INTRODUCTION
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At present, it is well established that the passage of ionizing particles through the cells causes certain rapid initial physical and physicochemical events, followed by relatively more stable chemical changes which in turn mediate the final biological effects (Korogodin 1966, Timofeeff-Ressovsky et al. 1968, Ahnström and Ehrenberg 1980, Singh and Singh 1982). In view of the extensive use of ionizing radiation, it is of interest to investigate the role of specific factors which can possibly modulate the initial physicochemical and biochemical reactions. Such studies have already resulted in the development of techniques, which can be employed for either enhancing or diminishing radiation-induced biological damage. In this connection, it would also be of interest to carry out studies on the mechanism of biological action of ionizing radiation.

Oxygen plays a vital role in the normal metabolism of both prokaryotic and eukaryotic cells (with the exception of obligate anaerobes). In radiobiology, "Oxygen effect" refers to the increase in the sensitivity of cells/organisms to the ionizing radiation as a result of increase in the concentration of oxygen from zero to a fixed value (Koch 1982). Oxygen is well known to enhance the sensitivity
of biological systems to ionizing radiation (Gray et al. 1953, Gray 1954, Howard-Flanders and Alper 1957, Suit et al. 1960, Thomlinson 1960, Elkind 1967). Furthermore, the clinical relevance of oxygen effect was also pointed out and brought to the attention of radiotherapists by Gray and colleagues (reviewed by Alper 1979).

The techniques available in the early 1960s were not adequate to study the oxygen-dependent reactions which occur within the time-span of microseconds. At that time, it had not been known whether the initiation of the sensitizing reactions involving oxygen in the irradiated cells was physicochemical or biochemical. It was at this juncture that Powers et al. (1960) introduced dry spores of Bacillus megaterium for radiobiological investigations. They successfully characterized three classes of damage, of which two were shown as oxygen-dependent. The credit also goes to Caldecott et al. (1957) for demonstrating that addition of oxygenated water to irradiated barley seeds resulted in the enhancement of damage (now termed as post-irradiation oxic damage) and to Conger (1961) for demonstrating that the radiation-induced, long-lived free radicals in the seeds provided a basis for the "slowly developing oxygen effect" in dry seeds. The involvement of the radiation-induced free radicals was further confirmed by studies with heat-shock which thermally anneals these and thereby

During the 1960s, a great deal of work had been initiated on chemical radioprotection and radiosensitization. However, these studies were not designed to address the effects of chemicals separately on the oxic and anoxic components of radiation damage to the cells and organisms. Further, a lot of data derived from the UV-studies were unfortunately extrapolated to the situations in which X-or gamma-photons constituted the radiant energy. Because of this, it was expected that caffeine, known as an inhibitor of photoreactivation (Sauerbier 1964, Cleaver 1968) and excision repair (Harm 1967, Lehman et al. 1975) would also be a sensitizer of X-or gamma-ray induced damage. However, Kesavan et al. (1973) corrected this fallacy by demonstrating that caffeine indeed exerts a radioprotective effect against the oxygen-dependent (oxic) pathways of damage, but potentiates an oxygen-independent (anoxic) pathway of damage. The reason for their gaining a new important insight was that they employed Caldecott's system (1957) with Powers et al. approach (1960).
Further work during the subsequent years established that oxygen effect is indeed physicochemical. The chemical modification of radiation-induced damage could also be, therefore, expected to be largely physicochemical. These aspects could be elucidated in the metabolically inert or slowly metabolizing systems. The relevance of these to metabolically active mammals is, however, no more doubtful (Powers 1982, Kesavan 1983).

From several publications during the period 1973-1985, it was fairly established that there is no better radioprotector than nitrogen and no greater radiosensitizer than oxygen. This paradigm prompted Kesavan and co-workers to probe into the possible molecular mechanism(s) which occur in the presence or absence of oxygen in the irradiated cells. Radiochemical events involving oxygen produce oxygen reduction products such as hydrogen peroxide and several organic peroxides. Oxygen is also involved in lipid peroxidation. In this regard, an interesting observation by Balachandran and Kesavan (1978) is that peroxidase activity registers an increase almost in parallel with seedling injury. Further, the role of peroxides in mediating radio-protection or radiosensitization has been variously reported. For example, Näslund et al. (1976b) have implicated the radiolytically produced hydrogen peroxide in the radio-
protection by aminothiols. On the other hand, radiolytically generated peroxides have been held responsible for cellular radiosensitization (Sobels 1963, Swartz 1973). Between these two extremities of views, Sah and Kesavan (Unpub.) have discussed the dual role of hydrogen peroxide in determining the cellular radiosensitivity. Kesavan and Powers (1985) observed dramatic increase in the concentration of hydrogen peroxide in the spores of Bacillus megaterium irradiated with caffeine in nitrogen and this increase was associated with the caffeine potentiation of the anoxic component of radiation damage induced by 50 kVp X-rays.

It should, however, be pointed out that in N₂O-saturated aqueous bacterial system, H₂O₂ alone is not capable of enhancing the radiation sensitivity without the co-operative action of hydroxyl radicals (OH·) (Powers 1972, Watanabe et al. 1981, 1982). The significant role assigned to H₂O₂ in mediating radiation damage would be evident from the double oxidation scheme proposed by Powers et al. (1972) and Cross et al. (1973).

This scheme is as follows:

\[
\begin{align*}
\text{RH}_2 + \text{OH}^\cdot & \rightarrow \text{RH}^\cdot + \text{HOH} \\
\text{RH}^\cdot + \text{H}_2\text{O}_2 & \rightarrow \text{R} + \text{OH}^- + \text{H}^+ + \text{OH}^\cdot
\end{align*}
\]

(1) (2)

In this, RH₂ is the target molecule (DNA) and R is the irreversibly altered configuration.
While this scheme assigns a destructive role for H$_2$O$_2$, the fact that it also exhibits a higher reactivity with electrons (Anbar et al. 1973, Behar et al. 1975) possibly suggests that it could compete with O$_2$ and reduce the formation of oxygen reduction products, such as hydroperoxy radicals (HO$_2^\cdot$) and superoxide anion radicals (O$_2^\cdot$). The production of these radicals and re-formation of H$_2$O$_2$ constitute a cycle in which many induced enzymes take part. With the commencement of active physiology and biochemistry during germination of the irradiated (in vacuo) dry seeds, many enzymes particularly catalase, superoxide dismutase (SOD) and peroxidase begin to exert their influence. These protective enzymes (superoxide dismutase, catalase and peroxidases) usually minimize damage, caused by superoxide anion radicals (O$_2^\cdot$), hydroxyl radicals (OH$^\cdot$) and hydrogen peroxides (H$_2$O$_2$), unless the rate of univalent oxygen reduction exceeds enzyme capacity (Vuillaume 1987). Thus it could be seen that hydrogen peroxide together with other organic peroxides (i.e. total peroxides) occupy a pivotal role between the physicochemical and biochemical reactions. The physicochemical reactions involving radiation-induced free radicals and oxygen to form peroxides are several and these have been described previously (Ewing 1983, Vuillaume 1987, Singh and Kesavan 1990b). When aqueous suspensions of spores and seeds or
actively metabolizing cells and organisms are irradiated under anoxic conditions, the formation of hydrogen peroxide is extremely small and is almost entirely due to the following reaction:

\[ \text{OH}^- + \text{OH}^- \rightarrow \text{H}_2\text{O}_2 \]  
\[ (k = 5.5 \times 10^9 \text{M}^{-1} \text{s}^{-1}) \text{ (Dorfman and Adams 1973)}. \]

In dry seeds (irradiated in vacuo and post-hydrated at 4°C for 8 hrs in oxic or anoxic water), the formation of OH\(^-\) can not be expected to be appreciable. The direct action of \(^{60}\text{Co}\)-gamma-rays on seeds would produce the following radicals:

\[ \text{RH}_2 \rightarrow \text{RH}^- + \text{H}^- + e^- \text{(trapped)} \]  
\[ \text{(target molecule)} \]  
\[ \text{(Kesavan et al. 1990)} \]

The diffusion of oxygen during post-hydration could result in RHOO\(^-\), ROO\(^-\) and O\(^2-\). Now the question arises regarding the influence of these oxygen reduction entities on the overall radiosensitivity and on the peroxidase enzyme. The formation of these oxygen reduction products is expected to be quite negligible when post-hydration media is oxygen-free, and data on peroxidase activity would be of considerable interest.

The initial seed moisture content is an important factor in seed radiobiology, which has considerable influence
on post-irradiation oxygen-dependent damage. It has been shown that post-irradiation oxygen-dependent damage decreases with increasing seed moisture content. However, seed moisture does not have appreciable effect on the post-irradiation oxygen-independent damage (Conger et al. 1966, 1968, Afzal and Kesavan 1979a).

The term "DNA repair" which is used in the title and content of this thesis encompasses all events involved in the restoration of the DNA from potential lesions induced by ionizing gamma-rays. If the intact DNA is denoted by RH$_2$ in the seeds, its form of potential but possibly reversible configuration is RH$^-$; and its irreversible damaged configuration is R or RHOO$^-$. Restoration of RH$_2$ occurs when RH$^-$ has been donated an electron (Adams 1967, Lohmann 1974). Many chemical radioprotectors are known to effect electron transfer for such restoration. In many other situations, the term DNA repair here could really mean its "protection" by the scavenging of harmful species (e.g. hydroxyl radicals, hydroperoxy radicals, superoxide anion, hydrogen peroxide etc.) by the chemical radioprotectors. The use of this term here is more comprehensive than that favoured by biochemists and is intended to emphasize that even the enzymatic repair of DNA is basically determined by the mode and magnitude
of the radiation-induced free radical reactions and recombi-
nations. This is evident from the fact that chemical radio-
protection against the oxic damage or the radiosensitization of anoxic damage is reflected in terms of chromosomal abe-
rrations and M$_2$ chlorophyll mutation (Kesavan and Ahmad 1974a, Jha and Kesavan 1986). More emphatically, Sah and Kesavan (1986) have shown that caffeine post-treatment which reduces the M$_1$ chromosomal aberrations in the barley shoot tip cells restores the DNA synthesis during the initial periods (12 hrs and 24 hrs) of seed germination; further the caffeine potentiation of anoxic damage is associated with reduced DNA synthesis. In the bone marrow cells of mice it has been shown (Paul and Kesavan, unpublished) that there is no discernible difference in the repair synthe-
sis following treatments with chemical radioprotectors and radiosensitizers. Hence, there is little substance in including the data on the DNA synthesis under different post-treatments. There is no doubt at all that the physico-
chemical reactions described in this thesis profoundly affect the damage or repair (restoration) of the target molecule, DNA (RH$_2$).

Using the known data as reference points, one can now examine the pathways by which certain chemicals exert radioprotection against oxic damage or potentiate anoxic damage.
The role of caffeine in radiation biology has been studied extensively in different biological systems. There are many conflicting reports on the radiosensitization or not by caffeine in wide ranging test systems (Yamamoto and Yamaguchi 1969, Wolff and Scott 1969, Ahnström 1974). But its differential role in barley system has been extensively studied by Kesavan and co-workers. It has been observed that caffeine affords radioprotection only if the $O_2^-$-sensitive sites are available in the irradiated seeds (Kesavan and Afzal 1975, Nadkarni and Kesavan 1975, Afzal and Kesavan 1979a) and that the concentration of caffeine must be optimal (Kesavan and Ahmad 1976).

In the literature, it has been shown that OH$^-$-scavengers like ethanol (EtOH), tertiary butyl alcohol ($t$-BuOH), dimethyl sulfoxide (DMSO), and glycerol are generally radio-protectors (Johansen and Howard-Flanders 1965, Sanner and Pihl 1969, Roots and Okada 1972, Chapman et al. 1973, Reuvers et al. 1973, Ewing and Powers 1976, Samuni and Czapski 1978, Afzal and Kesavan 1979b, Watanabe et al. 1981, Roots et al. 1982, Ewing and Kubala 1987) and those agents which react with the radiolytically produced electrons are anoxic radiosensitizers. Adams and Dewey (1963) were the first to describe the radiosensitization of the bacteria *Serratia marcescans* by ketonic agents like N-ethylmalamide, diketone
diacetyl and benzophenone. Subsequently, Tallentire et al. (1972) showed that increasing concentrations of the ketonic agents like diacetyl acetone, acetophenol and p-nitroacetophenone cause increase in the radiosensitivity of aqueous anoxic spores to an extent that is some 40 per cent of that achieved by $O_2$ alone. Further experiments by Tallentire and Jones (1973) with $KMnO_4$ which reacts with electrons (Achey and Duryea 1974) showed that $KMnO_4$ sensitizes the spores of Bacillus megaterium in anoxic phosphate buffer (pH=7.0) or water suspension to the lethal effects of gamma-rays. These findings of Tallentire and Jones (1973) were reinvestigated in barley seeds (Kesavan and Afzal 1975, Kesavan and Dodd 1976, Kesavan et al. 1978) and spores of Bacillus megaterium (Raghu and Kesavan, unpublished). What they found was that $KMnO_4$ exerts radioprotection against oxygen-dependent damage, but causes radiosensitization of oxygen-independent damage. The question raised by their observations is whether superoxide anion ($O_2^-$) formed by the reaction of $O_2$ with $e^-_{aq}$ or the perhydroxy radicals ($HCO_2^-$) formed by the reaction of $O_2$ with $H^+$ constitute at least a part of the oxygen effect. Obviously, there is need for more data on this aspect, especially on the peroxidase related events and therefore it has been included in the present study.
Ascorbic acid is known to exert a radioprotective effect against oxygen-dependent damage (Afzal and Kesavan 1977, Jha and Kesavan 1986) by its OH⁻-scavenging action (Bielski and Allan 1970). A suppression of radiation-and chemical-induced neoplastic transformation of C3H1OT² cells by ascorbic acid has also been reported by many authors (Benedict et al. 1980, Gol-Winkler et al. 1980, Rosin et al. 1980, Yasukawa et al. 1989). Apart from this, it is of interest because of its antagonistic effects on the radioprotective action of cysteamine. This is caused by its interference with the biochemical pathway involving cAMP (Näslund et al. 1976a).

Since its discovery in 1888, several cellular functions and roles have been attributed to glutathione (G-SH) (Kosower and Kosower 1978, Meister and Anderson 1983, Ziegler 1985, Frankenberg et al. 1987). This includes its role in cellular reduction processes and protection/detoxification from harmful oxidative species. Because ionizing radiation produces toxic free radical species, the role of glutathione (which can, in various ways, detoxify free radical damage) has been considered important in reducing the radiosensitivity. In particular, the free radical scavenging hypothesis set forth by Alexander and Charlesby (1954) and expanded upon later by Howard-Flanders (1960) predicts that at low oxygen
tension glutathione should provide substantial protection. Addition of exogenous glutathione prevents tumor development in a concentration-dependent manner in the murine skin cells (Rotstein and Slaga 1988). Depletion of glutathione by buthionine sulfoximine (BSO) has been shown to increase the sensitivity of hypoxic cells to the lethal effects of radiation (Bump et al. 1982, Hodgkiss and Middleton 1983, Clark et al. 1983, Xue et al. 1988) or on both hypoxic and aerobic cells (Biaglow et al. 1983, Mitchell et al. 1983, Astor et al. 1984, Biaglow and Varnes 1984, Dethlefsen et al. 1988).


With the rationale derived from the aforesaid review, the aim of the present investigation was to assess the
influence of chemicals which selectively react with electrons (H·, eaq) or with hydroxyl radicals (OH·) or with both on seedling injury, peroxidase activity and total peroxides in the 8-day-old barley seedlings. The inclusion of catalase is not for the reason that it enzymatically decomposes H2O2 (Sobels 1963, Brill 1966, Swartz 1973) but also that it appreciably reacts with the electrons and hydroxyl radicals (Anbar and Neta 1967, Anbar et al. 1973, Dorfman and Adams 1973). Post-treatment with catalase and hydrogen peroxide has yielded interesting results which are presented here and part of these has been recently published (Singh and Kesavan 1990b).

The original aim of using nitrous oxide (N2O) in the post-hydration medium was to convert the electrons into hydroxyl radicals (Keene 1964) and examine the expected enhancement in radiosensitivity as was reported for the aqueous suspension of Bacillus megaterium spores (Powers and Cross 1970, Ewing et al. 1974, Kesavan and Powers 1987), for vegetative cells of E.coli (Brustad and Wold 1976a, Samuni and Czapski 1978, Watanabe et al. 1981) and for mammalian cells (Roots et al. 1982). Apart from this, a lack of radiosensitizing effect by N2O has also been reported in lymphocytes (Antoku 1977), Chinese hamster V 79 cells (Ewing et al. 1986) and vegetative cells of bacteria (Mullenger and Ormerod 1969, Simone and Quintiliani 1977, Tilby et al. 1982).
Watanabe et al. (1982) have shown that cell concentration, dose-rate and irradiation temperature affect the radiosensitization of *Pseudomonas radiora* O-1 by N$_2$O. Ewing et al. (1974) and Brustad and Wold (1976b), however, reported that only a few strains of vegetative cells of bacteria and only two mutants of *E.coli* K-12, respectively was sensitized by N$_2$O, whereas others are not. However, in the dry seed studies, it was found that N$_2$O reduces the sensitivity in comparison with that of N$_2$. This is quite an unexpected observation of considerable implications to basic radiobiology and part of these results is just published (Singh and Kesavan 1990a).

The extensive studies on peroxidase activity and total peroxides reveal that radioprotection by chemicals in terms of seedling injury is associated with decreased enzyme activity and restoration of peroxides to the normal levels. Further, studies using nondenaturing polyacrylamide gel electrophoresis have revealed that post-irradiation oxic damage is associated with increased intensity of the peroxidase bands over those of the control and also with the formation of two entirely new bands. The chemical radioprotection is found to remove these two new bands. Further, chemical potentiation of anoxic damage resulted in an increase in peroxidase activity but there is no reappearance of the two new bands (Singh and Kesavan 1990c).
In the literature there are reports that heat-shock itself induces proteins (HSPs) in a wide range of test systems (Kapoor 1983, Nover 1984, Craig 1985, Lindquist 1986, Kapoor 1986) and some of these HSPs may be related to peroxidase. Therefore heat-shock was given immediately after irradiation of seeds and its effect was studied on peroxidase activity using spectrophotometric method and nondenaturing polyacrylamide gel electrophoresis.
MATERIALS AND METHODS

1. Test system:

All the experiments were carried out with healthy, pure-line barley seeds (Hordeum vulgare, hull-less strain IB 65) which were multiplied and maintained in the botanical field of School of Life Sciences, Jawaharlal Nehru University, New Delhi, for the past fifteen years. This barley strain is well known for its uniformity of growth and a very low incidence of chromosomal aberrations.

2. Chemicals:

All the chemicals used were of highest purity available and used without further purification.

2.1 Test Chemicals:

The effect of following chemicals were investigated.

(a) Chemicals which predominantly react with hydroxyl radicals (OH⁺):

(i) Tertiary-butyl alcohol (t-BuOH) (Sarabhai M. Chemicals, India).

(ii) Ethanol absolute (EtOH) (Merck, Darmstadt, F.R. Germany).

(iii) Superoxide dismutase (SOD) (specific activity 3400 units/mg protein) (Sigma Chemicals Co. St. Louis, MO, USA).
(iv) L-ascorbic acid (Sigma Chemical Co. St. Louis, MO, USA).

(b) Chemicals which predominantly react with electrons (H· and e⁻aq):

(i) Hydrogen peroxide (H₂O₂) (Merck, India).

(ii) Potassium permanganate (Glindia Limited, India).

(c) Chemicals which react with both hydroxyl radicals (OH⁻) and electrons (H· and e⁻aq):

(i) Caffeine (Sigma Chemical Co., St. Louis, MO, USA).

(ii) Catalase (specific activity 11,000 units/mg protein, 1 Unit = 1 µM H₂O₂ per min. at pH 7.0 and temperature 25°C) (Sigma Chemical Co. St. Louis, MO, USA).

(iii) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA).

(iv) Buthionine sulfoximine (BSO) (Sigma Chemical Co., St. Louis, MO, USA).

(v) Glutathione (G-SH) (Sigma Chemical Co., St. Louis, MO, USA).
2.2 Chemicals used for determining peroxidase activity and total peroxides:

Coomassie Brilliant Blue G-250 (CBB G-250), bovine serum albumin (BSA), Trisma hydrochloride [Tris-(hydroxymethyl) aminomethane hydrochloride], Trisma base [Tris-(hydroxymethyl) amino methane], were purchased from Sigma Chemical Co. St. Louis, MO, USA. Benzidine and ammonium molybdate were supplied by Rannal, Budapest, Hungary. Hydrogen peroxide from Mercks India, potassium iodide from Glindia Limited, India, orthophosphoric acid from Glaxo Laboratories, India, sodium hydroxide from Sarabhai M. Chemicals, India and potassium hydrogen phthalate from British Drug House, India, were used in the experiments.

2.3 Electrophoresis chemicals:

Acrylamide, N,N' methylene bis-acrylamide, ammonium persulphate, N,N,N',N'-tetramethylethylene diamine (TEMED), bromophenol blue, glycerol, Tris-buffer, β-mercaptoethanol, magnesium acetate, glycine, ethylenediaminetetraacetate (EDTA) and phenylmethyl-sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. St. Louis, MO, USA.
3. **Equilibration of seed moisture content:**

The seeds were equilibrated to the desired moisture content following the method of Kesavan *et al.* (1973), by keeping them bound in a piece of muslin cloth for the required period over fused calcium chloride (anhydrous) in a desiccator. To get seeds of higher moisture content, they were kept in desiccator containing glycerol instead of fused calcium chloride. The seed moisture content was determined as percentage difference between the initial weight and the final weight of seeds after drying them in an electric oven maintained at 100°C for 48 hrs.

4. **Evacuation and sealing:**

For dry seed experiments, about hundred seeds each were taken in 10 ml glass ampoules and evacuated at $10^{-2}$ Torr for 4 hrs. in a manifold glass vacuum system before being sealed off with a glass-working torch following the method of Kesavan *et al.* (1973). The ampoules were checked for any leakage by immersing them in water overnight. The loss of moisture during evacuation was about 0.05 to 0.1 per cent depending upon the initial seed moisture content. However, the final determination of seed moisture contents reported for each experiment was made after sealing off the ampoules from the glass-manifold vacuum unit.
5. **Irradiation:**

Glass ampoules containing the seeds in vacuo were exposed to different doses of gamma-rays using a gamma cell 4000 [source $^{60}\text{Co}$ 204 TB$_q$ (5500 Ci)] obtained from Bhabha Atomic Research Centre, Bombay, India. The exact dose of gamma-rays used in different experiments is specified in the legends for the tables and figures. The dose-rate was so chosen to get a post-irradiation oxygen enhancement factor (post-irradiation OER) between 3.5 to 5.5 depending upon the initial seed moisture content of the seeds. The dose-rate was determined at periodic intervals by Fricke $\text{Fe}^{+2}/\text{Fe}^{+3}$ dosimeter. At the beginning of this study the dose-rate was 0.50 Gy/sec. After recharging the gamma cell in August 1987, the dose-rate increased to 1.45 Gy/sec. During the final part (December 1989) of this study it had come down to 1.25 Gy/sec.

6. **Preparation of hydration media:**

Double-distilled water was taken in conical flask and degassed by boiling for 20 min. After this, the flask was rendered airtight with a stopper and then cooled to room temperature before being transferred either to a cold room at 4±1°C or an airconditioned chamber maintained at 25±1°C for a period of 10-12 hrs. The degassed water was saturated with oxygen, nitrogen (both from Indian Oxygen
Limited, New Delhi), or nitrous oxide gas (Medical Engineers India Limited, New Delhi) as described by Kesavan et al. (1978), by bubbling for 45 min. To increase the solubility of the gases, the water was kept in ice bath during the bubbling. The oxygen contamination in nitrogen and nitrous oxide gases were about 20-30 and 90-100 ppm respectively. The gas-saturated water was immediately transferred to a cold room or air-conditioned chamber. The requisite quantities of the test chemicals were dissolved in the O\textsubscript{2}-, N\textsubscript{2}- or N\textsubscript{2}O-saturated water to obtain the concentrations which are indicated in the tables, figures and histograms.

To study the development of oxygen effect in dry barley seeds, the oxygen content in the hydration medium was manipulated by mixing required volumes of oxygenated and oxygen-free water as reported by Sharma and Kesavan (1975). The oxygen content has been presented as percentage in the tables, and the equivalent molarities are provided in the text.

7. **Post-hydration treatment:**

7.1 **Experiments with dry (metabolically inert) seeds:**

Immediately after irradiation, the glass ampoules were broken open and the seeds were quickly plunged into 30 ml screw cap glass vials containing
oxygen-, nitrogen- or nitrous oxide-saturated water with or without appropriate concentrations of the test chemical(s) at 4±1°C or 25±1°C. Wherever necessary, the seeds, immediately after irradiation, were given a wet heat-shock for 90 sec. by immersing them in water bath maintained at 60°C and then transferred to the desired hydration media as described by Kesavan et al. (1973). Seeds were left in the hydration media for 8 hrs. and then washed thoroughly in running tap water. Immediately after this, the seeds were arranged (thirty seeds per petri-dish) in three petri-dishes (which formed three replications for each treatment) containing moist germination paper. These petri-dishes were first left in a dark chamber maintained at 25±1°C until the shoot tips emerged. Then, these were exposed to continuous illumination at 25±1°C.

7.2 Experiments with metabolizing seeds:

In these experiments, the seeds were pre-soaked in distilled water for 4 hrs. at 25±1°C and then they were transferred to fresh hydration media consisting of O₂-, N₂- or N₂O-saturated water with or without the test chemicals. Exactly 20 min. before the mid-point (2 hrs.) of a schedule of 4 hrs.
treatment, the vials containing the seeds were bubbled with $O_2^-$, $N_2^-$ or $N_2O$-saturated water and then exposed to desired doses of gamma-rays. All the treatments were carried out at $25\pm 1^\circ C$.

In another set of experiments, referred to as experiments with slowly metabolizing seeds, the metabolic activity of the seeds were slowed down by decreasing the temperature (about $4^\circ C$) of the treatment. These experiments were carried out as follows.

The seeds were pre-soaked in distilled water for 1 hr. at $4\pm 1^\circ C$ and then transferred to fresh hydration media saturated with $O_2$, $N_2$ or $N_2O$ gas for 1 hr. at the same temperature. These seeds were again transferred to a fresh hydration media consisting of $O_2^-$, $N_2^-$ or $N_2O$-saturated water with or without chemicals and were kept at $4^\circ C$ for 40 min. Then, these vials containing the seeds were bubbled with $O_2$, $N_2$ or $N_2O$ gas for 20 min. During this process the temperature was maintained at about $4^\circ C$ by keeping these vials in ice. Following this schedule of hydration, the moisture content of the seeds registered an increase from $9.3$ per cent to $22.0$ per cent. The vials were then exposed to desired doses of gamma-rays (doses are given in the tables), and post-hydrated for 30 min.
at 4±1°C and then washed thoroughly in running tap water and planted on moist germination paper in petri-dishes and cultured in a growth chamber as described for dry seed experiments.

8. **Assessment of radiation-induced seedling injury:**

After the eighth day (at 192 hrs.) the seedling growth was measured from the base of the first leaf to the top. Mean 8-day old seedling growth ± standard error (S.E.) was calculated from three replications of thirty seedlings for each treatment. The percentage seedling injury was obtained by calculating the percentage reduction in the seedling growth (Conger et al. 1968). For calculating percentage seedling injury the mean seedling growth of unirradiated seeds (with or without chemical treatment) was taken as control. Protection index was calculated and expressed as the ratio of the percentage seedling injury without and with the chemical(s). Likewise potentiation index was obtained as the ratio of percentage seedling injury with and without chemical(s). The post-irradiation oxygen enhancement factor was expressed as ratio of seedling injury in oxic water to that of seedling injury in anoxic water.
9. Determination of the rate of reactivity towards oxygen or decay of the radiation-induced oxygen-sensitive sites:

To determine the rate of development of oxygen-dependent damage at 4±1°C, the seeds were initially placed in oxygenated water. At specific intervals ranging from 5 min. to 30 min. of a post-hydration schedule of 480 min. at 4±1°C, the seeds were transferred to oxygen-free (means N₂- or N₂O-saturated) water thereby preventing any more development of oxygen-dependent damage. Similarly to determine the rate of disappearance of oxygen-dependent damage (decay of oxygen-sensitive sites) in the absence of oxygen, the seeds were initially placed in oxygen-free water. Then at the end of specific period of time ranging from 30 min. to 90 min. of a post hydration schedule of 480 min. at 4±1°C, the seeds were transferred to oxygenated water.

The oxygen-sensitive sites were calculated from 8-day old seedling growth following formula of Nadkarni and Kesavan (1975). Development of a particular component of damage was computed by following the changes with time between two damaged states i.e. irradiation and oxygen-free hydration to irradiation and oxic hydration. The rate of removal of oxygen-sensitive sites by oxygen i.e.
the rate of reaction of oxygen-sensitive sites with oxygen is demonstrated by plotting

\[ C = \frac{H_{to} - H_{ox}}{H_{ox-free} - H_{ox}} \times 100 \]

against time \( t \) in oxic water, where \( C \) is the radiation-induced oxygen-sensitive sites, \( H_{to} \) is the seedling growth following post-hydration in oxygenated water for a period \( t \) before transfer to oxygen-free water, \( H_{ox} \) is the seedling growth when the seeds are post-hydrated in oxygenated water for the complete post-hydration schedule of 480 min. and \( H_{ox-free} \) is the seedling growth when the seeds are post-hydrated in oxygen free (\( N_2 \)-or \( N_2O \)-saturated) water for the complete post-hydration schedule of 480 min. so that no post-irradiation oxygen-dependent damage occurs.

Similarly, the rate of decay of oxygen-sensitive sites in the absence of oxygen is demonstrated by

\[ C = \frac{H_{ox-free} - H_{tn}}{H_{ox-free} - H_{ox}} \times 100 \]

against time \( t \), where \( H_{tn} \) is the seedling growth when the seeds are post-hydrated in oxygen-free water for a period of \( t \) before transfer to oxic water.
10. **Extraction of Peroxidase (EC. 1.11.1.7):**

Peroxidase activity was assayed in 8-day old seedlings and embryos. In case of 8-day old seedlings, 250 mg of seedlings were homogenized in 5 ml of 0.05 M Tris buffer (pH=7.2) in a pre-chilled mortar and pestle at 4±1°C, but in case of embryos, 30 of them were scooped from the seeds (post-hydrated at 4±1°C for 8 hrs. with or without chemical(s) after delivering the desired dose of radiation) and homogenized in 3 ml of same buffer. The homogenate was centrifuged at 20,000 g at 4°C for 20 min. The supernatant was saved for peroxidase assay, total peroxides and protein estimations.

11. **Peroxidase assay**

Peroxidase activity was measured according to the procedure of Scandalia (1969). Peroxidase activity was estimated by recording a change in absorbance at 610 nm for 1 min. at intervals of 15 sec. after adding 0.05 ml of supernatant to 5 ml of reaction mixture. The reaction was monitored at 25±1°C. The assay mixture was prepared by adding 30 ml of 30 mM benzidine solution to 100 ml of 20 mM hydrogen peroxide. The benzidine solution was prepared by dissolving it in 1:4 (v/v) glacial acetic acid and water at 50°C, and cooling it to room temperature before mixing with hydrogen peroxide. An arbitrary unit of peroxidase activity was chosen as change in absorbance of 0.1/15 sec./mg protein.
12. **Protein estimation:**

Protein was estimated following the method of Bradford (1976) using bovine serum albumin as standard.

12.1 **Principle:**

Coomassie Brilliant Blue G-250 (CBB G-250) is a dye that binds proteins readily. The dye which is red in a free form with absorption maximum at 465 nm is converted to a blue form with the absorption maximum shifted to 595 nm, on binding proteins. Recent evidence (Compton and Jones, 1985) however, indicates that the dye infact exists in three different forms depending on the charge and it is the unprotonated catonic form that binds to the proteins. The dye interactions are chiefly with arginine residues. The protein-dye complex has a high extinction coefficient, thus leading to great sensitivity in measurement of protein.

The range of the assay is 1-10 μg protein/assay volume.

12.2 **Procedure:**

Bradford reagent was prepared as follows:

35.75 mg of CBB G-250 was dissolved in 12 ml of absolute alcohol. To this solution 27 ml of
Figure 1: A standard curve of protein using Bradford reagent. Different concentrations of protein (BSA) to the volume of 0.05 ml were thoroughly mixed with 0.95 ml of Bradford reagent. The optical density (O.D.) was measured at 595 nm.
CONCENTRATION OF BSA (µg)
orthophosphoric acid was added and stirred on a magnetic stirrer for about 10 min. The resulting solution was diluted to a final volume of 250 ml with distilled water, filtered twice through Whatman No.1 filter and stored in brown bottle.

Varying concentrations of standard BSA solution (0.2 mg/ml) were taken in 50 μl of distilled water. Water alone was taken as no protein control. Adequately diluted samples were also taken in the same volume. 950 μl of Bradford reagent was added to each tube (colour develops instantaneously which is quite stable for 1 hr). The absorbance was read at 595 nm 5 min. after adding the reagent. Protein concentration in the unknown samples were estimated by using the calibration curve prepared with standard BSA solution (Figure 1).

13. **Determination of total peroxides**:

The total peroxides were determined in 8-day old seedlings and embryos following the iodide method of Hochanadel (1952).

13.1 **Principle**:

Iodide ion is oxidized by H₂O₂ in neutral or slightly acid solution and the absorption of I⁻ is measured at 350 nm.
13.2 Procedure:

The iodide reagent was prepared immediately before using by mixing equal volumes of two solutions containing:

Solution A
6.6 g potassium iodide, 0.2 g sodium hydroxide and 0.02 g ammonium molybdate per 100 ml of water.

Solution B
2.0 g potassium hydrogen phthalate per 100 ml of water.

0.5 ml of supernatant was added to 2 ml of iodide reagent (1 ml of solution A and 1 ml of solution B) and absorbance of $I_3^-$ was recorded at 350 nm. The concentration of total peroxides was calculated as follows:

Concentration (in M) = \frac{OD}{26,900},

where 26,900 is molar extinction coefficient (\varepsilon) of $I_3^-$. 

14. Analysis of peroxidase activity using non-denaturing polyacrylamide gel electrophoresis:

500 mg of 8-day old seedlings were homogenized in buffer containing 20 mM Tris (pH=7.6), 3 mM Mg(OAC)$_2$, 10 mM
β-mercaptoethanol and 0.5 mM PMSF. The homogenate was
centrifuged at 20,000 g for 20 min. at 4°C and supernatant
was used for electrophoretic studies.

Gel electrophoresis was performed according to the
method of Laemmli (1970) with slight modifications. The
sample buffer was composed of 0.125 M Tris (pH=6.8), 15
per cent glycerol and 0.01 per cent bromophenol blue.
Electrophoresis was performed in 10 per cent acrylamide
gels. Separating gel was made of 10 per cent acrylamide,
0.26 per cent N,N' methylene bis-acrylamide and 0.375 M
Tris buffer (pH=8.8). The various components were mixed,
deaerated and polymerization was initiated by adding 0.01
per cent TEMED and 0.05 per cent ammonium persulphate.
This solution was quickly poured off to get a vertical
slab gel of 16.0 x 14.0 cm with 1.5 mm thickness. The
top of the gel was covered with water to stop aeration.
The stacking gels were composed of 4 per cent acrylamide,
0.10 per cent N,N' methylene bis-acrylamide, 0.01 per cent
TEMED and 0.05 per cent ammonium persulphate and 0.125 M
Tris (pH=6.8). The running buffer was composed of 0.024 M
Tris (pH=8.0), 0.19 M glycine.

Different samples, all containing equal protein (ranging
from 25-50 μg depending upon the size of the gel used)
were loaded and electrophoresis was carried out at a constant
voltage (200 V) in a cold room maintained at 4±1°C, until the bromophenol blue migrated to the bottom of the gel. After electrophoresis, the gel was first washed with water and then equilibrated with 1:4 (v/v) glacial acetic acid: water for 2 min. The colour development was seen by incubating the gel first with 15 mM benzidine solution for 3 min. followed by 20 mM hydrogen peroxide for 2 min. After colour development gels were photographed.