Title: RECOVERY FROM ULTRAVIOLET LIGHT-INDUCED DAMAGE IN MICROCOCCUS RADIODURANS

Abstract approved: Redacted for Privacy

Dr. A. W. Anderson

The fortification of a growth medium for Micrococcus radiodurans with N-Z-Case (a pancreatic digest of casein) resulted in a decrease in pigmentation and resistance to ultraviolet-irradiation. The decreased resistance was exhibited on the survival curve as a reduction in the extrapolation number from ~300 to ~20 with little change in the slope of the exponential portion of the curve.

The possibility that the decreased UV resistance resulted from a decrease in a protective or screening substance was ruled out by measuring the UV-induced conversion of thymine into thymine-containing dimers. Resistant and sensitive cells exposed to the same incident dose contained equivalent percentages of thymine-containing dimers.

Recovery, defined as the repair of sub-lethal damage and a return to normal multiplicity, was determined using paired dose studies. A bimodal recovery curve was obtained for both resistant and sensitive
populations irradiated to the same survival level.

The difference in the UV inactivation response expressed by the resistant and sensitive cultures was concluded to result from differences in recovery rates; full recovery, in both cell populations irradiated to equivalent survival levels, occurred in the same amount of time, even though the resistant cells had received approximately twice the dose and had suffered almost twice the damage as the sensitive cells.

A UV-induced lag in the completion of cytokinesis, with permanent inhibition at higher doses, was observed in _M. radiodurans_.

Recovery from Ultraviolet Light-Induced Damage in *Micrococcus radiodurans*

by

Ronald Douglas Ley

A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June 1971
APPROVED:

Redacted for Privacy

Professor of Microbiology
in charge of major

Redacted for Privacy

Head of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented June 30, 1970

Typed by Donna L. Olson for Ronald Douglas Ley
ACKNOWLEDGMENT

The author wishes to thank:

a patient Dr. A. W. Anderson,

a generous Dr. D. S. Nachtwey, and

an understanding wife, Yvonne.

Special thanks to Al Miller III for help in the preparation of this thesis.

This study was supported in part by a National Aeronautics and Space Administration Traineeship.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>HISTORICAL</td>
<td>3</td>
</tr>
<tr>
<td>The Organism</td>
<td>3</td>
</tr>
<tr>
<td>The Nature of Ultraviolet Light-Induced Lesions</td>
<td>6</td>
</tr>
<tr>
<td>The Biological Importance of Known Ultraviolet</td>
<td></td>
</tr>
<tr>
<td>Photoprodacts</td>
<td>8</td>
</tr>
<tr>
<td>Enzymatic Repair of UV-Induced Lesions</td>
<td>10</td>
</tr>
<tr>
<td>Photoreactivation</td>
<td>10</td>
</tr>
<tr>
<td>Dark Repair - Pyrimidine Dimer Excision</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MATERIALS AND METHODS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Microorganism</td>
<td>15</td>
</tr>
<tr>
<td>Culture Methods</td>
<td>15</td>
</tr>
<tr>
<td>Ultraviolet Irradiation</td>
<td>16</td>
</tr>
<tr>
<td>Survival Determination</td>
<td>17</td>
</tr>
<tr>
<td>Plating Procedure</td>
<td>19</td>
</tr>
<tr>
<td>Paired Dose Studies</td>
<td>19</td>
</tr>
<tr>
<td>Determination of Post-irradiation Resumption of</td>
<td></td>
</tr>
<tr>
<td>DNA Synthesis</td>
<td>20</td>
</tr>
<tr>
<td>Measurement of Pyrimidine Dimers</td>
<td>21</td>
</tr>
<tr>
<td>Post-irradiation Division Studies</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RESULTS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival Curves</td>
<td>25</td>
</tr>
<tr>
<td>Pyrimidine Dimer Formation</td>
<td>27</td>
</tr>
<tr>
<td>Recovery Curve Determination by Paired Doses</td>
<td>27</td>
</tr>
<tr>
<td>Full Recovery Time Determination</td>
<td>32</td>
</tr>
<tr>
<td>Post-irradiation DNA Synthesis</td>
<td>38</td>
</tr>
<tr>
<td>Post-irradiation Cell Division</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>The Paired Dose Technique</td>
<td>55</td>
</tr>
<tr>
<td>Post-irradiation DNA Synthesis Rates and Cell Division</td>
<td>61</td>
</tr>
<tr>
<td>Induced UV Sensitivity in PCNZ-Grown Micrococcus radiodurans</td>
<td>64</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Thymine-containing dimer formation in UV-irradiated PCNZ- and TGYM-grown M. radiodurans.</td>
<td>28</td>
</tr>
<tr>
<td>2.</td>
<td>Paired dose irradiations of <em>M. radiodurans</em> grown in PCNZ broth.</td>
<td>36</td>
</tr>
<tr>
<td>3.</td>
<td>Paired dose irradiations of <em>M. radiodurans</em> grown in TGYM broth.</td>
<td>37</td>
</tr>
<tr>
<td>4.</td>
<td>Percentages of potential colony-forming units showing single and multiple divisions.</td>
<td>43</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Modified Petri dish bottom used to hold cell suspensions during irradiation</td>
<td>18</td>
</tr>
<tr>
<td>2.</td>
<td>Survival curves for UV-irradiated Micrococcus radiodurans grown in PCNZ and TGYM broths</td>
<td>26</td>
</tr>
<tr>
<td>3.</td>
<td>Paired dose recovery curves for M. radiodurans grown in TGYM and PCNZ broths</td>
<td>30</td>
</tr>
<tr>
<td>4.</td>
<td>Survival curves for previously irradiated PCNZ-grown M. radiodurans</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Survival curves for previously irradiated TGYM-grown M. radiodurans</td>
<td>34</td>
</tr>
<tr>
<td>6.</td>
<td>The incorporation of $^3$H-thymidine into the trichloroacetic acid-insoluble fraction of UV-irradiated M. radiodurans grown in PCNZ broth</td>
<td>40</td>
</tr>
<tr>
<td>7.</td>
<td>The incorporation of $^3$H-thymidine into the trichloroacetic acid-insoluble fraction of UV-irradiated M. radiodurans grown in TGYM broth</td>
<td>41</td>
</tr>
<tr>
<td>8.</td>
<td>Cumulative percentages of M. radiodurans from PCNZ broth having undergone a single division after UV-irradiation</td>
<td>42</td>
</tr>
<tr>
<td>9.</td>
<td>Cumulative percentages of M. radiodurans from TGYM broth having undergone a single division after UV-irradiation</td>
<td>44</td>
</tr>
<tr>
<td>10.</td>
<td>Observed and predicted survival curves for TGYM-grown M. radiodurans</td>
<td>57</td>
</tr>
</tbody>
</table>
Micrococcus radiodurans, a pigmented, Gram-positive, tetra-
coccus is highly resistant to ultraviolet (UV) and ionizing radiations. The survival curves for this organism in response to ultraviolet-
and ionizing-irradiations are characterized by an initial shoulder,
followed by exponential inactivation of colony-forming ability at higher
doses. The magnitude of the shoulder greatly exceeds that expected
for a multiplicity of four cells/colony-forming unit.

The transfer of M. radiodurans from a Bacto-tryptone-glucose-
yeast extract-methione (TGYM) medium to a Bacto-tryptone-glucose-
yeast extract medium fortified with 0.5 percent N-Z-Case (PCNZ
medium) was shown to result in a decrease in this organism's genera-
tion time, pigmentation, and resistance to ultraviolet and ionizing
radiation (31). The Bacto-tryptone and N-Z-Case are both pancreatic
digests of casein. The decrease in UV resistance was observed as a
reduction in the shoulder width on the survival curve with little change
in the limiting slopes.

This study was undertaken to determine if the decreased resis-
tance to UV-irradiation was due to a decrease in the concentration of
a protective or screening substance or due to a reduced capacity for
recovery from UV-induced damage.

Measurement of the formation of UV-induced, pyrimidine dimers in resistant and sensitive cells was used as a form of internal dosimetry to detect possible differences in the amounts of protective or screening substances present in the two populations. Recovery capacity and recovery kinetics were measured using a paired dose technique.

The effects of UV-irradiation on DNA synthesis and cellular division were also studied in the sensitive and resistant cells.
HISTORICAL

The Organism

_Micrococcus radiodurans_, a non-spore forming, pink-pigmented, Gram-positive, tetracoccus is highly resistant to ultraviolet (UV) (10) and ionizing radiations (1). The inactivation of this organism in response to UV- and ionizing-irradiation is characterized by an initial "shoulder" which is followed by exponential inactivation at higher doses. The magnitude of the shoulder observed greatly exceeds that expected for a multiplicity of four cells/colony-forming unit.

The organism was originally isolated by Dr. A. W. Anderson (1) from heavily γ-irradiated cans of meat.

_Micrococcus radiodurans_ is not resistant to heat inactivation (9), and does not exhibit a resistance to Mitomycin C any greater than that found in _Escherichia coli_ (34). Mitomycin C has been shown to cross-link DNA _in vivo_ (23).

Electron micrographs of _M. radiodurans_ have shown an unusual multi-layered cell wall (65); while chemical studies have determined that L-ornithine, rarely found in bacterial cell walls, is the main diaminoacid in the mucopeptide complex (68).

The carotenoid pigments (29) of _M. radiodurans_ have been separated by thin-layer chromatography into eight (30) and five (64) components depending on the solvent systems and developing
techniques used. Attempts to correlate the high radiation resistance to the pigmentation of the organism have been inconsistent (29, 35, 37). Krabbenhoft, Anderson and Elliker (31) altered the radiation resistance of *M. radiodurans* by growth in different culture media; cultures with nutrient-induced UV sensitivity were also less pigmented and had a shorter generation time.

The UV radiation resistance of the organism could not be explained on the basis of an unusual DNA base composition, redundancy of the genetic material, or shielding of the DNA by cytoplasmic constituents. Setlow and Duggan (45) reported a G+C/A+T ratio of 1.6 for *M. radiodurans*; and although a relationship has been shown to exist between base content of bacterial DNA and resistance to X- and UV-irradiation (28), the high GC content was not considered to be responsible for the high UV resistance of the organism (45). In addition, Setlow and Duggan (45) found that *M. radiodurans* is less transparent to 265.2 nm UV light than is *E. coli*; this implicated shielding of the DNA as a possible factor in the UV resistance of the cells. They concluded that the high GC content of the DNA and lower transmission of UV light in *M. radiodurans* could possibly account for the observed resistance to thymine dimerization, but the difference was not sufficient to account for the unusually high resistance of *M. radiodurans* to cell inactivation.

Redundancy of genetic material does not appear to be a
resistance factor since the DNA content of \textit{M. radiodurans} was determined to be only one percent of the dry weight of the cell \cite{31} and 1.5-2.0 percent of the defatted dry weight \cite{38}. Whereas, the DNA of the more UV-sensitive \textit{E. coli} makes up one percent of the wet weight of the cell \cite{66}.

Using a multiple dose technique, Moseley and Laser \cite{37} showed that \textit{M. radiodurans} was capable of recovering from X-ray induced, sub-lethal damage. The recovery was evidenced by a previously irradiated population exhibiting a survival curve similar to an unirradiated population, if a suitable interval separated the initial dose from the survival curve determination. Moseley and Laser \cite{36} later used mixed doses of UV and ionizing radiation to show that there occurs in \textit{M. radiodurans} a similarity in the repair of damage from these two types of radiation.

A repair mechanism exists in \textit{M. radiodurans} which enzymatically removes UV-induced, pyrimidine dimers from the cells' DNA \cite{3}. Logarithmic phase cells excised \textasciitilde{}90 percent of the dimers within 90 minutes after receiving a dose of 5000 ergs/mm\textsuperscript{2}. Although maximum stationary phase cells exhibited little excision of dimers during the same time period, there was little difference in the UV sensitivity expressed by logarithmic and maximum stationary phase cells \cite{44}.

Moseley \cite{33} has studied the excision of UV-induced, pyrimidine
dimers from wildtype *M. radiodurans* and less resistant mutants. Although the mutant cells were capable of excising thymine-thymine dimers at the same rate found with wildtype cells, the mutants were slower in the excision of cytosine-thymine dimers.

*Micrococcus radiodurans*, like other bacteria studied, is subject to a UV-induced delay in DNA synthesis. With log phase *M. radiodurans*, DNA synthesis was repressed for 100 to 150 minutes after an incident dose of 3000 ergs/mm$^2$ (33, 44). DNA synthesis in the radiation resistant *E. coli* B/r was inhibited for 60 minutes after a dose of 200 ergs/mm$^2$ (56); a comparable delay in *M. radiodurans* would require $\sim$1000 ergs/mm$^2$ (33).

The action spectra for killing of most bacteria corresponds to the absorption spectra of nucleic acids. However, *Micrococcus radiodurans* exhibits a similar inactivation response to 280 nm radiation as to the 265 nm radiation (44); the 280 nm absorption is characteristic of proteins.

**The Nature of Ultraviolet Light-Induced Lesions**

Pyrimidine dimers are the most studied of the numerous photoproducts isolated from UV-irradiated DNA and solutions of purines and pyrimidines.

Ultraviolet-irradiation of thymine in frozen solution resulted in dimerization of the molecules through their 5 and 6 carbon atoms to
form a cyclo-butane ring (2). In addition, irradiation of di- and poly-
ucleotides has yielded not only thymine-thymine dimers (53), but
also dimers of thymine-cytosine (53), uracil-cytosine (13), thymine-
uracil (61), cytosine-cytosine (54), and uracil-uracil (63). Setlow
and Carrier (50) have identified thymine-thymine, thymine-cytosine,
and cytosine-cytosine dimers in UV-irradiated bacterial DNA.

In addition to the cyclobutane-type thymine dimers, a dimer
has been isolated from UV-irradiated thymidylyl-thymidine which was
joined by an ether linkage at the C-4 position (39).

Ultraviolet photoproducts of pyrimidines also result from the
addition of water at the double bond between the 5 and 6 carbon atoms.
In vitro hydration products have been reported for cytosine (27) and
uracil (17). Furthermore, cytidine hydrate has been shown to occur
in denatured and native DNA after UV-irradiation (16).

Because of the organism's sensitivity to 280 nm radiation, a UV
photoproduct of particular interest in the study of the inactivation of
*M. radiodurans* is the cross-linking of DNA to protein. Cross-linking
is measured by the inability to extract the bound DNA from the cell
(62); at a 99 percent killing dose for *E. coli* B/r, while only 0.1 per-
cent of the thymine was converted to thymine dimers, 11 percent of
the DNA was rendered unextractable (62). Newly synthesized DNA
appeared to be more sensitive to protein cross-linking; and under
thymine starvation, the sensitivity of *E. coli* 15 TAU to UV radiation
increased as did the sensitivity of the DNA to protein cross-linking (60). Photochemical (253.7 nm) addition of amino acids to \(^{14}\)C-uracil (58), and the addition of cysteine, tyrosine, and serine to DNA (59) are bases for the postulation of a model for DNA-protein cross-linking.

At high UV doses (exceeding the dose range of usual biological interest), cross-linking of complementary DNA strands and strand scission have been reported (14, 32). Many additional photoproducts have been detected, but not identified, after high UV doses.

The Biological Importance of Known Ultraviolet Photoproducts

Although the sheer volume of information may tend to overstate the importance of pyrimidine dimers as a biologically effective photoproduct, pyrimidine dimers have been correlated with UV-induced biological effects. The greatest support for pyrimidine dimers as biologically important lesions come from short-wavelength reversal and enzymatic photoreactivation studies.

The formation of pyrimidine dimers in DNA is a photochemically reversible reaction in which the amount of pyrimidine dimerized is dependent upon the wavelength of the incident radiation (48, 52). Thus, the dimer content of DNA previously exposed to 260 or 280 nm radiation is decreased if subsequently subjected to 239 nm radiation.
The ability of denatured DNA to act as a primer for in vitro DNA synthesis was drastically lowered by UV-irradiation at 280 nm (5). However, the reduced primer activity was partially reversed by subsequent exposure to 239 nm radiation. Therefore, decreasing the amount of pyrimidine dimers present was accompanied by a reduction in an effect of biological importance.

The transforming activity of Haemophilus influenzae DNA was reduced by large doses of 280 nm radiation and was partially reactivated with 239 nm radiation (55).

Enzymatic photoreactivation, generally occurring at wavelengths longer than 300 nm, has been shown to split cyclobutane-type pyrimidine dimers in deoxypolyribonucleotides (47, 48, 54, 69). In addition, the action of enzymatic photoreactivation will reverse the UV-induced inhibition of colony formation (56) and DNA synthesis (40, 56).

Short-wavelength reversal of UV-induced dimers overlaps with enzymatic photoreactivation of irradiated transforming DNA (47). The short-wavelength reversal of transforming DNA decreases its photoreactivability, and maximum photoreactivated DNA cannot be further reactivated by short-wavelength reversal. This indicates that the substate for enzymatic photoreactivation is the same photoproduct affected by short-wavelength reversal, the pyrimidine dimer.
An effect which could possibly result in mutagenesis in biological systems was found in the UV-irradiation of in vitro RNA coding systems. The formation of uridine hydrate in poly U resulted in the polyribonucleotide coding for serine instead of phenylalanine (17). In addition, cytosine hydrate was recognized as uracil or thymine in an in vitro polymerase system (16). If the same events occurred in DNA in vivo, mutagenesis by such coding shifts would be highly probable.

The UV-inactivation of E. coli B/r T⁻ has been correlated with the cross-linking of DNA and protein (58). However, these studies have been most successful with cells irradiated when frozen, and the importance of DNA-protein linking at temperatures of biological compatibility has yet to be determined.

Enzymatic Repair of UV-Induced Lesions

Photoreactivation

During photoreactivation, pyrimidine dimers formed by UV-irradiation are enzymatically monomerized when subjected to 330-430 nm radiation. The splitting of dimers has been shown to occur in vitro using cell-free extracts acting on irradiated transforming DNA (47) and in vivo in E. coli (56). The photoreactivation resulted in an increased activity of the transforming DNA and in a higher colony-forming ability in the irradiated E. coli. Although enzymatic photoreactivation has not been shown to occur in M. radiodurans, this
organism does possess a repair mechanism which can operate in the dark.

**Dark Repair - Pyrimidine Dimer Excision**

The ability to enzymatically remove UV-induced, pyrimidine dimers from DNA has been demonstrated in numerous cellular systems. Dimer excision has been shown to occur in several genera of bacteria (3, 46, 51) and in eucaryotic cell systems: the protozoan, *Tetrahymena pyriformis* (67), human skin fibroblasts and HeLa cells (41).

The repair mechanism, which involves the excision of UV-induced, pyrimidine dimers, was postulated by R. Setlow and Carrier (51) and by Boyce and Howard-Flanders (6). The mechanism has since been studied extensively. The steps thought to occur during the repair process have been discussed in reviews by R. Setlow (49) and Howard-Flanders (22).

The proposed repair sequence involves:

1) A single-strand incision near the pyrimidine dimer by a UV-specific endonuclease;

2) Excision of the dimer region by exonuclease activity;

3) Repair replication to replace the nucleotides removed during step 2; and

4) Joining of the "new" nucleotide sequence to the remainder
of the DNA strand by ligase action.

Experimental evidence in support of steps one and two of this repair sequence was found in the isolation and purification of an endonuclease and exonuclease from Micrococcus lysodeikticus which is specific for UV-irradiated DNA (18). Evidence for step three of the postulated sequence was provided by Pettijohn and Hanawalt (40) who found that the incorporation of 5-bromouracil (BU) into UV-irradiated bacteria occurs simultaneously at random positions in the genome. It is reasonable to associate repair replication with dimer excision, since this mode of replication was not observed in cells which had been photoreactivated (40), or in E. coli B<sub>8-1</sub>, which is unable to excise pyrimidine dimers (51).

Repair replication, as evidenced by the incorporation of BU, has been detected not only in bacteria, but also in PPLO (57), in Tetrahymena pyriformis (7), and in mammalian cells (8).

Evidence for step four of the repair sequence is found in the presence of a DNA ligase in E. coli which functions in normal DNA replication (15).

Attempts have been made to explain pre- and post-irradiation phenomena such as liquid holding recovery and photoprotection in terms of excision repair. If UV-irradiated E. coli B are held in phosphate buffer prior to plating on nutrient agar, higher surviving fractions are obtained than if the cells are plated immediately after
irradiation (42). This phenomenon, called liquid holding recovery (LHR), was thought to result from a growth-division delay which occurred upon plating and allowed time for enzymatic repair systems to function before the resumption of division (26). This hypothesis was disputed by Harm (20) who used inhibitors of excision-repair (caffeine and acriflavine) to show that LHR appears to take place during the holding period rather than after plating on the growth medium. However, this does not disprove a hypothesis that LHR results from increased repair in terms of increased repair time, the holding in buffer serving to extend the repair time available.

Survival can be also enhanced in *E. coli* B by subjecting the cells to 334 nm radiation prior to UV-irradiation (24). The photoprotection exhibited is thought to be due also to an induced division lag allowing time for repair.

In addition, a non-enzymatic photoreactivation takes place in *E. coli* B phr− (lacks enzymatic photoreactivation) when exposed to 334 nm illumination after UV-irradiation (25). The mechanism is thought to be similar to photoprotection and involve the induction of a growth-division delay.

The possible importance of a UV-induced delay in DNA synthesis allowing time for intracellular repair enzymes to function was shown by Hanawalt (19). He observed that temporary blockage of re-initiation of post-irradiation DNA synthesis in *Escherichia coli* TAU
resulted in a wide shoulder on the UV survival curve. The same strain in balanced growth exhibited exponential inactivation without the initial shoulder; the limiting slopes of the survival curves in both instances were approximately equal. Hanawalt concluded that by blocking the re-initiation of DNA synthesis the cells were given time to repair UV-induced damage, with inactivation at higher doses occurring due to saturation or inactivation of the repair system. The proposed relationship of repair to the lag in DNA synthesis finds experimental support in that E. coli B₉₋₁, which lacks the excision repair system, does not benefit from the delay in post-irradiation DNA synthesis (19).
MATERIALS AND METHODS

Microorganism

Wild-type *Micrococcus radiodurans* R₁ was obtained from the Oregon State University stock culture collection.

Culture Methods

The organism was grown on two types of media which had previously been used by Krabbenhoft et al. (31) in a study of the radiation resistance of *M. radiodurans*. These media were designated as TGYM and PCNZ, and the constituents of each are as follows:

**TGYM Medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Difco)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>DL - Methionine</td>
<td>20 mg</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

**PCNZ Medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Difco)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>N-Z-Case (Sheffield Chemical Co.)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1 liter</td>
</tr>
</tbody>
</table>
Each medium was adjusted to pH 7.0 and was distributed as 100 ml quantities into 250 ml DeLong culture flasks prior to sterilization. Cultures were maintained by transferring at 48 hour intervals using a one milliliter inoculum. The inoculated flasks were incubated at 34°C on a gyrotory shaker.

Only PCNZ cultures which had been transferred less than seven consecutive times were used in the following experiments. Those cultures which had exceeded seven transfers in PCNZ broth were discarded and a fresh PCNZ culture started with 1 ml from a TGYM culture. The reason for this limit on the number of transfers in PCNZ broth will be presented in the discussion.

**Ultraviolet Irradiation**

Maximum stationary phase cells (34-38 hours) were used for all irradiations. The cells were collected by centrifugation at 10,000 x G for 15 minutes, washed and suspended in 0.067 M phosphate buffer (pH 7.0). The cell density of the suspension was adjusted to meet the requirements of each experiment, but at no time were UV-irradiations carried out on cell suspensions which exceeded \(~10^7\) colony-forming units (CFUs)/ml. Colony-forming units/ml is not equivalent to cells/ml because this organism occurs in broth cultures primarily as tetrads.

The cell suspensions were irradiated by placing a 2.0 ml aliquot
in a flat-bottomed Petri dish which had been modified to reduce shadowing of the sample by the wall of the dish. For the modification, the center of a Petri dish was masked by a 5 cm disc, and the inside of the dish was heavily sprayed with adhesive-containing Fluoro-Glide (Chemplastic, Inc.). After the film had dried, the masking disc was removed, and the dish was ready for use. The modified Petri dish is shown in Figure 1. The 2.0 ml of cell suspension to be irradiated remained in the uncoated center of the dish as a uniform layer with a depth of approximately 1.0 mm.

The ultraviolet light source was a 30 watt germidical lamp (General Electric) which was positioned 16 cm from the surface of the cell suspension to give a dose-rate of 60 ergs/mm\(^2\)/sec. The dose-rate was checked at the beginning of each irradiation using a calibrated photoelectric intensity meter (Ultra-Violet Products, Inc.).

**Survival Determinations**

Irradiated and control cells were diluted appropriately in 0.067 M phosphate buffer (pH 7.0), and 0.1 ml of the sample was plated on TGYM or PCNZ agar (TGYM and PCNZ agar was prepared by adding 15 g of agar to one liter of the appropriate broth medium). In some cases, cells were diluted and plated before irradiation. Colony counts were made following incubation at 34 C for 48 hours.
Figure 1. Modified Petri dish bottom used to hold cell suspensions during irradiation. Top view.
Plating Procedure

For colony counts, cells were spread on agar by a method* utilizing glass beads instead of the conventional glass rod spreader. One-tenth of a ml of cell suspension was placed on the agar surface along with five, sterile glass beads (5 mm in diameter). The beads were propelled across the agar surface by briskly moving the plate through 5-10 horizontal oscillations comprised of 10-15 cm strokes. The plate was then rotated 1/4 turn and the oscillations repeated. This procedure was followed until the plate had been rotated through $360^\circ$. This method produces more evenly distributed colonies and less colony overlap than does the use of glass rod spreaders. Spreading by glass beads has the additional advantages of reducing the drudgery and time involved in spreading by glass rods. As many as ten stacked plates, each plate containing five beads, can be oscillated at one time to effect spreading of the bacterial suspension. One to ten plates can easily be spread in this manner in less than 30 seconds.

Paired Dose Studies

The paired dose procedures used by Elkind and Sutton (11) to measure recovery phenomena in X-irradiated Chinese hamster cells

*The origination of this method is unknown. It was used extensively at the U.S. Naval Radiological Defense Laboratory, San Francisco (F. A. Hodge, personal communication).
were adapted to study recovery from UV-induced, sub-lethal damage in *M. radiodurans*. Aliquots of TGYM- and PCNZ-grown cells were irradiated in phosphate buffer to a survival level of approximately 0.1. Samples (0.1 ml) of the irradiated suspensions were then spread upon plates of the respective solidified medium, and a second (challenge) dose or doses were administered following various intervals of incubation at 34°C. Survival levels were then determined as indicated above.

**Determination of Post-irradiation Resumption of DNA Synthesis**

Suspensions irradiated for DNA synthesis studies contained \( \sim 10^6 \) CFUs/ml. Two ml of the control and irradiated cells were collected by centrifugation, the supernatant removed by aspiration, and the cells resuspended in 0.2 ml of phosphate buffer to effect a ten-fold increase in cell density. A 0.15 ml aliquot of the concentrated cell suspension was added to 0.15 ml of double strength TGYM or PCNZ broth containing 10 µCi of \(^3\)H-thymidine (\(^3\)H-TdR), specific activity, 6.7 Ci/m mole (New England Nuclear) and placed in a 34°C water bath. The amount of radioactivity incorporated into the trichloroacetic acid-insoluble fraction of the cell was measured as a function of time by the following method of Bollum (4). At \( t_0 \) and 30 minute intervals thereafter, the suspensions were mixed well, and a 10 µl portion removed with a micro-syringe (Hamilton). The 10 µl sample was placed on a
filter paper disc (1.5 cm in diameter) and dropped into a beaker of cold 5% trichloroacetic acid. A number of discs can be placed into the same beaker as the radioactivity to be counted will be precipitated onto the disc. However, 10 ml of 5% trichloroacetic acid should be provided for each disc placed in the beaker. Ten minutes after the last disc was added to the beaker, the trichloroacetic acid was decanted and the discs washed twice with cold 5% trichloroacetic acid. Residual trichloroacetic acid and water were removed by washing the discs with 95% ethyl alcohol. The discs were then placed on absorbent paper and allowed to air dry. The radioactivity retained on the discs was measured by counting in a Packard Tri-Carb liquid scintillation counter using a toluene scintillation fluid. This scintillation fluid is a water-immiscible solution containing 2,5-diphenyloxazole (PPO), 5 g/l, and 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene (dimethyl-POPOP), 0.3 g/l, in toluene (reagent grade). The PPO and dimethyl-POPOP were obtained from New England Nuclear.

Measurement of Pyrimidine Dimers

In order to label DNA, cells were grown in the presence of 100 µCi of $^3$H-TdR (sp. act. 6.7 Ci/mmmole) in a volume of 0.5 ml. A 20 hour culture was incubated in the presence of $^3$H-TdR for 16 hours. The cells were collected by centrifugation, washed, and suspended at a density of $10^7$ CFUs/ml in 0.067 M phosphate buffer (pH 7.0).
Two ml aliquots of the labeled PCNZ and TGYM cell suspensions were exposed to doses of 0, 4000, and 8000 ergs/mm². The samples were centrifuged, washed in chilled, distilled water and suspended in 0.2 ml of water. Calf thymus DNA (0.08 ml at 4 mg/ml) was added to each sample, and then 0.2 ml of cold 10% trichloroacetic acid was added. The samples were mixed thoroughly and allowed to stand for 10 minutes. The resulting precipitate was collected by centrifugation and washed with 0.2 ml of 5% trichloroacetic acid. The precipitates were again collected by centrifugation, and the trichloroacetic acid supernatant fluid removed with a pipet. Residual trichloracetic acid was removed from the precipitates by washing with 95% ethyl alcohol, and the precipitates were allowed to air dry. The precipitate from each sample was taken up in 0.2 ml of 97+% formic acid and hydrolyzed in a sealed hydrolysis tube for 20 minutes at 175 C. The hydrolysate from each tube was spotted on strips of Whatman's No. 1 paper and developed by descending chromatography with a n-butanol: water (86:14) solvent system to separate thymine and thymine-containing dimers. Thymine and thymine-containing dimers have Rf values of 0.4 and 0.09, respectively, for this solvent system. Dimers which originally contained cytosine occur on the chromatogram as uracil-containing dimers due to the conversion of all cytosine to uracil during the hydrolysis procedure (5). The preceding procedure for the separation of thymine-containing
dimers from free thymine was that used by Moseley (33).

The dimer- and thymine-regions, located by UV absorption of unlabeled markers, were cut from the chromatogram and the activity eluted with water. The count rates of the eluants were determined by liquid scintillation counting in a dioxane-naphthalene scintillation fluid containing 7 g of PPO, 0.3 g of dimethyl-POPOP, and 100 g of naphthalene per liter of dioxane (reagent grade). The radioactivity in the dimer region was expressed as a percent of the total radioactivity in the trichloroacetic acid-insoluble fraction of the cell.

Post-irradiation Division Studies

PCNZ and TGYM grown cells were collected by centrifugation, washed, and suspended in phosphate buffer at a density of \( \approx 10^6 \) CFU/ml. Two ml of the suspensions were exposed to 4000 and 8000 ergs/mm\(^2\) for PCNZ and TGYM grown cells, respectively. Control and irradiated samples were collected by centrifugation and resuspended in 0.2 ml of phosphate buffer to give a density of \( \approx 10^7 \) CFUs/ml. A drop of the suspension was spread on a 2 x 2 x 0.5 cm block of the respective solidified medium and covered with a glass cover slip. A region within 5 mm of the edge of the agar block was scanned with a phase microscope (1000 X) to determine the number of cells making up each colony-forming unit and the frequency of the various cell numbers/CFU at the time of plating. At subsequent
time intervals thereafter, scans and cell counts were made to determine the number of divisions undergone by control and irradiated PCNZ and TGYM cells.
RESULTS

Survival Curves

Survival curves for TGYM- and PCNZ-grown *M. radiodurans* are given in Figure 2. A marked difference in resistance to UV-irradiation was evident, and extrapolation of the exponential portions of the curves to intercept the y-axis gave extrapolation numbers of \( \sim 20 \) and \( \sim 300 \) for PCNZ and TGYM cells, respectively. Sensitivity constants of \( 1.15 \times 10^{-3} \) ergs \(^{-1}\) mm \(^2\) for PCNZ cells and \( 1.05 \times 10^{-3} \) ergs \(^{-1}\) mm \(^2\) for TGYM cells were obtained from the slopes of the exponential portions of the curves. Using these empirically obtained extrapolation numbers and sensitivity constants, theoretical survival curves were calculated according to classical target theory in which multi-target inactivation kinetics exist using the following equation:

\[
S = 1 - (1 - e^{-kD})^n
\]

- \( S \) = surviving fraction
- \( k \) = sensitivity constant
- \( D \) = incident dose (in ergs/mm \(^2\))
- \( n \) = extrapolation number

Calculated survival curves are also given in Figure 2 for PCNZ- and TGYM-grown cells.

It is clear that the shoulders of the empirically determined
Figure 2. Survival curves for UV-irradiated *Micrococcus radiodurans* grown in PCNZ (●) and TGYM (○) broths. The dashed-line curves represent theoretical survival curves of the type $S = 1 - (1 - e^{-kD})^n$ where $k = 1.15 \times 10^{-3}$ ergs$^{-1}$ mm$^2$ and $n = 20$ for PCNZ cells, and $k = 1.05 \times 10^{-3}$ erg$^{-1}$ mm$^2$ and $n = 300$ for TGYM cells. Standard deviations are represented by vertical lines drawn through each point.
curves do not coincide with the calculated curves.

Since cytoplasmic shielding may be responsible for the different surviving fractions of PCNZ and TGYM cells exposed to the same incident dose, the percent thymine converted to thymine-containing, pyrimidine dimers was determined for both cell populations. The degree of dimerization functioned as an internal dosimeter.

Pyrimidine Dimer Formation

Percentages of thymine converted to thymine-containing dimers are given in Table 1. Insignificant differences (P = 0.05) in the percent thymine dimerized were observed for TGYM and PCNZ cells exposed to 4000 ergs/mm$^2$; however, the difference in the surviving fractions for TGYM and PCNZ cells exposed to 4000 ergs/mm$^2$ was highly significant (P = 0.01). The percentages of thymine converted to thymine-containing dimers are comparable to the results obtained by J. Setlow (43).

Since shielding did not appear to account for the difference in survival for the two populations, the paired dose technique was used to determine if the recovery capacity and recovery kinetics of PCNZ- and TGYM-grown cells differed.

Recovery Curve Determination by Paired Doses

Recovery determination by paired doses was based on the
Table 1. Thymine-containing dimer formation in UV-irradiated PCNZ- and TGYM-grown Micrococcus radiodurans.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Incident dose (ergs/mm²)</th>
<th>Net DPM of $^3$H in acid-insoluble fraction</th>
<th>Net DPM of $^3$H from dimer region</th>
<th>$%$ $^3$H in dimers$^a$ ± SD</th>
<th>Surviving fraction ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNZ</td>
<td>4000</td>
<td>49,290</td>
<td>721</td>
<td>1.46 ± .06</td>
<td>0.13 ± .02</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>58,469</td>
<td>1871</td>
<td>3.20 ± .13</td>
<td>-</td>
</tr>
<tr>
<td>TGYM</td>
<td>4000</td>
<td>102,104</td>
<td>1554</td>
<td>1.52 ± .06</td>
<td>0.60 ± .12</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>59,330</td>
<td>1780</td>
<td>3.00 ± .12</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ The radioactivity in the dimers was calculated as a percent of the total activity of the acid-insoluble cell fraction. SD = one standard deviation.
following assumption. If the survival level of a cell population exposed to paired doses increased with an increased time separation of the two doses, the cells are considered capable of recovery from UV-induced damage. To obtain recovery curves for PCNZ and TGYM cells, initial and challenge doses were separated by increasingly longer incubation periods; survival levels were determined by colony formation.

The initial doses used for PCNZ- and TGYM-grown cells, 4600 and 8000 ergs/mm\(^2\); respectively, reduced the surviving fractions of both cell suspensions to ~0.07 (Figure 2). Immediately after the irradiated suspensions were plated, and at hourly intervals thereafter, one plate of the TGYM cells and one plate of the PCNZ cells were exposed to a challenge dose of 2100 ergs/mm\(^2\). An incubation period of 1.5 hours between doses resulted in a 3-4 fold increase in survival over that level obtained when the paired doses were given consecutively to PCNZ and TGYM cells (Figure 3). Little change above the initial increase occurred when two and three hours of incubation separated the paired doses. A second increase, resulting in an additional 3-4 fold rise in survival level, occurred for both cell types when four, five and six hours of incubation separated the doses. In addition, a nutritive environment was apparently necessary for recovery since there was no increase in survival for PCNZ and TGYM cells held in phosphate buffer between exposures (Figure 3).
Figure 3. Paired dose recovery curves for Micrococcus radiodurans grown in TGYM broth (Graph A) and PCNZ broth (Graph B). Incubation between doses in phosphate buffer (●) and on TGYM or PCNZ agar (○). The initial doses for TGYM and PCNZ cells were 8000 and 4600 ergs/mm², respectively. The challenge dose was 2100 ergs/mm² in both cases. Standard deviations are represented by vertical lines drawn through each point.
INCUBATION TIME BETWEEN DOSES (HRS)

SURVIVING FRACTION

(A)

(B)
The survival increase obtained by the separation of doses may have resulted from recovery phenomena. However, the increase could have resulted from a change in resistance of remaining targets; an increase in target resistance having occurred during the incubation period. To determine which of the above possibilities was responsible for the observed survival increase, subsequent experiments were conducted to obtain full survival curves for a previously irradiated population. A change in target sensitivity would affect the slope of the exponential portion of the survival curve; whereas, recovery would result in the reappearance of a distinct shoulder on the curve.

Complete survival curves of TGYM and PCNZ cells were obtained 0, 2, and 6 hours after an initial irradiation. Samples (0.1 ml) of each cell suspension were spread on four plates of the appropriate medium. Plates 1, 2, 3 and 4 received challenge doses of 0, 800, 1600 and 2400 ergs/mm² for TGYM cells and 0, 700, 1400 and 2100 ergs/mm² for PCNZ cells, respectively. Additional plates of each medium were spread with the initially irradiated suspensions and incubated for two and six hours at 34 C before receiving challenge doses of 800, 1600, 2400 and 3200 ergs/mm² for TGYM cells and 700, 1400, 2100 and 2800 ergs/mm² for PCNZ cells.

A partial reappearance of a shoulder to the survival curve was observed for PCNZ- and TGYM-grown *M. radiodurans* when a two hour incubation period separated the initial irradiation (surviving
fraction < 0.1) from the subsequent challenge doses (Figures 4 and 5). The magnitudes of the shoulders indicated that full recovery from the initial irradiation had not occurred. However, PCNZ and TGYM cells exhibited a shoulder of greater magnitude than expected for full recovery when the initial and challenge doses were separated by a six hour interval. Therefore, the data indicated that full recovery had occurred between two and six hours after irradiation.

Full Recovery Time Determination

Full recovery for _M. radiodurans_ is defined to include the repair of sub-lethal, UV-induced damage and the return to normal multiplicity. The multiplicity factor is assumed to be four since this organism occurs primarily as tetrads.

An accurate determination of full recovery time is dependent on knowing the survival response of each cell population when the doses to be paired are given singly, as well as in a paired sequence. The single dose survival levels are used to calculate the maximum survival level attainable for cells which have fully recovered from the initial irradiation before receiving the challenge dose. In addition, accuracy is enhanced by using a challenge dose of such a magnitude that a large survival increase marks full recovery. The rationale supporting these requirements will be covered in the discussion on the paired dose technique as a measure of recovery. Since these
Figure 4. Survival curves for previously irradiated (SF = 0.1) PCNZ-grown *Micrococcus radiodurans* with incubation intervals of 0, 2 and 6 hours separating the survival curve determinations from the initial irradiation. Symbols: 0 hours (○), 2 hours (△) and 6 hours (□) of incubation. The dashed-line curve represents the inactivation response expected for cells which have fully recovered (repair of sub-lethal damage and return to normal multiplicity) from the initial irradiation.
Figure 5. Survival curves for previously irradiated (SF~0.1) TGYM-grown *Micrococcus radiodurans* with incubation intervals of 0, 2 and 6 hours separating the survival curve determinations from the initial irradiation. Symbols: 0 hours (O), 2 hours (Δ) and 6 hours (□) of incubation. The dashed-line curve represents the inactivation response expected for cells which have fully recovered (repair of sub-lethal damage and return to normal multiplicity) from the initial irradiation.
INCIDENT DOSE (ergs/mm$^2 \times 10^{-3}$)
requirements were not considered when obtaining the recovery curves of Figure 3, the data could not be used for an accurate determination of full recovery time.

Recovery from initial doses of 7200 or 8000 ergs/mm$^2$ for TGYM-grown cells and 4600 ergs/mm$^2$ for PCNZ-grown cells was determined using challenge doses of 5400 and 6000 ergs/mm$^2$ and 2000 ergs/mm$^2$ for TGYM and PCNZ cells, respectively. After the cell suspensions were subjected to the initial irradiations and samples plated, the respective challenge doses were administered to one plate of each medium at intervals of 0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 and 6.0 hours. The surviving fractions for the initial and challenge doses given alone were also determined.

Since the challenge dose was given to a cell suspension which had already been irradiated to a surviving fraction less than one, the maximum surviving fraction ($\text{SF}_{\text{max}}$) attainable for cells having received both the initial dose ($D_1$) and the challenge dose ($D_2$) was equal to the surviving fraction of $D_1$ ($\text{SF}_{D_1}$) multiplied by the surviving fraction of $D_2$ ($\text{SF}_{D_2}$); $\text{SF}_{\text{max}} = \text{SF}_{D_1} \times \text{SF}_{D_2}$. The $\text{SF}_{\text{max}}$ would be achieved when the surviving cells had completely recovered (repair and return to normal multiplicity) from $D_1$ before receiving $D_2$.

Recovery times are given in Tables 2 and 3 for PCNZ- and TGYM-grown cells, respectively. In experiment 1 with both cell types, full recovery from $D_1$ occurred when 2-4 hours separated $D_1$
Table 2. Paired dose irradiations of *M. radiodurans* grown in PCNZ broth.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D&lt;sub&gt;1&lt;/sub&gt; - t - D&lt;sub&gt;2a&lt;/sub&gt;</strong></td>
<td><strong>D&lt;sub&gt;1&lt;/sub&gt; - t - D&lt;sub&gt;2&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td>SF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SF&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4600 - 0 - 0</td>
<td>4600 - 0 - 0</td>
</tr>
<tr>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td>0 - 0 - 2000</td>
<td>0 - 0 - 2000</td>
</tr>
<tr>
<td>0.34</td>
<td>0.64</td>
</tr>
<tr>
<td>4600 - 0 - 2000</td>
<td>4600 - 0 - 2000</td>
</tr>
<tr>
<td>0.008</td>
<td>0.018</td>
</tr>
<tr>
<td>4600 - 2 - 2000</td>
<td>4600 - 2.0 - 2000</td>
</tr>
<tr>
<td>0.014</td>
<td>0.030</td>
</tr>
<tr>
<td>4600 - 4 - 2000</td>
<td>4600 - 2.5 - 2000</td>
</tr>
<tr>
<td>0.026</td>
<td>0.036</td>
</tr>
<tr>
<td>4600 - 6 - 2000</td>
<td>4600 - 3.0 - 2000</td>
</tr>
<tr>
<td>0.043</td>
<td>0.055</td>
</tr>
<tr>
<td>4600 - 2 - 2000</td>
<td>4600 - 3.5 - 2000</td>
</tr>
<tr>
<td>0.014</td>
<td>0.072</td>
</tr>
<tr>
<td>4600 - 4 - 2000</td>
<td>4600 - 4.0 - 2000</td>
</tr>
<tr>
<td>0.026</td>
<td>0.082</td>
</tr>
<tr>
<td>4600 - 5 - 2000</td>
<td>4600 - 5.0 - 2000</td>
</tr>
<tr>
<td>0.043</td>
<td>0.092</td>
</tr>
</tbody>
</table>

SF<sub>max</sub><sup>c</sup> = 0.02  
SF<sub>max</sub><sup>c</sup> = 0.07

<sup>a</sup>D<sub>1</sub> = initial dose in ergs/mm<sup>2</sup>  
<sup>b</sup>D<sub>2</sub> = challenge dose in ergs/mm<sup>2</sup>  
<sup>c</sup>t = time in hours separating D<sub>1</sub> and D<sub>2</sub>

<sup>b</sup>SF = surviving fraction for paired doses

<sup>c</sup>SF<sub>max</sub> = SF<sub>D1</sub> x SF<sub>D2</sub> = maximum surviving fraction attainable from the paired doses if the cells have completely recovered from the initial dose (D<sub>1</sub>) by the time the challenge dose (D<sub>2</sub>) is administered. When SF = SF<sub>max</sub><sup>c</sup>, the interval separating D<sub>1</sub> and D<sub>2</sub> represents the time necessary for surviving cells to effect full recovery from D<sub>1</sub>.

<sup>d</sup>The arrows indicate the approximate time at which SF = SF<sub>max</sub><sup>c</sup>. 

---

Table 2. Paired dose irradiations of *M. radiodurans* grown in PCNZ broth.
Table 3. Paired dose irradiations of *M. radiodurans* grown in TGYM broth.\(^a\)

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_1) - (t) - (D_2)</td>
<td>SF</td>
<td>(D_1) - (t) - (D_2)</td>
<td>SF</td>
</tr>
<tr>
<td>7200 - 0 - 0</td>
<td>0.21</td>
<td>8000 - 0 - 0</td>
<td>0.073</td>
</tr>
<tr>
<td>0 - 0 - 5400</td>
<td>0.37</td>
<td>0 - 0 - 6000</td>
<td>0.21</td>
</tr>
<tr>
<td>7200 - 0 - 5400</td>
<td>0.0006</td>
<td>8000 - 0 - 6000</td>
<td>0.0001</td>
</tr>
<tr>
<td>7200 - 2 - 5400</td>
<td>0.003</td>
<td>8000 - 2.0 - 6000</td>
<td>0.0006</td>
</tr>
<tr>
<td>7200 - 4 - 5400</td>
<td>0.093</td>
<td>8000 - 3.0 - 6000</td>
<td>0.0015</td>
</tr>
<tr>
<td>7200 - 6 - 5400</td>
<td>0.180</td>
<td>8000 - 3.5 - 6000</td>
<td>0.014</td>
</tr>
<tr>
<td>8000 - 4.0 - 6000</td>
<td>0.028</td>
<td>8000 - 5.0 - 6000</td>
<td>0.052</td>
</tr>
</tbody>
</table>

\(SF_{\text{max}} = 0.078\) \hspace{1cm} \(SF_{\text{max}} = 0.015\)

\(^a\)See Table 2 for definitions of terms, \(D_1\), \(D_2\), \(T\), SF, and \(SF_{\text{max}}\).
from $D_2$. For experiment 2, the interval between two and four hours was divided to more accurately focus on the recovery time. It appeared that full recovery had occurred approximately 3.5 hours after both TGYM- and PCNZ-grown *M. radiodurans* received the initial dose.

Since recovery encompasses a return to normal multiplicity, and assuming the bacterial genome is the UV-sensitive target, full recovery would require genome replication. Therefore, it was necessary to determine if the re-initiation of DNA synthesis after irradiation was compatible with a 3.5 hour full recovery time.

**Post-irradiation DNA Synthesis**

A lag in the uptake of $^3$H-TdR (Figures 6 and 7) was observed in unirradiated suspensions of PCNZ and TGYM cells. Measurable amounts of $^3$H-TdR were not found in the acid-insoluble fractions of both types of cells until 1-2 hours after placing the cells in fresh medium. The PCNZ and TGYM cells exhibited a UV-induced lag of approximately two hours, with measurable $^3$H-TdR uptake not having occurred until approximately three hours after placing the irradiated cells into a fresh medium.

In addition to the lag in $^3$H-TdR uptake, the rates of uptake were depressed in the irradiated suspensions. The relative uptake rates for the irradiated cells were calculated from the slopes of the linear
portions of the $^3$H-TdR incorporation curves for control and irradiated suspensions (Figure 6 and 7) as follows. The relative uptake rate was calculated as $\Delta$CPM for the irradiated suspension/ $\Delta$CPM for the control suspension over an equivalent time period. Relative uptake rates of 0.10 and 0.06 were calculated for PCNZ and TGYM cells, respectively.

To determine if the depressed rates of $^3$H-TdR uptake were due to a normal rate of DNA synthesis occurring only in the surviving cells or a reduced rate occurring in all cells, the post-irradiation division patterns of control and irradiated cells were followed. If all cells were capable of at least limited division after irradiation, one may assume that DNA synthesis was necessary and was therefore occurring at a reduced rate in all cells.

**Post-irradiation Cell Division**

Upon microscopic observation, approximately 90% of the colony-forming units (CFUs) plated appeared to be diplococci. Of the remaining 10%, <1% occurred as tetracocci and the other 9% appeared to be monococci.

The PCNZ-grown *M. radiodurans* receiving 4000 ergs/mm$^2$ showed a 3.0 hour UV-induced division delay in addition to the normal delay observed in non-irradiated cells (Figure 8). Within 5.6 hours after irradiation, 50% of the PCNZ CFUs had undergone one division.
Figure 6. The incorporation of $^3$H-thymidine into the trichloroacetic acid-insoluble fraction of UV-irradiated *Micrococcus radiodurans* grown in PCNZ broth. Incident dose: 4600 ergs/mm$^2$. Control (●) and irradiated (○) cells. Note difference in scale; left ordinate for non-irradiated cells is 10 times that of the right ordinate which was used to plot the uptake data for irradiated cells.
Figure 7. The incorporation of $^3$H-thymidine into the trichloroacetic acid-insoluble fraction of UV-irradiated Micrococcus radiodurans grown in TGYM broth. Incident dose: 8000 ergs/mm$^2$. Control (●) and irradiated (○) cells. Note difference in scale; left ordinate for non-irradiated cells is 10 times that of the right ordinate which was used to plot the uptake data for irradiated cells.
Figure 8. Cumulative percentages of *Micrococcus radiodurans* from PCNZ broth having undergone a single division after UV-irradiation. Incident dose: 4000 ergs/mm$^2$. Control (●) and irradiated (○) cells.
A CFU was scored as having undergone a division if one cell of the CFU had divided. After irradiation at 8000 ergs/mm$^2$, only 54% of the potential CFUs from TGYM broth divided a single time, with an irradiation-induced division lag of 3.4 hours (Figure 9). Table 4 gives the percentages of the potential CFUs from TGYM and PCNZ broths that were able to undergo single and multiple divisions following irradiation.

Table 4. Percentages of potential colony-forming units showing single and multiple divisions.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>UV-dose (ergs/mm$^2$)</th>
<th>% CFUs$^a$ undergoing 1 division$^b$</th>
<th>% CFUs resulting in micro-colony (50+ cells) formation$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 division</td>
<td>2 divisions</td>
</tr>
<tr>
<td>PCNZ</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>TGYM</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>54</td>
<td>35</td>
</tr>
</tbody>
</table>

$^a$ colony-forming units.

$^b$ this first division was later found to represent only a completion of cytokinesis.

$^c$ microcolony counts were taken 24 hours after irradiation.
Figure 9. Cumulative percentages of *Micrococcus radiodurans* from TGYM broth having undergone a single division after UV-irradiation. Incident dose: 8000 ergs/mm². Control (●) and irradiated (○) cells.
DISCUSSION

Micrococcus radiodurans grown in PCNZ medium are more sensitive to UV-irradiation than the same cells grown in the TGYM medium (see Figure 2). The survival curves do not follow the classical multi-target, single-hit type of inactivation; the initial slopes significantly differ from zero. Such a departure from theoretical expectations could result from a heterogeneous mixture of cell types that individually might show multi-target, single-hit inactivation (12), from a multi-hit type of response (12), from an interaction of saturatable recovery processes and damage processes (21), or from a combination of these and/or other factors. The complexity of the response and the limited amount of survival data precludes a complete analysis of the survival curves.

Despite the fact that the curves do not fit a classical inactivation response, the data clearly show that the apparent difference in sensitivity of the cells grown in the two media is expressed primarily as a difference in the width of the shoulder of the survival curves regardless of the shape; the final slopes of the curves do not significantly differ. The differences in shoulder width between survival curves might be attributable to any number of factors or combination of factors: for example, a difference in the multiplicity of organisms per colony-forming unit (CFU), a difference in number of sensitive sites (targets) per cell, a difference in the amount of a natural
protective or screening substance that is consumed when the cells are exposed to UV, or a difference in recovery capacity.

In a comparison of the survival curves of *M. radiodurans* grown in TGYM and PCNZ media, the difference in shoulder widths does not appear to result from a different multiplicity of organisms/CFU. If multiplicity accounted for the difference, there should be about 15 times as many TGYM cells as PCNZ cells per CFU. Microscopic examination revealed that both types of cells were plated as apparent diplococci. The apparent diplococci observed by light microscopy were later shown by electron microscopy to be made up of four distinct cells (C. Miller, personal communication).

One can reasonably assume that DNA is the target for UV-inactivation. Since the work of Krabbenhoft (31) indicates that TGYM cells contain the same amount of DNA as PCNZ cells, it is unlikely that a difference in the number of targets/cell is the basis of the difference in shoulder width.

The possibility that a difference in the amount of a consumable natural screening or protective substance might account for the difference in shoulder width is ruled out by the finding that a given incident dose of UV produces the same amount of damage; in both types of cells, 4000 ergs/mm$^2$ converts $\sim$1.5% of the thymine bases in DNA into thymine-containing dimers. Even if thymine-containing dimers are not the only, or even the primary, type of lesion responsible for
cell killing, the amount of dimerization can still serve as a kind of internal dosimeter or indicator of absorbed dose. Consideration of differences in the amount of a screening or protective substance other than a type destroyed during irradiation is apparently not necessary; differences in the amounts of a substance functioning in a filtering or energy sink capacity without being broken down during the irradiation would be exhibited as a change in the limiting slope of the survival curve. The limiting slopes obtained for TGYM and PCNZ cells do not differ significantly.

The similar levels of thymine-containing dimers found at a given dose suggests that the inherent sensitivity of the DNA of TGYM and PCNZ cells does not differ. However, this experiment does not rule out the possibility that the sensitivity of the DNA to lesion production other than pyrimidine dimers might vary. For example, a variation in sensitivity to DNA-protein cross-linking (59), or to cytosine-hydrate formation (16) might exist. Since a large body of evidence (49) implicates pyrimidine dimer formation as the major photochemical effect of UV on DNA, it is reasonable to expect that similar amounts of lethal damage are produced by similar doses given to PCNZ- and TGYM-grown cells.

Although factors such as multiplicity, target number, target sensitivity, and the presence of protective or screening substances appear to be ruled out as causing the difference in resistance between
PCNZ- and TGYM-grown *M. radiodurans*, the possibility of a difference in recovery capacity still exists. The paired dose technique was used to measure the amount of recovery occurring in both PCNZ and TGYM cells. This technique relies on the following assumptions: 1) if after a suitable time interval, survivors of a previous irradiation respond to a second dose as if no previous irradiation had occurred, the cells have completely recovered from the initial dose, and 2) during the time of recovery, the level of survival resulting from paired doses depends on the amount of radiation damage remaining from the initial dose plus the amount added by the challenge dose.

Since *M. radiodurans* occurs naturally as tetrads, the width of the survival curve shoulder is at least partially dependent on the multiplicity of organisms/CFU. At doses yielding less than ten percent survival of CFUs, it is likely that only one cell of the tetrad is viable (12). Therefore, the recovery that occurs after an initial dose that results in ten percent survival likely occurs in the one remaining potentially viable cell in the CFU. It would be desirable to study the recovery phenomenon on a single cell basis rather than on a tetrad basis. However, at present it is not known whether the survival curve for tetrads can be simply transformed to that for single cells by shifting the points a factor of four downwards as one can do with curves showing a classical multi-cell, multi-target, single-hit type of inactivation \[ S = 1 - (1 - e^{-kD})^{nN}, \] where \( S \) = survival, \( k \) = sensitivity
constant, \( D = \text{dose}, n = \text{target number}, \) and \( N = \text{multiplicity of cells/CFU} \).

For the above reasons, the kinetics of paired dose recovery in \textit{M. radiodurans} involves the return to normal radiation sensitivity -- i.e., the return to normal single cell sensitivity (unknown) plus a return to normal multiplicity. Therefore, full recovery in \textit{M. radiodurans} is defined to include repair from UV-induced damage and a return of the surviving cell(s) of a CFU to a normal multiplicity. Although multiplicity is generally intended to designate the number of cells per CFU, duplication of the sensitive target (the bacterial genome) without actual cell division would presumably be sufficient to increase the multiplicity factor.

Separation of the paired doses by increasing incubation periods yielded bimodal recovery curves (Figure 3) for both PCNZ- and TGYM-grown cells. It should be noted that no recovery occurred when the cells were held between doses in the non-nutrient environment of phosphate buffer. The initial mode of recovery occurred during the first three hours of post-irradiation incubation; the second mode occurred over a three hour period between three and six hours after the initial irradiation.

These curves indicate that during the time interval separating the paired doses, recovery was occurring. However, the increased survival could have resulted not from recovery, but from a decrease
in the sensitivity of remaining targets. If the latter were the case, the survivors of the initial irradiation would exhibit, upon subsequent irradiation, exponential inactivation with the limiting slope of the curve being less than that observed for a population which had not received the initial dose. On the other hand, if recovery were responsible for the increased survival obtained by dose separation, the survivors of the initial irradiation would exhibit, after a suitable recovery period, a survival curve shoulder in response to additional irradiation.

To determine if recovery, or a change in target sensitivity, was occurring in the cell population following UV-irradiation, complete survival curves were determined for previously irradiated populations at two time intervals after the initial dose. From these data, it is clear that recovery, and not a change in target sensitivity, was responsible for the increased survival obtained by dose separation; both PCNZ (Figure 4) and TGYM (Figure 5) cells exhibited a reappearance of the shoulder to the survival curve when two hours separated the initial irradiation from the survival curve determination. The reappearance of the shoulder was more obvious with the TGYM cells due to the greater magnitude of the normal survival curve shoulder. The shoulder widths obtained after a two hour recovery period were not of a magnitude expected for full recovery (dashed-line curves, Figures 4 and 5). Whereas the shoulder widths exceeded that predicted
for full recovery when six hours separated the survival curve determination from the initial dose.

It was apparent that full recoveries in PCNZ and TGYM cells were achieved between two and six hours after the initial irradiation; thus, doses were paired to determine a more exact recovery time. To promote an accurate determination of recovery time, it is necessary to know the inactivation response of each cell population to the doses given alone, as well as paired. These single dose survival levels are essential for calculating the maximum survival response attainable for cells which have fully recovered from the initial dose before receiving the challenge dose. Why these survival levels must be obtained for each cell population will be discussed in the section on the paired dose technique. Since single dose survival levels were not determined for the doses used to obtain the recovery curves in Figure 3, that data could not be used for an accurate recovery time determination; Figure 2 serves only to delineate the general shape of the recovery curves for PCNZ- and TGYM-grown *M. radiodurans*.

The recovery time obtained for both PCNZ and TGYM cells was approximately 3.5 hours (Tables 2 and 3) as measured by an increase in survival to a level dictated by the doses paired (see footnote Table 2). After the rapid increase in survival up to the level marking full recovery, the survival level continued to increase with increased intervals between the paired doses. The gradual increase was
probably associated with continued genome replication in excess of that required for full recovery. Genome replication beyond that necessary for a return to normal multiplicity may be also responsible for the exceedingly wide shoulders obtained for previously irradiated populations when six hours separated the survival curve determination from the initial dose (Figures 4 and 5).

Since full recovery in *M. radiodurans* requires a return to normal multiplicity, full recovery would thus require the post-irradiation resumption of DNA synthesis. Therefore, the incorporation of $^3$H-thymidine into the trichloroacetic acid-insoluble fraction of PCNZ and TGYM cells was determined as measure of post-irradiation DNA synthesis.

The resumption of DNA synthesis, as measured by $^3$H-thymidine uptake, was found to occur approximately three hours after the irradiation of both PCNZ and TGYM cells (Figures 6 and 7, respectively). However, DNA synthesis may have resumed actually prior to this time since pool effects could delay the incorporation of labeled thymidine into the DNA. Nonetheless, a three hour DNA synthesis resumption time is compatible with full recovery occurring within 3.5 hours after irradiation.

Two observations of considerable importance can be made from the data considered thus far: 1) TGYM-grown cells which had received almost twice the dose, and therefore suffered almost twice
the damage, were capable of expressing a survival level (\( \approx 0.1 \)) equal to the PCNZ-grown cells, and 2) full recovery in surviving TGYM and PCNZ cells at equivalent survival levels (\( \approx 0.1 \)) required the same amount of time, in spite of the fact that the TGYM-grown cells had suffered almost twice the damage as the PCNZ-grown cells.

Based on the first observation, it would seem logical to conclude that the TGYM cells possess a greater recovery capacity than the PCNZ cells. It is reasonable to assume that the recovery capacity of any cell would be a function of the rate at which recovery occurs and the recovery time available before the damage is fixed or expressed. Therefore, if the recovery capacity is greater in the TGYM-grown cells, it would be due to a higher recovery rate, since the TGYM cells are capable of recovering in the same amount of time from almost twice the dose and twice the damage received by PCNZ cells.

The work of Hanawalt (19) on the relationship existing between the UV sensitivity of bacteria and the DNA replication cycle is of interest in considering possible differences in the repair capacities of PCNZ and TGYM cells. Hanawalt observed that temporary blockage of re-initiation of post-irradiation DNA synthesis in Escherichia coli TAU resulted in a wide shoulder on the UV survival curve. The same organism in balanced growth exhibited an exponential inactivation without the initial shoulder; the limiting slopes of the survival curves in both instances were approximately equal. He concluded that
blocking re-initiation of DNA synthesis provided time for repair processes to function. This is supported by the observation that \textit{E. coli} Bs-1, which lacks the capacity to excise pyrimidine dimers from its DNA, did not benefit from the delay in DNA synthesis.

If repair time can influence the sensitivity of a culture, as indicated by Hanawalt (19), it is reasonable to assume that sensitivity would also be influenced by the maximum rate at which repair can occur; thus, the higher resistance of TGYM-grown \textit{M. radiodurans} to UV-irradiation could conceivably be due to a higher rate of repair.

In considering the role of pyrimidine dimer excision in the recovery of \textit{M. radiodurans}, an interesting observation can be made in regard to the recovery curves of Figure 3. These curves show that recovery, as evidenced by an increase in survival, occurs during the first 90 minutes after the initial irradiation; however, Boling and J. Setlow (3) had observed previously that \textit{M. radiodurans} from maximum stationary phase exhibit little excision of thymine-containing dimers in the 90 minutes immediately after UV-irradiation. This suggests, therefore, that the first mode of recovery (Figure 3) for this organism would occur by the repair of a lesion other than pyrimidine dimers. However, preliminary results have indicated that the stationary phase cells used for the experiments reported here are capable of dimer excision. It will be interesting to determine if there is a difference in rates of pyrimidine dimer excision between the
Additional observations can be made concerning the bimodality of the recovery curves obtained for PCNZ and TGYM cells. Since full recovery in *M. radiodurans* requires a repair of sub-lethal damage and a return to normal multiplicity, one might conclude that the first mode of survival increase is based on the repair of sub-lethal damage, and the second mode is based on genome replication resulting in an increase in the multiplicity factor. The resumption of DNA synthesis at approximately three hours after the initial irradiation would support a hypothesis that the second mode of survival resulted from genome replication. However, this does not seem to be entirely true; it seems unreasonable that the ten-fold increase in survival which occurred between the 3.0 and 3.5 hour dose separation intervals (Table 3, experiment 2) could result entirely from genome replication. Such a large survival increase based on an increase in multiplicity would require in excess of three rounds of replication occurring within 30 minutes; this seems unlikely, however, and the ten-fold survival increase occurring during this 30 minute interval of the second recovery mode probably resulted from a continued repair of sub-lethal damage in conjunction with an increase in multiplicity.

**The Paired Dose Technique**

It is desirable at this time to explore some of the hazards and
peculiarities encountered using the paired dose technique to measure recovery phenomena. An understanding of what changes in the cell population are being measured by paired doses is of value in the selection of doses to be paired and the evaluation of the data obtained.

Figure 10 shows observed and predicted survival curves for TGYM-grown M. radiodurans. Curve A is the experimentally determined inactivation of colony-forming ability of M. radiodurans by single-dose UV-irradiation. Curve B is the predicted single-dose inactivation response for the survivors of a previously irradiated population (SF = 0.07), provided suitable time has elapsed after the initial irradiation to allow for full recovery (repair of sub-lethal damage and a return to normal multiplicity).

It is obvious from Figure 8 that recovery, as measured by paired doses, is exhibited as an increase in survival obtained by the separation of two doses by an appropriate incubation period; however, a point which could cause considerable consternation to the unwary is the fact that the magnitude of the survival increase marking full recovery can be a function of the challenge dose used. For example, if a challenge dose of $2100 \text{ ergs/mm}^2$ is given immediately after an initial dose of $8000 \text{ ergs/mm}^2$, the survival level obtained would be $\sim 0.01$ (Figure 8). However, a survival level of $\sim 0.07$ is expected if sufficient time is provided between doses to allow for full recovery. Thus, full recovery from the initial dose is exhibited as a seven-fold
Figure 10. Observed and predicted survival curves for TGYM-grown Micrococcus radiodurans.

Curve A: empirically determined inactivation of colony-forming ability by single-dose UV-irradiation.

Curve B: the predicted inactivation response for a previously irradiated population (SF = 0.7) which was given sufficient time to fully recover from the initial dose.
increase in the survival level when a challenge dose of 2100 ergs/mm^2 is used. If, however, a challenge dose of 6000 ergs/mm^2 is used, the following results would be expected (from Figure 8). Application of the challenge dose immediately after the initial dose would give a survival level of ~0.00012; whereas, separation of the doses by a full recovery period would result in a survival level of ~0.024. Thus, full recovery from the 8000 ergs/mm^2 initial dose, as measured with a challenge dose of 6000 ergs/mm^2, results in a 200-fold increase in survival for the paired doses. The seven-fold survival increase obtained with a 2100 ergs/mm^2 challenge dose and the 200-fold increase obtained with the 6000 ergs/mm^2 challenge dose could be misinterpreted, at first glance, as representing vast differences in recovery capacity; while in fact, they represent the same degree of recovery.

The following precaution should be considered when determining the time necessary for full recovery. To accurately determine the recovery time, it is necessary to know the surviving fractions for the paired doses given singly, in addition to the survival obtained with the paired dose sequences. These single-dose survival levels are used to calculate the maximum surviving fraction ($SF_{\text{max}}$) which is attainable for cells which have fully recovered from the initial dose before receiving the challenge dose. The maximum surviving fraction is calculated as $SF_{\text{max}} = SF_{D1} \times SF_{D2}$ (See Results section). If the
surviving fraction for the initial dose ($D_1$) and the challenge dose ($D_2$) given singly are not obtained for each recovery time determination, but extracted from previously obtained survival curves, minor shifts in the population sensitivity can result in gross variations in the recovery times observed. The following example comes from the data reported in Table 2 for PCNZ-grown *M. radiodurans*. The survival levels obtained in experiment 2 (Table 2) for $D_1$ and $D_2$ were 0.11 and 0.64, respectively. Therefore, the $SF_{\text{max}}$ expected with full recovery occurring between doses equals $0.11 \times 0.64$, or 0.07; thus, the survival level for the paired doses equals $SF_{\text{max}}$ when ~3.5 hours separate the doses (Table 2). If the surviving fractions for $D_1$ and $D_2$ were taken from the survival curve in Figure 2 as 0.08 and 0.5, respectively, $SF_{\text{max}}$ would equal 0.04, and full recovery would be concluded as having occurred when only ~2.5 hours separated $D_1$ and $D_2$. The difference between 2.5 and 3.5 hours represents a sizable difference in recovery times. This clearly demonstrates the necessity of determining single-dose survival levels for the doses to be paired at the time of each experiment, particularly when reproducibility of culture sensitivity is difficult to achieve.

It may be possible to measure the rates at which recovery is occurring over the 3.5 hours necessary to effect full recovery in PCNZ- and TGYM-grown *M. radiodurans*. To do so will require that: 1) doses paired be of sufficient magnitude to give the largest increase
in survival as a measure of full recovery, and 2) to measure the survival increase at numerous time intervals during the 3.5 hour recovery period. As previously mentioned in the first part of the discussion, the largest increase in survival as a measure of recovery will be obtained when the initial and challenge doses are of sufficient magnitude to singly reduce the surviving fraction of the population to the exponential portion of the survival curve.

Correlation of the rate of pyrimidine dimer excision to the rate of recovery may be also possible if the recovery rate can be determined with some degree of reproducibility by paired doses. In addition, by increasing the initial dose after which recovery rates are determined, it may be possible to determine if the inactivation of repair processes occurs at high doses as proposed by Haynes (21).

The correlation of recovery rates to rates of dimer excision and the measurement of reduced recovery rates at high doses will require sensitive and reproducible determinations which may or may not be possible with the paired dose technique. In addition, for a correlation of dimer excision and recovery rates, it may be desirable to use an organism in which an increase in survival level by dose separation would not be enhanced by genome replication. Escherichia coli TAU blocked for the re-initiation of DNA synthesis would be such an organism. Under the condition of this system, increased survival obtained by dose separation would presumably be due to repair of UV-induced
lesions since no genome replication could have occurred during the interval separating the doses.

Post-Irradiation DNA Synthesis Rates and Cell Division

It is obvious from the data obtained on the resumption of DNA synthesis that the rate of synthesis is considerably lower in the irradiated populations. Relative DNA synthesis rates (irradiated to non-irradiated populations) of 0.10 and 0.06 were obtained for PCNZ and TGYM cells, respectively. The relative rates were determined from the slopes of the linear portions of the $^{3}$H-thymidine uptake curves in Figures 6 and 7 (note for Figures 6 and 7, the scale on the left ordinate for non-irradiated cells is ten times that of the right ordinate scale which was used to plot the uptake data for the irradiated cells).

The reduced rate of synthesis in the irradiated population could conceivably be due to a reduced rate of synthesis in all cells of the population (colony formers or non-colony formers) or due to a normal rate of synthesis occurring only in colony formers. At a survival level of 0.1, as measured by colony formation, only one cell of the tetrad was probably responsible for the colony formed. Therefore, at that survival level (0.1), only 0.025 of the total cell number actually survived. This would result in a relative synthesis rate of 0.025 if only the surviving cells were synthesizing DNA at a normal rate. The rates observed at a survival level of 0.1, however, were 2-4 times
higher than the 0.025 value; this indicates that it was not just the surviving cells that were responsible for the $^3$H-thymidine uptake.

The post-irradiation division patterns of irradiated PCNZ and TGYM cells were followed to determine if only colony-forming cells exhibit division activity. If, however, non-colony forming cells showed some post-irradiation cell division, it would be reasonable to assume that these cells were synthesizing DNA; this would support a hypothesis that DNA synthesis was occurring at a reduced rate in all cells.

One-hundred percent of the PCNZ colony-forming units (CFUs) and 54 percent of the TGYM CFUs appeared to have undergone a single division after irradiation to a survival level of 0.1 (Table 4). This first division was measured as the transition of an apparent diplococcus to a tri- or tetra-coccus. However, CFUs appearing as diplococci under the phase microscope have been observed by electron microscopy to have already undergone genome segregation and cross-wall synthesis for the formation of a tetrad (C. Miller, personal communication). Therefore, this first "division" was only a completion of cytokinesis and would not require DNA synthesis. The second division, which occurred in 34 and 35% percent of the originally plated PCNZ and TGYM colony-forming units, respectively, may have required DNA synthesis. With only 13 and 14 percent of the originally plated PCNZ and TGYM CFUs, respectively, able to form
microcolonies, the remaining 21 percent which had undergone the second division, but not microcolony formation, may be responsible for the higher than expected rates of DNA synthesis in the irradiated cells.

Neither the data obtained for the relative rates of DNA synthesis (irradiated/non-irradiated), nor the number of CFUs exhibiting cell division are sufficient to prove whether after irradiation, all cells are synthesizing DNA at a reduced rate, or only surviving cells are synthesizing DNA at a normal rate. These questions could probably be better answered using auto-radiographic techniques to visualize what percentage of the CFUs are taking up $^{3}$H-thymidine after irradiation.

It is interesting to observe from the data given in Figures 8 and 9 that the PCNZ- and TGYM-grown cells exhibit 3.0 and 3.5 hour UV-induced division delays, respectively, after irradiation to a survival level of $\sim$0.1. As previously mentioned, this first division from an apparent diplococcus to an apparent tri- or tetra-coccus seems to involve only cytokinetic processes; CFUs appearing as diplococci under the phase microscope have already undergone genome segregation and extensive cross-wall synthesis for formation of the tetrad. Therefore, this UV-induced division delay represents a sensitivity of cytokinetic processes to UV-irradiation. In addition, with only 54 percent of the TGYM cells completing cytokinesis after receiving an
incident dose of 8000 ergs/mm\(^2\) (Table 4) it is obvious that the cytokinetic processes are permanently inhibited at high doses.

**Induced UV Sensitivity in PCNZ-Grown Micrococcus radiodurans**

The factors involved in the reduction of pigmentation and radiation resistance of *M. radiodurans* when grown in PCNZ medium have not been elucidated. The Bacto-tryptone of the TGYM medium and the N-Z-Case of the PCNZ medium are both pancreatic digests of casein. The absence of DL-methionine and the presence of a higher concentration of yeast extract in the PCNZ medium have been shown not to be responsible for the lower resistance to \(\gamma\)-irradiation (31). The sensitivity of *M. radiodurans* to \(\gamma\)-irradiation was related to the concentration of N-Z-Case in the medium over a range of 0.01 to 0.5 percent; this suggests the N-Z-Case might also be responsible for the increased sensitivity seen in UV-irradiated cells.

The transfer of *M. radiodurans* from TGYM to the PCNZ medium may be comparable to a shift-up experiment in which a culture in balanced growth in a minimal medium is placed in an enriched medium. In this case, the PCNZ medium with its double concentration of casein digest would be the enriched medium. A shift-up is characterized by an increase in growth rate and a decrease in generation time. The generation time for *M. radiodurans* decreased from 50 to 38 minutes when transferred from TGYM to the PCNZ medium.
(30). During this shift, a change in concentration of constituents of various pathways could reduce pigmentation and repair capacity.

When *M. radiodurans* had been transferred in PCNZ broth several times, the reduced sensitivity to UV-irradiation began to decrease until the cells reached a level of resistance comparable to those cells maintained continuously in TGYM broth. For this reason, cultures which had been transferred in PCNZ broth over seven times were discarded and a fresh culture started from a TGYM broth culture.

It is possible that the N-Z-Case contains some unique property which is responsible for the decrease in pigmentation and radiation resistance. However, it would be interesting to determine if a shift from a TGYM broth to the same broth containing an additional 0.5 percent Bacto-tryptone would result in a reduction of radiation resistance. If a reduction occurs, it could be attributed to a shift into a richer medium containing twice the concentration of casein digest, and not a peculiar property of the N-Z-Case.
SUMMARY

The fortification of a growth medium for *Micrococcus radiodurans* with N-Z-Case (a pancreatic digest of casein) resulted in a decrease in pigmentation and resistance to ultraviolet-irradiation. The decreased resistance was exhibited on the survival curve as a reduction in the extrapolation number from \( \sim 300 \) to \( \sim 20 \) with little change in the slope of the exponential portion of the curve.

The possibility that the decreased UV resistance resulted from a decrease in a protective or screening substance was ruled out by measuring the UV-induced conversion of thymine into thymine-containing dimers. Resistant and sensitive cells exposed to the same incident dose contained equivalent percentages of thymine-containing dimers.

Recovery, defined as the repair of sub-lethal damage and a return to normal multiplicity, was determined using paired dose studies. A bimodal recovery curve was obtained for both resistant and sensitive populations irradiated to the same survival level.

The difference in the UV inactivation response expressed by the resistant and sensitive cultures was concluded to result from differences in recovery rates; full recovery, in both cell populations irradiated to equivalent survival levels, occurred in the same amount of time, even though the resistant cells had received approximately twice the dose and had suffered almost twice the damage as the sensitive
cells.

A UV-induced lag in the completion of cytokinesis, with permanent inhibition at higher doses, was observed in *M. radiodurans*. 
BIBLIOGRAPHY


