Chemical protection and reactivation of ultraviolet irradiated Shigella sonnei

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The University of Montana

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CHEMICAL PROTECTION AND REACTIVATION OF
ULTRAVIOLET IRRADIATED SHIGELLA SONNEI

by

MARIUS GLEN PEACOCK

A. B., MONTANA STATE UNIVERSITY, 1959

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1961

Approved by:

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Chairman, Board of Examiners

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Dean, Graduate School

MAY 8 1961

Date
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M. G. P.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Acknowledgments</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. STATEMENT OF THE PROBLEM</td>
<td>12</td>
</tr>
<tr>
<td>III. METHODS AND MATERIALS</td>
<td>13</td>
</tr>
<tr>
<td>IV. RESULTS</td>
<td>26</td>
</tr>
<tr>
<td>V. DISCUSSION AND CONCLUSIONS</td>
<td>60</td>
</tr>
<tr>
<td>VI. SUMMARY</td>
<td>69</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>70</td>
</tr>
<tr>
<td>AUTOBIOGRAPHY</td>
<td>75</td>
</tr>
<tr>
<td>TABLE</td>
<td>Title</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>I.</td>
<td>Composition of Erlandson and Mackey Synthetic Medium</td>
</tr>
<tr>
<td>II.</td>
<td>Comparison of the Susceptibility of Four Strains of <em>Shigella sonnei</em> to Ultraviolet Light</td>
</tr>
<tr>
<td>III.</td>
<td>Growth of <em>Shigella sonnei</em> (Strain CDC-5044-59) in the Synthetic Medium of Erlandson and Mackey</td>
</tr>
<tr>
<td>IV.</td>
<td>Compounds Which Protected <em>Shigella sonnei</em> from the Destructive Action of Ultraviolet Light</td>
</tr>
<tr>
<td>V.</td>
<td>Effect of Various Compounds on the Reactivation of <em>Shigella sonnei</em> after Exposure to Ultraviolet Light</td>
</tr>
<tr>
<td>VI.</td>
<td>Comparative Effects of Ultraviolet Light on &quot;Stock&quot; Cultures and &quot;Resistant&quot; Cultures of <em>Shigella sonnei</em></td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Depreciation Curve of Typical Low Pressure Ultraviolet Sources</td>
<td>18</td>
</tr>
<tr>
<td>2. Ultraviolet Killing Curves for Four Strains of <em>Shigella sonnei</em></td>
<td>28</td>
</tr>
<tr>
<td>3. Inactivation of <em>S. sonnei</em> Strain CDC-5044-59 by Ultraviolet Irradiation (in Saline)</td>
<td>29</td>
</tr>
<tr>
<td>4. Ultraviolet Killing Curves for Five Cell Concentrations of Strain CDC-5044-59 in Buffer</td>
<td>31</td>
</tr>
<tr>
<td>5. Effect of Size of Inoculum on the Growth of <em>S. sonnei</em> in Synthetic Medium</td>
<td>32</td>
</tr>
<tr>
<td>6. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Adenylc Acid</td>
<td>36</td>
</tr>
<tr>
<td>7. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Guanylic Acid</td>
<td>37</td>
</tr>
<tr>
<td>8. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Uridylc Acid</td>
<td>38</td>
</tr>
<tr>
<td>9. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Uridine</td>
<td>39</td>
</tr>
<tr>
<td>10. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Uracil</td>
<td>40</td>
</tr>
<tr>
<td>11. Protection of <em>S. sonnei</em> against the Lethal Action of Ultraviolet Light by Adenine</td>
<td>41</td>
</tr>
<tr>
<td>12. Protection of <em>S. sonnei</em> against the Lethal Action</td>
<td>41</td>
</tr>
</tbody>
</table>
of Ultraviolet Light by Cysteine ............... 42
13. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Sodium Thioglycollate .. 43
14. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Glutathione ............. 44
15. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Nicotinic Acid .......... 45
16. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Coenzyme I .......... 46
17. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Catalase ............... 47
18. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Adenosinetriphosphate .. 48
19. Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Penicillin G .......... 49
20. Restoration of Viability of Ultraviolet-inactivated S. sonnei by Adenylic Acid, Guanylic Acid and Uridylic Acid ........................................ 53
21. Restoration of Viability of Ultraviolet-inactivated S. sonnei by Adenine, Uridine and Uracil ...... 54
22. Restoration of Viability of Ultraviolet-inactivated S. sonnei by Glutathione, Sodium Thioglycollate and Cysteine HCl ........................................ 55
23. Restoration of Viability of Ultraviolet-inactivated S. sonnei by Coenzyme I, Niacin and Ascorbic
24. Restoration of Viability of Ultraviolet-inactivated S. sonnei by Adenosinetriphosphate, Catalase and Penicillin G
CHAPTER I

INTRODUCTION

One of the first laboratory observations of the effects of ultraviolet radiation on bacteria was published by Downes and Blunt in 1877. Since that time, a tremendous amount of research has been performed in order to understand the effects of radiation on microorganisms. Despite the extensive investigations performed during the past eighty-three years, our knowledge of the mechanism of action of ultraviolet radiation is not complete. Various aspects of ultraviolet radiation have been reviewed by Rahn (1930), Duggar (1936), Ellis (1941), Geise (1945, 1947, 1950), Latarjet (1946), Lea (1940, 1947), Grey (1947), Loofbourow (1948), Mitchell (1951), Errera (1953), Kelner et al. (1955), Zelle and Hollaender (1955) and Jagger (1958).

To our knowledge, no work has been published on the effects of ultraviolet radiation on the enteric pathogen, Shigella sonnei. As a matter of fact, very little research on the effects of ultraviolet light has been performed with the Shigellae. Sharp (1938) determined the least incident energy (ergs/mm²) necessary to kill 90% of several common pathogenic bacteria. The following organisms were tested: Escherichia coli, Streptococcus viridans, Streptococcus hemoyticus, Staphylococcus aureus, Staphylococcus albus, Shigella paradysenteriae, Serratia marcescens, Corynebacterium diphtheria and Bacillus anthracis. Shigella paradysenteriae required the least incident energy (168 ergs/mm²) for 90% killing action whereas B. anthracis required the most (452 ergs/mm²). Latarjet (1943) compared the primary actions of X-rays and ultraviolet
on the "paradysentery bacillus Y6R". No difference was found in the survival of the bacteria when irradiated with the ionizing and the non-ionizing radiation sources. The dose which gave a survival of 50% was 225 ergs/mm². Mukherji (1952) studied the effects of ultraviolet irradiation on the staining properties of gram-negative bacteria. He found that Shigella dysenteriae was rendered gram positive after one to two hours irradiation.

Any information concerning the effects of ultraviolet irradiation on Shigella sonnei should be of interest in view of the meager knowledge available concerning the effect of this physical agent on this organism. In order to be useful, a study of this type should encompass more than just the "killing" effect of ultraviolet light on S. sonnei. Some other aspects of the effects of ultraviolet light were also included in the present study, namely, studies on chemical protection and reactivation. Attempts were also made to isolate a mutant which was more resistant to ultraviolet radiation than the parent strain.

The mechanism of ultraviolet inactivation of cells is not clear and even controversial at this time. Henri (1914) postulated that the absorption of ultraviolet light by the nucleus was a primary cause of inactivation. He postulated and later showed that sublethal doses of ultraviolet light induced mutations. Crowther (1924, 1926) applied the target theory in explaining inhibition of mitosis in tissue culture cells observed by Strangeways and Oakley (1923). Gate (1928) associated adsorption of ultraviolet light by deoxyribonucleic acid (DNA) derivatives with the inactivation of microorganisms. He termed this phenomenon the "action spectrum" or relative effectiveness of different wave lengths of mono-
chromatic ultraviolet light. In essence, the action spectrum technique concerns the study of the relative effectiveness of different wave lengths which parallel the absorption spectrum of the biologically important molecules and hence furnishes evidence concerning the cellular components affected.

Loofbourow (1948) enlarged upon the action spectrum concept. The theoretical basis underlying the action-spectrum technique showed the following assumptions to be inherent:

1. The biological effect observed is, on the average, attributable to photochemical changes in a given number of molecules of an essential substance.

2. The quantum efficiency of the photochemical process is independent of wave length within the region studied.

3. The attenuation of the intensity of radiation before reaching the sensitive substance is either independent of wave lengths or so small in magnitude as to be ignored.

4. The relative absorption of suspected sensitive substances in the cell as a function of wave length either can be estimated with sufficient accuracy or can be assumed to be equivalent to the relative extinction coefficients of the suspected substances.

5. The reciprocity law is valid for the time of irradiation in

1. Beer's law of extinction coefficient, \( \alpha \), is defined by the following equation, in which \((m)\) is the concentration of the absorbent in moles/l, \((l)\) is the length of the light path in centimeters, and \((I_0)(I_{tr})\) are the intensities, respectively of the incident and transmitted light:

\[
I_{tr} = I_0 \times \alpha^m l
\]

2. Bunsen-Roscie reciprocity law states that the effect of exposure to radiation is a function of the total energy and is independent of intensity and time.

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the experiment. Zelle and Hollaender (1955) state that the reciprocity law holds for the inactivation of bacteria by ultraviolet radiation, if all other factors are held constant.

Rahn (1930) and Lea (1947) suggested that the inactivation of bacteria by both ionizing and ultraviolet radiation was due to the production of lethal mutations. Observations which support this theory are reviewed by Zelle (1955). The exponential killing curve indicated that unitary action or single event produced the effect. Independence of intensity and increasing dose required for inactivation with increasing ionization density, indicate the unit event to be a single ionization. The lethal effect appears, as Rahn pointed out, most logically to be a chemical change in a gene, because a change in a single gene could be lethal to the cell. There are numerous observations and exceptions which Zelle points out in his review. Zelle states that a number of mechanisms producing a number of different lethal effects will be found which may vary in relative importance in different bacterial strains, especially with regard to ultraviolet irradiation.

Heinmets et al. (1954, 1955) present a contrary point of view. They point out that such a view (single target theory) omits completely the general biological effects upon the cell and considers the cell to be essentially a particle which may survive or die. They suggest and present experimental data to show that ultraviolet radiations produce injury at many sites within the cell and that measurable changes occur in cellular metabolism and synthesis. Most of their work dealt with the tricarboxylic acid cycle and related processes. Oginsky et al. (1959) using long-wavelength ultraviolet radiation (350 to 400 mu) on E. coli
and *S. aureus* strains observed survival curves which suggested a process involving multiple events.

Other workers have postulated that the formation of toxic products such as ozone, peroxides and other compounds are responsible for the effects of ultraviolet light. Loofbourow (1943) found that the doses required to produce an appreciable degree of toxicity were far beyond those utilized in the bactericidal tests by direct irradiation of the organism. This toxic factor was significant in early experiments when organisms were irradiated on complex media and was considerably reduced when cells were irradiated in a suspension medium of buffer or saline. Wyss *et al.* (1948) failed to demonstrate an increase in mutation rate by direct treatment of bacteria with hydrogen peroxide, although positive results were obtained by Wagner *et al.* (1950) working with *Neurospora*. Hollaender and Zelle (1955) found that the mutation rate was increased when cells were grown in ultraviolet-irradiated or hydrogen peroxide-treated substrates. They indicated that it was difficult to assess properly the significance of these results as they relate to the induction of mutagenic effects by direct irradiation of the cells with ultraviolet. The wave lengths effective in producing mutagenic compounds in irradiated substrates lie mainly below 2,000 Å and are not present in the spectrum of the widely used germicidal lamps with glass envelopes or in monochromatic beams of ultraviolet radiation of the wave lengths most efficient in producing mutations by direct irradiation.

The literature search of chemical substances used in chemical protection and chemical reactivation studies indicated that a variety of compounds were active and many were not homologous agents.
Leif and Hebert (1960) used two ultraviolet absorbing compounds, urocanic acid and diplocolinic acid, and found that they could protect *Serratia marcescens* and *B. globigii* against ultraviolet radiation. They found greater protection when the organism was exposed in a liquid medium than in an aerosol.

Fuld, Procter and Goldblith (1958) found that sodium ascorbate protected in the order of 99.9% over controls in *Clostridium* and *Bacillus* spores. However, there was no protection of *B. stearothermophilus* spores with this compound. They suggested that the protection was due to the ability of sodium ascorbate to absorb light at 2550 Å.

Schoenborn (1956) tested many compounds for their ability to protect *Astasia longa* against ultraviolet irradiation. Sodium pyruvate, glutathione, potassium cyanide, dextrose, sucrose, 3 nucleic acid derivatives, 11 B-vitamins and 22 amino acids were tested for their ability to protect against ultraviolet light. Compounds which protected (when added to the cell suspensions just prior to irradiation) were: cysteine, phenylalanine, tryptophane, tyrosine, adenine, cytidylic acid, uracil and sodium pyruvate. None of these compounds protected when added after irradiation. Except for phenylalanine, solutions of these compounds also protected when used as filters between the ultraviolet source and the cell suspensions. Cysteine failed to protect when added just before irradiation, but caused good protection when irradiated cells were grown in its presence.

Wittingham and Stauffer (1956) found that *Penicillium chrysogenum* spores could be protected against the lethal effects of ultraviolet light by sodium azide, sodium cyanide, sodium fluoride, sodium nitrate,
sodium thiosulfate and atmospheric nitrogen. They postulated that the protection was due to oxygen depletion at critical sites in the cell, or to a repression of formation of organic peroxides.

Wainwright and Nevill (1955) protected Streptomyces T12 spores against loss of photoreactivation by treating spores with iodoacetate for periods of up to 6.5 hours before photoreactivation.

Thompson et al. (1951) were able to protect Bacillus anthracis, Escherichia coli and Micrococcus aureus from the mutagenic and lethal effects of X-ray, ultraviolet light and hydrogen peroxide with 0.5% sodium pyruvate.

Several investigators have found increased sensitivity to ultraviolet irradiation in the presence of certain compounds. Oginsky et al. (1959) found that there was a lethal action on long ultraviolet light on Staphylococcus aureus and Escherichia coli in the presence of 8-methoxypsoralen. Berger et al. (1952) showed that sodium azide could reduce the sensitivity of Chromobacterium violaceum, ATCC strain 7461, Escherichia coli and Micrococcus aureus to ultraviolet radiation. The degree of photoreactivation was also reduced by the compound.

Heinmets et al. (1953, 1954, 1955) have done some very careful and significant work on the chemical reactivation of ultraviolet inactivated Escherichia coli. Ultraviolet irradiated E. coli (B/r) was incubated in buffer with varying concentrations of metabolites and cofactors of the tricarboxylic acid cycle. They observed intensive restoration of cell viability in the presence of certain metabolites. In another paper they found alanine (among the amino acids) and several reducing agents to also be effective. They suggested that suppression of the
tricarboxylic acid cycle may be a significant cause of ultraviolet inactivation of microorganisms.

Ogg, Adler and Zelle (1956) found that the addition of hemin, a cytochrome system, and catalase to certain strains of \textit{E. coli} increased the survival rate following exposure to ultraviolet light. Ellison et al. (1954) found that sodium acetate reversed the effect of ultraviolet light; the degree of protection on \textit{E. coli} was directly proportional to the acetate concentration, up to 25 mg per ml.

When bacteria are exposed to radiation in either the high energy or the ultraviolet range the most prominent effect is the apparent killing of a percentage of the cells, the fraction killed being a function of the absorbed energy. The usual criterion for survival is the ability of the bacteria to form a colony visible to the eye when incubated following plating on ordinary culture media. There are many variable factors which will influence the "killing effect" of ultraviolet light on bacteria.

Roberts and Aldous (1949) showed a change in the type of survival curve (sigmoidal or exponential inactivation curve) by plating \textit{E. coli} on a synthetic medium instead of a complete medium. Harm and Stein (1952) showed a change by incubating the plates at 45°C after irradiation. Weatherwax (1956) obtained a change from an exponential to a sigmoidal type of inactivation curve by plating \textit{E. coli} (strain B) at pH 5.0 instead of at pH 8.0.

The effects of these variable factors such as temperature, pH of suspending medium, stage of growth, moisture content, enzymatic constitution, genetic constitution, post-irradiation treatment, etc. have
been reviewed by Zelle and Hollaender (1955).

Roberts and Aldous (1949) made careful studies before and after ultraviolet irradiation of various experimental conditions which affected the survival of *E. coli* (strain B). The shapes and slopes of the survival curves were markedly influenced by different conditions.

Zelle and Hollaender (1955) concluded that exponential or sigmoidal curves may be obtained following ultraviolet radiation, depending on the strain of bacteria and the technique of irradiation including precautions against clumping in the preparation of the bacteria to be irradiated.

Therefore, it appears to be imperative for the investigator to state in every detail the conditions under which an experiment is carried out.

In order to analyze the issue of cellular reactivation, it is desirable to discuss briefly the mode of biological action of ultraviolet irradiation. Heinmets and Lehman (1955) listed some factors that interfere with or excessively modify molecular organization and reactions that can be considered in the biological sense as injurious to or destructive to the cell. Some of the following changes may take place:

(a) inactivation and alteration of enzymes
(b) modification of organizers for synthetic processes
(c) alteration and decomposition of metabolites, intermediates or cofactors
(d) loss or modification of genetic elements

The molecular alteration produced within a cell may be reversible or irreversible. Ultraviolet irradiation produces both reversible and irreversible types of reactions in a cell (Heinmets et al. 1955). With
slight injury, there may be a prolonged lag phase, but after that, multiplication may proceed normally. Under conditions of greater injury, physiological disorganization may be rather extensive, and the cell cannot initiate synthetic and metabolic processes.

The principle of chemical reactivation is to add the necessary metabolites in proper concentrations to the normal culture medium. Then the cell is capable of performing synthetic processes again and viability is restored. Other experimental evidence in the literature supporting this view is that of Peacock and Hinshelwood (1948), Ellison et al. (1955) and Errera (1953).

The last phase of the present research involved an attempt to isolate a mutant which was more resistant to ultraviolet irradiation than the parent strain. A review of the literature for accounts of this type of mutant showed that their occurrence or the publications regarding their occurrence was quite rare. Witkin (1947) isolated a radiation-resistant mutant (B/r) from E. coli, strain B. The cultures derived from this mutant were characterized by considerably greater resistance to ultraviolet light than the corresponding control cultures. The resistant B/r strain was carried throughout 50 successive subcultures in broth, and for a period of two years on agar. During this time no change was observed in sensitivity of this strain to ultraviolet light. Differences were noted in growth rates between the two strains in the lag phase. The generation time was the same for both strains. Strain B/r had increased in resistance to penicillin and sodium sulfathiazole. Morse and Carter (1949) and Morse (1950) showed that E. coli B/r contained 3-4 times more DNA per cell than other E. coli strains. This
observation probably explains the resistance of this strain to ultra-violet radiation.
CHAPTER II

STATEMENT OF THE PROBLEM

Although the effects of ultraviolet irradiation on many organisms have been extensively studied, very little is known regarding the effect of this nonionizing radiation on *Shigella sonnei*.

This investigation is a study of some of the fundamental effects of ultraviolet irradiation on *S. sonnei* as well as the protection and reactivation of ultraviolet irradiated *S. sonnei* with various chemical compounds.
CHAPTER III

METHODS AND MATERIALS

I. General Methods and Materials

(1) Strains of Shigella sonnei employed

Four strains of *S. sonnei* were used throughout the course of this investigation. The major strain used (CDC-5044-59) was obtained from Dr. W. H. Ewing, Communicable Disease Center, Atlanta, Georgia. One strain was received from Dr. H. M. Gezon, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, which was isolated in April of 1960 from a patient named Leigh Halliwell. Strain # 21879-60 was received from Dr. G. G. Coffin, University of Maryland School of Medicine, Baltimore, Maryland. A fourth strain, "Formal Strain", was obtained from Dr. Arthur Abrams, Walter Reed Army Institute of Research, Washington, D. C. This strain was a transplant of strain # 13-3, a form I strain which was isolated overseas in 1943 and sent to the Walter Reed Army Hospital for serological identification.

(2) Culture media and methods employed

Two different culture media were used in this study. The synthetic medium of Erlandson and Mackey (1957) (See Table I) was used to grow cells for experiments and to maintain stock cultures. This medium was also employed in certain phases of this investigation to quantitate bacterial growth.

After irradiation of the bacterial suspensions, the number of surviving organisms was determined by serial dilution in M/15 phosphate buffer (pH 7.0), followed by "hockey-stick" plating 0.1 ml of dilution
**TABLE I**

**COMPOSITION OF ERLANDSON AND MACKEY SYNTHETIC MEDIUM FOR THE CULTIVATION OF SHIGELLA SONNEI**

<table>
<thead>
<tr>
<th>Substance</th>
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</tr>
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<tbody>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (Reagent Grade)</td>
<td>9.118 gm</td>
</tr>
<tr>
<td>K$_2$HPO$_4$ (Reagent Grade)</td>
<td>11.67 gm</td>
</tr>
<tr>
<td>NaCl (Reagent Grade)</td>
<td>8.5 gm</td>
</tr>
<tr>
<td>MgSO$_4$·7 H$_2$O (Reagent Grade)</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>Thiamin HCl (Nutritional Biochemical Corp.)</td>
<td>0.3 gm</td>
</tr>
<tr>
<td>Niacin (Merk, U. S. P.)</td>
<td>0.01 gm</td>
</tr>
<tr>
<td>Aspartic Acid (Nutritional Biochemical Corp.)</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>Glucose (Reagent Grade)</td>
<td>10.0 gm</td>
</tr>
</tbody>
</table>

pH adjusted to 7.0 with concentrated NaOH (50%) or concentrated H$_3$PO$_4$. The medium was dispensed into test tubes (1½ by 15 cm) in 10.0 ml amounts per tube. The tubes were capped with Morton stainless steel caps and autoclaved at 15 pounds pressure for 10-15 minutes.
samples on nutrient agar plates (1.5% agar).

(3) **Cell preparations**

A 24-hour culture of *S. sonnei* (CDC-5044-59) grown in the synthetic medium was washed three times with phosphate buffer (pH 7.0). The washed cells were resuspended in the buffer and adjusted to an optical density of 0.16 at a wavelength setting of 575 nm (Bausch and Lomb "Spectronic 20" colorimeter), resulting in a suspension containing about $1 \times 10^8$ colony-forming organisms per ml. One ml of this buffer cell suspension was used to inoculate ten ml of culture medium. It was experimentally found that the middle of the logarithmic growth stage was attained after 20-22 hour old cultures were used in the majority of experiments conducted.

Cell suspensions for chemical protection and chemical reactivation studies were prepared in the following way: the organisms were grown in the liquid synthetic medium, harvested after 20 hours, and washed 3 times by centrifugation with M/15 phosphate buffer (pH 7.0). Cells were resuspended in the buffer to produce an optical density of 0.16 (70% Transmittance). Five ml of this bacterial suspension was placed in special flat bottom pyrex petri dishes (antibiotic assay petri dishes). This sample gave an approximate depth of 0.5 mm in these petri dishes. If a chemical was to be used, the desired concentration of the chemical (in bu...r) was used to prepare the cell suspensions.

(4) **Counting Method**

The serial dilution method using M/15 phosphate buffer as diluent was used throughout this investigation. Pipettes were used only once. The error in viable count, which might result if pipettes were used for
more than one dilution might be considerable.

One-tenth ml of the desired dilution was pipetted onto the surface of nutrient agar plates. Bent glass rods (3.7 mm x 160 mm) in the shape of hockey-sticks were used to spread the aliquot over the entire surface of the agar. If the surface of the agar plates was too moist, the plates were inverted and incubated at 37 C for 24 hours. The number of colonies was counted (on the Quebec colony counter) and the number of viable organisms per ml in the original suspension was calculated.

(5) Ultraviolet Source and Dose Calculations

The light providing the 2537 Å radiation was a 15 watt, General Electric "germicidal" lamp, model #G 15T8. This lamp emits an intensity of 0.0038 watts/ft². (Calculations of intensity are based on graphs and information given in chapter 2, Radiation Biology, vol. 2: 41-94. Edited by A. Hollaender. McGraw-Hill Book Co., Inc., New York.)

\[
0.30 \text{ u.v. watts/ft}^2 = 300 \text{ milliwatts/ft}^2
\]

\[
\frac{\text{milliwatts/ft}^2}{0.929} = 322.9 \text{ microwatts/cm}^2
\]

\[
300 \text{ milliwatts/ft}^2 = 322.9 \text{ microwatts/cm}^2
\]

\[
\text{u.v. microwatts} = \text{ergs/sec}
\]

\[
\frac{322.9 \text{ microwatts/cm}^2}{0.1} = 3229 \text{ ergs/sec/cm}^2
\]

or 32.3 ergs/sec/mm²

To calculate the dose (Intensity x time) of ultraviolet irradiation imposed on a cell suspension 30 cm from the center of a General Electric #G 15T8, germicidal lamp, the intensity factor, 32.3 ergs/sec/mm² was multiplied by the number of seconds of exposure. This method of calculating the cumulative dose of ultraviolet radiation was used throughout
Bactericidal low-pressure ultraviolet bulbs depreciate rapidly during the first 100 hours of operation. In figure 1, the approximate depreciation of low-pressure sources is shown.

The lamp used in these experiments was estimated to have had approximately 1000 hours of operation before experiments were begun. However, there would be a relatively small amount of change in ultraviolet killing action even with extensive depreciation, since the ultraviolet killing action is an exponential rather than linear function of ultraviolet intensity (Buttolph, 1955).

(6) Irradiation Procedure

The aluminum foil-covered petri dishes were placed 30 cm from the center of the ultraviolet lamp. A twenty minute equilibrium period prior to irradiation was carried out to stabilize the emitted radiation of the lamp. The petri dishes were continuously agitated with a "home-made" shaker (frequency of oscillation, 84 one inch-strokes per minute) unless otherwise noted. For irradiation, the foil and regular top of the petri dishes were removed. Irradiation and sampling were carried out in the ultraviolet hood with nearly all visible light blocked out. All plating operations were conducted in the darkened laboratory, or in the dark with the aid of a 15 watt, 115-125 volt "Eagle" darkroom light with a yellow filter.

II. Experimental Methods and Materials

(1) Effect of ultraviolet light on Shigella sonnei

The CDC-5044-59 strain of S. sonnei was used in this study. Bacterial cell suspensions were prepared from 14- and 20-hour cultures.
The cells were washed and resuspended in 0.85% saline and in a phosphate buffer. In all experiments a 1/100 dilution of a cell suspension with an optical density of 0.0132 (97 % T) was used. This suspension resulted in approximately $1 \times 10^4$ to $1 \times 10^6$ colony-forming organisms per ml. Five ml of this cell suspension was irradiated in special petri dishes accompanied by shaking. Samples (0.1 ml) were removed at various time intervals, diluted and plated. Colonies visible to the eye, after incubation in the dark at 37 C for 24 hours, were taken as the criterion for survival. Controls for this experiment consisted of a growth control and a condition control. 0.1 ml of the unirradiated cell suspensions which were diluted and plated served as the growth controls. The condition controls consisted of diluting and plating 0.1 ml of a five ml sample of the prepared cell suspension, which were subjected to all the conditions of the experiment (i.e., shaking, time, etc.) except radiation. The conditions of the experiment which might possibly affect the survival of the bacteria were: agitation, the time interval between preparation of the cell suspensions and the final dilution and plating procedures, temperature of the ultraviolet hood, leakage of ultraviolet light, changes in pH, and the possible effects of an ozone atmosphere in the hood.

(2) Effect of ultraviolet light on various populations of S. sonnei

The effect of ultraviolet irradiation on various populations of S. sonnei (CDC-5044-59 strain) was determined using the same procedure as in (1). Thrice washed cells were resuspended in buffer and adjusted to obtain an O.D. (at 575 mu) of 0.46, 0.30, 0.19, 0.097, 0.022 and 0.0132 or a % transmittancy of 35, 50, 65, 80, 95, and 97, respectively.
Five ml of these various cell suspensions were irradiated under identical conditions. The % survivors were determined using the following formula:

\[
\% \text{ survivors} = \frac{N_d}{N_0} \times 100
\]

\(N_d\) is the number of survivors after the u.v. irradiation (Dark Survivors)
\(N_0\) is the number of viable cells before irradiation (Condition Control)

(3) Effect of ultraviolet light on saline and phosphate buffer and its subsequent effect on S. sonnei

It was necessary to determine whether the "killing" effect (loss of colony-forming ability) which the ultraviolet light produces is due to the direct effect of ultraviolet light on S. sonnei cells or due to some ultraviolet induced toxicity in the suspending medium.

Twelve- and 20-hour cultures were washed and resuspended in saline and/or buffer to obtain an optical density of 0.027 and 0.022. The growth controls were prepared by adding 1 ml of this suspension to 5 ml of saline, then diluting and plating. The ultraviolet treated experiment consisted of adding 1 ml of the cell suspension to 5 ml of saline or buffer which had been irradiated for five minutes. One ml of the 20-hour suspension was added to 5 ml of saline and irradiated for a 10-minute period in order to adequately allow interaction of cells and any possible toxic materials. A condition control consisted of 1 ml of the cell suspensions added to the unirradiated saline or buffer. After the period of agitation, dilutions and plating operations were carried out in the darkened laboratory. The ability of the cells to form colonies when exposed to ultraviolet-irradiated or unirradiated medium was compared. The cumulative dose of ultraviolet energy used
to irradiate the suspending medium was greater than that used in other experiments.

(4) Effect of inoculum size and growth of S. sonnei in the synthetic medium

In maintaining stock cultures of S. sonnei (CDC-5044-59) on the synthetic medium of Erlandson and Mackey (1957), it was found that a large inoculum was required to give a large number of viable cells within 24 hours. Erlandson and Mackey found that small inocula of S. flexneri produced growth which was initially slow, but the same total growth was eventually reached (after prolonged incubation) with inocula of various sizes. They stated that the results were similar with S. sonnei, however, no quantitative measurements were made for this strain. We thought that the effects of size of inoculum of strain CDC-5044-59 should be determined precisely. For example, it would be important to know the effects of various chemicals on the organism at different phases of the growth curve. It would also be important to use cells obtained from identical stages in the logarithmic growth phase throughout the chemical protection and reactivation experiments in order to compare results.

A 24-hour culture of strain CDC-5044-59 was washed three times with 0.85% NaCl. The washed cells were resuspended to obtain an O.D. of .52, .30, .16 and .046. One ml of these various cell suspensions was used as inocula in 10 ml of the media (in duplicate). Growth and rate of growth of the Shigella were determined by changed in the optical density of the cultures (Bausch and Lomb, "Spectronic-20", colorimeter). Samples were plated and growth was determined at three different inter-
vals during 32 hours of incubation at 37 C.

(5) Chemical protection of ultraviolet-irradiated S. sonnei

Six nucleic acid derivatives, two sulfhydryl compounds, a vitamin, several coenzymes, an enzyme and an antibiotic were tested for their ability to protect strain CDC-5044-59 from the "killing" effect of ultraviolet radiation. The cell suspensions were prepared in the desired concentration of the test substance (dissolved in buffer). The cell suspensions were irradiated with a cumulative dose of 484.5, 969, 1,453.5 and 1,938 ergs/mm². The controls for these experiments consisted of cell suspensions not containing the test chemical. These suspensions were exposed to ultraviolet light under conditions identical to the experiments of protection (described earlier). A second control was used to measure any changes in growth caused by the test chemicals added. This control was prepared by treatment of 5 ml of the chemical-buffer-cell suspension preparation to all conditions of the experiment except irradiation. Dilutions and plating operations were carried out as usual.

(6) Chemical reactivation of strain (CDC-5044-59) from the "killing" effects of ultraviolet radiation

The method of Heinmets et al. (1955) was followed closely in this phase of the research. Twenty-hour old cells from cultures prepared as described earlier, were resuspended to obtain an O.D. of 0.16. Five ml aliquots of the bacterial suspensions were irradiated in the special flat-bottomed petri dishes, during continuous agitation. After 15 and 30 seconds of irradiation, 0.1 ml samples were transferred to 9.9 ml M/15 phosphate buffer blanks. After 24 hours of incubation
at 37°C in buffer containing the various test substances. Samples were plated, and colony counts made after 24 hours of incubation.

Multiplication of the bacteria in buffer and also the effects of the chemical substrates on the bacteria were controlled by transferring 0.1 ml aliquots of unirradiated cell suspensions to the buffered substrate and observing colony counts after 24 hours of incubation.

Using this method (incubation in buffer for 24 hours), the multiplication of the bacteria was suppressed or kept at such a low level that it did not interfere with the observations of reactivation.

The general criterion for cellular recovery was the difference in the number of viable cells in the untreated and treated irradiated samples.

(7) Sensitivity of different strains of S. sonnei to ultraviolet irradiation

The "Gezon", "Formal", and # 21879-60 strains of S. sonnei were compared to the CDC-5044-59 strain for their sensitivity to ultraviolet radiation. The cell suspensions were prepared as described earlier. Twenty-hour cultures of the three strains were washed (3 times) with buffer and resuspended in buffer to obtain an O.D. of 0.16. The irradiation procedures previously described were used in this assay.

It was assumed that the organisms were in the middle of the logarithmic growth stage as in the case of strain CDC-5044-59. Growth curve studies in the synthetic media were not done with these strains. Controls used in this assay were the same as those used in establishing the killing curve for strain CDC-5044-59.

(8) Attempts to produce increased resistance against ultraviolet light in S. sonnei
Several different methods were used in the attempts to isolate a mutant of the parent strain CDC-5044-59 which was more resistant to ultraviolet irradiation.

The first method attempted was that used by Witkin (1947) in which she isolated the well known mutant, *Escherichia coli* B/r. In essence her method and results were as follows:

1. Approximately $5 \times 10^4$ washed cells of strain B were plated on a nutrient agar plate surface. The surface of the agar was irradiated with a ultraviolet dose of 1000 ergs/mm$^2$.

2. The four surviving colonies were inoculated into tubes of nutrient broth; at the same time, four control cultures were started by inoculating into broth, single colonies from a nonirradiated plate.

3. The sensitivity of the two sets of cultures were compared.

The cultures derived from the four survivors of the originally irradiated plate were characterized by considerably greater resistance to ultraviolet light than the corresponding control cultures (named strain B/r, strain B resistant to radiation). Witkin also compared growth rates at 37°C and susceptibility to penicillin and sodium sulfathiazole. Differences were noted in growth rates between the two strains in the lag phase. The B/r strain had a lag phase of 1 hour compared to 90 minutes for strain B. The generation time was the same, however. The B/r strain had developed an increase in resistance to penicillin and sodium sulfathiazole.

The method of Witkin was duplicated using the CDC-5044-59 strain in the present study.

A second method attempted was the irradiation of 5 ml of a cell
suspension of *S. sonnei* (O.D., 0.16) for 30 seconds. One ml of this
irradiated cell suspension was used to inoculate 10 ml of medium. The
culture was incubated at 37 C for 24-48 hours. The culture was centri-
fuged; the cells were washed 3 times with buffer and again resuspended
in buffer to obtain an O.D. of 0.16. This procedure was carried out
with increasing doses until growth no longer occurred after 48 hours
of incubation. Survival curves were determined after each increasing
dose and compared to the original killing curve. This method was
used in an attempt to produce a "step-wise" or "trained" mutant in
contrast to a "single-step" mutant.
CHAPTER IV

RESULTS

(1) Effect of ultraviolet irradiation on Shigella sonnei

Ultraviolet irradiation (wavelength of 2537 Å) of suspensions of S. sonnei strains in buffer resulted in rapid death of the cells as measured by loss of colony-forming ability. Results are recorded in Table II and figures 2 and 3. The incident energy necessary under the conditions of these experiments to produce 100% inhibition of colony formation for the four strains was as follows: strain CDC-5044-59, 1292 ergs/mm²; strain # 21879-60, 2261 ergs/mm²; "Gezon" strain, 1615 ergs/mm²; and the "Formal" strain, 1292 ergs/mm². The initial irradiation experiments with cell suspensions in saline showed little or no change from suspensions in buffer as far as the killing effect of ultraviolet irradiation was concerned.

The four strains showed varied susceptibility to ultraviolet irradiation, especially in the first 30 seconds of irradiation. The CDC-5044-59 strain showed a decreased sensitivity to the ultraviolet irradiation as compared to the other three strains tested. The # 21879-60 and the "Gezon" strains showed almost identical susceptibility to ultraviolet light with the exception that the # 21879-60 strain cell suspensions contained viable cells after 60 seconds (1938 ergs/mm²) of irradiation and the "Gezon" strain cell suspensions did not contain viable cells after 50 seconds (1615 ergs/mm²) of irradiation. The "Formal" strain cell suspensions showed the maximum sensitivity to the
TABLE II

Comparison of the susceptibility of four strains of *S. sonnei* to ultraviolet light (Average of 3 experiments)

<table>
<thead>
<tr>
<th>Strains Employed</th>
<th>Dose in ergs per mm²</th>
<th>% Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nd x 100 (Nd-dark survivors)</td>
</tr>
<tr>
<td>GDC-5044-59</td>
<td>0</td>
<td>100.0000</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>35.7600</td>
</tr>
<tr>
<td></td>
<td>646</td>
<td>6.0600</td>
</tr>
<tr>
<td></td>
<td>969</td>
<td>1.0300</td>
</tr>
<tr>
<td></td>
<td>1,292</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>1,615</td>
<td>0.0000</td>
</tr>
<tr>
<td># 21879-60</td>
<td>0</td>
<td>100.0000</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>3.7100</td>
</tr>
<tr>
<td></td>
<td>646</td>
<td>0.8030</td>
</tr>
<tr>
<td></td>
<td>969</td>
<td>0.0430</td>
</tr>
<tr>
<td></td>
<td>1,292</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>1,615</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
<td>1,938</td>
<td>0.0004</td>
</tr>
<tr>
<td>&quot;Gezon&quot;</td>
<td>0</td>
<td>100.0000</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>1.3900</td>
</tr>
<tr>
<td></td>
<td>646</td>
<td>0.8900</td>
</tr>
<tr>
<td></td>
<td>969</td>
<td>0.0310</td>
</tr>
<tr>
<td></td>
<td>1,292</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>1,615</td>
<td>0.0000</td>
</tr>
<tr>
<td>&quot;Formal&quot;</td>
<td>0</td>
<td>100.0000</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>0.8700</td>
</tr>
<tr>
<td></td>
<td>969</td>
<td>0.0770</td>
</tr>
<tr>
<td></td>
<td>1,292</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>1,615</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

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Ultra violet killing curves for four strains of Shigella sonnei.
Inactivation of S. sonnei strain CDC-5044-59 by ultraviolet irradiation (in saline).

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ultraviolet irradiation of the four strains tested.

(2) **The effect of ultraviolet irradiated saline and phosphate buffer upon the growth of Shigella sonnei**

Saline and phosphate buffer irradiated up to ten minutes (19,380 ergs/mm²) and allowed to interact with added washed cells for **ten minutes**, showed no decrease in viability when treated in this way. The cumulative dose of ultraviolet energy used to irradiate the saline or buffer suspending medium was greater than that used in other experiments. Therefore, any "killing effect" upon *S. sonnei* cells irradiated in these two suspending media was due to absorption of the ultraviolet energy by the cells or parts therein and not due to ultraviolet induced toxicity of the suspending medium.

(3) **Effect of ultraviolet light on various populations of *S. sonnei***

In figure 4 the "killing curves" of various populations of *S. sonnei* (CDC-5044-59) are compared. An ultraviolet dose of approximately 300 ergs/mm² separated the incident energy necessary for 99% inactivation for the five cell suspensions assayed. 775, 600, 575 and 480 ergs/mm², respectively, was required to inactivate 99% of the cells in suspensions containing $9.5 \times 10^8$, $1.4 \times 10^8$, $2.1 \times 10^7$, $4.0 \times 10^8$ and $4.4 \times 10^7$ cells per ml. Because of the limitations of the method used in performing the assay, no further assays were attempted for greater accuracy.

(4) **Effect of size of inoculum on the growth of *S. sonnei* in synthetic medium (Erlandson and Mackey)**

The rate of growth of strain CDC-5044-59 using different sizes of inocula is compared in figure 5. There was a considerable lag phase in the growth of strain CDC-5044-59 in the medium using smaller inocula.
Ultraviolet Killing Curves for Five Cell Concentrations of Strain CDC-5044-59 in Buffer

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FIGURE 5

Effect of Size of Inoculum on the Growth of S. cerevisiae in Phyto-liquid
The smaller inocula (2.8 x 10^2 cells per ml of culture) gave rise to a lag phase of about six times that of the larger inocula (2.2 x 10^6 cells per ml of culture). Growth with smaller inocula was initially slow, but the same total growth was eventually reached with all inocula. At the end of 32 hours, approximately 4.0 x 10^8 viable cells per ml of culture were obtained from all cultures. Table III lists the viable cell count per ml of culture after 8- and 32-hours incubation in the synthetic medium.

(5) Studies on the protection of S. sonnei (CDC-5044-59) against the lethal effects of ultraviolet light

A series of protection studies has shown that many of the compounds tested were able to reduce the destructive activity of the ultraviolet irradiation. Data from these studies are listed in Table IV and figures 6 through 19. Table IV lists the approximate number of viable cells per ml for each concentration after 60 seconds of irradiation (1,938 ergs/mm²). In all assays in the protection studies, the cells in control suspensions were inactivated after 40 seconds irradiation (1,292 ergs/mm²). The concentration of cells in the control suspensions before irradiation was approximately 1 x 10^9 cells per ml. Compounds tested which gave little or no protection were the sulfdryl compounds: cysteine hydrochloride, sodium thioglycollate and glutathione. The reducing properties of these compounds in the concentrations used did not exert any toxic effect on the growth of control suspensions under the conditions of the assays. The graphic representation of these assays are listed in figures 12, 13 and 14.

The addition of the enzyme catalase has been found to increase
TABLE III

Growth of *Shigella sonnei* (strain CDC-5044-59) in the synthetic medium of Erlandson and Mackey

<table>
<thead>
<tr>
<th>Optical Density of inocula (at 575 mu)</th>
<th>Viable Count of inocula (no./ml)</th>
<th>Optical Density after 8-hours of incubation</th>
<th>Viable Count after 8-hours of incubation</th>
<th>Optical Density after 32-hours of incubation</th>
<th>Viable Count after 32-hours of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>$2.2 \times 10^6$</td>
<td>0.82</td>
<td>$3.9 \times 10^8$</td>
<td>1.13</td>
<td>$3.5 \times 10^8$</td>
</tr>
<tr>
<td>0.07</td>
<td>$1.2 \times 10^6$</td>
<td>0.55</td>
<td>$1.1 \times 10^8$</td>
<td>1.19</td>
<td>$4.4 \times 10^8$</td>
</tr>
<tr>
<td>0.046</td>
<td>$8.0 \times 10^4$</td>
<td>0.69</td>
<td>$8.1 \times 10^4$</td>
<td>1.20</td>
<td>$3.9 \times 10^8$</td>
</tr>
<tr>
<td>0.022</td>
<td>$2.8 \times 10^3$</td>
<td>0.28</td>
<td>$2.8 \times 10^3$</td>
<td>1.15</td>
<td>$4.3 \times 10^8$</td>
</tr>
</tbody>
</table>
TABLE IV
Compounds which protected (or partially protected) Shigella sonnei (strain CDC-5044-59) from the destructive action of ultraviolet light.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrations used</th>
<th>Viable count (no per ml after 60 sec of irradiation*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenylc acid</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 10^6</td>
</tr>
<tr>
<td>guanylic acid</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 10^7</td>
</tr>
<tr>
<td>uridylic acid</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 10^7</td>
</tr>
<tr>
<td>uridine</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 10^7</td>
</tr>
<tr>
<td>uracil</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 0, 10^4</td>
</tr>
<tr>
<td>adenine</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 10^7</td>
</tr>
<tr>
<td>cysteine HCl</td>
<td>0.1, 0.5, 1.0 mg/ml</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>sodium thiglycollate</td>
<td>0.5, 1, 2 mg/ml</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>glutathione</td>
<td>0.5, 1, 2 mg/ml</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^7, 10^6</td>
</tr>
<tr>
<td>coenzyme I</td>
<td>10,100,1000 mcg/ml</td>
<td>10^3, 10^5, 10^7</td>
</tr>
<tr>
<td>catalase</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 0, 0</td>
</tr>
<tr>
<td>adenosine triphosphate</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^5, 10^7</td>
</tr>
<tr>
<td>penicillin &quot;G&quot;</td>
<td>10,100,1000 mcg/ml</td>
<td>0, 10^3, 0</td>
</tr>
</tbody>
</table>

* The approximate number of viable cells per ml are given for each concentration after 60 seconds of irradiation. The number of cells before irradiation was approximately 10^9 cells per ml. In all assays the ultraviolet irradiation destroyed all cells in the control suspensions after 40 seconds irradiation.
Protection of *S. sonnei* against the Lethal Action of Ultraviolet light by Guanylic Acid
FIGURE 8

Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Uridylic Acid
Protection of *S. sonnei* against the Lethal Action of Ultraviolet Light by Uridine
Protection of *S. sonnei* against the Lethal Action of Ultraviolet Light by Uracil
FIGURE 11

Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Adenine
FIGURE 13

Protection of S. pyogenes against the Lethal Action of Ultraviolet Light by Sodium Thioglycollate
FIGURE 14

Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Glutathione
FIGURE 15
Protection of B. subtilis against the lethal action of ultraviolet light by nicotinic acid.

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Protection of S. sonnei against the Lethal Action of Ultraviolet Light by Coenzyme I
FIGURE 17

Protection of *S. sonnei* against the Lethal Action of Ultraviolet Light by Catalase
Protection of *S. sonnei* against the Lethal Action of Ultraviolet Light by Adenosine Triphosphate
FIGURE 19

Protection of *S. coelic* against the Lethal Action of Ultraviolet Light by Penicillin G
the survival of *E. coli* (Monod *et al.*, 1949; Latarjet *et al.*, 1954; Miletic, 1954; Teti, 1954). In the present assays performed with catalase, no protection against the inactivation effects of ultraviolet irradiation on *S. sonnei* was observed. The significance of this observation will be discussed later.

The nucleotides (adenylic acid, guanylic acid, uridylic acid), nucleoside (uridine), purine base (adenine), nicotinic acid, coenzyme I, and adenosinetriphosphate protected *S. sonnei* against ultraviolet irradiation as compared to controls. The degree of protection with this group of compounds after 60 seconds of irradiation in terms of present survival (% survival = Dark survivors x 100) was small; less than 1% in most of the cases. It is significant, however, that after 60 seconds of ultraviolet irradiation under the conditions of the experiment; these compounds protected the cell suspensions to the extent that approximately $1 \times 10^7$ cells per ml of a total of approximately $1 \times 10^9$ cells per ml remained viable, while control suspensions showed no viable cells after 40 seconds of ultraviolet radiation.

The other compounds assayed which produced less protection than the above compounds, but still showed some protection were: penicillin G and cysteine hydrochloride.

(6) *Studies on the chemical reactivation of S. sonnei (CDC-5044-59) inactivated by ultraviolet light*

In the preceding portion of this thesis, the compounds to be tested for protective action were added before irradiation. In this study, aliquots of irradiated cell suspensions were added to the buffered chemical solutions, incubated for 24 hours and then assayed...
for viability. The concentration of the chemicals giving maximum protection in the prior protection studies was used in the reactivation study.

No attempt was made to perform systematic studies on the reactivation study because this would have required extensive experimental facilities which were not available. However, several experiments were performed in order to obtain some initial information on the subject: Table V represents such data, and it can be seen that some compounds were more effective in increasing viable cell counts than others. Of the compounds tested, niacin, ascorbic acid and sodium thioglycollate produced effects on the viability of the cells when incubated with normal unirradiated cells. Figures 20 through 24 give a graphic representation of the data presented in Table V.

Adenosinetriphosphate, catalase, sodium thioglycollate, glutathione and adenylic acid caused reactivation in magnitudes of approximately two log numbers of cells greater than buffer alone and approximately one log number of cells greater than the other compounds assayed. Of interest and perhaps of significance, are the differences noted in the magnitude of reactivation produced by the nucleotides. Adenylic acid and uridylic acid increased the viability of the irradiated cell suspensions by approximately two log numbers, whereas with guanylic acid there was only slight reactivation.

(7) Attempts to produce increased resistance to ultraviolet irradiation in S. sonnei

All attempts to isolate a mutant of strain CDC-5044-59 which possessed permanent increased resistance to ultraviolet irradiation
TABLE V

Effect of various compounds on the reactivation of *Shigella sonnei* exposed to ultraviolet light (969 ergs/sq.mm), subsequently incubated for 24 hours at 37 C, and than plated for cell counts.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Concentration</th>
<th>Viable Count (no./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td>1.0 x 10^3</td>
</tr>
<tr>
<td>Adenyllic acid</td>
<td>1,000 mcg/ml</td>
<td>3.0 x 10^5</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>1,000 mcg/ml</td>
<td>4.0 x 10^3</td>
</tr>
<tr>
<td>Uridyllic acid</td>
<td>1,000 mcg/ml</td>
<td>8.0 x 10^4</td>
</tr>
<tr>
<td>Uridine</td>
<td>1,000 mcg/ml</td>
<td>1.8 x 10^5</td>
</tr>
<tr>
<td>Adenine</td>
<td>1,000 mcg/ml</td>
<td>5.3 x 10^3</td>
</tr>
<tr>
<td>Uracil</td>
<td>1,000 mcg/ml</td>
<td>2.3 x 10^5</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.5 mgm/ml</td>
<td>5.0 x 10^5</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>1.0 mgm/ml</td>
<td>0</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>2.0 mgm/ml</td>
<td>2.6 x 10^5</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>100 mcg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Niacin</td>
<td>100 mcg/ml</td>
<td>0</td>
</tr>
<tr>
<td>Coenzyme I</td>
<td>1,000 mcg/ml</td>
<td>1.0 x 10^3</td>
</tr>
<tr>
<td>Catalase</td>
<td>10 mcg/ml</td>
<td>5.9 x 10^5</td>
</tr>
<tr>
<td>Adenosinetriphosphate</td>
<td>1,000 mcg/ml</td>
<td>6.2 x 10^5</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10 mcg/ml</td>
<td>6.0 x 10^3</td>
</tr>
</tbody>
</table>

(l mgm = 1435 units)
Restoration of Viability of Ultraviolet-inactivated *S. sonnei* by Adenylic Acid, Guanylic Acid, and Uridylic Acid
FIGURE 21

Restoration of Viability of Ultraviolet-inactivated S. sonnei by Adenine, Uridine, and Uracil
FIGURE 22

Restoration of Viability of Ultraviolet-inactivated *S. sonnei* by Glutathione, Sodium Thioglycollate and Cysteine HCl
Figure 24

Restoration of Viability of Ultraviolet-inactivated S. sonnei by Adenosine triphosphate, Catalase and Penicillin G
were unsuccessful. Approximately $5 \times 10^4$ washed cells were plated on nutrient agar plates. The surface of the agar was irradiated with an ultraviolet dose of 1,292 ergs/mm$^2$. Surviving colonies were inoculated into nutrient broth, and synthetic medium. Control cultures were started by inoculating nutrient broth and synthetic medium from a non-irradiated plate.

Of the 60 colonies isolated from numerous irradiated plate surfaces, several colonies possessed increased resistance as compared to control cultures. But these cultures maintained this resistance only through three to four transfers on nutrient agar slants or the liquid synthetic medium. Table VI shows the order of resistance through the first transfer. After the fourth daily transfer, there was no significant differences in resistance of the cultures.

The method used in attempting to produce a "trained" mutant could not be used because of the inherent ability of the synthetic medium to reactivate the irradiated cell suspensions used as inoculum. When 0.1 ml of a 5 ml cell suspension ($1 \times 10^8$ cells/ml), irradiated with a cumulative dose of 9,690 ergs/mm$^2$, was used to inoculate 9.9 ml of the synthetic medium, growth resulted in 20 out of 20 cultures repeatedly. When this inoculum was spread on nutrient agar plates, no growth resulted. Cell suspensions from these cultures showed no increased resistance to ultraviolet irradiation compared to the control cell suspensions.
TABLE VI

Comparative effects of ultraviolet light on "stock" cultures and "resistant" cultures of *S. sonnei*

<table>
<thead>
<tr>
<th>Cumulative u.v. Dose ergs/mm²</th>
<th>Viable cells/ml &quot;control&quot;</th>
<th>Viable cells/ml &quot;mutant&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$8.5 \times 10^7$</td>
<td>$1.0 \times 10^8$</td>
</tr>
<tr>
<td>323</td>
<td>$7.9 \times 10^5$</td>
<td>$2.1 \times 10^6$</td>
</tr>
<tr>
<td>646</td>
<td>$4.4 \times 10^5$</td>
<td>$1.8 \times 10^6$</td>
</tr>
<tr>
<td>969</td>
<td>$2.9 \times 10^3$</td>
<td>$8.9 \times 10^4$</td>
</tr>
<tr>
<td>1292</td>
<td>0</td>
<td>$3.1 \times 10^4$</td>
</tr>
<tr>
<td>1615</td>
<td>0</td>
<td>$2.9 \times 10^3$</td>
</tr>
<tr>
<td>1938</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
When an organism is "killed" by ultraviolet light, a whole spectrum of effects is probably produced. Bacteria and other microorganisms are the organisms of choice for such studies. Bacteria are single-celled, and their metabolic and genetic behavior can be readily studied. They are easy to manipulate because we can work with populations of millions of individuals, of which a majority can be affected by radiation in a short time, and the results of an experiment can usually be observed the day after exposure to irradiation.

However, the size of the bacterial cell presents a problem and limits the methods by which vital information can be obtained concerning the mechanism of destruction, region of the cell that has been damaged, and how this damage can be protected against or be repaired by chemical and physical agents. The cells are so small that we can see very little of their physical structure in the living state. The question of nuclear or cytoplasmic injury cannot be resolved with much certainty. If it were possible to irradiate a certain part of a bacterial cell and observe the visual and physiological effects of the irradiation, more valuable information could be obtained.

It became apparent that *Shigella sonnei* was easily inactivated by ultraviolet light (2,537 Å). Leif et al. (1960) compared the ultraviolet dose necessary to inactivate *S. aureus* under three different experimental conditions. The ultraviolet dose necessary to kill 99% of a cell suspension in an aerosolized state, on a nutrient agar
surface, and a thin film of liquid suspension, was 200, 1,000 and 2,750 ergs/mm$^2$, respectively.

It is impractical to compare results of inactivation experiments unless the experimental methods are performed identically. Much of the earlier work was done by irradiating cells spread upon an agar surface. More recent work has been done with aerosolized or liquid suspensions. The shape and slope of the survival curve are markedly influenced by different experimental conditions. Conditions such as temperature, pH, stage of growth, sensitivity of vegetative cells and spores, moisture content, enzymatic constitution, genetic constitution, etc., all influence the ultraviolet dose required for inactivation.

A comparison of the sensitivity of the four different strains of S. sonnei can be made. Survival curves obtained with the four strains showed exponential killing up to 99% inactivation. The CDC-5044-59 strain showed a lag in the ultraviolet inactivation during the first 10 seconds of irradiation. Two times the ultraviolet dose necessary to inactivate 99% of the "Formal" strain was necessary to inactivate 99% of the CDC-5044-59 strain. Witkin (1947) observed an exponential survival curve with E. coli B and a sigmoidal survival curve with E. coli B/r. Furthermore, three times the ultraviolet dose was required to inactivate 99% of the E. coli B/r population as compared to a similar E. coli B population. Witkin inferred from these results that within the framework of the target theory, the mutant E. coli B/r required multiple hits to cause lethality and that E. coli B required only a single hit.

It may be possible to explain the differences in the sensitivity
of the 4 strains of *S. sonnei* assayed to be a difference in genetic make up of the strains. No experimental proof of this is set forth or inferred.

The ultraviolet dose required to inactivate 99% of a population of \(9.5 \times 10^8\) cells/ml was approximately twice that necessary to inactivate \(2.1 \times 10^7\) cells/ml. The ultraviolet dose necessary for inactivation of the cells was not proportional to the number of cells present in the suspensions. This observation might be explained in several ways. There may have been clumping of some of the cells in the suspension. Some of the cells within the "clumps" retained their viability and were protected by outer cells absorbing the ultraviolet energy. A second explanation might concern the depth of penetration of ultraviolet irradiation. Although 90% absorption of the germicidal energy may require 10 feet distilled water, only a few thousands of an inch of milk or serum have the same capacity. (General Electric Technical Publication, LS-179, May, 1960, p. 12). It is conceivable that cells adhering to the bottom of the petri dishes under a 0.5 mm depth of phosphate buffer and bacterial cells may not receive sufficient germicidal energy to prevent cellular division or colony formation.

Since Witkin's experiment, many effects have been found that will change the form of the inactivation curve of *E. coli* B to approximate the curve of strain B/r. Roberts and Aldous (1949) showed such a change by plating the bacteria on synthetic media instead of on complete media. Anderson (1951) found such a change after photoreactivation of strain B. Harm and Stein (1952) showed this change by incubating the plates at 45 C after irradiation. Weatherwax (1956) obtained such
a change by plating strain B at pH 5.0 instead of pH 8.0.

In all, 15 chemicals were tested for their ability to protect *S. sonnei* cells against the lethal action of ultraviolet light. Our choice of the various chemicals was influenced in part by reports in the literature as to the effectiveness of these agents in protecting other organisms against ionizing and nonionizing radiations.

The first group of agents tested for protective action included nucleotides, a nucleoside, a pyrimidine base and a purine base. The nucleotides, adenylic acid and guanylic acid contain a purine base, ribose and phosphoric acid. Uridylic acid contains a pyrimidine base (uracil), ribose, and phosphoric acid. The nucleoside, uridine, contains a pyrimidine base (uracil) and ribose. Uracil and adenine contain only the pyrimidine and purine base respectively.

Referring back to Table IV it is interesting to note that the nucleotides, the nucleoside and the purine base gave a type of protection which was similar in magnitude and increased as their concentrations increased. But the pyrimidine base, uracil, did not follow this pattern. Sinsheimer and Hastings (1950) have found that irradiation with ultraviolet light for 15 hours at a pH of 7.0 caused a 63% loss in the maximum absorption of uracil.

Protection of *S. sonnei* cells with nucleic acid derivatives appears to be due to physical absorption of the germicidal energy by the nucleic acid derivatives. The ultraviolet absorption spectrum of nucleic acids and polynucleotides is characterized by a strong absorption maximum at about 2,600 Å, a minimum at about 2,300 Å, and continuously rising "end" absorption at below 2,300 Å (Loofbourow,
1940; Schlenk, 1949). Apparently, the molecules of these compounds coat the cells and absorb the energy. As their concentration increases, their efficiency as protecting agents increased, but complete protection could not be produced with the concentrations used.

The second group of compounds, the sulfhydryl compounds, did not show protection of S. sonnei cells. Cysteine hydrochloride, sodium thioglycollate and glutathione all contain the sulfhydryl radical (—SH) and are good reducing agents. If toxic oxidation products were formed in the suspensions, a reducing agent should neutralize these products. It should be stated again that those compounds were in contact with the cells during the irradiation procedures and then diluted out in the plating procedures. The time interval for these manipulations did not exceed 20 minutes. It is possible that these reducing molecules were not absorbed onto the cells where toxic free radicals and organic peroxides would be most effective. Jagger and Stapleton (1957) state that the killing action is enhanced in the presence of oxygen for X-rays, but not for ultraviolet rays. Errera, (1953) postulated that ultraviolet light produces toxic free radicals and organic peroxides inside the cells. Hollaender and Stapleton (1953) found that sulfhydryl compounds, i.e., cysteine, mercaptosuccinate, 2,3-dimercaptopropanol (BAL), and 2-(2-mercaptoethoxy)-ethanol protected cells against X-rays. No preincubation period at 37 C was required for maximum protection with the sulfhydryl compounds. They observed survival ratios up to 500 times those of the controls of E. coli B/r.

A single molecule of catalase can catalyze the degradation of
more than 5,000,000 molecules of hydrogen peroxide per minute (Pfeiffer, 1955). If toxic oxidation products are a factor in ultraviolet irradiation inactivation of phosphate buffer cell suspensions of S. sonnei, catalase in concentrations of 10,100 and 1000 mcg/ml did not protect against these toxic products. Conceivably, the catalase molecules may not have been adsorbed onto the cell before being diluted to a concentration at which they were ineffective.

Adenosinetriphosphate, coenzyme I and nicotinic acid produce a maximum absorption spectra at approximately 2,580 Å. The degree of protection afforded by these compounds approached that of the nucleic acid derivatives. The mechanism of protection is probably one of physical absorption of the ultraviolet energy, since this compound is a nucleotide.

Ascorbic acid in neutral solution has a strong absorption band at 2,650 Å, which shifts to 2,450 Å in acid solutions (Morton, 1942). As seen in Table IV, ascorbic acid did not protect against ultraviolet irradiation. This compound apparently does not absorb the germicidal waves of low pressure ultraviolet sources. Penicillin G showed slight protection against 60 seconds of ultraviolet irradiation in a concentration of 100 mcg/ml. Penicillin G (benzylpenicillin) has the structural formula C₆H₅CH₂CONH(C₇H₉ONS)COOH. The sodium salt of penicillin G is more stable in aqueous solution. It is doubtful that this protection is due to absorption of the ultraviolet energy by the penicillin molecules.

The work of Heinmets and Taylor (1951) showed that in the presence of oxygen, ultraviolet damage to frozen cells is essentially the
same as that for cells in the liquid state. Irradiation of cells (E. coli B) in the absence of oxygen (nitrogen atmosphere) showed 30% of the inactivation effect required the presence of oxygen. Jagger and Stapleton (1957) stated that the other 70% of the effect is due to other mechanisms. They also stated that 90% of the ultraviolet damage can be reversed or prevented. Puleston et al. (1957) showed that ultraviolet-irradiated S. faecalis R could utilize pyrimidines for growth. Cell suspensions of the streptococcus were irradiated for 30 minutes at a distance of 20 cm. Aliquots of these cells (in basal medium containing concentrations of pyrimidines varying from 0 to 333 micrograms/ml) were incubated at 30°C for 16 hours. They showed turbimetrically that growth of the suspensions containing 333 mcg/ml of the pyrimidines equalled unirradiated controls. It would have been interesting if they compared the "colony-forming-ability" of the control suspensions and the irradiated-pyrimidine suspensions.

In the reactivation studies the maximum reactivation produced by any compound was only a two log number increase (100 fold) of cells over the buffer control. Unirradiated cell suspensions produced an increase of five log number of cells compared to the buffer controls.

The nucleic acid derivatives showed a similar degree of reactivation. But the sulfhydryl compounds varied in their activity. Glutathione and sodium thioglycollate were able to repair some ultraviolet damage. It is not conceivable that any uninjured cells would be able to use these compounds for growth. The mechanism of reactivation of these compounds is probably the neutralization of toxic oxidative
products within the cells before injury could take place. These suspensions were incubated at 37°C for 24 hours. Molecules of the compounds should have been adsorbed onto the cells under these conditions.

It appeared that cysteine hydrochloride became toxic or inhibitory to the cells after irradiation injury. Sodium thioglycollate showed some inhibition of the control. But this inhibition was not produced in the irradiated cells. In fact, sodium thioglycollate produced a "restoration" to irradiation injury, although somewhat inhibitory in the buffer control.

Ascorbic acid and niacin in the concentrations used did not have any "restoration" effect. These two compounds also showed inhibition in unirradiated controls. The inability of these compounds to restore viability differs from that of cysteine hydrochloride. Ascorbic acid and niacin were toxic in the controls and this toxicity apparently carried over during the incubation period.

Coenzyme I and penicillin G did not show any restoration effect. Catalase and adenosinetriphosphate probably represent the two different types of reactivation. The catalase may have neutralized the toxic intracellular or extracellular oxidative products produced by ultraviolet irradiation, whereas, adenosinetriphosphate may have provided the high-energy bonds necessary to repair cellular depolymerization and other injury.

Information from these preliminary experiments would suggest that if there are two types of reactivation, i.e., one in which toxic oxidation products are neutralized and one in which the energy source
is provided to repair injury, than there should be approximately twice the reactivation when catalase and adenosinetriphosphate are both added. Heinmets et al. (1953, 1954, 1955) have shown that there is considerable reactivation of E. coli B/r when various combinations of the tricarboxylic acid cycle intermediates, coenzymes, and enzyme precursors were added to ultraviolet-irradiated cells in buffer.
CHAPTER VI

SUMMARY

1. The "killing" action of ultraviolet light on 4 strains of Shigella sonnei was studied.

2. Methods and procedures for protection and reactivation assays are discussed.

3. Nucleotides, a nucleoside, a pyrimidine base, a purine base, several sulfhydryl compounds, a vitamin, a growth factor, several coenzymes, an enzyme and an antibiotic were assayed for their ability to protect Shigella sonnei against the lethal effects of ultraviolet irradiation (2,537 Å).

4. These compounds were also assayed for their ability to restore the viability of ultraviolet-inactivated Shigella sonnei.

5. Attempts to isolate a mutant of Shigella sonnei which was more resistant to ultraviolet light met with failure.

6. The significance of the findings is discussed.
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