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# Biochemical Effects of 1,1-Dichloroethylene in Rats: Dissociation of Its Hepatotoxicity from a Lipoperoxidative Mechanism[1,2]

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Biochemical Effects of 1,1-Dichloroethylene in Rats: Dissociation of Its Hepatotoxicity from a Lipoperoxidative Mechanism. JAEGER, R. J., TRABULUS, M. J. and MURPHY, S. D. (1973). *Toxicol. Appl. Pharmacol.* **24**, 457–467. The dose-response and time course of hepatic injury following orally administered doses of 1,1-dichloroethylene (1,1-DCE) were studied. The following parameters were measured: hepatic glucose-6-phosphatase (G6Pase), serum alanine-α-ketoglutarate transaminase (SAKT), hepatic triglyceride (HTG), and pentobarbital sleeping time (PST). A comparison was made between 1,1-DCE and carbon tetrachloride ($CCl_4$) in terms of the ability of each to stimulate lipoperoxidation both in vitro and in vivo. The results indicate that 1,1-DCE causes G6Pase depression, SAKT and HTG elevation, and PST prolongation. Measurements of lipoperoxidative stimulatory activity indicated that 1,1-DCE was dissimilar to $CCl_4$ in terms of a proposed lipoperoxidative mechanism.

The chlorinated hydrocarbon, 1,1-dichloroethylene[3] (1,1-DCE), is used as a monomeric intermediate in the manufacture of plastics, particularly the Saran type.[4] Altman and Dittmer (1966) reported that 1,1-DCE has been isolated as an environmental contaminant in spacecraft and nuclear submarine atmospheres. Inhalation toxicity studies of 1,1-DCE indicate that it causes hepatic and renal damage (Irish, 1963; Prendergast *et al.*, 1967) and that, in general, the toxicity of 1,1-DCE may be considered both qualitatively and quantitatively comparable to carbon tetrachloride. Jenkins *et al.* (1972) noted that there was little information concerning the biochemical effects of 1,1-DCE and no information concerning the mechanism by which it exerts its hepatotoxic effects. They compared several biochemical indices of hepatoxic effects of 1,1-DCE with similar effects that have been reported for carbon tetrachloride ($CCl_4$) (Murphy and Malley, 1969). Jenkins *et al.* (1971) found that oral administration of both 1,1-DCE and $CCl_4$ caused a reduction in liver glucose-6-phosphatase (G6Pase) and increase in

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[3] 1,1-Dichloroethylene has also been called vinylidene chloride, or 1,1-dichlorethene.

[4] Pamphlet on vinylidene chloride monomer, Dow Chemical Company, Midland, Michigan, copyright 1968.

serum alanine-α-ketoglutarate transaminase (SAKT) activities in rats. Also, both chemicals caused increases in the stress responsive enzymes, liver alkaline phosphatase and liver tryosine transaminase.

This investigation was initiated to determine whether the qualitative similarities in response to $CCl_4$ and 1,1-DCE result from a similarity of mechanism. The mechanism of hepatotoxicity of $CCl_4$ has been studied extensively, and several proposals have been advanced to explain its hepatotoxic effects (Christie and Judah, 1954; Dianzani, 1954; Brodie and Maickel, 1963). The most recent of these, set forth by Butler (1961), Wirtschafter and Cronyn (1964) and later by Recknagel (1967), suggests that $CCl_4$ is metabolized in vivo by enzymes of the endoplasmic reticulum to a free radical intermediate, which in turn acts to initiate a process of lipid peroxidation within the membrane. The net result of such destructive events is the loss of unsaturated membrane lipid (Hochstein and Ernster, 1963) and a decrease in activity of enzymes associated with the endoplasmic reticulum; e.g., diminished microsomal G6Pase activity (Recknagel and Lombardi, 1961) and reduced activity of mixed function oxidases (Clifford and Rees, 1966). Subsequent cellular disruption leads to increased serum transaminase values (Rees and Sinha, 1960; Recknagel, 1967)

Since the mechanism of $CCl_4$ hepatotoxicity is well documented we examined 1,1-DCE induced liver injury in terms of similar parameters. We confirmed the time and dose-response effects of 1,1-DCE on G6Pase activity and SAKT reported by Jenkins *et al.* (1971, 1972) and added two additional tests of liver injury: pentobarbital sleeping time (PST) and liver triglyceride content. PST was determined since it may be considered a measure of the activity of the hepatic mixed function oxidase system. Liver triglycerides were measured because their elevation after $CCl_4$ is well known (Stettin and Salcedo, 1944). The in vivo and in vitro effects of 1,1-DCE and $CCl_4$ were compared in terms of the relative ability of each to stimulate the formation of malonyldialdahyde, a known product of lipoperoxidation (Dahle *et al.*, 1962). Finally, in vivo lipid peroxidation after oral doses of 1,1-DCE and $CCl_4$ was assessed by measurement of the amount of conjugated dienes present in extracts of endoplasmic reticulum lipid. This measurement is a sensitive indication of early, free-radical alteration of membrane lipid (Rao and Recknagel, 1968).

## METHODS

*Animals and treatments.* Male Holtzman rats, 250–350 g were used. They were housed in communal cages in an air-conditioned room, and were supplied with commercial rat chow[5] and water ad libitum. Food was withdrawn 28 hr before sacrifice or the rats were fasted overnight as noted. All animals were sacrificed between 12–2 PM except as noted.

Carbon tetrachloride[6] or 1,1-dichloroethylene[7] were mixed with an equal volume of corn oil.[8] Freshly prepared solutions were administered to rats by gavage at the appropriate dosages while the rats were very lightly, ether anesthetized. Control rats were handled in an identical manner but were given corn oil only.

Pentobarbital sodium[9] was administered ip at a dose of 30 mg/kg. Sleeping times were

[5] Purina Rat Chow, Ralston Purina Company, St. Louis, Missouri.
[6] Merck and Co., Rahway, New Jersey, reagent grade.
[7] K and K Laboratories, Inc. Plainview, New York. Highest purity.
[8] Mazola corn oil, Best Foods Division, CPC International Inc., Englewood, New Jersey.
[9] Nembutal Sodium, Abbott Laboratories, North Chicago, Illinois.

determined by measuring the time between the loss and regain of the righting reflex. The criteria for return of righting reflex was 3 spontaneous rightings within 1 min.

All animals were killed by cervical transection and exsangination. Blood was collected in centrifuge tubes for preparation of serum. The livers were immediately removed and rinsed in iced saline. They were blotted, weighed and homogenized in an aqueous solvent appropriate to the assay to be performed. A motor-driven Potter-Elvehjem homogenizer was used.

*Biochemical assays.* Liver glucose-6-phosphatase (G6Pase) activity was assayed as described by Murphy (1965). The assay procedure was modified slightly in that a 33% homogenate of liver (w/v) in 0.3 M sucrose, 3 mM EDTA was diluted to 1% with distilled water. This modification was necessary since lipoperoxidation measurements were made in the same homogenate, and 3 mM EDTA prevents spontaneous formation of conjugated dienes after sacrifice (Ghoshal and Recknagel, 1965). Control G6Pase activities were found to be unaffected by this modification.

Serum alanine-α-ketoglutarate transaminase (SAKT) was measured as outlined by Murphy and Malley (1969). In cases where greater than a 10-fold elevation of SAKT was suspected, serum samples were diluted appropriately. This was done to prevent substrate from being rate limiting at high enzyme activities. Within the range of dilution used, SAKT activity was found to be linear with enzyme concentration.

Triglyceride determinations on appropriately diluted liver homogenates were performed by the method of Carlson (1963).

Lipid peroxidation of rat liver homogenates after the addition of 1,1-DCE or $CCl_4$ to an in vitro system was measured by the thiobarbituric acid procedure. The conditions of the assay were essentially as described by Klaassen and Plaa (1969), except that 60, 75 and 90-min points were assayed following treatment of homogenates with the chlorinated hydrocarbon. A similar procedure was used when the chlorinated hydrocarbons were administered to rats in vivo. In this case, 1,1-DCE was given at 1 ml/kg and $CCl_4$ at 2.5 ml/kg. The animals were killed 1 hr later and homogenates were made. These were allowed to incubate aerobically for 60, 75 and 90 min.

In vivo lipid peroxidation was assessed by measurement of the amount of conjugated dienes present in extracts of microsomal lipid. The procedure used was similar to that reported by Klaassen and Plaa (1969). A 33% homogenate, usually 8 g of liver and 16 ml of iced 0.3 M sucrose–3 mM EDTA, was centrifuged at 4°C for 20 min at 9000 g. A 13-ml aliquot of the 9000 g supernatant was further centrifuged at 94,000 g for 60 min. The microsomal pellet was recovered and the supernatant fraction discarded. The pellet was resuspended in 2 ml of sucrose-EDTA solution. A 1-ml aliquot of this microsomal suspension was added to 19 ml of chloroform–methanol (2 : 1 v/v) and the mixture was shaken vigorously and allowed to stand for 30 min. All tubes were centrifuged, and the chloroform–methanol extract of microsomes was collected. Six milliliters of saline was added to this extract, the tubes were again shaken and were allowed to settle for several minutes. The tubes were centrifuged gently, and the aqueous–methanol phase was removed by aspiration. A 10-ml aliquot of the lower chloroform phase was removed, and 0.4 ml of methanol was added to clarify the solution. The optical density of this final extract was read at 243 nm in a Gilford Model 240 spectrophotometer and corrected for the absorbance of an appropriate reagent blank.

At high dosages or after the more prolonged time intervals after 1,1-DCE, livers became congested. In these circumstances activities or concentrations of liver constituents calculated per unit of fresh weight of liver would be erroneously low. To adjust for this a correction was made by calculating the liver activity per 100 g of body weight for all experiments (activity or concentration/g liver × g of liver/100 g of body weight). All comparisons were made with these units. It was felt that this would minimize errors due to dilution with blood or tissue fluid.

*Statistics.* Tests for significance of differences were made by the Student's t test or by the Mann Whitney U test. A $p$ value of less than 0.05 was considered statistically significant.

## RESULTS

### *G6Pase, SAKT and PST after 1,1-DCE*

Oral administration of 1,1-DCE to rats caused dose-dependent liver injury as measured by decreased hepatic G6Pase activity and increased SAKT activity (Fig. 1). Figure 2 shows that there was also a dose-dependent prolongation of PST. In this experiment, there was no prolongation of sleeping time at 100 mg/kg even though this dose produced detectable hepatic injury by G6Pase and SAKT criteria (Fig. 1).

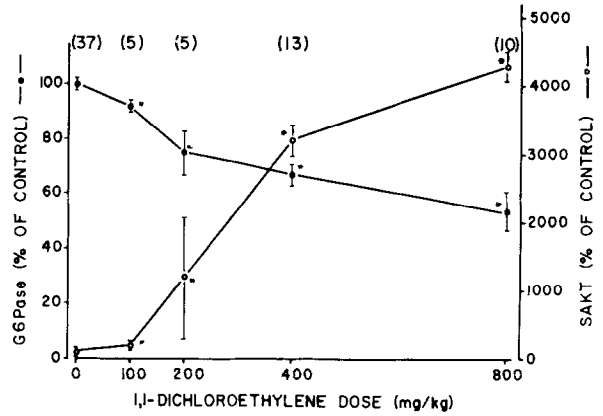


FIG. 1. Dose-response effect of 1,1-DCE on total hepatic G6Pase and SAKT. Rats, fasted and dosed as described in Methods, were sacrificed at 24 hr. In this and subsequent figures, an asterisk indicates significantly different from control at $p < 0.05$. The number in parentheses represents the number of animals used in each experiment. Control values for total liver G6Pase were 88.2 ± 1.5 mg $P_i$/hr/100 g body weight and for SAKT 0.28 ± 0.01 mg pyruvate/ml serum/hr.

### *1,1-DCE Effects on Liver Triglyceride*

Another indication of hepatotoxicity usually associated with $CCl_4$ intoxication is the appearance of large quantities of neutral fat within the liver (Stettin and Salcedo, 1944). In order to determine whether 1,1-DCE has a similar effect, triglyceride content of rat liver was measured 24 hr after increasing doses of 1,1-DCE (Fig. 3). As the dosage of 1,1-DCE was increased, the total amount of fat within the liver increased accordingly, and at 800 mg/kg, the amount of lipid present was almost doubled.

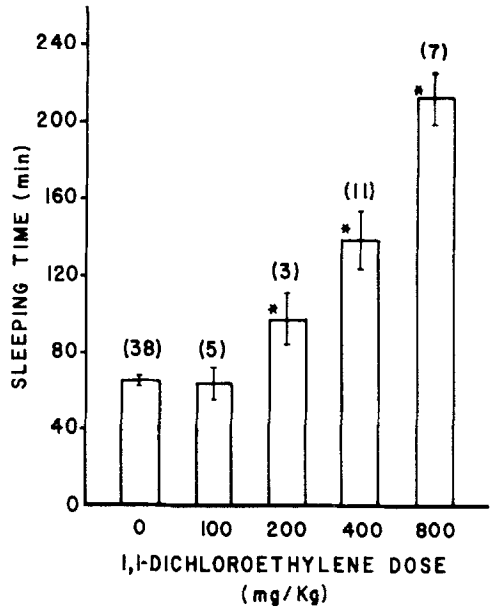


FIG. 2. Dose-response effect of 1,1-DCE on pentobarbital sleeping time (PST). The same rats used in Fig. 1 were used for these experiments. PST was determined between 20 and 24 hr.

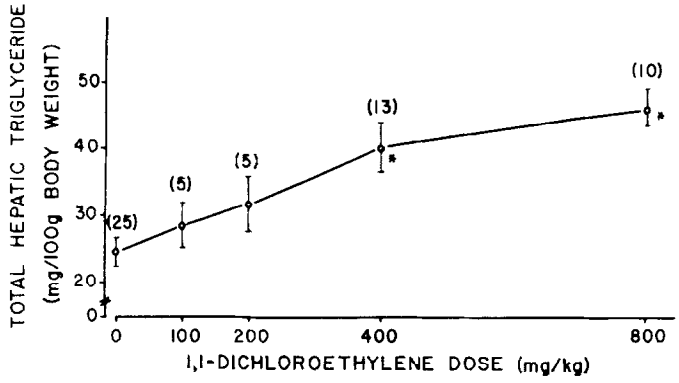


FIG. 3. Dose-response effect of 1,1-DCE on total heptatic triglycerides, 24 hr after dosing. The values plotted are corrected for changes in liver to body weight ratio. The uncorrected data were also significantly elevated at 400 and 800 mg/kg of 1,1-DCE (8.2 ± 0.7 mg triglyceride/g wet tissue weight in controls versus 11.1 ± 1.0 and 13.0 ± 1.0 for the two higher doses of 1,1-DCE).

*Time Course of G6Pase, SAKT and PST after 1,1-DCE*

Experiments were performed to determine the time course of hepatic and serum enzyme changes after a single oral dose of 1,1-DCE. The results (Fig. 4) indicate that there was no decrease in G6Pase activity 4 hr after 1,1-DCE while at 8 and 16 hr there was significant reduction in activity which did not decrease further by 24 hr. SAKT was slightly but significantly elevated at 4 hr and reached its maximal activity by 8 hr. Thereafter, the serum enzyme activity declined.

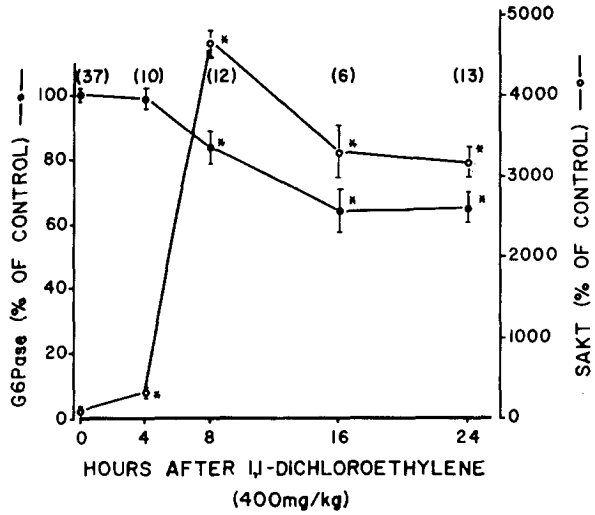


FIG. 4. Time-response effect of 1,1-DCE on total hepatic G6Pase and SAKT activity. All rats were fasted 28 hr before sacrifice. Values for control animals that had been given corn oil at various times before sacrifice were the same as in Fig. 1. There was no difference due to different times of corn oil treatment, and values for all control animals were pooled.

Pentobarbital sleeping time was determined in animals that had been previously dosed with 1,1-DCE. The results (Fig. 5) indicate that at 2–4 hr after 400 mg/kg of 1,1-DCE, there was a significant prolongation of PST. This prolongation of PST increased to a maximum between 12–16 hr. The sleeping times measured at 20–24 hr, 12–16 hr and 4–8 hr did not differ significantly ($p > 0.05$) from each other.

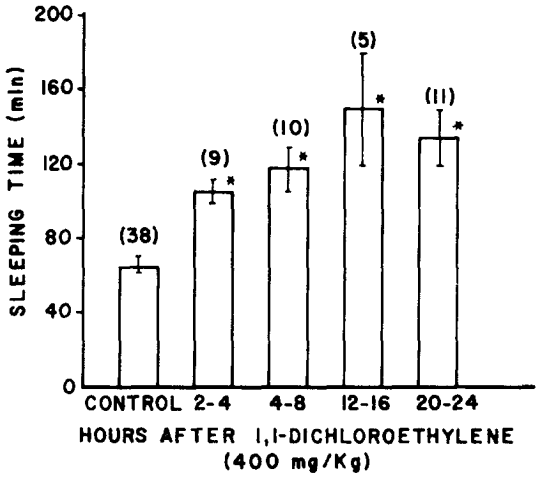


FIG. 5. Time-response effect of 1,1-DCE on pentobarbital sleeping time (PST). The rats described in Fig. 4 were given pentobarbital sodium, 30 mg/kg ip, 2–4 hr before sacrifice and the PST was determined.

*Lipoperoxidation after 1,1-DCE and $CCl_4$*

Experiments were performed to compare the effects of 1,1-DCE and $CCl_4$ in vitro on spontaneous lipoperoxidation as indicated by the production of malonyldialdehyde (MDA) in aerobic incubates of rat liver homogenates. The results of these experiments are shown in Fig. 6. Control homogenates only began to form MDA at greater than 60 min of incubation, and similar results were obtained for homogenates that had been treated with 5 $\mu$l of 1,1-DCE per flask. Five microliters of $CCl_4$ caused peroxidation to begin sooner than 60 min so that a measurable amount of MDA was detected at the first time point measured. All tissue blanks began from the same low level, which indicates that $CCl_4$ reduced the time interval for peroxidative destruction to become evident, i.e., less than 60 min.

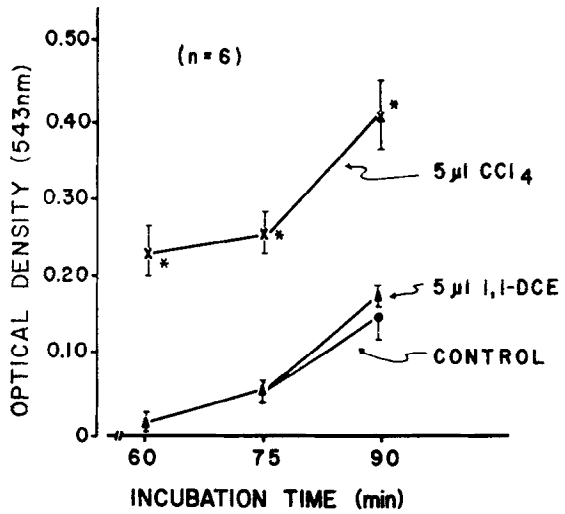


FIG. 6. Effect of $CCl_4$ and 1,1-DCE on in vitro lipid peroxidation. Whole liver homogenates from 6 male, nonfasted rats were treated with 5 $\mu$l of either 1,1-DCE or $CCl_4$. All values of $OD_{543nm}$ are corrected for the blank tissue absorption.

Another series of experiments was undertaken to determine the effects of in vivo treatment with 1,1-DCE or $CCl_4$ on in vitro lipid peroxidation. Rats were dosed in vivo with 1,1-DCE, $CCl_4$ or corn oil, the animals were killed 1 hr later and MDA production in aerobic liver incubates was measured after 60, 75 and 90 min of incubation. The dose of 1,1-DCE chosen was 1 ml/kg (12.5 mmoles/kg) or approximately three-fourths of the 24-hr oral LD50 (Jenkins *et al.*, 1972). Although $CCl_4$ was given at a higher absolute dose of 2.5 ml/kg (25.1 mmole/kg) it was a smaller proportion (one-half) of the 24-hr LD50 of $CCl_4$. The results of this experiment (Fig. 7) indicate that MDA production in liver homogenates of $CCl_4$-treated rats was significantly greater at 60 min than in liver homogenates from either control or 1,1-DCE-treated animals. As previously mentioned, the liver homogenate blanks at the start of in vitro incubation did not contain detectable MDA. In this experiment, it should be noted that unlike $CCl_4$ treatment, liver homogenates from 1,1-DCE-treated rats had reduced amounts of measurable MDA at 60, 75 and 90 min of incubation.

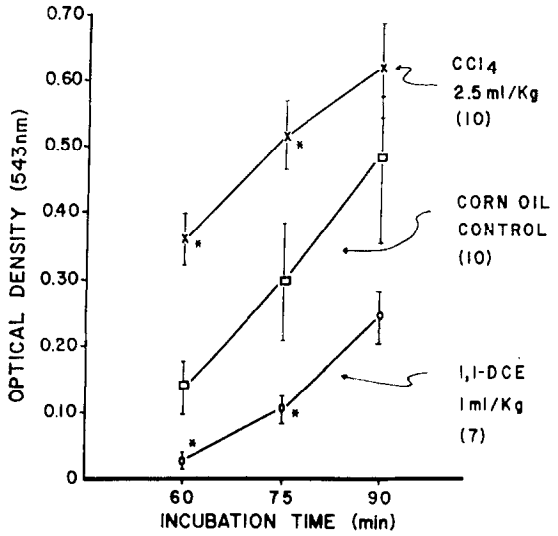


FIG. 7. Effect of in vivo treatment with 1,1-DCE or $CCl_4$ on in vitro lipid peroxidation. Rats were fasted overnight and dosed the following morning with corn oil (2.5 ml/kg), 1,1-DCE (1 ml/kg) or $CCl_4$ (2.5 ml/kg). One hour after dosing, the animals were sacrificed, and the liver homogenates were prepared. The experimental procedure was identical to the experiment in Fig. 6 except that exogenous addition of the chlorinated hydrocarbons was omitted.

Another measurement of hepatic lipid peroxidation is the amount of microsomal conjugated dienes present in vivo. Measurements of conjugated dienes were made in livers of rats killed 1, 4 and 20 hr after oral administration of 1,1-DCE or $CCl_4$. The results (Fig. 8) indicate that $CCl_4$ caused a large increase in the amount of conjugated dienes at 1 and 4 hr. However, 1,1-DCE did not increase conjugated diene levels at these times. By the 20th hr after dosing, the amount of conjugated dienes in livers of both $CCl_4$ and 1,1-DCE-treated rats was significantly below control amounts.

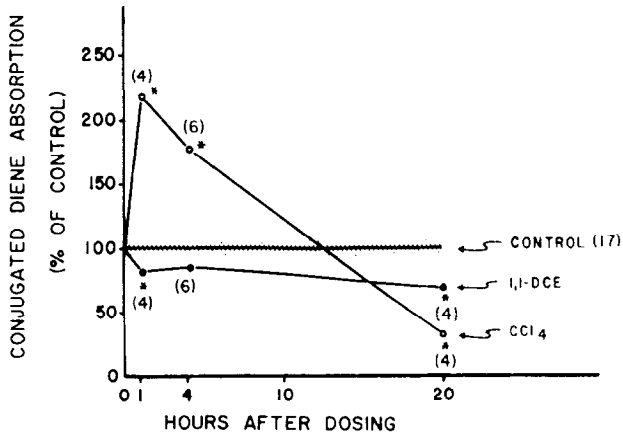


FIG. 8. Effect of $CCl_4$ and 1,1-DCE on microsomal conjugated dienes. Rats, fasted 20–28 hr before sacrifice, were dosed with corn oil, 1,1-DCE or $CCl_4$ as described for Fig. 7. At the indicated times after dosing, the animals were sacrificed (1–2 PM), their livers were removed, and conjugated dienes were measured as described in methods. The results shown in this figure represent the weight-corrected amounts expressed as a percentage of pooled control values (0.21 ± 0.01 $OD_{243nm}$/100 g body weight).

Hepatic G6Pase activity was measured in these same experiments. These values and the amounts of conjugated dienes at 1 and 20 hr are shown in Table 1. Table 1 also shows the results of measurements of G6Pase and conjugated dienes in livers of rats that had been given both $CCl_4$ at 1 hr and 1,1-DCE at 20 hr prior to sacrifice. The results indicate that prior treatment of rats with 1,1-DCE followed by $CCl_4$ results in a depression of glucose-6-phosphatase activity and an increase in conjugated dienes which is not significantly different from the effect of $CCl_4$ acting alone. These results indicate that the reduction in conjugated dienes which occurs 20 hr after 1,1-DCE does not decrease the amount of lipoperoxidative damage which occurs 1 hr after $CCl_4$.

TABLE 1

EFFECT OF 1,1-DCE PRETREATMENT ON SUBSEQUENT $CCl_4$ HEPATOTOXICITY[a]

| Treatment | G6Pase [% of respective control ± SE (*N*)] | Conjugated dienes [% of respective control ± SE (*N*)] |
|---|---|---|
| Corn oil | 100 ± 2 (20) | 100 ± 6 (18) |
| 1,1-DCE, 20 hr | 61 ± 5 (5) | 71 ± 8 (4) |
| $CCl_4$, 1 hr | 61 ± 6 (9) | 217 ± 11 (4) |
| 1,1-DCE, 20 hr / $CCl_4$, 1 hr | 73 ± 4 (5) | 220 ± 4 (5) |

[a] Rats were fasted 28 hr prior to sacrifice. Two groups of rats were treated with 1,1-DCE (1 ml/kg) and the other two groups were given equivalent doses of corn oil. One hour before sacrifice, one corn oil-treated and a 1,1-DCE-treated group of rats were given a dose of $CCl_4$ (2.5 ml/kg). The other two groups received doses of corn oil. All rats were sacrificed at 20 hr. Total hepatic G6Pase activity and conjugated dienes shown are weight-corrected and are expressed as a percentage of their respective control values. The control values for total liver G6Pase were: corn oil, 80.8 ± 1.7 mg $P_i$/hr/100 g body weight; and 1,1-DCE, 20 hr, 49.1 ± 2.4. Total liver conjugated diene control values were: corn oil, 0.21 ± 0.01 $OD_{243nm}$/100 g body weight; and 1,1-DCE, 20 hr, 0.15 ± 0.01. In the case of the combined treatment of 1,1-DCE, 20 hr and $CCl_4$, 1 hr, the value for 1,1-DCE, 20 hr represents the control value. The comparison between $CCl_4$, 1 hr and $\frac{(1,1\text{-DCE, 20 hr})}{(CCl_4, 1\text{ hr})}$ is not significant ($p > 0.05$).

## DISCUSSION

The results of this study confirm that 1,1-dichloroethylene and carbon tetrachloride share some qualitatively similar hepatotoxic actions. The aspects of liver damage that both chemicals have in common are: reduction of hepatic G6Pase activity, increased SAKT, increased PST, and increased amounts of hepatic triglyceride.

Our investigation of the time response of hepatic G6Pase and SAKT following 1,1-DCE treatment demonstrated a pattern of hepatotoxicity characterized by an early increase in SAKT at a time when there was only a relatively small decrease in G6Pase activity. SAKT was maximally elevated 8 hr after dosing and was significantly increased by 4 hr. At this time (4 hr) no decrease in G6Pase was detected. In contrast, Murphy and Malley (1969) reported that $CCl_4$ causes a significant decrease in G6Pase activity within 30 min of oral dosing. These workers were not able to show a statistically significant increase in SAKT activity until between the second and fifth hour after $CCl_4$ administration. Thus, the time course of effects on liver G6Pase and SAKT following oral doses of 1,1-DCE is different from that reported for $CCl_4$.

It appeared unlikely that 1,1-DCE caused an early prolongation of PST by a reduction in the rate of hepatic drug metabolism, since at the 4-hr interval, no evidence of injury to the hepatic endoplasmic reticulum was detected by reduced total G6Pase activity. Preliminary experiments on control and 1,1-DCE treated rats sacrificed at 3 hr did not show significant reduction in the rate of in vitro pentobarbital metabolism (unpublished observations). Some other nonhepatic factor, such as tissue distribituon of pentobarbital or an effect of toxic chemical stress following 1,1-DCE, may have been responsible for the early prolongation of PST that was observed.

The alterations in hepatic and serum enzyme activities after 1,1-DCE supports a qualitative similarity of effect with $CCl_4$. However, the temporal sequence of enzyme changes suggests a different mode of action for the two chemicals. This suggestion of mechanistic difference is better supported by experiments related to hepatic lipoperoxidation. It was shown that 1,1-DCE does not share any similarity with $CCl_4$ in the ability to stimulate MDA production in the liver after in vitro or in vivo treatment. In contrast to $CCl_4$, 1,1-DCE reduced the amount of MDA production in vitro following in vivo treatment.

Additional evidence against a $CCl_4$-like mechanism of action for the hepatotoxicity of 1,1-DCE comes from analysis of the amount of conjugated dienes present in extracts of microsomal lipid. While $CCl_4$ increased the amount of conjugated dienes, 1,1-DCE had no effect or slightly reduced it. Furthermore, 1,1-DCE pretreatment did not alter the effect of $CCl_4$ on this parameter or on decreases in hepatic G6Pase activity caused by $CCl_4$.

In conclusion, the evidence presented in this report is consistent with the hypothesis of Recknagel and Ghoshal (1966) that $CCl_4$ causes hepatic lipoperoxidative damage which may or may not be the direct cause for changes in liver enzyme activity. While 1,1-DCE produced some of the same liver and serum enzyme changes associated with $CCl_4$ hepatotoxicity, there was no evidence for mechanistic similarity. In the experiments reported here, we observed that 1,1-DCE caused an increase in the level of serum transaminase at a time when changes were not detected in hepatic G6Pase activity. The observation of a different temporal sequence of biochemical indices of hepatotoxicity suggests different sites of initial injury caused by $CCl_4$ and 1,1-DCE. That is, because 1,1-DCE causes an early rise in serum activities of a cytoplasmic liver transaminase, it may be speculated that this agent initially attacks the plasma membrane of the liver cell. Serum transaminase values would thus be expected to rise at an early time, and G6Pase activity would only be lost as cells became severely damaged or necrotic. Conversely, the rapid decline in G6Pase caused by $CCl_4$ as reported by Murphy and Malley (1969) and confirmed by us in separate experiments, only partially reported here (Table 1), indicates that $CCl_4$ most likely has its initial effect on the membrane of the endoplasmic reticulum as has been shown by other workers (Reynolds, 1965; Oberling and Rouiller, 1956).

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