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Deactivation of Triplet-Excited Riboflavin by Purine Derivatives: Important Role of Uric Acid in Light-Induced Oxidation of Milk Sensitized by Riboflavin

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The reactivity of purine derivatives (uric acid, xanthine, hypoxanthine, and purine) toward triplet-excited riboflavin in aqueous solution at pH 6.4 is described on the basis of kinetic (laser flash photolysis), electrochemical (square-wave voltammetry), and theoretical data (density functional theory, DFT). Direct deactivation of triplet-excited riboflavin in aqueous solution, pH 6.4 at 24 °C, in the presence of uric acid, xanthine, and hypoxanthine strongly suggests a direct electron transfer from the purine to the triplet-excited riboflavin with $k = 2.9 \times 10^9$ M^{-1} s^{-1} ($\Delta H^{\ddagger} = 14.7$ kJ mol^{-1}, $\Delta S^{\ddagger} = -15.6$ J mol^{-1} K^{-1}), 1.2×10^9 M^{-1} s^{-1} ($\Delta H^{\ddagger} = 34.3$ kJ mol^{-1}, $\Delta S^{\ddagger} = +45.3$ J mol^{-1} K^{-1}), and 1.7×10^8 M^{-1} s^{-1} ($\Delta H^{\ddagger} = 122$ kJ mol^{-1}, $\Delta S^{\ddagger} = +319$ J mol^{-1} K^{-1}), respectively. From the respective one-electron oxidation potentials collected in aqueous solution at pH 6.4 for uric acid ($E = +0.686$ vs normal hydrogen electrode, NHE), xanthine ($E = +1.106$ vs NHE), and hypoxanthine ($E = +1.654$ vs NHE), the overall free energy changes for electron transfer from the quencher to the triplet-excited riboflavin are as follows: uric acid ($\Delta G^{\circ} = -114$ kJ mol^{-1}), xanthine ($\Delta G^{\circ} = -73.5$ kJ mol^{-1}), hypoxanthine ($\Delta G^{\circ} = -20.6$ kJ mol^{-1}), and purine ($\Delta G^{\circ} > 0$). The inertness observed for purine toward triplet-excited riboflavin corroborates with its electrochemical inactivity in the potential range from 0 up to 2 V vs NHE. These data are in agreement with the DFT results, which show that the energy of the purine highest occupied molecular orbital (HOMO) (−0.2685 arbitrary unit) is lower than the energy of the semioccupied molecular orbital (SOMO) (−0.2557 a.u.) of triplet-excited riboflavin, indicating an endergonic process for the electron-transfer process. The rate-determining step for deactivation by purine derivatives can be assigned to an electron transfer from the purine derivative to the SOMO orbital of the triplet-excited riboflavin. The results show that uric acid may compete with oxygen and other antioxidants to deactivate triplet-excited riboflavin in milk serum and other biological fluids leading to a free radical process.

KEYWORDS: Riboflavin; uric acid; light oxidation; milk; kinetics; antioxidant

INTRODUCTION

The oxidation of milk causes nutritional losses and produces undesirable flavors and toxic compounds, and control of oxidation processes becomes central for consumers' acceptance of milk and dairy products (*1*−*3*). Milk oxidation may be promoted by enzymes, trace of transition metals ions, or exposure to light during production, transport, and storage (*1*−*3*). Milk and dairy products are important sources of riboflavin, and the high content of this vitamin makes light-induced oxidation sensitized by riboflavin the major factor promoting oxidation during the storage (*4*). Riboflavin, by absorbing visible light, generates a metastable (triplet) excited state from the initially populated singlet state by intersystem crossing that may react with triplet ground-state oxygen to form singlet oxygen by triplet−triplet annihilation, in a so-called type II photooxidation pathway, or it may react directly with a substrate such as protein (*4*) by accepting hydrogen or electrons and producing radicals through the type I photooxidation mechanism (*5*−*8*).

Uric acid, a natural low molecular weight antioxidant present in the milk serum, is formed from ruminal breakdown of microbial nucleotides and thereby its concentration can be controlled by intensity of the milk production and/or feeding regime (*9*−*11*). Østdal et al. (*10*) have recently demonstrate that uric acid is an important and efficient antioxidant to retard

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light-induced oxidation of milk and suggested that the antioxidant effect of uric acid is due to scavenging of radicals formed after riboflavin light excitation rather than direct quenching of excited states of riboflavin.

The purpose of the present work is to provide a better understand of the role of purine bases, especially uric acid, in light-induced oxidation of milk sensitized by riboflavin. Herein, the reactivity of selected purine derivatives (uric acid, xanthine, hypoxanthine, and purine) toward triplet-excited riboflavin has been examined by time-resolved laser flash photolysis, electrochemical techniques, and density functional theory (DFT) calculations.

EXPERIMENTAL SECTION

Chemicals. Trihydroxypurine (uric acid), dihydroxypurine (xanthine), hydroxypurine (hypoxanthine), *H*-imidazolylpyrimidine (purine), ascorbic acid, and riboflavin were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and used as received. The pH of aqueous solution is buffered to pH 6.4 (ionic strength = 0.2 M) with analytical grade Na_2HPO4/KH_2PO_4 (Merck, Darmstadt, Germany). The distilled water was purified on a Milli-Q Plus system (Millipore Corp., Bedford, MA). The nitrogen gas used for anaerobic experiments was of high purity (99.99%).

Laser Flash Photolysis. The third harmonic at 355 nm of a pulsed Q-switched Nd:YAG laser was used to pump a dye laser with Coumarin 120 (Lambda Physic, Germany) with an emission peak at 440 nm as dye (Spectron Laser System, Rugby, U.K.). The intensity of the laser pulse was approximately 3.5 mJ cm^{-2}. Samples were excited by the laser pulse (8 ns) in 1.0 × 1.0 cm fluorescence cuvettes from Hellma (Mullein, Germany) with a xenon arc pulse lamp from Applied Photophysics Ltd. (Leatherhead, U.K.) with appropriate UV cutoff filters to minimize the sample degradation by the monitoring light. The transmitted light entered a monochromator equipped with a R928 photomultiplier tube from Hamamatsu (Japan) and the signals were collected by use of a Phillips digital oscilloscope and transferred to the spectrometer workstation an LKS.50 system from Applied Photophysics Ltd. (Leatherhead, U.K.). Experiments were carried out on thermostated fresh solutions, and samples were previously purged with N_2 for 30 min. The main part of the experiment was performed at 24 °C, but temperature dependence was investigated between 20 and 44 °C.

Electrochemistry. Square-wave voltammetry was carried out in a Voltalab PGZ402 electrochemical system (Radiometer, Copenhagen, Denmark) connected to a personal computer using the proprietary software Voltamaster 2. Electrochemical oxidations were carried out in a three-electrode Pyrex glass with degassing facilities for bubbling nitrogen. The working electrode was a 0.62 cm × 1.0 cm boron-doped diamond film deposited on a silicon wafer produced by the Centre Suisse de Electronique et de Microtechnique SA (Neufchatel, Switzerland). The auxiliary electrode was a 2 cm^2 Pt foil, and unless otherwise stated, all measurements were carried out with an $Ag^+/AgCl$ electrode as reference.

Computational Methods. Density functional theory (DFT) calculations were performed with the molecules trihydroxypurine (uric acid), dihydroxypurine (xanthine), hydroxypurine (hypoxanthine), *H*-imidazolylpyrimidine (purine), and riboflavin, aiming at obtaining the following properties: ionization potential (IP), energies of the frontier HOMO (highest occupied molecular orbital), SOMO (semioccupied molecular orbital), and LUMO (lowest unoccupied molecular orbital), total energy (E_T), and bond dissociation enthalpies (BDE). The computational calculations for riboflavin were carried out for the isoalloxazine core, which is responsible for the flavin photochemistry and is assumed to be unperturbed by the removal of the ribityl chain.

Initially, the calculations were performed to determine the neutral structure for each molecule. The proposed structures were optimized and their total energies were compared. For our experimental conditions (pH = 6.4), uric acid and xanthine are found in monoanion form while hypoxanthine is found in the undissociated form. For these molecules, the same procedure to determine the more stable structure was adopted. The only exception was xanthine, for which similar calculations were previously performed by Rogstad et al. (*12*).

All the structures were optimized in solution by the DFT method with the B3LYP functional (*13*) and the 6-31+G(d) basis set. The solvent (water) effect was simulated with the integral equation formulation of polarizable continuum model (IEF-PCM) method (*14*, *15*), and the calculations were performed with the Gaussian 03 code (*16*). The absence of imaginary frequencies was used as a criterion to ensure that the optimized structures represent the minimum of the potential energy surface. BDE and IP were calculated by use of the expressions presented by DiLabio et al. (*17*).

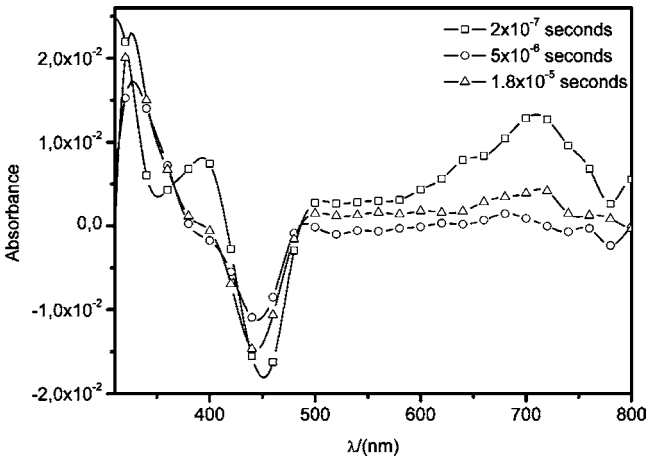


Figure 1. Time-resolved electronic absorption spectra (excited state minus ground state) for a deoxygenated solution containing riboflavin (80 μM) and uric acid (500 μM) following an 8 ns long 440 nm laser pulse.

RESULTS

Figure 1 shows the transient absorption spectra in the range of 300−800 nm obtained after 8 ns laser pulse (440 nm) of solutions containing riboflavin and the respective purine bases. The early spectrum (□), 2×10^{-7} s after the laser pulse, shows the triplet−triplet absorption band centered at 720 nm of triplet-excited riboflavin (*6*, *4*, *18*), used to monitor the triplet-state decay.

By monitoring the decay of the riboflavin triplet-state absorption band at 720 nm, which increased linearly with the quencher concentrations as shown in the inset of **Figure 2** for uric acid, the specific second-order rate constant could be calculated:

$$k_{obs} = k_T + k_q[\text{quencher}] \quad (1)$$

where k_T is the rate constant for the triplet-state self-decay and k_q is the second-order rate constant for the triplet-state deactivation by the quencher as presented in **Table 1**.

The specific rate constants obtained for excited-triplet riboflavin deactivation by uric acid, xanthine, and hypoxanthine are near diffusion control. This suggests that the purine bases may participate directly as antioxidants in the light-induced oxidation of milk by quenching triplet riboflavin, rather than acting as scavengers of radicals formed during the initial step of light-induced oxidation by reaction between triplet riboflavin and oxidation substrates, since their rate constants are of the same magnitude as deactivation by molecular oxygen (9.8×10^8 M^{-1} s^{-1}) and other substrates such as whey proteins (β-lactoglobulin = 3.6×10^8 M^{-1} s^{-1}) (*4*).

Additional evidence for direct deactivation by electron transfer is observed by monitoring the rate of excited triplet riboflavin decay at 720 nm versus the rate of formation of riboflavin anion radical at 340 nm (*18*). As can be seen in **Figure 3**, an intercept point at $t_{1/2}$ is observed, showing that formation of the riboflavin

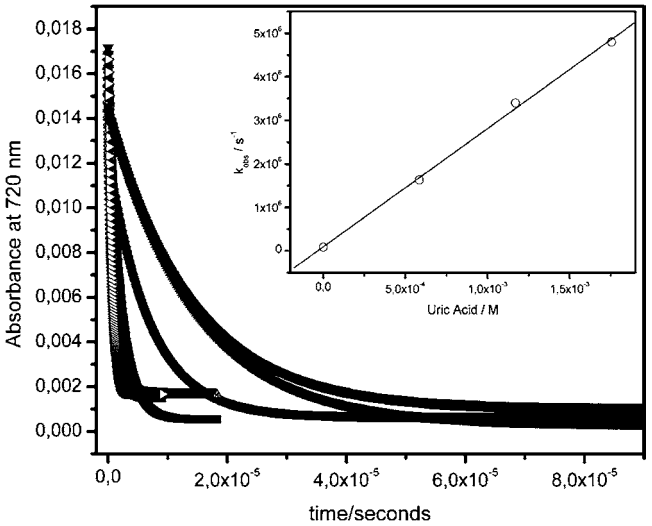


Figure 2. Transient absorption decays of triplet riboflavin (probe set to light of 720 nm) in N_2-saturated aqueous solution (pH 6.4) in the absence and presence of uric acid at four different concentrations. (Inset) Plot of k_{obs} versus uric acid concentration.

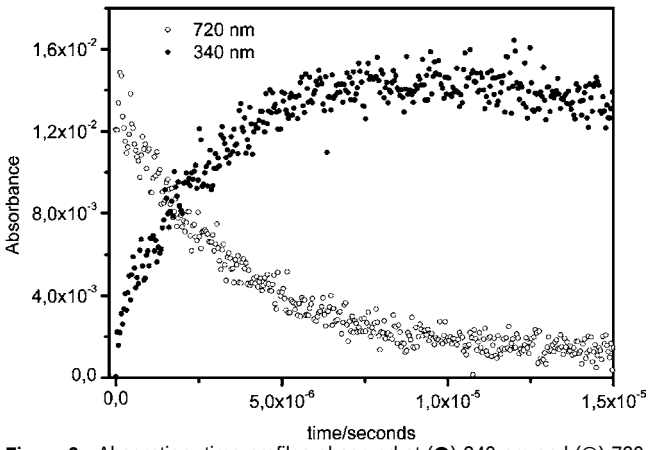


Figure 3. Absorption–time profiles observed at (●) 340 nm and (○) 720 nm after 440 nm laser photolysis of N_2-saturated aqueous solution at pH 6.4 containing riboflavin (80 μM) and uric acid (500 μM).

Table 1. Second-Order Rate Constant at pH 6.4 and 25 °C, Frontier Orbital Energy, Bond Dissociation Energy, and Activation Parameters for Triplet-Excited Riboflavin Deactivation by Purine Derivative

purine base	k_q (M^{-1} s^{-1})/ 10^9	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	E_{ox} (V vs NHE)	$\Delta G°$ (kJ mol^{-1})	HOMO energy (u.a.)	BDE (kJ mol^{-1})	pK_{a1}
uric acid	2.9 ± 0.14	14.7 ± 1.7	−15.5 ± 1.7	+0.686	−114	−0.1713	273.4	5.4
xanthine	1.2 ± 0.40	34.3 ± 11	+45.3 ± 6.8	+1.106	−73.5	−0.2004	374.8	7.4
hypoxanthine	0.17 ± 0.02	122 ± 18	+319 ± 31	+1.654	−20.6	−0.2505	396.1	8.9
purine					>0	−0.2685	435.3	

anion radical is synchronous with decay of the triplet state in the presence of purine derivatives. This behavior strongly suggests direct deactivation processed via electron transfer from the purine base to the triplet-excited riboflavin, followed by a fast protonation of the riboflavin anion radical ($pK_a = 8.3$). As expected for an electron-transfer mechanism as the rate-determining step, a lack of pH dependence on the rate of triplet-excited riboflavin deactivation by uric acid is observed when the pH is varied from 6.4 ($k = 2.9 \times 10^9$ M^{-1} s^{-1}) up to 9 ($k = 2.7 \times 10^9$ M^{-1} s^{-1}).

The suggested electron-transfer mechanism was further studied by taking into account the one-electron oxidation

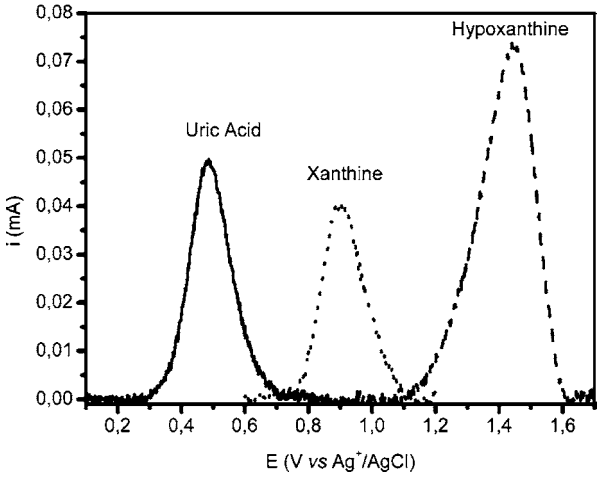


Figure 4. Square-wave voltammetry on boron-doped diamond electrode for uric acid (1.4×10^{-4} mol L^{-1}), xanthine (1.3×10^{-4} mol L^{-1}), and hypoxanthine (2.1×10^{-4} mol L^{-1}) in 0.2 mol L^{-1} phosphate buffer pH 6.4. $V = 500$ mV s^{-1}.

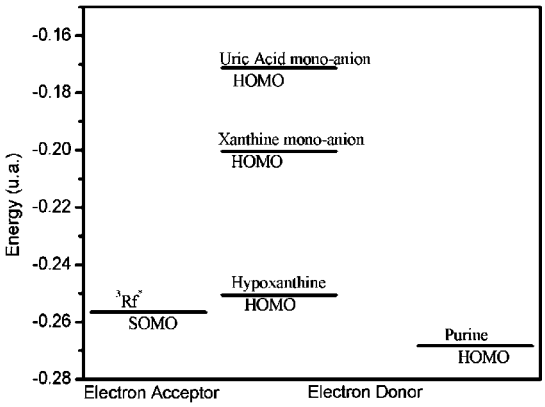


Figure 5. Frontier orbital: SOMO and HOMO levels of triplet-excited riboflavin and purine derivatives.

potential for each purine derivative. From the square-wave voltammetry data, shown in **Figure 4**, the free energy change, $\Delta G°$, for electron-transfer reaction was estimated (**Table 1**), according to the Rehm−Weller equation (*19*):

$$\Delta G° = 96.48(E_{ox} - E_{red} - e^2/\epsilon_a) - \Delta E_{0,0} \qquad (2)$$

where e^2/ϵ_a is the Coulombic term and can be neglected in aqueous medium, $\Delta E_{0,0}$ is the energy level of excited-triplet riboflavin (208.2 kJ mol^{-1}; *20*), E_{red} is the one-electron reduction potential for triplet-excited riboflavin (*21*), and E_{ox} is the one-electron oxidation potential for the purine bases.

From the free energy change for the electron-transfer process—uric acid ($\Delta G° = -114$ kJ mol^{-1}), xanthine ($\Delta G° = -73.5$ kJ mol^{-1}), and hypoxanthine ($\Delta G° = -20.6$ kJ mol^{-1})—an efficient intermolecular electron transfer from the quenchers to the triplet-excited states of riboflavin is expected. An exception was found for purine, for which according to the electrochemical experiments $\Delta G° > 0$ is expected, and thereby the electron-transfer rate is an endergonic process. The theoretical results support these findings. As can be seen in **Figure 5**, the energy of the purine HOMO (−0.2685 a.u.) is lower than the energy of the triplet riboflavin SOMO (−0.2557 a.u.), suggesting a nonspontaneous electron-transfer process from the purine HOMO to the triplet-riboflavin SOMO. Consistently, the correlation coefficient between the energy of the purine derivative's frontier orbital and the specific second-order rate constant was found to be close to unity.

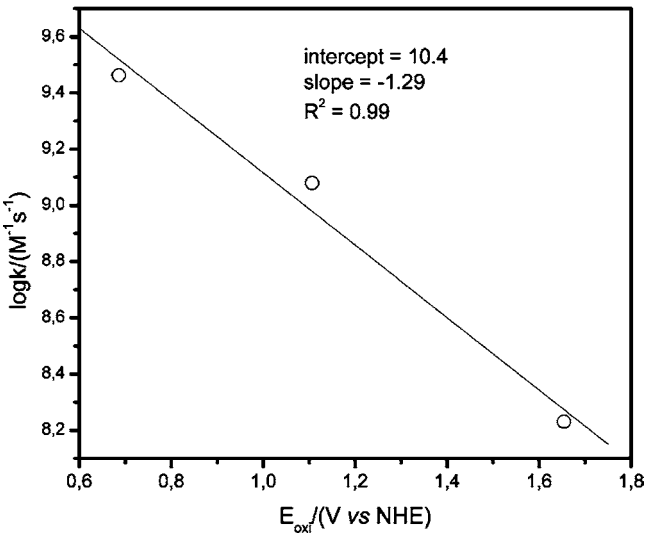


Figure 6. Plot of log k versus E_{ox} for deactivation of triplet-excited riboflavin by purine derivatives in aqueous solution at pH 6.4.

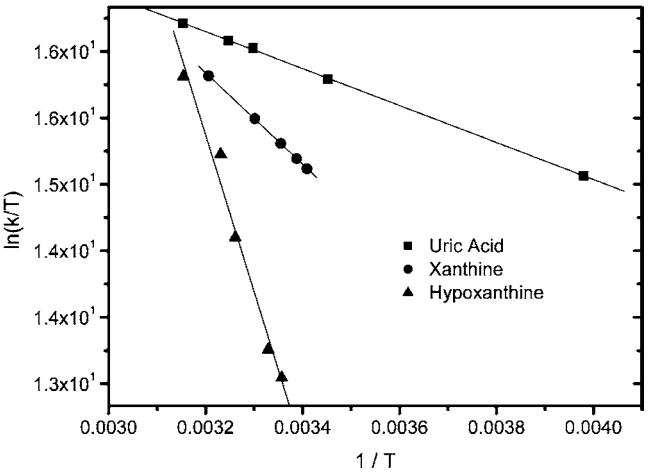


Figure 7. Arrhenius plot for triplet-excited riboflavin deactivation by purine derivatives.

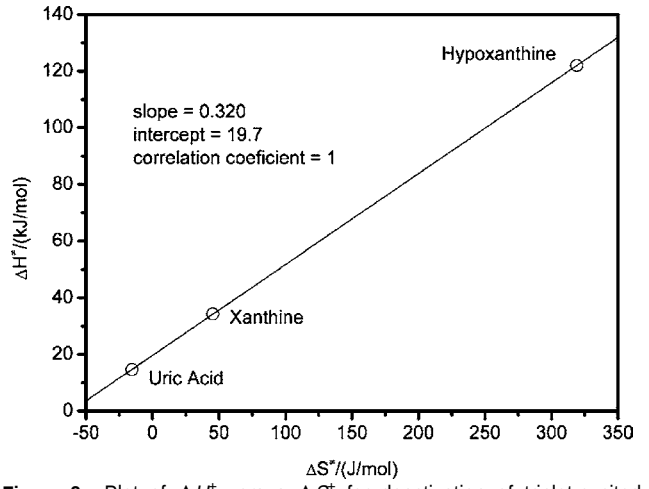
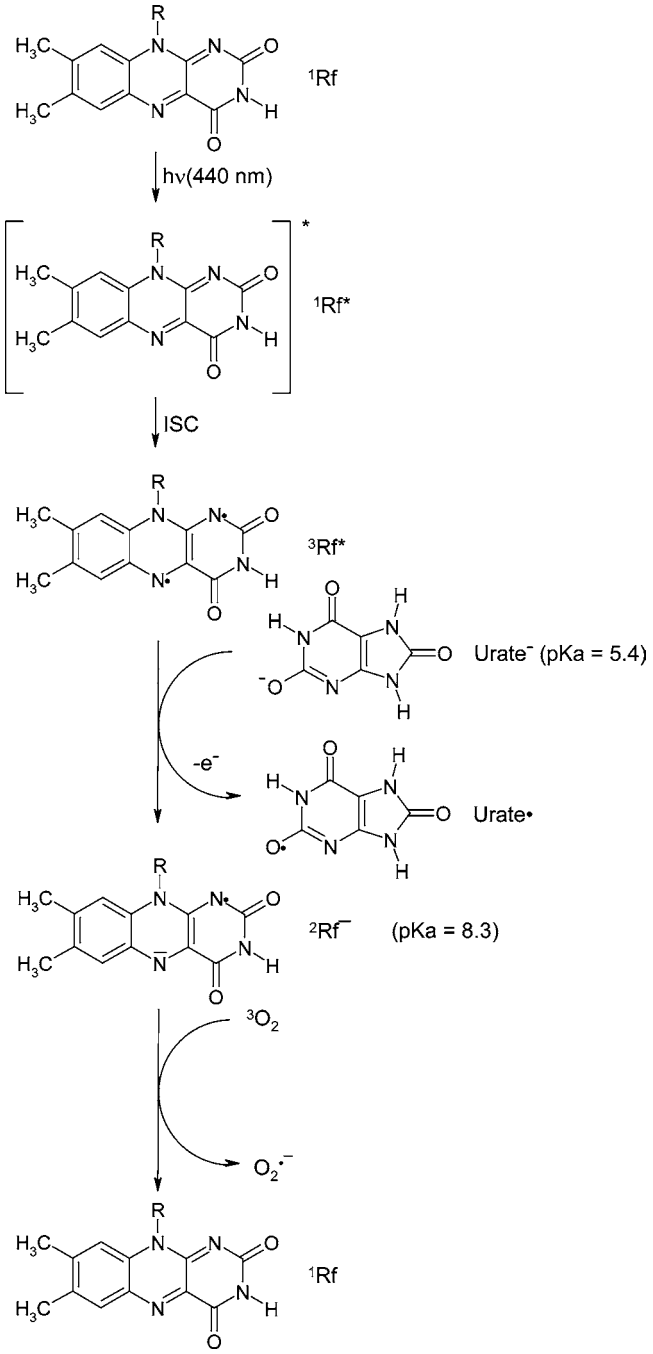


Figure 8. Plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$ for deactivation of triplet-excited riboflavin by purine derivative in aqueous solution at pH 6.4.

A linear free energy relationship (LFER) was found by plotting the one-electron oxidation potential, or free energy change, for uric acid, xanthine, and hypoxanthine versus the logarithm of the respective specific rate constant for triplet-excited riboflavin deactivation as shown in **Figure 6** with a correlation coefficient of 0.99, suggesting an electron transfer as the rate-determining step.

Scheme 1. Reaction Mechanism for Direct Deactivation of Triplet-Excited Riboflavin by Purine Derivatives



By plotting the BDE energy versus the second-order rate constant for triplet-state deactivation, a correlation coefficient of 0.83 is obtained, which suggests that an H-abstraction mechanism is unlikely to be operative in the system described here. Additionally, the free energy change for the H-abstraction mechanism from uric acid by triplet-excited riboflavin was calculated according to the Rehm−Weller (*19*) equation for H-atom transfer:

$$\Delta G^{\circ} = 96.48(E_{ox} - E_{red} - e^2/\epsilon_a) - \Delta E_{0,0} - 2.3RT [pK_{a(RibH\bullet)} - pK_{a(uric\ acid\bullet^+)}]$$

where the pK_a for riboflavin anion radical is 8.3 and the pK_a

for uric acid cation radical is 9.5. When the free energy changes are compared for the H-abstraction mechanism from uric acid to triplet-excited riboflavin (-48.4 kJ mol^{-1}) and for the electron-transfer mechanism (-114 kJ mol^{-1}), the H-abstraction mechanism is 65.6 kJ mol^{-1} more endergonic than the electron-transfer mechanism.

By use of transition-state theory, the activation parameters were investigated for deactivation of triplet riboflavin by purine derivatives by comparing the specific rate constants obtained at five different temperatures, as seen in **Figure 7**. The collected activation parameters for triplet-excited deactivation show that both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ vary along the series of purine bases, and a compensation effect is observed; that is, when $\Delta H^{\ddagger}$ increases, $\Delta S^{\ddagger}$ does so as well. A plot of $\Delta H^{\ddagger}$ versus $\Delta S^{\ddagger}$ is linear (**Figure 8**), suggesting that all the purine derivatives react by a common mechanism, that is, electron transfer from the quencher to triplet-excited riboflavin.

According to our findings, endogenous purine bases, particularly uric acid, are potentially antioxidants toward light-induced formation of singlet oxygen sensitized by riboflavin in milk. However, this antioxidant action promotes the formation of free radicals (urate radical, riboflavin neutral radical, and superoxide anion radical), which can be capable of trigger protein and lipid oxidation. Hence, the use of a special feeding regime to induce endogenous production of uric acid in milk (*9*−*11*) should be carefully evaluated since pro-oxidative effects may be present. Furthermore, the role of caffeine, an exogenous purine base, as antioxidant in coffee-based milk products should also be taken into account.

In conclusion, purine derivatives with accessible one-electron oxidation potentials (lower than +2 V vs NHE) to the triplet-excited riboflavin can directly deactivate the triplet state of riboflavin by an electron-transfer mechanism. Uric acid is shown to be a very efficient quencher for the triplet-excited state, and its role in the light-induced oxidation of milk should be reevaluated since it may directly react with triplet-excited riboflavin by a type I mechanism, as shown in **Scheme 1**. The present work shows that further studies should be carried out to provide a better understanding of the role of radicals produced in type I photooxidation sensitized by riboflavin since, as may be seen from **Scheme 1**, two free radical species may be formed per photon absorbed by riboflavin.

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