AN ABSTRACT OF THE THESIS OF

(Major professor)

An ecological study of a microorganism similar to that isolated in 1956 and designated as <u>Micrococcus radiodurans</u> indicated that the organism could be isolated from several different environments. By taking advantage of its radio-resistivity, high levels of gamma radiation were used in the isolation procedures to inactivate most of the non-resistant microflora. By this means, microorganisms resembling <u>M. radiodurans</u> were isolated from ground beef and pork sausage as well as from beef hides and water from Cox Creek (Albany, Oregon). Similar organisms could not be isolated, however, from soil, hay or fecal material. All of the environments tested were in the vicinity of the Nebergall Packing Plant, Albany, Oregon.

Pre-incubation and pre-inoculation experiments indicated that <u>M. radiodurans</u> did not compete well with the natural microflora present in meat and soil samples. The use of enrichment culture techniques and incubation of the samples before irradiation did not facilitate the isolation of M. radiodurans.

The presence of NZ-Case (a tryptic digest of casein) in the isolation medium produced more luxuriant growth than TGYM medium but the radiation sensitivity of <u>M</u>. <u>radiodurans</u> increased ten-fold in the former medium and the degree of pigmentation was less. Subsequent studies using thin layer chromatography indicated that the less resistant strain was lacking two of the eight pigments present in the more resistant culture.

The degree of resistance was inversely related to the concentration of NZ-Case in the growth medium at all of the levels tested. At the highest level of NZ-Case used (0.5 percent), the LD₅₀ dose was 350 k rads as compared to 700 k rads when TGYM was the growth medium. The resistance to ultraviolet radiation was also reduced.

Removal of NZ-Case from the growth medium resulted in a return of the high level of resistance and the reappearance of the missing pigments. A possible role of the pigments in the radiation resistance of this organism was thus suggested and described. In this role the pigments would serve as scavengers of toxic ions and radicals produced by radiation and thus protect the functionally important lipids and lipoproteins of the cell membranes from being altered with resultant release of cellular enzymes.

Spectral analysis of the eight pigments indicated that they were all closely related and were tentatively identified as lycopene, hydroxy lycopene or di-hydroxy lycopene. None of the pigments absorbed in the ultraviolet range. Pigments obtained from cells grown in NZ broth had the same spectral characteristics but were present in reduced concentrations.

SIGNIFICANCE OF PIGMENTS IN RADIATION RESISTANCE OF MICROCOCCUS RADIODURANS

by

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SIGNIFICANCE OF PIGMENTS IN RADIATION RESISTANCE OF MICROCOCCUS RADIODURANS

INTRODUCTION

Since the arrival of the atomic age, the effects of high levels of radiation on biological systems has received considerable attention. The more recent space age has prompted additional studies on the relative resistance of man and other organisms to 'outer space' radiation as attention has been given to the possible presence of forms of life which are highly resistant to radiation. The National Aeronautics and Space Administration (N. A. S. A.) is particularly interested in developing means to protect man from the harmful effects of radiation during outer space travel and in learning more about various possible mechanisms of radiation protection.

Although microorganisms vary greatly in their radiation resistance, in general they are more resistant that multicellular forms of life. This difference in radiosensitivity among microorganisms has been used to calculate variations in target volume and to study differences in their genetic properties such as degree of ploidy. Some of the other factors affecting radiosensitivity have been described, but many are unknown.

Since the discovery (4) of <u>Micrococcus</u> radiodurans, a pigmented, nonspore-forming bacterium showing extreme resistance to radiation, additional studies have been initiated to determine the mechanism(s) of radiation resistance of this and other microorganisms. However, these mechanism(s) which are responsible for the resistance still await discovery.

The original purpose of this investigation was to study the ecology of <u>M</u>. <u>radiodurans</u> and to find a possible source for the bacterium in nature. Upon using different cultural media to isolate this organism from various environments, it was observed that its radiation resistance could be significantly altered by changing cultural conditions. There was also an accompanying change in the degree of pigmentation of this bacterium in different media. This suggested that there was a relationship between pigmentation and radiation resistance.

The pigments of <u>M.</u> radiodurans have been tentatively identified as carotenoid in nature (59, p. 19). It is known that the carotenoid pigments perform an essential physiological function by protecting photosynthetic cells from the deleterious effects of chlorophyll-catalyzed photo-oxidations (17). From this it was considered that the carotenoid pigments of <u>M.</u> radiodurans may also protect but by serving as acceptors of damaging radicals and ions produced by ultraviolet and gamma radiations.

In view of the above information, an investigation was initiated to determine if the presence of specific bacterial pigments could be associated with the characteristic of radiation resistance in <u>M</u>. radiodurans. Such a correlation might help provide an explanation for radiation resistance in this organism.

REVIEW OF THE LITERATURE

Radiation - Use in Food Microbiology

The discovery of x-rays by Roentgen in 1895 was followed shortly by the observation that these rays could penetrate biological material and also bring about the death of living cells if applied for a sufficient length of time. Modern application of this latter observation has been used by medical radiologists to kill malignant cells. One of the first studies on the effects of x-rays on bacteria was published in 1896 (64, p. 307). However this and other reports gave essentially negative results because of low intensities and insensitive techniques used by early workers.

In 1930 a French patent was issued on the use of high energy radiation for processing foods (11, p. 420) and there subsequently followed numerous reports on the use of ionizing radiation to destroy microorganisms in highly contaminated foods (12; 14; 42, p. 1; 50;51; 55; 65; 80; 81, p. 900). Both industrial and federal agencies have since been interested in this means of cold pasteurization and sterilization of foods which are heat sensitive.

The use of ultraviolet (UV) irradiation in food preservation has not received as much consideration as other irradiation forms because of its low penetrating ability (58, p. 30). However, the Centrifilmer, a recently developed instrument in which fluids in the form of thin-flowing films may be exposed to UV energy, is now being used for the sterilization of blood plasma and vaccines (10; 72). It has also been tested, but with not complete success, for the sterilization of whole milk (18) and the pasteurization of egg liquids (54). The presence of highly radiation resistant microorganisms presents an obvious problem to further utilization of either ultraviolet or ionizing radiation for food processing.

Ionizing Radiation

Interaction with Matter

Radiation is the propagation or transmission of energy through space (34, p. 59). This may occur by wave motion or by means of atomic or subatomic particles moving at great velocities and set in motion by the action of electric fields or by emission from radioactive (unstable) substances (57, p. 304).

Radiant energy striking some material object may be absorbed, thus involving the interaction of the energy with matter. This interaction may affect changes in atomic electron configuration such as raising of the orbital electrons to higher energy shells, resulting in short-lived "activated atoms" (57, p. 305). This "excitation" may also occur when high energy particles pass near atoms, giving up energy, disturbing and reorganizing electrons holding constituent atoms together. One or several of the weakened bonds may rupture, releasing stable and unstable fragments into the environment.

The bonding energy of valence electrons in atoms is approximately ten electron volts (35, p. 92) while the binding energy of atoms in molecules (e.g., C-H bond) is approximately five electron volts (23, p. 35). As the absorption of energy increases over five or ten electron volts, it may cause the ejection of orbital electrons in some atoms and their conversion into ions or free radicals (57, p. 305). Other molecules may form secondary charged particles and cause additional ionization upon molecular collision. The innermost electrons require much higher energies for removal (58, p. 9-10). High speed atomic particles, however, carry higher quantum energies of from one to ten million electron volts (Mev). Electromagnetic radiation photons also carry large quantum energies; photons of gamma radiation from Cobalt-60 are about one Mev (78). This physical process of exposure and energy deposit in biological materials requires only 10⁻¹⁶ seconds (7, p. 1-5).

The excited and ionized molecules are very reactive, often seizing neighboring neutral molecules and exchanging an atom or group of atoms with it (58, p. 47). For example:

$$\begin{array}{rcrcrcrc} H_2O &+& O_2 &\rightarrow & H^* &+& *OH &+& *HO_2 \\ *OH &+& *OH &\rightarrow & H_2O_2 \\ H_2O &\rightarrow & H^* &+& *OH \end{array}$$

These and other chemical reactions and products may occur at a biologically essential site of the molecule. If the ionization or excitation which occurred was produced at a non-essential part of the molecule, it could still migrate to a vital site (38). This process of excitation and/or ionization lasts about 10^{-5} seconds (7, p. 1-5).

There then follows a biochemical stage which may last seconds to hours. Some of the molecular damage could be reversed without a permanent effect or the damage could be fixed and develop into biochemical lesions which are not deleterious (86). More frequently, however, the damage becomes irreversible and if it is not eliminated by a metabolic process, it could result in injury which might eventually kill the cell. This latter process may last from hours to days and is called a biological process. A review on the interaction of ionizing radiation with matter has recently appeared (7, p. 6-44).

Effects of Ionizing Radiation on Microorganisms

The advantages of using bacteria and other unicellular organisms to study radiation effects on cells are mainly those related to their simplicity of cultivation and the accompanying reduction in the time and cost of experimentation. This advantage, however, is minimized when we remember that we cannot examine the individual cells of an exposed culture with a reasonable degree of accuracy, and that what we actually are observing is population dynamics of a group of cells. It is also important to remember that the criteria of death differ with the organism studied. In bacteria, the ability to multiply and form colonies is usually studied and failure in this respect is termed "death". Nevertheless, a population of bacteria in which 99.9 percent were "dead" would retain its motility, most of its enzyme activity and its capacity to produce bacteriophage. Therefore, it has been suggested (96) that "inactivation" would be the more descriptive and preferred term.

Sensitive Site

Important contributions to our knowledge of radiation damage have arisen from studies on bacterial populations. The target theory was purposed from such a study (58, p. 72-78). This theory purposes a direct hit on a biologically essential site which results in cell inactivation. The nature of the "target" molecules has not yet been established. It is most often assumed that changes in deoxyribonucleic acid (DNA) are responsible (1; 16; 48; 73; 102) but there are some objections to this assumption (13; 25; 52; 60, p. 11; 67). Thus, it has been shown (7, p. 269) that the impact of an ionizing particle with a chromosome cannot be compared with the snapping of a telphone wire by a bullet. Model experiments with nucleoprotein gels show that structures of the dimensions of chromosomes are not broken into two parts by the passage of one ionizing particle through them. The fact that the sensitivity of DNA to breakage by x-rays can be modified by prior exposure to ionizing radiation and by exposure before or after irradiation to infra-red, also emphasizes that damage to the DNA strands are not the direct result of the passage of an ionizing particle, but are the result of a complex interplay of different factors in a living system which reacts to changes brought about in its environment (7, p. 268).

Enzyme Inhibition

A second theory which has been proposed to account for the mechanism of radiation damage in a biological system is that of interference with synthesis by enzyme inhibition (7, p. 270). A factor which argues against this purely biochemical mechanism is that chromosome damage is produced much more readily by radiation of high specific ionization. For example, a dose of five Mev alphaparticle may be ten to 20 times as effective as a similar amount of energy delivered by x-rays. This proves that chromosomal damage is not produced by inactivation of enzymes or alteration of isolated macromolecules as radiation of low specific ionization should then be most effective.

Enzyme Release

From the foregoing, it appears that neither the purely

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biochemical (inhibition of synthesis) nor the target (snapping of thread) hypothesis is completely acceptable. A third theory, considered a combined alternate of the two preceding ones, purposes a rupture of internal membranes which might disorganize the enzyme systems of the cell; i. e., ionizing radiation causes a disorganization of the pattern of macromolecules (smaller than DNA) in cellular and intracellular membranes (7, p. 272) with a resulting alteration in membrane permeability. These membranes may include the cell membrane as well as those surrounding important intracellular structures (nucleus, mitochondria, microsomes). Among the evidence to substantiate this theory is an abundant increase in the activity of many enzyme systems following irradiation (41; 56; 93) and change in the osmotic behavior in irradiated plant roots (95), mammalian skin (7, p. 414-417) and isolated mitochondria (71).

Within the cell, biochemical processes are controlled by the exact location of the enzyme and substrate, which prevents them from coming into contact, although they exist within the same cell. Some enzymes, such as DNAase, are outside the nucleus although the substrate is to be found in the nucleus. At least 60 percent of the acid phosphatase is associated with mitochondria or mitochondrial- like structures; glycerophosphate, which is a substrate of acid phosphatase, does not pass thru the "membrane" of isolated mitochondria (22).

All the intracellular membranes, both within the mitochondria and endoplasmic reticulum, appear to have the same structure. They are approximately 80 Å wide and three layers can be seen. These layers are believed to result from the association as in a sandwich of two protein-phospholipid complexes in such a way that their hydrophobic (fatty) parts come together in the center while the outsides are made up of the hydrophilic (protein) parts of the molecule (82). The selective permeability of cell membranes is believed to be in large part a reflection of their lipid and lipoprotein composition (89, p. 223). It is reasonable that the permeability of a structure of this type could be affected by ionization. However, the absence of know]edge on the vital initial steps of radiation damage makes the investigation of mechanisms of resistance a very speculative matter. It may happen that the study of resistance will throw some light on the mechanisms of damage.

Variation in Radiation Resistance Among Bacteria

Bacteria and other unicellular organisms are considerably more resistant to ionizing radiation than multicellular forms of life, but the reasons for this difference are not understood (7, p. 300). It is reasonable to believe that the higher degrees of specialization and intercellular organization of multicellular organisms will cause them to show a greater radiation sensitivity than unicellular organisms. It has been demonstrated (75) with tissue culture cells that once this intercellular dependancy is broken, the individual cell requires a greater radiation dose for inactivation.

The range of radiation resistance among bacteria is also extremely large, with a factor of about 50 separating the D_{10} (or 90 percent inactivation) dose of a pseudomonad from that of spores of Clostridium botulinum (96).

Spore-formers

Usually bacterial spores are the most radiation resistant. This phenomenon can be explained partially on the basis of decreased metabolic rate; however, <u>Micobacterium phlei</u> and <u>Azotobacter agilis</u>, which are very slow growing do not show marked radiation resistance (73). Other studies (99, p. 133) have shown that in <u>Bacillus</u> spores a cystine rich protective compound is synthesized which modifies the initial molecular changes caused by radiation. This compound was located in the spore coat so it is difficult to imagine its having any protective effect on DNA but rather its location and purposed function in radiation substantiate the theory of enzyme release mentioned previously. It has been suggested that the disulphide bond could supply an electron to fill a vacancy created by radiation in another part of a protein molecule (37); and that sulfhydryl groups can combine with proteins to give disulfide linkages which function as a harmless means of dissipating radiation energy (28).

Escherichia coli B and E. coli B/r

Comparison of a bacterial strain with a more radiation resistant mutant might be expected to throw some light on the mechanisms of resistance. However, extensive studies on <u>E. coli</u> B and its resistant mutant, B/r, have not been entirely fruitful (96). It has been shown that the two strains do not differ in nucleic acid content. Both strains are strongly influenced by post-irradiation conditions of medium, incubation temperature and other factors. Although both strains showed the same survival on synthetic media (3), richer, complex media gave rise to fewer survivors of <u>E. coli</u> B (2) and higher counts of <u>E. coli</u> B/r (92). This "medium effect" took place during the first few hours of incubation after irradiation, and it was concluded that the difference between the two strains lay not in the initial damage but in their metabolic processes immediately after irradiation.

It appeared that with <u>E. coli</u> B any factor which would slow down or inhibit protein synthesis would favor recovery after irradiation (32). This supported the theory (8) that death took place due to unbalanced growth when DNA synthesis could not keep pace with protein synthesis. This set of observations seems well established for <u>E. coli</u> B but it is not yet clear in what respects <u>E. coli</u> B/r differs from B and why it is more radiation resistant under certain conditions.

Achromobacter-Alcaligenes Group

Bacterial strains belonging to the <u>Achromobacter</u> and <u>Alcali</u>genes genera which have been isolated from poultry have a wide range of radiation resistance (LD₁₀ from 10 to 100 k rads) and yet taxonomic studies show they are closely related in most other characteristics (97). It was further found that most of the strains were sensitive to penicillin but about ten percent were resistant, and that the penicillin resistant strains were more sensitive to radiation. On this basis it was suggested that some property controlling penicillin resistance is at least partly responsible for radiation sensitivity. Since penicillin is known to act on the synthesis of cell wall material, it is possible that the variation in radiation resistance might correspond to differences in wall structure (96).

Micrococcus radiodurans

The most radiation resistant organism known is <u>M</u>. radiodurans which was isolated from cans of meat exposed to three to six M rad (4). Its inactivation curve shows a large "shoulder" (53) and becomes exponential only at very high doses; five to six M rads are needed to sterilize a culture. This microorganism is a non-sporeforming, pink-pigmented, tetracoccus which is not pathogenic or heat resistant but is resistant to ultraviolet irradiation (26) The LD_{50} dose to gamma radiation in phosphate buffer is 500 to 700 k rads.

A chemically defined medium found to support growth consisted of methionine as the only essential amino acid in combination with glucose, two vitamins and essential minerals (5).

Its radiation sensitivity can be greatly increased by irradiating in the presence of iodoacetic acid (61), iodoacetamide (21) or p-hydroxymercuribenzoate (15), and as all these compounds react with -SH groups, this finding would be consistent with the finding of protective sulfur compounds. It was also reported that the increased radiosensitivity of the cells in the presence of iodoacetic acid was accompanied by a decrease in pigment content.

Recent electron microscopy studies (96) suggest that the unusual resistance of this organism may be related to its unique cell wall which is multilayered and composed of a regular array of sub-units, hexagonally arranged. These cell walls have been found to contain a high proportion of lipids and to show structural lipoprotein layers similar to those found in the Gram-negative organism <u>Spirillum</u> <u>serpenes</u>(68). A new mucopeptide with L-ornithine as the main amino acid has also been isolated from the cell wall structure (100).

There is no direct evidence that DNA of <u>M</u>. radiodurans differs quantitatively or qualitatively from that of other bacteria (67). Recent reports indicate, however, that this organism has a remarkable ability to repair damage to its DNA by the formation of thymine dimers (84; 85).

The pigments of <u>M</u>. radiodurans have been identified as being of the carotenoid type, but attempts to correlate radiation resistance with the presence of these pigments have not been successful (53; 59, p. 25) nor consistent (66). However, the role of carotenoid pigments in photosynthetic organisms has been demonstrated clearly to be one of protecting the cell from the deleterious effects of chlorophyllcatalysed photo-oxidations (17). In a similar manner, the carotenoids of <u>M</u>. radiodurans could serve as scavengers or protective agents from the deleterious ions and radicals produced by ionizing radiation.

The reported isolation of other highly radiation resistant microorganisms with carotenoid type pigments provides additional evidence of the possible role of these compounds in the mechanism of resistance. A pink air contaminant (69) and an orange-brown coccus from haddock tissue (20) have been reported to be of comparable resistance to M. radiodurans.

Carotenoid Pigments of Microorganisms

As indicated previously the pigments of <u>M</u>. radiodurans have been identified as carotenoids. These compounds are found in all groups of organisms but are synthesized only by plants and microorganisms. They are characterized by possession of a bilaterally symmetrical C₄₀ skeleton consisting of four isoprene units (88, p. 221-223). These isoprenoid units have been shown to be formed from acetyl-CoA by condensation and conjugation reactions, and mevalonic acid occurs as an intermediate compound in the biosynthetic reactions.

The attribute of color of the carotenoids is largely determined by an extensive system of conjugated double bonds. Phytoene and phytofluene, which are colorless, contain three and five conjugated double bonds, respectively. In addition to the carotenoid hydrocarbons, there are many carotenoids that contain one or more oxygen atoms. These occur most frequently in the form of hydroxyl groups, but carbonyl, epoxy and methoxyl groups can also exist. For example, spirilloxanthin, which is the principal carotenoid from the stationary phase of a culture of <u>Rhodospirillum rumbrum</u>, has 13 conjugated double bonds and two methoxyl groups.

Stimulation of carotenogenesis by lipogenic substances (simple alcohols, glycols and glycerol) suggested a common biosynthetic pathway for the carotenoids and lipids (44). More recent evidence indicates clearly that mevalonic acid is a precursor both for squalene and the sterols as well as carotenoid pigments. The close association of carotenoids and lipids was more clearly presented by the observation that the carotenoids of bacteria are predominantly found with the lipids in a particulate fraction in cell extracts. Furthermore, controlled disintegration of the bacterial cell by destruction of the cell wall with lysozyme has shown that in two bacteria, <u>Micrococcus lysodeikticus</u> and <u>Sarcina lutea</u>, the carotenoids are exclusively membrane components (88, p. 225-228).

There are at least 32 different known carotenoid pigments occuring in bacteria. Experiments with growing cultures indicate various carotenoid intermediates can be isolated by controlling the growth conditions (45, p. 24-27; 98) and that these intermediates are less effective in preventing photo-oxidation than those carotenoids with a more extensive conjugated double bond system.

Recent reviews are available (46; 98) on the identification of specific carotenoid pigments in various solvents on the basis of their light absorption characteristics.

PART I AN ECOLOGICAL STUDY OF MICROCOCCUS RADIODURANS

Methods and Materials

The Organism

A strain of <u>Micrococcus radiodurans</u> isolated by Anderson (4) was used as a comparative organism throughout this study. As indicated in Anderson's original report, this organism was a Grampositive to Gram-variable, pink-pigmented coccus occuring predominantly in tetrads with a diameter of 1μ /cell. Although it was a nonsporeformer, it was six to eight times more resistant to gamma radiation than any other known organism. This unusual radiation resistance characteristic was the main criterion used in determining the presence of M. radiodurans from various environments.

Growth and Isolation Media

The standard medium used for culture maintenance was TGYM medium; this consisted of tryptone (Difco), 5.0 g; yeast extract (Difco), 1.0 g; glucose, 1.0 g; DL-methionine, 20 mg; Bacto agar, 15 g; tap water, 1000 ml; final pH, 7.0. Stock cultures were main-tained on TGYM agar slants incubated aerobically at 30 C for three days and then stored at 2 to 6 C.

The other medium used consistently throughout this study was

designated NZ medium and consisted of tryptone (Difco), 5.0 g; yeast extract, 2.5 g; glucose, 1.0 g; NZ-Case (Sheffield Chemical Co.), 5.0 g; Bacto agar, 15 g; tap water, 1000 ml; final pH, 7.0. This was the preferred medium used for the initial isolation and maintenance of a pigmented radiation resistant micrococcus from haddock tissues (20).

Sources of <u>M.</u> radiodurans Which Were Investigated

<u>Ground meat</u>. Samples of fresh ground beef and ground pork were obtained from Nebergall's Meat Plant at Albany, Oregon. Using sterile spatulas, these samples were packed into sterile 202 x 204 cans and returned to the laboratory packed in wet ice. Twenty gram samples of each type of meat were then packed into screw-capped, glass, radiation vials (25 x 55 mm) and placed in a holder adapted for the Cobalt-60 gamma radiation source at Albany, Oregon. Irradiation was conducted in air at wet ice temperatures. The total exposure dose was either 1 or 2 megarad (m rad). Unirradiated control samples were also provided.

Following irradiation, the entire contents of each glass vial were transferred to a sterile Waring blendor and blended with 200 ml of sterile phosphate buffer (0.067 M) for one minute.

The unirradiated controls were examined by making serial dilutions of a one ml aliquot of the blended sample in phosphate

buffer and plated in triplicate on both TGYM and NZ agar. The irradiated samples were examined by plating five ml of the blended sample in triplicate on both media. All plates were incubated aerobically at 30 C for four to eight days before being examined for total viable cell counts and for "typical" pink-pigmented colonies resembling <u>M.</u> radiodurans.

All of the pink colonies were transferred to slants and subsequently examined microscopically and compared with stock cultures of M. radiodurans for Gram-stain reaction and cellular morphology.

The main criterion used in determining the presence of organisms similar to <u>M.</u> radiodurans consisted of growing the new isolates as well as the stock <u>M.</u> radiodurans in either TGYM or NZ broth for 44 hours at 30 C on a gyrotory shaker. The cells were then harvested by centrifugation (7000 x G, 20 min.), washed twice and resuspended in phosphate buffer (0.067 M; pH, 7.0). Using a standard curve and a Baush and Lomb spectrophotometer, the density of the cell suspension was adjusted to contain approximately 1×10^8 cells/ml. Ten ml of cell suspension were pipetted into glass radiation vials and the cells were then exposed to the gamma radiation source either at Oregon State University or the U.S. Bureau of Mines facility at Albany, Oregon. A radiation exposure equivalent to 700,000 rads was used for comparative purposes since this is the reported (60, p. 57) LD₅₀ dose (the exposure dose resulting in 50 percent survival) for <u>M.</u> radiodurans in buffer. The number of survivors was determined by making viable plate counts. The plates were made in triplicate at each dilution and incubated for 72 hours at 30 C prior to counting on a Quebec colony counter. The percent survival was calculated and compared with the stock <u>M.</u> radiodurans.

In subsequent experiments, TGYM broth was used as the standard culture medium since NZ broth consistently produced cells which were approximately ten times more radiation sensitive. This latter observation was investigated more thoroughly in the second part of this thesis.

Preliminary experiments were conducted to determine the effect of pre- and post-incubation on the number of pink isolates which could be obtained from meat exposed to 2 m rad. In a similar manner, the effect of spice additives in pork sausage and the packaging operation for both types of meat were investigated. For this purpose, the samples were collected as previously indicated and handled as follows:

- a. Eight 20 g samples of freshly ground beef obtained directly from the meat grinder
- b. Eight 20 g samples of ground beef obtained from various parts of ten pre-packaged rolls
- c. Eight 20 g samples of spiced pork sausage from pre-packaged rolls
- d. Eight 20 g samples of freshly ground pork sausage without the spices

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- e. Four 20 g samples, each like b, c, and d above, respectively, except the samples were incubated for 18 hours at 30 C prior to irradiation
- f. Four 20 g samples, each like b, c, and d above, respectively, except the samples were incubated for 48 hours at 30 C after irradiation

All samples were irradiated at wet ice temperature; the total exposure time was 96 minutes for 2 m rads. The presence of pink micrococci in tetrads was determined as given in the previous section.

<u>Hides of Live Beef Animals.</u> Since microorganisms comparable in cellular morphology and radiation resistance to <u>M. radiodurans</u> could be isolated from ground meat, it was decided to determine potential sources of this organism. The possible sources which were investigated are listed below:

- 1. Ground Beef
- 2. Ground Pork, spiced and un-spiced
- 3. The Hides of Live Beef
- 4. Soil
- 5. Fecal Material
- 6. Hay
- 7. Water
 - a. Deep well water
 - b. Shallow well water
 - c. Albany municipal water
 - d. Cox Creek (Albany, Oregon)

An attempt was first made to look for this radiation resistant microorganism in the hides and hair of live beef animals. For this
purpose, sterile radiation vials were prepared each of which contained ten ml of TGYM broth. Sterile cotton swabs were used in swabbing the hides of 40 different beef animals at the Nebergall Meat Plant, Albany, Oregon. These animals had been held in feeding pens at Nebergall's for at least five days. Each swab was broken off individually into the vials containing broth and returned to the laboratory where the vial contents were mixed for one minute on a Vortex Jr. mixer. Once again, the use of high levels of gamma radiation were used to facilitate the isolation of M. radiodurans. For this purpose, all vials were exposed to 2 m rads at room temperature in air. The influence of pre- and post-incubation on the isolation procedure was determined by placing the vials at 30 C for zero, two or four days before irradiation or four days at 30 C after irradiation. The presence of viable pigmented cells was determined by making TGYM agar pour plates and incubating at 30 C for three to seven days. Microscopic examinations were made of all pink colonies which resembled M. radiodurans. Confirmatory indentification of selected isolates was made by growing the isolates in TGYM broth, irradiating them in phosphate buffer (700 k rads) and comparing their survival rate with that of M. radiodurans. The initial cell number was adjusted to approximately $1 \ge 10^8$ /ml by optical density measurements.

Preliminary experiments were also conducted to determine if irradiation of a cotton swab in TGYM broth had a deleterious effect on cell survival. A washed 44 hour culture of <u>M. radiodurans</u> was resuspended in fresh TGYM broth. Ten ml of this cell suspension was transferred to a radiation vial containing a sterile cotton swab and exposed to 700 k rads. The percent survival was calculated and compared with samples irradiated in the absence of a cotton swab.

Soil. Five hundred grams of top soil were collected from the cattle feed-lots and grazing areas at the Nebergall Packing Plant and returned to the laboratory in sterile covered cans. After the soil samples were air dried for 36 hours, the percent moisture was calculated to be ten percent and soil pH was 5.8. The water holding capacity of the soil was determined by supersaturating the soil in a Gooch crucible which was placed in a dessicating jar, the bottom half of which was filled with water, to allow the excess water to drain off.

After the air-dried soil had been passed thru a two mm screen, thirty portions (5g each) were weighed out into sterile radiation vials. To ten of the samples were added 2.5 ml of sterile phosphate buffer (this is equivalent to about 50 percent of the soil water holding capacity) plus one percent of the soils weight of glucose. Ten additional samples received phosphate buffer alone, and the remaining ten samples received no additives.

All samples were exposed to two m rads ionizing radiation at the Cobalt-60 source in Albany, Oregon. The dose rate was 965 k rads/ hour, and the exposure time was 124 minutes. After irradiation, the samples were returned to the laboratory. Four of the ten samples containing no additives were examined immediately for the presence of pink tetracocci by making duplicate pour plates at 10^{-3} 10^{-7} dilution on TGYM and NZ agar and incubating the plates aerobically at 30 C for three to ten days. The remaining six samples were incubated at 30 C for six and ten days prior to sampling. The soil samples which had received buffer alone or buffer plus glucose were handled in a similar manner. This entire experiment was repeated three times.

Enrichment culture techniques were also used. This consisted of inoculating ten g portions of soil into DeLong culture flasks containing 100 ml of TGYM broth and incubating on a gyrotory shaker at 30 C for 44 hours. Duplicate ten ml aliquots were then transferred to sterile radiation vials and exposed to 700 k rads. For control tests, a one percent inoculum of a 44 hour culture of <u>M</u>. radiodurans was inoculated into the soil-broth culture and incubated and irradiated in a similar manner. A second control consisted of inoculating $8 \ge 10^3$ cells/g into dry soil and attempting to re-isolate the organism with and without exposure of the soil sample to irradiation.

<u>Hay</u>. Hay samples totaling 100 g were collected at random from 20 different hay bales in the cattle feeding area at the Nebergall Packing Plant, Albany, Oregon. The samples were collected in covered sterile containers and returned to the laboratory. Samples weighing ten g each were placed in a sterile 500 ml Erlenmeyer flask and shaken vigorously for five minutes with 200 ml of sterile phosphate buffer. The liquid was decanted into a sterile container and subsequently ten ml portions were transferred into sterile radiation vials. These vials were then exposed to 700 k rads of gamma irradiation; the exposure time was 43.5 minutes.

After irradiation, the samples were examined for the presence of radiation resistant, pigmented tetracocci. For this purpose, duplicate pour plates were made at $10^{0} - 10^{6}$ dilutions on TGYM and NZ agar. The plates were examined periodically during a three week incubation period at 30 C. This experiment was repeated twice.

<u>Fecal Material.</u> Approximately 500 g of fecal material was collected at random from the floor of the cattle feeding lots at Nebergalls Packing Plant. Aliquots of this material were then distributed in five g portions into each of 20 sterile radiation vials. These samples were exposed to two m rads; exposure time was 124 minutes.

Following irradiation, the fecal samples were examined for the presence of pigmented tetracocci by the same procedure used for hay samples. This experiment was repeated twice.

<u>Water</u>. Ten liter samples of water were obtained as aseptically as possible in sterile glass carboys from the four sources of water used in the Nebergall plant. These sources were as follows:

- 1. Deep well (>100 ft.)-used for washing equipment
- 2. Shallow well (60-100 ft.)-used for washing equipment
- 3. Municipal water supply used for washing equipment and meat additive
- 4. Cox Creek used only for watering the livestock; runs parallel to and within 25 yds. of the meat plant

The samples were returned to the laboratory and examined within 24 hours for organisms similar to M. radiodurans.

In order to concentrate the microflora, the water samples were passed through Millipore filters (HA; 0.45 µ; 47 mm dia). However, all but the city water had to be pre-filtered through a sterile filter pad (Watman No. 1) in order to remove extraneous material which clogged the Millipore filter. After filtration, the Millipore filter pad was cut into four equal sections with a sterile forceps and scissors and placed in a radiation vial containing ten ml of sterile phosphate buffer. In a similar manner, the center portion (47 mm dia.) of the Watman filter paper was sectioned and prepared for irradiation. Two samples of ten and 20 ml each, respectively, of unfiltered water from each source was also irradiated. The vial contents were thoroughly dispersed prior to irradiation by mixing on a Vortex Jr. mixer for one minute.

The following table summarizes the number of samples and the manner in which they were handled prior to irradiation:

	No. of		
Water source	samples	<u>Ml samples</u>	Material irradiated
Deep well	4	1000 ml	Watman filter pad
Deep well	5	10 ml	unfiltered water
Deep well	5	20 ml	unfiltered water
Deep well	4	2000 ml	2 Millipore filters
Shallow well	4	1000 ml	Watman filter pad
Shallow well	5	10 ml	unfiltered water
Shallow well	5	20 ml	unfiltered water
Shallow well	4	2000 ml	Millipore filter pad
Municipal water	4	2000 ml	Millipore filter pad
Municipal water	5	l0 ml	unfiltered water
Municipal water	5	20 ml	unfiltered water
Cox Creek	4	1000 ml	Watman filter pad
Cox Creek	5	10 ml	unfiltered water
Cox Creek	5	20 ml	unfiltered water
Cox Creek	4	1000 ml	2 Millipore filters

All vials received 700 k rads at the Cobalt-60 source at Oregon State University; the exposure time was 56.5 minutes.

All samples were plated out in duplicate immediately after irradiation on TGYM and NZ agar at $10^{-1} - 10^{-3}$ dilution; in addition, one ml and five ml, respectively, were plated from each sample vial. During a three week incubation period at 30 C, all plates were examined every four days for the presence of pink colonies. All colonies which resembled <u>M</u>. <u>radiodurans</u> were picked and examined microscopically. Selected presumptive isolates were grown in broth culture and their radiation survival rate was determined as described previously.

A preliminary control experiment was conducted to determine

what effect, if any, the presence of Watman or Millipore filter paper had on the survival rate or irradiated M. radiodurans.

Results

Ground Meat

Irradiated samples of either ground beef or pork exposed to one m rad contained a total aerobic flora too numerous to count at the highest dilution used (10^{-6}) ; hence subsequent experiments involved the use of higher radiation levels. The total viable plate counts obtained from meat samples exposed to two m rads are shown in Table 1.

Sausage onposed to 2 m ruds gumma rudiationIsolationViable cells/g from irradiated
ground beefTGYM158 x 10⁴15 x 10⁴NZ178 x 10⁴27 x 10⁴

Table 1. Total aerobic viable cells/g^a of ground beef or pork sausage exposed to 2 m rads gamma radiation

^aCell counts represent the average of triplicate plates from each of three experiments incubated @ 30 C for four to eight days.

The number of pink colonies obtained from beef was 5/g while the irradiated pork samples had 20/g. Thus, although the total number of cells surviving two m rads was seven to ten times greater from ground beef, a larger percentage of pink-pigmented survivors was obtained from pork sausage. These results are in general agreement with those given in a previous report (4). Unirradiated meat samples of both types contained approximately 100-200 pink-pig-; mented cells per g.

As indicated in Table 1, the NZ medium consistently produced the largest number of survivors. Similarly, the pigmented colonies developed in four days at 30 C on this medium while eight days was required for the appearance of pink colonies on TGYM agar. Previous studies (20) also indicated that NZ medium was the best medium for isolating a radiation resistant micrococcus from haddock tissues. However, my observations from preliminary experiments indicated that cells grown in NZ broth are ten times more radiation sensitive than those grown in TGYM broth. Hence, for comparative resistance studies, all of the new isolates as well as <u>M. radiodurans</u> were grown in TGYM broth prior to irradiation.

Results from preliminary experiments indicated that incubation of fresh ground meat samples either before or after irradiation did not alter the number of pink isolates obtained which resembled <u>M</u>. <u>radiodurans</u>. Similarly, no increase in pink tetracocci was noted when spiced pork sausage or pre-packaged meat was examined.

A comparison of the radiation survival rates of several new isolates and <u>M</u>. radiodurans is given in Table 2. All of the new isolates which were radiation resistant were found to be cocci, $l \mu$

in diameter and occurring predominantly in tetrads with a salmonpink pigment. These characteristics were similar to those described for M. radiodurans (4).

Isolate		Viable cell	Viable cells/ml after		
No.	Source	0 k rads	700 k rads	Survival	
75	beef hide ^a	208×10^{6}	129 x 10 ⁶	62.0	
81	beef hide ^a	135×10^{6}	88×10^{6}	65.2	
w 7	Cox Creek ^b	110 x 10 ⁶	85×10^{6}	77.2	
w ₄	Cox Creek ^b	141×10^{6}	94 x 10 ⁶	66.7	
48	ground beef c	158×10^{6}	82×10^{6}	51.8	
30	ground pork ^C	104×10^{6}	62×10^{6}	59.6	
20	ground beef ^d	167×10^{6}	90 x 10 ⁶	54.0	
<u>M. radio-</u> durans	stock culture ^e	206 x 10 ⁶	109 x 10 ⁶	53. 2	

Table 2. Radiation sensitivities of shake cultures of <u>M</u>. radiodurans and several morphologically similar isolates obtained from various sources

^aObtained by exposing a cotton swab to 2 M rads which had previously been used to swab the hides of live beef.

^bObtained from creek water exposed to 700,000 rads.

^CObtained from meat previously exposed to 2 M rads.

^dObtained from unirradiated meat.

^eOregon State University, stock culture collection

Pink tetracocci were also isolated from ground beef which had not previously been exposed to gamma radiation. Their radiation resistance when suspended in phosphate buffer was similar to that of M. radiodurans (Table 2).

Live Beef Hides

The isolation of pigmented micrococci from the hides and hair of live beef animals was readily facilitated by the use of high levels of ionizing radiation. The results of these experiments are presented in Table 3.

The use of a four day pre-incubation period prior to irradiation resulted in an over-growth of non-pigmented aerobic sporeformers and no pink colonies. A two day pre-incubation permitted the recovery of 17 pink tetracocci. The range varied from less than one up to 20 cells/ml of the irradiated sample. When the swab samples were not incubated prior to irradiation, 114 pink isolates were obtained and 62 percent of these resembled <u>M</u>. <u>radiodurans</u> upon microscopic examination. All of the plates made from samples which were incubated for four days after irradiation had a reddish-pink color and most of the plates had more than 300 colonies.

Using isolates selected at random from non-incubated samples, their relation to <u>M</u>. radiodurans was confirmed by irradiating cell suspensions in buffer at the LD_{50} dose (700 k rads). The results of Table 2 indicate at least 60 percent survival of these isolates under these conditions.

	Pour plate dilution					
Animal No.	5:0	1:0	1:10	1:20	1:100	1:1000
			Group l ^a			
1						
2						
3						
4		All pla	ates were	discared	because	of
5		over-	growth of	non-nigm	ented mic	~ro-
6		organ	isms whic	h were n	ot microc	
7		8			00 111101 00	0001
8						
9						
			– b			
1.1			Group 2~			
	no grov	wth	_			
12	2	0	1	0	0	0
13	1	0	0	0	0	0
14	1	0	0	0	0	0
15	1	1	0	0	0	0
16	0	7	2	0	0	0
17	2	0	0	0	0	0
18	no grov	wth				
19	no growth					
20	no grov	wth	~			
		(Group 3 ^C			
21	a pure	culture o	of a yellow	, pigment	ted micro	cocci
22	a pure	culture d	of a yellow	, pigment	ted micro	cocci
23	19	4	0	0	0	0
24	7	1	0	0	0	0
25	15	0	0	0	0	0
26	6	5	0	0	0	0
27	5	1	1	0	0	0
28	19	9	0	0	0	0
29	6	2	0	0	0	0
30	10	2	1	0	0	0

Table 3. Total aerobic counts of pink-pigmented tetracocci obtained by gamma irradiation (2 m rads) of cotton swabs made from live beef hides

(continued on next page)

	Pour plate dilution						
Animal No.	5:0	1:0	1:10	1:20	1:100	1:1000	
Group 4 ^d							
31	TNTC ^e	TNTC	TNTC	TNTC	TNTC	TNTC	
32	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	
33	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	
34	TNTC	TNTC	TNTC	TNTC	297	105	
35	TNTC	TNTC	217	148	100	7	
36	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	
37	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	
38	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	
39	TNTC	TNTC	12	11	11	1	
40	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	

Table 3. (Continued)

^aSamples were incubated four days prior to irradiation followed by seven days incubation of pour plates @ 30 C.

^bSamples were incubated two days prior to irradiation followed by seven days incubation of pour plates @ 30 C.

^cSamples were not incubated prior to irradiation; pour plates were incubated seven days @ 30 C.

^dSamples were incubated four days after irradiation followed by three days incubation of pour plates @ 30 C.

^eIndicates too numerous colonies to count at the respective dilutions.

A control experiment indicated that the mere presence of a cotton

swab in pre-inoculated TGYM broth during irradiation did not alter

the percent survival of M. radiodurans.

Soil

Repeated attempts to isolate organisms resembling M. radio-

durans from irradiated soil samples provided negative results.

Neither the use of post-incubation periods of six to ten days for the

irradiated soil nor the presence of added nutrients (glucose) or moisture promoted the isolation of pigmented tetracocci. However, the results from a control experiment with pre-inoculated but unirradiated dry soil showed that of the 8000 cells/g originally inoculated, 3300/g could be recovered. This represented a 41 percent recovery rate.

After one week of incubation of plates made from non-incubated but irradiated soil samples, only five pink colonies were observed. Three of these developed from irradiated dry soil at 10^{-3} dilution. Microscopic examination revealed Gram-positive cocco-bacilli (0.25 x 2µ) occurring predominantly in pairs and short chains of two or three cells each. The other colonies were obtained from soil samples which had been moistened to 50 percent of its water holding capacity prior to irradiation. Their cellular morphology was the same as that just described.

After three weeks incubation of all plates, a total of 135 pink colonies were picked and examined microscopically using the Gramstain technique. Ninety-seven percent of the colonies consisted of Gram-variable rods $(0.5 \times 1.5 \mu)$ existing either singly or in pairs, and three percent of the colonies were composed of yeast cells. No pigmented tetracocci were found.

The use of enrichment culture techniques likewise did not facilitate the isolation of pigmented tetracocci from soil. However, a total viable cell count of $5 \ge 10^6/g$ was obtained after 700 k rads; of these, $3 - 10 \ge 10^3/g$ were found to be Gram-variable bacilli (0.75 \ge 1.5 μ) occurring singly and formed pink colonies on an agar surface.

The results from a control experiment indicated that no <u>M. radio-</u> <u>durans</u> cells could be recovered from a pre-inoculated 44 hour soilbroth culture exposed to 700 k rads. Attempts to isolate organisms resembling those of the inoculum from un-irradiated soil-broth cultures also were unsuccessful. The final pH of the soil-broth culture was 6.8.

Hay

No microorganisms could be isolated which resembled <u>M. radio-</u> <u>durans</u> in morphology or radiation resistance from irradiated hay samples at Nebergall's Meat Plant. A total aerobic viable plate count of 37×10^3 /ml and 79×10^3 /ml on TGYM and NZ agar, respectively, was obtained after ten days incubation at 30 C. The plates were not countable after ten days incubation due to bacterial overgrowth. Microscopic examination of the radiation survivors indicated that a majority were sporeformers.

Fecal Material

The experimental conditions used to isolate pigmented tetracocci from irradiated fecal material gave a total aerobic viable plate count of 40 x 10^4 g/ and 96 x 10^4 /g on TGYM and NZ media, respectively. However, microscopic examination of 50 selected colonies indicated that a majority of these survivors were sporeformers, and none of them were pigmented tetracocci. More than six days of incubation at 30 C resulted in plates which were overgrown and not countable.

Water Samples

Two weeks of incubation of pour plates made from irradiated water samples indicated the presence of ten to 25 pigmented tetracocci per ml. These were all obtained from creek water which had not been filtered. Attempts to concentrate these organisms by filtration were not successful even though results of a preliminary experiment indicated no decrease in survival rate of <u>M. radiodurans</u> irradiated in the presence of Watman or Millipore filter paper.

When selected presumptive isolates were irradiated (700 k rads) while suspended in phosphate buffer, they were found to possess the high degree of resistance characteristic of <u>M.</u> radiodurans (Table 2).

No microorganisms resembling <u>M</u>. radiodurans were isolated from the shallow or deep well water or from the municipal water supply used at the meat plant.

Discussion

An ecological study of a microorganism similar to that isolated

in 1956 by Anderson (4) and designated by him as <u>Micrococcus radio-</u><u>durans</u> indicated that the organism could be found in several different environments. It was readily isolated from ground beef and pork sausage at the Nebergall Packing Plant, Albany, Oregon. This was the same source from which it was originally isolated nine years ago. At that time it was shown to be highly radiation resistant. Numerous studies (15; 20; 21; 53; 61; 66; 96) since that time have substantiated that observation.

By taking advantage of its great radio-resistivity, high levels (2 m rad) of gamma radiation were used in the isolation procedures. This facilitated the inactivation of most of the microflora other than the most resistant types. However, approximately 160×10^4 microorganism/g of ground beef were recovered after the irradiation treatment. About one-tenth of this number were isolated from irradiated ground pork sausage (Table 1). These results were considered significant because until 1958, 2 m rads were considered to constitute an effective sterilizing dose for foods (70). Furthermore, previous reports (4) indicated that no microorganisms other than M. radiodurans were encountered in meat which had received 2 m rads. In the present study, the incidence of pigmented microorganisms resembling M. radiodurans varied between 5 - 20/g of irradiated meat. Hence, they comprised only a small fraction of the total number of survivors. The larger total number of survivors in the present study

may have been due to a larger initial number of organisms, the use of a different cobalt-60 source, different isolation media and a longer incubation period as compared to previous studies.

When the pigmented survivors were grown in pure culture and irradiated in phosphate buffer, their survival rate indicated a LD_{50} dose of approximately 700 k rads (Table 2). Microscopic examination of these pink-pigmented cultures revealed cocci (l μ , diameter) occurring as tetrads. These characteristics were similar to those previously reported for <u>M. radiodurans</u> (4; 20; 27; 53; 69). On this basis, and particularly the unusually high radiation resistance, the new isolates were considered as M. radiodurans.

When selected isolates among the other survivors of the irradiated meat were irradiated in buffer, their resistance was less than five percent of that for <u>M. radiodurans</u>. Evidently the meat substrate served as an excellent protective medium for these organisms, and this possibly could account for the high number of survivors. Recent reviews indicate that microorganisms are much more resistant when irradiated in a food as compared to a buffered suspension (70).

No apparent difference in susceptibility to radiation was discernible between strains of the resistant micrococcus that had been recovered from irradiated meat and those isolated from unirradiated meat and exposed to irradiation for the first time (Table 2). These observations were in accord with previous reports (4) and indicated that radiation resistance probably was a stable, inherent characteristic not acquired as a result of previous radiation exposures. The number of pink isolates obtained from unirradiated meat varied from 100-300/g.

The use of isolation media containing 0.5 percent NZ-Case resulted in more survivors from irradiated meat as compared to TGYM medium (Table 1). Similarly, the pigmented colonies developed within four days on NZ agar while the appearance of pink colonies on TGYM agar required a longer incubation period. A recent study (20) also indicated that NZ-medium was superior to six other media used for the isolation of a radiation resistant micrococcus from fish. However, additional observations from the present study indicated that cells grown in NZ broth were ten times more radiation sensitive when compared to TGYM broth. These cells were also noticeably less pigmented. Hence, TGYM broth was used as the culture medium for all comparative resistance studies of the new isolates.

Incubation of the meat samples either before or after irradiation did not increase the number of pink isolates obtained which resembled <u>M. radiodurans</u>. Evidently this organism does not compete well with other members of the microflora in this environment. The examination of spiced vs. non-spiced pork sausage and fresh vs. pre-packaged ground beef and pork did not result in an increased number of pink isolates. This suggested that neither the spices nor the packaging operation contributed to the presence of organisms resembling \underline{M} . radiodurans in the meat samples.

Pink-pigmented micrococci occurring in tetrads and possessing a high degree of radiation resistance in buffer were also isolated from beef hides (Table 3). Once again, ionizing radiation (2 m rads) facilitated the initial isolation of these organisms from cotton swabs which were used to brush the hides. These isolates were considered to be <u>M. radiodurans</u> on the basis of their radiation survival rate in buffer (Table 2).

Swab samples which were not incubated prior to irradiation produced 114 pink isolates of which 62 percent resembled <u>M. radiodurans</u> upon microscopic examination. Only one-tenth of this number could be obtained from swabs which had been pre-incubated in broth for two or four days prior to irradiation. This substantiates a previous statement that <u>M. radiodurans</u> does not compete well with other microorganisms in an enriched culture medium. When the swab samples were irradiated before incubation, however, the plates containing the recovery media presented a reddish-pink color due to the abundant growth of pigmented colonies. A majority of these proved to be Gram-positive micrococci upon microscopic examination. Hence, a post-incubation period appeared to offer a good method of isolating this organism from beef hides. The results of a control experiment indicated that the survival rate of M. radiodurans was not altered when cell suspensions were irradiated in the presence of sterile cotton swabs.

It was not possible to isolate organisms resembling M. radiodurans from soil, fecal material or hay samples obtained from the Nebergall plant. However, control experiments involving pre-inoculated soil samples (8000 cells/g) indicated that the experimental procedures allowed the re-isolation of M. radiodurans at a 41 percent recovery rate. These results suggested that microorganisms of this type were not present in the soil. Likewise, the use of pre- or post-incubation periods and the addition of nutrients or moisture to the soil did not promote the isolation of pigmented tetracocci, although non-spore forming bacilli which formed pink colonies were readily obtained. Evidence that M. radiodurans did not compete well with the microflora of a soil environment was provided from a second control experiment. By using an enrichment culture technique, it was found that cells of this type could not be re-isolated from a pre-inoculated 44 hour soilbroth culture. The use of radiation (700 k rads) did not alter this observation.

By irradiating water samples obtained from Cox Creek, Albany, Oregon, it was possible to isolate Gram-positive, pigmented tetracocci which proved to be just as resistant as <u>M. radiodurans</u> when irradiated in phosphate buffer (Table 2). Their incidence was 10 -25 per ml of water sample after 700 k rads. Attempts to concentrate these organisms by filtration techniques were unsuccessful although control experiments indicated that the presence of Millipore or Watman filter paper did not alter the recovery rate of irradiated cell suspensions. Cox Creek is used as a source of water for the livestock at Nebergall's Packing Plant. According to plant authorities, this water supply is pumped to the cattle holding pens and is used only for watering the livestock. The cattle are maintained at least 100 yards from the creek and in no case is refuse dumped into it. The samples in which the radiation resistant micrococci were found were obtained about 300 yards up-stream from the meat plant, and they were found in five different sampling areas.

The presence of <u>M</u>. radiodurans in this environment suggested a possible source and habitat of this organism. However, additional experiments should be conducted to confirm this observation and to determine if Cox Creek serves as an original source of this organism or if the organisms were fed into this creek from another source.

Summary

An ecological study of <u>M</u>. <u>radiodurans</u> indicated that microorganisms possessing the same morphological and radiation resistant characteristics of that organism could be isolated from ground beef and pork sausage. Further studies showed that it could also be isolated from beef hides and water from Cox Creek (Albany, Oregon). Similar microorganisms could not be isolated, however, from soil, hay or fecal material. The use of high levels of gamma radiation in the initial isolation procedures proved to be advantageous in inactivating most of the microflora and facilitating the isolation of <u>M. radio</u>durans.

Control experiments indicated that <u>M</u>. radiodurans did not compete well with the microflora present in ground meat, soil and beef hides. Pre-incubation before irradiation of meat and soil samples or enrichment culture techniques did not facilitate the isolation of <u>M</u>. radiodurans.

The presence of <u>M</u>. <u>radiodurans</u> in creek water suggested a possible source of this organism.

PART II MODIFICATION OF RADIATION RESISTANCE AND PIGMENT PRODUCTION BY GROWTH MEDIA

Ecological studies reported in Part I of this thesis involved the use of NZ medium to isolate <u>M. radiodurans</u> from various sources. Although it provided more luxuriant growth in less time than TGYM medium, it was observed that <u>M. radiodurans</u> was ten times more radiation sensitive when grown in NZ broth. It was also readily apparent that the least radiation resistant cells were also less brightly pigmented. This suggested that there may be a relationship between radiation resistance and degree of pigmentation. This possibility was investigated.

Methods and Materials

General Methods

A culture of <u>M.</u> radiodurans was provided from the stock culture collection of Oregon State University. It was maintained on slants of TGYM and NZ media. (The components of these media have been described in Part I).

Culture Conditions

Cells harvested from 44 hour TGYM or NZ broth cultures were used in all radiation experiments unless otherwise specified. These were prepared by using a one percent inoculum of a 44 hour culture and inoculating 100 ml of TGYM or NZ broth and incubating at 30 C on a gyrotory shaker.

Harvesting the Cells

The cells were harvested by centrifugation (7000 x G; 20 min.). They were then washed twice and resuspended in 100 ml of chilled, sterile phosphate buffer (0.067 M.; pH 7.0). The buffer solutions were never over 24 hours old. Cell density was adjusted with the aid of a spectrophotometer (Bausch and Lomb, Spectronic 20) and a viable cell-optical density curve. The spectrophotometer was used at 680 mµ with a red filter.

Radiation

The washed, resuspended cells were distributed in 10 ml quantities in duplicate, sterile radiation vials (25×55 mm) and irradiated at the Cobalt-60 source at Oregon State University at room temperature. Unless otherwise specified, the exposure dose was 700 k rads.

Recovery

Depending upon the initial growth medium, the cells surviving the irradiation exposure were recovered on either TGYM or NZ agar. All platings were made in triplicate and viable plate counts were made after 72 hours at 30 C. All cells which produced colonies under these conditions were considered viable and counted as survivors.

Particular Methods

Effect of Culture Media on Radiation Resistance. Broth cultures of <u>M</u>. radiodurans grown in TGYM and NZ media were obtained. After the cells were harvested and washed, their cell density was adjusted to approximately $1 \ge 10^8$ cells/ml. Ten ml aliquots were exposed in duplicate to a gamma radiation source and the number of survivors was determined on pour plates of TGYM or NZ agar. The percent survival was calculated.

Relative Effect of Growth and Recovery Medium on Radiation Resistance. It has been reported (2; 3; 32; 92) that the difference in radiation resistance of <u>E. coli</u> B and <u>E. coli</u> B/r lay not in the initial damage but in the metabolic processes after irradiation. Both strains are strongly influenced by the post irradiation conditions of recovery medium.

A ten-fold difference in resistance has been observed for \underline{M} . radiodurans grown in two different media before irradiation. Experiments were designed to determine the influence of recovery media on the radiation survival rate.

The cells were grown, harvested and irradiated as described

in the previous section. Cells initially grown in TGYM broth were recovered on TGYM agar for controls and also on NZ agar for comparative purposes. In a similar manner, cells initially grown in NZ broth were recovered on NZ and TGYM agar. In addition, the relative effect of using standard plate count agar (Difco) plus 0.5% NZ-Case vs an NZ medium made from the individual components was determined. All plates were incubated 72 hours at 30 C before they were counted. The initial cell concentration was adjusted to approximately $1 \ge 10^8$ cells/ml before irradiation.

<u>Comparative Effects of Various Additives in NZ and TGYM</u> <u>Media on Radiation Resistance.</u> Since NZ medium has certain components which are lacking in TGYM medium, experiments were designed to determine the effects of these individual ingredient differences on the radiation resistance of <u>M. radiodurans</u>. For this purpose, various broths were prepared which were variations of the standard TGYM and NZ media.

The components of the standard media were as follows:

TGYM (per lite:	<u>r)</u>	NZ (per liter)	
Bacto tryptone	5.0 g	Bacto tryptone	5.0 g
Glucose	1.0 g	Glucose	1.0 g
Yeast extract	1.0 g	Yeast extract	2.5 g
DL-methionine	20 mg	NZ-Case	
	0		

Variations of these two media were prepared as follows:

1. TGYM medium plus 0.5% NZ-Case

- 2. TGYM medium plus 0.15% yeast extract
- 3. NZ medium plus 20 mg DL-methionine

In addition, the standard TGYM and NZ medium were also prepared.

Cell cultures (44 hour) from each of these five media were prepared for irradiation as previously described and their relative radiation resistance was determined.

Effect of Different Levels of NZ-Case on Radiation Resistance. Previous experiments indicated that the presence of NZ-Case in the growth medium results in decreased radiation resistance of <u>M. radio-</u> <u>durans</u>. This experiment was designed to determine if there was direct relationship between the concentration of NZ-Case and the degree of radiation resistance.

A one percent inoculum of a 44 hour culture was used to inoculate a series of NZ media containing the following percent concentrations of NZ-Case: 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5. All media were inoculated in duplicate in DeLong culture flasks. The cells were harvested, irradiated (700 k rads) and the relative survival rate was determined as previously described. TGYM medium was used as a control and also as the recovery medium.

The Growth of <u>M</u>. radiodurans in TGYM and NZ Media. The difference in radiation resistance of <u>M</u>. radiodurans grown in two different media could be due to an altered growth rate in one medium.

This, in turn, could lead to "unbalanced" growth where the synthesis of one cellular component (e.g., DNA) cannot keep pace with the synthesis of another component (e.g., protein).

An altered growth rate would be readily detected from standard growth curves constructed for cells grown in the two different media. For this purpose, a 44 hour inoculum of <u>M</u>. radiodurans was grown in TGYM and NZ broth and used in inoculating duplicate flasks containing the two types of media. The cultures were incubated at 30 C on a gyrotory shaker. Viable plate counts were made every five hours during the first 30 hours of incubation and every eight to ten hours thereafter. The plating media was the same as the growth media. All plates were counted after 72 hours at 30 C.

<u>Resistance at Different Physiological Ages.</u> If "unbalanced" growth is responsible for the altered radiation resistance, then cells of different physiological ages should be markedly different in their resistance. Experiments were arranged to investigate this possibility.

Using a one percent inoculum of a 44 hour culture in each type of medium, DeLong culture flasks containing 100 ml of TGYM or NZ broth were inoculated every 12 hours for 56 hours and incubated at 30 C on a shaker. The time of inoculation was staggered so that cells of five different physiological ages could be harvested at the same time. After the cells had been washed twice and resuspended in phosphate buffer, they were irradiated and their survival rate was calculated as previously described.

Additional experiments to measure "unbalanced" growth were performed by measuring the relative amounts of DNA, RNA (ribonucleic acid) and total protein of <u>M. radiodurans</u> grown in the two different media and comparing them on a dry weight basis.

<u>DNA</u>. DNA measurements were made using cells which had been grown in the two different media, washed twice in saline and resuspended in saline (0.15 M) - versine (0.1 M) solution (5 ml/100 ml of culture) which previously had been adjusted to pH 8.0. DNA was liberated by hydrolyzing the cells (6 ml) with perchloric acid (2 ml; 11.7 N) at 70 C for ten minutes. The cells were mixed once for 30 seconds with a Vortex Jr. mixer during the heating period. After the mixture had cooled, it was centrifuged (3000 x G; 20 min) in plastic tubes and the supernatant was decanted into clean glass tubes.

The determination for DNA was made from the above supernatant according to the method described by Dische (24, p. 285). This method measures the amount of omega-hydroxylevlialdehyde produced by the acid hydrolysis of deoxyribose and develops a blue color in the presence of diphenylamine reagent. ^{*} The intensity of

Doubly recrystallized in 70 percent ethanol. One and a half grams of this was dissolved in 100 ml of glacial acetic acid and 1.5 ml of concentrated H_2SO_4 . On the day the reagent was to be used, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent required.

blue color was determined by the optical density at 600 m μ . This process was carried out by adding 3.0 ml of diphenylamine reagent to 1.0 ml of supernatant, mixing periodically for one hour and incubating at 30 C for 18 hours.

A DNA standard curve was prepared by dissolving calf thymus DNA (Sigma Chemical Corp.) in warm NaOH (5 mM). Perchloric acid (0.5 N) was used as a diluent.

<u>RNA</u>. Using the cell-free supernatant as prepared above, RNA was measured by the orcinol reaction (63) with some modifications. The orcinol reagent was prepared by dissolving one g of orcinol plus 0.5 g FeCl₃ in 100 ml of HCl (6.75 N). The final normality was 7.0 upon the addition of one ml of acid hydrolyzed cell supernatant. After steaming the cellular RNA-orcinol mixture for 20 minutes in screwcapped test tubes, it was cooled under running water. A green color developed due to the presence of free pentose sugars, and the optical density was measured at 660 mµ with a spectrophotometer (Bausch and Lomb, Spectronic 20). A RNA standard curve was prepared using yeast RNA (Cal-Biochem).

<u>Protein.</u> The percipitate from the cellular acid hydrolysis as described above was used in the determination of total protein. Ten ml of a three percent solution of NaOH was added to the percipitate to bring it into solution. The amount of protein was measured by the Biruet method (39). In this test, substances containing two or more peptide bonds form a purple complex with copper salts in alkaline solution. Albumin dissolved in NaOH (3 percent) was used in preparing a standard curve for protein.

pH. The relative acidity of the two media were compared by measuring the pH of the sterilized media before inoculation and after 44 hours of growth.

In order to compare the relative amounts of DNA, RNA and protein on a dry weight basis, measured quantities of cell culture were lyophilized for 24 hours (Virtis lyophilizer) and then weighed quantitatively on a Mettler balance.

Packed cell volumes were determined by centrifuging concentrated suspensions of cells in hematocrit tubes (3000 x G; 15 min).

Photomicrographs were taken of the cells grown in the two different types of media. These photographs were taken using a Leitz Ortholux microscope equipped with a Heine phase contrast condensor #74 and Pv Fl 70/1.15n objective. A Kodak High Contrast Copy film and "Mikas" camera attachment were used along with a green filter.

Dose-level Responses to Gamma Radiation

Since the LD_{50} dose is a frequently used index of the degree of radiation resistance of an organims, experiments were conducted to determine the relative LD_{50} dose of <u>M</u>. radiodurans grown in TGYM and NZ broth. The cells were grown, prepared for irradiation and

recovered as previously described. The radiation levels used were 0, 0.1, 0.2, 0.3, 0.5 and 0.7 m rads. All radiations were conducted at the Cobalt-60 source at Oregon State University.

Effect of Growth Media on Resistance to Ultraviolet

Previous reports (26) indicated that <u>M. radiodurans</u> was resistant to ultraviolet light as well as ionizing radiation. It also produced a sigmoidal survival curve when exposed to ultraviolet light. Since previous experiments reported in this thesis indicate a ten-fold decrease in resistance of this organism to gamma radiation when grown in NZ broth, additional experiments were conducted to determine if there was a similar change in resistance to ultraviolet light.

For this purpose, cells of <u>M.</u> radiodurans were grown, harvested and resuspended in phosphate buffer as previously described. After the cell density was adjusted to approximately $1 \ge 10^8$ cells/ml, appropriate dilutions were made so as to have approximately $1 \ge 10^4$ cells/ml. Serial dilutions were made from this latter cell suspension, and one ml aliquots were transferred in triplicate to sterile, flat bottom petri plates for exposure to ultraviolet light. The cell suspensions were uniformly spread over the surface of the plate by hand agitation.

The ultraviolet lamp was equipped with two 15 watt germicidal lamps (GE 15T8) and was used at a distance of 44 cm from the plates.

The exposure rate was 100 ergs/cm²/sec. The plates were gently agitated on a rotator (Eberbach) during the exposure periods varying from 15 to 210 seconds. At the higher initial cell concentrations, dilutions were made on the plates following ultraviolet exposure. The survival rate was determined by making pour plates with either NZ or TGYM agar.

Effect of Variation in Exposure Times to Two Different Media on Radiation Resistance

Since there was an inverse relationship between the degree of radiation resistance and concentration of NZ-Case in the growth medium, this suggested that the growth medium was possibly inhibiting or stimulating the synthesis of some vital cellular component which imparts radiation resistance to the cell.

An experiment to test this possibility was designed to show the reversibility of the alteration of radiation resistance. For this purpose, cells of <u>M</u>. radiodurans were grown for 44 hours in either NZ or TGYM broth, harvested, washed and resuspended in the same or opposite medium for either 3 or 44 hours. The cells were then washed free of the new medium and their relative survival rate to gamma radiation (700 k rads) was determined as previously described.

Study of the Pigments of <u>M. radiodurans</u> Using Thin Layer Chromatography

The pigments of M. radiodurans have been reported to be of the carotenoid type (53; 59, p. 20). Carotenoids are known to be closely related structurally and biosynthetically to lipids (36; 44; 45; 46; 88; 98) and as such they can be investigated using methods common for lipid analysis. One of the analytical methods which has become universally accepted within the last five years is thin layer chromotography. Its application to lipid analysis has been reviewed recently (74).

Since there was an apparent difference in the degree of pigmentation for cells of <u>M</u>. <u>radiodurans</u> possessing different degrees of radiation resistance, it was decided to use thin layer chromatography to study these pigments qualitatively and quantitatively.

<u>Preparation of Pigment Extracts.</u> Cells were grown in one liter quantities in TGYM and NZ broth as previously described. Following centrifugation, the cells were washed four times with distilled water and then lyophilized. Equal amounts (approximately 0.5 g) of the lyophilized cultures were weighed quantitatively and transferred to a 100 ml round-bottom reflux flask for extraction of the pigments. Folch's procedure (29) for the isolation and purification of lipids was used with several modifications.

A volume of chloroform-methanol (2:1; v:v) 20 times the weighed

cell mass was added to each reflux flask. The mixture was refluxed for 35 minutes using low heat and glass beads to avoid excessive bubbling. After the contents had been cooled under cold tap water, it was filtered thru a glass sintered funnel (coarse grade). The lipid filtrate was then washed with 0.2 of its volume of 0.73 percent NaCl with gentle agitation so as to avoid the formation of an emulsion. The mixture separated into two phases. As much as possible of the upper phase was removed by siphoning, and removal of its solutes was completed by rinsing the interface three times with small amounts (one ml) of pure solvents upper phase * in such a way as not to disturb the lower phase. Finally, the lower phase and the remaining rinsing fluid were made into one phase by the addition of methanol.

Excess water was removed by adding Na_2SO_4 in great excess (approximately 10 g) and allowing it to stand 15 minutes. The colored solution was now very clear and transparent. The Na_2SO_4 was removed by filtering the mixture thru a glass sintered funnel (coarse grade) and washing it three times with methanol to remove all the pigment. Nitrogen was bubbled thru the pigment extract to evaporate it to near dryness, and it was then evaporated to complete dryness under vacuum at 30 C. The dried material was immediately resuspended in

The pure solvent upper phase was prepared by mixing in a separatory funnel chloroform, methanol and water (8:4:3; v:v:v). This mixture separated into an upper and lower phase. The lower phase was discarded and the upper phase was used.

one ml of chloroform-methanol (2:1; v:v) and stored at -15 C. This material was used in running the thin layer chromatograms.

Preparation of Thin Layer Plates. Glass plates (20 x 20 cm) were thoroughly washed and then wiped dry with methanol. Silica gel G was applied with an adjustable applicator (Brinkman) to a thickness of 0.25 mm. After 15 minutes exposure to room temperature, the plates were activated by placing them at 100 C for at least four hours.

<u>Running the Chromatogram.</u> After numerous preliminary trials with various solvent systems, it was found that a mixture of benzene, methanol (absolute) and glacial acetic acid (87:11:2; v:v:v) gave the best pigment separation on thin layer.

Using a micro-pipette, the pigment extract was applied at a level of 40 gamma per spot on the activated thin layer plate. The size of the spot was kept small by quickly drying the extract with a steady flow of nitrogen, and in no instance did the spot exceed 30 mm². The chromatogram was run opposite to the direction of application of the silica gel.

The solvent system as previously described was prepared in duplicate 100 ml quantities. An antioxidant, 4-methyl-2, 6-Di-tertbutylphenol (BHT) was added at a level of 200 μ g/ml (101). One of the solvent mixtures was poured into a stainless steel trough which was adapted for development of the chromatogram by the "sandwich" technique. Numerous preliminary trials indicated that the best
pigment separation was obtained by initially having an extremely high degree of chamber saturation as provided by the "sandwich" apparatus. After the solvent front had advanced five cm, the chromatogram was quickly transferred to a covered glass chamber (34 x 21 x 10 cm) whose atmosphere had previously been saturated with the solvent. The solvent front was allowed to advance an additional seven cm at which time the chromatographic separation was terminated. The chromatogram was quickly transferred to a dry box which had previously been flushed and filled with nitrogen. By flushing the dry box six more times with nitrogen, the chromatogram was dried and the solvent vapors were removed. Utilization of a nitrogen atmosphere for the isolated, dried pigments was found necessary since exposure to the air caused their rapid oxidation and disappearance.

Pictures of these plates were taken by covering the chromatogram with Saran-Wrap and using a photocopy duplicator machine (A. B. Dick model 112).

All unsaturated lipids or lipid-like substances were readily apparent by developing the plates in an atmosphere of iodine vapors (62).

Elution. The pigmented spots were removed mechanically with an improvised vacuum zone collector. After the pigments were eluted with a mixture of absolute ethanol-methanol (1:1; v:v), they were analyzed spectrophotmetrically with a recording spectrophotometer (Cary, model 11) in the visible (325-800 mµ) and ultraviolet range (210-360 mµ). Quantitative estimates of the relative amounts of the pigments was calculated by determining the area under each absorption curve.

Additional Experiments with Thin Layer

Effect of Irradiation of Whole Cells on Pigmentation. M. radiodurans was grown, harvested and prepared for irradiation as previously described. Cells grown in TGYM broth received a dose of gamma irradiation which inactivated 99.9 percent of the exposed population. This dose was calculated to be 1.25 m rads. Cell cultures which served as controls were grown in TGYM and NZ broth and prepared in a similar manner but were unirradiated.

Each of the three cultures were then lyophilized and thin layer chromatograms were run on the extracted pigments as previously described.

Effect of Endogenous Metabolism on Radiation Resistance and Pigmentation. Cells of M. radiodurans which had been grown in either NZ or TGYM broth were harvested, resuspended in sterile phosphate buffer (0.067 M; pH 7.0) and placed on a gyrotory shaker at 30 C for ten hours. After centrifugation, the cells were resuspended in buffer and irradiated (700 k rads). Control cultures were handled in a similar manner except they were not shaken prior to irradiation. The relative survival rate was determined by the pour plate method. The degree of pigmentation was studied by the previously described thin layer chromatographic procedures.

Results

Effect of Culture Media on Radiation Resistance

The relative survival rate of irradiated <u>M</u>. <u>radiodurans</u> grown in two different media is given in Table 4. The percent survival represents the average of duplicate samples irradiated in four different experiments. The results indicated that cells grown in NZ broth were ten times less radiation resistant than those grown in TGYM broth.

Growth and Recovery	Viable cel	Percent	
Medium	0 k rads	700 k rads	Survival
TGYM	207×10^{6}	124×10^{6}	60.3
ΝZ	123×10^{6}	77×10^{5}	6.3

Table 4. The influence of two different growth media on the radiation resistance of \underline{M} . radiodurans

^aViable counts are the average of triplicate plates from four experiments.

Cell cultures grown in NZ broth produced a clear, brown colored liquid within 44 hours, while the TGYM broth was tan. Both media were the same color initially. The color change began to occur after approximately 20 hours incubation. Centrifuged cells grown in TGYM medium were bright pink, while those grown in NZ broth were a faded, dull pink color.

NZ-Case (Sheffield Chemical Co.) is a tryptic digest of casein in powder form. A typical analysis by the manufacturer is given in Table 5.

Moisture 3.5 % Ash 6.1 % 12.7 % Total Nitrogen Amino Nitrogen 5.1 % Amino N/Total N 40.2 % Sodium 2.45% Chloride 0.30% Iron 35.4 ppm Total Phosphates 2.37% pH (2% solution) 7.2 Solubility (clear, 30° C) 25 grams/1000 cc Clycine 1.8% Aspartic Acid 5.9 % Alanine 3.3% Glutamic Acid 22.8 % 5.5% Valine 10.6 % Proline Leucine 10.3% Histidine 3.05% Isoleucine 4.5% Tryptophane 1.4 % 6.7% Serine 4.6 % Phenylalanine Threonine 3.7% 3.5 % Tyrosine Lysine 6.5% Cystine 0.51% 3.6% Methionine Arginine 2.35%

Table 5. Typical analysis of NZ-Case as provided by the manufacturer (Sheffield Chemical Company, Norwich, New York)

Bacto-tryptone, the counterpart of NZ-Case used in the TGYM medium, has a typical analysis as shown in Table 6.

	Percent
Total nitrogen	13.14
Primary proteose N	0.20
Secondary proteose N	1.63
Peptone N	11.29
Ammonia N	0.02
Free amino N	4.73
Amide N	1.11
Mono-amino N	7.31
Di-amino N	3.45
Tryptophane	0.77
Tyrosine	4.39
Cystine	0.19
Organic sulfur	0.53
Inorganic sulfur	0.04
Phosphates	0.97
Chlorine	0.29
Sodium	2.69
Potassium	0.30
Calcium	0.096
Magnesium	0.045
Iron	0.0104
Ash	7.28
Ether soluble extract	0.30

Table 6. Typical analysis of Bacto-tryptone as provided by the manufacturer (Difco Laboratories, Detroit, Michigan)

As can be seen, NZ-Case has 2.5 times as much phosphates, 3 times as much iron and 3 times as much cystine as tryptone. NZ-Case also has relatively high concentrations of the aromatic amino acids as well as other component differences when compared to tryptone.

Effect of Growth vs Recovery Media on Radiation Resistance

The present survival of <u>M</u>. radiodurans was not altered when it was grown on one medium and recovered on the same or another

medium after irradiation (Table 7). Cells grown in TGYM broth and recovered on TGYM or NZ agar after irradiation (700 k rads) had approximately 60 percent survival. On the other hand, cells initially grown in NZ broth had approximately six percent survival regardless of whether they were recovered on NZ or TGYM medium. It was also observed that the survival rate was not altered appreciably by using either the dehydrated NZ agar (Difco) or the NZ agar medium made from the individual ingredients; there was a six percent recovery rate on the former and five percent recovery on the latter medium. The initial cell concentration was similar in all cases before irradiation. The results given in Table 7 are the average of three different experiments.

Effects of Major Component Differences of NZ and TGYM media on Radiation Resistance

<u>M. radiodurans</u> was grown in five different media to determine the effect of various additives on its resistance to gamma radiation. These additives were ones which represented major differences in NZ and TGYM media. The results of Table 8 indicate that the addition of DL-methionine to NZ broth at the same concentration used in TGYM broth did not produce increased radiation resistance. However, the addition of NZ-Case to TGYM medium reduced the resistance more than 30 fold, and the resistance was now comparable to

Growth	Recovery	Viable c	cells/ml ^d	Percent	
Medium	Medium	Before irradiation	After irradiation	Survival	
TGYM	TGYM ^a	207×10^{6}	124×10^{6}	60.0	
TGYM	NZ	189×10^{6}	121×10^{6}	64.0	
ΝZ	NZ ^b	123×10^{6}	77×10^{5}	6.3	
ΝZ	TGYM	140×10^{6}	86 x 10 ⁵	6.1	
NZ	NZ ^c	130×10^{6}	67×10^{5}	5 . 2	

Table 7. Effect of the growth vs recovery media on the survival of M. radiodurans exposed to700,000 rads of gamma radiation in phosphate buffer

^aMedium prepared from 0.5% tryptone, 0.1% yeast extract, 0.1% dextrose, 0.002% DL-methionine, 1.5% Bacto agar, pH adjusted to 7.0.

^bMedium prepared by using standard Difco Plate Count Agar plus 0.5% NZ-Case.

^CMedium prepared from 0.5% NZ-Case, 0.5% tryptone, 0.25% yeast extract, 0.1% dextrose,

1.5% Bacto agar, pH adjusted to 7.0.

^dViable counts are the average of triplicate plates from three different experiments.

	Viable	Percent	
Medium	Before radiation	After radiation	Survival
TGYM	43×10^{7}	164×10^{6}	38.1
NZ	41×10^7	85 x 10 ⁵	2.1
TGYM plus NZ (0.5%)	37×10^7	38×10^{5}	1.0
TGYM plus yeast extract (0.15%)	47×10^7	146×10^{6}	31.2
NZ plus DL- methionine (0.002%)	38×10^7	64×10^{5}	1.7

Table 8. Effect of various growth media on the survival of <u>M</u>. radiodurans exposed to 700,000 rads of gamma radiation in phosphate buffer

^aViable counts are the average of triplicate plates from two different experiments.

that observed for cells grown in NZ broth. When the yeast extract concentration was increased in TGYM to levels comparable to that in NZ medium, the resistance of the organisms was approximately the same as for those grown in the standard TGYM broth.

The initial cell concentration was approximately twice that used in previous experiments. Since increased cell concentrations are reported to cause increased radiation sensitivity (27), this may account for the ten percent decrease in resistance of cells grown in TGYM when compared to the results of previous experiments.

Effects of Various Levels of NZ-Case on Radiation Resistance

It was observed that an inverse relationship exists between the degree of radiation resistance of <u>M. radiodurans</u> and the concentration of NZ-Case in the growth media (Table 9). The higher the level of NZ-Case, the lower was the observed radiation resistance. At levels of 0.1 percent NZ-Case and above, the inverse relationship was linear (Figure 1). The survival of cells grown in the absence of NZ-Case was 67 percent while approximately five percent of the cells survived when they had been grown in the presence of 0.5 percent NZ-Case.

A decrease in the amount of pigmentation could also be observed visually for those cells grown in the higher levels of NZ-Case.

These results suggested that NZ-Case inhibited or stimulated

Concentration	Viable d	cells/ml ^a	Percent
of NZ-Case	Before radiation	After radiation	Survival
0 (TGYM)) 166×10^{6}	112×10^{6}	67.7
0.01	188×10^{6}	127×10^{6}	67.4
0.05	211 x 10 ⁶	93 x 10 ⁶	44.3
0.1	162×10^{6}	41×10^{6}	25.6
0.2	140×10^{6}	242×10^5	17.5
0.3	197×10^{6}	226×10^{5}	11.5
0.4	151×10^{6}	107×10^{5}	7.1
0.5	162 x 10 ⁶	76×10^5	4.5

Table 9.	Effect of various levels of NZ-Case in the growth medium
	on the gamma radiation resistance of M. radiodurans
	exposed to 700,000 rads in phosphate buffer

^aViable counts are the average of triplicate plates from duplicate irradiated samples of three different experiments.



Figure 1. The inverse relationship of gamma radiation resistance of M. radiodurans and the concentration of NZ-Case in the growth medium.

the synthesis of a metabolic factor associated with radiation resistance. The additional observation of a decrease in pigmentation with decreased resistance further suggested that the pigments of this organism were related to its radiation resistance.

The Growth of M. radiodurans in NZ and TGYM Media

<u>M.</u> radiodurans was grown in NZ and TGYM broth and viable plate counts were made periodically. This data was used in constructing standard growth curves (Figure 2).

When <u>M. radiodurans</u> was grown in NZ broth, it entered the death phase after 65 hours of incubation while the cells remained in the stationary phase for at least 96 hours when grown in TGYM medium. The rate of growth in the log phase was essentially the same for the two media. Although a larger number of viable cells were present at the end of the log phase, this probably was due to having twice as many cells per ml in the original inoculum.

Effect of Physiological Age on Resistance

Cells of <u>M.</u> radiodurans were grown in TGYM and NZ broth, harvested and irradiated at different physiological ages. As indicated in Table 10, cells grown in NZ broth were at least ten times more radiation sensitive than cells grown in TGYM at all physiological ages. The single exception to this general observation was that



Figure 2. Growth curves of <u>M</u>. radiodurans grown in NZ and TGYM broth @ 30 C on a gyrotory shaker.

Growth Medium	Culture (hrs.)	$\frac{\text{Viable cells/m}}{0 \text{ k rads}}$	l after exposure to 700 k rads	Percent Survival
TGYM	8	210 x 10 ⁶	281×10^{5}	13.3
	20	105×10^{6}	52×10^{6}	49.5
	32	136×10^{6}	48×10^{6}	35.3
	44	133×10^{6}	90 x 10 ⁶	67.5
	56	127×10^{6}	46×10^{6}	36.2
NZ	8	228×10^{6}	85×10^{5}	3.7
	20	164×10^{6}	70 x 10^5	4.3
	32	159×10^{6}	93 x 10 ⁵	5.8
	44	182×10^{6}	51×10^{5}	2.8
	56	144×10^{6}	32×10^5	2.2

Table 10. Effect of physiological age on gamma radiation resistance of \underline{M} . radiodurans when grown in NZ and TGYM broth and irradiated in phosphate buffer

the eight hour culture grown in TGYM broth was only twice as resistant as the NZ culture. With that exception, the survival rate for the TGYM cultures ranged from 35 to 67 percent; while the survival rate of the irradiated NZ cultures ranged from two to six percent at the different ages.

Effect of Altered Radiation Resistance on Certain Chemical Factors and Cell Morphology

The results of the chemical determinations which were made for cells of <u>M</u>. radiodurans of altered radiation resistance are given in Table 11.

Table 11.Chemical determinations of cells of M. radiodurans
grown in NZ and TGYM broth and possessing ten-fold
differences in gamma radiation resistance

Determination	TGYM culture	NZ culture	
DNA (percent, dry weight basis)	1.2	1.0	
RNA (percent, dry weight basis)	4. 5	4.5	
Protein (percent, dry weight basis)	47.0	48.3	
Growth medium pH, initial	7.0	7.0	
Growth medium pH, final	8.2	8.0	

From these results it was observed that the amount of DNA for both types of cultures was one percent on a dry weight basis. In a similar manner, the relative amounts of RNA and protein did not vary for the two cultures. There was 4.5 percent RNA and approximately 47.5 percent protein for both the highly radiation resistant and more radiation sensitive cultures.

The initial medium pH was 7.0 and final pH was 8.0 and 8.2 for a 44 hour culture in TGYM and NZ broth, respectively. In addition, the relative dry weight and the packed cell volume of the cells grown in TGYM were one-half of that for an equal volume of cells grown in NZ broth. This suggested that a 44 hour NZ culture produced twice the cell mass as a TGYM culture. Evidence that this increase in cell mass was due to higher numbers of cells and not an increase in cell size is apparent from Figures 2, 3 and 4. From Figure 2 it can be seen that there were twice as many cells in a 44 hour NZ culture; and Figures 2 and 3 show that there was no change in cell size or arrangement.

Gamma Radiation Dose-level Responses

The LD_{50} doses of <u>M</u>. <u>radiodurans</u> grown in the two different types of media were found to be significantly different. From Figure 5 it is apparent that the LD_{50} dose for cells grown in NZ broth was approximately 350 k rads compared to TGYM grown cells which required 700 k rads to inactivate 50 percent of the exposed population. A wide "shoulder" was observed in the survival curve for cells grown in TGYM; whereas, the survival curve became practically



Figure 3. Phase contrast photomicrograph of <u>M</u>. radiodurans grown in TGYM broth at 30 C for 44 hours. 3500x.



Figure 4. Phase contrast photomicrograph of <u>M</u>. radiodurans grown in NZ broth at 30 C for 44 hours. 3500x.



Figure 5. Dose survival for <u>M</u>. radiodurans grown in NZ or TGYM broth and exposed to gamma radiation while suspended in phosphate buffer. Irradiation was conducted in air at room temperatures at a dose rate of 713 k rads/hour.

exponential after 300 k rads for cells grown in NZ broth.

Effect of Growth Media on Resistance to Ultraviolet Light

When <u>M. radiodurans</u> was grown in NZ broth and suspended in phosphate buffer, its resistance to ultraviolet light was approximately ten times less than similar cells grown in TGYM broth. These results are presented in Figure 6. A sigmoidal survival curve was obtained for cells grown in TGYM broth. This was in agreement with previous reports (26). However, the rate of cellular inactivation was exponential for cells grown in NZ broth. This observation was similar to those reported for non-radiation resistant microorganisms (26).

Reversibility of Radiation Resistance

When <u>M. radiodurans</u> was grown in TGYM broth and then exposed to NZ broth for 44 hours, its radiation resistance was found to be one-tenth of that for cells exposed for three hours to NZ broth (Table 12). On the other hand, cells grown in NZ broth and then exposed to TGYM broth for either three or 44 hours were found to be equally radiation resistant.

A three hour incubation period allowed sufficient time for the production of only two or three generations of cells. On this basis and the results observed, it appeared that the precursors of factors related to radiation resistance were present in cells grown in NZ



Figure 6. Survival curves of <u>M</u>. radiodurans grown in NZ or TGYM broth and exposed to ultraviolet light while suspended in phosphate buffer. The exposure rate was 100 ergs/cm²/second.

Initial Growth	Exposure	Exposure	Viable cel	ls/ml after	Percent
Medium	Times (hrs.)	Medium	0 k rads	700 k rads	Survival
TGYM	3	NZ	$245 \ge 10^{6}$	118×10^{6}	48.0
ΝZ	3	TGYM	145×10^{6}	110×10^{6}	7 5. 4
TGYM	44	NZ	224×10^{6}	106×10^{5}	4 . 5
ΝZ	44	TGYM	135×10^{6}	95×10^{6}	70.3

Table 12. Change in the gamma radiation resistance of M. radiodurans by different exposure times to either TGYM or NZ broth. Cells were irradiated in phosphate buffer.

broth, but that the factors themselves could not be synthesized in this medium.

It was observed once again that cells grown in TGYM broth were more heavily pigmented than cells grown in NZ broth, and that this visually apparent change in pigmentation was reversible by exposing the cells to the two different media.

Study of Pigments by Thin Layer Chromatography

M. radiodurans grown in TGYM broth was found to have eight distinct pigments as shown in Figure 7. However, only six pigments could be observed in cells grown in NZ broth. The missing pigments were designated 1 and 4 because of their position on the chromatogram. Figure 8 is a colored photograph of the same chromatogram.

When the chromatogram shown in Figures 7 and 8 was developed in iodine vapors, numerous unsaturated lipids or lipid-like substances could be demonstrated (Figure 9). Most of these substances appeared to be closely associated with the pigments on the basis of their chromatographic separation. However, two or three additional spots could now be seen in areas remote from the colored pigments. These spots were particularly dense for cells grown in NZ broth and at least one of them demonstrated a slight green fluorescence in ultraviolet light. These additional spots were colorless under visible light prior to development in iodine vapors. They



Figure 7. Thin layer chromatogram of the pigments of M. radiodurans. Solvent system: benzene, methanol, glacial acetic acid (87:11:2; v:v:v). Chromatogram developed on Silica Gel G plates using both "sandwich" and tank developing chambers. Picture taken with photocopy duplicator machine. The cells were grown aerobically
@ 30 C in either TGYM or NZ broth.



Figure 8. Colored photograph of thin layer chromagram showing the pigments of <u>M. radio-</u> durans. The chromatogram was developed as described for Figure 7.



Figure 9. Thin layer chromatogram of the pigments and lipids of <u>M. radiodurans</u>. This is the same chromatogram as in Figure 7 except it was developed in iodine vapors to indicate colorless unsaturated compounds. (Note dense spots near the front for cells grown in NZ broth).

were found near the top of the chromatogram.

When these pigments were eluted from the chromatogram and analyzed spectrophotometrically, the absorption curves in the visible range were similar for all eight pigments (Figures 10 and 11). There was a maximum absorption peak at 475 mµ with two minor peaks at approximately 450 and 500 mµ. These absorption characteristics were typical for lycopene or its hydroxyl derivatives (46). The structure of lycopene is given in Figure 12. As can be seen, it is highly unsaturated and could well serve as a scavenger of ions and radicals produced by radiation. The structure of squalene is also presented to indicate the close relationship of lycopene to other lipids.

The pigments which were found in cells grown in NZ broth had absorption characteristics similar to those for corresponding pigments of TGYM grown cells but were present in only one-half the concentration. This was apparent from the optimal density spectral curves (Figures 10 and 11) as well as from Table 13.

Numerous attempts to characterize these pigments in the ultraviolet range (210-360 mµ) were unsuccessful.

Effect of Irradiation of Whole Cells on Pigmentation

When <u>M.</u> radiodurans was grown in TGYM broth and subsequently exposed to a dose of radiation lethal for 99.9 percent of the population, pigments 1 and 4 were missing upon chromatographic

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Figure 10. Spectrophotometric curves of pigments 1-4 of <u>M</u>. radiodurans eluted from thin layer chromatograms with ethanol-methanol (1:1; v:v). Curves were obtained using a Cary, Model 11.Chromatographic separation was the same as for Figure 7.



Figure 11.Spectrophotometric curves of pigments 5-8 of <u>M</u>. radiodurans eluted from thin layer chromatograms with ethanol-methanol (1:1; v:v). Curves were obtained using a Cary, Model 11. Chromatographic separation was the same as for Figure 7.



Figure 12. Chemical structure of lycopene and squalene (88). Note highly unsaturated, conjugated double bond system of lycopene.

Pigment No.	Area (cr O. D. cu TGYM	m ²) of 1rve NZ	Relative r conc. of pig TGYM	percent gments in NZ	Ratio of pig- ments in TGYM vs NZ media
1	3.12	b	7.2	^b	b
2	11.62	6.57	26. 8	37.4	1. 8:1
3	4.37	2.67	10.1	15.4	1. 6:1
4	2.06	 b	4.8	b .	b
5	5.57	1.93	12.8	11.0	2.9:1
6	10.55	3.34	24.4	19.0	3.2:1
7	1.47	0.96	3.4	5.5	1, 5:1
8	4.50	2.08	10.4	11.9	2.2:1

Table 13. Relative concentrations of the pigments of <u>M. radiodurans</u> when grown in NZ or TGYM broth^a

^aBased on spectral curves of pigments eluted from thin layer chromatograms (see Figures 10 and 11).

^bNot produced in cells grown in NZ broth.

analysis as well as pigments 6, 7 and 8 (Figure 13). It should be noted that pigments 1 and 4 were the same ones lacking in the less radiation resistant strain of this organism grown in NZ broth.

Effect of Endogenous Metabolism on Radiation Resistance and Pigmentation

When <u>M.</u> radiodurans was grown in NZ broth and then shaken in phosphate buffer (0.067 M, pH 7.0) for ten hours, its radiation resistance increased more than ten times (Table 14). Control experiments using cells grown in TGYM broth indicated no significant change in resistance when they were shaken in buffer.

Table 14. Survival rate of <u>M</u>. radiodurans grown in NZ or TGYM broth and resuspended in 0.067M phosphate buffer for 8-10 hours on a shaker prior to gamma irradiation (700 k rads).

Growth	Number of s	Percent	
medium	0 k rads	700 k rads	Survival
NZ broth	149 x 10 ⁶	52×10^5	.3. 5
NZ-shaken in buffer	$157 \ge 10^{6}$	$68 \ge 10^{6}$	43.3
TGYM broth	120×10^{6}	70×10^{6}	58.4
TGYM-shaken in buffer	131 x 10 ⁶	53×10^{6}	40.5

An analysis of the pigments by thin layer chromatography (Figure 14) indicated that pigments 1 and 4 were once again absent



Figure 13. Thin layer chromatogram of the pigments of <u>M</u>. <u>radiodurans</u> grown in NZ or TGYM broth. Solvent system: benzene, methanol, glacial acetic acid (87:11:2; v:v:v). Absorbent: Silica Gel G. Separation conducted in "sandwich" and tank chamber apparatus. TG-IRR indicates cells grown in TGYM and exposed to 1.25 m rads. NZ and TG cells were un-irradiated.



Figure 14. Thin layer chromatogram of the pigments of <u>M</u>. radiodurans grown in NZ broth. The culture on the right was shaken in phosphate buffer for 10 hours prior to chromatographic analysis of its pigments. Chromatographic procedures were the same as for Figure 13.





Figure 15. Thin layer chromatogram of the pigments and lipids of <u>M.</u> radiodurans grown in NZ broth. This is the same chromatogram as in Figure 14 except it was developed in iodine vapors. in cells grown in NZ broth but appeared after ten hours in those cells resuspended in buffer and placed on a shaker. Similar results with <u>Rhodospirillum rubrum</u> have previously been reported (46). When the preceding chromatogram was developed in iodine vapors (Figure 15), areas consistently obtained from NZ grown cells were now lacking when these cells were shaken in buffer. Since these areas were colorless until developed and showed a slight fluorescence with ultraviolet light, these results suggested that these areas were phytoene and/or phytofluene. These latter substances are also known precursors of the colored carotenoids (46; 79).

Discussion

The addition of NZ-Case (a tryptic digest of casein) to plate count agar has been reported (20) to provide an excellent isolation medium for obtaining an orange-brown micrococcus from irradiated haddock tissues. Smooth and rough variants of this new isolate and <u>M. radiodurans were grown in NZ broth and irradiated in phosphate</u> buffer. The radioresistance of the smooth strain as well as <u>M.</u> <u>radiodurans</u> was reported to be comparable. However, closer analysis of their survival curves indicates that <u>M. radiodurans</u> was at least twice as resistant at radiation levels below one m rad. Furthermore, the orange-brown isolates produced an exponential survival curve whereas M. radiodurans produced a sigmoidal survival curve.
The new isolates were further distinguished from <u>M. radiodurans</u> by being smaller in size and in being capable of reducing nitrate to nitrite and by hydrolyzing gelatin. It was also reported (20) that a less pigmented strain of the smooth variant was isolated and did not differ in its radioresistivity from the more highly pigmented variants. However, no data was provided as to the magnitude of difference in pigmentation or how this difference was measured or the results of their comparative radiation studies.

In the ecological studies reported in Part I of this thesis, an NZ-Case medium similar to the one reported above was used to investigate possible sources of M. radiodurans. Another medium, TGYM, was also used since it has been used consistently in studies of M. radiodurans (4; 26; 60). Although the NZ medium provided more growth in less time than TGYM medium, it was observed that M. radiodurans was ten times less radiation resistant when grown in the former medium and also less pigmented. Repeated experiments to confirm this observation were conducted and the relative radiation survival rates are given in Table 4. M. radiodurans grown in TGYM broth was consistently at least ten times more resistant as compared to NZ broth and centrifuged cultures were always more brightly pigmented. This suggested that there might be a relationship between the degree of pigmentation and radiation resistance. This was in agreement with previous reports (53) which

noted a reduction in resistivity and pigmentation for these cells when grown under reduced oxygen tension. Another report (61) indicated a rapid loss of pigment color in irradiated cells in the presence of iodoacetic acid and a corresponding decrease in radiation resistance. However, other investigations (59, p. 25; 66) suggest that pigments of <u>M. radiodurans</u> have little or nothing to do with its great resistance.

The pigments of M. radiodurans have been identified as carotenoids (53; 61). Diphenylamine has been successfully used as a specific inhibitor of carotenoid synthesis in photosynthetic bacteria (17). Lack of these colored pigments was accompanied by an increased susceptibility of the cells to photo-oxidation and an accumulation of carotenoid precursors. The results of the present study did not indicate what factor(s) may be associated with decreased pigment production. However, NZ-Case contains three times as much iron and two-and-a-half times as much phosphates as tryptone of the TGYM medium (Tables 5 and 6), and early reports (44) have indicated that the presence of phosphates and ferric ions inhibited carotenogensis. No biochemical mechanisms were presented to account for their observations. It can also be seen in Tables 5 and 6 that NZ-Case has 5.5 and 6.7 percent valine and serine, respectively, and the analysis of tryptone indicated no amino acids of this type. A recent study (43) presented evidence that these two amino acids increased the ultraviolet sensitivity of a yeast, Hansenula wingei.

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This same report indicated, however, that cystine and the aromatic amino acids protected this organism from ultraviolet inactivation. In the present study NZ-Case, which has a relatively high concentration of these amino acids, was found to increase the sensitivity of <u>M</u>. radiodurans to ultraviolet light (Figure 6).

A "recovery medium effect" has been described for radiation resistant and sensitive strains of E. coli (2; 3). A richer, more complex recovery medium produced higher counts of the resistant strain and fewer radiation survivors of the sensitive strain. It was concluded on the basis of these results that the difference between the two strains lay not in the initial radiation damage but in their metabolic processes immediately after irradiation. In the present study, no "recovery medium effect" was observed for M. radiodurans which was grown on TGYM or NZ medium and recovered on either of these two media (Table 7). Cells grown in TGYM broth had approximately 60 percent survival when recovered on TGYM or NZ agar, while cells grown in NZ broth had six percent survival on either recovery media. Hence, M. radiodurans was not influenced by the post-irradiation condition of recovery medium and the deleterious damage appeared to occur during the radiation process.

In studies to determine the relative effects of the major component differences of the two media on radiation resistance, it was found that the addition of NZ-Case to TGYM medium reduced the radiation resistance of <u>M. radiodurans</u> 30 fold. However, increasing the yeast extract concentration in TGYM to levels found in NZmedium did not reduce the resistance of the organism; nor did the addition of DL-methionine to NZ-medium increase the radioresistance. These observations provided evidence that some minor component of NZ-Case, such as iron, phosphorous or an amino acid, was responsible for the altered resistivity.

When <u>M. radiodurans</u> was grown in NZ-broth containing various levels of NZ-Case, an inverse relationship was observed between the concentration of NZ-Case and the radiation resistance. These results are presented in Table 9 and Figure 1. It appeared that as the medium became more enriched, the cultures became more radiation sensitive. This would agree with a previous suggestion (32) that a medium which stimulates protein synthesis does not favor recovery of <u>E. coli</u> after irradiation. This would suggest that an enriched medium causes cell death due to unbalanced growth (8) where DNA and protein synthesis cannot be properly regulated. Cells in this "abnormal" metabolic state probably would be more susceptible to radiation damage. This does not agree, however, with other reports (91) that an enriched culture medium produced the most radioresistant E. coli cells.

In addition to the altered radiation resistance, it was observed that cells grown in the higher levels of NZ-Case possessed the least pigments. This observation suggested that pigmentation and radiation resistance were, in some manner, related and NZ-Case was capable of altering these factors.

The growth characteristics of <u>M</u>. <u>radiodurans</u> in the two different media are presented in Figure 2 and indicate similar growth patterns. The initial cell inoculum from the NZ culture contained about twice as many cells as compared to the TGYM inoculum, and hence there were twice as many cells present at the end of the log period in the former medium. An earlier death phase for the NZ culture probably was a reflection of a larger number of cells depleting the available nutrients or producing toxic waste materials more rapidly than a lesser number of cells in the TGYM medium. If unbalanced growth was occurring in one of these media, it was not apparent from the standard growth curves. It should be noted that for most all comparative resistant studies, a 44 hour culture was used; from Figure 2 it can be seen that this represents about the middle of the stationary phase.

Further evidence that unbalanced growth did not occur in the NZ-medium was provided from experiments on the effect of physiological age on radiation resistance. From Table 10 it can be seen that, with one exception, at all the physiological ages tested, <u>M</u>. <u>radiodurans</u> was at least ten times more resistant when grown in TGYM broth as compared to NZ broth. The eight hour cultures in the two media, however, had only a four-fold difference in resistance. This variation may be attributed to the general increased sensitivity of microbial cultures in the early log phase of growth. The general observation that the radiation resistance of <u>M. radiodurans</u> does not change with age was in agreement with previous work (60, p. 42) although the survival rate was about twice as great in the present studies. This may be due to a difference in the growth media.

It has been suggested (91) that the sigmoidal survival curves (Figure 5) obtained for resistant cultures might be related to a multicellular or multi-nuclear state or to the initial and final pH of the growth media. The results of Table 11 indicated that there was no difference in the relative amounts of DNA, RNA, protein or medium pH for M. radiodurans when grown in the two different media. Thus, it did not appear that the ten fold difference in radiation sensitivity could be accounted for on the basis of quantitative difference in these chemical factors. The ratio of DNA:RNA agrees with literature values (19, p. 130) and the amount of DNA agreed with that previously reported (67). Evidence that the cell mass was the same for the two different cultures was provided from the total protein measurements as well as from the phase contract photomicrographs (Figures 3 and 4). These photographs also indicated that there were no changes in cellular arrangement in the less resistant cultures.

When M. radiodurans was grown in TGYM and irradiated in

buffer at different dosages, the survival curve was characterized by a wide "shoulder." However, the survival rate decreased rapidly and almost exponentially after 350 k rads for cells grown in NZ broth (Figure 5). The wide "shoulder" of sigmoidal survival curves are typically found in radiation resistant organisms (53; 61; 91) and are often referred to as multihit, multitarget or multiunit curves because they suggest a multiplicity of events required to bring about inactivation. Since the cells grown in NZ broth did not produce a wide "shoulder" in their survival curve, this suggested that the number of radiation sensitive units had been increased or the number of radioprotective units had been decreased by the growth media. From Figure 5 it can be seen that the LD_{50} dose was approximately 350 k rads for cells grown in NZ broth and 700 k rads for cells grown in TGYM broth. Hence, it is important to note that the resistance of M. radiodurans grown in NZ broth was still as great as that of Clostridium botulinum spores (96) but its radioresistance was now reduced by one-half.

A similar alteration in the survival curve was observed when cells of the two types of cultures were exposed to ultraviolet radiation while suspended in buffer (Figure 6). The survival curve for the TGYM grown cells was the same as previously reported (26). However, cells grown in NZ broth were approximately ten times less resistant. This degree of resistance was now comparable to those reported for nonsporeformers or the spores of <u>Bacillus globigii</u> when treated under similar conditions.

The response of <u>M</u>. <u>radiodurans</u> to ultraviolet light was similar in some respects to its response to gamma radiation. Mechanisms of resistance to ultraviolet light and to gamma rays may have some factors in common. It has been shown that both forms of radiation cause the formation of peroxides in the medium (49). Ultraviolet radiation in some cases causes radical reactions resembling those of ionizing radiations, but the big difference between the two types of radiation is most frequently depicted as one of ionization versus excitation of molecules. It is reasonable to assume that the conjugated double bond system of the carotenoid pigments would be altered by ultraviolet exposure as well as exposure to gamma rays. Hence, if the pigments are related in some way to radiation resistance, then similar survival curves would be expected for <u>M</u>. <u>radiodurans</u> exposed to the two different types of radiation.

Evidence that the growth media caused cellular biochemical alterations is presented in Table 12. Here it can be seen that the modification in radiation resistance can be reversed by a three hour exposure period to a different growth medium. Cells which had been grown in TGYM broth, washed and then exposed to NZ broth for three hours were ten times more radiation resistant as compared to a 44 hour exposure. On the other hand, cells which had been grown in NZ broth had similar radiation survival rates after either a three or 44 hour exposure to TGYM broth. No observations similar to these have been reported.

The generation times for <u>M</u>. <u>radiodurans</u> in the TGYM and NZ media were 50 and 38 minutes, respectively. This means that a three hour incubation period allowed time for the production of only two or three generations of cells. However, since the cell population exposed to gamma rays became resistant within three hours, this indicated that the precursors of factors related to increased resistance were present in cells originally grown in NZ broth, but that the factors themselves could not be synthesized in this medium.

Visual changes in pigmentation were also observed to be reversed by exposing the cells to the two different media. On the basis of this observation and the results previously discussed, it appeared once again that there was a relationship between the high level of radiation resistance and pigmentation.

Evidence that there was a difference in the pigments of <u>M</u>. <u>radiodurans</u> grown in the two different media was provided from the chromatogram shown in Figures 7 and 8. Eight distinct pigmented spots were obtained from the TGYM grown cells but the NZ grown cells had only six pigments. These results were consistently obtained by repeated experiments. A previous report (59, p. 15) indicated that this organism had six different carotenoid pigments when they were separated by column chromatography. Four of these six pigments were identified as lycophyll or zeaxanthin. Another report (53) indicated that <u>M. radiodurans</u> had only two carotenoid pigments when separated by paper chromatography and that these pigments had a spectral absorption peak at 478 mµ. It was also reported that the pigment was equally radio-sensitive inside the cell or extracted and isolated from other cell components. They were unable to show that the pigments could serve as energy sinks within the organism and thus account for the multihit inactivation curve. However, the number of pigment molecules was several orders of magnitude larger than necessary for this purpose.

The pigments extracted in the present study were found to oxidize very rapidly when exposed to air. Hence, antioxidants were used while running the chromatograms and the pigments were eluted in a nitrogen atmosphere. BHT has been reported to be the best antioxidant for the thin layer study of lipids (101). It did not interfere with the separation and identification of the pigments in the present study.

The development of thin layer plates in iodine vapors reportedly brings out unsaturated lipids (62). In the present study an iodine development of the thin layer plates containing the pigments indicated several additional spots in areas remote from the colored spots (Figure 9) near the top of the chromatograms. Since these spots were colorless and produced a green fluorescence under ultraviolet light, this suggested that they could represent phytoene and/or phytofluene. These are reported characteristics of these compounds which also serve as precursors of the colored carotenoids (46).

As can be seen from Figure 9, these spots were particularly dense for the NZ grown cells. However, when the cells were shaken in buffer, these spots disappeared and the missing pigments reappeared (Figures 14 and 15). This observation was not unlike that reported for the endogenous conversion of carotenoid pigments in Rhodospirillum rubrum (46). Other reports (17;88) indicated that diphenylamine (DPA) can cause an intracellular accumulation of colorless precursors of carotenoid pigments and when DPA was removed the colored pigments appeared and the colorless precursors, some which were identified as phytoene and phytofluene, disappeared. These investigators also noted that the photosynthetic bacteria were very sensitive to chlorophyll catalyzed photo-oxidation when the colored pigments were missing but that this sensitivity subsided when the pigments were synthesized that contained a more extensive conjugated double bond system.

In the present study, it was observed that the reappearance of the missing pigments was accompanied by a return of the high level of radiation resistance characteristic of <u>M. radiodurans</u> (Table 14). Further evidence of the role of the pigments in radiation resistance

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was provided when it was shown that the same pigments were absent from irradiated cells grown in TGYM medium as were absent in the cells grown in NZ broth. These results are shown in Figure 13.

On the basis of these results it appeared that pigments one and four (Figure 7) were responsible, at least in part, for the high level of resistance observed for <u>M. radiodurans</u> grown in TGYM. Since carotenoids are known to protect cells against photo-oxidation (17) and apparently function in the electron donor-acceptor complexes of both photosynthetic and non-photosynthetic bacteria (77), it is postulated that the pigments of <u>M. radiodurans</u> and particularly pigments one and four of Figure 7 serve as intracellular scavengers of harmful ions and radicals produced by ionizing radiation and thereby protect the cell. Furthermore, the conjugated double bond system characteristic of the colored carotenoids (see Figure 12 for structure of lycopene) presents a physical-chemical structure which should be capable of accepting radicals.

Tentative identification of the eight pigments by spectrophotometric methods indicated that all of the pigments had similar spectral characteristics (Figures 10 and 11) with a major absorption peak at 475 mµ and minor peaks at about 450 and 500 mµ. Literature data (46) indicate that these absorption characteristics were typical of lycopene, hydroxy lycopene or dihydroxy lycopene. Repeated attempts to demonstrate spectral absorption in the ultraviolet range were unsuccessful. All of these pigments have a conjugated double

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bond system with 11 double bonds and are orange or yellow-orange in color. As indicated previously, earlier studies (59, p. 15) indicated that the pigments of <u>M. radiodurans</u> were lycophyll and zeaxanthin. The present results agreed in part with these observations since another name for lycophyll is 3, 3' dihydroxy lycopene and this compound can be formed from zeaxanthin by opening the closed rings between carbons 1 and 6 and 1' and 6'. It should be noted that commercially synthesized lycopene has been found to have both a radiation protective and therapeutic effect when injected intraperitonically into mice (30). In this regard, lycopene was believed to function by stimulating hemopocetic recovery as far as hemin synthesis was concerned.

From Table 13 and Figures 10 and 11 it can be seen that all of the pigments were present in greater concentration in the cells grown in TGYM as compared to NZ broth. Pigments 1 and 4 constituted at least 12 percent of the total pigments present.

Carotenoid pigments are known to be present in relatively radiosensitive organisms such as <u>Micrococcus lysodiekticus</u> (88; 83) and <u>Sarcina lutea</u> (88). In these organisms the pigments were found exclusively in the cell membranes, and are also known to have spatial, structural and biosynthetic relationships with the lipids and lipoproteins of these membranes. (The structural similarity of lycopene and squalene, a precursor of sterols, is shown in Figure 12). Evidence from the literature indicates that these lipids play an important role in the semi-permeability characteristics of the cell membrane (89, p. 223) and that this function was significantly altered by radiation (7, p. 414 and 272; 71; 95) with an abundant increase in the activity of many enzyme systems (41; 56, p. 227; 93).

Accordingly, it is postulated that the carotenoid pigments, by accepting the deleterious ions and radicals produced by radiation, prevent an alteration of these functionally important lipids, and the resulting uncontrolled release of cellular enzymes is thereby prevented. It is in this manner that the pigments of <u>M. radiodurans may</u> have an important role in the radiation resistance of this organism.

Summary

The presence of NZ-Case (a tryptic digest of casein) in the growth media caused at least a ten-fold decrease in radiation resistance of <u>M. radiodurans</u> and a loss of two of the eight pigments shown to be present by thin layer chromatography. The level of radiation resistance was inversely related to the concentration of NZ-Case. The LD₅₀ dose for this organism was 700 k rads when grown in TGYM broth but it was only one-half as resistant when grown in NZ broth. The resistance to ultraviolet light was also reduced.

Spectral analysis of the eight pigments indicated that they were all closely related and were tentatively identified as lycopene or one of its derivatives. None of the pigments absorbed in the ultraviolet range.

Removal of NZ-Case from the growth media resulted in a return of the high level of resistance and the reappearance of the missing pigments.

A possible role of the pigments in the radiation resistance of M. radiodurans was described.

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