PHOTOREACTIVATION STUDIES ON AZOTOBACTER VINELANDII ATCC 12837

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PHOTOACTIVATION STUDIES ON AZOTOBACTER VINELANDII ATCC 12837

THESIS

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CHAPTER I

INTRODUCTION

Photoactivation Defined

Photoactivation refers to a phenomenon in which alterations, produced in biological systems by ultraviolet light, may be repaired or reversed by an exposure of the biological system to light of a wavelength which is greater than that of the damaging radiation. Ultraviolet light produces numerous effects in biological systems. The most important effects are morphological changes, delays in the onset of cell division, decreases in vigor, increase in mutation rates, and increases in cell death rates (22). It is doubtful that any single one of the above effects occurs separately. It is more probable that a combination of the above effects occurs when cells are exposed to ultraviolet light. Ultraviolet light is the only type of radiation which produces lesions in biological systems which can be photoreactivated by light of greater wavelength. Because of their ionizing properties, electromagnetic radiation with wavelengths smaller than the wavelength of ultraviolet light produces extensive damage to the cell and hence no photoreactivation. On the other hand, electromagnetic radiations of very large wavelengths have little or no effect of any kind on biological systems. The range of photoreactivating wavelengths, therefore, lies between 3150 and 5100 A (22).
There is a certain degree of specificity in various organisms for photoreactivating wavelengths which provide maximum response, but apparently no distinction is made with regard to interrelationships between photoreactivation light wavelength and type of ultraviolet light damage.

A search of the literature revealed several other terms which have been applied to the same phenomenon. Photoreactivation, photoreversal, and photorecovery are among the terms referred to in other reports (22). There has been a tendency in recent years to use only the term photoreactivation.

The general process for determining whether or not photoreactivation is possible in a given organism consists of four steps. The first step involves the exposure of the organism to a damaging dose of ultraviolet light. The second step involved in demonstrating photoreactivation requires the incubation of an aliquot of the ultraviolet light-damaged organisms in the dark. This aliquot serves as the control for the experimental system involved. The third step involves the exposure of another aliquot of the irradiated organism to the photoreactivating light. The fourth step involves the analysis of the two aliquots for survivors; increased survivor rate or decrease in mutation frequency in the light-treated aliquot indicates a reversal of the damage caused by the ultraviolet light, and therefore, the quality of photoreactivation.

This review of the pertinent literature has been concerned with wavelength of damaging as well as photoreactivating light
as the variables in the phenomenon. These are not the only factors which affect photoreactivation (22). Another important factor is the quantity of energy, as damaging radiation, which interacts with the biological system. Very large doses of ultraviolet light will cause irreversible damage to the biological system and photoreactivation will not be demonstrated in these cases. There are two explanations offered for this dramatic loss of photoreactivation capability: (1) such extensive damage that the capacity of the photoreactivating mechanism is exceeded, and (2) damage to the photoreactivation mechanism itself. Another factor is the time interval after exposure to ultraviolet light and before exposure to the photoreactivating light. The organism must be exposed to the photoreactivating light within five to thirty minutes after ultraviolet light irradiation before a decrease in the photoreactivability occurs (9). \(\text{\textsuperscript{27}}\) has reported that this time may be extended to twenty-four hours if the cells are kept chilled in ice during the intervening period. The explanation given for this observation is based on the theory that cells with damaged deoxyribonucleic acid (DNA) will not be capable of synthesizing protein at these low temperatures, and therefore that the alterations induced in the DNA by ultraviolet light will not be manifested by causing the synthesis of proteins incompetent with the viability of the cell. The time of exposure to the photoreactivating light also affects the amount
of photoreactivation. The amount of photoreactivation increases proportionately with an increase in total energy delivered as photoreactivating light. Since photoreactivation involves chemical reactions, the rate of the reaction is directly proportional to the amount of energy input. The pH of the culture medium affects the photoreactivability of the cells. Ultraviolet light causes ionization of essential cell constituents. The hydrogen ion concentration affects the stability of these ionized structures. Ionization of the structures will cause a change in the electronic configuration of the molecules. As a result, the molecules will change with respect to their attraction for other molecules. This change in attraction will alter cell constituents to the extent that they will not function properly. It has been unequivocally shown that photoreactivation in bacteria is greatly affected by the culture medium in which the organisms are grown prior to irradiation, and also by the medium used for survival assays. Roberts and Aldous (33) reported that the ultraviolet light sensitivity of cells of Escherichia coli K12 irradiated and then plated on nutrient broth was greater than those of cells plated on a chemically defined medium.

A search of the readily available literature revealed the fact that little or no work has been reported concerning the photoreactivation of bacteria in different physiological states other than those mentioned. Specifically, no report could be found on the presence or absence of photoreactivation in the
Azotobacter in the encysted state. No information could be found concerning photoreactivation in the Azotobacter growing in nitrogen-free media as opposed to those growing in complete media. The latter is of importance when the work of Whelden (42) is considered. This investigator found that ionizing radiation has widely differing effects on the Azotobacter existing in different physiological conditions.

Previous Works

Photoreactivation was first discovered by Kelner in 1949 (24). His report indicated the occurrence of photoreactivation in four different genera of microorganisms. These genera were Escherichia coli B/r, Streptomyces prisms ATCC 3326, Penicillium notatum, and Saccharomyces cerevisiae. The pioneer work done by Kelner with the above organisms furnished other investigators with information on the nature of ultraviolet-induced cell damage and the means whereby this damage may be diminished or completely reversed.

After Kelner's reports, a series of papers were written on photoreactivation which demonstrated that the phenomenon occurred universally (22). An attempt to illustrate the universal nature of photoreactivation is made in the following discussion. A large volume of literature was written about photoreactivation in many organisms ranging from the viruses to higher plants and animals. Photoreactivation has been studied intensely in certain viruses. Photoreactivation of the T bacteriophages of Escherichia coli B was reported by Dulbecco (31). Rabin and
Kleczkowski (2) demonstrated the occurrence of photoreactivation of some plant viruses, specifically in tobacco necrosis virus in the French bean and the spherical, tomato, bushy, stunt virus of *Nicotiana glutinosa*. The tobacco mosaic virus, on the other hand, could not be photoreactivated according to these investigators. Bawdon and Kleczkowski believed that there was a direct correlation between the relative amount of nucleic acid and the photoreactivability of the virus since the nucleic acid content of the tobacco mosaic virus is about one-third of that present in the other two viruses. On the other hand, Pfefferkorn et al. (31) reported that a pseudorabies virus and a Herpes simplex virus are equally photoreactivably, although the nucleic acid content of these two viruses is not dissimilar.

Several genera of bacteria have been investigated and shown to possess the quality of photoreactivation. Extensive research on this phenomenon has been reported using *Escherichia coli* and other closely related organisms. Many investigators, including Kelner (25), Witkin (43), Hollaender (21), and Novick and Sahl (30) participated in the research. Monod et al. (29) observed that *Escherichia coli* K12, irradiated with ultraviolet light in a citrate buffer and plated on a synthetic agar medium, was not photoreactivable. After the addition of catalase or ferrous sulfate, photoreactivation could be demonstrated. De Daurat et al. (8) in 1963 showed that *Acetobacter aerogenes* could also be photoreactivated. Several other bacterial species, including *Bacillus* and *Acetobacter*, have been reported to possess a
capacity for photoreactivation. The majority of quantitative data found in the literature on photoreactivation were compiled using the bacteria and viruses as experimental organisms.

The occurrence of the propensity for photoreactivation in the protozoa and algae was reported by several investigators. Chlamydomonas mackessi (80), Paramecium bursaria (14), Colpidium (15), Tetrahymena (?), and various other genera were shown to be capable of being reactivated. Hill (20) discovered that when ultraviolet light-irradiated cells of Euglena gracilis var. bacillaris were incubated in the dark in a nutrient medium which permitted cell division, they lost the ability to be photoreactivated. The rate of this loss increased with the dose of ultraviolet light administered.

Several fungi have been reported to be photoreactivable. Among these are the molds, Penicillium notatum (26), Penicillium chrysogenum (34), and Ustilago maydis (6). Koller (23) in 1949 reported that the yeast Saccharomyces cerevisiae could also be photoreactivated.

The insects can also be photoreactivated under the proper experimental conditions. Lethal and molt-retarding effects on nymphs of Oncopeltus fasciatus, the milkweed bug (41), produced by exposure to ultraviolet light, can be photoreactivated. Reccessive lethal mutations in the polar cap cells of Drosophila larvae have been photoreactivated (1). Von Borstel and Wolff (39) reported that after an exposure to a photoreactivating light,
the hatching ratio of ultraviolet light-irradiated wasp eggs produced by Habrobracon juglandis was increased.

Photoreactivation has been demonstrated in higher animals. Rieck (32) reported significant photoreactivation of the forelimb development in larvae of the salamanders, Amblystoma maculatum and Amblystoma opacum. Pigmentation changes in tadpoles of Rana pipiens and Rana catesbeiana as a result of exposure to ultraviolet light are also subject to photoreactivation (44). Griffen et al. (19) observed that the induction of ear tumors in Swiss mice is photoreactivable, but only if the ultraviolet light and the photoactivating light are used simultaneously. When the photoactivating light is applied after the ultraviolet irradiation, the usual method in all other systems, tumor induction increases. Doubt has been cast on these results. It is believed that the photoactivating light used contained light in the ultraviolet wavelength region, since Kelner and Taft (24), using monochromatic ultraviolet light, discovered photoreactivation of similar tumors in albino mice. In 1958, Jaggar (22) reported that the photoreactivation of tumors was not clearly demonstrated. Jaggar in 1967 stated that the phenomenon appears not to exist in mammalian tissues (personal communication) in clear opposition to the above mentioned studies.

Photoreactivation has been demonstrated in higher plants; Barden and Kleczewski (2) discovered that the first-formed
leaves of *Phaseolus vulgaris*, the pinto bean plant, became bronze colored and wilted upon exposure to ultraviolet light. If the leaves are exposed to visible light after irradiation with ultraviolet light, the bronze color will not appear and the leaves will not wilt. Dubrov (10) demonstrated the occurrence of photoreactivation in the epidermis of the onion, *Allium cepa*. Sokolov et al. (36) reported that leaves of the beet, *Beta vulgaris*, irradiated with ultraviolet light would turn greenish-brown. With an exposure to visible light, the greenish-brown color would not appear as intensely.

There are relatively few organisms which do not exhibit some type of photoreactivation (22). The greatest number of exceptions to photoreactivation occurs in the bacteria. Johnson (23) failed to find photoreactivation in *Bacillus cereus* when four other species of *Bacillus* and three other strains of *Bacillus cereus* were found to be photoreactivable (37, 38). Also, *Bacillus subtilis*, *Bacillus polymyxa*, and *Bacillus circulans* could not be photoreactivated (37, 38). Jaggar (22) believes that a possible reason for these diverse responses is that the bacilli are very sensitive to destructive action by the photoreactivating light. Bellamy and Germain (3) were unable to photoreactivate *Streptococcus faecalis* and *Streptococcus lactis*. Goodall et al. (16) could not photoreactivate *Hemophilus influenza* or *Diplodocus pneumoniae*. Goucher et al. (17) failed to photoreactivate *Azotobacter vinelandii*. 
strain 0. They were able to photoreactivate *Azotobacter chroococcum*, *Azotobacter* strain "Q", and *Azotobacter agile* strain A4.4. The latter organism is also designated *Azotobacter vinelandii* or *Azotobacter agile* variation vinelandii. In 1954, Goucher and Kocholaty (16) photoreactivated constitutive and adaptive respiratory systems in whole cells of *Azotobacter agile* A4.4.

A search of the readily available literature has revealed that the photoreactivation studies on *Azotobacter* species were done primarily or almost exclusively using nitrogen-free media, and that little or no work has been done regarding differences in the physiological condition of the *Azotobacter*. No reports could be found in the readily available literature concerning the photoreactivation of these nitrogen-fixing bacteria in different physiological states. No reports could be found concerning photoreactivation of the *Azotobacter* in the encysted state. No information could be found concerning photoreactivation in the *Azotobacter* growing in nitrogen-free media as opposed to those growing in complete media.

The purpose of this investigation is to study photoreactivation in different physiological conditions of the vegetative cell as well as the photoreactivation of the two morphological states of the *Azotobacter* cell: the vegetative cell and the cyst.
Photoreactivation Mechanisms

Several mechanisms for the photoreactivation of microorganisms have been proposed. Novick and Szilard (30) proposed the poison theory. They assumed that ultraviolet rays cause the formation of a poisonous chemical compound which is produced in proportion to the dose of ultraviolet light. The poison is present in two forms: one is light sensitive and the other is not. When the cells containing the ultraviolet-induced poison are exposed to light, part of the poison will deteriorate. This would allow the recovery of some of the organisms which would otherwise die. This explanation, while not based on fact, is compatible with laboratory observations.

Another theory of photoreactivation was reviewed by Jagger (22). This theory involves a chromophore and reactible site. A chromophore is defined as the site of absorption of the photoreactivating light. Several experiments support the existence of the chromophore either in the nucleus or cytoplasm of the cell. However, Blum et al. (5) asserted that the chromophore probably exists in the cytoplasm. In their report, it was pointed out that a delay in cell cleavage can be reversed by photoreactivating light when the irradiated spore of *Arthia papulosa* is introduced into the carinate half of an egg, and the artificial zygote is illuminated with visible light. The spore by itself is not photoreactivable under the experimental conditions stipulated. This is not presented as
conclusive proof that the chromophore exists in the cytoplasm due to the nature of the experiment. Shreb and Errera (35) studied photoreactivation of nucleate and enucleate halves of Amoeba proteus. Photoreactivation was approximately the same in each half. This experiment was interpreted as demonstrating the presence of the chromophore in the cytoplasm, although it does not exclude the possibility of the presence of the chromophore in the nucleus. In conclusion, it is believed that the chromophore exists in the cytoplasm, but no proof exists for the presence of a chromophore exclusively in the nucleus.

A reactivatable site is a crucial structure within the cell that suffers ultraviolet damage. Its composition is thought to be primarily DNA or RNA (22). Since ultraviolet radiation of wavelengths of approximately 2537 Å is absorbed strongly by nucleic acids, it is believed that the DNA molecule is prevented from functioning due to the formation of heat-stable bonds between the two DNA helices (22). These heat-stable bonds are composed of two adjacent thymine or cytosine residues which are chemically bonded to each other to form thymine or cytosine dimers (22). When these dimers are formed DNA synthesis is blocked. The blockage of DNA synthesis causes cell division to halt, thereby preventing the formation of bacterial colonies. The reactivatable site and the chromophore comply with laboratory data, but their existence is theoretical.
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27. ———, "Photoreactivation of Ultraviolet-Induced Bacterial coli, with Special Reference to the Dose Reduction Principle and to Ultraviolet-Induced Mutation," Journal of Bacteriology, LVII (July, 1949), 511-522.


CHAPTER II

METHODS AND MATERIALS

Stock Cultures

All of the experimentation was carried out using the soil organism, Azoarcus xylanolyticus, designated 12807 by the American Type Culture Collection. This culture was obtained from the Microbiology Stock Culture Collection, North Texas State University, Denton, Texas. This stock was maintained on Trypticase Soy slants as well as Trypticase Soy broth and Burk's nitrogen-free broth with daily transfers. Both the vegetative and encysted forms of Azoarcus xylanolyticus were utilized. The vegetative cells were grown in twenty-five milliliter quantities which were incubated at thirty degrees centigrade in a New Brunswick incubator-shaker for a period of twenty-four hours. The encysted forms were grown on Burk's butanol agar plates at thirty degrees centigrade for fourteen days in order to obtain one hundred percent encystment.

Culture Media

Four types of media were utilized in the research reported here. The first was Trypticase Soy media (1). For broth preparation, thirty grams of the dehydrated media were added to a liter of distilled water and autoclaved at one hundred and
twenty-one degrees centigrade for a period of fifteen minutes. Twenty grams of agar per liter of medium were added in order to prepare agar plates and culture tube slants. The second type of culture medium that was employed was Burk's nitrogen-free medium (5). This medium was prepared by the addition of the following chemicals to one thousand milliliters of distilled water:

**TABLE I**

**CONSTITUENTS OF BURK'S MEDIUM**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.2 gm.</td>
</tr>
<tr>
<td>CaSO$_4$·2H$_2$O</td>
<td>0.085 gm.</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>0.005 gm.</td>
</tr>
<tr>
<td>Na$_2$MnO$_4$·2H$_2$O</td>
<td>0.0003 gm.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10 gm.</td>
</tr>
</tbody>
</table>

The third type of culture medium was Burk's ammonium nitrate agar. The constituents of this medium are the same as those listed in Table I, but varying quantities of ammonium nitrate were added as a source of inorganic nitrogen. The fourth type of medium utilized was Burk's butanol agar (4). This medium was prepared by the addition of all of the chemicals listed in Table I (except the sucrose) to one thousand milliliters
of distilled water. Twenty grams of agar were also added per one thousand milliliters of medium. This medium was autoclaved at one hundred and twenty-one degrees centigrade for fifteen minutes. After sterilization, the medium was allowed to cool to approximately forty-five degrees centigrade. At this point, two milliliters of filter-sterilized n-butanol were added to the medium and it was poured into petri dishes. The plates were stored on the shelf for a period of four days before use.

Preparation of the Inocula

Vegetative cells were prepared by allowing them to grow in either twenty-five milliliters of Burk's nitrogen-free broth or twenty-five milliliters of Trypticase Soy broth for twenty-four hours in the incubator-shaker at thirty degrees centigrade. The cells were washed three times by centrifugation and resuspended in sterile distilled water. The optical density was adjusted to approximately 0.8-0.9 at 540 millimicrons in a Bausch and Lomb "Spectronic 20" colorimeter, using distilled water as the blank. The cell suspension was then chilled in ice (2).

Cysts of Azotobacter were produced by culturing the organism for fourteen days at thirty degrees centigrade on Burk's butanol plates. The degree of encystment in these cultures was determined by microscopic observation of the cysts. The cysts were removed from the agar and suspended
in sterile distilled water. The cysts were washed three times and resuspended in sterile distilled water. The optical density was adjusted to approximately 0.8-0.9 by dilution with sterile water. The suspended cysts were then chilled in ice (3).

Exposure to Ultraviolet Light

The vegetative and encysted cells were exposed to ultraviolet light in the same manner. All of the following irradiations were done in a room illuminated with a twenty-five watt, safety, yellow light bulb. The inactivating light was an ultraviolet lamp (Mineralight) which was mounted on a large ring stand to facilitate the regulation of the distance of the ultraviolet lamp from the bacterial cells. The height of the lamp was set at thirty-three centimeters from the cells. The energy output of the lamp was measured by a YSI Kettering Model "65" Radiometer and was adjusted to $1 \times 10^3 \text{ ergs/cm}^2\cdot\text{sec.}$ by adjusting the height of the ultraviolet lamp. The peak output of the lamp was 2537 Å. The cells were exposed to the inactivating light for the following periods of time: zero, thirty, sixty, ninety, and one hundred and twenty seconds.

Four-milliliter aliquots were removed from the chilled cell suspension and placed in sterile glass petri dishes where the depth of the cell suspension was less than one millimeter. The cells were then exposed to the light for previously determined periods of time and agitated constantly in order to minimize differences in exposure.
Photoreactivation Techniques and Preparation of a Dark Control

Immediately after exposure to ultraviolet light, three milliliters of the suspension were removed from the petri dish and divided equally between two sterile test tubes. One of the two tubes was completely wrapped in aluminum foil to block out all light. This tube served as the control. The two tubes were then inserted into the photoreactivation apparatus which is illustrated in Figure 1. The photoreactivation apparatus consisted of a water bath assembly with a 500-watt Sylvania photoflood light placed approximately four inches from the culture tubes. The energy output of the lamp at this distance was measured and found to be approximately $1.13 \times 10^8$ ergs/cm$^2$-sec. A plexiglass shield was placed between the water bath and the photoflood to prevent breakage of bulbs. All cells were left in the photoreactivation apparatus for the same period of time, approximately thirty minutes. The temperature of the water bath was maintained at approximately twelve to fifteen degrees centigrade during the whole time of the experiment.

Determination of Survival

In order to determine the number of Azotobacter cells killed and the number of cells photoreactivated, a means of counting the bacteria was needed. The spread-plate method was chosen because of its accuracy and convenience. The cells were plated in triplicate by making serial dilutions using sterile
Fig. 1—Diagram of the Photoreactivation Apparatus (3)
Water bath assembly for photoreactivation.
distilled water blanks. The plating medium was either Tryp-
ti-case Soy agar, Burk's nitrogen-free agar, or Burk's medium,
plus ammonium nitrate. The plates were incubated for forty-
eight hours in a dark incubator at thirty degrees centigrade.

Experimental Timing Schedule

To facilitate the operation of the experimentation, some
type of timing schedule was required. There were three require-
ments of the timing schedule. First of all, it was necessary
that it allow the cells to stay in the photoreactivating ap-
paratus for precisely thirty minutes. Secondly, it was necessary
that the cells be plated out as soon as possible after the irra-
diation with ultraviolet light. Thirdly, it was necessary that
the schedule conserve time. The schedule utilized in this study
can be seen in Table II.

The left-hand column represents decreasing time in minutes.
Column two represents the control tubes and the photoreactivation
tubes at various exposure periods. Column three represents the
amount of exposure of each sample to ultraviolet light. Column
four represents the staggered times at which the samples were
put into the photoreactivation apparatus. Column five represents
the staggered times at which the samples were removed from the
photoreactivation apparatus.
TABLE II
PHOTOREACTIVATION TIMING SCHEDULE

<table>
<thead>
<tr>
<th>Clock time (min.)</th>
<th>Tube number*</th>
<th>U. V. (in sec.)</th>
<th>In P. R.</th>
<th>Out P. R.</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>30R</td>
<td>30&quot;</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>90</td>
<td>30C</td>
<td>30&quot;</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>75</td>
<td>0R</td>
<td>0&quot;</td>
<td>75</td>
<td>45</td>
</tr>
<tr>
<td>75</td>
<td>0C</td>
<td>0&quot;</td>
<td>75</td>
<td>45</td>
</tr>
<tr>
<td>60</td>
<td>60R</td>
<td>60&quot;</td>
<td>60</td>
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<td>120&quot;</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>120C</td>
<td>120&quot;</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

*R = Photoreactivate
C = Dark Control
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CHAPTER III

Results

This thesis may be divided into two main divisions since there are two morphological states of Azotobacter vinelandii ATCC 12837. The first division involves an examination of photoreactivation in the encysted form. The second division involves a study of photoreactivation in the vegetative form. The division concerning photoreactivation in the vegetative cells may further be divided into several subdivisions since photoreactivation was examined in various physiological conditions of the vegetative cells.

Figures 2 and 3 are photomicrographs of the two morphological forms of Azotobacter vinelandii ATCC 12837, the vegetative form and the encysted form respectively, that were used in this study. It is interesting to note that the vegetative forms are pleomorphic rods, which are the smaller of the two forms. The encysted forms possess the typical, spherical central body with the large entine and exine of the cyst surrounding it. A further description of these forms has been presented in the literature (1).
Fig. 2—Photomicrograph of the vegetative forms of Azotobacter vinelandii ATCC 12837 (1000X phase contrast).
Fig. 3—Photomicrograph of the encysted form of *Azotobacter vinelandii* ATCC 12837 (1000X phase contrast).
The first study involves the photoreactivation of *Azotobacter vinelandii* ATCC 12837 cysts. Since the *Azotobacter* cyst is a very resistant form (1), one would anticipate a survival curve from exposure to ultraviolet light to decrease slowly as a function of time. Figure 4 illustrates the inactivation curve and photoreactivation curve for *Azotobacter vinelandii* ATCC 12837 when the cells were originally grown on Burk's butanol agar and immediately plated out on Burk's nitrogen-free agar. It is evident from Figure 4 that the cysts of *Azotobacter vinelandii* ATCC 12837 can be photoreactivated but that the amount of photoreactivation is not as great as that observed in the vegetative cells.

The second study is concerned with photoreactivation of the vegetative cells under various physiological growth conditions. A comparison of photoreactivation of the encysted form with that of the vegetative form may be made if the vegetative cells are treated as the cysts were in the previous section. Figure 4 illustrates a comparison of the photoreactivation curves and the inactivation curves of both the cysts and the vegetative cells when Burk's nitrogen-free medium is used for the original culture medium and the plating medium.
Fig. 4—A comparison of the inactivation and the photoreactivation curves of Azotobacter vinelandii ATCC 12837 vegetative and encysted cells. The curve on the left represents inactivation of cells and the curve on the right represents photoreactivation.
Figure 5 illustrates the inactivation and photoreactivation of Azotobacter vinelandi 1803 as a function of the physiological growth condition of the cells. When the cells are grown in Burk's nitrogen-free media and after irradiation are plated out on Trypticase Soy agar, no significant amount of photoreactivation occurs. In contrast, Figure 4 revealed that photoreactivation occurred when the cells were grown in Burk's nitrogen-free medium and subsequently plated out on Burk's nitrogen-free agar.

Furthermore, when the cells were grown in Trypticase Soy medium and after irradiation were plated out on Trypticase Soy agar, little or no significant amount of photoreactivation occurred. When the cells were grown in Trypticase Soy medium and after irradiation plated out on Burk's nitrogen-free agar, again no significant amount of photoreactivation occurred.

An attempt was made to determine if ammonium nitrate would have an effect on the photoreactivability of the vegetative cells. Ammonium nitrate was selected as a source of inorganic fixed nitrogen, since the ammonium ion is one of the intermediates in the nitrogen fixation pathway (2). Varying concentrations of ammonium nitrate were incorporated into the plating medium. All cells were originally grown in Burk's nitrogen-free medium. The results of the analysis are found in Figure 6.
Fig. 5—Inactivation and photoreactivation curves of Azotobacter vinelandii ATCC 12837 demonstrated as a function of the physiological condition of the cells when grown on different culture media. The curve on the left represents inactivation of cells and the curve on the right represents photoreactivation.
--- X --- Burk's Broth plated on TSA
--- O --- TSB plated on Burk's Agar
--- O --- TSB plated on TSA

Percent survival vs. irradiation time (sec.)

0 10 20 30 40 50 60 70 80 90 100

Irradiation Time (Sec.)
Fig. 6—Inactivation and photoreactivation of Azotobacter vinelandii ATCC 12577 when the cells were grown in Burk's nitrogen-free medium and plated on Burk's agar with varying concentrations of ammonium nitrate. The curve on the left represents inactivation of cells and the curve on the right represents photoreactivation.
ALL CELLS CULTURED IN SURK'S NITROGEN FREE BROTH

--- O --- PLATED ON SURK'S NITROGEN-FREE AGAR
--- X --- " " " +1 cm. NH₄NO₃/LITER
--- D --- " " " +2 " " "
--- X --- " " " +3 " " "
--- O --- " " " +4 " " "

Percen Survival

IRRADIATION TIME (SEC.)
Fig. 7--A comparison of the inactivation and photoreactivation of *Aerobacter vibrioformis* ATCC 12837 when immediately plated out after irradiation and when allowed to dark heal for twenty-four hours prior to plating. The curve on the left represents inactivation of cells and the curve on the right represents photoreactivation.
Inactivation & Photoreactivation of
dark healed cells

Inactivation & Photoreactivation
of non-dark healed cells
Figure 7 illustrates the effect of delayed DNA expression on the photoreactivability of Azotochacter vinelandii ATCC 12837. The delayed DNA expression was accomplished by keeping the cells under refrigeration in the dark for a period of twenty-four hours after photoreactivation attempts. The original culture medium was Trypticase Soy broth and the plating medium was Trypticase Soy agar. The results of the analysis are found in Figure 7.
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CHAPTER IV

Discussion

The encysted cells are much more resistant to ultraviolet light than are the vegetative cells. This is indicated in the research of Socolofsky and Wyss (4) and also in Figure 5 of this report. The same figure also illustrates that the encysted state, which is almost metabolically inactive, can be photoreactivated when exposed to the same treatment as the vegetative cells. A search of the readily available literature reveals that this is the first report of the photoreactivation of cysts. Nevertheless, a greater amount of photoreactivation occurs in the vegetative cell than in the cyst. It was previously reported that the vegetative cells could be photoreactivated on Burk's nitrogen-free media (2). No reports were found in the readily available literature concerning any change in the photoreactivability of Azotobacter with a change in the culture medium. It can be seen in Figure 6 that the culture medium has a startling effect on the shape of the inactivation and photoreactivation curves. It is inferred from these data that *Azotobacter vinelandii* ATCC 12837 grows on Trypticase Soy broth is more susceptible to ultraviolet light and that it has no capacity for photoreactivation. *Azotobacter vinelandii* ATCC 12837 grown in Burk's nitrogen-free broth and plated on
Trypticase Soy agar is more susceptible to ultraviolet light, and it also exhibits no capacity for photoreactivation.

The loss of ultraviolet resistance and photoreactivatability as determined in the data presented in Chapter III can be interpreted in one of two ways:

(1) The phenomenon may be the result of the healing of ultraviolet-induced lesions when the cells are plated on Burk's nitrogen-free medium as opposed to plating on Trypticase Soy agar where the DSA can express itself immediately. Healing due to delayed protein synthesis is a well known phenomenon.

(2) The phenomenon may also be the result of a fundamental difference in the physiological constitution of the organism brought about by its growth as a mesotroph on Burk's chemically defined medium as opposed to its growth on a heterotrophic basis on Trypticase Soy media.

A report by Roberts and Aldous in 1949 (3) presents evidence which supports the second interpretation. They found that in Escherichia coli the cells were more sensitive to ultraviolet light when grown on Nutrient agar than when they were grown on a chemically defined medium.

It was believed for a short time during this research that the nitrogen-fixing mechanism participated in the increased resistance to ultraviolet light and the increased rates of photoreactivation. If this were true, then the time required for nitrogen fixation would allow the DSA time enough to repair
itself. This viewpoint supports the first interpretation of
the loss of ultraviolet resistance and photoactivability.
This viewpoint was demonstrated to be wrong, according to the
data presented in Figure 7. Ammonium nitrate was incorporated
into Burk's nitrogen-free medium in varying concentrations.
Regardless of the concentrations of ammonium nitrate used, the
photoactivability of cells plated on nitrogen-free media and
those plated on ammonium nitrate media is approximately the
same. This indicates that ammonium nitrate has no effect on
the photoactivability of vegetative cells. Therefore, since
the ammonium ion is an intermediate in nitrogen fixation (5),
the nitrogen fixation mechanism has little or no effect on the
sensitivity of the vegetative cells to ultraviolet light or the
photoactivability of the vegetative cells.

It is well known that if bacterial cells are refrigerated
in the dark to delay protein synthesis, a certain amount of
recovery from ultraviolet irradiation may be obtained. If this
is the case for Azotobacter, then the first interpretation is
again supported. However, the data presented in Figure 8 do
not readily support this. The data appear to indicate that
only a small amount or no dark repair has occurred. This data
would then lead one to support the second interpretation that
the phenomenon is the result of a fundamental difference in
the physiological constitution of the organism brought about
by its growth as a mesotroph on Burk's chemically defined
media, as opposed to its growth on a heterotrophic basis on Trypticase Soy media. It is concluded then that only Azotobacter vinelandii ATCC 12837 cells grown autotrophically possess the capability for photoreactivation.

In 1964 Ben Gurin (1) described experiments with Escherichia coli K12 strains in which the growth was inhibited, after irradiation with ultraviolet light, in a minimal media by the addition of twenty-five micrograms of cysteine per milliliter. This amino acid and other reducing agents may reveal a clue which will allow one to understand the different sensitivities to ultraviolet light obtained on different culture media.
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CHAPTER V

SUMMARY

This thesis was written to study photoreactivation in different physiological conditions of the vegetative cell as well as the photoreactivation of the two morphological states of the encysted cell: the vegetative cell and the cyst. Vegetative cells grown in Burk's nitrogen-free broth and Trypticase Soy broth were irradiated with ultraviolet light at time intervals between zero seconds and one hundred and twenty seconds. They were then exposed to a high-intensity white light for thirty minutes. The cells grown in Burk's nitrogen-free broth were plated out on Burk's nitrogen-free agar plates and Burk's media, plus varying concentrations of ammonium nitrate as a source of nitrogen. These cells were also plated out on Trypticase Soy agar. The cells grown in Trypticase Soy broth were plated out on Burk's nitrogen-free agar and Trypticase Soy agar. Some of the cells grown in Trypticase Soy broth were refrigerated in the dark after photoreactivation to delay DNA expression and were subsequently plated out on Trypticase Soy agar. The encysted cells were obtained by plating vegetative cells out on Burk's butanol agar for fourteen days. The cells were washed off the plates
and suspended in sterile distilled water. These cysts were also exposed to ultraviolet light for the same periods, as were the vegetative cells. They were exposed to visible light for thirty minutes and then were plated out on Burk's nitrogen-free agar.

The results of the experimentation indicate that the cysts of Azotobacter vinelandii ATCC 12837 can be photoreactivated. The amount of photoreactivation, however, did not appear to be as great as that of the vegetative cells. Further work on the photoreactivation of Azotobacter vegetative cells was done. It was discovered that the amount of photoreactivation depends on the physiological state of the vegetative cell. It was inferred from the data that vegetative cells grown on Trypticase Soy broth were more susceptible to ultraviolet light and had no capacity for photoreactivation. Azotobacter cells grown in Burk's nitrogen-free broth and plated on Trypticase Soy agar are more susceptible to ultraviolet light and exhibit no capacity for photoreactivation. The data also indicates that the nitrogen-fixing mechanism is not involved in the loss of photoreactivation on different culture media.
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