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Effect of Pretreatment of Rats with Carbon Tetrachloride on Tolerance Development

THOMAS DAMBRAUSKAS AND HERBERT H. CORNISH

Department of Industrial Health and Institute of Industrial Health, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104

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The Effect of Pretreatment of Rats with Carbon Tetrachloride on Tolerance Development. DAMBRAUSKAS, THOMAS, and CORNISH, HERBERT H. (1970). *Toxicol. Appl. Pharmacol.* **17**, 83–97. To evaluate the effect of pretreatment of rats with CCl_4 on tolerance development to subsequent exposures, rats were exposed 25–7500 ppm of CCl_4 vapor or given CCl_4 orally, 3.25 mg/g body weight. Forty-eight hours later these rats were reexposed to a normally lethal 7500 ppm CCl_4 concentration. Mortality, blood clotting time, serum bilirubin, serum and liver triglycerides, and CCl_4 and $CHCl_3$ concentrations in tissues and in the whole rat were used as a measure of the response to CCl_4 exposure. Conversion of CCl_4 to $CHCl_3$ *in vitro* was also determined in normal and pretreated animals. It was found that animals exposed to 4000 ppm for 6 hr or given 3.25 mg/g of CCl_4 orally developed tolerance to subsequent normally lethal CCl_4 exposures. Serum bilirubin levels in animals protected by CCl_4 pretreatment were significantly lower than in those receiving only a single exposure to CCl_4. Blood clotting times in protected animals were normal, while in those not protected by pretreatment, they were greatly prolonged. Serum triglyceride levels in protected animals were normal whereas they were considerably depressed in the nonprotected animals. Liver triglycerides were elevated in both protected and nonprotected, but were not as high in the protected animals. Carbon tetrachloride conversion to $CHCl_3$ was slower in protected than in the nonprotected animals. Tolerance to CCl_4 developed in animals whose ability to metabolize CCl_4 had been depressed by pretreatment with the same compound. This suggests that the toxicity of CCl_4 is related to its metabolic pathway or to the production of toxic intermediates.

Exposure of living organisms to certain types of stress may have injurious or tolerance-developing effects depending upon the type and degree of stress. Tolerance development to ozone (Stokinger, 1965; Stokinger and Scheel, 1962), protection against lethal doses of X-irradiation by preexposure to ozone (Hattori *et al.*, 1963), reduction of radiation mortality through magnetic pretreatment (Barnothy, 1963), and protection against high doses of cadmium chloride by pretreatment with low doses (Terhaar *et al.*, 1965) have been reported. However, the effect of pretreatment and subsequent tolerance development is not uniform, and no general concept has been developed that would enable one to predict the effect of pretreatment on tolerance development or protection.

In the present study, the effect of pretreatment of rats with carbon tetrachloride on the development of tolerance to subsequent exposures was investigated.

METHODS AND MATERIALS

Male albino rats of the Sprague-Dawley[1] strain were used throughout the studies. The animals were maintained on an ad libitum diet of laboratory chow[2] except during the exposure and fasting period. Carbon tetrachloride was administered to animals by

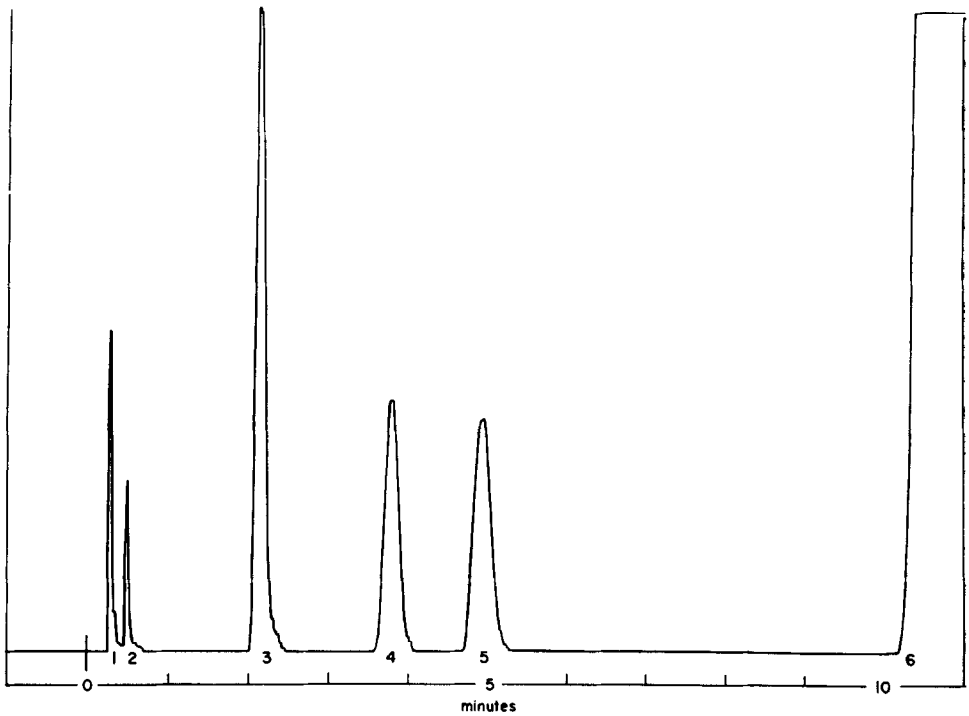

FIG. 1. Standard gas chromatogram. Peak 1 indicates CH_4; peak 2, CH_3Cl; peak 3, CH_2Cl_2; peak 4, CCl_4; peak 5, $CHCl_3$; and peak 6, toluene.

inhalation (Block and Cornish, 1958) at concentrations of 25–6000 ppm, 6 hr daily for periods of 1–5 days, and by the oral route at a dose of 3.25 mg/g. Animals were reexposed to lethal levels (7500 ppm of CCl_4) at various time intervals after the first exposure. Mortality data were procured after observation of treated animals for 14 days. Groups of normal control animals were included in all studies.

At appropriate time intervals after treatment, animals were sacrificed by stunning and decapitation. Liver and lungs were removed for histopathologic examination, and liver weights were determined. Liver and lung tissues were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin and Oil Red O for microscopic examination.

Blood clotting time was determined by the capillary tube method, and serum bilirubin by the standard Ehrlich's diazo procedure (Am. Assoc. Clin. Chem., 1953). The data were statistically processed by Duncan's New Multiple Range Test (Duncan, 1955).

Serum and liver triglycerides. Serum triglycerides were determined by the method of Van Handel and Zilversmit (1957), and liver triglycerides by the method of Butler *et al.*

[1] Spartan Research Animals, Inc., Haslett, Michigan.
[2] Purina.

(1961). Normal rat serum and liver triglyceride levels were determined on four, five rat-pool samples and on five individual rat samples. Using the *t* test for small samples, no significant difference between the mean liver triglyceride values of pooled and individual rat samples was found. Subsequent determinations of triglycerides were carried out on five rat-pool samples.

Carbon tetrachloride and chloroform analysis. Carbon tetrachloride and its metabolite, chloroform, were determined in rat tissues by microdiffusion and gas chromatographic techniques as described below.

A gas chromatograph[3] with hydrogen flame ionization detector was used for gas chromatographic analysis of chloromethanes. The stainless steel 5′ × 1/8″ column was filled with 5% Ucon-water[4] insoluble and 5% silicone[5] 550 w/w an Chromosorb P,[6] acid washed, mesh 45/60. The injector temperature at 80°C, column temperature at 45°C, carrier gas flow at 20 ml/min, hydrogen at 20 ml/min, and air flow delivered by a pump were kept constant at all times.

Mallinckrodt analytical reagent grade CCl_4, $CHCl_3$, and CH_2Cl_2, and Matheson CH_3Cl and CH_4 were used as standards for gas chromatography with toluene as a carrier solvent. The toluene, CCl_4, $CHCl_3$, and CH_2Cl_2 were purified by fractional distillation, and the purity was checked by gas chromatography.

The retention times of the various chloromethanes were determined by injection into the gas chromatograph of each component dissolved in toluene and by the injection of a number of mixtures of these compounds. Retention times were checked using two columns with different stationary phases. Figure 1 shows a typical chromatogram of the various chloromethanes.

The accuracy of the microdiffusion and gas chromatographic techniques was checked by the following procedure. Five-milliliter portions of diluted sulfuric acid (2:1) were introduced into 50-ml glass-stoppered Erlenmeyer flasks. A vial containing 1 ml of toluene was placed in each flask. Known amounts of pure CCl_4 and $CHCl_3$ or their mixtures were introduced below the surface of the sulfuric acid, and the flasks were immediately stoppered. Stoppers were lightly greased with Dow Corning silicone lubricant. Samples were shaken continuously for 18–26 hr at room temperature. Portions (0.2 μl) were withdrawn from each vial of toluene and injected into the gas chromatograph; recovery of CCl_4 and $CHCl_3$ was calculated by comparison of peak areas with the standard gas chromatogram. The recoveries of CCl_4 and $CHCl_3$ ranged from 96.5 to 103.5%.

The stability of CCl_4 in sulfuric acid was checked by placing CCl_4 in flasks prepared as above. No peaks other than that of CCl_4 were found on gas chromatograms even after a 10-day period.

For the determination of CCl_4 and $CHCl_3$ in tissues and in the whole rat, rats were sacrificed immediately after exposures by stunning and decapitation. Tissues were taken rapidly and placed in separate cold 50-ml Erlenmeyer flasks. In whole rat studies, animals were opened by a ventral incision and immediately placed in 500-ml Erlenmeyer flasks. Appropriate amounts of cold, (2:1) diluted sulfuric acid were added to digest the

[3] Wilkins Instrument and Research, Walnut Creek, California.
[4] Applied Science Laboratory, Inc., State College, Pennsylvania.
[5] Research Specialties Co., Richmond, California.
[6] Johns-Manville Corp., 22 E. 40th St., New York, N.Y.

tissues. Vials containing 1 ml of toluene were placed in each flask containing rat organs, and vials containing 2 ml of toluene were placed in flasks containing the whole rat to absorb CCl_4 and $CHCl_3$ released from tissues. After 24 and 48 hr shaking at room temperature, the quantity of chloromethanes in the toluene was determined as described above.

The *in vitro* conversion of CCl_4 to $CHCl_3$ by the liver of normal rats and by those exposed to 4000 ppm of CCl_4 48 hr prior to sacrifice was also studied (5 rats in each group). Normal and CCl_4-treated animals were sacrificed; livers were removed, sliced (approximately 2–3 mm in thickness), and placed in 50-ml Erlenmeyer flasks containing 6 ml of distilled water. One microliter of CCl_4 was added to each flask; flasks were sealed immediately and incubated for 24 hours at 37°C. At the end of the incubation period, a portion of gas was removed from each flask and subjected to gas chromatographic analysis for chloromethanes.

RESULTS

Mortality and Tolerance Development

The LD100 exposure to CCl_4 was found to be 7500 ppm for 6 hr. During reexposure studies described below, rats were exposed to 7500 ppm for 5.5 hr to allow survival for 24 hr in order to provide tissues for additional studies. Even at this level of exposure, usually not more than 2 of 20 rats survived for 14 days after exposure.

TABLE 1

EFFECT OF PRETREATMENT ON MORTALITY OF RATS EXPOSED TO CARBON TETRACHLORIDE

Pretreatment				Exposure to 7500 ppm of CCl_4 for 5.5 hrs.		
					Survival	
Number of runs	CCl_4 ppm	Exposure (hours)	Survival	Hours after pretreatment	Pretreated	Not pretreated (controls)
1	6000	6	11/20[a]	120	11/11[a]	2/20[a]
1	4000	6	20/20	96	20/20	2/20
1	4000	6	20/20	72	20/20	2/20
2	4000	6	20/20	48	20/20	1/20
1	4000	6	20/20	24	14/20	2/20
2	2000	6	20/20	48	16/20	4/20
2	1000	6	20/20	48	1/20	2/20
2	100	6	20/20	48	0/20	3/20
1	50	6	20/20	48	1/20	1/20
1	25	6	20/20	48	3/20	5/20
1	1000	6 (2 days)	24/24	48	6/24	4/24
1	400	6 (5 days)	24/24	48	5/24	5/24
1	50	8 (5 days)	24/24	48	6/24	5/24
1	25	8 (5 days)	24/24	48	4/24	4/24
1	3.25 mg/g	Oral	19/20	48	19/19	0/20
1	0 (Fasted, 48 hr)	—	20/20	48	0/20	2/20

[a] Number showing survival/number tested.

The effects of exposure to CCl_4 on the development of tolerance to subsequent lethal doses are summarized in Table 1.

Rats preexposed to 6000 ppm, 4000 ppm, and those given an oral 3.25 mg/g CCl_4 dose, 48 hours thereafter all developed tolerance to a normally lethal CCl_4 exposure. Only partial tolerance was developed (14/20) when rats were preexposed to 4000 ppm and after 24 hr reexposed to a normally lethal level of CCl_4. Similarly, partial tolerance (16/20) developed when rats were preexposed to 2000 ppm and after 48 hr reexposed to a normally lethal dose. No tolerance was developed when rats were preexposed to CCl_4 concentrations below 2000 ppm, whether after a single acute 6-hour preexposure or after extended 2–5 days preexposures.

A complete protective effect was still evident 96 hr after preexposure to 4000 ppm for 6 hr and 120 hr after a 6-hr preexposure to 6000 ppm.

Animals also were protected (19/20) against a normally lethal exposure to CCl_4 when preexposed to 4000 ppm of $CHCl_3$ for 1 hr, and against a normally lethal dose of $CHCl_3$ (4200 ppm for 5 hr) when they were preexposed to 4000 ppm of CCl_4 for 6 hr. Again, only partial protection (13/20) was observed when rats were preexposed to 2000 ppm of CCl_4 for 6 hr and 48 hr later reexposed to a normally lethal dose of $CHCl_3$.

Liver Weight

Slight decrease in liver weight was noted only immediately after the 6-hr single CCl_4 exposures and marked decrease only in fasted rat liver. No changes in liver weight were noted in rats receiving the two CCl_4 exposures.

Blood Clotting Time and Serum Bilirubin

Blood clotting times and serum bilirubin levels of rats exposed to CCl_4 or fasted and subsequently exposed to CCl_4 are presented in Table 2.

TABLE 2

BLOOD CLOTTING TIME AND SERUM BILIRUBIN OF RATS EXPOSED TO CARBON TETRACHLORIDE

CCl_4 ppm	No. of rats	Sacrificed hours after treatment	Serum bilirubin (mg/100 ml)		Blood clotting time (min)
			Direct	Total	
0	5	—	0.03 ± 0.01[a]	0.32 ± 0.16[a]	2.2 (2.0–2.5)[b]
4000[c]	5	24	0.14 ± 0.03[e]	1.02 ± 0.40[e]	1.7 (1.0–2.5)
7500[d]	5	24	2.09 ± 0.15[e]	3.79 ± 0.21[e]	21.0 (20.0–22.0)
4000[c] + 7500[d]	5	24	0.43 ± 0.07[e]	0.89 ± 0.14[e]	2.4 (2.3–2.5)
0 (Fasted, 48 hr)	5	24	0.09 ± 0.04	0.23 ± 0.13	2.1 (1.6–2.5)
7500[d] (Fasted, 48 hr)	5	24	2.52 ± 0.36[e]	6.00 ± 0.68[e]	44.0 (33.5–55.0)

[a] Mean ± standard deviation.
[b] Ranges in parentheses.
[c] Exposed for 6 hr.
[d] Exposed for 5.5 hr.
[e] Significantly different from normal, $P < 0.01$.

TABLE 3

TRIGLYCERIDES IN RAT SERUM AND LIVER AFTER SINGLE OR DOUBLE EXPOSURES TO CARBON TETRACHLORIDE

CCl_4 ppm	Triglycerides after single exposure: No. of 5 rat-pool runs	Triglycerides after single exposure: In liver (mg/g)	Triglycerides after single exposure: In serum (mg/100 ml)	Triglycerides after reexposure to 7500 ppm for 5.5 hr: Sacrificed, hours after exposure	Triglycerides after reexposure to 7500 ppm for 5.5 hr: No. of 5 rat-pool runs	Triglycerides after reexposure to 7500 ppm for 5.5 hr: In liver (mg/g)	Triglycerides after reexposure to 7500 ppm for 5.5 hr: In serum (mg/100 ml)
0	5	4.8 ± 0.8[a]	133.5 ± 24.2[a]	—	—	—	—
500[d]	1	7.6	48.5	0	1	15.9	14.4
	2	11.1 ± 1.6[b]	137.0 ± 2.4[b]	24	2	47.5 ± 0.1[b]	27.0 ± 10.4[b]
	2	8.5 ± 0.2	163.2 ± 4.2	48			
	2	6.3 ± 0.3	163.5 ± 17.5	72			
1000[d]	1	10.3	26.2	0	1	21.8	17.0
	2	16.3 ± 3.9	84.5 ± 3.0	24	2	53.4 ± 0.3	20.6 ± 2.4
	2	15.7 ± 3.0	110.7 ± 2.6	48			
	2	10.4 ± 0.0	127.5 ± 12.5	72			
4000[d]	1	16.6	16.5	0	2	36.6 ± 0.7	128.5 ± 2.5
	2	35.1 ± 2.7	32.7 ± 4.3	24	4	32.7 ± 7.3	124.4 ± 37.6
	2	36.6 ± 6.6	86.2 ± 2.7	48			
	2	18.4 ± 1.5	100.2 ± 15.2	72			
7500[e]	2	12.9 ± 1.2	2.6 ± 2.6	0	—	—	—
	3	48.0 ± 4.5	29.6 ± 18.6	24	—	—	—
0	1	4.6	36.4	0 (48)[c]	1	19.2	22.7
(Fasted, 48 hr)	2	6.9 ± 2.4	24.2 ± 0.8	24 (72)	2	38.1 ± 1.8	32.4 ± 9.6

[a] Mean ± standard error.
[b] Mean ± mean deviation.
[c] Total hours elapsed from the beginning of treatment.
[d] Exposed for 6 hr.
[e] Exposed for 5.5 hr.

Fasting or exposure of animals to 4000 ppm of CCl_4 had no effect on blood clotting time as shown in Table 2. Blood clotting time was prolonged only when rats were killed 24 hr after exposure to 7500 ppm, whether they were fasted or not. Animals preexposed to 4000 ppm of CCl_4 and subsequently exposed to 7500 ppm, had normal blood clotting times.

Twenty-four hours after exposure to CCl_4, all bilirubin levels were significantly elevated. However, rats receiving a single exposure to 7500 ppm had bilirubin levels 4 to 5 times higher than those of rats exposed to 4000 ppm for 6 and 48 hr later reexposed to 7500 ppm. A 48-hr fasting period had no effect on serum bilirubin levels. Fasted animals, however, appeared to be more susceptible to CCl_4 exposure as evidenced by the enhanced elevations of both direct and total serum bilirubin levels when animals were exposed to 7500 ppm after 48 hr of fasting.

Serum and Liver Triglycerides

Serum and liver triglyceride levels were determined in fasted rats and in those receiving single or double exposures to CCl_4. The results are presented in Table 3.

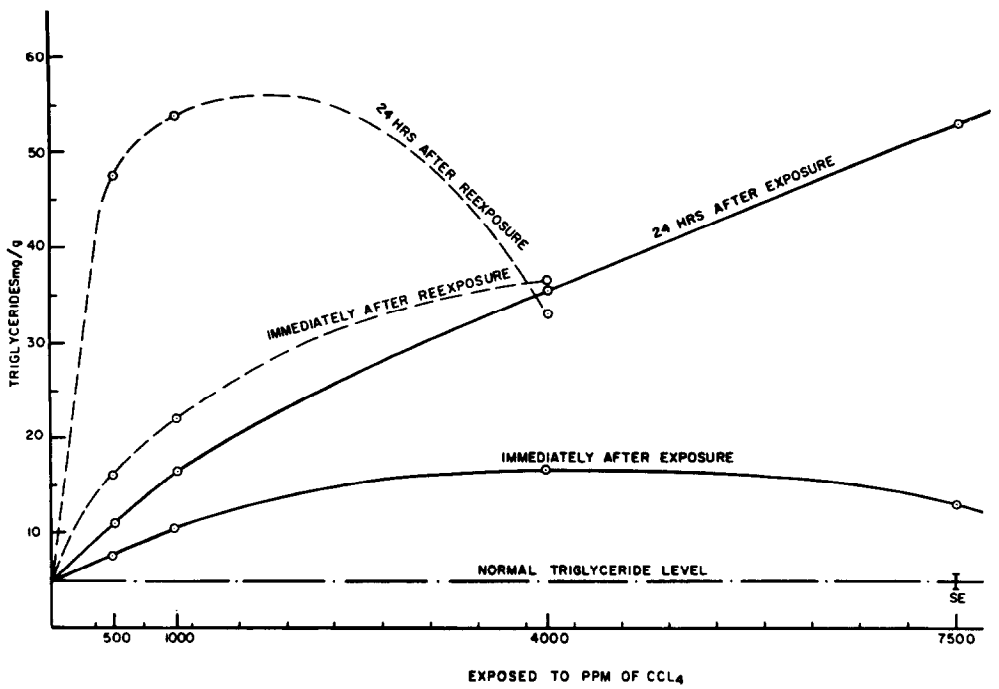

FIG. 2. Liver triglyceride levels of rats receiving single and double CCl_4 exposures. Solid line indicates single, and broken line double, exposure.

In general, liver triglyceride concentrations of rats exposed to various CCl_4 vapor concentrations were increased and serum triglycerides decreased progressively with increasing exposure concentrations. No changes in liver triglycerides were found in 48-hr fasted rats. After reexposure, changes in serum and liver triglyceride concentrations were somewhat greater than after the single exposure, with the exception of the rats preexposed to 4000 ppm and 48 hr later reexposed to 7500 ppm. In these animals, serum triglycerides were normal.

The liver and serum triglyceride levels in rats preexposed to CCl_4 concentrations as indicated in Table 3 and 48 hr later reexposed to 7500 ppm are illustrated graphically in Figs. 2 and 3.

Twenty-four hours after single 500–7500 ppm CCl_4 exposures, the increase in rat liver triglycerides (Fig. 2) was almost linear, varying directly with the dose. When rats were exposed to 500 or 1000 ppm for 6 hr (nonprotective dose) and after 48 hr were reexposed to 7500 ppm for 5.5 hr (lethal dose), liver triglyceride values increased sharply,

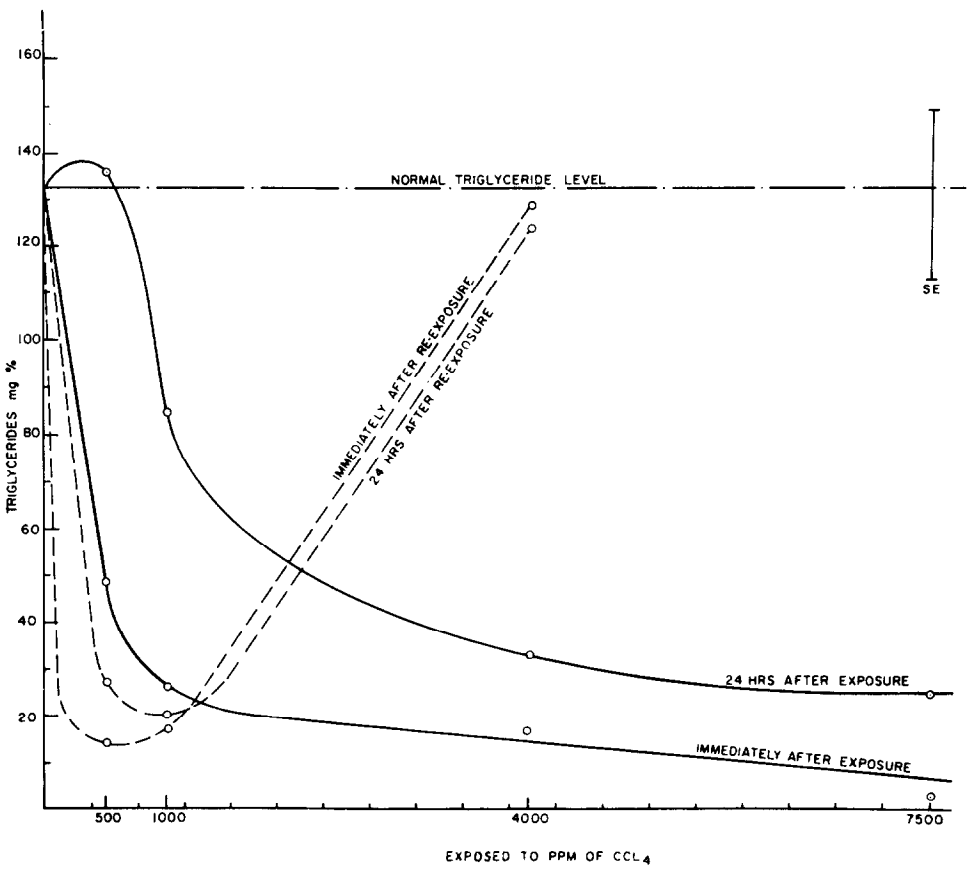

FIG. 3. Serum triglyceride levels of rats receiving single and double CCl_4 exposures. Solid line indicates single, and broken line double, exposure.

as compared to values after single exposures. However, liver triglycerides of rats exposed to 4000 ppm (protective dose) and reexposed to 7500 ppm remained at the same levels as in rats receiving a single 4000 ppm exposure.

Serum triglyceride changes in rats receiving protective and nonprotective exposures to CCl_4 were the opposite of those found in liver (Fig. 3). Immediately and 24 hr after single 500–7500 ppm exposures, triglyceride levels decreased progressively with increased exposure concentration. A still further decrease in serum triglycerides was found in rats exposed to 500–1000 ppm and 48 hr later reexposed to 7500 ppm. However,

in rats exposed to 4000 ppm and then reexposed to 7500 ppm, serum triglycerides increased and regained their normal levels.

Carbon Tetrachloride and Chloroform in Rat Tissues

Carbon tetrachloride was detectable in the tissues of rats after exposure to this compound (Fig. 4). In addition, chloroform was also found in the liver of exposed animals.

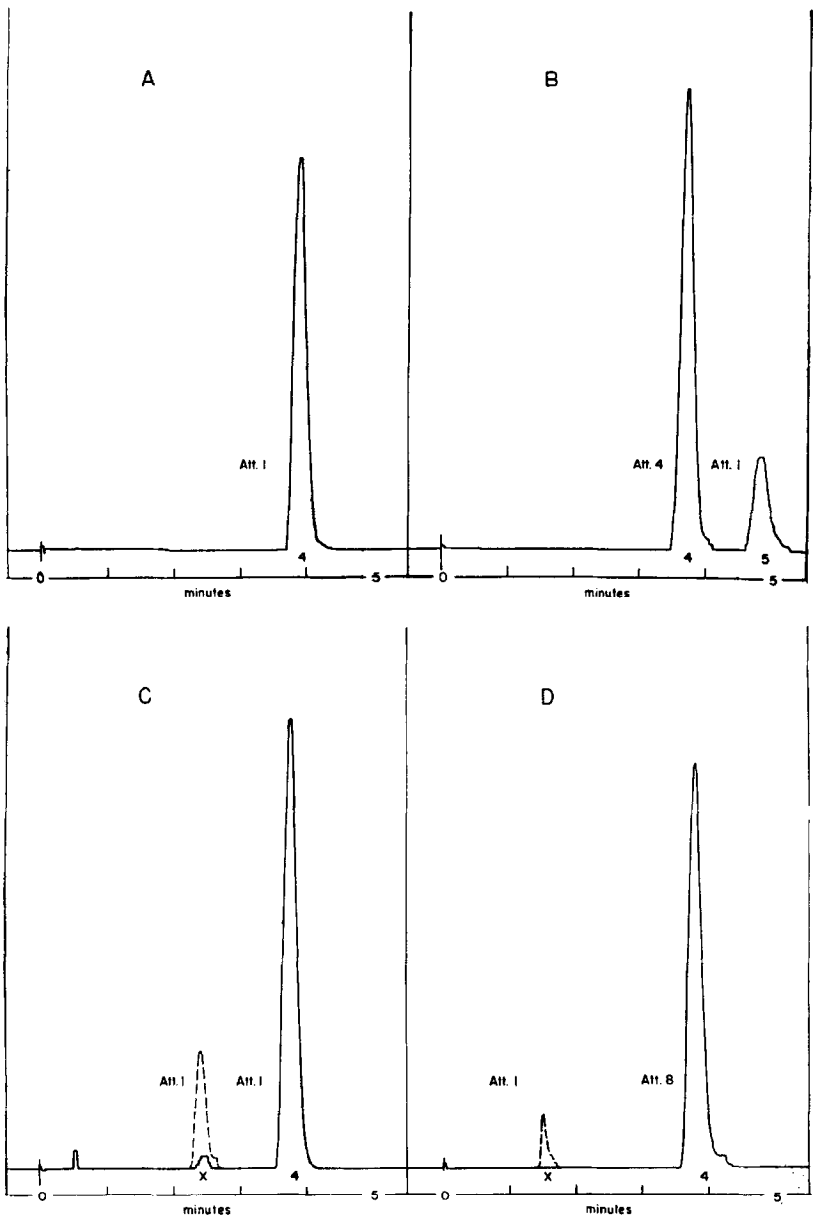

FIG. 4. Gas chromatogram of tissue after exposure to 4000 ppm of CCl_4. A indicates recovery of CCl_4 from blood; B, recovery of CCl_4 and $CHCl_3$ from liver; C, recovery of CCl_4 from kidney, D, recovery of CCl_4 from fat. Broken line peaks indicate an occasional appearance of unidentified components.

Figure 4 shows typical gas chromatograms of blood (A), liver (B), kidney (C), and fat (D) of animals exposed to 4000 ppm of CCl_4 and sacrificed immediately after exposure. Carbon tetrachloride is identified by peak 4 and chloroform by peak 5. An unidentified peak (broken line) appeared occasionally on chromatograms of kidney and fat of both control and exposed rats.

The concentration of CCl_4 and $CHCl_3$ found in rat organs is presented in Table 4.

TABLE 4

RECOVERY OF CARBON TETRACHLORIDE AND CHLOROFORM FROM RAT TISSUES IMMEDIATELY AFTER EXPOSURE TO CARBON TETRACHLORIDE

	Blood	Liver		Kidney	Fat	Brain
CCl_4 (ppm)	CCl_4 (μg/g)	CCl_4 (μg/g)	$CHCl_3$ (μg/g)	CCl_4 (μg/g)	CCl_4 (μg/g)	CCl_4 (μg/g)
4000[a]	40	146	9	219	1200	426
	75	129	10	228	1023	319
	82	135	12	277	2100	440
	36	87	7	229	1885	442
	85	182	9	213	2162	—
Mean ± SD	63.6[c] ± 23.7	135.8[c] ± 34.2	9.6 ± 1.80	233.2[c] ± 25.4	1674.0 ±527.4	406.7[c] ± 58.9
7500[b]	115	510	11	341	1795	970
	129	397	11	458	1715	980
	116	397	10	462	1750	1070
	157	428	11	455	1575	1080
	139	450	8	384	2445	—
Mean ± SD	131.2[c] ± 17.5	436.4[c] ± 46.8	10.2 ± 1.30	420.0 ± 54.7	1856.0 ± 339.4	1025.0 ± 58.0
4000[a]	157	635	7	490	3620	1010
+	167	478	8	497	4980	940
7500[b]	180	786	8	451	5930	985
	173	634	7	584	5180	835
	187	647	5	408	5900	—
Mean ± SD	172.8[c] ± 11.6	636.0[c] ± 109.8	7.0[c] ± 1.23	486.0 ± 65.3	5122.0[c] ± 940.4	942.5 ± 77.3

[a] Exposed for 6 hr.
[b] Exposed for 5.5 hr.
[c] Significantly different, $P < 0.01$.

After exposure to 4000 ppm, CCl_4 concentrations in all tissues, except fat, were, as expected, significantly lower than after exposures to 7500 ppm for 5.5 hr. In animals exposed to 4000 ppm and reexposed 48 hr later to 7500 ppm, the concentration of CCl_4 in blood, liver, and fat was significantly higher than in animals receiving a single exposure to 7500 ppm of carbon tetrachloride. The concentration of $CHCl_3$ in the liver of animals previously exposed to 4000 ppm and subsequently reexposed to 7500 ppm of CCl_4, although not of great magnitude, was significantly lower than in the animals receiving a single exposure to 7500 ppm.

Since animals receiving a single or double CCl_4 exposure might have different rates of absorption, distribution, and excretion of carbon tetrachloride and its metabolites, the concentration of CCl_4 and $CHCl_3$ in the whole rat was determined after single and double exposures to CCl_4. A composite chromatogram of a series of such determinations is shown in Fig. 5.

The carbon tetrachloride peak (4) and chloroform peak (5) were present on all chromatograms. Peaks marked by x are unidentified compounds, some of them also

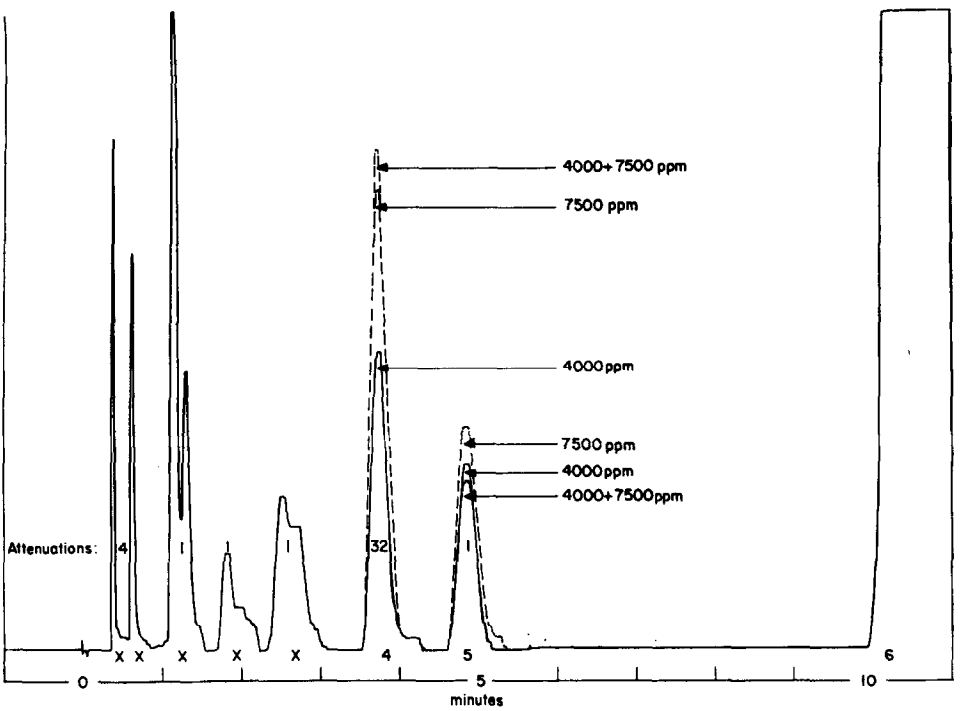

FIG. 5. Gas chromatogram indicating recovery of CCl_4 (peak 4) and $CHCl_3$ (peak 5) from whole rat after exposure to 4000 ppm of CCl_4. Peaks × denote unidentified components, some of which also appear in normal rats. Broken line peaks were transposed from other gas chromatograms after corresponding exposures to CCl_4.

appearing on chromatograms from normal animals. The recovery of CCl_4 and $CHCl_3$ from the whole rat is presented in Table 5.

Almost double the amount of $CHCl_3$ was found in animals receiving a single 7500 ppm exposure compared to the amount of $CHCl_3$ in animals after two exposures (4000 and 7500 ppm), whereas the total amounts of CCl_4 in these two groups of animals were quite comparable. The amount of $CHCl_3$ per gram of body weight was approximately the same, 1.23 ± 0.16 and 1.37 ± 0.07 μg/g, in the two groups of rats that survived, i.e., after a single 4000 ppm and double (4000 + 7500 ppm) exposures, respectively.

The effect of preexposure to CCl_4 on the conversion of CCl_4 to $CHCl_3$ *in vitro* was the same as *in vivo*; normal rat liver *in vitro* produced 13.7 ± 1.9 μg $CHCl_3$ per gram of liver, compared to 3.5 ± 1.2 μg/g produced by liver of rats sacrificed 48 hr after preexposure to 4000 ppm of CCl_4.

TABLE 5

RECOVERY OF CARBON TETRACHLORIDE AND CHLOROFORM FROM THE WHOLE RAT IMMEDIATELY AFTER EXPOSURE TO CARBON TETRACHLORIDE

CCl_4 (ppm)	Rat weight (g)	CCl_4		$CHCl_3$	
		Total (μg)	Per gram (μg)	Total (μg)	Per gram (μg)
4000[a]	219	14363	66	292	1.33
	200	16220	81	278	1.39
	228	12704	56	236	1.04
	194	12540	66	222	1.14
Mean ± SD	210	14031[c] ± 1641	67.2[c] ± 10.5	257.0 ± 33.3	1.23 ± 0.16
7500[b]	220	32700	149	507	2.30
	191	28400	149	413	2.16
	235	28255	120	408	1.74
	226	30310	134	422	1.87
Mean ± SD	218	29916 ± 2078	137.9 ±13.6	437.5[c] ± 46.7	2.02[c] ± 0.26
4000[a] + 7500[b]	189	30220	160	275	1.46
	205	29400	143	274	1.34
	199	33760	170	258	1.30
	203	24150	119	279	1.37
Mean ± SD	198	29382 ± 3968	148.0 ± 22.1	271.5 ± 9.3	1.37 ± 0.07

[a] Exposed for 6 hr.
[b] Exposed for 5.5 hr.
[c] Significantly different, $P < 0.01$.

Histopathology

A summary of the histopathologic findings is given below.

The lungs of exposed animals were comparable to those of control animals.

The following morphologic changes were found in liver:

1. At 24 hr after exposure to 4000 ppm for 6 hr: moderate to extensive centrilobular necrosis with infiltrative mononuclear cells; the necrotic areas were circumscribed by large vacuolated cells; there was extensive to severe lipid infiltration around the necrotic areas, several vacuolated areas which did not stain for fat.

2. At 48 hr after exposure to 4000 ppm for 6 hr: there was moderate centrilobular necrosis with marked vacuolization of adjacent parenchymal cells; slight to moderate lipid infiltration most pronounced around the necrotic areas.

3. At 24 hr after exposure to 7500 ppm for 5.5 hr: there was centrilobular cloudy swelling of hepatic parenchymal cells, hyaline and hydropic degeneration; in some rats there was nuclear pyknosis and karyorrhexis of hepatic parenchymal cells but no necrosis; moderate lipid infiltration most pronounced in periportal areas.

4. At 24 hr after double exposure (4000 ppm for 6 hr then 48 hr later exposed to 7500 ppm for 5.5 hr): there was slight to moderate centrilobular necrosis, widespread hydropic

degeneration and cloudy swelling of parenchymal cells, moderate lipid infiltration being most extensive in periportal areas.

DISCUSSION

Most animals died within 24–48 hr when exposed to 7500 ppm of CCl_4 for 5.5 hr or when preexposed to CCl_4 concentrations below 2000 ppm for 6 hr and 48 hr later re-exposed to 7500 ppm. Twenty-four hours after exposure these animals were obviously sick, with shabby fur, slow movement, and no food consumption.

Animals exposed to 4000 and 6000 ppm of CCl_4, or given 3.25 mg/g orally, developed tolerance and were protected against the acute toxicity of CCl_4 when reexposed 48 hr later to a normally lethal dose. These animals retained a healthy normal appearance and their food consumption was about 50% of normal. Though CCl_4-exposed rats did not eat as much as control rats, fasting apparently was not involved in protection since 48 hr fasted rats were not protected against the same lethal exposure to CCl_4.

For tolerance development and protection against a lethal dose of CCl_4, preexposure concentration (sufficient insult) and a minimum time interval (approximately 48 hr) prior to reexposure were the essential prerequisites.

Serum bilirubin concentration and blood clotting time seemed to parallel the degree of protection. Twenty-four hours after exposure to 7500 ppm for 5.5 hr, rats had markedly elevated bilirubin levels as well as greatly prolonged clotting times. When animals were pretreated with 4000 ppm of CCl_4 48 hr prior to the 7500 ppm exposure, bilirubin levels were only slightly elevated and blood clotting times were normal. Thus tolerance development was indicated not only in terms of decreased mortality, but also in biochemical parameters.

Development of fatty liver is a characteristic phenomenon associated with CCl_4 toxicity. Decreased serum triglyceride levels and concomitant elevations of liver triglycerides in animals treated with CCl_4 have been reported by a number of investigators (Heimberg *et al.*, 1962; Lombardi, 1965). During the investigation described in this paper, increase in liver triglycerides and a concomitant decrease in serum triglycerides were in direct correlation with the degree of exposure except in rats doubly exposed to CCl_4.

According to one recently advanced hypothesis, the earliest morphologic changes in the hepatic cell after CCl_4 intoxication, are swelling and disruption of the endoplasmic reticulum resulting in inhibition of protein synthesis. The inhibition of protein synthesis would prevent the formation and release of glycerides as lipoprotein complexes (Smuckler *et al.*, 1961; Seakins and Robinson, 1963; Lombardi, 1965).

In our studies, progressively higher CCl_4 exposures (500–7500 ppm) paralleled by greater accumulation of triglycerides in liver and concomitant decreases in serum might reflect the degree of the initial biochemical lesion and resultant greater depression of protein synthesis. The same, but more pronounced, triglyceride departure from the normals were observed after previously exposed rats were reexposed to 7500 ppm, with the exception of rats previously exposed to 4000 ppm. After exposure to 7500 ppm of CCl_4, liver triglycerides reached the highest level, 48.0 mg/g at 24 hr, and serum triglycerides the lowest level, 2.6 mg/100 ml immediately after exposure. At the same time, rats previously exposed to 4000 ppm and after 48 hr reexposed to 7500 ppm had normal serum triglyceride levels, 128 mg/100 ml, while liver triglyceride levels, though

elevated (32 mg/g), were about the same as after a single exposure to 4000 ppm (35 mg/100 ml).

The histopathologic findings in the various groups of animals do not appear to relate directly to the protection resulting from preexposure.

The protection afforded by pretreatment with carbon tetrachloride is apparently different from the tolerance developed to ozone as reported by Stokinger (1965), where the development of lung edema appeared to be a prerequisite for protection. Though lung irritation may be produced by high levels of CCl_4 exposure, there was no histologic evidence that such occurred in the present studies. In addition, the protective mechanism was evoked even when the first contact with CCl_4 was by the oral route.

Microscopic examination of the liver in several series of rats showed that in animals sacrificed 24 hr after receiving a single exposure to either 4000 ppm (6 hr) or 7500 ppm (5.5 hr), moderate centrilobular necrosis was evident only in the group exposed to 4000 ppm. Those animals exposed to 7500 ppm of CCl_4 in general showed cellular changes characteristic of earlier stages of degeneration including pronounced vacuolization of periportal parenchymal cells and some scattered pyknosis. The reexposure to 7500 ppm of rats previously exposed to 4000 ppm of CCl_4 did not induce significant additional morphologic changes in liver when compared to animals sacrificed 48 hr after a single 4000 ppm exposure. However, animals exposed to 7500 ppm died, and those exposed to 4000 and 7500 ppm survived. This is consistent with the hypothesis that the initial effect of CCl_4 on the liver is the induction of a biochemical lesion (Brauer *et al.*, 1961) and with the hypothesis that morphologic and functional liver changes are the results of the homolytic cleavage processes of carbon tetrachloride metabolism in the liver cells (Recknagel and Goshal, 1966; Butler, 1961).

Carbon tetrachloride concentrations in the various tissues did not appear to have a direct bearing on mortality. Rats protected by previous exposures to CCl_4 had consistently lower tissue levels of $CHCl_3$ than rats receiving a single lethal exposure to CCl_4. This suggested that the rate of conversion of CCl_4 to $CHCl_3$ was considerably depressed in the protected animals at the time of the second exposure.

Butler (1961) and Paul and Rubinstein (1963) reported the partial conversion of CCl_4 to $CHCl_3$ in rats. In the present studies, greater quantities of $CHCl_3$ were found in rats after a single 7500 ppm exposure than in those exposed to 4000 ppm and 48 hr later reexposed to 7500 ppm of CCl_4. The *in vitro* studies similarly show that the conversion of CCl_4 to $CHCl_3$ by normal rat livers is about four times faster than in rats previously exposed to 4000 ppm of CCl_4, suggesting that a direct relationship may exist between the rate of metabolism of carbon tetrachloride and its toxicity.

The formation of free radicals during metabolism of CCl_4 has been suggested by Butler (1961). Wirtschafter and Cronyn (1964) also studied the free radical mechanism and suggested that the toxicity of CCl_4 and $CHCl_3$ is the result of free radical cleavage and the subsequent combination of the free radicals with cell constituents. The protective action of various antioxidants (Gallagher, 1962) also lends support to the hypothesis that the breakdown of CCl_4 with the formation of free radicals may be the initial step in CCl_4 toxicity. For details of the various theories of CCl_4 toxicity, one should consult the comprehensive review by Recknagel (1967). In the present study, preexposure to CCl_4 decreased the rate of CCl_4 metabolism and might have protected the animal against subsequent high level exposures by diminishing the formation of active free radical

intermediates. A mechanism of toxicity, based upon the metabolism of CCl_4 is consistent with results obtained in the present study.

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