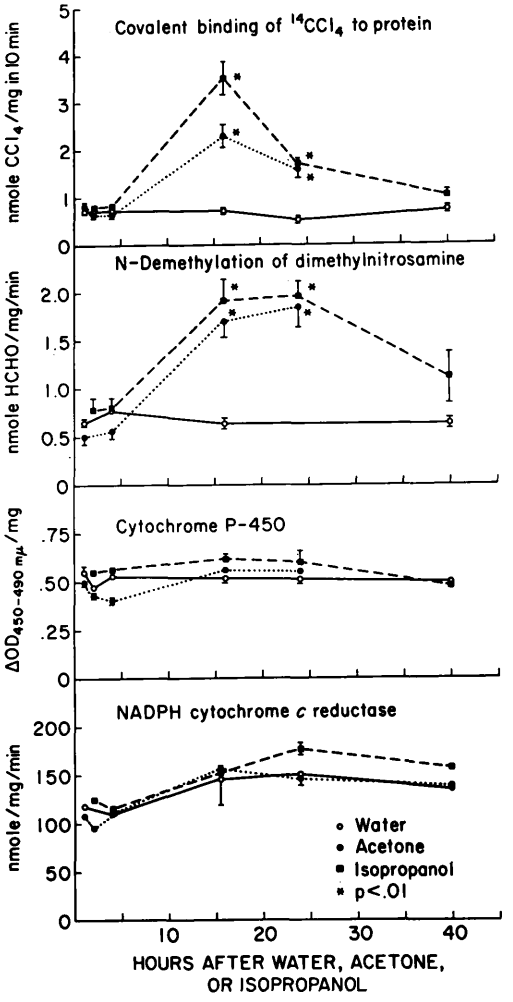

FIG. 1. Temporal effects of pretreatment of rats with acetone or isopropanol (2.5 ml/kg, orally in a 25% aqueous solution) on various indices related to hepatic microsomal function. Values are expressed as mean ± S.E. for 4 rats/group.

compared with the corresponding control values at any of the time intervals following administration of acetone or isopropanol.

The above results were confirmed in a subsequent experiment in which microsomes were prepared at 4 and 18 hr after administration of acetone or isopropanol. The covalent binding of $^{14}CCl_4$ and demethylation of DMN were enhanced to a similar extent at 18 hr, but were not significantly different from control at 4 hr. Cytochrome P-450 levels and NADPH cytochrome *c* reductase activity

TABLE I. The Effects of Pretreatment of Rats with Acetone or Isopropanol (2.5 ml/kg Orally in a 25% Aqueous Solution) on the Covalent Binding of $^{14}CHCl_3$ to Protein and the N-demethylation of Ethylmorphine.

	Hours after pretreatment	Water	Acetone	Isopropanol
Covalent binding of $^{14}CHCl_3$ to protein in picomoles/mg protein in 10 min	4	129 ± 12	240 ± 18	297 ± 26
	18	305 ± 5	637 ± 28[a]	581 ± 38[a]
N-demethylation of ethylmorphine in nmoles HCHO/mg protein/min	4	13.1 ± 1.7	12.9 ± 0.66	16.5 ± 1.2
	18	21.6 ± 1.7	19.2 ± 2.6	17.4 ± 1.4

Values are mean of 4 rats ± S.E.

[a] $p < 0.01$ when compared with 18-hr water pretreatment.

were unchanged at either time. In addition, the pretreatment with acetone or isopropanol produced a twofold increase in the covalent binding of $^{14}CHCl_3$ at 18 hr, but did not enhance the N-demethylation of ethylmorphine (Table I). No significant changes were observed at 4 hr.

No enhancement of covalent binding of $^{14}CCl_4$ or $^{14}CHCl_3$ occurred when acetone or isopropanol was added to the incubation media containing microsomes isolated from untreated animals (Table II). In fact, both acetone and isopropanol inhibited the covalent binding. A similar 20–50% inhibition by *in vitro* addition of acetone or isopropanol was observed in the N-demethylation of DMN.

Discussion. The mechanism by which acetone and isopropanol stimulates the covalent binding of $^{14}CCl_4$ and $^{14}CHCl_3$ and the metabolism of DMN remains unclear. The lack of any apparent increase during the first 4 hr or following *in vitro* addition suggests that an enhancement of enzymatic activity may occur as a result of an *in vitro* activation of the microsomal enzyme systems. However, this stimulation of drug metabolism appears to differ from that produced by the classical inducers of drug metabolism, such as phenobarbital or 3-methylcholanthrene (3-MC). Since acetone and isopropanol pretreatment produced no significant changes in the levels of microsomal protein or cytochrome P-450, in the activity of NADPH cytochrome *c* reductase or in the N-demethylation of ethylmorphine, these agents may be different from the phenobarbital type inducers of drug metabolism (16, 17). The lack of any change in cytochrome P-450 is not consistent with the type of induction produced after pretreatment with 3-MC (17). Pretreatment of rats with 3-MC reduces the rate of demethylation of DMN (18), decreases the covalent binding of $^{14}CCl_4$ to microsomal protein (Sipes, unpublished results) and blocks hepatic necrosis induced by CCl_4 (19). On the other hand, pretreatment with acetone or

TABLE II. Effect of the *In Vitro* Addition of Acetone or Isopropanol on the Covalent Binding of $^{14}CCl_4$ and $^{14}CHCl_3$ to Protein.

	Concentration	Picomoles/mg protein in 10 min $^{14}CCl_4$	$^{14}CHCl_3$
Acetone	0.00 *M*	576	107
	0.10 *M*	522	87
	0.43 *M*	566	83
	1.29 *M*	258	43
Isopropanol	0.00 *M*	576	107
	0.10 *M*	329	83
	0.43 *M*	382	74
	1.29 *M*	344	71

isopropanol enhances the rate of N-demethylation of DMN, increases the covalent binding of $^{14}CCl_4$ and potentiates the hepatotoxicity induced by CCl_4. Thus, the effects of pretreatment with acetone or isopropanol are opposite from the effects of 3-MC.

The increased covalent binding of $^{14}CCl_4$ and $^{14}CHCl_3$ metabolites and the N-demethylation of DMN do not appear to be caused by a direct action of either acetone or isopropanol on the liver endoplasmic reticulum. With either compound, the increase was not present 4 hr after administration, at which time the levels of acetone and isopropanol should have reached their maximum values. Moreover, acetone added to the incubation mixtures inhibited the covalent binding of $^{14}CCl_4$ and $^{14}CHCl_3$ metabolites. Thus, the stimulatory effects differ from the direct effects of acetone in increasing aniline hydroxylation as observed by Anders (20).

Whatever the mechanism of stimulation by acetone or isopropanol may be, it is noteworthy that these substances enhance not only the toxicity of CCl_4 as reported by Traiger and Plaa (6, 7) and others (5, 21, 22), but also enhance the covalent binding of $^{14}CCl_4$ to proteins and lipids *in vivo* (Maling and Saul, unpublished results). These findings are in agreement with those of Reynold (2) that there is a strong cause–effect relationship between the covalent binding of $^{14}CCl_4$ metabolites and CCl_4-induced toxicity. Further studies of the mechanism by which acetone or isopropanol enhances the covalent binding of $^{14}CCl_4$ to macromolecules may greatly aid in the understanding of CCl_4-induced hepatoxicity.

Summary. Rat liver microsomes isolated from rats pretreated orally with acetone or isopropanol possessed an enhanced capacity to bind $^{14}CCl_4$ and $^{14}CHCl_3$ covalently and to demethylate DMN. The N-demethylation of ethylmorphine was unchanged by this pretreatment. Since there was no increase in the level of microsomal protein, and microsomal cytochrome P-450 or the activity of NADPH-cytochrome *c* reductase, the mechanism of this enhanced metabolism remains unclear.

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