Stopped-Flow and Spectrophotometric Study on Radical Scavenging by Tea Catechins and the Model Compounds

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Radical scavenging of four tea catechins, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg) and (−)-eppigallocatechin gallate (EGCg), and the model compounds of their partial structure was examined against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical using stopped-flow and spectrophotometric methods. The number of DPPH radicals scavenged by a polyphenol molecule was larger than that of phenolic hydroxyl groups, suggesting that hydrogens which bond directly to the aromatic ring can also participate in radical scavenging. A model for the scavenging reaction was proposed in which the reaction proceeded with successive dehydrogenation from a polyphenol molecule. Analysis of the second order reaction rate constants and the activation parameters between DPPH and polyphenol at the early stage of the reaction showed that the values depended on the number of phenolic hydroxyl groups and their mutual position. Contribution of the A ring of catechins to the rate constants was estimated to be far smaller than that from the B ring. In the EGCg molecule, the B ring and the gallate group were not independent, but acted as a single group for DPPH radical scavenging.

Key words tea catechin; radical scavenging; stopped-flow method; 1,1-diphenyl-2- picrylhydrazyl

Tea catechins have recently been drawing attention because of their various pharmacological effects.[1—10] Especially interesting among them is an anti-carcinogenic effect. This effect is considered to be derived from the scavenging action of tea catechins on reactive oxygen species. We have also shown that tea percolates and the main constituent, epigallocatechin gallate, show a protecting effect against radiation-induced DNA scission, and the effect is derived from the scavenging of hydroxyl (OH) radicals produced from the surrounding water by radiation.[11—13] That is to say, tea catechins act as antioxidants like other polyphenols. Actually, many investigators have reported on the scavenging ability by using various physico-chemical methods. For example, Nanjo *et al.*[14] and Okuda *et al.*[15,16] measured the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability of many polyphenols, including tea catechins, by using ESR spectroscopy. They measured the concentration of polyphenols at which half the DPPH radicals were quenched and compared of their scavenging ability. On the other hand, Jovanovic *et al.*[17] measured the reaction rate of tea catechins with reactive radicals such as azidyl ($N_3\cdot$) or thiocyanate ($(SCN)_2^-\cdot$) by using pulse radiolysis and laser photolysis, and compared the ability of polyphenols as antioxidants from the reaction rate constants.

We think, however, that these results have not shown the overall mechanism of the radical scavenging reaction and emphasize that the ability of polyphenols to act as radical scavengers should be discussed from two points. One of them is the reaction rate with radicals, as usually seen in physico-chemical papers, and the other is the quantity of the radicals quenched by the antioxidant. The latter indicates how many radicals are scavenged by polyphenol molecule. A characteristic of polyphenols is that they have many phenolic hydroxyl groups and, as a result, have many active points in radical scavenging. This means that one polyphenol molecule can likely scavenge plural numbers of radicals. Therefore, it is necessary to discuss the antioxidant ability of tea catechins from the point of the quantity of scavenged reactive oxygen species, as well as from the reaction rate with them.

In this experiment, we used DPPH as a reactive free radical and measured spectrophotometrically the number of DPPH molecules scavenged by catechin molecule and the model compound. The reaction rate between them was measured by the stopped-flow method. These results were discussed from structural and kinetic points of view.

Experimental

Materials Four tea catechins, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg) and (−)-epigallocatechin gallate (EGCg), were used. These are the main constituents of green tea percolates, and the molecular structures are shown in Fig. 1. EC, EGC and ECg were purchased from Kurita Kogyo Co., Ltd., and EGCg was supplied from Mitsui Norin, Inc. They were separated from tea (*Camellia sinensis* L. O. KUNZE) and purified by HPLC, the purities of each being better than 98%.

These catechin molecules have the same flavonoid skeleton, and two or three resonant systems. These resonant systems were named A, B rings and a gallate group, as shown in Fig. 2. The A ring has two OH groups located at the *meta* position, so resorcinol can be a model compound for the A ring. However, phloroglucinol may be a more appropriate model, supposing that an oxygen atom in the central ring attaching to the A ring acts like an OH group on the electronic structure of the A ring. The B ring has two adjacent OH groups in EC and ECg, and three adjacent OH groups in EGC and EGCg molecules. Therefore, catechol and pyrogallol seem to be good models for them. The gallate group has a ester group in addition to three adjacent OH groups, so gallic acid and methyl gallate were chosen for the model. These model compounds, DPPH and solvents were obtained from Wako Pure Chemicals Co., and were guaranteed grade. As DPPH has low solubility in water, the mixture of ethanol and water (volume ratio, 2 : 1) was used as a solvent throughout all the experiments. The concentration of DPPH was fixed at 2×10^{-4} M. The same volume of DPPH solution and the solutions containing various concentrations of antioxidant were mixed in all cases.

Methods In order to examine the number of DPPH radicals scavenged by an antioxidant molecule, a spectrophotometric method was applied. 1.5 ml of DPPH solution was first put into the UV cell, the temperature was kept at 25±0.1 °C, then the same volume of solution containing one-tenth or less amount of antioxidant was added and mixed vigorously for several seconds using a stirrer. Then, the change of absorbance at 523 nm was recorded for an hour using a Hitachi U-3410 spectrometer. The number (n) of DPPH radicals scavenged by an antioxidant molecule was calculated by the following equation.

$$n=(\Delta A/A)\cdot[\mathrm{DPPH}]/[\mathrm{AO}] \quad (1)$$

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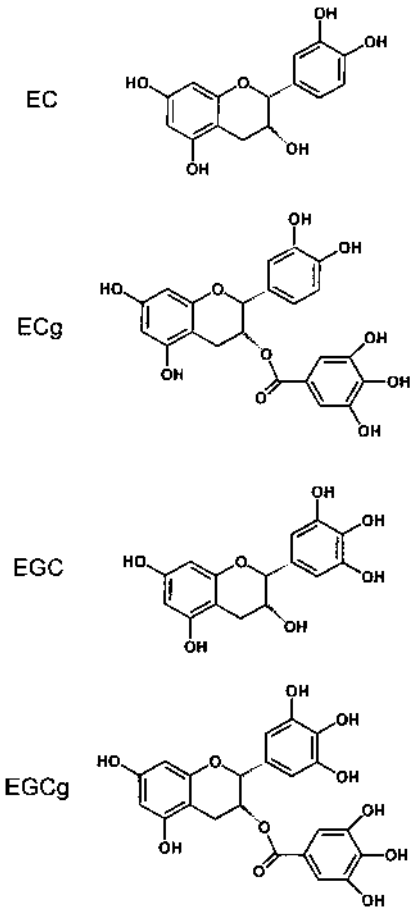

Fig. 1. Structures of Tea Catechins

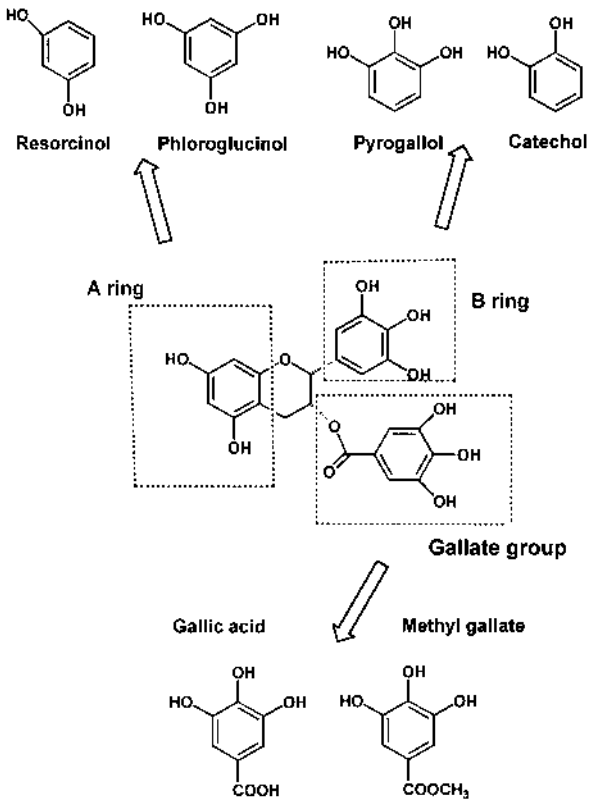

Fig. 2. Molecular Structures of a Catechin (EGCg) and the Model Compounds

Here, A is the absorbance at 523 nm of the solution without the antioxidant and ΔA is the decrease of the absorbance after adding the antioxidant. [DPPH] and [AO] are the concentrations of DPPH and the antioxidant, respectively.

For the purpose of obtaining second order reaction rate constants between DPPH and the antioxidant, a Model RA-401 UV stopped-flow spectrophotometer (Otsuka Denshi, Inc., Japan) was used, and a rapid change in absorbance was followed. In this experiment, both solutions were placed in a reservoir in which the temperature was maintained at the required value within ± 0.1 °C, and equal volumes of both solutions were blown into the mixing room instantaneously using high pressure N_2 gas, then introduced into a UV flow cell, and the change in absorbance at 523 nm followed for a period of less than 1 s.

Results and Discussion

Reactive oxygen species or free radicals of biological interest contain OH, alkyl (R·), alkoxy (RO·), alkylperoxy (ROO·), and superoxide anion (O_2^-·) radicals, *etc*. These radicals attack biomolecules and induce damage and disease to living organisms. Therefore, radical scavengers, a kind of antioxidant, play an important role in the living body; many natural substances which show antioxidant activity have been found and their radical-scavenging ability has been compared. For experiments to study this activity, it is necessary to construct a system for generating radicals. For example, the Fenton reaction and xanthine–xanthineoxidase system were used for the generation of OH and O_2^-·, and the radicals were spin-trapped in the presence of antioxidants.[18,19] The antioxidant competes with a spin-trapping agent for the consumption of the radicals, depending on the scavenging ability, so it was possible to estimate this ability from the degree of decrease of the spin adduct. However, it is difficult to control exactly the quantity of radicals generated , because the generating systems are affected by co-existing antioxidants, and estimation of the degree of radical scavenging by the oxidants becomes unreliable. DPPH is obtained as a stable solid, so it is easy to control the quantity of radicals.

As the reactivity of OH with polyphenols and other antioxidants is too large, the reaction rate is diffusion-controlled. Therefore, it becomes difficult to compare the reactivity of antioxidants from a structural point of view. In contrast, the reactivity of DPPH is far lower than OH, though it still has enough reactivity with polyphenols, so the rate is not diffusion-controlled but is ruled by the electronic structure of the antioxidants. From these points of view, DPPH seems a good substance for measuring the ability of antioxidants.

Figure 3 shows the molecular structure of DPPH. This is a kind of nitrogen-centered radical, but the reactivity is not so large as an oxygen-centered radical such as RO· and ROO· because of the widely-spreading resonant system. When this radical reacts with polyphenols, dehydrogenation occurs on polyphenol molecules and DPPH changes into DPPHn, the structure of which is shown in Fig. 3. DPPH is a deeply colored substance, but DPPHn is colorless; therefore, it is possible to estimate spectrophotometrically the changing ratio of DPPH into DPPHn. Figure 4 shows the spectral change of DPPH solution after mixing it with a solution of phloroglucinol. The peak at 523 nm is assigned to DPPH, and the peak intensity decreased with time, showing the progress of the hydrogenation, namely scavenging, with phloroglucinol. From this result, the absorbance at 523 nm becomes a useful measure of scavenging.

Figure 5 shows the change in the absorbance at 523 nm

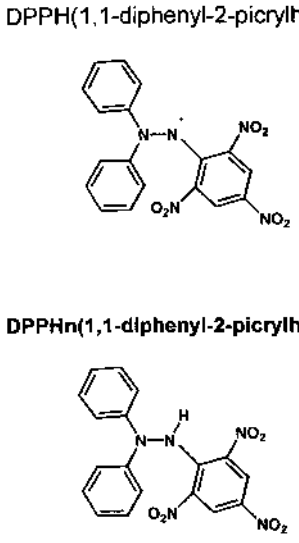

Fig. 3. Structures of DPPH and DPPHn

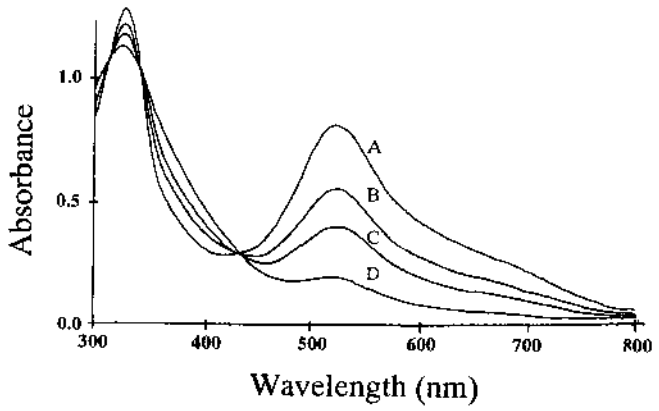

Fig. 4. Spectra of a DPPH Solution (1×10^{-4} M), 1 min (A), 6 min (B), 15 min (C) and 60 min (D) after Mixing with Phloroglucinol Solution (1×10^{-4} M)

for an hour after mixing with various polyphenols. Many of them showed a rapid decrease just after the mixing, then a slow decrease. This complex change demonstrates that plural stages, proceeding at different rates, are included in this process. Though the change in the absorbance did not finish for an hour, it became relatively mild at one hour. As the change in the absorbance of the DPPH solution without any antioxidants was very small for this period, no collection was performed on the data in Fig. 5. These changes are definitely induced by the reduction with polyphenols, therefore, it is possible from the decrease of the absorbance at one hour to calculate the number of DPPH radicals scavenged by one polyphenol molecule. These values are listed in Table 1, in addition to the number of phenolic groups in the molecule. The value of EGCg is close to that reported in ref. 14, though the value was discussed from another viewpoint. In all cases except for resorcinol, the numbers of scavenged DPPH were larger than that of the phenolic groups. This suggests that hydrogen atoms not existing as phenolic groups also work for scavenging.

We propose here a scheme for the progression of successive dehydrogenation from a polyphenol molecule using three adjacent OH-containing aromatic rings like EGC, ECg, EGCg, *etc*. in Fig. 6. The initial dehydrogenation may occur on the central OH group. If this is the case, prototropy from an adjacent OH group is easy, so the exact position of the radical might be not definitive. This semiquinone type radical must be more reactive than the original polyphenol molecule, so the process (2) proceeds rapidly, then the resulting biradical (III) changes into a quinone (IV). In this molecule, the remaining phenolic OH group is also activated by the presence of two carbonyl groups, and as a result, dehydrogenation occurs and a semiquinone (V) is formed. In this radical (V), it seems that the hydrogen atom which bonded directly to the aromatic ring is able to move on the oxygen atom possessing an unpaired electron, forming a carbon-centered radical (VI). Compounds V and VI are reversibly changeable, but VI is changed into VII by dimerization. It is known that teaflavin, red pigment in black tea, is formed by the dimerization of two catechin molecules, in which the hydrogen atom on the aromatic ring is removed and a condensed ring is formed.[20] Similarly, the amino acid tyrosine dimerizes to bityrosine with the direct coupling of two aromatic rings under an oxidative condition.[21] These results support that the process (5) really occurs. Processes (5) and (6) newly form phenolic OH groups, and contribute to further dehydrogenation, as shown in process (7). In such a way, all the hydrogen atoms are able to participate in radical scavenging. In the actual scavenging reactions, these processes shown in Fig. 6 might proceed in parallel and the degree of progression would depend on the ratio of the quantity of the radical and the polyphenol.

The reaction rates between DPPH and polyphenols were measured by using a stopped-flow method. As stated above, many processes possibly proceed simultaneously, so it be-

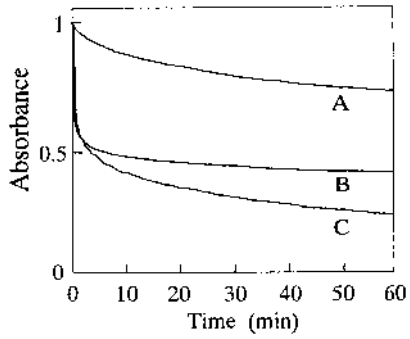

Fig. 5. The Change in Absorbance at 523 nm of a DPPH Solution after Mixing with Phloroglucinol (A), Methyl Gallate (B) and EGCg Solutions (C) (See text)

Table 1. The Numbers of Phenolic OH Groups in an Antioxidant Molecule and the Scavenged DPPH Molecules

Antioxidant	Phenolic OH	Scavenged DPPH
Resorcinol	2	1.6
Phloroglucinol	3	4.3
Catechol	2	4.0
Pyrogallol	3	6.2
Methyl gallate	3	7.9
Gallic acid	3	6.7
EGCg	8	14.5

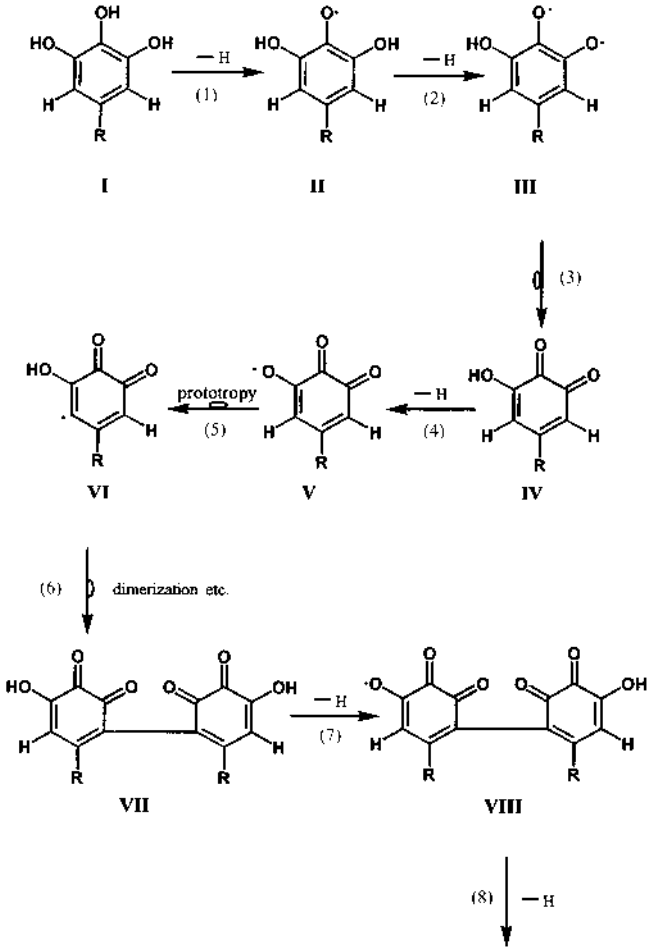

Fig. 6. A Model of Successive Dehydrogenation from a Polyphenol Molecule Proposed for the DPPH Radical Scavenging

comes difficult to analyze the rates. We therefore adopted a special condition in which the concentration of polyphenols is ten times larger than that of DPPH, in contrast to the case of the foregoing experiment. In this case, DPPH might be consumed rapidly in the early stages of the successive reactions shown in Fig. 6. We think only processes (1) and possibly (2) contribute to this scavenging of DPPH. The decrease in DPPH will proceed as a second-order reaction, as follows.

$$-d[\mathrm{DPPH}]/dt = k_2[\mathrm{AO}][\mathrm{DPPH}] \fallingdotseq k_2[\mathrm{AO}]_0[\mathrm{DPPH}] \quad (2)$$

Here, k_2 is the second-order reaction rate constant, and [AO] and [DPPH] are the concentrations of polyphenol and DPPH, respectively. $[\mathrm{AO}]_0$ is the initial concentration of the polyphenol. As the condition $[\mathrm{AO}] \gg [\mathrm{DPPH}]$ is applied, an approximation, $[\mathrm{AO}] \fallingdotseq [\mathrm{AO}]_0$, is possible; therefore, DPPH decreases as a pseudo-first-order reaction.

Figure 7 shows an example of the relationship between the logarithm of absorbance at 523 nm and time. An almost linear change was observed, suggesting that the above approximation holds with sufficient accuracy. However, a small deviation from the line was observed at a longer time region in each case. This was thought to be negligible and did not affect the following discussion. Therefore, the rate constants were calculated from the slopes of the tangential lines of

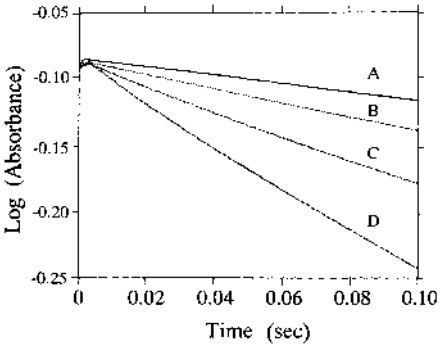

Fig. 7. The Change in Absorbance of DPPH Solution Measured by the Stopped-Flow Spectrometer after Mixing with a Catechol Solution Measured at 10 (A), 20 (B), 30 (C) and 40 °C (D)

Table 2. Second-Order Rate Constants between DPPH and Antioxidants

Antioxidant	$k_2(\times 10^3 \cdot \mathrm{s}^{-1} \cdot \mathrm{M}^{-1})$			
	10 °C	20 °C	30 °C	40 °C
Resorcinol	0.0015	0.0021	0.0046	0.0083
Phloroglucinol	0.0046	0.0081	0.016	0.031
Catechol	0.56	1.1	2.1	3.6
Pyrogallol	2.5	4.0	6.4	8.4
Methyl gallate	5.0	9.1	15	27
(Gallic acid)	0.15	0.38	0.73	1.4
EC	0.87	1.9	4.1	8.9
EGC	2.7	4.9	7.8	13
ECg	3.1	5.5	9.4	17
EGCg	3.3	5.6	9.2	15

these curves and $[\mathrm{AO}]_0$, and are listed in Table 2. The values of catechins are in the same order as those between tocopherols and the phenoxyl radical measured similarly by the stopped-flow method.[22] The rate constants of resorcinol and phloroglucinol, model compounds of the A ring, are very small . This suggests that the two phenolic OH groups positioned at the meta position have low reactivity; nevertheless, they have scavenging ability. Between them, the value of phloroglucinol is larger than that of resorcinol. This is the same tendency observed in the number of scavenged DPPH molecules, where resorcinol is the only substance: the number of scavenged DPPH is less than that of the phenolic OH group. Catechol has a far larger rate constant than resorcinol, yet they have same number of OH groups. This is caused by a difference in the positioning of two OH groups. That is to say, two adjacent OH groups at the ortho position interact and increase the rate. Pyrogallol has three adjacent OH groups, and the rate constant is larger than that of catechol. The value of methyl gallate, which similarly has three adjacent OH groups is further larger than that of pyrogallol, showing that the presence of an ester group serves to increase the rate. However, it is strange that the value of gallic acid becomes extremely small. The reason for this difference between methyl gallate and gallic acid is not clear, but it is conceivable that gallic acid molecules are associated by a hydrogen bond between the OH group and the carboxylic group and, as a result, dehydrogenation from the OH group is hindered. Accordingly, it is not appropriate to use gallic acid as a model of the gallate group of tea catechins.

Next, four tea catechins were compared. The value of EC

Table 3. Activation Parameters of the Reaction between DPPH and Antioxidants

Antioxidant	Activation entropy ($kJ \cdot mol^{-1} \cdot K^{-1}$)	Activation enthalpy ($kJ \cdot mol^{-1}$)
Catechol	−0.031	45.8
Pyrogallol	−0.072	30.5
Methyl gallate	−0.028	41.1
EC	−0.012	57.0
EGC	−0.043	38.4
ECg	−0.031	41.7
EGCg	−0.048	36.8

is smallest among them. If it is possible to assume that the A and B rings can independently act on the scavenging, the rate of the B ring might be far larger than that of the A ring, as estimated from the values of their model compounds, phloroglucinol and catechol or pyrogallol. This means that the contribution of the A ring to the rate constant is negligible under such condition where the concentration of polyphenol is far larger than that of DPPH, though it would work for scavenging under the reverse condition. The value of EC is similar to that of catechol, suggesting that the above estimation, the A ring being negligible, is correct. Javonovic *et al*. showed by using the pulse radiolysis method that the azidyl radical ($N_3\cdot$) reacts comparably with both A and B rings, in contrast to our case.[17] This difference seems to be derived from the difference in reactivity of $N_3\cdot$ and DPPH. As $N_3\cdot$ has the higher reactivity than that of DPPH, the reaction with the A ring became observable. This is consistent with the fact that the OH radical, the most reactive oxygen species, reacts with almost everything, independent of their reactivity.

Rate constants were compared among those catechins possessing three adjacent OH groups. They were a few times larger than that of EC, as the case between catechol and pyrogallol or methyl gallate, showing again that the presence of three adjacent OH groups is more reactive than two adjacent OH groups. The value of ECg is somewhat larger than that of EGC, so it appears that the gallate group has slightly higher reactivity than the B ring. This trend is also observable between the model compounds, that is to say, the value of methyl gallate is larger than that of pyrogallol. However, EGCg has both groups, so the radical scavenging behavior is interesting in this case. The constant of EGCg was close to the value of ECg, but a slight difference was measured. If the B ring and the gallate group of EGCg act independently on DPPH scavenging, the rate constant must become the sum of them. Therefore, the above result suggests that these two groups are not independent. In other words, when one group reacts with DPPH, the reaction of the other group at the same time is hindered. Therefore, it is likely that the B ring and gallate group form a new group, maybe through a weak interaction.

The activation parameters were obtained from k_2 values measured at different temperatures. Activation enthalpy, $\Delta H^{\ddagger}$, was calculated conventionally from the slopes of the curves obtained by plotting the logarithm of k_2 to the reciprocal of absolute temperature. The linearity was sufficient for obtaining reliable $\Delta H^{\ddagger}$ values. Then, activation entropy, $\Delta S^{\ddagger}$, was calculated using k_2 values at 30 °C. These values are listed in Table 3. EC and catechol have larger $\Delta H^{\ddagger}$ values than those possessing three adjacent OH groups, as expected from the comparison of k_2 values. Comparing the B ring and the gallate group of catechins or their model compounds, it is clear that the gallate group's $\Delta H^{\ddagger}$ value is larger than that of the B ring; namely, 41.7 (ECg)>38.4 (EGC), 41.1 (methyl gallate)>30.5 (pyrogallol). This result means that the k_2 values of the gallate group being larger than those of the B ring is caused by the larger $\Delta S^{\ddagger}$ values. However, it is difficult to discuss $\Delta S^{\ddagger}$ because the structure of the activated complex between DPPH and polyphenol is not defined at present. A difference was found between the $\Delta H^{\ddagger}$ values of the B ring and the model compounds; two adjacent OH groups, 57.0 (EC)>45.8 (catechol), three adjacent OH groups, 38.4 (EGC)>30.5 (pyrogallol). Catechins showed larger values than their model compounds in both cases. This difference seems to be derived from the effect of a substituting group on the electronic structure of the aromatic ring. In contrast, gallate groups did not show this difference: 41.7 (ECg)≒41.1 (methyl gallate). In this case, the effect of the substituting group is small, because it bonds to the end of an ester group far from the aromatic ring.

In the case of EGCg possessing B and gallate group, $\Delta H^{\ddagger}$ value was smaller than those of the B ring (EGC) and the gallate group (ECg). This is consistent with the speculation from the comparison of k_2 that the two groups are not independent. That is to say, the $\Delta H^{\ddagger}$ value is lowered through the interaction between them.

From these results and discussion, it was revealed that radical scavenging with polyphenols is complicated and must be considered mainly from two points: the quantity of radicals scavenged and the reaction rate. In this paper, only the reaction in the early stage of radical scavenging was discussed. However, many processes of dehydrogenation proceed in the actual reaction. Therefore, it is necessary to treat all processes statistically. When the radical scavenging *in vivo* is a problem, another viewpoint, for example the location of the antioxidant and the position of the radical generated, will be needed.

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