[Chem. Pharm. Bull. 17(9)1771—1777(1969)]

UDC 615.356.015 : 577.161.2

Modified Lipophilic Vitamin. II.[1] Effect of Tocopheronolactone on Lipid-Peroxidation[2]

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(Received September 6, 1968)

1) The effect of tocopheronolactone on two lipid-peroxidation systems, one is the enzymatic system coupled to NADPH oxidation and stimulated by ADP-Fe^{+2} mixture (NADPH-ADP-Fe^{+2} system), and the other is non-enzymatic system induced by ascorbate and Fe^{+2} (ascorbate-Fe^{+2} system), were studied.

2) The quinol form of tocopheronolactone inhibited the autoxidation of unsaturated fatty acid, but quinoid form of it, unlike ubiquinone and *p*-benzoquinone, did not show the antioxidant activity.

3) The enzymatic reduction of tocopheronolactone was inhibited by dicoumarol which peculiarly inhibit the quinone reductase.

4) Though antioxidant activity of tocopheronolactone on the ascorbate-Fe^{+2} lipid-peroxidation system come into effect through the prior reduction to quinol, its activity on the NADPH-ADP-Fe^{+2} lipid-peroxidation system seems to be caused by intact quinone. Such assumption could be introduced by taking the effect of dicoumarol on above two system into consideration.

5) Tocopheronolactone did not inhibit the NADPH-linked metabolism of aminopyrine and codeine.

Peroxidation of unsaturated fatty acid has been noticed as one of the physiological reactions which may subsequently lead to several diseases in various tissues.[4] Two main mechanisms of peroxidation have been proposed. One is non-enzymatic lipid-peroxidation which can be induced in the presence of oxygen by the incubation with various free radical initiators such as ferrous ion,[5] haemoprotein,[6] ascorbate,[7] or glutathione,[8] and the other is enzymatic lipid-peroxidation[9] which is taking place in microsomes under a certain condition similar to oxygenation coupled with the oxidation of NADPH. The latter peroxidative pathway is markedly stimulated by the addition of ADP or other compounds containing the pyrophosphate group, and also the presence of low concentration of Fe^{+2}.[9,10] α-Tocopherol is well known as antioxidant irrespective of such mechanism of lipid-peroxidation. In 1953 Simon, *et al.*[11] isolated tocopheronolactone from the urine of rabbit and human as a metabolite of α-tocopherol. The physiological significance of tocopheronolactone has been demonst-

1) Part I: M. Uchiyama, Y. Suzuki and K. Fukuzawa, *Yakugaku Zasshi*, **88**, 678 (1968).
2) This report has been presented at the 10th annual meeting of Japanese Conference for Biochemistry of Lipid, Tokyo, May 31, 1968.
3) Location: *Aobayama, Sendai.*
4) E.D. Wills, *Biochem. J.*, **99**, 667 (1966).
5) R.C. Mcknight, F.E. Hunter and W. Oehlert, *J. Biol. Chem.*, **240**, 3439 (1965).
6) A.L. Tappel, *J. Biol. Chem.*, **217**, 721 (1955).
7) A. Ottolenghi, *Arch. Biochem. Biophys.*, **79**, 355 (1959).
8) A.K. Schneider, E.E. Smith and F. E. Hunter, *Biochemistry*, **3**, 1470 (1964).
9) P. Hochstein and L. Ernster, *Biochem. Biophys. Res. Commun.*, **12**, 388 (1963); P. Hochstein, K. Nordenbrand and L. Ernster, *Biochem. Biophys. Res. Commun.*, **14**, 323 (1964).
10) A. Beloff-Chain, G. Serlupi-Crescenzi, R. Catanzaro, D. Venettacci and M. Balliano, *Biochim. Biophys. Acta*, **97**, 416 (1965).
11) E.J. Simon, A.E. Eisengart, L. Sundheim and A.T. Milhorat, *J. Biol. Chem.*, **221**, 807 (1956).

rated in some respects. Green, *et al.*[12] have reported that treatment of tocopheronolactone recovers the level of ubiquinone in the uterus of vitamin E-deficient rat more rapidly than α-tocopherol. Schwarz, *et al.*[13] have reported that the decline in respiration of necrotic liver degeneration, which is the outcome of feeding the α-tocopherol deficient diet, can be prevented by supplementing the diets with either α-tocopherol or selenium, or by injecting a solution of tocopherol directly into the circulation of the animal, but cannot be arrested by adding either α-tocopherol or selenium to the medium in which the slices are incubated. However tocopheronolactone or tocopheronic acid is completely effective *in vitro* in preventing respiratory decline. From these reports, it has been considered that vitamin E may function by being degraded to the quinoid metabolite such as tocopheronolactone.

We[1] have reported previously the antioxidative action of tocopheronolactone which is reduced in advance to a quinol form *in vivo* by tocopheronolactone-reductase[14] and thereafter behave as a trap for free radicals produced during the lipid-peroxidation. In the present experiment it became obvious that, without any reduction beforehand, tocopheronolactone itself can behave directly as an antioxidant on the enzymatic lipid-peroxidation.

Experimental

Materials——Amytal, dicoumarol, codeine and aminopyrine were commercially obtained. Ubiquinone-0 was synthesized by the method of Anslow, *et al.*[15] Ubiquinone-9, α-tocopherol and tocopheronolactone were obtained from Eisai Co., Ltd.

Preparation of Unsaturated Fatty Acids——Linseed oil was hydrolyzed with 5% of potassium hydroxide in ethanol. After removal of tocopherol by extraction with *n*-hexane the water layer was acidified with 6N HCl and extracted with *n*-hexane to obtain fatty acid mixture. After removing the solvent under reduced pressure in nitrogen the fatty acids were added to ten volumes of acetone and stand overnight at −20°. After removal of solid material by filtration, unsaturated fatty acid fraction was obtained upon evaporation of the filtrate. The composition of this fraction was analysed by gas-liquid chromatography (Table I).

Manometric Measurement——The reaction mixture indicated in Table II was shaken at 37° by means of Warburg manometric apparatus. Oxygen uptake caused by autoxidation of unsaturated fatty acids was measured manometrically.

Oxidative Polymerization——Fifteen ml of reaction mixture appeared in Table III was polymerized by incubation at 65° without agitation. The increase in viscosity of each sample was measured by Ostwald viscosimeter over 4 hr and compared with the increase in viscosity of controls containing only unsaturated fatty acids.

TBA Value——Lipid-peroxidation was estimated by a thiobarbiturc acid (TBA) reaction as described by Tappel.[16] To 2.0 ml sample of each reaction mixture was added 3.0 ml of 10% TCA solution (exceptionally 2.5 ml sample and 1.0 ml of 30% TCA solution in Table VII). After centrifugation, 2.0 ml of the supernatant was separated and heated together with 3.0 ml of 0.75% TBA solution at 100° for 10 min. The resultant red color was measured at 532 mμ.

Enzyme Activity for Drug Metabolism——The liver was homogenized in 4 volumes of 1.15% KCl solution by Potter-Elvehjem homogenizer with teflonpestle. The supernatant fraction was obtained by centrifuging the homogenate at $9000 \times g$ for 30 min. A reaction mixture of oxidative demethylation (see Table VII) containing 1.0 ml of this supernatant was incubated at 37° for 1 hr. The activities of the oxidative demethylation of codeine and aminopyrine were determined from the amount of formaldehyde formed. At the end of the incubation period 1.0 ml of 30% TCA solution and 1.0 ml of 1N H_2SO_4 were added to the reaction mixture. After centrifugation in removing protein, the supernatant was poured into distillating flask and made up to a volume of 5.0 ml. Three ml of distillate was taken and the amount of formaldehyde was determined with chromotropic acid method.[17]

12) J. Green and J. Bunyan, *Nature*, **190**, 318 (1961).
13) K. Schwarz, W. Mertz and E. J. Simon, *Biochim. Biophys. Acta*, **32**, 484 (1959).
14) J. Bunyan and J. Green, *Biochim. Biophys. Acta*, **49**, 420 (1961).
15) W.K. Anslow, J.M. Askley and H. Raistrick, *J. Chem. Soc.*, **1938**, 441.
16) A.L. Tappel and H. Zalkin, *Arch. Biochem. Biophys.*, **80**, 326 (1959).
17) R.M. Burton, *Method in Enzymology*, **3**, 247 (1957).

Result

Effect of Tocopheronolactone on the Autoxidation of Unsaturated Fatty Acid

It is known that ubiquinone, either in quinol form or in quinoid form inhibits autoxidation of unsaturated fatty acid.[18] Similar studies by Kaufmann and Garloff[19] showed that ubiquinone had greater antioxidant activity than α-tocopherol against emulsion of potassium linoleate in phosphate buffer at pH 7.2.

Table II shows the effect of tocopheronolactone and its quinol form on the heme-catalyzed oxygen uptake of unsaturated fatty acids in phosphate buffer at pH 7.4. Under these conditions, only quinol form of tocopheronolactone showed antioxidant activity.

Table I. Composition of Unsaturated Fatty Acid Fraction isolated from Linseed Oil

Fatty acid	Contents (%)	Fatty acid	Contents (%)
Palmitic acid	6.2	Linoleic acid	17.2
Oleic acid	27.1	Linolenic acid	49.5

Fatty acids were methylated with diazomethane and analyzed by a SHIMADZU GC-1B gas chromatograph with a hydrogen flame ionization detector. The column was 1.5 m×4 mm packed with 5% diethylenglycol succinate polyester coated 80—100 mesh Celite-545, and usually operated at 205°. Relative weight of each acid was determined by measurement of the peak area.

Table II. Tocopheronolactone and Related Compounds as Inhibitor of Hemin Catalyzed Lipid-Peroxidation

Inhibitor (5×10^{-3} M)	Oxygen uptake (μl/min)	Inhibition (%)
None	12.3	0
Tocopheronolactone	11.4	7.3
Reduced Tocopheronolactone	5.4	57.1
α-Tocopherol	4.5	63.4
Ubiquinone-9	8.3	32.5
Ubiquinone-0	5.6	54.5

The reaction mixture contained: 5×10^{-3} M of inhibitors, 1×10^{-4} M of hemin and 0.1 M phosphate buffer (pH 7.4) to make 2 ml per 15 ml Warburg flasks. The center cups contained 0.2 ml of 30% KOH. Incubation was carried out at 37°.

Effect of Tocopheronolactone on the Polymerization of Unsaturated Fatty Acid

Heating of linolenic acid in the presence of oxygen induces polymerization and increases viscosity.[18] The effects of tocopheronolactone and related compounds on the rate of oxidative polymerization of unsaturated fatty acids at 65° are shown in Table III. Ubiquinone-9, ubiquinone-0 and tocopheronolactone did not appreciably affect the polymerization. However, α-tocopherol, benzoquinone and reduced-tocopheronolactone were effective for inhibition of the lipid polymerization. Such ability of benzoquinone is well consistent with its utility as retarders of vinyl polymerization but the mechanism of action of the other two will be different from that of benzoquinone.

Inhibition of Tocopheronolactone Reductase

Bunyan, *et al.*[14] described the isolation of a pyridine nucleotide-tocopheronolactone reductase from rat liver. They suggested that tocopheronolactone could be the natural sub-

18) A. Mellors and A.L. Tappel, *Lipid*, **1**, 282 (1966).
19) H.P. Kaufmann and H. Garloff, *Fette Seifen Anstrichmittel*, **63**, 334 (1961).

Table III. Tocopheronolactone and Related Compounds as Inhibitor of the Oxidative Polymerization of Unsaturated Fatty Acid

Test substance (10^{-2}M)	$\frac{\text{Increase in viscosity over 4 hr}}{\text{Increase in viscosity of control over 4 hr}} \times 100$
Tocopheronolactone	76.1
Reduced Tocopheronolactone	42.3
α-Tocopherol	23.3
Ubiquinone-9	80.0
Ubiquinone-0	79.9
p-Benzoquinone	48.8
β-Naphthoquinone	78.0

The reaction mixture contained: 10^{-3} M of test substances and unsaturated fatty acid to make 15 ml. Incubation was carried out at 65° for 4 hr. The increase in viscosity of each sample was measured by Ostwald viscosimeters.

strate of a certain enzyme previously referred as vitamin K_1 and menadione reductase. We reported[1] that the activity of tocopheronolactone reductase was observed in 9000 × *g* supernatant fraction and not in mitochondria. The data in Table IV indicate that dicoumarol, which is a specific inhibitor of quinone reductase (1.6.5.1.), showed almost complete inhibition to the activity of tocopheronolactone reductase at 10^{-4}M concentration. Amytal, a inhibitor of NADH dehydrogenase, has slight inhibitory action on this system.

Table IV. Inhibition of Tocopheronolactone Reductase by Different Agents

Inhibitor	Concentration (M)	Activity (mμ moles/min/mg protein)	Inhibition (%)
Amytal	3.8×10^{-4}	97	29
Amytal	3.8×10^{-5}	138	0
Dicoumarol	7.3×10^{-4}	0	100
Dicoumarol	7.3×10^{-5}	26	81
Dicoumarol	7.3×10^{-6}	69	49
None		136	0

Incubation mixture contained: 0.1 ml of 3.65×10^{-2} M tocopheronolactone, 1 ml of 4.5×10^{-3} M NADH, 1 ml of 9.6×10^{-3} M Tris-HCl buffer (pH 7.4), 0.1 ml of 20000 × *g* supernatant (1.0 mg protein) and 0.25 M sucrose to make 3.2 ml. Incubation at 37° for 1 min.

Effect of Tocopheronolactone and Dicoumarol on Lipid-peroxidation of Ascorbate-Fe^{+2} System in 9000 × *g* Supernatant Fraction and in Mitochondria

Peroxidation of the endogenous unsaturated fatty acids in mitochondrial or microsomal fraction is stimulated with the existence of ferrous ion and ascorbate. In Table V are the data concerning the TBA value induced by ascorbate-Fe^{+2} system in mitochondria, 9000×*g* supernatant and boiled 9000×*g* supernatant fraction. α-Tocopherol inhibited lipid-peroxidation in these three fractions but tocopheronolactone was inhibitory only in 9000×*g* supernatant fraction in which a tocopheronolactone reductase exists. The antioxidative effect of tocopheronolactone in 9000×*g* supernatant was decreased by the addition of dicoumarol.

Effect of Tocopheronolactone on Lipid-peroxidation of NADPH-ADP-Fe^{+2} System in 9000×*g* Supernatant Fraction.

Hochstein, *et al.*[9] reported on the enzymatic lipid peroxidation of microsome that pyrophosphate group (as in ADP) and Fe^{+2} were required and NADPH could not be replaced by NADH. Tocopheronolactone was found to inhibit also such NADPH–ADP–Fe^{+2} system.

Table V. Effect of Tocopheronolactone and Dicoumarol on Ascorbic Acid and Ferrous Ions–Induced Lipid–Peroxidation in Mitochondria and $9000 \times g$ Supernatant (Sup.) of Rat Liver

Addition	TBA value		
	Mitochondria[a]	$9000 \times g$ sup.[b]	Boiled[c] $9000 \times g$ sup.
None	0.014	0.025	0.042
Fe^{+2}, ascorbate	0.148	0.153	0.282
Fe^{+2}, ascorbate, tocopheronolactone	0.178	0.178	0.266
Fe^{+2}, ascorbate, NADH	0.157	0.179	
Fe^{+2}, ascorbate, NADH, tocopheronolactone	0.190	0.040	0.304
Fe^{+2}, ascorbate, dicoumarol, tocopheronolactone		0.184	
Fe^{+2}, ascorbate, NADH, dicoumarol, tocopheronolactone		0.158	
Fe^{+2}, ascorbate, α-tocopherol	0.018	0.025	0.039

$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$; 6.4×10^{-5} M
tocopheronolactone; 4.5×10^{-4} M
NADH; 7.5×10^{-4} M
incubation at 37° for 10 min
ascorbate; 7.1×10^{-3} M
α-tocopherol; 2.9×10^{-4} M
dicoumarol; 3.7×10^{-4} M

a) incubation mixture contained: 0.5 ml of mitochondria (7 mg protein), 0.4 ml of 0.1 M Tris–HCl buffer (pH 7.4) and 0.25 M sucrose to make 4 ml
b) incubation mixture contained: 0.5 ml of $9{,}000 \times g$ supernatant (27 mg protein), 0.4 ml of 0.1 M Tris–HCl buffer (pH 7.4) and 0.25 M sucrose to make 4 ml
c) Boiling at 100° for 10 min.

However, in contrast to the ascorbate–Fe^{+2} lipid–peroxidation system, antioxidant effect of tocopheronolactone was insensitive to the system in the presence of dicoumarol (Table VI).

Table VI. Effect of Tocopheronolactone on NADPH–linked Lipid-Peroxidation in $9000 \times g$ Supernatant of Rat Liver

Addition	TBA value
None	0.024
ADP-Fe^{+2}	0.275
ADP-Fe^{+2}, NADPH	0.399
ADP-Fe^{+2}, NADPH, tocopheronolactone	0.047
ADP-Fe^{+2}, NADPH, dicoumarol	0.393
ADP-Fe^{+2}, NADPH, dicoumarol, tocopheronolactone	0.040
ADP-Fe^{+2}, NADPH, α-tocopherol	0.025
ADP-Fe^{+2}, NADPH, SKF-525A	0.086

ADP; 1.0×10^{-3} M
NADPH; 2.4×10^{-4} M
dicoumarol; 7.4×10^{-4} M
SKF-525FA; 1.3×10^{-4} M
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$; 1.3×10^{-6} M
tocopheronolactone; 1.8×10^{-4} M
α-tocopherol; 7.6×10^{-5} M
incubation mixture contained: 0.2 ml of $9000 \times g$ supernatant (7 mg protein), 0.2 ml of 0.1 M Tris-HCl buffer (pH 7.4) and 0.25 M sucrose to make 2.0 ml. incubation at 37° for 10 min

Effect of Tocopheronolactone on NADPH-ADP-Fe^{+2} System and Drug-metabolizing System

Ernster, *et al.*[20] have suggested that NADPH–ADP–Fe^{+2} system was coupled to the drug–detoxication system because drugs undergoing oxidative demethylation strongly inhibit the NADPH–linked peroxidation of lipid in microsomes.

Table VII shows that aminopyrine inhibits the NADPH–ADP–Fe^{+2} lipid–peroxidation system but codein has no effect upon the enzymatic lipid–peroxidation. Tocopheronolactone did not inhibit the drug–metabolizing system at the concentration completely inhibitory to the NADPH–ADP–Fe^{+2} system.

20) S. Orrenius, G. Dallner and L. Ernster, *Biochem. Biophys. Res. Commun.*, **14**, 329 (1964).

Slater[21] reported the similar observation that promethazine inhibits the microsomal demethylation of aminopyrine, but the concentration required was much higher than that found to depress the lipid–peroxidation of NADPH–ADP–Fe^{+2} system strongly.

Table VII. Effect of Tocopheronolactone on NADPH–linked Lipid–Peroxidation and Oxidative Demethylation in Rat Liver

Addition	Lipid-Peroxidation TBA Value	Demethylation formaldehyde (μ moles/hr.)
None	0.027	
ADP-Fe^{+2}	0.520	
Codein	0 039	1.92
Codein, ADP-Fe^{+2}	0.545	1.84
Codein, ADP-Fe^{+2}, tocopheronolactone	0.015	1.86
Codein, ADP-Fe^{+2}, α-tocopherol	0.018	1.90
Codein, ADP-Fe^{+2}, SKF-525A	0.092	0.76
Aminopyrine	0.019	2.42
Aminopyrine, ADP-Fe^{+2}	0.051	2.58

ADP; 5.0×10^{-4} M
α-tocopherol; 5.0×10^{-5} M
codein; 2.0×10^{-3} M
SKF-525A; 1.0×10^{-4} M
$FeSO_4(NH_4)_2SO_4\cdot6H_2O$; 6.0×10^{-6} M
tocopheronolactone; 5.0×10^{-5} M
aminopyrine; 8.0×10^{-3} M
incubation mixture contained: 1 ml of 9,000 × *g* supernatant (20 mg protein), 0.5ml of 0.1 M nicotinamide, 0.5 ml of 0.1 M semicarbazide, 0.5 ml of 5.0×10^{-2} M $MgCl_2$, 0.5 ml of 2.0×10^{-3} M NADP, 0.5 ml of 2.0×10^{-2} M G–6–P, 1 ml of 0.2 M phosphate buffer (pH 7.4) and H_2O to make 5 ml. incubation at 37° for 60 min

Discussion

Ascorbate–Fe^{+2} catalyzed lipid–peroxidation was inhibited by tocopheronolactone only in the presence of 9000 × *g* supernatant of liver. Since tocopheronolactone reductase is located in 9000 × *g* superanatant but not in mitochondria,[1] the above observation reveals that non–enzymatic lipid–peroxidation may be prevented by quinol form of tocopheronolatone. Such assumption is well compatible with the effect of reduced tocopheronolactone on UV-irradiated unsaturated fatty acids.[1] Dicoumarol inhibits the reduction of tocopheronolactone in 9000 × *g* supernatant and consequently diminishes the antioxidative potency against non–enzymatic peroxidation taking place in tissue preparation.

On the other hand, the antioxidant effect of tocopheronolactone on the NADPH–linked enzymatic lipid–peroxidation was not inhibited by the addition of dicoumarol. It is probable that tocopheronolactone reacts with a certain component of the NADPH–ADP–Fe^{+2} system without being reduced to quinol, thereby produces a diminished TBA value.

Lipid–peroxidation has so far been payed attention as a casual metabolic disorder introducing several diseases. However, the greater part of the study was done in connection with the non–ezymatic peroxidation occurring in various tissues. A few investigations taking the enzymatic lipid–peroxidation under consideration were reported: Slater[21] suggested that the protective effect of promethazine against the liver necrosis produced by carbontetrachloride is due to the inhibition of enzymatic lipid–peroxidation. F. Marks, *et al.*[22] have reported that the metabolism of estrone and the enzymatic lipid-peroxidation compete for a common factor.

If the drug–detoxication system and NADPH–ADP–Fe^{+2} system involve several common step as described by Ernster, tocopheronolactone must inhibit the NADPH–ADP–Fe^{+2} system

21) T.F. Slater, *Biochem. J.*, **106**, 155 (1968).
22) F. Marks and E. Hecker, *Hoppe–Seyler's Z. Physiol. Chem.*, **349**, 523 (1968).

after the point where the two system diverge, since tocopheronolactone showed no influence on drug-detoxicating activity (Table VII). NADPH–ADP–Fe^{+2} system is inhibited by amino pyrine. This inhibition is probably the result of competition between the two processes for common NADPH-oxidizing enzyme described above. However, codein had no effect on the NADPH–ADP–Fe^{+2} system. Similar result was reported by Gram, *et al.*[23] These findings may be explained by the assumption that several terminal enzyme system exist individually for drug metabolism and they have different affinities to the side of divergence of microsomal electron transfer chain.

Schwarz and Corwin, *et al.*[24] have suggested that initiation of respiratory decline in α-tocopherol deficient slices or homogenate is related to a disturbance in trace element balance, which lead to a loss of titratable sulfhydryl groups, and tocopheronolactone may protect these sensitive sulfhydryl site to 1—4 addition compounds or alternatively co-ordination compounds with a trace metal such as Manganese.

Utley[25] described that incubation of mouse liver microsomes with sulfhydryl reagents such as $HgCl_2$ or *p*-chloromercuribenzoic acid resulted in the formation of lipid-peroxidation, and this peroxidation may be consistent with the possibility that sulfhydryl-reacting agents produce a change in tertiary structure of microsomal Fe_x, thereby rendering the protein-bound iron available for catalysis of peroxiation of endogenous lipid.

Refer to these results mentioned above, we want to present the possibility that tocopheronolactone may prevent the occurrence of the abnormal reaction, such as enzymatic lipid-peroxidation, in consequence of protection of change in the sensitive redox system of microsomes.

Acknowledgement We wish to express our appreciation to Eisai Co., Ltd. for a generous gift of α-tocopherol and tocopheronolactone.

23) T.E. Gram and J.R. Fouts, *Arch. Biochem. Biophys.*, **114**, 331 (1966).
24) K. Schwarz, *Vitamin and Hormone*, **20**, 463 (1962); L.M. Corwin and K. Schwarz, *Arch. Biochem. Biophys.*, **100**, 398 (1963).
25) H.G. Utley and P. Hochstein, *Arch. Biochem. Biophys.*, **118**, 29 (1967).