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Redox Reactions of the Urate Radical/Urate Couple with the Superoxide Radical Anion, the Tryptophan Neutral Radical and Selected Flavonoids in Neutral Aqueous Solutions

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The kinetics of several processes involving the potential antioxidant role of urate in physiological systems have been investigated by pulse radiolysis. While the monoanionic urate radical, $^{\bullet}UH^{-}$, can be produced directly by oxidation with $^{\bullet}Br_2^{-}$ or $^{\bullet}OH$, it can also be generated by oxidation with the neutral tryptophan radical, $^{\bullet}Trp$, with a rate constant of 2×10^7 $M^{-1}s^{-1}$. This radical, $^{\bullet}UH^{-}$, reacts with $^{\bullet}O_2^{-}$ with a rate constant of 8×10^8 $M^{-1}s^{-1}$. Also, $^{\bullet}UH^{-}$ is reduced by flavonoids, quercetin and rutin in CTAB micelles at rate constants of 6×10^6 $M^{-1}s^{-1}$ and 1×10^6 $M^{-1}s^{-1}$, respectively. These results can be of value by providing reference data useful in further investigation of the antioxidant character of urate in more complex biological systems.

Keywords: Antioxidants, electron transfer, flavonoids, micelles, pulse radiolysis, tryptophan

INTRODUCTION

The deleterious effects of reactive oxygen species and their involvement in many pathologies, have been firmly established in appropriate model systems and *in vivo*.[1–3] The optimization of a natural defense system has thus been a critical development during the evolutionary processes of life. To this end, antioxidants have emerged to act as effective inhibitors of free radical reactions in biological systems through repair of harmful radicals or through the scavenging of oxyradicals and other activated oxygen species.[4,5] In addition to enzymatic detoxification, there exists

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for humans two categories of small antioxidant molecules that must be taken into consideration: (a) those antioxidants introduced by dietary uptake, such as vitamins E and C, carotenoids and flavonoids; (b) constitutive antioxidant molecules such as glutathione or uric acid, a hydrophilic metabolite of purine breakdown.[6] In this regard, the role of urate as an antioxidant remains unclear.

The efficacy of urate as an antioxidant is supported by its fairly high concentration (about 0.3 mM) in plasma and its excellent reactivity with singlet oxygen, as well as with the hydroxyl radical, $^{\bullet}OH$.[7] However, the urate radical seems to be fairly unreactive toward diatomic oxygen[8] and its antioxidative properties have been questioned on the grounds that urate sensitizes the free-radical inactivation of alcohol dehydrogenase.[9] Mechanisms by which uric acid may exhibit antioxidant activity have been investigated by several techniques, including pulse radiolysis[8] and electron spin resonance.[10] A one-electron redox potential of 0.59 V at neutral pH has been determined by pulse radiolysis[8] and the resulting urate anion radical, $^{\bullet}UH^{-}$, has been found to have an unpaired electron located primarily on the five-membered ring of the purine structure.[10]

It has recently been shown by some of us that depletion of plasma urate can be correlated with copper-induced peroxidation of plasma lipoproteins.[11] To better understand the antioxidative properties of plasma urate, we have extended pulse radiolysis studies to reactions involving urate with two putative species that may be formed during the *in vivo* lipoprotein oxidation, namely $^{\bullet}O_2^{-}$[12] and the tryptophan neutral radical resulting from the oxidation of Trp residues in apolipoproteins.[13–14] Additionally, we have monitored repair of $^{\bullet}UH^{-}$ by several flavonoids: quercetin, rutin and catechin, which have been shown to protect uric acid from the copper-induced oxidation in plasma.[11] It is found that the $^{\bullet}$Trp radical readily oxidizes urate to produce $^{\bullet}UH^{-}$ and that this radical reacts at a diffusion controlled rate with the $^{\bullet}O_2^{-}$ radical anion. Further, it is shown that of the flavonoids mentioned, $^{\bullet}UH^{-}$ is repaired only by quercetin and rutin with the concomitant formation of the flavonoid semi-reduced species.

EXPERIMENTAL

All chemicals were analytical grade and were used as received. Uric acid (UH_2), quercetin, catechin, rutin, tryptophan (Trp), cetyltrimethylammonium bromide (CTAB) and superoxide dismutase (SOD) from bovine erythrocytes were purchased from SIGMA (St. Louis, Mo, USA). The 10 mM phosphate buffer (pH 7.4) was prepared in pure water obtained with a milli Q system provided by Millipore. Solutions were saturated with pure N_2O or O_2.

Pulse Radiolysis

Pulse radiolysis measurements were carried out with the Notre Dame Radiation Laboratory 8-MeV linear accelerator, which provides 5 ns pulses of up to 30 Gy. In general, the doses used here were approximately 5–10 Gy. The principles of the detection system have been previously described.[15–16] A Corning O-51 optical filter, removing all wavelengths shorter than 350 nm, was placed in the analyzing light beam whenever possible.

Radical concentrations calculated from transient absorption data are referenced to $^{\bullet}(SCN)_2^{-}$ dosimetry. The extinction coefficient for $^{\bullet}(SCN)_2^{-}$ is taken to be $7580 \pm 60\ M^{-1}cm^{-1}$ at 472 nm, and the G value for $^{\bullet}OH$ in N_2O-saturated solution has been measured as 6.13 ± 0.09.[17] The G value is the number of radicals generated per 100 eV of absorbed energy, and such numbers may be recast as radical concentrations per unit radiation (e.g., a G value of 6.1 corresponds to a concentration of 6 μM/10 Gy).

All solutions were prepared with 0.1 M Br^{-} unless otherwise stated. Where necessary, differ-

ences in radical G value, arising from the dependence of spur scavenging on Br^- concentration, are taken into account. Additionally, production of superoxide radical by oxygen scavenging of H• is included in calculating the G value for $•O_2^-$. Numerical integrations carried out in analyses of rate data were conducted using the *Scientist* software from Micromath Scientific Software.

RESULTS AND DISCUSSION

Spectral and Kinetic Properties of the Urate Radical

In plasma at physiological pH, urate is a monoanion, designated by UH_2^-, with a pKa of 5.4 and bears a single negative charge on the oxygen linked to the C_8 carbon of the purine structure.[8] In pH 7.4 phosphate buffer, urate, UH_2^-, may be oxidized by $•Br_2^-$ or $•(SCN)_2^-$ radical anions,[8] leading to the urate radical monoanion, $•UH^-$, by loss of an electron from the negatively charged oxygen atom followed by loss of H^+, according to:

$$UH_2^- + •Br_2^- \rightarrow •UH_2 \qquad \text{(Ia)}$$

$$•UH_2 \rightarrow •UH^- + H^+ \qquad \text{(Ib)}$$

Oxidation by $•Br_2^-$ proceeds with a rate constant of 8.3×10^8 $M^{-1}s^{-1}$[8] and the ensuing $•UH^-$ spectrum is characterized by a transient absorption maximum at 320 nm with a shoulder at 370 nm.[8]

Urate is also known to react with •OH with a rate constant of 7×10^9 $M^{-1}s^{-1}$.[7] Under our experimental conditions, a rate constant of 4.3×10^9 $M^{-1}s^{-1}$ was determined. Figure 1 presents the transient absorption spectrum observed immediately after •OH reaction. It may be seen that this spectrum superimposes on that obtained by $•Br_2^-$ reaction. Assuming G values of 6.1 for $•UH^-$ generated by $•Br_2^-$ or •OH in N_2O-saturated solutions, a molar extinction coefficient of

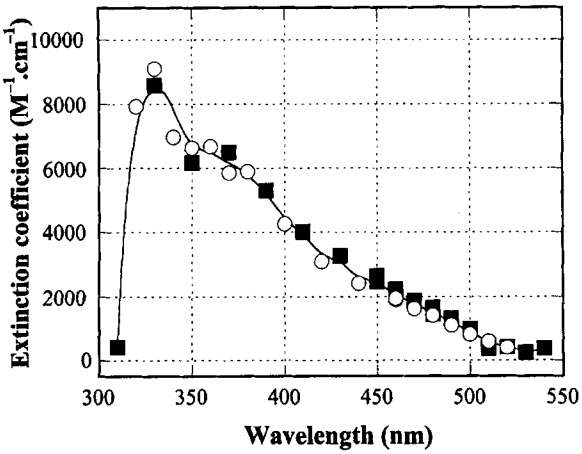

FIGURE 1 Transient absorption spectrum obtained at 22 °C immediately after pulse radiolysis of 1 mM urate in N_2O-saturated, 10 mM pH 7.4 phosphate buffer in the absence (○) or in the presence (■) of 0.1 M KBr. Dose per pulse was about 8 Gy.

$8{,}200 \pm 200$ $M^{-1}cm^{-1}$ can be estimated at 325 nm. The radical undergoes bimolecular decay and the rate constants are given in Table I. In neutral aqueous solutions, the reaction of •OH radicals with monoanionic urate could in principle occur either by hydrogen atom abstraction or by •OH radical addition reactions leading to species differing from that formed by one electron oxidation.[8,10] However, the present data suggest that the predominating reaction is, indeed, the hydrogen atom abstraction or the •OH addition to the urate ring followed by water elimination catalyzed by phosphate

$$UH_2^- + •OH \rightarrow •UH^- + H_2O \qquad \text{(II)}$$

leading to the same radical bearing a single negative charge at pH 7 as that obtained with the $•Br_2^-$ attack.[8] Indeed, it may be seen that the bimolecular reaction rate constants for the decay of the urate radical formed by •OH ($k = 2.5 \times 10^8$ $M^{-1}s^{-1}$) and by $•Br_2^-$ ($k_1 \sim 3.4 \times 10^8$ $M^{-1}s^{-1}$: see below) are very similar; the somewhat higher value for that from $•Br_2^-$ is consistent with the higher ionic strength of the Br^- containing solutions.

TABLE I Decay rate constant of urate radical under different experimental conditions

Conditions	Dose (Gy)	Reaction rate constants	
		$2k_1$ ($\times 10^{-8}$ $M^{-1}s^{-1}$)	k_2 ($\times 10^{-8}$ $M^{-1}s^{-1}$)
N_2O, no KBr*	8.1	2.5	–
N_2O*	3.6	3.4	–
N_2O**	21	4.2	–
N_2O, 10 mM CTAB*	8.5	11.5	–
O_2**	8.1	–	8.3
O_2**	4.3	–	4.0
O_2, 20 μM SOD*	7.5	3.2	–

k_1 and k_2 are the rate constants defined in Equation (1). Unless otherwise stated all the solutions contained 0.1 M KBr and 1 mM (*) or 100 μM (**) uric acid in pH 7.4 phosphate buffer. Solutions were saturated with N_2O or O_2 as indicated in the table. Temperature was 22 °C.

Urate Radical Generated by Repair of the Neutral Tryptophan Radical, •Trp

Copper-induced LDL oxidation is accompanied by a rapid destruction of the 37 Trp residues of apo B.[13,14] Formation of the •Trp radical has been suggested to be a key intermediate in this process. Since urate is present in plasma, it is interesting to look at a possible repair of •Trp by urate to produce the urate radical. Here, the •Trp radical was generated in N_2O-saturated pH 7.4 buffered aqueous solutions by reaction of 5 mM Trp with $^{\bullet}Br_2^-$.[18] The one-electron oxidation of Trp by $^{\bullet}Br_2^-$ occurs with a rate constant of 7×10^8 $M^{-1}s^{-1}$.[18] Therefore under our experimental conditions, essentially all $^{\bullet}Br_2^-$ reacts with Trp within a microsecond. The •Trp radical is characterized by a transient absorption at 520 nm with a molar extinction coefficient of 1750 $M^{-1}s^{-1}$.[19] In the absence of urate, the •Trp decays by second order kinetics at a rate of 6×10^8 $M^{-1}s^{-1}$ (data not shown). Addition of 100–500 μM uric acid provokes a pronounced acceleration of the •Trp decay which becomes pseudo-first order and is accompanied by a corresponding growth of the strong $^{\bullet}UH^-$ radical transient absorbance at 360 nm (data not shown). Figure 2 shows the decay of the •Trp radical in the absence and in the presence of 500 μM urate demonstrating the repair of •Trp by urate.

$$^{\bullet}Trp + UH_2^- \rightarrow Trp + {}^{\bullet}UH^- \qquad (III)$$

In the insert of Figure 2, the apparent first order rate constant of •Trp decay is plotted as a function of urate concentration. From this plot, a bimolecular reaction rate constant of 2×10^7 $M^{-1}s^{-1}$ can be determined. It should be noted that the occurrence of Reaction (III) is consistent with the redox potential of the •Trp/Trp couple which has been determined as 0.98 V at pH 7,[19] e.g., a value almost 0.4 V higher than that of the $^{\bullet}UH^-/UH_2^-$ couple.

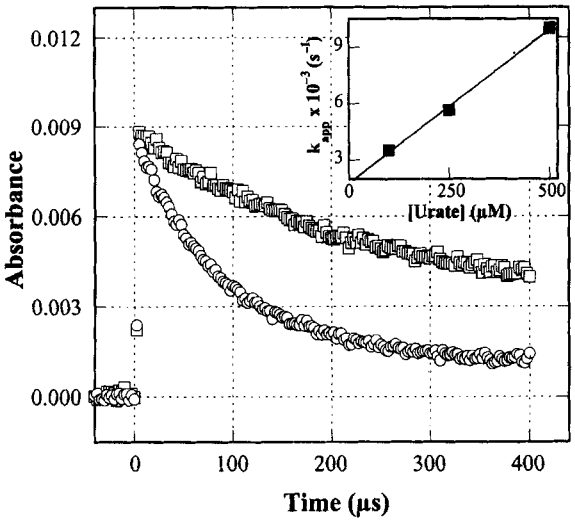

FIGURE 2 Decay of the •Trp radical measured at 520 nm at 22 °C after pulse radiolysis of 5 mM Trp (□) or 5 mM Trp + 500 μM urate (○) in N_2O-saturated, 10 mM pH 7.4 phosphate buffer containing 0.1 M KBr. Dose per pulse was about 8 Gy. (Insert) Apparent pseudo first order rate constant for the •Trp radical decay measured at 520 nm as a function of the urate concentration.

Reaction of the Urate Radical with the Superoxide Radical Anion

Superoxide radical is associated with various deleterious processes and, as mentioned above, its formation is implicated in lipoprotein LDL oxidation. This radical anion is produced *in vivo* by circulating phagocytic cells in plasma as part of their natural function.[12] Additionally, recent studies demonstrate that urate is almost as effective as the vitamin E analogue, Trolox and more effective than ascorbic acid in inhibiting LDL peroxidation.[20] It is of interest, then, to examine interactions between these two species, $^{\bullet}O_2^-$ and urate. It has been reported that the $^{\bullet}UH^-$ radical does not react with oxygen.[8] However it has not previously been determined whether this radical can react with $^{\bullet}O_2^-$ although the redox potential of the $^{\bullet}UH^-/UH_2^-$ couple is 0.59 V, well above the $O_2/^{\bullet}O_2^-$ redox potential of −0.33 V[8] which renders the "repair" of urate radical by $^{\bullet}O_2^-$ thermodynamically favorable. Willson *et al.* found[9] that $^{\bullet}O_2^-$ inactivates yeast alcohol dehydrogenase in the presence of urate. They proposed that $^{\bullet}O_2^-$ reacts with uric acid to yield urate-derived free radicals. To elucidate the fate of $^{\bullet}O_2^-$ in the presence of urate, radical behavior in O_2-saturated buffered solutions containing 0.1–1 mM urate and 0.1 M Br^- was studied by pulse radiolysis. Under these experimental conditions, $^{\bullet}OH$ radicals are scavenged by Br^- ions leading to $^{\bullet}Br_2^-$ (radiolytic yield 2.7) and subsequently, $^{\bullet}UH^-$. By contrast, hydrogen atoms and hydrated electrons, e_{aq}^-, react with oxygen[7] leading to $^{\bullet}O_2^-$ formation (radiolytic yield 3.3). The decay kinetics in Figure 3 demonstrate that $^{\bullet}UH^-$ definitely reacts with $^{\bullet}O_2^-$ since the fast decay rate is eliminated by the presence of 20 μM SOD in the solution. Kinetic data for this reaction were analyzed assuming involvement of the reactions:

$$^{\bullet}UH^- + {}^{\bullet}UH^- \rightarrow \text{product(s)} \quad (\text{rate: } k_1\ M^{-1}s^{-1}) \qquad (IV)$$

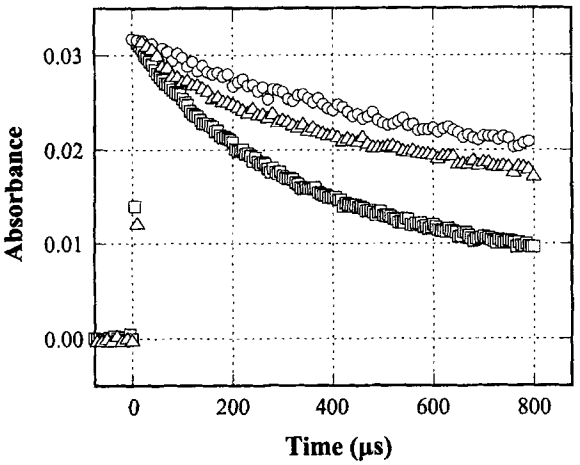

FIGURE 3 Decay of $^{\bullet}UH^-$ transient absorption recorded at 350 nm at 22 °C. Solution contained 1 mM urate in 10 mM pH 7.4 phosphate buffer with 0.1 M KBr: (□), oxygen saturated solution; (△), oxygen saturated solution containing 20 μM SOD. For comparison, a portion of the transient decay obtained in N_2O-saturated solution is included (○). This portion commences at the point where [$^{\bullet}UH^-$] corresponds to the initial [$^{\bullet}UH^-$] obtained in O_2-saturated solutions. Doses per pulse were about 8 Gy under all conditions.

$$^{\bullet}UH^- + {}^{\bullet}O_2^- \rightarrow \text{repair or product formation} \quad (\text{rate: } k_2\ M^{-1}s^{-1}) \qquad (V)$$

and according to the differential equation,

$$-d[^{\bullet}UH^-]/dt = 2k_1[^{\bullet}UH^-]^2 + k_2[^{\bullet}UH^-]\,[^{\bullet}O_2^-] \qquad (1)$$

In Equation (1), all radical concentrations were calculated from their initial G values using *Scientist* software for numerical integration. The k_1 value was determined separately using kinetic data from parallel experiments carried out with N_2O-saturated solutions. Results of the calculations are given in Table I. They show that the reaction of $^{\bullet}UH^-$ with $^{\bullet}O_2^-$ is fast but occurs at a rate somewhat lower than the value of 2×10^9 $M^{-1}s^{-1}$ obtained for the reaction of $Trp^{\bullet}$ radical with $^{\bullet}O_2^-$.[21] The quality of the fit between experimental and calculated decays is illustrated by Figure 4. With 1 mM uric acid, some limited scavenging of e_{aq}^- by urate may take place since the reaction rate constant of e_{aq}^- with uric acid

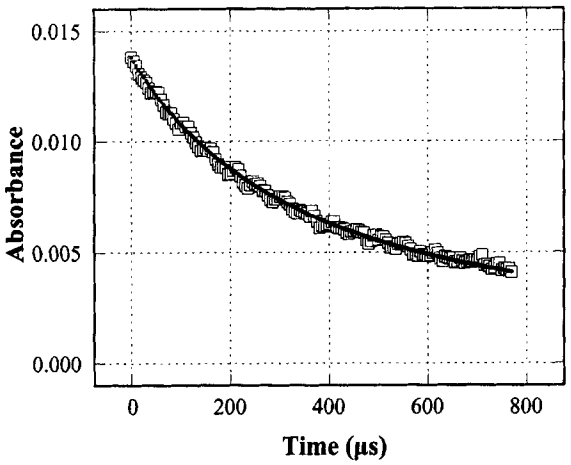

FIGURE 4 Decay of the $^{\bullet}UH^{-}$ radical measured at 350 nm obtained at 22 °C after pulse radiolysis of 1 mM urate in O_2-saturated, 10 mM pH 7.4 phosphate buffer containing 0.1 M KBr. The continuous line is the fit of the decay using Equation (1), where $2k_1 = 4.9 \times 10^8\ M^{-1}s^{-1}$ and $k_2 = 8.8 \times 10^8\ M^{-1}s^{-1}$. Dose per pulse was about 8 Gy.

at pH 5 (where the uncharged but not the negatively charged monoanonic species predominate) is about 1/3 of the reaction rate constant for the reaction of e^-_{aq} with O_2.[7] However the measured reaction rate constants for $^{\bullet}UH^{-}$ reaction with $^{\bullet}O_2^-$ or the simple second order reaction rate constant for the decay of $^{\bullet}UH^{-}$ in N_2O-saturated solution were similar under all urate concentration conditions (Table I). It should be noted that the rate constant for the decay of $^{\bullet}UH^{-}$ in CTAB micelles is significantly higher than that observed in plain buffer (Table I). Under these conditions, $^{\bullet}UH^{-}$ is most likely bound to the positively charged headgroup region of the micelles thereby localizing the reactive species as well as decreasing the net repulsion between negative charged radicals during Reaction (I). The bimolecular nature of the kinetics both in N_2O and O_2-saturated solutions was confirmed by the dose dependence of the second order decays. Thus, the reaction rate constants, k_1 and k_2, calculated from these decays by means of Equation (1) were all in good agreement within the expected experimental reproducibility (Table I).

Our results complement but do not contradict those of Simic and Jovanovic[8] since under their experimental conditions, using a gas mixture of N_2O and O_2, they could not form $^{\bullet}O_2^-$. Moreover, the "prooxidant activity" postulated by Willson and co-workers to be responsible for the inactivation of yeast alcohol dehydrogenase,[9] could be produced by Reaction (V) and not by reaction of urate with $^{\bullet}O_2^-$.

Reaction of the Urate Radical with Quercetin and Rutin

Since flavonoids such as quercetin, catechin and rutin are known to protect plasma urate from oxidation, it is of interest to investigate the possible occurrence of repair of urate radical species by such molecules.[11] The antioxidant properties of these flavonoids are well-established and their redox potential at pH 7 have been determined by pulse radiolysis to be 0.57, 0.6 and 0.33 V for catechin, rutin and quercetin, respectively.[22] Thus, in theory, only quercetin could repair $^{\bullet}UH^{-}$ for which the redox potential has been measured at 0.59 V.

We investigated the electron transfer from 500 μM catechin, rutin and quercetin to $^{\bullet}UH^{-}$ in N_2O-saturated pH 7.4 buffered aqueous solutions containing 0.1 M Br^- and 5 mM uric acid. Only catechin was soluble in plain pH 7.4 buffer. Therefore, in the case of rutin and quercetin, 10 mM CTAB was added to the buffer to solubilize these two flavonoids. In CTAB micelles, uric acid reacts at a rate constant of $8.4 \times 10^8\ M^{-1}s^{-1}$ with $^{\bullet}Br_2^-$ to form the $^{\bullet}UH^{-}$ radical stoichiometrically. This radical can decay by second order kinetics with a rate constant of about $1 \times 10^9\ M^{-1}s^{-1}$ (Table I). Figure 5 shows that upon addition of 500 μM quercetin, the decay of the transient absorption of the $^{\bullet}UH^{-}$ recorded at 360 nm is accompanied by a growth of transient absorption for semi-reduced quercetin at 600 nm. This wavelength of observation was chosen[22] because the $^{\bullet}UH^{-}$ radical does not absorb beyond 500 nm. Similar observations

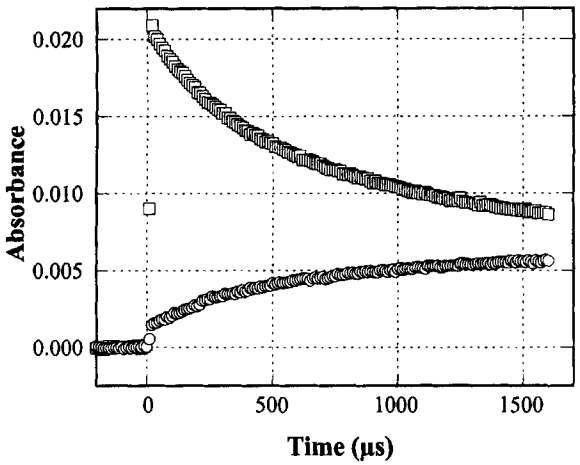

FIGURE 5 (□) Decay of the $^{\bullet}UH^{-}$ radical measured at 360 nm obtained at 22 °C after pulse radiolysis of 1 mM uric acid in O_2-saturated, 10 mM pH 7.4 phosphate buffer containing 0.1 M KBr, 10 mM CTAB and 500 μM quercetin. (○) is the corresponding growth of the semi-reduced quercetin radical measured at 600 nm.

TABLE II Rate constant (k_r) for the repair of the urate radical by flavonoids at pH 7.4 in CTAB micelles

Flavonoids	k_r ($M^{-1}s^{-1}$)
Catechin	$< 10^2$
Rutin	1×10^6
Quercetin	6×10^6

were made with rutin; but data in Table II demonstrate that the electron transfer rate was much slower for rutin. No transfer was observed with catechin. While the repair of the urate radical by quercetin was expected in view of its much lower redox potential, the repair by rutin does not follow expectations of non-reactivity based on the reported value of its redox potential measured by pulse radiolysis.[22] However, somewhat lower redox potentials of 0.36 V and 0.40 V (vs NHE) for rutin and catechin, respectively, have been obtained by cyclic voltammetry in neutral aqueous solution.[23] These lower values, which are similar, may explain the reactivity of rutin with the urate radical but cannot explain the lack of reactivity of catechin. It may be suspected that the electrostatic potential in the micellar headgroup region plays a role in the apparent change in the redox potential of water insoluble rutin and/or in the kinetics of the electron transfer process. Catechin, though the redox potential approaches that of rutin, is water-soluble and may not be subject to such influences. In addition, the much lower aqueous solubility of rutin will insure high local concentration in micelles, where reaction with solubilized $^{\bullet}UH^{-}$ may take place. This difference in concentration local to $^{\bullet}UH^{-}$ may well enhance the apparent reactivity of rutin while having no effect with catechin. These findings suggest the importance of determining whether such microenvironmental factors, which may be found in proteins, are also involved in the antioxidant properties of these and other flavonoids towards plasma proteins.

Acknowledgments

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