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Ubiquinone and Related Compounds. XXVIII.[1] Effect of the Metabolites of α-Tocopherol, Phylloquinone and Ubiquinone on the Stability of Rat-liver Lysosomal Membrane

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The effect on the stability of rat-liver lysosomal membrane was investigated with *trans*-2,3,5-trimethyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (E Acid-I), 2,3,5-trimethyl-6-(3′-carboxybutyl)-1,4-benzoquinone (E Acid-II), *trans*-2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (K Acid-I), 2-methyl-3-(3′-carboxybutyl)-1,4-naphthoquinone (K Acid-II), *trans,cis* mixture (3:1) of 2,3-dimethoxy-5-methyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (Q Acid-I) and 2,3-dimethoxy-5-methyl-6-(3′-carboxybutyl)-1,4-benzoquinone (Q Acid-II).

E Acid-I, E Acid-II, K Acid-I, K Acid-II and Q Acid-I, especially E Acid-I and K Acid-I, inhibited the release of acid phosphatase and β-glucuronidase from the heavy lysosomal fraction of rat-liver *in vitro*. Q Acid-II accerelated release of the hydrolases. Structure-activity relationships were considered.

Vitamin E deficiency in animals predominantly results in anemia and muscular dystrophy which seem to be based on membrane injury. Treatment with vitamin E causes a remission of these diseases.[3,4] Thus a number of observations have been reported concerning the possibility that membrane stabilization of vitamin E is related to the physiological action of this vitamin.[5] Since stabilization of the lysosomal membrane was observed *in vitro* with an α-tocopherol metabolite, 2,3,5-trimethyl-6-(5′-carboxy-3′-hydroxy-3′-methylpentyl)-1,4-benzoquinone lactone (tocopheronolactone) but not with α-tocopherol, the latter was thought to function as the membrane stabilizer after being metabolized into the former.[6] Recently, we made clear that α-tocopherol is metabolized into 2,3,5-trimethyl-6-(3′-carboxybutyl)-1,4-benzoquinone (E Acid-II) and probably into 2,3,5-trimethyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone.[7]

In the present study, we determined the membrane-stabilizing activity of an α-tocopherol metabolite and related compounds using rat-liver lysosome as a biological membrane model, and discuss structure-activity relationships.

Material and Method

Materials——*trans*-2,3,5-Trimethyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (E Acid-I), E Acid-II, *trans*-2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (K Acid-I), 2-methyl-3-(3′-carboxybutyl)-1,4-naphthoquinone (K Acid-II), a *trans*, *cis* mixture (3:1) of 2,3-dimethoxy-5-methyl-6-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-benzoquinone (Q Acid-I), 2,3-dimethoxy-5-methyl-6-(3′-carboxybutyl)-1,4-benzoquinone (Q Acid-II), their methyl esters (E Acid-I-Me, E Acid-II-Me, K Acid-I-Me, K Acid-II-Me, Q Acid-I-Me, Q Acid-II-Me), tocopheronolactone and 2,3,5-trimethyl-6-(3′-hydroxy-3′-methyl-

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5′-methoxycarbonylpentyl)-1,4-benzoquinone (methyl tocopheronate) were synthesized by procedures already described.[1,8] 2′,3′-Dihydro derivatives (dihydro E Acid-I and dihydro K Acid-I)[9] were obtained by catalytic reduction of the corresponding Acid-I, followed by oxidation with ferric chloride. Acetylsalicylic acid was of *Pharmacopoeia Japonica* grade.

Preparation of Lysosomal Suspension——Liver was obtained from female Sprague–Dawley rats weighing 200—280 g, 7 to 9 weeks old, after they were clubbed to death. The liver was washed with 0.25M sucrose, then homogenized twice with six times its weight of the same medium in a Potter–Elvehjem apparatus. The homogenate was filtered through three sheets of gauze and the filtrate was centrifuged at $800 \times g$ for 15 min. The sediment was similarly homogenized with 0.25M sucrose and centrifuged. The combined supernatants were centrifuged at $4300 \times g$ for 15 min. The sediment was washed with 0.25M sucrose and then suspended in an aliquot volume (1.0—0.8 ml/wet g of the starting liver) of the same medium. This suspension (about 20 mg protein/ml) was used as the "lysosomal suspension" throughout this study. All these procedures were carried out below 5°. Centrifugation was performed with Sorvall RC2-B, protein was determined with Folin–Ciocalteu reagent.[10]

Enzyme Assay——Acid Phosphatase (EC 3.1.3.2, Orthophosphoric Monoester Phosphohydrolase) was assayed as follows:[11] To the enzyme solution (0.1 ml), 0.15M acetate buffer (pH 5.5, 0.3 ml) and 0.0025M phenolphthalein diphosphate 5Na in the acetate buffer (0.1 ml) were added. After incubation at 37° for 30 min, the mixture was diluted with 0.12M glycin buffer (pH 11.2, 3 ml) and the phenolphthalein which resulted was determined by extinction at 530 mμ, using a Hitachi EPW-4 spectrophotometer.

β-Glucuronidase (EC 3.2.1.31, β-D-Glucuronide Glucuronohydrolase) was assayed as follows:[12] To the enzyme solution (0.1 ml), 0.15M acetate buffer (pH 4.5, 0.3 ml) and 0.0025M phenolphthalein mono-β-glucuronic acid in the acetate buffer (0.1 ml) were added, and the mixture was incubated at 37° for 30 min. The resulting phenolphthalein was similarly determined.

Effect on Lysosomal Hydrolases——A solution of lysosomal hydrolases was obtained by incubating a mixture of the lysosomal suspension (1 ml) and 0.04M Tris-acetate buffer (pH 7.4, 4 ml) at 37° for 90 min, followed by centrifugation at $4300 \times g$ for 15 min. To the resulting solution (5 ml), a solution (10 μl) of a test compound in N,N-dimethylformamide (DMF) or the solvent alone (10 μl for control), was added and the mixture was incubated at 37° for 90 min, then the hydrolase activities were assayed as described above.

Effect on Lysosomal Membrane Stability——Effect on membrane stability was assayed by determining the hydrolases released from the lysosomal fraction at 37° as already described.[6,13] To a mixture of the lysosomal suspension (1 ml) and 0.04M Tris-acetate buffer (pH 7.4, 4 ml), a solution (10 μl) of a test compound in DMF or the solvent alone (10 μl for control) was added and the mixture was incubated at 37°. An aliquot volume (1.5 ml) of the mixture was separated after 45, 90 and 135 min of incubation, and each was subjected to centrifugation ($4300 \times g$, 15 min) immediately after separation. The hydrolase activities in the resulting supernatant were assayed as described above and compared to that of the control.

Result and Discussion

Since organic solvents are preferably used for dissolving test compounds because of their lipophilic properties, the effects of a few solvents on lysosomal hydrolase activities and on the membrane stability have to be investigated in advance. Ethanol and DMF did not affect both hydrolase activities but tetrahydrofuran (THF) inhibited lysosomal β-glucuronidase. Under the conditions described in the experimental section, 1% of ethanol in the final incubation mixture slightly increased the release of acid phosphatase and β-glucuronidase from the lysosomal fraction, and 2% and 4% of it accerelated the release enormously, *i.e.*, the release of these enzymes increased with ethanol concentration and incubation time. In the mixture containing 1 to 4% of DMF, the release of β-glucuronidase was not affected but that of acid phosphatase was enhanced with DMF concentration and incubation time. One to 4% of THF increased the release of acid phosphatase, particularly when incubated for 45 and 90 min. From these results (Table I), test compounds were dissolved in DMF, the concentration of which was adjusted to become 0.2% in the final incubation mixture, throughout this study.

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Table I. Effect of Solvents on Release of Hydrolases from Lysosomal Fraction

Solvent	Concentration (%)	Hydrolase activity (%)					
		Acid phosphatase			β-Glucuronidase		
		45 min[a]	90 min	135 min	45 min	90 min	135 min
None	(control)	100	100	100	100	100	100
DMF	1	117	125	158	100	100	110
	2	152	149	191	99	95	110
	4	200	285	257	105	117	118
EtOH	1	—	107	113	103	114	112
	2	124	132	181	105	128	136
	4	179	376	454	137	225	199
THF	1	528	1190	484	57	62	79
	2	2900	1533	484	60	43	80
	4	2900	1578	484	34	26	84

a) incubation time

Table II. Effect of Test Compounds on Lysosomal Hydrolases

Compound[a]	Hydrolase activity (%)[b]	
	Acid phosphatase	β-Glucuronidase
E Acid-I	101±3[c]	102±1
Dihydro E Acid-I	102±1	104±3
E Acid-I-Me	105±1	107±1
E Acid-II	102±2	104±1
E Acid-II-Me	104±1	104±4
K Acid-I	106±6	102±1
Dihydro K Acid-I	101±2	107±3
K Acid-I-Me	101±3	104±1
K Acid-II	101±3	103±1
K Acid-II-Me	98±4	101±2
Q Acid-I	103±1	107±2
Q Acid-I-Me	101±5	103±4
Q Acid-II	97±6	103±1
Q Acid-II-Me	103±3	105±3
Tocopheronolactone	102	104
Methyl tocopheronate	96±2	100±1
Acetylsalicylic acid	103	98

a) Compounds were added to a final concentration of 2×10^{-4}M.
b) control: 100%
c) Expressed as the mean±standard errors ($n=3$).

Since all the test compounds did not inhibit the lysosomal hydrolases (Table II), their effects on the rat-liver lysosomal membrane were determined from the hydrolase activities released from the lysosomal fraction. With a slight modification of the conditions used for tocopheronolactone[6] and some *anti*-inflammatory drugs,[13] the rat-liver lysosomal fraction precipitated between $800\times g$ and $4300\times g$ was incubated with a test compound in 0.04M Tris-acetate buffer (pH 7.4) at 37° for 45, 90 and 135 min. Under these conditions, 2×10^{-3}M of acetylsalicylic acid inhibited the release of β-glucuronidase by 15—25% and that of acid phosphatase by 27—41%. Two$\times10^{-4}$M of tocopheronolactone inhibited the release of both hydrolases by 36—59% as already described[6,13,14] (Table III). Ubiquinone and phylloqui-

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none are metabolized to structurally-related compounds to the α-tocopherol metabolites.[7,8] Therefore, we used their metabolites and the mixture of the steric isomers, Q Acid-I, Q Acid-II, K Acid-I and K Acid-II, and the derivatives of them, as related compounds of α-tocopherol metabolites and their derivatives. All test compounds, except Q Acid-II which showed membrane-labilizing activity, inhibited the release of the hydrolases to a certain degree at 2×10^{-4} and 2×10^{-5} M.

TABLE III. Effect of Test Compounds on Release of Hydrolases from Lysosomal Fraction

Compound	Concentration (M)	Hydrolase release (%)[a]					
		Acid phosphatase			β-Glucuronidase		
		45 min[b]	90 min	135 min	45 min	90 min	135 min
E Acid-I	2×10^{-5}	68± 5[c]	67± 3	73± 3	72± 3	79±2	85±2 (20)[d]
	2×10^{-4}	74± 5	58± 4	70± 5	66± 4	67±3	77±3 (20)
Dihydro E Acid-I	2×10^{-5}	76± 3	70± 6	73± 4	73± 6	81±3	85±2 (3)
	2×10^{-4}	74± 6	60± 1	71± 4	73± 9	73±3	84±2 (3)
E Acid-I-Me	2×10^{-5}	72±11	69± 5	75± 6	82± 5	77±4	81±4 (5)
	2×10^{-4}	79± 4	66± 6	89± 6	92± 6	73±5	79±3 (5)
E Acid-II	2×10^{-5}	95±13	89± 8	100± 6	91± 4	89±4	91±3 (4)
	2×10^{-4}	93± 6	83±10	91±10	87± 5	84±6	87±5 (4)
E Acid-II-Me	2×10^{-5}	94±14	94± 7	106± 5	98± 8	95±3	101±4 (5)
	2×10^{-4}	97±12	96± 8	108± 7	96± 7	92±4	95±4 (5)
K Acid-I	2×10^{-5}	63± 6	49± 5	66± 4	62± 5	66±4	79±3 (13)
	2×10^{-4}	65± 4	55± 4	78± 9	63± 5	66±3	79±4 (11)
Dihydro K Acid-I	2×10^{-5}	76± 8	79± 5	84± 3	83± 6	88±1	93±1 (3)
	2×10^{-4}	84± 5	71± 3	81± 4	77± 5	81±3	89±1 (3)
K Acid-I-Me	2×10^{-5}	81±11	57±10	70± 6	77± 9	71±3	78±1 (4)
	2×10^{-4}	90±15	63± 9	83±15	74± 8	71±5	77±4 (4)
K Acid-II	2×10^{-5}	89±15	81±11	90± 3	84±12	86±6	93±4 (3)
	2×10^{-4}	75± 3	72± 8	83± 5	77± 3	78±6	85±3 (3)
K Acid-II-Me	2×10^{-5}	113±18	95± 7	100±12	100± 3	95±3	97±4 (4)
	2×10^{-4}	98±10	82± 4	78±11	90± 7	85±4	85±4 (4)
Q Acid-I	2×10^{-5}	108± 6	104± 6	115± 4	102± 5	101±4	103±2 (4)
	2×10^{-4}	73± 5	68± 7	100± 7	71± 6	81±5	93±3 (4)
Q Acid-I-Me	2×10^{-5}	89	90	100	91	91	100 (2)
	2×10^{-4}	71	71	92	70	74	88 (2)
Q Acid-II	2×10^{-5}	126± 5	136± 4	141± 7	120± 3	112± 1	114±2 (3)
	2×10^{-4}	116±14	165±22	219±25	116± 5	116± 1	120±6 (3)
Q Acid-II-Me	2×10^{-5}	94±11	90±11	107±12	96± 7	92± 5	103±7 (3)
	2×10^{-4}	75± 6	57± 2	83± 7	70±10	68± 4	85±3 (3)
Tocopheronolactone	2×10^{-5}	60± 4	63±13	70± 7	68±17	71± 5	81±3 (3)
	2×10^{-4}	50± 9	41±16	59±12	55±24	51±14	64±8 (3)
Methyl tocopheronate	2×10^{-5}	93	78	100	91	93	82 (2)
	2×10^{-4}	66	56	79	73	71	68 (2)
Acetylsalicylic acid	2×10^{-3}	96± 9	59± 5	73± 4	85± 5	75± 4	82±2 (3)

a) % of control, control: 100% b) incubation time c) standard error d) number of assays

Their structure-activity relationships in the lysosomal membrane stabilization were considered to be as follows: (a) The stabilizing activities of E Acid-I and K Acid-I were stronger than that of Q Acid-I, indicating that methoxyls on the quinone ring decreased the activity. (b) Since the activities of the Acid-II group (E Acid-II, K Acid-II, Q Acid-II) were lower than those of the corresponding Acid-I group (E Acid-I, K Acid-I, Q Acid-I), the structure of the side chain affects the activity. From the fact that E Acid-I, its 2′,3′-dihydro derivative and tocopheronolactone showed nearly equal activities, the difference in activities between E Acid-I and E Acid-II probably depends upon the carbon number rather than the double bond. (c) Since methyl tocopheronate showed activity nearly equal to E Acid-I-Me, 3′-hydroxy on the side chain hardly affected the activity. (d) Since differences in activities between the Acid-I group and the corresponding methyl esters were

small, free carboxylic acid seemed to be not necessarily essential for the activity of the Acid-I group. However, among the Acid-II group, conversion of the carboxyl into methyl ester decreased (E Acid-II-Me, K Acid-II-Me) or increased (Q Acid-II-Me) the stabilization. From these results, a clear-cut structural correlation in the lysosomal membrane stabilization was not obtained with respect to the carboxyl group.

Among the test compounds, K Acid-I and E Acid-I showed the strongest activities at 2×10^{-5}M and 2×10^{-4}M, respectively (Fig. 1a, b).

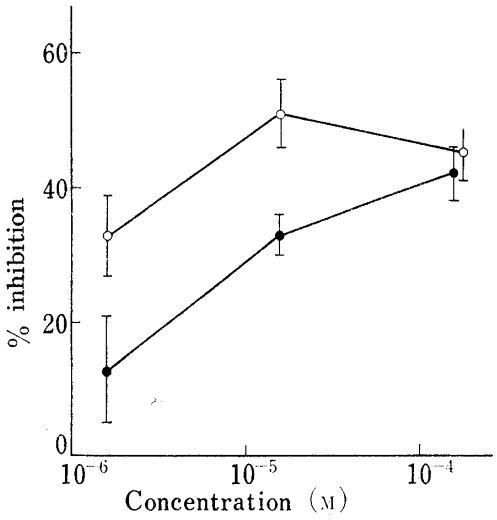

Fig. 1a. Dose-response Curves of K Acid-I and E Acid-I on Lysosomal Membrane Stabilization

ordinate: suppression of acid phosphatase release from lysosomal fraction during 90 min incubation
Each value represents the mean±standard errors.
—○—: K Acid-I, —●—: E Acid I

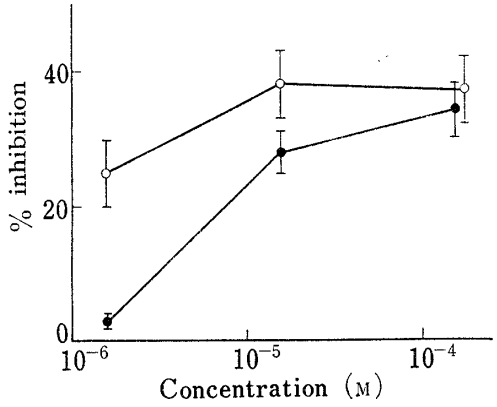

Fig. 1b. Dose-response Curves of K Acid-I and E Acid-I on Lysosomal Membrane Stabilization

ordinate: suppression of β-glucuronidase release from lysosomal fraction during 45 min incubation
Each value represents the mean±standard errors.
—○—: K Acid-I, —●—: E Acid-I

Tocopheronolactone has been reported to inhibit strongly the release of acid phosphatase, and slightly[6] that of β-glucuronidase. Under the conditions used, however, considerable inhibition of the release of β-glucuronidase was also observed with almost all of the quinone acids, including tocopheronolactone. Maximal suppression was observed at 90 min incubation for acid phosphatase, and at 45 and 90 min incubation for β-glucuronidase. When incubated for 135 min, suppression of the release was rather equivocal, especially for β-glucuronidase.

From our study, the metabolites of α-tocopherol, phylloquinone and ubiquinone, except Q Acid-II, were found to have the membrane-stabilizing activity. This activity of these metabolites may help elucidate the action mechanisms of these vitamins, since the membrane-stabilizing activities of these vitamins have been found *in vivo*[6,15,16] but not *in vitro*.[15,17]

Inflammation has been thought to be induced by the release of lysosomal hydrolases[18] and some *anti*-inflammatory drugs have been reported to have the membrane-stabilizing activity.[19] α-Tocopherol and the vitamin K group, though some discrepancy remains with

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the latter,[20] have shown *anti*-inflammatory effects.[21,22] An α-tocopherol metabolite, tocopheronolactone was also found to have *anti*-inflammatory effects against dextran- and carrageenin-induced paw edemas, being as effective as hydrocortisone acetate.[23] The strong stabilizing activity of E Acid-I and K Acid-I suggests that these quinone acids are formed metabolically and may play a part in the *anti*-inflammatory effect of the corresponding vitamin.

The immunoadjuvant activity of ubiquinone-7 may depend on the labilizing activity of Q Acid-II since intraperitoneal administration of ubiquinone-7 resulted in an increment of free forms of the splenic lysosomal hydrolases at 1 week after administration[16] and the adjuvant activity was reported to correlate to the labilization of the lysosomal membrane.[24]

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