The Mechanism of Synergism between Tocopherols and Trimethylamine Oxide in the Inhibition of the Autoxidation of Methyl Linoleate

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The synergistic effect of trimethylamine oxide (TMAO) on the antioxidant activity of γ -tocopherol (γ -Toc) to inhibit the autoxidation of methyl linoleate was investigated.

Formation of the reducing dimers of γ -Toc was found to be closely related to the synergism of TMAO with γ -Toc. γ -Toc diphenyl ether dimer and two γ -Toc biphenyl dimers were identified, and the latter were considered to be atropisomers with each other.

From the reaction of the hydroperoxide (LOOH) of methyl linoleate with TMAO, TMAO was found to act as a peroxide decomposer. TMAO produced methyl keto-octadecadienoate, which was formed only in the presence of γ -Toc and TMAO, from LOOH, and markedly decreased the POV of LOOH.

A possible mechanism of synergism between Toc and TMAO in the inhibition of the autoxidation of methyl linoleate was proposed.

Tocopherol (Toc) is widely present in vegetable oils and has been expected to replace some synthetic antioxidants such as BHA and BHT. Although the antioxidative activity of Toc is inferior to that of some synthetic antioxidants, it may be expected to have a marked antioxidative activity in a combination with proper synergists. Substances such as amino acid,^{1~3)} melanoidin,⁴⁾ flavonoids,⁵⁾ amines,⁶⁾ phospholipid,^{7~8)} ascorbic acid,^{9~10)} citric acid,^{10~12)} BHA,¹³⁾ trimethylamine oxide (TMAO), etc. have been known to be synergists of Toc. We have made much effort to clarify the mechanism of antioxidative synergism between Toc and TMAO, which exists in fish.

In a previous paper,¹⁵⁾ synergism between δ -Toc and TMAO in the inhibition of the autoxidation of methyl linoleate was found to be closely related to the formation of the reducing dimers of δ -Toc. Furthermore, methyl linoleate containing δ -Toc and TMAO characteristically formed methyl keto-octadecadienoate (abbreviated as keto acid), and its peroxide value (POV) did not increase much.

Kamiya^{16,17)} described synergism between a

free radical acceptor and a peroxide decomposer which occur in the following way. A free radical acceptor (*e. g.*, Toc or its dimers) acts on the peroxy radical of oil to form hydroperoxide. The hydroperoxide reacts with a peroxide decomposer to produce inactive compounds, which do not act as initiators of a radical chain reaction any longer.

Yamaguchi *et al.* reported that melanoidin acted as a synergist when coupled with Toc and decomposed peroxides in a hydrated system.^{4,18)} However, the synergistic mechanism has never been clarified.

The present paper deals with the reaction of α - and γ -Toc with TMAO during the autoxidation of methyl linoleate compared with that of δ -Toc.¹⁵⁾ The roles of TMAO in the formation of the keto acid from methyl linoleate and in decreasing POV were also investigated, and a possible synergistic mechanism between Toc and TMAO is proposed.

MATERIALS AND METHODS

Toc. *dl*- α -Toc (100%) and *d*- γ -Toc (98%) were

kindly provided by Eisai Co. and used without further purification.

Methyl linoleate. Methyl linoleate was the same as used in the previous paper.¹⁵⁾

Methyl linoleate hydroperoxide. The hydroperoxide (LOOH) was prepared from autoxidized methyl linoleate according to the method of Matsushita *et al.*,¹⁹⁾ and used after purification by TLC.

TMAO. TMAO (Tokyo Kasei Co.) of analytical grade was purchased.

Isolation of reaction products. Reaction products of α - and γ -Toc and methyl linoleate were isolated and their amounts were determined by weighing after purification according to the method described in the previous paper.¹⁵⁾

Measurement of POV. POV was iodometrically determined.

Evaluation of induction period. The experimental procedures were the same as those in the previous paper.¹⁵⁾

Decomposition of LOOH. A mixture of LOOH and TMAO or amine was kept under nitrogen in the dark at 50°C. The degree of the decomposition of LOOH was followed by measuring POV. After treatment, the reaction mixture was subjected to TLC with a solvent system, *n*-hexane-ethyl ether (8: 2, v/v).

TLC. Thin-layer plates (thickness, 0.5 mm) were prepared with Kieselgel HF₂₅₄ (Merck), and the chromatograms were examined under ultraviolet light and with reagents such as 2,4-dinitrophenylhydrazine and potassium iodide.

RESULTS

1. Effect of TMAO on the antioxidant activity of α - and γ -Toc

1) **Degradation of γ -Toc.** Changes in the amount of γ -Toc during the autoxidation of methyl linoleate with and without TMAO were followed by GLC,¹⁵⁾ and the results are shown in Fig. 1.

γ -Toc in the reaction mixtures treated with and without TMAO seemed to disappear within almost the same time. Methyl linoleate containing γ -Toc and TMAO needed a longer induction period of 26 days to initiate propagation even after a drastic decrease in the amount of γ -Toc compared with that of 9 days

FIG. 1. Degradation of γ -Tocopherol during the Autoxidation of Methyl Linoleate.

Methyl linoleate containing 0.1% of γ -Toc and 0.05% of TMAO was kept in the dark at 60°C. ○, without TMAO; ●, with TMAO.

in the absence of TMAO. The results suggest that synergism between γ -Toc and TMAO mainly depends on the interaction of the reducing dimers derived from γ -Toc with TMAO in the autoxidizing oil.²⁰⁾

2) **Changes in the amounts of γ -Toc and its dimers.** Three dimers of γ -Toc were obtained when methyl linoleate containing 4% of γ -Toc and 2% of TMAO was autoxidized in the dark at 50°C, and they were identified to be 5-(γ -tocopheryl-oxy)- γ -tocopherol (γ -Toc diphenyl ether dimer) and two 5-(γ -tocopheryl)- γ -tocopherol (γ -Toc biphenyl dimers) which are existing as atropisomers with each other as described in a previous paper.²⁰⁾ Changes in the amounts of γ -Toc and its dimers were followed in the presence or absence of TMAO, and the results are shown in Fig. 2.

The induction period for the autoxidation of methyl linoleate was about 30 days in the absence of TMAO, and γ -Toc and its dimers disappeared in about 35 days, while the period in the presence of TMAO was more than 150 days and remarkable synergism was observed. The change in POV proceeded almost in the same manner as in the case of δ -Toc reported previously.¹⁵⁾

Figure 3 shows the relationship between the amount of the keto acid formed and the consumption of γ -Toc and its dimers. The amounts of keto acid formed only in the pre-

FIG. 2. Changes in the Amounts of γ -Tocopherol and Its Dimers during the Autoxidation of Methyl Linoleate with and without TMAO.

Autoxidized methyl linoleate of a commercial product was purified by passing through a silica gel column to remove peroxides. The purified methyl linoleate containing 4% of γ -Toc and 2% of TMAO was autoxidized in the dark at 50°C. ●, total amounts of γ -Toc and its dimers; ○, γ -Toc; △, γ -Toc biphenyl dimers; □, γ -Toc diphenyl ether dimer.

FIG. 3. Relationship between the Formation of Methyl Keto-octadecadienoate and the Consumption of γ -Tocopherol and Its Dimers during the Autoxidation of Methyl Linoleate Containing TMAO.

The incubation conditions were the same as those described in Fig. 2. ○—○, γ -Toc; ●—●, γ -Toc and its dimers; ○---○, methyl keto-octadecadienoate.

sence of TMAO were 0.8, 2.4, and 7.3% of the initial weight of methyl linoleate at 16, 40, and 80 days reaction, respectively.

3) *Changes in the amounts of α -Toc and keto acid.* Figure 4 shows changes in the amounts of α -Toc and keto acid during the autoxidation of methyl linoleate at 50°C.

FIG. 4. Degradation of α -Tocopherol and Formation of Methyl Keto-octadecadienoate during the Autoxidation of Methyl Linoleate.

Methyl linoleate containing 4% of α -Toc and 2% of TMAO was autoxidized in the dark at 50°C. ●—● and ○—○, α -Toc with and without TMAO, respectively; ○---○, methyl keto-octadecadienoate.

α -Tocopheryl ethane, a dimer formed from the reaction of α -Toc with TMAO as described in a previous paper²⁰⁾ was not observed. NMR analysis showed that a complex between α -Toc and methyl linoleate was present in the reaction products, but its structure could not be determined.

A noteworthy feature in the presence of TMAO was the formation of a large amount of keto acid, and about one-third of methyl linoleate changed into keto acid at 28 days after the initiation of reaction.

2. Reaction of LOOH with TMAO

In order to clarify the causes of the inhibition of the increase in POV and the pathway for keto acid, investigated were reactions of TMAO with LOOH which is the first oxidation product in the autoxidation of methyl linoleate and is produced by reaction between an antioxidant and methyl linoleate peroxy radical.^{21~23)}

1) *Decomposition of LOOH with TMAO.* The results are shown in Fig. 5. As LOOH is unstable, it decomposed under nitrogen at 50°C and the decrease in POV was observed. The drastic decrease in POV in the presence of TMAO indicates that LOOH was rapidly decomposed and TMAO acted as a peroxide

FIG. 5. Decomposition of Methyl Linoleate Hydroperoxide (LOOH) with TMAO.

LOOH (1 g) with and without TMAO (100 mg) was kept under nitrogen in the dark at 50°C.

Initial POV of LOOH was 5240.

○, without TMAO; ●, with TMAO.

decomposer.^{16,17)}

2) TLC of LOOH treated with TMAO.

As shown in Fig. 6, a characteristic spot was observed at R_f 0.4 corresponding to keto acid, and its NMR spectrum coincided with that of an authentic sample.¹⁵⁾ However, no spot at R_f 0.4 was observed in LOOH without TMAO.

FIG. 6. Thin-layer Chromatograms of Methyl Linoleate Hydroperoxide Treated in the Presence or Absence of TMAO.

TLC: Kieselgel HF₂₅₄, *n*-hexane-ethyl ether (8:2). Detection: A, UV light at 254 nm; B, 2,4-DNPH; C, potassium iodide.

Sample: 1, original LOOH; 2, LOOH incubated without TMAO; 3, LOOH incubated with TMAO.

Samples 2 and 3 were the same as those in Fig. 5 (incubated for 5 days).

3) Decomposition of LOOH with amines.

As shown in Table I, tri-*n*-octylamine and stearylamine, showing synergism with Toc,²⁴⁾ acted as a potent decomposer, but diphenylamine, known as a peroxide decomposer,²⁵⁾ rather inhibited the decomposition of LOOH. On TLC (Fig. 7) the formation of much keto acid was observed in LOOH treated with TMAO followed by tri-*n*-octylamine.

TABLE I. DECOMPOSITION OF METHYL LINOLEATE HYDROPEROXIDE WITH AMINES

LOOH (45 mg) was incubated with amine (1 M) under nitrogen in the dark at 50°C, and POV (%) after 3 days of incubation was expressed as the percentage of the initial POV of LOOH (100).

	POV (%)
LOOH (original) ^a	100
TMAO	4.3
Tri- <i>n</i> -octylamine	0
Stearylamine	0
Diphenylamine	49.7
LOOH (incubated)	37.9

^a Initial POV was 5240.

FIG. 7. Thin-layer Chromatograms of Methyl Linoleate Hydroperoxide Treated with Amines.

TLC: the same as in Fig. 6.

Detection: A, potassium iodide; B, 2,4-DNPH.

Sample: 1, original LOOH; 2, TMAO; 3, tri-*n*-octylamine; 4, stearylamine; 5, diphenylamine; 6, LOOH incubated.

Samples were the same as in Table I.

*: diphenylamine.

DISCUSSION

The effect of TMAO on the antioxidant activity of α - and γ -Toc in methyl linoleate was investigated.

δ -Toc was found to have remarkable synergism with TMAO and to form its reducing dimers and keto acid from methyl linoleate, as found similarly in the case of δ -Toc reported previously.¹⁵⁾ On the contrary, synergism between α -Toc and TMAO could not be expected because TMAO accelerated the autoxidation of methyl linoleate in an early stage of the reaction. The POV of the oil with TMAO, therefore, increased more rapidly than without TMAO within 1 day, but this relation was reversed after 2 days.

Although synergism between γ -Toc and TMAO was observed under the conditions described in Figs. 1 and 2, large differences in the rate of the decrease of γ -Toc in both cases might be related to the concentration of Toc,^{26,27)} temperature^{27,28)} and the degree of autoxidation of the substrate. In the last case, it was observed that γ -Toc decreased to near zero after 6 days when commercial methyl linoleate (POV, *ca.* 15) was autoxidized under the conditions described in Fig. 2.

An initial concentration of 4% of γ -Toc is approximately equivalent to 100 mmol/kg. When γ -Toc (AH) reacts with the peroxy radical (LOO $\cdot$), a hydrogen atom of an OH group of the AH is abstracted and a A $\cdot$ radical is given. It readily combines each other and forms a reducing dimer (AAH). In this case, the total effective amounts of AH and AAH should be equivalent to about 150 mmol/kg. If 1 mol of methyl linoleate is autoxidized, it forms initially the alkyl radical (L $\cdot$), which reacts rapidly with oxygen to give the LOO $\cdot$ radical, and consumes 1 mol of γ -Toc or its dimers to form 1 mol of LOOH, which decomposes to produce 1 mol of keto acid. Therefore, in the experiment in Fig. 3, if AH and AAH are completely consumed, 150 mmol of keto acid should be formed. However, as shown in Fig. 3, about 20 mmol/kg of the dimers remained in the reaction mixture, whereas about 230 mmol/kg of keto acid was formed after the reaction for 80 days. Furthermore, the amount of γ -Toc decreased gradually, whereas its dimers was found to be kept at a constant concentration. These results suggest

that the autoxidation of methyl linoleate should be inhibited owing to synergism between the reducing dimers and TMAO after γ -Toc disappeared, and that most reducing dimers in the presence of TMAO may be regenerated by repeated reduction.

From the reaction of LOOH with TMAO, it was found that the inhibition of the increase in POV and the formation of keto acid mainly depends on the action of TMAO as a peroxide decomposer. A similar mechanism is also considered in the case of synergism between δ -Toc and TMAO, described in the previous paper.¹⁵⁾

Although various pathways have been reported for the formation of keto acid by reacting LOOH with some substances,^{25,29~33)} it is considered that TMAO produces keto acid from LOOH in the following way:

An active intermediate, (TMAO-H) $\cdot$, which is assumed to be formed when TMAO abstracts H $\cdot$ from the alkoxy radical (LO $\cdot$) to give keto acid (L=O), may react easily with an oxidation product of Toc dimer (AA $\cdot$) to regenerate an initial reducing dimer (AAH) which repeatedly serves as an antioxidant. Although a remarkable synergistic effect could be obtained by combining an antioxidant with a peroxide decomposer,^{16,17,33)} its mechanism has never been clarified in the case of edible oils and fats. Phenol,¹⁷⁾ amine,³³⁾ melanoidin,¹⁸⁾ *etc.* have been reported as peroxide decomposers, and they are reducing matters. However, TMAO differs from them in the fact that it acts as a potent prooxidant under some conditions.¹⁴⁾

From the results, a possible mechanism of synergism between Toc (mainly γ - and δ -Toc)

FIG. 8. A Possible Mechanism of Synergism between Tocopherols and TMAO.

and TMAO is likely to be concluded as follows (Fig. 8). Synergism between Toc and TMAO is mainly due to that between the reducing dimers of Toc (AAH) and TMAO. AAH is consumed by offering $H\cdot$ to $LOO\cdot$ radical to give $AA\cdot$ radical and LOOH. TMAO then reacts with LOOH to produce keto acid and an active intermediate, $(TMAO-H)\cdot$, which offers $H\cdot$ to $AA\cdot$ radical, thus AAH is regenerated.

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REFERENCES

- 1) J. Janicki and M. Gogolewski, *Przemysl. Spozywczy*, **12**, 417 (1958) [*C. A.*, **53**, 15602d (1959)].
- 2) Y. Watanabe and Y. Ayano, *J. Jap. Soc. Food Nutr.*, **25**, 219 (1972).
- 3) E. Yuki, Y. Ishikawa and T. Yoshiwa, *Yukagaku*, **23**, 497, 714 (1974).
- 4) N. Yamaguchi and M. Fujimaki, *J. JPN. Soc. Food Sci. Technol.*, **21**, 13 (1974).
- 5) N. Yamaguchi, *ibid.*, **22**, 270 (1975).
- 6) H. S. Olcott, *ibid.*, **11**, 544 (1964).
- 7) L. M. Smith, E. N. Frankel, W. Haab and E. L. Jack, *J. Dairy Sci.*, **41**, 472 (1958).
- 8) C. Kanno, K. Yamauchi and T. Tsugo, *Agric. Biol. Chem.*, **34**, 1652 (1970).
- 9) G. Pongracz, *Internat. J. Vit. Nutr. Res.*, **43**, 517 (1973).
- 10) A. L. Tappel, W. D. Brown, H. Zalkin and V. P. Maier, *J. Am. Oil Chem. Soc.*, **38**, 5 (1961).
- 11) E. N. Frankel, P. M. Cooney, H. A. Moser, J. C. Cowan and C. D. Evans, *Fette, Seifen Anstrichm.*, **61**, 1036 (1959).
- 12) S. Masuyama, *Yukagaku*, **14**, 692 (1965).
- 13) L. Dugan, *J. Am. Oil Chem. Soc.*, **33**, 527 (1957).
- 14) E. Yuki, Y. Ishikawa, I. Yamaoka and T. Yoshiwa, *J. JPN. Soc. Food Sci. Technol.*, **20**, 411 (1973).
- 15) Y. Ishikawa, E. Yuki, H. Kato and M. Fujimaki, *Agric. Biol. Chem.*, **42**, 703 (1978).
- 16) Y. Kamiya, *Yukagaku*, **14**, 672 (1965).
- 17) Y. Kamiya, "Yuki Sanka Hanno," Gihodo Pub. Co., Tokyo, 1973, pp. 85, 338.
- 18) N. Yamaguchi and Y. Okada, *J. JPN. Soc. Food Sci. Technol.*, **15**, 187 (1968).
- 19) S. Matsushita, M. Kobayashi and Y. Nitta, *Agric. Biol. Chem.*, **34**, 817 (1970).
- 20) Y. Ishikawa and E. Yuki, *ibid.*, **39**, 851 (1975).
- 21) N. Uri, "Autoxidation and Antioxidants," Vol. 1, ed. by W. O. Lundberg, Interscience Pub., 1961, pp. 134~6.
- 22) W. O. Lundberg, Symposium on Foods, Lipids and Their Oxidation, ed. by H. W. Schultz *et al*, Avi Pub. Co., 1962, pp. 36~42.
- 23) J. Pokorny, *Can. Inst. Food Technol. J.*, **4**, 68 (1971).
- 24) Y. Ishikawa, E. Yuki, H. Kato and M. Fujimaki, *Yukagaku*, **26**, 765 (1977).
- 25) L. Reich and S. S. Stivala, "Autoxidation of Hydrocarbons and Polyolefins, Kinetics and Mechanisms," translated by K. Matsuzaki and Z. Osawa, Maruzen Pub. Co., Tokyo, 1972, p. 152.
- 26) C. Kanno, M. Hayashi, K. Yamauchi and T. Tsugo, *Agric. Biol. Chem.*, **34**, 878 (1970).
- 27) L. T. Kováts and E. Berndorfer-Krasznér, *Nahrung*, **12**, 407 (1968).
- 28) Y. Ishikawa and E. Yuki, *Yukagaku*, **23**, 710 (1974).
- 29) Y. Ogata, "Yuki Kasankabutsu no Kagaku," Nankodo Pub. Co., Tokyo, 1971, pp. 6, 27.
- 30) M. Hamberg, *Lipids*, **10**, 87 (1975).
- 31) G. J. Garssen, J. F. G. Vliegthart and J. Boldingh, *Biochem. J.*, **122**, 327 (1971).
- 32) G. Streckert and H. J. Stan, *Lipids*, **10**, 847 (1975).
- 33) G. Scott, "Atmospheric Oxidation and Antioxidants," Elsevier Pub. Co., 1965, pp. 188~203.