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Effect of Sodium Copper Chlorophyllin on Lipid Peroxidation. VII.[1] Effect of Its Administration on the Stability of Rat Liver Lysosomes

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The effect of administration of sodium copper chlorophyllin (Cu–Chl–Na), a mixture of copper chelates of chlorophyll derivatives, on the peroxidative and hypo-osmotic fragility of lysosomes was investigated using the lysosome-containing ($3500 \times g$) fraction of rat liver. When Cu–Chl–Na was given by *i.p.* injection 24 h prior to sacrifice, both ascorbic acid-induced lipid peroxidation in $3500 \times g$ fraction and the concomitant release of acid phosphatase and aryl sulfatase from lysosomes were inhibited to similar extents. These inhibitory effects were correlated with the content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na in the $3500 \times g$ fraction. Further, the administration of Cu–Chl–Na significantly depressed the release of the two lysosomal enzymes caused by the incubation of $3500 \times g$ fraction in 0.18 M sucrose medium. These results suggest that Cu–Chl–Na may function *in vivo* as a membrane stabilizer of liver lysosomes through its antioxidative and direct actions on the lysosomal membranes.

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

In relation to the impairment of biomembranes resulting from lipid peroxidation, the labilization of lysosomes has been regarded as an important factor causing damage to the structural and functional parts of the cell through the release of lysosomal hydrolytic enzymes.[2] Such peroxidative impairment of lysosomes has been assumed to be involved in some pathological events such as muscular dystrophy produced by vitamin E deficiency,[3] radiation damage[2a,4] and photosensitive dermatitis.[5] The release of lysosomal hydrolases is also postulated to play an important role in mediating the inflammatory process.[6] In recent *in vitro* studies,[7] we have shown that sodium copper chlorophyllin (Cu–Chl–Na), a mixture of copper complexes of chlorophyll derivatives, which acts as a lipid antioxidant,[8] protects rat liver lysosomes from peroxidative lysis by inhibiting the lipid peroxidation, and we also showed that it has a direct inhibitory action on the hypo-osmotic labilization of lysosomal membranes.

It was, therefore, of interest to investigate whether Cu–Chl–Na brings about *in vivo* stabilization of lysosomal membranes through its antioxidative and direct actions on the membranes. In this paper, we describe the results of experiments in which Cu–Chl–Na was administered to rats in order to assess its influence on the peroxidative and hypo-osmotic fragility of liver lysosomes.

Materials and Methods

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 isotonic saline, was injected *i.p.* into the rats. Control animals were treated with an equivalent volume (10 ml/kg) of the saline vehicle. All animals were fasted for about 18 h prior to sacrifice, but were given tap water freely.

Preparation of Liver Lysosome-Containing ($3500 \times g$) Fraction——The rats were killed by decapitation at the

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designated time intervals after the administration of Cu–Chl–Na or vehicle. The livers were perfused *in situ* with ice-cold 0.25 M sucrose solution to remove blood. The 3500 × *g* fraction was prepared from liver homogenate in 0.25 M sucrose–40 mM Tris-acetate buffer (pH 7.4) by the procedures described previously,[7] and finally suspended in the same solution at a concentration of 5 mg protein per ml. The protein concentration was measured by the method of Lowry *et al.*[9]

Assay of Lysosomal Membrane Stability——The labilization and stabilization of lysosomes were assayed by determining the release of acid phosphatase (Pase) and aryl sulfatase (Sase) as lysosomal marker enzymes, as described in the previous paper.[7] Experimental details on the reaction mixture are given in the legends to figure and table. After incubation of the reaction mixture at 37 °C for 30 min, 0.5 and 1.5 ml aliquots were removed for the assays of lipid peroxidation and of marker enzymes released from lysosomes, respectively. The total activities of the two enzymes were assayed using the suspension of 3500 × *g* fraction treated with 0.2% Triton X-100 at 37 °C for 30 min. The enzyme release was expressed as percent of the total enzyme activity.

Analytical Methods——Acid Pase (EC 3.1.3.2) and aryl Sase (EC 3.1.6.1) activities were determined with sodium *p*-nitrophenyl phosphate[10] and dipotassium *p*-nitrocatechol sulfate,[11] respectively, as substrates. The degree of lipid peroxidation was assayed by the determination of malondialdehyde produced according to the thiobarbituric acid (TBA) method as described previously,[7] and was expressed as TBA value (absorbance at 532 nm/ml of reaction mixture). To measure the content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na in liver 3500 × *g* fraction, suspensions of the fractions from Cu–Chl–Na-treated and control rats were diluted with the above sucrose-buffer to a concentration of 2 mg protein per ml. The content was then estimated by difference spectrophotometry as described in the previous paper,[1] and expressed in terms of μg eq to Cu–Chl–Na per mg protein. Statistical significance was determined by means of Student's *t*-test.

Results and Discussion

When the difference spectrum for the 3500 × *g* fraction of rat liver 1 h after a single injection of Cu–Chl–Na (100 mg/kg, *i.p.*) minus that of control rat liver was recorded between 590 and 700 nm, an absorption maximum was observed at *ca.* 632 nm and the spectral profile was almost identical with that of authentic Cu–Chl–Na added to the 3500 × *g* fraction of control rats. This indicates that the administered Cu–Chl–Na or substance(s) derived from Cu–Chl–Na was taken into the liver 3500 × *g* fraction. Then, the time course of change in its content was examined. As shown in Fig. 1, the content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na increased progressively and reached a maximum 24 h after the administration of Cu–Chl–Na; this level was maintained up to 48 h, but began to decrease 72 h after dosing.

Our previous studies demonstrated that incubation of the 3500 × *g* fraction of rat liver with ascorbic acid (AsA) produced a marked increase in the release of acid Pase and aryl Sase from lysosomes in parallel with the increased formation of lipid peroxides, and both alterations were abolished by adding EDTA, indicating that the AsA-induced lipid peroxidation is responsible for the labilization of lysosomes.[7] Thus, the effects of Cu–Chl–Na administration on the AsA-induced formation of lipid peroxides and release of lysosomal marker enzymes were investigated using the hepatic 3500 × *g* fraction of rats *i.p.* dosed with

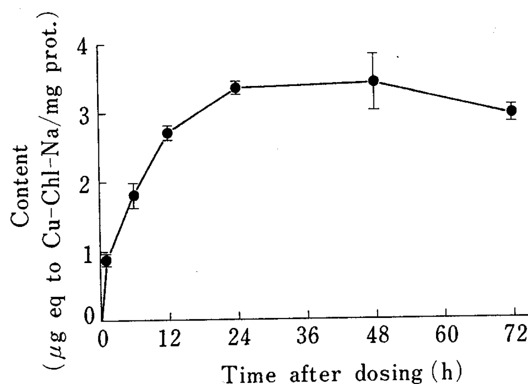

Fig. 1. Time Course of the Content of Cu–Chl–Na or Substance(s) Derived from Cu–Chl–Na in the 3500 × *g* Fraction of Rat Liver after the Injection of Cu–Chl–Na

Rats received a single intraperitoneal injection of Cu–Chl–Na at a dose of 100 mg/kg and were sacrificed at various time intervals after dosing. Each point represents the mean ± S.E. (vertical bars) for 3—4 animals.

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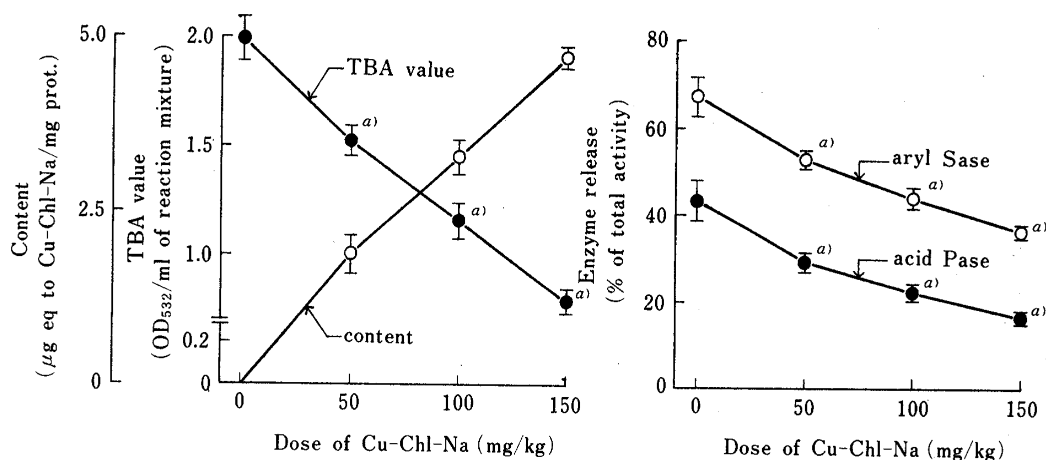

Fig. 2. Effects of Cu–Chl–Na Administration on Lipid Peroxidation and Lysosomal Enzyme Release in the 3500×*g* Fraction of Rat Liver Stimulated by AsA

Rats were given Cu–Chl–Na intraperitoneally at the doses indicated and were sacrificed 24 h after the injection. The reaction mixture, consisting of 3500×*g* fraction (2.5 mg protein/ml), 0.25 M sucrose, 0.5 mM AsA and 40 mM Tris-acetate buffer (pH 7.4) in a total volume of 3.0 ml, was incubated at 37 °C for 30 min. Each point represents the mean±S.E. (vertical bars) for 5 animals. *a*) Significantly different from control (0 mg/kg) at a *p* value of 0.05 or less.

TABLE I. Effect of Cu–Chl–Na Administration on Lysosomal Enzyme Release from the 3500×*g* Fraction of Rat Liver Incubated with 0.18 M Sucrose

Cu–Chl–Na treatment	Acid Pase		Aryl Sase	
	% release of total activity	Dec. (%)	% release of total activity	Dec. (%)
Control	13.4±1.2		19.9±1.4	
50 mg/kg	11.7±2.0	12.7	14.0±2.7	29.6
100 mg/kg	8.7±0.7[a)]	35.1	11.3±0.7[a)]	43.2
150 mg/kg	7.0±0.4[a)]	47.8	10.8±0.7[a)]	45.7

Treatment of rats was carried out as described in the legend to Fig. 2. The reaction mixture, consisting of 3500×*g* fraction (2.5 mg protein/ml), 0.18 M sucrose and 40 mM Tris-acetate buffer (pH 7.4) in a total volume of 3.0 ml, was incubated at 37 °C for 30 min. Results are expressed as the mean±S.E. for 4—6 animals.

a) Significantly different from control at a *p* value of 0.01 or less.

Cu–Chl–Na at a single dose of 50, 100 or 150 mg/kg. Based on the results shown in Fig. 1, the rats were sacrificed 24 h after dosing. The results are summarized in Fig. 2. The TBA value in 3500×*g* fraction 30 min after the incubation with AsA at 37 °C was significantly lowered by Cu–Chl–Na administration in a dose-dependent manner. This lowering effect was well correlated with the content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na in the 3500×*g* fraction which increased linearly with increase in the dose of Cu–Chl–Na. In addition, the AsA-induced release of acid Pase and aryl Sase decreased dose-dependently in rats injected with Cu–Chl–Na. At each dose of Cu–Chl–Na, the degree of decrease in lysosomal enzyme release was almost the same as that in lipid peroxidation. On the basis of these results, in the *i.p.* Cu–Chl–Na-treated rats, Cu–Chl–Na or substance(s) derived from Cu–Chl–Na may be distributed among not only mitochondria but also lysosomes contained in hepatic 3500×*g* fraction in a form capable of acting as an antioxidant, thereby preventing

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the lipid peroxidation-induced disintegration of the lysosomal membranes followed by the release of the lysosomal contents.

In *in vitro* experiments,[7] Cu–Chl–Na was shown to decrease the accelerated release of acid Pase and aryl Sase during incubation of the liver $3500 \times g$ fraction in a hypo-osmotic medium, *i.e.*, 0.18 M sucrose medium. Thus, the changes in the release of the two lysosomal enzymes in this medium were examined with the hepatic $3500 \times g$ fraction from rats 24 h after an *i.p.* injection of Cu–Chl–Na. As shown in Table I, when the fraction was incubated for 30 min, the release of acid Pase and aryl Sase from lysosomes was significantly reduced by 100 or 150 mg/kg of Cu–Chl–Na. This result suggests that Cu–Chl–Na or substance(s) derived from Cu–Chl–Na existing in hepatic lysosomes possesses the ability to stabilize directly the lysosomal membranes.

In conclusion, the results described in this paper suggest that Cu–Chl–Na may function *in vivo* as a lysosomal membrane stabilizer through its antioxidative and direct actions on the membranes.

References and Notes

1) Part VI: M. Sato, K. Imai, R. Kimura and T. Murata, *Chem. Pharm. Bull.*, **32**, 716 (1984).
2) *a*) I. D. Desai, P. L. Sawant and A. L. Tappel, *Biochim. Biophys. Acta*, **86**, 277 (1964); *b*) A. L. Tappel, *Fed. Proc.*, **32**, 1870 (1973).
3) *a*) A. L. Tappel, H. Zalkin, K. A. Caldwell, I. D. Desai and S. Shibko, *Arch. Biochem. Biophys.*, **96**, 340 (1962); *b*) I. D. Desai, C. C. Calvert, M. L. Scott and A. L. Tappel, *Proc. Soc. Exp. Biol. Med.*, **115**, 462 (1964).
4) E. D. Wills and A. E. Wilkinson, *Biochem. J.*, **99**, 657 (1966).
5) *a*) T. F. Slater and P. A. Riley, *Nature* (London), **209**, 151 (1966); *b*) W. Torinuki, T. Miura and M. Seiji, *Nippon Hifuka Gakkai Zasshi*, **89**, 77 (1979).
6) *a*) A. Janoff and B. W. Zweifach, *J. Exp. Med.*, **120**, 747 (1964); *b*) G. Weissmann, *Arthritis Rheum.*, **9**, 834 (1966); *c*) W. Seegers and A. Janoff, *J. Exp. Med.*, **124**, 833 (1966).
7) M. Sato, K. Konagai, R. Kimura and T. Murata, *Chem. Pharm. Bull.*, **31**, 3665 (1983).
8) *a*) M. Sato, N. Iguchi and T. Murata, *Yakugaku Zasshi*, **97**, 268 (1977); *b*) M. Sato, N. Iguchi, T. Kotani and T. Murata, *Eisei Kagaku*, **23**, 23 (1977).
9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) M. Nakanishi, H. Imamura and K. Goto, *Yakugaku Zasshi*, **90**, 557 (1970).
11) K. S. Dodgson and B. Spencer, *Biochem. J.*, **65**, 668 (1957).

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