EFFECT OF SCAVENGERS ON RADIATION-SUSCEPTIBLE SITES IN FIBRINOGEN

by H. N. KAMAT *and* SHANTOO GURNANI, *Biochemistry and Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay*-400085

The characteristics of emission and absorption spectra of the irradiated fibrinogen suggest that the aromatic residues of fibrinogen are susceptible to radiation effects. Quantum-yield of the fluorescence decreases progressively with increasing irradiation doses. Although, small decrease in the absorption-maximum is noticeable there is proportionate increase in absorption in 240-270 nm region as a function of dose of γ-irradiation thus, indicating that aromatic residues probably are not degraded but are modified as a result of radiation. Potassium iodide, the $OH^{\cdot}$ radical scavenger prevents this modification and provides protection to the biological activity to the extent of 80–90 per cent. Potassium nitrate, the e^{-} scavenger, protects neither modification nor biological activity. Data clearly indicate that $OH^{\cdot}$ radicals produced as a result of radiolysis of water, attack aromatic side-chains in the fibrinogen molecule.

INTRODUCTION

Haemorrhage occurs in all the animals exposed to ionizing radiations. The bleeding disorders could arise due to the damage caused to the coagulation system of the circulating blood. The failure of the clotting process may be the result of the structural changes in the circulating protein molecules which participate in coagulation. It is therefore important to understand how—radiation alters the chemical properties of the blood proteins. One of the most important protein constituents of clotting, fibrinogen, has been subjected to γ-radiations *in vitro* for these studies.

It has been recently reported from our laboratory that biological and physico-chemical properties of the purified fibrinogen are altered when it is exposed to radiation either in solid or in solution form (Kamat and Nadkarni 1972). In this paper we discuss susceptible sites in fibrinogen and how these are modified by radiation.

MATERIALS AND METHODS

Bovine fibrinogen (Fraction I) and thrombin were obtained from Armour Laboratories Inc. and Mann Research Laboratories Inc., USA, respectively. The fraction I was further purified to 98 per cent clottable protein by the method of Laki (1957).

Fibrinogen, 1 mg/ml in 0.3 M KCl (*p*H 7.4) was exposed to γ-radiation under cold conditions (0–4°C) as detailed earlier (Kamat and Nadkarni 1972). Measurement of clotting-time and clottability has also been described previously (Kamat and Nadkarni 1972).

UV spectra were obtained on double beam spectrophotometer, DB Perkin Elmer, between 230–340 nm. Fluorescence spectra were obtained using Aminco-Bowman

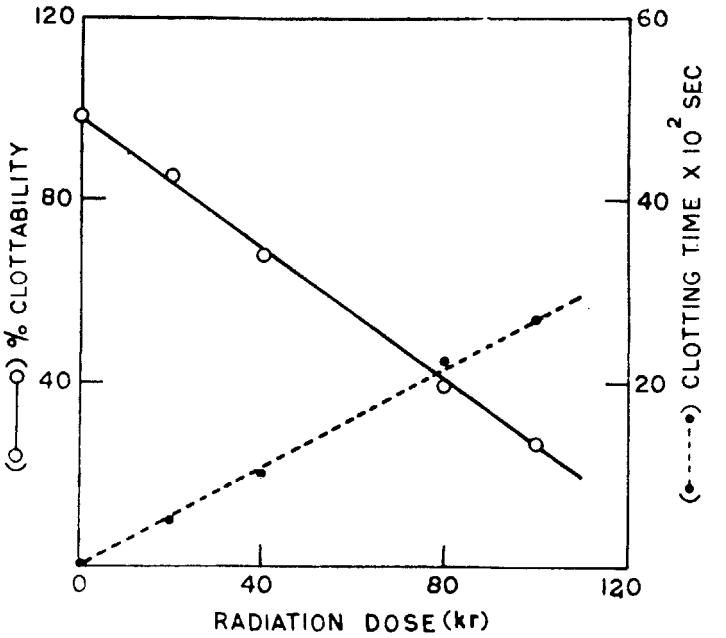

FIG. 1. Effects of radiation on the biological activity of fibrinogen as a function of radiation dose.

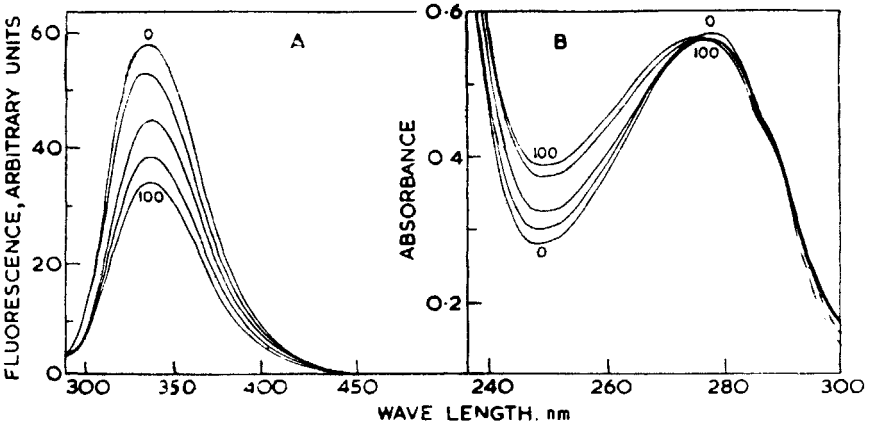

FIG. 2. A, fluorescence spectra of irradiated fibrinogen, 100 μg/ml; B, uv spectra of irradiated fibrinogen, concentration, 500 μg/ml. Numbers shown on spectra indicate irradiation dose in kr. Concentration shown refers to that used for spectral studies.

spectrofluorometer. Irradiated fibrinogen solutions were diluted with 0.3 M KCl solution (*p*H 7.4) appropriately for taking either type of the spectra. High concentration of salt was used to prevent any aggregation of fibrinogen.

Tryptophan was determined by the method of Spies *et al.* (1948). Protein was estimated by Lowry's Folin-Phenol reagent (Lowry *et al.* 1951).

Results and Discussion

The effect of radiation on the biological activity of fibrinogen is shown in the Fig. 1. The clotting time of the unirradiated fibrinogen was very short, 7–10 sec and since all the fibrinogen molecules participate in the process it's clottability was 100 per cent. The clotting-time of the irradiated fibrinogen, however, was prolonged several folds and it's clottability was reduced progressively as exposure-dose was increased. At 100 kr clotting-time was increased to 2500 sec and clottability came down to 28 per cent.

Since our earlier studies indicated damage to the aromatic amino acids we investigated these in further detail, in particular, we focussed attention on tryptophan which is known to be relatively more sensitive to ionizing and non-ionizing radiation effects (Korgaonkar and Donde 1962; Gurnani *et. al.* 1966; Gurnani 1968; Lynn and Orpen 1969; Lynn 1970, 1971; Winchester and Lynn 1970; Grossweiner and Usui 1971). The clear indication of damage to the aromatic residues was obtained from uv and fluorescence spectra of the irradiated fibrinogen shown in Fig. 2.

There was no change in the overall shape of the absorption spectra. Very slight decrease in the maximum region was noticed but there was distinct increase in the minimum region, 245–252 nm, as well as a shift of 2–3 nm towards red (Fig. 2 B). However, a progressive decrease in the emission maximum, 335 nm, was seen with the increase in the radiation dose (Fig. 2 A). Increase in the minimum region in the absorption spectra and decrease in the fluorescence maximum were proportionate to radiation dose. Since spectral characteristics of the proteins are due to their content of aromatic residues, it is reasonable to conclude from spectral data that as the radiation dose is raised, larger number of aromatic residues are either degraded or modified. In order to confirm this observation, one of the aromatic amino acids, tryptophan, was exposed to radiation under the same conditions as fibrinogen. Results are shown in Fig. 3. The absorption and the emission spectra of the free tryptophan also changed as a result of irradiation. The changes were almost identical to those observed in the irradiated fibrinogen.

From this data it was evident that the amino acid, tryptophan, was definitely affected by the increasing radiation dose which was further supported when residual tryptophan was measured by calorimetric method. It can be seen in Fig. 4 that per cent loss of tryptophan increased with increase in the radiation dose. It may be noted that the tryptophan in fibrinogen appeared to be relatively more sensitive to radiation-damage than the free tryptophan under the same conditions. This could be due to the relative concentration of the tryptophan in the fibrinogen and in the free form. The latter concentration was four times as much as the former during irradiation. We, therefore, concluded that one of the radiation susceptible sites in fibrinogen was tryptophan. Although, the evidence for the modification of the

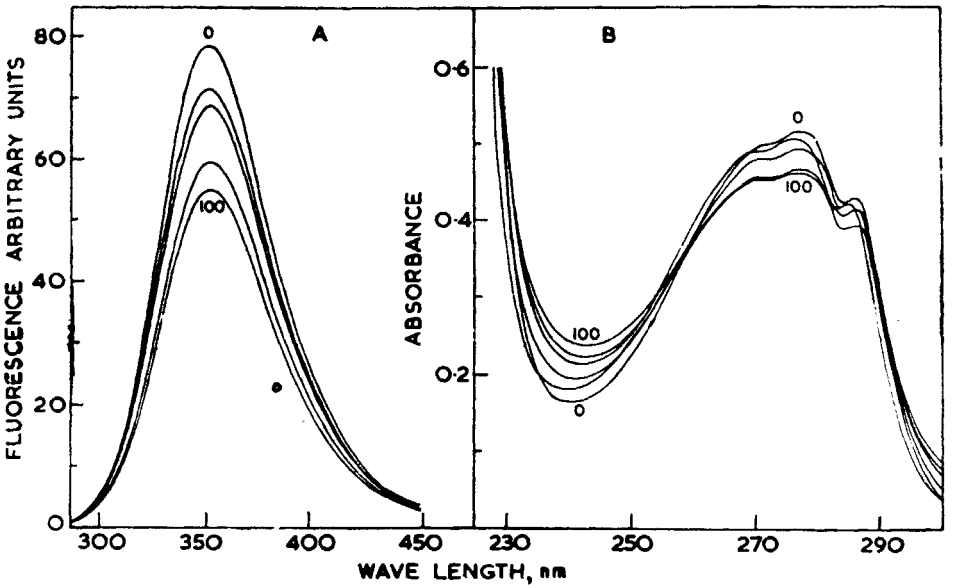

FIG. 3. A, fluorescence spectra of free tryptophan, 4 μg/ml; B, uv spectra of free tryptophan 20 μg/ml. Numbers shown on spectra indicate irradiation dose in kr. Concentration shown refers to that used for spectral studies.

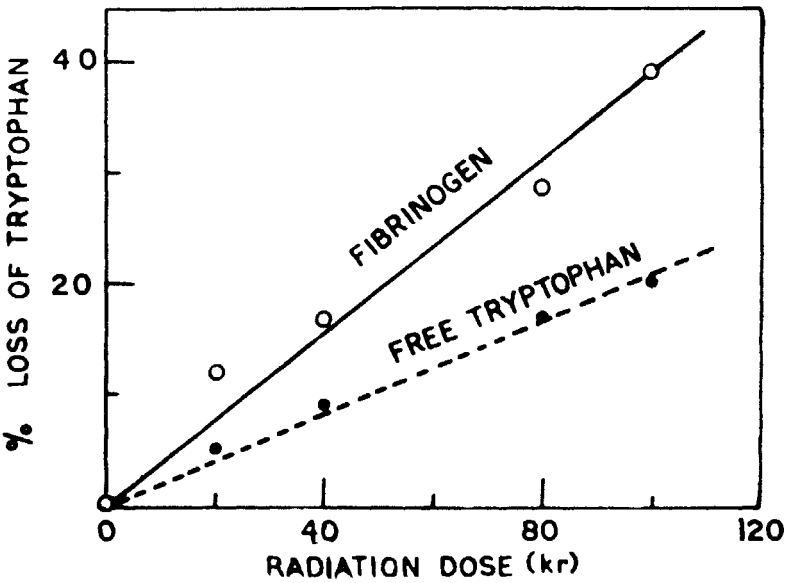

FIG. 4. Residual tryptophan measured by calorimetric method after the irradiation of solutions of fibrinogen and free tryptophan. Fibrinogen 1 mμ/ml. free tryptophan, 1×10^{-3} M was exposed to radiation as mentiones in the experimental section.

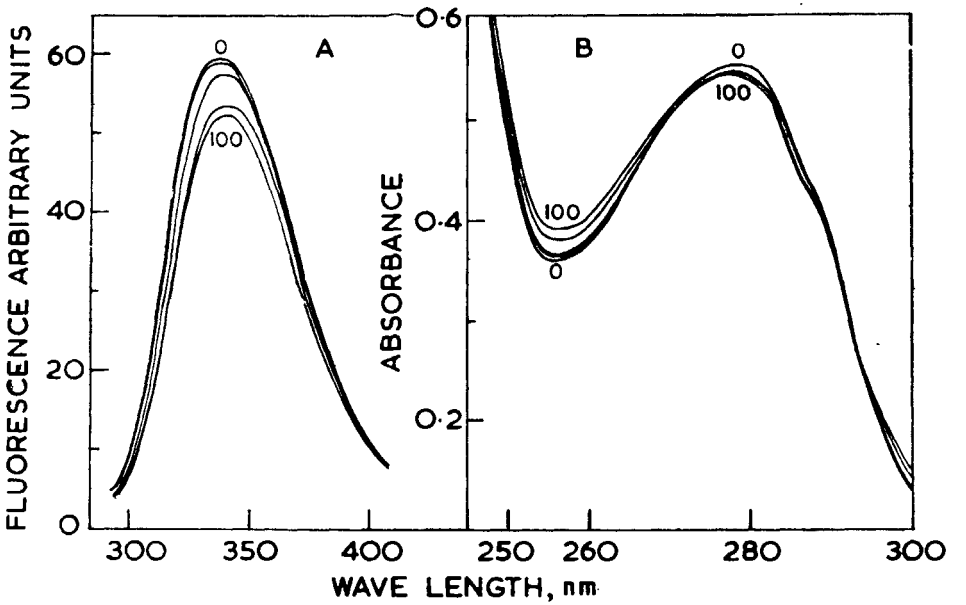

FIG. 5. A, fluorescence spectra of fibrinogen, irradiated in presence of 1×10^{-3} M KI (For spectra, Fibrinogen, 100 μg/ml); B, uv spectra of fibrinogen irradiated in presence of 1×10^{-3} M KI (For spectra fibrinogen, 500 μg/ml).

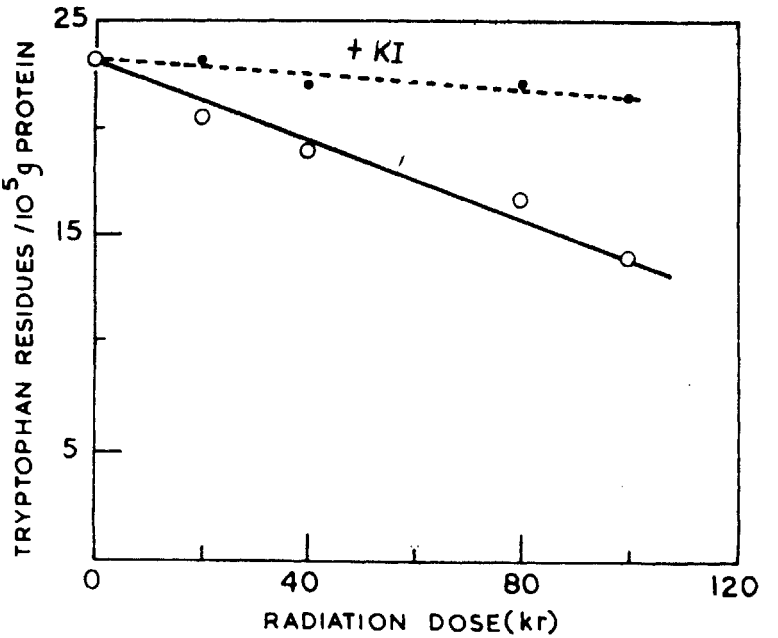

FIG. 6. Protection of tryptophan observed when irradiation was carried out in presence of KI 1×10^{-3} M.

tryptophan residues is presented, that does not exclude the possibility of the other aromatic residues being also destroyed due to radiation along with tryptophan.

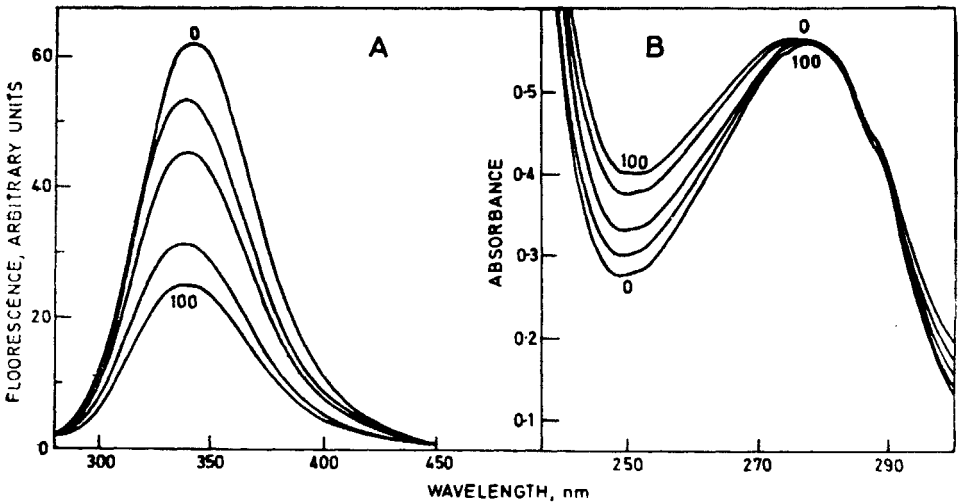

FIG. 7. A, Fluorescence spectrum of fibrinogen irradiated in presence of 1×10^{-3} M $NaNO_3$. For spectra Fibrinogen, 100 μg/ml, B, uv spectra of fibrinogen irradiated in presence of 1×10^{-3} M $NaNO_3$ (For spectra Fibrinogen 500 μg/ml).

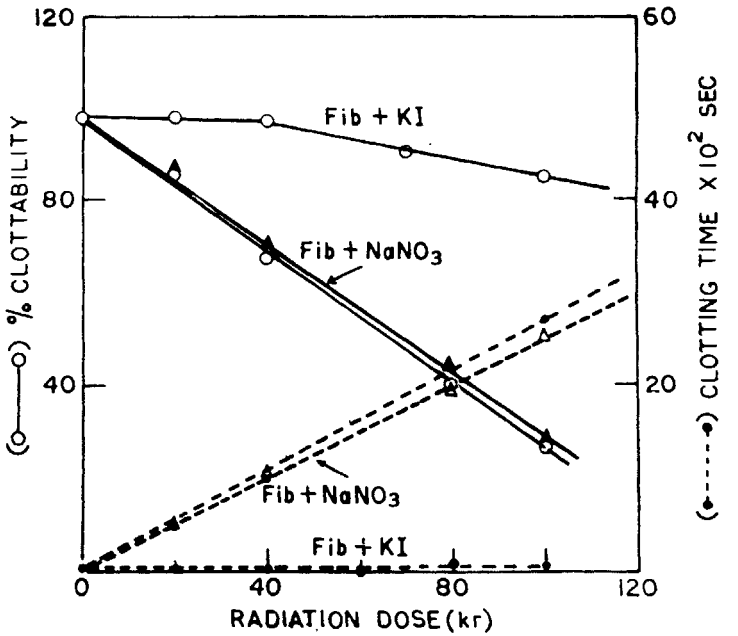

FIG. 8. Protection of biological activity of fibrinogen observed when irradiation was carried out in presence of either KI (1×10^{-3} M) or $NaNO_3$ (1×10^{-3} M).

We then looked at the other aspect of this problem. Since radiolysis of water leads to the production of hydroxyl radicals ($OH^{\cdot}$) and electrons (e^{-}) it was interesting to examine whether the tryptophan residues in fibrinogen were modified and/or

degraded by either of the radiolytic products of water. It is now established that radiation does not follow a single uniform mechanism in damaging the biological systems. In fact radiation damage is affected by a variety of factors which determine the ultimate mode of its action. Biological macromolecules could be sensitive to the action of either of both radiolytic species of water. In order to distinguish between either mechanism, fibrinogen was exposed to radiation in the presence of KI, the $OH^{\cdot}$ radical quencher, and $NaNO_3$—the electron sweeper. It can be seen in Fig. 5 that KI, (1×10^{-3}M) protects the tryptophan residues in fibrinogen during irradiation, to the extent of 85–90 per cent. Similar protective effect of KI on free tryptophan was observed. This is also evident from the calorimetric data on the tryptophan (Fig. 6). $NaNO_3$ (1×10^{-3}). (Fig 7) which sweeps away electrons on the other hand had no effect either on spectral characteristics or on tryptophan content of fibrinogen estimated by other methods.

It is interesting to note that protection of tryptophan leads to protection of biological activity as depicted in Fig. 8. Our experiments provide evidence that at least some tryptophan residues participate in the clotting process. The evidence clearly suggests that $OH^{\cdot}$ radicals produced during irradiation of the aqueous solution of fibrinogen modify the tryptophan side chain in the fibrinogen molecule.

Acknowledgements

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