Antioxidant Potential of Gallocatechins. A Pulse Radiolysis and Laser Photolysis Study

Slobodan V. Jovanovic,*,† Yukihiko Hara,‡ Steen Steenken,§ and Michael G. Simic||

Contribution from the Department of Chemistry, University of Ottawa, 10 Marie Curie, Ottawa, Ontario K1N 6N5, Canada, Food Research Laboratory, Mitsui Norin, 2-1-17 Fujieta, Fujieta, Shizuoka Pref., 426 Japan, Max-Planck-Institut für Strahlenchemie, 34-36 Stiftstrasse, D-45470 Mülheim a.d. Ruhr, Germany, and Techlogic Inc., 9404 Bac Place, Gaithersburg, Maryland 20877

Received May 8, 1995[⊗]

Abstract: Gallocatechins and catechins, which are constituents of green tea, and related, simpler single-ring model compounds undergo one-electron oxidation by the azidyl radical ($k = (1.4-4.8) \times 10^9$ M^{-1} s^{-1}), which was used as a model one-electron, rapid oxidant. The initial oxidation leads to the formation of a mixture of A- and B- (or C-) ring phenoxyl radicals. This finding was confirmed by comparison with the spectra of 3,5-dihydroxyanisole (the model for A ring) and methyl gallate (the model for B or C ring) radicals and by photoionization experiments in which only the B-ring radical of epigallocatechin was generated, as expected from its lower ionization potential. The A-ring phenoxyl radical is converted to the B- (or C-) ring phenoxyl radical by inter- and intramolecular electron and proton transfer. The activation parameters clearly indicate solvent-assisted *inter*molecular electron and proton transfer, whereas *intra*molecular transfer in epigallocatechin gallate radicals is suggested to proceed through an intermediate molecular complex formation. Acid–base equilibria of parent gallocatechins ($pK_{a1} > 8.0$) are significantly altered in the corresponding phenoxyl radicals ($pK_{r1} = 4.4-5.5$). The low reduction potentials of gallocatechin radicals, $E_7 = 0.42$ V (which is lower than that of vitamin E radicals, $E_7 = 0.48$ V), are responsible for their antioxidant efficacy, which may include the repair of vitamin E radicals. These low reduction potentials also imply high susceptibility of parent gallocatechins to rapid oxidation in aerated aqueous media. The reactivity of epigallocatechin gallate with superoxide radical at pH 7, $k = 7.3 \times 10^5$ M^{-1} s^{-1}, is one of the highest measured rates of *reduction* of superoxide radical by any chemical antioxidant. In this reaction, superoxide is converted to hydrogen peroxide, thus eliminating the redox cycling that may be involved in the corresponding *oxidation* reaction. The high rates of quenching of singlet oxygen by gallocatechins in acetonitrile, $k = (1.1-2.2) \times 10^8$ M^{-1} s^{-1}, are comparable to quenching by vitamin E, $k = 5 \times 10^8$ M^{-1} s^{-1}.

Introduction

Physiological antioxidant protection is invoked as one of the major defense mechanisms in fighting free radical induced, mediated, and promoted disorders. Furthermore, aging and diseases such as autoimmune disorders, cancer, arthritis, and cardiovascular diseases have a free radical component.[1-5] The proper function of the immune system appears to depend on the level of dietary antioxidants.[3,6-8] While mildly beneficial in the treatment of various pathological conditions, the most significant role of antioxidant protection is believed to be its role in disease prevention. With increasing environmental challenges, efficient prevention of damage requires maintenance of the highest possible levels of chemical defense agents. It is a widespread misconception that antioxidant vitamins and provitamins (α-tocopherol, ascorbic acid, and β-carotene) are the only protective agents against free radical damage. Failure to observe beneficial effects of antioxidant vitamin supplementation in severely challenged individuals[9] has resulted in underestimation of the significance of antioxidants. Antioxidant vitamins represent only the most studied component of the overall antioxidant defense system, which includes all available antioxidants, ranging from various nutrients, such as polyphenols,[10-12] to metabolites, such as uric acid[1,13] and 5-hydroxytryptophan.[14,15] The importance of an individual antioxidant in averting a particular pathological condition would depend on its availability at a site of oxidative challenge and its reactivity with damaging free radical oxidants.

The antioxidant role of gallocatechin and catechin derivatives has been neglected, particularly from a mechanistic point of view. Historically, gallocatechins were first used and studied in vegetable tanning. The original process, in which animal skin was treated with an infusion of oak bark for a period of 3–6 months, has been modified over the years to incorporate

* To whom the correspondence should be addressed.
† University of Ottawa.
‡ Mitsui Norin.
§ Max-Planck-Institut für Strahlenchemie.
|| Techlogic Inc.
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

(1) Ames, B. N.; Cathcart, G. R.; Schwiers, E.; Hochstein, P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6858.
(2) Ames, B. N. *Science* **1983**, *221*, 1256.
(3) *Antioxidant Nutrients and Immune Functions*; Bendisch, A., Phillips, M., Tangerdy, R. P., Eds.; Plenum Press: New York, 1990; Vol. 262.
(4) *Oxidative Damage and Repair*; Davies, K. J. A., Ed.; Pergamon Press: New York, 1991.
(5) Rice-Evans, C.; Diplock, A. T. *Free Radical Biol. Med.* **1993**, *15*, 77–96.
(6) Greenspan, H. C. *Med. Hypotheses* **1993**, *40*, 85.
(7) Sandstrom, P. A.; Tebbey, P. W.; Van Cleave, S.; Buttke, T. M. *J. Biol. Chem.* **1994**, *269*, 798.
(8) Screck, R. S.; Rieber, P.; Baenerle, P. A. *EMBO J.* **1991**, *10*, 2247.
(9) Heinonen, O. P.; Albanes, D. N. *N. Engl. J. Med.* **1994**, *330*, 1029.
(10) Jovanovic, S. V.; Steenken, S.; Tosic, M.; Marjanovic, B.; Simic, M. G. *J. Am. Chem. Soc.* **1994**, *116*, 4846.
(11) Larson, R. P. *Phytochemistry* **1988**, *278*, 969.
(12) Six, P. *Inform* **1994**, *5*, 679.
(13) Simic, M. G.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1989**, *111*, 5778.
(14) Simic, M. G.; Al-Sheikhly, M.; Jovanovic, S. V. *Nutritional Aspects of Food Processing*; Bibl. Nutr. Dieta: Basel, 1989; pp 288.
(15) Jovanovic, S. V.; Steenken, S.; Simic, M. G. *J. Phys. Chem.* **1990**, *94*, 3583.

0002-7863/95/1517-9881$09.00/0

drum processing and vegetable tannins from other sources.[16] The underlying principle of *tanning* is the capability of vegetable tannins to precipitate proteins by forming insoluble molecular complexes. It was subsequently discovered that tannins, which are polyphenols, readily form molecular complexes with a variety of biomacromolecules, such as alkaloids and polysaccharides. This property of vegetable tannins, which is a cause of the astringency of polyphenol-rich foods, is believed to be responsible for the reported beneficial effects of medicinal teas and other beverages containing plant extracts. Although the benefits of various herbal extracts in treating pathological conditions are widely recognized and utilized,[16-22] the mechanism of action of plant polyphenols is still elusive.

Gallocatechins are secondary metabolites of higher plants[16] because they do not appear to be necessary for growth or maintenance of the plants that produce them. Because of their astringency, gallates serve to deter insects and other organisms harmful to the plant. In addition, they also have antibacterial and antiviral properties.[16,21] The supplementation of chicken with tea polyphenols[23] appears beneficial for the caecal flora. Recent study[24] shows that both epigallocatechin gallate and theaflavin digallate inhibited significantly the infectivity of influenza virus *in vitro*. Given the concentration of gallocatechins in edible plants and various plant products, it is important to understand their action in the gastrointestinal tract.

In this study we investigated the free radical chemistry of gallocatechins from green tea and of appropriate model compounds for various ring structures in complex polyphenols. Reduction potentials and spectral and acid−base properties of gallocatechin and catechin phenoxyl radicals were investigated because these radicals mediate their oxyl radical scavenging action. The antioxidant activity of the major green tea gallocatechins was also fully established with superoxide radical and singlet oxygen.

Materials and Methods

(−)-Epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epigallocatechin gallate were produced from green tea by Mitsui Norin, Inc., as detailed elsewhere.[17,20] Methyl gallate, 2-chloroethanol, Trolox C, rutin, and gallic acid (Aldrich), 3,5-dihydroxyanisole (Fluka), 4-methoxyphenol, sodium azide, phosphate and borate buffers, NaOH, $HClO_4$, and potassium thiocyanate (Merck), ferrocene monocarboxylate (Strem), phenazine (Eastman), and acetonitrile omnisolv-grade (BDH) were used as received. Water was purified through a Millipore Milli-Q system. All solutions were prepared freshly before each experiment. High-purity (>99.99%) N_2O, N_2O:O_2 (4:1), O_2, and N_2 were purged through solutions either to enable conversion of e_{aq}^- to the hydroxyl radical,[25] to generate singlet oxygen and superoxide radical, or to exclude oxygen to prevent its interference with radical reactions.

The 3 MeV Van-de-Graaff pulse radiolysis equipment with optical detection at the Max-Planck-Institute für Strahlenchemie[26] was used for the pulse radiolysis studies. A 2 cm Suprasil quartz cell with temperature variation through a thermostatically controlled liquid jacket was used for sample irradiation. The spectra of the radicals and the dissociation constants were measured at 5−10 Gy/pulse, whereas the rate constants were determined at considerably lower 1−2 Gy/pulse to minimize interference from radical−radical reactions. Thiocyanate dosimetry was used in dose determinations, assuming $G[(SCN)_2^{\bullet-}] = 6.0$ in N_2O-saturated 10 mM KSCN aqueous solutions.

Lumonics EX-510 excimer laser operated with $Kr/F_2/He$ (~5 ns, 100 mJ/ pulse, 248 nm) at the University of Ottawa[27,28] was used for photoionization experiments. The sample was flown through a 7 × 7 mm Suprasil quartz cell from a container (also used to saturate solution with the appropriate gas), to avoid depletion of absorbing species. The signal from a photomultiplier was fed into a Tektronix digital oscilloscope interfaced to a Macintosh data station, where a Labview program was used for data acquisition and processing.

Singlet oxygen quenching was monitored with a 1270 nm photodiode. Singlet oxygen was generated by energy transfer from excited triplet state of phenazine (excited at 355 nm by a ~15 mJ pulse from a Surelite Nd-YAG laser, $\phi(^1O_2) = 0.83$[29]). In the absence of any reactive solutes, $\tau(^1O_2) = 80\ \mu s$. Addition of appropriate concentrations of gallocatechins lead to the shorter lifetimes of $^1O_2$, due to the quenching. From the slope of the quenching plot, the second-order rate constant of the reaction of $^1O_2$ with a polyphenol derivative is obtained.

Superoxide radical was generated in N_2O:O_2 (4:1)-saturated 0.1 M HCOONa aqueous solutions at pH 7 in the following sequence of reactions:[25]

$$H_2O \rightsquigarrow e_{aq}^-,\ {}^\bullet OH,\ {}^\bullet H,\ H_3O^+,\ \ldots\ldots$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^\bullet OH + OH^- + N_2 \qquad k = 9\times10^9\ M^{-1}\ s^{-1}$$

$${}^\bullet OH + HCOO^- \rightarrow H_2O + CO_2^{\bullet-} \qquad k = 2.5\times10^9\ M^{-1}\ s^{-1}$$

$${}^\bullet H + HCOO^- \rightarrow H_2 + CO_2^{\bullet-} \qquad k = 2\times10^8\ M^{-1}\ s^{-1}$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \qquad k = 1\times10^9\ M^{-1}\ s^{-1}$$

Since $[N_2O] \sim 20$ mM, $[O_2] \sim 0.26$ mM, and $[HCOO^-] = 0.1$ M, at 1 atm, 20 °C, in aqueous solutions, the superoxide radical is produced within $\leq 1\ \mu s$.

Semiempirical calculations were carried out using the AM1 SCF-MO method as implemented into the SPARTAN program supplied with a Silicon Graphics workstation. The most probable structures, which appear in this paper, were derived by optimizing the geometry of all possible isomers and selecting the one with the lowest semiempirical heat of formation.

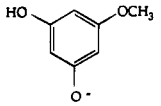
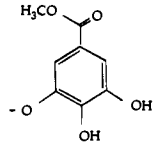
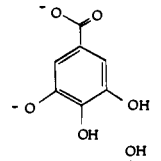
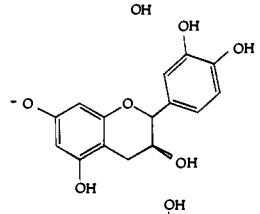
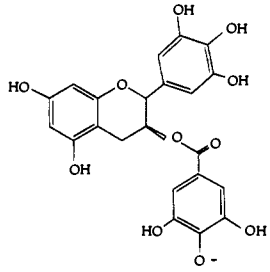
The structures and dissociation constants of selected phenol derivatives used in this study are summarized in Table 1.

Results and Discussion

A. Free Radical Chemistry of Gallocatechins. Generation of Phenoxyl Radicals. The free radical chemistry of gallocatechins was investigated in detail to fully characterize phenoxyl radicals, which are the transient products of their antioxidant action. The following techniques are often used for exclusive generation of phenoxyl radicals: (1) One-electron oxidation of a phenol derivative by strong transient oxidants, such as $SO_4^{\bullet-}$, $^\bullet N_3$, and $Br_2^{\bullet-}$, which is a technique normally employed in pulse radiolysis experiments in aqueous solutions, (2) H-atom abstraction from phenolic OH, e.g. by *tert*-butylalkoxyl generated by

(16) Haslam, E. *Plant Polyphenols. Vegetable Tannins Revisited*; Cambridge University Press: Cambridge, U.K., 1989.
(17) Matsuzaki, T.; Hara, Y. *Nippon Nogeikagaku Kaishi* **1985**, *59*, 129.
(18) Ammar, N.; El Diwany, A.; Osman, N.; Gaafar, S.; Amin, N. *Arch. Pharmacol. Res.* **1990**, *13*, 211.
(19) Sato, Y.; Terazawa, M.; Ishikawa, H. *Shokuhin Kogyo* **1984**, *27*, 42.
(20) Hara, Y.; Matsuzaki, T.; Suzuki, T. *Nippon Nogeikagaku Kaishi* **1987**, *61*, 803.
(21) Wagner, H. In *Flavonoids: Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Chapman and Hall: London, 1982; p 589.
(22) Suzuki, S.; Moroe, M.; Uchida, Y. *Shokuhin Kogyo* **1983**, *26*, 57.
(23) Terada, A.; Hara, H.; Nakajyo, S.; Ichikawa, H.; Hara, Y.; Fukai, K.; Kobayashi, Y.; Mitsuoka, T. *Microb. Ecol. Health Dis.* **1993**, *6*, 3.
(24) Nakayama, M.; Suzuki, K.; Toda, M.; Okubo, S.; Hara, Y.; Shimamura, T. *Antiviral Res.* **1993**, *21*, 289.
(25) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis: London, 1987.
(26) Jagannadham, V.; Steenken, S. *J. Am. Chem. Soc.* **1984**, *106*, 6542.
(27) Scaiano, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 7747.
(28) Scaiano, J. C.; Tanner, M.; Weir, D. *J. Am. Chem. Soc.* **1985**, *107*, 4396.
(29) Gorman, A. A.; Rodgers, M. A. J. In *Handbook of Organic Photochemistry*; Scaiano, J. C., Ed.; CRC Press: Boca Raton, FL, 1989; p 229.

Table 1. Dissociation Constants of Gallocatechins and Phenol Models at 20 °C

phenol	structure of anion	pK_{a1}[a]	pK_{a2}[a]	pK_{a3}
3,5-Dihydroxy-anisole		9.30	11.3	
Methyl gallate		8.03	11.6	
Gallic acid		8.73[b]	12.4[b]	
Epicatechin		8.64[c]	9.41[c]	11.26; 13.4[c]
Epigallocatechin gallate		7.75	8.0	n.m.[d]

[a] Determined spectrophotometrically in this study. Estimated to be accurate to ±0.05 pH units. [b] From ref 46. [c] From ref 10. [d] Gallocatechins are extremely unstable in alkaline media which precluded accurate measurements of higher pK_a values.

Table 2. Rate Constants of Oxidation of Gallocatechins and Model Phenols with Transient Oxidants, Determined by Pulse Radiolysis in Aqueous Solutions at pH 7 and 20 °C

phenol derivative (Ar-OH)	oxidant (X)	k(X + Ar-OH),[a] $M^{-1}s^{-1}$
3,5-dihydroxyanisole	$^{\bullet}N_3$	1.4×10^9
methyl gallate	$^{\bullet}N_3$	4.2×10^9
epicatechin	$^{\bullet}N_3$	4.0×10^9
epigallocatechin	$^{\bullet}N_3$	4.7×10^9
epicatechin gallate	$^{\bullet}N_3$	4.7×10^9
epigallocatechin gallate	$^{\bullet}N_3$	4.8×10^9
	$(SCN)_2^{\bullet-}$	4.2×10^8
	$CH_2CH_2O^{\bullet}$	4.6×10^7

[a] Accurate to ±10%.

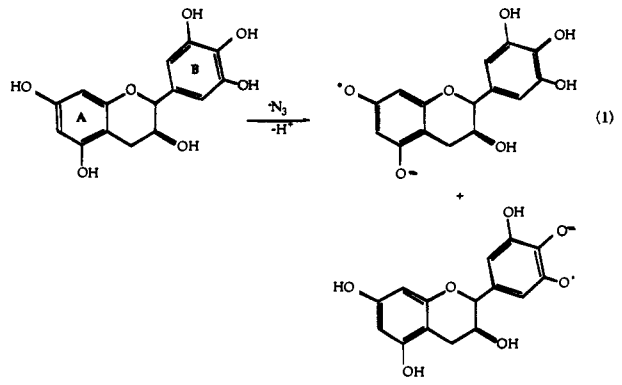
laser photolysis of *tert*-butyl peroxide in nonaqueous media, or (3) direct photoionization of a phenol derivative in polar solutions.

We used one-electron oxidation by the azidyl radical, $^{\bullet}N_3$, and direct photoionization by a 248 nm laser to generate phenoxyl radicals from gallocatechins. The rate constants of the reaction of the azidyl radical with polyphenols and simpler model phenols are summarized in Table 2. The reactivities of a few other transient oxidants with epigallocatechin gallate are included in Table 2 for comparison.

The rates of oxidation of polyphenols by the azidyl radical are close to diffusion controlled, reflecting their favorable electron-donating properties. 3,5-Dihydroxyanisole is oxidized at a somewhat lower rate, very probably as a consequence of higher reduction potential of its radicals (see Table 7). The rates of one-electron oxidation of epigallocatechin gallate by thiocyanate and formyl radicals are lower, as may be expected from the relatively lower reduction potentials of these oxidants (see below).

In simple phenols both chemionization (one-electron oxidation by strong transient oxidants) and photoionization yield the same phenoxyl radical. To our surprise, one-electron oxidation of the catechin derivatives by the azidyl radicals results in the formation of both a short-lived and a long-lived transient (Figure 1A), whereas the 248 nm laser-induced photoionization of epigallocatechin produces only the long-lived transient (Figure 1B). We have investigated this phenomenon using 3,5-dihydroxyanisole (Figure 2) and methyl gallate (Figure 2) as the models for the A and B ring of gallocatechins, respectively.

Apparently, two transients are formed upon initial oxidation of the catechin derivatives because $^{\bullet}N_3$ attacks on both A and B rings, as illustrated by the reaction with epigallocatechin at pH 7 (reaction 1). The site of attack is readily deduced from



(1)

the similarity of the UV−vis spectra (Figures 1 and 2) of the A ring (λ_{max} = 490 and 550 nm at pH 3 and 7, respectively) and B ring (λ_{max} = 310 and 340, 420 nm at pH 3 and 7, respectively) phenoxyl radicals with those of 3,5-dihydroxyanisole and methyl gallate radicals. The ratio of the attack on the A ring over that on the B ring (approximately 1:2) is independent of the reduction potential and charge of the oxidant, since the phenoxyl spectra produced upon the reaction of epigallocatechin gallate with neutral azidyl (E_7 = 1.33 V) and formyl ($E_7 \sim$ 1 V) radicals and negatively charged thiocyanate (E_7 = 1.25 V) and bromide (E_7 = 1.7 V) radicals are qualitatively and quantitatively similar.

In the 248 nm laser flash photolysis of aqueous epigallocatechin at pH 7 only the B ring is photoionized because of its relatively lower ionization potential (the results of geometry optimization give IP(3,5-dihydroxyanisole) = 9.12 eV vs IP(methyl gallate) = 9.00 eV). The quantum yield of photoionization, ϕ = 0.066 (based on ϕ = 0.29[30] for photoionization of KI), is very low. The spectrum of the photochemically generated phenoxyl radical is the same as that of the longer-lived transient in the pulse radiolysis experiment, thus confirming the assignment of the longer-lived transient.

Intramolecular Electron Transfer. Subsequent to the one-electron oxidation of polyphenols, the resorcinol phenoxyl (at the A ring) transforms to the gallate phenoxyl (at the B ring) via inter- and intramolecular electron transfer. This is concluded from a parent concentration- *in*dependent and a dependent process. The rates of these electron transfer processes were measured in acidic, neutral, and alkaline media to determine the influence of the protonation states of parent polyphenols and corresponding phenoxyl radicals on the rates of electron transfer. The results are summarized in Table 3.

(30) Calvert, J.; Pitts, J. N., Jr. *Photochemistry*; J. Wiley and Sons: London, 1966.

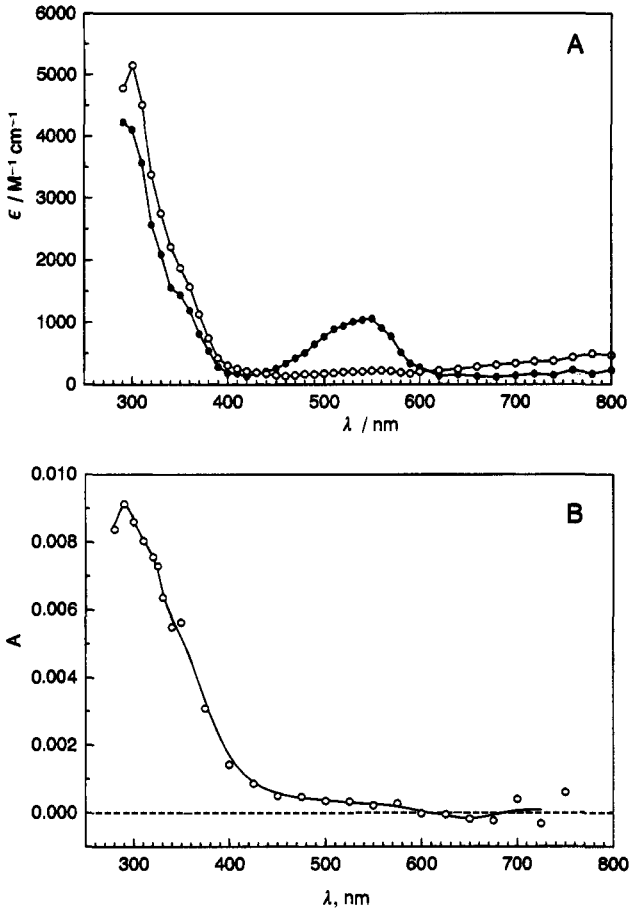


Figure 1. Absorption spectra of epigallocatechin phenoxyl radicals (A) recorded immediately upon one-electron oxidation by $^{\bullet}N_3$ (●) and upon completion of secondary electron-transfer process (○) (in an N_2O-saturated aqueous solution of 22 mM NaN_3, 3.5 mM phosphate buffer, and 0.046 mM EGC, at pH 7 and 20 °C) and (B) upon photoionization of epigallocatechin (in an O_2-saturated aqueous solution of 0.26 mM EGC, at pH 7 and 20 °C).

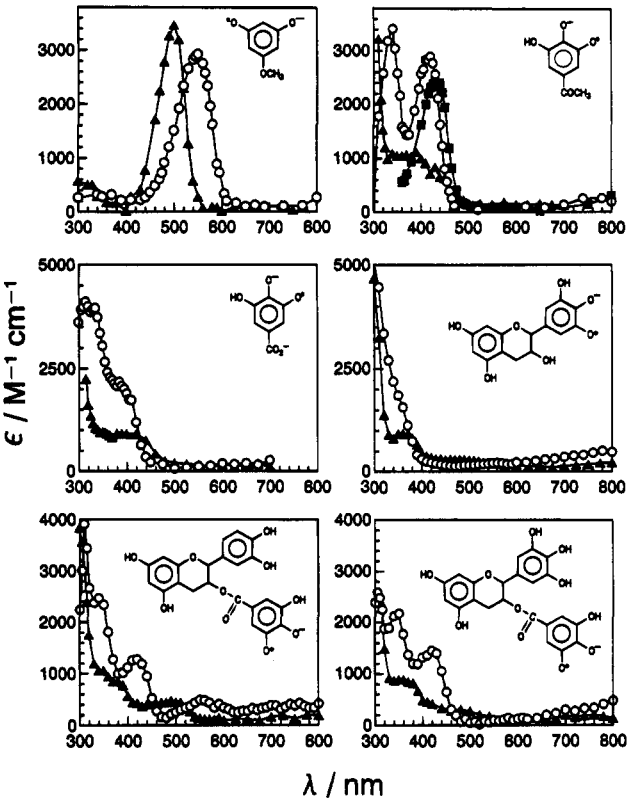
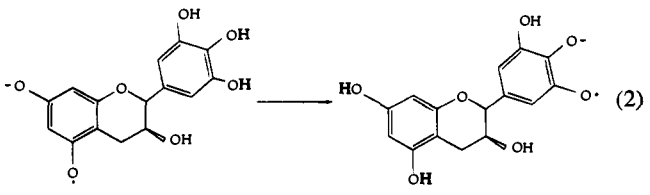


Figure 2. Absorption spectra of the neutral (▲) and negatively charged phenoxyl radicals (○) obtained upon $^{\bullet}N_3$-induced one-electron oxidation of polyphenols and model phenols (in N_2O-saturated aqueous solutions of ~10 mM NaN_3, ~3 mM phosphate buffer, and ~0.1 mM phenol derivative at 20 °C). The spectrum of the dianion methyl gallate radical (■) (at pH 10) is also shown.

The rates of both inter- and intramolecular "positive hole" migration from the A to B (or C) ring of the catechin derivatives depend on the ionization of the phenol groups in the B (or C) ring of parent phenols and corresponding phenoxyl radicals. The lowest rates are measured in acidic media, where both rings are fully protonated (see Table 1) and where oxidation yields neutral phenoxyl radicals (see Table 5), whereas the highest rates are observed in alkaline media, where the B (or C) ring is deprotonated and the oxidation yields phenoxyl radical anions. Independent of the experimental conditions and the catechin derivative, the secondary electron transfer processes proceed to completion, leaving the B- (or C-) ring phenoxyl radicals as the final transient products. This may be expected from ~0.3 V reduction potential difference (equilibrium constant $K \sim 10^5$) between the A and B (or C-) rings in the radicals of catechin derivatives (assuming reduction potentials similar to those of the model 3,5-dihydroxyanisole and methyl gallate radicals, see Table 7). The dramatic rate increase with pH in the range from 7 to 10 may be explained by both a decrease in reorganization energy and a higher driving force for electron transfer because of the deprotonation of the parent phenol. For example, the reduction potential difference between the 3,5-dihydroxyanisole and methyl gallate radicals increases from ~0.3 V at pH 7 to ~0.4 V at pH 10.

Intermolecular electron transfer rates (Table 3) increase slightly with the difference in the reduction potentials of A and B rings in the radicals, e.g. $k = 1.06 \times 10^7$ M^{-1} s^{-1} for epicatechin with $\Delta E = 0.84$ (3,5-dihydroxyanisole) − 0.6 (dihydroxybenzoic acid)[10] = 0.24 V vs $k = 3.8 \times 10^7$ M^{-1} s^{-1} for epigallocatechin with $\Delta E = 0.84 - 0.43 = 0.41$ V (see Table 7) at pH 7. However, these rates are lower than expected from the high thermodynamic driving force. In addition, considerably higher rates of intermolecular electron transfer in epigallocatechin gallate, e.g. $k = 3.5 \times 10^8$ M^{-1} s^{-1} at pH 7, point to the possible attenuation of the rate constant by unfavorable steric effects. This entropic restriction is alleviated in epigallocatechin gallate radicals by the presence of another, freely rotating electron-rich trihydroxyphenol ring and favorable acid−base equillibria (see Table 5).

It should be emphasized that one or two protons are exchanged subsequent to the electron transfer at any pH. For example, the intramolecular electron and proton transfer reaction in the epigallocatechin radical at pH 7 is shown here:



Consequently, a considerable solvent reorganization is required for intermolecular electron transfer, whereas the equivalent of an H-atom is transferred in the intramolecular process. The mechanism of secondary electron transfer processes was investigated in the $^{\bullet}N_3$ + epigallocatechin gallate and $^{\bullet}N_3$ + epigallocatechin systems at pH 7 in the temperature range from 0 to 60 °C, and the results are shown in Table 4.

Table 3. Rate Constants of Inter-[a] and Intramolecular[b] Electron Transfer Reactions of Gallocatechins Determined by Pulse Radiolysis of Aqueous Solutions at 20 °C

	$k_{intermolecular}$, $M^{-1}s^{-1}$ [c]			$k_{intramolecular}$, $M^{-1}s^{-1}$ [d]		
gallocatechin	pH = 3	pH = 7	pH = 10	pH = 3	pH = 7	pH = 10
epicatechin	n.m.[e]	1.06×10^7	1.1×10^7	n.m.	2300	5×10^4
epigallocatechin	2.2×10^7	3.8×10^7	1.6×10^8	3000	7000	1.3×10^5
epigallocatechin gallate	1.3×10^8	3.5×10^8	n.m.	5000	1×10^4	1.5×10^5

[a] Reaction is between *m*-semiquinone-type radical and the catechol-type part of a second parent molecule. [b] Reaction is between *m*-semiquinone-type radical and the catechol-type part of the same molecule. [c] Estimated to be accurate to ±10%. [d] Accurate to ±20%. [e] Not measured.

Table 4. Activation Parameters of the Electron Transfer Reactions of Polyphenol Radicals Determined by Pulse Radiolysis of Aqueous Solutions in the Temperature Range from 0 to 60 °C at pH 7

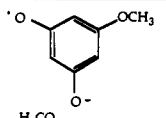
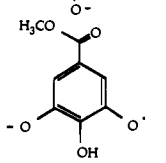
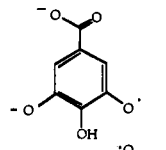
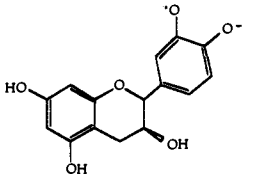
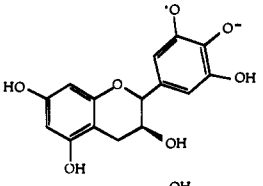
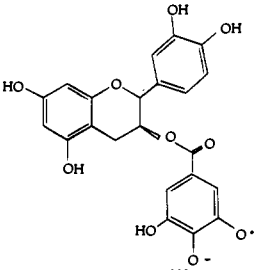
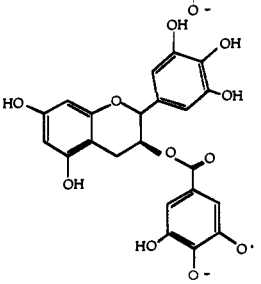
	intermolecular		intramolecular	
polyphenol	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu
epigallocatechin	1.7	−18	9.2	−11
epigallocatechin gallate	2.2	−14	17	14

The activation parameters of the intermolecular electron transfer reaction are similar in both systems. Small activation enthalpies, $\Delta H^{\ddagger} \sim 2$ kcal/mol, and negative activation entropies, $\Delta S^{\ddagger} \sim -15$ eu, are consistent with the "entropy control" as a consequence of considerable solvent reorganization in the transition state, which is necessary to accommodate proton exchange.[31,32]

The activation parameters of the intramolecular process, however, are significantly different in these closely related gallate systems, with a positive activation entropy for the epigallocatechin gallate system and negative for the epigallocatechin system. In the $^{\bullet}N_3$ + epigallocatechin system, the negative activation entropy, $\Delta S^{\ddagger} = -11$ eu, indicates a large solvent reorganization, which is expected for solvent-assisted electron transfer. Conversely, a rather large activation enthalpy, $\Delta H^{\ddagger} = 17$ kcal/mol, and a positive activation entropy, $\Delta S^{\ddagger} = 14$ eu, in the $^{\bullet}N_3$ + epigallocatechin gallate system, suggest bond formation and breakage in the transition state, which points to an H-atom transfer rather than an electron transfer reaction. However, a direct H-transfer from the C to A ring in the epigallocatechin gallate radical would require a highly ordered transition state, where these rings are brought in the close proximity. This means effective loss of rotational degrees of freedom with the accompanying loss of activation entropy. On the basis of the conformational analysis of the anisole- and gallate-centered epigallocatechin gallate radicals which clearly shows that the B and C rings are stacked at ~30 nm distance in the most stable conformation, we propose the formation of molecular complex between the epigallocatechin gallate radical and water molecules as a stable intermediate. The measured activation parameters would then correspond to the dissociation of this complex, and the mechanism of the intramolecular process in the $^{\bullet}N_3$ + epigallocatechin gallate system would amount to an inner-sphere electron transfer.

Spectral and Acid–Base Properties of Phenoxyl Radicals. One-electron oxidation of gallocatechins and other polyhydroxyphenols by the azidyl radical affords rapid generation of corresponding phenoxyl radicals for study of their spectral and acid–base properties. The spectra of phenoxyl radicals change with the pH of the solution, giving rise to the titration curves as shown in Figure 2, from which the dissociation constants of the phenoxyl radicals (summarized in Table 5) were obtained.

Table 5. Acid–Base Properties of Phenoxyl-Type Radicals Determined by Pulse Radiolysis of Aqueous Solutions at 20 °C

phenoxyl from	structure of phenoxyl anion	pK_r[a]
3,5-Dihydroxyanisole		6.7
Methyl gallate		4.4; 9.2
Gallic acid		5.0
Epicatechin		4.6[b]
Epigallocatechin		5.5
Epicatechin gallate		4.3
Epigallocatechin gallate		4.4; 5.5

[a] Refers to conjugate acid of phenoxyl anion. Accurate to ±0.1 pH unit. [b] From ref 10.

The pK_a value of the phenoxyl radical from methyl gallate is 0.6 units lower than that from gallic acid, as a consequence of the electron-withdrawing ester group in the former radicals. The

(31) Jovanovic, S. V.; Jankovic, I.; Josimovic, L. *J. Am. Chem. Soc.* **1992**, *114*, 9018.
(32) Neta, P.; Huie, R. E.; Maruthamuthu, P.; Steenken, S. *J. Phys. Chem.* **1989**, *93*, 7654.
(33) Deeble, D. J.; Parsons, B. J.; Phillips, G. O.; Schuchmann, H.-P.; von Sonntag, C. *Int. J. Radiat. Biol.* **1988**, *54*, 179.

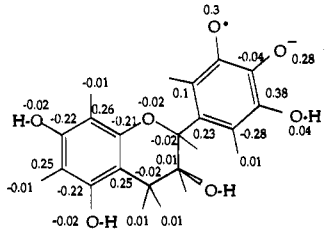
Table 6. Electron Transfer Reactions of Polyhydroxyphenols and Corresponding Radicals Studied by Pulse Radiolysis in Aqueous Solutions at pH 7 and 20 °C

$$^{\bullet}A + D \underset{k_r}{\overset{k_f}{\rightleftharpoons}} A^- + D^{\bullet+}$$

acceptor (•A) from	donor (D)	k_f, $M^{-1}s^{-1}$	k_r,[a] $M^{-1}s^{-1}$	K_{kin}[b]	K_{abs}[b]	ΔE, V[c]
3,5-dihydroxyanisole	4-methoxyphenol	6.4×10^6	1.4×10^5	46	49	0.099
rutin	methyl gallate	8.3×10^5	7×10^4	12	n.m.	0.06
methyl gallate	ferrocene monocarboxylate	1.9×10^6	4×10^5	5	2	0.04
trolox c	epigallocatechin	3.3×10^4	2×10^3	16	8	0.06
trolox c	epigallocatechin gallate	3.3×10^4	3×10^3	11	5	0.05

[a] Rate constants of forward electron transfer reactions are accurate to ±10% and to ±20% for reverse reactions. [b] Equilibrium constants from kinetics, $K_{kin} = k_f/k_r$, and equilibrium absorbancies of radicals, K_{abs}. [c] The reduction potential difference between the redox pairs of acceptor and donor is calculated from the arithmetic mean of equilibrium constants, K, using the Nernst equation, $\Delta E = 0.059 \log K$. The values are estimated to be accurate to ±0.02 V.

Chart 1. Calculated Unpaired Electron Density in the Epigallocatechin Radical Anion



pK_a values are similar to those previously reported,[33] for *n*-propyl gallate, pK_a = 4.1, and for pyrogallol, pK_a = 5.1, which have similar electronic structures.

The spectra of gallocatechin phenoxyl radicals are qualitatively similar to the spectra of corresponding model phenoxyls, namely those derived from 3,5-dihydroxyanisole, gallic acid, methyl gallate, and 3,4-dihydroxybenzoate. This is expected because of the lack of conjugation of the chromophores in the catechin derivatives, so that the radical centers behave as isolated entities, "not seeing" the rest of the molecule. Such behavior is also reflected in the acid–base properties of the phenoxyl radicals, which exhibit dissociation constants similar to those of the model phenoxyls (Table 5).

The titration curve of epigallocatechin gallate radicals could not be fitted with a single pK_a value. A two pK_a fit revealed the presence of two radicals (Table 5), probably at both gallate moieties. The AM1 conformation search of the epigallocatechin radical anion clearly shows that the two gallate rings are stacked at a distance of ~30 nm in the most stable conformers. It is conceivable that such a structure would be further stabilized through hydrogen bonding with water molecules, which may promote proton and electron delocalization within such a molecular complex.

Higher pK_a values of the radicals derived from polyphenols and gallic acid could not be accurately determined because these compounds are unstable in alkaline media. However, it is conceivable that the second pK_a value of the polyphenol radicals with the gallate ester moiety is similar to pK_a = 9.2 of the methyl gallate radical. The second pK_a of epigallocatechin radicals may be estimated as ~10.3, assuming that ΔpK_a(epigallocatechin–methyl gallate radicals) ~ 1.1 derived for the first set of pK_a values remains the same for the second set.

The difference in the pK_a values of parent phenols and phenoxyl radicals, which amounts to ~4 pH units, suggests a high degree of delocalization of the unpaired electron in the radicals. Such delocalization of unpaired electrons is common in various substituted phenoxyl radicals.[10,34–36] This is further corroborated by the results of the AM1 geometry optimization of the phenoxyl radicals (shown below for the epigallocatechin radical anion) which clearly indicate the spread of unpaired electron density throughout the phenol rings.

Table 7. One-Electron Reduction Potentials of Polyhydroxyphenoxyl Radicals at pH 7 and 20 °C

phenoxyl from	E_m, V[a]	E_c, V[b]
3,5-dihydroxyanisole	0.84	0.85
methyl gallate	0.56	0.56[c]
epigallocatechin	0.42	0.43[d]
epigallocatechin gallate	0.43	0.43[d]

[a] Measured reduction potential vs NHE estimated to be accurate within ±0.04 V. [b] Calculated reduction potential using the formula.[34] $E_7 = 0.95 + 0.31\Sigma\sigma^+$. The σ^+ and σ values are taken from ref 47. [c] Geometry optimization of the gallate radical anion shows clearly that the radical is in *meta* position, whereas the negative charge is almost equally distributed between the other two hydroxy groups. To account for such structure we used the arithmetic mean of σ^+ values for *ortho* [$\sigma^+_o(OH) = -0.78$, $\sigma^+_o(O^-) = -1.96$] and *meta* [$\sigma^+_m(OH) = -0.02$, $\sigma^+_m(O^-) = -0.47$] hydroxy and hydroxide groups and added $\sigma^+_m(COOCH_3) = 0.37$. [d] Using the same reasoning as above, $\Sigma\sigma^+ = -1.62$ for the gallate phenoxyl. The σ^+ of the rest of the epigallocatechin molecule is taken as $\approx\sigma^+_m(CH_3) = -0.07$.

Reduction Potentials of Gallocatechin Radicals. The efficacy of gallocatechins as antioxidants in biological systems strongly depends on their ability either to intercept and inactivate damaging free radicals before they reach vital cell components or to chemically repair damaged biomolecules or bioradicals thus reversing the damage. Because of their high solubility in water, gallocatechins are expected to act as antioxidants in polar aqueous phase, where one-electron transfer is likely to be the dominant reaction mechanism. The electron transfer reactions of gallocatechins and polyhydroxyphenols with the redox standards Trolox C (E_7 = 0.48 V vs NHE),[36] ferrocene monocarboxylate (E_7 = 0.53 V),[37] rutin (E_7 = 0.6 V),[10] and 4-methoxyphenol (E_7 = 0.74 V)[15] were studied in aqueous solutions at pH 7 and 20 °C, and the results are summarized in Tables 6 and 7.

The highest reduction potential is that of 3,5-dihydroxyanisole radicals. The reduction potential of the methyl gallate radicals is higher than those of polyphenols, probably as a result of electron-withdrawing effect of the ester group. The relatively low reduction potentials of epigallocatechin and epigallocatechin gallate radicals are due to the B ring, in which the *para* position is occupied by the electron-donating methyl group. Taking into account the results of the geometry optimizations and assuming

(34) Jovanovic, S. V.; Tosic, M.; Simic, M. G. *J. Phys. Chem.* **1991**, *95*, 10824.
(35) Lind, J.; Shen, X.; Eriksen, T. E.; Merenyi, G. *J. Am. Chem. Soc.* **1990**, *112*, 479.
(36) Steenken, S.; Neta, P. *J. Phys. Chem.* **1982**, *86*, 3661.
(37) Farragi, M.; Weinraub, D.; Broitman, F.; DeFelippis, M. R.; Klapper, M. H. *Radiat. Phys. Chem.* **1988**, *32*, 293.

Table 8. Reactivities of Superoxide Radicals with Polyhydroxyphenols Measured by Pulse Radiolysis at pH 7 and 20 °C

phenol derivative (Phe-OH)	$k(O_2^{\bullet-} + \text{Phe-OH})$,[a] $M^{-1}\,s^{-1}$
3,5-dihydroxyanisole	$<10^4$
methyl gallate	2.5×10^5 [b]
gallic acid	3.4×10^5
epigallocatechin	4.1×10^5
epicatechin gallate	4.3×10^5
epigallocatechin gallate	7.3×10^5
epicatechin	6.8×10^4
catechin	6.4×10^4 [b]

[a] Estimated to be accurate to ±10%. [b] From ref 10.

the additivity of the Brown substituent constants, we calculated the reduction potentials of the phenoxyl radicals using the formula from ref 34. The results are included in Table 7. It should be noted that a very good agreement between the measured and calculated reduction potentials confirms the validity of this empirical relation for phenoxyl radicals.

The reduction potentials of epigallocatechin and epigallocatechin gallate radicals are lower than that of vitamin E radicals, which means that these major polyphenol components of green tea are able to repair vitamin E radicals under physiological conditions. Despite the low redox potential difference of $\Delta E \sim 0.06$ V, that is $K \sim 17$, we believe that the electron transfer from gallocatechins to the vitamin E radical would be efficient because the lifetime of the vitamin E radical in cell membranes is probably several orders of magnitude greater than those of gallocatechin radicals in the surrounding medium. The radical–radical reactions disrupt the electron transfer equilibrium by consuming the gallocatechin radicals, thus driving the reaction to the complete repair of vitamin E.

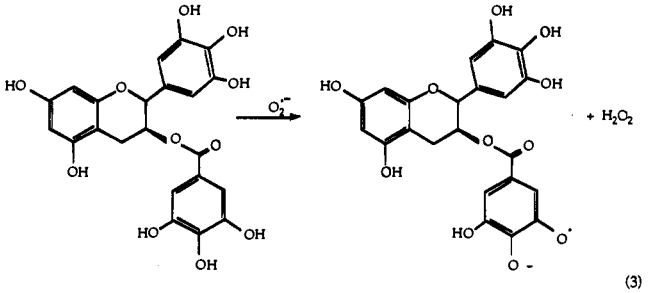
B. Gallocatechins as Antioxidants. Reaction with the Superoxide Radical. The superoxide radical is present in all aerobic organisms. Although unreactive with amino acids and DNA bases,[38,39] superoxide yields the considerably more reactive hydroxyl, •OH, which is the most damaging free radical in biosystems, in the metal-catalyzed oxidation of hydrogen peroxide (the Haber–Weiss–Fenton reaction).[25] Superoxide and its conjugate base, hydroperoxyl, are capable of initiatiating membrane phospholipid chain oxidation.[38] In addition, the product of the reaction of nitric oxide and superoxide, peroxynitrite, in its protonated form, may also initiate lipid peroxidation.[40] Normally the concentrations of superoxide in blood and/or intercellular fluid are low, enabling chemical (ascorbate, vitamin E) and biochemical defense mechanisms (e.g., superoxide dismutase, catalase) to inactivate radicals and remove harmful products. However, in pathological conditions, such as oxidative stress, ischemia-reperfusion injury, and inflammation, when the concentration of superoxide is several orders of magnitude higher, more protection is needed to prevent damage and subsequent severe biological consequences. Because of the apparent importance of efficient scavenging of superoxide for their antioxidant function, we determined the reactivities of superoxide with gallocatechins and polyhydroxyphenols. The results are summarized in Table 8.

The rates of the reactions of various phenol derivatives with the superoxide radical depend on their electron-donating properties. For example, the rate of the reaction with catechin ($E_7 = 0.57$ V)[10] or epicatechin, $k = 6.8 \times 10^4\ M^{-1}\,s^{-1}$, is higher than with 3,5-dihydroxyanisole ($E_7 = 0.84$ V), $k < 10^4\ M^{-1}\,s^{-1}$, but lower than with epigallocatechin ($E_7 = 0.42$ V), $k = 4.1 \times 10^5\ M^{-1}\,s^{-1}$. Epigallocatechin gallate is one of the most efficient scavengers of the superoxide radical, $k = 7.2 \times 10^5\ M^{-1}\,s^{-1}$. The rate constant is approximately two times higher than that of either epigallocatechin or epicatechin gallate, which may be explained by the presence of two antioxidant sites (two gallate moieties) in the epigallocatechin gallate molecule. As already mentioned, the presence of an additional freely rotating gallate ring may facilitate the formation of the transition complex for electron transfer.

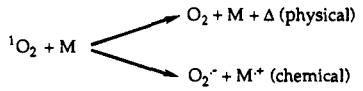
Table 9. Reactivities of $^1O_2$ with Polyphenols, Determined by Laser Flash Photolysis of Acetonitrile Solutions at 20 °C

phenol derivative, Phe-OH	$k(^1O_2 + \text{Phe-OH})$,[a] $M^{-1}\,s^{-1}$
catechin	1.1×10^8
epicatechin	9.6×10^7
epigallocatechin	1.1×10^8
epicatechingallate	2.2×10^8
epigallocatechin gallate	2.2×10^8

[a] Accurate to ±15%.



Singlet Oxygen Quenching. The lowest excited state of molecular oxygen, $^1O_2$ ($^1\Delta_g$), is of considerable interest because of its relatively long lifetime in condensed media, which translates into propensity to cause biological damage.[3,25,29] It oxidizes amino acids, fatty acids, and nucleosides to *endo*-peroxides,[3,4,29,41] thus causing cell death and mutations. Interestingly, this efficacy in cell killing is the basis of the photodynamic therapy (type II mechanism), in which cancer cells are killed by dye-sensitized oxidation.[4,41] $^1O_2$ is also implicated in numerous undesirable oxidations, such as "weathering" of polymeric products. $^1O_2$ is generated[4,29] upon quenching of triplet sensitizers by molecular oxygen, O_2 ($^3\Sigma_g^-$), thermal cleavage of endoperoxides, enzymatic oxidations, etc. In general, the quenching of $^1O_2$ may be physical and/or chemical:



Phenol derivatives scavenge singlet oxygen by both physical and chemical processes.[41–44] The overall rate constants of the $^1O_2$ quenching by gallocatechins were determined by laser flash photolysis in oxygen-saturated acetonitrile solutions at 20 °C, by using phenazine as the sensitizer (Table 9).

Gallocatechins rapidly quench $^1O_2$, $k \sim 10^8\ M^{-1}\,s^{-1}$. The rate constant of the reaction of $^1O_2$ with epigallocatechin gallate, $k = 2.2 \times 10^8\ M^{-1}\,s^{-1}$, is comparable to that of vitamin E, $k = 5 \times 10^8\ M^{-1}\,s^{-1}$.[29,42] It is conceivable that the mechanism of quenching is similar to that of other phenolic compounds.[43,44]

(38) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041.
(39) Afanas'ev, I. *Superoxide Ion: Chemistry and Biological Implications*; CRC Press: Boca Raton, FL, 1989; Vols. I and II.
(40) Feldman, P. L.; Griffith, O. W.; Stuehr, D. J. *Chem. Eng. News* **1993** (December 20), 26.
(41) Houba-Herin, N.; Calberg-Bacq, C. M.; Piette, J.; Van de Vorst, A. *Photochem. Photobiol.* **1982**, *36*, 297.
(42) Gorman, A. A.; Gould, I. R.; Hamblett, I.; Standen, M. C. *J. Am. Chem. Soc.* **1984**, *106*, 6956.
(43) Luiz, M.; Gutierez, M. I.; Bocco, G.; Garcia, N. A. *Can. J. Chem.* **1993**, *71*, 1247.
(44) Thomas, M. J.; Foote, C. S. *Photochem. Photobiol.* **1978**, *27*, 683.

In aqueous media, where gallocatechins are expected to act as antioxidants, there is a possibility of intermediate generation of the superoxide radical.[42,45] However, since gallocatechins are very efficient scavengers of $O_2^{\bullet-}$ (see Table 8), the overall reaction would neutralize all active oxygen species at the greater expense of the gallocatechins.

Conclusions

The reduction potential of the radicals of simple catechin and gallo derivatives of green tea ($E_7 = 0.42$ V) was found to be less than that of vitamin E ($E_7 = 0.48$ V). This electron-donating property may involve gallocatechins both in redox defense and in restitution of vitamin E. Hence, gallocatechins may play an important role in the physiological defense of the gastrointestinal tract.

Many natural phytochemicals contain both catechin and gallo groups within the same molecule. As shown in this work, the attack of oxidants is distributed within these groups. Furthermore, the catechin radicals would lose an unpaired electron to gallo groups either intra- or intermolecularly. The free radical transfer rates depend on parameters such as the molecular structure of gallocatechins, pH (state of protonation), and gallocatechin concentration.

In addition, the reactivities of gallocatechins with the superoxide radical at pH 7, $k \sim 10^6$ M^{-1} s^{-1}, are among the highest reported rates of *reduction* of superoxide radicals. Superoxide is reduced to hydrogen peroxide, which eliminates potentially hazardous redox cycling (for example, $Fe^{3+} + e^- \rightarrow Fe^{2+}$) promoted by oxidation of superoxide and formation of another transient reductant.

The high antioxidant potential of gallocatechins is confirmed by singlet oxygen quenching. The rates of quenching in acetonitrile, which may be viewed as a suitable model for the cell membrane, $k = (1.1-2.2) \times 10^8$ M^{-1} s^{-1}, are comparable to that of vitamin E, $k = 5 \times 10^8$ M^{-1} s^{-1}.

The impact of physiological antioxidant activities of gallocatechins and other nutritional antioxidants on longevity and illnesses, such as cancer and cardiovascular diseases, has not yet been fully and unequivocally established in this emergent area of research. The mechanistic observations presented in this paper provide a quantitative basis for studying the antioxidant role of gallocatechins in physiological defense systems.

Acknowledgment. We are indebted to Karen A. Taylor for editorial assistance and to Dr. Alain St. Amant for the use of Silicon Graphic work station. S.V.J. would like to acknowledge the generous support of Prof. J. C. Scaiano in finishing the manuscript.

JA9514813

(45) Saito, I.; Matsuura, T.; Inoue, K. *J. Am. Chem. Soc.* **1983**, *105*, 3200.

(46) Serjeant, E. P.; Dempsey, B. *IUPAC Ionization Constants of Organic Acids in Aqueous Solutions*; Pergamon Press: Oxford, U.K., 1979.

(47) Hansch, C.; Leo, A.; Taft, R. W. *Chem. Rev.* **1991**, *91*, 165.