SECTION H, MICROBIOLOGY

Photodynamic Response in Microorganisms

R. L. HUDDLESTON[1] and J. B. CLARK,

University of Oklahoma, Norman

Photodynamic action is a term used to designate a variety of detectable changes in various types of cells when the cells are irradiated with visible light in the presence of a sensitizing dye and molecular oxygen. The subject is often expanded to include photosensitivity diseases. A most comprehensive coverage of the field and literature prior to 1941 is found in Blum (1941). In a more recent review, Clare (1956) covers the nature and possible mechanism of photodynamic action in biological systems, and the role of such action in various diseases. Many cell systems have been used in the study of photodynamic action, and the literature on such studies involving microorganisms is too vast to be covered here. In many respects, microorganisms are ideal tools to use in an extended study of this phenomenon and perhaps in the final elucidation of the mechanism, or mechanisms, of action involved.

MATERIALS AND METHODS

The organisms used in this study were: *Nocardia corallina*, ATCC 4273, maintained on fructose agar; *Staphylococcus aureus* FDA 209 *(Micrococcus aureus)*, maintained on nutrient agar; and *Escherichia coli*, University of Oklahoma culture collection, also maintained on nutrient agar. *N. corallina* and *Staph. aureus* were incubated at 29 C and used when 36 to 48 hr old while *E. coli* was grown at room temperature and harvested when 24 hr old.

Dyes used were erythrosin B, CI 773; methylene blue, CI 922; and acriflavine. They were used in dilutions of 1:20,000 (w/v) in 0.015 M phosphate (pH 7) or in 0.015 M acetate (pH 4, 5, and 6) buffers. The solutions were prepared within 6 hr of use and stored in the dark. Each dye was tested for toxicity to the cells used in the absence of light and found to be nontoxic in the concentrations used.

Organisms to be irradiated were transferred from agar slants to water blanks containing glass beads and were shaken on a vibrating machine for 10 min to disperse clumps. When *N. corallina* was used, this suspension was transferred to a sterile tube and centrifuged at low speed to remove any remaning clumps. The centrifugate or shaken suspension was stained with methylene blue and examined microscopically for clumps, and only suspensions of at least 90% single cells were used. The cell concentration at this point was adjusted to approximately 10,000 per ml.

The remainder of the procedure was done in the dark or in light transmitted through a filter containing the sensitizing dye. Two ml of the cell suspension were added to 18 ml of the dye solution and the resulting suspension was stored in the dark for the length of time previously found to give maximum sensitization. At the end of this period, 1 ml of the suspension was removed to a 99 ml water blank as a control. The dye-organism suspension was then transferred to a Prickett tube for irradiation.

The Prickett tube was mounted parallel to and 2 cm from a 30-watt Sylvania daylight fluorescent lamp. The light intensity at this point was

[1]Present address: Central Research Laboratory, Continental Oil Company, Ponca City, Oklahoma.

found to be 650 foot candles as measured with a quartz Weston Illumination Meter, Model 758.

After irradiation was initiated, 1-ml samples were removed from the dye-organism suspension at predetermined time intervals and dispersed in 99-ml water blanks which were immediately placed in the dark. Dispersion of the organisms in the Prickett tube was maintained by pipette agitation. This dispersion was found to give adequate oxygenation of the cells to yield uniform results.

Immediately after irradiation, 0.1-ml amounts of the dilutions were plated in quintuplicate. The plates were then incubated at the same temperature at which the organisms were grown prior to irradiation.

Dark effect of the dyes used, effect of light transmitted by the filter containing the sensitizer dyes, and inhibitory effects of the buffer solutions were tested. The short lag-period between sample taking and plating was also checked for effect on survival responses.

RESULTS

Dark contact time—In most reports on photodynamic action, no mention is made of the time the cells were left in contact with the photosensitizing dye before visible irradiation was started. This dark contact time has undoubtedly been quite variable, depending on experimental procedures used, and in many cases, there is no evidence that the time was kept constant from one experiment to another. Hyman and Howland (1940) reported that the time required for lysis of paramecia was independent of the period of contact between the dye and the organisms in the dark prior to irradiation. In other reports such as Kaplan (1950) and Baugh and Clark (1959), definite, and somewhat arbitrary, dark contact times were used.

In this work, the effect of dark contact time was determined using two dyes, erythrosin B and methylene blue. If the period of dark contact between the cells and erythrosin B was only long enough to permit dispersion of the organisms in the dye (about 30 sec), there was 95% survival after 1 hr of subsequent visible irradiation. As shown in Fig. 1, the subsequent lethality of the visible irradiation increased as the dark contact time was increased up to a maximum of 6 min. Dark contact times greater than 6 min resulted in a decrease in the lethality of the visible irradiation.

When methylene blue was used as the sensitizing dye, somewhat different results were obtained (Fig. 2). There was almost immediate sensitization of the cells which resulted in killing 45% of them after 8 min of visible irradiation. A prolonged dark contact time caused a slight increase in lethality of the visible radiation, the maximum effect occurring after 8 min dark contact time. Additional dark contact time caused a reduction in the lethal effect of the visible light.

The mechanism involved in this dark contact reaction is unknown. It can be speculated that adsorption of the dye at the cell surface is involved. The reaction apparently does not proceed in the presence of visible light, indicating that the photochemically altered dye molecule is unable to carry out the reactions involved. It is believed that the dark contact reaction is a significant step in the series of reactions involved in photodynamic action and it should be considered in all photodynamic experiments.

Effect of dye type on inactivation kinetics—It has been reported that, with the acid dye erythrosin B, Gram-positive organisms exhibited multi-event inactivation kinetics when irradiated with visible light. Gram-negative organisms, however, were photodynamically inactivated at a single-
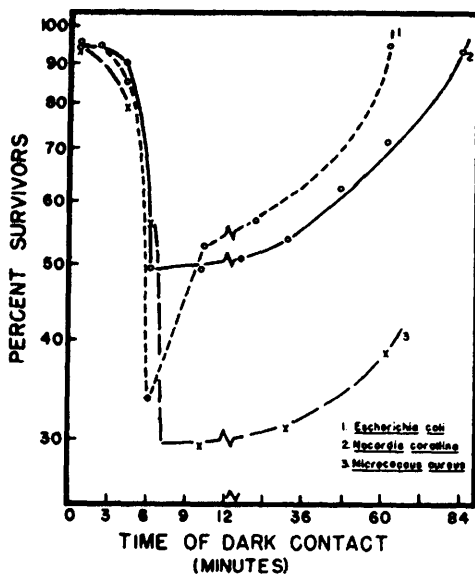
Fig. 1. Survival reponses after eight minutes of irradiation with varying times of dark sensitization using erythrosin B.
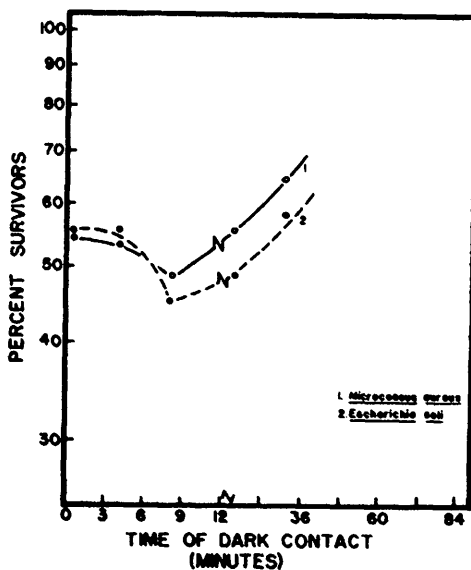

Fig. 2. Survival responses after eight minutes of irradiation with varying times of dark sensitization using methylene blue.

event rate. This was evidenced by the shape of the survival curves of Gram-positive and Gram-negative organisms under identical experimental conditions (Baugh and Clark, 1959). When *N. corallina* or *Staph. aureus* were irradiated in the presence of a basic dye, they were found to react as did Gram-negative organisms in an acidic dye. The inactivation rate of *Staph. aureus* with acriflavine as the dye is shown in Fig. 3. The inactivation rate of *N. corallina* in the presence of acriflavine or methylene blue was similar to that shown in Fig. 3. These results suggest that the acidic or basic properties of the dye molecule are of significance in the determination of the kinetics of the photodynamic action. This may involve the relative ease of adsorption of the dye molecule on the cell surface. However, the results on dark contact time reported previously in this communication indicates that at least part of the adsorption takes place before the dye-cell complex is irradiated with visible light. The kinetics reported here are observed after an adequate dark contact time and in the presence of the inactivating light. Thus, it appears more proable that the multi-event reaction does not directly involve adsorption. The true nature of the reactions involved is not resolved by these experiments.

Effect of pH of dye solution—The pH of the solution in which the dye-cell complex was suspended during the dark-contact reaction and the visible irradiation was found to have a pronounced effect on the survival of the cells. In each case the pH used was tested on cells alone to determine if any toxic effect occurred during the time of contact used in the experiments. The only toxic effect was found with *E. coli* at pH 4, and in such cases the toxic effect was subtracted from the apparent photodynamic effect to give a better indication of the photodynamic effect alone. With the basic dye, erythrosin B, and *Staph. aureus*, a multi-event response was found which was less pronounced at pH 4. A gradual increase in photodynamic killing was found as the pH was increased to 7 (Fig. 4). When erythrosin B was used with *E. coli*, a single-event response was found with a similar pH effect (Fig. 5). The corrected pH 4 line appears to be out of place in Fig. 5 and it is doubtful if full correction for pH toxicity was applied to this line. With methylene blue and *Staph. aureus*, a single-event response was also found and again the photodynamic killing rate was increased as the pH was increased from 4 to 7. If methylene blue was used with *E. coli*, a response very similar to that found with *Staph. aureus* was obtained.

Effect of catalase on photodynamic action—Hydrogen peroxide has been implicated as a lethal factor in photodynamic action, and a mechanism based on peroxide production has been presented (Clare, 1956). The protective action of catalase has been used as a means of indicating the lethal effect of hydrogen peroxide produced during photodynamic action.

When a solution made from crystalline catalase was mixed with the dye-cell complex, no effect was found with *Staph. aureus* or *E. coli*. However, a decided protective action was found when *N. corallina* was used (Fig. 6).

DISCUSSION

On the basis of the results reported in this communication, it is easy to speculate on several aspects of photodynamic action. However, at best, these results are only suggestive. As pointed out by Clare (1956) the establishment of the term "photodynamic action" by Tappeiner and Jodlbauer (1904) was perhaps unfortunate and misleading. The multitude of effects, ranging from enzyme inactivation to human diseases, now associated with the term "photodynamic action" appear to involve several different basic mechanisms. The fact that catalase should have a pronounced effect on photodynamic action in one organism and not in two others
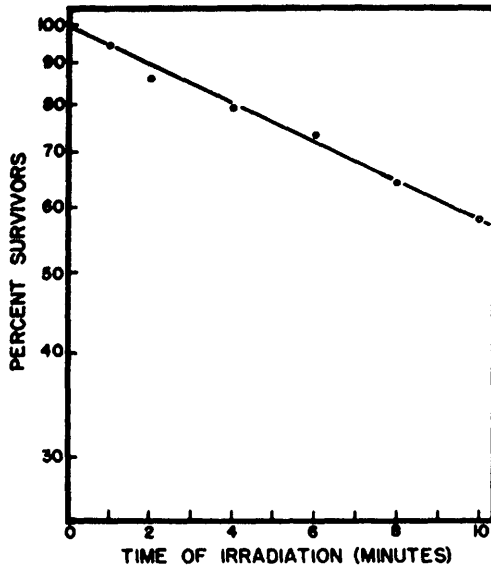
Fig. 3.   The photodynamic response of *Staphylococcus aureus* with acri-
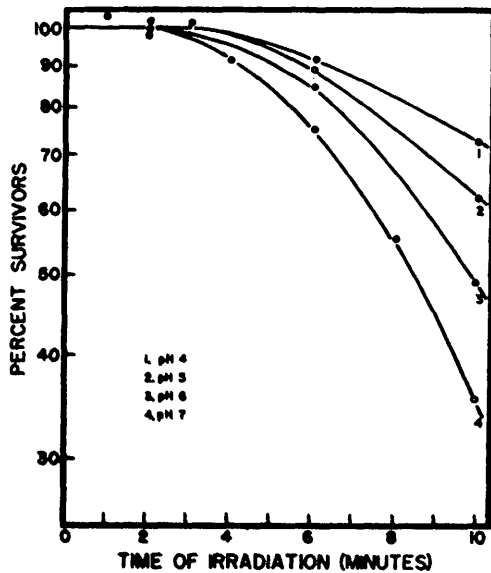flavine at pH 7.

Fig. 4.   The effect of pH on the photodynamic response of *Staphylococcus
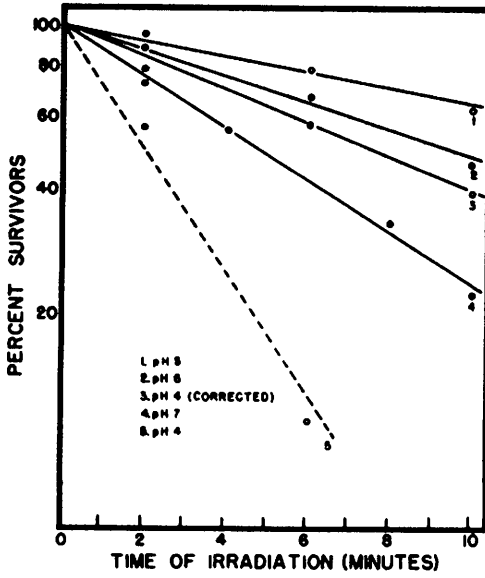aureus* with erythrosin B.

Fig. 5.   The effect of pH on the photodynamic response of *Escherichia coli* with erythrosin B.
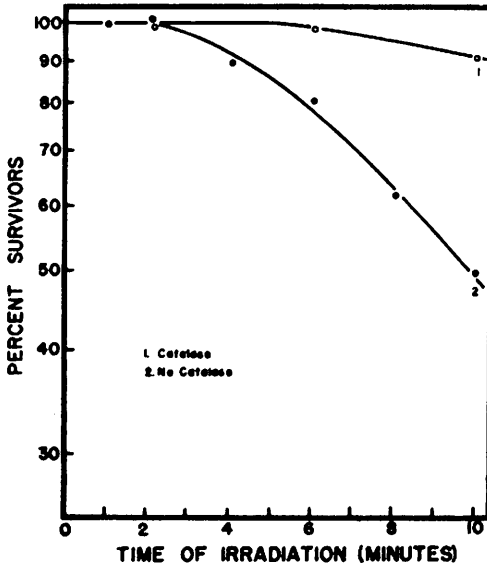

Fig. 6.   The effect of catalase on the photodynamic killing of *Nocardia corallina*.

under identical experimental conditions is suggestive that the mechanism may even differ in different organisms.

It has been well established that one aspect of photodynamic action is the adsorption of the sensitizing dye on the cell surface. The experiments on dark contact time suggest that adsorption takes place prior to irradiation with visible light. Since it was found that, once visible irradiation is started, cell survival is dependent on the prior dark contact time, it appears that the light-activated dye molecule is not adsorbed. If it were, adsorption to a maximum should continue in the presence of light, and no effect of variation in dark time contact would be found. The decrease in the lethal action of light after optimum dark contact time cannot yet be explained.

The effect of pH on the subsequent lethal effect of the light may involve either adsorption during the dark contact time period or some reaction during the actual irradiation, or perhaps both. The cell-dye suspension was maintained at stated pH values during both dark period and during irradiation, and no attempt was made to resolve whether the action occurred in the dark or during irradiation. Heinmets, et al. (1952) found that the adsorption of methylene blue by *E. coli* was affected by pH and concluded that dye binding to the bacteria is essentially ionic. They stated that "dye is absorbed essentially according to a Langmuir isotherm and can be removed by ionic substitutions."

The effect of dye type in affecting the kinetics of photodynamic inactivation may also involve dye adsorption to the cell. With acid dyes, Gram-positive cells are inactivated by a multi-event mechanism. Gram-negative cells are inactivated by a single-event mechanism with either type of dye. It is probable that the relationship between the isoelectric point of the cell and the nature of the dye affects dye adsorption, which could account for these differences in mechanism.

SUMMARY

In studies on photodynamic response with *Nocardia corallina, Staphylococcus aureus*, and *Escherichia coli*, the time the cells were left in contact with the sensitizing dye in the dark affected the subsequent photodynamic response of the system. The kinetics of inactivation depended on the Gram reaction of the organism, the nature of the sensitizing dye, and the pH of the system. Catalase was found to prevent photodynamic inactivation in *Nocardia corallina,* but not in the other two species studied.

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