

## STUDIES ON THE MECHANISM OF THE PHOTSENSITIZED INACTIVATION OF *E. COLI* AND REACTIVATION PHENOMENON\*

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(Received for publication, June 6, 1952)

Photosensitized inactivation of microorganisms has been the subject of numerous studies, and various hypothetical reaction mechanisms have been proposed for the interpretation of the phenomenon. In spite of such extensive experimentation and theorizing, there still exists uncertainty concerning the inactivation processes. No attempt will be made to review the subject since it has been analyzed and treated by Blum (1) and Arnow (2). It is evident that photosensitized inactivation is dependent on a variety of experimental factors. However, the literature reveals that the influence of such factors has been determined in many diverse and isolated experiments which makes the over-all evaluation of the data difficult. It was thought that, by studying a single system under various experimental conditions, a more satisfactory interpretation could be found. A methylene blue and *E. coli* system was selected, since the methylene blue oxidation-reduction cycle represents a reversible process, and the associated structural changes in the molecule are known.

### *Experimental Methods and Results*

*Experimental Material and Irradiation Procedures.*—*E. coli* B/r were grown in liquid synthetic M-9 medium (3) with aeration. The culture was centrifuged and resuspended in the desired solvent. After irradiation, assays were made by diluting the suspension in saline and plating on nutrient agar containing  $4 \times 10^{-6}$  gm. per ml. of gentian violet.

G. E. H-4 and DH-1 mercury lamps were used as irradiation sources. The test tubes containing the samples were placed in a water bath 5 cm. from the source. During the irradiation oxygen bubbled through the solution, providing a constant oxygen tension and continuous agitation of the suspension. Occasionally, different procedures were used. These will be described in other sections of the paper.

The methylene blue used was U.S.P. grade commercial product which contained not less than 98.5 per cent methylene blue chloride.

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*Absorption of Methylene Blue by Bacteria and the Rate of Photosensitized Inactivation.*—Absorption of the dye by the bacteria is influenced by various factors such as pH, salt concentration, the presence of certain specific cations, and dye concentration (1, 4, 5). Gilbert and Blum have studied the rose bengal uptake by the red blood cells and have analyzed various interaction mechanisms between dye and cell (6). Oster and McLaren have performed similar studies on the dye absorption by the tobacco mosaic virus (7). Experiments indicate that dye is absorbed essentially according to a Langmuir isotherm and can be removed by ionic substitutions. Hydrogen ion is most effective and, by varying the pH of the medium, the quantities of dye absorbed on the bacteria can be effectively controlled. Harris has shown that there is a definite relationship between the uptake of the acid dye and the electrophoretic mobility of the cell (5). Our experiments with methylene blue (basic dye) and *B. coli* indicate a similar relationship. Absorbed methylene blue also produced a reduction of electrophoretic mobility. In view of the existing evidence, one may assume that dye binding to the bacteria is essentially ionic.

In Fig. 1, curves 1 and 2 represent the absorption spectrum of the bacteria and the dye respectively. The former exhibits only a generalized absorption and the latter has absorption peaks at 668 and 615  $m\mu$  wave lengths. The peak at 615  $m\mu$  represents dimmer absorption. When the bacteria are suspended in the dye solution (phosphate buffer, pH 9.0), having final concentrations similar to the values given for curves 1 and 2, a new absorption curve results (curve 3). It is evident that the main peak, at 668  $m\mu$ , is reduced by mixing of the two components. Curve 4 represents spectral absorption of the supernatant solution, after the bacteria have been sedimented from the solution by centrifugation. This curve also exhibits typical methylene blue absorption characteristics. When the dye is eluted from the sedimented bacteria, by lowering the pH of the solution, again the typical methylene blue absorption curve results. This indicates that the dye molecule is not altered in the process of absorption and elution, and that the fractional impurities of the dye are not preferentially absorbed by the bacteria. Curve 5 results when optical density values of curves 1 and 4 are subtracted from curve 3. This difference curve represents, approximately, the spectral absorption of the dye molecules being associated with the bacteria. Here the main peak, at 610  $m\mu$ , is evidently related to the spectral absorption of the absorbed dye. The magnitude of this peak can be varied, depending on the amount of the dye absorbed by the bacteria, but the peak position is essentially constant. However, even in the case in which practically all the dye has been absorbed by the bacteria, there is always present the original dye peak at the 668  $m\mu$  region, but dispersion of the curve around the maxima is wider than that of the original dye. When the experiment described is carried out at pH 2.0, only relatively small changes in the absorption spectra of the dye-bacteria mixture take place, since relatively few dye molecules are

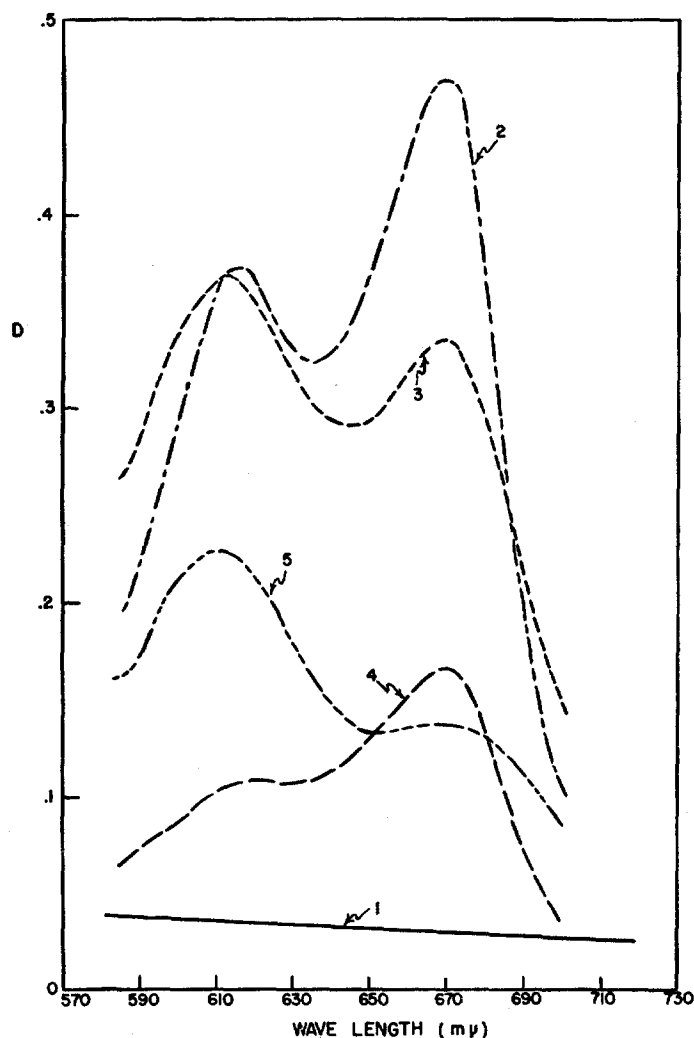


FIG. 1. Effect of the bacteria on methylene blue absorption spectrum. Curve 1, optical density of bacterial suspension ( $1.4 \times 10^{10}$  organisms per ml.); curve 2, optical density of  $1 \times 10^{-4}$  M methylene blue in 0.025 M phosphate buffer at pH 9; curve 3, optical density of the dye and the bacteria mixture at final concentration similar to the values given for curves 1 and 2; curve 4, supernatant of the dye and the bacteria mixture from which the bacteria were removed by centrifugation for 30 minutes at 2500 R.P.M.; curve 5, calculated curve after subtracting the optical density values of curves 1 and 4 from curve 3.

absorbed on the bacterial surface in the presence of large numbers of hydrogen ions. This also indicates, that the dispersion of the dye-bacteria absorption

curve at pH 9, is not caused by the scattering of bacteria, but by the absorbed dye molecules.

Since the methylene blue absorption spectrum is altered in the presence of the bacteria, it can be assumed that such a change is produced by the ionic binding of the dye on the cell surface. The bond formation between the dye and cell structure shifts the absorption peak toward the shorter wave length, which is similar to changes observed on molecular aggregation of the dye and interaction with colloids (8, 9). The new absorption peak at  $610\text{ m}\mu$  results from such an interaction phenomenon. However, the original dye peak at  $668\text{ m}\mu$  still exists even when practically all the dye has been absorbed from the medium by the cells. This could be interpreted to mean that a relatively large fraction of the dye is absorbed by the cells, and molecules may exist after penetration of the cell wall as free dye in solution. However, elution experiments (Fig. 2) indicate that in buffered solutions the dye is absorbed essentially in a reversible manner. An alternative interpretation could be founded on the assumption, that the binding of the dye to the cell alters the structural symmetry of the dye molecule and produces changes in the orbital pathways of the oscillating electron. This view seems to be supported by the evidence when the absorption spectra of the symmetrical crystal violet and unsymmetrical malachite green are compared (10).

The reversible character of the dye absorption by the bacteria in buffered solutions is demonstrated by curve 3 in Fig. 2. Here a certain amount of dye was absorbed by bacteria at pH 7 and dye eluted with the buffer solutions at various pH values. It is evident that hydrogen ion effectively displaces the dye molecules from the bacterial surface.

When dye and bacteria are mixed in buffer solutions at various pH values, and the amount of the absorbed dye determined by sedimenting the bacteria by centrifugation, and measuring the remaining dye in the supernatant, the difference value represents absorbed dye. Fig. 2, curve 2, shows that increasing dye absorption is associated with decreasing hydrogen ion concentration. When such bacteria-dye mixtures, at various pH values, are exposed to light and the number of surviving organisms determined, an S-shaped inactivation curve, represented in Fig. 2, curve 1, results. It is indicated that the inactivation rate increases with decreasing hydrogen ion concentration, although a saturation effect appears at high pH values.

*Influence of the Methylene Blue Concentration on the Inactivation Rate.*—When a constant amount of bacteria is mixed with different concentrations of dye and exposed to light for the same period, the relationship between the dye concentration and photosensitizing effect can be established. Curve 1, on Fig. 3, shows that the inactivation rate as a function of the dye concentration has a maximum in the  $10^{-5}\text{ M}$  concentration region.

In order to evaluate the dye concentration effect on the inactivation rate,

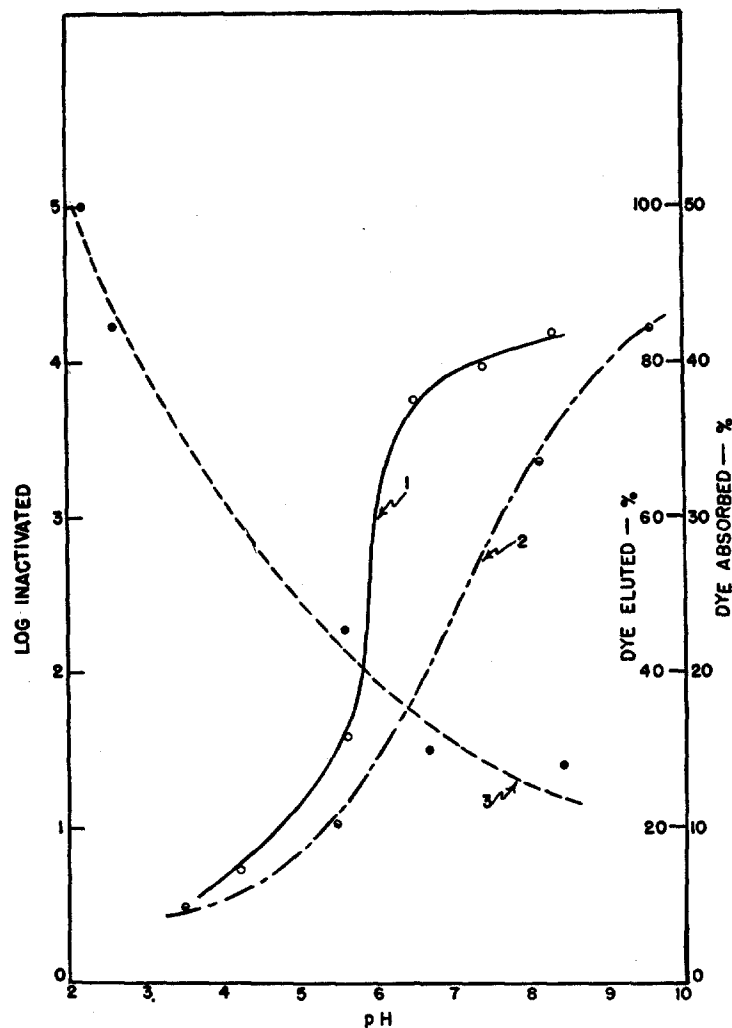


FIG. 2. Absorption of the methylene blue and inactivation of *E. coli* B as function of pH. Bacteria suspended in  $6.6 \times 10^{-4}$  M methylene blue solutions at the various pH values of the phosphate buffers (0.033 M); after 1 hour the suspension is sedimented by centrifugation for 20 minutes at 3000 R.P.M. and the optical density values for 668  $m\mu$  determined; the difference between the original dye concentration and supernatant represents the amount of the absorbed dye. *E. coli* suspended ( $9.5 \times 10^8$  per ml.) in buffered methylene blue solutions and irradiated for 10 minutes; phosphate buffers (0.025 M) at varying pH contained  $6.7 \times 10^{-5}$  M methylene blue.

Curve 1, bacterial inactivation as a function of pH; curve 2, absorption of the dye by the bacteria as a function of the pH; curve 3, bacteria and dye mixed in the phosphate buffer (0.1 M) pH 7 and after 90 minutes' standing sedimented by centrifugation. Sediment resuspended in 0.1 M phosphate buffers at various pH values. After 60 minutes' standing the suspension was centrifuged and the dye concentration determined in the supernatant by spectrophotometry; relative dye solution as a function of the pH is shown by curve 3.

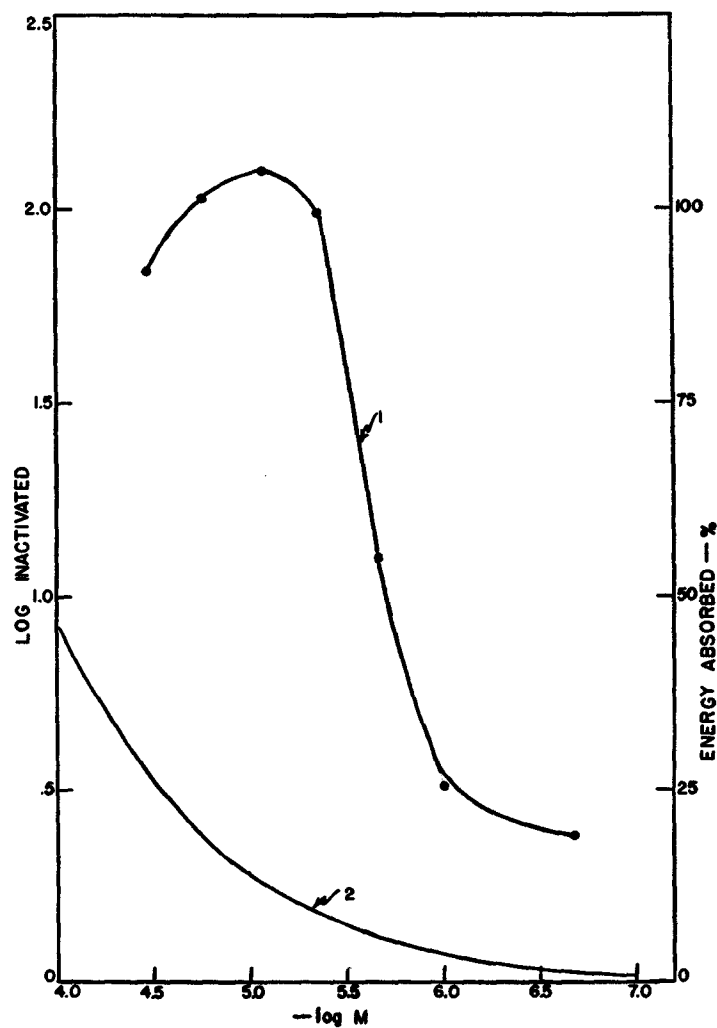


FIG. 3. Influence of dye concentration. Constant amount of bacteria mixed with different concentrations of dye in phosphate buffer at pH 7 (0.075 M). Dye left in contact with bacteria for 15 minutes, oxygenated, and exposed to the light for 5 minutes at 5°.

Curve 1, log of the inactivated bacteria as a function of the dye concentration (log scale); curve 2, total energy absorbed by the free dye in the solution from the H-4 lamp spectrum which has passed through a 1 cm. water filter; thickness of the dye solution 13 mm.

some measurements were made on light absorption by dye solutions. Incident and transmitted light energies were measured with a thermopile, and, in order

to remove the infrared spectrum the light was passed through a 10 mm. water layer. The amount of polychromatic spectral energy absorbed by the dye solution as a function of concentration is shown by curve 2 on Fig. 3. In  $1 \times 10^{-4}$  to  $1 \times 10^{-7}$  M dye concentration range, the absorbed energy declines from 47 per cent to a practically insignificant value. This indicates, that the optimum dye concentration is an artifact, which is produced by the filtering effect of the dye. At the higher dye concentrations the optical density of the system increases to such a degree, that the amount of light reaching the cell-dye complex is drastically reduced.

*Inactivation of E. coli-B/r as a Function of Exposure Time.*—The dose-survival relation is presented by curve 1 on Fig. 4. Such irradiation did not produce any measurable change in the absorption spectrum of the dye-bacteria mixture. In order to demonstrate the possible small changes in the methylene blue spectrum, serum albumin was selected as an optically clear medium for additional experiments. Curve 2 on Fig. 4 shows the optical density change of the methylene blue-serum albumin mixture at  $668 \text{ m}\mu$  as the function of exposure time. After the exposure the ultraviolet absorption spectrum of serum albumin indicated a slight, general increase in optical density values.

*Frozen State Inactivation of Bacteria by Photosensitization.*—It is well known (1) that, in the presence of various chemical compounds which can act as hydrogen donors, methylene blue, when exposed to light, can be reduced to leuco form. It was questioned whether such photochemical reaction would also be possible in a rigid medium and, if this were true, such a phenomenon would help also to interpret the basic mechanism of photosensitization. Experiments indicated that when a methylene blue and pyruvic acid solution was frozen solid at  $-20^\circ$  and subsequently exposed to the H-4 lamp, the sample lost its blue color and that after melting, the solution was also colorless. This color conversion was faster when solutions, previous to their exposure to light, were saturated with nitrogen or carbon dioxide. Since it is known that a reversible color loss of methylene blue is produced by the transfer of hydrogen, this was construed as meaning that the methylene blue molecule can act in a rigid medium as a hydrogen acceptor, provided that the donor molecule is sufficiently close. Since it has been observed that bacteria can be inactivated in a rigid medium by ultraviolet irradiation, and partial inactivation also produced by the 300 to 400  $\text{m}\mu$  spectral region (11), it was decided to perform some photosensitized inactivation experiments in the frozen state.

In Fig. 5 the log of surviving organisms is presented as a function of exposure time. Liquid samples were saturated with  $\text{O}_2$ , frozen at  $-25^\circ$ , and irradiated.  $\text{O}_2$ -saturated samples indicate a 2.8 log unit decrease of surviving bacteria after 5 minutes' irradiation. Longer exposure times indicate a saturation phenomenon which is typical in optically dense medium. Due to the crystallization, the optical density of the dye-bacteria mixture increases with

decreasing temperature (11). This phenomenon makes it difficult to perform the experiments at low temperatures. The most satisfactory results are obtained at temperatures a few degrees above the freezing point, since here, the

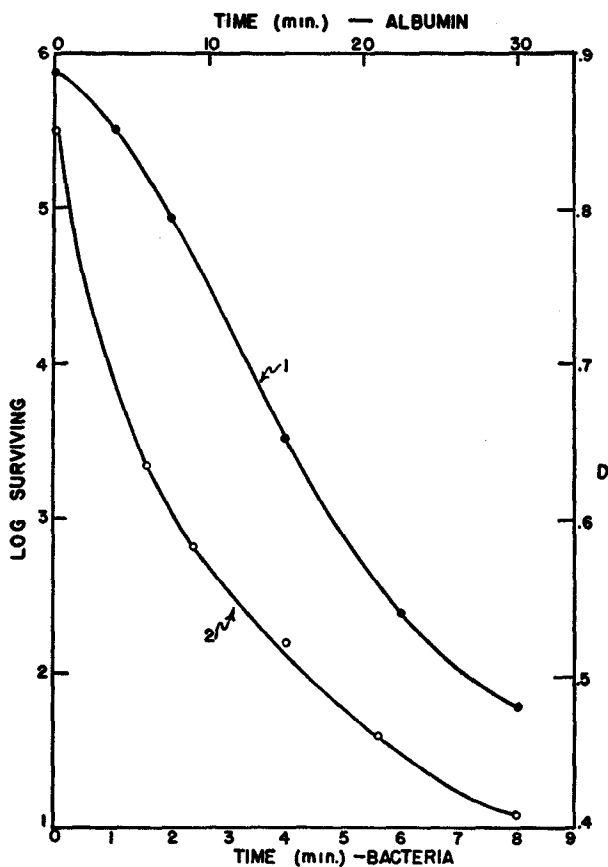


FIG. 4. Inactivation as function of exposure time. Bacteria suspended in water, methylene blue added yielding a final concentration of  $8 \times 10^{-5}$  M dye. Suspension exposed to the H-4 lamp and samples removed at various time intervals. For comparison methylene blue and serum albumin mixture is exposed to the H-4 lamp and the optical density determined at various time intervals at the  $668 \text{ m}\mu$  wave length.

Curve 1, log of the surviving organisms as a function of the exposure time (lower time scale); curve 2, optical density ( $D$ ) of methylene blue in the serum albumin solution as a function of the exposure time.

degree of crystallization of ice is smaller. The experiments presented in Fig. 5 were performed at  $-25^\circ\text{C}$ . in order to be quite sure that no free water was left in the bacteria-dye mixture.

A sample saturated with  $\text{CO}_2$  exhibits only 0.5 log unit reduction of the



surviving organism after a 10 minute irradiation. The major part, if not all, of this inactivation is produced by direct action of the spectrum of the H-4 lamp on the bacteria and by the freezing process itself (11). Samples, saturated with

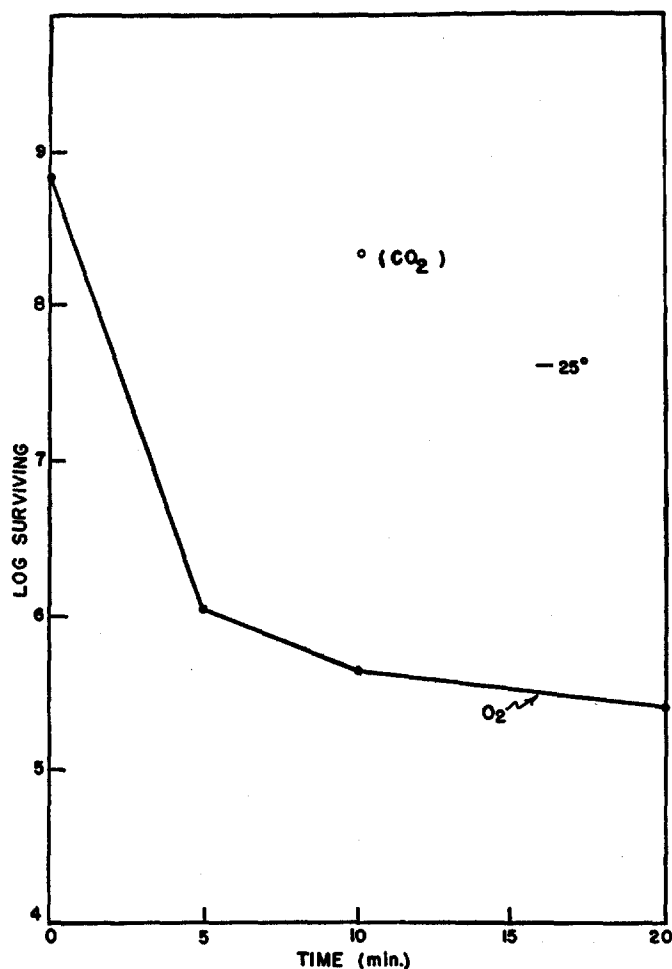


FIG. 5. Dose-survival relation in frozen state. Bacteria and methylene blue solutions saturated with oxygen, mixed, and frozen at  $-25^{\circ}$ ; final dye concentration  $8 \times 10^{-6}$  M; samples irradiated for the various time intervals. One sample saturated with  $\text{CO}_2$  and irradiated for 10 minutes.

$\text{O}_2$ , are also subject to such direct irradiation action, but such inactivation is small when compared with the inactivation produced by photosensitization.

*Influence of Dissolved Gases in Photosensitized Inactivation.*—It is generally accepted that the presence of oxygen is essential for photosensitized inactivation.

tion (1). However, some disagreement still exists on the subject. Since any attempt to analyze fundamental mechanisms of photodynamic action is impossible without a definite conclusion concerning the influence of oxygen, a few experiments were performed to clarify this subject.

In oxygen-saturated solutions, inactivation proceeds at a relatively rapid rate (Fig. 4). By using the Thunberg tube technique, dissolved air was first replaced by carbon dioxide and, later, it was removed by evacuation. Then bacteria and dye were mixed and irradiated while being continuously evacuated. The control sample had a log count of 8.9. After 5 and 20 minute irradiation periods, the corresponding log counts for the surviving bacteria were 8.54 and 8.36 respectively. Essentially similar results were obtained by using carefully purified nitrogen. Obviously, the methods used to bubble gases through the suspension can displace only free gas in the solution, and removal of all gas residues, associated with bacterial structure, cannot be accomplished by such methods. It seems that the small initial inactivation produced is the result of the residual  $O_2$ , associated with the bacterial structure. Frozen state inactivation experiments also present evidence that part of the  $O_2$  is bound to the cell. Here, inactivation proceeds extensively only if the suspension, prior to freezing, contained oxygen. Suspensions saturated with  $CO_2$  exhibited only minor initial inactivation, outside the killing effect of light itself (11). In oxygen-saturated water at  $20^\circ$ , the average distance between  $O_2$  molecules is about 120 A. In the frozen state, at such an average distance, only very small fractions of the molecules would be close enough to participate in photochemical reaction. The obvious explanation would be that part of the  $O_2$  is associated with the bacterial structure, which is removed only partially by bubbling some other gas through the system.

*Action Spectrum.*—We performed studies on the methylene blue-bacteria system in the spectral range from 350 to 1100  $m\mu$ . For such purposes two lamps were used. The short wave region was obtained by using a 400 watt G. E. mercury lamp, and the long wave region by using a 600 watt photographic flood lamp. For isolating spectral regions, various filter combinations were used. In order to exclude the infrared region, aqueous copper chloride filters were used with the following glass filters:<sup>1</sup> 365, 425, and 525  $m\mu$  peak and

<sup>1</sup> Corning filters with the following designations were used:

Peak filters	Color specification Nos.
365	7-83
425	5-74
525	4-64
Cut-off filters	
540	3-67
625	2-59

540 and 625  $m\mu$  cut-off. Incident and absorbed spectral energies were determined for the same filter systems with a thermopile. The bacteria were irradiated for 30 minutes and oxygen was bubbled through the sample during the exposure. The bacterial suspension was adjusted photometrically to be in the range of  $1 \times 10^{-7}$  organisms per ml., and the methylene blue concentration in the final solution was  $8 \times 10^{-6}$  M.

There seem to be two active spectral regions. Wave lengths slightly above 300  $m\mu$  produced some minor inactivation, but the main inactivation was observed when 540 and 625 cut-off filters were used. The wave length regions, 540 to 700  $m\mu$  and 610 to 780  $m\mu$ , were isolated and the respective incident energies were 150 and 203 (determined in arbitrary units). The log inactivation values are respectively 1.77 and 1.94. Other experimental regions were 350 to 510, 480 to 570, and 700 to 1000  $m\mu$ , and none of them indicated significant inactivation. Also, a liquid methylene blue filter (1.3 cm. thick and dye concentration  $2 \times 10^{-4}$  M) was used which absorbed almost all of the energy at the 668  $m\mu$  peak. However, here also, a 2.11 log unit inactivation of bacteria was produced. A few experiments were performed in which the samples, in addition to the usual irradiation, were simultaneously exposed to infrared radiation, but no increase in the inactivation rate was observed.

In photosensitized inactivation, a large number of dye molecules are involved (average range  $1 \times 10^7$  dye molecules per cell) and relatively long irradiation at high intensity is required. Methylene blue itself has a relatively high spectroscopic extinction coefficient and the dye-bacteria complex exhibits only a shift and some wider dispersion around the peak. High interaction efficiency between photon and bacteria-dye complex indicates that a large number of photons are required for an inactivation process. Action spectrum studies support the view that predominant inactivation takes place in the wave length region in which the dye-bacteria complex absorbs the energy. The 700 to 1000  $m\mu$  region, in which  $O_2$  has an absorption peak, did not produce inactivation, indicating that  $O_2$  molecules, at such an excitation level, are not capable of interacting with an unexcited dye-bacteria complex. The slight inactivation, with the 365 peak filter, does not represent true photosensitized inactivation, since it is well known that irradiation with this spectral region at high intensities is capable of inactivating bacteria, and absorbed dye may increase this inactivation.

*Influence of the Secondary Reaction Products.*—Blum (1) has reviewed and analyzed the role of peroxides formed in photosensitized processes, and suggests that  $H_2O_2$  is a by-product which may act as an oxidizing agent; its importance in the total process would not appear to be great. Weil and Maher (12) performed photochemical studies and presented evidence that hydrogen peroxide is formed during the reoxidation of the reduced methylene blue.

It was questioned whether peroxide so formed could produce bacterial in-

activation. Since peroxide is a secondary reaction product, not involving radiation energy, the following experimental procedure was selected. Dextrose was added to the bacteria and dye mixture and the suspension saturated with nitrogen. Subsequently the sample was incubated at 37° until all the methylene blue was reduced by the bacteria to the leuco form, and then the dye was reoxidized by saturating the suspension with oxygen. The appearance of the blue color indicated that the dye was oxidized. In order to be sure that absorbed dye was also oxidized, the bacterial suspension was immediately centrifuged and a deep blue sediment was formed. Plating the bacterial samples before and after reoxidation of the dye did indicate that there was no change in the number of viable organisms. Similarly, irradiation, in the presence of reduced dye, did not change the bacterial count.

*Influence of Irradiation on the Bacterial Ability to Reduce Methylene Blue.*—Since methylene blue molecules are absorbed on the surface of the bacteria, it was questioned whether the photochemical injury, produced by irradiation, could manifest itself in some change in the biological characteristics of the bacterial surface. Only one type of experiment was performed. It has been shown that bacteria can reduce methylene blue in the presence of various hydrogen donors, and that such activity is associated with the dehydrogenase type of enzymes (13, 14).

The Thunberg technique was used for methylene blue reduction studies. At 37°, in the presence of pyruvic, lactic, formic, and succinic acids, *E. coli* were capable of reducing the methylene blue within the time range of 8 to 15 minutes. However, when the organisms were completely inactivated by photosensitization, no visible color change occurred after 12 hours of incubation, indicating the loss of the ability to reduce the methylene blue.

*Reactivation of the Inactivated Bacteria.*—*E. coli* B and B/r, rendered non-viable by ultraviolet irradiation, can be reactivated by exposure to light (15). In addition *E. coli* B can be reactivated by heat (16, 17) and *E. coli* B/r by catalase and ferrous sulfate, when added after irradiation (18, 19). Our attempts to photoreactivate *E. coli* B and B/r, which had been inactivated by photosensitization, were unsuccessful. Complete removal of dye from the bacteria was possible only by hydrogen ions at such high concentrations that the bacteria were inactivated by the ions themselves. Other ionic substitution methods also failed. Further attempts, by reducing the absorbed methylene blue metabolically and subsequently exposing bacteria covered with inert leuco methylene blue to an H-4 lamp spectrum, also proved to be unsuccessful.

However, when *E. coli* B and B/r after irradiation were stored for 24 hours, prior to plating, a partial recovery took place. Subsequently the influence of several factors, such as exposure time, storage time, and incubation temperature, was studied. It was observed that when irradiation was too extensive,

no reactivation occurred. The phenomenon of reactivation could be demonstrated consistently. However, there were always considerable quantitative fluctuations in the recovery rate, even when carefully controlled experiments were repeated. Latarjet and Caldas observed a similar phenomenon in enzymatic reactivation (19). More studies are needed to determine the cause of such fluctuations. Since only a relatively small number of experiments were performed, no definite statement can be made, but reactivation seemed to be affected by the concentration of the irradiated sample. Higher concentrations were more favorable for reactivation. This seems to be similar to the original observation of Monod *et al.* (18), who observed that the number of dead bacteria influenced ultraviolet recovery rate.

Fig. 6, curve 1, shows the usual dose-survival curve when irradiation takes place in an optically dense medium. After irradiation, one part of the sample was plated immediately, and two others stored at 5 and 30° for 24 hours and then plated. It is evident that storage at 5° produced a minor recovery of the *E. coli* B, but a more pronounced effect is achieved by storage at 30°. In order to rule out the possibility that the multiplication was not the cause of such an increase, various unexposed control samples were stored under similar conditions, but no increase of bacterial count was observed. Further experiments indicated that a still higher heat reactivation took place at 37°. Strain *E. coli* B/r also was subject to such a recovery. However, the storage time strongly influenced the recovery rate. After 4 hours of storage at 5 and 30°, no recovery was observed in either case. In Fig. 7, reactivation of *E. coli* B and B/r is presented as a function of irradiation time. It is evident that at intermediate exposure time the total number of reactivated cells have the highest value. Since there is no nutrient material present in the dye-bacterial mixture, it is suggested that the increased number of viable cells is a result of bacterial recovery. To rule out the possibility that dead bacterial cells serve as a source of food for the surviving organisms, the following control experiment was performed. A control bacterial suspension was diluted to a level at which the number of cells present was in the range comparable to that of an irradiated sample. To this suspension an equal number of ultraviolet-killed organisms were added, bringing the number of cells back to the original value. Such a sample was stored and plated in a fashion similar to that in which the irradiated samples were stored and plated. Instead of an increase, a reduction of surviving organisms took place. Such a decrease is also exhibited by the unexposed control samples. Care has to be taken that the bacteria are entirely killed by ultraviolet irradiation otherwise such cells could themselves be reactivated.

It was questioned whether the addition of some chemicals which are related to metabolic dehydrogenation could influence cellular recovery. *E. coli* B/r and dye mixture was irradiated and subsequently various chemicals added

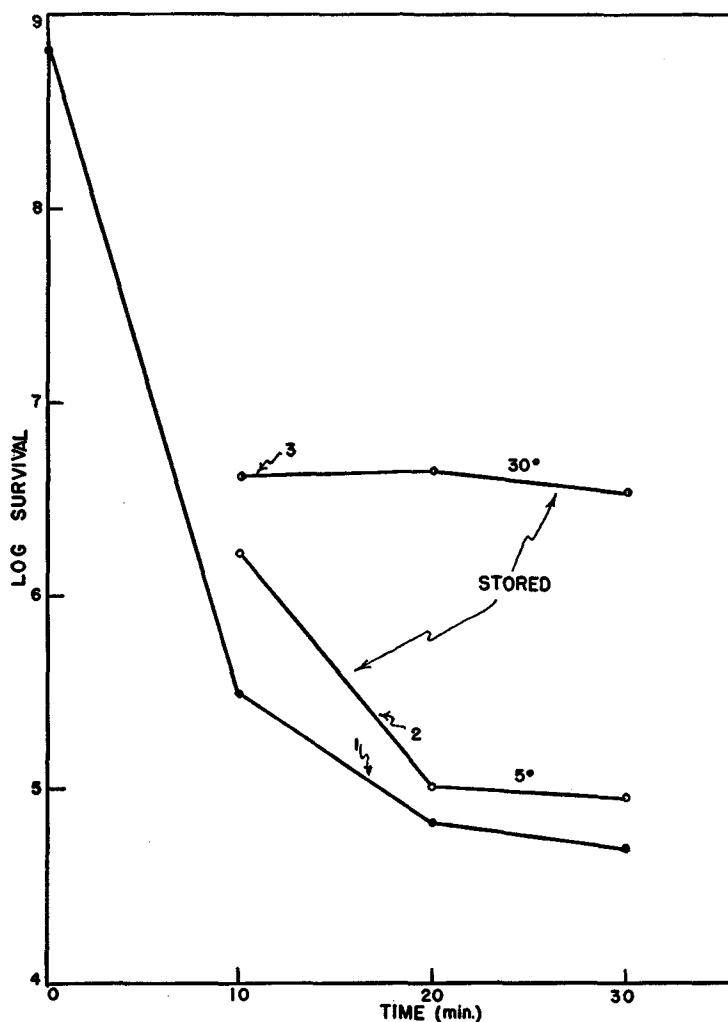


FIG. 6. Influence of incubation temperature on reactivation. *E. coli* B and methylene blue ( $8 \times 10^{-6}$  M) mixture exposed to the H-4 lamp for various time intervals. Part of the sample plated immediately. The other part stored at 5 and 30° for 24 hours and plated. Colonies counted after 48 hours.

Curve 1, bacteria exposed to the H-4 lamp and plated immediately; curve 2, bacteria exposed to the H-4 lamp, stored 24 hours at 5°, and plated; curve 3, bacteria exposed to the H-4 lamp, stored 24 hours at 30°, and plated.

(final concentration  $3.5 \times 10^{-8}$  M) to the suspension. Part of the sample was plated immediately, and the other part stored at 37° for 24 hours and then plated. Table I presents the experimental data. It is evident that the addition

of various hydrogen donors has, on the average, slightly increased the heat recovery of *E. coli* B/r. An exception seems to be uracil which reduced the

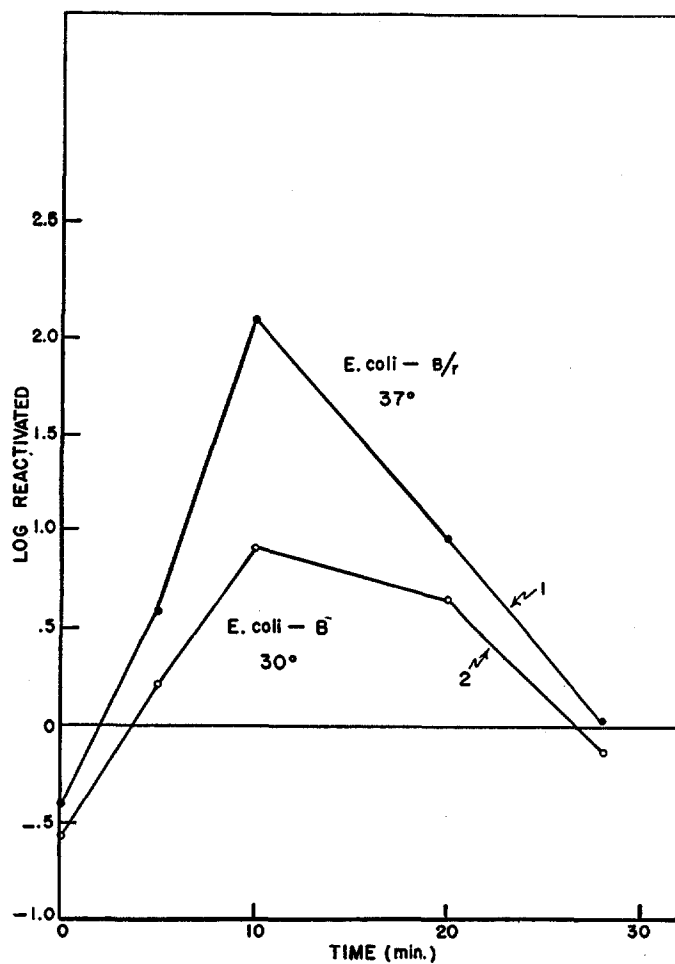


FIG. 7. Influence of radiation dose on reactivation. Bacteria ( $1 \times 10^7$  cells per ml.) and methylene blue ( $8 \times 10^{-6}$  M) irradiated for various time intervals. Part of the sample plated out immediately; the other part stored for 24 hours and then plated.

Curve 1, *E. coli* B/r stored at 37° for 24 hours; curve 2, *E. coli* B stored at 30° for 24 hours. Both curves show the increase of the colony count after 24 hour storage.

number of viable cells. However, the data are preliminary in character and extensive studies are needed to evaluate the procedure and the value of a particular compound.

TABLE I  
*Influence of Various Chemicals on Heat Recovery of E. coli B/r*

Exposure time. . . 20 minutes  
 Dye concentration. . .  $8 \times 10^{-6}$  M  
 Storage temperature. . . 37°  
 Storage time. . . 24 hours  
 Bacteria suspended in 0.1M phosphate buffer at pH 7.

Sample	Added chemicals ( $3.5 \times 10^{-3}$ M)	Plated after irradiation	Plated after 24 hr. storage at 37°
1	Buffer alone	$4.0 \times 10^8$	$1.4 \times 10^6$
2	Sodium pyruvate	$4.0 \times 10^8$	$2.4 \times 10^6$
3	Formic acid	$4.0 \times 10^8$	$3.0 \times 10^6$
4	Succinic acid	$4.0 \times 10^8$	$8.6 \times 10^6$
5	Dextrose	$4.0 \times 10^8$	$3.6 \times 10^6$
6	Lactic acid	$4.0 \times 10^8$	$2.4 \times 10^6$
7	Uracil	$4.0 \times 10^8$	$3.8 \times 10^6$
8	Mixture of substances (samples from 2 to 7)	$4.0 \times 10^8$	$1.7 \times 10^6$
9	Unexposed control	$4.7 \times 10^7$	$8.9 \times 10^5$

#### DISCUSSION

The phenomenon of frozen state inactivation definitely supports the concept that only absorbed dye is photobiologically effective since at  $8 \times 10^{-6}$  M dye concentration the average distance between the randomly distributed dye molecules is about 590 Å, and in such a case the number of the dye molecules, being in the distance range of a few Angstroms from the bacteria, would be too small to produce photochemically significant changes. Oster and McLaren produced photosensitized inactivation of the tobacco mosaic virus and they came to a similar conclusion, that the dye must be bound to the surface of the virus particle in order to be photochemically active (7). Blum and Gilbert have shown, that under appropriate conditions photosensitized hemolysis by rose bengal is directly proportional to the amount of dye taken up by the red cell (20). It is also evident that absorbed photon energy is essential for photosensitized inactivation, since dye reoxidation experiments indicate that secondary reaction products, such as peroxides, are not capable of inactivating the bacteria.

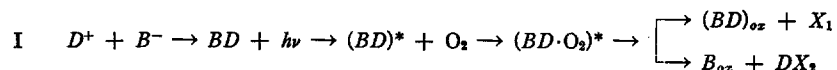
In view of the collected experimental data, it is desirable to analyze the basic reaction mechanisms of photosensitized inactivation. Blum (1) has critically reviewed the literature and discussed the relative advantages of various photochemical mechanisms in interpreting the mass of experimental data. It seems that our experiments have provided data which further reduce the number of possible modes of reaction mechanisms. The evidence that photochemical reactions and photosensitized inactivation can take place in the frozen state, excludes all possible modes of interaction in which energy



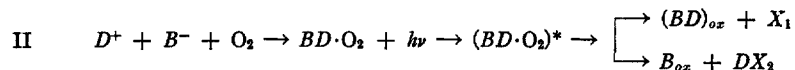
is transferred from one molecule to another by collision. In order to have reaction take place in the rigid medium, all participating elements would have to be situated in very close proximity. The evidence that the  $2 \times 10^{-4}$  M methylene blue filter, which absorbs 95 per cent of the energy at 668 m $\mu$ , still permits inactivation to proceed with comparable rates, indicates that the action spectrum coincides with the dye-bacteria absorption curve rather than with the dye absorption alone. The following general scheme for photosensitized inactivation could be presented:

\*—excited state  
*D*—dye molecule  
*B*—bacteria  
*O*<sub>2</sub>—oxygen molecule  
*X*—unknown reaction product  
*hν*—photon  
*ox*—oxidized state

The first step would be a dye-bacteria complex formation by ionic binding (other modes may be also possible); the complex would absorb a photon and in the excited state would react with O<sub>2</sub>; the scheme would be as follows:—



However, experiments with the frozen state suggest that O<sub>2</sub> may form a loose complex with other participating elements, prior to the excitation, and after the absorption of a photon a more permanent type of O<sub>2</sub> binding may be formed. The fate of the dye molecule is unknown; it is possible that after the excitation process, the O<sub>2</sub> interaction dye molecule is dissociated from the complex. The following would be the scheme of such type of reaction:—



Fiala (21) performed some polarographic and spectroscopic studies on photosensitized reactions with serum proteins using fluorescent dyes. His conclusions for serum denaturation involved mechanisms essentially similar to those presented in schemes I and II. Our experiments with the frozen state suggest that scheme II is the more probable.

It is of interest to find out which regions of the bacterial cell are essentially injured by photosensitized inactivation. Experiments indicate that dye molecules are predominantly absorbed on the cell surface. This would suggest that photobiological injury would be produced at the site of the absorption. This is supported by the evidence that photosensitized inactivation is also accompanied by loss of the ability to reduce methylene blue in the presence of various hydrogen donors. Since methylene blue molecules can be reduced, and

reoxidized, while absorbed on the cell surface, this would indicate that the cell membrane could be the predominant site of the injury. Brandt *et al.* (22) studied the effect of radiations on galactozymase formation in yeast. Using rose bengal as a photosensitizer, they observed that, after the exposure to light, entry of materials into the cell was in some way reduced or prevented. They took into account some other experimental data, and came to the conclusion that photosensitized injury seems to be confined to the cell surface. Question will arise, as to which of the structural elements would be involved in such a photochemical injury. Prolonged irradiation usually produces an irreversible inactivation and this could indicate an extensive disorganization of the cell surface. However, the reactivation phenomenon suggests that, in an earlier phase, such injury is reversible. Since the reactivation is so strongly dependent on temperature and time (Fig. 6), it is suggestive that such a recovery is closely associated with the metabolic activity of the cell. Such a recovery may constitute a repair process of injured structural elements, reduction of photooxidized metabolites, or formation of new adaptive structures to restore cellular functions and activity.

The concept of the general reaction mechanism does not provide any further clue as to the detailed reactions taking place at the cell surface. It is assumed that the primary photobiological manifestations have to be interpreted through photochemical processes. Weil *et al.* (23) have shown that tyrosine, tryptophan, histidine, and cysteine were highly reactive during photosensitized oxidation. The rest of the amino acids were relatively stable. It has to be expected that photobiological action, at least in part, could be related to alterations of such chemically active compounds. The experimental evidence supports this view. Both photosensitized inactivation and photooxidation indicate that an increasing pH accelerates reaction rates. Increased inactivation is associated with increased dye absorption by the bacteria. However, the inactivation rate increases much faster than the rate of dye absorption. It is suggestive that dye absorbed at high pH values is bonded to structural elements which are more sensitive to the photochemical action. Methylene blue forms a complex with the bacterial structure through negatively charged groups of these structural elements. The greatest bacterial inactivation seems to take place where photochemically more sensitive groups form complexes with the dye. At lower pH values the carboxyl group is the main dye absorber, but this group is relatively stable against photooxidation (23), and the bacterial inactivation rate is relatively small in the low pH region.

#### SUMMARY

In order to find a more satisfactory interpretation of the phenomenon of photosensitized inactivation of bacteria, studies were performed under various experimental conditions on methylene blue and *E. coli*. In summary the findings are as follow:—

1. The dye is absorbed by the bacteria according to the Langmuir isotherm and can be removed by ionic substitutions; the dye binding to the bacteria is predominantly ionic; the dye-bacteria complex produces a new absorption peak in the 610  $\mu$  wave length region, and the action spectrum corresponds to the spectral absorption of the dye-bacteria complex.

2. There is an optimum dye concentration range for the photosensitized inactivation.

3. Photosensitized inactivation of bacteria can take place both in the frozen and liquid states and the presence of oxygen is essential to the inactivation process.

4. Hydrogen peroxide, formed by reoxidation of the reduced methylene blue, does not inactivate bacteria.

5. Following the photosensitized inactivation, *E. coli* lose their ability to reduce the methylene blue in the presence of various hydrogen donors, suggesting that enzymes are involved in the inactivation process.

6. Bacteria inactivated by photosensitization can be reactivated by prolonged storage after irradiation; the recovery rate increases with increasing temperature (maximum 37°), and is also influenced by the presence of various hydrogen donors.

In view of collected experimental data, the basic reaction mechanisms are analyzed in photosensitized inactivation. The first step of the reaction seems to be excitation of the dye-bacteria, or dye-bacteria oxygen complex, by a photon which produces an activated complex. In such a state, molecular oxygen is capable of producing an oxidizing reaction, which results in the inactivation of the bacteria. Some aspects of the detailed reactions taking place at the cell surface are discussed.

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