Flavonoids as Antioxidants

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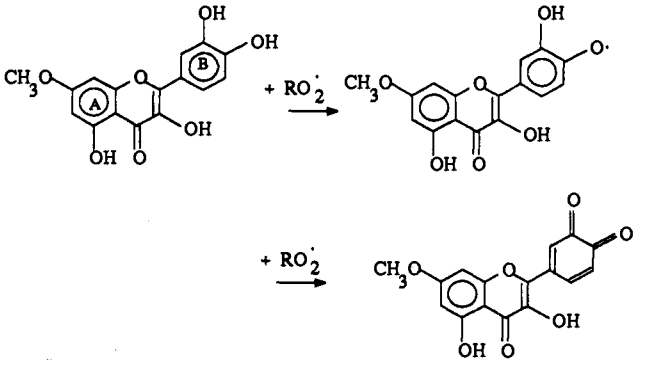
Abstract: Spectral, acid–base, and redox properties of the phenoxyl radicals derived from 3,4-dihydroxybenzene derivatives and selected flavonoids were studied by pulse radiolysis of aqueous solutions. From the pH-dependent changes in the phenoxyl spectra, the dissociation constants were derived. The pK_a values for the deprotonation of the 3′-OH group in the catechin (pK_a = 4.6) and rutin (pK_a = 4.3) radicals are similar to the pK_a value of the 3,4-dihydroxybenzoate radicals, pK_a = 4.2, which is expected from their similar electronic structures. Deprotonation of 5- and 7-OH in the catechin and rutin and of 5-OH in the hesperidin radicals has no effect on the radical spectra, which is explained by the inefficient coupling of the A-ring of the flavonoid radicals with the unpaired electron. Because of favorable reduction potentials of the phenoxyl radicals, E_7 = 0.56–0.7 V vs NHE, flavonoids may act as efficient antioxidants of alkylperoxyl and superoxide/hydroperoxyl radicals. The ac kinetic conductivity method was developed for the measurements of the low reaction rate constants of the superoxide radical reactions with flavonoids and phenols in aqueous solutions at pH 10. The rates of the superoxide radical reactions with flavonoids, $k = 3 \times 10^2$–5.1×10^4 M^{-1} s^{-1}, depend on the redox properties and the charge of the flavonoids. The highest rates are measured for the oxidation of quercetin and rutin, whereas the lowest are those for the B-ring monosubstituted derivatives, with substantially higher redox potentials. Uncharged catechin at pH 7 reacts at $k = 6.6 \times 10^4$ M^{-1} s^{-1}, whereas the rate at pH 10, where catechin is doubly negatively charged, is approximately 4 times lower, $k = 1.8 \times 10^4$ M^{-1} s^{-1}. The activation parameters of the oxidation of rutin and trolox at pH 10 and methyl gallate at pH 7 were determined in an attempt to understand why the rates of the superoxide reactions are low despite high driving forces of $\Delta E \geq 0.4$ V. Low activation enthalpies, $\Delta H^{\ddagger}$ = 2.3–3.6 kcal/mol, and negative activation entropies, $\Delta S^{\ddagger}$ = −25–28 cal/(mol K), point to an inner-sphere electron-transfer mechanism.

Introduction

Flavonoids are phenol derivatives present in substantial amounts (0.5–1.5%) in plants.[1] Because of the importance of plants and food products derived from plants and plant extracts in human nutrition, their potential antioxidant activity has been investigated ever since their discovery. The studies of the distribution of radiochemically labeled compounds[2] revealed that the major portion of ingested flavonoids is present in the gastrointestinal tract (e.g. 44.2% of ingested quercetin), before they are excreted in a bile. Consequently, it is reasonable to assume that the action of flavonoids as biochemical antioxidants takes place during vigorous oxidative processes in digestion.

In earlier studies,[3] the ability of flavonoids to inactivate organic peroxyl radicals was found to be at least comparable to if not better than that of conventional phenolic antioxidants (e.g. butylhydroxytoluene (BHT) and butylhydroxyanisole (BHA)). It is interesting to note that a consecutive two-electron oxidation was postulated[3] as the mechanism of the peroxyl radical reaction with 3′,4′- and 2′,5′-dihydroxyflavonols to yield corresponding quinones. The first one-electron oxidation produces the flavonoid phenoxyl radical, which subsequently scavenges another peroxyl radical:



Apparently, organic peroxyl radicals ***selectively*** attack the B ring of any 3′,4′- or 2′,5′-dihydroxyflavonoid. The ability of flavonoids to inactivate linoleic acid peroxyl radicals was demonstrated[4] in aqueous micellar solutions at pH 11.5. In addition, it was shown[5] that flavonoids are efficient scavengers of (alkoxyl) *tert*-butoxyl radicals. Somewhat controversial are the reports on the ability of flavonoids to inactivate the superoxide radical. For example, various natural and synthetic flavonoids were found[6] to be efficient superoxide scavengers, whereas other authors[4] reported that superoxide was completely unreactive with querectin and kaempferol at pH 11.5 and reacted as a reducing agent (similar

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to $CO_2^{\bullet-}$) at pH 8.5. It was recently reported[7] that the superoxide radical is slowly but efficiently inactivated by the phenol derivatives with relatively high redox potentials, e.g. phenol ($k = 5.8 \times 10^2$ M^{-1} s^{-1}), guaiacol ($k = 2.5 \times 10^3$ M^{-1} s^{-1}), and eugenol ($k = 8.3 \times 10^3$ M^{-1} s^{-1}). On the basis of the rather low reduction potentials[8] of the flavonoid phenoxyl radicals, which are similar to and lower than that of the trolox C (a water-soluble analog of vitamin E) radical at pH 13.5, it is expected that the abilities of the parent flavonoids to act as antioxidants are at least comparable to that of trolox. Furthermore, trolox inactivates alkylperoxyl and superoxide radicals in neutral media,[9-11] and the flavonoids are expected to have similar antioxidant properties. To provide quantitative assessment of the antioxidant potential of flavonoids, we investigated the electron-transfer reactions of various polyhydroxyphenols in neutral and alkaline solutions. Spectral, acid–base, and redox properties of the polyhydroxyphenoxyl radicals were determined by optical pulse radiolysis in aqueous solutions. To resolve the inconsistency of published data,[6-8] the reactivities of various flavonoids with the superoxide radical were measured by kinetic conductivity at pH 10 and by optical pulse radiolysis at pH 7. The activation parameters of selected superoxide radical reactions were also determined.

Materials and Methods

All chemicals were of analytical grade and were used as received. Hesperetin, hesperidin, and morin were obtained from Sigma; rutin, quercetin, catechin, galangin, 3,4-dihydroxybenzoic acid, 3,4-dihydroxycinnamic acid, trolox, fisetin, and 4′,5,7-trihydroxyflavanone were the products of Aldrich; 4-methoxyphenol, (3,4-dihydroxyphenyl)alanine (DOPA), 3,4-dihydroxybenzaldehyde, NaOOCH, KBr, KSCN, and 2-propanol were obtained from Merck; and kaempferol was obtained from ICN Biochemicals. Water was purified through a Millipore Milli Q system. All solutions were freshly prepared before experiments and were used immediately. Special precautions were taken in the preparation of alkaline solutions, because of the well-known sensitivity of polyhydroxyphenols to oxygen at high pH. In addition, the concentration of ions had to be kept at the minimum for better conductance measurements. For pulse conductivity experiments, alcohol-soluble flavonoids (catechin, quercetin, morin, galangin, 4′,5,7-trihydroxyflavanone, and kaempferol) and 4-methoxyphenol were first dissolved in 2-propanol and the mixtures were then diluted with an aqueous solution (pH 10) saturated with a N_2O:O_2 = 4:1 gas mixture. When necessary, the pH of the solution was adjusted to 10 with 0.1 N NaOH (Merck). Flavonoids and phenol derivatives less soluble in 2-propanol were dissolved in N_2O-saturated aqueous 0.26 M propanol at pH 10. Typically the pH of the solution fell to ~7–8 upon dissolving a flavonoid. The pH was readjusted to 10 by careful titration of the solution with 0.1 N NaOH (Merck), and the solution was then saturated with the N_2O:O_2 = 4:1 gas mixture by passing the gas through the solution for 5 min. In most cases the solutions were stable for more than ~30 min, depending on the flavonoid concentration, which was sufficient for the rate determinations. To minimize potential errors arising from the thermal oxidation of phenols in alkaline media, at least three independent rate measurements at pH 10 were done for at least three concentrations of a phenol per measurement. pH 7 in the optical measurements was maintained using the phosphate buffer (Merck). The pH variation in the determinations of the dissociation constants of various phenoxyl radicals was achieved by the addition of $HClO_4$ or NaOH at appropriate concentrations to the phosphate-buffered aqueous solutions.

A 3-MeV van de Graaff pulse radiolysis apparatus at the Max-Planck-Institute für Strahlenchemie[12] was used for optical and conductivity measurements. A 2-cm Suprasil quartz cell was used for the optical whereas a 10-MHz ac bridge was used for the conductance measurements. Both the optical and conductivity cells were thermostatically controlled. The thiocyanate dosimetry[13] was used in the optical whereas aqueous dimethyl sulfoxide[12] at pH 4 was used for the dose determinations in the conductance measurements. The doses were typically from 0.5 to 1.5 Gy/pulse in the rate constant determinations, from 5 to 10 Gy/pulse when the absorption spectra of the radicals were recorded by optical pulse radiolysis, and from 1 to 2 Gy/pulse in the pulse conductivity experiments.

A 1.85×10^5 GBq ^{60}Co source at The VINCA Institute was used for γ irradiations. The dose rate was 47 Gy/min as determined by the Fricke dosimetry. The absorbed doses were adjusted so that the consumption of oxygen would not exceed 30%, taking $G(-O_2) \leq 6$.

The yield of total peroxides upon irradiation of aqueous solutions of various polyhydroxyphenols was determined by the iodide[14] and titanium(IV)[15] methods. For the iodide measurements, 5 mL of polyhydroxyphenol solution was mixed with 20 mL of freshly prepared reagent (a 1:1 mixture of 100 mL of solution containing 6.6 g of potassium iodide, 0.02 g of ammonium molybdate, and 0.2 g of sodium hydroxide and 100 mL of solution containing 2 g of potassium hydrogen phthalate). The concentration of peroxide was derived from the absorbance at 350 nm, $\epsilon = 26\,000$ M^{-1} cm^{-1}, measured against the polyhydroxyphenol solution without the reagent. The titanium(IV) method is based on the oxidation of Ti(IV) to Ti(VI) by H_2O_2 and is considered more specific for the determination of hydrogen peroxide. A 1-mL portion of the Ti(IV) reagent (3.6 g of potassium titanium oxalate and 100 mL of concentrated sulfuric acid in 1000 mL of water) was mixed with 9 mL of the irradiated polyhydroxyphenol solution and the absorbance determined at 400 nm, $\epsilon = 940$ M^{-1} s^{-1}, against the polyhydroxyphenol solution without the reagent.

The dissociation constants of various ionizable groups in the phenol derivatives are summarized in Table 1.

Results and Discussion

Flavonoid Phenoxyl Radicals. (a) Spectral and Acid–Base Properties. Low solubility of most flavonoids in neutral aqueous media precludes accurate measurements of the reduction potentials of the flavonoid phenoxyl radicals at biologically relevant pH values. Therefore we chose quercetin-3-*O*-rutinose (rutin), hesperitin-7-*O*-rutinose (hesperidin), and catechin, whose solubilities permit such measurements. We also investigated water-soluble 3,4-dihydroxyphenol derivatives, such as 3,4-dihydroxybenzoic acid, (3,4-dihydroxyphenyl)alanine, and 3,4-dihydroxycinnamic acid, as suitable model compounds for the flavonoids.

The flavonoid phenoxyl radicals were generated by the bromide radical ion induced oxidation of flavonoids in aqueous solutions

$$Br_2^{\bullet-} + FVH\text{–}OH \rightarrow FVH\text{–}OH^{\bullet+} + 2Br^- \quad (1)$$

By analogy with other phenoxyl radicals,[17] the flavonoid phenoxyl radical cation rapidly loses a proton to form the neutral radical

$$FVH\text{–}OH^{\bullet+} \rightarrow FVH\text{–}O^{\bullet} + H^+ \quad (2)$$

The rate constants of reaction 1 were pH dependent because of the ionization of various phenol groups in the flavonoids. For example, the rate of $Br_2^{\bullet-}$ oxidation of rutin increases from 2×10^7 at pH 3 to 2.1×10^8 M^{-1} s^{-1} at pH 7 because pK_a(rutin) = 7.1 (for a complete list of pK_a values of flavonoids, see Materials and Methods). The rate constants of one-electron oxidations of various flavonoids at pH 7 are summarized in Table 2.

The bromide radical ion induced oxidations of flavonoids ($k \sim 10^7$–10^8 M^{-1} s^{-1}) produce rapid generation of the flavonoid phenoxyl radicals, for the investigation of their spectral, acid–base, and redox properties.

The absorption spectra of the flavonoid phenoxyl radicals were similar to those obtained by the $^{\bullet}N_3$-induced oxidation at pH

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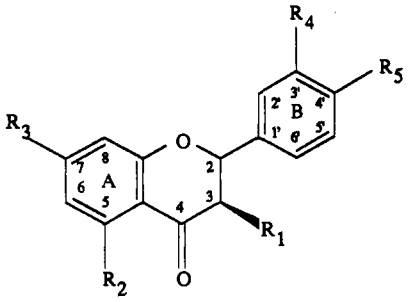
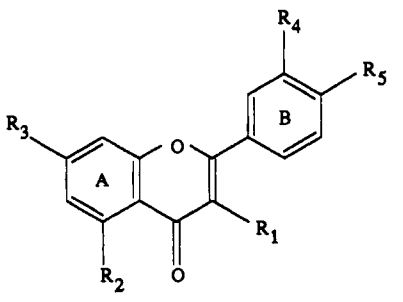
Table 1. Dissociation Constants of the Phenol Derivatives at 20 °C



flavanone



flavone

phenol derivative	R_1	R_2	R_3	R_4	R_5	pK_1	pK_2	pK_3	pK_4
catechin[a]	OH	OH	OH	OH	OH	8.64[b]	9.41[b]	11.26[b]	13.26[b]
				Flavanones					
hesperetin	H	OH	OH	OH	OCH_3	6.67[c]	8.76[c]	11.54[c]	
hesperidin	H	OH	rutinose	OH	OCH_3		8.9[c]	11.24[c]	
4',5,7-trihydroxyflavanone	H	OH	OH	H	OH	6.7[c]	9.1[c]		13.05[c]
				Flavones					
galangin	OH	OH	OH	H	H	6.8[c]	9.4[c]		
fisetin	OH	H	OH	OH	OH		8.87[c]	10.31[c]	13.2[c]
kaempferol	OH	OH	OH	OH	H	8.2[b]	9.5[b]		
quercetin	OH	OH	OH	OH	OH	6.74[b]	9.02[b]	11.55[b]	
rutin	rutinose	OH	OH	OH	OH	7.1[c]	9.15[c]	11.65[c]	
morin	OH	OH	OH	H[d]	OH	3.46[c]	8.1[c]		
				Phenols					
3,4-dihydroxybenzaldehyde						7.21[b]	11.8[b]		
3,4-dihydroxybenzoic acid						8.67[b]	11.74[b]	4.48[b] (COOH)	
3,4-dihydroxycinnamic acid						7.6[c]	11.85[c]	4.44[c] (COOH)	
trolox C						11.91[e]			

[a] Catechin is flavan-3-ol, without the keto group at C4. [b] Taken from ref 16. [c] Determined spectrophotometrically. Estimated to be accurate to ±0.01. [d] Morin has the second OH group at the 2'-position. [e] From ref 8.

Table 2. Rate Constants for the $Br_2^{\bullet-}$ Oxidation of the Flavonoids at pH 7

flavonoid, FH–OH	$k(Br_2^{\bullet-} + FH–OH)$,[a] $M^{-1} s^{-1}$
catechin	9×10^7
rutin	2.1×10^8
hesperidin	4.8×10^7

11.5.[18] The spectrum of one-electron-oxidized hesperidin did not show any appreciable absorption above 300 nm. This is similar to the previously published spectrum of hesperetin phenoxyl radicals.[18] The absorption spectra of hesperidin phenoxyl radicals did not change in the pH range from 3 to 14, as expected from the lack of ionizable groups in the B-ring. Being electron richer than ring A, the B-ring of flavonoids is an apparent target of any oxidant. Consequently, one-electron oxidation of hesperidin at pH 7 generates the "neutral" 3'-phenoxyl radical. The state of protonation of the A-ring did not appreciably influence the spectral characteristics of the hesperidin 3'-phenoxyl radical, which means that the pK_a values of the 5-OH group in the radical and in the parent compound are similar.

The spectra of phenoxyl radicals derived from rutin, catechin, and selected 3,4-dihydroxybenzoate derivatives change in the pH range from 3 to 7, giving rise to titration curves. These spectral changes were used to determine the dissociation constants of the phenoxyl radicals, which are summarized in Table 4. As an illustration, the spectra of various forms of rutin radicals and the pK_a curve derived from the pH-dependent absorbancy changes are given in Figure 1.

The dissociation constants of phenoxyl radicals derived from catechin and rutin (Table 4) are similar to those of the 3,4-dihydroxybenzoate and 3,4-dihydroxycinnamate radicals, as expected from their similar electronic structures. The absorption spectra of the 4'-phenoxyl radicals of rutin and catechin did not change appreciably in the pH range from 7 to 14. The 5- and 7-OH groups are too distant from and/or inefficiently coupled with the positions of higher unpaired spin density to influence the acid–base properties of the radical. Consequently, it may be suggested that the pK_a values of 5- and 7-OH in rutin and catechin are similar to those of the corresponding phenoxyl radicals.

(b) Reduction Potentials. The reduction potentials of the phenoxyl radicals derived from rutin, catechin, hesperidin, DOPA, 3,4-dihydroxycinnamic, and 3,4-dihydroxybenzoic acid were determined at pH 7 with reference to the trolox redox couple (E_7 = 0.48 V).[8] The procedure is given in detail elsewhere,[8,19] and only the highlights will be presented here. The phenoxyl radicals were generated by the $Br_2^{\bullet-}$-induced oxidations of the flavonoids and trolox in aqueous solutions at pH 7, 20 °C. Since 3'- or 4'- (or 4-) phenoxyl radicals derived from 3',4'- (or 3,4-) dihydroxyphenol derivatives either have very low absorbencies at 425 nm (the maximum of the trolox phenoxyl absorption), i.e. catechin, hesperidin, 3,4-dihydroxycinnamate, 3,4-dihydroxybenzoate, and DOPA radicals, or have high absorbencies at 450 nm (minimal absorption of the trolox phenoxyl), such as radicals from rutin, the equilibrium data were derived from the measurements at these two wavelengths. The results are summarized in Tables 3 and 4.

The reduction potentials of flavonoids depend strongly on the electron-donating properties of the substituents in the B-ring. The highest reduction potential, E_7 = 0.72 V, was measured for the hesperidin phenoxyl radical. In fact, this value is similar to E_7 = 0.73 V[19] for the 4-methoxyphenoxyl radical, which is also a monomethoxyphenoxyl radical. The reduction potentials of the phenoxyls from catechin and rutin are lower, because of the electron-donating 3'-O^- substituent. The reduction potential of

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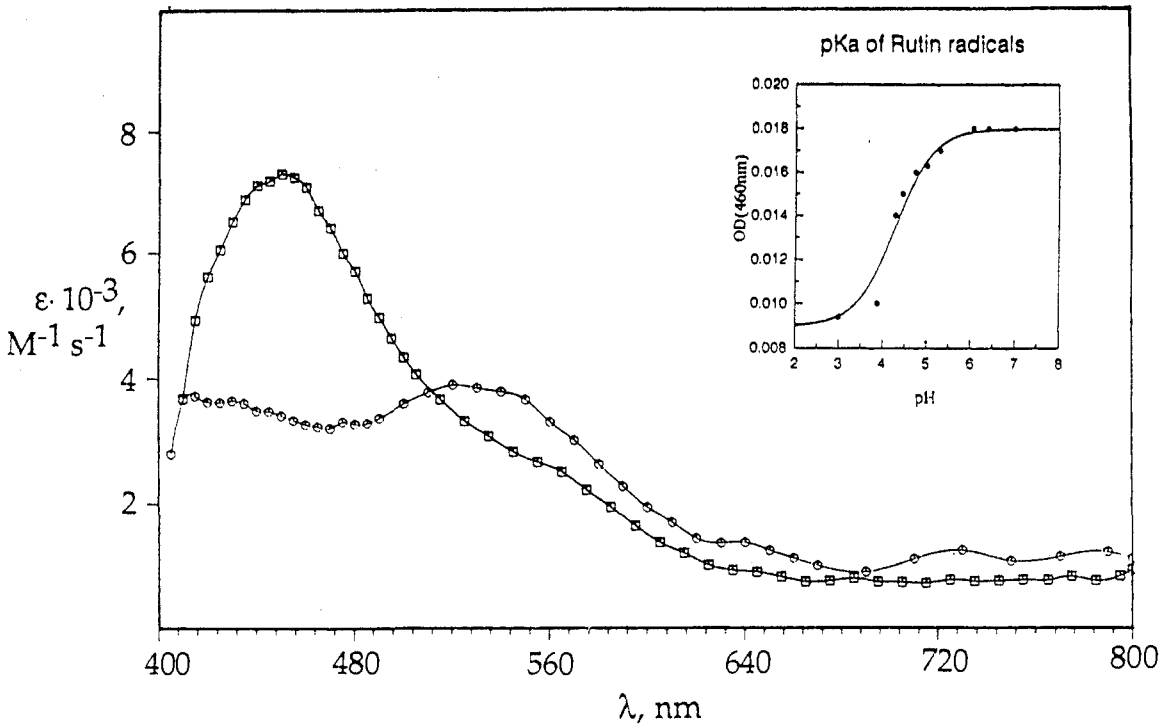

Figure 1. Absorption spectra of the rutin phenoxyl radicals, obtained upon $Br_2^{\bullet-}$ oxidation of rutin (0.1 M KBr, 1 mM rutin, dose/pulse 2 Gy): □, pH 7; ○, pH 3. Inset: absorbance at 460 nm as a function of pH. The solid curve is a fit for $pK_a = 4.3$.

Table 3. Electron-Transfer Reactions of Polyhydroxyphenoxyl Radicals Monitored by Pulse Radiolysis of Aqueous Solutions at pH 7, 20 °C

acceptor, A•, from	donor, D	k_f,[a] M^{-1} s^{-1}	k_4,[a] M^{-1} s^{-1}	K_{kin}	K_{abs}	ΔE,[b] V
catechin	trolox	2.0×10^8	6×10^6	33	33	0.09
rutin	trolox	3.6×10^6	4×10^4	90	80	0.12
hesperidin	trolox	1.9×10^8	irreversible	n.a.	n.a.	>0.2
hesperidin	rutin	5.0×10^7	4×10^5	125	83	0.12
3,4-dihydroxybenzoate	trolox	6.1×10^7	5×10^5	122	110	0.12
3,4-dihydroxycinnamate	trolox	2.6×10^5	3×10^4	9	9	0.06
DOPA	trolox	7.4×10^4	n.a.	n.a.	21	0.09

[a] Estimated to be accurate to ±10% for the reactions in favorable directions and to ±30% for the others. [b] Calculated from the arithmetic mean of the two equilibrium constants, using the Nernst equation, $\Delta E = 0.059 \log K$.

Table 4. One-Electron-Reduction Potentials and pK_a's of the Phenoxyl Radicals Determined by Pulse Radiolysis of Aqueous Solutions at pH 7, 20 °C

radicals from	pK_a	E_7(measd),[a] V	E_7(calcd),[b] V
hesperidin		0.72	
3,4-dihydroxybenzoic acid	4.2	0.60	0.58
rutin	4.3	0.60	
DOPA	4.5[c]	0.57	0.60
catechin	4.6	0.57	
3,4-dihydroxycinnamic acid	4.6	0.54	0.55

[a] Estimated to be accurate to ±0.02 V. [b] The reduction potential of phenoxyl radicals was calculated using the formula[19,21] $E_7 = 0.95 + 0.31\sum\sigma^+$. [c] This value is lower than the published[22] $pK_a = 4.7$. We believe that our value is more accurate since the published value was determined in the system where the DOPA semiquinone radical was generated by the •OH radical induced oxidation of DOPA in which the acid–base and electron-transfer equilibria are probably distorted due to the OH adducts present.

the catechin radicals, $E_7 = 0.54$ V, is 0.06 V lower than $E_7 = 0.6$ V of the rutin radicals, presumably because $\sigma^+(CH{=}CH_2) = -0.16$ at C1 in rutin is less negative than $\sigma^+(CH_3) = -0.3$ for catechin radicals.

The reduction potentials of the radicals derived from 3,4-dihydroxybenzene derivatives decrease with the electron-donating power at C1. For example, $E_7 = 0.60$ V of dihydroxybenzoic acid radicals ($\sigma^+ = 0$) is higher than $E_7 = 0.54$ V of dihydroxycinnamate radicals ($\sigma^+ = -0.16$). The opposite holds for pK_a values of the radicals, since more electronegative substituents decrease the proton-donating ability of the ionizable group, which results in increased pK_a.

On the basis of low $E_7 = 0.54$–0.7 V of the flavonoid and 3,4-dihydroxybenzene radicals, it is expected that corresponding parent compounds efficiently inactivate alkylperoxyl, $E_7 = 1.05$ V,[9] and superoxide/hydroperoxyl radicals, $E_7 = 0.94$ V.[20] (The latter value probably underestimates the reduction potential of the $O_2^{\bullet-}/O_2^{2-},H^+$ couple at pH 7, since it must be higher than the potential of the alkylperoxyl radical, $E_7 = 1.05$ V.[10]) However, the reduction potential of the trolox radical, $E_7 = 0.48$ V is lower, which means that the oxidation of vitamin E by flavonoid radicals is thermodynamically feasible. Flavonoids should act as antioxidants in the digestive tract. There is a very small probability of the reaction of flavonoid radicals with vitamin E, since very little vitamin E is present in the cell membranes of the digestive tract organs. On the other hand, the generation of various highly damaging oxyl radicals with $E > 1$ V, which may be efficiently inactivated by flavonoids, is highly probable during digestion.

Reaction with $O_2^{\bullet-}$. (a) The Method. The reactions of flavonoids with $O_2^{\bullet-}$ were monitored by kinetic conductivity in the following system: 0.26 M 2-propanol, a N_2O:O_2 = 4:1 mixture, pH 10. The reasons for doing the experiments at pH 10 were (a) the decay of $O_2^{\bullet-}$ at pH ≤10 is too fast for accurate measurements of slow reactions, such as those with flavonoids, and (b) the flavonoids are considerably more soluble at pH 10. The following set of reactions may be envisaged:

$$H_2O \rightsquigarrow OH, H, e_{aq}^-, H_3O^+, \ldots \quad (3)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \quad (4)$$

$$H_3O^+ + OH^- \rightarrow 2H_2O \quad (5)$$

$$^{\bullet}OH + CH_3CH(CH_3)OH \rightarrow H_2O + CH_3C^{\bullet}(CH_3)OH \quad (6)$$

$$CH_3C^{\bullet}(CH_3)OH + O_2 \rightarrow CH_3C(OO^{\bullet})(CH_3)OH \quad (7)$$

$$CH_3C(OO^{\bullet})(CH_3)OH + OH^- \rightarrow O_2^{\bullet-} + (CH_3)_2CO + H_2O \quad (8)$$

$$2O_2^{\bullet-} + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (9)$$

$$O_2^{\bullet-} + FVH\text{–}O^- \rightarrow O_2^{2-} + FVH\text{–}O^{\bullet} \quad (10)$$

$$O_2^{2-} + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (11)$$

$$OH^- + FVH\text{–}O^- \rightarrow H_2O + FV\text{–}O^{2-} \quad (12)$$

$$pK_a(H_2O_2) = 11.9$$

Upon generation of $O_2^{\bullet-}$ (reaction 8)

$$\Delta\kappa = \Lambda(O_2^{\bullet-}) - \Lambda(OH^-) \quad (13)$$

which means that the initial conductivity will be negative, because the equivalent conductivity of the superoxide anion is considerably lower than that of OH^-.[23] The superoxide radical is extremely long-lived in alkaline media, because of the low rate of reaction 9. In our system, the initial negative conductivity decayed within 100 s for doses in the range from 0.1 to 1.5 Gy/pulse. This enables determination of reaction rates of the superoxide higher than 0.1 s^{-1} (i.e., at the concentration of 1 mM solute, rate constants of ≥ 100 M^{-1} s^{-1} for reaction of $O_2^{\bullet-}$ with solute can be determined).

The flavonoids are ionized at pH 10, in either the A-ring or the B-ring (for a complete list of the pK_a values, see Materials and Methods). Consequently, their reaction with $O_2^{\bullet-}$ (reaction 10) results in the reduction of superoxide and the formation of the flavonoid phenoxyl radical and hydrogen peroxide dianion. At pH 10, the hydrogen peroxide dianion reacts with water, or any other proton donor, to give hydrogen peroxide (reaction 11). Because of the buffering capacity of most flavonoids at pH 10, the hydroxide ion formed is exchanged with the flavonoid ion (reaction 12). The overall conductivity change is

$$\Delta\kappa = \Lambda(FV\text{–}O^{2-}) - 2\Lambda(FVH\text{–}O^-) \approx 0 \quad (14)$$

which is indeed detected for trolox, galangin, hesperetin, and hesperidin.

Phenoxyl radicals derived from the 3′,4′-dihydroxyflavonoids deprotonate at pH 10, because the pK_a of the 3′-hydroxy group is typically 4–5 (for pK_a values, see previous section)

$$FVH\text{–}O^{\bullet} + OH^- \rightarrow FV\text{–}O^{\bullet-} + H_2O \quad (15)$$

The overall conductivity change is negative, because of relatively higher equivalent conductivity of the hydroxide ion

$$\Delta\kappa = \Lambda(FV\text{–}O^{2-}) + \Lambda(FV\text{–}O^{\bullet-}) - 2\Lambda(FVH\text{–}O^-) - \Lambda(OH^-) \approx \Lambda(FV\text{–}O^{\bullet-}) - \Lambda(OH^-) \quad (16)$$

as detected for the reactions with rutin, quercetin, catechin, fisetin, and morin.

Most flavonoids have one pK_a value in the pH range from 9 to 11 (see Table 1), which means that they act as weak buffers at pH 10. The 2-propanol peroxy radical may react with any base to eliminate the superoxide radical.[24] Consequently, the concentrations of flavonoids were adjusted to minimize the buffering effect in the rate determinations. Such an effect was not observed in the formate system (10 mM NaOOCH, N_2O:O_2 = 4:1 mixture, pH 10), where superoxide was produced upon the direct electron transfer from the formate radical ion to oxygen. However, in the presence of 10 mM NaOOCH, the ac bridge is difficult to balance, and the rate of the superoxide decay is higher. In most cases, the use of the 2-propanol system to generate the superoxide radical is more advantageous.

Table 5. Rate Constants for the Reduction of the Superoxide Radical by Flavonoids and Phenols, Determined by Pulse Conductivity in Aqueous Solutions at 20 °C, pH 10

phenol derivative, Phe–OH	$k(O_2^{\bullet-} + Phe\text{–}OH)$,[a] M^{-1} s^{-1}	E_7,[b] V
catechin	1.8×10^4	0.57
4′,5,7-trihydroxyflavanone	$\sim 3 \times 10^2$	
fisetin	1.3×10^4	
quercetin	4.7×10^4	0.6
rutin	5.1×10^4 [c]	0.6
hesperetin	5.9×10^3	
hesperidin	2.8×10^4 [c]	0.72
kaempferol	2.4×10^3	~0.95
morin	1.6×10^3	~0.95
galangin	8.8×10^2	
3,4-dihydroxybenzaldehyde	1.1×10^4	0.83
4-methoxyphenol	1.8×10^4	0.73
trolox	5.8×10^3 [d]	0.48

[a] Estimated to be accurate to ±25%. At ionic strength μ = 0.5–1 mM unless otherwise stated. [b] The reduction potential of the phenoxyl radicals at pH 7. Calculated using the formula[19,21] $E_7 = 0.95 + 0.31\Sigma\sigma^+$, and σ^+(flavone) = 0 and σ^+(flavanone) = –0.1. [c] Ionic strength was μ = 1–3 mM. [d] Ionic strength was μ = 5 mM, but the rate was unaffected by the variation in the salt concentration, since trolox was not ionized at pH 10 (pK_a = 11.9).[8]

Table 6. Yields of Hydrogen Peroxide Determined upon γ Radiolysis of Aqueous Solutions of 10 mM NaOOCH and 0.5 mM Flavonoid at pH 7 and of 0.26 M 2-Propanol and 0.5 mM Flavonoid at pH 10, 20 °C

	$G(H_2O_2)$ at pH 7		$G(H_2O_2)$ at pH 10	
flavonoid, F	KI	Ti	KI	Ti
rutin	6.25	6.02	7.10	5.76
quercetin	6.18	6.05	7.24	6.41
catechin	6.28	5.98	7.16	6.21
morin	6.18	5.88	7.09	6.21
hesperidin	6.31	6.21	6.89	6.02
galangin	6.08	5.63	7.29	6.34
NaOOCH	3.69	3.75	n.m.	n.m.
2-propanol	n.m.	n.m.	3.90	3.88

(b) Kinetics and Mechanism. The rate constants for the reactions of the superoxide radicals with various flavonoids and some phenol derivatives determined by the kinetic conductivity method described above are summarized in Table 5. The radiation chemical yields of hydrogen peroxide determined upon γ radiolysis of aqueous solutions of flavonoids at pH 7 and 10 are presented in Table 6.

The reactions of $O_2^{\bullet-}$ with the flavonoids were monitored as the increase of the initially negative conductance change, which is ascribed to the formation of the superoxide radical (reaction 8). In order to verify that the observed conductance change corresponds to the ***reduction*** of the superoxide radical, we measured the yield of hydrogen peroxide (Table 6). In our system, the radiation yield of the superoxide radical equals the total radical yield; that is, $G(O_2^{\bullet-}) = G_{e^-} + G_{OH} + G_H = 2.7 + 2.8 + 0.55 = 6.05$. If superoxide is unreactive with flavonoids, as reported previously,[4,18] then $G(H_2O_2) = G_{H_2O_2} + {}^1/_2G(O_2^{\bullet-}) = 0.7 + {}^1/_2 \times 6.05 = 3.7$, where $G_{H_2O_2} = 0.7$ is the molecular yield of hydrogen peroxide. In the ***oxidation*** of superoxide, that is when an oxidant

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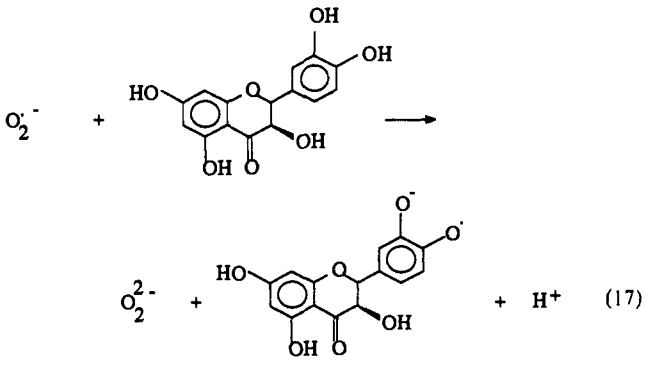
(24) Illan, Y.; Rabani, J.; Henglein, A. *J. Phys. Chem.* **1976**, *80*, 1558.

accepts an electron from the superoxide radical, $O_2^{\bullet-}$ is converted to oxygen and only molecular hydrogen peroxide (produced in spurs from the recombination of •OH radicals) may be measured; i.e., $G(H_2O_2) = G_{H_2O_2} = 0.7$. In the ***reduction*** of $O_2^{\bullet-}$, when an antioxidant donates an electron to superoxide (e.g., reactions 10 and 11), $G(H_2O_2) = G_{H_2O_2} + G(O_2^{\bullet-}) = 0.7 + 6.05 = 6.75$. Since $G(H_2O_2) \approx 6.7$ was measured in all reactions, it may be concluded that flavonoids ***reduce*** (antioxidant action) the superoxide radical in the pH range from 7 to 10.

The reactivities of flavonoids with the superoxide radical (Table 5) apparently depend on their redox properties, which were highly sensitive to the substitution of the B-ring. The highest rates of reaction of the superoxide radical are determined for 3′,4′-dihydroxyflavones, quercetin and rutin, whereas the lowest are observed for monohydroxy-substituted kaempferol and 4′,5,7-trihydroxyflavanone. The low rate of the superoxide reaction with galangin, which is unsubstituted in the B-ring, $k = 8.8 \times 10^2$ M^{-1} s^{-1}, apparently reflects an inferior antioxidant ability of the A-ring. The great difference among the rates of $O_2^{\bullet-}$ reactions with tetrahydroxyflavones, quercetin, and morin originates from substituent effects on their redox properties. Because of unfavorable meta-hydroxy substitution, $k(O_2^{\bullet-} + \text{morin}) = 1.6 \times 10^3$ as compared with $k(O_2^{\bullet-} + \text{quercetin}) = 4.7 \times 10^4$ M^{-1} s^{-1}.

In addition to the redox properties, the reactivity of superoxide with the flavonoids depends on their charge. For example, the rate constant for the reaction of superoxide with uncharged catechin at pH 7 (determined by optical pulse radiolysis)



$$k = (6.6 \pm 0.6) \times 10^4\ M^{-1}\ s^{-1} \qquad (17)$$

is approximately 4 times higher than the corresponding rate constant at pH 10, $k = 1.8 \times 10^4$ M^{-1} s^{-1}, where catechin is doubly negatively charged (see Table 1). Furthermore, $k = 1.1 \times 10^4$ M^{-1} s^{-1} for the reaction with singly negatively charged 3,4-dihydroxybenzaldehyde is similar to the rate constant for catechin oxidation, despite the ~0.3 V higher reduction potential of the 3,4-dihydroxybenzaldehyde phenoxyl (Table 5). Electrostatic repulsion of the negatively charged superoxide and deprotonized flavonoids apparently results in the decrease of the reaction rate constant by the factor of 2 for each additional negative charge in a flavonoid.

It is, however, more difficult to understand the observed difference between the rates of the $O_2^{\bullet-}$ reactions with flavonoids and phenols, such as 4-methoxyphenol and trolox. In spite of their relatively higher redox potentials (see Table 5) and unfavorable electrostatic effect (most flavonoids are either singly or doubly negatively charged at pH 10, while trolox is uncharged), flavonoids and 4-methoxyphenolate react faster with superoxide than trolox. Moreover, the rate constants are quite low, $k = 3 \times 10^2$–10^5 M^{-1} s^{-1}, which is surprising on the basis of the magnitude of the driving force $\Delta E > 0.4$ V for these reactions. This redox potential difference was calculated by assuming that the reduction potential of the superoxide radical is at least 0.02 V higher than $E_{10} = 0.78$ V[10] of the methylperoxyl radical. To understand these effects better, the activation parameters of the reactions of the superoxide radical with rutin and trolox at pH 10 and with methyl gallate at pH 7 were determined, respectively, by the pulse conductivity and optical pulse radiolysis of aqueous solutions. The results are summarized in Table 7.

Table 7. Activation Parameters of the Reactions of the Superoxide Radical with Rutin and Trolox Determined by Pulse Conductivity at pH 10 and with Methyl Gallate Measured by Optical Pulse Radiolysis at pH 7

substrate	k, M^{-1} s^{-1} (t = 20 °C)	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol K)	E_a, kcal/mol
rutin	5.1×10^4	3.6	−25	4.5
trolox	5.8×10^3	3.6	−28	4.5
methyl gallate	2.4×10^5	2.3	−26	2.9

The mechanism of the reaction of superoxide with the various phenol derivatives is ***one-electron transfer*** with concerted proton transfer in the transition state.[11] This is concluded from the negative activation entropy, which is a consequence of the solvent reorganization to accommodate the proton transfer in the transition state of electron transfer. That the reaction is entropy controlled is evident from the low activation enthalpies. As a further example, an order of magnitude difference in the rates of oxidation of trolox and rutin originates from the more negative activation entropy in the case of the reaction with trolox. It is possible that this more negative activation entropy is a consequence of steric hindrance for the reaction of superoxide with the phenoxy group in trolox (fully substituted phenol ring). A similar entropy-controlled electron-transfer mechanism was proposed[10] for the reactions of alkylperoxyl radicals.

Conclusions

The acid–base and redox properties of flavonoids render them convenient biochemical antioxidants. Because of their relatively low pK_a's (4–5), the radicals derived from 3′,4′-dihydroxyflavonoids are negatively charged at pH 7, which should effectively retard both their reaction with and passage through the negatively charged phospholipids in cell membranes. The reduction potentials of the flavonoid radicals, $E_7 = 0.5$–0.7 V, are higher than that of trolox, $E_7 = 0.48$ V,[8] which means that their reaction with vitamin E is thermodynamically feasible. However, since flavonoids are expected to act as antioxidants in the digestive tract, where the concentration of vitamin E is small, there is very little chance for such reaction. On the other hand, the reduction potentials of flavonoid radicals are lower than those of alkylperoxyl radicals, $E_7 = 1.05$ V,[10] and the superoxide radical, $E_7 = 0.94$ V,[20] which means that flavonoids may inactivate these damaging oxyl species and prevent deleterious consequences of their reactions.

The ac kinetic conductivity method was developed for the determination of the low rate constants for reactions of superoxide radicals. Superoxide is generated selectively upon pulse irradiation of a 4:1 N_2O/O_2-saturated aqueous solution of 0.26 M 2-propanol at pH 10. In the absence of any reactive solutes, $O_2^{\bullet-}$ decays within 100 s. This enables the measurement of reaction rates of the order of 0.1 s^{-1}. Providing that the ionic strength is kept below 10 mM, this method is applicable to intensely colored flavonoid solutions, which are otherwise difficult or impossible to handle by optical pulse radiolysis.

The flavonoids reduce the superoxide radical in the pH range from 7 to 10. Albeit slow, these reactions constitute an efficient inactivation of the superoxide radical to produce hydrogen peroxide and the flavonoid radicals. The mechanism of these reactions appears to be electron transfer with concerted proton transfer. This mechanism of the superoxide inactivation is similar to that of the reactions of the alkylperoxyl radicals,[10,11] which emphasizes the entropy control in one-electron-transfer reactions of peroxyl radicals.

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