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A KINETIC AND MECHANISTIC APPROACH TO PROTECTION AND REPAIR OF *TERT*-BUTOXYL RADICALS INDUCED URACIL RADICALS BY CHLOROGENIC ACID

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ABSTRACT: The rates of oxidation of uracil and chlorogenic acid (CGA) by *tert*-butoxyl radicals have been studied by measuring the absorbance of uracil at 258 nm and CGA at 328 nm spectrophotometrically. *tert*-butoxyl radicals are generated by the photolysis of *tert*-butyl hydroperoxide in presence of *tert*-butyl alcohol to scavenge $^{\bullet}$OH radicals. The rates and the quantum yields (ϕ) of oxidation of CGA by *t*-BuO$^{\bullet}$ radicals have been determined in the absence and presence of varying concentrations of uracil. An increase in the concentration of uracil has been found to decrease the rate of oxidation of CGA suggesting that uracil and CGA compete for *t*-BuO$^{\bullet}$ radicals. Using competitive kinetic studies, the rate constant for the reaction of *t*-BuO$^{\bullet}$ radical with uracil has been found to be 4.43×10^{8} dm^{3} mol^{-1} s^{-1}. The quantum yields (ϕ_{expt}) have been calculated from the experimentally determined rates of oxidation of CGA under different experimental conditions. Assuming that CGA acts as a scavenger of *t*-BuO$^{\bullet}$ radicals only, the quantum yields (ϕ_{cal}) have been theoretically calculated. ϕ_{expt} and ϕ_{cal} values suggest that CGA not only protects uracil from *t*-BuO$^{\bullet}$radicals but also repairs uracil radicals formed by the reaction of uracil with *t*-BuO$^{\bullet}$radicals.

INTRODUCTION: Reactive oxygen species (ROS) are continuously produced in all living beings, especially in higher organisms as a result of normal cellular metabolism, phagocytosis, and inflammation and by other exogenous factors like ionizing radiations and xenobiotics[1-4]. In living cells, when the formation of intracellular reactive oxygen species exceeds the cells' antioxidant capacity, oxidative stress can arise, resulting in damage to cellular macromolecules such as proteins, lipids and DNA[5-7]. DNA is a particularly sensitive cellular target because of the potential to create cumulative mutations that can disrupt cellular homeostasis.

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In this case, the reactive oxygen species can lead to the formation of single and double-strand breaks, as well as induce chemical and structural modifications to purine and pyrimidine bases, and also to 2′-deoxyribose [8, 9].

Oxidative DNA damage has been considered as an important promoter of cancer, besides being implicated in the normal process of aging[10, 11].

Organic peroxides form an important part of various chemical, pharmaceutical and cosmetic products. Upon reduction or oxidation by the cytochrome P450 enzyme family, by other heme proteins and by low molecular weight metal ion complexes, these peroxides produce alkoxyl and hydroxyl radicals. The lethal effects of the hydroxyl radicals on DNA and its constituents have been extensively studied[4] but relatively little is known about the biological effects of alkoxyl radicals and the key cellular targets for these

species. The alkoxyl radicals were shown to react with the nucleobases and nucleosides as well as antioxidants, histone proteins and amino acids[12]. Recent studies [13, 14] have demonstrated that the exposure of cultured cells to alkoxyl radicals resulted in the generation of DNA strand breaks though the mechanism of damage has not been elucidated. Organic oxygen radicals in particular alkoxyl radicals may participate in metabolic and pathological processes.

tert-butyl hydroperoxide (*t*-BuOOH) [15-17] is a well known oxidant that has been used as a model oxygen-centered radical for the study of mechanisms of oxidative cell injury in mammalian cells and to investigate mechanisms of its interaction with polyphenols which on homolysis produces $^{\bullet}$OH and *t*-BuO$^{\bullet}$ radicals.

These *t*-BuO$^{\bullet}$ radicals were found to readily cross cellular membranes and initiate lipid peroxidation, affect cell membrane integrity, damage protein, DNA, and result in cell injury in hepatocytes and rat liver[16, 18]. Previous studies on the reactivity of *tert*-butoxyl radicals suggest that these species might be expected to attack both the sugar and the base moieties of DNA[19]. The experimental evidence indicates that base radicals also contribute to it by transfer of their radical sites from base moiety to sugar moiety. Strand breaks are considered to be a very serious kind of damage to DNA[20, 21].

By definition, an antioxidant is a compound which, when present at concentrations much lower than those of oxidisable substrate(s), delays to a significant extent, or even inhibits, oxidation of the substrate(s). Phenolic antioxidants are particularly attractive as prophylactic agents due to their high prevalence in the diet and also due to their pluripharmacological effects [22, 23].

The pharmacological actions of phenolic antioxidants stem mainly from their free radical scavenging and metal chelating properties as well as their effects on cell signalling pathways and on gene expression[24]. Plant-derived phenolics represents good sources of natural antioxidants, however, further investigation on the molecular mechanism of action of these phytochemicals is crucial to the evaluation of their potential as prophylactic agents. There is emerging interest in the use of naturally occurring antioxidants for the preservation of foods and in the management of a number of pathophysiological conditions, most of which involve free radical damage. During the last decade natural antioxidants, particularly phenolics, has been under very close scrutiny as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases and also aging[25-27]. Moreover, restrictions over the use of synthetic antioxidants BHA and BHT in food further strengthen the concept of using naturally occurring compounds as antioxidants.

CGA, the quinic acid ester of caffeic acid, is a major phenolic compound in coffee. It is present in a wide variety of fruit and vegetables including tomatoes, spinach, broccoli, asparagus, white grapes, pears and peaches. CGA is found to be a potent ROS and RNS radical scavenger[28, 29]. In the presence of CGA, the extent of peroxynitrite mediated DNA damage decreased in a dose-dependent manner suggesting that CGA can inhibit the formation of single strand breaks in supercoiled pBR322 DNA by efficiently scavenging peroxynitrite radicals in peroxidase-containing systems *in- vivo*[30].

Recently, *in-vivo* studies suggested that CGA provides beneficial effects during ischemia-reperfusion injury of rat liver and paraquat-induced oxidative stress in rats[31]. Thus, studies involving CGA assume importance due to its presence in many dietary phytochemicals in higher concentrations.

Hydroxycinnamic acid derivatives such as CGA have been identified as good antioxidants for reduction and repair of OH-adducts of pyrimidines *via* electron transfer. It has been demonstrated[32] that CGA can either scavenge $^{\bullet}$OH radical or repair oxidizing $^{\bullet}$OH radical adduct of 2'-deoxyguanosine-5'-monophosphate (dGMP). Unlike most antioxidants, products of CGA formed by reaction with free radicals were rapidly broken down further to the products which were not able to generate any free radical which is the beneficial

nature of the antioxidant. It is in this background the kinetic study of oxidation of CGA in the presence of uracil, which is used as a model for DNA, have been carried out to with a view to assess the protection offered by CGA towards oxidation of uracil by *t*-BuO$^{\bullet}$radicals and also repair, if any, by CGA towards uracil radicals.

MATERIALS AND METHODS:

Uracil and CGA were purchased from sigma and used as received. All solutions were prepared afresh using double distilled water. *tert*- Butyl hydroperoxide (*t*-BuOOH) was used as received from Merck-Schuchardt of Germany. There is no contamination of other peroxides in the assay of the sample. *t*-BuOOH was estimated by iodometric method[33].

The irradiations were carried out at room temperature in a quantum yield reactor model QYR-20 supplied by Photophysics, England attached with 400 W medium pressure mercury lamp. The quartz cuvette containing the sample was irradiated and the irradiations were interrupted at definite intervals of time and the absorbance was noted. The light intensity corresponding to the irradiating wavelength (254nm) was measured using peroxydisulphate chemical actinometry[34].

On photolysis, *t*-BuOOH is activated at 254 nm to generate $^{\bullet}$OH and *t*-BuO$^{\bullet}$ radicals by homolytic cleavage of –O-O-bond[35]. The $^{\bullet}$OH radicals produced have been scavenged using sufficient concentration of *t*-BuOH[36]. In a typical kinetic run the aqueous reaction mixture of uracil, *t*-BuOOH and *t*-BuOH was taken in a specially designed one-centimeter path length quartz cuvette, suitable for both irradiations and absorbance measurements. The absorbance measurements were made at the λ_{max} of uracil (258 nm) on a Chemito UV-Visible spectrophotometer (model 2100).

The photochemical reaction of CGA in the presence of *t*-BuOOH and other additives, viz., *t*-BuOH and uracil, has been followed by measuring the absorbance of CGA at 328 nm at which uracil is totally transparent.

It is known that *t*-BuOOH is activated to radical reaction by the absorption of light at 254 nm[34]. However, the substrates used in the present work, viz., CGA and uracil have strong absorption in this region. But in the absence of *t*-BuOOH, CGA, uracil or CGA-uracil mixture has not undergone any observable chemical change on shining the light. Even though a small fraction of the total light intensity is absorbed by *t*-BuOOH directly in the presence of uracil and/or CGA, a considerable chemical change has been observed with uracil as well as CGA.

If uracil and CGA act as only inner filters, the rate of the reaction of uracil or CGA with *t*-BuO$^{\bullet}$ radicals would have been decreased with increase in concentration of uracil or CGA but the results are contrary to this. One another fact against the inner filter concept is that the rate of oxidation of CGA in the presence of uracil would have been much less than the experimentally observed values (Table 3). Hence, we propose that the excited states of CGA and uracil 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. Therefore, the light intensity at 254 nm has been used to calculate the quantum yields of oxidation of uracil as well as CGA under different experimental conditions.

RESULTS AND DISCUSSION:

The oxidation of uracil by *t*-BuO$^{\bullet}$ radicals has been carried out by irradiating the reaction mixture containing known concentrations of uracil and *t*-BuOOH in the presence of sufficient amount of *t*-BuOH to scavenge the $^{\bullet}$OH radicals completely[34].The reaction was followed by measuring the absorbance of uracil at 258 nm (λ_{max} of uracil) with time. The initial rates and quantum yields of oxidation of uracil by *t*-BuO$^{\bullet}$ are presented in **Table 1**.

The initial rates of photooxidation of CGA by *t*-BuOOH in presence of *t*-BuOH have been calculated[37] from the plots of absorbance of CGA at 328 nm *vs* time using microcal origin computer program on a personal computer. UV-visible absorption spectra of CGA in presence of *t*-BuOOH and *t*-BuOH at different irradiation times were recorded **(Fig.1)**. In order to find the protection offered to uracil by CGA towards oxidation by *t*-BuO$^{\bullet}$, the reaction mixture

containing known concentrations of uracil, *t*-BuOOH and *t*-BuOH was irradiated in presence of varying concentrations of CGA. The reactions were followed by measuring the absorbance of CGA at 328 nm **(Fig.2)** at which uracil is transparent and the rate data are presented in **Table 2**. The photooxidation of CGA by *t*-BuO$^{\bullet}$ radicals at different concentrations of uracil was also studied **(Fig.3)** and the data are presented in **Table 3**.

The oxidation rate of uracil in the presence of *t*-BuOH refers exclusively to the reaction of *t*-BuO$^{\bullet}$ radicals with uracil. These rates have been found to increase with increase in concentration of uracil as well as *t*-BuOOH. The quantum yield values are also found to increase with increase in [uracil] as well as [*t*-BuOOH] **(Table 1)**.

TABLE 1 - EFFECT OF [*T*-BUOOH] AND [URACIL] ON THE RATE AND QUANTUM YIELD OF PHOTOOXIDATION OF URACIL BY *T*-BUOOH IN THE PRESENCE OF LIGHT IN AQUEOUS NEUTRAL MEDIUM

10^5 × [Uracil] (mol dm^{-3})	10^3 × [*t*-BuOOH] (mol dm^{-3})	10^9 × Initial rate (mol dm^{-3} s^{-1})	Quantum yield (φ)
1.0	5.0	1.143	0.00007
2.0	5.0	2.082	0.00013
5.0	5.0	4.214	0.00028
8.0	5.0	4.802	0.00032
10.0	5.0	5.463	0.00036
5.0	10.0	6.317	0.00042
5.0	15.0	7.489	0.00049

Light Intensity = 2.7168 × 10^{15} quanta s^{-1}, λ_{max} = 258 nm, pH ~ 7.5, Temperature = 298 K, [t-BuOH] = 1.0 mol dm^{-3}

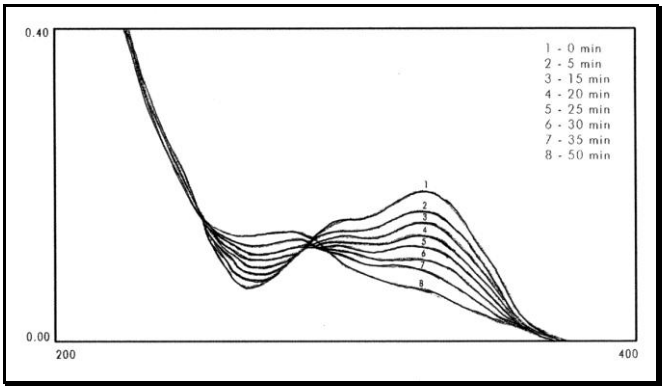


FIG. 1: ABSORPTION SPECTRA OF PHOTOOXIDATION OF CGA IN THE PRESENCE OF *T*-BUOOH AT DIFFERENT IRRADIATION TIMES

[CGA] = 1 × 10^{-5}mol dm^{-3}, [*t*-BuOOH] = 5 × 10^{-3}mol dm^{-3}, Light Intensity = 2.7168 × 10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, temperature = 298K

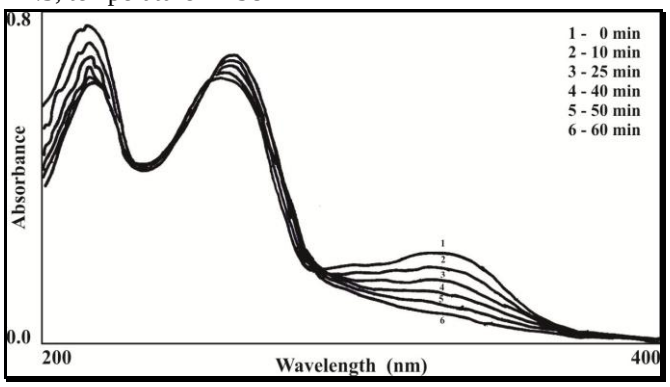


FIG. 2 - ABSORPTION SPECTRA OF PHOTOOXIDATION OF CGA IN THE PRESENCE OF *T*-BUOOH AND URACIL AT DIFFERENT IRRADIATION TIMES

[CGA] = 1 × 10^{-5}mol dm^{-3}, [*t*-BuOOH] = 5 × 10^{-3}mol dm^{-3}, [uracil] = 5 × 10^{-5} mol dm^{-3}, Light Intensity = 2.7168 × 10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, temperature = 298K

The rate of oxidation of CGA has been found to increase with increase in concentration of CGA[37]. The quantum yields of oxidation of CGA have been calculated from the initial rates and the light intensity at 254 nm. These values are also found to increase with increase in concentration of CGA. Having known the rates of *t*-BuO$^{\bullet}$ radical reactions with uracil as well as CGA under varying experimental conditions, both uracil and CGA are introduced for the competitive studies with *t*-BuO$^{\bullet}$ radical.

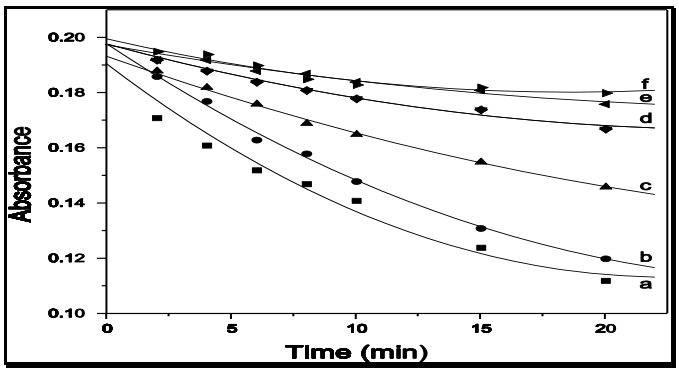


FIG. 3: EFFECT OF VARYING CONCENTRATIONS OF URACIL ON THE PHOTOOXIDATION OF CGA (1.0 × 10^{-5} mol dm^{-3}) IN THE PRESENCE OF *t*-BuOOH (5 × 10^{-3} mol dm^{-3}) AT 298 K

[uracil] = (a) 0.0, (b) 5 × 10^{-5} mol dm^{-3}, (c) 8 × 10^{-5} mol dm^{-3}, (d) 1 × 10^{-4} mol dm^{-3}, (e) 5 × 10^{-4} mol dm^{-3}, (f) 8 × 10^{-4} mol dm^{-3}, (g) 1 × 10^{-3} mol dm^{-3}, Light Intensity = 2.7168 × 10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5

Aqueous solutions of reaction mixture containing CGA, *t*-BuOOH and *t*-BuOH were irradiated in presence of varying concentrations of uracil **(Fig.3)**. The initial rates and quantum yields of

oxidation of CGA by *t*-BuO$^{\bullet}$ radicals were found to decrease with increase in concentration of uracil **(Table 3)**.

Comparison of the initial rates and quantum yields of oxidation of CGA in presence and absence of uracil clearly indicate that the initial rates and quantum yields of oxidation of CGA are substantially decreased in presence of uracil **(Table 3)**. These observations clearly demonstrate that uracil and CGA are in competition for *t*-BuO$^{\bullet}$ radicals.

The rate constant of the reaction of *t*-BuO$^{\bullet}$ with CGA has been reported to be 3.20×10^{9} dm^{3} mol^{-1} s^{-1} under similar experimental conditions of the present work[37]. The rate constant for the reaction of *t*-BuO$^{\bullet}$ with uracil has been calculated by the CGA competition method, which is very similar to the one chosen by Akhalaq *et al*[38] to determine the rate constant for the reaction of $^{\bullet}$OH radicals with polyhydric alcohols in competition with KSCN. In the present study, solutions containing CGA and varying amounts of uracil in presence of *t*-BuOOH and *t*-BuOH were irradiated for two minutes and the decrease in absorbance of CGA was measured.

The decrease in absorbance of CGA reflects the amount of *t*-BuO$^{\bullet}$ radicals that has reacted with CGA. From the known rate constant of the reaction of CGA with *t*-BuO$^{\bullet}$ radical[37] under similar experimental conditions of the present work (k_{CGA} = 3.20×10^{9} dm^{3} mol^{-1}s^{-1}), the rate constant of *t*-BuO$^{\bullet}$ radical reaction with uracil (k_{uracil}) can be calculated using the following equation:

$$\frac{[\text{Absorbance of chlorogenic acid}]_0}{[\text{Absorbance of chlorogenic acid}]_{uracil}} = 1 + \frac{k_{uracil}\,[uracil]}{k_{\text{chlorogenic acid}}\,[\text{chlorogenic acid}]} \quad (1)$$

In Eq. (1), [Absorbance of CGA]$_o$ and [Absorbance of CGA]$_{uracil}$ are the absorbance values of CGA in the absence and presence of uracil respectively, at the same interval of time. Experiments of this kind can be carried out with great accuracy. Using Eq. (1) the rate constant for the reaction of *t*-BuO$^{\bullet}$ radical with uracil (k_{uracil}) has been calculated at different concentrations of CGA and uracil and the average of these is found to be 4.43×10^{8} dm^{3} mol^{-1} s^{-1}.

As CGA has strong absorption at 258 nm, it is not possible for the direct determination of protection and repair offered to uracil by CGA. However, one can calculate indirectly the extent of protection offered to uracil by CGA from competition kinetic studies measured at 328 nm, λ_{max} of CGA. The method is as follows: When the system containing uracil, CGA and *t*-BuOOH in the presence of *t*-BuOH is irradiated, the probability of *t*-BuO$^{\bullet}$ radicals reacting with uracil {$p_{(t\text{-}BuO^{\bullet}+\ uracil)}$ }is calculated using the following equation:

$$p_{(t\text{-}BuO^{\bullet}+\,uracil)} = \frac{k_{uracil}\,[uracil]}{k_{uracil}\,[uracil] + k_{\text{chlorogenic acid}}\,[\text{chlorogenic acid}]} \quad (2)$$

If CGA scavenges only *t*-BuO$^{\bullet}$ radicals and does not give rise to any other reaction (e.g. reaction with uracil radicals), the quantum yield of oxidation of CGA (ϕ_{cal}) at each concentration of uracil may be given by equation:

$$\phi_{cal} = \phi^{o}_{expt} \times p \quad (3)$$

Where ϕ^{o}_{expt} is the quantum yield of oxidation of CGA in the absence of uracil, and *p* is the probability given by Eq. (2).

The calculated quantum yield (ϕ_{cal}) values at different uracil concentrations are presented in **Table 3**. The data show that the ϕ_{cal} values are lower than the experimentally measured quantum yield (ϕ_{expt}) values. This indicates that more number of CGA molecules is consumed in the system than expected and the most likely route for this is H atom donation by CGA to uracil radicals. In **Table 3**, are presented the fraction of *t*-BuO$^{\bullet}$ radicals scavenged by CGA at different concentrations of uracil. These values refer to the measure of protection offered to uracil due to scavenging of *t*-BuO$^{\bullet}$ radicals by CGA. Using the ϕ_{exptl} values, a set of values, viz., ϕ' values have been calculated from Eq. (4) and are presented in **Table 3**.

$$\phi' = \frac{\phi_{expt}}{p} \quad (4)$$

Where ϕ's represent the experimentally found quantum yield values if no scavenging of uracil radical by CGA occurs. In the absence of any

"repair" of uracil radicals by CGA, the ϕ′values should all be equal to ϕ^{o}_{expt}. The observed increase in ϕ′ with increasing uracil concentration (Table 3) clearly indicates that repair of uracil radicals does occur. The extent of repair may be quantified by the following equation:

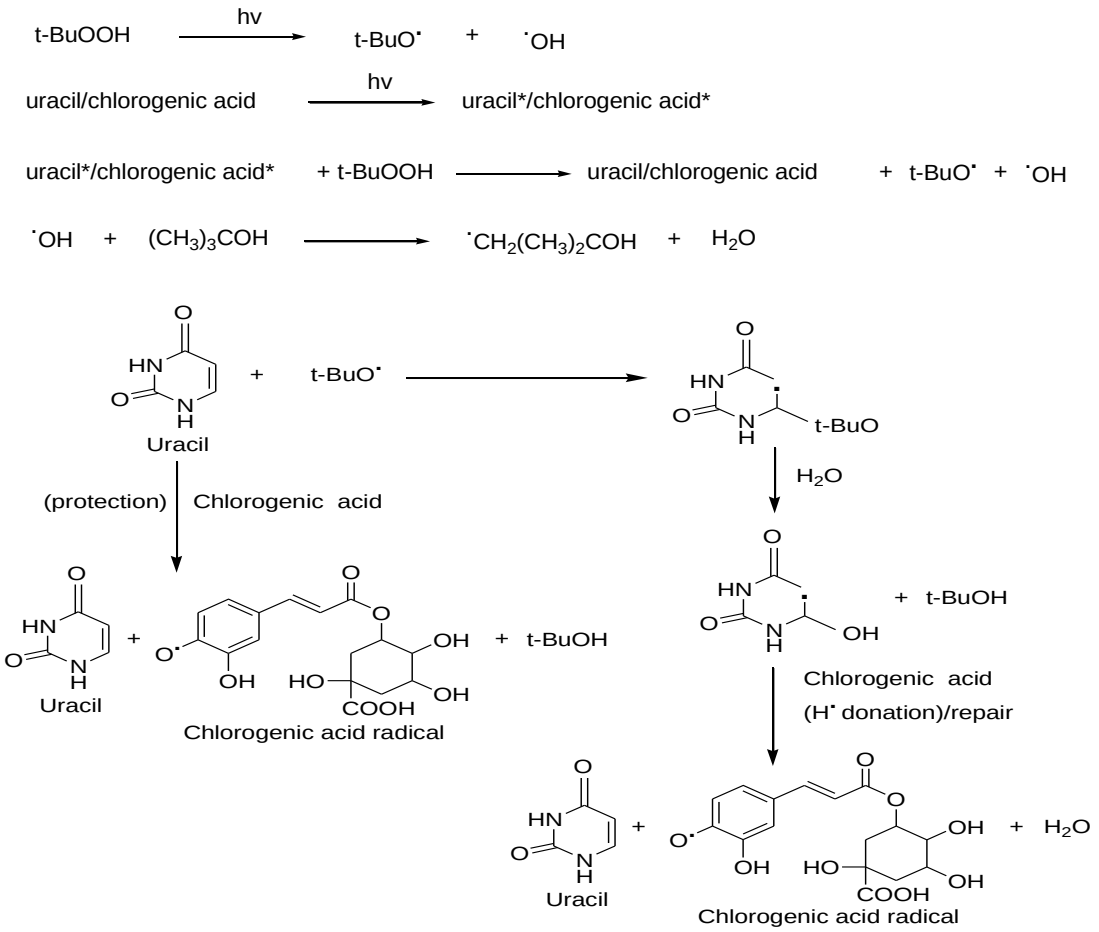
$$\% \text{ Repair} = \frac{(\phi' - \phi^{o}_{expt})}{\phi^{o}_{expt}} \times 100 \qquad (5)$$

TABLE 2: EFFECT OF VARYING [CGA] ON THE RATE AND QUANTUM YIELD OF PHOTOOXIDATION OF CGA BY *T*-BUOOH IN THE ABSENCE AND PRESENCE OF URACIL IN *T*-BUOH -WATER (1:4 v/v) MEDIUM

10^5 × [CGA] (mol dm^{-3})	10^5 × [uracil] (mol dm^{-3})	10^9 × Rate (mol dm^{-3} s^{-1})	Quantum yields ϕ
2.0	0.0	9.6908	0.00644
1.0	0.0	7.0008	0.00465
0.8	0.0	5.2798	0.00351
0.5	0.0	2.7845	0.00185
2.0	5.0	8.1370	0.00541
1.0	5.0	5.9565	0.00396
0.8	5.0	2.3726	0.00158
0.5	5.0	1.6541	0.00110

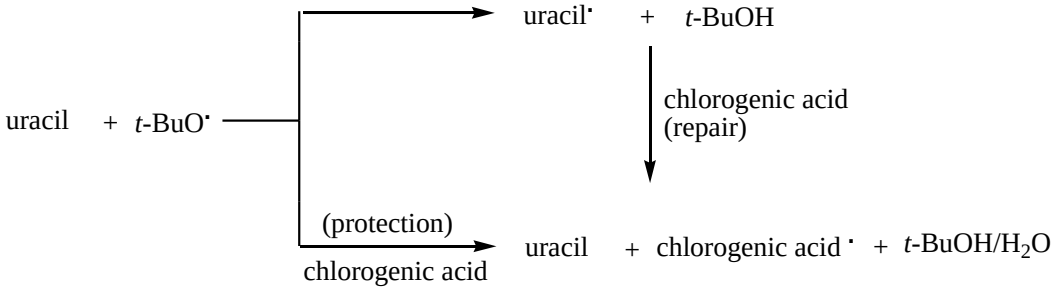
[t-BuOOH] = 5×10^{-3}mol dm^{-3}, Light Intensity = 2.7168×10^{15} quanta s^{-1} λ_{max} = 328 nm, pH ~ 7.5, Temperature = 298 K

The data on percentage repair is presented in **Table 4**. The experimentally determined quantum yield (ϕ_{expt}) values are higher than the quantum yield (ϕ_{cal}) values calculated using Eq. (3) under the assumption that CGA acts only as a *t*-BuO $^{\bullet}$radical scavenger. This shows that CGA acts not only as an efficient scavenger of *t*-BuO$^{\bullet}$ radicals, but also as an agent for the repair of uracil radicals. The repair reaction of CGA is explained in terms of the H donation as shown in **scheme 1**.



SCHEME - 1

The results obtained in the present study **(Table 3)** indicate that uracil radicals are efficiently repaired by CGA to the extent of ~32 % at about 10μM of CGA concentration. This type of repair reactions by CGA has been reported in the oxidation of nucleobases by *t*-BuO$^{\bullet}$ radicals[40, 41]. The *t*-BuO$^{\bullet}$ radicals react with uracil preferentially by adding to either end of the C_5-C_6 double bond giving the C_5-*t*-BuO and C_6-*t*-BuO adduct radicals similar to adduct radicals from the reaction of uracil with $^{\bullet}$OH radicals. The C_6-*t*-BuO adduct radicals are found to be oxidizing in nature which are formed in lower concentration compared to C_5-*t*-BuO adduct radicals with reducing nature. During the oxidation of uracil by *t*-BuO$^{\bullet}$ radicals, formation of both oxidizing and reducing radicals has been observed. Since CGA can effectively repair only oxidizing radicals, the extent of repair is low for uracil compared to other nucleobases. Thus, CGA is found to repair the transient oxidizing radicals of uracil efficiently upto ~32 %. The protection of uracil and repair of uracil radicals are summarized in the following scheme.



The CGA radicals were generated in the process of protection of uracil and repair of uracil radicals. These radicals were reported[42,43] to have short life time, extremely unstable and rapidly converted to unknown compounds of physiological pH[44]. If CGA radicals reacted with uracil, then ϕ_{expt} would have been less than ϕ_{cal} values. Contrary to this, the ϕ_{cal} values were lower than the experimentally found quantum yield values (ϕ_{expt}) **(Table 3)**. This supported our contention that the CGA radicals might not be involved in oxidative stress in our experimental conditions.

TABLE 3: EFFECT OF VARYING [URACIL] ON THE RATE AND QUANTUM YIELD OF PHOTOOXIDATION OF CGA IN THE PRESENCE OF *T*-BUOOH IN *T*-BUOH-WATER 1:4 (v/v) NEUTRAL MEDIUM

10^5 × [uracil] (mol dm^{-3})	10^9 × Rate (mol dm^{-3} s^{-1})	ϕ_{expt}	ϕ_{cal}	p	ϕ'	% scavenging	% repair
0.0	7.0008	0.004656	0.004656	1.0000	0.004656	100.0	0.00
5.0	6.5915	0.004384	0.004276	0.9185	0.004772	91.85	2.51
8.0	6.3408	0.004217	0.004076	0.8756	0.004816	87.56	3.44
10.0	6.2489	0.004156	0.003954	0.8492	0.004894	84.92	5.11
50.0	4.1938	0.002779	0.002467	0.5298	0.005264	52.98	13.1
80.0	3.5254	0.002344	0.001924	0.4132	0.005674	41.32	21.9
100.0	3.3249	0.002211	0.001678	0.3604	0.006135	36.04	31.8

[CGA] = 1.0 × 10^{-5}mol dm^{-3}, [*t*-BuOOH] = 5.0 × 10^{-3}mol dm^{-3}, Light Intensity = 2.7168 × 10^{15} quanta s^{-1}, λ_{max} = 328 nm, pH ~ 7.5, Temperature = 298 K

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