RESULTS
Riboflavin causes $K^+$ leakage:

The incubation of fresh human RBC with increasing concentration of riboflavin (25-150 μM) in fluorescent light for six hours resulted in progressive loss of $K^+$ (Fig. 3). $K^+$ leakage started at 25 μM riboflavin, and at 50 μM riboflavin about 50% $K^+$ was leaked out in the medium; further increase in riboflavin concentration led only to slight increase in $K^+$ loss, where at 150 μM riboflavin about 65% $K^+$ loss was observed. In the light protected control samples under same conditions, no $K^+$ loss was observed. For further experiments 50 μM riboflavin was used unless otherwise stated.

Riboflavin-Cu(II) causes hemolysis:

The percent $K^+$ loss and hemolysis of human RBC by photoactivated riboflavin and increasing concentrations of Cu(II) are shown in Fig. 4. There was an initial increase in extracellular $K^+$. At 25 μM Cu(II) and 50 μM riboflavin 60% $K^+$ loss was observed compared to 50% caused by riboflavin alone. It stabilized at 50 μM Cu(II) where 75% $K^+$ was observed. The maximum $K^+$ loss was observed in the presence of Cu(II) which is 30% more than when RBC were incubated with riboflavin alone. The presence of Cu(II) also induced significant hemolysis in addition to $K^+$ loss. It was appreciable at 50 μM Cu(II) (20%), then increased with increasing the concentration of Cu(II). The maximum of 45% hemolysis was however, obtained at 100 μM Cu(II). Further increase in Cu(II) did not lead to any significant increase in hemolysis.

$K^+$ loss and hemolysis are time dependent processes:

The percent $K^+$ loss and hemolysis in presence of 50 μM riboflavin and 100 μM Cu(II) were determined as a function of time (Fig. 5). $K^+$ loss started immediately after the incubation. One hour of incubation with riboflavin and Cu(II) resulted in about 18% of the $K^+$ loss in the medium without any significant hemolysis. The hemolysis started after a lag of 1 hour incubation in fluorescent light in the presence of both riboflavin and Cu(II). Increasing the time of incubation to 2 hours resulted in the release of 38% of cellular $K^+$ with 5% hemolysis. Further increase in the time of incubation caused more
Fig. 3. Effect of different riboflavin concentrations on $K^+$ loss. RBC were incubated in 3 ml reaction mixture containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 25-150 μM riboflavin to give 0.5 % hematocrit. The percentage of $K^+$ loss was measured after six hours of incubation in fluorescent light (○) and in dark (■).
Fig. 4. Percent of $K^+$ loss and hemolysis of RBC induced by riboflavin and different concentrations of Cu(II). RBC were incubated with 50 $\mu$M riboflavin and increasing concentrations of Cu(II). Hemolysis (●) and $K^+$ loss (■) were monitored after six hours of incubation in fluorescent light.
Fig. 5. Effect of increasing time of incubation on the K$^+$ loss and hemolysis of RBC induced by riboflavin alone and riboflavin-Cu(II). RBC were incubated with 50 μM riboflavin and 100 μM Cu(II) for different time intervals. (□) K$^+$-loss, (○) K$^+$-loss (without Cu(II)), (■) hemolysis, (●) hemolysis (without Cu(II)).
K⁺ loss and hemolysis. After 6 hours of incubation, 80% of the K⁺ was lost with 45% hemolysis. In the absence of Cu(II), RBC did not exhibit any hemolysis even after prolonged incubation. In the light-protected control sample, loss of K⁺ or hemolysis was not observed in the presence of both riboflavin and Cu (II) (not shown).

The effect of different metals on RBC hemolysis:

As the presence of Cu(II) resulted in significant hemolysis, it was of interest to see the effect of other metal ions on RBC. The effect of different metals on RBC hemolysis in the presence of riboflavin is shown in Fig. 6. Metals like Fe(III), Co(II), Mn(II), Zn(II) and Ni(II) when used with riboflavin instead of Cu(II), did not cause significant hemolysis. Among various metals tested Fe(II) caused 28% hemolysis after 6 hours of incubation in fluorescent light and that is about half of what we observed when Cu(II) was present in the reaction.

The role of Cu (I):

To investigate the role of Cu(I) in hemolysis reaction, bathocuproine the specific Cu(I)-sequestering agent was included in the reaction containing riboflavin and Cu(II) (Fig. 7). Bathocuproine specifically binds to Cu(I) if it is produced in the reaction thereby preventing it to participate in the reaction if present. Ten μM bathocuproine was able to inhibit the hemolysis by 40% considering the 45% hemolysis caused by riboflavin-Cu(II) systems as 100% hemolysis. Complete inhibition of hemolysis was achieved at 30 μM bathocuproine.

Hydroxyl radical is the main reactive oxygen specie involved in RBC hemolysis:

Several free radical scavengers were included in the reaction to identify the major ROS participating in the RBC hemolysis (Fig. 8). Different scavengers like KI, a scavenger of triplet oxygen; SOD, a scavenger of superoxide radical; catalase, scavenger of hydrogen peroxide; β-carotene, scavenger of both nitric oxide and singlet oxygen, and mannitol and thiourea, both scavengers of hydroxyl radical were used. KI showed 60% inhibition,
Fig. 6. Effect of different metals on RBC hemolysis induced by riboflavin. RBC were incubated with 50 μM riboflavin and 100 μM of Fe(III), Mn(II), Ni(II), Co(II) or Zn(II) for 6 hours in fluorescent light.
Fig. 7. Effect of bathocuproine on the hemolysis of RBC induced by riboflavin and Cu(II). RBC were incubated with 50 μM riboflavin, 100 μM Cu(II) and varying concentrations (10-50 μM) of bathocuproine. After 6 hours of incubation in fluorescent light, hemolysis was determined and compared with a control lacking bathocuproine.
Bathocuproine (µM)
Fig. 8. Inhibition of RBC hemolysis by various free radical scavengers induced by riboflavin and Cu(II). RBC were incubated with 50 μM riboflavin and 100 μM Cu(II) and 0.1 mM of either mannitol, thiourea, KI, β-carotene or 20 μg/ml of SOD or catalase. The incubation was for six hours in fluorescent light.
SOD showed 52% inhibition, β-carotene and catalase did not show any significant inhibition. Mannitol and thiourea showed 63% and 93% inhibition respectively. The reaction was incubated for 6 hours in fluorescent light with riboflavin and Cu(II).

**Sodium azide stimulate RBC hemolysis caused by riboflavin-Cu(II) system:**

The incubation of human RBC in fluorescent light for 3 hours with 50 μM riboflavin, 100 μM Cu(II), and increasing concentration of sodium azide resulted in progressive loss of K⁺ and a significant hemolysis (Fig. 9). There was an initial increase in extracellular K⁺ and at 100 μM sodium azide 100% K⁺ was lost out in the medium. At this stage 60% hemolysis was observed. At 150 μM sodium azide, maximum hemolysis (75%) was obtained. Further increase in sodium azide concentration did not result in any significant increase in hemolysis.

The percent hemolysis was also determined in the presence of riboflavin, sodium azide and increasing Cu(II) concentrations. Increasing the Cu(II) concentration resulted in increase the rate of hemolysis which stabilized at 100 μM Cu(II) (Fig. 10). The above reaction when incubated in dark did not show any hemolysis. However, in the absence of Cu(II) and in the presence of both riboflavin and sodium azide, no hemolysis was observed even after a prolonged incubation (data not shown).

The percent K⁺ loss and hemolysis in the presence of 50 μM riboflavin, 100 μM Cu(II) and 150 μM sodium azide were determined as a function of time of incubation under fluorescent light (Fig. 11). After 2 hours of incubation almost all K⁺ was leaked out in the medium with 35% hemolysis. Increasing the time of incubation to 4 hours resulted in 75% hemolysis. In light protected control samples, no K⁺ loss or hemolysis was observed even after prolonged incubation (not shown).

**Involvement of Cu (I) in the reaction:**

In order to investigate whether Cu(I) plays a role in hemolysis caused by riboflavin, Cu(II) and sodium azide. The Cu(I) sequestering agent, bathocuproine was included in
Fig. 9. Effect of increasing concentrations of NaN₃ on K⁺ loss and hemolysis. RBC were incubated in 3 ml buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 50 μM riboflavin, 100 μM Cu(II) and 25 -200 μM NaN₃ to give 0.5 % hematocrit. The percentage of K⁺ loss (●) and hemolysis (■) were determined after 3 hours of incubation in fluorescent light.
Fig. 10. Effect of increasing concentration of Cu(II) on RBC hemolysis induced by riboflavin and NaN₃. RBC were incubated with 50 μM riboflavin, 150 μM NaN₃ and increasing concentration (25-150 μM) of Cu(II). The percent hemolysis was measured after 3 hours of incubation in fluorescent light (●) and in the dark (■).
Fig. 11. Effect of increasing time of incubation on K⁺ loss and the hemolysis induced by riboflavin, Cu(II) and NaN₃. RBC were incubated with 50 μM riboflavin, 100 μM Cu(II) and 150 μM NaN₃ for different time intervals in fluorescent light and K⁺ loss (●) and hemolysis (■) were determined.
the reaction (Fig. 12). The effect of bathocuproine was determined by measuring the inhibition of RBC hemolysis. Complete inhibition of hemolysis was achieved at 60 μM bathocuproine.

**Effect of various metals:**

To find out whether the effect of riboflavin, Cu(II) and sodium azide is restricted to the presence of Cu(II) or other metals can substitute Cu(II), different metals were used in place of Cu(II) (Fig. 13). Among these metals Fe (III) was causing 48% hemolysis in the presence of riboflavin and sodium azide. Other metals like Co(II), Mn(II), Ni(II) and Zn(II) were causing only 20-30% hemolysis.

**Inhibition by free radical scavengers:**

Several free radical scavengers were used to identify the major ROS participating in RBC hemolysis by riboflavin, Cu(II) and sodium azide (Fig. 14). Scavengers like SOD, catalase and β-carotene did not show significant inhibition. However, KI gave 38% inhibition of hemolysis. While thiourea the scavenger for hydroxyl radical was very effective showing 85% inhibition to hemolysis.

**BSA degradation by riboflavin, Cu (II) and sodium azide:**

We have also studied the effect of sodium azide on BSA in the presence of riboflavin and Cu(II) (Fig. 15, a & b). This was done to find out whether the effect of sodium azide is restricted to RBC system or it can function in vitro and on other systems also. Riboflavin alone caused only a slight degradation of BSA (lane 2) while in the presence of Cu(II) caused some enhancement of BSA degradation (lane 3). However, BSA is completely degraded when sodium azide is included in the reaction (lane 4) and this effect is almost completely inhibited by thiourea (lane 5). The degradation of BSA is enhanced in all cases after increasing the time of incubation from 2 hours (Fig. 15, a) to 4 hours (Fig. 15, b). Irradiation of BSA in the presence of sodium azide alone has no effect (data not shown).
Fig. 12. Effect of bathocuproine on the hemolysis of RBC induced by riboflavin, Cu(II) and NaN₃. RBC were incubated with 50 µM riboflavin, 100 µM Cu(II), 150 µM NaN₃ and varying concentrations (10-80 µM) of bathocuproine. After 3 hours of incubation in fluorescent light, percent hemolysis was determined and compared with a control lacking bathocuproine.
Fig. 13. Effect of different metals on RBC hemolysis induced by riboflavin and NaN₃. RBC were incubated with 50 μM riboflavin, 150 μM NaN₃ and 100 μM of either Fe(III), Co(II), Mn(II), Ni(II) or Zn(II) for 3 hours in fluorescent light.
Fig. 14. Effect of various free radical scavengers on RBC hemolysis induced by riboflavin, Cu(II) and NaN₃. RBC were incubated with 50 μM riboflavin, 100 μM Cu(II), 150 μM NaN₃ and 0.1 mM of either thiourea, potassium iodide, β-carotene or 20 μg/ml of SOD or catalase. After incubation for 3 hours in fluorescent light percent hemolysis was determined.
Fig. 15. Enhancing effect of NaN₃ on BSA degradation induced by riboflavin and Cu(II). BSA (2 mg/ml) was incubated under fluorescent light for 3 hours with 50 μM riboflavin, 100 μM Cu(II), 150 μM NaN₃ and 0.1 mM thiourea. After 3 hours of incubation in fluorescent light, 30 μg protein was analyzed by 10% SDS-PAGE. BSA alone (lane 1); BSA and riboflavin (lane 2); BSA, riboflavin and Cu(II) (lane 3); BSA, riboflavin, Cu(II) and NaN₃ (lane 4); BSA, riboflavin, Cu(II), NaN₃ and thiourea (lane 5).
Absorption spectra of riboflavin in the presence of different scavengers:

The spectral changes in riboflavin induced by light under different conditions were recorded (Fig. 16). Riboflavin as mentioned before exhibits a visible spectrum with a major peak of absorbance at 440 nm and a minor peak at 370 nm. Incubation of riboflavin under fluorescent light for 30 minutes caused complete disappearance of absorption peak at 440 nm. The presence of Cu(II) inhibits the photodegradation and restore the peak to some extent. These observations are in agreement with the previous reports (Jazzar and Naseem, 1996). However, the addition of sodium azide to the reaction containing riboflavin and Cu(II) significantly inhibited the photodegradation and restored the 440 nm peak of absorbance to about 85%.

Riboflavin induced lipid peroxidation in human RBC:

The objective of the following study is to investigate the effect of riboflavin on MDA production, which is the major end product in lipid peroxidation. As evident from Fig. 17, the exposure of human RBC to riboflavin in fluorescent light resulted in the formation of MDA which is progressively increased with increasing riboflavin concentration. Significant amount of MDA (3.2 nmol.) was observed with 50 μM riboflavin, after this there was stabilization.

Addition of Cu (II) to photoilluminated riboflavin is known to generate hydroxyl radical via metal-catalyzed Haber-Weiss reaction. In our previous experiments addition of Cu(II) was shown to enhance K⁺ loss and induced hemolysis. Here we are interested to find out whether Cu(II) also has an enhancing effect on the production of MDA by riboflavin. It was clearly seen that the addition of Cu(II) resulted in the enhanced production of MDA (Fig. 18). Increasing the Cu(II) concentration to 100 μM resulted in about 9 nmol. of MDA compared to 4 nmol. MDA formed in absence of Cu(II).

Enhancement of MDA production by sodium azide:

The effect of sodium azide on MDA production was also studied in the presence of
Fig. 16. Absorption spectra of riboflavin under different conditions. Riboflavin alone at zero time (1), riboflavin alone after 30 minutes of incubation (2), riboflavin and Cu(II) after 30 minutes of incubation (3), riboflavin, Cu(II) and NaN3 after 30 minutes of incubation under visible light (4). The concentration of riboflavin, Cu(II) and sodium azide were 50 μM, 100 μM and 150 μM respectively.
Fig. 17. Effect of increasing concentrations of riboflavin on lipid peroxidation. 100 μl of packed RBC (10% hematocrit) were suspended in 1 ml of 10mM Tris-HCl, pH 7.4, 0.15 M NaCl containing 10-90 μM riboflavin. The amount of MDA was determined after 4 hours of incubation in fluorescent light (■) and in dark (●).
Fig. 18. Effect of increasing concentration of Cu(II) on lipid peroxidation induced by riboflavin. RBC were incubated with 50 μM riboflavin and 25-150 μM Cu(II) for 4 hours in fluorescent light.
riboflavin and Cu(II) (Fig. 19). Increasing sodium azide concentration resulted in an increase MDA production. At 150 μM sodium azide about 14 nmol of MDA was formed.

Photoilluminated riboflavin and riboflavin-Cu(II) induced MDA formation was determined as a function of time (Fig. 20). There was a slow increase in MDA formation in both the cases of riboflavin and riboflavin-Cu(II) followed by rapid increase in MDA formation attaining the maximum of 4 nmol and 9 nmol within 3 hour with riboflavin alone and riboflavin-Cu(II) system respectively. Addition of sodium azide to riboflavin-Cu(II), resulted in increased MDA formation to about 12 nmol after 3 hours of incubation.

Effect of various metals on MDA formation in the presence of riboflavin:

Cu(II) was replaced by other metals like Fe(III), Co(II), Mn(II), Ni(II) and Zn(II) to find the ability of these metals to induce formation of MDA (Fig. 21). Fe(II) was most effective giving 7 nmol of MDA formation followed by Mn(II), Ni(II), Co(II) and Zn(II). The reaction was incubated for 3 hours in fluorescent light.

Involvement of free radicals in MDA production:

Several free radical scavengers were used to find the major free radical species responsible for causing lipid peroxidation in both riboflavin and riboflavin-Cu(II) systems (Figs. 22 & 23). In case of MDA formation by riboflavin alone (Fig. 22), catalase was the most effective scavenger showing 60% inhibition followed by SOD with 45% inhibition and KI showing 38% inhibition. Thiourea and β-carotene have no significant effect on MDA formation caused by riboflavin alone.

When Cu(II) is included in the reaction, the effect of scavengers has followed a different pattern (Fig. 23). Thiourea was the most effective scavenger showing 62% inhibition followed by catalase giving 43%, KI giving 32%, SOD giving 24% and β-carotene giving 3%. The incubation time was for 3 hours in fluorescent light.
Fig. 19. Effect of increasing concentration of NaN₃ on lipid peroxidation. RBC were incubated with 50 µM riboflavin, 100 µM Cu(II) and 25-200 µM NaN₃ for 4 hours in fluorescent light.
Fig. 20. Effect of increasing time of incubation on RBC lipid peroxidation induced by riboflavin, Cu(II) and sodium azide. RBC were incubated with riboflavin (●), riboflavin and Cu(II) (■), and riboflavin, Cu(II) and NaN3 (▲) for different time intervals in fluorescent light. The concentrations of riboflavin, Cu(II) and sodium azide were 50 μM, 100 μM and 150 μM respectively.
Fig. 21. Effect of different metals on lipid peroxidation induced by riboflavin. RBC were incubated with 50 μM riboflavin and 100 μM Fe(III), Co(II), Mn(II), Ni(II) or Zn(II) for 4 hours in fluorescent light.
Fig. 22. Inhibition of lipid peroxidation by various free radical scavengers induced by riboflavin. RBC were incubated with 50 μM riboflavin and 0.1mM of either thiourea, KI or β-carotene or 20 μg/ml SOD or catalase. The incubation was for 4 hours in fluorescent light.
Fig. 23. Inhibition of lipid peroxidation by various free radical scavengers induced by riboflavin and Cu(II). RBC were incubated with 50 μM riboflavin, 100 μM Cu(II) and 0.1mM of either thiourea, KI or β-carotene or 20 μg/ml SOD or catalase. The incubation was for 4 hours in fluorescent light.
Involvement of Cu(I) in the reaction:

Bathocuproine the Cu(I) sequestering agent was used to investigate the role of Cu(I) on the MDA production (Fig. 24). Bathocuproine when included in the reaction inhibited the formation of MDA. Increasing the concentration of bathocuproine resulted in decreasing the MDA formation. At 40 μM bathocuproine, 70% inhibition in MDA formation was achieved. Further increase in bathocuproine concentration did not cause further inhibition. The reaction was incubated for 3 hours in fluorescent light.

Effect of riboflavin on human RBC membrane protein:

The human RBC membrane was prepared as described in the “methods” and was used the same day. The membrane was treated with riboflavin and subjected to SDS in 3-15% gradient PAGE. When the membrane was incubated in fluorescent light with 50 μM riboflavin for different time intervals (Fig. 25), there was a remarkable decrease in the intensity of all bands. The decrease in the intensity of different bands was significant after 4 hours of incubation. Even after 2 hours of incubation there was noticeable decrease in the intensity of spectrin followed by slight decrease in the intensity of other bands.

Cu(II) enhanced the degradation of membrane protein:

The effect of time of incubation in fluorescent light with 25 μM riboflavin and 50 μM Cu(II) on RBC membrane polypeptides is shown in Fig. 26. Increase in the time of incubation led to progressive loss of spectrin followed by appearance of high molecular weight protein. The high molecular weight protein retained at the top in the control lane is also disappearing slowly, with increase in the intensity of band 7. At the end of 40 minutes the intensity of band 7 has increased very significantly compared to control lane.
Fig. 24. Effect of bathocuproine on RBC lipid peroxidation. RBC were incubated with 50 μM riboflavin, 100 μM Cu(II) and 10-80 μM bathocuproine for 4 hours in fluorescent light. A control sample in the absence of bathocuproine was taken as 100% lipid peroxidation.
Fig. 25. Effect of increasing time of incubation on the degradation of membrane polypeptides induced by riboflavin. Human RBC membrane was prepared as described in the methods. Membrane was exposed to 50 μM riboflavin in fluorescent light for different time intervals.

Lane a: membrane alone.
Lane b: membrane + riboflavin after 30 minutes
Lane c: membrane + riboflavin after 60 minutes
Lane d: membrane + riboflavin after 120 minutes
Lane e: membrane + riboflavin after 240 minutes
Fig. 26. Effect of increasing time of incubation on the degradation of membrane polypeptides induced by riboflavin and Cu(II). Human RBC membrane was prepared as described in the methods. Membrane was exposed to 25 μM riboflavin 50 μM Cu(II) in fluorescent light for different time intervals.

Lane a: membrane alone.
Lane b: membrane + riboflavin + Cu(II) after 10 minutes
Lane c: membrane + riboflavin + Cu(II) after 20 minutes
Lane d: membrane + riboflavin + Cu(II) after 30 minutes
Lane e: membrane + riboflavin + Cu(II) after 40 minutes
Hydrolysis of calf thymus DNA by riboflavin and riboflavin-Cu(II) system:

Riboflavin upon photoillumination is known to generate sensitive sites in DNA (Naseem et al., 1988). The effect was enhanced in the presence of Cu(II). The reaction was assessed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by pea seed nuclease (Naseem and Hadi, 1987). Control experiment has established that heat denatured DNA undergoes 100% hydrolysis following treatment with pea seed nuclease whereas native DNA resulted in around 5% conversion. We have determined the DNA hydrolysis as a function of riboflavin concentration (Fig. 27). At 100 μM riboflavin, about 55% of DNA were hydrolyzed. Increasing the concentration up to 200 μM riboflavin resulted in about 78% of DNA hydrolysis. The reaction was incubated for 4 hours in fluorescent light.

Addition of 30 μM Cu(II) to 100 μM riboflavin resulted in the enhancement of DNA hydrolysis to 100% (Fig. 28). The percent hydrolysis increased almost linearly upto 4 hours after a lag of 30 minutes for both riboflavin and riboflavin-Cu(II) system (Fig. 29).

The effect of sodium azide on DNA hydrolysis:

It was of interest to find out whether sodium azide a known singlet oxygen scavenger has the same enhancing effect on DNA hydrolysis (Fig. 30), as in case of BSA or K⁺ loss and hemolysis of human RBC. Surprisingly sodium azide did not enhance the DNA hydrolysis by riboflavin and Cu(II), on the contrary it caused slight inhibition.

Aminophylline induces hemolysis:

The incubation of human RBC with 50 μM riboflavin and increasing concentration of aminophylline for 2 hours in fluorescent light resulted in progressive loss of K⁺ and significant hemolysis (Fig. 31). There was an initial increase in the extracellular K⁺, and at 48 μg/ml aminophylline 100% K⁺ leaked out in the medium. At this stage 62% hemolysis was also observed. However, further increase in aminophylline concentration to 80 μg/ml caused about 18% increase in hemolysis. No hemolysis was observed after
Fig. 27. Effect of increasing concentration of riboflavin on DNA hydrolysis. 500 μg of native calf thymus DNA incubated with 25-200 μM riboflavin in a final volume of 1 ml of 0.05 M Tris-HCl, pH 7.4. The reaction was incubated for 2 hours in fluorescent light (○) and in the dark (■). The % DNA hydrolysis was recorded after treatment with pea seeds nuclease. A control sample of denatured DNA after treatment with pea seeds nuclease was taken as 100% hydrolysis.
Fig. 28. Effect of increasing concentration Cu(II) on DNA hydrolysis induced by riboflavin. 500 μg native calf thymus DNA was incubated with 100 μM riboflavin and 5-40 μM Cu(II) for 3 hours in fluorescent light.
Fig. 29. Effect of increasing time of incubation on DNA hydrolysis induced by riboflavin and Cu(II). 500 μg native calf thymus DNA was incubated with 100 μM riboflavin alone (●), and 100 μM riboflavin with 30 μM Cu(II) (■) for different time intervals in fluorescent light.
Fig. 30. Effect of NaN₃ on DNA hydrolysis induced by riboflavin and Cu(II). 500 µg native calf thymus DNA was incubated with 100 µM riboflavin, 30 µM Cu(II) and 25-150 µM NaN₃ for 2 hours in fluorescent light.
Fig. 31. Riboflavin-aminophylline combination induced K⁺ loss and hemolysis of human RBC. RBC were incubated in 3 ml of buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 50 μM riboflavin and 16-112 μg/ml aminophylline to give 0.5% hematocrit. The reaction was incubated for 2 hours.

(■) K⁺ loss in fluorescent light.
(●) Hemolysis in fluorescent light.
(▲) Hemolysis in dark.
prolonged incubation time in the absence of aminophylline or in parallel sample containing riboflavin and aminophylline when incubated in dark, suggested that the reaction is light mediated.

The percent K$^+$ loss and hemolysis were found to be time dependent (Fig. 32). After 1 hour of incubation in fluorescent light, 100% of K$^+$ was leaked out in the medium with 45% hemolysis. Increasingly the time of incubation to 2 hours resulted in 80% hemolysis.

**Involvement of free radicals in the reaction:**

Since photoilluminated riboflavin mediates various damaging reactions via ROS, the possible involvement of ROS was tested using different free radical scavengers. Thiourea and KI gave 70% and 23% inhibition to RBC hemolysis respectively, when present with riboflavin and aminophylline in the reaction (Fig. 33). Other scavengers like SOD, β-carotene and catalase did not show significant inhibition.

**Absorption spectra of riboflavin in the presence of aminophylline and thiourea:**

The spectral changes in riboflavin induced by light under different conditions were recorded (Fig. 34). Riboflavin exhibits a visible spectrum with a major peak of absorbance at 440 nm and a minor peak at 370 nm. Incubation of riboflavin under fluorescent light for 30 minutes caused disappearance of absorption peak at 440 nm suggesting photodegradation of riboflavin. The presence of aminophylline did not have any effect on the disappearance of the peak at 440 nm. Interestingly, the addition of thiourea to the riboflavin-aminophylline mixture inhibited the photodegradation of riboflavin and restored the peak at 440 nm to a significant extent, thiourea has also inhibited RBC hemolysis significantly as shown above (Fig. 33)

**Absorbance spectra of aminophylline in the presence of riboflavin and different scavengers:**

To determine whether aminophylline undergoes any structural change or exhibits
Fig. 32. Effect of increasing time of incubation on $K^+$ loss and hemolysis of RBC induced by riboflavin and aminophylline. RBC were incubated with 50 $\mu$M riboflavin and 80 $\mu$g/ml aminophylline for different time intervals in fluorescent light. (■) $K^+$ loss; (●) hemolysis.
Fig. 33. Inhibition of human RBC hemolysis by various free radical scavengers. RBC were incubated with 50 μM riboflavin and 80 μg/ml aminophylline and 0.1 mM of either potassium iodide, thiourea or β carotene or 20 μg/ml of SOD or catalase. The incubation was for 2 hours in fluorescent light.
Fig. 34. Absorption spectra of riboflavin in the presence of aminophylline under different conditions. 50 μM riboflavin alone at zero time (1), riboflavin and 80 μg/ml aminophylline at zero time (2), riboflavin alone after 1 hour of incubation under fluorescent light (3), riboflavin with aminophylline after 1 hour of incubation (4), riboflavin with aminophylline and 0.1 mM thiourea after 1 hour of incubation (5), riboflavin and 0.1 mM thiourea after 1 hour of incubation (6).
binding to riboflavin during irradiation, the absorption spectrum of aminophylline was recorded between 240-300 nm (Fig. 35). Aminophylline exhibit a UV spectrum with a peak at 270 nm. Irradiation of aminophylline in fluorescent light for more than 2 hours did not cause any change in the absorption peak at 270 nm. Addition of 50 μM riboflavin to the reaction caused a decrease in the peak within 10 minutes of irradiation. Moreover, the absorption peak of aminophylline completely vanished in the presence of riboflavin after 30 minutes of incubation in fluorescent light. However, the addition of sodium azide or KI to the medium inhibited this photodegradation and almost restored the peak at 270 nm to 90%, while thiourea did not cause any inhibition to aminophylline degradation.

BSA degradation by riboflavin-aminophylline system:

We have studied the effect of riboflavin-aminophylline combination using BSA as a target molecule (Fig. 36). This was studied to find out whether the effect of this combination is restricted to RBC damage or it functions in vitro and on other systems also. A slight change in BSA took place when it was incubated with riboflavin alone, while the presence of aminophylline with riboflavin, caused almost complete degradation of BSA into small peptides. The effect is shown by the smearing of the major band. The presence of thiourea in the reaction significantly inhibited BSA degradation. The irradiation of BSA in the presence of aminophylline alone had no effect (data not shown).

Scanning electron microscopy of RBC treated with riboflavin, Cu(II), sodium azide and aminophylline:

When RBC were treated with riboflavin alone and in the presence of various agents, they undergo certain changes in their morphology. Incubation of RBC with riboflavin alone resulted in the formation of spherical form of RBC called spherocytes (Fig. 39). RBC were incubated with 50 μM riboflavin for 2 hours in fluorescent light. No clear holes or damage was observed in this case.
Fig. 35. The absorption spectra of aminophylline illuminated with riboflavin under different conditions. The spectra of 80 µg/ml aminophylline was recorded between 240-300 nm after irradiation with fluorescent light in the presence of 50 µM riboflavin. Aminophylline alone at zero time (1), aminophylline and riboflavin at zero time (2), aminophylline and riboflavin after 10 minutes of incubation (3), aminophylline with riboflavin after 30 minutes of incubation (4), aminophylline and riboflavin with NaN₃ (5) or KI (6) after 30 minutes of incubation, aminophylline, riboflavin and thiourea after 30 minutes of incubation (7).
Fig. 36. Degradation of BSA induced by riboflavin and aminophylline. 2 mg/ml BSA was incubated with 50 μM riboflavin and 80 μg/ml aminophylline in fluorescent light for 2 hours.

Lane a: BSA alone.
Lane b: BSA + riboflavin.
Lane c: BSA + riboflavin + aminophylline.
Lane d: BSA + riboflavin + aminophylline + 0.1mM thiourea.
Fig. 37. A scheme for the photodegradation of riboflavin-aminophylline and RBC damage in fluorescent light. ISC = Intersystem conversion; $^1$S = singlet state; $^3$T = triplet state; $^* = n \rightarrow \pi^*$; $R$ = CHO-H-CHO-H-CHO-H-CHO-CH$_2$OH
Riboflavin → Light → 1 → ISC → Part conversion → Return to ground state

Damage to aminophyline → \(!^{1}O_{2} + ^{1}O_{2}\)

Damage to RBC membrane → Hemolysis

Hydroxylated riboflavin → •OH + unknown oxidized product

Inhibition by NaN₃ & KI

Inhibition by thio urea
Fig. 38. Scan electron microscopy of normal RBC.
Fig. 39: Scan electron microscopy of riboflavin-treated RBC. RBC (0.5% hematocrit) were incubated with 50 μM riboflavin for 2 hours in fluorescent light. The sample was then prepared as described in the methods before using the SEM.
When RBC were incubated with 50 μM riboflavin and 100 μM Cu(II), the RBC undergo more advanced changes where irregular spikes appeared at the surface of RBC (Fig. 40). These structures are called acanthocytes.

The changes recorded when RBC were incubated with 50μM riboflavin, 100 μM Cu(II) and 150 μM sodium azide are shown in Fig. 41. The membrane is showing drastic changes and is completely damaged.

Addition of aminophylline to riboflavin has been shown to enhance the damaging effect of riboflavin. The structural changes that are seen in RBC when incubated with 50 μM riboflavin and 80μg/ml aminophylline are shown in Fig. 42. RBC surface is modified with equally spaced projections over the entire surface. This form of RBC is called “echinocytes”.
Fig. 40: Scan electron microscopy of RBC treated with riboflavin-Cu(II) system. RBC (0.5% hematocrit) were incubated with 50 μM riboflavin and 100 μM Cu(II) for 2 hours in fluorescent light. The sample was then prepared as described in the methods before using the SEM.
Fig. 41: Scan electron microscopy of RBC treated with riboflavin, Cu(II) and NaN₃. RBC (0.5% hematocrit) were incubated with 50 µM riboflavin, 100 µM Cu(II) and 150 µM NaN₃ for 2 hours in fluorescent light. The sample was then prepared as described in the methods before using the SEM.
Fig. 42: Scan electron microscopy of RBC treated by riboflavin-aminophylline system. RBC (0.5% hematocrit) were incubated with 50 \( \mu \text{M} \) riboflavin and 80 \( \mu \text{g/ml} \) aminophylline for 2 hours in fluorescent light. The sample was then prepared as described in the methods before using the SEM.