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Photoilluminated riboflavin/riboflavin-Cu(II) inactivates trypsin: Cu(II) tilts the balance

E Husain[1] R A Fatima[1], I A F Ali[2] and I Naseem[1*]

[1]Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202 002 (U.P.), India

[2]Faculty of Pharmacy, Al–Najah National University, Nablus, Palestine

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Riboflavin (RF) upon irradiation with fluorescent light generates reactive oxygen species like superoxide anion, singlet and triplet oxygen, flavin radicals and substantial amounts of hydrogen peroxide (H_2O_2). H_2O_2 can freely penetrate cell membrane and react with a transition metal ion like Cu(II), generating hydroxyl radical via the modified metal-catalyzed Haber-Weiss reaction. Earlier, it was reported that trypsin-chymotrypsin mixture served as an indirect antioxidant and decreased free radical generation. Thus, in the present study, we used photoilluminated RF as a source of ROS to investigate the effect of free radicals on the activity of trypsin. We also compared the damaging effect of photoilluminated RF and RF-Cu(II) system using trypsin as a target molecule. RF caused fragmentation of trypsin and the effect was further enhanced, when Cu(II) was added to the reaction. Results obtained with various ROS scavengers suggested that superoxide radical, singlet and triplet oxygen were predominantly responsible for trypsin damage caused by photoilluminated RF. On the other hand, when Cu(II) was added to the reaction, hydroxyl radical was mainly responsible for trypsin damage. A mechanism of generation of various ROS in the reaction is also proposed. Trypsin did not show any antioxidant effect with RF alone or with RF–Cu(II) combination.

Keywords: Riboflavin, Trypsin, Copper, Reactive oxygen species

Riboflavin (RF), when exposed to light, absorbs energy and gets excited to its singlet and triplet state. With molecular oxygen present in the reaction, RF generates various ROS. These ROS are known to damage red blood cell membrane and induce haemolysis[1], damage lens[2], and increase protein cross-linking[3]. The photodegradation of RF involves both photolysis and photoaddition reactions simultaneously, leading to lumichrome and cyclodehydroriboflavin, as major products[4]. The effect of a combined dosing regimen of RF and metalloporphyrins with the aim of increasing the efficiency of phototherapeutic treatment of hyperbilirubinemia is also studied[5]. Occular neovascular disease, an important cause of blindness today, was used as a model to evaluate the vaso-occlusive potential of photodynamic therapy with RF[6].

RF supplementation might also be a useful adjunct to therapy in migraine prevention[7]. Dermal injection of RF and exposure to near UV/visible radiation decrease the dermal pigment of blue nevi, which are recalcitrant to laser therapy[8]. Oxidative modification of cellular constituents including lipids, proteins and nucleic acids was also implicated in the etiology of various pathological conditions and in ageing[9,10]. The presence of RF and UV light selectively enhance damage to guanine bases in DNA *in vitro*[11]. RF induces DNA damage, mainly 7,8 dehydro-8-oxo-2-deoxy guanosine formation[12]. In addition, proteins are the other important cellular constituents damaged by RF. The binding studies suggested that RF interacts non-covalently with BSA[13]. The spectroscopy and photophysics of RF is of considerable interest, due to its biological relevance[14].

Transition metal ions, especially copper and iron frequently have unpaired electrons, and are excellent catalysts, playing a key role in the generation of strong reactive species from the less reactive ones by catalyzing the formation of hydroxyl radical from hydrogen peroxide (H_2O_2) via modified metal-catalyzed Haber-Weiss reaction[15]. Iron also behaves in a similar manner to copper by generating hydroxyl radical, which damages the protein when generated in its vicinity, resulting in the loss of activity[15] . But the damage by Fe(III) is less pronounced. This may be

*Corresponding author
E-mail: imrananaseem@yahoo.com
Tel: (91 9719069125)

Abbreviations: CAT, catalase; MNL, mannitol; RBC, red blood cell; RF, riboflavin; ROS, reactive oxygen species; SOD, superoxide dismutase; TU, thiourea; UV-A, ultra -violet-A

due to the fact that Cu salts react to form hydroxyl radical, with a much greater rate constant than Fe salts while other divalent metal ions do not show any significant activity[16].

The photoexcitation of RF may occur both *in vitro* and *in vivo* in the organs and tissues that are permeable to light such as the eyes and skin and may damage other cell matrix components causing inflammation and aging[17]. RF in the presence of Cu(II) causes breakage of calf-thymus DNA[18] and induces haemolysis of human RBC[1]. The adult human body contains about 80 mg of copper[16], which is absorbed in the intestine and transported by albumin to the liver. After hepatic uptake, it may be incorporated into copper-containing enzymes or ceruloplasmin, which can be transported into the blood[19]. Cells take up and degrade ceruloplasmin, to obtain copper from it[20]. Thus, copper available in the system enhances the generation of ROS.

Trypsin-chymotrypsin mixture has been shown to be effective anti-inflammatory agent and the preparation was found to reduce oxidative damage in burn patients by reducing the formation of free radicals[21]. It was further proved that this mixture reduces stress to the liver by decreasing the levels of marker enzymes in the serum and consequently preventing tissue destruction. Conclusively, the combination behaves as an indirect antioxidant. Thus, it was of interest to examine the activity of trypsin alone in conditions of oxidative stress. In the present study, the effect of phtoilluminated RF alone and that of RF-Cu(II) was investigated using trypsin as the target molecule and the enzyme was found to be susceptible to oxidative damage.

Materials and Methods

Materials

Trypsin, (EC 3.4.21.4) and $CuCl_2$ were from SRL, India, while riboflavin (RF) and neocuproine were from Sigma Chemical Co. USA. All other chemicals used were of the highest purity grade available.

Methods

Trypsin assay

Trypsin was assayed by the method of Kunitz[22] using casein as the substrate. Reaction mixture in a final volume of 3 ml contained 10 mg casein, 10 m*M* sodium phosphate buffer (*p*H 7.4) and 30 µg trypsin. The reaction mixtures were incubated at 37°C for 30 min, and then terminated by the addition of 1 ml of 10% TCA. The samples were then centrifuged for 10 min at 2500 rpm to remove the undigested protein and the supernatant was used for determining the acid soluble peptides by the method of Lowry[23].

Inactivation of trypsin by photoilluminated RF alone and RF-Cu(II) system

Trypsin (30 µg) was incubated with increasing concentrations of RF (25-150 µ*M*) in total volume of 3 ml in 10 m*M* sodium phosphate buffer (*p*H 7.4). The reaction mixtures were irradiated with 800 lux of cool fluorescent light for 30 min at room temperature. Trypsin was then assayed as described.

In subsequent experiments, metals like Cu(II), Fe(III), Co(II), Zn(II), Mn(II), Ni(II) and Hg(II) were included in the reaction. Free radical scavengers such as sodium azide (NaN_3), potassium iodide (KI), thiourea (Tu), mannitol, superoxide dismutase (SOD) and catalase (CAT) were also included in subsequent experiments to determine the nature of ROS generated.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed essentially by the method of Laemmlli[24] using mini slab gel apparatus. Reaction mixtures containing 25 µg of trypsin were exposed with RF alone, RF with Cu(II), RF with neocuproine [specific chelator of Cu(I)] and RF with Cu(II) in the presence of neocuproine in fluorescent light for 2.5 h. Various scavengers were also used in some experiments. Treated samples containing 25 µg of protein were electrophoresed on 15% (w/v) SDS-PAGE.

Silver staining

Protein bands were detected by silver staining of the gel using method of Merill[25]. The gels were soaked in 30 ml of 50% acetone for 10 min and treated with solutions containing 30 ml of distilled water and 50 µl of 10% sodium thiosulphate. Next, they were washed with distilled water and then soaked in a solution containing 0.4 ml of 20% silver nitrate, 0.3 ml of 37% formaldehyde and 30 ml of distilled water for 8-10 min. After washing with distilled water again, gels were transferred to a solution 0.6 g of sodium carbonate, 12.5 µl of 10% sodium thiosulphate and 12.5 µl of 37% formaldehyde to make them alkaline. After 10-20 s, gels were suspended in 1% glacial acetic acid for 1 min to stop the reaction and were finally washed with distilled water.

Absorption spectroscopy

Absorption spectra of reaction mixture containing 75 μ*M* RF alone in a total volume of 3 ml was recorded before and after 2 h of incubation in fluorescent light in the range of 300 to 500 nm using Cintra 10 Spectrophotometer. Spectra of samples containing 75 μ*M* RF and 150 μ*M* $CuCl_2$ were also recorded before and after incubation. Neocuproine 300 μ*M*, was also employed to read spectral changes in the spectra of RF alone and RF-Cu(II). The incubation time for all absorption spectroscopy experiments was 2 h unless otherwise stated. Scavengers were also used in spectral experiments. The scavengers included were NaN_3 (scavenger of singlet oxygen), thiourea (TU) and mannitol (MNL) (scavenger of hydroxyl radical), KI (scavenger of triplet oxygen), SOD (scavenger of superoxide anion) and CAT (scavenger of H_2O_2).

Statistical analysis

Data were analyzed by the Student's t-test. Each experiment was repeated thrice and the values expressed as average of three independent replicates.

Results

Trypsin was incubated with RF and irradiated with visible light for 30 min. A progressive decrease in trypsin activity was observed with increasing concentrations of RF from 20 μ*M* to 150 μ*M*, with a complete loss of activity observed at 150 μ*M* RF (Table 1). With 75 μ*M* RF and increasing concentrations of Cu(II), inactivation of trypsin was achieved at the ratio of 1:2, more specifically at 75 μ*M* RF and 150 μ*M* Cu(II) (Table 1). Irradiation of trypsin with Cu(II) alone had no effect (data not shown). In subsequent experiments, the above mentioned ratio of 75 μ*M* RF and 150 μ*M* Cu(II) was used. All the reactions were incubated in fluorescent light, unless otherwise mentioned.

The decrease in the activity of trypsin caused by RF alone and in the presence of Cu(II) was monitored as a function of time of illumination in fluorescent light (Table 2). Irradiation for 5 min in the presence of RF alone resulted in only 5% loss of trypsin activity, but when Cu(II) was also included 58% loss of activity was observed. The enzyme was almost completely inactivated, in the presence of RF and Cu(II) after 25 min of incubation while with RF alone, it still retained 40% activity.

The effect of other metals like Co(II), Zn(II), Mn(II), Ni(II), Hg(II), and Fe(III) on the activity of trypsin in the presence of 75 μ*M* RF was also investigated. Only Fe(III) was able to catalyze Haber-Weiss reaction, and resulted in 88% inhibition of trypsin activity at the concentration tested (150 μ*M*). Other metals exhibited no significant inhibitory effect on the catalytic activity of trypsin, when compared to irradiation with RF alone which exhibited 50% loss of activity (data not shown). The efficiency of Cu(II) over other transition metal ions is due to the fact that it catalyzes the modified Haber–Weiss reaction, resulting in more deleterious ROS, whereas other metals, except Fe(III) are not good substitutes for this reaction[16].

Inactivation of trypsin was also studied in the presence of different free radical scavengers to determine the major ROS involved in the reaction with RF alone and RF in the presence of Cu(II). In the reaction containing RF alone, SOD was most effective and resulted in maximum prevention of trypsin inactivation (83%), followed by NaN_3 (70%),

Table 1—Inactivation of trypsin by RF and RF + Cu(II) as a function of increasing concentration [Trypsin (10 μg/ml) was incubated with increasing concentration of RF (A) and 75 μ*M* RF + increasing concentration of Cu(II) (B) in fluorescent light for 30 min. Enzyme assay was carried out as detailed in 'Materials and Methods'. Data are average of triplicate samples from 3 different experiments]

(A)		(B)	
RF (μ*M*)	% Remaining activity	Cu (II) (μ*M*)	% Remaining activity
20	80	20	80
50	60	50	45
75	45	75	20
100	40	100	10
125	20	125	5
150	5	150	0

Table 2—Inactivation of trypsin by RF and RF + Cu(II) as a function of time [Trypsin (10 μg/ml) was incubated with (A): 75 μ*M* RF alone; and (B): 75 μ*M* RF+150 μ*M* Cu(II) and irradiated in fluorescent light for different time intervals. Enzyme assay was carried out as detailed in 'Materials and Methods'. Data are average of triplicate samples from 3 different experiments]

Incubation time (min)	% Remaining trypsin activity	
	(A)	(B)
5	95	42
10	84	30
15	80	28
20	70	25
25	60	5
30	40	2

KI (63%) and CAT (60%). Whereas when Cu(II) was added to the above reaction, TU was most effective and resulted in maximum prevention of inactivation (88%), followed by MNL (42%), NaN_3 (38%), KI (29%), CAT (25%) and SOD (20%) (Table 3).

SDS-PAGE of trypsin after photoillumination in presence of RF alone or RF-Cu(II) was performed. Degradation of trypsin by RF is shown in (Fig. 1A). Protein degradation was visible at 25 μ*M* RF (lane b) and became very significant at 200 μ*M* RF, with the main band almost disappearing (lane f). In a parallel experiment, trypsin was photoilluminated with 75 μ*M* RF and increasing Cu(II) concentrations (50-150 μ*M*) for 2.5 h (Fig. 1B). Protein degradation was evident at 75 μ*M* Cu(II) (lane d) and the protein band altogether disappeared at a Cu(II) concentration of 150 μ*M* (lane f). In the experiment performed in the presence of various free radical scavengers, almost complete inhibition of trypsin degradation by RF was observed when SOD, KI, NaN_3 and CAT were included in the reaction (Fig. 2A). Whereas in the case of RF-Cu(II), TU was most effective in inhibiting the degradation of trypsin, followed by KI and NaN_3 (Fig. 2B).

Cu(I) sequestering agent neocuproine was employed to investigate whether Cu(II) is converted to Cu(I) in the photodegradation process initiated by RF. The degradation pattern of trypsin (Fig. 3) by RF alone (lane b) was found to be similar to that observed when neocuproine was also used with RF (lane c). However, degradation patterns of samples treated with RF and Cu(II) alone (lane d) and in combination with neocuproine were remarkedly different (lane e). The presence of neocuproine led to almost complete inhibition of degradation, suggesting the involvement of Cu(I) in RF-Cu(II)-mediated damage of trypsin.

The spectral changes in RF induced by light under different conditions were recorded (Fig. 4, 5). RF exhibited a visible spectrum with a major peak of absorbance at 440 nm and a minor peak at 370 nm. Incubation of RF under fluorescent light for 2 h caused almost complete disappearance of absorption peak at 440 nm. The presence of Cu(II) restored the

Table 3—Inactivation of trypsin by RF and RF + Cu(II) in the presence of free radical scavengers [Trypsin (10 μg/ml) was incubated with (A): 75 μ*M* RF + 50 m*M* radical scavengers; and (B): 75 μ*M* RF + 150 μ*M* Cu(II) + 50 m*M* radical scavengers respectively and irradiated in fluorescent light for 30 min. Enzyme assay was carried out as detailed in 'Materials and Methods'. Data are average of triplicate samples from 3 different experiments]

Scavengers	% Activity of trypsin	
	(A)	(B)
KI	63	29
NaN_3	70	38
TU	35	88
MNL	40	42
SOD	83	20
CAT	60	25

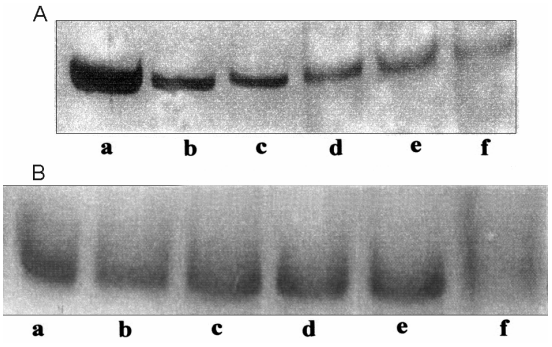

Fig. 1—(**A**): Modification of trypsin as a function of increasing concentration of RF on 15% SDS-PAGE [Trypsin (25 μg/ml) was incubated with increasing concentration of RF and irradiated for 2.5 h in fluorescent light and then analyzed by SDS-PAGE. Lane a, trypsin alone; and lanes b-f, trypsin + 50, 75, 100, 150 and 200 μ*M* RF, respectively]; and (**B**): Modification of trypsin as a function of increasing Cu(II) concentration on 15% SDS-PAGE [Trypsin (25 μg/ml) was incubated with 75 μ*M* RF and increasing Cu(II) concentration for 2.5 h in fluorescent light and then analyzed by SDS-PAGE. Lane a, trypsin alone; lane b, trypsin + RF; and lanes c-f, trypsin + RF and 50, 75, 100 and 150 μ*M* Cu(II), respectively]

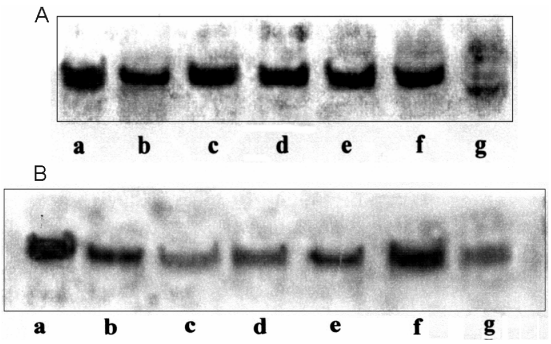

Fig. 2—SDS-PAGE analysis of trypsin photoilluminated with (A): RF; and (B): RF+Cu(II) in the presence of free radical scavengers [Trypsin (25 μg/ml) was incubated with (A): 75 μ*M* RF; and (B): 75 μ*M* RF-150 μ*M* Cu(II) and various free radicals scavengers for 2.5 h in fluorescent light, respectively. The samples were then analyzed by SDS-PAGE. (A): Lane a, trypsin alone; lane b, trypsin+RF; and lanes c-g, trypsin + RF + KI/NaN_3/CAT/SOD/TU; and (B): Lane a, trypsin alone; lane b, trypsin+RF; lane c, trypsin+RF+Cu(II); and lanes d-g, trypsin + RF + Cu(II) + NaN_3/KI/TU/SOD. The concentration of free radical scavengers was 50 m*M* whereas, 20 μg/ml SOD or CAT was used]

peak partially. In the presence of neocuproine, RF degradation was inhibited significantly (Fig. 6), confirming the role of Cu(I) as an essential intermediate in the reaction.

Discussion

RF is widely distributed in human tissue and fluids in free and conjugated form. It has also found widespread application in the food industry both as a nutrient and coloring agent. Our previous report shows that RF upon photoexcitation generates singlet and triplet excited states, which via energy transfer generates singlet and triplet oxygen and RF gets partly demoted to its original state[15]. Superoxide anion is also generated when molecular oxygen and water are present in the medium. It seems that the superoxide anion is not directly derived from RF, as SOD was found ineffective in preventing photodegradation of RF. In the presence of Cu(II), however when H_2O and molecular O_2 are also available in the medium hydroxyl radical and hydroxyl ions are generated via Fenton-like reaction, which presumably gives rise to H_2O_2 and Cu(II) gets reduced to Cu(I) in the process[15]. This property is also exhibited by a number of other endogenous metabolites such as uric acid[26] and L-DOPA[27].

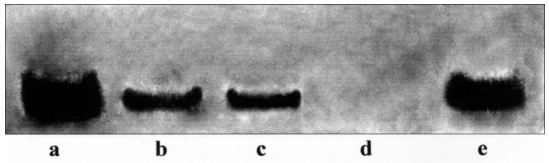

Fig. 3—SDS-PAGE of trypsin photoilluminated with RF, RF+Cu(II) in presence of Cu(I) sequestering agent neocuproine [Trypsin (25 µg/ml) was incubated with 75 µ*M* RF alone and together with 150 µ*M* Cu(II) in the presence of 300 µ*M* neocuproine for 2.5 h in fluorescent light. The samples were then analyzed by SDS-PAGE. Lane a, trypsin alone; lane b, trypsin + RF; lane c, trypsin+RF+neocuproine; lane d, trypsin + RF + Cu(II); and lane e, trypsin + RF + Cu(II) + neocuproine]

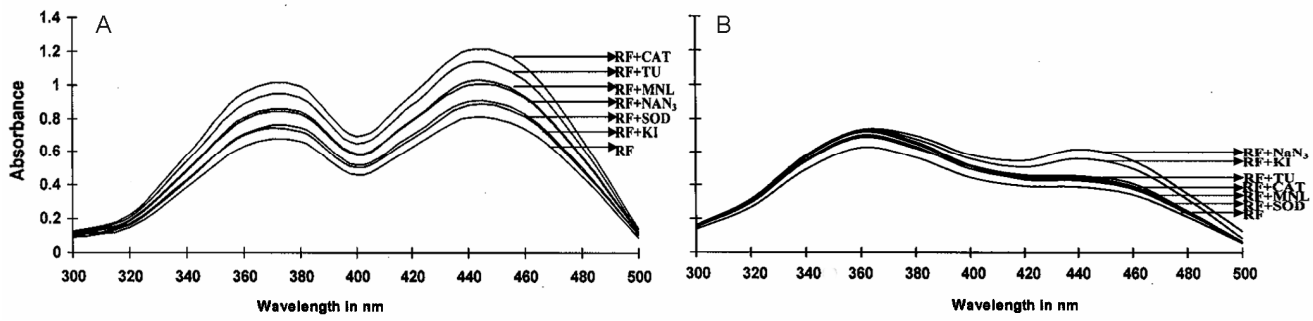

Fig. 4—Absorption spectra of RF under different conditions [RF alone; and RF with radical scavengers (KI, NaN_3, TU, SOD, CAT and MNL) at (A): zero time; and (B): 2 h of incubation under fluorescent light. (A): (1) RF+CAT; (2) RF+TU; (3) RF+MNL; (4) RF+NaN_3; (5) RF+SOD; (6) RF+KI; and (7) RF alone; and (B): (1) RF+NaN_3; (2) RF+KI; (3) RF+TU; (4) RF+CAT; (5) RF+MNL; (6) RF+SOD; and (7) RF alone. The concentration of RF and radical scavengers were 75 µ*M*, 50 m*M*. 20 µg/ml SOD or CAT was used]

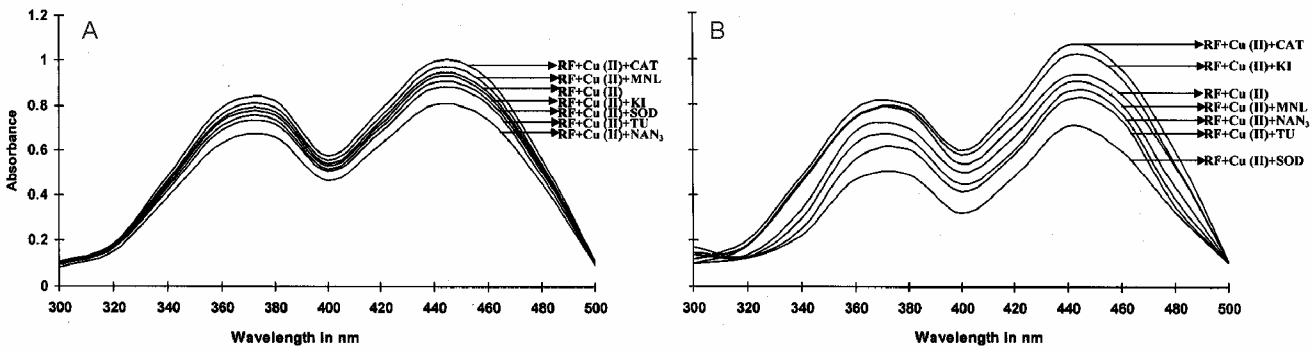

Fig. 5—Absorption spectra of RF+Cu(II) under different conditions [RF + Cu(II); and RF + Cu(II) in the presence of radical scavengers (KI, NaN_3, TU, SOD, CAT, MNL) at (A): zero time; and (B): 2 h of incubation under fluorescent light. (A): (1) RF+Cu(II)+CAT; (2) RF+Cu(II)+MNL; (3) RF+Cu(II); (4) RF+Cu(II)+KI; (5) RF+Cu(II)+SOD; (6) RF+Cu(II)+TU; and (7) RF+Cu(II)+NaN_3. (B): (1) RF+Cu(II)+CAT; (2) RF+Cu(II)+KI; (3) RF+Cu(II); (4) RF+Cu(II)+MNL; (5) RF+Cu(II)+NaN_3; (6) RF+Cu(II)+TU; and (7) RF+Cu(II)+SOD. The concentration of RF, Cu(II) and radical scavengers were 75 µ*M*, 150 µ*M*, 50 m*M* respectively. 20 µg/ml SOD or CAT was used]

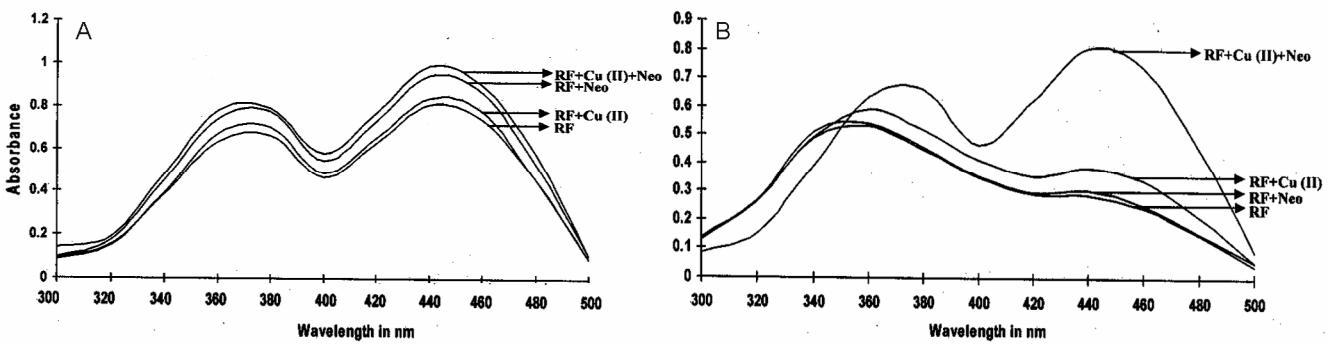

Fig. 6—Absorption spectra of RF alone; and RF+Cu(II) in presence of neocuproine under different conditions [RF alone; RF + Cu(II); RF + neocuproine; and RF + Cu(II) + neocuproine at (A): zero time; and (B): 2 h of incubation under fluorescent light. (A): (1) RF + Cu(II)+neocuproine; (2) RF + neocuproine; (3) RF+Cu(II); and (4) RF alone. (B): (1) RF + Cu(II) + neocuproine; (2) RF + Cu(II); (3) RF + neocuproine; and (4) RF alone. The concentration of RF, Cu(II) and neocuproine were 75 μ*M*, 150 μ*M*, 300 μ*M* respectively]

Singlet and triplet oxygen generated in the reaction are possibly directly derived from RF, hence the presence of KI and NaN_3 resulted in the complete restoration of peak at 440 nm. As these species are not involved in any other reaction, they may be predominantly involved in protein damaging reaction. However, in the presence of Cu(II), hydroxyl radical seems to be predominant in damaging protein, but it may not be directly derived from RF, as TU was effective in inhibiting photodegradation, when Cu(II) was present. But, it is certain that Cu(II) is reduced to Cu(I) in the process, as neocuproine completely inhibited the photodegradation of RF in the presence of Cu(II) as well as degradation of trypsin, while with RF alone, it had no effect on either spectra of RF or on the degradation of trypsin.

In conclusion, the present study suggests that singlet and triplet oxygen are the major protein damaging species that are directly derived from photoilluminated RF via direct energy transfer. However, hydoxyl radical appears to be the predominant damaging species that is generated via H_2O_2, when RF is illuminated in the presence of Cu(II).

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