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KINETIC AND MECHANISTIC STUDY OF OXIDATION OF PURINES BY SODIUM CHROMATE AND HYDROGEN PEROXIDE SYSTEM IN PRESENCE OF ASCORBIC ACID AND PROTECTION BY RIBOFLAVIN

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ABSTRACT

The oxidation of purines viz., adenine and guanine with sodium chromate and hydrogen peroxide (Cr(VI) - H_2O_2) system in presence of ascorbic acid at pH 8 has been carried in TrisHCl buffer. The reactions are followed by measuring the absorbance of purine bases at their respective λ_{max}. The rates of oxidation of purines increased with increase in [chromate], [hydrogen peroxide] and [ascorbic acid]. The plot log (initial rate) versus log [chromate], and log (initial rate) versus log [ascorbic acid] was found to be linear with slope less than one indicating fractional order dependence on [chromate] and [ascorbic acid]. The fractional order dependence of reaction rate on [ascorbic acid] and [chromate] indicates that reaction might be proceeding through the initial formation of a complex between ascorbic acid and Cr (VI). The first order dependence of rate of oxidation on [H_2O_2] suggests that Chromium-Ascorbate complex might react with H_2O_2 in the rate determining step via Fenton like mechanism to generate OH radicals. An increase in [purine] at constant [chromate], [hydrogen peroxide] and [ascorbic acid] has no significant increase of rate of oxidation suggests that OH radicals react with purines in a fast step to form product. It has been found that riboflavin protects guanine and adenine to the extent of 74.04% and 83.77% at 64 μM concentration of riboflavin from OH radicals.

Key words: Oxidation of purines, Chromium (VI), Hydrogen peroxide, Ascorbic acid, Chromium-Ascorbate complex, Protection by riboflavin.

INTRODUCTION

Chromate, Cr(VI), has been established as a human carcinogen, although its mechanism of action has not been clearly defined[1,2]. The reaction of chromate with DNA creates a number of putative lesions in cellular systems including inter- and intrastrand

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cross-linked adducts, DNA–protein cross-links, DNA strand breaks, abasic sites, and oxidized nucleic acid bases[3-7]. The tetrahedral anionic conformation of the +6 oxidation state of chromium facilitates active transport into cell systems through the phosphate and sulfate cellular transport systems[8].

However, it is reported that Cr(VI) is not the oxidation state that reacts with isolated DNA, reduction of Cr(VI) by cellular reductant has been thought to be an important step in the mechanism of Cr(VI)-induced DNA damage[8,9]. Cr (V) species has been reported to be generated in the reduction of Cr(VI) by various biological systems[8], in particular microsomes[10,11], mitochondria[12], superoxide radical[13], certain flavoenzymes[14-16], mitochondrial electron transfer chain complexes[17], ascorbate[18,19] and thiol- and diol-containing molecules[8,20-22]. Regarding the mechanism of Cr(VI) Carcinogenicity, Jennette[10] postulated that Cr(V) is the ultimate carcinogenic form of chromium compounds.

The first evidence for the role of $Cr^{V} (O_2)_4^{3-}$ in the Cr (VI) - mediated OH radical generation was reported by Kawanishi et al.[23] They observed ($Cr^{V} (O_2)_4^{3-}$) formation by E.S.R from a mixture containing 40 mM Na_2CrO_4 and 400 mM H_2O_2 at pH 8. However, the concentrations used for both Cr(VI) and H_2O_2 were orders of magnitude higher than in any vivo estimate. Later Aiyar et al.[24] reaffirmed the Kawanishi model of $^{\bullet}OH$ radical generation from a mixture of Cr(VI) and H_2O_2 i,e tetraperoxochromium (V) complex ($Cr^{V} (O_2)_4^{3-}$) was the species responsible for OH radical generation.

Shi et al.[25] studied using ESR and spin trapping techniques and found that ($Cr^{V} (O_2)_4^{3-}$) is not formed in any significant quantity in the reaction of chromate with biologically relevant reductants such as glutathione, NADPH, Ascorbate, Vitamin B_2. Decomposition of $Cr^{V} (O_2)_4^{3-}$ or its reaction with H_2O_2 does not generate any significant amount of $^{\bullet}OH$ radicals. The major Cr(V) Species formed are complexes of Cr(V) with reductant moieties as ligands. These Cr(V) complexes generate $^{\bullet}OH$ radicals from H_2O_2 via Fenton like reaction.

Treatment of the cells with vitamin B2 (Riboflavin) prior to chromate treatment led to an increase in DNA single strand breaks over that observed upon treatment with chromate alone[26], presumably due to an increase in chromium (V)-related hydroxyl radical production. On the other hand, incubation of the V-79 cells with vitamin E, a hydroxyl radical scavenger, prior to chromate treatment led to a decrease in DNA single-strand breaks[27].

In animal studies, chromium (VI)-induced DNA damage has been found to be tissue-dependent. DNA- interstrand crosslinks and DNA-protein crosslinks were detected in the livers of chick embryos treated with chromium (VI), whereas in the red blood cells, DNA

damage was primarily in the form of strand breaks[28]. This may reflect the fact that chromium (VI) reduction occurs by different metabolic pathways in the various tissues.

One way to obtain information about these species is by studying the products of the reaction of chromate with DNA, or individual nucleotides, nucleosides, nucleic acid bases to get an insight into the mechanism of oxidation of DNA constituents. In the present study an attempt has been made to investigate the mechanism of damage to DNA constituents by OH radicals produced from chromium (VI) with hydrogen peroxide (Cr(VI) - H_2O_2) system in presence and absence of naturally available antioxidants.

EXPERIMENTAL

Adenine, guanine sodium chromate and ascorbic acid were from sigma and used as such. The solutions were prepared afresh with double distilled water, standardized and de-aerated by bubbling nitrogen. The hydrogen peroxide solution was standardized using the method described in the literature[29]. The concentration of purines was determined by measuring the absorbance at their respective λ_{max} from the known absorption coefficient values. In a typical reaction required amounts of purines viz., adenine/ guanine and H_2O_2 solution were then injected as aqueous solution into the mixture of ascorbic acid and sodium chromate solutions present in a 1-cm path length quartz cuvette suitable for absorbance measurements. The progress of the reactions were followed by measuring the absorbance at λ_{max} of purines on double beam UV-visible Spectrophotometer model T60U of Lab India at different time intervals from which rate of oxidation of purines were calculated.

The HPLC system used for analysis of products includes schimadzu LC-10AT equipment with dual piston pump system, a solvent programmer and Reodhyne injector model 7725 fitted with 20 μL loop. A prepacked octadecylsilyl silica gel ODS hypersil column 25 cm × 0.46 cm, mean particle size 5 μm was used. The column effluents were monitored at 280nm, using variable wavelength SPD-10A UV-Visible detector equipped with 8 μL flow cell and attached to C-R7Ae plus chromatographic integrator. Samples were eluted with aqueous solutions containing 5% (v/v) methanol and buffered with 10 mM KH_2PO_4 solution adjusted to pH 8. Before use the phosphate buffer was filtered through Millipore type HA 0.45 μm membrane filter. All mobile phases were degassed using a vaccum pump. The solvent flow rate was kept at 0.5ml/min and all the HPLC runs were carried out at ambient temperatures.

RESULTS AND DISCUSSION

The oxidation of purines viz., adenine and guanine with sodium chromate and hydrogen peroxide (Cr(VI) - H_2O_2) system in presence and absence of ascorbic acid at pH 8

has been carried in TrisHCl buffer. Purines were not oxidized even with highest concentration of sodium chromate (1 × 10^{-4} M) and hydrogen peroxide (5 × 10^{-3} M) system in the absence of ascorbic acid but in presence of ascorbic acid (1 x 10^{-5} M), purines were oxidized even with concentration as low as of sodium chromate (1 × 10^{-8} M) and hydrogen peroxide (5 × 10^{-3} M). The oxidation of purine bases with Cr(VI) - H_2O_2 system in presence of ascorbic acid were carried out at different [chromate] varying from 2 × 10^{-4} to 1 × 10^{-8} M keeping [ascorbic acid], [purine] and [H_2O_2] constant. The plot of log (initial rate) versus log [chromate] was found to be linear with slope less than one indicating fractional order dependence on chromate (Fig. 1). The effect of [ascorbic acid] varying between 1 × 10^{-5} – 4 × 10^{-5} M was studied keeping [H_2O_2], [purine] and [chromate] constant. The plot of log (initial rate) versus log [ascorbic acid] was found to be linear with slope less than one indicating fractional order dependence on ascorbic acid (Fig. 2). An increase in [H_2O_2] was found to increase the rate of oxidation and order with respect to [H_2O_2] has been found to be unity from the slopes of log (initial rate) versus log [H_2O_2]. The concentration of H_2O_2 was varied between 1 × 10^{-3} – 1 × 10^{-2} M. An increase in [purine] at constant [chromate], [hydrogen peroxide] and [ascorbic acid] has no significant effect on rate of oxidation of purines. The decrease of absorbance of purine in the reaction mixture at different intervals of time has been obtained by using chromate, hydrogen peroxide and ascorbic acid mixture as reference in double beam spectrophotometer. From these the rates of oxidation of purines were calculated from the plots of absorbance versus time using microcal origin computer program on personal computer.

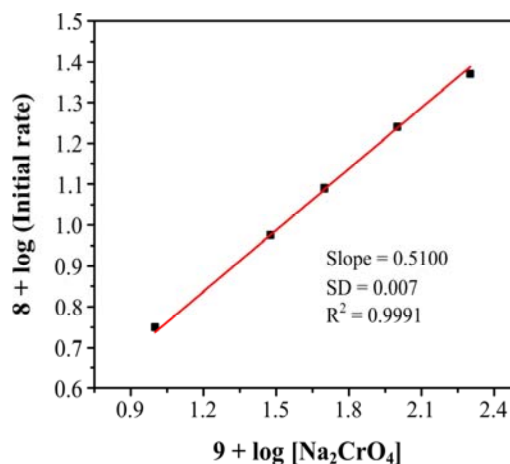


Fig. 1: Order in [Chromate] in the oxidation of guanine with Cr(VI)- H_2O_2 system in presence of ascorbic acid

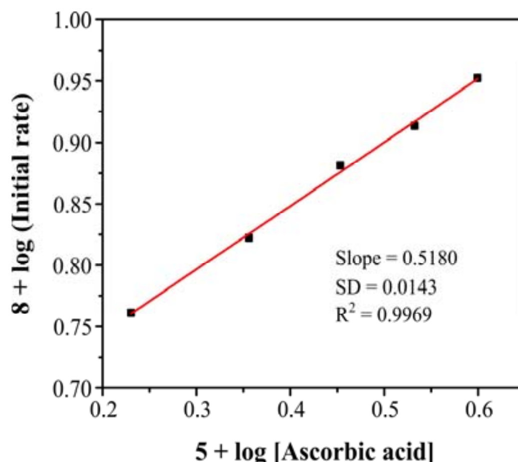


Fig. 2: Order in [Ascorbic acid] in the oxidation of guanine with Cr(VI)- H_2O_2 system in presence of ascorbic acid

Shi et al.[25] reported that as low as 25 mM of $K_2Cr_2O_7$ and 25 mM H_2O_2 were required to generate detectable amount of ($Cr^V(O_2)_4^{3-}$) ions at physiological pH (pH = 7.2). In contrast in presence of certain flavoenzymes such as GSSG-R, even 0.5 mM of $K_2Cr_2O_7$ and 1 mM NADPH generated intense Cr(V) ESR signal which was assigned to Cr(V)-NADPH complex formation[14-16]. The Cr(V)-complex formation is much more efficient than ($Cr^V(O_2)_4^{3-}$) formation from direct reaction of Cr(VI) and H_2O_2[25]. Mixture of $K_2Cr_2O_7$ (10 mM) and ascorbic acid (10 mM) in phosphate buffer (pH = 7.2) generate an E.S.R signal at g = 1.9794, which was assigned to Cr(V)-Ascorbate complex[25,18,19]. No ($Cr^V(O_2)_4^{3-}$) ESR Signal was detected. When H_2O_2 and DMPO (as a spin trap) were added a 1:2:2:1 quartet with hyperfine splitting of $a_N = a_H$ = 14.9G was observed. Based on this splitting[20] the 1:2:2:1 signal was assigned to the DMPO/OH adduct, as evidence of OH radical generation. Upon addition of H_2O_2, however, Cr(V) became non-detectable indicating that OH radicals were generated in the reaction between Cr(V)-ascorbate complex and H_2O_2 via a Fenton-like mechanism[25].

In the present work, the fractional order dependence of reaction rate on [ascorbic acid] and [chromate] indicates that reaction might be proceeding through the initial formation of complex between ascorbic acid and Cr (VI) to give Cr(V)-ascorbate complex. The first order dependence of rate of oxidation of purine on [H_2O_2] suggests that Cr(V)-Ascorbate complex reacts with H_2O_2 in the rate determining step via Fenton like mechanism to generate OH radicals. With the addition of ethanol oxidation of purines decreased indicating that OH radicals are generated in our system. The rate constant of OH radical with ethanol is reported to be $1.8 \pm 0.2 \times 10^9$ $dm^3mol^{-1}s^{-1}$ [30] and the rate constant of OH radical

with adenine and guanine are reported to be 6.1 x 10^9 $dm^3mol^{-1}s^{-1}$ and 9.2 x 10^9 $dm^3mol^{-1}s^{-1}$, respectively[31,32]. Hence in this system there might be a competition between purine and ethanol for OH radicals. The relative amounts of OH radicals reacting with purine decreases with increasing [ethanol]. An increase in [purine] at constant [chromate], [hydrogen peroxide] and [ascorbic acid] has no effect on rate of oxidation suggests that OH radicals react with purines in the fast step to form product. It has been reported that OH radicals attack purines viz., guanine and adenine at C8 –Position[33-36]. According to Pullman[37], the calculated charge density and localization energy values of electrophillic attack at various carbon atoms in purine ring suggest C8 position is more favourable for electrophillic attack. When OH radicals attack guanine nucleobase in DNA, three types of adduct radicals viz., C4-OH, C5-OH and C8-OH are formed. The C4-OH and C5-OH adduct radicals revert back to guanine by gaining an electron from the medium, whereas C8-OH adduct radical initiates two types of DNA damage based on environmental conditions. The C8-OH adduct radical under oxidative conditions yields 7,8-dihydro-8-oxo guanine; under reducing conditions gain of electron and proton results in imidazole ring opening and 2,6-diamino-5-formamido-4-hydroxy pyrimidine is formed[38,39]. The oxidation product of guanine has been identified as 8-Oxoguanine by HPLC with 5% methanol buffered with 10 mM phosphate buffer at pH 8 with ODS column at flow rate of 0.5 mL/min. The retention times of guanine and 8-oxoguanine has been found to be 8.4 min and 4.1 min, respectively which is confirmed by the authentic sample. Therefore it is suggested that OH radicals generated in our system attacks purines at C8 position to form Product.

Effect of pH

With increase in pH rate of oxidation of purines have been found to increase with Cr(VI)-H_2O_2 system in presence of ascorbic acid. The Cr(V)-Ascorbate complex at g_{iso} = 1.9791 is much more stable in moderately acidic solution (pH = 3-6) than in neutral and alkaline solutions[40]. Bokare and Choi[41] reported that tetraperoxochromate (V) ($Cr^V(O_2)_4^{3-}$) production is reduced with increase in pH in the reaction of Cr(VI) (2 mM) and hydrogen peroxide (20 mM). The production of ($Cr^V(O_2)_4^{3-}$) is immediate at pH = 3, but was markedly reduced at pH = 6 and negligible at pH = $\geq$ 8. When H_2O_2 is added to chromate solution the absorption peak at 352 nm (lambda max of chromium) decreases and absorbance above 500 nm increases indicating the formation of ($Cr^V(O_2)_4^{3-}$). The increase in absorbance above 500 nm is decreased as pH is increased from pH = 3-6 and negligible at pH = 8. Increase in rate of oxidation of purines with pH clearly shows that oxidation occurs by OH radicals generated by reaction of H_2O_2 with Cr(V)-ascorbate complex via Fenton like reaction (Table 1 & 2).

Table 1: Effect of [pH] on rate of oxidation of guanine by Cr(VI)-H_2O_2 system in presence of ascorbic acid in aqueous solutions

$[H_2O_2] = 5.0 \times 10^{-3}$ mol dm^{-3}, [Guanine] = 3.0×10^{-5} mol dm^{-3},
$[Na_2CrO_4] = 1.0 \times 10^{-7}$ mol dm^{-3}, [Ascorbic acid] = 2.5×10^{-5} mol dm^{-3}.
Temperature = 298 K

S. No.	pH	$10^7 \times$ (Initial rate) (mol dm^{-3} s^{-1})
1	8.0	0.715
2	6.0	0.4010
3	4.5	0.3453

Table 2: Effect of pH on rate of oxidation of adenine by Cr(VI)-H_2O_2 system in presence of ascorbic acid in aqueous solutions

$[H_2O_2] = 5.0 \times 10^{-3}$ mol dm^{-3}, [Adenine] = 2.5×10^{-5} mol dm^{-3},
$[Na_2CrO_4] = 1.0 \times 10^{-4}$ mol dm^{-3}, [Ascorbic acid] = 2.5×10^{-5} mol dm^{-3}.
Temperature = 298 K

S. No.	pH	$10^7 \times$ (Initial rate) (mol dm^{-3} s^{-1})
1	8.0	0.546
2	6.0	0.321
3	4.5	0.128

Anti oxidant activity of Riboflavin

The initial rates of oxidation of purines by Cr (VI) - H_2O_2 system in presence of ascorbic acid have been found to decrease with increase in [Riboflavin] indicating that riboflavin is protecting the purines from OH radicals generated with Cr (V)-ascorbate complex reaction with H_2O_2 via Fenton like mechanism [Table 3, 4] & [Fig. 3, 4]. In this system there is a competition between purine and riboflavin for OH radicals. The relative amounts of OH radicals reacting with purine decreases with increasing [riboflavin]. The rate constant of riboflavin with hydroxyl radical has been reported to be $1.2 \pm 0.05 \times 10^{10}$ dm^{3}mol^{-1}s^{-1} over the pH range 1-11[42]. The rate constant of hydroxyl radical with ascorbic acid at physiological pH has been reported to be 2.7×10^5 dm^{3}mol^{-1}s^{-1}.[43] This indicates that ascorbic acid is not specific hydroxyl radical scavenger at physiological pH. Since the rate constant of OH radical with riboflavin is much higher than rate constant of OH radical with

ascorbic acid, it is understood that ascorbic acid does not compete for OH radicals under the experimental conditions.

Table 3: Effect of [Riboflavin] on the rates of oxidation of guanine with Cr(VI)-H_2O_2 system in presence of ascorbic acid

[H_2O_2] = 5.0 × 10^{-3} mol dm^{-3}, [Guanine] = 3.00 × 10^{-5} mol dm^{-3},
[Na_2CrO_4] = 1.0 × 10^{-7} mol dm^{-3}, [Ascorbic acid] = 2.5 × 10^{-5} mol dm^{-3}
pH = 8, Temp. = 298 K

S. No.	10^6 x [Riboflavin] (mol dm^{-3})	10^8 x rate (mol dm^{-3} s^{-1})	p	% Scavenging
1	0.00	7.150		
2	4.00	4.850	0.848	15.13
3	8.00	3.900	0.737	26.28
4	16.0	2.812	0.583	41.62
5	32.0	1.862	0.412	58.78
6	64.0	1.031	0.259	74.04

Table 4: Effect of [Riboflavin] on the rates of oxidation of adenine with Cr(VI)-H_2O_2 system in presence of ascorbic acid

[H_2O_2] = 5.0 × 10^{-3} mol dm^{-3}, [Adenine] = 2.5 × 10^{-5} mol dm^{-3},
[Na_2CrO_4] = 1.0 × 10^{-4} mol dm^{-3}, [Ascorbic acid] = 2.5 × 10^{-5} mol dm^{-3}
pH = 8, Temp. = 298 K

S. No.	10^6 x [Riboflavin] (mol dm^{-3})	10^8 x rate (mol dm^{-3} s^{-1})	p	% Scavenging
1	0.00	5.460		
2	2.00	3.562	0.861	13.89
3	4.00	2.687	0.756	24.39
4	8.00	1.717	0.607	39.22
5	16.00	0.987	0.436	56.35
6	32.00	0.552	0.279	72.07
7	64.00	0.338	0.162	83.77

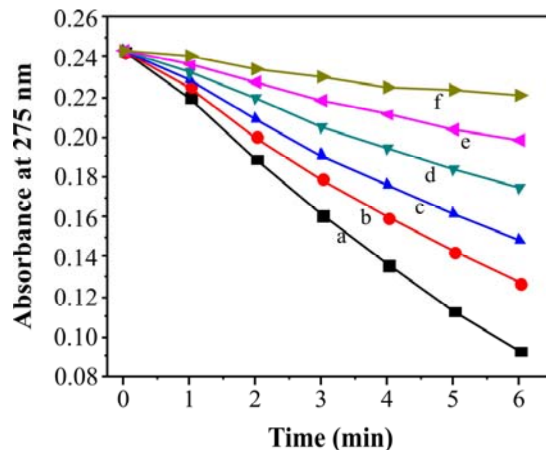


Fig. 3: Effect of Riboflavin on the oxidation of guanine with Cr(VI)-H_2O_2 system in presence of ascorbic acid

$[H_2O_2] = 5.0 \times 10^{-3}$ mol dm^{-3}, [Guanine] = 3.0×10^{-5} mol dm^{-3},
$[Na_2CrO_4] = 1.0 \times 10^{-7}$mol dm^{-3},
[Riboflavin] = (a) 0.00 (b) 4.0×10^{-6} mol dm^{-3} (c) 8.0×10^{-6} mol dm^{-3} (d) 16.0×10^{-6} mol dm^{-3} (e) 32.0×10^{-6} mol dm^{-3} (f) $64.0.0 \times 10^{-6}$ mol dm^{-3}

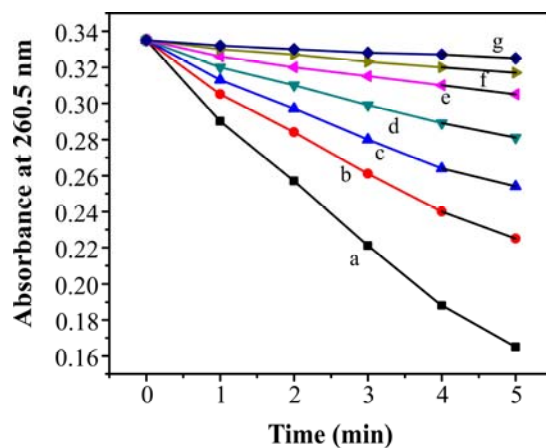


Fig. 4: Effect of Riboflavin on the oxidation of adenine with Cr(VI)-H_2O_2 system in presence of ascorbic acid

$[H_2O_2] = 5.0 \times 10^{-3}$mol dm^{-3}, [adenine] = 2.5×10^{-5} mol dm^{-3},
$[Na_2CrO_4] = 1.0 \times 10^{-4}$ mol dm^{-3},
[Riboflavin] = (a) 0.00 (b) 2.0×10^{-6} mol dm^{-3} (c) 4.0×10^{-6} mol dm^{-3} (d) 8.0×10^{-6} mol dm^{-3} (e) 16.0×10^{-6} mol dm^{-3} (f) $32.0. \times 10^{-6}$ mol dm^{-3} (g) $64.0.0 \times 10^{-6}$ mol dm^{-3}

The rate constant of the reaction of the OH radical with adenine and guanine were reported[30,31] to be 6.1 x 10^9 $dm^3mol^{-1}s^{-1}$ and 9.2 x 10^9 $dm^3mol^{-1}s^{-1}$, respectively. The rate constant of the reaction of the OH radical with riboflavin has been calculated by the purine competition method at pH 8 in phosphate buffer, which is very similar to the one chosen by Akhalaq et al.[44] to determine the rate constant for the reaction of OH radicals with polyhydric alcohols in competition with KSCN. Solutions containing purines with Cr(VI)-H_2O_2 system in presence of ascorbic acid and varying amounts of riboflavin were made to react for 4 min and decrease of absorbance of purines was measured. The decrease of absorbance of purine reflects the number of OH radicals that have reacted with purine. From the rate constant of reaction of purines with OH radicals ($k_{\text{Adenine + OH}}$ = 6.1 x 10^9 $dm^3mol^{-1}s^{-1}$) and ($k_{\text{Guanine + OH}}$ = 9.2 x 10^9 $dm^3mol^{-1}s^{-1}$. The rate constant of OH radicals with riboflavin ($k_{\text{Riboflavin + OH}}$) can be calculated using equation (1).

$$\frac{[\text{Absorbance of Purine}]_0}{[\text{Absorbance of Purine}]_{\text{Riboflavin}}} = 1 + \frac{k_{(OH + Riboflavin)}\,[Riboflavin]}{k_{(OH + \text{Purine})}\,[\text{Purine}]} \quad \ldots(1)$$

Where $[\text{Absorbance of purine}]_0$ and $[\text{Absorbance of purine}]_{\text{riboflavin}}$ indicate the decrease in the absorbance of purine in the absence and presence of riboflavin respectively, in the same interval of time. Experiments of this kind can be carried out with great accuracy. The rate constant for the reaction of OH radical with riboflavin has been calculated with five different concentrations of riboflavin and average value obtained is 1.23 x $10^{10}dm^3$ $mol^{-1}s^{-1}$.

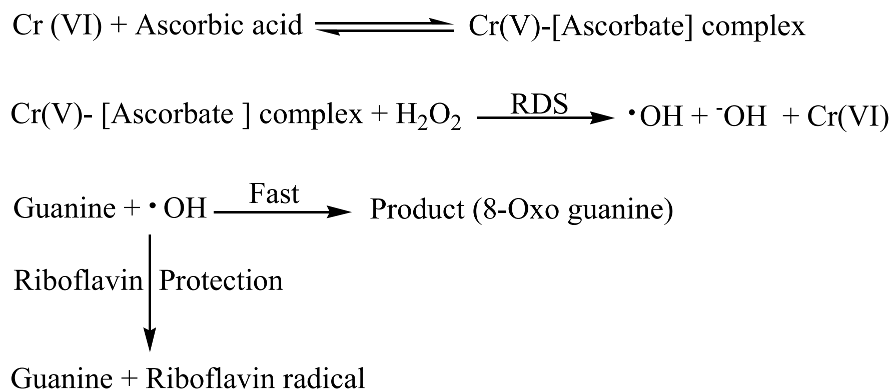
The probability of OH radicals reacting with purines {p (OH + purine)} is calculated using the following equation.

$$p(\text{OH + Purine}) = \frac{[\text{Purine}]\,k_{\text{purine}}}{[\text{Purines}]k_{\text{purine}} + [\text{Riboflavin}]k_{\text{Riboflavin}}} \quad \ldots(2)$$

k_{Purine} and $k_{\text{riboflavin}}$ are the rate constants of OH radical with purine and riboflavin, respectively.

From the rate constant of OH radical with riboflavin and Purine (Equation (2)), the fraction of OH radicals scavenged by riboflavin (Percentage scavenged = (1 – p) x 100) at different [riboflavin] were calculated (Table 3 & 4). These values were a measure of protection of purine due to scavenging of OH radicals by riboflavin. It has been found that riboflavin is protecting guanine and adenine to the extent of 74.04% and 83.77% at 64 µM concentration of riboflavin from OH radicals.

Based on the above results and discussion the following mechanism has been proposed taking guanine as an example in **Scheme 1**.



Scheme 1

CONCLUSION

The present study exhibit that under biologically relevant conditions, mixture of Cr(VI) and H_2O_2 does not oxidize purines by OH radicals generated in this system but Cr(VI) Concentration as low as 1×10^{-8} M and H_2O_2 5×10^{-3} M could oxidize purines in presence of ascorbic acid. Riboflavin is found to protect guanine and adenine oxidation by OH radicals to the extent of 74.04% and 83.77% at 64 µM concentration of riboflavin.

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