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CHEMICAL MANUFACTURERS ASSOCIATION VINYLIDENE CHLORIDE PROGRAM PANEL COMMENTS ON PROPOSED TEST RULE FOR 1,1-DICHLOROETHYLENE (VINYLIDENE CHLORIDE) OPTS-42082 51 Fed. Reg. 28840 (August 12, 1986)

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1 1-DICHLOROETHYLENE HEPATOTOXICITY: EFFECT OF ALTERED THYROID FUNCTION AND EVIDENCE FOR THE SUBCELLULAR SITE OF INJURY

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Male Sprague-Dawley rats were exposed for 4 hr to 1,1-dichloroethylene (1,1-DCE) by inhalation following an 18 hr fast. They were killed at 6 hr. Under these circumstances, prior thyroidectomy caused a decrease in the severity of the injury as measured by elevation of serum alanine-α-ketoglutarate transaminase (AKT), while thyroxine pretreatment (50 μg per rat sc for 7 days) enhanced the hepatotoxicity of 1,1-DCE as measured by lethality and serum AKT elevation. Chemical thyroidectomy using either propylthiouracil (30 mg/kg po for 7 days) or methimazole (15 mg/kg po for 7 days) also provided a degree of protection. Thyroidectomy, surgical or chemical, was associated with an increase in hepatic glutathione concentration, while thyroxine decreased the hepatic concentration of this nucleophile. Livers from rats exposed to 1,1-DCE were found to have decreased in vitro oxygen uptake when supplied with succinate and ADP. This form of injury preceded the elevation in serum AKT, which occurred at 4 hr. Subcellular fractionation of livers from fed or fasted rats exposed to air or 1,1-DCE showed that the heavy and light mitochondrial fractions from fasted, exposed rats had the largest decrease in glutathione concentration relative to the other groups. 1,1-DCE exposure was associated with decreased glutathione concentration, but subfractions from fasted rats generally had the largest decreases. Serum sorbitol dehydrogenase, a cytoplasmic marker, was positively correlated to changes in serum ornithine carbamoyl transaminase, a mitochondrial marker, suggesting that mitochondrial damage may occur before or at the same time as cytoplasmic membrane rupture. These latter findings are consistent with previously reported histologic observations. Based on these data, a mechanism for 1,1-DCE is proposed.

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INTRODUCTION

1,1-Dichloroethylene (vinylidene chloride), 1,1-DCE, is an hepatotoxin with an 8 hr threshold limit value at 10 ppm. While no published experimental evidence has yet indicated that this compound is a carcinogen, a report by Viola suggests that in chronic experiments, tumors may develop in animals repeatedly exposed to concentrations of 1,1-DCE that are acutely hepatotoxic to fasted rats (Jaeger et al., 1974a). These concentrations do not produce gross hepatic injury in fed rats.

Our interest has centered on the differential sensitivity of fed and fasted rats in their response to hepatic injury following inhalation of 1,1-DCE at concentrations as low as 200 ppm for 4 hr. In several reports we have shown that diminished glutathione concentrations at the time of exposure appear to be associated with increased sensitivity to hepatic injury (Jaeger et al., 1974a, 1974b).

Szabo et al. (1974) suggested that thyroidectomized animals were resistant to the toxic effects of organomercury compounds as a result of an elevation of tissue sulfhydryl concentration following thyroid removal. Based on this, we hypothesized that alterations in hepatic glutathione concentration resulting from altered thyroid function, caused surgically, chemically, or by thyroxine pretreatment, were inversely related to the development of hepatic injury following inhalation exposure to 1,1-DCE. Since the thyroid has been shown to be one regulator of metabolic activity in animals, we also measured the effects of 1,1-DCE on the time course of oxygen utilization of liver homogenate when succinate and ADP were supplied as substrate and cofactor, respectively. Our interest in these biochemical measurements was based on the morphologic observations of Reynolds et al. (1975) showing that early injury following 1,1-DCE was associated with *mitochondrial* damage. Thus we hypothesized that metabolic activity of this organelle should be affected early in the course of injury. We attempted to determine the serum enzyme activity of cytoplasmic and *mitochondrial* origin to establish whether liver mitochondria were the site of initial attack by 1,1-DCE or its metabolites.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague-Dawley rats were obtained from Holtzman, Madison, Wisconsin, or Charles River, Wilmington, Massachusetts. They were housed in air-conditioned quarters with a 12 hr light-dark cycle, and were given Purina Rat Chow and tap water *ad libitum*. Sham operation, surgical thyroidectomy, or drug pretreatment was initiated 7 days before exposure. Sodium thyroxine (Sigma Chemical Co.) in water (0.5 cm^3) was given sc

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(total daily dose 50 μg). Propylthiouracil (Aldrich Chemical Co.) or methimazole (Eli Lilly & Co.) was given daily (po) at the dose designated. As indicated, the rats were fasted from 4 p.m. of the day before exposure. The animals were killed by cervical transection 5–7 hr (average, 6 hr) after onset of exposure.

Following cervical transection, the animals were exsanguinated and the blood 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 high-speed homogenizer (Polytron) or a Potter-Elvehjem homogenizer was used.

Inhalation Exposure

Exposures took place in 30 liter dynamic chambers, as described by Leach (1963) and as used previously (Jaeger et al., 1974a). The chambers were ventilated at 20 liter/min under positive pressure in a ventilated cabinet. Concentrations of 1,1-DCE on the exhaust side of the chamber were determined by using a Hewlett-Packard gas chromatograph and an electronic integrator. The column was Poropak Q (Waters Associates) operated with nitrogen as the carrier. 1,1-DCE (MEHQ grade) purchased from the Dow Chemical Co. was used for the exposures and was diluted with *n*-hexanes to serve as the gas chromatographic standard.

Biochemical Assays

Serum alanine-α-ketoglutarate transaminase (AKT) and liver glutathione (GSH) were measured as previously described (Jaeger et al., 1974a). Sorbitol dehydrogenase (SDH) was measured as described by Korsrud et al. (1972) and ornithine carbamoyl transferase (OCT) was determined by the method of Snodgrass and Parry (1969). Oxygen uptake was measured manometrically in a final volume of 3 ml using freshly prepared liver homogenates (10%) supplemented with ADP (1.5 m*M*) and succinate (10 m*M*). Protein concentration was determined by the method of Lowry (1951).

Subcellular Fractionation

After removal from the animal, the liver was weighed and further operations were performed in a cold room (4°C). The liver was minced in a tissue press (fine screen), and 6 g of the mince was placed in a size C Teflon glass homogenizer to which 18 ml of a solution consisting of 0.25 *M* sucrose, 0.01 *M* Tris, and 0.002 *M* EDTA, pH 7.4, buffer had been added. A 25% homogenate was made by using an electric drill set to 400 rpm; four up-and-down strokes were employed. The homogenate was filtered through two layers of surgical gauze. Centrifugation was by the method of Hook et al. (1972).

TABLE 2. Effect of Thyroidectomy, Thyroxine Pretreatment, or a Combination of Both on Hepatic Glutathione Concentration Following 1,1-DCE[a]

	Glutathione (mg GSH/100 g body weight)[b]		
Exposure	No pretreatment	Thyroidectomy	Thyroxine
Air only	4.76 ± 0.28 (4)	5.44 ± 0.20[c] (4)	3.97 ± 0.16[d] (4)
1,1-DCE	3.23 ± 0.30[e] (4)	3.59 ± 0.43[e] (5)	2.75 ± 0.41[e] (4)

[a] See Table 1 and text for details.
[b] Mean ± SEM. Values are corrected for increased liver/body weight ratio associated with 1,1-DCE-induced injury.
[c] This value was 1.71 ± 0.04 mg GSH/g liver compared to a control value of 1.35 ± 0.04 mg/g, a difference statistically significant at the 0.05 level. When corrected for liver/body weight ratio, which was not different between the groups, the difference was not significant ($0.10 < p < 0.05$) because of the larger variability of the data.
[d] Significantly less than air-only, no-pretreatment group ($p < 0.05$), 1.11 ± 0.03 mg GSH/g liver.
[e] $p < 0.05$ compared to respective air controls.

6 hr or were sacrificed *in extremis* following exposure to 1,1-DCE shows a comparable absolute decline in GSH concentration in each category.

To determine whether the protective action of thyroidectomy was due to the absence of the thyroid gland and not to some combination of thyroidectomy and parathyroidectomy, a series of experiments was conducted with the antithyroid agents propylthiouracil and methimazole These data, which are shown in Table 3, indicate that the two antithyroid drugs were comparable to thyroidectomy in their protection against the hepatotoxic effects of 1,1-DCE. We observed (data not shown) that the two pretreatments were associated with significant elevations in liver GSH concentration, confirming that decreased thyroid function (surgical removal or chemical inhibition) was associated with increased liver sulfhydryl content.

Table 4 shows that a time-related decrease in oxygen uptake precedes large changes in the serum AKT activity. In these two experiments, while the absolute activity of the oxygen uptake differs, the time-related correlation is consistent. The decreased oxygen uptake precedes large changes in the serum AKT activity. That is, the changes in the serum AKT activity, while small but showing a time-related trend, are slight at times when oxygen uptake inhibition is substantial.

In view of these observations—that the thyroid appeared to regulate the amount of GSH in the liver and that oxygen uptake, presumably due to mitochondrial activity, was diminished at early times of injury—it

Statistics

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

RESULTS

The effect of thyroidectomy or thyroxine pretreatment on serum AKT elevation following air or 1,1-DCE exposure is shown in Table 1. The results from rats exposed to 1,1-DCE indicate that thyroidectomy provided significant protection against the toxic effects of 1,1-DCE, while thyroxine pretreatment significantly potentiated the toxicity observed after exposure to this chemical. In the thyroxine-treated group, three of the four rats exposed died as a result of the exposure. When this portion of the experiment was repeated, the thyroxine-treated rats again died, suggesting that their increased sensitivity to 1,1-DCE precluded survival to 6 hr after exposure to 2,000 ppm 1,1-DCE.

Table 2 shows the effect of thyroidectomy or thyroxine pretreatment on hepatic GSH concentration following air or 1,1-DCE exposure. It can be seen that thyroidectomized animals had a significantly higher liver GSH concentration than control animals when GSH was expressed per gram of liver. Thyroxine pretreatment resulted in a significant reduction in GSH concentration compared to that in controls. A comparison of the data from air-exposed animals with the results from animals that were killed at

TABLE 1. Effect of Thyroidectomy or Thyroxine Pretreatment on Serum AKT Elevation Following 1,1-DCE Exposure[a]

	Serum AKT (mg pyruvate/ml-hr)[b]		
Exposure	No pretreatment	Thyroidectomy	Thyroxine
Air only	0.30 ± 0.02 (4)	0.32 ± 0.02 (4)	0.35 ± 0.02 (4)
1,1-DCE	9.16 ± 1.58[c] (4)	2.94 ± 1.12[c] (4)	30.30[d] (1)

[a] Fasted Charles River rats were exposed to air only or to 2,000 ppm 1,1-DCE for 4 hr and were killed at 6 hr. Only the 6 hr survivors were used. The number of rats in each group is given in parentheses.

[b] Mean ± SEM.

[c] $p < 0.05$ compared to unexposed controls.

[d] Three of four rats at this concentration died. This dose of thyroxine was supermaximal at enhancing 1,1-DCE toxicity. However, no lower doses of thyroxine were tested.

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TABLE 5. Effect of 1,1-DCE Exposure on Subcellular Distribution of Glutathione[a]

	μg GSH/mg protein[b]						
	Fed rats			Fasted rats			
Fraction	Air	1,1-DCE	Absolute decrease	Air	1,1-DCE	Absolute decrease	Fed/fasted ratio
Whole homogenate	4.12 ± 0.27	2.58 ± 0.22[c]	1.54	3.95 ± 0.15	1.98 ± 0.17[c]	1.97	0.81
Nuclei	2.77 ± 0.13	1.76 ± 0.17[c]	1.01	2.58 ± 0.17	1.12 ± 0.06[c]	1.46	0.67
Heavy mitochondria	2.76 ± 0.44	2.13 ± 0.59	0.63	3.91 ± 0.29[d]	1.67 ± 0.25[c]	2.24	0.56
Light mitochondria	2.21 ± 0.29	1.39 ± 0.23[c]	0.82	2.73 ± 0.20	1.02 ± 0.14[c]	1.71	0.59
Microsomes	2.39 ± 0.18	1.14 ± 0.22[c]	1.25	1.63 ± 0.07[d]	0.59 ± 0.11[c]	1.04	0.75
Supernatant	11.62 ± 0.36	7.19 ± 1.23[c]	4.43	10.17 ± 0.39	4.74 ± 1.22[c]	5.43	0.76

[a]Fasted Holtzman rats (three per group) were exposed to 200–300 ppm 1,1-DCE for 4 hr. They were killed at 6 hr.
[b]Mean ± SEM.
[c]$p < 0.05$ compared to air group.
[d]$p < 0.05$ compared to fed-air group.

TABLE 3. Effect of Propylthiouracil or Methimazole Pretreatment on Serum AKT Activity Following 1,1-DCE Exposure[a]

Treatment	Serum AKT (mg pyruvate/ml-hr)[b]	
	Air	1,1-DCE
Vehicle	0.38 ± 0.11 (3)	13.52 ± 1.14[c] (4)
Propylthiouracil[d]	0.28 ± 0.06 (6)	4.08 ± 1.23[c,e] (9)
Methimazole[f]	0.31 ± 0.03 (5)	2.61 ± 0.81[c,e] (10)

[a] Fasted Charles River rats were exposed to 1,1-DCE at 2,000 ppm for 4 hr and killed at 6 hr.
[b] Mean ± SEM.
[c] $p < 0.05$ compared to nonexposed control.
[d] 30 mg/kg po for 7 days.
[e] $p < 0.05$ compared to vehicle plus 1,1-DCE group.
[f] 15 mg/kg po for 7 days.

became of interest to determine the distribution of GSH within the subcellular fractions of rat liver from fed or fasted rats exposed to air or 1,1-DCE. The results, shown in Table 5, provide data on the effect of 1,1-DCE exposure on subcellular GSH concentrations in nuclei, mitochondria, microsomes, and cytoplasm compared to those in whole homogenate. It can be seen from these data that 1,1-DCE exposure caused substantial decreases in GSH concentrations in all cell fractions from both fed and fasted rats. In absolute terms, the largest decrease occurred in the supernatants from both exposed groups (4.43 and 5.43 μg GSH/mg for fed and fasted rats, respectively). The fasted rats exposed to 1,1-DCE had

TABLE 4. Effect of 1,1-DCE Exposure on Liver Homogenate Oxygen Uptake and Serum AKT[a]

Time from start of exposure to death (hr)	Oxygen uptake[b] (μl O_2/mg protein per 30 min)		Serum AKT[b] (mg pyruvate/ml-hr)	
	Experiment I	Experiment II	Experiment I	Experiment II
Nonexposed controls	6.87 ± 1.07 (3)	10.12 ± 0.55 (9)	0.19 ± 0.04	0.18 ± 0.01
1	5.25 ± 0.32 (5)		0.25 ± 0.01	
2	4.66 ± 0.52 (5)	7.91 ± 0.72 (3)[c]	0.27 ± 0.04	0.23 ± 0.03
3	4.20 ± 0.56 (5)		0.30 ± 0.05	
4 (end exposure)		6.42 ± 0.86 (3)[c]		3.66 ± 0.72[c]
6		3.73 ± 0.98 (3)[c]		7.65 ± 1.51[c]

[a] Fasted Holtzman rats were exposed to 200–300 ppm 1,1-DCE and killed at the indicated times.
[b] Mean ± SEM.
[c] $p < 0.05$ compared to nonexposed controls.

to the mitochondria, if not a cause of cellular damage, is closely related to the observed plasma membrane injury.

DISCUSSION

The results given above suggest that the thyroid gland regulates the concentration of glutathione in the liver and support a previously described hypothesis (Szabo et al., 1974). Thyroidectomy, propylthiouracil, and methimazole, which elevated hepatic GSH concentrations, resulted in protection against 1,1-DCE-induced liver injury, while thyroxine pretreatment, which resulted in a significant decrease in hepatic GSH concentration, was associated with a high mortality. In the latter case a substantial serum AKT elevation was seen in the surviving animal. Subsequent experiments confirmed that thyroxine increased the sensitivity of rats to 1,1-DCE so that 2,000 ppm for 4 hr was a superlethal concentration.

Thyroid hyperfunction is known to enhance several aspects of metabolic activity, including the MFOS (Vesell et al., 1975). Thus, it is possible that thyroxine stimulated hepatic metabolism of 1,1-DCE to a more toxic form in addition to its effect on GSH concentration. This cannot be excluded as a possible cause of the enhanced toxicity observed in thyroxine-pretreated animals. However, in previous work we showed that hepatic MFOS induction protected against 1,1-DCE toxicity in direct proportion to the increase in cytochrome P-450 (Reynolds et al., 1975).

An hypothesis relating specific mitochondrial damage to hepatic injury after 1,1-DCE is supported by the observation of an early decline in oxygen uptake in liver homogenates at times of slight serum enzyme elevation. The two experiments described here were repeated using mitochondria isolated from fasted Charles River rats exposed to 1,1-DCE, rather than whole liver homogenates. Similar results were obtained (R. J. Jaeger manuscript in preparation). Observations of GSH changes in the mitochondria of fasted compared to fed rats exposed to 1,1-DCE also support the hypothesis of a specific mitochondrial lesion at an early stage of liver injury. The simultaneous elevation of SDH and OCT is consistent with this hypothesis.

A clear understanding of the role of the MFOS and the mitochondria in the pathogenesis of 1,1-DCE toxicity must await more information on the metabolism of 1,1-DCE and its reactivity or the reactivity of its metabolites with GSH. Based on the GSH measurements reported here, one may speculate that if MFOS activity is important to the bioactivation of 1,1-DCE, the observation that the microsomes from fasted rats exposed to air contained lower GSH concentrations than the microsomes from air-exposed fed rats permits us to suggest that larger amounts of reactive or toxic metabolites are able to escape from this site of activation in fasted rats and reach the mitochondria. Because of this, the GSH decreases

larger absolute GSH concentration reductions, with the exception that the values for the microsomal fractions in the two exposed groups were of similar magnitude (1.25 and 1.04 μg GSH/mg for fed and fasted rats, respectively).

Also shown in Table 5 is a fed/fasted ratio, calculated as the fractional decrease of the GSH concentration in fasted rats (exposed divided by control) divided by the fractional decrease of GSH concentration in fed rats (exposed divided by control). If the same fractional decrease occurred in both groups, the ratio would be 1. Supporting the observation noted above, that fasted rats had a larger absolute decline in GSH than did fed rats, this fractional decrease ratio is less than 1 for the various fractions. Further, the fractional decrease ratio is greatest in the two mitochondrial fractions (0.56 and 0.59), which suggests that this cellular component was most severely affected by the reactive metabolite or toxic substance produced *in vivo* following 1,1-DCE exposure.

Because the data show that mitochondrial GSH concentrations were reduced to a greater extent than those in other fractions and because a decrease in oxygen uptake appeared to precede changes in serum AKT activity, we used two biochemical assays to verify that early injury had occurred to the mitochondria. One enzyme measured was serum SDH, a cytoplasmic marker with greater sensitivity to hepatic injury than elevation in serum AKT activity (Curtis et al., 1972; Jaeger et al., 1975a). The second enzyme, serum OCT, is a mitochondrial marker. Its appearance in the serum, if correlated with the appearance of SDH, would suggest that damage to the mitochondria occurred at the same time as or preceded that to the plasma membrane. The data in Table 6 indicate that significant serum SDH elevations occurred simultaneously with significant elevations of serum OCT. These two enzymes were related to one another with a correlation coefficient of 0.66 ($p < 0.05$). From this we infer that injury

TABLE 6. *Effect of Exposure on Serum Sorbitol Dehydrogenase (Cytoplasmic Marker) and Serum Ornithine Carbamoyl Transferase (Mitochondrial Marker)*[a]

	Serum activity	
Exposure	SDH[b] (IU/ml)	OCT[b] (Mol citrulline/ml-min)
Air (4)	7.4 ± 1.4	2.8 ± 1.0
1,1-DCE (10)	4,111 ± 604[c]	100.7 ± 28.7[c]

[a] Fasted Holtzman rats were exposed to 200–300 ppm 1,1-DCE for 4 hr and killed at 6 hr.
[b] Mean ± SEM.
[c] $p < 0.05$ compared to air group.

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in the mitochondria from fasted rats (2.24 and 1.71 μg GSH/mg protein) were significantly greater than those from fed rats (0.63 and 0.82 μg GSH/mg protein).

Further speculation on the toxicity of 1,1-DCE and its mechanism of action is warranted. D. Henschler (University of Würzburg, Germany) has suggested that a metabolic intermediate may be chloroacyl chloride, which could be derived from the dichlorooxirane of 1,1-DCE. Bonse et al. (1975) were unable to synthesize this compound, although they did synthesize chloroethylene oxide, the oxirane intermediate of vinyl chloride. Spontaneous rearrangement of chloroacyl chloride would lead to chloroacetic acid, a compound isolated from extracts of the isolated perfused liver after exposure to 1,1-DCE (Reichert and Bashti, 1976). Monochloroacetic acid has been reported by Hayes et al. (1973) to possess some toxicologic properties similar to those of monofluoroacetic acid, which is a specific mitochondrial toxin (Peters, 1958). In a preliminary communication, we suggested that monochloroacetic acid is toxic to mitochondria by a process of lethal synthesis—that is, through formation of chlorocitric acid—and we find that hepatic citric acid levels are elevated in livers of fasted rats exposed to 1,1-DCE (Jaeger and Coffman, 1977). Alternative explanations for the data reported here are possible, but the hypothesis that lethal synthesis may occur coupled with mitochondrial injury provides a basis for a productive line of further investigation.

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