REACTIONS OF DYE MOLECULES IN PHOTODYNAMIC INACTIVATION OF *ESCHERICHIA COLI* B

by

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INTRODUCTION

The mutual effects of light and certain dyes on biological material were first observed during the last years of the nineteenth century. In 1900 Oscar Raab was the first to publish the information that demonstrated increase in dye toxicity that was due to the light it absorbed.

Since the initial rash of publications that followed Raab's observations, interest in this phenomenon, known as photodynamic action, was sporadic. Many investigators studied the effects of illumination with a great variety of dyes. As a consequence, very little integrated information was available when Blum began his studies. Through the efforts of Blum and his associates, many of the chemical and physical characteristics of the reactions were determined. Blum terminated an active period of research on photodynamic action when he published a comprehensive review on the subject in 1941. The effect of this excellent review was profound; it appeared that only the most difficult and theoretical phases of photodynamic action were left unconquered and most biologists turned their interests elsewhere.

In recent years, however, there has been a resurgence of interest in this field of photochemistry with many workers, including Blum, reinvestigating the mechanism of the reaction, the nature of the reactants that are involved, and the location of the reaction.

During all the years that photodynamic action was investigated, little interest was taken in the fate of the dye molecule. Since changes in the dye molecule and the location of the dye molecule

as it undergoes these changes might add to the growing knowledge of the reaction mechanisms of photodynamic action, a study of changes in the dye molecule and changes in the relationship between the dye molecule and the treated material was initiated.

For these investigations, a well established photodynamic system was chosen. Recent investigations with Escherichia coli and methylene blue made this the system of choice.

The characteristics of photodynamic inactivation of E. coli were established. From this information, standard procedures were designed that enabled physical determinations of the changes in the dye and dye-cell relationship.

REVIEW OF LITERATURE

While observing the effect of the dye acridine on paramecia, Oscar Raab (Blum, 1941) noted that the time required to kill these protozoa varied greatly from day to day. Examination of all known factors failed to explain the inconsistent results of the experiments. Raab soon discovered that an unsuspected variable, the intensity of light in the laboratory, caused the observed variations. In subsequent examinations of this phenomenon, Raab definitely proved that death of paramecia in dye solution was caused not by the dye molecules or light alone, but by some mutual action of both. Raab was able to characterize this reaction, to some extent, by observing that close contact of dye molecules to the paramecia was necessary to enable the reaction to proceed. The light emitted by illuminated dye solution had no effect on the microorganisms if they were in another vessel.

According to Blum (1941), these observations came at the beginning of the twentieth century, a time when great interest in the biological effect of light had been stimulated by the therapeutic use of light. Soon, many investigators attempted to explain light-produced phenomena on the basis of Raab's work. This interest led to early recognition of the role of atmospheric oxygen in this reaction. In experiments with red blood cells and infusoria, Straub (in Blum, 1941), Joslbauer and Tappeiner (in Blum, 1941) found that oxygen was necessary in this reaction. During these investigations, probably in an attempt to group all observed phenomena in one class, Tappeiner coined the term "photodynamic action". This term has been used extensively ever since. Therefore, photodynamic action can be defined as reactions that are typified by a change in the system under study that does not occur when the system is treated with low concentrations of dye solutions or visible light alone.

In the examination of red blood cell hemolysis induced by a photodynamic system of light and eosin, Schmidt and Norman (1920) found that the amino acids tyrosine and tryptophan offered marked protection against hemolysis. The selection of amino acids as test material resulted from observation of the relative protective properties of gelatin, egg white and blood serum. Of these three only gelatin offered no protection. Since this protein contains no tyrosine, tryptophan or cystine, and only small amounts of phenylalanine and histadine, Schmidt postulated that these amino acids were substrates in photodynamic action inasmuch as neither glucose nor starch offered protection. Further investigations by

Schmidt and Norman (1922) led to the conclusion that the reducing ability of these easily oxidized amino acids accounted for their inhibition of photodynamically induced hemolysis. Interest in the possible role of amino acids in the photodynamic process led to investigation of their photosensitized oxidation. Using a number of amino acids as substrates, Harris (in Blum, 1941) found that only tyrosine and tryptophan displayed appreciable oxygen uptake and that gelatin was not oxidized. Further in vitro studies by Carter (1928) demonstrated that fatty acids were not readily oxidized. Ring compounds, notably tyrosine, were oxidized at a monomolecular rate. These results indicated that contact between the dye (in these experiments methylene blue or hematoporphyrin) and the substrate was necessary for the reaction to take place. The increase of surface in the reaction vessel, however, did not increase the rate of oxidation. Additional evidence that this reaction was characterized by physical contact between the photosensitizing dye and the substrate was supplied by Rask and Howell (1928). Their findings substantiated the work by many of the earlier investigators. In their studies, Rask and Howell examined in vitro the photodynamic action of hematoporphyrin on turtle heart. Tissue damage was limited to the half of the heart that received light, indicating that no toxic substances were produced. They were unable to inhibit the light-induced tissue damage with peptone solution, as might be postulated from the results of Harris, Schmidt and others.

These early investigations established the principal characteristics of photodynamic action: (1) atmospheric oxygen was

consumed, (2) close physical contact between dye molecule and substrate was necessary for the transfer of the light energy absorbed by the dye to the oxidizable substrate and (3) the substrate appeared to be comprised of certain cyclic amino acids. With these facts established, Blum initiated a series of studies of photodynamic action on red blood cells. Using fluorescein dyes, Blum was able to produce hemolysis in the dark with previously irradiated dye solution. He found that after exposure to sunlight, the dye solutions contained a small proportion of peroxide which enhanced the hemolytic action of the dye solution. However, further examination of the role of peroxide formation in photodynamic action by Blum (1930b) indicated the amount of peroxide formed during irradiation was insufficient to produce hemolysis. In this research Blum (1930b) observed hemolysis by non-irradiated eosin. He distinguished this dark reaction from photodynamic hemolysis by the mechanisms involved. In general, light-induced hemolysis occurs in very dilute solutions of a photosensitizing dye, whereas the dye concentration necessary to initiate hemolysis in the dark was much higher. The effect of temperature on rose bengal-sensitized hemolysis, reported by Blum, Pace and Garret (1937), also indicated a different mechanism. The Q_{10} of dark hemolysis varied between a slightly negative value to fifteen, whereas the Q_{10} of photodynamic hemolysis was uniform at all dye concentrations tested and had a value of approximately 1.2. Another difference, and perhaps the most important in distinguishing these two reactions, was demonstrated when the dark reaction was shown to proceed in the absence of oxygen (Blum, 1941).

It was soon established that factors other than those outlined by Blum would affect photodynamic action. Turner (1932) found that borate accelerated hemolysis of yellow eosin-sensitized erythrocytes while phosphate retarded this reaction. The addition of magnesium and calcium ions did not change the per cent hemolysis. In their investigation of light stimulation of eosin-stained muscle, Lillie, Hindrichs and Kosman (1935) found that sodium salts increased the contraction of the muscle when exposed to light but did not cause an increase in oxygen uptake of the tissue. In these experiments calcium ions proved to be a strong inhibitor of the reaction. Kosman and Lillie (1925) made observations on the oxygen consumption of muscle and nerve tissue. They found that protein-free muscle extract exhibited no oxygen uptake whereas fat-free muscle extract did show oxygen consumption. The presence of calcium, sodium or cyanide ions did not affect the rate of oxygen uptake. Their observation that boiling did not alter oxygen consumption could mean that only the amino acid content controls photodynamic oxidation or that the protein in their muscle extract was denatured in preparation and any steric formation of the protein molecules that may be important in photodynamic action was altered before boiling.

As with various cations and anions, the hydrogen ion activity of the liquid containing dye molecules and a suspended or dissolved substrate was found to have an effect on photodynamic processes. Turner (1932) and Blum (1930) observed that the change in the amount of hemolysis produced by fluorescein dye-sensitized erythrocytes was related to (Ht). Beck and Nichols (1937) concluded that

the penetration of dyes into paramecia was favored by a change in pH and thereby enhanced photodynamic damage to the cell. The effect of (H^+) on the photodynamic process, Blum (1941) suggested, was due to the effect on the uptake of dye by the cell surface.

To establish the nature of a photochemical reaction, the light energy and wave length characteristics of the reaction must be determined. In photodynamic action, the response of a system under study should be limited to the wave lengths of light that are absorbed by the dye or the dye-substrate complex. The absorption spectrum should correspond, on a quantitative basis, with the action spectrum of the process. By determining the threshold at which eosin-treated plant roots would demonstrate phototropism, Blum and Scott (1933) were able to establish the action spectrum of this system.

Continued investigations designed to demonstrate the adherence of photodynamic action to photochemical principles by Blum and Hyman (1939a) demonstrated that photodynamic hemolysis obeyed the reciprocity law. This law, often referred to as the Bunsen-Roscoe law (Blum, 1941), states that the product of duration of irradiation and the intensity of irradiation is equal to a constant. This law follows from the equivalence law which states that the first step in any photochemical reaction is the absorption of a quantum of energy by an atom or molecule in the reaction system (Blum, 1941). Blum and Scott (1933) demonstrated the adherence of photodynamic action to these laws in their study of phototropism of plant roots. Another photochemical measurement that contributes to the understanding of photodynamic action is the determination of quantum

requirements. Determination of the number of ergs of monochromatic light that are required to produce a specific photochemical reaction often supplies information that may be used in establishing the mechanism of the reaction. A study of the quantum requirements of photodynamic hemolysis of red blood cells enabled Blum (1941) to calculate the number of quanta that were required to hemolyse a single erythrocyte. Blum (1941) found that the number of quanta to bring about this change in a single red blood cell was close to ten billion (10^{10}) at all test concentrations of dye (rose bengal or erythrosine) adsorbed to the cell. In an earlier presentation, Blum and Hyman (1939b) found that photodynamic effectiveness was not proportional to the concentration of the dye taken up by the cell. In the case of photodynamic hemolysis, only a small per cent of the cell surface was required to be covered by dye to initiate the hemolytic reaction. This observation led Blum and Hyman (1939) to conclude that the dye molecule acted repeatedly in the transfer of absorbed energy to the reaction site.

During the years that Blum and fellow workers were establishing the photochemical nature of photodynamic action, other investigators were concerned with the determination of the substrates that were oxidized in these reactions. With the use of Warburg respirometers, Meyer (1933a) studied that oxygen uptake of pyruvic acid in a light reaction sensitized by eosin. Pyruvic acid was oxidized to oxalic acid. The oxidation rate was proportional to both the concentration of the substrate and the logarithm of the concentration of the sensitizing dye. Extending his studies, Meyer (1933b) observed the oxidation of ergosterol. He found that

in an eosin-sensitized photooxidation, one mole of oxygen was taken up per mole of substrate whereas in a chlorophyll-sensitized photooxidation, only one-half mole of oxygen was taken up per mole of substrate.

The study of photodynamic action on bacteria was initiated early. In attempts to elucidate the mechanism of this reaction, Joalbauer and Tappeiner, in 1905 (Blum, 1941), studied the effect of oxygen on rose bengal, methylene blue and phenosafranine-sensitized action of Bacillus proteus (Proteus vulgaris). T'Ung (1935), using methylene blue, studied the photodynamic action on a variety of gram positive and gram negative bacteria. T'Ung found great variation in response to photodynamic action, but noted that the gram negative bacteria were more resistant to photodynamic action than gram positive bacteria. Focusing his efforts on pneumococcus, T'Ung (1936) concluded from serologic tests and mouse protection studies that photodynamically treated pneumococci were more antigenic than untreated controls.

More recent investigations of photodynamic action, using bacteria as an experimental biological substrate, has been directed toward the determination of the mechanism of the photochemical reactions that are involved. With this aim in mind, Heinmets, Vinegar and Taylor (1952) studied the inactivation of E. coli. These investigators observed that the dye, methylene blue, was adsorbed by the bacteria in a predominantly ionic fashion. The dye-bacteria complex that was formed had an absorption curve that differed from that of methylene blue alone by the presence of a new peak in the 610 mu wave length range. This research also

substantiated observations made by other investigators that there was an apparent optimal dye concentration, that the presence of oxygen was essential to the inactivation process and that hydrogen peroxide, although produced by the reoxidation of methylene blue, does not inactivate the bacteria. The inactivation was shown to take place in a frozen state, thereby reiterating the necessity of a dye-substrate complex formation before initiation of photodynamic action. In a study of the photosensitization of E. coli B and B/r and Stephylococcus aureus with long wave ultra violet and 8-methoxypsoralen, Oginsky, Green, Griffith and Fowlks (1959) found that this system varied a great deal from the methylene blue--E. coli system studied by Heinmets. Molecular oxygen did not stimulate the reaction. Stepwise increases in reaction temperatures resulted in a proportional decrease in the lethal effect of the compound tested. Another basic difference was observed when the investigators demonstrated that pH had little effect on the reaction. This reaction appeared to be quite different from any other that had been investigated.

By correlating some of the old data with recent studies of photooxidation of amino acids and proteins, Fowlks (1959) stated that a sequential relationship appeared. The initial step that was observed in the reaction was a formation of a dye-protein complex. This was often associated with a shift in the absorption spectrum of the dye as Heinmets, Vineger and Taylor (1952) observed in their studies with an E. coli-methylene blue system.

After this initial step, the oxidation of the amino acids was shown to follow a definite sequence. The observations made by

Weil, Gordon and Burchert (1951) on the methylene blue-sensitized oxidation of amino acids, proved that histidine was the most readily oxidized. Tryptophan was the next most easily oxidized followed by tyrosine and cystine. In these oxidations, it was observed that tryptophan was altered by the opening of the pyrrole ring and that tyrosine was altered to form 3,4 dihydroxy phenylalanine. Fowlks (1959) reported that the disulfide bond in cystine was broken by some unknown mechanism. Other amino acids were altered only after extensive reaction had taken place in the system under study.

In the photodynamic changes that occur in proteins, Weil et al (1951) found that peptide bonds were not broken as a primary reaction. This was also shown by Weil and Seibles (1955) in the photodynamic inactivation of ribonuclease. It was observed that the decrease in enzyme activity was due to the photooxidation of the histidine residue in the protein. There was no evidence of peptide bond destruction. Also, in the riboflavin-sensitized inactivation of lysozyme, tryptophan destruction proceeded and paralleled inactivation (Fowlks, 1959).

The historical development that preceeded this discussion of the mechanism of photodynamic action may be considered a statement of the variables that are important in this reaction. The contributions of these investigators have led to the formulation of reaction equations that represent the process. Of the many schemes that have been proposed, the over-all reaction given by Blum and Kauzmann (1953) served best as a simple representation. Using the symbols D as the dye molecule, S as the substrate and hv/D as the

dye molecule which captures the light quantum, the reaction was written as follows:

$$(1) \quad h\nu/D + O_2 + S \rightarrow S_{ox} + D$$

As shown, the dye molecule appears on both sides of the equation. This suggested the reappearance of unaltered dye after it had taken part in the reaction and indicated that the dye was part of a cyclic reaction and therefore a few dye molecules could react repeatedly to bring about the change associated with photodynamic action.

This generalized reaction was expanded in the scheme of Heinmets, Vinegar and Taylor (1952). Using the symbols previously mentioned in addition to X for unknown reaction product and * as an excited state, the following reactions were postulated.

$$(1a) \quad D + S \rightarrow DS + h\nu \rightarrow (DS)^* + O_2 \rightarrow (DS{\cdot}O_2)^* \begin{cases} (DS)_{ox}X_1 \\ S_{ox} + DX_2 \end{cases}$$

In this reaction, the formation of a dye-substrate complex followed by the absorption of a photon resulted in an excited state complex that reacted with oxygen. However, Heinmets et al (1952) suggested that oxygen may form complexes with other reactants. Under these altered circumstances, the following reaction was outlined.

$$(2) \quad D + S + O_2 \rightarrow DS{\cdot}O_2 + h\nu \rightarrow (DS{\cdot}O_2)^* \begin{cases} (DS)_{ox} + X_1 \\ S_{ox} + DX_2 \end{cases}$$

This reaction did not explain the fate of the dye molecule which may be dissociated from the complex. However, Fisla (Fowlks, 1959) found that a dye-protein complex is first formed and this complex was transformed into a dye-protein compound after irradiation.

The oxygen in the system, Fowlks (1959) suggested, may merely regenerate the dye. The reaction scheme proposed by Fiala agreed with the reaction that was given by Heinmets et al.

The location of photosensitized reactions within a cell, whether the cell membrane, the cytoplasm, cytoplasmic organelle, or nuclear material, was not determined in many cases. However, the location of these reactions was suggested by many investigators to be on the cell surface. Blum (1941), in his studies of photodynamic hemolysis, suggested that the photosensitized reaction took place at the surface of the erythrocyte. Observation of a shift in the absorption peaks of the dye after complexing with E. coli, led Heinmets, Vinegar and Taylor (1952) to conclude that this ionic binding was at the cell surface. However, these same investigators observed a reactivation phenomenon which suggested repair of cytoplasmic damage. Another indication that cytoplasmic damage resulted from photodynamic action, (Fowlks, 1959), was demonstrated in the loss of motility of paramecia as a result of photodynamic damage. Any damage to the nucleus by photodynamic action was ruled out because Paramecium caudatum can remain motil after removal of the nucleus. On the other hand, damage to the nucleus was demonstrated in this organism by the delay in division that resulted from sub-lethal photodynamic treatment. In this case, the action spectrum of the delay in division closely followed the absorption spectrum of nucleoportein. Another apparent cytoplasmic effect was demonstrated by Giese and Crossman (1946). Their studies with paramecia demonstrated an increased sensitivity to heat following sub-lethal treatment which indicated a cytoplasmic

effect. Another indication of penetration of the dye molecules was found in studies of the photosensitized inactivation of coliphage T-2. Helprin and Hiatt (1959) found that the rate of inactivation of the coliphage was a function of the time of incubation of the phage in the dye solution. This observation suggested the presence of a semi-permeable membrane around the T-even phages. Therefore, in the photosensitized inactivation of T-even phages, it appeared that the reaction was not limited to the surface of the organism. However, Yamamoto (1958), in studies of the kinetics of photodynamic inactivation of phage, found competitive inhibition of methylene blue photosensitization by crystal violet. He concluded that there were definite sites on the phage surface for which the dye molecules competed.

It appeared to Freeman and Giese (1952) that a combination of surface and cytoplasmic reactions took place in the photodynamic inactivation of yeast. They measured the oxygen uptake of living and steamed yeast cells and observed that there was a twenty minute lag before the living cells began photosensitized oxygen uptake, whereas there was no such lag period when dead cells were treated. They concluded that photodynamic injury began at the outside of the cell and progressed inward. This conclusion was reinforced by the demonstrated increase in the concentration of irreversibly bound dye that paralleled increase of exposure.

It appears then, that photodynamic action is a sum of many complex reactions that may take place simultaneously or separately at the surface or deep within a cell. The reaction appears photochemical in nature with complex kinetic relationships to the

concentrations of photosensitizer, substrate, and oxygen. The characteristics of the light, such as intensity and wave length, contribute to the rate of reaction as do the expected environmental factors, i.e., hydrogen ion concentration and cation or anion concentrations. Typical photochemical reactions do not show a change in reaction rate with a change in reaction temperature. There is some indication, however, that photodynamic action may be an atypical photochemical reaction in this respect, since the over-all reaction is dependent on temperature in a few instances. Since the other photochemical characteristics have not been determined in most investigations, a great deal has been left to speculation.

MATERIALS AND METHODS

Test Organism and Culture Conditions

The test organism in these experimenta was Escherichia coli B. Cultures were maintained in triplicate on nutrient agar slants at four degrees Centigrade and transfers made at ten to fourteen day intervals. Cultural purity was monitored by observation of the Gram stain reaction, cell morphology and colony appearance on eosin-methylene blue agar plates. Test cells were harvested from L-broth (10 g. Bacto Tryptone, 5 g. yeast extract, 5 g. NaCl and 1 g. glucose in 1000 ml. distilled water, pH 7.0) by centrifugation after eighteen to twenty hours incubation at 28° C in shaken 250 milliliter Erlenmeyer flasks. These cells were washed three times with cool, neutral 0.85 per cent sodium

chloride solution and then resuspended in 0.01 molar, pH 7.0 phosphate buffer (K_2HPO_4-KH_2PO_4). The cells were starved by aeration for one hour on a rotary shaker, washed and resuspended in either 0.01 M phosphate buffer or saline of appropriate test pH. The cells were stored at 4° C, for no longer than twenty-four hours, before testing. The test suspension, containing approximately 10^8 cells per milliliter, was prepared by diluting the stock suspension to the extent that a one to ten dilution of the test suspension registered an optical density of 0.20 at 600 mu in a Bausch and Lomb Spectronic 20 photoelectric colorimeter.

Dye Solutions

Dilute solutions of test dyes were prepared by dissolving desiccator dried dye in distilled water. Solutions of rose bengal (National Aniline and Chemical Co., C.I. 779, 88 per cent dye content) and methylene blue chloride (National Aniline and Chemical Co., 88 per cent dye content, certification number NA 4) of 8.76×10^{-4}M and 4.99×10^{-4}M concentrations respectively, were prepared as stock solutions. These were stored in glass stoppered reagent bottles at room temperature.

Light Source

The light energy for these experiments was supplied by General Electric, 150 watts, 120 volts tungsten filament projector flood lamps. A single lamp was used in small test volume experiments and a bank of four lamps, wired in parallel, was used when a large area was illuminated. The spectral emission of tungsten

light sources is primarily infra red (Hollsender, 1956). Very little input energy is converted into light of the visible spectrum range, and only a small fraction is emitted as ultra violet rays. Either deep stationary water filters or two centimeter deep running water filters were used to remove a major portion of the infra red rays. The glass lens of the lamp, the pyrex sides of the water filters and the reaction vessels effectively reduced the ultra violet irradiation to below significant levels.

Apparatus

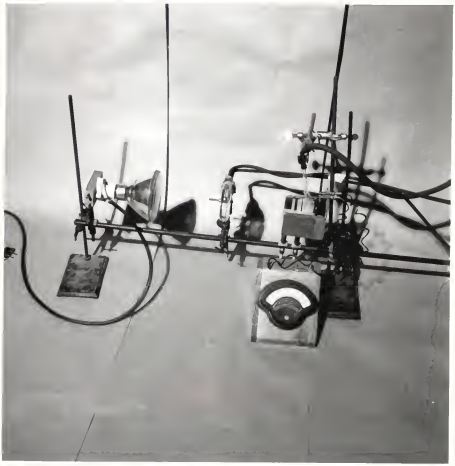
For short term illumination studies, the apparatus shown in Plate I was used. As Plate II illustrates, the equipment consisted of a light energy source (projector lamp), a running water filter to absorb the infra red radiations and a reaction vessel holder. These components were clamped to a horizontal rod supported by two ring stands. Compressed air was fed into the reaction flask through a capillary tube. The bubbling action thus produced supplied oxygen for the reaction and mixed the cell suspension to equalize the exposure of each cell. The reaction flask holder, as shown in Plate III in a schematic drawing, was constructed to hold a Carrel flask as the reaction vessel and a Luxtron photocell. When in use, the photocell was held in the compartment directly behind the reaction vessel. The light intensity was measured by observing the current produced across the poles of the photocell.

For long term illumination studies the running water filters were inadequate in absorbing the infra red irradiations. For

EXPLANATION OF PLATE I

A photograph of the equipment used to determine photodynamic inactivation of Escherichia coli B.

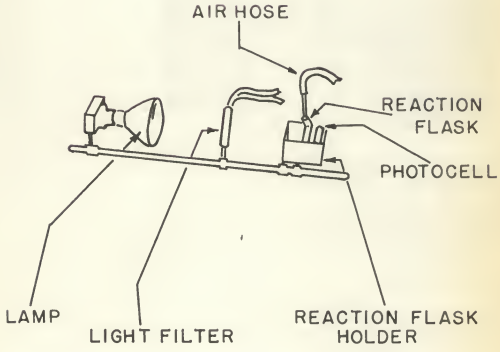
PLATE I



EXPLANATION OF PLATE II

An outline drawing showing the details of the equipment used to determine photodynamic inactivation of Escherichia coli B.

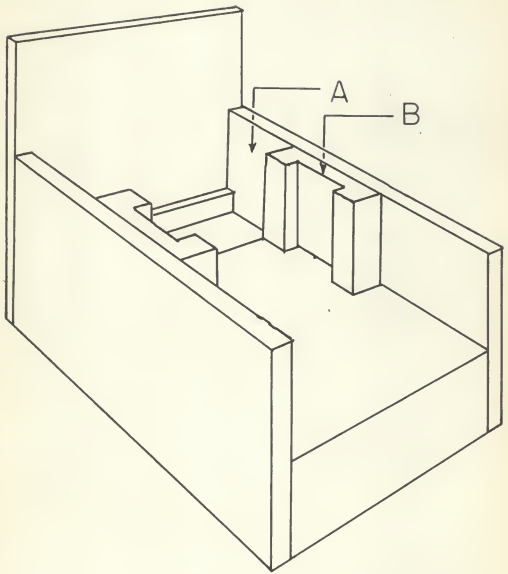
PLATE II


AIR HOSE
REACTION FLASK
PHOTOCELL
LAMP
LIGHT FILTER
REACTION FLASK HOLDER

EXPLANATION OF PLATE III

Details of the reaction flask and photocell support. The photocell was supported in compartment A; the flask in compartment B.

PLATE III



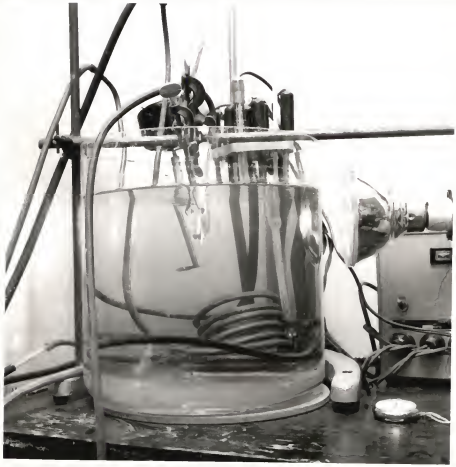
this reason the reaction flask was partially submerged in a constant temperature water bath. The photograph in Plate IV shows the arrangements by which the reaction vessel and the light source were clamped to the horizontal supporting rod. Temperature equilibrium was maintained in the water bath by the use of a copper tube cooling coil that carried cold tap water.

Manometric measurement of photooxidation was made with the use of a modified Warburg water bath as shown in Plate V. A light bar, consisting of four General Electric projector flood lamps attached to a wooden beam, was secured over the center of the water bath. The lamps were ten centimeters from the surface of the water. The interior of the water tank was covered with heavy aluminum foil to reflect and therefore distribute the light throughout the water bath. The temperature of the waterbath was held at thirty plus or minus two tenths degree Centigrade ($30\pm0.2^{\circ}$ C) by augmenting the temperature controlling heating element with a cooling coil. Constant volume Warburg manometers were used with fifteen milliliter reaction flasks. In all experiments, the volume of the fluid in each flask was three and two tenths milliliters. Three milliliters of the fluid were the reaction mixture that consisted of selected proportions of dye, cell suspension, substrate and/or buffer. The carbon dioxide evolved during the reaction was adsorbed by 10 per cent potassium hydroxide that saturated a cylinder of filter paper supported by the center well of the reaction flask.

EXPLANATION OF PLATE IV

Apparatus used in long term illumination studies showing the immersed reaction flask, the water bath, and the external light source.

PLATE IV



EXPLANATION OF PLATE V

Arrangements of lights used with the Warburg apparatus in manometric studies of photodynamic oxygen uptake studies.

PLATE V

Determination of Photodynamic Inactivation

Methylene blue or rose bengal solution was added to a fifteen milliliter sample of a test suspension of E. coli B. The amount of dye added to the cell suspension varied with the test. In tests with rose bengal, the pH was 6.0 and with methylene blue the pH was 7.5. The dye-bacteria mixture was then placed in the reaction vessel and aerated for five to ten minutes to assure equilibration of temperature and dye uptake by the bacterial cells. During the equilibration, an opaque shield protected the cell suspension from the light. The light source was also allowed an equilibrating period. A stop watch was used to determine the time of exposure. After treatment, a one milliliter aliquot of the test cell suspension was removed and diluted in a ninety-nine milliliter water blank. This dilution stopped the reaction. The remainder of the cell suspension was used for other tests to be described in the following sections. Further dilutions and pour plates were made in triplicate using nutrient agar as the plating medium. The same reaction flask was used for all the determinations. The flask was rinsed with two small quantities of test suspension before each determination. This eliminated any variables caused by the poor optical quality of the reaction flasks' faces. Randomization of samples with respect to exposure time, controlled sequence effect. No efforts to maintain aseptic or sterile techniques were made other than in cultural work. With such a large number of organisms, 10^8 per milliliter, the few contaminants that would become part of the population under study

were not significant. The plates were incubated in the dark at thirty-seven degrees Centigrade for forty-eight hours and then the colonies counted with the aid of a Quebec colony counter. The average of these counts was used as the viable cell count of the survivors. Two controls were maintained. The first was a dark control that monitored any possible change in the number of cells resulting from any toxic reaction due to the dye alone. An aliquot of the first sample placed in the reaction flask was used to determine a zero time count and an aliquot of the sample that was used for the final test sample, after an aeration period equal to the maximum time that any sample was treated in the reaction flask, was used as a maximum time control. In this manner the control was biased toward a maximum possible dark reaction. Also, the effect of the light alone on the cell suspension was controlled. A fifteen milliliter sample of the test suspension of cells, without dye, was placed in the reaction vessel and illuminated for a period of time equal to the maximum time period of illumination given to any of the samples that were tested. The viable cell count of samples taken at the beginning and end of illumination was determined.

Dye Adsorption

To determine quantitatively the changes of the concentration of dye in solution in equilibrium with the dye adsorbed to the bacterial cell, the adsorption curves of the test dyes in the visible and ultra violet spectral ranges were determined with a Beckman D U spectrophotometer. The maxima were recorded and the

change in optical density as a function of concentration was measured. This information was used to calculate the molar extinction coefficients for each dye at each maximum. Also, the absorption curves obtained served as standards of comparison and were used to determine any change in the absorption curves of the dyes caused by the reaction under study. Since methylene blue is easily reduced, the absorption curve and the molar extinction coefficient were determined on totally reduced dye. Ten milliliters of stock methylene blue were placed in a one-hundred milliliter volumetric flask. The dye was reduced by heating this sample of dye to boiling after the addition of one gram of sodium sulfite. Constant aeration of this reduced dye solution for five hours failed to cause any discernible oxidation. However, dilution in distilled water aided oxidation of the leuco form of the dye. For this reason, dilutions were carried out in one per cent sodium sulfite solution that had been boiled and cooled. This solution also served as a blank in the spectrophotometric determination of absorption spectrum and molar extinction coefficient.

Using the absorption maxima obtained with the Beckman D U spectrophotometer, dye optical density - concentration relationships were determined in a Bausch and Lomb Spectronic 20 photoelectric colorimeter. When a large number of dye concentration determinations were made, the Bausch and Lomb instrument was used. The Beckman D U was employed for the determination of changes in absorption curves and measurement of changes in concentration of ultra violet absorbing compounds, i.e., the change in the ultra violet absorption curves of the dyes.

The determination of dye concentration changes and absorption spectrum alternations were made on the same samples used in the determination of photosensitized inactivation. Twelve milliliter samples of this test suspension, after withdrawal of the dilution sample, were placed in fifteen milliliter centrifuge tubes. After sedimentation of the cells by centrifugation at high speed for fifteen minutes in a Sorvall angle head centrifuge, the clear supernatant was examined with either the Spectronic 20 or the Beckman D U.

Another characteristic of dye adsorption by treated and control cells was evaluated by the study of adsorption isotherms. For these investigations, a large volume of treated cells was obtained by substituting a one-hundred milliliter Kolle flask for the reaction vessel usually employed. These cells were illuminated under the same conditions that were maintained for small volume experimentation but for a longer duration. Five milliliter aliquots of test cell suspension, both treated and control, were added to selected amounts of dye solution in centrifuge tubes. The concentration of unadsorbed dye was determined by calculating the amount of dye in the supernatant. After cell sedimentation, the number of viable cells in each milliliter of test suspension was determined. The amount of dye adsorbed by the cells was calculated and the information was used in the determination of adsorption isotherms.

RESULTS

Inactivation Curves

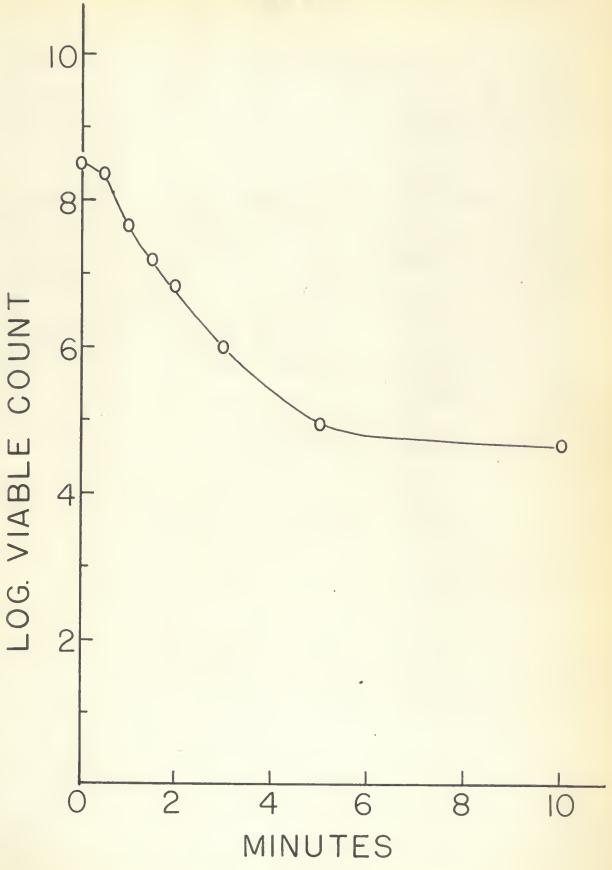
Character of Curves. The effect of a photodynamic action on E. coli B cells was partially characterized by the determination of the decrease in viable cell count as a function of exposure time. Death curves were determined to establish the presence of typical photodynamic action. The decrease in the number of viable cells, shown in Plate VI, was a non-linear function of exposure time on a semi-logarithmic plot. After a lag the inactivation proceeded at a decreasing rate until a minimum was reached. This minimum appeared as a plateau that occurred after the viable count was decreased by more than 99.99 per cent.

Light Intensity. Inasmuch as light intensity is an important variable in photodynamic action, death curves were determined at different light intensities to ascertain the effect they had on the extent of the lag at the initiation of the reaction and on the level of the plateau near the end of the reaction. Different light intensities were achieved by placing the light source at distances of 30, 35, 45, and 60 centimeters from the reaction vessel. The effects these changes had on the character of the inactivation are shown in Plate VII. At a distance of 60 centimeters inactivation was negligible. As the distance was decreased, the lag shortened and the rate of inactivation increased. However, the rapid inactivation rate at the 30 centimeter distance was interrupted by a high plateau at which the per cent decrease in the number of test cells was relatively low. Therefore, an

EXPLANATION OF PLATE VI

The decrease in visble cell counts of Escherichia coli B during photodynamic inactivation with methylene blue.

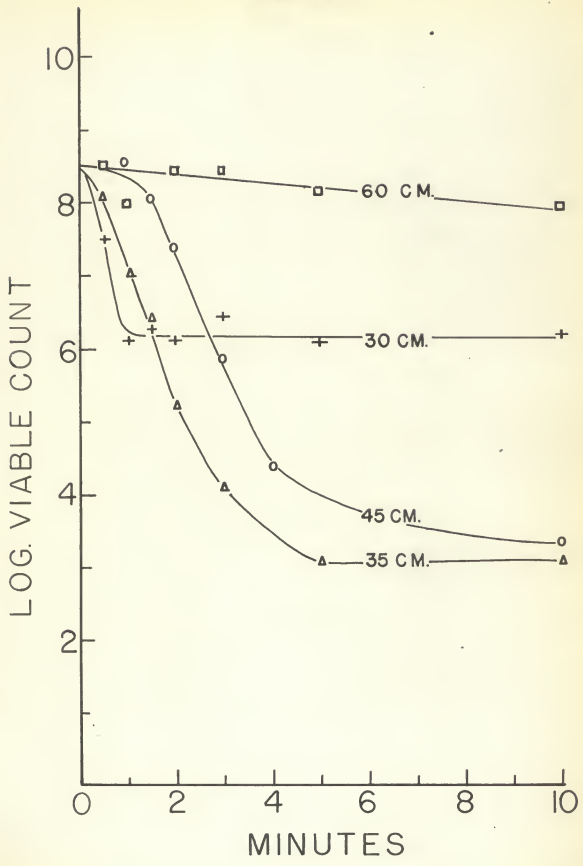
PLATE VI



EXPLANATION OF PLATE VII

The characteristics of Escherichia coli B inactivation curves at light to reaction flask distances of 30, 35, 45, and 60 cm.

PLATE VII


10
8
6
4
2
LOG. VIABLE COUNT
60 CM.
30 CM.
45 CM.
35 CM.
0
2
4
6
8
10
MINUTES

intermediate distance of 40 centimeters was chosen as a standard test distance. In this way the intensity of light was held constant.

The data used to construct the inactivation curves shown in Plate VI and VII are presented in appropriately labeled tables in the appendix.

Dye Absorption Changes

Methylene Blue Absorption Spectrum. The absorption spectrum of methylene blue in aqueous solution, shown in Plate VIII, was characterized by absorption maxima at 246 mu and 291 mu in the ultra violet and 610 mu and 662 mu in the visible range. The optical density readings at the wave lengths at which observations were made are shown in Table 1. The maxima were not altered in position by the addition of 0.2 M phosphate buffer at pH between 7.5 and 6.0 or the addition of sodium chloride (to 0.85 per cent). The wave lengths of the peaks compared well with the values of 667.5 mu and 608.4 mu given in the International Critical Tables (1930).

Light Induced Spectrum Changes. Observations were made of the changes in absorbancy at the three principal wave lengths of 662 mu, 291 mu and 246 mu, that occurred in the supernatants of cell suspensions subjected to photodynamic action. The percentages of change in optical density that were calculated from experimental measurements, as shown in Table 2, were plotted against the duration of illumination. These curves, presented in Plate IX, indicated striking changes in the concentration of unadsorbed dye.

EXPLANATION OF PLATE VIII

The light absorption spectrum of an aqueous solution of methylene blue.

PLATE VIII

Table 1. Optical density values of 1.497×10^{-4}M methylene blue at wave lengths between 200 mu and 700 mu.

mu	O.D.	mu	O.D.	mu	O.D.	mu	O.D.
700	.075	605	.600	300	.440	246.5	.286
690	.195	600	.550	292	.612	246	.290
680	.490	590	.405	291.5	.612	245.5	.286
675	.705	580	.290	291	.615	245	.285
670	.895	560	.160	290	.600	244	.275
665	1.030	540	.080	289	.585	242	.260
662	1.060	520	.050	288	.570	240	.230
660	1.050	500	.045	286	.530	238	.210
655	1.000	480	.033	285	.510	235	.170
650	.950	460	.020	280	.400	230	.140
640	.775	440	.010	275	.315	225	.125
630	.665	400	.013	270	.250	220	.120
625	.643	380	.013	265	.195	215	.132
620	.625	360	.017	260	.170	210	.142
615	.623	340	.055	255	.193	205	.170
612	.630	320	.150	250	.255	200	.200
610	.628	310	.200	247	.283		

Table 2. Photodynamic changes in absorbancy in supernatant of a reaction mixture consisting of 2.2×10^8 cells ml^{-1} 1.25×10^{-5}M methylene blue and buffer, pH 7.5.

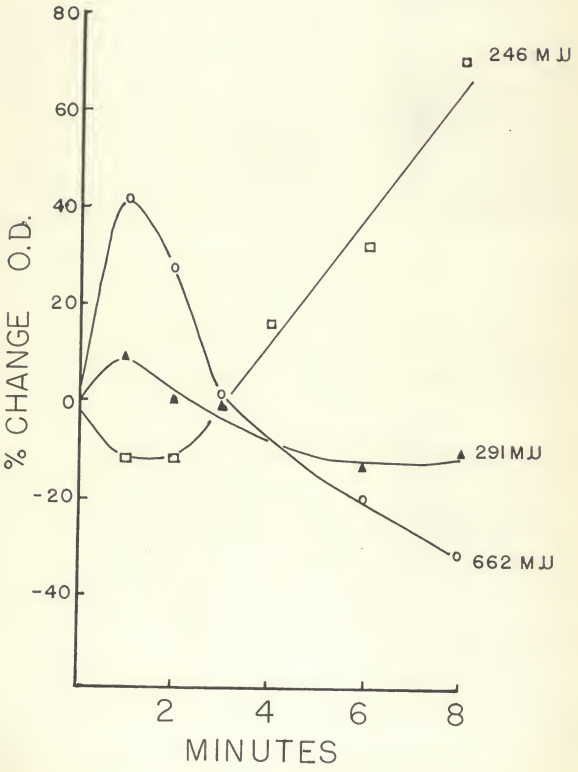
Time	A_{662}	% Change	A_{291}	% Change	A_{246}	% Change
0	.450	0.0	.357	0.0	.430	0.0
1	.640	42.2	.390	9.0	.380	-11.6
2	.575	27.8	.360	0.8	.380	-11.6
3	.460	2.2	.355	-0.6	.500	16.3
6	.360	-20.0	.288	-19.3	.570	32.6
8	.310	-31.1	.325	-8.7	.735	70.9

However, in an experiment that was designed to control the changes in absorbancy of methylene blue caused by light alone, a rapid decrease in absorbancy at 662 mu was observed. The change was

EXPLANATION OF PLATE IX

Changes in light absorption of reaction mixture supernatants that accompanied the methylene blue-sensitized photodynamic inactivation of Escherichia coli B.

PLATE IX


80
60
40
20
0
-20
-40
% CHANGE O.D.
246 MJJ
291 MJJ
662 MJJ
2
4
6
8
MINUTES

restricted to this wave length and occurred in phosphate buffer but not in saline. As shown in Plate X and in Table 3, this change appeared to be a constant linear function of time. Another sample of methylene blue in phosphate buffered solution was illuminated for ten minutes and the absorption spectrum between 200 mu and 700 mu determined. No change in the ultra violet absorption curve was found and an interesting shift in the position of the visible spectrum maximum was observed. The absorption curve, shown in Plate XI, had a maximum at 645 mu.

Table 3. Changes in observed values of absorbancy in methylene blue solutions caused by illumination.

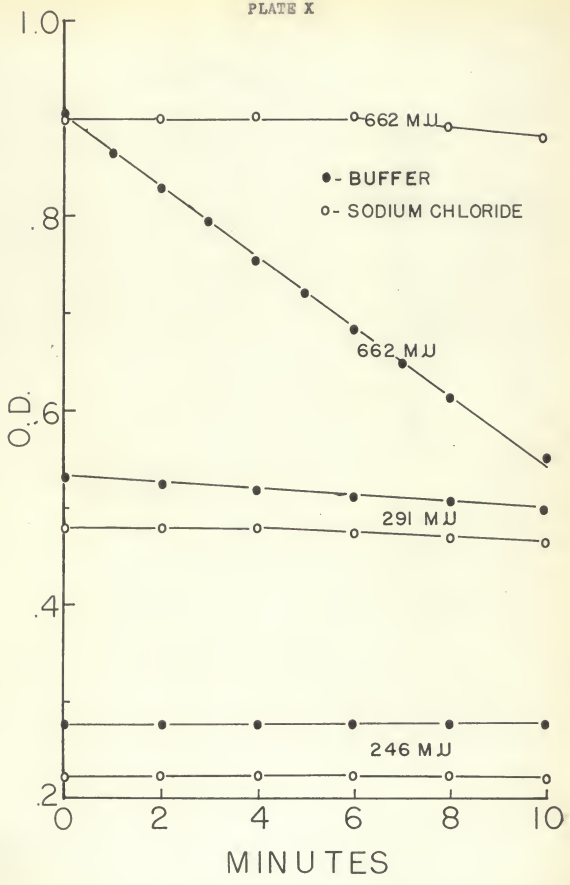
	Phosphate Buffer Solution			Sodium Chloride Solution		
Minutes	A_{662}	A_{291}	A_{246}	A_{662}	A_{291}	A_{246}
0	0.905	0.532	0.280	0.905	0.480	0.225
1	0.865	-	-	-	-	-
2	0.830	0.528	0.275	0.910	0.480	0.228
3	0.795	-	-	-	-	-
4	0.755	0.520	0.275	0.905	0.478	0.228
5	0.723	-	-	-	-	-
6	0.685	0.514	0.280	0.900	0.475	0.231
7	0.650	-	-	0.890	-	-
8	0.615	0.508	0.280	0.880	0.470	0.227
10	0.555	0.508	0.275		0.465	0.227

The role of this "blue shift" in the changes in absorbancy that occurred in photodynamically treated cell suspension supernatants was investigated. Absorption measurements of supernatants from illuminated and non-illuminated cell suspensions in buffered methylene blue solution produced the visible spectrum curves shown in Plate XII. After eight minutes of illumination two

EXPLANATION OF PLATE X

The effect of illumination on phosphate buffered and saline solutions of methylene blue.

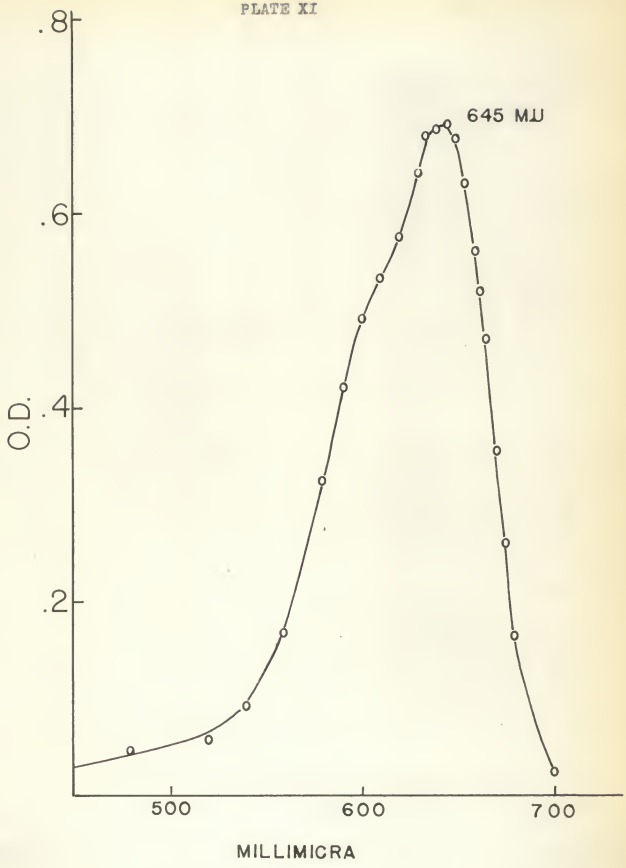
PLATE X


1.0
.8
.6
.4
.2
O.D.
662 MJJ
●- BUFFER
o- SODIUM CHLORIDE
662 MJJ
291 MJJ
246 MJJ
0
2
4
6
8
10
MINUTES

EXPLANATION OF PLATE XI

The post illumination spectrum of buffered methylene blue solution showing the extent of the blue shift from the normal maximum at 662 mu.

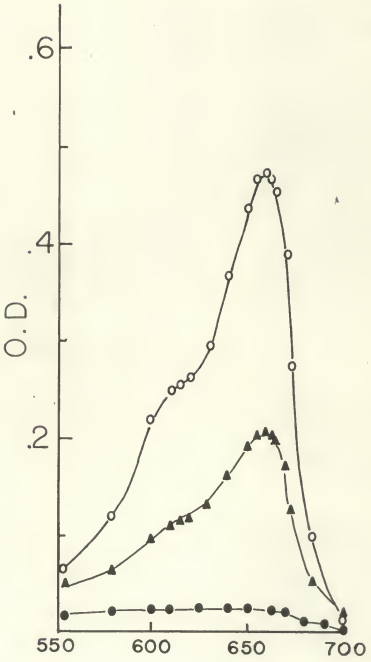
PLATE XI



EXPLANATION OF PLATE XII

The visible absorption spectra of normal (open circles) chemically reduced (closed circles) and photodynamically treated (triangles) methylene blue solutions.

PLATE XII



changes were apparent. A notable change was the marked decrease in absorbancy at 662 mu. The concomitant blue shift was minor; it changed the maximum only 2 mu to a new peak at 660 mu. The more notable change in the absorption spectrum occurred in the ultra violet range. Plate XIII shows the observed disappearance of the 246 mu and 291 mu peaks and the appearance of a new peak at 255 mu. These changes in the absorption spectrum of supernatants were observed in both sodium chloride and phosphate buffer solutions that served as suspending media. Table 4 shows the changes observed in phosphate buffer supernatants.

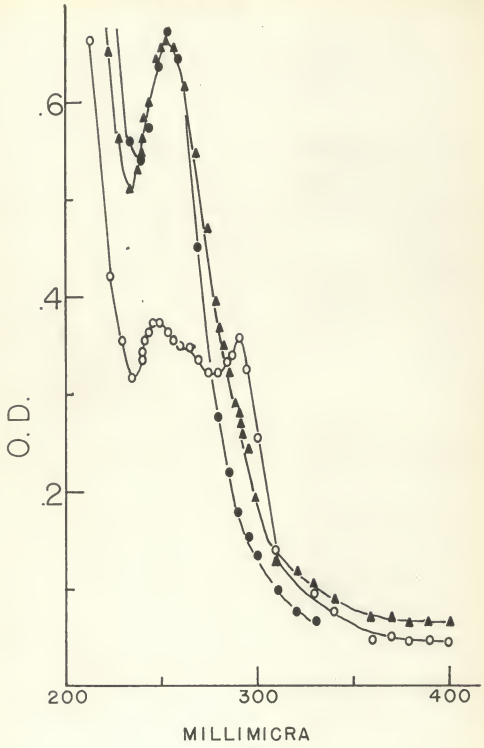
Table 4. Spectra of illuminated and non-illuminated methylene blue phosphate buffer solutions.

Time (minutes)			Time (minutes)		
mu	0	8	mu	0	8
700	0.015	0.020	300	0.255	0.193
685	0.100	0.055	295	0.326	0.241
675	0.275	0.130	292	0.350	0.270
670	0.390	0.175	291	0.357	0.280
665	0.445	0.200	290	0.356	0.290
662	0.473	0.205	286	0.338	0.325
660	0.470	0.207	284	0.333	0.348
655	0.468	0.205	282	0.325	0.370
650	0.438	0.192	280	0.320	0.395
640	0.365	0.162	275	0.323	0.470
630	0.292	0.132	270	0.335	0.550
620	0.263	0.120	265	0.348	0.615
615	0.258	0.118	260	0.350	0.650
610	0.250	0.110	256	0.356	0.662
600	0.215	0.098	255	0.362	0.662
580	0.120	0.068	254	0.363	0.662
560	0.065	0.050	252	0.370	0.653
400	0.043	0.066	250	0.373	0.648
390	0.046	0.068	246	0.375	0.600
380	0.046	0.068	244	0.365	0.582
370	0.050	0.073	242	0.355	0.560
360	0.048	0.075	241	0.343	0.550
340	0.073	0.093	240	0.335	0.535
330	0.098	0.105	235	0.315	0.512
320	0.115	0.116	230	0.355	0.562
310	0.140	0.133	225	0.420	0.650

EXPLANATION OF PLATE XIII

The ultra violet absorption spectra of normal (open circles), chemically reduced (closed circles) and photodynamically treated (triangles) methylene blue solutions.

PLATE XIII



In an attempt to explain the origin of the 255 mu absorption peak, methylene blue was reduced by sodium sulfide and the absorption spectrum of the reduced dye determined. The reduced dye had no appreciable absorbance in the visible range. However, the ultra violet absorption curve of the chemically reduced dye matched the photodynamically-produced ultra violet absorption curve. The comparisons are shown in Plates XII and XIII. To rule out ultra violet-absorbing material derived from bacterial cells as the origin of the 255 mu peak, the ultra violet absorbancy of a cell suspension was measured. The ultra violet absorption maximum was at 255 mu. However, a large number of cells, 1.2×10^7 per milliliter, was necessary to give appreciable ultra violet absorption - many more than would be expected to remain in the supernatant after centrifugation.

Dye Reduction and Cell Inactivation

Photodynamic Reduction. To determine the relationship between methylene blue reduction and photodynamic inactivation of E. coli B cells, a series of concomitant measurements of methylene blue absorbancy changes and cell death rates were made. The results of one such set of determinations of absorbancy changes, as shown in Plate XIV, illustrates the changes that occurred at 662 mu, 291 mu, 255 mu and 246 mu. The changes in absorbancy that were observed at 662 mu, 291 mu and 246 mu were of the same order as those observed previously. The absorbancy readings and the calculated per cent change of absorbancy are shown in Table 5.

EXPLANATION OF PLATE XIV

Changes in the light absorption of reaction mixture supernatants that accompanied the methylene blue-sensitized photodynamic inactivation of Escherichia coli B.

PLATE XIV

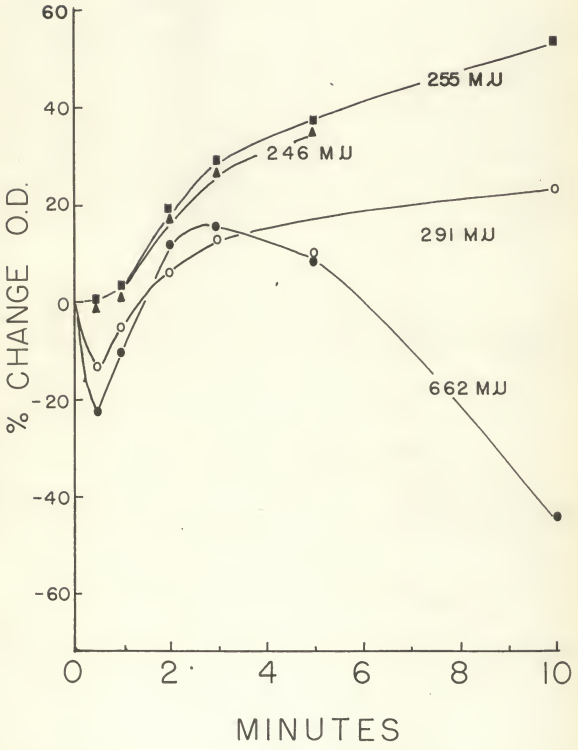

60
40
20
0
-20
-40
-60
% CHANGE O.D.
255 MJJ
246 MJJ
291 MJJ
662 MJJ
0
2
4
6
8
10
MINUTES

Table 5. Percentage change of optical densities of methylene blue that was observed during photodynamic inactivation of E. coli B.

Minutes	A_{662}	%	A_{291}	%	A_{255}	%	A_{246}	%
0	.290	0	.305	0	.600	0	.550	0
0.5	.225	-22.4	.265	-13.1	.600	0	.541	-0.9
1	.260	-10.4	.290	-4.9	.620	3.3	.570	3.6
2	.325	12.1	.325	6.6	.710	18.3	.650	18.2
3	.335	15.5	.345	13.1	.775	29.2	.710	29.1
5	.320	10.3	.335	9.8	.820	36.7	.735	33.6
10	.162	-44.1	.375	22.9	.920	53.0	-	-

The increase in absorbancy at 255 mu had the characteristics of an inverted inactivation curve. When the logarithm of the number of surviving cells and the inverted optical density changes were plotted against the same time axis as shown in Plate XV, Figure 1, the similarity in the curves was apparent. The lag periods were approximately the same in length and the curvatures were of similar magnitude. These data were transformed to linear relationships by plotting the logarithm of the number of surviving cells and the inverted optical density changes against the logarithm of the minutes of exposure time. In this log-log plot shown in Plate XV, Figure 2, the lag period was omitted.

Statistical Analysis. When the optical density values at each time interval were plotted against the logarithm of the number of surviving cells at the corresponding time of exposure, the plot shown in Plate XVI was obtained. This plot suggested a linear correlation between the measured variables.

By the method of least squares (Daniels, 1928), an expression

EXPLANATION OF PLATE XV

Fig. 1. The photodynamic increase in optical density of reduced methylene blue and decrease in viable cell count as a linear function of time.

Fig. 2. The photodynamic increase in optical density of reduced methylene blue and decrease in viable cell count as a logarithmic function of time.

PLATE XV

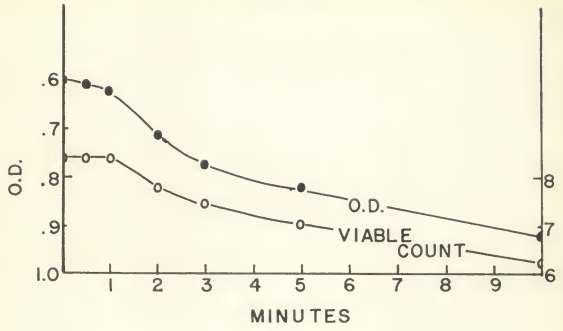


FIG. 1

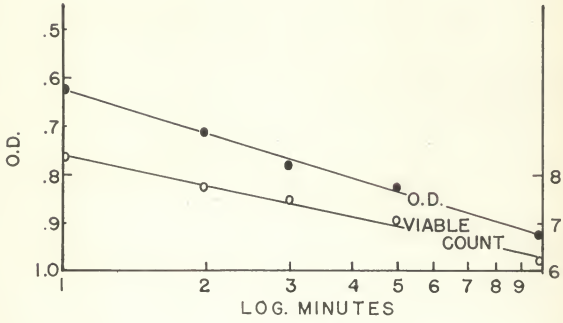
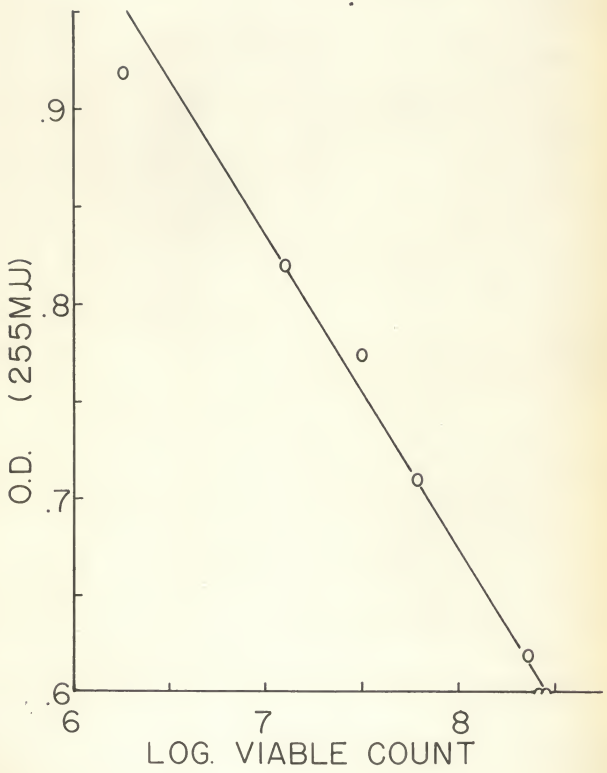


FIG. 2

EXPLANATION OF PLATE XVI

Correlation of optical densities of reduced methylene blue and values of logarithm of viable count.

PLATE XVI



for the change in optical density as a function of the logarithm of the exposure time was calculated. Equation 1 represents the reduction of the logarithmic function to an equation of the first degree.

(1) $O.D. = 0.297 \log t + 0.622$

As shown in Table 6, this equation was used to transform calculated values of optical density into the curvilinear function. The experimentally determined values of optical density fit this calculated equation as shown in Plate XVII, Figure 1. Statistically, the calculated equation fit the experimental results significantly at a probability of less than 0.01.

Table 6. Calculated and observed values of optical density.

Minutes	Calculated O.D.	Observed O.D.
1	.622	.620
2	.711	.710
3	.764	.775
4	.801	-
5	.829	.820
6	.853	-
7	.873	-
8	.890	-
9	.950	-
10	.919	.920

Similar calculations were made with the data on cell inactivation. Equation 2 represents the change in the logarithm of the number of surviving cells as a function of the logarithm of exposure time.

(2) Log No. = -2.07 log t + 8.443

Plate XVII, Figure 2, shows the plots of the calculated and observed values. As shown in Table 7, these values agreed well. Also, equation 2 was a good statistical fit.

With the data obtained from these least squares calculations, the correlation coefficient (Snedecor, 1956) was calculated. The value obtained, -0.993, demonstrated a significant correlation between the increase in optical density at 255 mu and the decrease in the viable cell count.

Table 7. Calculated and observed values of log. number of survivors of photodynamic action.

Minutes	Calculated Log. No.	Observed Log. No.
1	8.44	8.38
2	7.82	7.79
3	7.46	7.50
4	7.19	-
5	6.99	7.11
6	6.83	-
7	6.69	-
8	6.57	-
9	6.47	-
10	6.37	6.26

Upon repeating this experiment, the results shown in Plate XVIII were obtained. These data, as in the previous experiment, gave straight line plots when the optical density or the logarithm of the number of surviving cells was plotted against the logarithm of the minutes of treatment. Equations 3 and 4 were calculated and found to fit the experimental results significantly.

EXPLANATION OF PLATE XVII

Fig. 1. The fit of observed values to the curve calculated from: $O.D. = 0.297 \log t + 0.622$.

Fig. 2. The fit of observed values to the curve calculated from: $\text{Log No.} = -2.07 \log t + 8.443$.

PLATE XVII

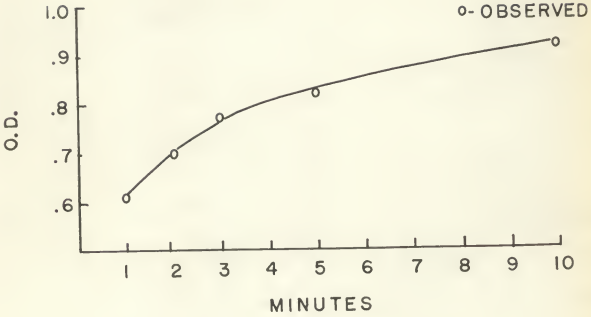


Fig. 1

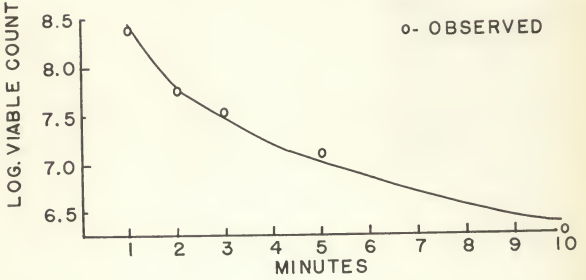
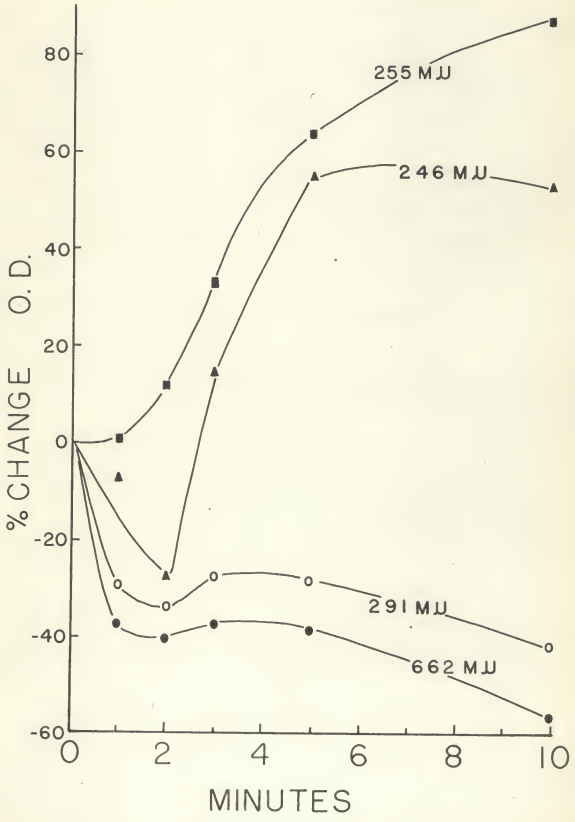


Fig. 1

EXPLANATION OF PLATE XVIII

Changes in light absorption of reaction mixture supernatants that accompanied methylene blue-sensitized photodynamic inactivation of Escherichia coli B.

PLATE XVIII


80
60
40
20
0
-20
-40
-60
% CHANGE O. D.
255 MJJ
246 MJJ
291 MJJ
662 MJJ
0
2
4
6
8
10
MINUTES

(3) Log. No. = -1.269 log. t + 7.56

(4) O.D. = 0.252 log. t + 0.274

With these data the coefficient of correlation was calculated as -0.984, which was significant at a probability of 0.05. The comparison between the experimental and calculated values is shown in Tables 8 and 9. Tables showing the calculation of the regressions and of the correlations appear in the appendix.

Table 8. Calculated and observed values of optical density at 255 mu measured during photodynamic inactivation of E. coli B.

Minutes	Calculated O.D.	Observed O.D.
1	.374	.380
2	.449	.405
3	.494	.480
4	.526	-
5	.550	.590
6	.570	-
7	.587	-
8	.601	-
9	.614	-
10	.626	.670

Manometric Studies

Photodynamic Oxygen Consumption. Photodynamic uptake of oxygen by living cells was very small, compared to endogenous respiration, in a three milliliter reaction mixture that contained 5×10^8 cells, 9.8×10^{-6}M methylene blue and phosphate buffered to pH 7.5. However, cells that were inactivated by heat at 60° C for thirty minutes and then incorporated into a reaction

mixture identical to that used with living cells, did show a definite oxidative response to illumination. Plate XIX shows oxygen consumed by 5×10^8 dead cells. As a comparison, the oxygen uptake of 0.629 mg of l-tyrosine was also plotted in Plate XIX.

Table 9. Calculated and observed values of log. number of survivors of photodynamic action.

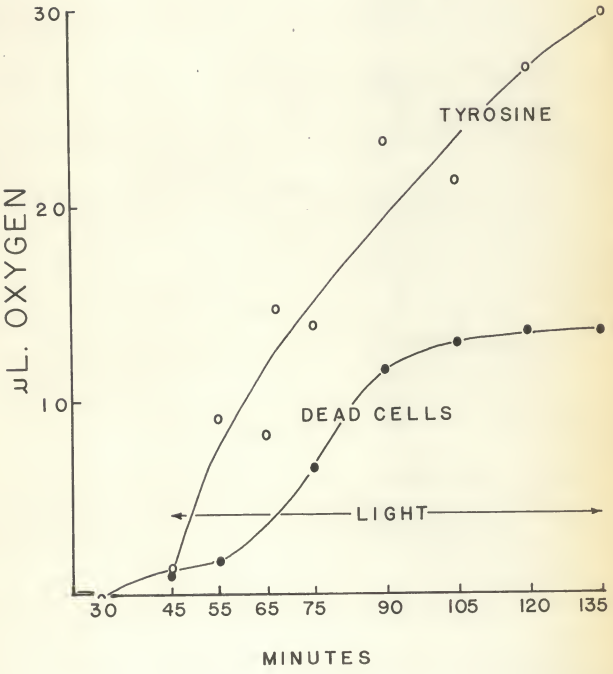
Minutes	Calculated Log. No.	Observed Log. No.
1	7.56	7.43
2	7.18	7.26
3	6.96	7.08
4	6.79	-
5	6.67	6.72
6	6.57	-
7	6.49	-
8	6.41	-
9	6.35	-
10	6.29	6.18

The low order of oxygen uptake by dead cells that had been sensitized by methylene blue suggested an examination of photodynamic oxygen uptake with a more active dye, rose bengal. Cells were either pretreated with dye or photodynamic action. A sample of dead cells was placed in buffered rose bengal solution at pH 6.0. The final concentration of dye was 1.75×10^{-5}M. This mixture was divided into two parts; one was illuminated for ten minutes while the other was stored in the dark for the same period of time. After this pretreatment, both samples were washed in pH 7.0 buffer. The supernatant of the fourth washing gave no visual evidence that indicated the presence of dye. Plate XX

EXPLANATION OF PLATE XIX

Oxygen uptake by l-tyrosine and dead Escherichia coli B cells during rose bengal-sensitized photodynamic action.

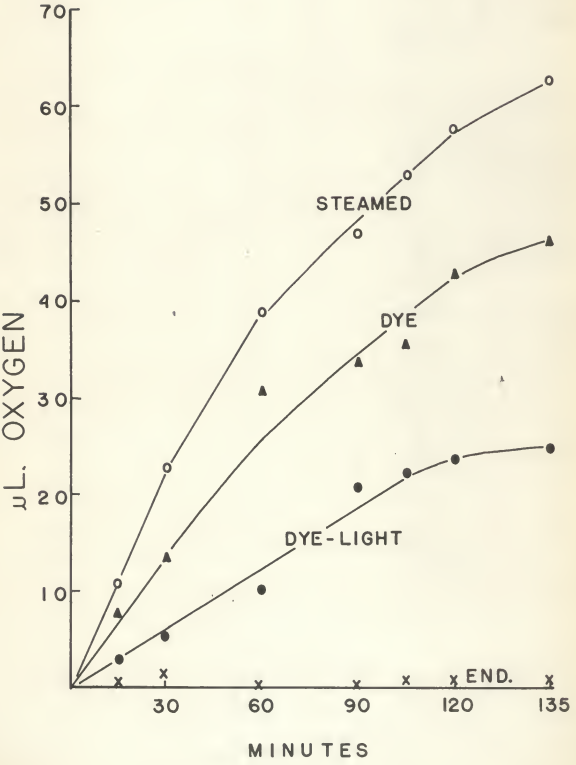
PLATE XIX


30
20
10
µL. OXYGEN
TYROSINE
DEAD CELLS
LIGHT
30
45
55
65
75
90
105
120
135
MINUTES

EXPLANATION OF PLATE XX

Rose bengal-sensitized oxygen uptake of steamed, dye pretreated and photodynamically pretreated cells.

PLATE XX


70
60
50
40
30
20
10
µL. OXYGEN
STEAMED
DYE
DYE-LIGHT
END.
30
60
90
120
135
MINUTES

shows the photodynamic oxygen consumption of dead cells, dye pretreated cells, and photodynamically pretreated cells. The reaction mixture, buffered with phosphate to pH 6.0, contained 4×10^8 cells and 1.46×10^{-5}M rose bengal.

Cell suspensions of E. coli B that were actively oxidizing glucose reacted to photodynamic action as shown in Plate XXI. Buffered rose bengal was dumped from the side arm of the reaction flask and mixed with the contents of the main compartment. The reaction mixture then consisted of 7.5×10^7 cells, 0.07 M glucose and 1.46×10^{-5}M rose bengal. The dye was dumped at 10, 15, and 20 minute intervals before the illumination was interrupted at 65 minutes. After 55 minutes, the flasks were again illuminated for the final 60 minutes of observation. Since the dye may initiate deleterious reactions without absorption of light energy, a dark reaction control was observed. The reaction flask was masked with aluminum foil to exclude all light.

Dye Adsorption

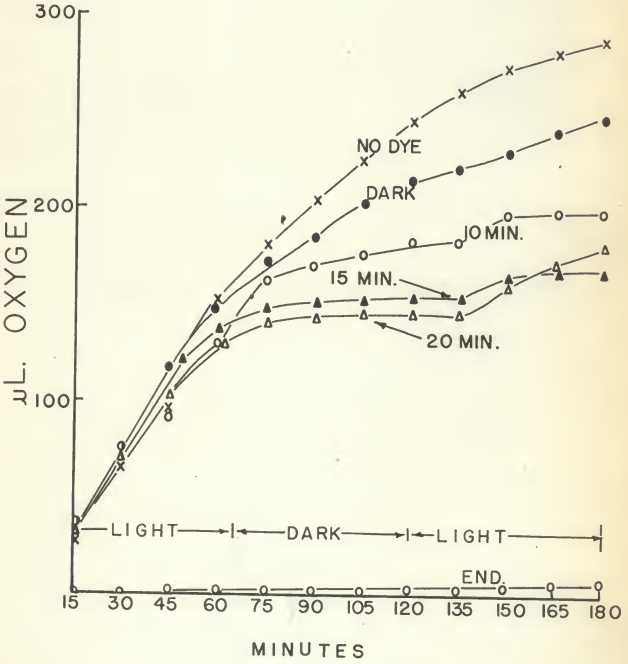
Rose bengal was diluted in a buffered suspension of E. coli B and illuminated. The changes in absorbancy of the supernatant was measured at 545 mu, the absorption maximum of the dye. The uptake of rose bengal in moles per cell was calculated. Table 10 shows the calculated decrease in concentration of the dye in the supernatant and the apparent increase in uptake of the dye by the cells that resulted from photodynamic action. Plate XXII illustrates this uptake.

Cells subjected to photodynamic action in rose bengal solutions

EXPLANATION OF PLATE XXI

The effect of rose bengal and light on the oxygen uptake of suspensions of Escherichia coli B in buffered glucose solutions.

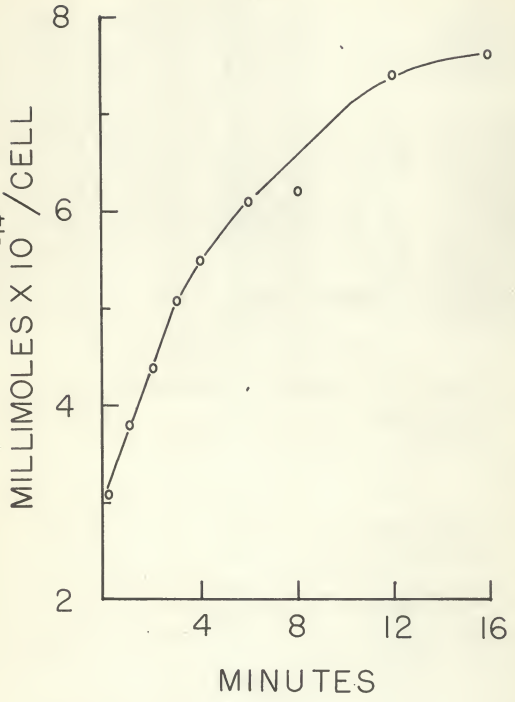
PLATE XXI


300
200
100
µL. OXYGEN
NO DYE
DARK
10 MIN.
15 MIN.
20 MIN.
LIGHT
DARK
LIGHT
END
15
30
45
60
75
90
105
120
135
150
165
180
MINUTES

EXPLANATION OF PLATE XXII

The uptake of rose bengal by *Escherichia coli* B cells during rose bengal-sensitized photodynamic action.

PLATE XXII



were capable of adsorbing methylene blue. Rose bengal-treated cells were mixed with methylene blue solutions buffered to pH 7.5. The concentration of methylene blue in the supernatant from these cell suspensions was determined by observing the optical density at 662 mu. Although this procedure did not elute all of the rose bengal and thereby allow maximum adsorption of methylene blue, it did measure the change in the ability of the cell suspension to adsorb methylene blue as a result of photodynamic action. Plate XXIII shows the increase in the number of millimoles of dye adsorbed to each cell that resulted from rose bengal-sensitized photodynamic action. The only changes in the absorption spectrum of rose bengal during such experiments were general decreases in absorbancy at the 545 mu maximum and at the 240-260 mu shoulder. These changes were due to decrease in concentration of the dye as a result of adsorption to bacterial cells.

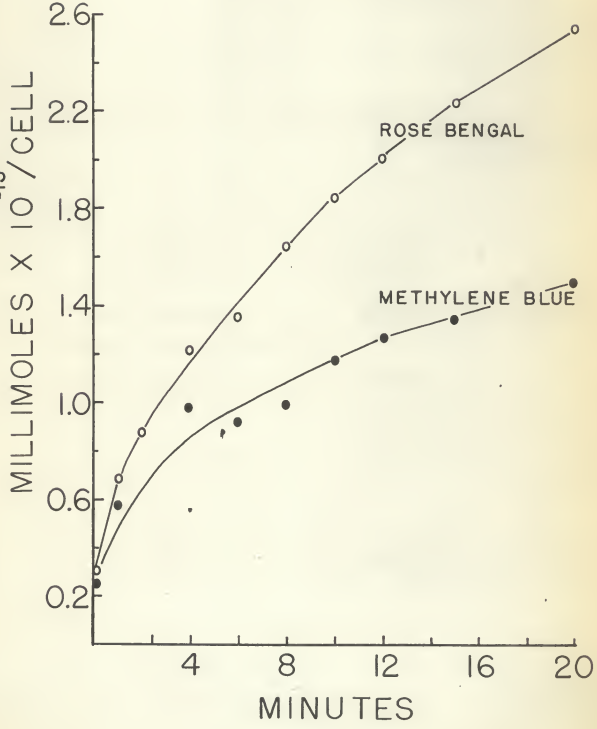
Table 10. The adsorption of rose bengal by 2.2 X 10^{8} cells/ml. from 2.9 X 10^{-5}M dye solution.

Minutes	Supernatant Conc. X 10^{-5}M	Dye Adsorbed X 10^{-14}mM/Cell
0	2.22	3.1
1	2.05	3.8
2	1.94	4.4
3	1.78	5.1
4	1.69	5.5
6	1.56	6.1
8	1.53	6.2
12	1.28	7.4
16	1.23	7.6

EXPLANATION OF PLATE XXIII

The uptake of rose bengal and methylene blue by Escherichia coli B cells as a result of rose bengal-sensitized photodynamic action.

PLATE XXIII


2.6
2.2
1.8
1.4
1.0
0.6
0.2
MILLIMOLES X 10 -13 /CELL
ROSE BENGAL
METHYLENE BLUE
4
8
12
16
20
MINUTES

Adsorption Isotherms

The amount of rose bengal adsorbed by cells from several concentrations of buffered dye solutions was determined. Two sets of determinations were made. In the first set, a comparison of dye adsorption was made between living cells and photodynamically treated cells. In the second set, the comparison was made between living and heat-killed cells.

In the measurements with photodynamically treated cells, the sensitizing dye, rose bengal, was diluted in buffered cell suspension to a final concentration of 2×10^{-5}M. The dye-bacteria mixture was divided into one-hundred milliliter aliquots. One was illuminated for thirty minutes while the other served as a dark control. Additional dye was then added to duplicate five milliliter samples of treated and untreated cell suspensions after which the concentration of dye in the supernatants was determined spectrophotometrically.

In the second set of determinations, the buffered cell suspension was divided before the addition of the dye. One aliquot was held at 60° C for thirty minutes. The buffered cell suspensions were then used to dilute rose bengal to a final concentration of 2.5×10^{-5}M. Additional dye was then added and the dye concentration in the supernatant determined.

Freundlich's isotherm, equation 1, represents the relationship between the amount adsorbed and the concentration.

(1) $\frac{x}{m} = kc^{n}$

where x = weight of material adsorbed by m number of cells

c = concentration in solution

n = constant

k = another constant

This equation can be transformed to the linear function shown in equation 2.

(2) $\log x/m = n \log c + \log k$

The values of c, x, and x/m that were determined with heat-killed and photodynamically treated cells appear in Tables 11 and 12. Plots of log x/m versus log c gave the straight lines shown in Plate XXIV. Living cell adsorption information did not yield straight line plots with the use of Freundlich's equation.

Table 11. Adsorption isotherm data for illuminated cell suspension.

	m = 4 X 10^9 cells ml^{-1}		
Sample	C mg X 10^{-3} ml^{-1}	X mg X 10^{-2}	x/m 10^{-11} mg $cell^{-1}$
1	1.22	4.62	1.16
	1.15	4.69	1.17
2	10.20	7.30	1.82
	10.80	6.72	1.68
3	15.50	7.88	1.97
	15.50	7.88	1.97
4	21.20	7.94	1.98
	21.20	7.94	1.98
5	27.00	8.06	2.02
	26.60	8.41	2.10
6	48.30	10.10	2.52
	36.70	21.70	5.42

EXPLANATION OF PLATE XXIV

Application of Freundlich's adsorption equation to the uptake of rose bengal by heat-killed and photodynamically inactivated Escherichia coli B cells.

PLATE XXIV


C X 10⁻⁴ HEAT KILLED
HEAT KILLED
PHOTODYNAMIC ACTION
C X 10⁻³ PHOTODYNAMIC ACTION
X/M X 10⁻¹¹
30
20
10
8
6
4
2
1
2
4
6
8
10
20
40
60
80
100

Table 12. Adsorption isotherm data for heat-killed cell suspensions.

	m = 2.15 X 10^9 cells		
Sample	C mg X 10^{-4} ml^{-1}	X mg X 10^{-2}	x/m 10^{-11} mg $cell^{-1}$
1	2.88	7.01	3.26
	3.62	6.93	3.22
2	5.48	18.43	8.57
	5.76	18.40	8.55
3	10.38	23.78	11.06
	10.10	23.81	11.07
4	15.12	29.15	13.56
	14.72	29.19	13.58
5	20.91	34.41	16.00
	20.91	34.41	16.00
6	43.22	55.54	25.83
	42.04	55.66	25.89

DISCUSSION

The results of these experiments indicated that the role of the dye molecule in photodynamic action was not limited to the simple cyclic process that was given by Blum and Kautzmann (1953). Deviation from Blum's scheme was indicated by the observed changes in optical density of supernatant methylene blue, as illustrated by Plate IX, that accompanied photodynamic action. These results suggested two possible reactions that would involve the dye molecule: (1) a change in the dye molecule and (2) a change in the dye-cell relationship.

Changes in the dye molecule during photodynamic action was reported by Heinmets *et al* (1952). They observed a decrease in

the optical density of methylene blue during the illumination of serum albumin which was accompanied by a general increase in the ultra violet absorption by the albumin. In the experiment illustrated in Plate XIII, the changes in absorbancy that occurred at 662 mu was accompanied by the appearance of a new absorbancy maximum at 255 mu. Unfortunately, Heinmets et al (1952) did not report any changes in the ultra violet absorption of the dye.

The relationship of the reduction of methylene blue to photodynamic inactivation, suggested by the similarity in the optical density and inactivation curves shown in Plate XV, was supported by other factors. Statistical calculations, made with data collected during two experiments, gave correlation coefficients of -0.993 and -0.984. These results were interpreted as demonstrating the existence of a definite relationship between the two reactions; that is to say, the decrease in viable cell count was inversely related (proportional?) to the increase in concentration of reduced methylene blue.

Although the results point toward the existence of this relationship, several other factors must be considered before these changes can be related to each other. The most important factor suggesting a relationship was the observation that dye reduction and accumulation occurred only during illumination. The samples to be illuminated for various time intervals were selected at random with the use of a table of random numbers (Snedecor, 1959). As pointed out by Wilson (1952), this design eliminated possible error due to sequence of treatment. The zero time sample proved to be an adequate control for dye reduction that might

result from endogenous respiration.

The change in the dye molecule that was represented by the "blue shift" in the absorption maximum (see Plate XI), was not operative during photodynamic action. However, this reaction may possibly play an important role in photodynamic action. This shift, as it occurred in phosphate buffer solution, may be a form of photobleaching. Photobleaching occurs during illumination of dye solutions that contain a trace of reducing substance, but does not occur in the presence of a higher concentration of the reducing substance (Frank and Livingston, 1941). Rothstein (1959) stated that phosphates may be important as functional groups in binding sites that are vital to bacterial metabolism. If methylene blue molecules were bound to the bacterial cell through some dye-phosphate linkage, a reaction similar to the "blue shift" might operate in photodynamic action.

The experimental results indicated the operation of reactions that integrated methylene blue reduction into the photodynamic action scheme. Such reactions were suggested by Frank and Livingston (1941). The light energizing reaction(s) could be followed by the reaction shown in equation 1.

(1) $AH + DH_2 \rightleftharpoons DH_3 + A$

The dye semiquinone (DH_2) reacts reversibly with a partially oxidized reducing agent (AH) to give a reduced dye (DH_3) and an oxidized reducing agent (A). The semiquinone may undergo the reversible reaction shown in equation 2.

(2) $2\,DH_2 \rightleftarrows D + DH_3$

In this case the reaction products are dye (D) and leuco base (DH_3). The operation of either reaction would lead to the formation of a reduced dye and thereby explain, in part, the experimental results. The reverse of these reactions, the oxidation of reduced dye by oxygen, is slow (Michalis in Green, 1946); therefore, the reduced dye would accumulate.

Changes in the cell-dye relationship were indicated by the changes in the supernatant dye concentration, shown in Plate XIV. If these changes were due solely to dye reduction, the decrease in dye concentration, measured as absorbancy at 662 mu, would have been proportional to the increase in concentration measured at 255 mu. This simple relationship did not exist. Also 662 mu measured concentration changes were apparent before the initiation of photodynamic action, i.e., during the lag period that preceeded the initiation of dye reduction and cell inactivation. Furthermore, all experiments of this nature gave evidence suggesting that the adsorptive ability changes by the cells that occurred during photodynamic action followed a definite sequence. Plate XIV shows a decrease in adsorption of dye in the first thirty seconds of reaction followed by an increase in adsorption that continued for two to three minutes.

Therefore, two changes in the cell-dye relationship existed; one that preceeded photodynamic action and another that accompanied photodynamic action.

The first could be due to light-induced reactions that were

either not injurious to the cell or easily reversed. Polar groups of protein on the surface of the cell are possible binding sites for dye. Destruction of these through a photooxidative reaction similar to that given by Weil and Seibles (1955) could lead to changes in adsorptive ability without serious damage to the cell. Reversible changes, less damaging to the cell than the inactivation changes that Heinmets et al (1952) observed, could also account for these observations.

Oxygen uptake studies, suggested by the work of Freeman and Giese (1952), showed that the changes in the dye-cell relationship that accompanied photodynamic action were similar to the change that results from heat inactivation of cells. Plate XIX and Plate XX show the definite oxidative response to photodynamic action by heat-killed and photodynamically treated cells. Both treatments exposed a limited amount of photodynamically reactive materials. The experimental results shown in Plate XXI also demonstrate this point. Illumination destroyed the suspension's ability to oxidize glucose and exposed a limited quantity of photodynamically active material that was oxidized during the terminal period of illumination.

The results of the experiments, shown in Plates XXII, XXIII, and XXIV, on dye uptake by photodynamically treated cells and heat-killed cells, demonstrated that photodynamic treatment causes changes in the dye-cell relationship that approach the change in this relationship that is caused by heat inactivation. Dye adsorption by heat-killed and photodynamically treated cells followed the adsorption isotherm of Freundlich (Hartmen, 1939) whereas dye

adsorption by living cells did not follow known adsorption laws. These adsorption observations corroborate Finkelstein's and Bartholomew's observations (1960) that heat-killed E. coli adsorbed crystal violet in accordance with Freunlich's isotherm.

The dye adsorption observed in these experiments could be due to either the formation of the dye-protein bonds that Fowlks (1959) reported or to the formation of dye-reaction products bond that Heinmets et al (1952) include in their photodynamic action scheme.

Regardless of the true nature of the reaction, photodynamic action caused changes in the adsorption of dye by the cells.

SUMMARY

Investigations of the fate of dye molecules in photodynamic inactivation of E. coli B demonstrated that this reaction was accompanied by changes in the methylene blue dye molecule and changes in the adsorptive ability of the cells towards methylene blue and rose bengal.

The results show that the changes in the dye molecule during photodynamic action were limited to reduction of the dye. The observed shift in the absorption spectrum that resulted from illumination of methylene blue in phosphate buffer did not occur during photodynamic action. The observed accumulation of reduced methylene blue in the supernatants of photodynamically treated cell suspensions may have been due to two factors: (1) the lack of suitable adsorptive sites on the bacterial surface and (2) a very slow rate of leuco base oxidation.

A change in the dye-cell relationship as the result of photodynamic action was observed. The changes in the dye adsorption during photodynamic action with methylene blue, and the increase in dye adsorption during photodynamic action with rose bengal suggested surface reactions that were followed by penetration of the dyes into the cytoplasm of the treated cells.

It was concluded that the reactions of the dye molecules were not limited to oxidation-reduction cycles; they took part in diverse reactions that started at the surface of the cell and terminated in the cytoplasmic remnants of disorganized, inactive cell.

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APPENDIX

Table 13. Photodynamic inactivation of E. coli B in 4.9×10^{-5}M methylene blue.

Photodynamic Action		Controls	
Time (min.)	Count	Illuminated Count	Dye-Dark Count
0.0	3.8×10^8 4.4×10^8 3.2×10^8	4.7×10^8 3.6×10^8 3.0×10^8	4.3×10^8 3.2×10^8 3.4×10^8
0.5	2.2×10^8 1.6×10^8 2.0×10^8	-	-
1.0	4.3×10^7 5.4×10^7 5.5×10^7	-	-
1.5	1.8×10^7 1.4×10^7 1.5×10^7	-	-
2.0	6.0×10^6 6.3×10^6 5.3×10^6	-	-
3.0	1.2×10^6 1.0×10^6 1.0×10^6	-	-
5.0	0.7×10^5 1.1×10^5 0.8×10^5	4.0×10^8 3.1×10^8 3.6×10^8	-
10.0	9.0×10^4 4.0×10^4 3.0×10^4	4.8×10^8 3.0×10^8 3.4×10^8	3.1×10^8 3.6×10^8 4.4×10^8

Table 14. Photodynamic inactivation of E. coli B in 4.9×10^{-5}M methylene blue, 30 cm. light path.

Time (min.)	Count	Ave. Count	Ave. Count X 10
0.0	6.0×10^7 4.0×10^7 4.2×10^7	4.7×10^7	4.7×10^8
0.5	3.3×10^6 4.4×10^6 4.6×10^6	4.1×10^6	4.1×10^7
1.0	1.3×10^5 1.9×10^5 7.0×10^4	1.3×10^5	1.3×10^6
1.5	2.5×10^5 1.9×10^5 2.3×10^5	2.2×10^5	2.2×10^6
2.0	8.0×10^4 2.3×10^5 6.7×10^4	1.3×10^5	1.3×10^6
3.0	5.1×10^5 4.0×10^5 1.0×10^6	3.1×10^5	3.1×10^6
5.0	8.7×10^4 3.2×10^5 1.8×10^5	1.9×10^5	1.9×10^6
10.0	2.8×10^5 2.2×10^5 2.4×10^5	2.5×10^5	2.5×10^6

Table 15. Photodynamic inactivation of E. coli B in 4.9×10^{-5}M methylene blue, 35 cm. light path.

Time (min.)	Count	Ave. Count
0.0	5.5×10^{8} 3.9×10^{8} 3.8×10^{8}	4.4×10^{8}
0.5	1.7×10^{8} 1.2×10^{8} 1.3×10^{8}	1.4×10^{8}
1.0	1.2×10^{7} 9.8×10^{6} 1.2×10^{7}	1.1×10^{7}
1.5	2.4×10^{6} 2.8×10^{6} 2.4×10^{6}	2.6×10^{6}
2.0	1.8×10^{5} 1.8×10^{5} 1.6×10^{5}	1.7×10^{5}
3.0	1.3×10^{4} 1.3×10^{4} 1.2×10^{4}	1.3×10^{4}
5.0	1.1×10^{3} 1.3×10^{3} 1.4×10^{3}	1.3×10^{3}
10.0	7.9×10^{2} 1.3×10^{3} 1.4×10^{3}	1.2×10^{3}

Table 16. Photodynamic inactivation of *E. coli* B in 4.9×10^{-5}M methylene blue, 45 cm. light path.

Time (min.)	Count	Ave. Count
0.0	4.4×10^8 3.6×10^8 4.0×10^8	4.0×10^8
0.5	3.4×10^8 3.6×10^8	3.5×10^8
1.0	3.6×10^8 3.9×10^8 3.2×10^8	3.6×10^8
1.5	1.4×10^8 1.2×10^8 1.4×10^8	1.3×10^8
2.0	2.7×10^7 3.0×10^7 2.7×10^7	2.8×10^7
3.0	9.6×10^5 9.2×10^5 8.0×10^5	8.9×10^5
5.0	2.5×10^4 2.6×10^4 2.9×10^4	2.7×10^4
10.0	3.8×10^3 2.1×10^3 1.5×10^3	2.5×10^3

Table 17. Photodynamic inactivation of E. coli B in 4.9×10^{-5}M methylene blue, 60 cm. light path.

Time (min.)	Count X 10^7	Ave. Count	Ave. Count X 10
0.0	3.4 3.8 3.5	3.60×10^7	3.6×10^8
0.5	3.6 3.8 3.6	3.70×10^7	3.7×10^8
1.0	1.1 1.1 0.82	1.00×10^7	1.0×10^8
1.5	6.2 6.4 5.7	6.10×10^7	6.1×10^8
2.0	2.7 2.7 2.4	2.80×10^7	2.8×10^8
3.0	1.9 2.5 2.7	2.40×10^7	2.4×10^8
5.0	1.2 1.0 1.7	1.30×10^7	1.3×10^8
10.0	8.7 7.5 7.6	7.90×10^6	7.9×10^7

Table 18. Optical densities in visible and ultra violet range of buffered methylene blue after ten minutes illumination - "Blue Shift".

mu	O.D.	mu	O.D.
700	.025	360	.025
680	.165	350	.033
675	.260	340	.050
670	.355	330	.090
665	.470	320	.110
662.5	.520	300	.290
660	.560	295	.410
655	.630	292.5	.455
650	.675	290	.470
645	.690	287.5	.460
640	.685	285	.430
635	.680	280	.350
630	.640	270	.207
620	.575	265	.170
610	.533	260	.145
600	.490	255	.150
590	.420	250	.185
580	.325	247.5	.198
560	.170	245	.210
540	.095	242.5	.206
520	.060	240	.188
480	.048	235	.135
440	.025	230	.105
400	.015	225	.085
350	.025	220	.075

Table 19. The absorption spectrum of sodium sulfite-reduced 9.96×10^{-6}M methylene blue.

mu	O.D.	mu	O.D.
700	.005	340	.052
690	.010	330	.067
680	.013	320	.076
670	.022	310	.100
665	.025	300	.135
662	.026	295	.155
660	.027	290	.180
650	.027	285	.220
640	.027	280	.275
625	.027	279	.450
610	.027	260	.645
600	.027	256	.675
580	.022	255	.680
560	.020	250	.640
400	.033	245	.575
380	.034	240	.545
360	.038	235	.560
350	.045	230	.720

Table 20. Statistical Calculations: optical density changes as a function of log time.

O.D.	Log Time					
Y	X	y	x	y^2	x^2	xy
.620	0	-.149	-.496	.0222	.2460	.0739
.710	.301	-.059	.196	.00348	.0384	.01156
.775	.477	.006	.016	.00086	.00026	.000096
.820	.699	.051	.203	.0026	.0412	.0104
.920	1.000	.151	.504	.0228	.2540	.0761
$\Sigma Y=3.845$	$\Sigma X=2.479$	0	0		$\Sigma x^2=0.5798$	$\Sigma xy=0.17205$
$\bar{Y}=0.769$	$\bar{X}=0.496$					

$$b = \frac{\Sigma xy}{\Sigma x^2} = \frac{0.17205}{0.5798} = 0.297$$

$$\hat{Y} - \bar{y} = b(X-\bar{x})$$

$$\hat{Y} = b(X-\bar{x}) - \bar{y}$$

$$\hat{Y} = .247x - (.297)(.496) + .769$$

$$\hat{Y} = .297 \log t + .622$$

$$O.D. = .297 \log t + .622$$

Y	$\hat{Y}$	$Y-\hat{Y}$ $d_{y\cdot x}$	$d_{y\cdot x}^2$
.62	.622	-.002	.000004
.71	.711	-.001	.000001
.775	.764	.011	.000121
.82	.830	-.010	.0001
.92	.919	.001	.000001
			$\Sigma d_{y\cdot x}^2=.000227$

$$S_{y\cdot x}^2 = \Sigma d_{y\cdot x}^2/n-2 = \frac{.000227}{3} = .0000757$$

$$S_{y\cdot x} = \sqrt{S_{y\cdot x}^2} = \sqrt{.0000756} = .008695$$

$$S_b = \frac{S_{y\cdot x}}{\sqrt{\Sigma x^2}} = \frac{.008695}{\sqrt{.5798}} = 0.01141$$

$$t = \frac{b}{S_b} = \frac{.297}{0.01141} = 26.03^{**}$$

Table 21. Statistical Calculations: log viable count as a function of log time.

Log Viable Count: Y	log t: X	y	x	x^2	xy
8.38	0	0.97	-0.495	0.2450	-0.480
7.79	.301	0.38	-0.194	0.0376	-0.074
7.50	.477	0.09	-0.018	0.0003	-0.0016
7.11	.699	-0.30	0.204	0.0416	-0.0612
6.26	1.000	-1.15	0.505	0.2550	-0.581
37.04	2.477				-1.198

$\overline{Y} = 7.408$ $\overline{X} = 0.495$

$$\Sigma x^2 = .5795$$

$$\Sigma xy = -1.198$$

$$Y = -2.07x + 8.443$$

$$\text{Log No.} = -2.07 \log t + 8.443$$

Table 22. Statistical Calculations: correlation of change in O.D. at 255 mu and change in visble cell count.

X_1 (log visble count)	x_1	x_1^2
8.38	.97	.941
7.79	.38	.144
7.50	.09	.008
7.11	-.30	.090
6.26	-1.15	1.322
ΣX_1=37.04		Σx_1^2=2.505
$\bar{X}_1$= 7.408		

X_2	x_2	x_2^2	x_1x_2
.62	-.149	.0222	-.1445
.71	-.059	.00348	-.0224
.775	.006	.000036	.00054
.82	.051	.0026	-.0154
.92	.151	.0228	-.1736
ΣX_2=3.845		Σx_2^2=.0511	Σx_1x_2=-.3553
$\bar{X}_2$=.769			

$$r = \frac{\Sigma x_1 x_2}{(\Sigma x_1^2)(\Sigma x_2^2)} = \frac{-.3553}{(2.505)(.0511)} = \frac{-.3553}{.1280}$$

$$r = -0.993$$

$$t = r\sqrt{n-2}/1-r^2$$

$$t = -.993\sqrt{3}/1-(.993)^2$$

$$t = 14.536^{**}$$ 3 degrees of freedom

Table 23. Raw data from a repeated determination of optical density at 255 mu - viable cell count correlation.

Time (min.)	$A_{662.5}$	%	A_{290}	%	A_{255}	%	A_{246}	%	Average Viable Cell Count
0	.78	0.0	.480	0.0	.360	0.0	.400	0.0	4.7×10^7
1	.49	62.8	.340	70.6	.380	105.6	.375	93.8	2.7×10^7
2	.47	60.2	.320	66.7	.405	112.5	.395	73.8	1.8×10^7
3	.49	62.8	.355	73.9	.480	133.3	.460	115.0	1.2×10^7
5	.48	61.5	.350	72.9	.590	163.9	.620	155.0	5.2×10^6
10	.35	44.9	.280	58.3	.670	186.1	.575	143.8	1.5×10^6
15	.35	44.9	.285	59.4	.770	213.9	.505	126.2	1.4×10^6

Table 24. Average microliters of oxygen uptake: illumination initiated at forty-five minutes, photosensitized with methylene blue.

Time (min.)	Tyrosine	Dead Cells
15	0.0	0.0
30	0.0	0.0
45	1.42	1.29
55	9.17	1.92
65	14.83	8.40
75	13.47	6.47
90	23.31	11.65
105	21.17	12.95
120	26.83	13.58
135	29.73	13.58

Table 25. Rose bengal-sensitized oxygen uptake on pretreated steam-killed E. coli B suspensions: photodynamic and dye pretreatment - time duration of twenty minutes in 1.75 X 10^{-5}M rose bengal.

Time (min.)	Steamed: Dye Added	Steamed: No Dye Added	Photodynamically Pretreated	Dye Pretreated
15	10.99	1.25	3.25	7.08
30	22.60	1.97	5.85	14.10
60	39.40	0.56	13.60	31.10
90	47.20	0.56	20.80	33.90
105	53.00	1.27	22.60	36.10
120	57.40	1.97	24.00	43.20
135	60.10	1.97	25.20	45.90

Table 26. The effect on microliters of oxygen uptake by the addition of rose bengal to respiring E. coli B cells.

Time (min.)	Endogenous	Dark	10 min.	15 min.	20 min.	No Dye
15	1.33	33.8	33.0	31.9	30.2	36.6
30	1.33	76.4	68.6	73.2	69.3	76.1
45	2.66	117.7	92.7	122.4	104.6	102.9
60	2.66	148.5	135.9	138.3	131.0	150.9
75	3.99	173.5	163.8	148.9	143.6	181.9
90	3.99	185.2	170.2	151.6	144.9	203.0
105	3.99	204.0	176.5	152.9	147.4	224.2
120	5.32	214.6	181.6	154.3	146.2	246.8
135	5.32	219.0	182.9	154.3	147.4	260.8
150	5.32	229.0	198.0	163.6	161.3	272.1
165	7.98	239.0	196.8	167.6	171.4	280.6
180	9.30	248.0	196.8	167.6	181.4	287.6

Table 27. Adsorption of rose bengal and methylene blue by E. coli B cells treated photodynamically with rose bengal.

Time (min.)	Rose Bengal 10^{-13} Millimoles Dye per Cell	Methylene Blue 10^{-13} Millimoles Dye per Cell
0	.30	.260
1	.68	.590
2	.88	-
4	1.22	.963
6	1.35	.930
8	1.65	.997
10	1.85	1.190
12	2.02	1.260
15	2.23	1.360
20	2.53	1.490

REACTIONS OF DYE MOLECULES IN PHOTODYNAMIC INACTIVATION OF *ESCHERICHIA COLI* B

by

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A. B., Miami University, 1955

ABSTRACT OF A THESIS

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Photodynamic action, the increase in dye toxicity caused by light absorption, has been studied since the turn of the century. Some of these studies suggested that the dye was involved in complex reactions with biological substrates. For this reason the fate of two photodynamically active dyes (methylene blue and rose bengal) in the photodynamic inactivation of Escherichia coli B was investigated. Dilute dye solutions were added to cell suspensions. These cells were buffered to pH values which enhanced dye adsorption. Portions of these mixtures were placed in reaction vessels that were either located behind running water filters or partially submerged in a constant temperature water bath. During illumination with a tungsten filament projector lamp, the reaction mixture was aerated and mixed by a stream of air bubbles. After predetermined time intervals, determinations of viable cell counts, dye adsorption determinations or dye absorption spectra measurements were made. Similar arrangements were used for oxygen uptake studies. Spectrophotometric measurements demonstrated that illumination, in the absence of cells, caused a shift in the absorption maximum of methylene blue from its normal position at 662 mu toward the blue end of the visible spectrum. Although this "blue shift" did not occur when cells were added to the reaction mixture, other changes in the dye solution were observed. Methylene blue was reduced during the course of photodynamic inactivation of the bacterial cells. The rate of accumulation of the reduced dye in the reaction mixtures was related to the rate of decrease in the viable cell count. Correlation coefficients, calculated from data collected from two independent experiments, indicated the presence

of a significant relationship between these two variables. This change in the dye molecule was not the only reaction that involved the dye in photodynamic action. Both methylene blue and rose bengal were increasingly adsorbed during the course of this reaction. Manometric studies of oxygen uptake by living and dead cells during rose bengal-sensitized photodynamic action demonstrated a greater amount of substrate susceptible to photooxidation in the dead cells than in the living cells. Dye adsorption studies demonstrated similar Freundlich isotherm plots for both dead and photodynamically inactivated cells. These observations indicated dye molecule penetration of the cell during the course of photodynamic action. These observations suggested that photodynamically active dye molecules take part in complex surface and oxidation-reduction reactions that are initiated at the surface of Escherichia coli B cells and terminated in the cytoplasmic remnants of inactive cells.