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Stopped-Flow Investigation of Antioxidant Activity of Tocopherols

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It was observed by ESR measurement that the oxidation of α-, β-, γ-, and δ-tocopherols (vitamin E) with a stable phenoxyl radical in benzene immediately gives corresponding tocopheroxyl radicals. The rates of reaction of α-, β-, γ-, and δ-tocopherols with the stable phenoxyl radical in ethanol solution have been determined spectrophotometrically using stopped-flow technique, as a model reaction of tocopherols with unstable free radicals (ROO·, RO·, and HO·) in biological systems. The second-order rate constants obtained are $(5.12\pm0.36)\times10^3$ (α-Toc), $(2.24\pm0.04)\times10^3$ (β-Toc), $(2.42\pm0.16)\times10^3$ (γ-Toc), and $(0.51\pm0.01)\times10^3$ (δ-Toc), $M^{-1}\,s^{-1}$ in ethanol at 25.0 °C. The relative rates agree well with those obtained from studies of the reactivities of tocopherols toward poly(styrylperoxyl) and galvinoxyl radicals by O_2 consumption and by ESR method, respectively. The results suggest that the relative reactivities, that is, relative antioxidant activities of tocopherols do not depend on the kinds of unstable free radicals reacted.

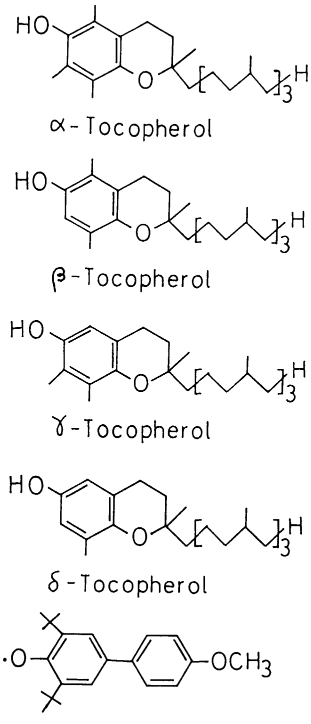
Vitamin E (α, β-, γ-, and δ-tocopherols) is known to scavenge peroxyl, alkoxyl, and hydroxyl radicals.[1,2] The above free radical scavenging actions by vitamin E have been ascribed to the initial reaction of the phenolic hydroxyl group with the production of a tocopheroxyl* radical.[3,4] Therefore, several investigators have measured the oxidation rates k_1 of α-tocopherol by peroxyl radicals (ROO·), using different experimental methods such as chemiluminescence, pulse radiolysis, and O_2 consumption.[5-10]

$$\text{ROO}\cdot + \text{Toc} \longrightarrow \text{ROOH} + \text{Toc}\cdot \qquad (1)$$

However, considerable variations in the values of k_1 have been observed when different experimental methods were used, because the techniques employed to measure k_1 for α-tocopherol are indirect.[11]

Recently, Burton et al. have measured rate constants for reactions of the α-, β-, γ-, and δ-tocopherols with poly(styrylperoxyl) radicals, using O_2 consumption method.[11,12] They suggested that the reactivity of the tocopherols towards peroxyl radicals parallels their biological activity (α > β > γ > δ-tocopherol). Further, Niki et al. have measured the rate constants for reactions between galvinoxyl radical incorporated into dimyristoyl phosphatidylcholine (abbreviated to PC) liposomes and α-, β-, γ-, and δ-tocopherols incorporated into different dimyristoyl PC liposomes by ESR technique.[13] The observed pseudo-first-order rate constants of tocopherols decreased in the order of α > β ≅ γ > δ-tocopherol.

In this study we have used a stopped-flow technique to examine the reaction of tocopherols with a stable phenoxyl radical, i. e., 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (PhO·) (see Fig. 1), as a model reaction of tocopherols with unstable free radicals (ROO·, RO·, and HO·) in biological systems. The reactions were performed in the presence of excess tocopherol in ethanol, giving the pseudo-first-order rate constants, k_{obsd}. The second-order rate constants, k_s, may be immediately calculated from the k_{obsd} values, using the relation, $k_{obsd} = k_s[\text{Toc}]$. That is, the k_s value can be directly obtained from the above stopped-flow experiments. Therefore, this method will give more reliable values of rate constants than the other methods. By comparing the relative reactivities of α-, β-, γ-, and δ-tocopherols found by different experimental methods, we discussed the antioxidant activity of tocopherols.


α-Tocopherol

β-Tocopherol

γ-Tocopherol

δ-Tocopherol

Phenoxyl

Fig. 1. Molecular structures of α-, β-, γ-, and δ-tocopherols and phenoxyl radical (PhO·).

Experimental

d-α-, d-β-, d-γ-, and d-δ-tocopherols were kindly supplied from Eisai Co., Ltd.

The 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (PhO·)

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* The phenoxyl radicals generated from tocopherols are named in this paper as widely used tocopheroxyl radicals for convenience rather than tocopheryloxyl radicals that conform to rules of nomenclature.

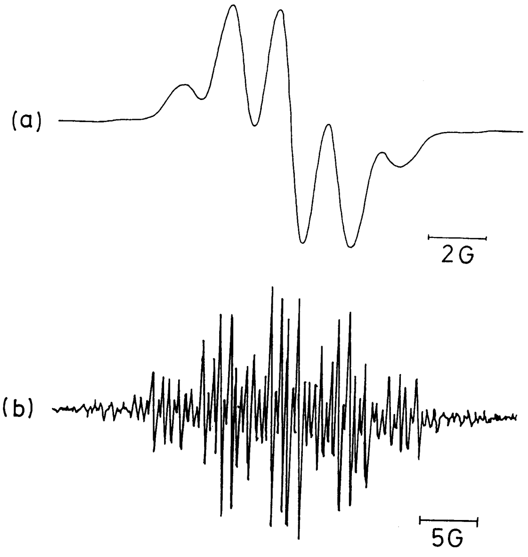

Fig. 2. ESR spectra of (a) phenoxyl radical and (b) α-tocopheroxyl radical at 25 °C in benzene under vacuum.

(abbreviated to "phenoxyl" hereafter) was prepared according to the method of Müller et al.[14] Radical concentration of the phenoxyl radical was obtained from the results of the paramagnetic susceptibility measurements at 20 °C, after correcting for the diamagnetic contribution ($\chi_{\mathrm{dia}}=-0.220\times10^{-3}$ emu mol^{-1}) calculated by the Pascal's method. The value was 100% for phenoxyl, assuming the Curie law.

The ESR measurements were carried out using a JES-FE-2XG spectrometer with 100 KHz magnetic field modulation. UV. visible spectra were recorded on a JASCO UVIDEC-1 spectrophotometer. The stopped-flow data were obtained on a UNISOKU stopped-flow spectrophotometer Model RS-450 by mixing equal volumes of ethanol solutions of phenoxyl and tocopherol. The oxidation reactions were studied under pseudo-first-order conditions, and the observed rate constants, k_{obsd}, were calculated in the usual way using a standard least-squares analysis. All measurements were performed at 25.0 ± 0.5 °C.

Results and Discussion

ESR Studies of the Oxidation of Tocopherols with Phenoxyl Radical. Figure 2(a) shows an ESR spectrum of the phenoxyl radical in benzene at 25 °C under vacuum.[15] When two benzene solutions containing 2.50 mM phenoxyl and 5.00 mM α-tocopherol (1 : 1 in volume), respectively, were mixed at 25 °C under vacuum, phenoxyl disappeared instantaneously, and a new spectrum due to α-tocopheroxyl radical appeared (Fig. 2(b)). The above reaction can be represented by the following equation.

$$\mathrm{PhO}\cdot + \mathrm{Toc} \xrightarrow{k_s} \mathrm{PhOH} + \mathrm{Toc}\cdot \qquad (2)$$

Similarly, β-, γ-, and δ-tocopherols were reacted with the phenoxyl, and the ESR spectra due to β- and γ-tocopheroxyl radicals were observed. However, in the case of δ-tocopherol, the radical is very unstable, and the clear ESR spectrum cannot be observed. The observed hyperfine splittings are listed in Table 1. There have been many ESR studies of tocopheroxyl radicals published.[16] However, the spectrum is fairly complex and has only recently been completely resolved, first by Mukai (one of the present authors) et al.,[3,17] and, then, by Matsuo et al.[18] and Tsuchiya et al.[19] The hyperfine splittings in Table 1 show good accordance with those reported previously.[3,17]

Table 1. Hyperfine Couplings (a_i^{H}) of Tocopheroxyl Radicals in Benzene at 25 °C

	a_5^{H}	a_7^{H}	a_4^{H}	a_8^{H}
α-Toc	6.04G	4.52G	1.48G	0.93G
β-Toc	6.39	4.63	1.76	0.81
γ-Toc	5.97	4.80	1.17	1.17
δ-Toc	—	—	1.52	1.04

Stopped-Flow Studies of the Reaction Rates of Tocopherols with Phenoxyl. The phenoxyl radical is stable in the absence of tocopherol, and shows absorption peaks at $\lambda_{\max}=376$ nm ($\varepsilon=17600$ M^{-1} cm^{-1}) and 580 nm ($\varepsilon=4280$ M^{-1} cm^{-1}) in ethanol. The phenol precursor of the phenoxyl and α-tocopherol show absorption peaks at $\lambda_{\max}=267$ nm ($\varepsilon=19500$ M^{-1} cm^{-1}) and $\lambda_{\max}=295$ nm ($\varepsilon=3610$ M^{-1} cm^{-1}), respectively, in ethanol; no absorptions were observed in the visible region.

By adding an ethanol solution of α-tocopherol (5.00 mM) to an ethanol solution of phenoxyl (0.10 mM) (1 : 1 in volume) at 25.0 °C, the absorption spectrum of the phenoxyl immediately changed to that of α-tocopheroxyl. The absorption spectrum of α-tocopheroxyl shows weak absorption peaks at 425 and 405 nm.[20] However, the α-tocopheroxyl is unstable, and the absorption peaks rapidly disappeared. Similarly, the phenoxyl radical has been reacted with excess β-, γ-, and δ-tocopherols, showing rapid decrease of the absorption spectrum, respectively. On the other hand, the absorption spectra of the corresponding tocopheroxyls were not observed in these cases, because these tocopheroxyls are more unstable than α-tocopheroxyl.

Therefore, the oxidation rates of tocopherols by phenoxyl were studied spectrophotometrically using stopped-flow technique in the presence of excess tocopherol in ethanol. The rate was measured by following the decrease in absorbance at 376 and 580 nm of phenoxyl radical. The time dependence of the decrease in absorbance at 376 nm observed when 0.10 mM ethanol solution of phenoxyl is mixed with 5.00 mM ethanol solution of α-thcopherol (1 : 1 in volume; final concentration of phenoxyl is 0.05 mM) is shown in Fig. 3. A typical Guggenheim plot versus time was linear to >90% completion, as shown in Fig. 4. The pseudo-first-order rate constants, k_{obsd}, obtained by varying the concentration of phenoxyl and α-tocopherol are presented in Table 2. The stopped-flow reactions were performed at 25.0 ± 0.5 °C.

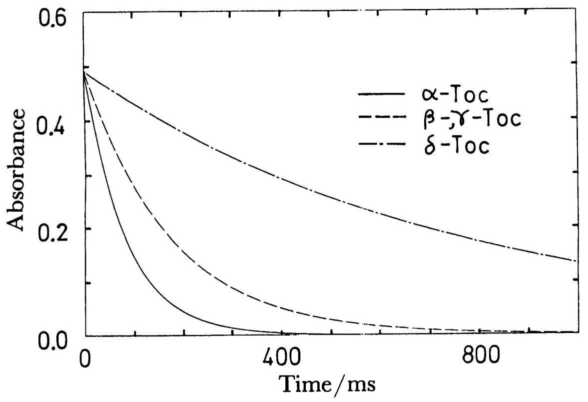

Fig. 3. The decay of phenoxyl radicals for the reaction of phenoxyls with α-, β-, γ-, and δ-tocopherols in ethanol at 25.0 °C.
$[\mathrm{PhO\cdot}]_{t=0}$ 0.05 mM and $[\mathrm{Toc}]_{t=0}$ 2.50 mM. At 376 nm.

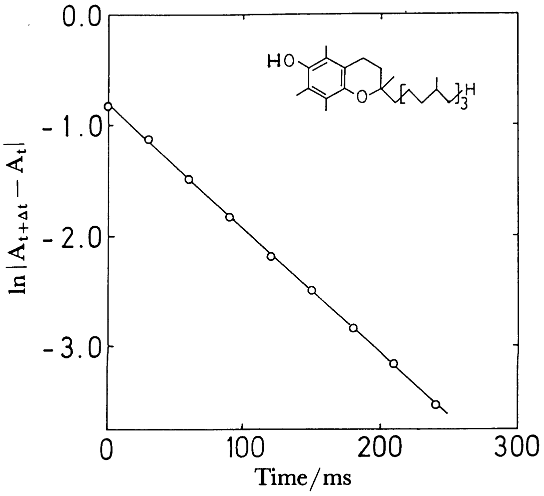

Fig. 4. First-order Guggenheim plot of the data of α-tocopherol in Fig. 3; $k_{\mathrm{obsd}}=12.0\,\mathrm{s}^{-1}$.

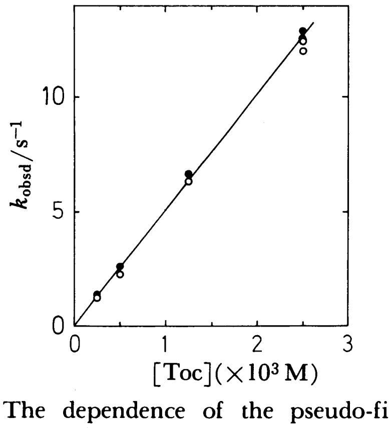

Fig. 5. The dependence of the pseudo-first-order rate constant, k_{obsd}, on [α-Toc] in ethanol. (○): observed at 376 nm. (●): observed at 580 nm.

Table 2. Pseudo-First-Order (k_{obsd}) and Second-Order (k_s) Rate Constants for Oxidation of α-Tocopherol by the Phenoxyl Radical in Ethanol at 25.0 °C

$\lambda_{\max}$	Ratio [PhO·]:[Toc]	k_{obsd} s^{-1}	$10^{-3}k_s$ $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$
376 nm	2 :50	12.5	4.98
	1.5:50	12.4	4.94
	1[a]:50	12.0	4.76
	1 :25	6.34	5.15
	1 :10	2.62	5.31
	1 : 5	1.29	5.22
580 nm	2 :50	12.5	4.98
	1.5:50	12.5	4.98
	1 :50	12.9	5.10
	1 :25	6.65	5.41
	1 :10	2.61	5.29
	1 : 5	1.32	5.34

a) $[\mathrm{PhO\cdot}]_{t=0}=0.05$ mM. For PhO·, see text and Fig. 1.

The pseudo-first-order rate constants, k_{obsd}, observed at 376 and at 580 nm were linearly dependent on the concentration of tocopherol as shown in Fig. 5. Therefore, the rate law is expressed as

$$-\mathrm{d}[\mathrm{PhO\cdot}]/\mathrm{d}t = k_{\mathrm{obsd}}[\mathrm{PhO\cdot}] = k_s[\mathrm{Toc}][\mathrm{PhO\cdot}], \tag{3}$$

where k_s is the second-order rate constant for oxidation of tocopherol by phenoxyl radical. The k_s values were calculated from k_{obsd} values in 12 independent experiments (0.25—2.50 mM tocopherol), respectively, and listed in Table 2. The average value for k_s was $(5.12\pm0.36)\times10^3\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ in ethanol at 25.0 °C. Similarly, the second-order rate constants, k_s, were obtained for β-, γ-, and δ-tocopherols. For each tocopherol, k_s was measured in 4—12 independent experiments. The experimental error in k_s values was $<\pm7\%$ in every case. The values of the k_s observed are listed in Table 3. The results listed in Table 3 demonstrate that α-tocopherol is about two times as reactive as β-tocopherol, whereas β-tocopherol is, within our limits of error, as reactive as γ-tocopherol. However, δ-tocopherol is only ca. 10% as reactive as α-tocopherol.

Century and Horwitt reported that biological activities of tocopherols decrease in the order of $\alpha>\beta>\gamma>\delta$, and that in vivo and in vitro tests of their relative biological activities change depending on the kinds of test (see Table 2 in Ref. 21).[21] As described in a previous section, Burton et al. have reported absolute second-order rate constants, k_s', for the reaction of α-, β-, γ-, and δ-tocopherols with poly(styrylperoxyl) radicals, using the O_2 consumption method (see Table 3).[11,12] The suggested that the relative magnitude of the second-order rate constant, k_s', of α-, β-, γ-, and δ-, tocopherols is in agreement with in vivo and in vitro tests of their relative biological activities which yield: $\alpha>\beta>\gamma>\delta$-tocopherol.[21] Niki et al. have measured the pseudo-first-order rate constants (k_f) (see Table 3) for the reaction between galvinoxyl radical incorporated into dimyristoyl PC liposomes and α-, β-, γ-, and δ-tocopherols incorporated into different dimyristoyl PC liposomes, by ESR technique.[13] By comparing the k_f value for α-tocopherol with those found for β-, γ-, and δ-tocopherol, they suggested that the reactivities of

Table 3. Second-Order Rate Constants (K_s) and Relative Rate Constants (k_s/k_s (α-Toc)) for Oxidation of Tocopherols by Phenoxyl Radical in Ethanol at 25.0 °C

	Present Work $10^{-3} k_s$ ($M^{-1} s^{-1}$)	k_s/k_s (α-Toc)	Ingold et al.[a] $10^{-6} k'_s$ ($M^{-1} s^{-1}$)	k'_s/k'_s (α-Toc)	Niki et al.[b] $10^4 k_f$ (s^{-1})	k_f/k_f (α-Toc)
α-Toc	5.12±0.36	(1.00)	3.24±0.15	(1.00)	10.1[c]	(1.00)
β-Toc	2.24±0.04	(0.44)	1.66±0.33	(0.51)	5.44	(0.54)
γ-Toc	2.42±0.16	(0.47)	1.59±0.42	(0.49)	5.00	(0.50)
δ-Toc	0.51±0.01	(0.10)	0.65±0.13	(0.20)	4.76	(0.47)
Technique	Stopped-flow Spectrophotometer		O_2 Consumption		ESR	
Radical	Phenoxyl[d]		Poly(styrylperoxyl)		Galvinoxyl	
T/°C	25		30		30	

a) See Ref. 11 and 12. b) See Ref. 13. c) Experimental errors are not indicated. d) The structure is shown in Fig. 1.

tocopherols toward galvinoxyl radical decrease in the order of $\alpha > \beta \cong \gamma > \delta$.

In the present work, we have measured the rate constants k_s for the reaction of α-, β-, γ-, and δ-tocopherols with the stable phenoxyl radical in ethanol, using a stopped-flow spectrophotometer. As listed in Table 3, the relative k_s values ($\alpha : \beta : \gamma : \delta = 1.00 : 0.44 : 0.47 : 0.10$) obtained by the stopped-flow technique are in good agreement with the values (1.00 : 0.51 : 0.49 : 0.20) obtained by the O_2 consumption method, except for δ-tocopherol, although the absolute k_s values are about 600 times smaller than those for the reaction of tocopherols with poly(styrylperoxyl) radical in chlorobenzene.[11,12] Further, the above relative magnitude of the second-order rate constants, k_s, of α-, β-, γ-, and δ-tocopherols is also in satisfactory agreement with that (1.00 : 0.54 : 0.50 : 0.47) of the pseudo-first-order rate constant, k_f, obtained by ESR technique, except for δ-tocopherol.[13] The results suggest that the relative reactivities, that is, relative antioxidant activities obtained by the three different experimental methods agree well with each other. In addition, the above results indicate that the relative antioxidant activities do not depend on the kinds of radicals (phenoxyl, poly(styrylperoxyl), and galvinoxyl radical) used, while the absolute rates are considerably different from each other. As described above, biological activities of tocopherols decrease in the order of $\alpha > \beta > \gamma > \delta$, and in vivo and in vitro tests of their relative biological activities change depending on the kinds of test.[21] For instance, the relative biopotencies of α-, β-, γ-, and δ-tocopherols have been reported as 100, 25, 18, and 0.3 in an erythrocyte hemolysis test and as 100, 55, 5, and 4 in respiratory decline test, respectively.[21] The result shows that the relative biological activity and antioxidant activity of tocopherols do not always agree with each other, suggesting that the function of tocopherols in biological system is not simple, but manifold.

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References

1) P. Schudel, H. Mayer, and O. Isler, "The Vitamins," 2nd ed, ed by W. H. Sebrell, Jr. and R. S. Harris, Academic Press, New York (1972), Vol. 5, pp. 168—218.

2) M. L. Scott, "The Fat-Soluble Vitamins," ed by H. F. Deluca, Plenum Press, New York (1978), pp. 133—210.

3) K. Mukai, N. Tsuzuki, S. Ouchi, and K. Fukuzawa, *Chem. Phys. Lipids*, **30**, 337 (1982).

4) M. T. Craw and M. C. Depew, *Rev. Chem. Intermediates*, **6**, 1 (1985).

5) A. A. Kharitonova, Z. G. Kozlova, V. F. Tsepalov, and G. P. Gladyshev, *Kinet. Katal.*, **20**, 593 (1979).

6) J. E. Packer, T. F. Slater, and R. L. Willson, *Nature (London)*, **278**, 737 (1979).

7) Ye. B. Burlakova, Ye. N. Kukhtina, I. P. Ol'khovskaya, I. K. Sarycheva, Ye. B. Sinkina, and N. G. Khrapova, *Biophysics*, **24**, 989 (1980).

8) G. W. Burton, Y. Le. Page, E. J. Gabe, and K. U. Ingold, *J. Am. Chem. Soc.*, **102**, 7791 (1980).

9) M. G. Simic, *J. Chem. Educ.*, **58**, 125 (1981).

10) E. Niki, R. Tanimura, and Y. Kamiya, *Bull. Chem. Soc. Jpn.*, **55**, 1551 (1982).

11) G. W. Burton and K. U. Ingold, *J. Am. Chem. Soc.*, **103**, 6472 (1981).

12) G. W. Burton, L. Hughes, and K. U. Ingold, *J. Am. Chem. Soc.*, **105**, 5950 (1983).

13) E. Niki, J. Tsuchiya, Y. Yoshikawa, Y. Yamamoto, and Y. Kamiya, *Bull. Chem. Soc. Jpn.*, **59**, 497 (1986).

14) E. Müller, A. Schick, and K. Scheffler, *Chem. Ber.*, **92**, 474 (1959).

15) A. Rieker and K. Scheffler, *Liebigs Ann. Chem.*, **689**, 78 (1965).

16) T. Ozawa, A. Hanaki, S. Matsumoto, and M. Matsuo, *Biochim. Biophys. Acta*, **531**, 72 (1978), and references cited therein.

17) K. Mukai, N. Tsuzuki, K. Ishizu, S. Ouchi, and K. Fukuzawa, *Chem. Phys. Lipids*, **29**, 129 (1981).

18) M. Matsuo and S. Matsumoto, *Lipids*, **18**, 81 (1983).

19) J. Tsuchiya, E. Niki, and Y. Kamiya, *Bull. Chem. Soc. Jpn.*, **56**, 229 (1983).

20) W. Boguth and H. Niemann, *Internat. Z. Vit. Forschung*, **39**, 429 (1969).

21) B. Century and M. K. Horwitt, *Fed. Proc. Am. Soc. Exp. Biol.*, **24**, 906 (1965).