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Mechanism of Deactivation of Triplet-Excited Riboflavin by Ascorbate, Carotenoids, and Tocopherols in Homogeneous and Heterogeneous Aqueous Food Model Systems

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Tocopherols (α, *β*, *γ*, and *δ*) and Trolox were found to deactivate triplet-excited riboflavin in homogeneous aqueous solution (7:3 v/v *tert*-butanol/water) with second-order reaction rates close to diffusion control [k_2 between 4.8×10^8 (*δ*-tocopherol) and 6.2×10^8 L mol^{-1} s^{-1} (Trolox) at 24.0 ± 0.2 °C] as determined by laser flash photolysis transient absorption spectroscopy. In aqueous buffer (pH 6.4) the rate constant for Trolox was 2.6×10^9 L mol^{-1} s^1 and comparable to the rate constant found for ascorbate (2.0×10^9 L mol^{-1} s^{-1}). The deactivation rate constant was found to be inferior in heterogeneous systems as shown for α-tocopherol and Trolox in aqueous Tween-20 emulsion (approximately by a factor of 4 compared to 7:3 v/v *tert*-butanol/water). Neither *β*-carotene (7:3 v/v *tert*-butanol/water and Tween-20 emulsion), lycopene (7:3 v/v *tert*-butanol/water), nor crocin (aqueous buffer at pH 6.4, 7:3 v/v *tert*-butanol/water, and Tween-20 emulsion) showed any quenching on the triplet excited state of riboflavin. Therefore, all carotenoids seem to reduce the formation of triplet-excited riboflavin through an inner-filter effect. Activation parameters were based on the temperature dependence of the triplet-excited deactivation between 15 and 35 °C, and the isokinetic behavior, which was found to include purine derivatives previously studied, confirms a common deactivation mechanism with a bimolecular diffusion-controlled encounter with electron (or hydrogen atom) transfer as rate-determining step. $\Delta H^{\ddagger}$ for deactivation by ascorbic acid, Trolox, and homologue tocopherols (ranging from 18 kJ mol^{-1} for Trolox in Tween-20 emulsion to 184 kJ mol^{-1} for ascorbic acid in aqueous buffer at pH 6.4) showed a linear dependence on $\Delta S^{\ddagger}$ (ranging from −19 J mol^{-1} K^{-1} for Trolox in aqueous buffer at pH 6.4 to +550 J mol^{-1} K^{-1} for ascorbic acid in aqueous buffer pH 6.4). Among photooxidation products from the chemical quenching, lumicrome, α-tocopherol quinones and epoxyquinones, and α-tocopherol dimers were identified by ESI-QqTOF-MS.

KEYWORDS: Riboflavin; tocopherols; carotenoids; ascorbate; chemical quenching; laser flash photolysis

INTRODUCTION

Light exposure results in quality deterioration of most foods and beverages. Milk and other dairy products are among the most sensitive due to the high content of riboflavin, vitamin B_2, which is an efficient photosensitizer for oxidative processes (*1*−*3*). Oxidation in milk affects the nutritive value and produces off-flavors, in effect reducing the shelf life of most dairy products. The flavor of beer is likewise affected by riboflavin-sensitized formation of off-flavor upon light exposure (*4*).

Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine) is a water-soluble vitamin widely present in meat products, eggs, dairy products, and some vegetable foods. The photoreactivity of riboflavin and other flavins (10-alkyl-7,8-dimethyl-isoalloxazines) has been extensively studied due to the involvement in a large number of biological processes (*5*−*10*). Riboflavin has been shown to act as a sensitizer in biological systems for both the so-called type I and type II photo-oxidation mechanisms. In milk and dairy products, the type II (singlet oxygen) mechanism is suggested to be responsible for light-induced oxidation sensitized by riboflavin (*11*). However, as has been recently reported by us (*12*, *13*), a type I (free radical) mechanism also should be taken into account and may be a major pathway for milk photo-oxidation sensitized by riboflavin.

Lipids, proteins, and vitamins are known to undergo riboflavin-sensitized oxidation (*1*, *11*, *13*). For dairy products, proteins and amino acids are oxidized prior to the lipids, and the "burnt feather" off-flavor from methionine photo-oxidation precedes the "cardboard" off-flavor from lipid oxidation (*2*).

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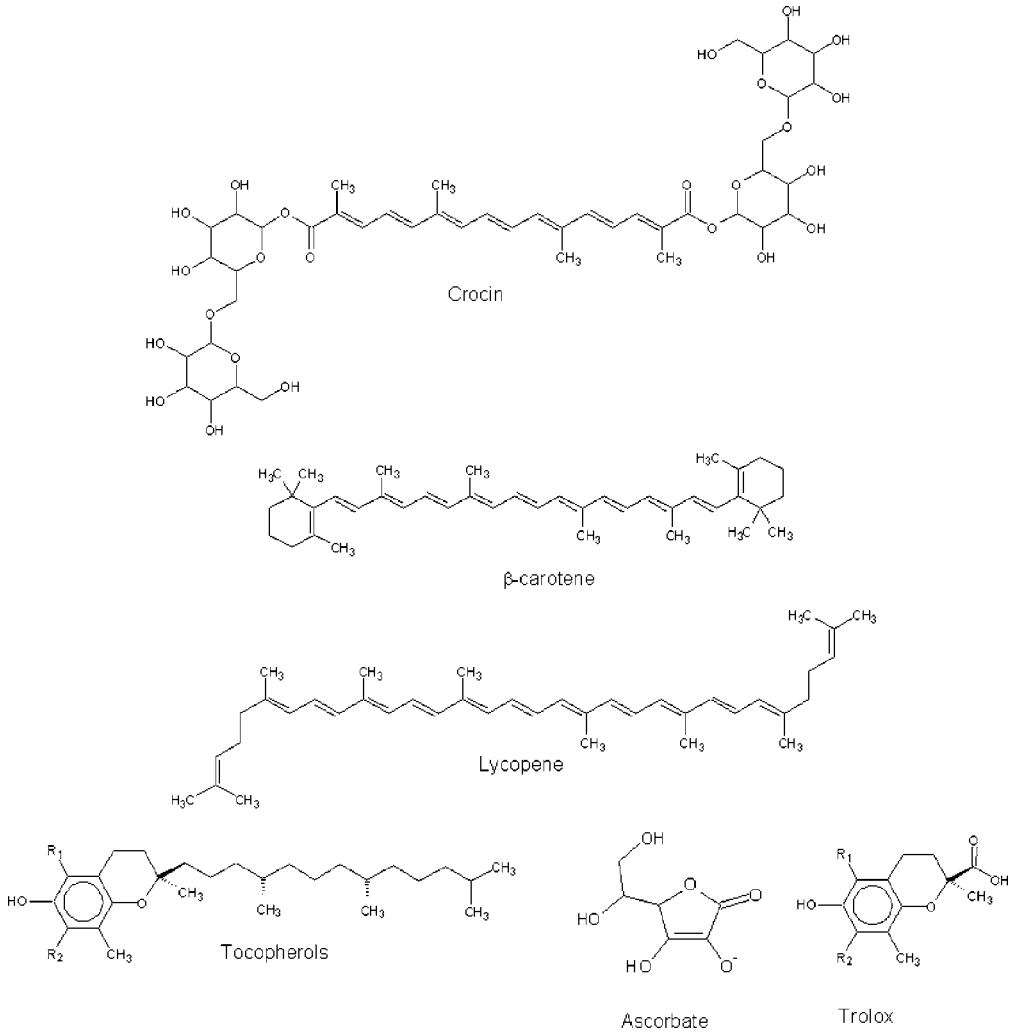
Chart 1. Chemical Structures for the Potential Quenchers for Triplet-Excited Riboflavin Studied



It is well documented that the content in milk of low molecular weight antioxidants such as tocopherols, carotenoids, and uric acid can be manipulated by changing the ruminant diet (*14*). The antioxidant effect of uric acid in milk under light exposure has been assigned to its action as a triplet-excited riboflavin quencher (*15*). Although the ability of vitamin E and β-carotene to delay light-induced oxidation processes in milk and dairy products has been widely studied, the mechanisms of their antioxidative effect remain speculative (*16*). Recently, we suggested that singlet oxygen formation from triplet-excited riboflavin is unlikely to happen, because various substances present in milk at the actual concentration have been shown to deactivate triplet-excited riboflavin at diffusion-controlled rates and more rapidly than oxygen (*12*, *15*). Compounds such as free amino acids, peptides, whey proteins, purine bases, and phenolics are present at higher concentration in most products than dissolved oxygen, and the type I mechanism becomes the major pathway for the formation of reactive intermediates during milk photo-oxidation.

Herein, we report a mechanistic study of the antioxidant action of α-, β-, γ-, and δ-tocopherol, Trolox, ascorbate, β-carotene, lycopene, and crocin on the early steps of the light-induced oxidation sensitized by riboflavin. The chemical structures of the studied compounds are shown in **Chart 1**. The compounds were selected to include ascorbate, tocopherols, and a carotenoid naturally occurring in milk and also the water-soluble vitamin E analogue Trolox and the water-soluble carotenoid crocin in order to include studies in homogeneous aqueous solution.

MATERIALS AND METHODS

Chemicals. Chloroform, *tert*-butanol, formic acid, and tocopherol-Kit for biochemistry (α, β, γ, and δ) were from Merck (Darmstadt, Germany) and used as received. Analytical grade ascorbic acid, Tween-20, riboflavin, and Trolox were obtained from Sigma-Aldrich (St. Louis, MO). The carotenoid β-carotene and lycopene were kindly provide by Roche (Avedøre, Denmark) and used without further purification. Crocin was obtained from Apin Chemicals Limited (Oxon, U.K.). Water was purified on a Millipore Q-plus purification train (Millipore, Bedford, MA).

Laser Flash Photolysis Kinetics. Laser flash photolysis experiments were carried out with an LKS.50 spectrometer from Applied Photophysics (Leatherhead, U.K.). The third harmonic at 355 nm of a pulsed Q-switched Nd:YAG laser was used to pump a dye laser (Spectron Laser System, Rugby, U.K.) using Coumarin 120, which has an emission peak at 440 nm. The intensity of the laser pulse was approximately 3 mJ cm^{-2}. An R928 photomultiplier tube from Hamamatsu (Hamamatsu, Japan) was used to detect the transient absorption (300−800 nm). Appropriate UV cutoff filters were used to minimize the sample degradation by the monitoring light. The samples were excited in 0.5 × 1.0 cm fluorescence curvettes from Hellma (Mulheim, Germany). All samples were prepared using fresh solutions and purged with high-purity N_2 before each experiment. Unless otherwise stated, samples were thermostated at 24.0 ± 0.2 °C. Under all conditions the triplet-excited riboflavin decayed in the absence or

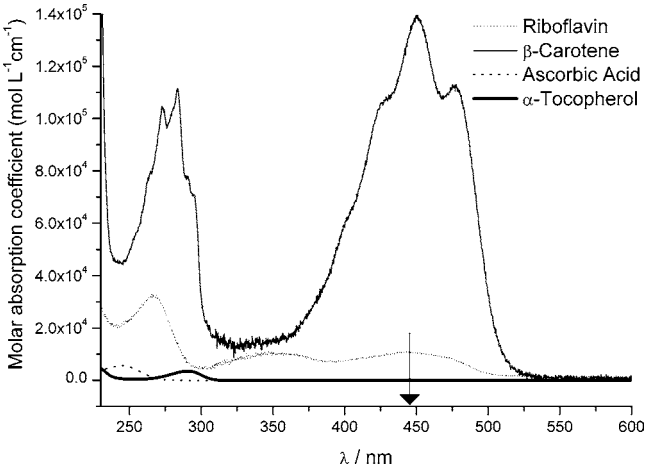


Figure 1. Absorption spectra of riboflavin, β-carotene, ascorbic acid, and α-tocopherol in *tert*-butanol/water 7:3 v/v at room temperature. Arrow indicates the laser excitation wavelength used in the present investigation.

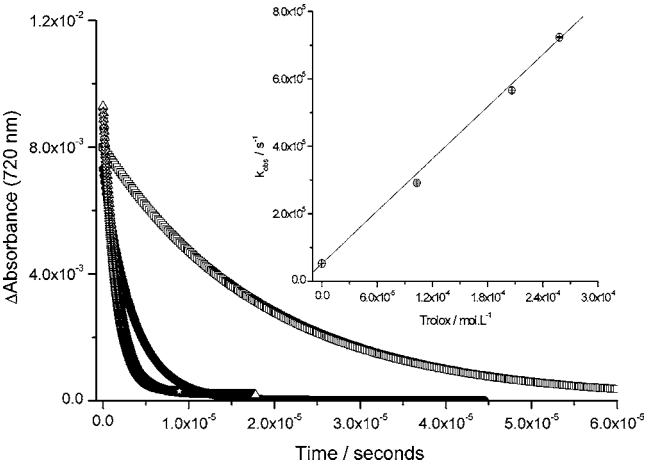


Figure 2. Transient time profile for triplet-excited riboflavin observed at 720 nm (24.0 ± 0.2 °C) in the absence and presence of different concentrations of Trolox in anaerobic aqueous buffer at pH 6.4. (Inset) Observed first-order rate constants for decay of triplet-excited riboflavin as function of Trolox concentration (mol L^{-1}).

presence of quenchers by first-order kinetics, and the decay curve was fitted using the Applied Photophysics Spectra Kinetic Workstation v.4.63 and the following equation with a floating end point: $f(x) = P(1)\ e^{-P(2)x} + P(3)$ to obtain the first-order rate constant k_{obs} [$P(2)$]. Temperature dependence for all quenching reactions was investigated at three to four temperatures in the temperature range of 15−35 °C. The ground-state absorption spectra recorded for control of concentration and light absorption were made on a Cintra 40 GBC spectrophotomer (GBC Scientific Equipment Pty Ltd., Victoria, Australia) or on a Hitachi U-4100 (Hitachi High-Technologies Corp., Tokyo, Japan).

Electrospray Ionization Mass Spectrometry. Mass spectra were measured utilizing a hybrid quadrupole reflector orthogonal time-of-flight high resolution, QqTOF, Ultima mass spectrometer (Waters MicroMass, Manchester, U.K.) equipped with a Z-spray type nanoelectrospray ion source. All experiments were performed in the positive mode detection. Samples were irradiated under steady-state conditions using a high-pressure Hg lamp with a water filter and an interference filter, which was used to select light at 436 nm with an intensity of 9.6 $\times$ 10^{16} photons per second as determined by ferric oxalate actinometry. Irradiations were performed in air-saturated *tert*-butanol/water 7:3 v/v solution in 1 cm $\times$ 1 cm quarts cells (Hellma) for 30 min. The photolysis solution contained riboflavin (112 μM) and α-tocopherol (4.13 mM). The general conditions for ESI-QqTOF-MS were as

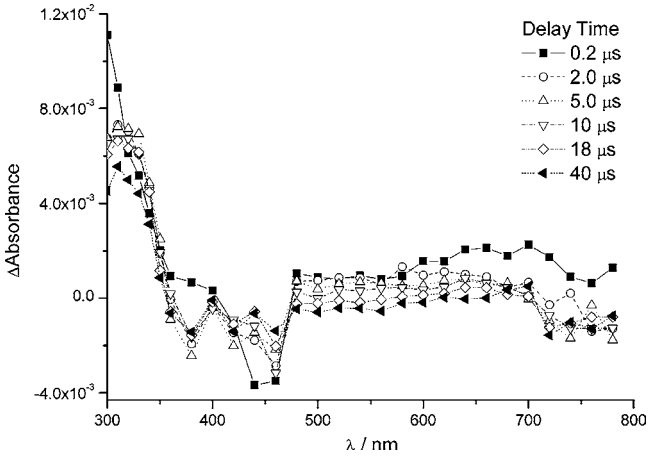


Figure 3. Transient absorption spectra (excited state minus ground state), recorded at different delay times following the laser pulse (8 ns) for an anaerobic aqueous solution containing riboflavin (70 μM) and Trolox (967 μM) at 24.0 ± 0.2 °C.

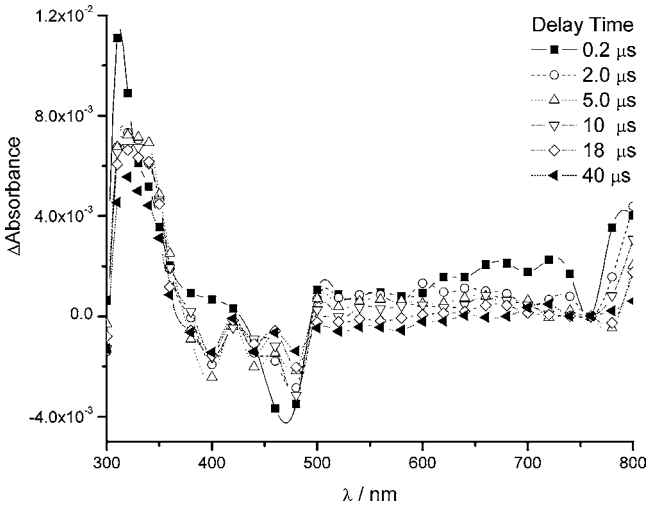


Figure 4. Transient absorption spectra (excited state minus ground state), recorded at different delay times following laser pulse (8 ns) for an anaerobic aqueous Tween-20 emulsion containing riboflavin (70 μM) and α-tocopherol (1.1 mM) at 24.0 ± 0.2 °C.

follows: direct infusion flow rate, 0.5 μL min^{-1}; accumulation time, 1 s; scan range, *m*/*z* 50−1000; capillary voltage, 3.6 kV; cone voltage, 100 V; source temperaturee, 80 °C; desolvation gas flow, 390 L h^{-1}; desolvation temperature, 150 °C; cone gas flow, 32 L h^{-1}. For ESI-(+)MS analysis, 0.5 μL of formic acid was added to the sample (500 μL) to yield 0.1% as final acid concentration.

RESULTS AND DISCUSSION

The yellow color of dairy products is attributable to the presence of β-carotene and riboflavin (see **Figure 1**). Exposure to light of riboflavin solutions generates the lowest energy riboflavin excited singlet state, which by efficient intersystem crossing yields the very reactive triplet-excited state, a biradical that has an oxidation potential of 1.7 V versus NHE (*17*). The half-life of this highly reactive species depends on the solvent and was found to have the value of 14 ± 2 μs in aqueous buffer at pH 6.4 (see **Figure 2**), 27 ± 3 μs in *tert*-butanol/water 7:3 v/v, and 15 ± 3 μs in aqueous Tween-20 emulsion, respectively. Notably, the same half-life was found for homogeneous aqueous buffer at pH 6.4 and the aqueous Tween-20 emulsion, which is in agreement with the location of riboflavin in the aqueous phase.

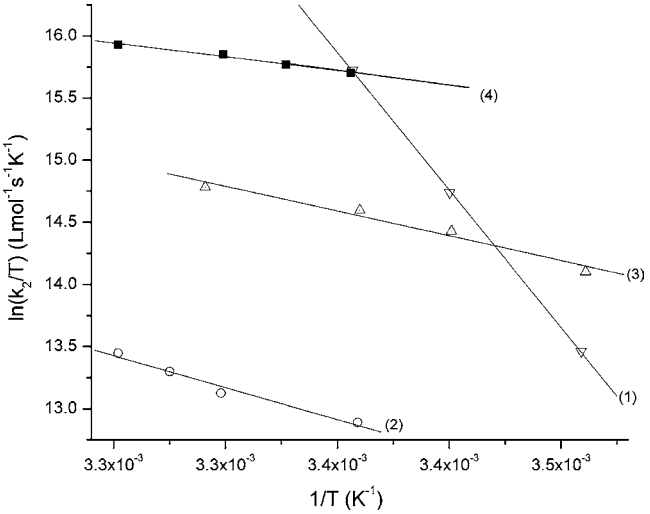


Figure 5. Temperature dependence of triplet-excited riboflavin quenching by (1) (ascorbate in aqueous buffer at pH 6.4, (2) Trolox in aqueous Tween-20 emulsion, (3) Trolox in *tert*-butanol/water 7:3 v/v, and (4) α-tocopherol in aqueous Tween-20 emulsion).

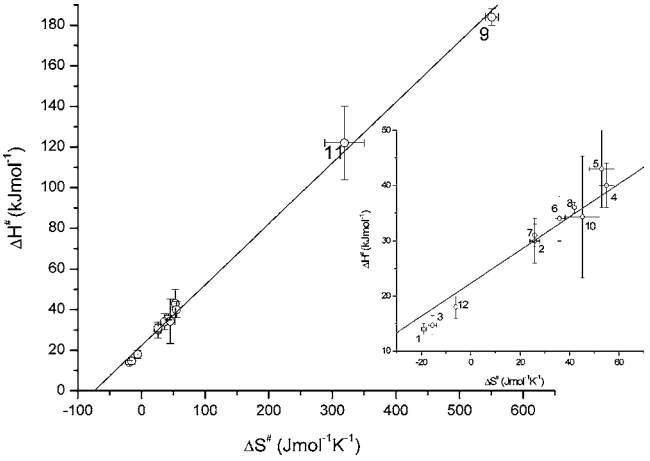


Figure 6. Isokinetic plot for chemical quenching of triplet-excited riboflavin by ascorbate, purine bases, Trolox, and homologue tocopherols in different solvent systems: (1) Trolox in aqueous buffer at pH 6.4; (2) Trolox in *tert*-butanol/water 7:3 v/v; (3) Trolox in aqueous Tween-20 emulsion; (4) α-tocopherol in *tert*-butanol/water 7:3 v/v; (5) α-tocopherol in aqueous Tween-20 emulsion; (6) β-tocopherol in *tert*-butanol/water 7:3 v/v; (7) γ-tocopherol in *tert*-butanol/water 7:3 v/v; (8) δ-tocopherol in *tert*-butanol/water 7:3 v/v; (9) ascorbate in aqueous buffer at pH 6.4; (10) xanthine; (11) hypoxanthine; (12) uric acid in aqueous buffer at pH 6.4 from ref *10*. The inset is an enlargement of part of the plot.

The transient difference absorption spectrum obtained after 445 nm light-pulse excitation with 8 ns of duration confirmed the formation of triplet-excited riboflavin in all three solvent systems as may be seen for an aqueous solution in **Figure 3** and an aqueous Tween-20 emulsion in **Figure 4**. The transient difference absorption spectra are similar to those previously reported using the same experimental method (*12*, *15*).

Addition of various concentrations (up to 1 mM) of β-carotene, lycopene, or crocin to oxygen-free solutions containing 50 μM riboflavin in *tert*-butanol/water 7:3 v/v did not affect the decay rate of triplet-excited riboflavin as probed at 720 nm. However, the initial population of the triplet-excited state of riboflavin was seen to decrease significantly. The decrease in triplet-excited population of riboflavin may be explained by an inner-filter effect, as has been also observed for a dairy spread product (*18*). **Figure 1** illustrates the spectral overlap of

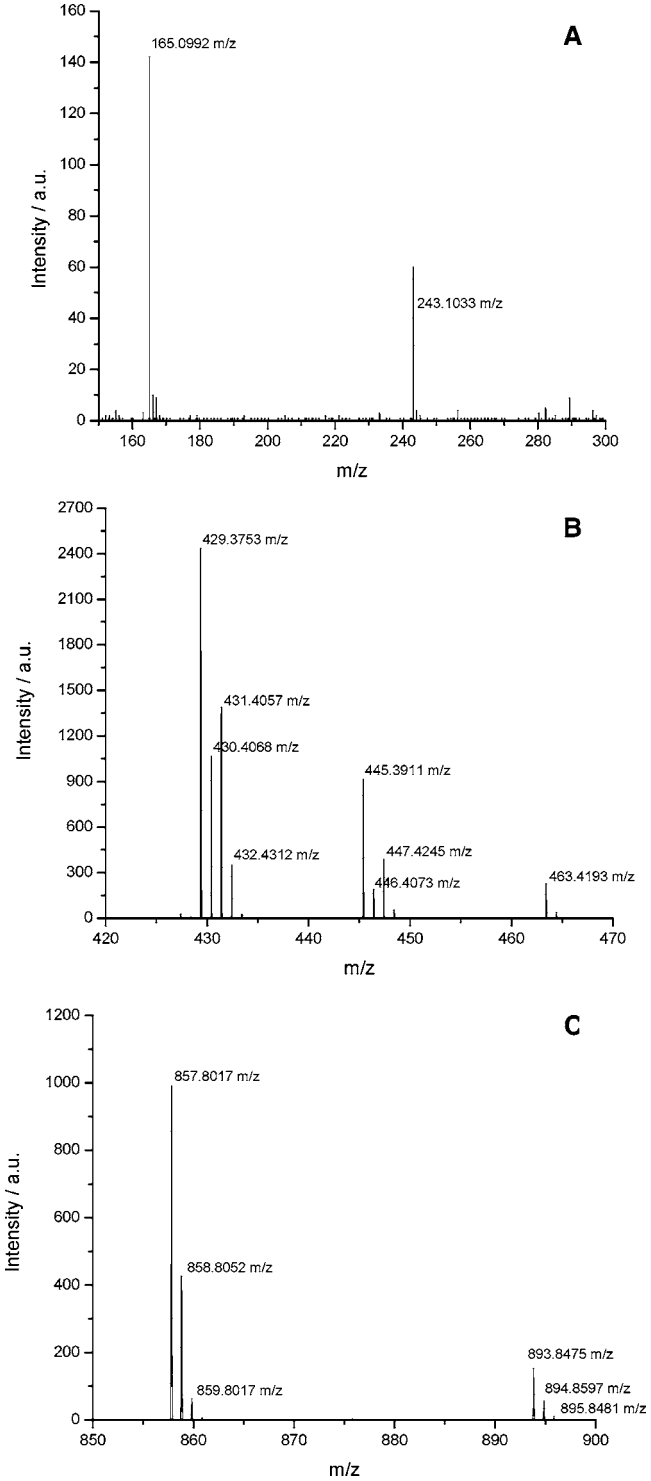


Figure 7. Positive electrospray ionization mass spectra following steady-state photolysis of α-tocopherol (4.1 mM) and riboflavin (112 μM) in aerobic *tert*-butanol/water 7:3 v/v at room temperature: (**A**) spectrum from *m*/*z* 150 to 300; (**B**) spectrum from *m*/*z* 420 to 470; (**C**) spectrum from *m*/*z* 850 to 900. For photoproduct identification, see text.

β-carotene and riboflavin for a *tert*-butanol/water 7:3 v/v solution of riboflavin, Trolox, α-tocopherol, ascorbic acid, and β-carotene.

In contrast, ascorbate, Trolox, and the homologue tocopherols did not affect the initial population of triplet-excited riboflavin, in agreement with the lack of spectral overlap (**Figure 1**), but strongly decreased the half-life of triplet-excited riboflavin as seen for Trolox in aqueous buffer at pH 6.4 (**Figure 2**). From

Table 1. Second-Order Rate Constant, k_2, for Triplet-Excited Riboflavin Deactivation by Ascorbate, Trolox, and Homologue Tocopherols at 24.0 ± 0.2 °C in Different Anaerobic Solvents Together with Literature Values for Bond Dissociation Energy (BDE) for Phenolic Hydrogen and Ionization Potential (IP)

quencher	k_2 (L mol^{-1} s^{-1})	solvent	BDE[a] (kJ mol^{-1})	IP[a] (kJ mol^{-1})
ascorbate	$(2.0 \pm 0.3) \times 10^{9}$[b]	aqueous buffer, pH 6.4 (0.2 M)		
Trolox	$(2.6 \pm 0.3) \times 10^{9}$	aqueous buffer, pH 6.4 (0.2 M)		
Trolox	$(6.2 \pm 0.1) \times 10^{8}$	*tert*-butanol/water 7:3 v/v		
Trolox	$(1.9 \pm 0.2) \times 10^{8}$	Tween-20 emulsion		
α-tocopherol	$(5.4 \pm 0.1) \times 10^{8}$	*tert*-butanol/water 7:3 v/v		
α-tocopherol	$(1.1 \pm 0.1) \times 10^{8}$	Tween-20 emulsion	317.1	669.7
β-tocopherol	$(5.1 \pm 0.2) \times 10^{8}$	*tert*-butanol/water 7:3 v/v	332.0	680.2
γ-tocopherol	$(5.1 \pm 0.2) \times 10^{8}$	*tert*-butanol/water 7:3 v/v	332.9	683.1
δ-tocopherol	$(4.8 \pm 0.1) \times 10^{8}$	*tert*-butanol/water 7:3 v/v	340.7	693.1

[a] Gas-phase values from ref *20*. [b] Bimolecular rate constant agreeing with that reported in the literature (*9*).

the exponential decays, first-order rate constants were calculated and further found to depend linearly on quencher concentration as seen in the inset of **Figure 2** for Trolox in aqueous buffer at pH 6.4. The second-order quenching constants derived from linear regression of such a plot are collected in **Table 1** for ascorbate, Trolox, and homologue tocopherols for the three solvent systems investigated.

From **Table 1** it may be seen that ascorbate, Trolox, and the homologue tocopherols deactivate triplet-excited riboflavin with second-order rate constants close to the diffusion limit and accordingly competitive with the deactivation by molecular oxygen ($k = 9.8 \times 10^8$ M^{-1} s^{-1}), bovine whey proteins ($k \sim 10^8$ M^{-1} s^{-1}), uric acid ($k = 2 \times 10^9$ M^{-1} s^{-1}), and phenolic antioxidants from plant material ($k \sim 10^9$ M^{-1} s^{-1}) (*12*, *15*, *19*). A slight difference in reactivity is observed among the homologue tocopherols, which can be assigned to an electron donation effect from methyl groups in α ($R_1 = R_2 = CH_3$ in **Chart 1**), β ($R_1 = CH_3$, $R_2 = H$), γ ($R_1 = H$, $R_2 = CH_3$), and δ ($R_1 = R_2 = H$) positions increasing the reactivity in the following order: Trolox > α-tocopherol > β ≈ γ > δ. This order of reactivity corroborates reported values of bond dissociation energy for phenolic hydrogen (BDE) and ionization potential (IP) (**Table 1**), which are the molecular properties of particular importance for H-atom abstraction deactivation and the single-electron-transfer mechanism, respectively (*20*). Notably, the same order of reactivity was observed for the reaction of tocopherols with peroxyl radicals (*21*). Trolox shows to be slightly more effective than α-tocopherol in the deactivation of the triplet-excited riboflavin despite the difference in structure related only to a substitution of the "phytyl tail" of α-tocopherol by a carboxylic group in Trolox (**Chart 1**). Likewise, the presence or absence of the phytyl tail in α-tocopherol results only in a very small difference in the calculated BDE and IP values when replaced by a methyl group in the same position (*20*). Thus, the difference in reactivity of Trolox [$(6.2 \pm 0.1) \times 10^8$ M^{-1} s^{-1}, *tert*-butanol/water 7:3 v/v] and α-tocopherol [$(5.4 \pm 0.1) \times 10^8$ M^{-1} s^{-1}, *tert*-butanol/water 7:3 v/v] toward triplet-excited riboflavin may be related to an increase of molecular mobility of the smaller Trolox with a carboxylic group compared to the long phytyl tail. To follow the formation and disappearance of transient species (triplet-excited riboflavin, riboflavin radical, and phenoxyl radicals), the time-resolved difference absorption spectra were recorded in the spectral region from 300 to 800 nm (**Figures 3** and **4**). In spite of the spectral overlap due to the strong bleaching of riboflavin ground state, it is possible to observe the formation of the tocopheroxyl radical with a maximum absorption centered at 430 nm in all of the solvent systems investigated. The action of tocopherols as quenchers of triplet-excited riboflavin is accordingly the result of a single-electron transfer followed by rapid deprotonization or an H-atom transfer from the phenoxyl group to the triplet-excited state of riboflavin.

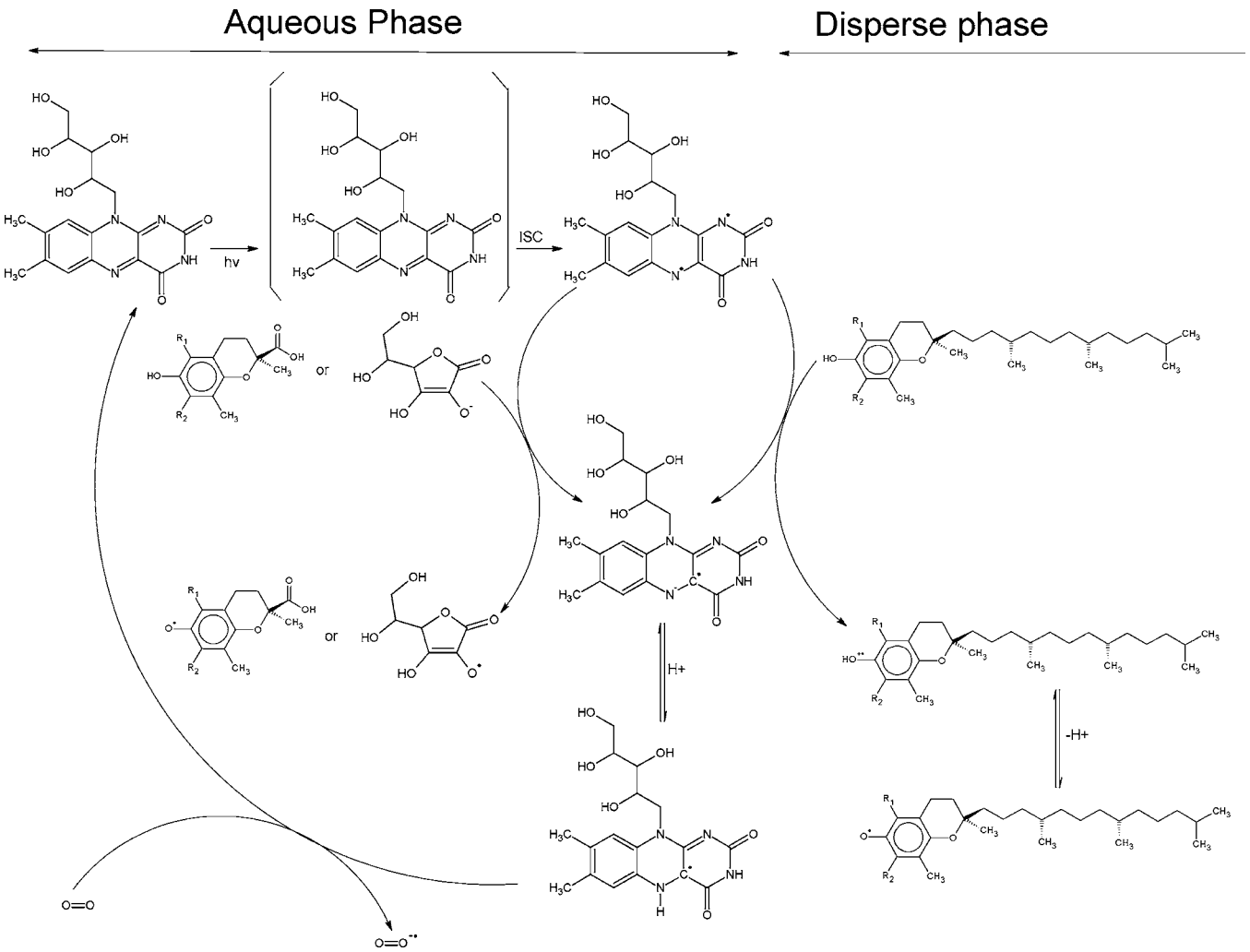
Table 2. Activation Parameters for the Triplet-Excited Riboflavin Deactivation by Ascorbate, Trolox, and Homologue Tocopherols in Different Anaerobic Solvents at 25 °C

quencher	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	solvent
ascorbate	184 ± 4	551 ± 10	aqueous buffer, pH 6.4 (0.2 M)
Trolox	14 ± 1	−19 ± 1	aqueous buffer, pH 6.4 (0.2 M)
Trolox	30 ± 4	26 ± 2	*tert*-butanol/water 7:3 v/v
Trolox	18 ± 2	−6 ± 0.2	Tween-20 emulsion
α-tocopherol	40 ± 4	55 ± 3	*tert*-butanol/water 7:3 v/v
α-tocopherol	43 ± 7	53 ± 5	Tween-20 emulsion
β-tocopherol	34 ± 4	36 ± 2	*tert*-butanol/water 7:3 v/v
γ-tocopherol	31 ± 2	26 ± 1	*tert*-butanol/water 7:3 v/v
δ-tocopherol	36 ± 1	42 ± 1	*tert*-butanol/water 7:3 v/v

Recent studies on controlled photo-oxidation (*22*) of milk samples have shown that samples containing high amounts of α-tocopherol are more stable in relation to protein oxidation and also that high amounts of carotenoids and tocopherols do not prevent lipid oxidation, but can delay protein oxidation. Because many studies (*1*, *2*) have indicated that milk whey protein photo-oxidation sensitized by riboflavin is responsible for the early developed off-flavor in product exposed to light, our present findings corroborate the photo-oxidation studies in real milk samples, in view of the fact that tocopherols may compete with bovine whey proteins to quench triplet-excited riboflavin and thus delay the protein oxidation.

Solvent has an effect on the reaction rate of quenching triplet-excited riboflavin as may be seen from **Table 1**. For Trolox, homogeneous solutions of water and *tert*-butanol/water 7:3 v/v may be compared, and the ratio between rate constants is approximately 4 and probably reflects the difference in viscosity for which the inverse ratio is approximately 6 (*23*). For emulsion systems, the deactivation rate is also observed to decrease in agreement with a bimolecular quenching reaction. To obtain more mechanistic information, the laser flash experiments were repeated at three or four temperatures for each quencher and various concentrations. From the temperature dependence of k_2 and using transition-state theory, the activation parameters were calculated (see **Figure 5** for examples) and may be found in **Table 2**. The variation in enthalpy of activation is high. For Trolox the lowest energy barrier for quenching is in water and the highest in the *tert*-butanol/water mixture. Ascorbate has a very high enthalpy of activation, which, however, is compensated by large positive entropy of activation. This compensation effect is seen to be more general and to apply for all of the quenching reactions of triplet-excited riboflavin studied as may

Scheme 1. Proposed Reaction Mechanism for Triplet-Excited Riboflavin Deactivation in a Heterogeneous Aqueous Tween-20 Emulsion in the Presence of α-Tocopherol and in a Homogeneous Aqueous Solution in the Presence of Ascorbate



be seen from **Figure 6**, which confirms an isokinetic behavior for all six quenchers and in the three different media. The linear dependence of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ provides strong evidence for a common reaction mechanism for the nine reactions studied. Remarkably, the purine derivatives previously studied, that is, uric acid, xanthine, and hypoxanthine, also follow the isokinetic behavior as seen from **Figure 6**, providing further evidence for a common reaction mechanism (*15*).

Photoproducts of the quenching were studied for riboflavin and α-tocopherol in *tert*-butanol/water 7:3 v/v by steady-state photolysis. The photoproducts were identified by direct infusion mass spectrometry as may be seen **Figure 7**. From the mass spectrum of **Figure 7** lumicrome was identified (*m*/*z* 243.1033, −64.4 ppm of the calculated value for $C_{12}H_{11}O_2N_4$) as the main degradation product of riboflavin, whereas α-tocopherol was oxidized to orthoquinone methide (*m*/*z* 429.3753, 6 ppm of the calculated value for $C_{29}H_{49}O_2$), quinone (*m*/*z* 447.4245, 92 ppm of the calculated value for $C_{29}H_{51}O_3$), epoxyquinone (*m*/*z* 463.4193, 88.7 ppm of the calculated value for $C_{29}H_{51}O_4$), and the tocopherol spirodimer (*m*/*z* 857.8017, 74 ppm of the calculated value for $C_{58}H_{97}O_4$) (*24*, *25*). Also, an unknown dimer with a possible molecular formula of $C_{58}H_{101}O_6$ is observed at *m*/*z* 893.8475 (−98.7 ppm of the calculated value), which product ion spectrum yielded one major fragment at *m*/*z* 429.4176 and another at *m*/*z* 447.4504. These photoproducts all involve initial single-electron or hydrogen-atom transfer followed by further redox reactions and identify the quenching process as a chemical quenching rather than physical. We then arrive at the following mechanism for quenching of triplet-excited riboflavin. The quencher is approaching ^{3}Rib* in a process close to diffusion, the barrier depends on the viscosity and, for the homogeneous system, on the spatial arrangement of the reactants. However, the mechanism is the same and involves electron transfer or hydrogen atom abstraction in the transition state for which the high-energy barrier is compensated by positive entropy reflecting the loss of organization in the transition state. This compensation is especially seen for ascorbate, and for this hydrophilic quencher we suggest that the transition state involves loss of hydration, increasing the entropy. The mechanism is illustrated by **Scheme 1**, although, on the basis of the available thermodynamic data (**Table 2**), we cannot differentiate between single-electron transfer and hydrogen atom abstraction. However, the purine derivatives were previously shown experimentally and by ab initio calculations to quench triplet-excited riboflavin by single-electron transfer in aqueous solution. The isokinetic behavior observed to include purine derivatives (**Figure 6**) supports the assignment of an electron-transfer mechanism to all of the quenching reactions studied.

Carotenoids were not found to be quenchers. A comparison between the structures of the tocopherols and the carotenoids shows that both have conjugated systems, but that tocopherols in contrast to the carotenoids have an electron-rich functional group, which facilitates electron donation to the strong oxidant

triplet-excited riboflavin. Carotenoids are as the tocopherols efficient quenchers of singlet oxygen but mainly support a physical process with energy transfer in the excited state (*11*).

In conclusion, ascorbate, Trolox, and tocopherol were found to quench triplet-excited riboflavin, which may be formed by light exposure of milk. The tocopherols located in the disperse lipid phase in milk will protect the lipid against oxidation but the reduced riboflavin generates a radical in the aqueous phase. For ascorbate, Trolox, and uric acid two radicals may be formed in the aqueous phase, leading to depletion of water-phase antioxidants rather than depletion of lipid-phase antioxidants as was observed for buttermilk (*26*).

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