CHAPTER VI

CONCLUSIONS
A great deal of radiobiological research is aimed at a better understanding of the mechanisms underlying cellular radiosensitivity. Various levels of radiosensitivity are known to exist even among one particular group of organisms. Different factors determining cellular responses to irradiation have been broadly classified into three categories as physical, chemical and biological (Szumiel, 1981). Physical factors include the radiation quality, dose rate and the temperature at which irradiation is performed. Chemical factors consist of the levels of oxygen and endogenous radiosensitizers and radioprotectors, whereas biological factors comprise of intracellular factors affecting intrinsic radiosensitivity of a given organism. Factors which contribute to the intrinsic cellular radiosensitivity have remained an area of extensive research in order to understand the reasons for the differences in the levels of radiosensitivities among different biological systems.

In the present investigation, an attempt has been made to study the ionizing gamma radiation induced biochemical and structural changes in three closely related Gram negative organisms which differ in their radiosensitivities. The three organisms selected for the study were Escherichia coli, Escherichia coli B/r (a radioresistant strain) and Shigella flexneri. The $D_{10}$ values (dose required for 90% inactivation) for these three different organisms were found to be 8, 35 and 30 krad respectively. On the basis of the $D_{10}$ values and the doses required for 6 log cycle reductions in viable count,
E. coli was found to be the most sensitive and S. flexneri, the most resistant towards the action of ionizing radiation.

The effects of ionizing radiation on DNA, proteins, enzymes and membrane lipid peroxidation have been studied and compared in an effort to understand the radiation induced biochemical differences between these cultures. The DNA of E. coli and E. coli B/r was found to be more sensitive to ionizing radiation induced inactivation than the DNA from S. flexneri. The doses required for maximum DNA damage were found to be 5, 20 and 80 krad for E. coli, E. coli B/r and S. flexneri respectively. The differences in the radiosensitivities of DNA could be an important reason for the differences in cellular radiosensitivity. The alkaline density gradient sedimentation profiles of control and irradiated DNA from S. flexneri revealed that irradiation of DNA may result in fragmentation at lower doses and cross-linking or aggregation of DNA molecules (or fragmented DNA molecules) at higher doses. This observation is different from that made in studies on alkaline density gradient sedimentation profiles of irradiated E. coli DNA (Nair et al., 1975). Radiation induced crosslinking in DNA and proteins has been reported in other systems (Yamamoto, 1976; Dizdaroglu and Simic, 1985; Simic and Dizdaroglu, 1985).

Besides DNA, cell membranes constitute an important target for ionizing radiation induced damage. Example of correlation between bacterial membrane properties and cellular radiation response are known (Suzuki and Akamatsu, 1978, 1980; Suzuki et al., 1982; Suzuki, 1985). Radiation induced changes in
membranes include disturbances in active and passive transport systems especially those for $K^+$, $Na^+$ and $Ca^{2+}$ and molecules of DNA precursors; membrane depolarization and decrease in viscosity, alteration in membrane bound enzymes and cross-linking of membrane proteins (as reviewed by Szumiel, 1981; Nakazawa et al., 1984; Yukawa and Nakazawa, 1983). Membrane damage is usually characterized in terms of lipid peroxidation and its consequences on membrane functions are accepted as an important effect of irradiation (Buege and Aust, 1978).

The whole cell lipid peroxidation levels upon gamma irradiation of *E. coli*, *E. coli* B/r and *S. flexneri* were studied and were found to be very similar. There was an increase in the peroxidation with increasing radiation dose in all the three organisms. The extent of lipid peroxidation was, however, different in all the three organisms. Maximum (7.36 fold) over that of unirradiated cells lipid peroxidation was observed in radioresistant *E. coli* B/r at a dose of 500 krad whereas radiosensitive *E. coli* and *S. flexneri* cells showed lower levels of lipid peroxidation (3 fold and 2 fold respectively) at the same dose.

The studies on the periplasmic acid phosphatase from these organisms suggested at least a quantitative difference in their periplasmic protein content. The radiation responses of acid phosphatases from all the three organisms were found to be different. The *E. coli* enzyme showed a decline in activity with increasing radiation dose. The acid phosphatase activity
was found to be inhibited at lower doses in case of \textit{E. coli} B/r and higher doses were stimulatory. No significant change in the acid phosphatase activity was observed upon irradiation of \textit{S. flexneri} cells. Irradiation resulted in significant alteration in some of the kinetic properties of the periplasmic phosphatase of \textit{E. coli} and \textit{S. flexneri} cells. The change in the kinetic properties of an enzyme could possibly be due to radiation induced conformational changes in the enzyme. Enzyme kinetic properties of membrane bound ATPase from \textit{S. typhi} has been shown to get altered upon irradiation of membrane fractions in presence of the radiosensitizer ascorbic acid (Kapila, 1985).

The study on the effect of gamma radiation cell free extracts on cytoplasmic proteins revealed that the proteins from \textit{E. coli} B/r were more sensitive to radiation induced damage than those from \textit{E. coli} or \textit{S. flexneri}.

Various enzymes like superoxide dismutase (SOD), catalase and peroxidase are known to scavenge free radicals and hydrogen peroxide (as reviewed by Fridovich, 1975, 1976, 1978). These enzymes are endogenous radioprotectors and hence contribute to the intrinsic cellular radiosensitivity. The effect of gamma radiation on these enzymes has also been studied. The polyacrylamide gel electrophoretic profiles of control and irradiated peroxidase and superoxide dismutase from \textit{E. coli}, \textit{E. coli} B/r and \textit{S. flexneri} have been compared. In both the radiosensitive as well as the radioresistant strains of \textit{E. coli}, two distinct activity bands of peroxidase were detectable while only one activity band was observed in \textit{S. flexneri} cells. The peroxidases
from radiosensitive *E. coli* and *S. flexneri* cells were found to be more resistant to radiation inactivation than the peroxidase from *E. coli* B/r. In the former organisms, the enzymes were found to retain their activities on the gel even after having been exposed to a dose of 500 krad, whereas no activity band of the *E. coli* B/r peroxidase was observed after irradiation at a dose of 200 krad. Similarly, the polyacrylamide gel electrophoretic profiles of superoxide dismutases were found to be different in these organisms. The SOD from *E. coli* cells was found to be more sensitive to radiation induced inactivation as compared to that from *E. coli* B/r and *S. flexneri* cells. This suggests that SOD plays a more important role in radioprotection in *E. coli* B/r cells than peroxidase, whereas both SOD and peroxidase appear to be involved in defense against radiation damage in case of *E. coli* and *S. flexneri*.

Thus, the study indicated that the whole cell lipid peroxidation, protein damage and damage to peroxidase is higher in radioresistant *E. coli* B/r compared to *E. coli* and *S. flexneri*. This indicates that in case of *E. coli* and *S. flexneri*, the endogenous radioprotectors play an important role as secondary defense mechanisms. It is known that *E. coli* B/r is more efficient in its DNA repair mechanisms (Nair et al., 1975; Dertinger and Jung, 1970h) and hence its primary defense mechanism is sufficient to repair radiation induced damage, unlike in the two other organisms. However, this could only be conclusively claimed by studying the repair mechanisms in all the three organisms.
The radiation induced biochemical changes have also been correlated with the morphological and ultrastructural alterations occurring upon irradiation of *E. coli*, *E. coli* B/r and *S. flexneri* cells. Notable differences, mainly in the form of deformation of cells, cytoplasmic shrinkage and clumping of cells were observed in the studies conducted to examine radiation induced morphological changes. The qualitative nature of the radiation induced ultrastructural damage appeared to be the same in all the three organisms. It was observed mainly in the form of cytoplasmic shrinkage, deformation of cells, aggregation of cytoplasmic contents and chromosomal matter, an increase in the waviness of the cell wall and cell membrane structure, infolding of the membrane, an increase in the electron density of cells and appearance of ghost cell (cells without cytoplasmic contents). There was no significant repair of radiation induced damage to the morphology and structure of these organisms when they were allowed to regrow in a post irradiation growth medium for a period of 60 minutes. However, *E. coli* B/r showed a greater number of normal cells as compared to radiosensitive *E. coli* or *S. flexneri* cells.

Radioisotopes and radiation have immense potential in maintaining the health of man and his environment. Ionizing radiation has been successfully employed in the past in applications like cancer therapy, medical product sterilization and food preservation (Krishnamurthy, 1980). In recent years, studies have been directed towards the application of gamma radiation in the area of environmental management. Radiation
disinfection of wastewaters and municipal sludge is one such example (Krishnamurthy, 1980, 1981; Kapila et al., 1981; Suess et al., 1983; Watanabe and Takehisa, 1983; Iya and Krishnamurthy, 1984; Hashimoto et al., 1986; Pandya et al., 1987).

An attempt has also been made to study the possibility of employing ionizing radiation for hygienisation and recycling of sewage sludge from a local treatment plant. The results obtained indicated that a dose of 1000 krad could bring about a reduction of 5.3 to 6.7 log cycles in all the sewage samples collected from different points of the treatment plant. Complete removal of bacteria was not possible even at a dose of 1000 krad probably due to the association of bacteria with wastewater solids. A dose of 500 krad was, however, found to reduce the total coliforms count of sewage samples by 5 log cycles. A dose of 0.3 to 0.5 Mrad has been reported to reduce coliforms in sewage to undetectable levels (Watanabe and Takehisa, 1983). The radiation inactivation patterns of certain bacterial pathogens usually found in sewage have been compared. The study was performed with the cell suspensions in phosphate buffer as well as in sewage samples. It was found that the doses required for bacterial inactivation in sewage were higher than those required for inactivation of buffer suspended cells indicating a protective effect of sewage samples towards radiation induced killing of bacterial pathogens.
Various chemicals (at concentrations non-toxic to cells) have been tested in an effort to find a radiosensitizer that can reduce the gamma ray dose required for bacterial inactivation in sewage samples. Two Gram negative organisms, *E. coli*, an index organism of sewage pollution and *S. flexneri*, a human pathogen were selected for this study. Amongst all the chemicals tested in buffer, only ferric chloride (100 ppm) could sensitize *E. coli*. Most other compounds were found to be radioprotective towards *E. coli*. Ferric sulphate (5 ppm), ammonium chloride (10 ppm) and ascorbic acid (100 ppm) proved to be good radioprotectors for *E. coli*. Most of the compounds, unlike in the studies on *E. coli*, showed varying degrees of radiomodification towards *S. flexneri*. Ferric sulfate (5 ppm) and sodium arsenate (0.025 M) were found to be good sensitizers of *S. flexneri* whereas ferrous sulphate (5 ppm), ferric chloride (100 ppm), barium carbonate (5 ppm) and copper sulphate (5 ppm) were moderate sensitizers. Mercury chloride (0.01 ppm), sodium chloride (0.1 M) and sodium sulphate (0.1 M) were moderate radioprotectors whereas barium acetate (3 ppm) was found to be a good radioprotector of *S. flexneri*. Thus, the same chemical was found to exert different radiomodifying effects on different organisms. The efficiency of various chemicals as radiosensitizers was evaluated in sewage itself. All the chemicals except lead nitrate (10 ppm) were found to protect *S. flexneri* cells in sewage. The difference in the radiomodifying effect of a chemical in buffer and in sewage could be due to the complex chemical composition of sewage.
The above study indicated that although treatment with gamma radiation could be a feasible method of ridding sewage sludge of indigenous pathogenic organisms, the constitution of individual sewage systems should be given careful consideration before applying the method on a large scale.

Domestic or industrial wastewater treatment generates sludge which is rich in organic substances, macro- and micro-nutrients and pathogenic microorganisms. Toxic substances, especially heavy metals may also be present in comparatively large concentrations (Mininni and Santori, 1987). The safe disposal of sewage sludge has become a serious problem in recent years in terms of public health and ecological balance. This is mainly because disposal options are fast diminishing while the quantum of sewage sludge generated is rapidly increasing at an alarming rate (Krishnamurthy, 1980). Beneficial recycling of sewage and sludge to recapture its source value is the only other alternative consistent with environmental safety and public health (Iya and Krishnamurthy, 1984; Mininni and Santori, 1987; Yeager and Ward, 1981).

An attempt has also been made to study the effect of gamma irradiated sludge on plant growth to examine the fertilizer value of irradiated sludge and the possibility of recycling it for agricultural purposes. Two agriculturally important plants, rice (*Oryza sativa* L. var GR-3) and chickpea (*Cicer arietinum*) were selected for this study.
A beneficial effect of irradiated sludge on the growth of rice plants was observed. There was an increase in the shoot length, the root length, the fresh weight and the dry weight of plants grown in soil supplemented with irradiated sludge. Irradiated sludge was also found to enhance the levels of total proteins, total soluble sugars and starch content of rice plants by 1.5, 1.25 and 2.17 fold respectively as compared to those grown in soil alone. The total chlorophyll content of rice plants grown in soil containing irradiated sludge was also found to be significantly enhanced.

In case of chickpea plants, the irradiated sludge had no significant effect on the shoot length, the root length, the fresh weight and the dry weight of the plants as compared to those grown in soil alone. However, there was a 1.12, 1.49 and 1.56 fold increase in the total proteins, total soluble sugars and starch content respectively of chickpea plants grown in soil containing irradiated sludge as compared to those grown without sludge. The chlorophyll content of chickpea plants grown in soil supplemented with irradiated sludge was also found to be 1.8 fold higher than in those grown without any sludge. The unirradiated sludge showed no detrimental effect on the growth of rice plants and even had a beneficial effect in terms of all the growth parameters. The irradiation of sludge further enhanced the growth of rice plants suggesting a beneficial effect of irradiation. However, in chickpea plants, the unirradiated sludge was found to be inhibitory to the growth of plants. The shoot length, the root
length, the fresh weight and the dry weight of chickpea plants were found to be reduced when grown in soil supplemented with unirradiated sludge as compared to control plants grown only in soil. An inhibition of 36% in the starch content was observed in plants grown in soil containing unirradiated sludge as compared to those grown without any sludge. However, this inhibition of the sludge on the growth of chickpea plants was removed upon gamma irradiation suggesting the radiation induced inactivation of toxic compounds in sludge.

The yield of rice and chickpea plants grown in soil supplemented with irradiated sludge were also determined. Rice plants grown in soil containing irradiated sludge showed a 17% increase in the total number of filled seeds and a 25% increase in the weight of total filled seeds per plant indicating a higher yield as compared to plants grown in soil containing unirradiated sludge. In case of chickpea, even though the total number of pods produced per plant were less in soil containing irradiated sludge compared to control plants grown in soil alone, the protein content of pods produced in plants grown in soil supplemented with irradiated sludge was found to be enhanced by 71% indicating an improvement in the quality of pods produced on plants grown in the presence of irradiated sludge.

Thus, this study revealed that the gamma irradiated sludge can be safely recycled for agricultural applications. Irradiation of sludge results in enhancement of the growth and yield of plants and also removes the toxic compounds present which otherwise could inhibit plant growth. The exact mechanism underlying the improvement of plant growth in the presence of
irradiated sludge is difficult to explain but it may be due to the changes in physico-chemical properties of sludge upon irradiation. Irradiation of the sludge was found to result in an increase in the inorganic and organic content of the sludge. Increased levels of phosphate and sulfate content were also observed in irradiated sludge as compared to unirradiated sludge. This could possibly be correlated with the growth improvement of plants grown in soil supplemented with irradiated sludge. The positive effects of using sludges in agriculture are related to the presence of nitrogen, phosphorous and organic matter (Mininni and Santori, 1987). Irradiation could also possibly stimulate the growth of plants by detoxifying the toxic substances present in sludge.
In continuation with the study presented here, future work may be performed -

1) To compare the rates of DNA degradation, DNA synthesis and DNA repair (using radiolabelled compounds) upon irradiation of \textit{E. coli}, \textit{E. coli} B/r and \textit{S. flexneri}.

2) To compare and characterize the nature of the membrane damage in terms of membrane lipids and membrane proteins in \textit{E. coli}, \textit{E. coli} B/r and \textit{S. flexneri}.

3) To purify peroxidase, catalase and superoxide dismutase from these organisms and to study their kinetics upon gamma irradiation.

4) To study gamma irradiation damage in \textit{E. coli}, \textit{E. coli} B/r and \textit{S. flexneri} by scanning electron microscopy, which will in particular highlight the radiation-induced changes in membrane topology.

5) To study the gamma radiation induced repair of morphological and ultrastructural damage in \textit{E. coli}, \textit{E. coli} B/r and \textit{S. flexneri} at lower radiation doses and allowing longer post-irradiation incubation times for the repair process. Also, to segregate the viable from the dead cells by differential centrifugation and to selectively study the repair of viable cells.

6) To screen more chemicals to find an ideal radiosensitizer which could be employed for sludge hygienisation by radiation on a commercial scale.
7) To standardise the ratio of soil to irradiated sludge, which would yield maximum beneficial results for agriculture. Also, to check the effect of gamma irradiated sludge on other agriculturally important plants in order to generalise the results obtained with rice and chickpea plants and to understand in detail, the biochemical mechanisms of stimulation or inhibition of plant growth when grown in soil supplemented with irradiated or unirradiated sludge.

8) To carry out physico-chemical analysis of gamma irradiated sludge in detail to understand the irradiation effects on sludge.