AN ABSTRACT OF THE THESIS OF

JASPER DAMON MANES, JR. for the MASTER OF SCIENCE
(Name) (Degree)

in RADIOLOGICAL HEALTH presented on MARCH 29, 1967
(Major) (Date)

Title: THE SITE OF ACTION OF RADIO-LETHAL COMPOUNDS ON MICROCOCCUS RADIODURANS

Abstract approved Redacted for Privacy
Dr. David Willis

Iodoacetic acid and diphenylamine were used in this study to elucidate the mechanisms of resistance of Micrococcus radiodurans to radiation. This organism, a gram positive, non-spore forming, salmon-pink, tetracoccus has a LD_{50} of 213,000 R when grown in the absence of radio-modifying agents.

M. radiodurans, when grown in media containing iodoacetic acid, was more resistant to ultraviolet light, but its LD_{50} for X-rays was not changed. The presence of diphenylamine in the growth media did not alter the sensitivity to ultraviolet light, but did reduce the LD_{50} for X-rays to 134,000 R.

The effect of diphenylamine and iodoacetic acid upon the colored carotenoid composition was analyzed by thin layer chromatography. Cells grown in the absence of radio-modifiers possessed seven pigment bands. Cells grown in the presence of iodoacetic acid

demonstrated an increase in pigment band number one, but lacked pigment components number six and seven. Diphenylamine did not alter the colored carotenoid composition of M. radiodurans.

Thin layer chromatography was used to monitor the effect of radio-modifying agents on extractable lipid composition of M. radiodurans. Iodoacetic acid did not change the extractable lipids of this microorganism. Cells incubated in the presence of diphenylamine had an additional lipid band, as well as larger amounts of fatty acids.

Gas liquid chromatography was used to examine the fatty acid composition of M. radiodurans. Cells grown in the presence of iodoacetic acid possessed two compounds not found in the other cells. Diphenylamine increased the saturated: unsaturated C-16 fatty acid ratio by approximately three fold.

The results indicate that there is a relationship between the carotenoid pigments and the resistance of the test organism to ultraviolet light, but not to X- and gamma rays. In addition, it appears that the unsaturated lipids, in particular C-16, protect M. radiodurans from the harmful effects of X- and gamma rays. There was no correlation between fatty acid content and ultraviolet light sensitivity.

The Site of Action of Radio-lethal Compounds on *Micrococcus radiodurans*

by

Jasper Damon Manes, Jr.

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1967

APPROVED:

Redacted for Privacy

Associate Professor of General Science
in charge of major

Redacted for Privacy

Chairman of the Department of General Science

Redacted for Privacy

Dean of Graduate School

Date thesis is presented _____

Typed by Gwendolyn Hansen for Jasper Damon Manes, Jr.

ACKNOWLEDGMENT

A special thanks is extended to Dr. A. W. Anderson for his patience and understanding. Also, I am indebted to Dr. David Willis for his assistance in editing.

This work was made possible by the United States Public Health Service, which supplied funds for the investigation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

THE SITE OF ACTION OF RADIO-LETHAL COMPOUNDS ON MICROCOCCUS RADIODURANS

INTRODUCTION

The objective of this study was to identify the molecules responsible for the extreme radiation resistance of Micrococcus radiodurans. Because of prior investigations, particular emphasis was placed on the fatty acid and carotenoid pigment content (6, 20, 21, 22, 24, 26, 40). Iodoacetic acid and diphenylamine, radio-modifying agents, were used to alter the radio-protective mechanism of M. radiodurans. Alterations of this microorganism's chemical composition was monitored by thin layer and gas chromatographic analysis. Changes in radio-resistance were analyzed by the use of a pour plate technique. This study differs from previous experimentation in that the cells were grown in the presence of a radio-modifying compound, rather than merely irradiated in a medium containing the agent. In addition, earlier investigations used either gamma or X-irradiation exclusively; however, this study utilized both ultraviolet light and X-irradiation sources (6, 20, 21, 22, 26, 40).

REVIEW OF LITERATURE

Interaction of Ultraviolet and X-irradiation with Biological Material

Ultraviolet light and X-rays are electromagnetic radiations of short wavelength. The absorption of ultraviolet light is dependent upon the particular molecular configuration of the absorbing material. The energy of an absorbed quantum of ultraviolet is stored in the molecule and is released in many different reactions. Some of these lead to chemical changes while others result in physical alterations (3, p. 6, 7). Organic peroxides and hydrogen peroxide have been reported as products of the interaction of ultraviolet light with biological material (41). Excited atoms are also products of ultraviolet irradiation and are distributed throughout the irradiated medium. The energy per quantum is on the order of several electron volts.

X-rays are of a much shorter wavelength than ultraviolet light and hence possess a greater energy content per quantum (several thousand electron volts). The interaction between X-rays and matter results in ionization and excitation. The ejection of an electron results in a positively charged atom or molecule (hence the term "ion pair"). Eventually this primary electron becomes attached to another molecule or atom producing a negative ion. Usually, the

X-ray quantum has an energy content in excess of the amount necessary to eject an electron. This surplus energy is imparted to the ejected electron in the form of kinetic energy. The excess energy of the primary electron can be dissipated in forming secondary ion pairs. Thus, tracks of ion pairs are produced along the path of the primary electrons. A fundamental difference in the interaction of ultraviolet light and X-rays with matter is the spatial distribution of reaction products. X-rays are absorbed independently of molecular configuration, i.e., absorption is dependent on the atomic number of the absorbing atoms and the electron density of the material. Atoms are excited by X-rays when the energy imparted to the atom is not sufficient to eject an electron. Excited atoms are short-lived products with a lifespan of less than 10^{-10} seconds. "Free radicals" are the intermediate products between excitations or ions and the final radiochemical product. They are highly chemically reactive as they possess an unpaired electron (3, p. 12-20).

Effect of Ultraviolet Light and X-rays on Microorganisms

The absorption of electromagnetic radiation causes changes at the molecular level of the cell. Figure 1 depicts the sequential steps starting with the interaction of the quantum of electromagnetic radiation that leads to the observable changes. Chemical changes are rapid; however, the observable manifestations of damage (mutation

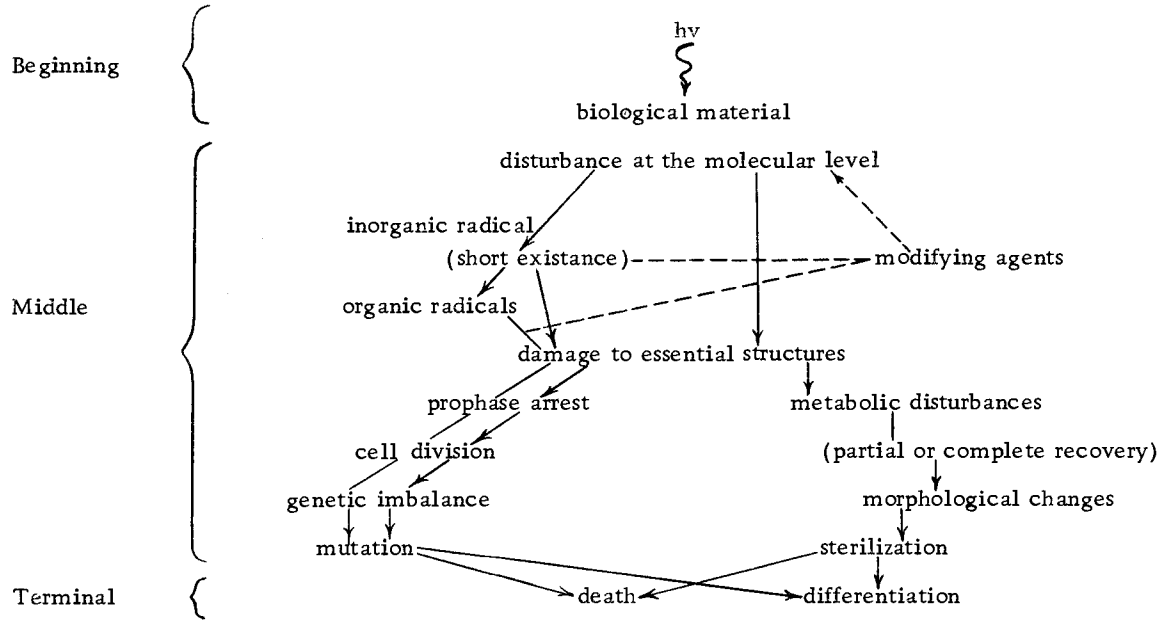

Figure 1. The sequential steps of damage produced by radiation.

and inactivation) require processes of cell metabolism which take a relatively long period of time. It is possible to divide the sequence of steps leading to the observable lesions into three periods (beginning, middle, and terminal). The beginning phase includes the instant of irradiation and the resulting primary reactions. The middle interval covers the time from the primary reaction to the inception of visible effects. During this time the inhibition of deoxyribonucleic acid (DNA) synthesis is predominant. A visible alteration or lesion of the cell denotes the terminal phase (23).

Until recent years investigators assumed that the major portion of radiation effects resulted from chemical changes produced by direct action in biologically important molecules (14). Direct action means that the molecule(s) being studied is undergoing changes itself as the result of an interaction with an electron or other atomic particle (3). The discovery of post-treatment effects and the influence of oxygen and other modifying agents implicated indirect action as another principal means of radiation damage (41). Indirect action occurs when the molecule(s) being investigated does not absorb the energy but receives this energy by transfer from another molecule (3).

Mutation and inactivation are the two visible effects of the radiation response of microorganisms. Inactivation (death) of a microorganism means that the organism is unable to produce a

visible colony after exposure to radiation (32). A limiting condition is that the organisms are plated on the same growth media that they grew on before irradiation. This means that inactivations results from both mutational and non-mutational disturbances. Inactivation is not a single step process, but rather consists of a sequence of steps (41). The portion of inactivation attributable to lethal mutations is difficult to assess from present data. Modifying agents exert parallel effects on both mutation and inactivation phenomena (36).

Two non-mutational processes responsible for inactivation are (a) damage to enzyme synthesis and (b) alteration of the DNA-synthesizing mechanism. Stapleton, Sbarra, and Hollaendar (39) have shown that E. coli B/r was more resistant to radiation when previously cultured on a minimal nutrient medium. These authors suggest that inactivation can result from a temporary alteration in the cell's ability to synthesize certain adaptive enzymes. Another possible non-mutational process that may be responsible for the inactivation of microorganisms is the production of a poison by the interaction between the radiation and organism's environment (36).

Mutational disturbances are changes in the organism's genetic material that may be carried over into succeeding generations. If the damage to the genetic material is extensive, it may result in the loss of the reproductive ability of the organism.

Microorganisms are generally more resistant to the effects of

radiation than multicellular organisms (41). The reported lethal dose that inactivates 50% (LD_{50}) of the organisms for different bacterial species ranges from kilo-rads to mega-rads. This variation among microorganisms is the result of many factors. One of the radio-protective factors is the formation of spores. A notable exception is M. radiodurans, which is a non-spore forming, salmon-pink, tetracoccus. It has an LD_{50} for gamma rays of 8×10^5 rads when irradiated in phosphate buffer. It is characterized by a sigmoidal survival curve with a large shoulder (2). A unique multi-layer cell wall is also a feature of this bacterium (40).

Metabolic studies of M. radiodurans indicated that methionine (sulfur containing) is the only amino acid essential for survival (17). Several sulfur-containing compounds were demonstrated among the end products of respiration. One of these was hydrogen sulfide and the other a mercaptoalkylamine (11). The latter is a radio-protective compound. The carotenoid pigments of M. radiodurans were characterized by Lee (26). Studies using white mutants of this organism to determine the role of colored carotenoids in radio-resistance have yielded varying results (21, 33). The possibility of genetic changes (other than pigmentation) cannot be ruled out as a factor in this variability. Krabbenhoft (24), working with M. radio-durans, correlated the absence of certain carotenoid pigment components with enhanced radio-sensitivity. The unsaturated lipids

of the C-12 through C-18 range constitute the vast majority of the extractable lipids (28). These may be important in radio-resistant mechanisms dependent on free radical scavenging. Mice were protected by post-irradiation injections of an extract prepared from M. radiodurans. This extract is rich in lipid and lipid-like material (7, 21). M. radiodurans is sensitized to radiation by compounds that combine with sulfhydryl groups (25, 10). On the other hand, the DNA of M. radiodurans does not differ greatly in composition and molecular conformation from that of other microorganisms (31, 37).

Modification of Radiation Effects

The first report of a modified radiation response was by Schwartz (14) in 1909, when he demonstrated the effect of ischemia on the radiation sensitivity of the human skin. In the years 1920 to 1930 the fundamental phenomena associated with the effects of oxygen, temperature, and hydration on radiation sensitivity were investigated. Modification of the indirect effects of radiation was studied by chemical means using pure homogeneous solvent-solute systems. The studies showed that the addition of a second solute to the pure chemical system decreased the damage to the first solute. The competition between the two solutes for the activated water (indirect action product) is believed to explain these findings (15). In 1940 the terms protection and protective effect (increased resistance to

radiation effects when given before exposure to radiation) were introduced (14). During the period 1940 to 1942 investigations demonstrated that the radiation damage to isolated biological molecules, such as crystalline enzymes, could be reduced by addition of specific substances, such as cysteine (3). In vivo chemical protection was first reported in 1941 by Patt, et al. (34), who observed the protective qualities of cysteine. Dale (8) measured the "protective power per molecule" of a group of substances and reported on the unique ability of various sulfur-containing compounds to inactivate free radicals. Thiols were later shown to have a high radioprotective power per molecule (14). Certain non-sulfur-containing compounds were demonstrated to possess a high order of radioprotective power (3).

Enhanced sensitivity to radiation was first shown in studies which compared the lethal effect of radiation when cells were exposed in different atmospheric gases (14). The absence of oxygen reduced the damage produced by radiation, and respiratory inhibitors such as cyanide were shown to have a protective action. Later many other agents such as iodoacetamide and iodoacetic acid were shown to have a sensitizing action (4, 9).

The phenomena of "protection and sensitization" are two closely linked aspects of radio-sensitivity. Previous experiments indicate that there is a physiological level of chemical protection in the cell

which may be altered in either direction (23). Modifying agents are believed to compete for indirect action products; however, Alper (1) proposed a scheme whereby modification could possibly involve direct effects. The radiation response may be changed by procedures operating either before, during, or after the exposure to radiation. Pre- and post-irradiation procedures bring about effects that are different in effect as well as in magnitude. Pre-treatment modifiers are thought to influence the action of free radicals and excitation products of radiation. Post-treatment agents seem to be related to the cell's repair systems and its adaptive-enzyme mechanisms. Protection or sensitization of a particular compound depends on the reactivity of the new radical produced by the interaction of the chemical agent and the original free radical. If it is more reactive toward the essential molecules of the cell, increased sensitivity will be observed (14).

Sites of Action

The site of action of a modifying agent may be identical with the inherent radio-resistant mechanism of the cell. The carotenoid pigments, unsaturated lipids, and sulfhydryl groups were implicated by previous investigations as possible radio-resistant systems of M. radiodurans (4, 6, 7, 24, 25, 28).

The carotenoids represent the most widespread group of

naturally occurring pigments. They are essentially plant pigments. The majority of the bacterial carotenoids are xanthophylls rather than carotenes. Carotenoids are composed of approximately forty carbon atoms per molecule. A characteristic feature of the carotenoids is an isoprenoid polyene chain which is responsible for the red or yellow color exhibited (18, 38).

The exact role of the carotenoids in bacterial cells is not known. Diphenylamine, which selectively blocks colored carotenoid synthesis, was used to attempt to determine the function of carotenoids (18, 37). It was noted that cells grown in the presence of diphenylamine were devoid of colored pigments and were inactivated on exposure to visible light. This indicated that the carotenoids protected the photosynthetic apparatus from irreversible photo-oxidation (37). A radio-resistant function was postulated for the carotenoids due to the radical-scavenging ability of the unsaturated bonds of the molecule (37). In addition, these unsaturated isoprenoid chains are able to transfer energy along the length of the chain; hence, a radio-protective mechanism may function by transferring the energy produced by the absorption of radiation from an essential to a non-essential cellular component.

Radiochemical experiments with sulfhydryl-containing compounds indicate that they are capable of out-competing essential cell components for indirect radiation products. Later, the

radio-protective power of sulfhydryl-containing compounds (glutathione, cysteine) was demonstrated in vivo (34). Heavy metal-containing compounds, such as phenylmercuriacetate and hydroxy-mercuribenzoate, combine with sulfhydryl groups. These compounds were used to sensitize microorganisms to radiation effects (7).

The work of Lee and Krabbenhoft (24, 25) implied that the colored carotenoid pigments might act as a (part of the) mechanism for radio-resistance in M. radiodurans. The next step in testing this hypothesis was the blockage of the colored carotenoid formation before exposure to ultraviolet light and X-irradiation. Diphenylamine and citral (methylheptonone) were chosen since they were shown to block colored carotenoid synthesis in some photosynthetic fungi and bacteria. Certain sulfhydryl-containing molecules of M. radiodurans were postulated to protect this organism from radiation. In a separate experiment, iodoacetic acid, which combines with sulfhydryl groups, was added to the growth medium and its effect on the radio-resistance of M. radiodurans was observed.

MATERIALS AND METHODS

General Procedures

The culture of M. radiodurans used in this study was obtained from the Oregon State University stock culture collection. The culture was maintained in the viable state on Bacto-tryptone-glucose-yeast extract-methionine-agar slants. Microscopic examination and plating of the stock culture revealed that it was free from contaminants throughout the study.

Culture Conditions

The basic culture medium employed was designated TGYM and consisted of the following ingredients.

Bacto-tryptone (Difco)	5.0 g/liter
Glucose (Baker analyzed grade)	1.0 g/liter
Yeast extract (Difco)	1.0 g/liter
DL-Methionine (Matheson, Coleman and Bell	20.0 mg/liter
Tap water	1000.0 ml/liter

A solid medium was prepared by adding 20 g/liter of agar-agar (Difco) to the TGYM broth. A Corning pH meter (model 7) was used to monitor the hydrogen ion concentration of the media. Sodium hydroxide (1.0 N) and hydrochloric acid (1.0 N) were used to

maintain the pH (7.0 ± 0.1) of the solution. Each 250 ml DeLong culture flask was filled with 100 ml of TGYM broth. A 1.0% inoculum of M. radiodurans from a 24 hour-old broth culture was pipetted into each DeLong flask. The culture was aerated on a rotary-shaker. The temperature was maintained at 30° C during the 24 hour incubation period.

Harvesting the Cells

At the termination of the incubation period, the cell suspension was transferred to 200 ml sterile Nalgene centrifuge bottles. The cells were harvested by centrifugation at 7,000 x G for ten minutes and were washed twice with sterile 0.1% Bacto-peptone (Difco) solution. A standard curve was constructed to approximate the number of viable cells per ml by using various dilutions of the 24-hour-old cell suspension. The percent transmission was recorded and plotted against the log (number of viable cells/ml) recovered by pour plate technique at that dilution. The cell suspensions irradiated contained between $1.5\text{-}3.0 \times 10^8$ cells/ml.

Radiation Procedures

The cells were irradiated on flat bottomed Petri dishes. Since two different sources of radiation were employed, the techniques varied for each source.

Ultraviolet Light. The ultraviolet light source was a thirty watt germicidal lamp (General Electric) located 26 cm above a rotating platform which was leveled and rotated at the rate of one rotation/sec. This insured an even thickness of cell suspension and compensated for the heterogenous field. A light meter (General Electric) equipped with a Luckiesh-Taylor germicidal attachment was used to confirm the exposure rate, which was 51 ergs/cm^2/sec. Two ml of cell suspension was placed in a sterile flat bottomed plate and exposed for the desired period of time. The plates were left uncovered to prevent attenuation of the ultraviolet light. The high dose necessary to inactivate M. radiodurans reduced the problem of air-borne contamination by other microorganisms.

X-ray. The source of X-rays was a General Electric Maxitron capable of continuous operation at 300 kVp and 20 mA. The exposure rate of 1580 R/min at 28 cm was produced at 300 kVp, 20 mA, and 0.25 mm aluminum filtration. An R meter (Victoreen) with a 250 R ionization chamber was used to determine the exposure rate. A flat bottomed Petri dish was loaded with 20 ml of the cell suspension and placed on a rotating platform 28 cm from the target of the X-ray tube. A visible light mounted in the tube housing allowed definition of the X-ray field. The field overlapped the Petri dish by 4 cm. The exposures ranged from 60 to 180 min.

Determining the Survivors

After exposure to ultraviolet light or X-irradiation, the cell suspension was diluted with sterile 0.1% peptone water until there were sufficient cells to produce between 30 and 300 visible colonies per plate. The cells were agitated on each dilution for a minimum of 30 seconds to prevent clumping. The number of viable cells was determined by using a pour plate technique. TGYM agar was the recovery medium. Each sample was plated in triplicate. The plates were incubated at 30° C for 72 hrs before recording the number of colonies per plate with the aid of a Quebec darkfield colony counter.

Special Procedures

Iodoacetic acid and diphenylamine were added separately in varying concentrations to TGYM broth to determine their influence on the radio-resistance of M. radiodurans. The iodoacetic acid solution was prepared by adding 100 mg of iodoacetic acid to 100 ml of sterile distilled water. The diphenylamine solution was prepared by adding 50 mg of diphenylamine to 100 ml of sterile distilled water. The iodoacetic acid and diphenylamine were added to the TGYM broth before inoculation with M. radiodurans. The cells grown in the presence of diphenylamine were washed with 0.1%

Bacto-peptone that contained the same concentration of diphenylamine as the culture medium. This was necessary as removal of the inhibitor, diphenylamine, would permit the cell to produce colored pigments.

Pigment and Extractable Lipid Analysis

M. radiodurans was grown in 500 ml DeLong culture flasks containing 300 ml aliquots of TGYM broth or altered TGYM broth. The cells were inoculated, incubated, and harvested as outlined above. The only exception was that distilled water was substituted for the 0.1% Bacto-peptone in washing the cells. A slurry of the cells was made by adding enough distilled water to permit the cells to be poured into a round-bottomed 100 ml flask.

Preparation of the Carotenoid Pigment Extract. The pigments were extracted as follows:

1. Freeze dry the cells.
2. Gently reflux the dried cells for 30 minutes with a chloroform: methanol solution (2:1;v:v). The weight of the solution is 20 times that of the freeze-dried cells.
3. After refluxing, decant the material.
4. Return the solid material to the flask. Place the supernatant in a clean separatory funnel.
5. Wash the supernatant with 0.73% sodium chloride solution

using 0.2 the volume of the supernatant; gently agitate to prevent formation of an emulsion.

6. Allow the mixture to form two phases and remove the top phase with a siphon.

7. Prepare a mixture of chloroform: methanol (absolute): water (8:4:3;v:v:v). Discard the bottom phase and use one ml of the top phase to wash the lipid extract. Gently agitate the system. Allow the material to stand until two distinct phases appear. Remove the top phase by siphon. Repeat this washing step twice.

8. Add methanol to the bottom phase (Step 6) until a clear solution is obtained.

9. Add sodium sulfate and sodium bicarbonate in generous amounts to absorb the remaining water. Decant.

10. Evaporate the supernatant to dryness by bubbling nitrogen through the extract.

11. Add one ml of dried[1] chloroform: methanol (2:1;v:v). Seal the container and store in a refrigerator until ready to use.

Preparation of the Thin Layer Plate. Glass plates (20 x 20 cm),

---

[1] The solution is dried by adding generous amounts of sodium sulfate and sodium bicarbonate then decanting to remove the drying agents.

after being scrubbed with a cleansing powder and allowed to soak overnight in a strong detergent solution, were rinsed with distilled water and air-dried. Silica gel G (30 g of gel mixed with 60 ml of distilled water) was spread to a thickness of 0.25 mm with a Brinkman applicator. The plates were air-dried at room temperature, after which time they were activated by drying in an oven for two or three hours at 105° C. An oven maintained at 50° C was used to store the activated thin layer plates until needed, at which time they were removed and allowed to cool to room temperature.

Preparation of the Chromatogram. The gel was removed for three centimeters along three edges of each plate. The bottom, left undisturbed, was chosen as the best of the two sides that were parallel to the direction of gel application. (The solvent migration, therefore, progressed at an equal rate for all samples, because it was perpendicular to the direction of gel application.) A U-shaped glass rod was placed along the periphery of the gel, with its open side along the bottom of the plate. A Desaga spotting guide was placed over the gel. Six 30 mm^2 spots of extract, each containing 100 gamma, were placed three cm from the bottom of the plate with a micropipette. The spotting guide was removed and the loaded plate was placed under an infrared lamp to dry. A clean glass plate of the same size was positioned over the glass rod and secured on the sides and top with three clips. This created a sandwich

chromatogram.

The sandwich was carefully and evenly dipped into the solvent (benzene:methanol:acetic acid; 37:11:2;v:v:v) in a vertical position so that 1/2 cm of the gel was immersed. This left the spotted areas 2 1/2 cm above the solvent in the tray. The chromatogram was removed from the solvent at which time the solvent approached the upper fifth of the gel. After removing the plate, the sandwich was dismantled by removing the supporting clips. Upon drying, the plate was wrapped in plastic wrap to preserve the pigments from oxidation. The total lipid distribution was observed by spraying the plate with chromic acid, and then placing it in an oven to char until the organic areas were darkened. The results were photographed with a Polaroid camera. A recording densitometer was also used to analyze the thin layer chromatogram.

The basic problem of the sandwich apparatus is that of the solvent system going out of phase. This problem was compensated for by (1) placing an infrared lamp about three feet from the blank side of the plate and heating the sandwich during solvent migration, and (2) by dismantling the sandwich apparatus after the solvent had migrated six to eight cm and placing the loaded plate into a large chamber containing about 30 ml of solvent.

Total Fatty Acid Analysis

M. radiodurans was grown, incubated, harvested, and prepared as described in the pigment and extractable lipid analysis section.

Preparation of the Total Fatty Acid Extract. The fatty acid extract was prepared as follows;

1. Prepare carotenoid pigment extract as previously described; after drying, resuspend in 20 ml of methanol: benzene: conc. sulfuric acid (17:2:1;v:v:v).
2. Gently reflux the material for one hour.
3. Stop the reaction with water.
4. Extract the fatty acid methyl esters with 60 ml of distilled n-hexane (4 x 15 ml).
5. Dry the extract with sodium sulfate and sodium bicarbonate.
6. Evaporate to dryness.
7. Re-suspend in distilled n-hexane. Store the extracts at 0° C until used.

Analysis of the Total Fatty Acids. Two microliter samples of each extract were introduced into a recording gas chromatograph. The gas chromatograph using a 10% ethylene-glycol-succinate column, was set at 180° C and operated isothermally. Helium was employed as the carrier gas. Standards were analyzed to determine

the unknown fatty acids of the microorganism grown in the three different media.

RESULTS AND DISCUSSION

The Growth of Micrococcus radiodurans

M. radiodurans was grown in TGYM broth as previously described. One milliliter sample was removed from the culture with a sterile pipette. The number of viable cells per milliliter was confirmed by pour plate technique. M. radiodurans demonstrated exponential growth until 15 hours post-inoculation (Figure 2). No lag phase was observed as previously reported for this organism (13). After 20 to 25 hours post-inoculation the cell population leveled off to approximately 3×10^8 viable organisms per milliliter of medium. Twenty-four hour cultures were used throughout the study as they gave the most reproducible results. At this time (24 hours) the organisms were in the early stationary phase of growth. They were suspended in 0.1% sterile Bacto-peptone during harvesting and irradiation as it is non-toxic to M. radiodurans. Previous investigations utilized phosphate buffer solution which is toxic to the bacterium (12, 24).

Toxicity of IAA, DPA, and Citral

The toxic effect of iodoacetic acid on M. radiodurans are shown in Figure 3. This organism's tolerance of iodoacetic acid is

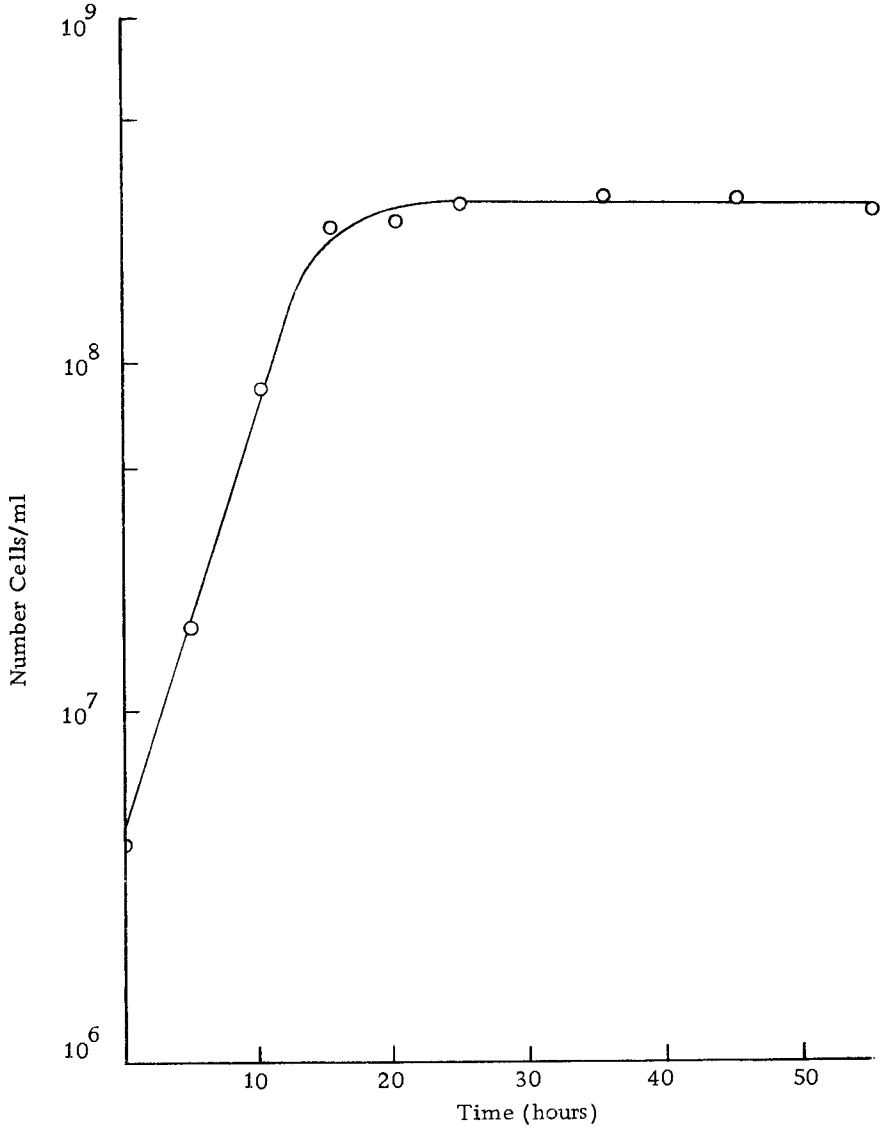

Figure 2. The growth of M. radiodurans in TGYM broth at 30° C.

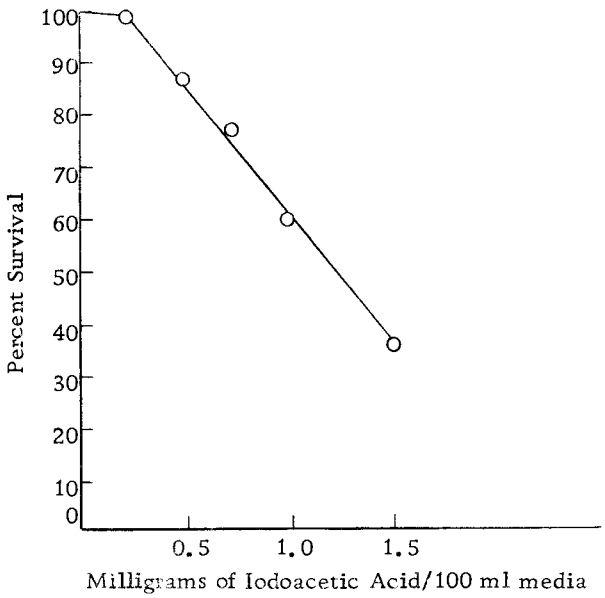

Figure 3. The toxicity of iodoacetic acid to M. radiodurans.

extremely high. When iodoacetic acid is added to the TGYM growth medium, molecules possessing a sulfhydryl linkage (-SH) are thought to be converted to those having an (-S-I-S-) linkage. The (-S-I-S-) linkage is similar to the disulfide linkage (-S-S-). Most bacteria cannot rapidly metabolize the disulfide linkage; therefore, the toxicity of iodoacetic acid may result from the formation of the (-S-I-S-) linkage in essential nutrient molecules. A concentration of 0.75 mg iodoacetic acid per 100 ml was used throughout this study.

The toxic effects of diphenylamine are exhibited in Figure 4. Diphenylamine is relatively insoluble in water. For this reason, the toxicity of diphenylamine was not investigated beyond 2.0 mg per 100 ml of medium. Although previous work with photosynthetic bacteria demonstrated diphenylamine to be a colored carotenoid inhibitor, diphenylamine exhibited little effect on the pigment producing ability of M. radiodurans. Diphenylamine is believed to block the terminal steps of colored carotenoid synthesis. This block of the pigments causes the accumulation of more saturated polyenes (18). M. radiodurans is able to acclimate itself to the presence of diphenylamine by shunting its metabolic pathway around this block. Diphenylamine was still of interest, however, as it could be used to study the relationship between the pigment precursors (polyenes) and radio-resistance.

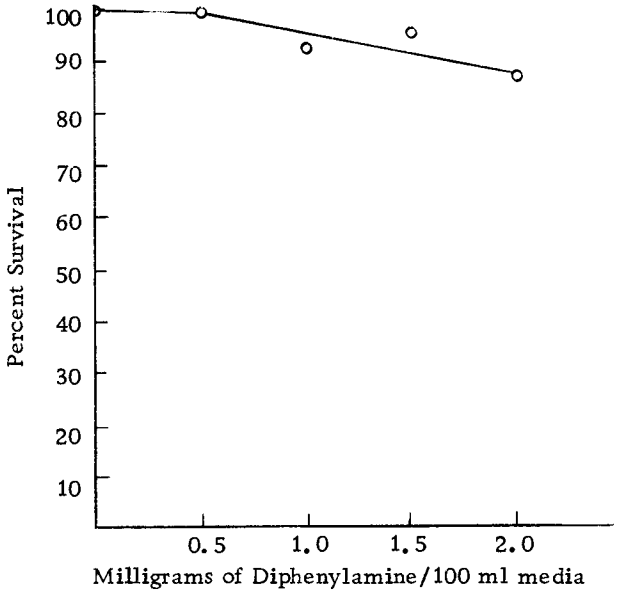

Figure 4. The toxicity of diphenylamine to M. radiodurans.

Citral was extremely toxic to M. radiodurans as shown in Figure 5. This compound was investigated because of its reported ability to competitively inhibit colored carotenoid pigment formation in other microorganisms (29). However, M. radiodurans was able to form a colored pigment in the presence of 20 microliters of citral per 100 ml of medium. The toxicity of citral cannot be explained at the present time. Since citral was extremely toxic, it was decided to omit it in investigating the relationship between colored carotenoids of M. radiodurans and radio-resistance.

Effect of IAA and DPA on Ultraviolet Light Response of M. radiodurans

M. radiodurans was cultured in TGYM broth, TGYM broth + 0.75 mg iodoacetic acid per 100 ml of medium, and TGYM both + 1.0 mg diphenylamine per 100 ml of medium as previously described. The TGYM and TGYM + IAA grown cells were irradiated in sterile 0.1% Bacto-peptone. Diphenylamine (1.0 mg per 100 ml) was added to the 0.1% Bacto-peptone water which was used to suspend the diphenylamine cultured cells. The cells were exposed to ultraviolet light for periods of up to five minutes. The effect of ultraviolet light exposure on M. radiodurans is presented in Figure 6. TGYM grown cells exhibited a survival curve similar to that obtained by other investigators (12, 24). The survival curve for cells cultured

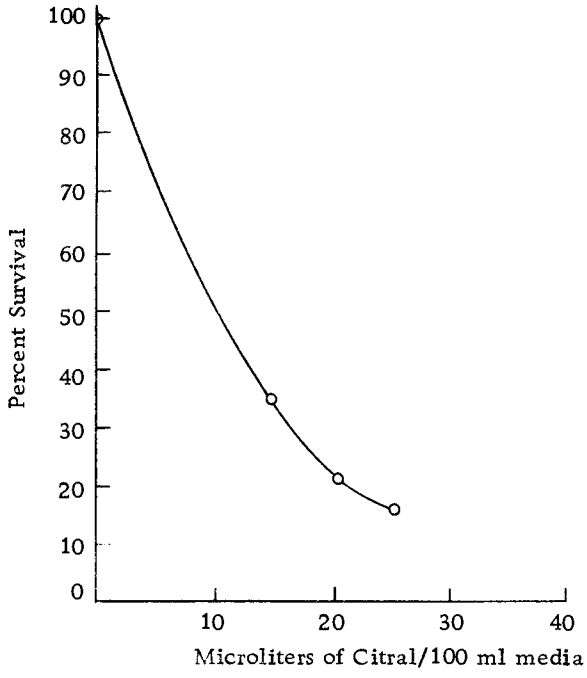

Figure 5. The toxicity of citral to M. radiodurans.

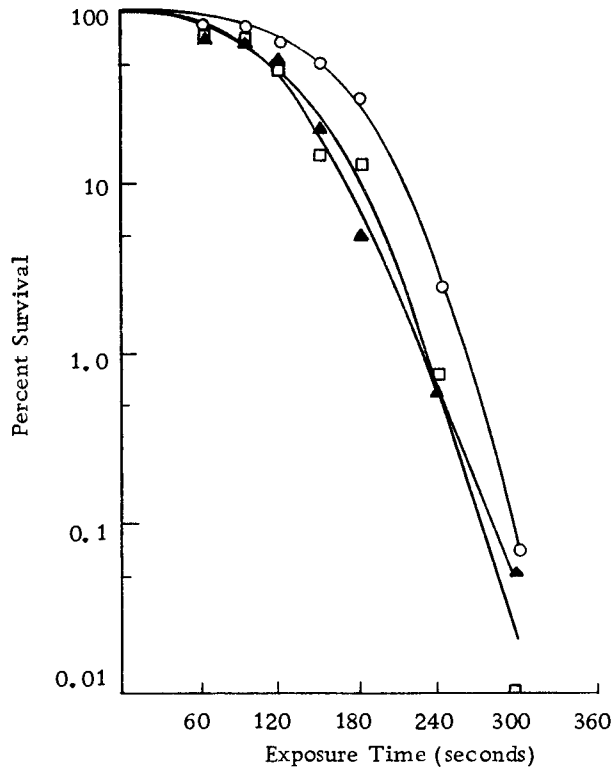

Figure 6. The effect of iodoacetic acid and diphenylamine on the ultraviolet light sensitivity of M. radiodurans. (The exposure rate was 51 ergs/cm^2/sec. M. radiodurans was grown in - TGYM, - TGYM + IAA, and - TGYM + DPA.)

in the presence of diphenylamine did not vary significantly from the survival curve for the TGYM-grown cells. Alteration of the pigment precursors of M. radiodurans had little effect on ultraviolet survival. M. radiodurans, when grown in a medium containing iodoacetic acid, demonstrated an increased resistance to the damaging effects of ultraviolet light as shown in Figure 6. Iodoacetic acid may alter the ultraviolet light response by several means: (a) It may penetrate the cell wall and interact with or absorb the electromagnetic radiation and/or its products, or (b) it may alter the population (mutational change) of microorganisms. Further investigation of the modifying action of iodoacetic acid was prompted by this finding.

Effect of Iodoacetic Acid During Ultraviolet Light Irradiation

Cells cultured in TGYM broth were harvested and suspended in 0.1% sterile peptone water containing 0.75 mg iodoacetic acid per 100 ml. The cells were kept in contact with iodoacetic acid for 30 minutes prior to 150 second exposures of ultraviolet light. Table 1 shows that TGYM grown cells incubated in the presence of iodoacetic acid prior to ultraviolet light exposure are more resistant than the control cells. Thus, iodoacetic acid may modify the radiation response by selective absorption of ultraviolet light. This hypothesis is tested in the following experiment.

Table 1. The effect of pre- and post-growth addition of iodoacetic acid on the ultraviolet light sensitivity of M. radiodurans. (Ultraviolet light exposures were 150 seconds at an exposure rate of 51 ergs./cm^2/sec. The concentration of iodoacetic acid was 0.75 mg/100 ml. of media.)

Culture Treatment	Percent Survival
1. Control-cells grown in TGYM broth and irradiated in 0.1% peptone.	31
2. Cells grown in TGYM but irradiated in presence of iodoacetic acid.	46
3. Cells grown in the presence of iodoacetic acid but irradiated in 0.1% peptone.	48
4. Cells grown and irradiated in the presence of iodoacetic acid.	67

Effect of Iodoacetic Acid in the Growth Medium on Ultraviolet Light Sensitivity

M. radiodurans was grown in various concentrations of iodoacetic acid (0 to 1.5 mg per 100 ml) and irradiated in 0.1% peptone solution. Table 1 shows that these cells survived ultraviolet light exposure better than the control. It was assumed that washing the cell removed the iodoacetic acid from the cell mass. Since Lee (27) demonstrated that iodoacetic acid does not penetrate the bacterial cell wall, this increase in survival may not be explained

by selective absorption of ultraviolet light.

Effect of Iodoacetic Acid During Growth on Ultraviolet Light Irradiations

M. radiodurans was cultured in concentrations of iodoacetic acid ranging from 0 to 1.5 mg per 100 ml of media. After harvesting, the organisms were re-suspended in 0.1% peptone water containing the same concentration of iodoacetic acid as the particular growth medium. Table 1 shows that the presence of iodoacetic acid during growth and ultraviolet light irradiation gave the greatest increase in the radio-resistance of M. radiodurans.

The Modifying Action of Iodoacetic Acid

Figure 7 reports the ultraviolet light sensitivity of M. radiodurans relative to the survival of M. radiodurans from iodoacetic acid treatment. Figure 8 corrects these results for the toxicity of iodoacetic acid. Data from Figures 3 and 7 and Table 1 were used to construct Figure 8.

> Curve A represents the "expected" ultraviolet light survival of iodoacetic acid-treated cells, if these treatments are independent of each other. The "expected" survival is the product of the survival of the separate treatments.

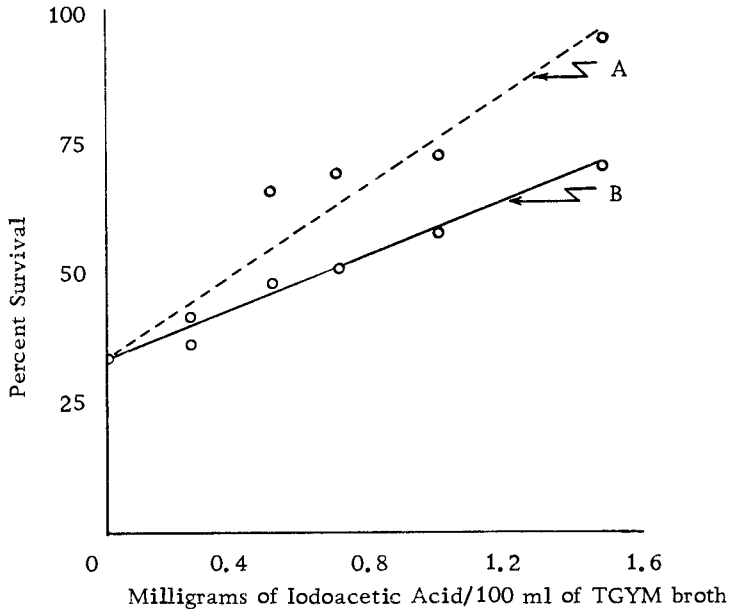

Figure 7. The protective effect of iodoacetic acid. (M. radiodurans was exposed to two and one half minutes ultraviolet light. A- Cells grown and irradiated in the presence of iodoacetic acid. B- Cells grown in the presence of iodoacetic acid but irradiated in 0.1% peptone solution.)

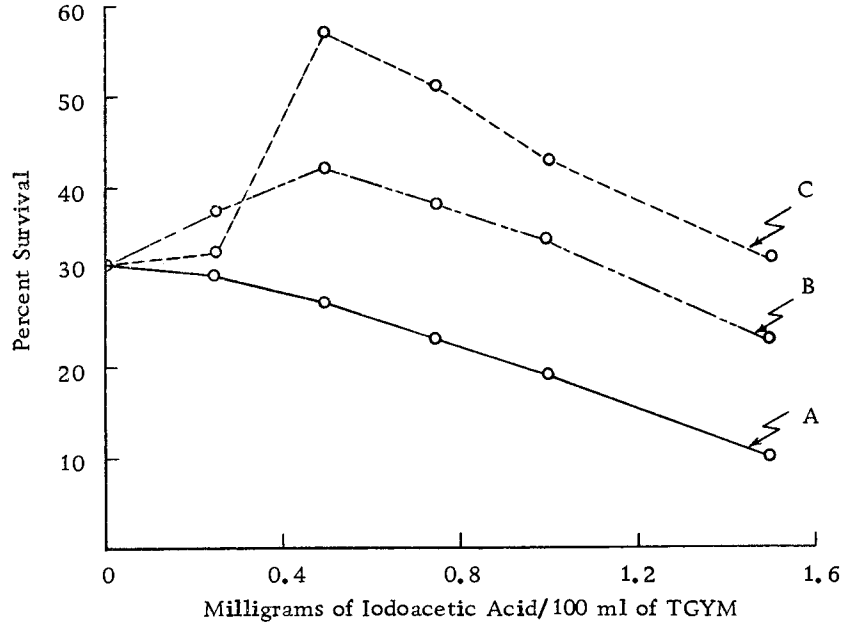

Figure 8. The protective effect of iodoacetic acid upon ultraviolet light exposed cells. (A - "Expected survival" B - Cells grown in iodoacetic acid but irradiated in 0.1% peptone solution. C - Cells grown and irradiated in iodoacetic acid.)

$$\text{"Expected" survival} = \frac{N(IAA)}{N(O)} \times \frac{N(UV)}{N(O)}$$

$\frac{N(IAA)}{N(O)}$ = fraction surviving iodoacetic acid treatment

$\frac{N(UV)}{N(O)}$ = fraction surviving ultraviolet light treatment

Curve B shows the actual survival of M. radiodurans treated with ultraviolet light and iodoacetic acid.

$$\text{Actual survival} = \frac{N(IAA \cdot UV)}{N(IAA)} \times \frac{N(IAA)}{N(O)}$$

$\frac{N(IAA \cdot UV)}{N(IAA)}$ = fraction surviving combined treatment relative to those surviving iodoacetic acid

$\frac{N(IAA)}{N(O)}$ = fraction of cells surviving iodoacetic acid

Curve C is similar to B except that the cells were treated with iodoacetic acid during growth and ultraviolet irradiation.

The results show that ultraviolet light and iodoacetic acid do not act independent of one another. The areas bounded by curves A and B and A and C represent the "protective" effect of iodoacetic acid. A maximum "protective" action is noted at a concentration of 0.5 mg iodoacetic acid per 100 ml of TGYM in both curves B and C. Apparently iodoacetic acid does supply some agent or precursor necessary for greater radio-resistance or increase repair of damage. The modifying actions of iodoacetic acid and diphenylamine were further investigated by exposure of M. radiodurans to X-irradiation.

Effects of Iodoacetic Acid and Diphenylamine on the X-ray Response of Micrococcus radiodurans

The cells were grown, harvested, and irradiated as previously described. Figure 9 shows the survival curve for M. radiodurans grown in TGYM broth at 30° C. Table 2 shows the LD_{50} values for the cells cultured in TGYM broth, TGYM broth + 0.75 mg iodoacetic acid per 100 ml of medium, and TGYM broth + 1.0 mg of diphenylamine per 100 ml of medium. M. radiodurans, when cultured and irradiated in the presence of diphenylamine, exhibited an increase in radio-sensitivity. Cells cultured in TGYM and suspended in 0.1% peptone water containing iodoacetic acid were more sensitive to X-rays than the controls. This result agrees with that obtained in earlier investigation (5, 7). M. radiodurans, when cultured in a medium containing iodoacetic acid and irradiated in 0.1% peptone water, demonstrated a small but insignificant increase in radio-resistance.

Since the resistance of M. radiodurans to ultraviolet light and X-irradiation was changed by the addition of diphenylamine and iodoacetic acid to the growth medium, thin layer chromatography was used to investigate the effect of the substances on the lipids and pigments of the bacterium. It was hoped that examination of the pigments and lipids would add insight to the mechanisms of radio-resistance.

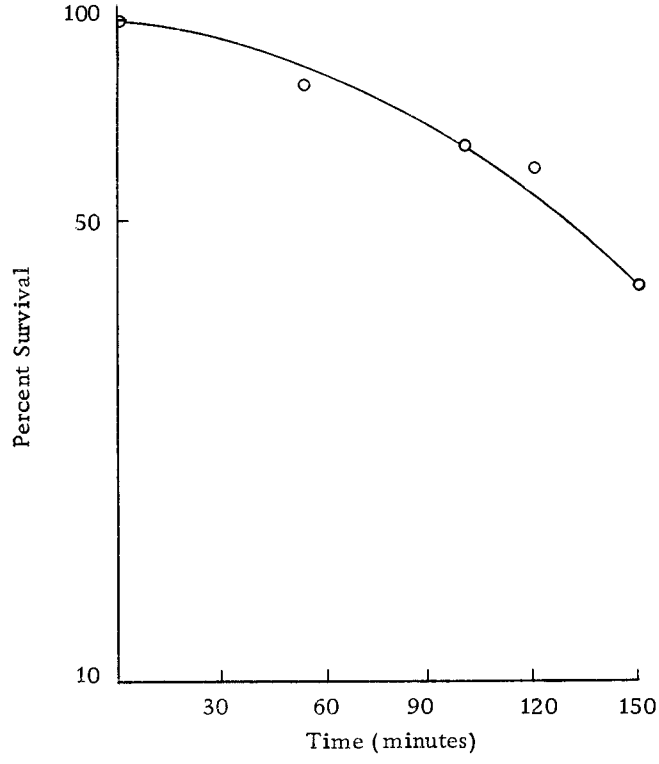

Figure 9. X-ray response of M. radiodurans grown in TGYM broth. (The exposure rate was 1580 R/minute.)

Table 2. Effect of growth media on the X-ray sensitivity.

MEDIA	LD_{50} for X-rays
TGYM broth	213,000 R
TGYM broth + 1.0 mg diphenylamine	134,000 R
TGYM broth + 0.75 mg iodoacetic acid	165,000 R
TGYM broth + 0.75 mg iodoacetic acid but washed and irradiated in peptone water	221,000 R

Effects of Iodoacetic Acid and Diphenylamine on Colored Carotenoid Pigments

The pigments of cells grown in TGYM broth, TGYM broth + 0.75 mg iodoacetic acid per 100 ml of medium and TGYM broth + 1.0 mg of diphenylamine per 100 ml of medium were extracted and prepared for thin layer chromatography. After completion of the chromatograms, a recording densitometer was used to measure the pigment components. Figures 10, 11, and 12 are tracings of these recordings. An analysis of these revealed that the cells cultured in iodoacetic acid lacked peaks numbers six and seven. Although M. radiodurans lacked these pigments when grown in the presence

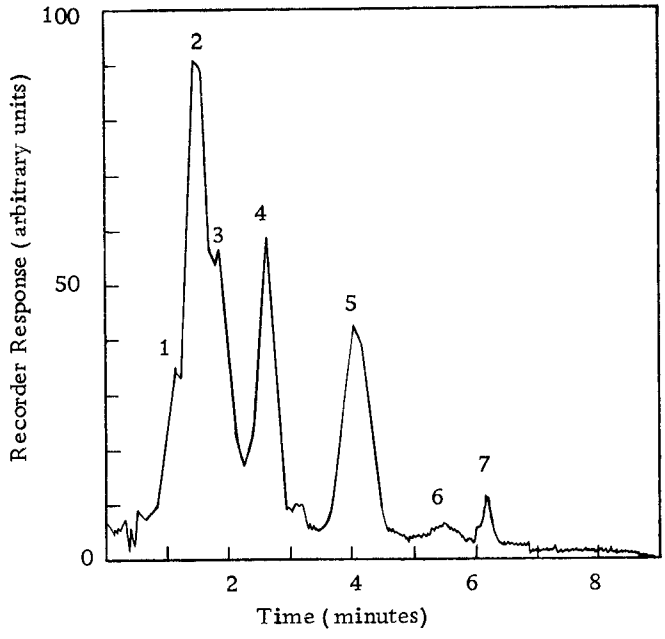

Figure 10. Densitometric recording of the carotenoid pigments of M. radiodurans grown in TGYM broth.

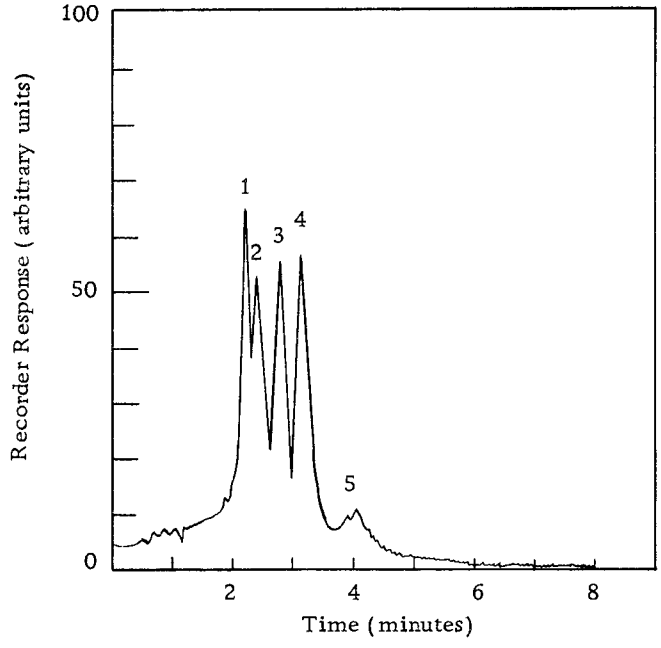

Figure 11. Densitometric recording of the carotenoid pigments of M. radiodurans grown in TGYM broth + 0.75 mg iodoacetic acid/100 ml medium.

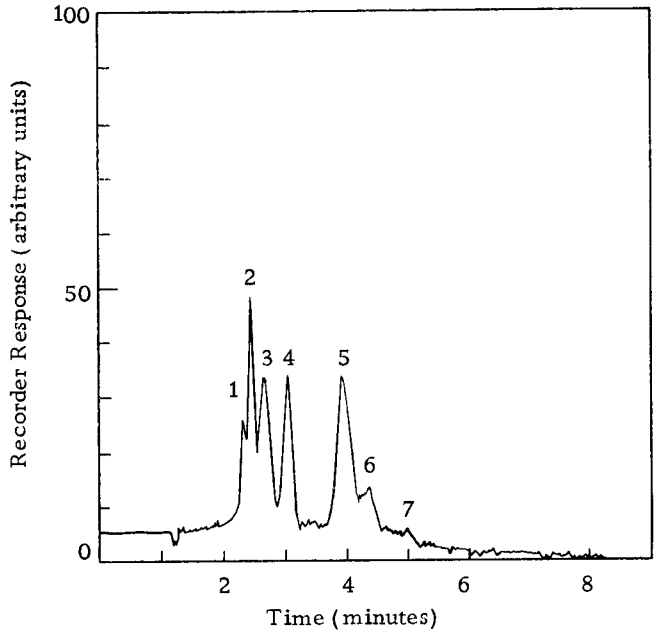

Figure 12. Densitometric recording of the carotenoid pigments of M. radiodurans grown in TGYM broth + 1.0 mg diphenylamine/100 ml medium.

of iodoacetic acid, it did contain large amounts of pigment component number one. Krabbenhoft's work demonstrated that increased sensitivity to ultraviolet light and gamma rays was related to the absence of pigment components number one and four (5). The observation that greater amounts of pigment component number one was formed when the cells were grown in the presence of iodoacetic acid seem to strengthen the postulation that colored carotenoid pigments are involved in the radio-protective mechanism of M. radiodurans. This study supports the postulation for radio-resistance mechanism involving carotenoid pigments for ultraviolet light irradiation, but the carotenoid pigments do not seem to be involved in the protective mechanism for X-irradiation.

The addition of diphenylamine to the growth medium did not change the pigment components. If colored carotenoids protect M. radiodurans from the damaging effects of X-rays, the pigment composition should have changed in light of the increased sensitivity to X-rays. Thus, these two experiments suggest that colored carotenoids do not play a role in the resistance of the organism to the effects of X-rays. An examination of the total extractable lipids was made to look for another possible difference between cells grown in the presence of iodoacetic acid and of diphenylamine.

Effect of Iodoacetic Acid and Diphenylamine on the Total Extractable Lipids

Thin layer chromatograms were prepared by spraying the pigment chromatograms with chromic acid and charring until the portions containing carbon were dark. Densitometric recordings were made of the chromatograms. Figures 13, 14, and 15 are tracings of these recordings. Iodoacetic acid did not change the total extractable lipid composition of M. radiodurans. Cells grown in the presence of diphenylamine possessed an extra lipid band number 10 and large amounts of fatty acids. Diphenylamine is thought to block the terminal steps of dehydrogenation of the colored carotenoid precursors. This extra lipid band may thus be due to the accumulation of pigment precursors produced by the diphenylamine's presence in the growth medium. Unsaturated lipids have been implicated in radio-protection of M. radiodurans because of their radical scavenging ability. Thus, M. radiodurans would be more sensitive to the damaging effects of X-rays if production of the unsaturated lipids were prohibited. As radical scavenging is only important with ionizing radiation, little change in the radiosensitivity would be observed with ultraviolet light. The excess amounts of fatty acids produced by the presence of diphenylamine suggested a further investigation.

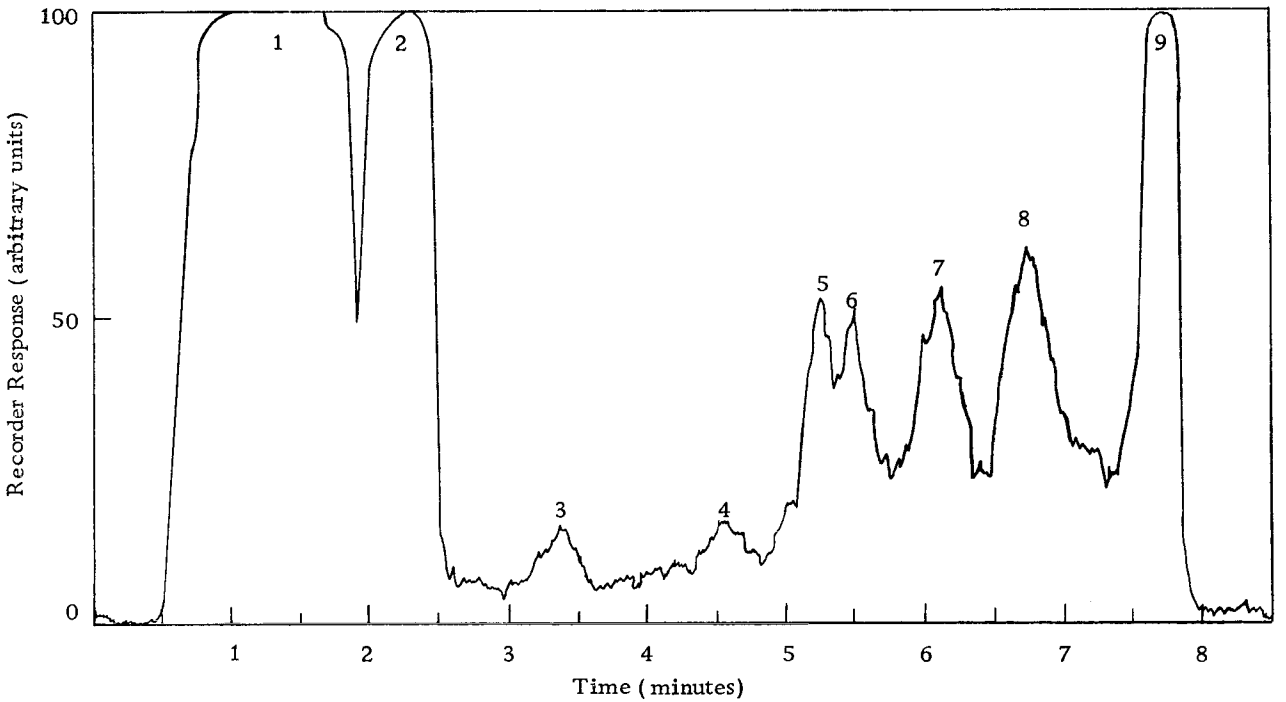

Figure 13. Densitometric recording of a thin layer chromatogram of total extractable lipids of M. radiodurans grown in TGYM broth.

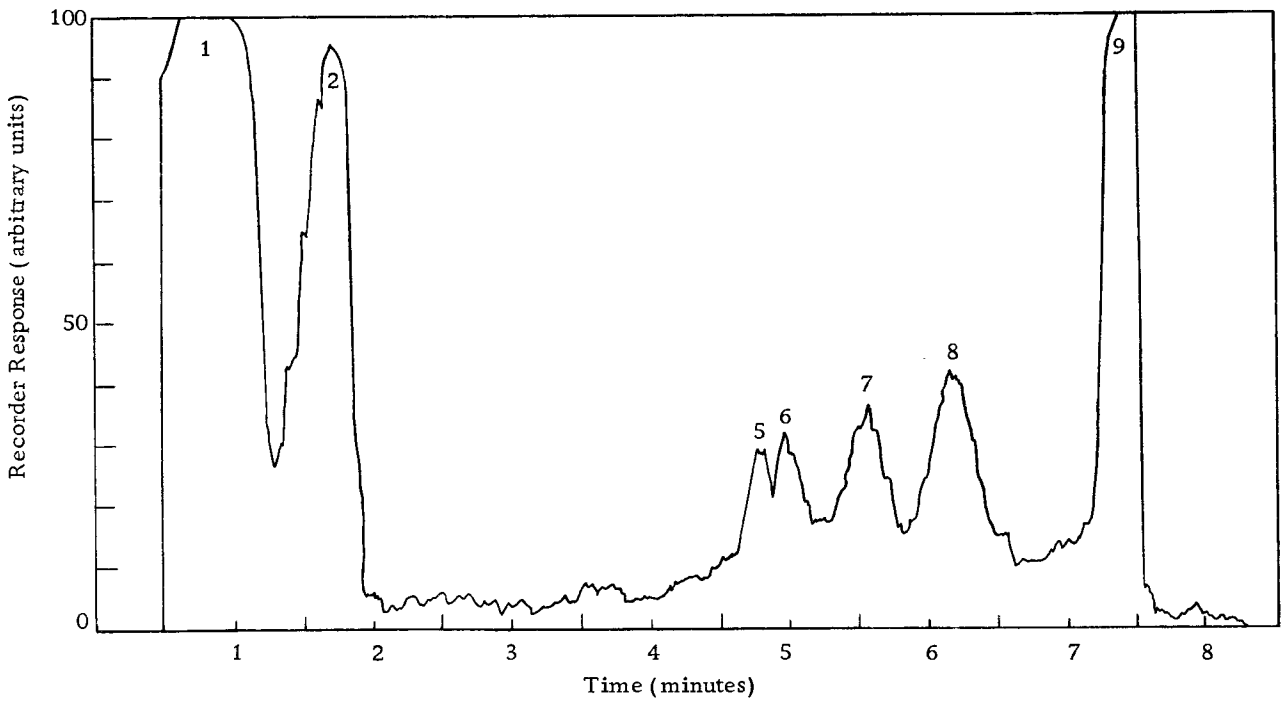

Figure 14. Densitometric recording of a thin layer chromatogram of the total extractable lipids of M. radiodurans grown in TGYM broth + iodoacetic acid.

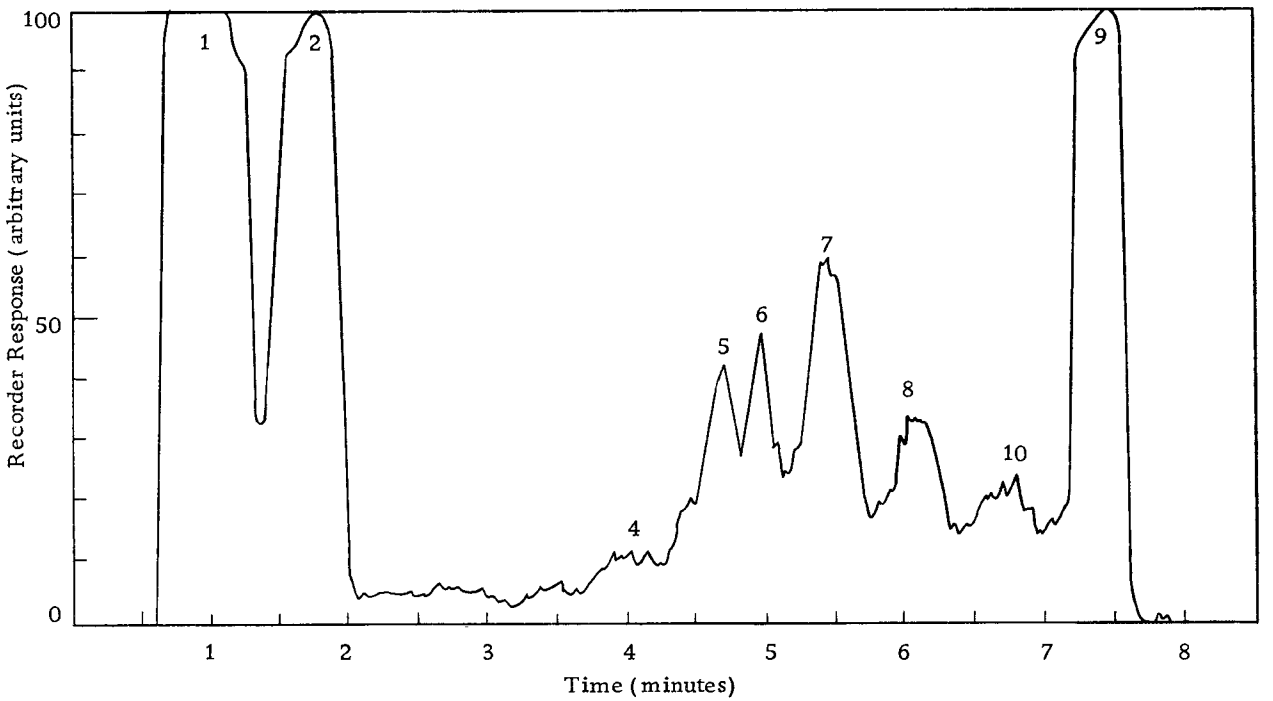

Figure 15. Densitometric recording of a thin layer chromatogram of the total extractable lipids of M. radiodurans grown in TGYM broth + diphenylamine.

Effect of IAA and DPA on the Total Fatty Acid Composition

Table 3 shows that a difference was observed between the cells grown in iodoacetic acid and those grown in TGYM broth. Diphenylamine alters the relative concentrations of C-16:0 (palmitic acid) and C-16:1 (palmitoleic acid). The saturated form (C-16:0) is present in a greater concentration than that observed in control cells (Table 4). Thus, diphenylamine may sensitize M. radiodurans to X-rays by inhibition of certain unsaturated lipid and fatty acid compounds. Cells treated with iodoacetic acid possessed two peaks number 3 and 5 not found in the other cells.

Table 3. Total fatty acid composition of M. radiodurans grown in three different growth media. (Gas chromatography was used to determine the compositions. A 10% ethylene-glycol-succinate column was employed.)

Peak Number	Compound*	Percent Composition		
		TGYM	IAA	DPA
1	12:0	-	0.92	0.25
2	13:0	3.58	1.92	0.50
3	Unknown	-	1.32	-
4	14:0	2.86	1.72	2.01
5	Unknown	-	2.54	-
6	15:0	2.29	2.11	2.51
7	15:1	3.00	3.17	2.26
8	16:0 +	3.00	3.43	1.46
9	16:0	9.30	11.39	21.54
10	16:1	54.4	46.57	47.05
11	17:0	3.15	4.79	2.84
12	17:1	7.01	7.20	4.52
13	18:0	4.29	3.70	1.53
14	18:1	7.15	9.21	13.53

* Number of carbon atoms in acid: number double bonds
+ = BRANCHED CHAIN

Table 4. The ratio of unsaturated: saturated C-16 fatty acid for M. radiodurans grown in three different growth media.

Growth Medium	C-16:0/C-16:1
TGYM	0.171
IAA	0.245
DPA	0.458

SUMMARY AND CONCLUSIONS

M. radiodurans was grown in the presence of diphenylamine and iodoacetic acid to determine their effect on the radio-resistance of this microorganism. It was found that:

(1) The presence of iodoacetic acid in the growth medium increased M. radiodurans resistance to ultraviolet light, but had little effect when X-rays were the source of irradiation.

(2) Thin layer chromatograms of the colored carotenoid pigments revealed that the iodoacetic acid-cultured cells contained a large amount of pigment component number one. M. radiodurans lacked pigment components numbers six and seven when cultured in media containing iodoacetic acid.

(3) Diphenylamine's presence in the growth media showed little effect on the cells' resistance to ultraviolet light, however, increased sensitivity to the damaging effects of X-rays was observed.

(4) Thin layer chromatograms of the total extractable lipids demonstrated that M. radiodurans exhibited an extra lipid band and large amounts of fatty acids when grown in the presence of diphenylamine.

(5) Gas chromatography was used to examine the fatty acid

composition of M. radiodurans. A relatively large amount of C-16 saturated fatty acid was produced by M. radiodurans cultured in the presence of diphenylamine.

Although M. radiodurans exhibits a great tolerance to both ultraviolet light and X-irradiation, the mechanisms of damage are not the same. Therefore, two separate mechanisms are postulated to protect M. radiodurans. It may be concluded that:

(1) Colored carotenoid pigment number one may be responsible for the extreme tolerance of M. radiodurans to ultraviolet light. Since M. radiodurans may be able to split the iodine atom from the iodoacetate molecule and incorporate the acetate in the synthesis of pigment number one, iodoacetic acid may act as a precursor to colored carotenoid pigment formation.

(2) Unsaturated lipids and fatty acids may protect M. radiodurans from ionizing radiation by scavenging free radicals and ions.

BIBLIOGRAPHY

1. Alper, Tikvah. The modification of damage caused by primary ionization of biological targets. Radiation Research 5:573-586. 1956.

2. Anderson, A. W. et al. Studies on a radio-resistant micrococcus. I. The isolation, morphology, cultural characteristics and resistance to gamma radiation. Food Technology 10:565-577. 1956.

3. Bacq, Z. M. and Peter Alexander. Fundamentals of radiobiology. 2d ed. Oxford, Pergamon, 1961. 555 p.

4. Bridges, B. A. The chemical sensitization of Pseudomonas species to ionizing radiation. Radiation Research 16:232-242. 1962.

5. Bridges, B. A. and T. Horne. The influence of environmental factors on the microbial effect of ionizing radiations. Journal of Applied Bacteriology 22:96-115. 1959.

6. Bruce, A. K. Extraction of the radioresistant factor of Micrococcus radiodurans. Radiation Research 22:155-165. 1964.

7. Bruce, A. K. and W. H. Malchman. Radiation sensitization of Micrococcus radiodurans, Sarcina lutea, and Escherichia coli by p-Hydroxymercuribenzoate. Radiation Research 24:473-481. 1965.

8. Dale, W. M. The effect of X-rays on the conjugated protein d-amino-acid oxidase. Biochemical Journal 36:80-85. 1942.

9. Dean, C. J. The radiosensitization of bacteria by iodoacetamide. British Journal of Radiology 35:73-74. 1962.

10. Dean, C. J. and Peter Alexander. Sensitization of radioresistant bacteria to X-rays by iodoacetamide. Nature 196: 1324-1326. 1962.

11. Deeney, Anne O. The role of methionine in the metabolism of Micrococcus radiodurans. Ph. D. thesis. Corvallis, Oregon State University, 1963. 118 numbered leaves.

12. Duggan, Dennis E. Physiological studies on radiation resistant bacteria occurring in food. Ph. D. thesis. Corvallis, Oregon State University, 1961. 174 numb. leaves.

13. Duggan, D. E. et al. Ultraviolet exposure studies on a gamma radiation resistant Micrococcus isolated from food. Food Research 24:376-382. 1958.

14. Eldjarin, L. and E. Pihl. Mechanisms of protection and sensitizing action. In: Mechanisms in radiobiology, Vol. 2. ed. by M. Effesce and A. Forssberg. New York, Academic Press, 1960. p. 231-296.

15. Fricke, Hugo, Edwin J. Hart and Homer D. Smith. Chemical reactions of organic compounds with X-ray activated water. Journal of Chemical Physics 6:229-240. 1938.

16. Friedwald, W. R. and R. S. Anderson. Influence of extraneous protein and virus concentration on the inactivation of rabbit papilloma virus by X-rays. Journal of Experimental Medicine 74:463-472. 1941.

17. Harkinson, D. R. et al. Utilization of carbohydrates and amino acids by Micrococcus radiodurans. Canadian Journal of Microbiology 6:289-298. 1960.

18. Jensen, S. L. Biosynthesis and function of carotenoid pigments in microorganisms. Annual Review of Microbiology 19:163-182. 1965.

19. Kelner, A. et al. Symposium of radiation effects on cells and bacteria. Bacteriological Reviews 19:22-44. 1955.

20. Kiga, M., Y. Ando and H. Koike. Enhancement of radiobiological effect by malonic and maleic acids. Science 122: 331-332. 1955.

21. Kilbourn, Joan P. Use of a radiation resistant organism as a protectant from lethal effects of irradiation in mice. Ph. D. thesis. Corvallis, Oregon State University, 1963. 212 numb. leaves.

22. Kilburn, R. E., W. O. Bellamy and S. A. Terni. Studies on a radiation-resistant Sarcina sp. Radiation Research 9:207-215. 1958.

23. Kimball, R. F. Non-genetic effects of radiation on microorganisms. Annual Review of Microbiology 11:199-220. 1957.

24. Krabbenhoft, Kenneth L. Significance of pigments in radiation resistance of Micrococcus radiodurans. Ph. D. thesis. Corvallis, Oregon State University, 1965. 120 numb. leaves.

25. Lee, Jong Sun. Investigation of factors influencing radiation resistance of Micrococcus radiodurans. Ph. D. thesis. Corvallis, Oregon State University, 1963. 91 numb. leaves.

26. Lee, J. S. Spectrophotometric characterization of the carotenoid pigments isolated from Micrococcus radiodurans. Master's thesis. Corvallis, Oregon State University, 1962. 31 numb. leaves.

27. Lee, J. S., A. W. Anderson and P. R. Elliker. The radiation sensitizing action of N-ethylmaleimide and iodoacetic acid on a radiation-resistant Micrococcus. Radiation Research 19: 593-598. 1963.

28. Lewis, Norman F. Studies on the lipid and fatty acid composition of Micrococcus radiodurans. Master's thesis. Corvallis Oregon State University, 1965. 53 numb. leaves.

29. Mac Kinney, G. et al. Carotenoid production in Phycomyces. Journal of the American Chemical Society 74:3456-3457. 1952.

30. Moseley, B. E. B. The variation in X-ray resistance of Micrococcus radiodurans and some of its less pigmented mutants. International Journal of Radiation Biology 6:489. 1963.

31. Moseley, B. E. B. and A. H. Schein. Radiation resistance and deoxyribonucleic acid base composition of Micrococcus radiodurans. Nature 203:1298-1299. 1964.

32. Niven, C. F., Jr. Microbiological aspects of radiation preservation of food. Annual Review of Microbiology 12:507-524. 1958.

33. Noaman, A. Mohamed et al. Radiosensitization of Streptococcus faecalis and Escherichia coli. Journal of Food Science 29: 80-86. 1964.

34. Patt, H. M. et al. Cysteine protection against X-irradiation. Science 110:213-214. 1949.

35. Payne, J. I. and J. N. Campbell. The effect upon Micrococcus violagabriellae of short-term exposure to ultraviolet light. Journal of General Microbiology 38:343-356. 1965.

36. Rubin, B. A. Growth and mutation of bacteria during continuous irradiation. Journal of Bacteriology 67:361-368. 1954.

37. Schein, A. H. The Deoxyribonucleic Acid of Micrococcus radiodurans. Biochemical Journal 101:647-650. 1966.

38. Stanier, R. Y. and Germaine Cohen-Bazeire. Inhibition of carotenoid synthesis in photosynthetic bacteria. Nature 181: 250-252. 1958.

39. Stanier, R. Y., Michael Doudoriff and Edward A. Adelberg. Microbial world. 2d ed. Englewood Cliffs, Prentice Hall, 1963. 753 p.

40. Stapleton, G. E., A. J. Sbarra and A. Hollaender. Some nutritional aspects of bacterial recovery from ionizing radiations. Journal of Bacteriology 70:7-15. 1955.

41. Thornley, Margaret J. Radiation resistance among bacteria. Journal of Applied Bacteriology 26:334-345. 1965.

42. Zelle, M. R. Genetics of microorganisms. Annual Review of Microbiology 9:45-85. 1955.