Effects of Copper(II) Ions on the $SO_4^{-\cdot}$ Radical Induced Oxidation of Cytosine and Its Derivatives

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The reactions of $SO_4^{-\cdot}$ radicals with cytosine and its derivatives have been studied in dilute aqueous solutions. The results show that the nature of the group attached to N-1 of the base greatly influences the reaction sequences. Presence of copper(II) ions greatly affects the reaction pathways, where it is observed that the substrate consumption increases drastically indicating initiation of some chain reactions. Release of unaltered base from cytidine and deoxycytidine also increases under these conditions.

The mutagenic and lethal effects of ionizing radiation in biological systems are thought to involve the induction of chemical changes within essential biomolecules such as DNA. There is considerable evidence that the hydroxyl radical produced by water radiolysis, is partly involved in the inactivation of radiation induced molecular changes.[1,2] Such changes may be modified in the presence of various agents. There is considerable evidence that direct deposition of energy within DNA[3] may result in chemical changes to compliment the role of water radicals produced in the vicinity of DNA. The use of $SO_4^{-\cdot}$ radicals to mimic, to some extent, the direct energy deposition may assist in our understanding of the resulting molecular processes after energy deposition within DNA. Studies on the reactions of $SO_4^{-\cdot}$ radicals with uracil and its derivatives have been carried out by various workers[4—8] but the exact sequences of the reactions are unknown. Little is known about the reaction sequences of $SO_4^{-\cdot}$ with thymine and cytosine. The chemistry of cytosine is more interesting due to the amino group, which on deamination is converted to uracil. This sort of reactions results in irreversible changes within DNA leading to mutation. It is therefore facinating to study the reactions of $SO_4^{-\cdot}$ with cytosine and its derivatives.

$SO_4^{-\cdot}$ radical is a strong oxidant compared to other oxidizing radicals such as ·OH, $Cl_2^{-\cdot}$, $Br_2^{-\cdot}$, $O_2^{-\cdot}$, etc. It is of interest to find its reaction sequences with DNA and its constituent base. $O_2^{-\cdot}$, a by product of oxygen consumption in respiring cells is supposed to be a deleterious agent. In view of the low reactivity of $O_2^{-\cdot}$ towards biologically important molecular constituents,[9,10] it has been suggested that $O_2^{-\cdot}$ rather act as precursor of more powerful oxidants. But $O_2^{-\cdot}$ in the presence of transition metal ions (by the modified metal catalyzed, Haber–Weiss mechanism) generates highly reactive ·OH radicals.[11] Copper-catalyzed degradation of nucleic acid by H_2O_2, involving base destruction and base liberation, such as single and double strand scissions, is abundantly documented.[12,13] From such studies it is evident that the role of metal ions in oxidizing DNA and its constituents is highly deleterious. This leads to the present study of oxidation of cytosine and its derivatives in the presence of copper(II) ions by $SO_4^{-\cdot}$ radicals in dilute aqueous solutions.

The present investigation has some biological relevance, since copper(II) ions which may be available in cell nuclei, as constituents stabilizing nuclear structure by interaction with non-histone proteins, may play a major role in the direct effects of ionizing radiation on DNA and its constituents.

Experimental

Cytosine, cytidine, and deoxycytidine were purchased from Sigma chemicals. Potassium peroxodisulfate, copper sulfate and *t*-butyl alcohol used were of Anala R grade. Methanol used was of HPLC grade. Triply distilled water was used to prepare all the solutions for radiolysis. A phosphate buffer 10^{-2} mol dm^{-3} was used for maintaining pH at 6.6. Argon gas of high purity was used for flushing the solutions. A ^{60}Co γ-source (dose rate 7.2 Gy min^{-1}) was used for irradiating the solutions. The dose rate was measured by Fricke dosimetry.

Analysis: Spectrophotometric Measurements: The absorption spectra of cytosine and its derivatives, both in the presence and in the absence of copper(II) ions were recorded on Shimadzu UV 2101 PC absorption spectrophotometer.

Fluorimetric Measurements: All the fluorimetric measurements were carried out on Hitachi F-4010 fluorescence spectrophotometer. The emission spectra of irradiated samples of cytosine and its derivatives in presence and absence of copper(II) ions when excited at 265 nm and 340 nm respectively have been recorded.

HPLC Measurements: The radiolytic degradation of cytosine, cytidine, and deoxycytidine were determined by separating each of them from their radiolysed products by HPLC, both in the presence and absence of copper(II) ions. The release of unaltered cytosine from cytidine and deoxycytidine was also ascertained by separating it from cytidine, deoxycytidine and their respective radiolysed products separately. The separations were carried out by using waters liquid chromatograph with an ODS C_{18} column.

Results

All the solutions that were radiolyzed contained cytosine or its derivatives as substrates of concentrations 10^{-4} mol dm^{-3}, 10^{-2} mol dm^{-3} phosphate as buffer at pH 6.6, 10^{-2} mol dm^{-3} *t*-butyl alcohol to scavenge OH, and 10^{-3} mol dm^{-3} $K_2S_2O_8$, and 5×10^{-5} mol dm^{-3}

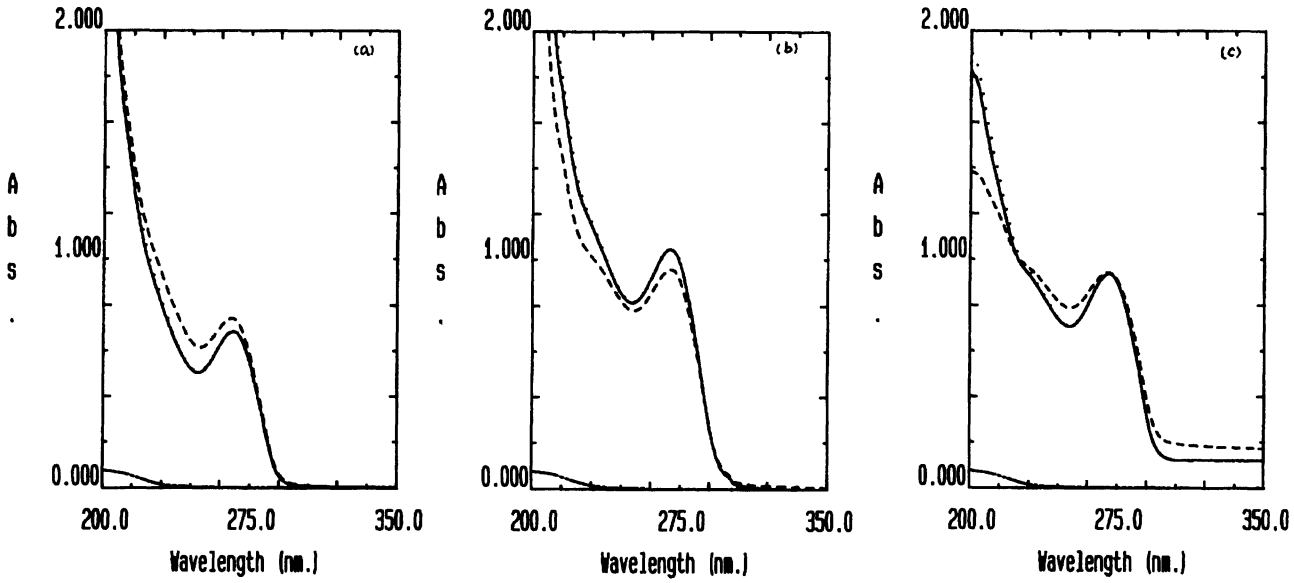

Fig. 1. The absorption spectra of 1×10^{-4} mol dm^{-3} (a) cytosine, (b) cytidine, and (c) deoxycytidine recorded (—) in absence and (- - -) in presence of 5×10^{-5} mol dm^{-3} $CuSO_4$. (····) is the additive spectra of the substrate and 5×10^{-5} mol dm^{-3} $CuSO_4$ and (–··–) is absorption spectra of 5×10^{-5} mol dm^{-3} $CuSO_4$ in each case.

copper(II) ions wherever necessary. The rate constants of e_{aq}^- with HPO_4^-, $H_2PO_4^-$, $S_2O_8^{2-}$, and copper-(II) ions are such that it mainly reacts with $S_2O_8^{2-}$ to produce mainly $SO_4^{-\bullet}$ radicals. The rate constants for reactions of $SO_4^{-\bullet}$ with *t*-butyl alcohol[14] and $H_2PO_4^{2-}$ or $H_2PO_4^{2-}$ are sufficiently low[15] such that about 95% of the $SO_4^{-\bullet}$ reacted with the pyrimidine. From Fig. 1 (a—c) it is evident that the spectra of cytosine and its derivatives show little changes on addition of spectrum of copper ions to each of them respectively. However, when the spectra of cytosine and its derivatives were recorded in copper containing solutions, though there is no shift in the absorption maxima there are significant changes in the absorption from 270 to 200 nm in case of cytosine and cytidine. Changes in absorption are also observed from 350 to 290 nm and from 250 to 200 nm in case of deoxycytidine. From these observations it is evident that there is some interaction between the base molecules and copper ions.

To understand the nature of the reactions of $SO_4^{-\bullet}$ with cytosine and its derivatives in the absence and in the presence of free or complexed copper(II) ions, substrate degradation and base release were followed by HPLC. A typical plot for the substrate degradation and unaltered base release with absorbed dose is shown in Fig. 2 (a,b). *G*-values for substrate degradation and base release were determined from the initial slopes of the respective plots and are given in Table 1.

To get an insight into the nature of the products formed and their yields on reaction of $SO_4^{-\bullet}$ with cytosine and its derivatives, fluorescence emission spectra of the same substrates irradiated separately in aqueous solutions containing $K_2S_2O_8$ and *t*-Butyl alcohol in presence and in absence of copper(II) ions when excited at 265 and 340 nm were monitored. The nature of the emission spectra of cytosine in presence and absence of copper(II) ions were similar. However, the fluorescence intensities of samples containing copper(II) ions were higher. Similar results were also obtained for cytidine and deoxycytidine. The results are summarized in Table 2.

The emission maxima of irradiated cytosine and deoxycytidine were at 400 nm and that of cytidine was at 370 nm when excited at 265 nm (Fig. 3a), but the emission maxima for all these three substrates were at 385 nm when excited at 340 nm (Fig. 3b and Figs. 4a and 4b). As mentioned earlier no corrections have been made for the fluorescence quenching by Copper(II) ions and each spectra presented has been obtained after subtraction of emission spectra of unirradiated from the irradiated samples under similar conditions in each case.

Discussion

To study the reactions of $SO_4^{-\bullet}$ with cytosine and its derivatives, dilute aqueous solutions containing the cytosine and its derivatives, potasium peroxodisulfate and *t*-butyl alcohol were irradiated using Co-60 γ-rays. The concentrations of the substrates were chosen such that among the primary water borne radicals OH, H, and e_{aq}^- [reaction (1)] the hydrated electrons and the hydrogen atoms react with the peroxodisulfate ion to give $SO_4^{-\bullet}$ radicals [reaction (2)], and the hydroxyl radicals are scavenged by *t*-butyl alcohol [reaction (3)]. The $SO_4^{-\bullet}$ is left to react with cytosine and its derivatives thus producing the base radicals of interest:

$$H_2O \rightarrow OH,\ H,\ e_{aq}^-,\ H^+,\ H_2O_2,\ H_2 \quad (1)$$

$$e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{-\bullet} + SO_4^{2-} \quad (2)$$

$$OH + (CH_3)_3COH \rightarrow CH_2C(CH_3)_2OH + H_2O \quad (3)$$

Table 1. The G Values of Freed Cytosine and Consumption of Cytosine and Its Derivatives on Radiolysis in Dilute Aqueous Solutions Containing $K_2S_2O_8$ and t-Butyl Alcohol in Presence of Various Additives in Deaerated Conditions

Substrate	Additive	Substrate consumption	Unaltered base release	Cu(I) yield
Cytosine	Nil	2.9	—	—
	$CuSO_4$	5.2	—	4.2
Cytidine	Nil	3.5	2.7	—
	$CuSO_4$	7.1	6.7	1.4
	$[Cu^{II}(edta)]$	5.8	4.9	U.d.
	$[Cu^{II}(nta)]$	5.4	4.46	U.d.
Deoxycytidine	Nil	3.0	.26	U.d.
	$CuSO_4$	7.7	2.68	.85
	$[Cu^{II}(edta)]$	6.7	U.d.	U.d.
	$[Cu^{II}(nta)]$	6.38	U.d.	U.d.

U.d.: undetermined.

Table 2. The Fluorescence Excitation and Emission Wavelengths of Cytosine and Its Derivatives after Reacting with OH and $SO_4^{-\bullet}$ Radicals in Dilute Aqueous Solutions

Substrate	Radical	E_x[c]	E_m[a]	R.F.In[b] at E_m	Excitation maxima	at E_m[a]
Cytosine	OH	265	385		236 & 330	386
		265*			228 & 288*	386*
		340	385			
	$SO_4^{-\bullet}$	265	401	0.3	228 & 330	386
		265*	401*	0.54*	227 & 341*	386*
		340	385	1.0		
		340*	385*	4.1*		
Cytidine	$SO_4^{-\bullet}$	265	362	0.7	241 & 302	390
		265*	370*	0.85*	234 & 300*	390*
		340	385	0.4		
		340*	385*	0.5*		
Deoxycytidine	$SO_4^{-\bullet}$	265	397	1.3	240 & 340	396
		265*	402*	1.5*	239 & 339*	396*
		340	385	2.6		
		340*	385*	3.0*		

* in presence of copper ions. a) E_m: Emission wavelength in nm. b) R.F.In: Relative fluorescence intensity in A.B.U. c) E_x: Excitation wavelength in nm.

Previous studies by Hazra and Steenken[16] with unsubstituted cytosine showed that $SO_4^{-\bullet}$ reacts with cytosine forming a cation radical which rapidly undergoes deprotonation at N_1 forming N centered radical species [reactions (4,5)] (Chart 1). However, when hydrogen at N_1 is substituted with ribose or deoxyribose no deprotonation reaction from N_1 is possible. For deoxycytidine O'Neill and Davies[7] suggested the formation of radical centred at NH_2 of cytosine [reactions (6, 7)]. Both the radicals (I & II) are oxidizing in nature. Similar reactions of cytidine are not much studied.

As an alternative pathway there is a possibility of formation of $SO_4^{-\bullet}$ adduct which rapidly is converted to OH adduct radical by reaction with water [reactions (8, 9,10)] (Chart 2). This OH adduct radical could also be generated from the cation radicals formed in reaction (4) through reaction (11). In such adducts OH may be added to either 5- or 6-positions. But it is known that OH adds predominantly to the 5-position which is, however, reducing in nature. This is a minor process and majority of the cations decay through deprotonation leading to the formation of oxidizing species like I & II as shown in reactions (5) and (7).

From Table 1 it is obvious that when $SO_4^{-\bullet}$ radical induced oxidation of aqueous solutions of cytosine and its derivatives is carried out in the absence of copper(II) ions, the G-values of the substrate degradation were comparable with the $G(SO_4^{-\bullet})$; these results are not

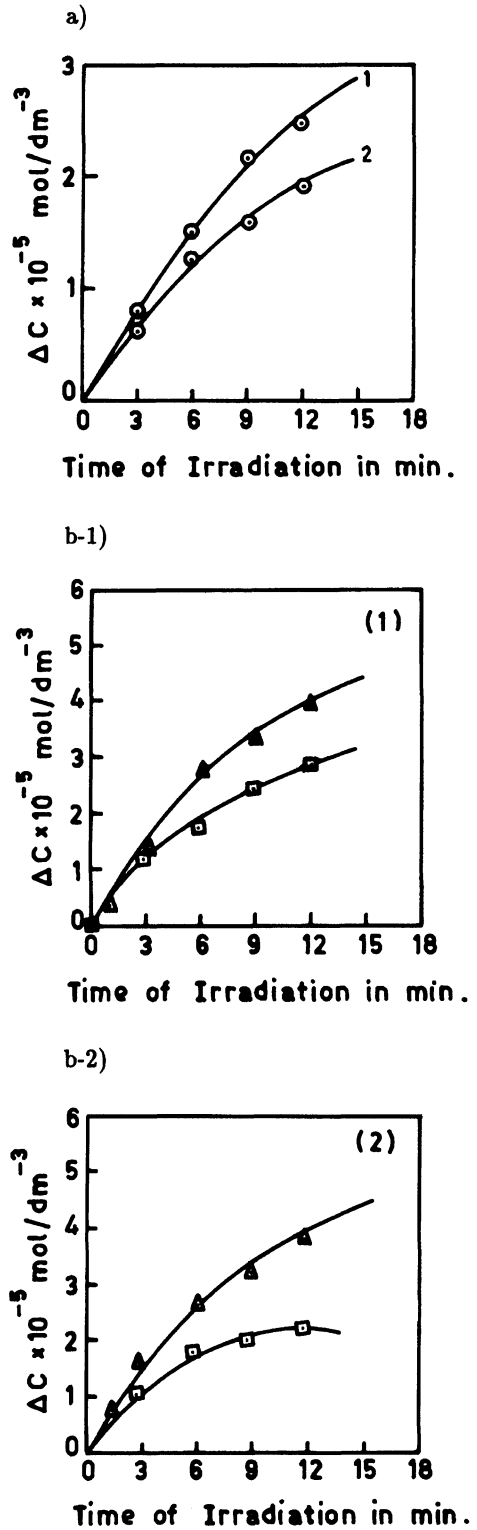

Fig. 2. (a) Extent of (1) cytidine degradation, (2) release of unaltered cytosine, on radiolysis of aqueous solutions of cytidine (1×10^{-4} mol dm^{-3}) containing $S_2O_8^{2-}$ (1×10^{-3} mol dm^{-3}). (b) Extent of (1) cytidine degradation, (2) release of unaltered cytosine on radiolysis of aqueous solutions of (1×10^{-4} mol dm^{-3}) cytidine containing $S_2O_8^{2-}$ (1×10^{-3} mol dm^{-3}) in the presence of (5×10^{-5} mol dm^{-3}) (△) $CuSO_4$, (□) Cu(II)EDTA.

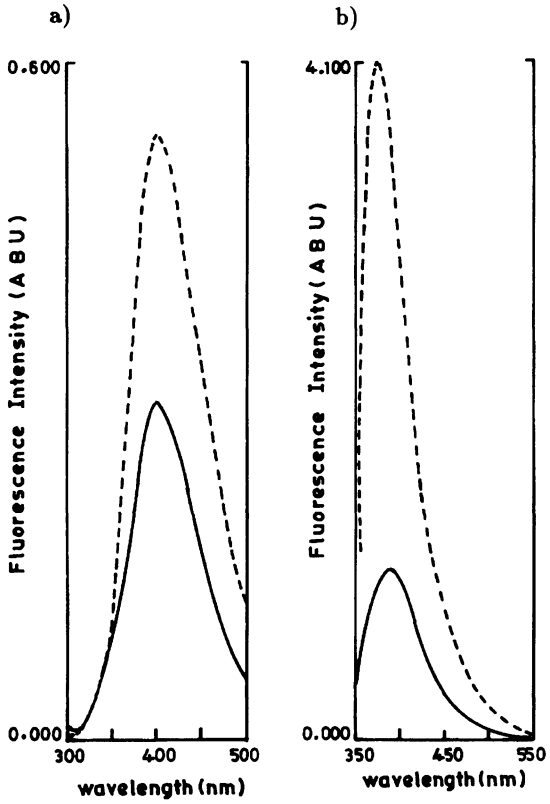

Fig. 3. Fluorescence emission spectra of (1×10^{-4} mol dm^{-3}) cytosine irradiated at dose of 72 Gray in absence (—) and in presence (---) of (5×10^{-5} mol dm^{-3}) $CuSO_4$ in aqueous solutions containing $S_2O_8^{2-}$ (a) when excited at 265 nm (b) when excited at 340 nm.

similar to those obtained by reactions of $SO_4^{-\cdot}$ with uridine and deoxyuridine, where the observed high G-values may be thought of arising from the occurrence of chain reactions.[5,6,8] However, when similar reactions of cytosine and its derivatives were carried out in presence of copper(II) ions; the G-values for the substrate degradation are much higher than the G-values of $SO_4^{-\cdot}$. This can be explained only if some sort of chain reactions are also thought of to be occurring in the presence of copper(II) ions. These results suggest that the radicals responsible for the substrate degradation in the presence and in the absence of copper(II) ions are different. The radicals formed in the presence of copper(II) ions should be reducing in nature so that they can reduce $S_2O_8^{2-}$ to generate more $SO_4^{-\cdot}$ which initiates the chain reactions. This can only be possible if the deprotonation reactions (5) and (7) become less favorable compared to the formation of the OH adduct radicals by reactions (10) and (11) which are reducing in nature. A sequence of reactions can be suggested accordingly,

$$S_2O_8^{-2} + e_{aq}^- \longrightarrow SO_4^{-\cdot} + SO_4^{2-} \quad (12)$$

$$Cyt + SO_4^{-\cdot} \xrightarrow{k_1} Cyt^{\cdot} + SO_4^{2-} \quad (13)$$

$$Cyt^{\cdot} + S_2O_8^{2-} \xrightarrow{k_2} Products + SO_4^{-\cdot} \quad (14)$$

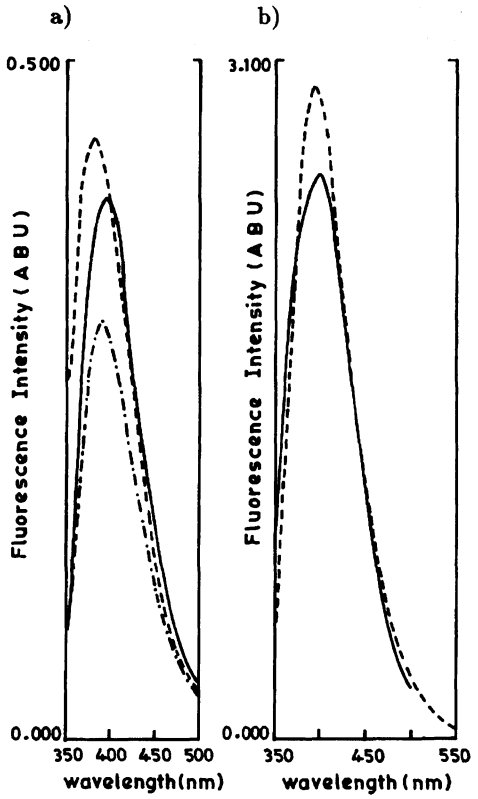

Fig. 4. Fluorescence emission spectra of (1×10^{-4} mol dm^{-3}) (a) cytidine, (b) deoxycytidine irradiated at a dose of 72 Gray in dilute aqueous solutions containing $S_2O_8^{2-}$ in the absence (——) and in the presence (- - -) of (5×10^{-5} mol dm^{-3}) $CuSO_4$; and in the presence of (—·—) of (5×10^{-5} mol dm^{-3}) [Cu^{II}(edta)] when excited at 340 nm.

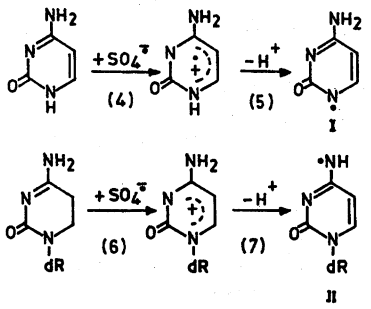

Chart 1.

$$Cyt^{\bullet} + Cu(II) \xrightarrow{k_3} Products + Cu(I) \quad (15)$$

$$Cyt^{\bullet} + Cyt^{\bullet} \xrightarrow{k_4} Dimers \quad (16)$$

where $Cyt^{\bullet}$ is the reducing radical formed in each case.

The reactions (13) and (14) comprise a chain reaction, and this accounts for the relatively high G(-substrate) values. Chain termination is by reaction of the radical with Cu(II) [reaction (15)] and by combination of two of the free radicals involved [reaction (16)] and as is expected for a chain reaction of this sort G(-substrate) is found to be inversely proportional to the square root of the dose rate where the substrate may be cytosine,

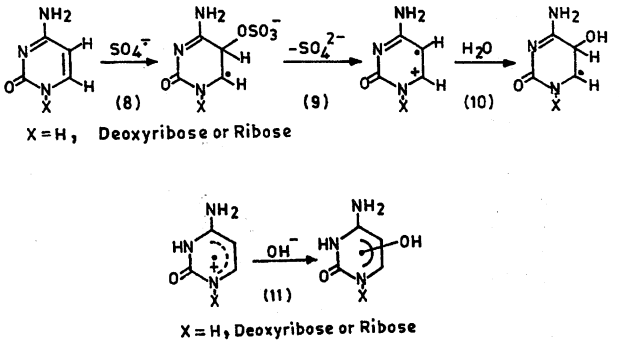

Chart 2.

cytidine or deoxycytidine (Fig. 5). Here it is also worth mentioning that the yields of dimers and Cu(I) are highest in case of cytosine where the substrate degradation is the least which confirms that formation of dimers and Cu(I) are the chain terminating steps.

To explain these results it has been assumed that weak associations are formed between the copper(II) ions and cytosine and its derivatives. The minor changes observed in the absorption spectra of cytosine and its derivatives in the presence and in the absence of Copper(II) ions are indicative of such associations. Previous studies by Albert[17] in 1953 also suggested that weakly associated complexes of first transition metal ions with the basic sites of the nucleosides do exist. Albert and later Frieser[18] and his collaborators were infact unable to measure the proton from the basic sites in the presence of such ions. Under such conditions, it may then be assumed that the cations of cytosine and its derivatives undergo hydrolysis reactions [reaction (11)] rather than deprotonation reactions.

The difference in the extent of degradation of cytosine, cytidine, and deoxycytidine may be governed by the life times of the radicals involved and their reducing ability. This may be further influenced by the stereochemistry of the complexes formed between the base molecules and copper ions. These may be the determining factors for the yields of dimers and Cu(I) which in turn are the terminating steps of the chain reactions involved. It is however, important to mention here that the yields of unaltered cytosine released from cytidine are very high in presence of copper(II) ions compared to deoxycytidine which might be due to the difference in the type of sugar attached to the base molecule.

This possibility of complexation and its role in initiating chain reactions was further supported by the observation that the substrate consumption and the unaltered base release from cytidine and deoxycytidine decreased in the presence of copper(II) complexes like [Cu^{II}(edta)] and [Cu^{II}(nta)]. This happens because EDTA and NTA (nitrolotriacetic acid) form very strong complexes with copper(II) ions and so its availability to form association with the nucleotides decreases.

It is known that some of the radiolytic products of cytosine and its derivatives are fluorescent and to get

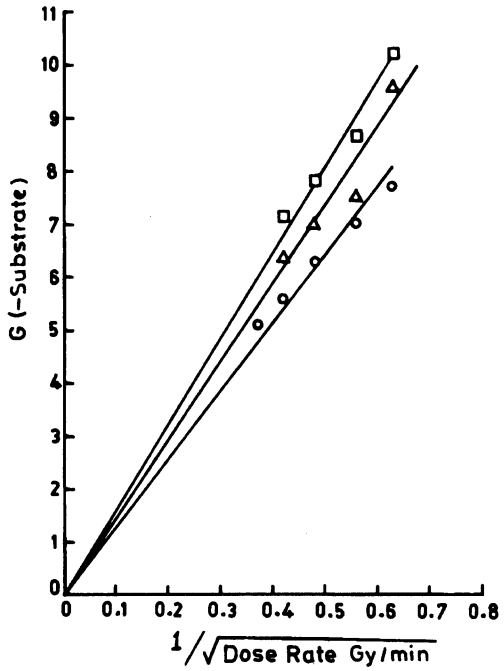

Fig. 5. A plot of G(-substrate) against inverse of square root of the dose rate, ⊙ cytosine, ⊡ cytidine, and △ deoxycytidine.

an insight into the nature of the products formed the fluorescence emission and excitation spectra of cytosine irradiated in N_2O saturated conditions in aqueous solutions and when irradiated in N_2 saturated conditions in solutions containing $K_2S_2O_8$ and t-butyl alcohol both in presence and absence of copper ions were compared. From Table 2 it can be seen that when OH is the reacting radical the irradiated solutions when excited at 265 nm as well as at 340 nm show emission maxima at 385 nm and the excitation spectrum of the same at emission wavelength 386 nm show excitation maxima at 234 and 330 nm. However, from Table 2 it can also be seen that when copper(II) ions are present in experimental solutions during radiolysis the excitation maxima are shifted to 228 and 288 nm. This has been reported to be due to the restriction in formation of dimers in the presence of copper(II) ions.[19,20] It should be mentioned here that these excitation maxima do not change with change in emission wavelength. When $SO_4^{-\cdot}$ is the reacting radical the samples when excited at 265 nm show emission maxima at 400 nm in case of cytosine (Fig. 3a) and deoxycytidine, and at 370 nm in case of cytidine (Table 2). However, when excited at 340 nm all samples show emission maxima at 385 nm (Figs. 3b, 4a, and 4b). In each case higher fluorescence intensities without shift in emission maxima were observed for samples irradiated in presence of copper ions, which indicate an increased formation of similar products in presence of copper ions as pointed earlier. The excitation spectrum of the samples containing cytosine when monitored at emission wavelength 386 nm show excitation maxima at 228 and 330 nm when $SO_4^{-\cdot}$ is the reacting radical which are very similar to those obtained when OH is the reacting radical. This leads to the conclusion that the fluorescent products formed are the same when OH and $SO_4^{-\cdot}$ are the reacting radicals and may be thought of arising from similar intermediates. It is important to mention here that in case of cytidine excitation spectrum at emission wavelength 386 nm show excitation maxima at 241 and 302 nm which are close to those obtained in case of cytosine in the presence of copper ions when OH is the reacting radical, where there is a shift in the excitation wavelength from 330 to 288 nm due to the restriction in formation of dimers. A look at Fig. 4a and Table 2 will show that the fluorescence intensities at 385 nm are very low compared to cytosine and deoxycytidine when excited at 340 nm which means the yields of dimers are very low in case of cytidine. Further investigations are in progress.

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