Kinetics of oxidation of thymine by \( \text{r-BuO}' \) radical — Protection and repair by caffeic acid

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Received 9 April 2006; revised 17 August 2006

The kinetics of oxidation of thymine and caffeic acid by \( \text{r-BuO}' \) radicals has been studied by the photolysis of \( \text{r-BuOOH} \) in the presence of \( \text{t-BuOH} \). The rates and the quantum yields \((\phi)\) of oxidation of caffeic acid by \( \text{r-BuO}' \) radicals have been determined by varying concentrations of thymine. An increase in [thymine] has been found to decrease the rate of oxidation of caffeic acid suggesting that thymine and caffeic acid compete for \( \text{r-BuO}' \) radicals. From competition kinetics, the rate constant of \( \text{r-BuO}' \) - thymine reaction has been calculated to be \( 5.3 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{s}^{-1} \). The experimentally determined quantum yields \((\phi_{\text{cal}})\) and the calculated yields \((\phi_{\text{exp}})\) of oxidation of caffeic acid, assuming that caffeic acid reacts only with \( \text{r-BuO}' \) radicals, suggest that caffeic acid not only protects thymine from \( \text{r-BuO}' \) radicals but also repairs thymine radicals formed by the reaction of \( \text{r-BuO}' \) radicals.

IPC Code: Int. Cl. C07B33/00

Hydroperoxides, such as \( \text{r-BuOOH} \) and cumene hydroperoxide, are rapidly metabolized via two major processes. The first of these, which is the normal metabolic route and the major detoxification pathway, involves two-electron reduction at the expense of glutathione in a reaction catalyzed by the enzyme glutathione peroxidase\textsuperscript{1}. The other process is one-electron reduction of peroxide to give free radicals, which is significant in the presence of high concentrations of the hydroperoxide. The free radicals are formed by either reduction or oxidation of the hydroperoxide by the cytochrome P450 enzyme family\textsuperscript{2,3}, by other heme proteins and by low molecular weight metal ion complexes\textsuperscript{4}. The generation of radical species from these compounds is directly linked to their tumor-promoting activity\textsuperscript{5}. But, relatively little is known about the biological effects of these radicals and the key cellular targets for these species. The exposure of cultured cells to hydroperoxides is reported to result in the generation of DNA strand breaks\textsuperscript{6,7} though the mechanism of damage has not been elucidated. In particular, it is not clear whether the observed damage arises from the direct interaction of the hydroperoxide derived radicals with DNA or not. Similarly, it is not known whether strand breakage is the major form of DNA damage, or whether like \( \cdot \text{OH} \) induced damage, attack at the base moiety is also important\textsuperscript{9,10}. Sudha et al.\textsuperscript{11} studied the reactions of \( \text{SO}_4'^- \) with adenosine and reported that the base moiety is preferentially attacked by \( \text{SO}_4'^- \) rather than sugar moiety. The reactions of similar radical species, \( \text{PO}_4'^- \) with thymine have also been reported\textsuperscript{12}. Previous studies on the reactivity of tertiary butoxy radicals suggest that this species might attack both the sugar and the base moieties\textsuperscript{13} of DNA.

\( \text{r-BuO}' \) radicals have been generated here by steady state photolysis of tert-butyl hydroperoxide \( (\text{t-BuOOH}) \) in the presence of tert-butanol \( (\text{t-BuOH}) \), which scavenges \( \cdot \text{OH} \) radicals in aqueous solution\textsuperscript{14}. The reactions of \( \text{r-BuO}' \) radicals with thymine have been studied to understand the mechanism of oxidation. These reactions have also been studied in the presence of caffeic acid to get an insight into the nature of transient thymine radicals produced and to assess any possible protection due to caffeic acid.

Experimental

Thymine and caffeic acid were purchased from Sigma and used as received. All the solutions were prepared afresh using double distilled water. \( \text{t-BuOOH} \) was procured from Merck-Schuchardt of Germany. No contamination of other peroxides was found during the assay of the sample. \( \text{t-BuOOH} \) was estimated by iodometric method\textsuperscript{15}. Srinivasan Griffin Rayonet type photochemical reactor, containing four 18 W medium pressure mercury lamps arranged in a circular way, was used. Irradiations of the samples were carried out at room temperature. The quartz cuvette containing the sample was exposed to light from both sides of the transparent surfaces and the irradiations were uniform throughout the bulk of the solution. The irradiations were interrupted at regular intervals of time to determine the absorbance. The light intensity corresponding to the irradiating wavelength (254 nm) was measured using peroxysulphate chemical actinometry\textsuperscript{16,17}. On
photoysis, \( t\)-BuOOH got activated to generate \('OH\) and \( t\)-BuO\(^\cdot\) radicals by homolytic cleavage of O-O bond\(^{18}\). The 'OH radicals produced have been scavenged using sufficient concentration of \( t\)-BuOH\(^{14}\). In a typical reaction, the aqueous reaction mixture containing the desired concentration of thymine, \( t\)-BuOH and \( t\)-BuOOH was taken in one-centimeter path length quartz cuvette, suitable for both irradiation and absorbance measurements. The absorbance measurements were made on a Chemito 2100 UV-vis spectrophotometer.

The photochemical reaction of caffeic acid in the presence of \( t\)-BuOOH and other additives, viz. \( t\)-BuOH and thymine, has been followed by measuring the absorbance at 310 nm.

It is known that \( t\)-BuOOH is activated to radical reaction by the absorption of light at 254 nm\(^{19}\). However, the substrates used in the present work, viz. caffeic acid and thymine have strong absorption in this region. But, in the absence of \( t\)-BuOOH, neither caffeic acid, nor thymine, or caffeic acid - thymine mixture undergoes any observable chemical change on exposure to light. Even though a small fraction of the total light intensity is absorbed by \( t\)-BuOOH directly in the presence of thymine and/or caffeic acid and also \( t\)-BuOH, a considerable chemical change is observed with thymine and caffeic acid. It is also observed that the rates of oxidation of thymine or caffeic acid in the presence of \( t\)-BuOOH are found to increase with increase in [thymine] or [caffeic acid] (Tables 1 and 2). If thymine and caffeic acid act as only inner filters, the rates of the reaction of thymine or caffeic acid with \( t\)-BuO\(^\cdot\) would have decreased with increase in [thymine] or [caffeic acid]. Hence, we propose that the excited states of caffeic acid and thymine act as sensitizers to transfer energy to \( t\)-BuOOH to produce radical species. This type of sensitizing effect has been proposed in similar systems earlier\(^{19,20}\). Therefore, the light intensity at 254 nm has been used to calculate the quantum yields of oxidation of thymine as well as caffeic acid under different experimental conditions.

### Results and discussion

The oxidation of thymine by \( t\)-BuO\(^\cdot\) radicals has been carried out by irradiating the reaction mixture containing known concentrations of thymine and \( t\)-BuOOH in the presence of sufficient amount of \( t\)-BuOH to scavenge the 'OH radicals completely\(^{14}\). The reaction was followed by measuring the absorbance of thymine at 264 nm (\( \lambda_{\text{max}} \) of thymine) with time. The initial rates of oxidation of thymine were calculated from the initial absorbance versus time using microcal origin computer program on a personal computer (Table 1). The quantum yields of oxidation of thymine were calculated from the initial rates of oxidation of thymine and the light intensity at 254 nm. The rates and quantum yields of oxidation of thymine increased with increase in concentration of thymine as well as \( t\)-BuOOH (Table 1).

The rates of oxidation of caffeic acid by \( t\)-BuO\(^\cdot\) in the presence of \( t\)-BuOH have been calculated using absorbance data of caffeic acid at 310 nm. The quantum yields of oxidation of caffeic acid have been calculated from the initial rates (at 254 nm). These rates and quantum yields of oxidation of caffeic acid have been found to increase with increase in [caffeic acid] as well as [\( t\)-BuOOH] (Table 2).

#### Table 1 — Effect of \([t\)-BuOOH] and [thymine] on the rate and quantum yield of photooxidation of thymine by \( t\)-BuOOH using \( t\)-BuOH in presence of light in aqueous neutral medium ([\( t\)-BuOH] = 1 M, light intensity = \( 3.51 \times 10^3\) quanta s\(^{-1}\), \( \lambda_{\text{max}} = 264 \) nm, \( pH = 7.5\), temp. = 298 K)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>( 10^3 \times [\text{thymine}] ) mol dm(^{-3})</th>
<th>( 10^3 \times [t)-BuOOH] mol dm(^{-3})</th>
<th>( 10^3 \times \text{rate} ) mol dm(^{-3}) s(^{-1})</th>
<th>( \phi_{\text{expt}} )</th>
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#### Table 2 — Effect of \([t\)-BuOOH] and [caffeic acid] on the rate and quantum yield of photooxidation of caffeic acid by \( t\)-BuOOH using \( t\)-BuOH in presence of light in aqueous neutral medium ([\( t\)-BuOH] = 1 M, light intensity = \( 3.51 \times 10^3\) quanta s\(^{-1}\), \( \lambda_{\text{max}} = 310 \) nm, \( pH = 7\), temp. = 298 K)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>( 10^3 \times [\text{caffeic acid}] ) mol dm(^{-3})</th>
<th>( 10^3 \times [t)-BuOOH] mol dm(^{-3})</th>
<th>( 10^3 \times \text{rate} ) mol dm(^{-3}) s(^{-1})</th>
<th>( \phi_{\text{expt}} )</th>
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Having known the rates of t-BuO· radical reactions with thymine as well as caffeic acid separately under varying experimental conditions, both thymine and caffeic acid were introduced for the competitive studies with t-BuO· radicals. As caffeic acid absorbs strongly at 264 nm, it is not possible to determine the competition strongly at 264 nm, it is not possible to determine change of absorbance of thymine due to oxidation by t-BuO· at this wavelength and the protection offered to thymine by caffeic acid could not be estimated. However, one can calculate the extent of protection offered to thymine by caffeic acid from competition kinetics. The measurements were made at 310 nm, λmax of caffeic acid. At this wavelength thymine was transparent. Aqueous solutions of the reaction mixture containing caffeic acid, t-BuOOH and t-BuOH were irradiated in the presence of varying [thymine]. The variation of absorbance at 310 nm (λmax of caffeic acid) with time is shown in Fig. 1. The initial rates and quantum yields of oxidation of caffeic acid by t-BuO· radical in the presence of thymine have been calculated and found to decrease with increase in concentration of thymine (Table 3). Comparison of the initial rates and quantum yields of oxidation of caffeic acid in the presence and absence of thymine clearly indicate that the initial rates and quantum yields of oxidation of caffeic acid substantially decrease in the presence of thymine (Table 4), showing thereby that thymine and caffeic acid are in competition for t-BuO· radicals.

The rate constant for the reaction of t-BuO· with caffeic acid (k confiscate = 8.15 x 10^8 dm^3 mol^-1 s^-1) has been calculated using the reported rate constant for the reaction of t-BuO· with adenosine^13 to be 1.40 x 10^8 dm^3 mol^-1 s^-1 under similar experimental conditions. The rate constant for the reaction of t-BuO· with thymine has been calculated by the caffeic acid competition method, which is very similar to the one chosen by Akhalaq et al.\(^{21}\) to determine the rate constant for the reaction of ·OH radicals with polyhydric alcohols in competition with KSCN. In the present study, solutions containing caffeic acid and varying amounts of thymine in the presence of t-BuOOH and t-BuOH were irradiated for 2 min and the decrease in absorbance of caffeic acid was measured. The decrease in absorbance of caffeic acid reflects the number of t-BuO· radicals that have reacted with caffeic acid. The rate constant for the reaction of t-BuO· with thymine (k thymine) is

![Fig. 1 — Plot of absorbance versus time (Effect of [thymine] on the photooxidation of caffeic acid in the presence of tert-butyl hydroperoxide ([caffeic acid] = 5.00 x 10^-4 mol dm^-3, [t-BuOOH] = 5.00 x 10^-3 mol dm^-3, [t-BuOH] = 1.00 mol dm^-3; [thymine] = (a) 0.00, (b) 2.00 x 10^-3 mol dm^-3, (c) 4.00 x 10^-3 mol dm^-3, (d) 8.00 x 10^-3 mol dm^-3, (e) 10.0 x 10^-3 mol dm^-3, Light intensity = 3.51 x 10^4 quanta s^-1).)](image)

Table 3 — Effect of varying [thymine] on the quantum yield of photooxidation of caffeic acid in the presence of t-BuOOH in aqueous neutral medium ([caffeic acid] = 2 x 10^-3 M, [t-BuOOH] = 5 x 10^-3 M, [t-BuOH] = 1 M, light intensity = 3.51 x 10^4 quanta s^-1, λmax = 310 nm, pH = 7, temp. = 298 K)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>10^4 x [thymine] mol dm^-3</th>
<th>10^8 x rate mol dm^-3 s^-1</th>
<th>Ψexp</th>
<th>ρ</th>
<th>Ψcal</th>
<th>Ψ%</th>
<th>% Scavenging</th>
<th>% Repair</th>
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</table>
The calculated \( \varphi_{\text{cal}} \) values at different [thymine] are given in Table 3. The data show that \( \varphi_{\text{cal}} \) values are lower than the experimentally measured \( \varphi_{\text{expt}} \) values. This indicates that more of caffeic acid is consumed in the system than expected and the most likely route for this is reaction of caffeic acid with thymine radicals. The fraction of \( t-BuO^* \) radicals scavenged by caffeic acid at different [thymine] are given in Table 3. These values refer to the measure of protection of thymine due to scavenging of \( t-BuO^* \) radicals by caffeic acid. Using the \( \varphi_{\text{expt}} \) values, a set of values, viz. \( \varphi' \) have been calculated from Eq. (4) and are given in Table 3.

\[
\varphi' = \frac{\varphi_{\text{expt}}}{p} 
\]

The \( \varphi' \) values indicate the quantum yields corrected for the \( t-BuO^* \) radicals scavenged by thymine. In other words, \( \varphi' \) values indicate the quantum yield values of oxidation of caffeic acid if no scavenging of \( t-BuO^* \) radicals by thymine occurs. Thus, in the absence of any reaction (repair) of thymine radicals with caffeic acid, \( \varphi' \) values should all be equal to \( \varphi_{\text{expt}} \). However, the increase in \( \varphi' \) with increasing thymine concentration (Table 3) clearly indicates that thymine radicals react with caffeic acid. This reaction is called repair reaction of thymine radicals by caffeic acid. The extent of repair may be quantified by:

\[
\% \text{Repair} = \left( \frac{\varphi_{\text{expt}} - \varphi'_{\text{expt}}}{\varphi'_{\text{expt}}} \right) \times 100 \quad \ldots (5)
\]

The data on percentage repair is presented in Table 3. The experimentally determined \( \varphi_{\text{expt}} \) values are higher than the \( \varphi_{\text{cal}} \) values calculated using Eq. (3) under the assumption that caffeic acid acts only as a \( t-BuO^* \) radical scavenger. This shows that caffeic acid acts not only as an efficient scavenger of \( t-BuO^* \) radicals, but also as an agent for the repair of thymine radicals. It is, therefore, obvious that caffeic acid reacts not only with \( t-BuO^* \) radicals but also with thymine radicals. Thymine reacts with \( t-BuO^* \) to form tert-butoxy adduct radical, which on hydrolysis gives oxidizing C6-OH adduct radical. This oxidizing adduct radical captures an electron from caffeic acid and repaired by dehydroxylation to give the parent molecule thymine and caffeic acid radical as shown in Scheme 1.

Table 3 shows that thymine radicals are efficiently repaired by caffeic acid to the extent of 48% at about
Scheme 1

\[
\begin{align*}
\text{t-BuOOH} & \xrightarrow{\text{hv}} \text{t-BuO}^+ + \cdot \text{OH} \\
\text{thymine / caffeic acid} & \xrightarrow{\text{hv}} \text{thymine}^* / \text{caffeic acid}^* \\
\text{thymine}^* / \text{caffeic acid}^* + \text{t-BuOOH} & \xrightarrow{} \text{thymine} / \text{caffeic acid} + \text{t-BuO}^+ + \cdot \text{OH} \\
\cdot \text{OH} + (\text{CH}_3)_2\text{COH} & \xrightarrow{} \cdot \text{CH}_2(\text{CH}_3)\text{COH} + \text{H}_2\text{O}
\end{align*}
\]
20 µM of caffeic acid concentration. This type of repair reactions by caffeic acid have been reported in the oxidation of adenine by SO$_4$$^{2-}$. It is known that caffeic acid can repair the transient oxidizing radicals efficiently. The percentage repair found in the present study suggests that the oxidation of thymine by t-BuO$^·$ radicals is via oxidizing transient radicals on thymine to the extent of 48% at 20 µM of caffeic acid and 1 mM thymine. It is reported that t-BuO$^·$ radical attacks C5-C6 double bond of pyrimidine moiety of nucleic acid. Even though C5 position is relatively electron rich compared to C6, due to the steric hindrance of methyl group at C5 position, t-BuO$^·$ radical could attack at C6. The C5-yl radical formed by attacking at C6 are oxidizing, while produced by addition to C5 (C6-yl radical) are reducing. The percentage repair obtained during the studies shows that the transient oxidizing radicals formed in the oxidation of thymine by t-BuO$^·$ radical are to the extent of 48%. Such type of repair reactions are reported in the oxidation of thymine by PO$_4$$^{3-}$ in presence of DTT and SO$_4$$^{2-}$ in presence of caffeic acid. However, the percentage of oxidizing radicals generated by PO$_4$$^{3-}$ and SO$_4$$^{2-}$ with thymine are different. In short, the reactions of protection of thymine and repair of thymine radicals are as given in Scheme 2.

![Scheme 2](image)

**Acknowledgement**

M Adinarayana is thankful to UGC, New Delhi, for awarding the Major Research Project. The authors thank Prof P Jayaprakash Rao, Department of Chemistry, Osmania University, for helpful discussions.

**References**