Mechanism of free radical oxidation of caffeine in aqueous solution

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The oxidation of caffeine with persulfate and hydroxyl radicals in aqueous solution has been studied by EPR spectroscopy and HPLC analysis. In both cases the formation of 1,3,7-trimethyluric acid is observed as the main final product. A C8–OH radical adduct is postulated as the intermediate after reaction with $OH^{\bullet}$, and leads to the final product after further oxidation. This radical is too short-lived to be observed by EPR. After oxidation of caffeine with $SO_4^{\bullet -}$ its radical cation is detected by EPR. This radical reacts with water to produce the above mentioned C8–OH radical adduct after deprotonation. In the presence of dibasic phosphate a spectrum attributed to the C8-phosphate radical adduct is observed. This radical results from the nucleophilic attack of the buffer on the radical cation of caffeine. Hydrolysis and oxidation of the phosphate radical intermediate results in the formation of 1,3,7-trimethyluric acid.

Introduction

Caffeine is a naturally occurring purine present in coffee, tea, cocoa and cola nuts. Because of the immense consumption of caffeine containing beverages, research has focused attention on its physiological effects. Caffeine has been reported to have antimutagenic, antifungal, antiviral and antioxidant properties and to be an efficient scavenger of oxygen free radicals.[1–3]

There is only limited information on the reaction of caffeine with oxidizing free radicals. The main product of this reaction is 1,3,7-trimethyluric acid (8-hydroxycaffeine),[4,5] resulting from site-specific hydroxylation on C8. However, products from monodemethylation at N1, N3 and N7, like theobromine, paraxanthine and theophyline, respectively, were also detected.[5] Under Fenton conditions both demethylation and hydroxylation at C8 occur, yielding mainly 1-methyluric acid and 1,3-dimethyluric acid.[6]

Although the ultimate products of the oxidative degradation of caffeine are known, almost nothing is known about the possible radical intermediates or about the reaction mechanisms. In this work we studied by EPR spectroscopy the radicals formed in the oxidation of caffeine with persulfate and hydroxyl radicals photolytically generated in a flow system. The final products of these reactions were also investigated by reversed-phase HPLC and, by combining both results, mechanisms for the radical-induced oxidative degradation of caffeine are proposed.

Results and discussion

HPLC results

The products of the reaction of caffeine with $OH^{\bullet}$ in a neutral medium were studied by HPLC with photodiode array detection. The main product observed is 1,3,7-trimethyluric acid [Fig. 1(*a*)], suggesting the formation of an OH radical-adduct of caffeine at C8 (**2**$^{\bullet}$, see Scheme 1) that yields 1,3,7-trimethyluric acid (**4**) after further oxidation. The attack of $OH^{\bullet}$ at this position is a typical pattern of the reaction of this radical with purines, as is already well documented for unsubstituted xanthine,[7] isocaffeine[8] and the DNA purines adenine[9] and guanine.[10] Products resulting from monodemethylation of caffeine, *e.g.* theophyline and theobromine, have also been detected in trace amounts. In the experiments here reported it was also observed that the amount of 1,3,7-trimethyluric acid formed upon reaction of caffeine with $OH^{\bullet}$ decreases for irradiation times longer than one hour. The decrease or even disappearance of 1,3,7-trimethyluric acid is accompanied by the formation of new products with shorter HPLC retention times. This is in good agreement with the high capacity of $OH^{\bullet}$ to oxidize uric acid,[11] a behavior similar to that found on the reaction of this radical with 8-hydroxyadenine.[12]

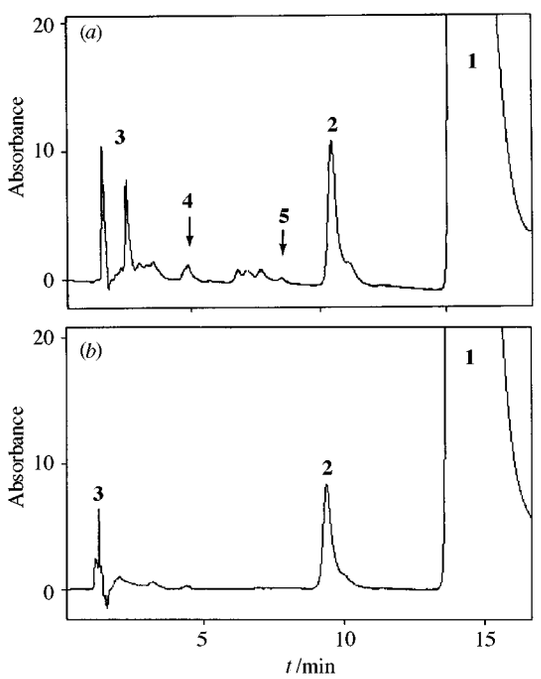
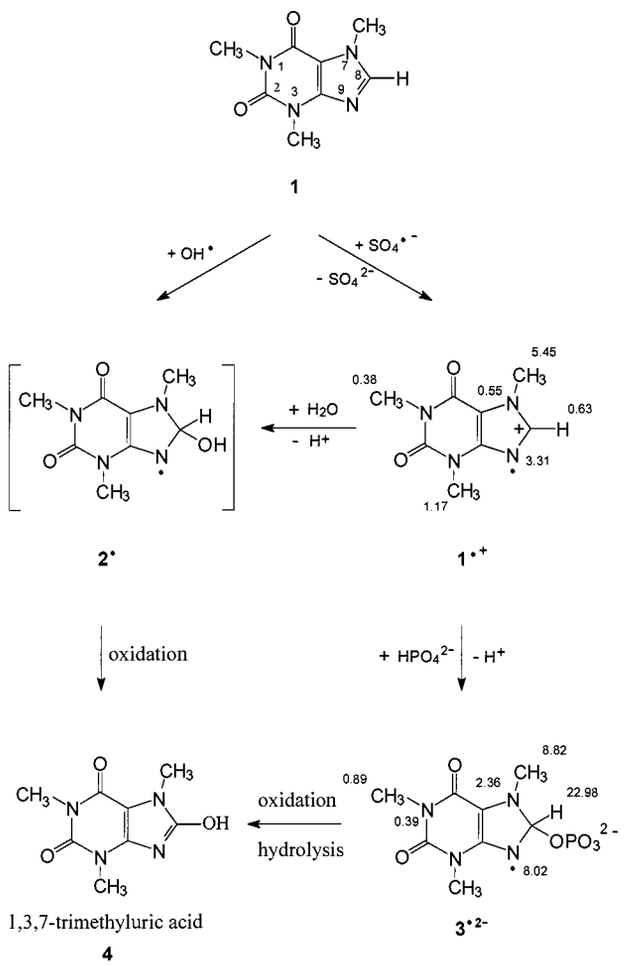


Fig. 1 Optical chromatograms at 280 nm of 0.5 mM caffeine solutions buffered with 10 mM phosphates at pH 6.9, containing (*a*) 1 mM H_2O_2 and (*b*) 1 mM $K_2S_2O_8$, after 10 min, UV-irradiation. **1**: caffeine; **2**: 1,3,7-trimethyluric acid; **3**: unretained solutes; **4**: theobromine; **5**: theophyline.

The reaction of caffeine with $SO_4^{\bullet -}$ was also studied by the method described above. This radical is known to react with purines mainly by electron transfer, producing one-electron oxidized species as primary products, *i.e.* radical cations. After UV-irradiation of caffeine solutions containing $K_2S_2O_8$ at pH 7–9, the formation of the corresponding 8-hydroxylated compound, 1,3,7-trimethyluric acid, was observed as the only product [Fig. 1(*b*)]. The presence of this compound is consistent with the reaction of the radical cation with water to yield an OH radical-adduct at C8 after deprotonation, formally identical to the one produced upon reaction of caffeine with $OH^{\bullet}$ (**2**$^{\bullet}$). Further oxidation of this radical results in the final product. A similar reaction mechanism was recently found for the $SO_4^{\bullet -}$ oxidation of isocaffeine (1,3,9-trimethylxanthine), which



Scheme 1 Mechanism of caffeine (**1**) oxidation by the hydroxyl and sulfate radicals. Hyperfine coupling constants are shown (in G) for $\mathbf{1}^{\bullet+}$ and $\mathbf{3}^{\bullet 2-}$. Constants not mentioned are within linewidth.

produces 1,3,9-trimethyluric acid as the final product.[8] The presence of phosphate buffer increased the yield of 1,3,7-trimethyluric acid (see EPR results). Above pH 10 the presence of uric acid was not detected, since uric acids are readily decomposed by persulfate in basic conditions.[13]

EPR results

We extensively studied the reaction of caffeine with $OH^{\bullet}$ produced by the photolysis of 4-mercaptopyridine *N*-oxide or H_2O_2 by EPR and no radicals were ever detected. Changes in the relative concentration of the reactants, in pH or in the flow rate of the solution also proved to be unsuccessful. The previously postulated 8-OH radical adduct of caffeine (**2**$^{\bullet}$) probably disproportionates fast to give 1,3,7-trimethyluric acid and to regenerate caffeine. A similar mechanism was found for the $OH^{\bullet}$ oxidation of hypoxanthine to xanthine and of xanthine to uric acid.[7,14] Under these circumstances the radical **2**$^{\bullet}$ is too short-lived in aqueous solution to be observed by EPR.

Oxidation of caffeine with photolytically generated $SO_4^{\bullet-}$ gave rise to a weak EPR spectrum (Fig. 2) with $g = 2.003\,92$ and hyperfine coupling constants of one hydrogen, two non-equivalent nitrogens and three sets of three equivalent hydrogens (CH_3 groups). This spectrum was attributed to the radical cation of caffeine $\mathbf{1}^{\bullet+}$ and its intensity was enhanced by the presence of buffer (except dibasic phosphate, see below). The best results were obtained with 60 mM succinate at pH = 8. The radical is stable down to pH = 4 but the spectrum disappears at pH values higher than 11.5.

In order to assign the hyperfine coupling constants caffeine selectively deuteriated on the methyl groups was used. EPR spectra of the radical cation derived from 1-CD_3-caffeine and

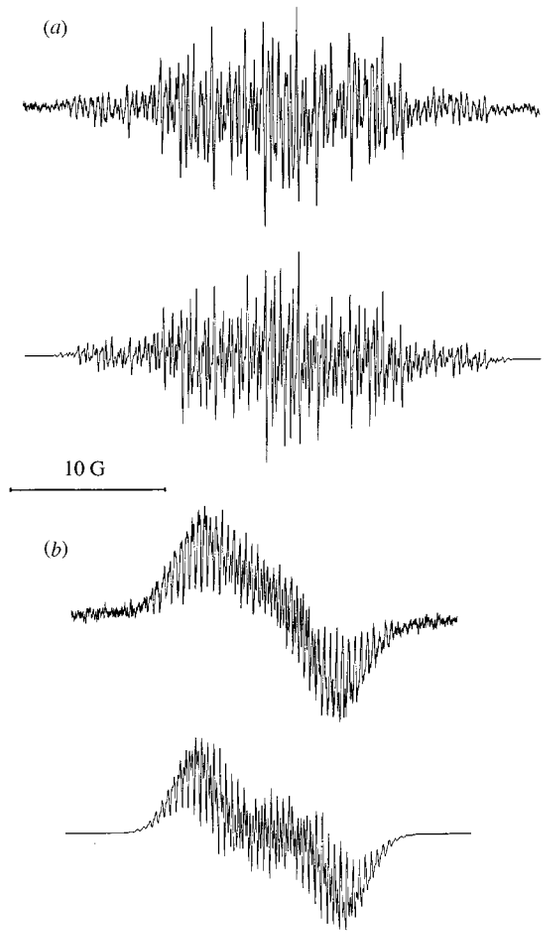


Fig. 2 EPR spectra of the radical cation ($\mathbf{1}^{\bullet+}$) of (*a*) caffeine and (*b*) 7-CD_3-caffeine and corresponding simulations. For coupling constants see Scheme 1 and text.

7-CD_3-caffeine unequivocally assigned the hydrogen hyperfine constants of the methyl groups (see Scheme 1). In the spectra of the 7-CD_3-caffeine radical cation, the 7-methyl hydrogen coupling constants were replaced by deuterium constants with magnitudes that agree with the theoretical ratio $a_D/a_H = 0.153$ ($a_D = 0.82$ G). In the spectra of the 1-CD_3-caffeine radical cation the deuterium constants are within the linewidth. The largest nitrogen hyperfine coupling constant was assigned to N9 by resonance considerations. These results agree with the ones from Lenard and McDowell. These authors studied the radical cation of caffeine in crystals of caffeine hydrochloride at 77 K by ENDOR and obtained hyperfine coupling constants of 6.9 G for 7-CH_3 and 1.91 G for 3-CH_3. All the other hydrogen constants were smaller than 1 G and were not determined.[15]

The radical cation of caffeine reacts with water to give the 8-OH radical adduct **2**$^{\bullet}$, as mentioned above. The weak spectrum of the radical cation is indicative of its short lifetime. The presence of the conjugated base of the buffer, acting as a negatively charged counterion, may somewhat protect the radical cation from the reaction with water. This would explain the increase in the intensity of the spectrum in the presence of buffer. The decrease of the EPR signal intensity with increasing pH indicates that the reaction of the radical cation with OH^- is faster than with water. At pH values higher than 11 the radical cation is already too short-lived to be detected by EPR.

The use of phosphate as a buffer between pH = 8 and pH = 12 resulted in the observation of a much wider and more intense EPR spectra (Fig. 3) with $g = 2.003\,84$ and coupling constants characteristic of three different nitrogens, two different sets of three equivalent hydrogens (CH_3 groups) and a very large hydrogen coupling constant (*ca.* 23 G). The magnitude of the hydrogen coupling constant and the fact that the intensity

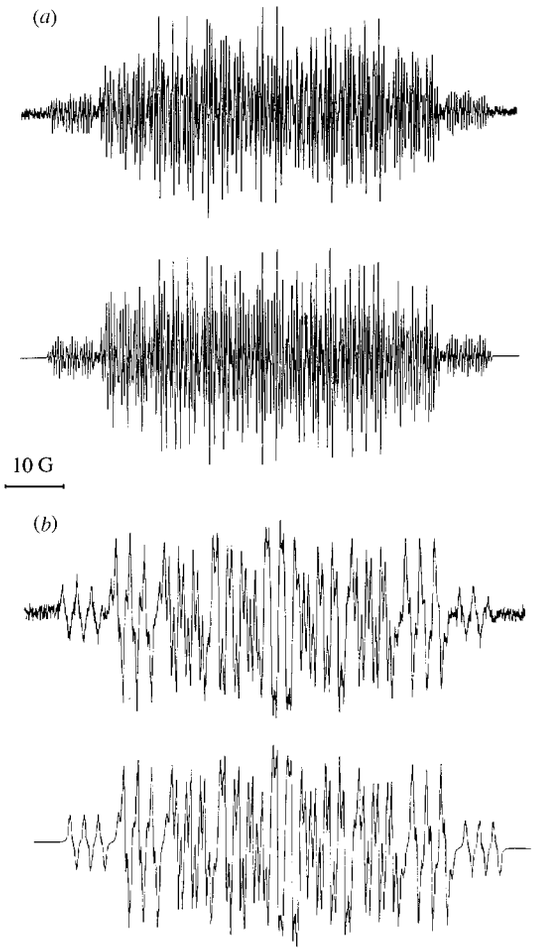


Fig. 3 EPR spectra of the 8-phosphate radical adducts ($\mathbf{3}^{\bullet 2-}$) of (*a*) caffeine and (*b*) 1-CD^3-caffeine and corresponding simulations. For coupling constants see Scheme 1 and text.

of the spectra increased with increasing concentration of phosphate led us to assign these spectra to the caffeine-8-phosphate radical adduct ($\mathbf{3}^{\bullet 2-}$). This radical results from the nucleophilic attack of dibasic phosphate on the radical cation. No ^{31}P hyperfine coupling constant was observed. The assignment of the hyperfine coupling constants was made as for the radical cation. The deuterium constants obtained for 7-CD_3 and 1-CD_3 were 1.35 G and 0.13 G, respectively.

Competition between water and dibasic phosphate for reaction with radical cations was previously found in the oxidation of 1-methyluracils with $SO_4^{\bullet -}$ and phosphate radical adducts were also observed.[16] The dibasic phosphate seems to be the only nucleophile capable of competing with water for the reaction with the radical cation. At pH values lower than 7, where phosphate is in the monobasic form, and with all the other buffers used (acetate, borate, succinate and sulfate) only the spectra of the radical cation were observed. No spectra were detected at pH >12, indicating that the OH^- reacts faster with the radical cation than the phosphate di- or tri-anion.

The yield of 1,3,7-trimethyluric acid (**4**) formed after the oxidation of caffeine by the persulfate radical increased in the presence of phosphate buffer. The caffeine-8-phosphate radical adduct formed under these circumstances undergoes hydrolysis yielding 1,3,7-trimethyluric acid after further oxidation. Since dibasic phosphate reacts faster with the radical cation than water, the overall effect of the phosphate is to catalyze the transformation of the radical cation into **4**. The shorter lifetime of the radical cation in the presence of phosphate decreases the kinetic competition from possible reactions of this radical not resulting in **4**. Therefore, the yield of this final product is increased.

Experimental

Caffeine (BDH) was recrystallized from water and dried at 100 °C. 1-CD_3-Caffeine and 7-CD_3-caffeine were prepared by methylation of theobromine and theophyline, respectively, with [$^2H_3$]methyl iodide (Aldrich, 99.5+ atom % D) in aqueous basic solution. Both products were extracted with chloroform and recrystallized from water.

For the HPLC experiments the solutions to be irradiated were prepared immediately before use in Millipore Milli-Q water and contained 0.5 mM caffeine, 1 mM $K_2S_2O_8$ or 0.1 mM 4-mercaptopyridine *N*-oxide or 0.1 mM H_2O_2, with or without 10–50 mM Na_2HPO_4. UV-photolysis was carried out over 1–90 min using a Hanau 150 W high pressure Hg lamp and 2.5 ml quartz cells placed at 5 cm from the lamp. Analyses of the irradiated solutions were performed by a HPLC system consisting of a Shimadzu LC-10AS pump, a Reodhyne model 5125 injector fitted with a 100 μl loop, a reversed-phase (octadecylsilane) Merck analytical column and a Shimadzu SPD-M10A diode array optical detector set to the range 200–300 nm. Samples were eluted with aqueous solutions containing 10–20% (v/v) methanol and buffered with 5–10 mM $NH_4H_2PO_4$.

Solutions for EPR spectroscopy were typically 3 mM in caffeine, 20 mM in potassium persulfate (Merck) and 0.06 to 0.1 M in buffer. Water purified by a millipore Milli-Q system was used in all experiments. For the generation of the $OH^{\bullet}$ radical, 3 mM of 4-mercaptopyridine *N*-oxide[14,17,18] or 20 mM H_2O_2 were used. The solutions were deaerated with argon, thermostatted at 293 ± 2 K and allowed to flow at a constant rate into a quartz flat cell placed in the EPR cavity. The *in situ* photolysis was performed with an optically focused high-pressure Hg–Xe UV lamp. X-Band EPR spectra were recorded in a Bruker ER200D spectrometer.

Acknowledgements

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