COMPARATIVE STUDY OF LETHAL PHOTOSENSITIZATION OF *SARCINA LUTEA* BY 8-METHOXYPSORALEN AND BY TOLUIDINE BLUE

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ABSTRACT

MATHEWS, MICHELINE M. (Department of Bacteriology, University of California, Berkeley). Comparative study of lethal photosensitization of *Sarcina lutea* by 8-methoxypsoralen and by toluidine blue. J. Bacteriol. **85**:322–328. 1963.—A comparative study has been made of the photokilling of *Sarcina lutea* by 8-methoxypsoralen (8-MOP) and by toluidine blue. It has been found that photosensitization by 8-MOP differs from photosensitization by toluidine blue, in that it has a temperature coefficient of less than one, and that the presence of oxygen is not necessary for, and even is deleterious to, the photosensitization, the psoralen being destroyed by its presence. It has previously been shown that the presence of carotenoid pigments protects the cells of *S. lutea* from lethal photosensitization by toluidine blue; it was found that the presence of these pigments has no protective effect in photosensitization with 8-MOP. Studies on the lethal photosensitization of *S. lutea* with toluidine blue suggested that the primary sensitive site of the photokilling was the protein of the cell membrane, as manifested by the destruction of membrane enzyme activity and the regulation of permeability. It has been found that photokilling by 8-MOP has no effect on these functions. A study was made on the effect of photokilling by 8-MOP on the production of penicillin-resistant mutants as an indication of an alteration in the cellular deoxyribonucleic acid (DNA) by the psoralen. Psoralen photosensitization resulted in the development of many penicillin-resistant mutants. On the basis of the findings reported in this paper, it is suggested that photosensitization of *S. lutea* by 8-MOP does not reflect damage to cellular protein, as does toluidine blue, but rather damage to cellular DNA.

It has long been known that certain organic pigments can photosensitize cells. When one of these pigments is present in the cell, exposure to wavelengths of light that are normally innocuous provokes severe cellular damage or death. Sensitizing pigments can be either endogenous metabolic products (e.g., porphyrins or chlorophylls) or exogenous natural or synthetic compounds able to penetrate the living cell (e.g., toluidine blue or eosin). In most cases, the sensitized cell can be damaged by light only in the presence of free oxygen, and it has been generally believed that the role of the sensitizer is to catalyze intracellular photo-oxidations (Blum, 1941).

Recent work, primarily conducted with bacteria, has shown that the carotenoid pigments of the cell can act as protective agents against pigment-catalyzed photo-oxidations. This was first shown in photosynthetic bacteria, where genetic or physiological elimination of carotenoid pigments renders the cell highly susceptible to lethal photo-oxidation by its own bacteriochlorophyll (Sistrom, Griffiths, and Stanier, 1956; Cohen-Bazire and Stanier, 1958). Carotenoidless mutants of nonphotosynthetic bacteria have also been found to be far more sensitive to both exogenous and endogenous photosensitizers than are cells of the wild type (Kunisawa and Stanier, 1958; Mathews and Sistrom, 1959*b*, 1960). The studies of Mathews and Sistrom (1959*b*, 1960), conducted with a carotenoidless mutant of *Sarcina lutea*, showed that the primary site of photo-oxidative damage by toluidine blue is the cytoplasmic membrane, as manifested both by derangements of permeability and by selective destruction of membrane-bound enzymes. Since the carotenoid pigments of the wild type are exclusively found in the membrane (Mathews and Sistrom, 1959*a*), their protective action can

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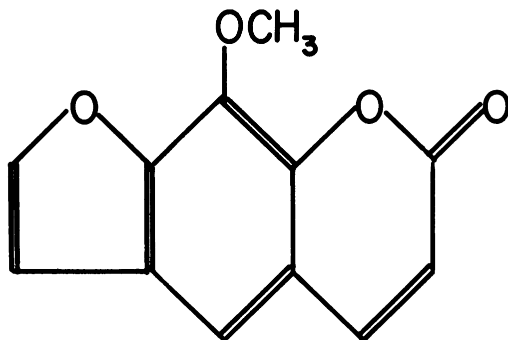

FIG. 1. *Formula of 8-methoxypsoralen.*

best be interpreted as resulting from a minimization of local photo-oxidative damage, although the mechanism involved is still unknown.

Fowlks, Griffith, and Oginsky (1958) recently found that certain furocoumarins of plant origin, notably 8-methoxypsoralen (8-MOP, Fig. 1), can cause a lethal photosensitization of bacteria to long wavelengths of ultraviolet light. The photosensitizing action of 8-MOP differs from that of previously known photosensitizing dyes in several respects, notably in the absence of a requirement for free oxygen (Oginsky et al., 1959). This suggests that the site of its cellular action and the means by which photosensitization is effected may be quite different from those characteristic of such photosensitizers as toluidine blue. Accordingly, a systematic comparison was conducted of the effects of 8-MOP and toluidine blue on *S. lutea*, the organism employed in earlier work on photosensitization by toluidine blue (Mathews and Sistrom, 1960).

MATERIALS AND METHODS

All experiments were conducted with *S. lutea* ATCC 9341a and a colorless mutant obtained by ultraviolet irradiation of the parent strain, previously used in studies on photosensitization by toluidine blue (Mathews and Sistrom, 1960). The same general methods were employed in the present work.

For experiments with 8-MOP, the cells were harvested in the exponential phase of growth, washed once with water, and resuspended in water. The quantity of cells used in these experiments was in the same range as that used in the experiments in which these organisms were exposed to toluidine blue (Mathews and Sistrom, 1960). Solutions of 8-MOP were made up by dissolving the psoralen first in acetone and then in water, as described by Oginsky et al. (1959). The cells were exposed to light either in petri dishes (as described by these authors), in test tubes, or in special flat-sided vessels with a light path of 15 mm. Provision was made in all methods for bubbling the cell suspensions with gas mixtures. To determine the number of surviving organisms after irradiation, viable counts were performed by spreading 0.1 ml of cell suspensions, appropriately diluted, on nutrient agar (Difco) plates. The colonies were counted after 48 hr of incubation at 30 C.

The light source used for experiments with the 8-MOP was a General Electric "Blacklight" fluorescent lamp. This lamp has a maximal output of light between 3,300 and 4,300 A. For experiments with toluidine blue, a General Electric reflector flood lamp was used. All experiments involving light exposure were conducted in a darkened area, where the maximal light intensity did not exceed 5 ft-c. Viable counts were also performed in this area.

The enzyme assays used, and the manner of calculating and expressing the results, are described in a previous paper (Mathews and Sistrom, 1959*a*). An additional method for the measurement of succinic dehydrogenase was also used; it consisted of measuring oxygen uptake manometrically at 30 C in the presence of succinate and phenazine methosulfate (PMS; Cohen-Bazire and Kunisawa, 1960). The manometer flasks contained, in a final volume of 1.4 ml, 60 μmoles of sodium succinate, 100 μmoles of potassium phosphate (pH 7.4), 10 μmoles of $MgSO_4$, and 2 mg of PMS. The reaction was started by tipping in substrate and PMS from the sidearm.

The 8-MOP was provided by the Paul B. Elder Co., Bryan, Ohio. Toluidine Blue O was obtained from Matheson Co., Inc., East Rutherford, N. J. All other chemicals used were reagent grade. The gases used were commercial products.

RESULTS

The exposure of cell suspensions of the colorless mutant of *S. lutea* to long-wave ultraviolet light in the presence of 8-MOP results in the death of more than 99% of the cells within 2 min (Fig. 2). In the absence of either light or

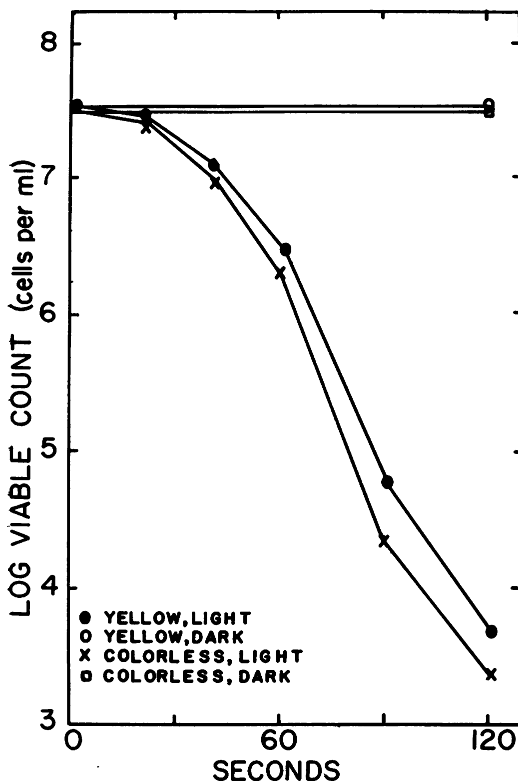

FIG. 2. *Effect of exposure of colorless and pigmented cells of Sarcina lutea to light from a General Electric "Blacklight" bulb in the presence of 6.25 μg/ml of 8-methoxypsoralen.*

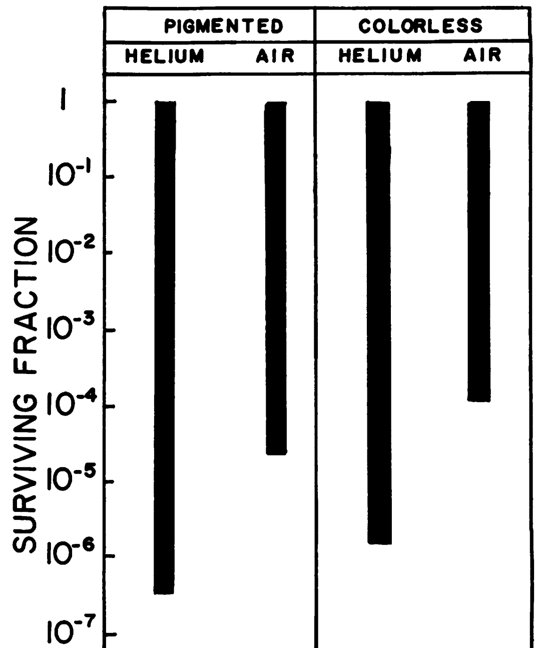

FIG. 3. *Effect of exposure of the pigmented and the colorless strains of Sarcina lutea to 6.25 μg of 8-methoxypsoralen and blacklight for 2 min in the presence of air and of helium.*

psoralen, the cells are not killed; it may be concluded that the killing is due to photosensitization by the 8-MOP.

Carotenoid pigments and psoralen photosensitization. It has been demonstrated that the carotenoid pigments of *S. lutea* can protect the cells against lethal photosensitizations by toluidine blue (Mathews and Sistrom, 1960). However, as shown in Fig. 2, the pigmented and colorless strains of *S. lutea* are killed at essentially the same rate by exposure to long-wave ultraviolet light in the presence of 8-MOP. It can be concluded that carotenoid pigments offer no protection against photosensitization by 8-MOP.

Role of oxygen in photosensitization by 8-MOP. Mathews and Sistrom (1960) showed that oxygen is required for the photokilling of the colorless mutant of *S. lutea* by toluidine blue. The results of the experiments of Oginsky et al. (1959) on *Staphylococcus aureus* and *Escherichia coli* indicated that the presence of oxygen was not necessary for photokilling by 8-MOP. To determine whether this was also the case in the psoralen photokilling of *S. lutea*, the following experiment was performed. Two separate suspensions of cells were exposed to 6.25 μg/ml of 8-MOP and blacklight; one was gassed with helium (shown to be oxygen-free by mass spectrometer analysis), and the other with air. Each tube was bubbled with its respective gas for 5 min before exposure to light; gassing was continued during light exposure and was not stopped until after the light was turned off. The results of this experiment (Fig. 3) demonstrate that oxygen is not needed for photosensitization by 8-MOP, thus confirming the findings of Oginsky et al. (1959).

The data presented in Fig. 3 also show that the degree of photokilling is less in the presence of air (or oxygen) than in the presence of helium. To investigate this phenomenon of apparent protection by oxygen in more detail, on the hypothesis that the psoralen might be destroyed by the oxygen even before reaching the cells, the following experiment was devised. Three tubes, each containing 6.25 μg/ml of 8-MOP, were treated as follows. Tube 1 was bubbled with

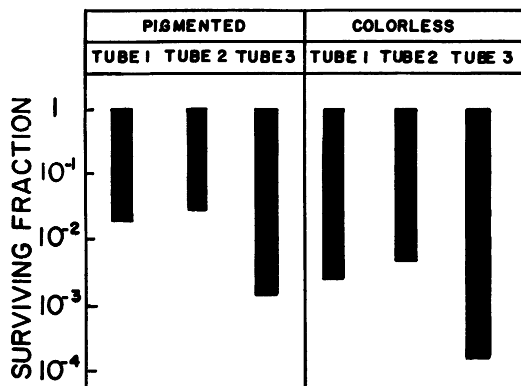

FIG. 4. *Effect of oxygen on the extent of photokilling of the pigmented and colorless strains of Sarcina lutea by 6.25 μg/ml of 8-methoxypsoralen and blacklight. Numbers refer to tubes described in the text.*

oxygen in the dark for 5 min, then with oxygen-free helium for 2 min, and bacteria were then added to the tube; tube 2 received the bacteria, then was bubbled in the dark with oxygen and helium as described for tube 1; tube 3 received the bacteria, but was not bubbled. After receiving the described treatments, all three tubes were exposed to blacklight for 2 min in closed tubes with no gassing. The results of this experiment (Fig. 4) show that there is no significant difference between the degrees of killing in tubes 1 and 2. These results suggest that the apparent "protective" effect of oxygen may result from destruction of the psoralen before it has entered the cell.

Primary sensitive site of psoralen photosensitization. The results of these experiments confirm the fact that photosensitization by 8-MOP is unlike that mediated by such dyes as toluidine blue. In view of this difference, an attempt was made to locate the site of lethal photosensitization in cells treated with 8-MOP. Experiments were performed in which cell suspensions of each strain, containing 6 to 7 mg (dry wt) of cells/ml, were photosensitized by toluidine blue (2×10^{-5} M, 3 hr of tungsten light exposure) and by 8-MOP (25 μg/ml, 5 min of blacklight exposure); their respiration in the presence of two different substrates was measured before and after exposure to each photosensitizer and light. In further experiments, cell suspensions, containing the same amount of cells per ml, were broken in a Raytheon 10-kc sonic oscillator (technique described by Mathews and Sistrom, 1959*a*) after exposure to each photosensitizer and light, and the activities of several enzymes were measured in the total extract. In addition, a sample of each cell suspension exposed to photosensitizer but not to light was also broken, and the total extract was assayed. In each case, viable counts were done, immediately after treatment with the respective photosensitizer, to determine the degree of killing. It can be seen from Table 1 that enzyme activity is not destroyed in either the pigmented or the colorless cells treated with 8-MOP or in the pigmented cells treated with toluidine blue; however, the enzyme activity of the colorless cells treated with toluidine blue, whether in whole cells or in extracts, is greatly diminished.

TABLE 1. *Effect of exposure to 8-methoxypsoralen and exposure to toluidine blue on the enzyme activity of whole cells and extracts of the pigmented and colorless strains of Sarcina lutea*

Enzyme	Exposure to toluidine blue		Exposure to 8-methoxypsoralen	
	Pigmented	Colorless	Pigmented	Colorless
	%	%	%	%
In extracts				
Succinic dehydrogenase	100*	30	89	89
Reduced diphosphopyridine nucleotide oxidase	99	26	92	98
Adenosine deaminase	95	35	90	89
In whole cells				
Succinoxidase	100	8	96	97
Pyruvic oxidase	100	7	100	97

* Figures are percentage of activity of respective dark control remaining after exposure of cells or extracts to respective dye and light.

To determine whether treatment with 8-MOP causes any changes in the permeability of the cells, the spectra of the solutions in which the cells in the experiments just described were photosensitized were taken, in a Cary model 14 recording spectrophotometer, to see if there was any material absorbing at 260 mμ released from the cells. It was found that there was no increase in absorption at 260 mμ in the solutions in which either the colorless or the pigmented cells treated

TABLE 2. *Effect of ultraviolet irradiation, exposure to 8-methoxypsoralen, and exposure to toluidine blue on the development of penicillin-resistant mutants of the colorless and pigmented strains of Sarcina lutea*

Photosensitizer	Radiation	Strain of organism	Number of resistant cells per 10^9 survivors	
			Irradiated	Dark control
None	Short wavelength UV	Pigmented	6,000	0.1
		Colorless	466,000	0.03
Toluidine blue	Visible	Pigmented	0.03	0.07
		Colorless	20	0.04
8-Methoxypsoralen	Long wavelength UV	Pigmented	12,000,000	0.08
		Colorless	3,200,000	0.07

with 8-MOP were suspended. With the cells treated with toluidine blue, an increase in 260-mμ absorbing material was found only in the solution in which the colorless cells were treated with the dye.

The results of the experiments just described indicate that two important functions of the cell membrane, namely the regulation of permeability and enzymatic activity, are unaffected by an exposure to 8-MOP and blacklight sufficient to kill over 99% of the cells. These findings suggest that photokilling by psoralen does not reflect primary damage to the cell membrane. It was then decided to determine whether photosensitization by 8-MOP had any effect on the nucleic acids of the cell. Experiments were devised to determine whether exposure to 8-MOP had any effect on the frequency of occurrence of penicillin-resistant mutants, and to compare its effects to those of a known mutagen, ultraviolet light, and to those produced by treatment with toluidine blue. In these experiments, three separate batches of cells, containing 7–9 × 10^9 cells/ml, were treated as follows. Batch A was suspended in 50 μg/ml of 8-MOP and irradiated with a blacklight bulb, batch B was suspended in 1.25 × 10^{-3} M toluidine blue and irradiated with a reflector flood lamp, and batch C was irradiated with an ultraviolet lamp (2,537 A), each for a time sufficient to kill more than 99% of the cells. A portion of each batch was removed prior to irradiation and kept in the dark. Irradiated and nonirradiated cells were each plated on 50 nutrient agar plates, containing 0.2 units/ml of penicillin, and incubated at 30 C in the dark. These experiments were done with both the pigmented and the colorless strain. The results (Table 2) show that a large number of mutants were obtained from the cells treated with 8-MOP.

Discussion

Several differences between photosensitization of *S. lutea* by 8-MOP and by toluidine blue are apparent from the results presented in this paper. The first difference is in the role of oxygen in the photosensitization; the evidence presented in Fig. 3 and 4 shows quite definitely that oxygen is not necessary for photosensitization by 8-MOP, and that its presence may indeed diminish the photosensitization by destroying the 8-MOP. Oginsky et al. (1959) also found that oxygen was not necessary for, and seemed to diminish, psoralen sensitization of *E. coli* and *S. aureus*, but did not further investigate this finding.

These authors also reported that, as the temperature at which the photosensitization with 8-MOP was carried out was decreased, the amount of killing increased; a temperature coefficient (Q_{10}) of less than 1. We found this to be true also for the 8-MOP photokilling of *S. lutea*. In an experiment performed in the range of 5 to 30 C, the Q_{10} was found to be 0.45. These results are markedly different from those obtained in the photokilling of *S. lutea* with toluidine blue; here, there is no change in the amount of killing when the temperature is lowered (Q_{10} of 1; Mathews and Sistrom, 1960).

It can be seen from Fig. 2 that the rate of killing of the pigmented strain is essentially the same as that of the colorless strain; it can thus be concluded that the carotenoid pigments offer no protection against photosensitization by 8-MOP. This stands in contrast to earlier findings (Mathews and Sistrom, 1960) concerning

photosensitization by toluidine blue, which kills the colorless organism but not the pigmented organism.

It has also been shown that in cells treated with toluidine blue two important functions of the cell membrane, the regulation of permeability and the activities of enzymes shown to be associated with the cell membrane, are destroyed. It can be seen from the data for the permeability experiments that this function is unaffected in both the pigmented and the colorless cells treated with 8-MOP. In addition, it can be seen from Table 1 that succinoxidase and reduced diphosphopyridine nucleotide oxidase activities are also unaffected in cells treated with 8-MOP. It should be pointed out, however, that Pathak and Fellman (1960) found that succinic dehydrogenase activity was destroyed in rat liver mitochondria and in guinea pig skin exposed to 8-MOP and light.

Included in Table 1 are data for two enzymes, adenosine deaminase and pyruvic oxidase, which are located in the soluble fraction of the cell; these are also not affected by treatment with psoralem. However, in the colorless cells treated with toluidine blue, their activity is markedly decreased; but in the pigmented cells similarly treated, there is no decrease in activity. This finding may be explained by postulating that the permeability barrier of the dye-treated pigmented cells is intact, and that not enough dye is allowed to enter the cells to photosensitize the soluble enzymes. On the other hand, the permeability barrier of the dye-treated colorless cells is destroyed, and dye enters the cell and destroys the soluble enzymes.

In sum, then, the photosensitization of *S. lutea* by 8-MOP does not require the presence of oxygen, is temperature-dependent, is not prevented by the presence of carotenoid pigments, and does not result in destruction of cellular protein.

The finding that cellular protein was not affected by treatment with 8-MOP sufficient to kill over 99% of the cells leads to the conclusion that the destruction of cellular protein is not the cause of the lethal action of 8-MOP. An attempt was then made to determine whether the deoxyribonucleic acid (DNA) of the cell was damaged in photosensitization by 8-MOP. It is well known that the DNA of the cell is affected in one kind of photosensitization, that by ultraviolet light. For this reason, the effect on the production of mutants (an indication of alterations produced in the DNA) was determined for the two photosensitizers under study, and for ultraviolet light. It can be seen from the data presented in Table 2 that 8-MOP does indeed increase the frequency of mutants; it can be concluded, then, that 8-MOP affects the nucleic acids of the cell.

It can also be seen from Table 2 that toluidine blue seems to have some effect on the mutation rate; this would be in agreement with the work of Kaplan (1956), who found an increase in s-mutation in *Serratia marcescens* treated with toluidine blue. The findings of Mathews and Sistrom (1960) indicate, however, that this plays no major role in the photokilling of *S. lutea* by toluidine blue, since this killing is completely prevented by the presence of the carotenoid pigments.

The results presented in Table 2, although they indicate a wide variation in the number of mutants produced by the three photosensitizers, cannot be used to compare their relative efficiencies as mutagens, since a detailed study of the relationship of killing curve to mutation frequency has not been made.

It is suggested, then, that photokilling by 8-MOP is quite different from photokilling by toluidine blue, and that the mode of action of 8-MOP in the photokilling of *S. lutea* is not to destroy cellular protein, but to cause alterations in the nucleic acids of the bacteria exposed to it.

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