Discussion
Acridine dyes, especially proflavine, are photodynamic agents known to generate different types of reactive oxygen species upon illumination with visible light only when a macromolecule such as DNA is present in the reaction (Leupold and Kochevar, 1997). These reactive oxygen species are known to target DNA and other biomolecules (Leupold and Kochevar, 1997; Kochevar and Buckley, 1990). Although the exact mechanism of damage to DNA by proflavine is not well characterized, there are indications that proflavine is a type 1 photosensitizer in which its electronically excited state reacts directly with DNA (Piette et al., 1981; Vande Vorste et al., 1976). Both single- and double-strand breaks of plasmid DNA by photoilluminated proflavine have been observed (Shafirovich, 1999).

Our spectral studies indicate that upon photoillumination proflavine undergoes photodegradation and in the process reactive oxygen species are generated. Significantly, these reactive oxygen species are generated even in the absence of a macromolecule. This is in contrast to the observations of Piette and co-worker (Piette et al., 1978; Piette et al., 1979; Piette et al., 1981; Piette et al., 1982) who found that the reactive oxygen species are generated upon photoillumination of proflavine but only when a macromolecule like DNA is also present in the reaction (Kochevar and Dunn, 1990). The addition of sodium azide, potassium iodide and thiourea significantly inhibited the photodegradation of proflavine. The effect is most pronounced with potassium iodide indicating the \( \text{O}_2^- \) is the major species generated in the reaction. The spectral studies also indicate that the presence of Cu (II) during photoillumination of proflavine partially restores the proflavine peak, but does not cause enhancement in the production of ROS.

The formation of \( \text{OOH} \) and \( \cdot \text{OH} \) during the DNA strand breakage reaction have been proposed by Piette et al. (1982). According to their proposed mechanism, proflavine may also give rise to \( \text{O}_2^- \) either by direct reaction between the electron ejected by excited proflavine and molecular oxygen or by the decomposition of \( \text{OOH} \).

We have shown that around 55% inhibition to NBT reduction reaction occurred when SOD was present in the reaction confirming the formation of \( \text{O}_2^- \) in addition to other reactive oxygen species in the reaction. However, several authors have implicated a highly reactive \( \cdot \text{OH} \) derived from \( \text{O}_2^- \) as the ultimate reactive species (Fridovich, 1978; Halliwell et al., 1980; Halliwell, 1981). The \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) can generate \( \cdot \text{OH} \) by either
modified Haber-Weiss (1) or by Fenton (2) reactions provided a metal ion is present in the latter reaction.

\[
\begin{align*}
O_2^- + H_2O_2 & \rightarrow \cdot OH / \cdot OH + O_2 \quad (1) \\
H_2O_2 + Fe(II)/Cu(I) & \rightarrow \cdot OH + \cdot OH + Fe(III)/Cu(II) \quad (2)
\end{align*}
\]

It is also known that the generation of the \(O_2^-\) may lead to the formation of \(H_2O_2\). The addition of a second electron to \(O_2^-\) gives the peroxide anion \((O_2^{-2})\) which has no unpaired electron and is not a radical. However, at neutral pH the peroxide ion immediately gets protonated to give \(H_2O_2\). Alternatively, in aqueous solution the \(O_2^-\) undergoes dismutation to form \(H_2O_2\) and \(O_2\) (Halliwell and Gutteridge, 1984):

\[
2O_2^- + 2H \rightarrow H_2O_2 + O_2 \quad (3)
\]

We have shown through the deoxyribose reaction the ability of proflavine to generate \('OH\) alone as well as in presence of Cu(II) supporting the finding of Piette and co-workers (Piette et al., 1981; Piette et al., 1982). However, in contrast to their observations the \('OH\) is generated even in the absence of DNA.

Most of the study that have been carried out by Piette and co-workers (Piette et al., 1978; Piette et al., 1979; Piette et al., 1981; Piette et al., 1982) have shown that induction of radical is possible only in the presence of macromolecule as DNA and this was reported to induce the formation of peroxide radical and singlet oxygen. Van de Vorst et al. (1976) however, have suggested the involvement of superoxide and formyl radical in the same photoilluminated reaction mixture. Our observation indicates that the photogeneration of \(O_2^-\) from proflavine was faster in presence of dsDNA. While both ssDNA and RNA were actually inhibitory. The spectral studies of proflavine in presence of these molecules suggest that the presence of ds DNA in the reaction helps in restoring the structure of proflavine more than RNA. This is probably due to the fact that proflavine intercalates with ds DNA. Radical produced in the process probably attack the DNA in the vicinity of proflavine binding before they can be detected. It is well known that intercalation of planar ligands between DNA base pairs involves partial unwinding of the double helix (Waring, 1970). Therefore, interstrand covalent crosslinks were expected to block intercalation. In the cross-linked dsDNA the stimulatory effect on \(O_2^-\) formation was not observed. Availability of guanine residues
may be still necessary for the stimulatory effect of dsDNA on the photogeneration of O$_2^\cdot$. Comparative observation using alkylated and depurinated DNA on the generation of O$_2^\cdot$ by proflavine has clearly shown the better stimulatory effect of alkylated DNA than that of depurinated DNA. This result indicates that the presence of guanine residues in DNA is essential for the stimulatory effect of dsDNA on O$_2^\cdot$ generation of by proflavine in visible light. This again supported the idea of Piette et al. (1978) who showed that intercalation of acridine dyes in DNA under light treatment favours the formation of free radicals.

A survey of literature revealed that interaction of proteins with oxygen free radicals both in vivo and in vitro results in enhanced hydrophobicity due to modifications of amino acids, hence, possible increase in the susceptibility to proteolysis (Wolff and Dean, 1986; Davies and Doroshow, 1986; Davies et al., 1987). Wolff and Dean (1986) have shown that OH attack on proteins leads to conformational changes and therefore, enhanced susceptibility to enzymatic proteolysis. It is well reported that limited protein oxidation can increase hydrophilicity while further oxidation by radical attack can increase hydrophobicity (Kim et al., 1985). In our observation however, susceptibility to tryptic proteolysis of BSA was decreased in presence of proflavine and Cu(II) indicating the possibility of modification of specific amino acids, lysine and arginine which are the amino acids residues that are recognized by trypsin. Others have reported that these amino acids are modified after radical attack (Kang et al., 1985), supporting our observation.

BSA is known to bind to a variety of endogenous and exogenous ligands (Thiessen et al., 1972; Brodersen, 1974). When a ligand binds to protein at or around tryptophan it quenches its native fluorescence (Chignell, 1972). The quenching of fluorescence of tryptophan residues is diagnostic of the binding of ligand to a tryptophan or alternatively of a conformational change caused by interaction (Levine, 1977). BSA and three other proteins differing in their tryptophan content, namely lysozyme, invertase and trypsin, have been used for fluorescence studies. A shift in $\lambda_{\text{max}}$ for tryptophan fluorescence together with quenching was observed in all the cases, suggesting the binding of proflavine to these proteins in the vicinity of tryptophan residues. The shift in $\lambda_{\text{max}}$ also suggests that these tryptophan residues are in hydrophobic environment (Ahmed et al., 1994). This is consistent with at least the
known crystallographic structure of lysozyme. The shift in \( \lambda_{\text{max}} \) was maximum with invertase followed by BSA and lysozyme and minimum with trypsin. This suggests that the tryptophan of trypsin is in the least hydrophobic environment.

As the pattern of fluorescence quenching was not fully in accordance with the number of tryptophan residues in the proteins, it is therefore proposed that proflavine being hydrophobic in nature shows binding characteristics to BSA similar to quercitin (Ahmed et al., 1994). Scatchard analysis of the data showed that the proflavine binding capacity was in the order of invertase > trypsin > lysozyme > BSA, reflecting the different tryptophan content (Campbell and Dwek, 1984). The emission spectra of BSA recorded after addition of increasing proflavine concentrations also indicate that proflavine either binds to tryptophan directly or modifies the conformation of BSA, leading to shielding of tryptophan as evidenced by the progressive decrease in emission intensity.

Proteins are the key targets of reactive oxygen species leading to their oxidation which may sometimes control cellular remodeling and growth. (Dean et al., 1997). Protein fragmentation has previously been reported following exposure to \( \cdot \text{OH} \) and \( \cdot \text{O}_2^- \) in presence of oxygen. The fragmentation process was suggested to involve hydrogen abstraction by \( \cdot \text{OH} \) from amino acid \( \alpha \)-carbon atoms, followed by reaction with \( \text{O}_2 \) to produce peroxyl species oxygen. Decomposition of \( \alpha \)-carbon peroxides was proposed as the mechanism for protein fragmentation oxygen (Garrison et al., 1962). Davies and Delsignore (1987) have shown fragmentation of BSA upon exposure to \( \cdot \text{OH} \) and \( \cdot \text{O}_2^- \) under aerobic condition which is broadly consistent with the above proposed mechanism. We, therefore, studied the effect of reactive oxygen species generated upon photoillumination of proflavine on BSA. Proflavine alone upon photoillumination, caused pronounced degradation of BSA. The addition of Cu(II) in the reaction significantly enhanced this degradation. Spin trapping technique provided evidence for the production of \( \cdot \text{OH} \) during proflavine mediated degradation of DNA (Piette et al., 1982). We have shown that \( \cdot \text{OH} \) are involved in degradation of BSA by photoilluminated proflavine since the addition of thiourea (a scavenger of \( \cdot \text{OH} \)) to either proflavine or proflavine-Cu(II) significantly inhibited this degradation. However, there is strong evidence for the formation of \( \text{O}_1^1 \), \( \text{O}_2^3 \) also as sodium azide and potassium iodide also inhibited protein degradation reactions to a significant extent. This was
Further confirmed using two other enzymes with different structure properties. First was a glycoprotein, namely invertase, which was also fragmented by proflavine alone and the presence of Cu(II), enhanced this fragmentation as revealed in SDS-PAGE. However, in case of monomeric nonglycoprotein, trypsin, proflavine led to intramolecular crosslinking, while the presence of Cu(II) significantly decreased the extent of crosslinking. Our results are comparable to the finding of Sara et al. (1984) who have suggested that multimeric proteins when exposed to free radical generating systems show visible fragmentation, while crosslinking was predominant in case of monomeric proteins. The work carried out by Davies and Delsignore (1987) points to the production of bityrosine upon exposure to \( \text{OH} \) and \( \text{O}_2^- \) which result in late degradation of the protein with the formation of aggregate. But in case of glycosylated protein, the fragmentation induced by proflavine alone is slow which could be due to the presence of attached carbohydrates. However, Wolff and co-workers (Wolff and Dean, 1987; Wolff et al., 1989) have proposed that the presence of Cu(II) could catalyse the auto-oxidation and hence the fragmentation of the protein is expected in a system that generates \( \text{OH} \).

Proflavine has been reported to form hydrogen bonds with some amino acids with one of its amino groups or with the protonated ring nitrogen (Conte et al., 1998). We have shown the evidence for the binding of proflavine to proteins through fluorescence quenching studies. The fluorescence of proteins decreased with increasing proflavine concentration. Decreased fluorescence reflects changes in the aromatic side-chain composition, possibly a loss of conjugation from tryptophan or other aromatic amino acids (Gutteridge and Wilkins, 1983).

We have used copper in our studies since it is an essential trace element that is distributed throughout the body. It is present in many tissues including liver and kidney where its concentration is relatively high (Burkitt et al., 1996). Copper forms the essential redox-active centre in a variety of metalloproteins such as ceruloplasmin, Cu,Zn superoxide dismutase, cytochrome c oxidase, tyrosinase and ascorbate oxidase. Normal serum contains up to 8 \( \mu \text{M} \) loosely bound copper which is available for redox reaction (Bukitt et al., 1996; Rowley and Halliwell, 1983). When bathocuproine, a Cu(I) sequestering agent, was used in the degradation reaction of BSA that contains Cu(II) it has given significant inhibition showing the involvement of Cu(I). Thus it appears that
binding of proflavine leads to formation of proflavine-protein-Cu(II) complex, which upon photoexcitation via charge transfer is converted into oxidized proflavine-protein-Cu(I), resulting in generation of reactive oxygen species which then mediate protein fragmentation.

The results of our work with proteins showed that photoilluminated proflavine caused significant structural and functional alterations. The accumulation of catalytically inactive or less active, more heat labile forms of many enzymes is an index of cellular aging (Dreyfus et al., 1978; Rothstein, 1977). Invertase catalyses the hydrolysis of sucrose to mixture of glucose and fructose or the so called invert sugar. The enzyme is of considerable industrial significance especially in the production of fructose containing syrups. Yeast invertase is a glycoprotein with about half its mass contributed by the high mannose type of the oligosaccharide chains (Trimble and Maley, 1977). Most of the glycoenzymes (Lis and Sharon, 1993) including invertase (Lehle et al., 1979) do not require their glycosyl residues for their catalytic functions. The presence of oligosaccharides did not protect the enzyme from attack by radicals which were generated from the photoilluminated proflavine. The loss of activity was due to proflavine derived free radical mediated damage to protein. Metal-catalysed oxidation systems are reported to inactivate a wide range of enzymes (Bauskine et al., 1991; Stadtman, 1991), and invertase is known to bind to Cu(II), hence, the presence of Cu(II) in the reaction is expected to enhance the degradation of protein. Presence of Cu(II) in the reaction mixture resulted in enhanced degradation of protein to smaller peptide. This is in agreement with the finding that radical attack on glycoproteins stimulates peptidolysis (Cooper et al., 1985). Wolff and co-worker (Wolff and Dean, 1987; Wolff et al., 1989; Hunt et al., 1988) have shown that hydroxyl radical and Cu(II) also catalyze the autoxidation of sugars which can then participate in the modification of proteins.

Proflavine is known to bind competitively to trypsin (Brantner et al., 1976) and prevents substrate binding. The rate of the formation of enzyme-inhibitor (proflavine) complex is equal to the rate of formation of enzyme substrate complex, hence high concentration of substrate is required for the formation of enzyme substrate complex when an inhibitor like proflavine is also present in the reaction. BSA the substrate for trypsin in our study, was always used in excess for this reason. Even so, the proteolytic activity of trypsin was shown to be markedly decreased, probably due to free radical
mediated damage to specific amino acids in the protein by proflavine. The loss of activity of trypsin was more pronounced when Cu(II) was also included in the reaction. The degradation of BSA by proflavine was higher than trypsin as shown by SDS-PAGE analysis. Same observation was recorded by other investigators. After exposing trypsin to \( \cdot \text{OH} \) (Davies, 1987) no polypeptide fragmentation was observed; rather intramolecular bi-tyrosine was produced by reaction of two tyrosyl radicals or a tyrosyl radical plus a tyrosine molecule (Prutz et al., 1983). As the trypsin degradation following exposure to proflavine or proflavine and Cu(II) was not very significant, this could also be due to the production of intramolecular bi-tyrosine, as the band intensity or migration is not much affected on SDS-PAGE.

Glycopolypeptides are effective scavengers of \( \cdot \text{OH} \) when they are generated in the system. Such scavenging action is expected from the high sugar content of the glycopolypeptides. This scavenging action may be of primary importance in protecting the respiratory and gastrointestinal tracts from adventitious radicals in smoke or those produced by normal cellular action (Cross et al., 1984). Other reports have shown that peroxide radical attack on glycoproteins occurs largely at the histidine residues causing its modification (Cooper et al., 1985). Wolff and co-workers (Wolff and Dean, 1987; Wolff et al., 1989; Hunt et al., 1988) have proposed that hydroxyl radical and Cu(II) catalyze auto-oxidation of the sugars which can then participate in the modification of protein. Modification of proteins by radical attack have been previously reported by Gantchev and Van Lier (1995) where they have shown that radical generated by photosensitizer like tetrasyphonated metallophthalocyanines can change the activity of catalase. It also caused conformational changes in the enzyme. Singlet oxygen and other radical species can participate in the photosensitizer induced inactivation of enzymes as exemplified by other studies on purified catalase, or catalase within cells (Gantchev and Van Lier, 1995). Our studies have supported the above mentioned report of Cross et al. (1984) for our target enzyme, the heavily glycosylated invertase, which has shown a 43% decrease of activity after five hours of exposure to proflavine and light. This resistance could be due to the presence of carbohydrates on the surface of the enzyme which upon reacting with various reactive oxygen species produced by proflavine may themselves participate in the scavenging of \( \cdot \text{OH} \) produced in the system. The enzyme has shown enhanced inactivation in presence of proflavine and Cu(II), such decrease in the activity could be due to the attached carbohydrate. Invertase can bind to Cu(II)
directly and the activity of invertase is inhibited, or the presence of bound Cu(II) directed the reactive oxygen species generated in the reaction to target the active site, thereby, inhibiting the enzyme (Bernfeld, 1955). This was confirmed by the use of metal chealating agent like EDTA. The inactivation of the enzyme was slow in the presence of EDTA. Comparing this study with the non glycosylated enzyme trypsin we have shown that after photoillumination with same system for only twenty minutes trypsin showed 40% residual activity, and was completely inactivated after one hour of incubation with proflavine and light. The enzyme seems to be directly effected by the ROS generated from proflavine or proflavine and Cu(II). The effect being slightly more but not significant when Cu(II) is present. Trypsin has shown resistance to degradation on SDS-PAGE under similar condition compared to BSA probably for different reasons, which supports the observation of Davies (1987) suggesting the formation of bi-tyrosine crosslinks as discussed earlier.

From our studies on proflavine we propose the following probable mechanism for the generation of various reactive oxygen species from photoexcited proflavine (Figure 39). Proflavine upon photoillumination is excited to singlet state which gives rise to triplet state through inter system crossing. When H₂O and O₂ are present in the reaction photoexcited proflavine can then give rise to O₂³ and O₂¹ through direct energy transfer (pathway (I)). These O₂³ and O₂¹ can participate in the protein degradation reaction. Through an alternative pathway (II), the photoexcited proflavine can accept an electron from molecular oxygen and give rise to cationic radical which further reacts with molecular oxygen and give peroxide radical. This peroxide radical can, in the presence of H₂O, give 'OH or 'OOH and in the process Cu(II) may be reduced to Cu(I) if present in the reaction, and proflavine then returns to the ground state. Polyaromatic systems are well known to go for facile photoxidation in presence of light and oxygen to give peroxide radical. Furthermore, photochemical electron transfer reactions between transition metal ions and anions or water molecules are well established (Spinks et al., 1964), moreover, exposure of water to pulse of electrons can cause ionization and excitation within 10⁻¹⁶ second (Halliwell and Gutteridge, 1985) as seen in the following example:

\[ \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + \text{e}^- + \text{H}_2\text{O}^* \quad (1) \]
\[ \text{H}_2\text{O}^* \rightarrow \text{H} + \text{HO} \quad (2) \]
Figure 39. Scheme for the production of free radicals from the photoilluminated proflavine.
Proflavine

I Light

Pathway (II)

O₂

Cation radical (stable)

Pathway (I)

Part conversion

Inter System Crossing

Energy transfer

'O₂

Damage Biological Macromolecules

 Peroxide

H₂O

H₂O

HOO⁻

Cu²⁺
Where is H$_2$O$^*$ represent an excited water molecule. And within the same timescale H$_2$O$^*$ also react to give 'OH

$$\text{H}_2\text{O}^* + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^* + \cdot \text{OH} \quad (2)$$

The 'OH and 'OOH, in addition to O$_2^*$ and O$_2^+$, are also available in the reaction to attack the target molecule and cause further damage.

The above study gains significance because proflavine is structurally similar to tacrine and quinacrine, drugs approved for the treatment of Alzheimer's disease and malaria, respectively (Plymale and de la Iglesia, 1999; Motten et al., 1999). Studies using human hepatocytes with tacrine and proflavine show subcellular changes and mitochondrial dysfunction (Plymale and de la Iglesia, 1999). Proflavine and 7-hydroxy tacrine have been implicated as potential precursors of reactive metabolites (Madden et al., 1995). A toxic role of oxidized proteins, rather than oxidized lipids, has been recently proposed in the etiology of Alzheimer's disease (Sigman et al., 1979). As tacrine is known to be converted to 7-hydroxy tacrine, the risk of protein oxidation leading to precipitation of disease will increase. Moreover, the ability of proflavine to inactivate certain types of micro-organisms can give possible use of photosterilization against contamination with pathogenic bacteria in water model ecosystems, Kussoviski et al. (2001) have studied the survival of Salmonella dublin and heterotrophic bacteria in freshwater microcosms after exposing them to proflavine and sun light.

In conclusion, the data presented here confirm that proflavine has a photosensitizing action and in the presence of visible light generates various reactive oxygen species which can attack proteins leading to modification. The effect was enhanced in the presence of Cu(II). Further studies using in vivo system are, however, needed to confirm the damaging effect of proflavine.