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MODIFIED LIPOPHILIC VITAMIN—IV

STABILIZING EFFECT OF α-TOCOPHEROL AND TOCOPHERONOLACTONE ON MOUSE LIVER LYSOSOMES *IN VIVO* AND *IN VITRO**

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Abstract—The effect of α-tocopherol and tocopheronolactone on the stability of mouse liver lysosomal membrane *in vitro* and *in vivo* was examined by measurement of the enzyme activity released from lysosomes. Tocopheronolactone is a stronger inhibitor of the release of acid phosphatase from lysosomes *in vitro* than is α-tocopherol, but when given *in vivo*, α-tocopherol was as effective as tocopheronolactone in stabilizing the lysosomes. There was no clear-cut structure–activity correlation with α-tocopherol and its derivatives, tocopheronolactone, α-tocopherylquinone, and 6-hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman, with respect to stabilization of lysosomal membrane. Examination of the variation of potency with the amount given indicated that tocopheronolactone showed a stronger stabilization effect with increasing concentration, whereas α-tocopherol showed a biphasic effect, effecting stabilization at a low concentration of 10^{-6} to 10^{-4} M but labilization at a higher concentration of 5×10^{-4} M. Acid phosphatase and β-glucuronidase, which were derived similarly from the lysosomes, differed in the degree of inhibition of their release by tocopheronolactone. This fact seems to suggest that these two enzymes are present in different lysosomal particles, in different parts of the same particle, or are activated by different mechanism.

SINCE de Duve *et al.*[1] introduced the lysosomes as a group of new subcellular organelles in 1955, their function in many of the physiological and pathological changes, such as intracellular digestion, autolysis, and tissue damage has become clear. Lysosomal enzymes become redistributed either because of membrane injury or autophagic phenomena during encephalomalacia, muscular dystrophy, liver necrosis, and autolysis of kidneys, all because of vitamin E deficiency.[2–4]

Stabilization of lysosomal membrane, labilized by vitamin A deficiency or by administration *in vivo* of α-tocopherol, has also been reported.[5] However, addition of α-tocopherol *in vitro* fails to stabilize the lysosomes, or rather results in labilization.[6] This fact suggests that the stabilization of lysosomal membrane by α-tocopherol *in vivo* is because of the secondary production of α-tocopherol. Tocopheronolactone was isolated by Simon *et al.*[7] in 1956 as a metabolite of α-tocopherol from rabbit and human urine; it showed a marked antioxidant activity equivalent to α-tocopherol.[8,9] Recent theories suggest that vitamin E may produce its effect by being decomposed into a quinonoid metabolite like tocopheronolactone.[10] In fact, tocopheronolactone has been found to have a much more rapid effect than α-tocopherol in the revival of ubiquinone levels in the rat uterus; there is also a report that, although the decline of

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respiration in necrotic liver because of vitamin E deficiency can be recovered by administration *in vivo* but not *in vitro* of α-tocopherol, tocopheronolactone is entirely effective even *in vitro*.[11] This evidence suggests that the principle responsibility for the stabilization of lysosomal membrane by α-tocopherol *in vivo* might be tocopheronolactone. Therefore, the effect of tocopheronolactone on the stabilization of lysosomal membrane was studied, together with that of cortisone, which has been shown both to stabilize the membranes of lysosomes[6,12] and to act, *in vivo*, as an antioxidant in retinol-induced disruption of lysosomes.[12]

EXPERIMENTAL

Materials. Cortisone acetate was purchased from Nihon-Merk-Banyu Co. Ltd. Tocopheronolactone, α-tocopherol, α-tocopherylquinone and 6-hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman were supplied by Eisai Co. Ltd.

Preparation of lysosomal fractions. Female ddy-strain mice weighing 28–30 g were used. After decapitation and bleeding, the liver was rapidly removed and washed in ice-cold 0·44 M sucrose. After weighing, the liver was cut into small pieces and homogenized in 9 vol. of ice-cold 0·44 M sucrose using a Potter–Elvehjem glass homogenizer. The homogenate was centrifuged at 2000 *g* for 5 min and the supernatant was further centrifuged at 13,000 *g* for 10 min. After washing once, the sediment was resuspended in the 0·44 M sucrose to give 0·1 g (for investigation of acid phosphatase activity) or 0·4 g (for β-glucuronidase) of liver equivalent per milliliter and used as lysosomal fraction.

Preparation of enzyme samples. The enzyme sample was prepared as follows: To 10 ml of lysosomal preparation was added 0·15 ml of 8% Triton X-100 and the suspension was centrifuged immediately at 13,000 *g* for 10 min. The resulting supernatant was used as the enzyme sample.

Effect of neutral buffers on the stability of acid phosphatase. Studies were made of the effect of several neutral buffers on the stability of acid phosphatase during incubation. To aliquots of the enzyme sample, equal volumes of buffer (pH 7·4) and sucrose (0·25 M sucrose in 0·02 M citrate buffer, Tris-HCl buffer, or phosphate buffer) were added. After incubation for various time intervals, an aliquot of 1·0 ml was removed for enzyme assay.

Direct effect of drugs on enzyme activity. By incubation of chemicals with acid phosphatase or β-glucuronidase, direct effects of chemicals on these enzymes were examined. Incubation mixtures consisted of 1·5 ml of the enzyme sample, 1·5 ml of 0·02 M citrate buffer (pH 7·4)–0·25 M sucrose medium and 0·05 ml of chemical solution in ethanol. Incubation was carried out for 60 min at 37° and aliquots of 1·0 ml were immediately subjected to enzyme assay.

Effect in vitro *of drugs on the release of lysosomal enzymes.* Free activity was determined, as well as enzyme activity released in the supernatant during incubation of lysosomes. The incubation mixture consisted of 1·5 ml of the lysosomal preparation, 1·5 ml of 0·02 M citrate buffer (pH 7·4)–0·25 M sucrose medium and 0·05 ml of a chemical solution in ethanol. After the mixtures were made up in shaking flasks (designated as the zero time of incubation), the flasks were incubated at 37°. At definite time intervals, the incubation mixtures were centrifuged at 13,000 *g* for 10 min and 1·0 ml of the resulting supernatant was then subjected to enzyme assay to determine

the free activity. For the determination of free plus bound activities, 1·0 ml of the incubation mixture was used without centrifugation for enzyme assay. Total activity was determined as follows: In order to liberate the enzymes completely from lysosomes, 0·05 ml of 4% Triton X-100 was added to the mixture before incubation. Subsequent handling was the same as for the determination of free activity.

Enzyme assay. Activity of acid phosphatase (EC 3.1.3.2, Orthophosphoric monoester phosphohydrolase) was measured using sodium *p*-nitrophenyl phosphate as the substrate. The concentration of the substrate, acetate buffer (pH 5·0), and sucrose, was 25, 50, and 350 mM, respectively, and the total volume was 4·0 ml. Incubation was carried out for 10 min at 37° and the reaction was terminated by addition of 1·0 ml of 10% sodium hydroxide solution. After adequate dilution with distilled water, the extinction at 420 mμ was measured using a Shimazu Multiconvertible Spectrophotometer Double-40.

Activity of β-glucuronidase (EC 3.2.1.31, β-D-glucuronide glucurono-hydrolase) was measured according to the conditions reported by Gianetto and de Duve[13] with slight modifications. The concentration of the substrate (*p*-nitrophenyl glucuronide), acetate buffer (pH 5·0), and sucrose was 12·5, 50, and 350 mM, respectively, and the total volume was 4·0 ml. After incubation for 10 min at 37°, the reaction was stopped by addition of 1·0 ml of 2% sodium hydroxide solution. In this case, the extinction at 420 mμ was measured 10–20 min after addition of alkaline solution because slight decomposition of unreacted substrate occurred after prolonged standing.

Treatment in vivo *with tocopheronolactone and α-tocopherol.* The mice maintained on vitamin E deficient diet* (vitamin E content was 4 μg/10 g of diet) for 5 months were used. α-Tocopherol and tocopheronolactone were dissolved in cotton seed oil and injected at 50 μmoles (0·1 ml) per mouse i.p. As a control, cotton seed oil was injected at 0·1 ml per mouse i.p. After 12 hr from the time of administration of α-tocopherol or after 15 min from the time of administration of tocopheronolactone, the mice were sacrificed and the livers were removed. Stability of the lysosomal membrane was investigated as described in the preceding section.

RESULTS AND DISCUSSION

Effect of neutral buffers on the stability of acid phosphatase. It was found that incubation over a long period of time tended to lower the activity of enzymes that were released into the supernatant from lysosomes during incubation. In order to obtain the optimal buffer to (a) prevent the inactivation of enzymes and (b) maintain high enzyme activity, the effect of several neutral buffers on the stability of acid phosphatase was investigated.

As shown in Fig. 1, greatest activity was obtained by the use of a citrate buffer. Because of this finding, citrate buffer (pH 7·4) was used as the incubation medium for lysosomes in all subsequent experiments.

Direct effect of α-tocopherol and its derivatives on enzyme activity. In the examination of the stability of lysosomal membrane to various chemicals, it is possible that the variation of enzyme activity in the supernatant fraction might not reflect the stability of the membrane but result from the direct effect of the chemicals on the

* Purchased from Clea Co. Ltd.

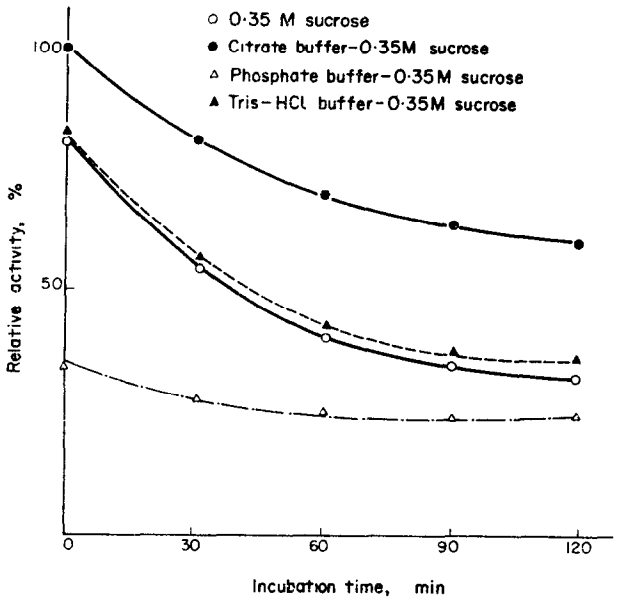

FIG. 1. Effect of neutral buffers on the stability of acid phosphatase. The enzyme preparation used was the supernatant obtained from lysosomal suspension containing 0·12 per cent Triton X-100. Pre-incubation mixture: 0·01 M buffer (pH 7·4), 0·35 M sucrose, and enzyme preparation at 50 mg of liver equivalent per milliliter. Incubation was carried out at 37°.

released enzyme. To test this possibility, studies were made of the effect of the drugs employed upon acid phosphatase and β-glucuronidase activity.

As shown in Table 1, acid phosphatase was hardly affected at drug concentrations of 10^{-4} M. It was slightly inhibited by 5×10^{-4} M α-tocopherol, α-tocopherylquinone, and 6-hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman. On the other hand, β-glucuronidase was not affected by these chemicals at concentrations of 10^{-4} to 5×10^{-4} M.

Effect of α-tocopherol and its derivatives on the release of lysosomal enzymes. It has been reported that α-tocopherol *in vitro* labilizes lysosomal membrane,[6] while another report showed that α-tocopherol added *in vitro* stabilized the lysosomes labilized by vitamin A deficiency.[14] In order to resolve these discrepancies, the stabilizing effect of α-tocopherol and its derivatives on lysosomal membrane was examined. The lysosomes were incubated in the presence of α-tocopherol and its metabolite, tocopheronolactone, and the activity of acid phosphatase found in the supernatant fraction was followed periodically. The results are shown in Fig. 2.

In the control, acid phosphatase activity detected in the supernatant increased linearly. α-Tocopherol, at a concentration of 10^{-4} M, inhibited this increase slightly; under these conditions tocopheronolactone at 10^{-4} M markedly inhibited the increase of acid phosphatase in the supernatant fraction to a greater extent than did either α-tocopherol or 5×10^{-4} M of cortisone acetate.

The decrease in enzyme activity detected in the supernatant fraction in the presence of tocopheronolactone and α-tocopherol may be taken as a measure of the stabilization of lysosomal membrane by these compounds, since it had been found in previous

TABLE 1. DIRECT EFFECT OF TOCOPHERONOLACTONE, α-TOCOPHEROL, α-TOCOPHERYLQUINONE, 6-HYDROXY-2-CARBOXYETHYL-2,5,7,8-TETRAMETHYLCHROMAN AND CORTISONE ACETATE ON LYSOSOMAL ENZYME ACTIVITY*

Compound	Concentration (M)	Enzyme activity			
		Acid phosphatase		β-glucuronidase	
		Specific activity (mμmoles/min/mg protein)	Relative activity (% of corresponding control)	Specific activity (mμmoles/min/mg protein)	Relative activity (% of corresponding control)
		means ± S.E.		means ± S.E.	
None (control)		60·4 ± 5·9	100 (4)	3·95 ± 0·29	100 (3)
Tocopheronolactone	10^{-4}	56·7 ± 5·0	93·9 (3)	4·08	103·7 (2)
α-Tocopherol	10^{-4}	57·8 ± 5·3	95·7 (3)	4·12	104·8 (2)
	5×10^{-4}	48·8	80·8 (1)	4·19	106·7 (2)
α-Tocopherylquinone	10^{-4}	59·3 ± 5·5	98·1 (3)	4·13	105·1 (2)
	5×10^{-4}	53·8	89·0 (1)		
6-Hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman	10^{-4}	59·1 ± 5·6	97·9 (3)	4·03	102·0 (2)
	5×10^{-4}	52·2	86·4 (1)		
Cortisone acetate	10^{-4}	61·1 ± 5·1	101·2 (3)		
	5×10^{-4}	61·1	102·5 (1)	4·10	104·0 (2)

* Figures in brackets indicate the number of samples used. The enzyme preparation used was the same as in Fig. 1. Incubation mixture: 0·01 M citrate buffer (pH 7·4), 0·35 M sucrose, 1·7% ethanol, and enzyme preparation at 50 mg (acid phosphatase) or 200 mg (β-glucuronidase) of liver equivalent per milliliter. Incubation for 60 min at 37°.

experiments that these compounds do not directly affect the enzyme. Since the free plus bound activities were decreased by tocopheronolactone and α-tocopherol, to the same degree as the free activity (Table 2), it seems likely that release of the enzyme is inhibited by stabilization of lysosomes.

Compounds other than tocopheronolactone showed almost the same effect of inhibiting the release of acid phosphatase as well as β-glucuronidase into the supernatant fraction. However, tocopheronolactone did not show such a marked inhibition of the release of β-glucuronidase as that of acid phosphatase. These findings seem to support the hypothesis of Verity *et al.*[15] that these two enzymes are present either in different lysosomal particles, in different parts of the same particle, or are activated by different mechanism.

Dose–response curves and structure–activity relationship of α-tocopherol and its derivatives on lysosome stabilization. As indicated in Fig. 3, α-tocopherol showed a biphasic action depending upon its concentration: about 10 per cent stabilization at a concentration of 10^{-4} M and a labilization at 5×10^{-4} M. α-Tocopherylquinone, formed by cleavage of the chroman ring in α-tocopherol to form a benzoquinone, does not show stabilization of lysosomal membrane. Instead, labilization of the membrane increased with increasing concentration. 6-Hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman, formed by shortening the side chain in α-tocopherol, showed a pattern of dose–response curve and stabilizing activity about equal to that of cortisone acetate. In contrast, tocopheronolactone, formed by modifying the chroman ring of

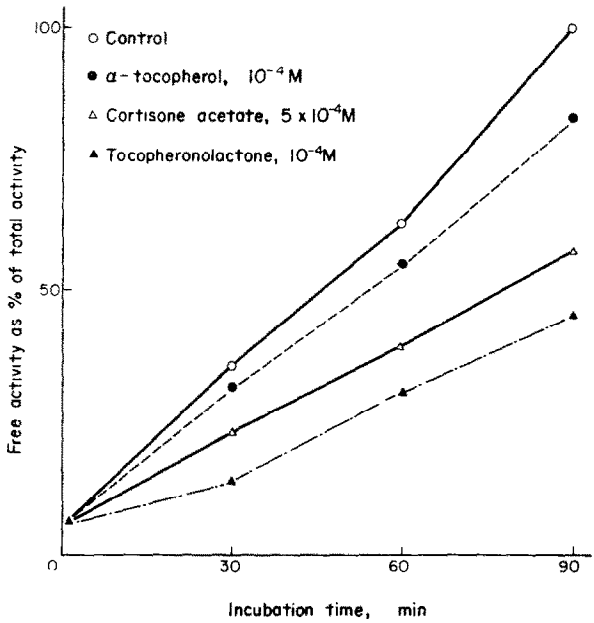

FIG. 2. Effect of tocopheronolactone, α-tocopherol and cortisone acetate on release of acid phosphatase from lysosomal fraction. Ordinate: Free activity of acid phosphatase detectable in the supernatant. Incubation mixture: 0·01 M citrate buffer (pH 7·4), 0·35 M sucrose, 1·7 per cent ethanol, and lysosomal fraction at 50 mg of liver equivalent per milliliter. Incubation was carried out at 37°.

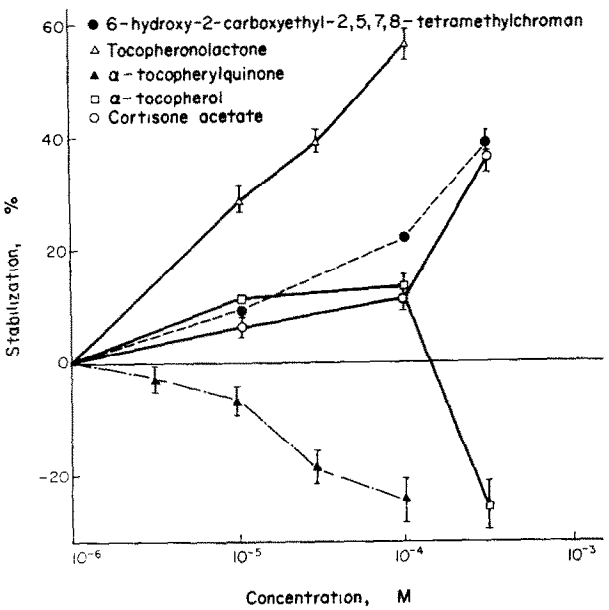

FIG. 3. Dose–response curve of tocopheronolactone, α-tocopherol, α-tocopherylquinone, 6-hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman and cortisone acetate on lysosome stabilization. Ordinate: Suppression of acid phosphatase release from lysosomal fraction during 30-min incubation at 37°. The incubation mixture was the same as in Fig. 2. The following standard errors were not illustrated in the figure in order to avoid confusion on the figure: ±1·1 for cortisone acetate 10^{-4} M; ±1·5 and ±1·1 for 6-hydroxy-2-carboxyethyl-2,5,7,8-tetramethylchroman 5×10^{-4} M and 10^{-5} M; ±1·1 and ±0·8 for α-tocopherol 10^{-4} M and 10^{-5} M respectively.

TABLE 2. EFFECT OF TOCOPHERONOLACTONE, α-TOCOPHEROL, α-TOCOPHERYLQUINONE, 5-HYDROXY-2 CARBOXYETHYL-2,5,7,8-TETRAMETHYLCHROMAN AND CORTISONE ACETATE ON RELEASE OF ACID PHOSPHATASE AND β-GLUCURONIDASE FROM LYSOSOMAL FRACTION*

Compound	Concentration (M)	Free and bound activity released during incubation			
		Acid phosphatase		β-glucuronidase	
		Free	Free + Bound	Free	Free + Bound
		(% of corresponding free activity of control ± standard errors)			
None (control)		100 (5)	183·1 ± 4·1 (5)	100 (4)	222·8 ± 8·1 (4)
Tocopheronolactone	10^{-4}	47·5 ± 0·5 (4)	127·4 ± 5·9 (4)	90·9 ± 0·9 (3)	199·6 ± 1·1 (3)
α-Tocopherol	10^{-4}	85·6 ± 2·5 (3)	169·6 ± 0·7 (3)	93·3 ± 1·1 (3)	204·5 ± 3·6 (3)
	5×10^{-4}	126·9 ± 5·8 (3)	209·5 ± 4·4 (3)	138·7 (2)	
α-Tocopherylquinone	10^{-4}	124·4 (2)	218·8 (2)	108·3 (2)	238·7 (2)
6-Hydroxy2-carboxyethyl-2,5,7,8-tetramethylchroman	0^{-4}	81·2 ± 1·1 (4)	142·8 ± 1·8 (4)	87·4 ± 1·1 (3)	193·8 ± 0·9 (3)
Cortisone acetate	5×10^{-4}	63·1 ± 3·0 (4)	133·1 ± 1·1 (4)	79·2 ± 0·9 (3)	186·9 ± 1·6 (3)

* Figures in brackets indicate the number of samples used. Free activity means the activity detectable in the supernatant of incubation mixture. Free plus bound activities mean the activity detectable in the incubation mixture without centrifugation. Incubation mixture: 0·01 M citrate buffer (pH 7·4), 0·35 M sucrose, 1·7% ethanol, and lysosomal fraction at 50 g (acid phosphatase) or 200 mg (β-glucuronidase) of liver equivalent per milliliter. Incubation was carried out for 30 min at 37°.

α-tocopherol to a benzoquinone and shortening its side chain to a lactone ring, showed stabilization of lysosomal membrane about four times that of cortisone acetate at the same concentration of 10^{-4} M. These results failed to show any clear-cut structure–activity relationship by α-tocopherol and its derivatives in the stabilization of lysosomal membrane. Destruction of lysosomes by α-tocopherol at higher concentrations might be because of the detergent like activity of the lipoid side-chain of α-tocopherol on lysosomal membrane

Effect in vivo *of tocopheronolactone and α-tocopherol on lysosomes.* Distribution of tocopheronolactone and α-tocopherol in mouse organs has already been reported.[16] According to this report, incorporation of tocopheronolactone into liver is much more rapid than that of α-tocopherol, and the largest amount was present 15 min after its intraperitoneal administration. The maximum incorporation of α-tocopherol appeared 12 hr after its administration.

Considering these findings, the stabilization effect of tocopheronolactone and α-tocopherol was therefore examined by killing the mice 15 min after the administration of tocopheronolactone and 12 hr after that of α-tocopherol. As shown in Table 3, tocopheronolactone markedly stabilized lysosomes, as was the case *in vitro*, while α-tocopherol showed far stronger stabilizing action *in vivo* than its maximum activity *in vitro* shown in Fig. 3. The fact that α-tocopherol was as effective as tocopheronolactone in stabilizing lysosomal membrane *in vivo*, together with the result *in vitro*

TABLE 3. EFFECT *IN VIVO* OF TOCOPHERONOLACTONE AND α-TOCOPHEROL ON RELEASE OF ACID PHOSPHATASE FROM LYSOSOMAL FRACTION*

Administration	Relative activity		No. of experiments
	Free	Total	
	(% of corresponding free activity of control ± standard errors)		
Control (vitamin E deficiency)	100	226·0 ± 15·5	5
Tocopheronolactone	57·0 ± 6·3	202·1 ± 9·4	3
α-Tocopherol	62·7	214·5	2

* Each mouse was injected i.p. with 50 μmoles per mouse of drugs. Detailed treatment *in vivo* is described in the text. Incubation mixture: 0·01 M citrate buffer (pH 7·4), 0·35 M sucrose, and lysosomal fraction at 50 mg of liver equivalent per milliliter. Incubation was carried out for 45 min at 37°.

shown in Figs. 2 and 3, seems to suggest that tocopheronolactone is the active metabolite of α-tocopherol, with respect of the stabilization of lysosomes.

Tappel *et al.*[17] have carried out extensive studies on the mechanism of lysosome labilization in experimental muscular dystrophy produced by vitamin E deficiency. They consider that the primary action in the production of such a dystrophy is a lipid peroxidation that damages cells or their subcellular constituents, such as the lysosomes. Based on this view, and from the previously reported fact that tocopheronolactone shows a strong antioxidation activity, inhibition of lipid peroxidation, which initiates damage to lysosomal membrane, might be responsible for stabilization of the membrane.

On the other hand, Desai *et al.*[18] found that while the release of lysosomal enzymes in vitamin E deficient chick muscle is inhibited by the administration of methionine, the tissue still shows a strong lipid peroxidizability and assumed that lipid peroxidation is not an initiator of damage to lysosomal membrane. Sharma and Krishna Murti[19] stated that the release of enzymes that occurs during incubation of lysosomes *in vitro* is because of factors other than lipid peroxidation. Since there seems to be no correlation between the extent of antioxidation activity[8] and stability of lysosomal membrane by tocopheronolactone, α-tocopherol, and α-tocopherylquinone, the stabilizing effect of these compounds on lysosomal membrane is not that of an antioxidant, but is probably a direct action on the lysosomal membrane which becomes stabilized by virtue of the interaction between membrane lipid[20] or sulfhydryl groups of membrane protein[21] and these compounds. It is also possible to consider, as Seeman[22] has hypothesized, that the compounds are inserted into the lipid membrane of lysosomes and expand the membranous layers, thereby affecting its stabilization.

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REFERENCES

1. C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX and F. APPELMANS, *Biochem. J.* **60**, 604 (1955).
2. A. O. JIBRIL and P. B. MCCAY, *Nature, Lond.* **205**, 1214 (1965).
3. H. ZALKIN, A. L. TAPPEL, K. A. CALDWELL, S. SHIBKO, I. D. DESAI and T. A. HOLLIDAY, *J. biol. Chem.* **237** (8), 2678 (1962).

4. T. Moore, I. M. Sharman, M. G. Stanton and J. T. Dingle, *Biochem. J.* **103**, 923 (1967).
5. O. A. Roels, M. Trout and A. Guha, *Biochem, J.* **97**, 353 (1965).
6. C. de Duve, R. Wattiaux and M. Wibo, *Biochem. Pharmac.* **9**, 97 (1962).
7. E. J. Simon, A. Eisengart, L. Sundheim and A. T. Milhonat, *J. biol. Chem.* **221**, 807 (1956).
8. J. Green and J. Bunyan, *Nature, Lond.* **190**, 318 (1961).
9. K. Fukuzawa, Y. Suzuki and M. Uchiyama, *Chem. pharm. Bull., Tokyo* **17**, 1761 (1969).
10. J. Green and D. Mckale, in *Biochemistry of Quinone* (Ed. R. A. Morton), p. 261. Academic Press, London & New York (1965).
11. K. Schwarz and E. J. Simon, *Biochim. biophys. Acta* **32**, 484 (1959).
12. G. Weissmann and L. Thomas, *Recent Prog. Horm. Res.* **20**, 215 (1964).
13. R. Gianetto and C. de Duve, *Biochem. J.* **59**, 433 (1955).
14. A. Guha and O. A. Roels, *Biochim. biophys. Acta* **111**, 364 (1965).
15. M. A. Verity, R. Caper and W. J. Broun, *Biochem. J.* **109**, 149 (1968).
16. K. Fukuzawa, Y. Suzuki and M. Uchiyama, *Chem. pharm. Bull., Tokyo* **17**, 2164 (1969).
17. A. L. Tappel, H. Zalkin, K. A. Caldwell, I. D. Desai and S. Shibko, *Archs Biochem. Biophys.* **96**, 340 (1962).
18. I. D. Desai, C. C. Calvert and M. L. Scott, *Archs. Biochem. Biophys.* **108**, 60 (1964).
19. S. K. Sharma and C. R. Krishna Murti, *Biochem. Pharmac.* **15**, 2025 (1966).
20. G. Weissman, in *The Interaction of Drugs and Subcellular Components in Animal Cells* (Ed. P. N. Campbell), p. 203. Churchill, London (1968).
21. K. Schwartz, *Vitam. Horm.* **20**, 463 (1962).
22. P. Seeman, *Biochem. Pharmac.* **15**, 1753 (1966).