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Effect of Sodium Copper Chlorophyllin on Lipid Peroxidation. V.[1] Effect on Peroxidative Damage of Rat Liver Lysosomes

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Incubation of a heavy lysosome-containing ($3500 \times g$) fraction from rat liver with ascorbic acid resulted in a marked increase in the formation of lipid peroxides, and a concomitant increase in the release of two marker enzymes, acid phosphatase and aryl sulfatase, from lysosomes. These simultaneous increases in the lipid peroxidation and the lysosomal enzyme release were completely inhibited by adding ethylenediamine tetraacetic acid, indicating that the ascorbic acid-induced lipid peroxidation is responsible for the labilization of lysosomes. Sodium copper chlorophyllin (Cu-Chl-Na) caused a concentration-dependent inhibition of both the lipid peroxidation and the release of marker enzymes which were stimulated by ascorbic acid, and the dose-inhibition curves showed nearly the same pattern. Cu-Chl-Na also prevented the stimulation of lipid peroxidation and release of aryl sulfatase by ferrous ion. These findings indicate that Cu-Chl-Na has the ability to protect liver lysosomes from peroxidative damage, and this effect is ascribed to an inhibition of lipid peroxidation. Cu-Chl-Na also moderately inhibited the release of two marker enzymes from lysosomes in 0.25 and 0.18 M sucrose media, suggesting a direct stabilizing effect of Cu-Chl-Na on the lysosomal membrane.

Keywords——sodium copper chlorophyllin; lipid peroxidation; antioxidative action; ascorbic acid; rat liver lysosome; lysosomal enzyme release; lysosomal membrane stabilization

In the previous papers of this series,[2] we have reported that sodium copper chlorophyllin (Cu-Chl-Na), a mixture of copper complexes of chlorophyll derivatives, showed an inhibitory effect on the peroxidation of lipids in rat liver homogenate and of a mixture of linolenic and linoleic acids. In addition, Cu-Chl-Na was as effective as α-tocopherol in reducing 1,1-diphenyl-2-picrylhydrazyl, a model compound of free radicals, suggesting that the antioxidative effect of Cu-Chl-Na is attributable to its action as a radical scavenger.

It is well known that the peroxidation of membrane lipids leads to loss of the integrity of membrane structure and functions.[3] Many investigations[4] have shown that the lysis of red cells or of subcellular organelles such as mitochondria and lysosomes is frequently attributable to lipid peroxidation in their membranes, and that the peroxidative damage of membranes is prevented by inhibitors of lipid peroxidation such as α-tocopherol, tinoridine and mepacrine.

This paper deals with *in vitro* studies on the inhibitory effect of Cu-Chl-Na on the peroxidative damage of lysosome membranes in rat liver. In addition, the effect of Cu-Chl-Na on the hypo-osmotic lysis of hepatic lysosomes is described.

Materials and Methods

Chemicals——Cu-Chl-Na, ascorbic acid (AsA) and 2-thiobarbituric acid (TBA) were purchased from Wako Pure Chemical Industries, Ltd. Sodium *p*-nitrophenyl phosphate and dipotassium *p*-nitrocatechol sulfate were obtained from Nakarai Chemicals Co., Ltd. All other chemicals were of reagent grade.

Animals——Male rats of Wistar strain weighing 200—220 g were used. The animals were starved for about 18 h prior to sacrifice, but were given water *ad libitum*.

Preparation of Liver Lysosome-Containing ($3500 \times g$) Fraction——The lysosome-containing fraction was prepared according to the method of Tanaka and Iizuka[5] with a slight modification. Rats were killed by decapitation, and the livers were perfused with ice-cold 0.25 M sucrose solution *in situ* to remove blood. The livers were quickly excised, rinsed with the same solution, and homogenized in 6 volumes of ice-cold 0.25 M sucrose-40 mM Tris-acetate buffer (pH 7.4) by means of 6 strokes in a Potter–Elvehjem type homogenizer with a Teflon pestle. The homogenate was then centrifuged at $700 \times g$ for 10 min, and the resultant supernatant fraction was recentrifuged at $3500 \times g$ for 15 min. After being washed once with the sucrose-buffer solution, the resulting sediment ($3500 \times g$ fraction) was resuspended in the same buffer solution to give 0.4 g of liver equivalent per milliliter, and the suspension was used as lysosome-containing fraction.

Assay of Lysosomal Membrane Stability——The labilization and stabilization of lysosomes were assayed by determining the release of lysosomal marker enzymes, *i.e.*, acid phosphatase (Pase) and aryl sulfatase (Sase). A typical reaction mixture consisted of a suspension of $3500 \times g$ fraction (0.2 g of liver eq/ml), 0.25 M sucrose and 40 mM Tris-acetate buffer (pH 7.4) in a total volume of 3.0 ml. Other experimental conditions for the reaction mixture are given in each figure and table. The incubations were carried out aerobically at 37 °C with constant shaking (100 cycles/min). At definite time intervals, a 1.5 ml aliquot of the reaction mixture was removed and centrifuged at $24000 \times g$ for 15 min. The resultant supernatant was then used for the assay of marker enzymes released from lysosomes. Another 0.5 ml aliquot was withdrawn from the mixture to determine the degree of lipid peroxidation. The total activity of those enzymes was assayed using the supernatant obtained by centrifuging a suspension of $3500 \times g$ fraction incubated at 37 °C for 30 min in 0.2% Triton X-100-40 mM Tris-acetate buffer (pH 7.4). The enzyme release was expressed in terms of per cent of the total enzyme activity.

Enzyme Assay——Acid Pase (EC 3.1.3.2) activity was assayed by the method of Nakanishi *et al.*[6] using sodium *p*-nitrophenyl phosphate as a substrate. Aryl Sase (EC 3.1.6.1) activity was determined as described by Dodgson and Spencer[7] using dipotassium *p*-nitrocatechol sulfate as a substrate.

Assay of Lipid Peroxidation——Lipid peroxidation was measured by determining malondialdehyde formed in the reaction mixture according to the method of Fong *et al.*[4e] with a slight modification. To a portion (0.5 ml) of the reaction mixture, 0.5 ml of 20% trichloroacetic acid, 2.0 ml of distilled water and 1.0 ml of 0.7% TBA were added, and the mixture was then heated at 60 °C for 90 min. When the mixture had cooled, 1.0 ml of 70% trichloroacetic acid was added and the whole was mixed gently. Chloroform (3 ml) was then added and the mixture was shaken vigorously to remove dispersed lipid, then centrifuged. The absorbance of the clear pink supernatant fraction was determined at 532 nm, and expressed in terms of TBA values. The interference with the TBA color reaction by sucrose[4a] was negligible under the above conditions.

When necessary, the data were analyzed statistically by using Student's *t*-test.

Results

Relationship between Lipid Peroxidation and Lysosomal Enzyme Release

The relationship between the formation of lipid peroxides and the lysosomal enzyme release in the $3500 \times g$ fraction of rat liver was studied with AsA and ethylene diamine tetra-acetic acid (EDTA) as a stimulator and an inhibitor, respectively, of lipid peroxidation. As shown in Fig. 1, incubation of the $3500 \times g$ fraction at 37 °C without AsA scarcely affected the TBA value during the period up to 60 min, and resulted in a slight and spontaneous release of acid Pase and aryl Sase from lysosomes present in the $3500 \times g$ fraction. The addition of 0.5 mM AsA to the reaction mixture led to a marked stimulation of lipid peroxidation and the TBA value increased continuously throughout the incubation period. In parallel with the elevation of TBA value, the release of the two lysosomal enzymes occurred rapidly and reached a level of more than 80% of the total enzyme activity after incubation for 60 min. When 0.1 mM EDTA was added to the reaction mixture, the AsA-stimulated lipid peroxidation was completely inhibited and, simultaneously, the release of acid Pase and aryl Sase was depressed to the control level. At a concentration of 0.1 mM, EDTA did not directly inhibit the activity of the marker enzymes.

Effects of Cu-Chl-Na on Lipid Peroxidation and Lysosomal Enzyme Release

Figure 2 shows the effects of Cu-Chl-Na at various concentrations on the formation of lipid peroxides and on the release of acid Pase and aryl Sase which occur during the incubation of the $3500 \times g$ fraction with AsA. Cu-Chl-Na showed a dose-related inhibition of the AsA-stimulated lipid peroxidation and at concentrations of 0.5×10^{-3}% or above, almost

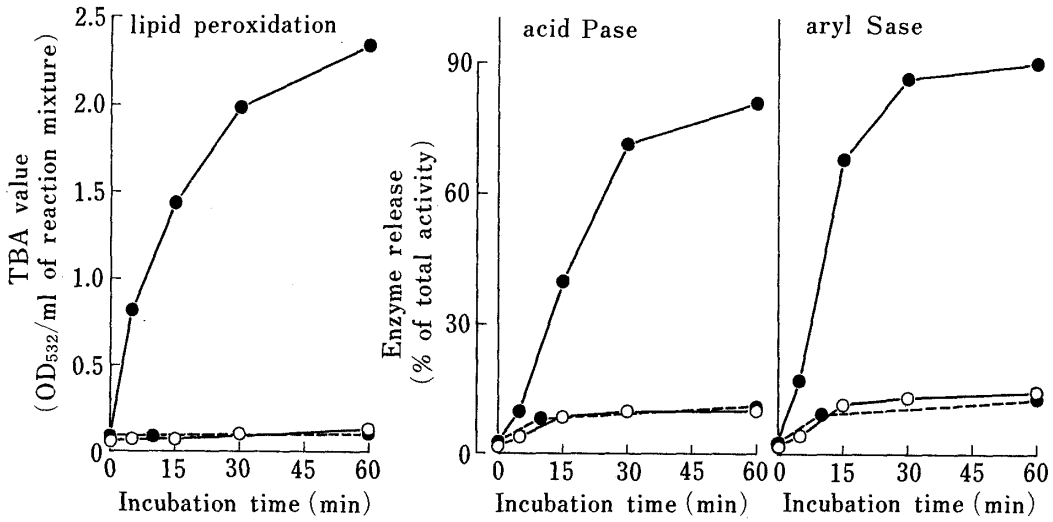

Fig. 1. Time Course of Lipid Peroxidation and Lysosomal Enzyme Release in $3500 \times g$ Fraction of Rat Liver in the Presence of AsA

The reaction mixture, consisting of $3500 \times g$ fraction (0.2 g of liver eq/ml), 0.25 M sucrose and 40 mM Tris-acetate buffer (pH 7.4) in a total volume of 3.0 ml, was incubated at 37 °C. Each point represents the mean for 3—4 separate experiments.
—○—, control; —●—, 0.5 mM AsA; ---●---, 0.5 mM AsA + 0.1 mM EDTA.

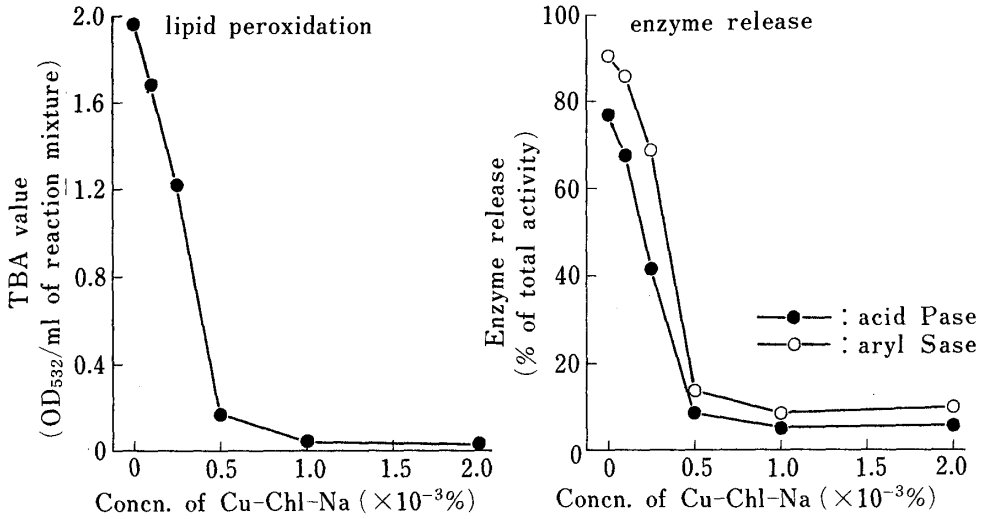

Fig. 2. Effects of Cu-Chl-Na on Lipid Peroxidation and Lysosomal Enzyme Release in $3500 \times g$ Fraction of Rat Liver Stimulated by AsA

The reaction mixture consisted of the same components as described in the legend to Fig. 1, except that 0.5 mM AsA was added, and it was incubated at 37 °C for 30 min. Each point represents the mean for 4 separate experiments.

complete inhibition was observed. The decrease in the release of the two marker enzymes with increase in the concentration of Cu-Chl-Na occurred with nearly the same pattern as that in the TBA value. Cu-Chl-Na at the concentrations tested exerted no direct inhibitory effect on acid Pase or aryl Sase.

The effect of Cu-Chl-Na on the lysosomal enzyme release was studied in the presence of ferrous ion. As shown in Fig. 3, the addition of 10 μM ferrous ion to the reaction mixture markedly elevated the TBA value in the $3500 \times g$ fraction, and increased the release of aryl Sase from lysosomes by about 90%. Cu-Chl-Na at a concentration of 1×10^{-3}% showed complete inhibition of both the lipid peroxide formation and the enzyme release which were induced by ferrous ion.

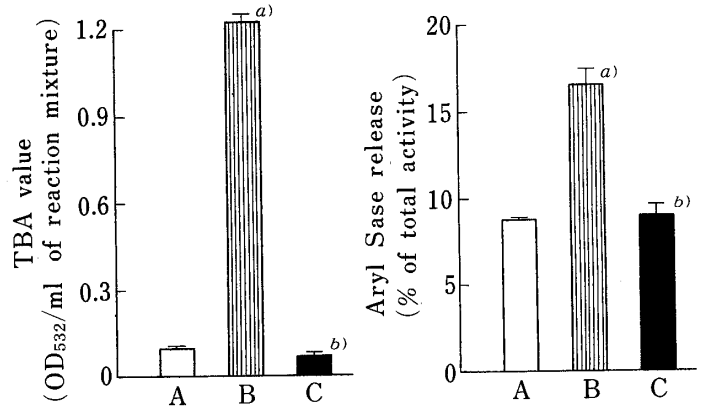

Fig. 3. Effects of Cu-Chl-Na on Lipid Peroxidation and Aryl Sase Release in $3500 \times g$ Fraction of Rat Liver in the Presence of Fe^{2+}

The composition of the reaction mixture was the same as described in the legend to Fig. 1. Incubation was carried out at 37 °C for 60 min. Results are expressed as the mean ± S.E. for 3 separate experiments.
A, no addition; B, 10 μM $FeSO_4$; C, 10 μM $FeSO_4 + 1 \times 10^{-3}$ % Cu-Chl-Na.
a) $p < 0.02$ in A *vs.* B.
b) $p < 0.01$ in B *vs.* C.

TABLE I. Effect of EDTA and Cu-Chl-Na on Lysosomal Enzyme Release from $3500 \times g$ Fraction of Rat Liver

Incubation conditions		Acid Pase		Aryl Sase	
		% release of total activity	Effect (%)	% release of total activity	Effect (%)
0.25 M sucrose +0.5 mM AsA	Control	60.5±2.2		84.4±1.8	
	0.1 mM EDTA	5.6±0.6[a)]	−91	10.5±1.3[a)]	−88
	1×10^{-3} % Cu-Chl-Na	4.0±0.4[a)]	−93	7.4±0.7[a)]	−91
0.25 M sucrose	Control	5.5±0.3		10.5±0.6	
	0.1 mM EDTA	6.2±0.3	+12	11.8±0.6	+13
	1×10^{-3} % Cu-Chl-Na	3.8±0.2[a)]	−32	8.3±0.5[a)]	−20
0.18 M sucrose	Control	17.7±1.2		29.4±1.3	
	0.1 mM EDTA	19.6±1.6	+10	33.3±1.7	+13
	1×10^{-3} % Cu-Chl-Na	13.2±1.1[a)]	−26	21.8±1.6[a)]	−26
0.125 M sucrose	Control	40.1±1.4		68.5±1.9	
	0.1 mM EDTA	43.3±1.4	+8	71.7±5.8	+5
	1×10^{-3} % Cu-Chl-Na	36.4±1.5	−9	62.2±2.6	−9

The reaction mixture consisted of the same components as described in the legend to Fig. 1 except that various concentrations of sucrose were added. Incubation was carried out at 37 °C for 30 min. Results are expressed as the mean ± S.E. for 3—4 separate experiments.
a) Significantly different from the control at a *p* value of 0.05 or less.

Effect of Cu-Chl-Na on Lysosomal Enzyme Release in Hypo-Osmotic Media

To determine whether Cu-Chl-Na has a direct action on lysosomal membranes, the effect of Cu-Chl-Na on the release of lysosomal enzymes during incubation of the $3500 \times g$ fraction in media containing various concentrations of sucrose was examined in comparison with that of EDTA. The results are given in Table I. Decreasing the concentration of sucrose accelerated the release of acid Pase and aryl Sase from lysosomes, while no elevation of the TBA values was observed (data not shown). EDTA at 0.1 mM caused no significant change in the release of either enzyme at any sucrose concentration, which was different from the result

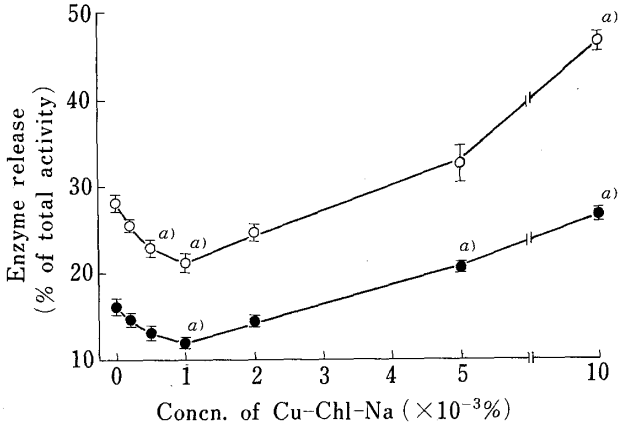

Fig. 4. Effect of Cu-Chl-Na on Lysosomal Enzyme Release from 3500 × *g* Fraction of Rat Liver Incubated with 0.18 M Sucrose

The experimental conditions were as described in Table I except that the concentration of sucrose was 0.18 M. Each point represents the mean ± S.E. (vertical bars) for 4 separate experiments.
—●—, acid Pase; —○—, aryl Sase.
a) Significantly different from the control (0%) at a *p* value of 0.02 or less.

obtained in the presence of AsA. On the other hand, the addition of 1×10^{-3}% Cu-Chl-Na significantly inhibited the release of the two marker enzymes in 0.25 and 0.18 M sucrose media by about 20—30%, which was much lower than the inhibition of the enzyme release in the presence of AsA. In 0.125 M sucrose, however, Cu-Chl-Na had no significant effect.

The effect of Cu-Chl-Na at various concentrations on the lysosomal enzyme release in 0.18 M sucrose medium is shown in Fig. 4. The release of both acid Pase and aryl Sase was decreased with increasing concentration of Cu-Chl-Na up to 1×10^{-3}%, at which an about 26% decrease was observed in each case. At high concentrations of 5×10^{-3}% or above, however, Cu-Chl-Na increased the release of the two lysosomal enzymes, and showed an about 66% increase of both enzymes at 10×10^{-3}%.

Discussion

Several investigators[8)] have shown that the addition of hydrogen peroxide, a labilizer of lysosomal membranes, to a suspension of the heavy lysosome-containing fraction (700—3500 × *g*) from rat liver stimulated not only the release of lysosomal enzymes such as acid Pase and aryl Sase but also the lipid peroxidation in the 700—3500 × *g* fraction. Furthermore, it was shown that antioxidants such as tinoridine or α-tocopherol inhibited the lysosomal enzyme release, but the lysosome-stabilizing activity of those drugs was not necessarily proportional to the inhibitory activity on the lipid peroxidation.[8a)] Thus, it seems unlikely that the labilization of lysosomes by hydrogen peroxide is attributable only to the peroxidation of their membrane lipids.

When the 3500 × *g* fraction of rat liver was incubated in the presence of AsA, a stimulator of lipid peroxidation, the release of two marker enzymes, acid Pase and aryl Sase, from lysosomes was markedly accelerated in parallel with the increased formation of lipid peroxides. These simultaneous increases in lipid peroxidation and lysosomal enzyme release were abolished by the addition of EDTA, which neither directly affected the activity of marker enzymes nor directly stabilized the lysosomal membrane. From these findings, it is assumed that AsA stimulates the peroxidation of membrane lipids of lysosomes contained in the 3500 × *g* fraction, thereby resulting in damage to their membranes, followed by the release of lysosomal enzymes.

As shown in Fig. 2, the addition of Cu-Chl-Na inhibited both the lipid peroxidation in the 3500 × *g* fraction and the lysosomal enzyme release which were induced by AsA. In addition, the dose-response curve of Cu-Chl-Na for the inhibition of lipid peroxide formation showed almost the same pattern as that for the inhibition of the release of lysosomal marker enzymes. The ferrous ion-induced lipid peroxidation and release of aryl Sase were also

depressed in the presence of Cu-Chl-Na. Cu-Chl-Na did not act as an inhibitor of the two marker enzymes. These results suggest that Cu-Chl-Na possesses the ability to protect the lysosomes present in the $3500 \times g$ fraction from peroxidative lysis through an inhibition of lipid peroxidation in the membrane.

On the other hand, as shown in Table I, Cu-Chl-Na moderately inhibited the release of both marker enzymes from lysosomes in the $3500 \times g$ fraction incubated in 0.25 and 0.18 M sucrose buffer solutions, suggesting a direct stabilizing action of Cu-Chl-Na on the lysosomal membrane. In 0.18 M sucrose medium, Cu-Chl-Na exhibited biphasic effects on lysosomes, *i.e.*, it decreased the release of lysosomal marker enzymes at low concentrations and increased the release at high concentrations. Lysosomal membrane stabilizers such as anti-inflammatory drugs are well known to possess such biphasic effects.[8,9] Therefore, it is considered that the mode of lysosome-stabilizing action of Cu-Chl-Na is similar to that of the anti-inflammatory drugs.

References and Notes

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