CHAPTER III

BIOCHEMICAL RESPONSE OF M.RADIOPHILUS EXPOSED TO VARIOUS PHYSICAL-CHEMICAL AGENTS

INTRODUCTION

In the previous chapter results were presented to show that as compared to Escherichia coli cells, cells of M.radiophilus were highly resistant to the treatments known to cause damage to cellular DNA. Suggestive evidence was also obtained to indicate that DNA repair machinery could be wholly or substantially responsible for the bacterium to display such a high resistance to DNA damaging conditions. Although it is well known that radiations and radiomimetic agents affect cell viability by the primary injury to DNA (1) it is not known to what extent other important constituents of the cell - for instance, proteins, cell membrane - are damaged by these agents and whether any recovery processes occur if such components are injured. Such phenomenon cannot be studied in sensitive and moderately resistant bacteria such as E.coli since the organism would loose viability at much lower doses. The highly radiation resistant organism like M.radiophilus hence offers an ideal system to study the effects of radiations and similar agents on a variety of cellular processes. The study of the effect of radiations and other DNA-damaging agents on cellular processes in a highly radio-resistant microorganism is also worthwhile since such studies may throw light on certain special attributes which may contribute either directly or indirectly to the high resistance that the bacterium exhibits.

In the studies to be described in this chapter the effects of gamma radiation, UV radiation and MMS treatment were investigated on three classes of cellular processes, namely, (1) DNA degradation; (2) DNA, RNA and protein syntheses; and (3) degradation and recovery of carotenoid pigments.

It is known that gamma radiation exposure in many bacteria including E.coli causes degradation of cellular DNA (2 - 5). This process is a subject of much study and has been also suggested to be associated in some manner with DNA repair systems (6, 7). Thus mutants such as uvr D^- and rec A^- of E.coli exhibit extensive DNA degradation compared to wild type after irradiation (8). It would be of interest to study how this process is triggered in M.radiophilus at high doses of gamma radiation and other DNA damaging treatments.

DNA dependent processes such as DNA replication, RNA synthesis and, in turn, protein synthesis are expected to be impaired after the damage to cellular DNA (9). It would be of interest to ascertain how these processes in M.radiophilus respond to high doses of radiations.

One striking effect of radiation in M.radiophilus as in M.radiodurans reported in literature, is the loss of carotenoid pigments thus resulting in the bleaching of

characteristic pink colour of this bacterium (10). It would be of interest to know the mechanism by which the pigment is destroyed by radiation, the protective effect, if any, afforded by this pigment against the radiation-induced loss of cell viability, and the mechanism by which the regeneration of carotenoid pigment is brought about by post-irradiation growth.

MATERIALS AND METHODS

Bacterial strains Media and culture conditions Cells of M.radiophilus, M.luteus and E.coli were grown in TGYM medium as described in the previous chapter.

Chemicals Dithiothreitol, ATP, dATP, dCTP, dGTP, dTTP, calf thymus DNA, Chloramphenicol, PPO and POPOP were obtained from Sigma Chemicals Co., St. Louis, U.S.A.

Radioactive chemicals (Methyl-^{3}H)-thymidine (specific activity 6 Ci/mM), ^{3}H-uridine (specific activity 5.7 Ci/mM), DL-1-^{14}C-leucine (specific activity 54 mCi/mM) were obtained from the Isotope Group of this Research Centre. (2-^{14}C) TTP (specific activity 50 mCi/mM) was obtained from New England Nuclear Corporation.

Irradiation and chemical treatment Treatment of bacterial cells with MMS, and exposure of the cells to UV- or gamma-radiations were done as described in the previous chapter.

Degradation of DNA

The DNA of M.radiophilus was labelled by growing cells (10^7 cells/ml) in TGYM medium containing 15 µCi/ml ^{3}H-thymidine for 16 hours at 30°C. The cells were harvested, washed with sodium phosphate buffer (0.1 M, pH 7.0) and suspended in the same buffer. Aliquots of the suspensions were exposed to gamma radiation, UV radiation or treated with MMS as described earlier. The bacterial cells after irradiation or chemical treatment were centrifuged out, washed and incubated in TGYM medium at 30°C. Aliquots were withdrawn at various intervals on Whatman No.3 paper discs (2.5 cm dia.) and cells were killed by 3 drops of 3% formalin. After drying for overnight the paper discs were washed 3 times with 10% TCA, followed by ethanol, and acetone (11). The discs were dried and the radioactivity of the samples was measured in Beckman LS-100 Liquid Scintillation System with 10 ml of scintillation flour consisting of 0.4% PPO and 0.05% POPOP in toluene.

Macromolecular syntheses

To study the syntheses of DNA, RNA, and protein the bacterial cells were suspended in TGYM medium at the density of 10^8 cells/ml. The cell suspensions were incubated at 30°C with 15 µCi/ml each of ^{3}H-thymidine, ^{3}H-uridine or ^{14}C-leucine. At various intervals, 0.1 ml aliquots were transferred to Whatman No.3 paper discs and TCA-insoluble radioactivity (due to incorporation of radioactive precursors in DNA, RNA or protein) was determined as described earlier.

Determination of RNA synthetic rate

Cells of M.radiophilus were exposed to 200 krad of gamma radiation in sodium phosphate buffer as described earlier. The unirradiated and irradiated cells were incubated in TGYM broth at 30°C. Aliquots of 0.5 ml of the cell suspensions were transferred to 0.5 ml TGYM medium containing ^{3}H-uridine (15 µCi/ml) and incubated for 2 min at 30°C. At the end of 2 min pulse of the radioactive precursor, 5 ml cold 10% TCA was added to precipitate the macromolecules. TCA-insoluble radioactivity of the samples due to incorporation of radioactivity in uridine was determined as described earlier.

Extraction and analysis of the pigment

The pigments of M.radiophilus exposed to different doses of gamma radiation were extracted with a solvent mixture of chloroform and methanol (2:1 v/v) at 4°C (10). The procedure involved repeated treatments of the bacterial cells with the solvent mixture (about 3 times) for quantitative extraction.

The pigments of M.luteus exposed to different doses of gamma radiations was quantitatively extracted by repeated treatments (about 3 times) with methanol at 0°C.

The pigments isolated from both the strains were analysed in a Shimadzu spectrophotometer MPS 502 using respective solvent systems as references.

Studies on regeneration of pigments

Log phase cells of M.radiophilus were suspended in 0.1 M phosphate buffer and exposed to different doses of gamma radiation. Irradiated cells were washed and incubated in the TGYM medium in absence or presence of chloramphenicol (20 µg/ml) at 30°C. Aliquots of 5 ml removed at various time intervals, harvested and pigment extraction and analysis ~~was~~ were carried out as described earlier.

DNA polymerase assay

Preparation of crude extract: E.coli or M.radiophilus cells were suspended in a spheroplasting medium consisting of Tris-HCl buffer (0.06M, pH 7.6) at the density of 10^9 cells/ml. The suspensions were incubated with lysozyme (5 mg/ml) at 0°C for 30 min. At the end of incubation, the spheroplasts were centrifuged out and suspended in the Tris-HCl buffer and were subjected to ultrasonic treatment for 20 min as described in the previous chapter. Aliquots of the sonicates were exposed to 200 krad at 0°C. Unirradiated and irradiated sonicates were centrifuged and the supernatants were treated as crude enzyme preparations.

Heat inactivation of calf thymus DNA: Calf thymus DNA was dissolved in 0.1 M Tris-HCl (pH 7.0) buffer at a concentration of 0.5 mg/ml. This was incubated in a boiling water bath for 10 min and chilled immediately.

Enzyme assay: The assay mixture of 0.1 ml contained 5 mM Tris-HCl buffer (pH 7.8). 8.0 mM $MgCl_2$, 5 mM DTT, 0.3 mM ATP, 0.05 mM each of dATP, dCTP, dGTP and 2-^{14}C-TTP (specific activity 10 mCi/mM) and 100 µg crude enzyme protein preparation (12). To one set of samples heat denatured calf thymus DNA was added to the final concentration of 100 µg/ml. After incubation at 37°C for 1 hour the reaction was terminated by adding 0.5 ml 10% cold TCA. The TCA-insoluble radioactivity was collected on Whatman Glassfibre GF/c filter paper discs (2.5 cm dia.) washed subsequently with 10% cold TCA, ethanol and acetone and dried. Radioactivity on each sample was determined as described earlier.

RESULTS

Effect of gamma radiation on the growth of M.radiophilus

As seen in Fig. 1 gamma radiation at the dose of 200 krad - which is a non-lethal dose - resulted in the lower rate of growth of M.radiophilus cells in TGYM medium. Thus although this radiation dose - which is quite high when compared with its effect on viability of E.coli cells - is in the shoulder region of the survival curve, its effect on cell division is quite significant. Such lower cell growth can be interpreted as the consequence of division delay and may be attributed to the temporary impairment of synthesis of proteins concerned with cell division.

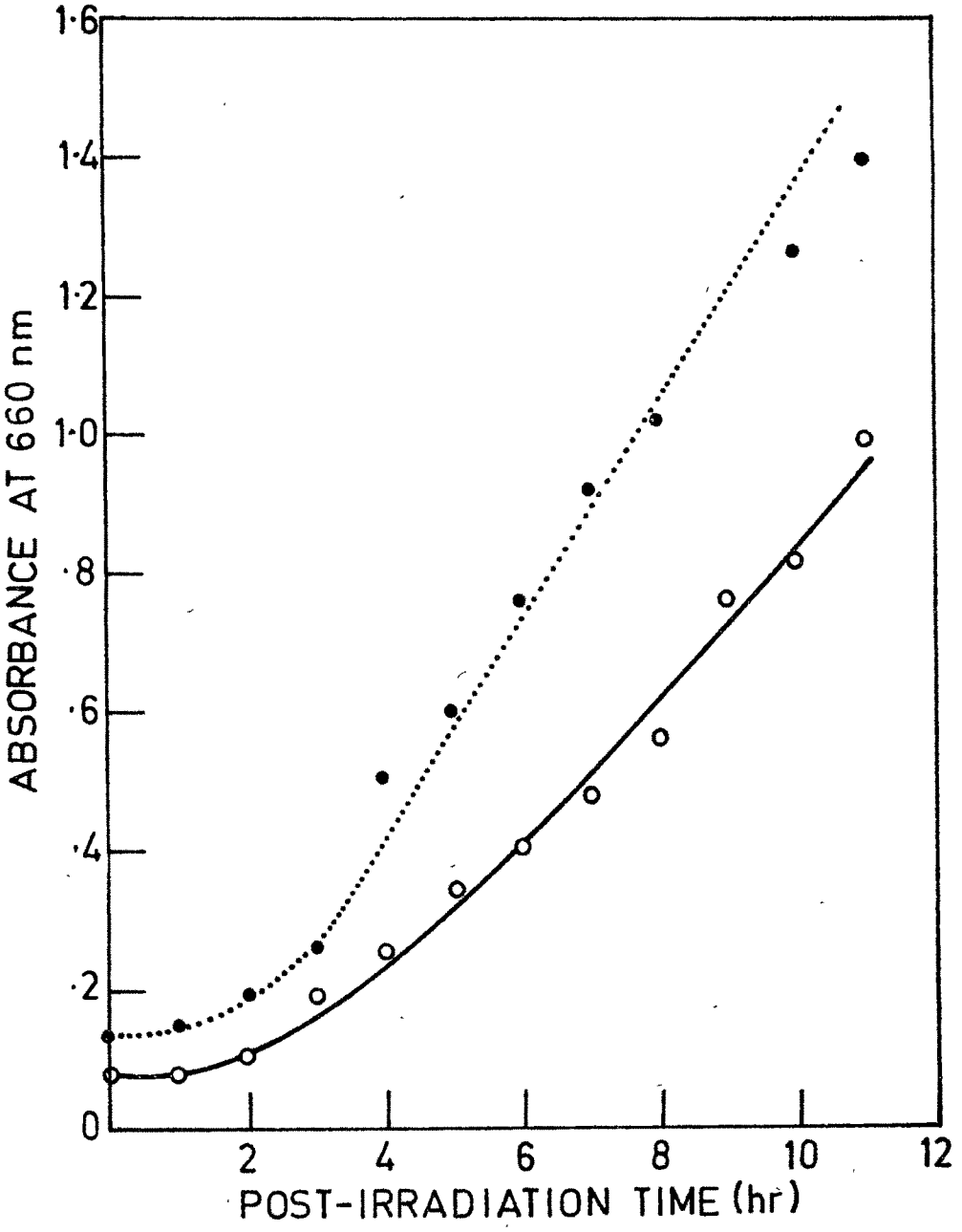

Figure 1. Growth of M.radiophilus cells after exposure to gamma radiation. Log phase cells of M.radiophilus were suspended in 0.1 M sodium phosphate buffer pH 7.0 at the density of 1 x 10^8 cells/ml and exposed to 200 krad gamma radiation. The irradiated cells and unirradiated control cells were incubated at 30°C in TGY broth and the absorbance at 660 nm of the cultures was measured at various intervals. (...●...), unirradiated; (-o-), 200 krad.

DNA degradation during post-irradiation incubation

Experiments were conducted to examine degradation of DNA in M.radiophilus cells (whose DNA was pre-labelled with ^{3}H-thymidine) after exposure to gamma radiation, MMS treatment and UV irradiation. The results are shown in Figs. 2, 3 and 4. As will be seen in Fig. 2 and 3, at the doses of gamma radiation (200 krad) and MMS (50 mM) which lie in the shoulder regions of the respective survival curves (see chapter II), the DNA degradation during the post-irradiation/treatment incubation of 3 to 4 hr was negligible. On the other hand, following exposure of the cells to UV radiation in the shoulder region (630 J/m^2) the DNA degradation during post-irradiation incubation was quite substantial (18% of the total cellular DNA). When the cells were exposed to gamma radiation at the dose of 500 krad - the D_{10} value - the DNA degradation was found to be extensive amounting to about 28% of the total cellular DNA. The UV radiation dose of 1260 J/m^2 (D_{10} value) resulted in drastic degradation of DNA to the extent of 44% of the total DNA. These results indicate that the process of DNA degradation is stimulated to different degrees by different cellular DNA damaging agents.

Macromolecular synthesis in M.radiophilus after exposure to DNA damaging agents

Experiments were conducted to see if high doses of gamma radiation, MMS and UV radiation have any effect on DNA-dependent cellular processes namely,

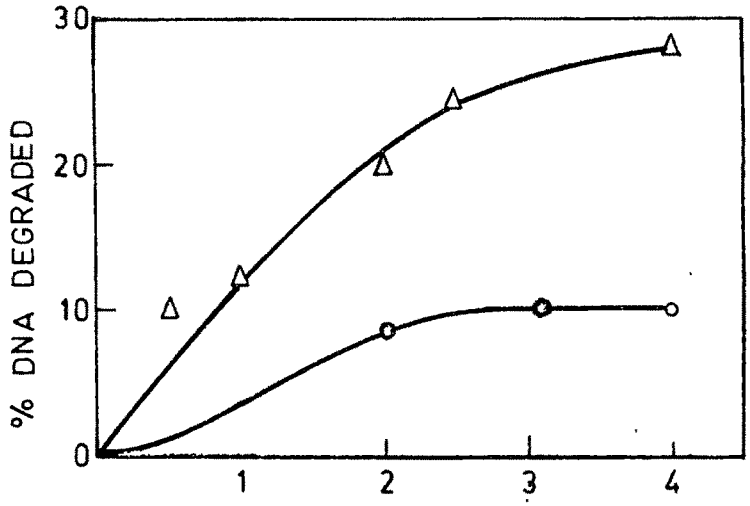

Figure 2.

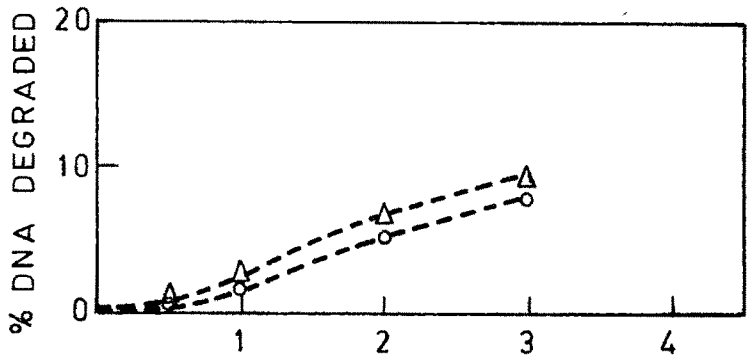

Figure 3.

Fig.2. DNA degradation in gamma-irradiated M.radiophilus. The DNA of M.radiophilus was labelled by growing in TGYM broth containing ^{3}H-thymidine (15 µCi/ml; sp. activity 6000 mCi/mM). Cells harvested from log phase cultures were exposed to various doses of gamma radiation, washed and incubated in TGYM broth at the density of 10^{8} cells/ml at 30°C. Aliquots of 0.2 ml were removed at different intervals and the TCA insoluble radioactivity was determined. Per cent loss of TCA insoluble radioactivity is considered as the per cent DNA degraded.
(-o-), 200 krad; (-Δ-), 500 krad.

Fig. 3. DNA degradation in MMS-treated M.radiophilus. The DNA of M.radiophilus was pre-labelled with ^{3}H-thymidine as described in Fig.2. Cells were suspended in TGYM broth (10^{8} cells/ml) containing various concentrations of methyl methanesulphonate (MMS). The MMS-treated cells were washed free off MMS, incubated in TGYM broth and DNA degradation was followed at various times as described in Fig. 2.
(--o--), 10 mM; (--Δ--), 50 mM.

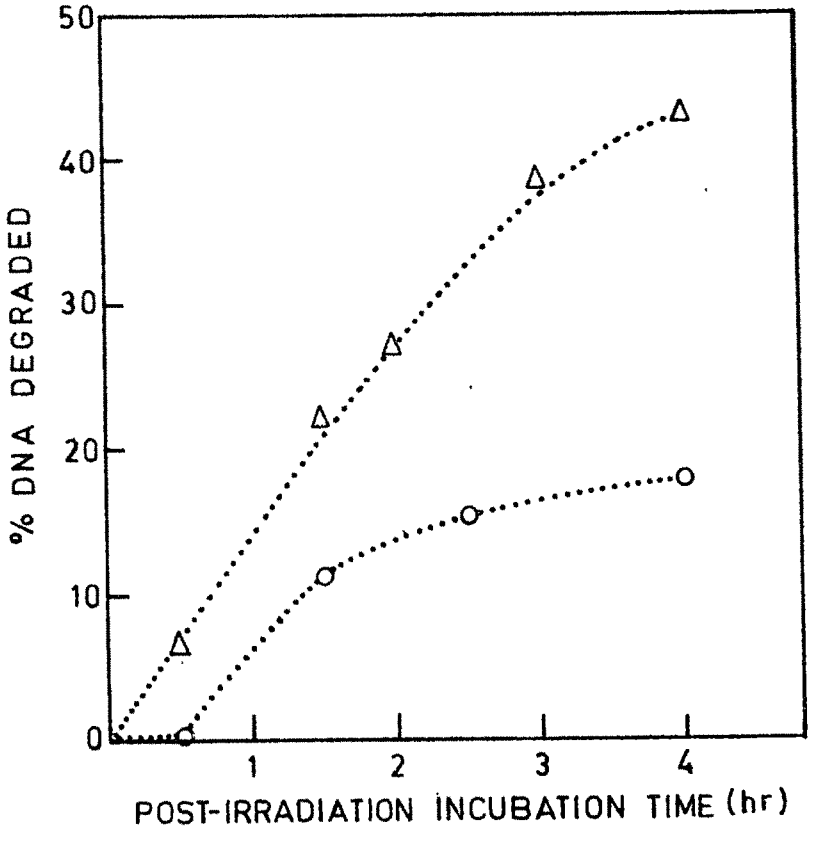

Figure 4. DNA degradation in UV irradiated M.radiophilus. The DNA of M.radiophilus was pre-labelled with ^{3}H-thymidine as described in Fig.2. Aliquots of 7 ml cell suspensions (10^{8} cells/ml) were exposed to various doses of UV radiation in glass petri dishes (9 cm dia.). After UV exposure cells were incubated in TGYM broth and degradation of DNA during post-irradiation incubation was determined as described in Fig.2. (..o..), 630 J/m^{2}; (..Δ..), 1260 J/m^{2}.

DNA, RNA and protein syntheses. The results are illustrated in Fig. 5, 6 and 7. It will be seen that gamma irradiation results in reduction in the syntheses of DNA, RNA and protein (as studied by following incorporation of radioactive precursors for 4 hr).even at the doses within the shoulder region. RNA synthesis seems to little more sensitive than DNA synthesis at the doses up to 250 krad but beyond this dose range DNA synthesis shows a considerable reduction. The patterns of inhibition of the macromolecular syntheses in MMS treated and UV irradiated cells are similar to those of gamma irradiation excepting that at all the doses studied DNA synthesis is more affected than RNA synthesis which is more affected in turn than protein synthesis.

Since RNA synthesis was affected to a greater degree than DNA synthesis in cells irradiated at 200 - 250 krad dose range it was thought of interest to examine the synthetic rate of RNA at different post-irradiation time by pulse-labelling of RNA for 2 min. The results are illustrated in Fig. 8. The results clearly show that RNA synthesis (chain-initiation as well as chain-elongation) is affected considerably and the restoration of the synthetic rates is not apparent even during incubation for 120 min.

The most intriguing aspect of the results mentioned above is that the three macromolecular syntheses

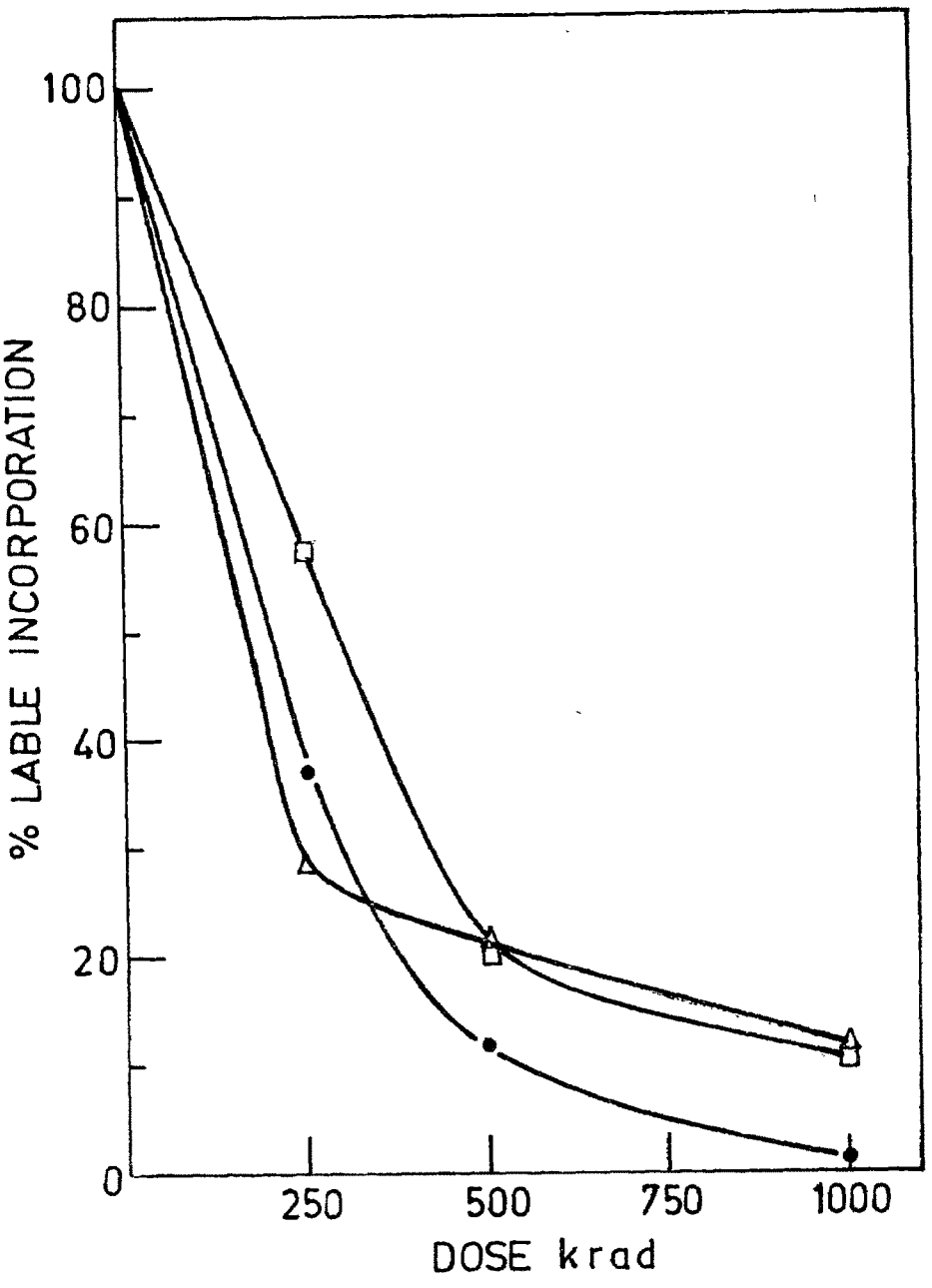

Figure 5. Effect of gamma irradiation on macromolecular syntheses in M.radiophilus cells. Cell suspensions (10^8 cells/ml) were exposed to different doses of gamma radiations and incubated in TGYM broth containing the radioactive precursor (15 μCi/ml): ^{3}H-thymidine, ^{3}H-uridine and ^{14}C-leucine, respectively, for studying the synthesis of DNA, RNA and protein. At the end of 4 hr incubation aliquots were removed for determination of radioactivity in respective macromolecules.(-●-), DNA; (-△-), RNA; (-□-), protein.

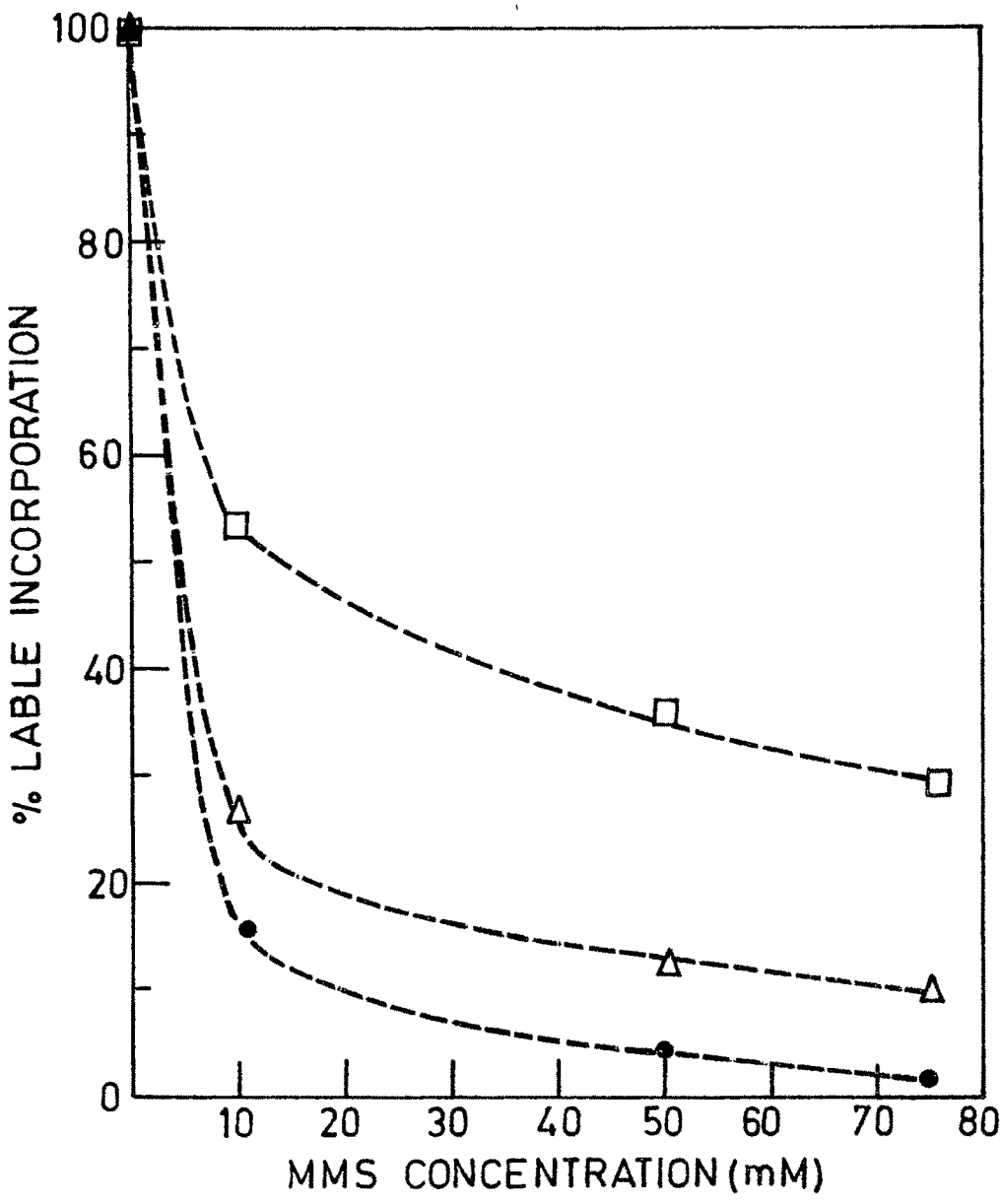

Figure 6. Effect of methyl methane sulphonate on DNA, RNA and protein syntheses in M.radiophilus. Log phase cultures of M.radiophilus were harvested, washed and suspended in TGYM broth (10^8 cells/ml) containing 10 mM MMS. Cultures were washed free off MMS and syntheses of macromolecules were followed by determining incorporation of radioactive precursors as explained in Fig. 6. (--●--), DNA; (--△--), RNA; (--□--), protein.

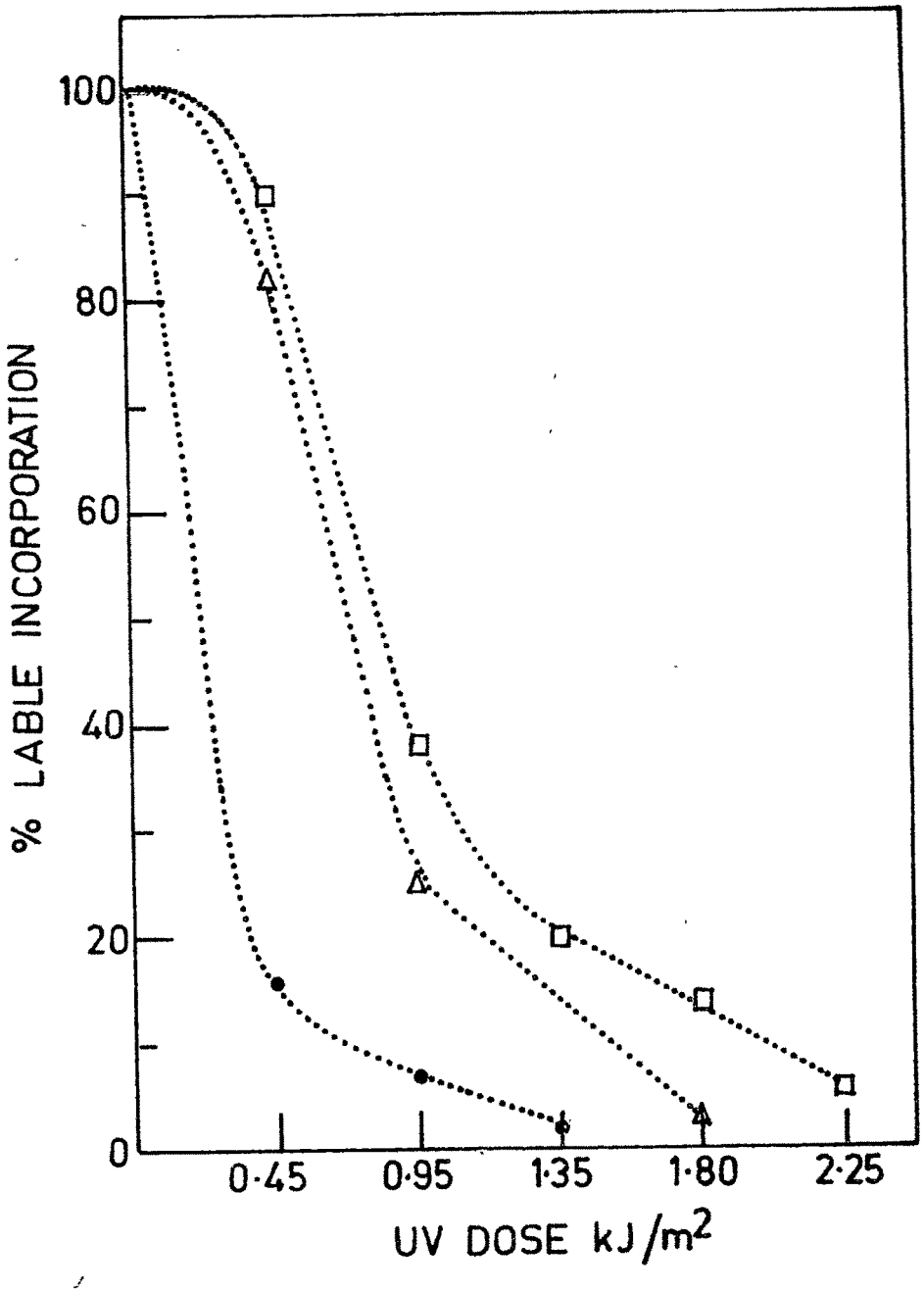

Figure 7. Effect of UV radiations on DNA, RNA and protein syntheses in M.radiophilus. Log phase cells were harvested, washed and suspended in 0.1 M phosphate buffer at 10^8 cells/ml. Aliquots (7 ml) layered in open petri dishes were exposed to various doses of ultraviolet radiation. The syntheses of macromolecules were followed by determining incorporation of radioactive precursors as described in Fig. 5. (..●..), DNA; (..△..), RNA; (..□..), protein.

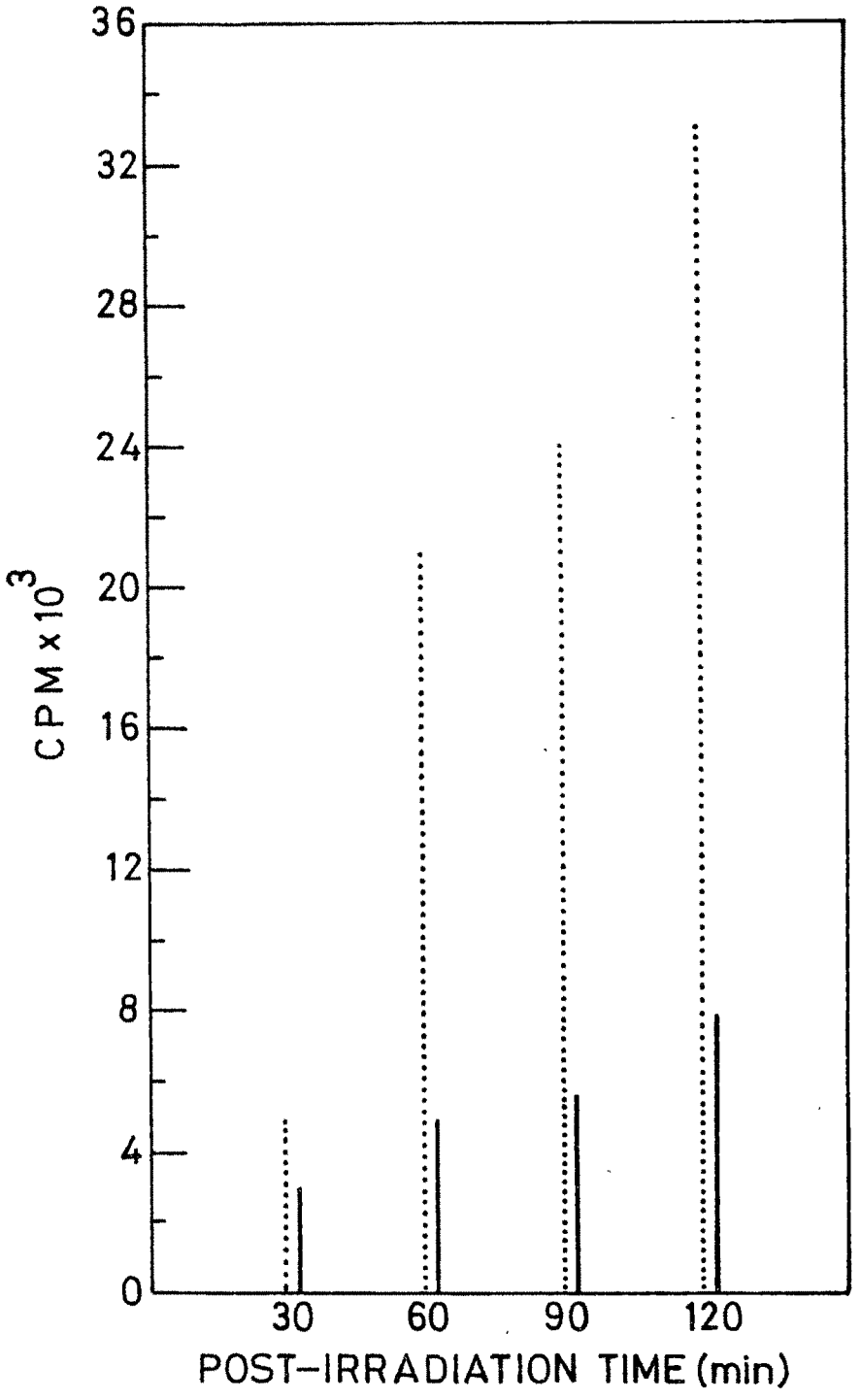

Figure 8. RNA synthetic rates in 200 krad gamma-irradiated cells of M.radiophilus during post-irradiation incubation. Log phase cells of M.radiophilus were suspended in 0.1 M phosphate buffer (pH 7.0) at the density of 10^8 cells/ml and exposed to 200 krad. The irradiated cells and unirradiated control cells were incubated in fresh TGYM broth. Aliquots of 0.5 ml of cell cultures were removed at different intervals and transferred to 0.5 ml TGYM broth containing 15 μCi/ml of ^{3}H-uridine. After 2 min pulse, the incorporation was stopped by adding cold 10% TCA. The precipitates were collected on Whatman No.3 paper discs and radioactivity counted. ....., Control; ———, 200krad.

are quite resistant at the doses which drastically affect these syntheses in moderately resistant bacterium like E.coli. The results of an experiment on the reduction of macromolecular syntheses in gamma irradiated E.coli along with the reduction in survival are compiled in Table I. For comparison the results of the experiments described above on gamma irradiated M.radiophilus are also tabulated in Table II. Thus although the macromolecular syntheses in M.radiophilus are unquestionably more resistant to gamma radiation compared to those in E.coli, it will be apparent that for a given survival fraction (for example 10% survival), the reduction in macromolecular synthesis is considerably more in M.radiophilus compared to E.coli.

Effect of gamma irradiation on M.radiophilus and E.coli DNA polymerases in vitro

Since DNA synthesis in M.radiophilus cells showed considerable resistance to gamma radiation compared to that in E.coli, an experiment was designed to ascertain whether the DNA polymerisation enzymes in M.radiophilus were also more resistant to gamma irradiation in vitro compared to similar enzyme from E.coli. Sonicated lysates of M.radiophilus and E.coli were prepared by converting the cells to spheroplasts using lysozyme and subjecting the spheroplasts so prepared to ultrasonication. These lysates were exposed to 200 krad gamma radiation and then assayed for DNA polymerase activity with and

Table I

REDUCTION IN SURVIVAL AND MACROMOLECULAR SYNTHESES IN E.COLI EXPOSED TO GAMMA RADIATION

Dose	Survival	Synthesis of: DNA	RNA	Protein
(krad)	(Per cent reduction)			
30	90	66	10	52
60	99	79	75	64
90	99.9	97	83	74

Table II

REDUCTION IN SURVIVAL AND MACROMOLECULAR SYNTHESES IN *M. RADIOPHILUS* EXPOSED TO GAMMA RADIATION

Dose	Survival	Synthesis of DNA	Synthesis of RNA	Synthesis of Protein
(krad)	(Per cent reduction)			
250	0	62	65	42
500	90	87	67	67
750	99.5	95	83	86
1000	99.998	98.5	88	91

without exogenous addition of calf-thymus DNA. The requirements for the DNA polymerase assay are given in Table III. It will be seen that DNA polymerase activity is totally absent in the absence of any of the four deoxyribonucleoside triphosphate ~~nucleotide~~ precursors. However, even without exogenous addition of DNA substantial activity is apparent with E.coli and M.radiophilus cell extracts. This would suggest that the enzyme uses endogenous DNA as its template. Addition of exogenous heat-denatured calf-thymus DNA nevertheless causes further stimulation in the enzyme activities. The results on the effect of gamma irradiation on *in vitro* DNA polymerase activities are given in Table IV. Irradiation apparently does not inhibit DNA polymerase activities from both the organisms. On the other hand this treatment enhances the activities of both the polymerases as studied in the presence or absence of exogenous DNA. Probably this increase is the consequence of gamma radiation-induced strand breaks in DNA by creating new non-specific initiation sites as suggested by the work of Billen and Hewitt in E.coli (13) and Matsuyama in permeabilised cells of M.radiodurans (14). However the striking increase in the gamma irradiated E.coli DNA polymerase after the addition of exogenous DNA remains unexplained. Further work with purified DNA polymerases could throw more light on this puzzling observation.

Table III

DNA POLYMERASE ASSAY OF CELL-FREE EXTRACTS FROM E.COLI AND M.RADIOPHILUS

E.coli	p moles of ^{14}C-TMP incorporated
Complete*	327
- Cell free extract	0.0
- dATP	0.0
- Calf thymus DNA	201
M.radiophilus	
Complete*	159
- Cell free extract	0.0
- dATP	0.0
- Calf thymus DNA	115

* The complete assay systems contained 5 mM Tris-HCl buffer pH 7.8, $MgCl_2$ 8 mM, DTT 5 mM, ATP 0.33 mM, dATP 50 mM, dGTP 50 mM, dCTP 50 mM, ^{14}C-TTP 50 mM, heat-denatured calf thymus DNA 100 µg/ml and bacterial cell-free extracts equivalent to 100 µg protein. The reaction was carried out for 1 hr at 37°C.

Table IV

DNA POLYMERASE ACTIVITY BY SONICATED CELL-FREE EXTRACTS OF M.RADIOPHILUS AND E.COLI: EFFECT OF GAMMA IRRADIATION

Source	Dose (krads)	DNA polymerase activity *	
		- Calf thymus DNA	+ Calf thymus DNA
E.coli	0	201	327
	200	524	823
M.radiophilus	0	116	159
	200	322	326

* p moles of ^{14}C-TMP incorporated into DNA under the assay conditions.

Cells were suspended in 0.06 M Tris-HCl buffer (pH 7.6) at the density of 10^9 cells/ml, spheroplasts were prepared with lysozyme subjected to ultrasonication and the lysates were exposed to gamma rays in air at 0°C.

Effect of gamma radiation *in vivo* and *in vitro* on carotenoid pigments of *M.radiophilus* cells

The loss of carotenoid pigments in *M. radiodurans* and *M.radiophilus* cells after exposure to sub-lethal doses of gamma radiation is a well known phenomenon (10). Studies were devoted to examine this phenomenon further in *M.radiophilus*. ~~further~~. The pigments of *M.radiophilus* are a group of carotenoids similar to those in *M.radiodurans* though on thin-layer chromatography the pigments of the two organisms show distinct differences (15). The radiation-induced loss of the β-carotene has been suggested to be due to destruction of the conjugated double-bonds (15). The loss of carotenoid pigments from *M.radiophilus* cells upon the exposure to various doses of gamma radiation is depicted in Fig. 9. The loss of pigments at 200 krad is to the extent of 35 - 40% and at the dose of 1000krad the reduction in the carotenoid pigment is to the extent of 85% (see also Fig. 12). The loss in the pigment is instantaneous which implies that the mechanism is probably photo-chemical destruction of β-carotene rather than some indirect (enzymatic ?) action. In further experiments it was shown that gamma irradiation *in vitro* of carotenoid pigments extracted from *M.radiophilus* (in chloroform : methanol) resulted in almost 80% loss of the pigment (see Fig. 12). This result implied that as compared to gamma irradiation *in vivo*, gamma irradiation of pigments *in vitro* is far more effective in the destruction of carotenoids suggesting

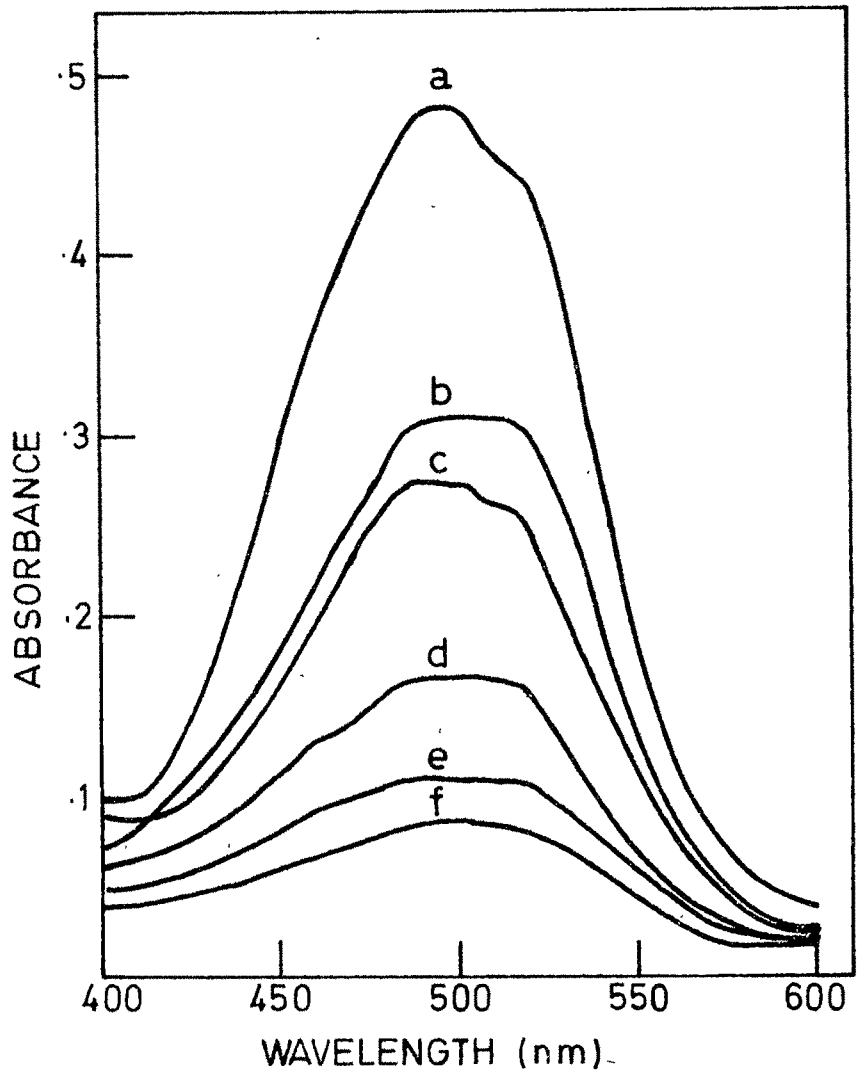

Figure 9. Effect of gamma radiation of M.radiophilus cells on carotenoid pigments. Log phase cells were harvested, washed and suspended in 0.1 M phosphate buffer (pH 7.0) at the density of 10^9 cells/ml and exposed to different doses of radiation. Carotenoid pigments from unirradiated and irradiated cells were extracted with chloroform : methanol (2:1 v/v) mixture at 4°C. Spectral analyses of the extracts were carried out in a Shimadzu spectrophotometer (MPS50L) using the same solvent as the reference. (a), 0 krad; (b), 200 krad; (c), 400 krad; (d), 600 krad; (e), 800 krad; (f), 1000 krad.

that the cellular environment could afford some protection. Since M.radiophilus is a highly radio-resistant bacterium, it was thought of interest to ascertain whether a bacterium with normal radio-susceptibility could also offer similar protection to its carotenoid pigments against radiation onslaught. For this purpose, a strain of Micrococcus luteus was chosen. The pigments of this lemon-yellow coloured organism are also composite carotenes though differ from those of M.radiodurans and M.radiophilus (16). The effect of radiation on the pigments of this organism are depicted in Fig. 10. It will be seen that the spectral characteristics of M.luteus significantly differ from those of carotenes from M.radiophilus. At 15 krad the loss of pigment (in vivo) is about 30% and at about 90 krad about 75% of carotenoids are destroyed. In vitro irradiation of the carotenoids extracted from M.luteus (in methanol) (Fig. 11) indicate that carotenoid pigments of this organism are more or less equally sensitive to those of M.radiophilus irradiated in vitro (see also Fig. 12). Thus as seen in Fig. 12 M.radiophilus afford much greater protection to its carotenoid pigments against gamma-irradiation than M.luteus.

Regeneration of carotenoid pigments in gamma irradiated M. radiophilus cells

The next set of experiments were designed to study the extent of regeneration of carotenoid pigments in M.radiophilus cells. For this

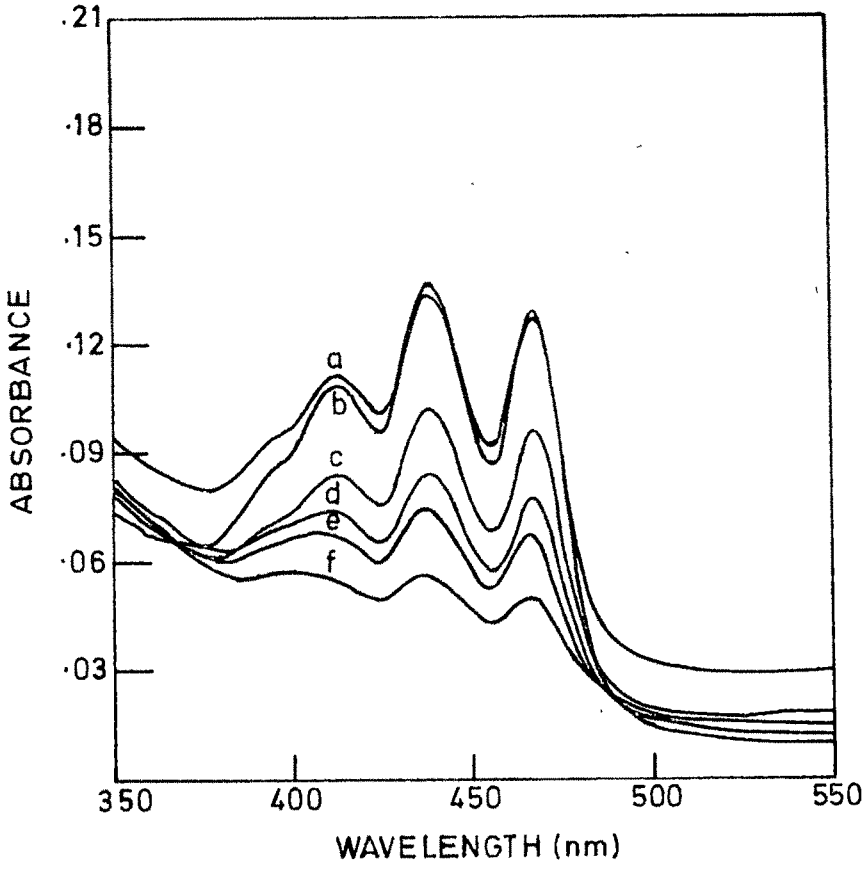

Figure 10. Effect of gamma radiation of M.luteus cells on carotenoid pigments. Log phase cells were suspended in 0.1 M sodium phosphate buffer pH 7.0 at the density of 10^9 cells/ml and aliquots (5 ml) were exposed to different doses of gamma radiation. The pigments were extracted from irradiated and unirradiated control cells with methanol at 4°C and spectral analyses were carried out in a Shimadzu spectrophotometer. (a), unirradiated; (b), 15 krad; (c), 30 krad; (d), 45 krad; (e), 75 krad; (f), 90 krad.

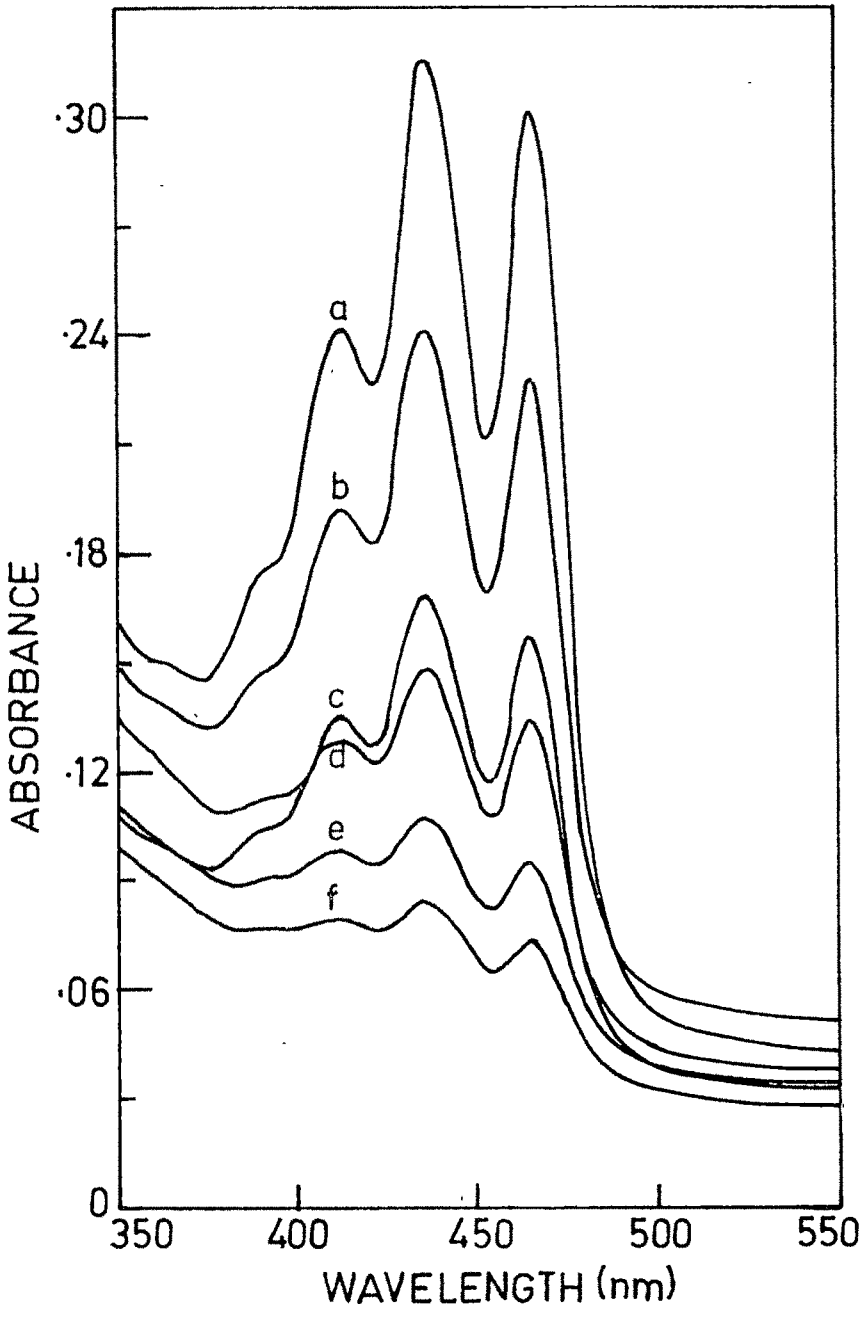

Figure 11. Effect of gamma radiation in vitro on carotenoid pigments isolated from M.luteus. The pigments from 50 ml log-phase cultures of M.luteus were extracted as described in the text and aliquots of the solution of the extracted pigments were exposed to gamma radiation at different doses and spectral analyses were carried out. (a), unirradiated; (b), 15 krad; (c), 30 krad; (d), 75 krad; (e), 90 krad; (f), 120 krad.

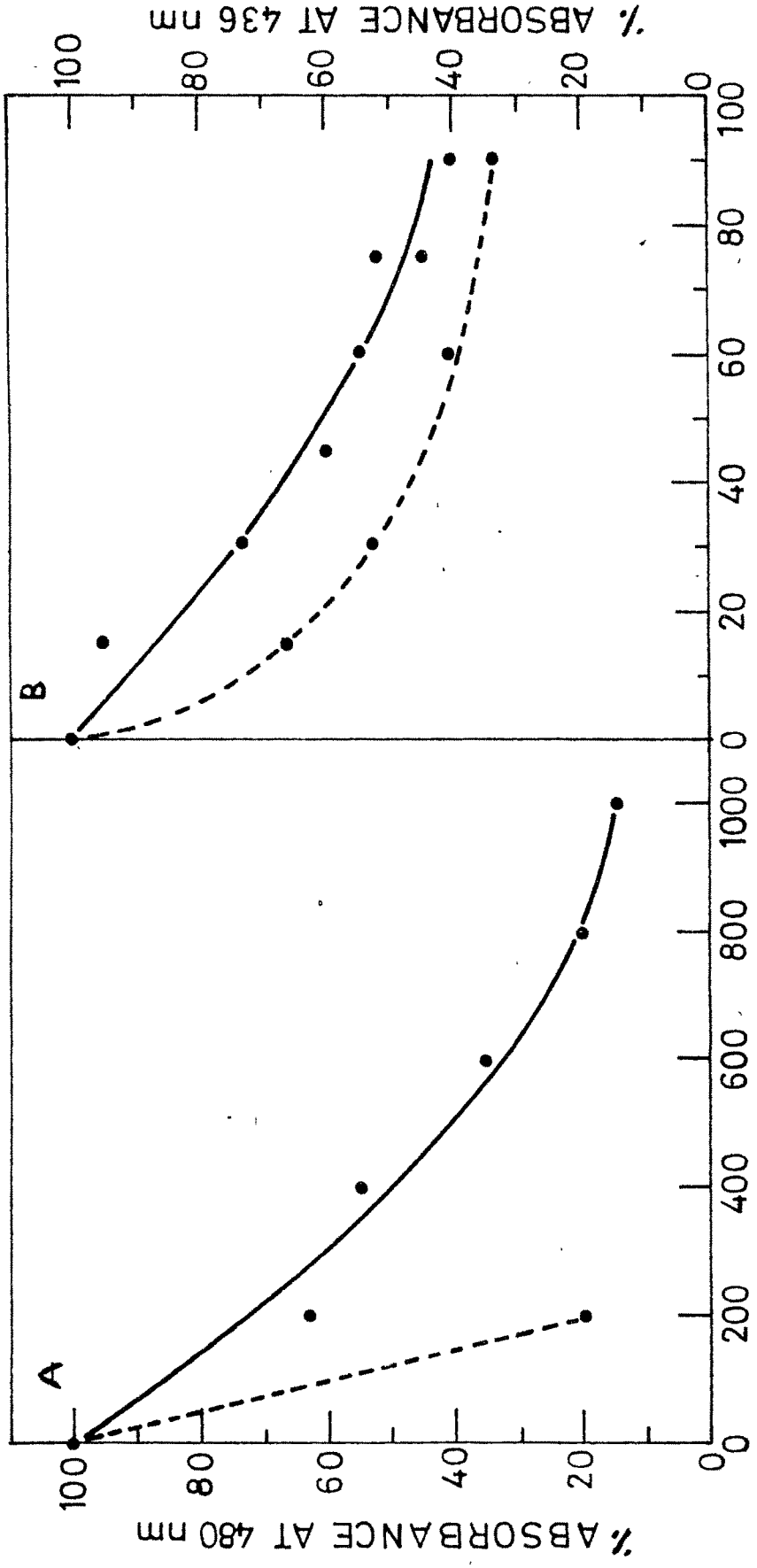

Figure 12. Effect of gamma irradiation in situ and in vitro on destruction of carotenoid pigments of M.radiophilus (A), and M.luteus (B). (-●-), in situ; (-·●·-), in vitro.

purpose irradiated cells were incubated in TGYM broth and reappearance of carotenoids was followed. As seen in Fig. 13 and Table V 20 - 25% of the pigments are destroyed at 100 krad and after post-irradiation incubation for 15 min there is a complete recovery. In fact incubation of these cells for further 30 min resulted in the synthesis of pigments to the extent of 130% over the unirradiated controls. The recovery of pigments could be observed even after the dose of 500 krad but some reduction in the rate of carotenoid synthesis is apparent. It is further seen that while chloramphenicol does not arrest the recovery of pigments in the cells irradiated at 200 krad, a partial inhibition in the regeneration process is discernible in the case of 500 krad irradiated cells. These results suggest that irradiation may have inactivated some enzymes in the carotenoid synthetic pathway and that the recovery of carotenoid in 500 krad-irradiated cells could be associated with the de novo synthesis of these enzymes.

In the next set of experiments the factors contributing to the regeneration of carotenoid pigments in irradiated cells were investigated. The regeneration could simply be the manifestation of carotenoid turnover, i.e., synthetic and breakdown of the pigments. To ascertain whether this is so, M. radiophilus cells were incubated in the presence of nicotine. This alkaloid has been shown to inhibit the enzymes involved in the cyclization steps in

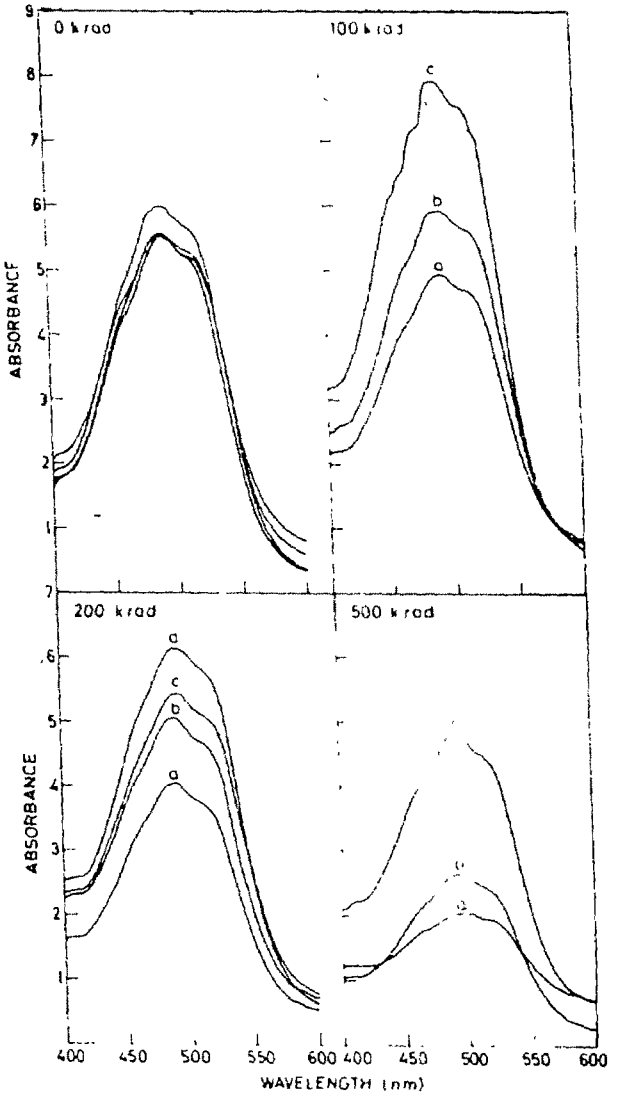

Figure 13. Regeneration of carotenoid pigments in M.radiophilus during post-irradiation incubation in growth medium. Log phase cells were harvested, washed and suspended (10^9 cells/ml) in 0.1 M phosphate buffer (pH 7.0) and exposed to different doses of gamma radiation, as indicated in the fig. Control and irradiated cells were transferred to TGYM medium and incubated at 30°C. At different intervals, 5 ml aliquots were removed for the determination of cellular carotenoid pigment levels by quantitative extraction and spectral analysis. a - Irradiated; b - Irradiated and incubated for 15 min; c - Irradiated and incubated for 30 min; d - Irradiated and incubated for 60 min.

Table V

REGENERATION OF CAROTENOID PIGMENT IN GAMMA IRRADIATED *M. RADIOPHILUS* CELLS

Post-irradiation incubation in TGYM broth(30°C) (min)	Cellular levels of carotenoids (per cent unirradiated control)*		
	0 krad	200 krad	500 krad
0	100	62.5	40
60	100	100	87
60, with chloramphenicol**	100	100	58

*Carotenoid content of unirradiated cells : 2.5 µg equivalent of β-carotene per 10^9 cells.

**20 µg/ml

the /3-carotene synthetic pathway. It has been demonstrated that growth of M.radiophilus and M.radiodurans cells in the presence of nicotine results in complete bleaching (17). The bleached cells are however as radiation resistant as pigmented cells indicating that carotenoid pigments had no protective effect on M.radiophilus cells (10).

In the present studies, cells were incubated in nicotine to arrest any de novo synthesis of carotenoids. The results illustrated in Fig. 14 indicate that there is an inhibition of carotenoid synthesis after the addition of nicotine. However no loss of pigment already present in the cells could be discernible even during prolonged growth in the presence of nicotine. These results rule out the possibility that the carotenoid pigments, once synthesized, are degraded in the cell. It follows then that the cellular level of carotenoid pigment may be maintained by the feed-back mechanism. The recovery of carotenoid pigments in irradiated cells may then be interpreted as resulting from the removal of /3-carotene which may have (feed-back) inhibitory action on the early enzyme(s) of /3-carotene biosynthetic pathway.

DISCUSSION

The studies presented in this chapter have revealed some significant changes in cellular process

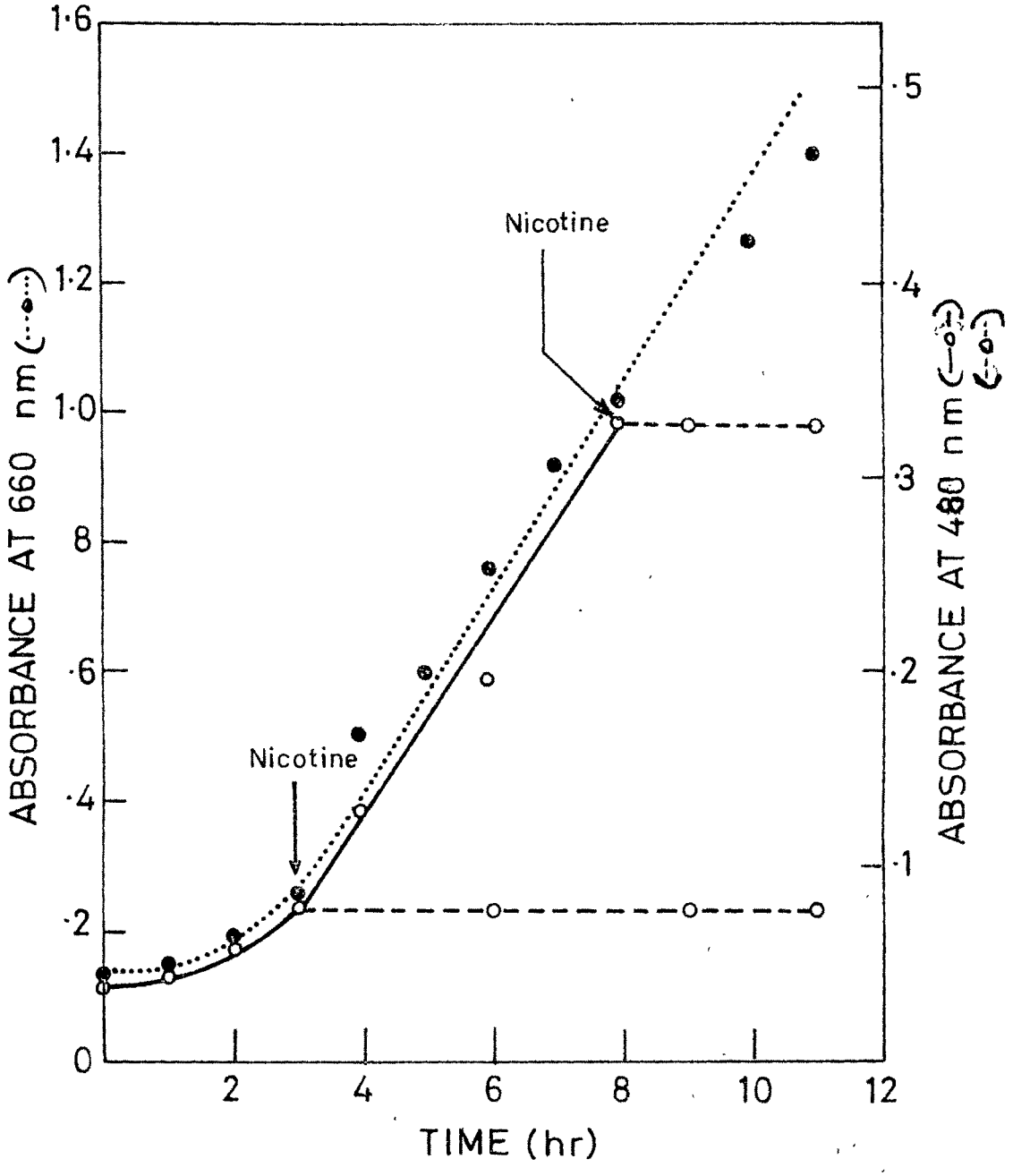

Figure 14. Effect of nicotine on the levels of carotenoid pigments during growth of M.radiophilus. Nicotine (10^{-2} M) was added to growing culture in TGYM broth as indicated in the figure. At various times aliquots of the culture were withdrawn for the determination of cell density (Absorbance at 660) and for the analysis of pigment (extracted as described in Fig. 10, absorbance at 480 nm). Arrows indicate time of addition of nicotine.

that are brought about at very high doses of radiations and DNA acting chemicals. Such studies can be done only in a highly radiation resistant bacterium like M.radiophilus as most other bacteria loose their viability at these doses.

At the doses of gamma/UV-radiation and MMS in the respective shoulder regions, the patterns of DNA degradation are found to be surprisingly different. Whereas in the case of UV radiation a distinct triggering of DNA degradation is observable at a shoulder region dose, in the case of gamma radiation and MMS no degradation is discernible. As described in the introductory chapter, specific enzymes have been identified which recognise different lesions created in DNA. It would seem likely that differences in DNA degradation provoked by different agents may probably be related to the presence or absence of such enzymes. This hypothesis however cannot explain the equally efficient repair of DNA damaged by diverse types of physical and chemical treatments.

At higher doses of UV radiation, gamma radiation and MMS (D_{10} values) extensive DNA degradation is observed and this could be partly related to the loss of cell viability. Needless to say, the doses that are required for substantial DNA degradation in M.radiophilus (30 - 40% degradation) are very much higher than those required in the case of E.coli and most other bacteria.

Studies on gamma/UV radiations and MMS treatment on the macromolecular syntheses demonstrated that these processes are more resistant as compared with those in E.coli. However some interesting aspects have come to light. Thus at equivalent survivals (for example D_{10} values) DNA synthesis in M.radiophilus seems to be more sensitive than that in E.coli. This phenomenon can be explained as follows. Although at higher doses, cellular DNA of M. radiophilus may not have been damaged, some amount of injury would have been inflicted on the non-DNA components leading to impairment in macromolecular syntheses. Such impairment could be due to inactivation of cellular enzymes, damage to energy generating processes (18), damage to membrane and in turn to membrane associated processes (19, 20). The impairment was also apparent in RNA synthesis and there were no recovery signs in this process even at 2 hr after gamma radiation dose in the shoulder region (200 krad). Thus on one hand the cells show phenomenal resistance to radiations and other similar reagents, while on the other, macromolecular syntheses are strikingly inhibited even at non-lethal doses. It may be noted that despite severe impairment in macromolecular syntheses, the cells eventually recover, which is indicative of the operation of some kind of restorative processes. The most plausible mechanism that can be proposed is that the damaged components including enzymes could be replaced by de novo synthesis of

these components. This can be possible only in a resistant microbial cell such as M.radiophilus which after heavy doses of gamma radiation would have either undamaged DNA or a repaired DNA.(22). Some degree of evidence to such a proposition comes from the experiments on regeneration of carotenoid pigments in M.radiophilus cells exposed to 500 krad gamma radiation. The recovery of this pigment during post-irradiation incubation requires protein synthesis as evidenced from studies with chloramphenicol.

Although gamma radiation dose of 200 krad substantially inhibited DNA synthesis (as also syntheses of other macromolecules) in M.radiophilus cells, the same dose given in vitro to the cell-free extract of M.radiophilus had no effect on DNA polymerase activity. Thus impairment of some other important cellular function, for example, energy production, may be instrumental in the observed impairment of DNA synthesis in gamma irradiated M.radiophilus cells. Further some cellular factors elaborated as a result of gamma radiation may be responsible for impaired synthesis of DNA and other macromolecular syntheses. It is a common observation reported in many studies that certain cellular components are more resistant to in vitro irradiation as compared to in vivo irradiation. Thus it has been shown in this laboratory that mitochondria of Tetrahymena pyriformis are far more resistant in vitro than in vivo (23). Also it has been reported in this laboratory that

lipopolysaccharidal endotoxin of Salmonella typhimurium is more sensitive to gamma radiation in vivo than in vitro (24). Lysosomal structures in mammalian cells have also been found to be more sensitive to radiation in vivo than in vitro (25).

The studies on the radiation-induced loss of carotenoid pigments have led to some new insights into the recovery processes as well as in the regulation of cellular processes. Carotenoid pigments were susceptible to gamma radiation at the doses in shoulder region and hence one would have expected that the presence of such a radiation susceptible component might mitigate to a certain extent the radiation injury to the cell. However it appears that carotenoid pigments do not afford any protective effect on M.radiophilus cells against gamma radiation attack. The experiments on regeneration of carotenoid pigments together with those with nicotine- an inhibitor of the cyclization step in β-carotene synthesis (17) - provide compelling evidence to suggest that the recovery of carotenoid pigment in gamma irradiated cells is due to the lifting of the normal feed-back inhibition of β-carotene synthetic pathway by its final product rather than the result of the turnover of carotenoid pigments. Indeed experiments with nicotine clearly show that there is no such turnover of carotenoid pigments.

It has already been mentioned in the earlier part of the discussion that the regeneration of carotenoid pigments in cells irradiated at 500 krad (D_{10} dose), but not at 200 krad, partly requires concomitant protein synthesis. This requirement seems to be due to the inactivation of some of the enzymes of carotene biosynthesis. The inactivation at 500 krad would seem not to be a phenomenon of specific inactivation but general inactivation of cellular proteins and other components. This can be surmised from the observation that macromolecular syntheses were severely impaired at this dose.

One very interesting phenomenon that came to light in the present study is that carotenoid pigments extracted from M.radiophilus were far more sensitive to radiation in vitro compared to ~~when~~ carotenoids irradiated with in the cells. Conceivably the cellular environment seems to exert a kind of protective effect on radiation-induced destruction of carotenes. When similar carotenoid pigment extracted from the radiation sensitive M.luteus was examined for its radiosusceptibility in vitro and in vivo, another interesting phenomenon was noticeable. It was found that the sensitivity of carotenoid pigments of M.luteus to gamma irradiation in vitro was comparable to that of M.radiophilus carotenoid pigments. In contrast to the results of M.radiophilus carotenoid pigment irradiated in vivo, carotenoid pigments of M.luteus could be protected only marginally by the cellular

environment against radiation onslaughts. These results would imply that M.radiophilus cells may contain a component, probably a protein, which may protect carotenoid pigment from radiation attack and that the radiosensitive M.luteus may not be endowed with such kind of protective components. It would be of interest to know whether the radiation resistant microorganism such as M.radiophilus also has components (either in greater number or in greater protective activity) which protect a number of other sensitive constituents (including perhaps DNA ?) from high doses of radiation and presumably also from high doses of other DNA damaging agents.

The final chapter of the thesis is devoted to understanding the mechanism by which M.radiophilus cell manages to protect or repair damage to its cellular DNA inflicted by a variety of stresses.

SUMMARY

Studies were conducted on the effects of high doses of gamma radiation, UV radiation and MMS treatment on various cellular processes in M.radiophilus a bacterium found to be highly resistant to radiations and other DNA acting agents. These treatments affected the process of DNA degradation in a differential manner. While gamma radiation and MMS treatment (in the shoulder regions of respective survival curves) caused no appreciable DNA degradation, UV irradiation (in the shoulder region) induced extensive DNA degradation. The doses required for the inhibition of macromolecular syntheses in M.radiophilus were much higher (~~for~~ e.g. 200 krad gamma radiation) as compared to those reported to be required for the inhibition of macromolecular syntheses in E.coli (30 krad). However the results indicated that for a given reduction in survival, macromolecular syntheses were inhibited to a greater extent in M.radiophilus than in E.coli. These results reveal that at high radiation doses other non-DNA cellular components could have been affected. Since however the cells do not seem to lose their viability at these doses, it may be inferred that the inactivated non-DNA components could have been replaced by de novo syntheses. Other studies revealed that although DNA synthesis in M.radiophilus cells is greatly impaired at the shoulder region dose of 200 krad gamma radiation, this dose does not inactivate DNA polymerase in vitro.

Gamma radiation at 100 to 500 krad range resulted in the progressive loss of carotenoid pigments in M. radiophilus. The carotenoid pigments extracted from M.radiophilus cells by chloroform : methanol mixture (2:1) contained predominantly /3-carotenes. The pigment was found to be much more sensitive to gamma irradiation in vitro compared to gamma irradiation in situ, indicating that the cellular environment of M.radiophilus affords a kind of protection to the pigment from radiation. Similar studies were also conducted on a carotenoid pigment of M.luteus. This pigment had spectral properties similar to but not identical to carotenoid pigments of M.radiophilus. The studies reveal that while the sensitivity of this pigment to gamma irradiation in vitro was similar to that of M.radiophilus in vitro the cellular environment of M.luteus afforded only a marginal protection to its pigment from gamma radiation attack.

Post-irradiation incubation of M.radiophilus cells led to a rapid regeneration of the carotenoid pigment. The recovery of the pigment in the cells irradiated up to 200 krad did not require de novo synthesis of protein whereas that in 500 krad irradiated cells required synthesis of proteins. Addition of nicotine, a known inhibitor of the cyclising steps in /3-carotene pathway, to growing cells of M.radiophilus stopped synthesis of carotenoid pigments without affecting cell growth. The nicotine addition however did not result in the disappearance of carotenoid

pigment already synthesized. Taken together these results would imply that the regeneration of carotenoid pigments seen during post-irradiation incubation is the case of removal of feed-back inhibition of the β-carotene pathway rather than manifestation of the cellular turnover of carotenoids.

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