EFFECT OF "DRUGS FOR LIVER DISEASE" ON HEPATOTOXIC ACTION OF CARBON TETRACHLORIDE

I. CHANGES OF LYSOSOMAL ENZYME LEVELS AND EFFECT OF PROTOPORPHYRIN ON THE LEVELS

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Abstract—In order to clarify the action of drugs for liver disease, the effect of protoporphyrin (PP) on CCl_4 -induced liver injury was studied. Attention was given to the levels of lysosomal enzymes, some components of the liver, and inhibition of enzymes and lysis of lysosomal membranes by lipid peroxides. Administration of PP to CCl_4 -poisoned rats was found to prevent the decrease in lysosomal lipolytic enzyme level in the liver, but not in other enzyme levels tested. The inhibition of lipolytic enzyme by CCl_4 administered may be partially involved in lipid accumulation in the liver. A dose of PP administered to CCl_4 -poisoned rats for 8 days depressed the neutral lipid content in the liver nearly to the control value. Methyl linoleate hydroperoxide (hydroperoxide) at a lower concentration of $10^{-6}\%$ inhibited the lipolytic enzyme activity by 30% and in concentrations ranging from 10^{-4} to $10^{-3}\%$ inhibited β -glucuronidase activity. Addition of PP to the medium containing 10^{-6} to $10^{-5}\%$ hydroperoxide and α -tocopherol reduced the enzyme inhibition further than in the absence of PP. The hydroperoxide in concentrations varying from 10^{-8} to $10^{-3}\%$ caused a partial lysis of liver lysosomal membranes, but addition of PP slightly reduced the damage by the hydroperoxide in concentrations lower than $10^{-5}\%$.

The existence of lysosomes containing a variety of acid hydrolases in rat liver, first recognized in 1955 by de Duve et al. (1), drew attention to the possibility that the segregation of such hydrolytic enzymes might be a mechanism for control and restriction of autolysis. There is a fair amount of evidence that lysosomes are concerned with the process of intracellular digestion and are involved in various physiological and pathological phenomena of autolysis and necrosis (2). Much biochemical work has been done in attempts to elucidate the pathogenesis of the liver injury produced by CCl_4 (3-5). It is known that administration of a suitable dose of CCl_4 to rats results in a severe centrilobular type of necrosis and fatty degeneration of the liver (6), profound metabolic disturbances such as changes in the hepatic level of enzymes (7) and defective protein synthesis in the injured liver (8, 9). Dianzani showed that after CCl_4 poisoning, there was evidence for uncoupling of oxidative phosphorylation, loss of mitochondrial pyridine nucleotides, and lowering of the liver content of adenosine 5'-triphosphate (5, 10). It is also shown that lysosomes are involved in the later scavenging process of liver injury (11).

At present, drugs such as protoporphyrin (PP), are clinically used for liver diseases. However, little is known of the effects of these drugs on the lysosomal enzymes of an injured liver. To clarify their action, the effects of PP on the decreased lysosomal enzyme

levels and fatty accumulation in rat liver during CCl_4 intoxication were studied, and in addition the enzyme inhibition and the lysis of lysosomal membranes with methyl linoleate hydroperoxide (hydroperoxide) were investigated herein.

MATERIALS AND METHODS

Materials: Protoporphyrin disodium salt was a gift from Dozin Iyakukako. p-Nitrophenyl phosphate disodium salt and di-p-nitrophenyl phosphate lithium salt from Seikagaku Kogyo Co., Ltd., tributyrin, methyl linoleate and DL- α -tocopherol from Wako Junyaku Co., Ltd. and phenolphthalein glucuronide cinchonidine from Calbiochem were also used in this experiment.

Animals: Male Wistar rats weighing about 100 g, maintained on Oriental diets, MF, for 3–4 days prior to the experiments, were used. The animals were divided at random into 3 groups and each group included 4–5 rats. Food was provided *ad libitum*. (A) Controls were treated for 3 days with daily s.c. injections of olive oil at a dose of 0.1 ml/100 g body weight and with daily oral administrations of water (about 0.5 ml/100 g body weight) given 2 hr after the olive oil injection. These animals were also given water orally once a day from day 4 till day 8 (Control-rats). (B) Animals were treated for 3 days with daily s.c. injections of a mixture of 0.1 ml of CCl_4 and 0.1 ml of olive oil/100 g body weight and with daily oral administration of PP (0.3 mg/100 g body weight) solution which was given 2 hr after the injection of the mixture. The animals were also given PP orally once a day from day 4 till day 8 (PP-rats). (C) Animals were treated as described in B), except that water was given instead of the drug solution (CCl_4 -rats).

All animals were sacrificed by decapitation 2 hr after the final administration of the drug or water at 3 and 8 days, respectively. The doses of the drug to rats were double the clinically effective daily doses.

Preparation of liver lysosomal fractions: The lysosomal fractions (heavy lysosomal fractions) were prepared by a modification of the method of Tanaka (12). The liver was removed, weighed and homogenized in 4 volumes of ice-cold 0.25 M sucrose, using 5 strokes of the Teflon pestle. The homogenate was centrifuged at $800 \times g$ at 2°C for 15 min. The precipitate was discarded and the supernatant obtained was centrifuged for 15 min at $6,000 \times g$. The sediment (lysosomal fraction) was washed with ice-cold 0.25 M sucrose and recentrifuged under the same conditions, and then the washed sediment was resuspended in the sucrose to make a solution containing 1.25 g of liver equivalent per ml. The supernatant obtained was further centrifuged for 15 min at $15,000 \times g$ and the upper layer was used for the assays of the enzyme activity of unsedimentable fractions.

Preparation of hydroperoxide: Hydroperoxide was prepared by the oxidation of methyl linoleate according to the method of Banks et al. (13).

Assays of enzymes: The lysosomal fractions were sonicated for 3 min at 10 kc under chilling with ice-NaCl and centrifuged for 15 min at $15,000 \times g$. The supernatant was used for the assay of each enzyme. Triton X-100 was not used as a labilizer of lysosomal enzymes, because the activities of lipolytic enzyme [EC 3.1.1.3] and β -glucuronidase [EC 3.2.1.31]

were partially inhibited by this surfactant at a final concentration of 0.1–0.5% (v/v): At 0.1%, 7 and 37% decrease in the activities was given, respectively, while acid phosphatase [EC 3.1.3.2] activity was not inhibited (unpublished data).

Acid phosphatase activity was assayed according to the procedure of Symons et al. (14) with a slight modification. Total volume of 3.5 ml contained 1 ml each of 0.015 M p-nitrophenyl phosphate, 0.09 M citrate buffer of pH 4.8 and 0.1 M NaOH and 0.5 ml of the diluted supernatant.

β -Glucuronidase activity was assayed by the same method as described by Fishman et al. (15) except that 1 ml of acetate buffer was used.

The method of Dole (16) was adopted for the assay of lipolytic enzyme activity. Incubation mixture consisted of 1 ml of 0.1 M tributyrin dispersed in a 5% solution of gum acacia, pH 8.6, 1 ml of 0.1 M tris-HCl buffer, pH 8.6, containing 4% albumin and 0.5 ml of the enzyme solution. The reaction was stopped by adding 5 ml of isopropanol-n-heptane-0.05 M H_2SO_4 (40: 10: 1, by volume) containing 1.5% polyoxyethylene lauryl alcohol (Brij 35) (17).

Phosphodiesterase [EC 3.1.4.1] activity was assayed according to the method of Björk (18).

One unit of enzyme was defined as the amount of enzyme which was able to liberate 1 μ mole of product per min under the conditions tested.

Determination of lipid peroxides and sulfhydryl groups: Lipid peroxides were measured by the thiobarbituric acid reaction with the modification of Zalkin and Tappel (19) to avoid interference by sucrose. The lysosomal preparation was subjected to extraction in an equal volume of 10% trichloroacetic acid. After centrifugation, 4 ml of the supernatant was reacted with 1.25 ml of 0.75% thiobarbituric acid at 80°C for 10 min.

Sulfhydryl groups were assayed according to the method of Ellman (20).

Determination of protein concentration: Protein concentration of each sample was determined by the method of Lowry et al. (21).

Extraction and determination of neutral lipids and phospholipids in liver homogenates: Extraction of lipids was done according to the method of Colbeau et al. (22). The lipid extract was fractionated into neutral lipids and phospholipids by column chromatography on silicic acid (1 g of silicic acid+0.5 g of Celite 545) (22). The eluates were evaporated to dryness in vacuum at room temperature and the residues were weighed.

All assays were carried out two or three times.

RESULTS

Effect of PP on changes of lysosomal enzyme levels in CCl_4 -poisoned liver

As shown in Table 1, a single daily dose of CCl_4 for 3 days significantly decreased the lipolytic enzyme activity in both the lysosomal and the unsedimentable fractions of CCl_4 -rats (a decrease of 45–60% as compared with the control group), although the decreased lipolytic enzyme activity partially recovered at 8 days. The enzyme activity was increased about 17% in PP-rats as compared with that in CCl_4 -rats. Alterations in β -glucuronidase

TABLE 1. Effect of CCl_4 and PP on enzyme activities in lysosomal fractions of rat liver

Enzymes	Rats	3 days		8 days	
		Fractions		Fractions	
		Lysosomal	Unsediment	Lysosomal	Unsediment
Lipolytic enzyme	Control-rats	344 $\pm$ 36	54 $\pm$ 0.2	290 $\pm$ 35	57 $\pm$ 0.8
	PP-rats	214 $\pm$ 26	32 $\pm$ 0.4	216 $\pm$ 12	45 $\pm$ 0.4
	CCl_4 -rats	154 $\pm$ 18*†	28 $\pm$ 0.3*	164 $\pm$ 20**	37 $\pm$ 0.7*†
Acid phosphatase	Control-rats	509 $\pm$ 30	214 $\pm$ 5	478 $\pm$ 19	168 $\pm$ 10
	PP-rats	455 $\pm$ 47	229 $\pm$ 5	448 $\pm$ 3	174 $\pm$ 14
	CCl_4 -rats	448 $\pm$ 53	242 $\pm$ 8	443 $\pm$ 4	196 $\pm$ 10
β -Glucuronidase	Control-rats	21.0 $\pm$ 1.5	1.38 $\pm$ 0.01	18.0 $\pm$ 1.7	1.38 $\pm$ 0.11
	PP-rats	17.8 $\pm$ 1.5	1.60 $\pm$ 0.03	16.5 $\pm$ 1.0	1.55 $\pm$ 0.23
	CCl_4 -rats	15.8 $\pm$ 0.2‡	1.58 $\pm$ 0.12‡	15.8 $\pm$ 0.5	1.66 $\pm$ 0.21
Phosphodiesterase	Control-rats	5.1 $\pm$ 0.2	6.5 $\pm$ 0.1	4.8 $\pm$ 0.2	5.0 $\pm$ 0.2
	PP-rats	4.8 $\pm$ 0.4	6.1 $\pm$ 0.2	4.4 $\pm$ 0.1	4.9 $\pm$ 0.2
	CCl_4 -rats	4.9 $\pm$ 0.2	5.8 $\pm$ 0.2	4.5 $\pm$ 0.1	5.1 $\pm$ 0.1

Rat weight was 100–135 g. Activities are expressed as nmoles of product per min per mg of lysosomal protein. Each value represents the mean of four rats $\pm$ standard error.

* $p < 0.01$ in control vs. CCl_4 -rats

† $p < 0.05$ in PP-rats vs. CCl_4 -rats

‡ $p < 0.05$ in control vs. CCl_4 -rats

activity in both the fractions of each group were also determined. The activities in lysosomal fractions of PP-rats and CCl_4 -rats at 3 days decreased by 15% and 25%, respectively, and that in unsedimentable fractions showed an increase due to the release of intralysosomal enzyme. This result closely paralleled that obtained by Ruediger and Wilhelm (23). Only a slight decrease in acid phosphatase activity was found in PP-rats and CCl_4 -rats. PP, however, had little effect on the recovery of acid phosphatase and β -glucuronidase levels, decreased during CCl_4 intoxication. On the other hand, no significant alterations in phosphodiesterase activity were found in any group. Two other experiments produced the same results.

Changes in the amount of lipids, sulphydryl groups and peroxides

The effect of CCl_4 and PP on the components in rat liver and lysosomes was examined. The amount of peroxides was found to show little or no change in all the animals at 3 and 8 days (Control-rats at 3 and 8 days were 12 and 10 optical density (OD), PP-rats at 3 and 8 days were 13 and 9 OD, CCl_4 -rats at 3 and 8 days were 13 and 11 OD/g protein, respectively). Although in PP-rats and CCl_4 -rats at 3 days, sulphydryl content increased by 20–30% per mg of protein as compared with that of the control group (the contents at 3 and 8 days were 1.48 and 1.63 μg SH/mg protein, respectively), there was a fall in the content to the control value in all the animals at 8 days.

Table 2 shows the amount of neutral lipids and phospholipids in liver homogenates of all groups. The phospholipid content in CCl_4 -rats at 3 days was found to decrease by 27% and the neutral lipid content to increase about 3 times that of Control-rats. There was also a significant increase in the neutral lipid content in PP-rats at 3 days. In rats

TABLE 2. Effect of CCl₄ or PP on the amount of neutral and phospholipids in liver

Rats	3 days		8 days	
	Phospho-lipids (mg)	Neutral lipids (mg)	Phospho-lipids (mg)	Neutral lipids (mg)
Control-rats	27.7 ± 1.2	12.6 ± 1.2	28.9 ± 0.8	11.0 ± 0.9
PP-rats	23.2 ± 2.5	45.7 ± 4.0*	28.8 ± 1.7	14.1 ± 2.0
CCl ₄ -rats	20.1 ± 2.2‡	33.2 ± 2.0*	26.5 ± 1.4	22.4 ± 1.6*†

The liver weight was 4.5–6.5 g. Each value represents the mean of five rats ± standard error and is expressed as mg/g wet weight of liver.

* $p < 0.01$ in control vs. CCl₄-rats ‡ $p < 0.05$ in control vs. CCl₄-rats

† $p < 0.05$ in PP-rats vs. CCl₄-rats † $p < 0.05$ in control vs. PP-rats

dosed with PP for 8 days, however, the neutral lipid content in the liver was depressed nearly to the control value, indicating that the drug was capable of diminishing the lipid accumulated by CCl₄ intoxication but did not prevent the lipid accumulation.

Effect of a single dose of PP on lysosomal enzyme activities

Since the increase in lipolytic enzyme level caused by administration of PP to CCl₄-poisoned rats is considered to be due to induction or activation of the enzyme, the enzyme activities in liver lysosomal fractions of rats given the drug for 3 and 8 days were determined in comparison with those of Control-rats. It was found that the enzyme activities hardly increased, although the lipolytic enzyme activity was slightly enhanced (11%) at 8 days. This result suggests that PP does not play a great role in the induction or activation of lysosomal enzymes *in vivo*.

In an experiment to clarify whether or not liver lysosomal enzymes are activated with PP, PP was added to the assay medium in concentrations varying from 10⁻⁵ to 10⁻³%. The results indicated that PP does not increase the activities.

In vitro experiments using hydroperoxide, α -tocopherol and PP

Protective effect of PP on the hydroperoxide-induced inhibition: The inhibitory effect of hydroperoxide on the activities of lipolytic enzyme, acid phosphatase and β -glucuronidase in lysosomal fractions was determined *in vitro*, and results are shown in Fig. 1. It was found that hydroperoxide at a low concentration of 10⁻⁶% inhibited the lipolytic activity by approximately 30% and in concentrations ranging from 10⁻⁵ to 10⁻³% by 40%. When PP at a final concentration of 10⁻⁴% was added to the medium, the inhibitory effect of hydroperoxide was reduced by 11% at its lower concentration (at 10⁻⁶%, $p < 0.05$), however, the effect was not reduced at its higher concentrations. β -Glucuronidase activity was slightly inhibited at lower concentrations of hydroperoxide and was inhibited by 70% at a higher concentration of 10⁻³%. However, addition of PP slightly reduced the inhibition of β -glucuronidase activity at lower concentrations of hydroperoxide, while at its higher concentrations, the inhibitory effect was not reduced. The protecting effect of PP on hydroperoxide inhibition was not enhanced even at a higher concentration of 10⁻³%, suggesting that the action may be due to an indirect protecting effect on lipolytic enzyme and β -glu-

FIG. 1. Effect of hydroperoxide and PP on enzyme activities in isolated lysosomal fractions of rat liver.

Treatment of the lysosomal fractions and assay methods of the enzyme activities are described in the text. The concentrations of PP and hydroperoxide were $10^{-4}\%$ and 10^{-6} to $10^{-3}\%$, respectively, in the medium. Open symbols represent data with hydroperoxide and closed symbols those with hydroperoxide and PP.

○, ●: lipolytic enzyme activity
 △, ▲: acid phosphatase activity
 □, ■: β -glucuronidase activity

FIG. 2. Effect of α -tocopherol and PP on lipolytic enzyme inhibition by hydroperoxide.

The liver lysosomal fractions were sonicated and centrifuged as described in the text. The concentrations of PP, hydroperoxide and α -tocopherol in the medium were $10^{-4}\%$, 10^{-6} to $10^{-3}\%$ and $10^{-4}\%$, respectively. Hydroperoxide and α -tocopherol were mixed in 2 ml of ethanol for 30 sec and diluted with water. The lipolytic enzyme activity is expressed as percent activity to that of control.

●: with hydroperoxide △: with hydroperoxide and PP
 ○: with hydroperoxide and α -tocopherol
 ×: with hydroperoxide, α -tocopherol and PP

curonidase rather than a direct action against hydroperoxide.

Inhibitory effect of α -tocopherol and PP on the hydroperoxide-induced inhibition: It is obvious from the results described above that PP has a protecting effect on the enzyme inhibition by hydroperoxide (Fig. 1). To clarify the mechanism of the protection, the lipolytic enzyme activity in liver lysosomal fractions was assayed in the presence of 10^{-6} to $10^{-3}\%$ hydroperoxide with α -tocopherol, PP or the antioxidant and the drug added. The result is shown in Fig. 2. α -Tocopherol reduced the inhibitory effect by approximately 9%, as also demonstrated by other workers (19, 24). It scarcely reduced, however, the inhibition at higher concentrations of 10^{-4} to $10^{-3}\%$ hydroperoxide. The lesser effect of the added antioxidant may be due to the fact that it was suspended in water and consequently the contact with hydroperoxide was not sufficient. Addition of PP to the medium containing α -tocopherol caused further reduction of the inhibitory action at lower concentrations of hydroperoxide by about 17%, while a single addition of PP reduced the inhibition to 10%. The experiments were carried out three times and all results were in good agreement. This result also agreed reasonably well with the protecting effect of PP

obtained from an *in vivo* experiment (Table 1).

Effect of hydroperoxide and PP on lysosomal membranes: In order to investigate whether or not PP has the effect of stabilizing lysosomal membrane, the activity of acid phosphatase which was released from lysosomes during incubation with or without PP was determined. The enzyme release from the particles was slightly enhanced with increasing concentrations of hydroperoxide, as shown in Fig. 3. Hydroperoxide in concentrations ranging from 10^{-5} to $10^{-3}\%$ increased the lytic action by about 10 to 13%. PP was found to prevent slightly the lytic action in lower concentration of 10^{-6} to $10^{-5}\%$ hydroperoxide, while it gave no significant protecting effect on the lysis at higher concentrations.

FIG. 3. Release of acid phosphatase from lysosomes by hydroperoxide or the compound and PP.

Two ml of lysosomal fractions, 1.5 ml of 0.25 M sucrose in 0.04 M tris-HCl buffer (pH 7.4), 0.5 ml of hydroperoxide suspension (10^{-6} to $10^{-3}\%$ in the medium) and 1 ml of PP ($10^{-4}\%$ in the medium) in 0.25 M sucrose were incubated at 37°C for 90 min under shaking. After centrifuging at $15,000\times g$ for 20 min, acid phosphatase activity of the supernatant was assayed.

●: activity released by hydroperoxide
○: activity released by hydroperoxide and PP

DISCUSSION

In this study, PP was administered to CCl_4 -poisoned rats for 8 days, since it has been documented that regeneration of CCl_4 -poisoned liver and recovery of the enzyme activities were completed in 8 days (25).

PP is said to be clinically effective and is known to enhance the oxidative catalytic action as shown by an increased rate of oxygen uptake by liver mitochondria (26), to prevent decrease in catalase levels (27), and to be useful for the oxidation of fatty acids (28), but its action on lysosomes of injured liver and against fat accumulation and necrosis in the liver have not yet been made clear.

It has been suggested that CCl_4 is activated in the endoplasmic reticulum and in the mitochondria of the liver to a more toxic product, possibly a free radical form. As a consequence of this activation, extraneous free radicals are produced that induce the formation of lipid peroxides in neighboring lipid-rich membranes and the structure and function of the membranes are disturbed (6, 29).

To clarify the action of PP, experiments were carried out. As a result of the present study, a single daily dose of CCl_4 (0.1 ml/100 g body weight/day) for 3 days was found to decrease the levels of some enzymes in rat liver lysosomal fractions. This decrease may be attributed to partial inactivation of enzymes, especially in lipolytic enzyme, by lipid peroxides and to release of the enzymes into serum in response to the membrane damage, although the decrease in the enzyme activities of liver is reported to be mainly concerned with

a transfer of enzymes into the blood (25). The most marked decrease of the enzyme activities in CCl_4 intoxication was found in the lipolytic enzyme activity of both lysosomal and unsedimentable fractions. The phenomenon may be due to the relatively high sensitivity of this enzyme to lipid peroxides (Fig. 1), because lipase which has an affinity for lipids appears to bind to lipid peroxides and be easily inactivated, while acid phosphatase, which is not a lipid-bound enzyme, may be hardly inactivated by lipid peroxides. The decrease in acid phosphatase and β -glucuronidase levels and their release from the particles into unsedimentable fractions were also found to a slight extent in CCl_4 -rats. The minor differences are consistent with results of many studies (23, 25, 30). Phosphodiesterase, which hydrolyzes 3',5'-cyclic AMP (31), was not affected by administration of CCl_4 . Administration of PP to CCl_4 -poisoned rats was found to increase the lipolytic enzyme level in liver lysosomal fractions and to a lesser extent decrease the unsedimentable activity. However, PP had little effect on the recovery of other enzyme levels tested.

It has been postulated that the formation of triglycerides in the liver is not interfered with in the CCl_4 -poisoned animal, but the mechanism for hepatic triglyceride secretion is inhibited or destroyed, and triglycerides accumulate in the liver (32). The inhibition of lipolytic enzyme activity, which may be able to hydrolyze the accumulated triglycerides, may cause further increase of fat accumulation in the liver. A similar view was presented by Ugazio and Torrielli (33). This concept is consistent with the fact that the quantity of neutral lipids in the liver nearly paralleled the changes of lipolytic enzyme levels (Table 2). A single dose of CCl_4 caused a rapid accumulation of neutral lipids in the liver. However, PP dosed for 8 days produced a marked decrease in neutral lipids. This may be attributed to the increase in the lipolytic enzyme activity and the stimulating effect of PP on tissue respiration (26) after the incorporation of PP into cellular components (34). The action of PP indeed seems to play an important role in the restoration of liver functions.

As a result of determination of peroxide content in lysosomal fractions, the amount of peroxides was hardly changed by administration of CCl_4 . A possible explanation for this phenomenon is that oxidative enzyme systems involving free radical intermediates have not been detected in the particles, and as a consequence, activation of CCl_4 presumably may not occur in the particles (6). A stabilizing effect of α -tocopherol on lysosomal membranes is suggested by the results of Zalkin et al. (35). The present result (Fig. 1) indicated that hydroperoxide at a lower concentration of $10^{-6}\%$ inhibited the lipolytic activity and in higher concentrations inhibited β -glucuronidase activity. α -Tocopherol at a final concentration of $10^{-4}\%$ was found to bring about 9% reduction of the inhibitory effect on the lipolytic enzyme at a lower concentration of hydroperoxide. PP added to the medium together with α -tocopherol further reduced this inhibition. This may be ascribed to the fact that PP stabilizes endogenous antioxidant materials in the particles. The reason for the protecting effect being abolished at higher concentrations of the hydroperoxide can be interpreted as the result of the decreased amount of α -tocopherol and other antioxidants, which react with lipid peroxides and free radicals (36).

These experiments on the effect of PP on lysosomal fractions lead to the conclusion

that administration of PP decreases the leakage of lysosomal lipolytic enzyme and prevents inactivation of the enzyme, to a lesser extent, and also diminishes neutral lipids accumulated in the liver during CCl₄ intoxication. One of the mechanisms may be that PP protects the lysosomal enzyme and the membranes from lipid peroxides and free radicals. However, PP administered contributed little to the recovery of β -glucuronidase and acid phosphatase activities decreased during CCl₄ intoxication.

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