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Chemistry and Physics of Lipids 146 (2007) 26–32
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CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

Kinetics of the reaction by which natural vitamin E is regenerated by vitamin C

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Received 9 June 2006; received in revised form 4 December 2006; accepted 22 December 2006
Available online 30 December 2006

Abstract

The rate constant and activation energy of the regeneration reaction of natural vitamin E by vitamin C were determined with a double-mixing stopped-flow spectrophotometer. The formation of vitamin C radical was observed in the absorption spectrum. The kinetic effect of methyl substitution on the aromatic ring of vitamin E radical indicates that partial charge-transfer plays a role in the reaction. Since a substantial deuterium kinetic isotope effect was not found, the tunneling effect may not play an important role under the present experimental conditions.

Keywords: Vitamin E regeneration; Vitamin C; Reaction rate; Activation energy; Tocopherol; Ascorbate

1. Introduction

Although no theory advanced to account for aging is generally accepted, one of the promising is the free radical theory (Harman, 2001) postulating that aging is in part caused by lipid peroxyl radicals (LOO•'s) formed by the reactions of lipids and oxygen (Cutler, 1984; Burton and Ingold, 1986; Niki, 1987, 1989; Diplock et al., 1989). The living body, however, has a way to scavenge LOO• and thus to help prevent aging: it is the so-called antioxidant reaction of vitamin E (*d*-tocopherols, TocH's). Evidence for this is that the lifetimes of mammalian species are proportional to their plasma levels of a TocH (Cutler, 1984).

Fig. 1 shows the scheme of LOO• production and of a part of the antioxidant reaction of TocH (Burton and Ingold, 1986; Niki, 1987, 1989; Diplock et al., 1989). A lipid radical (L•) is first formed from lipid (LH), usually because of radicals, light, heat, metal (Fukuzawa et al., 1991) or irradiation. The L• radical reacts with oxygen to produce LOO•, and the reaction of LOO• and TocH results in production of lipid hydroperoxide (LOOH) and a vitamin E radical (tocopheroxyl radical, Toc•). Fig. 2 shows the molecular structures of natural TocH's (α-, β-, γ- and δ-TocH's), each of which has a hydroxychroman ring and a phytyl side chain that stabilizes TocH in the cell membrane. These TocH's differ from one another only in the number and positions of the methyl groups on the aromatic ring. The most biologically active of these four TocH's is the fully methylated α-TocH.

The Toc• produced in the above-mentioned reaction reacts with LH and LOOH and again produces L• and LOO• (Fig. 1). This prooxidant action of TocH (Cillard et al., 1980; Terao and Matsushita, 1986) is suppressed when TocH is regenerated by the reaction of Toc• with

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doi:10.1016/j.chemphyslip.2006.12.001

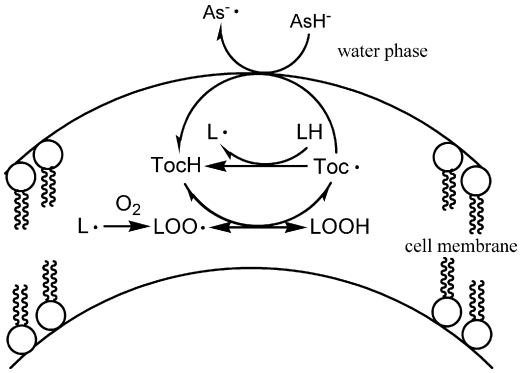

Fig. 1. Scheme of LOO• production and of part of the antioxidant, prooxidant and regeneration reactions of TocH in the cell membrane.

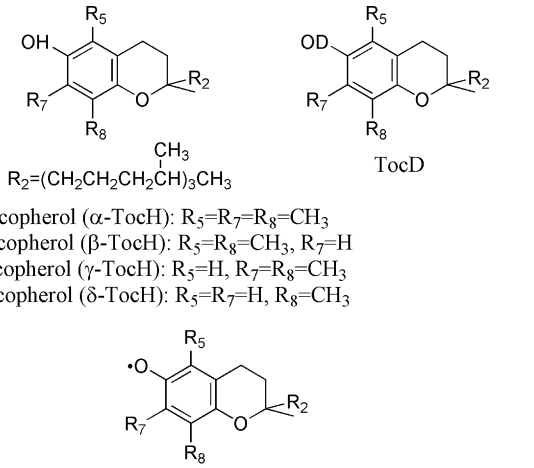

Fig. 2. Structures of molecules used in the present work.

vitamin C (ascorbate monoanion, AsH^-) at the interface of the cell membrane and the water phase (Packer et al., 1979; Scarpa et al., 1984; Niki et al., 1984; Mukai et al., 1987, 1989, 1991; Sato et al., 1990; Bisby and Parker, 1995; Watanabe et al., 1999) (Fig. 1).

All of the above-mentioned reactions of TocH are essentially proton- or hydrogen-transfer reactions:

$$\mathrm{TocH} + \mathrm{LOO^\bullet} \rightarrow \mathrm{Toc^\bullet} + \mathrm{LOOH}, \quad (1)$$

$$\mathrm{Toc^\bullet} + \mathrm{LH} \rightarrow \mathrm{TocH} + \mathrm{L^\bullet}, \quad (2)$$

$$\mathrm{Toc^\bullet} + \mathrm{LOOH} \rightarrow \mathrm{TocH} + \mathrm{LOO^\bullet}, \quad (3)$$

$$\mathrm{Toc^\bullet} + \mathrm{AsH^-} \rightarrow \mathrm{TocH} + \mathrm{As^{-\bullet}}, \quad (4)$$

where $As^{-\bullet}$ denotes the dehydroascorbate monoanion radical. Reaction (3) is the reversal of reaction (1). It would be interesting to study the tunneling effect in the proton or hydrogen transfer of these reactions.

In previous studies using a conventional stopped-flow spectrophotometer (Mukai et al., 1987, 1989, 1991; Nagaoka et al., 2000; Nagaoka, 2004), we investigated the kinetics of reaction (4) by using 5,7-diisopropyl-Toc• or 7-*t*-butyl-5-isopropyl-Toc• (Fig. 2) because the Toc•'s originating from natural α-, β-, γ- and δ-TocH's are so short-lived that the kinetics of their reaction (4) cannot be examined with the conventional method. The results obtained using 5,7-diisopropyl-Toc• showed that the tunneling effect plays an important role in reaction (4) (Nagaoka et al., 2000; Nagaoka, 2004). Bisby and Parker (1995) studied the kinetics of reaction (4) using α-Toc• and laser flash photolysis, but the formation of $As^{-\bullet}$ has not yet been observed nor has the effect of methyl substitution on the aromatic ring of Toc• been examined. In the work presented here we have observed the decays of α-, β- and γ-Toc•'s and the formation of $As^{-\bullet}$ by using a double-mixing stopped-flow spectrophotometer, which allows us to examine the kinetics of the reactions of short-lived radicals. We have determined the rate constants and activation energies for reaction (4) and examined the deuterium kinetic isotope effect.

2. Experimental procedures

2.1. Sample preparation

The structures of the molecules studied in this work are shown in Fig. 2. The *d*-α-, *d*-β-, *d*-γ-, and *d*-δ-TocH's kindly supplied by Eisai Co., Ltd. were used without further purification. We prepared 2,6-di-*t*-butyl-4-(4-methoxyphenyl)phenoxyl (ArO•) as reported in a previous paper (Rieker and Scheffler, 1965). In aqueous

solutions around pH 7, vitamin C exists mostly as the monoanion (AsH^-) in which the proton at the 3-position is dissociated (Fig. 2) (Mukai et al., 1991). Accordingly, sodium ascorbate (Na^+AsH^-) was used in the present work. Na^+AsH^- (guaranteed reagent) was obtained from Nacalai Tesque and was used without further purification. Ethanol-d_0 (EtOH) was obtained from Wako, dried and purified by distillation. Water-d_0 (H_2O) was purified by ion exchange. Ethanol-d_1 (C_2H_5OD, EtOD) of 99% purity and water-d_2 (D_2O) of 99% purity were purchased from Cambridge Isotope Laboratories and used without further purification.

When TocH is dissolved in EtOD, the hydrogen atom of the OH group is easily replaced by a deuteron to yield the deuterated molecule TocD. This replacement was confirmed by proton NMR. When Na^+AsH^- is dissolved in D_2O, the hydrogen atoms of the OH groups are easily replaced by deuterons to yield the deuterated molecule Na^+AsD^-. This replacement was confirmed by the proton NMR spectrum of Na^+AsH^- in DMSO showing peaks due to the hydrogen atoms of the OH groups and by the disappearance of those peaks when a small amount of D_2O was added.

In the experiments evaluating the kinetics of reaction (4), EtOH (EtOD) and H_2O (D_2O) were mixed in a 5:1 volume ratio to obtain a mixed solvent EtOH/H_2O (EtOD/D_2O) because Toc$^\bullet$ is alcohol-soluble and Na^+AsH^- is water-soluble. An EtOH/H_2O (EtOD/D_2O) solution of Na^+AsH^- (Na^+AsD^-) was produced by first dissolving Na^+AsH^- in H_2O (D_2O) and then adding EtOH (EtOD), and an EtOH/H_2O (EtOD/D_2O) solution of TocH (TocD) was produced by first dissolving TocH in EtOH (EtOD) and then adding H_2O (D_2O).

2.2. Measurements

The reactions studied in the present work were the following ones:

$$\text{TocH (TocD)} + \text{ArO}^\bullet \rightarrow \text{Toc}^\bullet + \text{ArOH (ArOD)}, \quad (1')$$

$$\text{Toc}^\bullet + \text{Na}^+\text{AsH}^- (\text{Na}^+\text{AsD}^-) \xrightarrow{k} \text{TocH (TocD)} + \text{Na}^+\text{As}^{-\bullet}, \quad (4')$$

where k denotes the second-order rate constant for reaction (4′).

The kinetic data of reaction (4′) were obtained using a Unisoku double-mixing stopped-flow spectrophotometer Model RSP-1000-03F. The experimental error in the temperature was less than ±0.5 °C. First, equal volumes of EtOH/H_2O (EtOD/D_2O) solutions of TocH (TocD) and ArO$^\bullet$ were mixed under a nitrogen atmosphere (reaction (1′)). The change in the absorption spectrum measured during reaction (1′) is shown in Fig. 3. Although ArO$^\bullet$ was stable in the absence of TocH (TocD), when an EtOH/H_2O (EtOD/D_2O) solution with excess TocH (TocD) was added to the ArO$^\bullet$ solution, the ArO$^\bullet$ absorption peak disappeared immediately, the Toc$^\bullet$ peak appeared, and the isosbestic points were observed. Since δ-Toc$^\bullet$ is very unstable, its peak was not seen in the absorption spectra measured during reaction (1′).

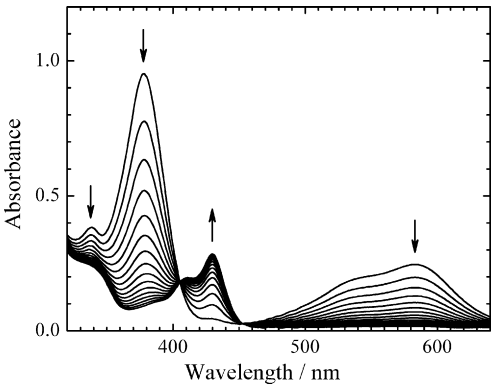

Fig. 3. Change in absorption spectrum (at 20-ms intervals) during the reaction of α-TocH and ArO$^\bullet$ in EtOH/H_2O at 25 °C (reaction (1′)). $[\alpha\text{-TocH}]_{t=0} = 0.839$ mM. The arrows indicate the decrease in the absorbance of ArO$^\bullet$ and the increase in the absorbance of α-Toc$^\bullet$.

Reaction (1′) yielded the maximum concentration of Toc$^\bullet$ after about 1 s in EtOH/H_2O (about 10 s in EtOD/D_2O), and the resultant mixture and an EtOH/H_2O (EtOD/D_2O) solution of Na^+AsH^- (Na^+AsD^-) were mixed under a nitrogen atmosphere (reaction (4′)). The change in absorption spectrum during reaction (4′) is shown in Fig. 4: the Toc$^\bullet$ absorption peak disappeared,

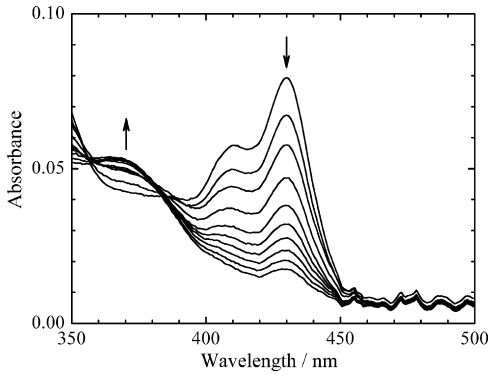

Fig. 4. Change in absorption spectrum (at 2-ms intervals) during the reaction of α-Toc$^\bullet$ and Na^+AsH^- in EtOH/H_2O at 25 °C (reaction (4′)). $[Na^+AsH^-]_{t=0} = 38.5$ μM. The arrows indicate the decrease in the absorbance of α-Toc$^\bullet$ and the increase in the absorbance of $Na^+As^{-\bullet}$.

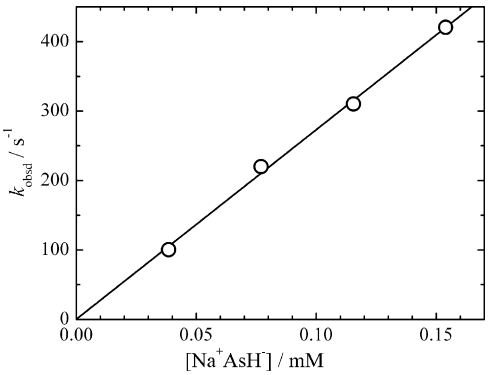

Fig. 5. Dependence of k_{obsd} on [Na^+AsH^-] in the reaction of α-Toc$^\bullet$ and Na^+AsH^- in EtOH/H_2O at 25 °C (Eq. (5)), where [Na^+AsH^-] stands for the concentration of Na^+AsH^- in the solution. The value of k_{obsd} was obtained by monitoring the decrease in the absorbance of α-Toc$^\bullet$ at 430 nm (Fig. 4).

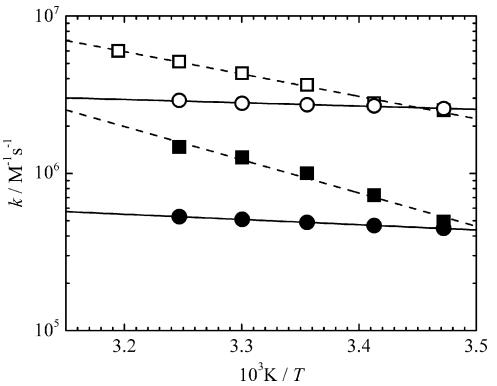

Fig. 6. Arrhenius plots of the k's for the reactions of α-Toc$^\bullet$ with Na^+AsH^- in EtOH/H_2O (open circles), α-Toc$^\bullet$ with Na^+AsD^- in EtOD/D_2O (closed circles), β-Toc$^\bullet$ with Na^+AsH^- in EtOH/H_2O (open squares), and β-Toc$^\bullet$ with Na^+AsD^- in EtOD/D_2O (closed squares).

the $Na^+As^{-\bullet}$ (Fig. 2) peak appeared, and the isosbestic points were observed.

Pseudo-first-order conditions were assumed in the analyses (Mukai et al., 1987, 1989, 1991; Nagaoka et al., 2000; Nagaoka, 2004), and the absorption decay of Toc$^\bullet$ in reaction (4′) was well characterized by a single-exponential decay. The pseudo-first-order rate constant (k_{obsd}) was determined by evaluating the decrease in the absorbance of Toc$^\bullet$. As shown in Fig. 5, k_{obsd} was linearly dependent on the concentration of Na^+AsH^- (Na^+AsD^-). The location of the intercept depends on the rate of the dimerization reaction (Toc$^\bullet$ + Toc$^\bullet$ → Toc − Toc) (Watanabe et al., 1999, 2000). The rate equation is thus expressed as

$$-\frac{d[Toc^\bullet]}{dt} = k_{obsd}\,[Toc^\bullet] = k[Na^+AsH^-\ (Na^+AsD^-)][Toc^\bullet], \quad (5)$$

where [X] (X = Toc$^\bullet$, Na^+AsH^- or Na^+AsD^-) stands for the concentration of X in the solution. The k value was obtained by plotting k_{obsd} against [Na^+AsH^- (Na^+AsD^-)] (Fig. 5) and using a standard least-squares analysis.

The temperature dependences of the k's for α-Toc$^\bullet$ and β-Toc$^\bullet$ were measured (Fig. 6), but the temperature dependence of the k for γ-Toc$^\bullet$ could not be examined with our apparatus because γ-Toc$^\bullet$ is unstable. Only its k value in EtOH/H_2O at 25 °C was obtained in the present study.

3. Results and discussion

It is found experimentally that for many reactions a plot of the logarithm of the reaction rate constant (log k) against the reciprocal of temperature (1/T) (an Arrhenius plot) is a straight line (Atkins and de Paula, 2002). This behavior is normally expressed mathematically in the following way:

$$\log k = \log A - \frac{E}{2.303RT}, \quad (6)$$

where E, A and R, respectively, denote the activation energy, frequency factor and gas constant. Linear relationships between log k and 1/T can be seen in the Arrhenius plots of the k's for the reaction of Toc$^\bullet$'s with Na^+AsH^- and Na^+AsD^- (Fig. 6). Let the k's for the reactions of a Toc$^\bullet$ with Na^+AsH^- and of the Toc$^\bullet$ with Na^+AsD^-, respectively, be k^H and k^D. The values of k at 25 °C and the average ratio of k^H to k^D (k^H/k^D) at temperatures from 15 to 35 °C are listed in Table 1 along with the values of E and log A.

For Na^+AsH^- at 25 °C the order of the k values is as follows: α-Toc$^\bullet$ < β-Toc$^\bullet$ ≈ γ-Toc$^\bullet$. Thus the k value for α-Toc$^\bullet$, having three electron-donating methyl groups, is less than the k values for β-Toc$^\bullet$ and γ-Toc$^\bullet$, each having two electron-donating methyl groups, and the k values for β-Toc$^\bullet$ and γ-Toc$^\bullet$ are close to each other. The weaker the electron-donating property of the substituent in Toc$^\bullet$, the larger the k value. These results suggest that the following partial charge-transfer plays a role in reaction (4):

$$Toc^\bullet + AsH^- \rightarrow [(Toc^\bullet)^{\delta-}\cdots(AsH^-)^{\delta+}] \rightarrow TocH + As^{-\bullet}. \quad (7)$$

A similar charge-transfer in reaction (1) was suggested by the results of stopped-flow spectroscopy (Nagaoka et al., 1992, 2000; Nagaoka, 2004) and femtosecond spectroscopy (Nagaoka and Ishihara, 1996) as well as by

Table 1
E, $\log A$, k at 25 °C and k^H/k^D values for the reactions of various tocopherol radicals with ascorbic acid and ascorbate monoanions

Reaction	k at 25 °C ($M^{-1}\,s^{-1}$)	k^H/k^D	E (kJ/mol)	$\log A$[a]
α-Toc$^\bullet$ + Na^+AsH^-	2.73×10^6	5.6	4.0 ± 0.4	7.1 ± 0.1
α-Toc$^\bullet$ + Na^+AsD^-	4.87×10^5	–	6.4 ± 0.1	6.8 ± 0.1
β-Toc$^\bullet$ + Na^+AsH^-	3.65×10^6	3.9	27.2 ± 1.3	11.3 ± 0.2
β-Toc$^\bullet$ + Na^+AsD^-	1.00×10^6	–	40.5 ± 3.5	13.1 ± 0.6
γ-Toc$^\bullet$ + Na^+AsH^-	3.81×10^6	–	–	–
α-Toc$^\bullet$ + ascorbic acid[b]	4.97×10^4	7.97	18.9 ± 0.5	–

[a] Because $\log A$ was obtained by extrapolating the linear $\log k$ vs. $1/T$ plot in a limited $1/T$ range around room temperature to the intercept, the $\log A$ values thus obtained have a large uncertainty.
[b] In SDS micelles (Bisby and Parker, 1995).

time-dependent density functional theory (Duan et al., 2004).

It is interesting that β- and γ-TocH's are more effective in antioxidant regeneration (reaction (4)) than α-TocH, which is the most biologically active of the four natural TocH's shown in Fig. 2. In fact, Willett suggested that γ-TocH has important antioxidant and other properties not shared by α-TocH (Willett, 2003), and Jiang et al. (2001) noted that γ-TocH is the major form of vitamin E in the United States diet and deserves more attention.

Substantial deuterium kinetic isotope effects on the k and E for reaction (4′) were not found under the present experimental conditions. The values of k^H/k^D do not exceed the maximum semiclassical ratio [6–8 (Bell, 1980; Kwart, 1982)], nor does the E difference between the reaction of α-Toc$^\bullet$ with Na^+AsH^- and the reaction of α-Toc$^\bullet$ with Na^+AsD^- exceed the maximum semiclassical difference [1.3–4.2 kJ/mol (Bell, 1980; Kwart, 1982)]. These results show that although the tunneling effect plays an important role in reaction (1′) (Nagaoka et al., 1992, 2000; Nagaoka, 2004), it does not play an important role in reaction (4′) under the present experimental conditions. The E difference between the reaction of β-Toc$^\bullet$ with Na^+AsH^- and that the reaction of β-Toc$^\bullet$ with Na^+AsD^- is discussed in the paragraph after the following one.

In the work reported in previous papers (Mukai et al., 1987, 1989, 1991; Nagaoka et al., 2000; Nagaoka, 2004), we used 5,7-diisopropyl-Toc$^\bullet$ or 7-*t*-butyl-5-isopropyl-Toc$^\bullet$ (Fig. 2) in the studies of reaction (4) (reaction (4″)):

5, 7-diisopropyl-Toc$^\bullet$ + Na^+AsH^- (Na^+AsD^-)
→ 5, 7-diisopropyl-TocH (TocD) + $Na^+As^{-\bullet}$, (4″-1)

7-*t*-butyl-5-isopropyl-Toc$^\bullet$ + Na^+AsH^- (Na^+AsD^-)
→ 7-*t*-butyl-5-isopropyl-TocH (TocD) + $Na^+As^{-\bullet}$. (4″-2)

The absence of the tunneling effect in α- and β-Toc$^\bullet$'s is not consistent with the important role the tunneling effect plays in reaction (4″-1) (Nagaoka et al., 2000; Nagaoka, 2004). This inconsistency seems to be a result of steric hindrance due to the two isopropyl groups at the 5- and 7-positions of the Toc$^\bullet$. This steric hindrance prevents access of Na^+AsH^- (Na^+AsD^-) to the O$^\bullet$ at the 6-position of 5,7-diisopropyl-Toc$^\bullet$ (Fig. 2) in solutions and gives large E's in reaction (4″-1) [11.1 kJ/mol for Na^+AsH^- and 24.2 kJ/mol for Na^+AsD^- (Nagaoka et al., 2000)]. Tunneling allows the proton to cut a corner on the potential surface with the high energy barrier, and the proton tunneling takes place below the transition state in reaction (4″-1). In reaction (4′) for natural TocH's, in contrast, since the steric hindrance giving large E's is absent, the proton prefers to jump semiclassically over the low energy barrier (E = 4.0 kJ/mol for α-Toc$^\bullet$ + Na^+AsH^- and 6.4 kJ/mol for α-Toc$^\bullet$ + Na^+AsD^-) rather than to tunnel through it. As a result, the tunneling effect does not play an important role in reaction (4′) *in vitro*. *In vivo*, however, the energy barrier of reaction (4) would be higher than that in a uniform solution because the collisions between Toc$^\bullet$ in the cell membrane and AsH^- in the water phase (Fig. 1) would be less frequent than the collisions between the free Toc$^\bullet$ and AsH^- in a uniform solution. Accordingly, owing to the high energy barrier *in vivo*, the proton tunneling might take place below the transition state in reaction (4). In fact, the E value of reaction (4″-1) for Na^+AsH^- in a micellar dispersion (29.7 kJ/mol), which is a model of the cell membrane, is much larger than that in a uniform solution (11.1 kJ/mol) (Nagaoka et al., 2000). Bisby and Parker (1995) also reported high E's for reaction (4) in micelles and bilayers (17.3–28.8 kJ/mol) and low E's for reaction (4) in uniform solutions (2.2–2.3 kJ/mol). Miyazaki and Kumagai (2004) also concluded that the tunneling effect plays an important role in mutation-suppression by vitamin C.

The E and A values of β-Toc$^{\bullet}$ are much larger than those of α-Toc$^{\bullet}$ (Table 1), and there seems to be a large deuterium kinetic isotope effect on E for β-Toc$^{\bullet}$. At present we do not have an unambiguous explanation for this, but a possible reason is the isokinetic relationship (compensation effect) (Leffler, 1955; Nagaoka, 1987), in which the correlation between the enthalpy and entropy changes in a series of related reactions results in E being proportional to log A.

4. Conclusions

The k and E values of reaction (4′) for Toc$^{\bullet}$'s originating from natural TocH's were determined with a double-mixing stopped-flow spectrophotometer. The formation of $Na^{+}As^{-\bullet}$ was observed in the absorption spectrum. The kinetic effect of methyl substitutions on the aromatic ring of Toc$^{\bullet}$ indicates that partial charge-transfer plays a role in reaction (4′). Since a substantial deuterium kinetic isotope effect was not found, the tunneling effect may not play an important role under the present experimental conditions.

Acknowledgments

We thank Eisai Co., Ltd. for the generous gifts of *d*-α-, *d*-β-, *d*-γ and *d*-δ-TocH's. We also thank Dr. Umpei Nagashima of National Institute of Advanced Industrial Science and Technology for his valuable discussion and to Mr. Hiroyuki Shiode of Ehime University for his kind help in our experiments. This work was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas "Applications of Molecular Spins" (Area No. 769 and Proposal No. 15087104) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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