ETHANOL POTENTIATION OF CARBON TETRACHLORIDE HEPATOTOXICITY: POSSIBLE ROLE FOR THE *IN VIVO* INHIBITION OF ALDEHYDE DEHYDROGENASE

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Abstract—1. A potentiation of CCl_4-induced hepatotoxicity was observed in rats pretreated with ethanol 18 hr prior to CCl_4 exposure.

2. Hepatic microsomal aldehyde dehydrogenase (ALDH) was significantly inhibited in animals sacrificed 1 hr following the sequential exposure, however, no more so than in those animals receiving CCl_4 alone.

3. The animals receiving ethanol alone had ALDH activity similar to vehicle treated controls.

4. Twenty-four hours following a potentiating dose of ethanol and CCl_4 an 81 and 57% decline in NAD^+-dependent microsomal and mitochondrial ALDH activity was observed, respectively.

5. Similar results were observed for microsomal and mitochondrial $NADP^+$-dependent ALDH activity.

6. The decline in membrane-bound ALDH was greater in potentiated animals than in those receiving CCl_4 alone.

7. A relatively smaller decline in cytosolic ALDH activity was observed in CCl_4 treated rats with or without ethanol pre-exposure.

8. The data suggest that inhibition of membrane bound ALDH may be one of the major mechanisms of *in vivo* potentiation of CCl_4-induced hepatotoxicity by ethanol.

INTRODUCTION

The ability of aliphatic alcohols to potentiate carbon tetrachloride (CCl_4) hepatotoxicity in experimental animals is well established (Cornish and Adefuin, 1967; Traiger and Plaa, 1971, 1974; Traiger and Bruckner, 1976; Cantillena et al., 1979). For ethanol, maximum potentiation occurs in rats when alcohol administration (5 g/kg p.o.) precedes the CCl_4 treatment by 18 hr (Traiger and Plaa, 1971). Despite numerous attempts to determine the mechanism of alcohol potentiation, the knowledge of specific potentiating events remain unknown.

Early investigations examined different alcohols or their metabolites as potentiators of CCl_4 hepatotoxicity (Traiger and Plaa, 1971, 1973; Traiger and Bruckner, 1976; Cantillena et al., 1979; Hewitt and Plaa, 1979; Dietz and Traiger, 1979; Curtis and Mehendale, 1980). Based on the observations that (a) pyrazole enhanced potentiation of ethanol-CCl_4 hepatotoxicity (Traiger and Plaa, 1971), (b) ethanol concentrations in blood of CCl_4-treated rats following an oral potentiating dose of ethanol were virtually undetectable (Traiger and Plaa, 1972) and (c) acetaldehyde administered intraperitoneally, 18 hr prior to CCl_4 failed to potentiate CCl_4 hepatotoxicity

(Traiger and Plaa, 1973), it was suggested that acetaldehyde does not play a significant role in ethanol-CCl_4 interactions. In contrast to these observations Strubelt et al. (1974) reported that 16 hr following an oral dose of 5 g/kg of ethanol, rat blood levels of ethanol were approximately 75 and 25% of peak blood levels in fed and fasted rats, respectively. Similarly, Sato et al. (1981) reported that 18 hr following 5 g/kg ethanol p.o. in the rat, relatively large amounts of ethanol were found in the blood (0.8–3.9 mM) and in the liver (1.1–4.8 mM). Therefore, the possibility exists that acetaldehyde is still being generated 18 hr after ethanol treatment. Acetaldehyde was also shown to accumulate in mice receiving CCl_4 and a subsequent dose of ethanol (Hjelle and Petersen, 1981). CCl_4-dependent inhibition of hepatic cytosolic and mitochondrial aldehyde dehydrogenase (ALDH) was implicated to explain these observations. However, the possible inhibition of microsomal ALDH was not considered.

Besides acetaldehyde, the production of several other cytotoxic aldehydes during *in vivo* ethanol/acetaldehyde- or CCl_4-stimulated lipid peroxidation is well established (Ugazio et al., 1976; Valenzuela et al., 1980; Stege, 1982; Muller and Sies, 1982; Benedetti et al., 1980, 1982, 1984). These aldehydes are hepatotoxicants (Lieber et al., 1981; Bird et al., 1982; Benedetti et al., 1980, 1982, 1984), act as growth inhibitors (Chio and Tappel, 1969; Lieber, 1980; Dianzani, 1982) and impair the regenerating capacity of the liver. Since endoplasmic reticulum not only represents a major site of CCl_4 activation (Recknagel, 1983), but also significantly contributes to the overall

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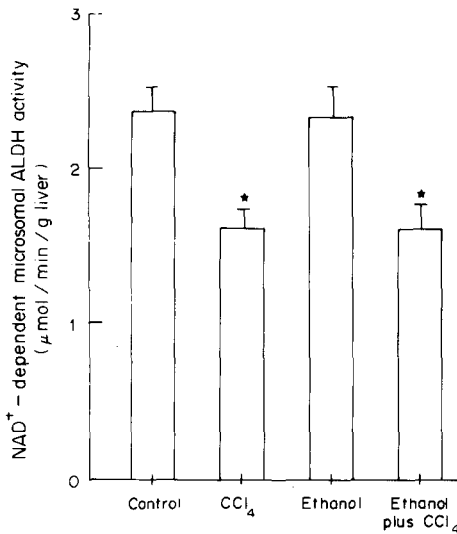
Fig. 1. Effect of CCl_4, ethanol and ethanol plus CCl_4 pretreatment on the rat hepatic NAD^+-dependent microsomal ALDH activity. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl_4 alone (once, 1 ml/kg, i.p.) and killed 1 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl_4 (1 ml/kg, i.p.) and killed 1 hr later. Control rats received vehicle only. Values represent mean $\pm$ SEM for 4 rats. *Statistically significant ($P \leqslant 0.05$) from appropriate control. **Statistically significant ($P \pm 0.05$) from CCl_4 treatment alone.

Animals were anesthetized with CO_2 while blood was collected from the posterior vena cava for SGPT determination. Blood acetaldehyde levels were obtained with slight modification of the procedure described by Anderson *et al.* (1981). Blood sample (3 ml) obtained by a heart puncture was placed in a 500 ml Erlenmeyer flask containing 0.1 M thiourea (12 ml) dissolved in 0.1 N HCl. One milliliter of 0.15 M semicarbazide in 0.2 M sodium phosphate buffer, pH 7.0 was placed in a 10 ml beaker. The semicarbazide containing beaker was lowered into the head space of the Erlenmeyer flask, and the latter was then sealed. After heating at 40°C for 18 hr, 0.45 ml of the acetaldehyde-semicarbazide adduct was sealed in a 25 ml reaction flask containing 100 μl of concentrated HCl. At this time 100 μl of a 1:10,000 dilution of isopropanol was added as an internal standard. The reaction flask was heated at 40° for 90 min. A 5 ml head space sample was analyzed by gas chromatography for acetaldehyde content using a (6 ft × 0.4 mm i.d.) column containing Poropak Q. The Varian model 3700 gas chromatograph equipped with a flame ionization detector was operated under following conditions: carrier gas 20 ml/min; inlet, column and detector temperature were 190°, 150°, and 190°C, respectively.

The washed liver subcellular fractions were prepared and assayed for total ALDH as described previously (Kenel and Kulkarni, 1985). The specific activity was corrected for protein recovery and is expressed as mol/min/g liver. SGPT assays were performed according to the manufacturer's instructions. Protein was determined according to the Biuret method as described by Gornall *et al.* (1949). Statistical analysis consisted of analysis of variance followed by Duncan's multiple range test. Significance was assumed at $P \leqslant 0.05$.

hepatic acetaldehyde and other toxic aldehyde generation and detoxication (Tottmar *et al.* 1973; Horton and Barrett, 1975), an examination of microsomal ALDH during CCl_4 metabolism is viewed important. Our *in vitro* experiments reported earlier (Kenel and Kulkarni, 1985) indicated that rat hepatic microsomal and mitochondrial ALDH inhibition occurs concomitantly with CCl_4 metabolism. In this paper, we present evidence for significant inhibition of membrane-bound ALDH activity in ethanol and CCl_4 exposed rats.

MATERIALS AND METHODS

Amytal, pyrazole, deoxycholic acid, $NADP^+$, NADPH, NAD^+ NADH, semicarbazide, and the reagents for the determination of serum glutamate pyruvate transaminase (SGPT) were obtained from Sigma Chemical Company, St Louis, MO. Thiourea was purchased from Alfa products, Danvers, Mass. Reagent grade acetaldehyde was obtained from Matheson, Coleman, and Bell of Norwood, Ohio. CCl_4 was a Fisher product. One hundred percent ethanol was obtained from Aaper Alcohol and Chemical Co., Louisville, KY.

Male 200–250 g Sprague–Dawley rats (Charles River, Portage, MI) were housed two per cage and allowed free access to Purina Rat Chow and water. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl_4 alone (once, 1 ml/kg, i.p.) and killed 1 or 24 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl_4 (1 ml/kg, i.p.) and killed 1 or 24 hr later. Both control and treated animals were fasted for 8 hr before the ethanol treatment to eliminate differences in food consumption. Ethanol and CCl_4 were administered as 50% solutions in distilled water and corn oil, respectively. Control rats received equal volumes of vehicle only.

RESULTS

The effects of *in vivo* ethanol-CCl_4 administration to rats on *in vitro* NAD^+-dependent liver microsomal ALDH are shown in Fig. 1. An 18 hr exposure to ethanol alone did not significantly alter ALDH activity while CCl_4 treatment after 1 hr resulted in a 37% ($P \leqslant 0.05$) loss. The animals receiving both treatments showed a 34% decline in ALDH activity which was not statistically different from CCl_4 treatment alone. The blood levels of acetaldehyde, 1 hr following CCl_4 exposure in ethanol pretreated animals (Table 1) were not significantly elevated over ethanol alone treated animals.

Figures 2–4 show the effects of *in vivo* ethanol-CCl_4 exposure on *in vitro* microsomal, mitochondrial, and cytosolic ALDH activities respectively, 24 hr following CCl_4 treatment. NAD^+-dependent microsomal ALDH (Fig. 2) was significantly decreased (52%) in animals exposed to CCl_4 alone. Ethanol alone also caused an 11% loss in activity. Hepatotoxicity of CCl_4 was potentiated by ethanol as indicated by SGPT values (Fig. 5), and in these animals an 81% decline in microsomal NAD^+-dependent ALDH was

Table 1. Blood acetaldehyde levels in CCl_4 and ethanol treated rats

| | Blood acetaldehyde (nmol/ml) | | |
Vehicle Control	CCl_4 alone	Ethanol alone	Ethanol + CCl_4
23 ± 6.3	21 ± 4.4	35 ± 5.2	37 ± 6.0

Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl_4 alone (once, 1 ml/kg, i.p.) and killed 1 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl_4 (1 ml/kg, i.p.) and killed 1 hr later. Control rats received vehicle only. Each value represents mean ± SEM for 4 rats.
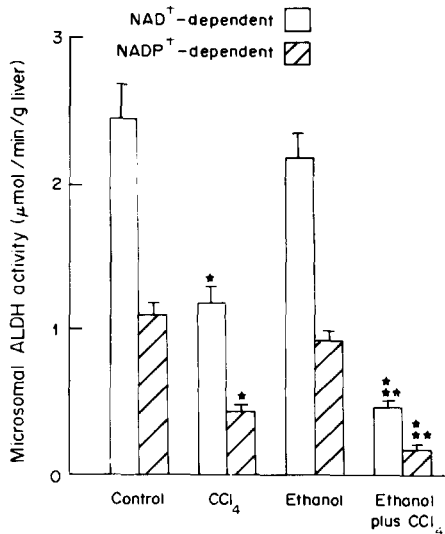
Fig. 2. Effect of CCl₄, ethanol and ethanol plus CCl₄ pretreatment on the rat hepatic microsomal ALDH activity. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl₄ alone (once, 1 ml/kg, i.p.) and killed 24 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl₄ (1 ml/kg, i.p.) and killed 24 hr later. Control rats received vehicle only. Values represent the mean ± SEM for 4 rats. *Statistically significant ($P < 0.05$) from appropriate control. **Statistically significant ($P \leqslant 0.05$) from CCl₄ treatment alone.
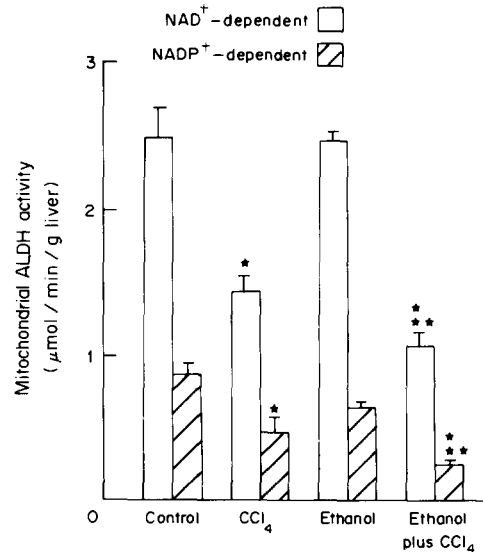

Fig. 3. Effect of CCl₄, ethanol and ethanol plus CCl₄ pretreatment on the rat hepatic mitochondrial ALDH activity. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl₄ alone (once, 1 ml/kg, i.p.) and killed 24 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl₄ (1 ml/kg, i.p.) and killed 24 hr later. Control rats received vehicle only. Values represent the mean ± SEM for 4 rats. *Statistically significant ($P \leqslant 0.05$) from appropriate control. **Statistically significant ($P \leqslant 0.05$) from CCl₄ treatment alone.

observed (Fig. 2). The NAD⁺-dependent mitochondrial ALDH (Fig. 3) declined 42% in CCl₄ treated rats but remained essentially unchanged in ethanol treated animals. In ethanol + CCl₄ treated rats the observed decline of 57% in NAD⁺-dependent mitochondrial ALDH was significantly different from CCl₄ alone treated animals. Finally, ethanol treatment alone resulted in a non-significant decline (5%) in NAD⁺-dependent cytosolic ALDH (Fig. 4). In ethanol-CCl₄ treated animals the observed 48% decline in NAD⁺-dependent cytosolic ALDH, as compared to rats receiving corn oil only, was not significantly different from the decline of 40% due to CCl₄ alone.

Essentially similar trends in inhibition of microsomal, mitochondrial, and cytosolic ALDH were observed for the NADP⁺-dependent activity (Figs 2, 3 and 4).

DISCUSSION

With dismissal of the recently advanced hypothesis based on alterations in cell calcium homeostasis (Recknagel, 1983), the question of which biochemical mechanism is responsible for CCl₄ hepatotoxicity remains unanswered. Earlier our laboratory demonstrated that within 5 min the *in vitro* metabolism of CCl₄ leads to a significant inhibition of rat microsmal and mitochondrial ALDH activity (Kenel and Kulkarni, 1985). Furthermore, literature reports have shown that lipid peroxidation initiated by CCl₄ (Rao and Recknagel, 1968) and covalent binding of ¹⁴C from *in vivo* administered ¹⁴CCl₄ (Rao and Recknagel, 1969) are at or near maximum within 15 min

of dosing. Therefore, an objective of this study was to determine if *in vivo* CCl₄ exposure results in an inhibition of ALDH. It was proposed (Kenel and Kulkarni, 1985) that as a consequence, acetaldehyde
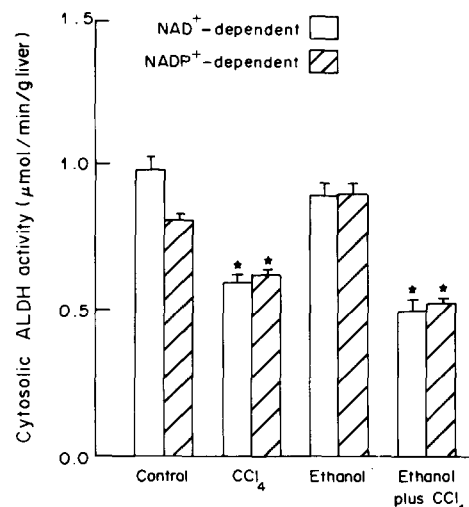

Fig. 4. Effect of CCl₄, ethanol and ethanol plus CCl₄ pretreatment on the rat hepatic cytosolic ALDH activity. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl₄ alone (once, 1 ml/kg, i.p.) and killed 24 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl₄ (1 ml/kg, i.p.) and killed 24 hr later. Control rats received vehicle only. Values represent the mean SE for 4 rats. *Statistically significant ($P \leqslant 0.05$) from appropriate control. **Statistically significant ($P \leqslant 0.05$) from CCl₄ treatment alone.
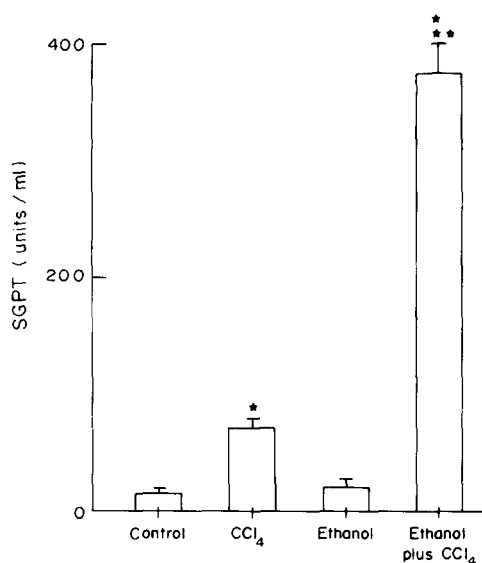
Fig. 5. Effect of CCl_4, ethanol and ethanol plus CCl_4 pretreatment on the rat hepatic serum SGPT values. Rats were treated with either ethanol alone (once, 5 g/kg, p.o.) and killed 18 hr later, or CCl_4 alone (once, 1 ml/kg, i.p.) and killed 24 hr later, or ethanol (5 g/kg, p.o.) followed 18 hr later by CCl_4 (1 ml/kg, i.p.) and killed 24 hr later. Control rats received vehicle only. Values represent the mean ± SEM for 4 rats. *Statistically significant ($P \leqslant 0.05$) from appropriate control. **Statistically significant ($P \leqslant 0.05$) from CCl_4 treatment alone.

and other cytotoxic aldehydes would accumulate in the hepatocytes and this may serve as a conceivable mechanism of ethanol potentiation of CCl_4 hepatotoxicity. If the ALDH inhibition hypothesis is valid then inhibition should occur not only shortly after CCl_4 poisoning, but be persistent and cumulative at the time of maximum hepatotoxicity. Therefore, ALDH activity was evaluated at 1 and 24 hr following CCl_4 expsosure in ethanol pretreated rats.

Microsomal ALDH was studied because, if an early cellular effect was to occur, it would most likely occur at the well accepted site of CCl_4 metabolism, the endoplasmic reticulum. Our data in Figure 1 show that 1 hr following CCl_4 treatment there is a significant decline in microsomal ALDH activity. This suggests that at this stage of hepatotoxicity the observed inhibitory effects of CCl_4 on ALDH are probably due to the biochemical events rather than the results of hepatocyte necrosis. In an earlier study on the time course of the CCl_4-induced decrease in mitochondrial ALDH, Hjelle et al. (1983) reported no significant inhibition 2 hr after exposure. However, a maximum depression of about 56% in ALDH activity was observed by 6 hr. The possible effects on the microsomal ALDH were not considered. In this study, a sequential exposure to ethanol and CCl_4 was found to result in a significant inhibition of microsomal ALDH. However, the magnitude of inhibition was nearly equal to that observed in rats exposed to CCl_4 alone (Fig. 1). This may be related to the protection rendered by various defense systems. At this early stage of CCl_4 poisoning, the available content of reduced glutathione and other tissue anti-

oxidants as well as ethanol induced glutathione reductase and glutathione peroxidase activities (MacDonald, 1973) apparently provide necessary partial protection.

Our data on the alteration of microsomal and mitochondrial ALDH 24 hr after CCl_4 exposure differ considerably from those reported by Hjelle et al. (1981). In contrast to the significant inhibition noted in our study, these authors did not observe any inhibition of microsomal and mitochondrial total ALDH. Only mitochondrial low K_m ALDH activity towards formaldehyde was reported to be decreased by 43–46%. This descrepancy in the results can partly be explained by the differences in the methodology used. Hjelle et al. (1981) neither incorporated into assay medium rotenone which is necessary to block mitochondrial NADH oxidase (Tottmar et al., 1973) nor used detergent to release latent ALDH activity (Horton and Barrett, 1975). Furthermore, the assays were performed at 7.4 pH even though the reported optimum pH for ALDH is 8.8 (Tottmar et al., 1973; Horton and Barrett, 1975). As a result of the suboptimal assay conditions employed the total ALDH activities noted by Hjelle et al. (1981) for the hepatic microsomes and mitochondria from control rats appear to be about 3 to 7 fold lower than those reported in the literature (Tottmar et al., 1973; Horton and Barrett, 1975) or observed in our studies. In any case, the ethanol + CCl_4 treated animals sacrificed 24 hr following CCl_4 exposure exhibited a significantly greater inhibition of microsomal and mitochondrial ALDH than the CCl_4 treated animals (Figs 2 and 3). Apparently, increased rate of in vivo CCl_4 metabolism due to induction of microsomal cytochrome P-450 in the ethanol pretreated animals (Sato et al., 1981) results in increased CCl_4-stimulated lipid peroxidation and/or covalent binding of free radical intermediates that overwhelm the cellular defenses and ultimately leads to a drastic decline in the activity of membrane bound ALDH. Since the animals were sacrificed at a time (24 hr following CCl_4 treatment) when hepatotoxicity is maximally expressed, the observed decline in ALDH may be due to cellular necrosis and/or due to cumulative ALDH inhibition in dying hepatocytes resulting from subsequent passes of CCl_4 through the hepatic tissue.

Since the blood acetaldehyde levels observed (Table 1) in ethanol and CCl_4 exposed rats were not elevated at 1 hr, it appears that both ethanol and acetaldehyde were largely eliminated prior to CCl_4 treatment or that the remaining ALDH activity and possibly other enzymes were sufficient to metabolize any acetaldehyde being generated at that time. However, it should be pointed out that how well blood levels reflect acetaldehyde contents of hepatocytes is unknown. It is, therefore, conceivable that localized levels of acetaldehyde and other cytotoxic aldehydes may reach toxic levels within mitochondria and the endoplasmic reticulum. In view of the observed ALDH inhibition a significant accumulation of one or more cytotoxic aldehydic products of lipid peroxidation could occur in the early stages of ethanol-CCl_4 toxicity. These toxic aldehydes, besides inhibiting ALDH, may play some contributory role in the genesis of enhanced CCl_4 hepatotoxicity. In conclusion, our data suggest that in vivo inhibition

of membrane bound ALDH may represent one of the major mechanisms responsible for CCl$_4$ hepatotoxicity and its potentiation by ethanol pretreatment. This hypothesis, however, does not exclude the involvement of other contributory factors reported by other investigators (Recknagel, 1983; Smith et al., 1983). Which ultimate toxicant(s) is (are) involved in the observed ethanol potentiation of CCl$_4$ hepatotoxicity remains to be determined.

SUMMARY

Recently Recknagel (1983) concluded that none of the previously proposed biochemical mechanisms of action satisfactorily explains genesis of CCl$_4$ hepatotoxicity. In our in vitro studies (Kenel and Kulkarni, 1985) we observed a significant inhibition of membrane-bound ALDH occurring concomitantly with CCl$_4$ bioactivation. Both covalent binding and lipid peroxidation were found to contribute to the inhibitory process. We have extended these observations and now demonstrate a significant in vivo inhibition of hepatic membrane-bound ALDH during early as well as acute stages of hepatotoxocity in CCl$_4$ or CCl$_4$ plus ethanol treated rats. It is proposed that in vivo ALDH inhibition may be the early event that links CCl$_4$ metabolism to the reported cascade of biochemical lesions leading to hepatotoxicity. Although the involvement of cytotoxic aldehydes other than acetaldehyde is most likely, further studies are needed to identify the ultimate toxicant(s) and its biochemical mechanism of action.

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