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Effect of Sodium Copper Chlorophyllin on Lipid Peroxidation. VIII.[1] Its Effect on Carbon Tetrachloride-Induced Liver Injury in Rats

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The present study was carried out to investigate the effectiveness of sodium copper chlorophyllin (Cu–Chl–Na) as an *in vivo* lipid antioxidant by examining its effect on CCl_4-induced hepatic injury. Rats received two *i.p.* injections of Cu–Chl–Na (50 or 100 mg/kg) 18 and 2 h prior to CCl_4 administration (1 ml/kg, *p.o.*). It was found that in the pretreated rats, there was effective depression of both the CCl_4-induced lipid peroxidation in hepatic microsomes and the CCl_4-induced increase of lipid peroxides in whole liver at 0.5 and 24 h after the dosing of CCl_4, respectively. Cu–Chl–Na pretreatment also clearly inhibited the elevation of serum levels of glutamic oxaloacetic and glutamic pyruvic transaminases and isocitric dehydrogenase, and the increase in liver triglyceride level caused by CCl_4. In addition, the hepatotoxin-induced decrease of hepatic tryptophan pyrrolase activity was substantially prevented in the rats pretreated with Cu–Chl–Na. Since the CCl_4-induced peroxidation of liver lipids is generally accepted to be involved in the pathogenesis of its hepatotoxicity, the above *in vivo* antioxidative effect of Cu–Chl–Na is considered to play an important role in its protection of rats against the liver damage produced by CCl_4.

Keywords——sodium copper chlorophyllin; lipid peroxidation; antioxidative effect; liver injury; carbon tetrachloride

It has been generally considered that lipid peroxidation *in vivo* is involved in basic deteriorative mechanisms which are responsible for various events, such as the aging process,[2] some phases of atherosclerosis,[3] and liver injury induced by chemicals (CCl_4,[4] ethanol,[5] orotic acid,[6] *etc.*).

In our previous studies,[7] sodium copper chlorophyllin (Cu–Chl–Na), a mixture of copper chelates of chlorophyll derivatives, was demonstrated to have an antioxidative effect on the peroxidation of lipids in rat liver homogenate and of a mixture of linolenic and linoleic acids, possibly due to the action of Cu–Chl–Na as a radical scavenger. We have recently reported that in *i.p.* Cu–Chl–Na-treated rats, Cu–Chl–Na or some substance(s) derived from Cu–Chl–Na is distributed among the hepatic subcellular organelles such as microsomes and lysosomes in a form capable of exerting an antioxidative activity, thus inhibiting the peroxidative deterioration of their membranes.[1,8]

Therefore, it seemed to be valuable to investigate further the effectiveness of Cu–Chl–Na as an *in vivo* lipid antioxidant. In the present study, we chose CCl_4-induced liver injury in rats as a model of the impairment of liver functions caused by lipid peroxidation, and examined the effect of Cu–Chl–Na pretreatment on this tissue injury.

Experimental

Animal Treatments——Male Wistar rats weighing about 200 g were maintained on a commercial chow and tap water *ad lib.* Cu–Chl–Na (Wako Pure Chemical Ind., Ltd., Tokyo), dissolved in 0.9% saline solution, was injected *i.p.* into the rats. Control animals received an equivalent volume (10 ml/kg) of the vehicle. Thereafter, the pretreated animals were administered CCl_4 *p.o.* at a dose of 1 ml/kg as a mixture with olive oil (1 : 1, v/v). Corresponding

controls were given the same amount of olive oil alone. The dose of Cu–Chl–Na and the time-schedule of the above treatments were as noted in the legends to figures and tables. All rats were starved for about 18 h prior to CCl_4 administration, and killed by decapitation at the designated time intervals after CCl_4.

Assay of Hepatic Lipid Peroxidation——The quantitative estimation of CCl_4-induced lipid peroxidation *in vivo* in hepatic microsomes was done by measuring diene conjugation absorption of lipid extracts of the fraction, as described by Rao and Recknagel.[9] In addition, lipid peroxides in whole liver were assayed by the determination of 2-thiobarbituric acid (TBA) reactants, presumably malondialdehyde, according to the colorimetric method of Masugi and Nakamura,[10] and were expressed in terms of TBA value.

Analytical Procedures——Assays of serum enzymes, *i.e.*, glutamic oxaloacetic and glutamic pyruvic transaminases (GOT and GPT, respectively) and isocitric dehydrogenase (ICDH), were performed by the methods of Reitman and Frankel[11] and McLean and McLean,[12] respectively. Liver triglyceride was determined as described by Block and Jarrett[13] using hepatic lipids extracted by the procedure of Folch *et al.*[14] The total activity of liver tryptophan pyrrolase was measured in the presence of hematin, a cofactor of the enzyme, according to the method of Maki *et al.*[15] When necessary, the significance of differences between values was assessed by using Student's *t*-test.

Results and Discussion

It has been shown that the peroxidation of rat liver microsomal lipids induced *in vivo* by CCl_4 occurs as early as 5 min after its administration, as evidenced by the appearance of diene conjugation absorption, which is a manifestation of lipid peroxidation.[9,16] Thus, the effect of Cu–Chl–Na pretreatment on the microsomal lipid peroxidation in rat liver was examined at 0.5 h following CCl_4 dosing (1 ml/kg, *p.o.*). Cu–Chl–Na was given *i.p.* at a dose of 50 or 100 mg/kg twice, at 18 and 2 h prior to CCl_4. As shown in Fig. 1, the difference spectra between hepatic microsomal lipids from the rats treated with a mixture of CCl_4–olive oil and olive oil alone showed the conjugated diene absorption at *ca.* 240 nm. Pretreatment of the rats with Cu–Chl–Na clearly decreased this absorption, the extents of the decrease being 55% at 50 mg/kg and 66% at 100 mg/kg. The effect of Cu–Chl–Na pretreatment on the CCl_4-increased formation of lipid peroxides in whole liver was further studied by determining TBA-reactive substances 24 h after CCl_4 administration to the rats, when the impairment of

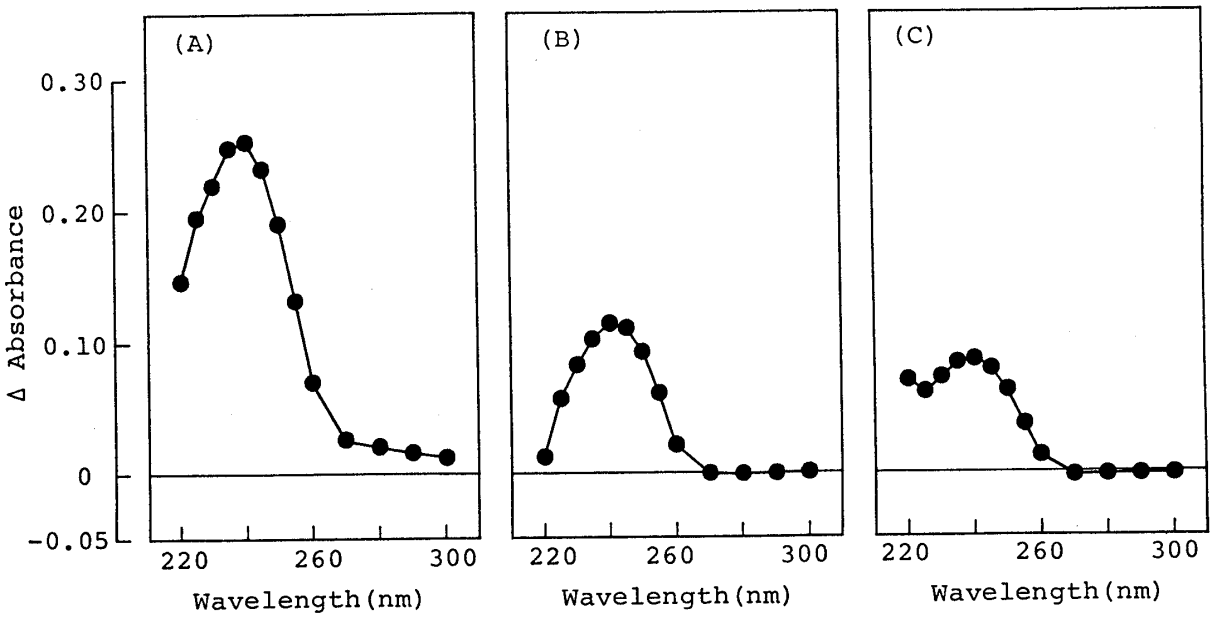

Fig. 1. Effect of Cu–Chl–Na Pretreatment on CCl_4-Induced Lipid Peroxidation *in Vivo* in Liver Microsomes of Rats

Rats were injected *i.p.* with Cu–Chl–Na (A, 0 mg/kg; B, 50 mg/kg; C, 100 mg/kg) twice at 16 h intervals. At 2 h after the second injection of Cu–Chl–Na, rats received a *p.o.* administration of CCl_4 (1 ml/kg) and were sacrificed 0.5 h later for the determination of difference spectra (CCl_4-treated minus untreated rats) of liver microsomal lipids. Each point represents the mean of 4 separate experiments.

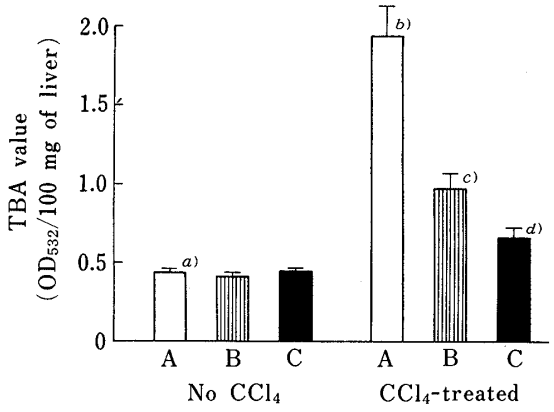

Fig. 2. Effect of Cu–Chl–Na Pretreatment on TBA-Reactive Substance Level in Rat Liver after CCl_4 Dosing

Treatment of rats was carried out as described in the legend to Fig. 1 except that they were sacrificed 24 h after the dosing of CCl_4. Results are expressed as the mean ± S.E. for 7 animals. Cu–Chl–Na pretreatment: A, none; B, 50 mg/kg × 2; C, 100 mg/kg × 2. Statistical significance of the differences: $p < 0.001$ in *a*)–*b*), *b*)–*c*), and *b*)–*d*).

TABLE I. Effect of Cu–Chl–Na Pretreatment on Serum Enzyme Levels in CCl_4-Treated Rats

Cu–Chl–Na pretreatment (mg/kg, *i.p.*)	GOT activity (Karmen units/ml)		GPT activity (Karmen units/ml)		ICDH activity (nmol NADPH/ml/min)	
	Non-treated	CCl_4-treated	Non-treated	CCl_4-treated	Non-treated	CCl_4-treated
None	78.5 ± 5.9[a)]	34793 ± 2313[b)]	18.4 ± 3.7[a)]	15832 ± 1247[b)]	9.1 ± 0.7[a)]	4462 ± 452[b)]
50 × 2	—	23986 ± 2276[c)]	—	10500 ± 866[c)]	10.9 ± 1.3	2880 ± 276[c)]
100 × 2	—	15964 ± 1378[d)]	—	6514 ± 841[d)]	13.0 ± 1.7	1985 ± 465[d)]

Treatment of rats was carried out as described in the legend to Fig. 1 except that they were sacrificed 24 h after the dosing of CCl_4. "Non-treated" means that the rats received no CCl_4. Each value represents the mean ± S.E. for 4—7 animals. Statistical significance of the differences: $p < 0.02$ in *a*)–*b*), *b*)–*c*), and *b*)–*d*).

hepatic functions should have occurred.[4b)] The results are given in Fig. 2. In the non-poisoned (olive oil-treated) group, the dosing of Cu–Chl–Na produced no change in the hepatic TBA value. The treatment with CCl_4 caused 4.4-fold elevation of TBA value in the control (saline-pretreated) rat liver, and this elevated value was significantly reduced in the rats pretreated with Cu–Chl–Na at 50 and 100 mg/kg by 50 and 66%, respectively.

In conjunction with our previous observation[1,8)] that *i.p.* administered Cu–Chl–Na or some substance(s) derived from Cu–Chl–Na is incorporated in several subcellular fractions of liver, the above results indicate that Cu–Chl–Na acts *in vivo* as a hepatic lipid antioxidant, and consequently, pretreatment with this drug brings about an effective inhibition of the peroxidation of liver lipids generated by CCl_4 dosing.

Next, we investigated the effect of Cu–Chl–Na on CCl_4-induced liver damage by measuring serum GOT, GPT and ICDH levels, liver triglyceride content, and hepatic tryptophan pyrrolase activity as indicators of the damage. These indicators were assessed in parallel 24 h after the administration of CCl_4 to the Cu–Chl–Na-pretreated rats. As shown in Table I, in the CCl_4-poisoned group, Cu–Chl–Na pretreatment significantly depressed the drastic elevation of the three serum enzyme activities caused by CCl_4 (a measure of hepatic necrosis), and the extent of the depression increased with increasing dose of Cu–Chl–Na. The results in Table II show that CCl_4 treatment produced a marked increase in liver triglyceride level, a characteristic of the liver injury, and this increase was clearly inhibited by pretreatment with Cu–Chl–Na at 50 and 100 mg/kg. In addition, both doses of Cu–Chl–Na considerably ameliorated the marked CCl_4-induced decrease in hepatic tryptophan pyrrolase activity (Table II), which is regarded as an index of the damage to hepatic protein synthesis caused by CCl_4.[15)]

These findings demonstrate that Cu–Chl–Na affords effective protection against the liver

TABLE II. Effects of Cu–Chl–Na Pretreatment on Triglyceride Content and Tryptophan Pyrrolase Activity in Liver of CCl_4-Treated Rats

Cu–Chl–Na pretreatment (mg/kg, *i.p.*)	Liver triglyceride (mg/g liver)		Tryptophan pyrrolase (μmol kynurenine/g liver/h)	
	Non-treated	CCl_4-treated	Non-treated	CCl_4-treated
None	$9.0 \pm 0.8^{a)}$	$52.5 \pm 4.7^{b)}$	$4.75 \pm 0.28^{a)}$	$1.26 \pm 0.19^{b)}$
50×2	9.3 ± 0.9	$28.9 \pm 2.8^{c)}$	4.62 ± 0.20	$3.16 \pm 0.56^{c)}$
100×2	12.4 ± 1.8	$30.1 \pm 3.9^{d)}$	4.29 ± 0.28	$3.34 \pm 0.37^{d)}$

Treatment of rats was carried out as described in the legend to Fig. 1 except that they were sacrificed 24 h after the dosing of CCl_4. "Non-treated" means that the rats received no CCl_4. Each value represents the mean $\pm$ S.E. for 5—6 animals. Statistical significance of the differences: $p < 0.01$ in *a*)–*b*), *b*)–*c*), and *b*)–*d*).

injury induced *in vivo* by CCl_4. Through histological techniques, McCloskey and McGehee[17] observed that when Cu–Chl–Na was injected *s.c.* into rats before CCl_4 administration, a significant decrease occurred in the amount of liver necrosis caused by the hepatotoxin. The present results, obtained by biochemical techniques, support their findings. Our results are also similar to the observation reported by several workers that several antioxidants such as α-tocopherol and *N*,*N*′-diphenyl-*p*-phenylenediamine, when administered to rats prior to CCl_4, have a preventive effect on necrosis and fat accumulation in the CCl_4-poisoned rat liver.[16,18]

There has been considerable work by many investigators indicating that the CCl_4-induced peroxidation process of the structural lipids of liver cells, mainly in microsomes, may be one of the principal causes of the hepatotoxicity of CCl_4.[4] Accordingly, it is considered that the preventive effect of Cu–Chl–Na on the CCl_4-induced liver damage is probably a consequence of the *in vivo* lipid antioxidative action mentioned above.

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