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Kinetic Studies of the Free Radical-Scavenging Actions of Tocopherol Metabolites (α-, γ-, and δ-Carboxyethyl-6-hydroxychroman) and Trolox in Ethanol and Micellar Solutions

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The reaction rates (k_s) of tocopherol metabolites (α-, γ-, and δ-CEHC) and Trolox with aroxyl radical have been measured in ethanol and micellar solution by a stopped-flow spectrophotometer, and the k_s values obtained were compared with those reported for tocopherols (α-, β-, γ-, and δ-tocopherol, TocH) and tocol. The rate constants (k_s) increased in the order of Tocol < δ-CEHC < δ-TocH < γ-CEHC < Trolox ∼ γ-TocH ∼ β-TocH < α-CEHC < α-TocH in ethanol. The antioxidants that have lower oxidation potentials (E_p) showed higher reactivities. The k_s values of α-, β-, γ-, and δ-tocopherol and tocol in micelle remained constant between pH 4 and pH 10 and decreased rapidly at pH 11∼12 by increasing pH value. On the other hand, the k_s values of α-CEHC, γ-CEHC, and Trolox showed notable pH dependence. As a result of the detailed analysis of the pH dependence of the rate constants (k_s), the structure–activity relationship in the free radical-scavenging action of the tocopherol metabolites and Trolox has been clarified.

KEYWORDS: Free radicals; tocopherol metabolites; CEHC; tocopherol; Trolox; antioxidant activity; reaction rate; pH dependence; stopped-flow spectrophotometer; pK_a

INTRODUCTION

Tocopherols (α-, β-, γ-, and δ-tocopherol, TocH) are well-known as lipophilic antioxidants. The antioxidant actions of tocopherols have been ascribed to the oxidation reaction of a phenolic hydroxyl group, producing the corresponding tocopheroxyl (Toc$^•$) radicals (reaction 1). The mechanism involved has been studied extensively by several investigators (*1–4*).

$$\mathrm{LOO}^• + \mathrm{TocH} \xrightarrow{k_{inh}} \mathrm{LOOH} + \mathrm{Toc}^• \qquad (1)$$

γ-Tocopherol is the major form of tocopherols in many plant seeds but has received relatively little attention as compared with α-tocopherol, because γ-tocopherol is found in blood and tissues at concentrations much lower than that of α-tocopherol (*5–9*). Tissue concentrations of α- and γ-tocopherols are influenced by both dietary intake and their rates of elimination (*5, 10*). γ-Tocopherol is rapidly metabolized to γ-CEHC [2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman] (see **Figure 1**), whereas α-tocopherol is maintained in the plasma and little is metabolized to α-CEHC [2,5,7,8-tetramethyl-2-(β-carboxyethyl)-6-hydroxychroman] (*10–12*).

Wechter et al. (*13*) reported that the main urinary metabolites of tocopherols are γ-CEHCs, and they proposed a physiological role for this substance in the urinary excretion of sodium (natriuresis). The metabolites (α-, γ-, and δ-CEHC) from α-, γ-, and δ-tocopherol (*14, 15*) and from α- and γ-tocotrienol (*16*) have also been found in human urine and plasma (*17, 18*). Both the tocopherols and the tocotrienols are metabolized by side chain degradation, which starts with a hydroxylation of the ω-methyl group and is followed by β-oxidation. γ-CEHC has natriuretic activity, but α-CEHC has no such activity (*13*). Both γ-tocopherol and γ-CEHC, but not α-tocopherol, inhibit cycloxygenase activity and, thus, possess antiinflammatory properties (*19*).

Yoshida et al. (*20*) reported that the reactivities of α- and γ-CEHC toward peroxyl radicals are the same as those of the corresponding α- and γ-tocopherols in acetonitrile solution. Furthermore, it has been found that α- and γ-CEHC scavenge aqueous radicals more efficiently but that they inhibit the lipid peroxidation within the liposomal membranes less efficiently than the corresponding α- and γ-tocopherols. It has been reported that α-CEHC shows antioxidant activities similar to those of Trolox, a synthetic water-soluble vitamin E homologue

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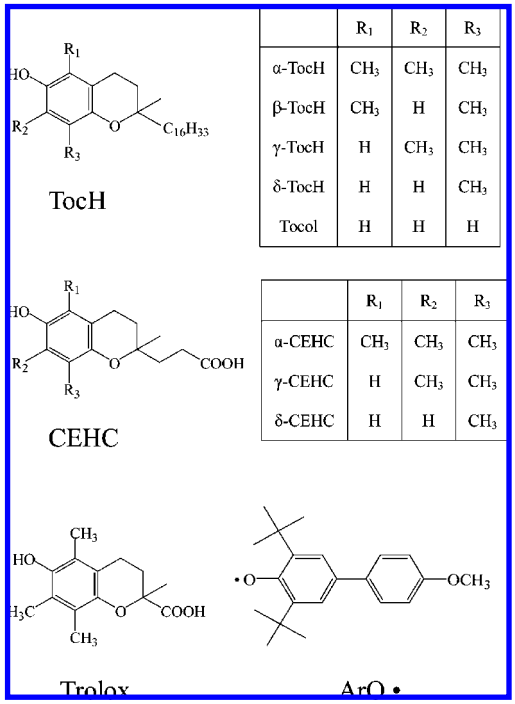

	R_1	R_2	R_3
α-TocH	CH_3	CH_3	CH_3
β-TocH	CH_3	H	CH_3
γ-TocH	H	CH_3	CH_3
δ-TocH	H	H	CH_3
Tocol	H	H	H

	R_1	R_2	R_3
α-CEHC	CH_3	CH_3	CH_3
γ-CEHC	H	CH_3	CH_3
δ-CEHC	H	H	CH_3

Figure 1. Molecular structures of tocopherols (α-, *β*-, *γ*-, and *δ*-TocH), tocol, tocopherol metabolites (α-, *γ*-, and *δ*-CEHC), Trolox, and aroxyl radical (ArO•).

(*21*). However, the kinetic study of the antioxidant activity of α- and *γ*-CEHC has not been performed, as far as we know.

In previous works, we measured the reaction rates (k_s) of α-, *β*-, *γ*-, and *δ*-tocopherols with 2,6-di-*t*-butyl-4-(4′-methoxyphenyl)phenoxyl (aroxyl, ArO•) (see **Figure 1**) in ethanol (eq 2) using stopped-flow spectrophotometry (*22, 23*). ArO• can be regarded as a model for active oxygen radicals (LOO• and others) in biological systems.

$$\mathrm{ArO^{\bullet} + TocH \xrightarrow{k_s} ArOH + Toc^{\bullet}} \qquad (2)$$

The second-order rate constants (k_s) obtained were 5.12×10^3 (α-TocH), 2.24×10^3 (*β*-TocH), 2.42×10^3 (*γ*-TocH), and 1.00×10^3 (*δ*-TocH) M^{-1} s^{-1} in ethanol at 25.0 °C (see **Table 1**). The relative rates (α:*β*:*γ*:*δ* = 100:44:47:20) agreed well with those obtained in studies on the reactivity of tocopherol toward poly(peroxystyryl)peroxyl radicals (100:41:44:14) in chlorobenzene using the O_2 consumption method (eq 1) (*1*). The result suggests that the relative reactivity of tocopherol in solution probably does not depend on the type of oxyradicals (ArO• and LOO•) used (*23–25*).

In the present work, to clarify the structure–activity relationship in the scavenging reaction of free radicals by tocopherol metabolites (α-, *γ*-, and *δ*-CEHC) and Trolox, we have measured the reaction rates (k_s) of these antioxidants with ArO• in ethanol and aqueous Triton X-100 micellar (5.0 wt %) solutions. The rate constants obtained in micellar solution were pH-dependent because of the dissociation of phenolic hydroxyl and carboxyl groups in these antioxidants. The measurement of the oxidation potential (E_p) has also been performed by a cyclic voltammetry technique.

MATERIALS AND METHODS

Chemicals. α-, *γ*-, and *δ*-CEHCs (racemic compounds) were kindly supplied from Eisai Co. Ltd. Trolox was commercially available. ArO• radical was prepared according to the method of Rieker et al. (*26*).

Measurements. The kinetic data were obtained with a Unisoku model RSP-1000 stopped-flow spectrophotometer by mixing equal volumes of solutions of antioxidants and ArO• under nitrogen atmosphere (*27*). The shortest time for mixing two solutions and recording the first data point (that is, dead time) was 10–20 ms. The reaction was monitored with either single wavelength detection or a photodiode array detector attached to the stopped-flow spectrophotometer. All measurements were performed at 25.0 ± 0.5 °C. Experimental errors in the rate constants (k_s) are estimated to be about 5 and 8% in ethanol and micellar solutions, respectively.

Cyclic voltammetry was performed at 20 °C under an atmosphere of nitrogen with platinum electrodes and a Ag/Ag^+ reference electrode in acetonitrile (dried over P_2O_5) containing 0.1 M tetrabutylammonium perchlorate with a BAS model CV-50W cyclic voltammetric analyzer. Under these conditions, ferrocene as a standard sample has a half-wave potential ($E_{1/2}$) of +40 mV. The observed E_p values are summarized in **Table 1**. The experimental error in E_p values was ±10 mV at maximum.

RESULTS

Rates of the Aroxyl Radical-Scavenging (k_s) of α-, *γ*-, and *δ*-CEHC and Trolox in Ethanol. Measurements of the rate constant (k_s) for the reaction of aroxyl (ArO•) radical with α-, *γ*-, and *δ*-CEHC and Trolox were performed in ethanol solution (eq 2). The decay rate of ArO• radical was measured by following the decrease in absorbance at 376 nm of the ArO• (*22*). The pseudo-first-order rate constants (k_{obsd}) at 376 were linearly dependent on the concentration of CEHC ([CEHC]), and thus, the rate equation is expressed as

$$-\mathrm{d[ArO^{\bullet}]}/\mathrm{d}t = k_{obsd}[\mathrm{ArO^{\bullet}}] = k_s[\mathrm{CEHC}][\mathrm{ArO^{\bullet}}] \qquad (3)$$

where k_s is the second-order rate constant for oxidation of CEHC by ArO• radical. The rate constants (k_s) were obtained by plotting k_{obsd} against [CEHC], as shown in **Figure 2**. The k_s values obtained are summarized in **Table 1**, together with those obtained for α-, *β*-, *γ*-, and *δ*-tocopherol (TocH) and tocol. As is clear from the k_s values listed in **Table 1**, the rate of the scavenging reaction of ArO• increases in the order of

$$\begin{aligned}\text{tocol} < \delta\text{-CEHC} < \delta\text{-TocH} < \gamma\text{-CEHC} \\ < \text{Trolox} \sim \gamma\text{-TocH} \sim \beta\text{-TocH} < \alpha\text{-CEHC} \\ < \alpha\text{-TocH}\end{aligned} \qquad (4)$$

The rate constants (k_s) of α-, *γ*-, and *δ*-CEHC are 1.65, 1.30, and 1.49 times smaller than those of α-, *γ*-, and *δ*-TocH, respectively. The rate constants (k_s) of α-CEHC and Trolox are 1.65 and 2.30 times smaller than that of α-TocH, respectively.

When the logarithm of the rate constant (log k_s) was plotted as a function of the oxidation potentials (E_p) of the antioxidants, a good linear correlation was observed, as shown in **Figure 3**; the antioxidants that have smaller E_p values show higher reactivities (*23, 27*).

Rates of the Aroxyl Radical-Scavenging (k_s) of α-, *γ*-, and *δ*-CEHC and Trolox in Micellar Solution. Measurements of the rate constant (k_s) for the reaction of ArO• with α-, *γ*-, and *δ*-CEHC and Trolox were performed at various pH values in Triton X-100 (5.0 wt %) micellar solution. The rate was measured by following the decrease in absorbance at 378 nm of the ArO• radical. The k_s values obtained are summarized in **Tables 1** and **2**, together with those obtained for α-, *β*-, *γ*-, and *δ*-TocH and tocol (*27*). In those cases in which pH < 4 or pH > 13, the ArO• radical and/or antioxidants are unstable, and measurement of the k_s value was unsuccessful.

With increasing pH values, the rate constants (k_s) of α-CEHC decreased rapidly from 1.75×10^5 M^{-1} s^{-1} at pH 4.0 to 6.20

Table 1. Second-Order Rate Constants (k_s) for the Reaction of Tocopherols, Their Metabolites, and Trolox with ArO$^\bullet$ Radical in Ethanol and Triton X-100 Micellar (5.0 wt %) Solutions at 25.0 °C, the Relative Rate Constants [100 k_s(TocH)/k_s(α-TocH), %], and Peak Oxidation Potentials (E_p)

	ethanol	ratio (%)	micelle	ratio (%)	E_p (mV)
antioxidant	k_s (M^{-1} s^{-1})	100 k_s(TocH)/k_s (α-TocH)	k_s (M^{-1} s^{-1}) (pH 7.4)	100 k_s(TocH)/k_s (α-TocH)	vs Ag/AgNO$_3$
α-CEHC	3.10×10^3	66	6.20×10^4	12	500
γ-CEHC	1.86×10^3	38	2.44×10^4	4.8	590
δ-CEHC	0.67×10^3	13	5.04×10^3	0.98	680
Trolox	2.23×10^3	44	3.07×10^{4a}	6.0	530
α-tocopherol	5.12×10^3	100	5.14×10^5	100	490
β-tocopherol	2.24×10^3	44	1.05×10^{5a}	21	550
γ-tocopherol	2.42×10^3	47	1.00×10^{5a}	20	560
δ-tocopherol	1.00×10^3	20	1.49×10^{4a}	2.9	640
tocol	0.56×10^3	11	3.53×10^{3a}	0.69	720

[a] The k_s values at pH 7.0 (see ref *27*).

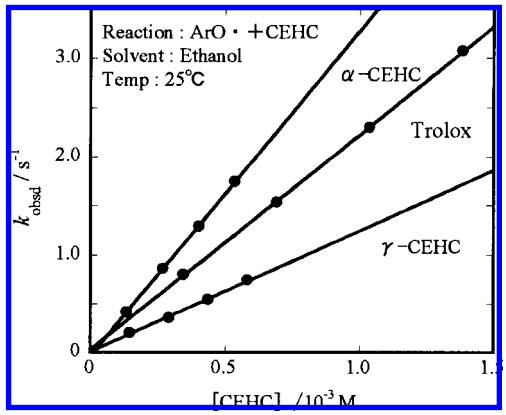

Figure 2. Dependence of k_{obsd} on [CEHC] (or [Trolox]) in the reaction of CEHC (or Trolox) with ArO$^\bullet$ in ethanol at 25.0 °C (eq 3).The value of k_{obsd} was obtained by analyzing the decrease in the absorbance of ArO$^\bullet$ at 376 nm.

$\times 10^4$ M^{-1} s^{-1} at pH 7.4 and remained constant between pH 7 and 13, as shown in **Figure 4A**. Similarly, the k_s values of γ-CEHC decrease rapidly between pH 4 and 7, are constant at pH 8–9, and decreased gradually at pH 12–13 (**Figure 4B**). On the other hand, the rate constants (k_s) of Trolox decreased with increasing pH, showed similar values at pH 7–9, and increased until pH 13 (**Figure 4C**). The pH dependence of the reaction rates observed for these antioxidants will be discussed later.

As reported in a previous work (*27*), the rate constants (k_s) of α-, β-, γ-, and δ-TocH and tocol are pH-independent and show similar values at pH 4–10 and decrease rapidly at about pH 11, respectively. At pH 7.4, the rate constants increase in the order of

$$\text{tocol} < \delta\text{-CEHC} < \delta\text{-TocH} < \gamma\text{-CEHC} < \text{Trolox} < \alpha\text{-CEHC} < \gamma\text{-TocH} \sim \beta\text{-TocH} < \alpha\text{-TocH} \quad (5)$$

The ratios of the rate constants [100 $\times$ k_s(TocH)/k_s(α-TocH)] at pH 7.4 change from 100 (α-TocH) to 0.69 (Tocol) in micellar solution, as listed in **Table 1**. The k_s values of α-, γ-, and δ-CEHC are 8.29, 4.10, and 2.96 times smaller than those of α-, γ-, and δ-TocH at pH 7.4 in micellar solution, respectively. The rate constants (k_s) of α-CEHC and Trolox are 8.29 and 16.7 times smaller than that of α-TocH, respectively. The order of the rate constants (k_s) of α-CEHC and γ-TocH ($\sim$ β-TocH) in micelle is different from that in ethanol.

When the logarithm of the rate constant (log k_s) at pH 7.4 was plotted as a function of E_p of the antioxidants, a good linear correlation was not obtained (see Figuer **3A**), because the k_s values for α-, γ-, and δ-CEHC and Trolox are pH-dependent. A detailed discussion will be performed in the following section.

Analyses of the pH Dependence on the Reaction Rates (k_s) of α- and γ-CEHC and Trolox. γ-CEHC is dibasic and can exist in three different molecular forms, that is, undissociated form (γ-CEHC-H_2), monoanion (γ-CEHC-H^-), and dianion (γ-$CEHC^{2-}$), depending on the pH value (see **Figure 5B**). The equilibrium reactions of γ-CEHC have the form

$$\gamma\text{-CEHC-H}_2 \overset{K_{a1}}{\rightleftharpoons} \gamma\text{-CEHC-H}^- \overset{K_{a2}}{\rightleftharpoons} \gamma\text{-CEHC}^{2-} \quad (6)$$

If the pK_{a1} and pK_{a2} values of γ-CEHC are reported, the mole fractions (f) present as the γ-CEHC-H_2 molecule and the γ-CEHC-H^- and γ-$CEHC^{2-}$ ions may be calculated as a function of pH (see **Figure 4B**) (*27–29*). The analytical concentration (C_a) is given as

$$C_a = [\gamma\text{-CEHC-H}_2] + [\gamma\text{-CEHC-H}^-] + [\gamma\text{-CEHC}^{2-}] \quad (7)$$

If we assume that k_{s1}, k_{s2}, and k_{s3} are the reaction rates for γ-CEHC-H_2, γ-CEHC-H^-, and γ-$CEHC^{2-}$ forms of γ-CEHC, respectively, the total rate k_s will be expressed as

$$k_s = k_{s1}f(\gamma\text{-CEHC-H}_2) + k_{s2}f(\gamma\text{-CEHC-H}^-) + k_{s3}f(\gamma\text{-CEHC}^{2-}) \quad (8)$$

The pK_{a1} and pK_{a2} values of γ-CEHC have not been reported, as far as we know. We assumed that the OH group in γ-CEHC has the same pK_{a2} value as that in γ-TocH (pK_{a1} = 12.7) (*27*). The rate constants (k_s) of γ-CEHC remained constant between pH 8 and pH 11, as shown in **Figure 4B**. Therefore, at pH 8–11, only the monoanion form of γ-CEHC exists in solution, that is, f(γ-CEHC-H^-) = 1, and we can immediately determine the k_{s2} value. As observed for γ-TocH (*27*), the k_s value of γ-CEHC decreased rapidly at pH 12, because dianion form γ-$CEHC^{2-}$ does not have any OH proton to reduce ArO$^\bullet$. The k_{s3} value is considered to be small as compared to k_{s2} and negligible. The electron transfer from electron-rich dianion form to ArO$^\bullet$ was not observed. By comparing the observed pH dependence of k_s with the pH dependence of mole fraction calculated, the values of k_{s1} and pK_{a1} were determined. As shown in **Figure 4B**, a good accordance between the observed k_s and the simulation curve was obtained, suggesting that each reaction rate (k_{si}) and pK_{ai} value (see **Table 3**) estimated is reasonable. The k_{s1} value

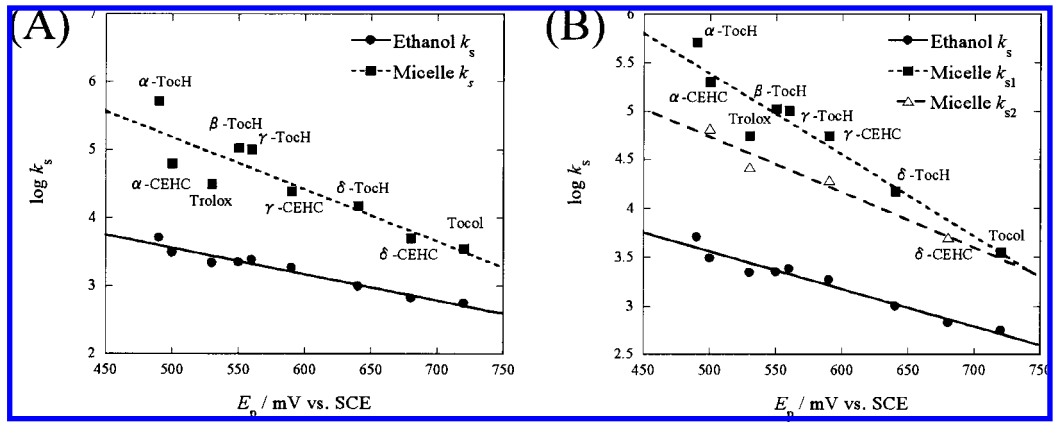

Figure 3. (**A**) Plots of log k_s vs E_p for α-, β-, γ-, and δ-tocopherol, tocol, α-, γ-, and δ-CEHC, and Trolox in ethanol (closed circle) and in micellar solution (at pH 7.4) (closed square). (**B**) Plots of (i) log k_s vs E_p and (ii) log k_{s1} vs E_p for α-, β-, γ-, and δ-tocopherol, tocol, α-, γ-, and δ-CEHC, and Trolox in ethanol (closed circle) and in micellar solution (closed square), respectively (see text). (iii) Plots of log k_{s2} vs E_p (open triangle) (see text).

Table 2. pH Dependence of the Second-Order Rate Constants (k_s) for the Reaction of α- and γ-Tocopherol, α- and γ-CEHC, and Trolox with ArO• Radical in Triton X-100 Micellar Solution (5.0 wt %) at 25.0 °C

	k_s (M^{-1} s^{-1})				
pH	α-tocopherol	α-CEHC	γ-tocopherol	γ-CEHC	Trolox
4.0	5.28×10^5	1.75×10^5		5.46×10^4	5.01×10^4
5.0	5.25×10^5	1.16×10^5		5.38×10^4	4.07×10^4
6.0	4.93×10^5	8.87×10^4	9.93×10^4	3.81×10^4	3.38×10^4
7.0	5.12×10^5		10.0×10^4	2.74×10^4	3.07×10^4
7.4	5.14×10^5	6.20×10^4		2.44×10^4	
8.0	5.32×10^5		9.40×10^4		2.80×10^4
8.5					2.69×10^4
9.0	5.32×10^5	6.33×10^4	10.1×10^4	1.93×10^4	2.64×10^4
9.5					3.02×10^4
10.0	5.25×10^5	6.78×10^4	10.2×10^4	2.15×10^4	3.32×10^4
10.5					3.81×10^4
11.0	4.87×10^5	6.69×10^4	9.19×10^4	1.75×10^4	3.99×10^4
12.0	4.76×10^5	6.44×10^4	7.17×10^4	1.54×10^4	4.90×10^4
13.0	3.00×10^5	6.73×10^4	3.99×10^4	8.67×10^3	6.79×10^4

is 2.8 times as large as the k_{s2} value. The result indicates that the reaction rate k_{si} decreases by increasing the anionic character of γ-CEHC.

α-CEHC is also dibasic. The reaction rates (k_{s1} and k_{s2}) and pK_{a1} value of α-CEHC were determined similarly, by assuming that the pK_{a2} value of α-CEHC is the same as that (pK_{a1} = 13.1) in α-TocH and the k_{s3} value for dianion (α-CEHC^{2-}) is small and negligible. As shown in **Figure 4A**, a good accordance between the observed k_s and the simulation curve was obtained at pH < 12. The k_{s1} value is 3.0 times as large as the k_{s2} value. The value of pK_{a1} (=5.0) obtained for α-CEHC is smaller than that (=6.0) for γ-CEHC. As shown in **Figure 4A**, the k_s value at pH 13 is similar to that at pH 12. The decrease of the k_s value at pH > 12 was not observed, although we repeated the measurement. If the k_{s3} value is similar to the k_{s2} value in α-CEHC or the pK_{a2} value in α-CEHC is larger than 13.1, we can explain the pH dependence of the k_s value at pH > 12. The reason for such a disagreement is not clear at present.

pH dependence of the k_s values of Trolox has also been analyzed similarly. The reaction rates k_{s1}, k_{s2}, and k_{s3} for three different molecular forms of Trolox were determined, by varying the k_{si} (i = 1–3) values and pK_{a1} and pK_{a2} values. The values obtained are listed in **Table 3**. The values of pK_{a1} (=5.00) and pK_{a2} (=11.5) obtained for Trolox are similar to those (=4.4 and 11.9) reported by Amorati et al. (*30*). The k_{s1} value is about 2.1 times as large as the k_{s2} one. Furthermore, the k_{s3} value is 2.6 times as large as the k_{s2} value, although the dianion form of Trolox has no OH proton to reduce the aroxyl radical. We can expect the electron transfer between ArO• radical and Trolox^{2-}, as reported for the reaction of ArO• (or LOO•) with caffeic acid, cinnamic acid, and chlorogenic acid, recently (*30–32*). However, the details are not clear at present.

DISCUSSION

Correlation between the Logarithms of the Rate Constants (k_{s1} and k_{s2}) for Undissociated and Monoanion Forms of α-, γ-, and δ-CEHC and Trolox and Peak Oxidation Potentials (E_p). As reported in previous works, the scavenging rate (k_s) of ArO• by tocopherol derivatives and biological hydroquinones in ethanol solution increases as the total electron-donating capacity of the alkyl substituents on the aromatic ring increases (*23, 24, 27, 33*). The logarithms of the rate constants (log k_s) of the antioxidants were found to correlate with their peak oxidation potentials (E_p). As shown in **Figure 3A**, similar good correlation was observed for the antioxidants including α-, β-, γ-, and δ-TocH, tocol, α-, γ-, and δ-CEHC, and Trolox in ethanol with a gradient of −3.85/V (correlation coefficient = 0.976); the antioxidants that have smaller E_p values show higher scavenging rates. The result suggests that the transition state in the above ArO•-scavenging reaction by antioxidants has the property of a charge-transfer intermediate (*23, 24, 34*). On the other hand, such a good correlation between log k_s and E_p was not observed for the k_s values obtained in micellar solution (at pH 7.4) (see **Figure 3A**), because three different molecular forms coexist in solution; thus, the k_s values show notable pH dependence. Therefore, the semilogarithm plot for the rate constants of the undissociated form (k_{s1}) of the above antioxidants vs E_p values was performed. As shown in **Figure 3B**, a good correlation was observed with a gradient of −8.37/V (correlation coefficient = 0.959). Similarly, log k_{s2} was also found to correlate with E_p with a gradient of −5.68/V (correlation coefficient = 0.975). The E_p values of monoanion and undissociated forms are considered to be similar to each other, as the COO^- group in α-, γ-, and δ-CEHC and Trolox is electronically insulated from the phenol ring, which is certainly the source of the one-electron oxidation. The measurement of the k_s value for δ-CEHC was performed only at pH 7.4. We can expect that δ-CEHC will take mainly monoanion form (δ-CEHC-H$^-$) at pH 7.4. Therefore, the k_s value (that is, the k_{s2} value) for δ-CEHC is also included in the above log k_{s2} vs E_p plot. As the results of the detailed analyses of the pH dependence of the rate constants (k_s), the

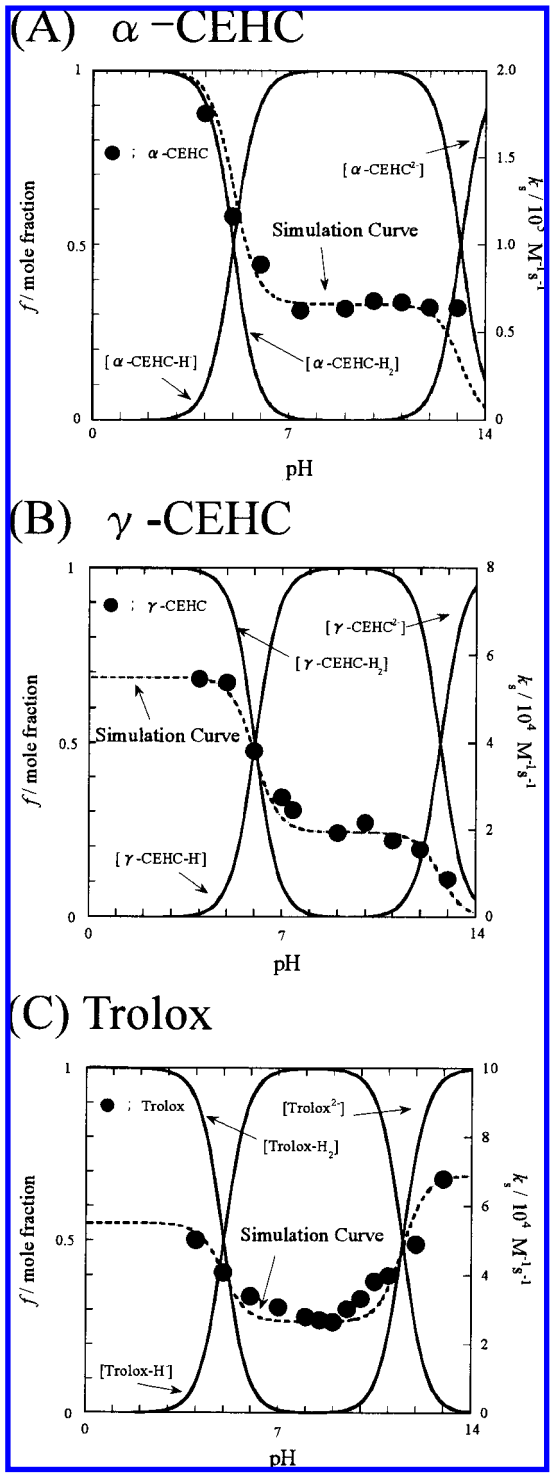

Figure 4. (**A**) Plots of second-order rate constant (k_s) for α-CEHC vs pH (closed circle) and of mole fraction (f) of three α-CEHC species (α-CEHC-H_2, α-CEHC-H^-, and α-CEHC^{2-}) vs pH (solid line). The dotted line is a simulation curve for k_s. (**B** and **C**) Similar plots for γ-CEHC and Trolox.

structure–activity relationship in the free radical-scavenging action of vitamin E metabolites (CEHC) and Trolox has been clarified.

Comparison between the Aroxyl Radical-Scavenging Activities of Tocopherols and Their Metabolites (CEHC) in Ethanol and Micellar Solutions. It has been reported that α- and γ-CEHC have the same reactivities toward peroxyl radicals and exert the same antioxidant activities against lipid peroxidation in CH_3CN as the corresponding parent tocopherols,

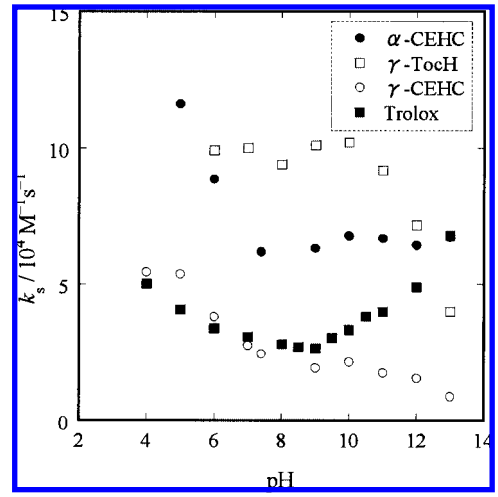

Figure 5. Plots of second-order rate constant (k_s) for the reaction of γ-tocopherol (open square), α-CEHC (closed circle), γ-CEHC (open circle), and Trolox (closed square) with ArO$^\bullet$ radical vs pH.

Table 3. Reaction Rates, k_{si} (i = 1–3), for Undissociated, Monoanion, and Dianion Forms and pK_{ai} (i = 1 and 2) Values of Vitamin E, Their Metabolites, Tocol, and Trolox in Triton X-100 Micellar Solution (5.0 wt %) at 25.0 °C

antioxidant	k_{s1} (M^{-1} s^{-1})	k_{s2} (M^{-1} s^{-1})	k_{s3} (M^{-1} s^{-1})	pK_{a1}	pK_{a2}
α-CEHC	2.00×10^5	6.60×10^4	0 (or 6.60×10^4)	5.0	13.1
γ-CEHC	5.50×10^4	1.95×10^4	0	6.0	12.7
δ-CEHC		$\sim 5.04 \times 10^3$			
Trolox	5.50×10^4	2.65×10^4	6.90×10^4	5.00 (4.4[a])	11.5 (11.9[a])
α-tocopherol	5.12×10^5	0		13.1	
β-tocopherol	1.05×10^5	0		12.8	
γ-tocopherol	1.00×10^5	0		12.7	
δ-tocopherol	1.49×10^4	0		12.6	
tocol	3.53×10^3	0		12.6	

[a] The values were reported by Amorati et al. (ref *30*).

respectively, although the k_{inh} values have not been determined (*20*). The antioxidant effect decreased in the order of α-TocH ∼ α-CEHC > γ-TocH ∼ γ-CEHC. On the other hand, the present result indicates that the aroxyl radical-scavenging rates (k_s) of α-, γ-, and δ-CEHC are 1.65, 1.30, and 1.49 times smaller than those of the α-, γ-, and δ-TocH, respectively, in ethanol, as listed in **Table 1**. Furthermore, the k_s values of α-, γ-, and δ-CEHC are 8.29, 4.10, and 2.96 times smaller than those of α-, γ-, and δ-TocH at pH 7.4 in micelle solution, respectively. The differences are more notable in micellar solutions.

As shown in **Figures 3** and **5** and as listed in **Tables 1** and **2**, the k_s values of α-TocH, α-CEHC, and Trolox having three methyl substituents at 5-, 7-, and 8-positions of phenol ring decreased in the order of α-TocH > α-CEHC > Trolox in ethanol and in micelle solution over almost the whole pH range. In fact, the E_p values of α-TocH, α-CEHC, and Trolox increased in the order of α-TocH < α-CEHC < Trolox. Similarly, the k_s values of γ- and δ-CEHC are smaller than those of the corresponding γ- and δ-TocH, and the E_p values of γ- and δ-CEHC are larger than those of the corresponding γ- and δ-TocH. The increase in E_p of Trolox will be due to the interaction between the COOH group and the 1-oxygen atom at the chroman ring, that is, probably due to the formation of intramolecular hydrogen bond and/or the inductive effect of the COOH group. The COOH groups in α-, γ-, and δ-CEHC molecules are not π-conjugated to the phenol ring, suggesting that the effects of COOH group on the electronic state of these

CEHC are small and negligible. The formation of the intermolecular hydrogen bond between COOH and OH groups in α-, γ-, and δ-CEHC molecules may contribute to the change of the electronic state of CEHC, that is, the E_p value. It is well-known that the COOH group forms dimers due to strong intermolecular homonuclear O–H···O double hydrogen bonds (*35*). Such a dimer formation between CEHC molecules may also affect the reaction rates (k_s). On the other hand, the values of hyperfine splitting constants (a_i^H), that is, the unpaired electron distributions (ρ_i^H) of α-CEHC$^\bullet$ radical obtained by ESR measurement, are very similar to those of α-Toc$^\bullet$ radical in toluene; no meaningful changes in unpaired spin distribution by replacing a long alkyl chain ($C_{16}H_{33}$) with a carboxyethyl group (–CH_2CH_2COOH) have been found (*36*). The reason for the difference between the free radical-scavenging activities of tocopherols and their metabolites in homogeneous ethanol solution is not clear at present.

The aroxyl radical-scavenging rates (k_s) for α-CEHC (3.36×10^3 M^{-1} s^{-1}), γ-CEHC (1.94×10^3 M^{-1} s^{-1}), and δ-CEHC (0.67×10^3 M^{-1} s^{-1}) in ethanol are larger than those reported for epicatechin (1.32×10^2 M^{-1} s^{-1}), epigallocatechin gallate (3.36×10^2 M^{-1} s^{-1}), caffeic acid (5.54×10 M^{-1} s^{-1}), and rutin (1.42×10 M^{-1} s^{-1}), which are well-known as representative polyphenols (*32, 37*). However, the scavenging rates (k_s) are smaller than that for ubiquinol-10 (4.70×10^3 M^{-1} s^{-1}) (*27*). Similarly, the k_s values for α-CEHC (6.20×10^4 M^{-1} s^{-1}), γ-CEHC (2.44×10^4 M^{-1} s^{-1}), and δ-CEHC (5.04×10^3 M^{-1} s^{-1}) in micelle solution (at pH 7.4) are larger than those obtained for epicatechin (8.03×10^2 M^{-1} s^{-1}), epigallocatechin gallate (3.20×10^3 M^{-1} s^{-1}), caffeic acid (3.68×10^2 M^{-1} s^{-1}), and rutin (2.28×10^2 M^{-1} s^{-1}) but are smaller than that for ubiquinol-10 (1.25×10^5 M^{-1} s^{-1}) (*27, 32, 37*). The tocopherol metabolites (α-, γ-, and δ-CEHC) show high activity for the scavenging of free radicals in ethanol and micellar solutions.

Effect of the Reaction Field on the Aroxyl Radical-Scavenging Activity. As described in the Introduction, the relative rates [$100 \times k_s$(TocH)/k_s(α-TocH)] of the k_s values for the reaction with ArO$^\bullet$ radical (α:β:γ:δ:tocol = 100:44:47:20) in ethanol (*22, 23*) are similar to the relative rates [$100 \times k_{inh}$(TocH)/k_{inh}(α-TocH)] of the k_{inh} values for the reaction with LOO$^\bullet$ radical (100:41:44:14) in chlorobenzene (*1*). On the other hand, the relative rates of the k_s values (α:β:γ:δ:tocol = 100:44:47:20:11) in ethanol are very different from that in micellar solution (100:21:20:2.9:0.69) (see **Table 1**) (*27*).

The solvent effect on the reaction rates (k_s) of α-tocopherol with ArO$^\bullet$ radical has been studied in a previous work (*33*). The k_s values are 5.12×10^3 (ethanol), 1.44×10^4 (diethyl ether), 9.52×10^4 (benzene), and 1.94×10^5 M^{-1} s^{-1} (*n*-hexane) for α-tocopherol. The k_s value of α-tocopherol in *n*-hexane is 38 times as large as that in ethanol. When the logarithm of the rate constant (log k_s) of α-tocopherol was plotted as a function of the reciprocal of the solvent dielectric constants ($1/\epsilon$), it gave a straight line. The result indicates that the aroxyl radical-scavenging rates of the α-tocopherol change notably, depending on the polarity of solvents, that is, the reaction field that the α-tocopherol reacts with free radical (ArO$^\bullet$). The polarity of the reaction field of the tocopherols in micellar solution will increase in the order of α- < β- < γ- < δ-tocopherol < tocol, because the hydrophilic property of tocopherol molecules will increase by decreasing the number of methyl substituents. The k_s values of tocopherols in micelle will decrease with increasing the polarity of the reaction field. The difference between the relative ratios (100:44:47:20:11) in homogeneous ethanol and (α:β:γ:δ:tocol = 100:21:20:2.9:0.69) in inhomogeneous micellar solution will be due to the effect of the reaction field (*27*).

As reported in a previous work (*27*) and as listed in **Table 2**, the rate constants (k_s) of α-, β-, γ-, and δ-tocopherols and tocol remained constant between pH 4 and pH 10 and decreased rapidly at pH 11–12 by increasing the pH value. On the other hand, a notable pH dependence of k_s values was observed for α- and γ-CEHC and Trolox. For example, the k_s values of γ-CEHC decrease rapidly with increasing pH value from 4.0, remain constant at pH 8–10, and then decreased at pH 11. We can expect similar reaction rates (k_{s1} and k_{s2}) for undissociated and monoanion forms of γ-CEHC, because the electronic structure of the chroman ring in each form will be similar to each other. However, the k_{s1} value is 2.8 times larger than the k_{s2} value. The polarity of monoanion form of γ-CEHC is higher than that of undissociated form, and thus, the reaction field of monoanion form with ArO$^\bullet$ radical will be more polar than that of undissociated form. Consequently, the value of k_{s2} is smaller than that of k_{s1} in γ-CEHC. Similarly, the k_{s2} values of α-CEHC and Trolox are 3.0 and 2.1 times smaller than the k_{s1} values, respectively (see **Table 3**). This is the reason why the k_s values of α- and γ-CEHC and Trolox decrease with increasing pH value at pH 4–7.

The lipophilicity of α-CEHC, γ-CEHC, α-TocH, γ-TocH, and Trolox was discussed by measuring the partition coefficient between water and 1-octanol of the antioxidants (*20*). The partition coefficient decreased in the order of α-TocH (3.36) > γ-TocH (3.14) > α-CEHC (2.26) > γ-CEHC (1.83) > 0 > Trolox (–0.97), indicating that the lipophilicity decreased in the order of α-TocH > γ-TocH >> α-CEHC > γ-CEHC >> Trolox. The order of the lipophilicity (that is, the polarity of the reaction field) is similar to that of the free radical-scavenging rates (k_s) at pH 7.4 in micellar solution (see eq 5). As a conclusion, the aroxyl radical-scavenging rates (k_s) in micellar solution reflect (i) the electron-donating capacity (that is, the oxidation potential (E_p) of tocopherols (α-, β-, γ-, and δ-TocH) and their metabolites and (ii) the effect of the reaction field.

As described in the Introduction, tissue concentrations of α- and γ-tocopherol are influenced by both dietary intake and their rates of elimination (*5, 10*). γ-Tocopherol is rapidly metabolized to γ-CEHC, whereas α-tocopherol is maintained in the plasma and little is metabolized to α-CEHC (*10–12*). In fact, urine contains more γ-CEHC than α-CEHC despite the fact that the plasma α-tocopherol concentration exceeds that of γ-tocopherol by a factor of 5–10, suggesting that γ-tocopherol having smaller numbers of methyl substituents preferentially undergo phytyl chain oxidation relative to α-tocopherol (*38*). It has been suggested that the concentrations of vitamin E metabolites (α- and γ-CEHC) index the fraction of "free" tocopherols not incorporated into LDL particles (*39, 40*), although this has not been conclusively proven. As described above, the γ-tocopherol molecule has a lower lipophilicity than that of α-tocopherol, and thus, the reaction field of γ-tocopherol will be different from that of α-tocopherol. The difference in the lipophilicity may affect the rates of the enzymatic oxidation reaction of the phytyl chain in α- and γ-tocopherol, although the details are not clear at present.

Wechter et al. (*13*) reported that γ-CEHC has natriuretic activity, but α-CEHC has no such an activity. The γ-CEHC molecule has a lower lipophilicity than that of α-CEHC, and thus, the interaction between γ-CEHC molecules and water-soluble Na ions will be stronger than that between α-CEHC and Na ions. Furthermore, the pK_a value (6.00) of γ-CEHC is larger than that (5.00) of α-CEHC. As shown in **Figure 4**, at

pH 7.4, α-CEHC shows only the monoanion form. However, γ-CEHC takes both monoanion and undissociated forms. Such a difference in the pK_a value may also affect the physiological property of α- and γ-CEHC. Further investigation will be necessary to clarify the reason for the difference in the natriuretic activity of α- and γ-CEHC.

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