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Selected Paper

Determination of the Reaction Rate Constants of Antioxidants with the Hydroxyl Radical by a Rapid-Flow ESR Method

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The reaction rate constants of antioxidants with the hydroxyl radical ($HO^\bullet$) were determined by a rapid-flow ESR method. ESR spectra of the antioxidant radical formed by a reaction with $HO^\bullet$ generated from the $Ti^{3+} + H_2O_2$ system were measured. When an antioxidant and ethanol were mixed with $HO^\bullet$, a superposed spectrum of the 1-hydroxyethyl radical and antioxidant radical was obtained. The intensity ratio of the signals of these radicals was calculated from the doubly integrated curve, and then the ratio of the reaction-rate constant of the antioxidant with $HO^\bullet$ to that of ethanol was obtained. The ratios of pyrogallol, gallic acid, catechol, phloroglucinol, resorcinol, and methanol were 19, 17, 11, 1.5, 1.2, and 0.56, respectively. The dissociation energies of the bonds in antioxidant molecules were obtained by MO calculations, which demonstrated that phenoxy, hydroxymethyl, and 1-hydroxylethyl radicals were formed by the reactions with $HO^\bullet$, as expected from their spectra. The relationship between the relative activation energies obtained from the rate constants and the bond-dissociation energies showed that the Evans–Polanyi equation holds in polyphenol series, but the line was shifted from that of alcohols. This suggested that the structures of the transition state of alcohols were stabilized by a polar effect.

Excessive amounts of reactive oxygen species generated in an organic body damage biomolecules, resulting in various diseases.[1,2] Among such species; because the hydroxyl radical ($HO^\bullet$) has an especially strong reactivity, it is the most dangerous. Recently, antioxidants that effectively scavenge $HO^\bullet$ have been sought from natural substances, and many methods for evaluating the radical scavenging activity have been proposed. We have also studied the activity of catechins extracted from green tea and proposed some alternatives.[3–7] A spin probe method is useful[8,9] in which an appropriate $HO^\bullet$ radical generating system is used, and the generated $HO^\bullet$ is trapped by a spin-trapping agent. When an antioxidant is mixed in this system, it competes with the trapping agent with reference to the reaction with $HO^\bullet$. As a result, the amount of the spin adducts decreases, and the degree of the decrease is considered a measure of the radical scavenging activity. Generally, a Fenton reaction ($Fe^{2+} + H_2O_2$) is used as a $HO^\bullet$ radical generating system, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is used as a trapping agent. This method, however, has serious problems. For example, such an antioxidant as a polyphenol coordinates with Fe^{2+} and affects the reaction of $HO^\bullet$ generation. In addition, the spin adduct, DMPO-OH, is scavenged by the antioxidant, resulting in a decrease of the antioxidant, which is mistaken to be the result of the scavenging of $HO^\bullet$. Therefore, we need to obtain direct information about the reaction of $HO^\bullet$ with the antioxidants to accurately evaluate the scavenging activity.

Pulse radiolysis is a proven method for studying the rapid reactions of $HO^\bullet$ with antioxidants,[10–12] but it generally uses UV absorption spectra. Since ESR allows for the direct observation of radicals, we believe that it is a more reliable source for studying such radical reactions. In previous papers, we proposed a rapid-flow ESR method that uses a dielectric mixing resonator[13] and analyzes quantum-mechanically the structure of short-lived radicals formed with $HO^\bullet$.[14] In the present paper, we discuss the application of this method to measure radical scavenging activity, because it provides more reliable data than a conventional ESR method. Furthermore, the bond dissociation energies (BDE) in antioxidant molecules were obtained by MO calculations for confirming which bond is dissociated with $HO^\bullet$ and for recognizing the relationship between the activation energy of the reaction and the BDEs.

Experimental

Materials. As a $HO^\bullet$ radical generating system, the reaction of Ti^{3+} with H_2O_2 was used. $HO^\bullet$ is formed according to the following formulae:

$$Ti^{3+} + H_2O_2 \rightarrow Ti^{4+} + OH^- + HO^\bullet. \quad (1)$$

Ti^{3+} is very unstable, and it changes into Ti^{4+} after reacting with dissolved oxygen. Therefore, we used a commercial $TiCl_3$ in a HCl solution (WAKO), where Ti^{3+} was stabilized under acidic conditions. Because such antioxidants as catechol, pyrogallol, resorcinol, phloroglucinol, and gallic acid are considered to be model compounds of the partial structures of tea catechins, they were measured. All of the reagents were obtained from WAKO and were of guaranteed grade.

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Method. A rapid-flow ESR method using a dielectric mixing resonator was described in previous papers.[13,14] Two aqueous solutions were prepared. Solution A contained 1.0×10^{-2} M $TiCl_3$, 2.5×10^{-1} M ethanol (EtOH), 0–1.0 M polyphenol, and 1.9×10^{-1} M H_2SO_4. Solution B contained 8.0×10^{-2} M H_2O_2 and 1.9×10^{-1} M H_2SO_4. Methanol (MeOH) was mixed instead of polyphenol to ascertain the method's accuracy, and for comparing with polyphenols. These two solutions were inserted into 25 mL syringes and set on a syringe pump. After being mixed, the flow rates of the two solutions were 8, 10, 14, 20, 24, and 30 mL/min. They corresponded to the flow times from the mixing chamber to the center of the resonator: 5.0, 4.0, 2.9, 2.0, 1.7, and 1.3 ms. ESR spectra were measured by a Bruker EMX spectrometer at room temperature under the following conditions: frequency, 9.65 GHz; center field, 344.2 mT; sweep width, 10.0 mT; modulation frequency, 100 kHz; modulation width, 0.13 mT; number of measuring points, 512; conversion time, 41 ms; sweep time, 21 s; power, 8 mW.

Calculation of the Bond Dissociation Energy. The bond dissociation energy (BDE) was calculated as the energy difference for the reaction polyphenol $\rightarrow$ polyphenol$^{\bullet}$ + H$^{\bullet}$, where polyphenol$^{\bullet}$ is the corresponding phenoxyl radical. The basic calculation method was essentially the same as described in Ref. 15. The geometries of the closed-shell species were optimized at the B3LYP/6-311+G(2d, 2p) level of theory. For the radicals, a restricted open-shell (ROB3LYP) approach was utilized. The energy of the hydrogen atom is exceptionally set to its exact value, -0.50000 au. The energies of all species were corrected using unscaled zero-point energies. All of the calculations were carried out with the Gaussian 98 system of programs.[16]

Results and Discussion

Method for Obtaining the Ratio of Reaction Rate Constants. We have already reported[13] that a singlet spectrum was observed when solution A containing no reactants, such as polyphenols or alcohols, was mixed with solution B, showing that free HO$^{\bullet}$ was actually formed according to Eq. 1. When MeOH and EtOH were added to solution A, the spectra of Figs. 1A and B were observed. The triplet absorption of A is attributed to the hydroxymethyl radical ($CH_2{}^{\bullet}OH$) and the double quartet absorption of B is assigned to a 1-hydroxyethyl radical ($CH_3CH^{\bullet}OH$). The hyperfine splitting constants are same as those reported.[17] The result shows that a hydrogen atom was abstracted from the carbon on which the hydroxyl group was attached, according to the following equations:

$$HO^{\bullet} + CH_3OH \rightarrow CH_2{}^{\bullet}OH + H_2O, \quad (2)$$

$$HO^{\bullet} + CH_3CH_2OH \rightarrow CH_3CH^{\bullet}OH + H_2O. \quad (3)$$

When both MeOH and EtOH were mixed with solution A, the spectrum of Fig. 1C was obtained, and it was ascertained to be the superposition of Figs. 1A and B based on the simulation spectrum. This means that HO$^{\bullet}$ reacted competitively with MeOH and EtOH. Since the double integration of the spectrum of Fig. 1C was easily obtained, as shown in Fig. 2A, it was possible to estimate the ratio of the quantity of $CH_2{}^{\bullet}OH$ and $CH_3CH^{\bullet}OH$ from the doubly integrated curve. In the central region, signals due to both radicals are superposing, but the signals of $CH_2{}^{\bullet}OH$ do not interfere with the four lines of $CH_3CH^{\bullet}OH$ on both sides. The sum of the intensities

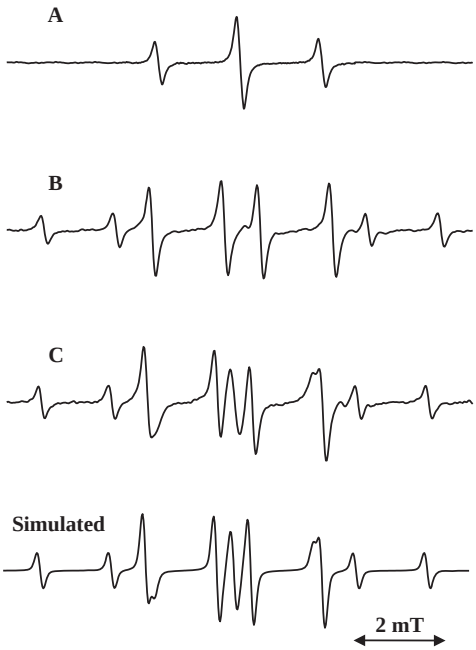

Fig. 1. ESR spectra of radicals formed by reactions with MeOH (A), EtOH (B), MeOH + EtOH (C), and simulation of C. [MeOH] = [EtOH] = 2.5×10^{-1} M.

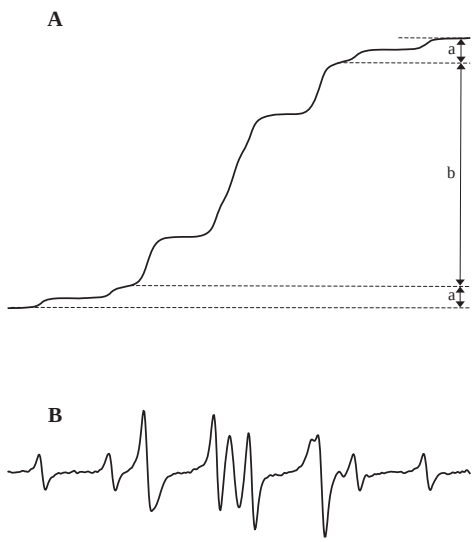

Fig. 2. Doubly integrated curve (A) and original spectrum (B) of the MeOH + EtOH sample.

of these four lines is one fourth of the total intensity of $CH_3CH^{\bullet}OH$ because the spectrum is a double quartet due to three equivalent and other protons. The intensity of $CH_2{}^{\bullet}OH$ is calculated by subtracting three times the intensity of the four lines from the intensity of the central region. Thus, it is possible to estimate the intensity ratio of $CH_2{}^{\bullet}OH$ and $CH_3CH^{\bullet}OH$ by measuring the intensities of the four lines and of the central region. In the case of polyphenols, same procedure was used.

The ratio of the reaction rate constants (k) of MeOH and EtOH with HO$^{\bullet}$ can be calculated using the above intensity ratio as follows. Establishing the rate constant of Eqs. 2 and 3 as k_{Me} and k_{Et}, respectively, the reaction rates are expressed as:

$$d[CH_2{}^{\bullet}OH]/dt = k_{Me}[MeOH][HO^{\bullet}], \quad (4)$$

$$d[CH_3CH^{\bullet}OH]/dt = k_{Et}[EtOH][HO^{\bullet}]. \quad (5)$$

Here, disappearance of $CH_2{}^{\bullet}OH$ and $CH_3CH^{\bullet}OH$ was assumed to be negligible. For understanding, let's consider a simple model in which radical formation begins at $t = 0$ in the mixing chamber; then ESR is measured at $t = T$ in the center of the resonator. The time T is called the flow time, and is obtained by dividing the distance between the mixing chamber and the resonator by the flow rate of the solution in the capillary of the resonator.

Dividing T into small time domains $(\Delta T)_n$,

$$\sum_n (\Delta T)_n = T. \quad (6)$$

In each $(\Delta T)_n$, the quantities of the radicals generated, $\Delta[CH_2{}^{\bullet}OH]_n$ and $\Delta[CH_3CH^{\bullet}OH]_n$, are calculated using Eqs. 4 and 5, as follows:

$$\Delta[CH_2{}^{\bullet}OH]_n = d[CH_2{}^{\bullet}OH]/dt \cdot (\Delta T)_n = k_{Me}[MeOH][HO^{\bullet}] \cdot (\Delta T)_n, \quad (7)$$

$$\Delta[CH_3CH^{\bullet}OH]_n = d[CH_3CH^{\bullet}OH]/dt \cdot (\Delta T)_n = k_{Et}[EtOH][HO^{\bullet}] \cdot (\Delta T)_n. \quad (8)$$

Since $[HO^{\bullet}]$ might be time-dependent and is unknown, it is impossible to calculate above quantities. However, the ratio of them is independent on $[HO^{\bullet}]$ and $(\Delta T)_n$, and is always constant, as easily known from

$$\Delta[CH_2{}^{\bullet}OH]_n/\Delta[CH_3CH^{\bullet}OH]_n = (k_{Me}/k_{Et}) \cdot [MeOH]/[EtOH]. \quad (9)$$

Since the quantity of the radical measured at $t = T$ is the sum of the radicals formed in each $(\Delta T)_n$, the ratio of the radicals measured by ESR, $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{obs}$, is

$$\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{obs} = \sum_n \Delta[CH_2{}^{\bullet}OH]_n \Big/ \sum_n \Delta[CH_3CH^{\bullet}OH]_n = (k_{Me}/k_{Et}) \cdot [MeOH]/[EtOH]. \quad (10)$$

The ratio $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{obs}$ equals the ratio of their ESR intensities, and is obtained by the above mentioned method. Since [MeOH]/[EtOH] are the ratio of the initial concentrations, it is possible to calculate k_{Me}/k_{Et}.

Figure 3 shows $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{obs}$ as a function of flow time. The ratio depended slightly on the flow time, which means that disappearance of $CH_2{}^{\bullet}OH$ and $CH_3CH^{\bullet}OH$ was actually not negligible. The flow time should be minimized to obtain an exact value of k_{Me}/k_{Et}. Therefore, we obtained the value $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{t=0}$ by extrapolating the lines in the figure to zero time.

The values $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{t=0}$ were measured while changing the concentration ratio, [MeOH]/[EtOH]. Figure 4 shows the relationship between $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{t=0}$ and [MeOH]/[EtOH]. The slope of this line gives k_{Me}/k_{Et}. It was 0.56, being consistent with that calculated from reference data.[19] The excellent linearity proves the reliability of this method.

Rate Constants of Polyphenols. The reaction rate constants between $HO^{\bullet}$ and the polyphenol molecules are obtained in the same way. Figure 5 shows the ESR spectra of the radicals formed by mixing solution A containing various polyphenols and solution B. In these cases, singlet absorption due to $HO^{\bullet}$ was not observed, which assured that the reaction of polyphenol with $HO^{\bullet}$ proceeded completely. The broad spectra of catechol and gallic acid radicals were analyzed by a spectral simulation using a Bloch equation, as mentioned in our previous paper.[14] Though a few mechanisms of $HO^{\bullet}$ attack were considered, it was concluded that H-atom abstraction from the phenolic hydroxyl group (abbreviated as ϕ-OH) occurred. However, the radical position was not fixed on a ϕ-OH, and it moved among various ϕ-OHs by repeating the attachment and detachment of hydrogen ions to the radical. Such interconversion between several limiting radical structures broadened the ESR signals. In the case of resorcinol, phloroglucinol, and pyrogallol, it is also considerable that $HO^{\bullet}$ abstracts a hydrogen atom from ϕ-OH, and that similar interconversion occurs, as shown in Fig. 6. A spectral simulation using a Bloch equation was, however, not carried out be-

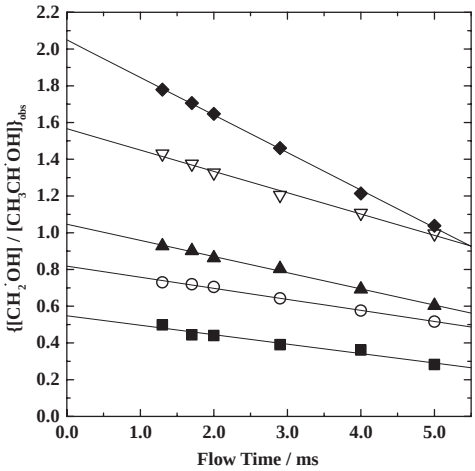

Fig. 3. Relationship between $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{obs}$ and the flow rate. [EtOH] = 2.5×10^{-1} M, [MeOH] = ■; 2.5×10^{-1} M, ○; 3.8×10^{-1} M, ▲; 5.0×10^{-1} M, ▽; 7.5×10^{-1} M, ◆; 1.0 M.

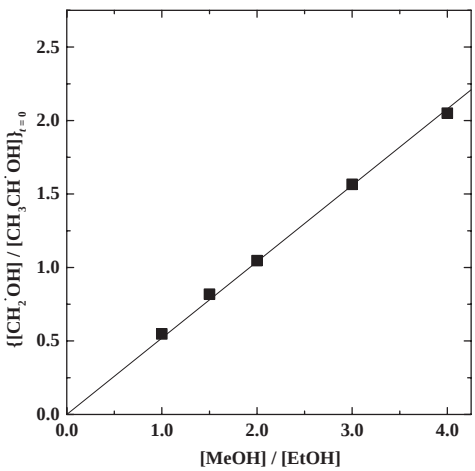

Fig. 4. Relationship between $\{[CH_2{}^{\bullet}OH]/[CH_3CH^{\bullet}OH]\}_{t=0}$ and [MeOH]/[EtOH].

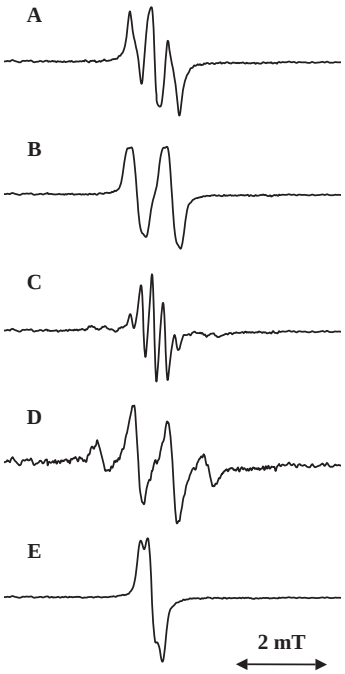

Fig. 5. ESR spectra of polyphenol radicals formed by reactions with HO•. A, catechol (5.0×10^{-2} M); B, pyrogallol (2.5×10^{-2} M); C, resorcinol (1.0×10^{-1} M); D, phloroglucinol (1.0×10^{-1} M); E, gallic acid (5.0×10^{-2} M).

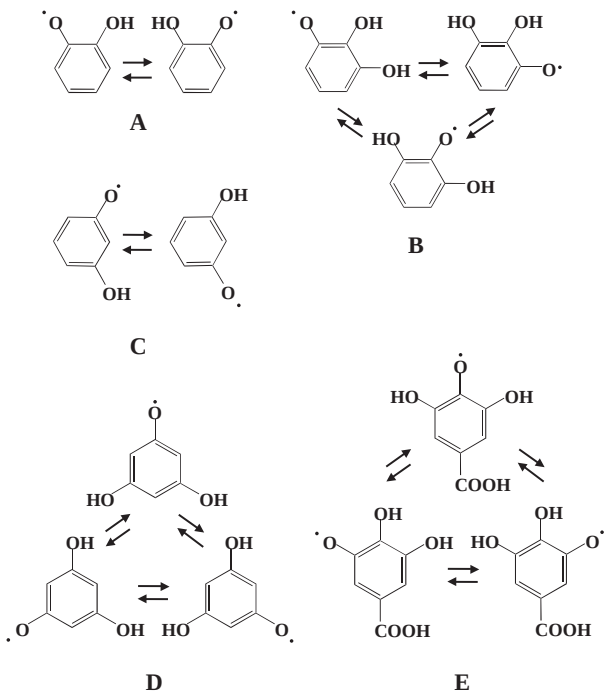

Fig. 6. Structure of polyphenol radicals and interconversion between limiting structures. A, catechol; B, pyrogallol; C, resorcinol; D, phloroglucinol; E, gallic acid.

cause our aim was to obtain the reaction rate constants.

Figure 7 shows the ESR spectra when solution A contained EtOH and each polyphenol. All of the spectra were in the superposition of $CH_3CH^{\bullet}OH$ and the polyphenol radicals (polyphenol•), ascertained by a simulation, where the measured data

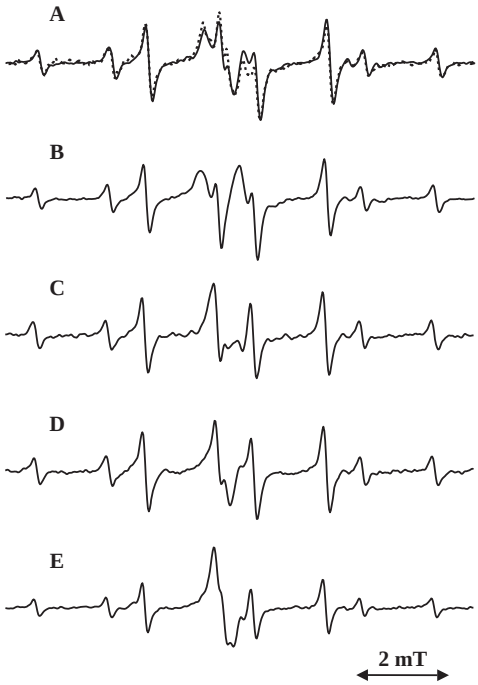

Fig. 7. ESR spectra of polyphenol + EtOH samples. [EtOH] = 2.5×10^{-1} M; A, catechol (5.0×10^{-2} M); B, pyrogallol (2.5×10^{-2} M); C, resorcinol (1.0×10^{-1} M); D, phloroglucinol (1.0×10^{-1} M); E, gallic acid (5.0×10^{-2} M). The dotted line in A is the superposed spectrum of $CH_3CH^{\bullet}OH$ (Fig. 1B) and catechol• (Fig. 5A).

of Fig. 5 were used (results were not shown). The signal intensity ratios of polyphenol• and $CH_3CH^{\bullet}OH$, namely $\{[\text{polyphenol}^{\bullet}]/[CH_3CH^{\bullet}OH]\}_{obs}$, were calculated from the double-integration curves, as in the case of $CH_2^{\bullet}OH$. They were plotted against the flow time, and the $\{[\text{polyphenol}^{\bullet}]/[CH_3CH^{\bullet}OH]\}_{t=0}$ was obtained by extrapolation to zero time. The value of $\{[\text{polyphenol}^{\bullet}]/[CH_3CH^{\bullet}OH]\}_{t=0}$ was plotted as a function of [polyphenol]/[EtOH], as shown in Fig. 8. The ratios of the reaction rate constants of polyphenols with HO• to k_{Et} were calculated from the slopes of the lines, and are shown in Table 1. Pyrogallol and gallic acid possessing three neighboring ϕ-OHs have large values. The value of catechol possessing two neighboring ϕ-OHs is smaller than pyrogallol, but larger than resorcinol and phloroglucinol, in which ϕ-OHs are located in meta-positions. All the polyphenols have larger values than alcohols. This trend is the same as in the case of scavenging the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, though the mutual differences are larger in the case of DPPH.[18]

In this experiment, the reaction of EtOH with HO• was used as a reference. It has been reported that k_{Et} was $1.6–2.2 \times 10^9$ $M^{-1}\,s^{-1}$,[19] and so k_{Me} was calculated to be $0.9–1.2 \times 10^9$ $M^{-1}\,s^{-1}$ using the k_{Et} value and the observed ratio. The reactions having such large rate constants are usually considered to be diffusion-controlled. That is to say, the collision between HO• and the reactive site, namely C–H in alcohols and ϕ-OH in polyphenols, always reaches the dehydrogenation from them without any activation energy. However, if this is the case, it is impossible to explain the results given in Table 1. For example, catechol and resorcinol have the same molecular

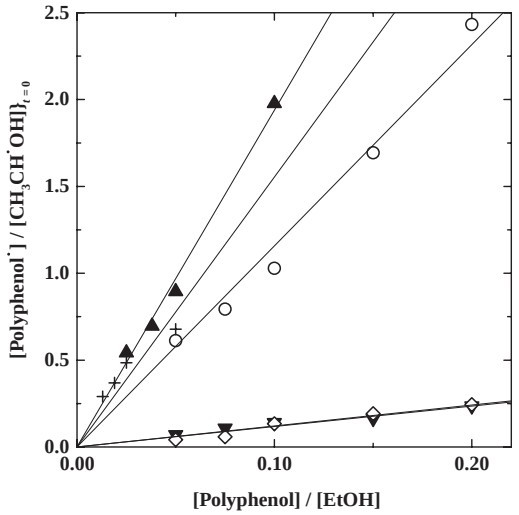

Fig. 8. Relationship between $\{[\text{polyphenol}^\bullet]/[CH_3CH^\bullet OH]\}_{t=0}$ and [polyphenol]/[EtOH]. ○, catechol; ▲, pyrogallol; ▼, resorcinol; ◇, phloroglucinol; +, gallic acid.

Table 1. Ratios of the Reaction Rate Constants, Relative Activation Energies, and Bond Dissociation Energies

Compound	k_x/k_{Et}	$\bar{k}_x/\bar{k}_{Et}$	$\Delta E_x - \Delta E_{Et}$ /kcal mol^{-1}	BDE /kcal mol^{-1}
Pyrogallol	19	38	−2.16	69.3
Gallic acid	17	34	−2.09	72.0
Catechol	11	11	−1.42	74.5
Phloroglucinol	1.5	1.0	0	82.8
Resorcinol	1.2	1.2	−0.11	82.6
Ethanol	1.0	1.0	0	92.0
Methanol	0.56	0.37	0.58	94.1

weights and two reactive sites, namely ϕ-OH, but the k_x values are different by one order of magnitude. This suggests that the dehydrogenation process requires some activation energy.

In the above calculation of the rate constant, we used the concentration of the reactant. However, because all of the alcohols and polyphenols have plural reactive sites, we must divide the rate constant by the number of reactive sites to compare their relative reactivity. In the case of EtOH and MeOH, the constant measured must be divided by 2 and 3, respectively, because they have two and three equivalent C–H bonds, from which dehydrogenation occurs. It is absolutely certain that the numbers are 2, 2, and 3 in the case of catechol, resorcinol, and phloroglucinol, respectively. However, since three ϕ-OHs in pyrogallol and gallic acid molecules are not equivalent, it is not easy to estimate the contribution of each ϕ-OH to the reaction. We assumed that the central ϕ-OH reacts exclusively, based on the BDE shown in Fig. 9, so the contributions from both side ϕ-OHs were neglected. These reaction rate constants, designated as $\bar{k}_x$, are given in Table 1.

Relationship between Relative Activation Energies and BDEs. It is natural to consider that the dehydrogenation of alcohols occurs with minimum activation energy when HO$^\bullet$ approaches from the opposite direction of the C–H bond. In the case of polyphenols, a similar arrangement is also considerable between ϕ-OH and HO$^\bullet$. Then, C–H or O–H bond scission is necessary to transfer hydrogen atoms to HO$^\bullet$, forming water molecules. In these reactions, the hydrogen atoms might combine with the two neighboring atoms in the transition state in the order of C···H···O–H and ϕ-O···H···O–H. These structures reduce the energy of the transition state; as a result, the activation energy becomes smaller than the BDE. Considering the reaction based on the Arrhenius equation, it was postulated that the frequency factors are not so different among the reactants, because the reaction mechanism of each reactive site is almost the same. Therefore, the difference between the rate constants is attributed to the difference of the activation energy. If so, we can obtain the difference of the activation energy of the reactant (ΔE_x) from that of EtOH (ΔE_{Et}) by the following equation:

$$\bar{k}_x/\bar{k}_{Et} = \{A \cdot \exp(-\Delta E_x/RT)\}/\{A \cdot \exp(-\Delta E_{Et}/RT)\} = \exp\{-(\Delta E_x - \Delta E_{Et})/RT\}. \quad (11)$$

Here, ΔE_x and ΔE_{Et} are the activation energies of the reactions of each reactant and EtOH with HO$^\bullet$, respectively. A, R, and T are the frequency factor, the gas constant, and the absolute temperature, respectively. The values $\Delta E_x - \Delta E_{Et}$ were calculated according to Eq. 6 using the observed rate-constant ratios. They are given in Table 1.

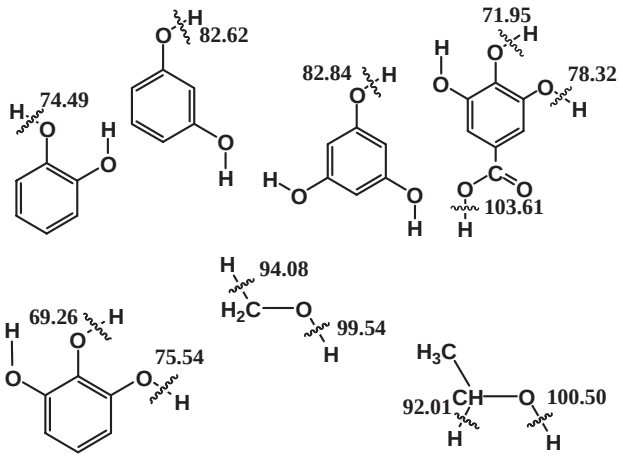

Fig. 9. Bond dissociation energies of polyphenols and alcohols.

The bond-dissociation energies of the main bonds in each reactant were obtained by MO calculations, and are shown in Fig. 9. By comparing the analysis of the ESR spectra and the BDE, it was apparent that dehydrogenation occurred from the weakest C–H or O–H bonds in the molecule, which are ϕ-OH in polyphenols and C–H groups of alcohols, respectively. Since the value of the O–H bond in the product a water molecule is 119 kcal/mol,[20] which is much larger than either of the C–H or O–H bonds from which dehydrogenation occurs, all of the reactions mentioned above are highly exothermic and proceed easily. The reaction energy, ΔH, is $BDE_x - 119$; BDE_x means the BDE of the weakest bond. Comparing $\Delta E_x - \Delta E_{Et}$ with $BDE_x - BDE_{Et}$, it is clear that the activation energies become smaller as BDE decreases. This coincides with Hammond's postulate,[21] and it is expected that the Evans–Polanyi equation[22] holds. This equation implies a linear relationship between the activation energies and the heat of the re-

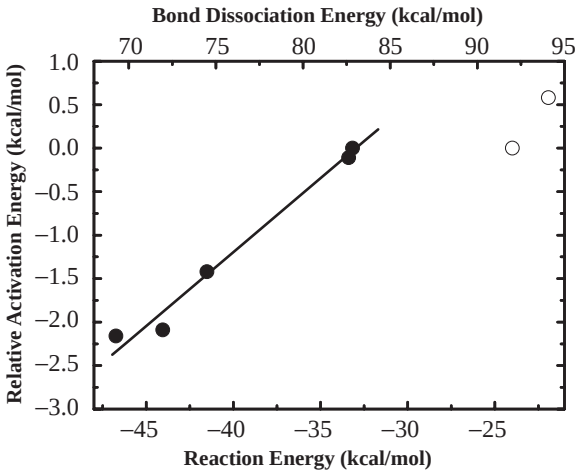

Fig. 10. Relationship between relative activation energy, heat of reaction (or bond dissociation energy). ●, polyphenol; ○, alcohol.

action among the same series of reactions. Figure 10 shows the relationship between $E_x - \Delta E_{Et}$ and $BDE_x - BDE_{Et}$. Linearity between $\Delta E_x - \Delta E_{Et}$ and $BDE_x - BDE_{Et}$ must be observed to hold the Evans–Polanyi equation. It is clear that the values of polyphenol are on a straight line, but the values of the alcohols are apart from the extended line; they look as if they are on another line lying downward, though the line was drawn tentatively using only two points. This result means that the activation energies of alcohols are lower than those expected from extrapolation of the values of the polyphenols. The term "polar effect" captures this lowering effect.[22] That is to say, if the transition state has a polar structure, it has stabilized and, as a result, the activation energy is lowered. Comparing the structures of the transition states, C···H···O of alcohols are more polar than O···H···O of polyphenols. Therefore, the transition states of alcohols are more stabilized than polyphenols, and alcohols have lower activation energies than those expected from the Evans–Polanyi plot of polyphenols.

Conclusion

These results confirm that this method for measuring the rate constants between polyphenols or alcohols and $HO^{\bullet}$ is useful for examining radical-scavenging or their antioxidizing activity, which was ascertained by a MO calculation.

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