

LETHAL PHOTSENSITIZATION OF BACTERIA WITH 8-METHOXYPsorALEN TO LONG WAVE LENGTH ULTRAVIOLET RADIATION¹

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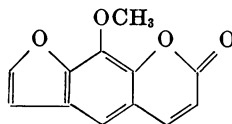
Severe, or even lethal, cellular damage can follow the conjoint action of a chemical photosensitizer and effective frequencies of the electromagnetic spectrum. The fact that bacteria, as well as other cellular systems, are subject to photosensitization by such compounds as methylene blue was known fifty years ago (Reitz, 1908). The relatively limited number of studies of bacterial photosensitization over the years since then has been concerned primarily with the photodynamic action of methylene blue. Recently, Siström *et al.* (1956) demonstrated a lethal photosensitizing effect of bacteriochlorophyll in the absence of carotenoids in *Rhodospseudomonas spheroides*. The photosensitizing action of both dyes and bacteriochlorophyll is dependent primarily on wave lengths in the red region of the visible spectrum.

Another group of photosensitizing compounds was disclosed by the finding that certain furcoumarins of plant origin, particularly 8-methoxypsoralen, are useful in the deliberate photosensitization of the skin of patients with vitiligo (Fahmy and Abu-Shady, 1947), and indeed, the compounds were present in certain folk remedies used for this skin disease in India for at least 3000 years and in Egypt almost as long (Pathak, 1958). While early studies with 8-methoxypsoralen used sunlight as the radiation source, Musajo *et al.* (1954) demonstrated with an artificial light source the effectiveness of frequencies in the long wave length ultraviolet region in producing typical skin responses of erythema and edema. Clinical studies on the use of 8-methoxypsoralen to augment the normal tanning process

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of light sensitive skin have also been reported (Fitzpatrick *et al.*, 1955). That the photosensitization effect of psoralens is not peculiar to human skin was clearly shown by the strikingly increased susceptibility of several bacterial species to long wave length ultraviolet irradiation in the presence of these compounds, as measured by paper-disk diffusion assay (Fowlks *et al.*, 1958). Such irradiation normally requires far more incident energy to kill bacterial cells than does short wave length ultraviolet (Hollaender, 1943); the obvious effect of the psoralens appeared to be a reduction in the amount of energy required for killing bacterial cells with long wave length ultraviolet radiation.



The studies reported in the present paper were conducted primarily for exploration of the mode of action of the psoralens, particularly of 8-methoxypsoralen, the psoralen derivative which is most readily available and which has been most investigated clinically. Investigations were made of the effects of variations in the three components of the photosensitization system: bacterial cells, 8-methoxypsoralen, and light, and in the environmental conditions during and after irradiation. From this accumulation of information, it has been possible to derive a clearer definition of the characteristics of 8-methoxypsoralen photosensitization, and to compare these with some characteristics both of methylene blue photosensitization and of irradiation with long wave length ultraviolet alone.

MATERIALS AND METHODS

The bacterial cultures employed were *Staphylococcus aureus* strain UOMS, and *Escherichia*

coli strains B and B/r.³ Unless otherwise indicated, the bacterial cells were grown on nutrient broth (1.0 per cent peptone, 0.3 per cent beef extract, 0.5 per cent NaCl) in 100-ml aliquots in 300-ml bottles incubated 16 to 22 hr at 37 C without shaking. After centrifugation, the cells were suspended in 0.85 per cent saline to an optical density at 520 m μ of approximately 0.320 for *S. aureus* and 0.360 for *E. coli*, resulting in suspensions with about 6×10^8 colony-forming organisms per ml. Solutions of 8-methoxypsoralen, containing 50 μ g per ml, were freshly prepared for all experiments by dissolving 5 mg in 0.5 ml acetone, diluting with 100 ml saline, heating the solution to boiling, and then cooling to room temperature. Equal volumes of cell suspension and 8-methoxypsoralen solution were mixed at the start of an experiment, giving a final concentration of 3×10^8 bacterial cells and 25 μ g 8-methoxypsoralen per ml. These mixtures were kept in foil covered or low actinic glass Erlenmeyer flasks until samples were irradiated.

The light providing long wave length ultraviolet radiation was a model 70 Glo-Craft lamp (Switzer Bros., Cleveland, Ohio). The predominant output of the light source was found by the use of a wedge interference filter to be in the region between 350 to 400 m μ , which included the 365.4-m μ band of the mercury high pressure arc spectrum. A photometric survey of the target field 15 cm from the Wood's filter on the light source showed variations up to 100 per cent in relative intensities between extreme values and up to 20 per cent between adjacent small areas. Therefore, a relative intensity map of the light field was plotted using a photometer with a movable probe, and an area which had intensity difference of less than 10 per cent was marked with a 60-mm circle. This circle was the target area used in all irradiation studies. During many of the experiments, the output of the light was monitored with the photometer and was never observed to vary more than 5 per cent throughout the experiment. The absolute intensity when measured with a uranyl oxalate actinometer was 6×10^5 ergs/cm²/min.

Samples (5 ml) of the suspensions of bacteria with 8-methoxypsoralen were irradiated in

standard 60-mm pyrex petri dishes at room temperature, unless otherwise noted. For irradiation, the regular top of the petri dish was replaced with a 3-mm thick, 100-mm diameter, optical quality, fused quartz disk. Irradiation in a gaseous atmosphere other than air was carried out using petri dish bottoms modified by the addition of a short side arm, 4 mm in diameter, near the upper rim. The gases, nitrogen, medical helium, and oxygen, were commercial products. The gas flows were saturated with water, and then monitored at 1 L per min into the modified petri dish through the side arm. The gas escaped between the petri dish rim and the quartz disk. The contents of the petri dish chamber were flushed with gas for 3 min prior to irradiation and then irradiated without interruption of gas flow. All preparatory and plating operations were conducted in dimmed diffuse light.

Comparative experiments were also carried out with the classical system of photodynamic action, methylene blue and visible light. The dye used was methylene blue USP, medicinal (National Aniline and Chemical Company, certification no. NA-5, dye content 87 per cent), freshly prepared in saline solution at 75 μ g dye per ml. These solutions were mixed with the bacterial suspensions in the same manner as the 8-methoxypsoralen solutions, giving a final dye concentration of 37.5 μ g per ml. In the case of methylene blue, however, the mixtures were irradiated with a 300 w tungsten bulb at a distance of 20 cm, rather than with the usual ultraviolet light source.

After irradiation of the bacterial suspensions, the number of surviving organisms was determined by serial dilution in distilled water, followed by pour plating of dilution samples with an agar medium containing 0.5 per cent peptone, 0.3 per cent beef extract, 0.1 per cent yeast extract, 0.1 per cent glucose, 0.05 per cent K₂HPO₄, and 1.5 per cent agar. Duplicate, and occasionally triplicate, plates were made for all appropriate serial dilutions. The elapsed time from completion of the irradiation to pouring of the plate never exceeded 5 min, and was in the majority of instances less than 2 min. The plates were incubated in the dark at 37 C for 48 hr before counting. Results are expressed as the average of the counts.

Some variation was encountered, particularly in the early studies, in the surviving fraction

³ We are indebted to Dr. George Stapleton, Oak Ridge National Laboratory, for transfers of the *Escherichia coli* strains, and to the Paul B. Elder Company, Bryan, Ohio, for the 8-methoxypsoralen.

values obtained in similar experiments on different days. It is quite likely that a considerable amount of this variation can be attributed to fluctuation in the ambient temperature during irradiation, since experiments reported later in this paper show that the killing rate is dependent on temperature. In later experiments, when the temperature of irradiation was more carefully controlled, considerably less variation was observed. However, the same conclusions could consistently be drawn from the different experiments, even with some day to day variations in absolute surviving fraction values.

RESULTS

Long wave length ultraviolet irradiation of suspensions of *S. aureus* strain UOMS, and *E. coli* strains B and B/r, in the presence of 8-methoxypsoralen resulted in rapid death of the cells, as measured by loss in colony-forming ability (figure 1). The photosensitizing lethal action of 8-methoxypsoralen was clearly shown by a series of control experiments. No detectable effect on the viable count was obtained on exposure of the bacterial cell suspensions for as long as 10 min, either to 8-methoxypsoralen in the absence of irradiation, or to irradiation in the absence of the compound. Furthermore, in experiments with *S. aureus*, simultaneous irradiation of bacterial cells and 8-methoxypsoralen in separate containers for 120 sec, followed by immediate mixing and subsequent contact for 120 sec did not produce any decrease in the viable count compared to that of an unirradiated control cell suspension. It was thus apparent that the simultaneous presence of 8-methoxypsoralen and radiation was necessary for the lethal effect.

The greater sensitivity of *S. aureus* to the photosensitizing lethal action of 8-methoxypsoralen in these experiments is in agreement with our earlier findings (Fowlks *et al.*, 1958) on the greater susceptibility of gram-positive than of gram-negative organisms to the photosensitizing action of a variety of furocoumarins assayed by a paper-disk diffusion method. The shape of the survival curves obtained with both bacterial species suggested that multiple events were required for a lethal effect. Although such a curve with *S. aureus* might have been a consequence of cumulative kills needed to inactivate a colony-forming cluster, this was not likely with *E. coli*, since the *E. coli* strains used were found by microscopic examination to consist of single cells.

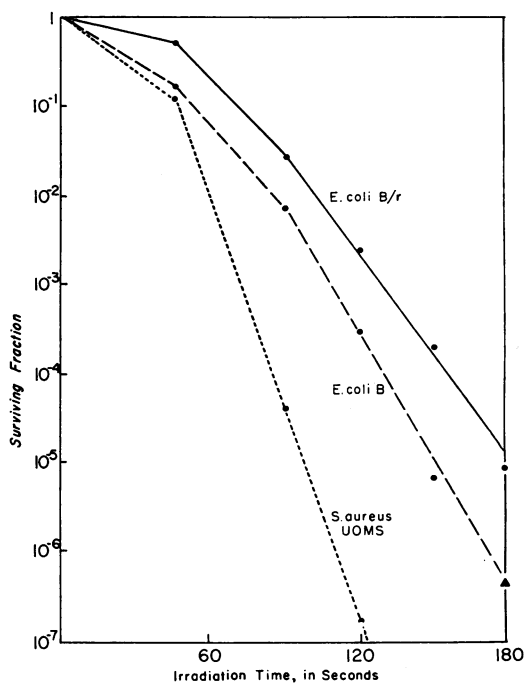


Figure 1. Surviving fractions of *Staphylococcus aureus* and *Escherichia coli* as a function of time of irradiation with long wave length ultraviolet in the presence of 8-methoxypsoralen.

The greater resistance of *E. coli* strain B/r than of *E. coli* strain B to both X-ray and short wave length ultraviolet radiation (Witkin, 1947) was also reflected in its greater resistance to photosensitization by 8-methoxypsoralen to long wave length ultraviolet radiation. This greater resistance of *E. coli* strain B/r to 8-methoxypsoralen photosensitization was observed under a variety of environmental conditions described hereafter. The observations with 8-methoxypsoralen extend the range of agents to which *E. coli* strain B/r exhibits greater resistance, but do not contribute to understanding of the mechanisms of resistance. One difference in the characteristics of the organisms was consistently noted in our studies of *E. coli* strain B and B/r. *E. coli* strain B regularly showed about 1.5 greater viable cell counts than B/r on the plating of cell suspensions made to equivalent optical density at 520 m μ . It should be pointed out that, in agreement with the findings of Witkin (1947), *E. coli* strain B/r reached a somewhat lower final viable count than B in nutrient broth after 16 to 21 hr incubation. However, this difference was pre-

sumably eliminated by adjustment of both B and B/r suspensions in saline to the same optical density at 520 $m\mu$, with consequently smaller volumes of B/r suspension harvested per unit of medium. No gross difference in morphology of the two organisms was apparent in gram stained preparations.

Effect of molecular oxygen. The definition of photodynamic action, as stated by Blum (1941), is "the sensitization of a biological system to light by a substance which serves as a light absorber for photochemical reactions in which molecular oxygen takes part." The sensitization of bacteria to light by methylene blue is in agreement with the above definition for photodynamic action, since Heinmets *et al.* (1952), in studies with *E. coli*, showed that the action spectrum included the absorption maxima of methylene blue and its complexes with bacterial cells, and that the lethal effect was markedly increased by the presence of molecular oxygen.

It was of interest, therefore, to determine whether the photosensitization of bacteria by 8-methoxypsoralen conformed to Blum's definition of photodynamic action by virtue of its acceleration by, or dependence on, the presence of molecular oxygen. Suspensions of *S. aureus* and the two strains of *E. coli* were irradiated in the presence of oxygen, nitrogen, or helium with two systems: 8-methoxypsoralen with long wave length ultraviolet, and methylene blue with visible light. No effect on the viable count was obtained when the radiation sources in the two systems were exchanged. The survival values obtained on irradiation with the proper light sources in the presence of oxygen and nitrogen are shown in figure 2; the data obtained with helium were essentially identical to those with nitrogen. Since the light sources were different and the relative intensities of the effective wave lengths are unknown, the effectiveness of the two compounds cannot be directly compared. However, the identical experimental gassing technique demonstrated that the presence of molecular oxygen markedly accelerated the death rate with methylene blue, whereas no such effect was observed with 8-methoxypsoralen. Indeed, the presence of molecular oxygen consistently retarded the lethal action of the compound as compared to that of nitrogen and helium, and time studies indicated that the rate of death of all three organisms tested was somewhat lower

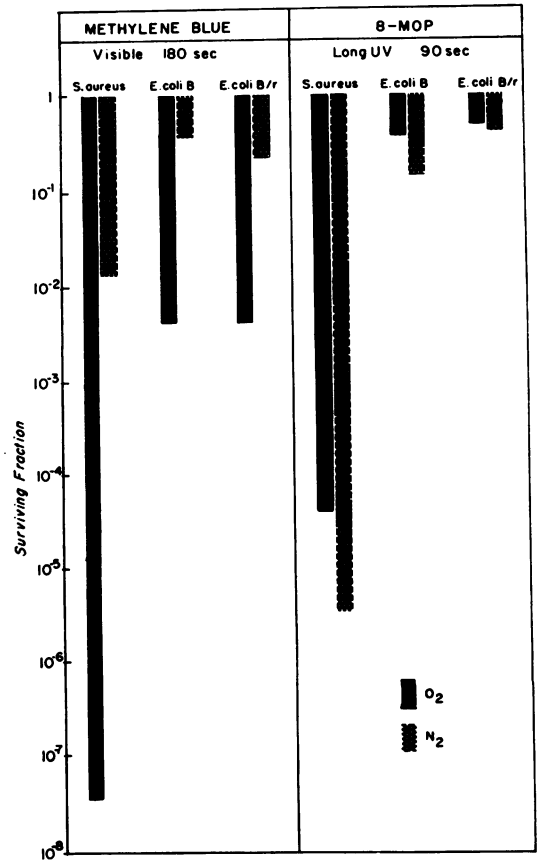


Figure 2. Effect of gaseous environment on surviving fractions of *Staphylococcus aureus* and *Escherichia coli* during irradiation with visible light in the presence of methylene blue, or with long wave length ultraviolet (UV) in presence of 8-methoxypsoralen (MOP).

in oxygen than in the other two gases. The photosensitization of bacteria by 8-methoxypsoralen thus does not appear to be true photodynamic action as defined by Blum, but represents rather a type of photosensitization not accelerated by molecular oxygen.

No consistent difference has been observed between the susceptibilities of *E. coli* strains B and B/r to photosensitization by methylene blue with visible light, in contrast to the results obtained with 8-methoxypsoralen. It may be that our experiments with the dye were not sufficiently extensive to reveal a slight difference in susceptibility between the two strains; but a difference of the magnitude shown with respect

to photosensitization by 8-methoxy-psoralen would certainly have been detected.

The presence of molecular oxygen during growth, as well as during irradiation, was found by Hollaender *et al.* (1951) to increase considerably the sensitivity of *E. coli* suspensions to X-irradiation. Since the lethal effect of 8-methoxy-psoralen photosensitization had not been stimulated by the presence of oxygen during irradiation, it was considered likely that accessibility of air during culture growth also would not increase the sensitivity of the harvested resting cell suspensions. To test this hypothesis, the two strains of *E. coli* were grown under three different degrees of availability of air; the data concerning 8-methoxy-psoralen photosensitization of resting cell suspensions harvested after growth in these different environments are shown in table 1. The sensitivity of *E. coli* strain B was not increased by the degree of aeration during growth, and *E. coli* strain B/r appeared to be even slightly less sensitive when grown aerobically. Thus the availability of molecular oxygen, during either irradiation or growth, did not augment the photosensitization of *E. coli* by 8-methoxy-psoralen, in sharp contradistinction to its reported effects on X-irradiation and on photosensitization by methylene blue.

Effect of 8-methoxy-psoralen concentration. The standard procedure, in which bacterial suspensions were mixed with an equal volume of solution containing 50 μg of 8-methoxy-psoralen per ml, had been imposed to some extent by the

TABLE 1

Effect of availability of air during growth on photosensitization of Escherichia coli resting cell suspensions by 8-methoxy-psoralen

Growth Condition	Surviving Fraction	
	<i>E. coli</i> strain B	<i>E. coli</i> strain B/r
Aeration.....	0.10	0.84
Standard.....	0.11	0.58
Anoxia.....	0.09	0.50

Growth conditions: *aeration*, 100 ml medium in 500-ml Erlenmeyer flask on reciprocal shaker; *standard*, 100 ml medium in 300 ml bottle in stationary position; *anoxia*, 250 ml medium in 300 ml bottle in stationary position. Irradiation 60 sec in air.

limited aqueous solubility of the compound (about 60 μg per ml). The final concentration of the mixtures, 25 μg 8-methoxy-psoralen per ml, provided an amount of furocoumarin adequate to result in significant and reproducible lethal action on irradiations of 15 sec or more. The relationship between concentration and lethal effect was explored by the use of lower concentrations. Both *E. coli* strains B and B/r were subjected to irradiation for periods varying from 45 to 120 sec. In figure 3 are presented data for the effect of 8-methoxy-psoralen concentration on strain B irradiation for 60 sec, and strain B/r for 120 sec. At 25 μg per ml, the standard concentration, these different periods of irradiation gave approximately the same lethal effect on both organisms in these experiments. However, the plots obtained with the two organisms had opposite curvature, so that the compound reached almost maximal effectiveness on strain B at 10 μg per ml, whereas more than 10 μg per ml was required to effect significant killing of B/r. At shorter periods of irradiation than those illustrated in figure 3, the plots of the response to concentration appeared to be more nearly straight lines. The reality of this shift to a linear response was difficult to assess, since the total amount of killing at low concentrations and short irradiation periods was relatively slight, and

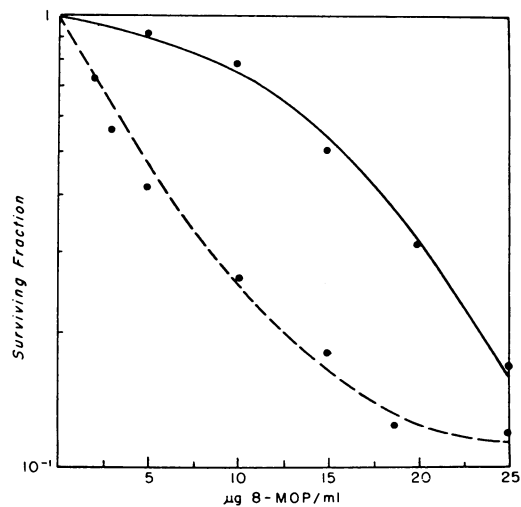


Figure 3. Surviving fractions of *Escherichia coli* strains B and B/r as a function of 8-methoxy-psoralen (MOP) concentration. ---, Strain B, irradiated 60 sec; —, strain B/r, irradiated 120 sec.

definite fixing of the points a formidable task. On the basis of the data obtained in these experiments, the fortuitous choice of irradiation of all cell suspensions in the presence of 25 μg 8-methoxypsoralen per ml was confirmed as a routine procedure.

Action spectrum. A critical discussion of the interpretation of action spectra for ultraviolet irradiation of biological materials has been given by Looftbrow (1948). This interpretation is highly dependent upon the concept of a chromogen as the primary absorber of the effective frequencies, and should apply in principle to the interpretation of the action spectrum of a photosensitization. In general, only for specific absorption bands will any absorbed photon with sufficient energy be equally effective in producing a biological response. A plot of biological activity against wave length should resemble the absorption spectrum of the chromogen over the effective frequencies.

The absorption maxima of the 8-methoxypsoralen spectrum appear at 303, 247, and 215 $\text{m}\mu$ with the absorption coefficient increasing and the bands narrowing as the wave length decreases. On the other hand, suspensions of the bacterial cells used show no well defined absorption peaks below 400 $\text{m}\mu$ (except for a shoulder at approximately 260 $\text{m}\mu$), and the absorption coefficient increases continuously as the wave length decreases. Since there is no obvious absorption maximum of the components of the photosensitization system within the wave lengths available from the light source at 350 to 400 $\text{m}\mu$, it was considered that a cell:8-methoxypsoralen complex with an absorption maximum in this region might have been formed in the mixture. This type of change in absorption spectrum due to cell:photosensitizer complex has been reported for *E. coli* strain B/r mixed with methylene blue (Heinmets *et al.*, 1952). The spectral shift resulted in a reduction of intensity of the 670- $\text{m}\mu$ band of methylene blue and the formation of a new maximum at 610 $\text{m}\mu$. Therefore, absorption spectra from 220 to 400 $\text{m}\mu$ were obtained of suspensions of B and B/r in 8-methoxypsoralen solution, and of each of the components of the mixtures separately. The spectrum of a mixture of cells and the compound was essentially the same as the sum of the spectra of the separate components. It was apparent that if a cell:8-methoxypsoralen complex was indeed formed, it

did not produce a change in the absorption spectrum in this region.

An attempt was therefore made to determine the action spectrum by resort to methods for the reduction of intensity of certain wave lengths. The customary quartz plate was replaced by a series of Corning color filters differing in transmittance. The surviving fraction of a bacterial cell:8-methoxypsoralen suspension irradiated through these filters was compared to the relative amount of energy received by an equal aliquot of ferrioxalate solution (Hatchard and Parker, 1956) irradiated through the same filters under identical conditions. A plot of the surviving fraction under a given filter against the relative intensity transmitted by the same filter resulted in a straight line (figure 4) for almost all of the points, as would be anticipated from adherence to the reciprocity law. A marked displacement from this line would occur only if the filter had cut off a portion of the band that was particularly effective. However, no such displacement was apparent, indicating that all frequencies with relatively high intensity available from the light may be equally effective. The major portion of the wave lengths transmitted by a few of the filters used in these experiments is longer than the 390 $\text{m}\mu$ upper limit of the absorption spectrum of the compound. Since it appears that wave lengths longer than 390 $\text{m}\mu$ are as effective as those shorter than 390 $\text{m}\mu$, it is reasonable to consider that 8-methoxypsoralen is not the primary chromogen in this system. On the other hand, in sufficiently high concentration, the compound can absorb some effective wave lengths from the spectrum of the radiation source. When a layer of solution (50 μg per ml) 1.5 times as deep as the cell:8-methoxypsoralen suspension (25 μg per ml) was interposed as a filter, 46 per cent of *E. coli* strain B/r was killed in 60 sec compared to 82 per cent killed when a similar layer of saline was interposed. It might be noted that this screening by the compound itself may be one of the reasons for the nonlinear response to increasing concentrations (figure 3).

Effect of pH. The experiments of Heinmets *et al.* (1952) on methylene blue photosensitization of *E. coli* demonstrated that as alkalinity increased, more dye was adsorbed by the cells and consequently a greater lethal effect was observed. They found the fraction surviving irradiation at pH 8 to be smaller by 3 log units

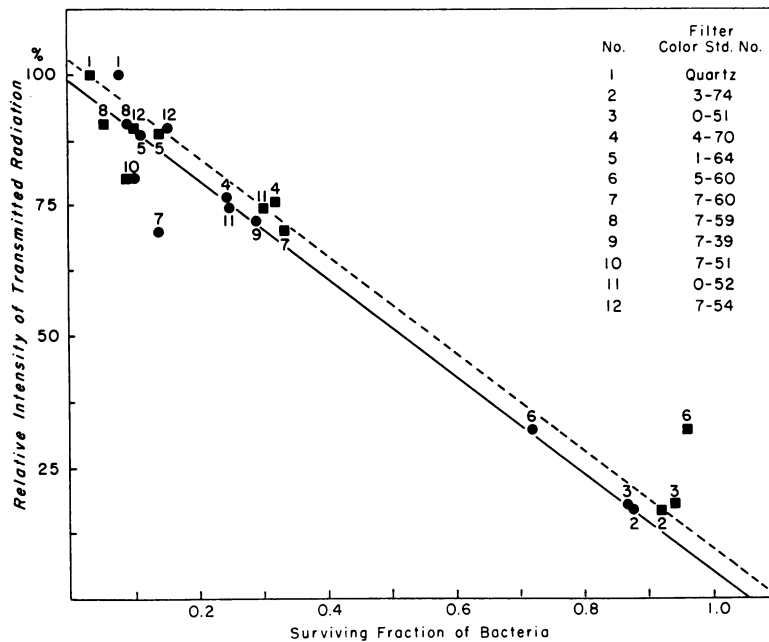


Figure 4. Comparison of the relative intensity of light transmitted by Corning color filters with the surviving fractions of *Escherichia coli* strain B (●—●) after 60 sec irradiation, and *E. coli* strain B/r (■—■) after 90 sec irradiation, through the same filter. Intensity transmitted by standard quartz filter arbitrarily set as 100 per cent.

than the fraction surviving irradiation at pH 5. It was therefore of interest to determine whether such an effect of pH could be demonstrated with 8-methoxypsoralen photosensitization. For these experiments, the bacterial suspensions were made up to twice their usual concentration in saline, and mixtures prepared with 1 volume of cell suspension, 1 volume of 0.2 M phosphate buffer of appropriate pH, and 2 volumes of 50 μ g 8-methoxypsoralen per ml solution. The pH values of the mixtures ranged from 4.3 to 8.7. Irradiation of such strain B mixtures, for either 60 or 90 sec, resulted in essentially the same killing effect at all pH values, acid or alkaline. However, irradiation of *E. coli* strain B/r mixtures for 60 or 90 sec resulted in gradually increasing lethality as the pH increased; for example, at 90 sec, the surviving fraction was 0.48 at pH 5.2, 0.23 at pH 7.1, and 0.088 at pH 8.7. Comparable values for *E. coli* strain B at the same pH values were 0.038, 0.036, and 0.025. The reasons for the differences in response to pH changes on strains B and B/r photosensitization have not been explored, but might possibly be caused either by increased adsorption of 8-methoxypsoralen at

alkaline pH by B/r, or by an increase in sensitivity of the targets in this organism. However, even with strain B/r, the decrease in surviving fraction at more alkaline pH values is of much smaller magnitude than that observed by Heinmets *et al.* on the same organism with methylene blue.

Effect of temperature during irradiation. Photodynamic action on sensitive biological systems has been reported (Blum, 1941) to have a temperature coefficient only slightly greater than 1.0, the theoretical coefficient for photochemical reactions. During the course of our studies, it came to our attention that Graevskii (1952) reported the lethal action of methylene blue on *Bacterium coli commune* (*E. coli*) to have a temperature coefficient of 1.6 to 1.25 over the range from 0 to 37 C. It therefore seemed possible that the 8-methoxypsoralen photosensitization might have a temperature coefficient of similar order, rather a coefficient closely approaching 1.0, and would therefore be more temperature dependent than had been heretofore considered.

To test this presumption in simple fashion, an *S. aureus*:8-methoxypsoralen suspension was

chilled in cracked ice for about 5 min and then irradiated. The results were most surprising, since the chilled suspension contained far fewer survivors than a control suspension irradiated at room temperature. The temperature coefficient appeared to be less than 1.0, a most unexpected finding in comparison with the results on methylene blue. Detailed study of the temperature coefficient was therefore obligatory, for which a different procedure than the usual was devised. The bacterial cell:8-methoxypsoralen suspension was placed in a 2-oz opal glass ointment jar which had been previously brought to the desired temperature in a water bath; the suspension was then allowed to equilibrate to this temperature for 10 min, and then was irradiated in the jar at the standard target area. The heavy walls of the ointment jar provided satisfactory thermal control since the temperatures of the suspensions checked after irradiation were found to agree, within a maximum of two degrees, with the temperature of equilibration.

The results obtained using this procedure with both strains B and B/r plus 8-methoxypsoralen, and with *E. coli* strain B plus methylene blue, are shown in figure 5. The contrasting effects of temperature changes on the lethality of the two photosensitizations are readily apparent. The temperature coefficients (Q_{10}) calculated for methylene blue action were 1.6, both for the range 11 to 21 C, and 21 to 31 C, in reasonable agreement with the data of Graevskii. The temperature coefficients calculated for 8-methoxypsoralen action were less than 1.0, as one could predict from the curves in figure 5. With *E. coli* strain B, over the 0 to 10 C range, the Q_{10} was 0.88; at 10 C intervals from 10 to 45 C, the Q_{10} averaged 0.67, with a standard deviation of ± 0.06 . With *E. coli* strain B/r, over the 0 to 10 C range, the Q_{10} was 0.79; at 10 C intervals from 10 to 45 C, the Q_{10} averaged 0.44, with a standard deviation of ± 0.06 . These relatively constant temperature coefficients over the range of biologically significant temperatures suggest the possibility of one or more competing reactions with constant temperature coefficients above 1.0. Competing reactions which conceivably could have such an effect might involve dissociation of 8-methoxypsoralen from cell constituents either at the surface or within the protoplast, metabolic activity of the cell at the time of irradiation, or

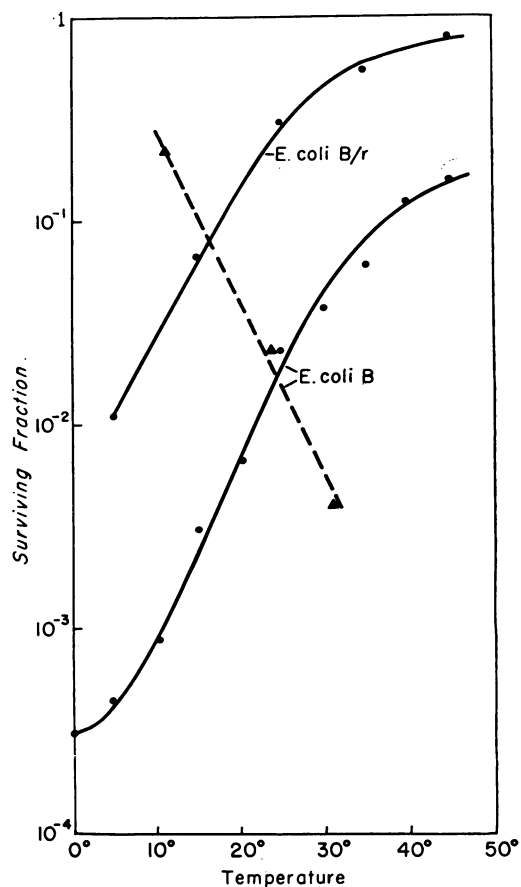


Figure 5. Effect of temperature during irradiation of *Escherichia coli* strains B and B/r with long wave length ultraviolet in presence of 8-methoxypsoralen, and of strain B with visible light in presence of methylene blue. ●—●, 60 sec irradiation in presence of 8-methoxypsoralen; ▲—▲, 180 sec irradiation in presence of methylene blue.

recovery processes immediately subsequent to irradiation. However, it should be emphasized that these effects of temperature occur only if the complete mixture is irradiated at that temperature. Cooling the mixture to 5 C as rapidly as possible after irradiation at room temperature, and holding the mixture at 5 C for 15 min thereafter, had no effect on the viable count, as compared to controls plated immediately after irradiation. Neither did a precooling period, with return to room temperature just before irradiation, affect the count of survivors.

Effect of culture age. Bacterial cells in the lag

or early logarithmic phases of the growth curve have been considered to be more sensitive than cells in the late logarithmic and stationary phases to a variety of deleterious effects. In order to determine whether cell sensitivity to the lethal action of 8-methoxypsoralen was dependent on culture age, measurement was made of the rate of growth of the *E. coli* strains under the standard procedure for preparation of cell suspensions. The growth curves obtained are shown in figure 6. Samples of B and B/r cultures were removed at 0, 60, 150, and 330 min of incubation, centrifuged at 4 C, suspended in saline to the standard density and irradiated in the 2-oz ointment jars at 27 C. *E. coli* strain B was found to be more susceptible to photosensitization by 8-methoxypsoralen in the logarithmic phase of growth, whereas strain B/r was more susceptible in the lag phase (figure 6). No increase in resistance during the late lag and early logarithmic phases, such as was observed by Stapleton (1955) with X-irradiation of *E. coli* strain B/r, was detected in our experiments. Actually, these were periods when the cells were most susceptible to photosensitization, suggesting that sensitivity was greatest during

time of high metabolic activity. There was some indication that it was not metabolism *per se*, but rather metabolic activity of a particular sort, since allowing cells harvested at the stationary phase to oxidize glucose for 20 min immediately prior to irradiation had no effect on the surviving fraction. The periods of higher susceptibility to 8-methoxypsoralen photosensitization are those when there is intense activity in the synthesis of new cellular material, particularly of ribonucleic acid and protein, and it may be that the physiological state of the organism which conditions susceptibility to the photosensitization is a consequence of such synthetic activity.

Nutrient media and survival. The fraction of *E. coli* strain B/r surviving after X-irradiation is dependent on the medium on which the organism is grown (Stapleton *et al.*, 1955). These authors found that growth on simple synthetic media prior to X-irradiation provided for better survival than growth on complex media, whereas the latter were superior for outgrowth of survivors after irradiation. It was suggested that survival was more likely if the cell facing the irradiation had a greater complement of enzymes, or if it had more opportunity to make new en-

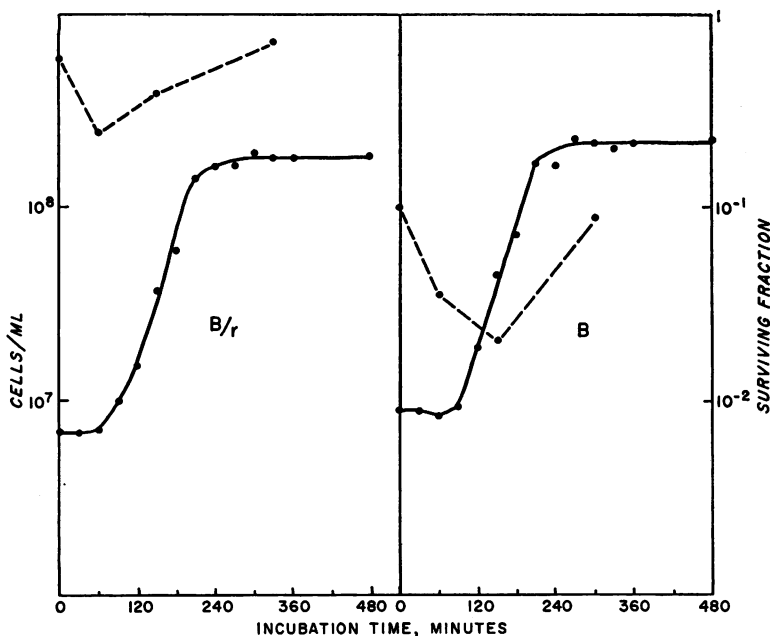


Figure 6. Effect of culture age on the lethality of photosensitization of *Escherichia coli* strains B and B/r by 8-methoxypsoralen. —, Growth curves (cells per ml); ---, lethality of photosensitization (surviving fraction after 60 sec irradiation).

zymes in the recovery process. Since these conclusions would seem to be relevant to many lethal agents whose mode of action appears to be relatively nonspecific, it was considered that the effects of growth on simple or complex media on survival of bacterial cells subjected to 8-methoxy-psoralen photosensitization might perhaps provide some information about the mechanism of damage to the cell.

The effects of plating medium on the outgrowth of strain B/r photosensitized by 8-methoxy-psoralen were determined by the use of Davis A synthetic agar (Davis and Mingioli, 1950) used alone, or supplemented with 0.5 per cent peptone, 0.3 per cent beef extract, or 0.1 per cent yeast extract, or with all three together. These concentrations of the supplements were equivalent to those in the standard complex plating medium. The surviving fractions of cells grown on nutrient broth after 60 and 90 seconds of irradiation were determined for each plating medium by comparison with the counts of unirradiated suspensions on the same medium. As with X-irradiated *E. coli* (Stapleton *et al.*, 1955), it was found that the surviving fraction of photosensitized *E. coli* on synthetic medium alone was considerably less than on standard complex medium. Supplementation improved the performance of the synthetic agar as an outgrowth medium, with beef extract, yeast extract, peptone, and the mixture ranged in order of increasing effectiveness. However, even the addition of all three supplements did not bring the surviving fraction value up to that on the standard complex medium. For example, the surviving fraction after 90 sec irradiation was 0.21 on standard agar, 0.065 on synthetic agar supplemented with all three materials, and 0.0066 on synthetic agar alone. This finding suggested the possibility that one or more of the components of the synthetic medium actually exerted an inhibitory effect on outgrowth; the only substances in this medium absent in the complex medium were Na-citrate, $(\text{NH}_4)_2\text{SO}_4$, and KH_2PO_4 . It is perhaps possible that autoclaving of the supplements with one or more of these substances during preparation of the medium decreased the amount of the factors stimulatory to outgrowth after irradiation.

Attempts were made to substitute ingredients of known composition for the natural product supplements to the synthetic plating medium. No significant activity in promoting recovery

was obtained with any of the following used in varying amounts, singly or together: vitamin-free casamino acids, adenine, guanine, cytosine, thymine, uracil, and a mixture of nicotinic acid, riboflavin, panthothenic acid, pyridoxal, thiamine, folic acid, and biotin. The nature of the factor or factors present in the complex materials which promote recovery of *E. coli* subjected to photosensitization has not been further investigated. It is furthermore not known if the promotion of recovery was indeed due to the enhancement of new enzyme synthesis; the results of these limited experiments do not conflict with this hypothesis.

Our experimental results are however at variance with those reported for X-irradiated cells on the protective effect of growth on synthetic medium prior to irradiation. For these experiments, *E. coli* strains B and B/r were transferred daily for 5 days on the synthetic agar medium, and then inoculated into synthetic broth; comparable inoculations were made from nutrient agar cultures into nutrient broth. The cells prepared from each medium were irradiated with 8-methoxypsoralen, and plated on both the standard complex agar and the synthetic agar. As shown in figure 7, the synthetic-grown cells of both strains B and B/r exhibited as poor recovery on both media as did nutrient broth-grown cells on synthetic agar. The metabolic equipment of synthetic-grown cells apparently was incapable either of taking advantage of the presumed "recovery factors" of the complex agar, or of conferring superiority in the ordeal of irradiation. One might possibly consider that the irradiation of 8-methoxypsoralen photosensitized cells harvested from either simple or complex medium results in an equal degree of damage of the same target(s), but that complex-grown cells are more capable of integrating the externally supplied supplement factors into the metabolic recovery process.

Effects of the damage were readily discernible even by simple metabolic studies. Suspensions of strains B and B/r harvested from nutrient broth were irradiated for 60 sec in the presence of 8-methoxypsoralen, and then samples were transferred as rapidly as possible to Warburg flasks for manometric measurement of oxygen uptake with glutamate, succinate, pyruvate, malate, and acetate as substrates. The oxidation rates were lower by 19 to 70 per cent than those of control

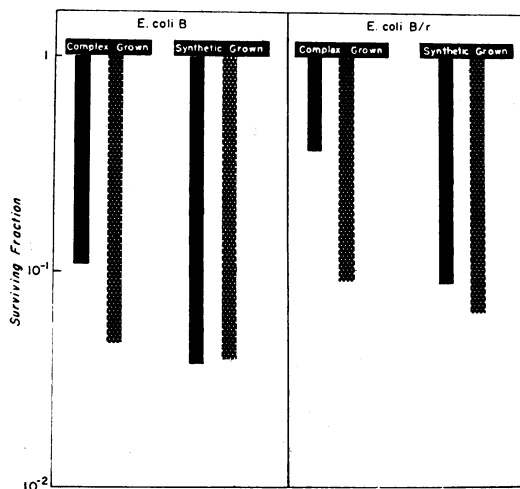


Figure 7. Comparative influence of the composition of growth and plating media on the lethality of photosensitization of *Escherichia coli* strains B and B/r by 8-methoxypsoralen. Solid bars = surviving fraction as plated on standard complex agar; dotted bars = surviving fraction as plated on synthetic agar. Sixty sec irradiation.

unirradiated suspensions during the first 60 min, and remained generally at these lower rates on continued incubation for 240 min thereafter. The effects on these enzymatic systems are probably not directly correlated with the lethal action, since there was a somewhat greater effect on the oxidation rates of strain B/r than on those of strain B. It should be emphasized that while metabolic studies of this order may provide helpful information for further exploration, they are just as likely to present evidence only of the indirect effects of the true mode of action. The explanation for the lethality of 8-methoxypsoralen photosensitization to long wave length ultraviolet may be considerably more complex than direct inactivation of respiratory enzymes.

DISCUSSION

The manifest effect of the presence of 8-methoxypsoralen on long wave length ultraviolet irradiation of bacteria is the marked reduction in the amount of incident energy required for killing the cells. In the present studies, such irradiation in the absence of 8-methoxypsoralen for as long as 10 min had no effect whatever on the viable count. Since the absolute intensity was determined to be 6×10^5 ergs/cm²/min, the

incident energy required for lethal action in this system must therefore have been greater than 6×10^6 ergs/cm². The amount of energy required is probably considerably greater than this, as indicated by the results obtained by Hollaender (1943), who used a system emitting radiation from 350 to 490 m μ , with the major output in the 365-m μ band and only a trace above 436 m μ . With this radiation source, he found that 50 per cent killing of a strain of *E. coli* required 5×10^8 ergs/cm². Our experiments show that with similar irradiation in the presence of 25 μ g 8-methoxypsoralen per ml, only 3.2×10^5 ergs/cm² were required for 50 per cent killing of strain B, and 5.5×10^5 ergs/cm² for strain B/r. Thus, the effect of 8-methoxypsoralen on bacterial cells is indeed a photosensitization, and results in sufficiently critical damage to the cellular physiology for lethal consequences.

This photosensitization of bacteria by 8-methoxypsoralen to long wave length ultraviolet irradiation raises the question whether the normal lethal action of this portion of the spectrum may be due to endogenous substances with an action similar to the furocoumarins. If the furocoumarins are present, they must occur in much lower concentration than in the experiments here described. To our knowledge, these compounds have not so far been reported to be present in bacteria. Their distribution in the plant kingdom is primarily in the Rutaceae, Leguminosae, and Umbelliferae (Geissman and Hinreiner, 1952). Furocoumarins have been shown to be effective inhibitors even in the dark of lettuce seed germination, seedling, and root growth (Rodighiero, 1954), as well as inhibitors of the growth of tomato seedlings, whose roots, immersed in nutrient solution containing furocoumarin, were protected from light (Bennett and Bonner, 1953). Our earlier experiments (Fowlks *et al.*, 1958) showed that a few of the furocoumarins were slightly toxic to some bacteria even in the dark, although markedly greater effects were observed when exposure to these compounds, and to other furocoumarins nontoxic in the dark, was accompanied by exposure to long wave length ultraviolet radiation. If furocoumarins are present in bacteria, their concentration would have to be low enough to be ineffective not only on dark incubation of cells, but also on irradiation of cells with long wave length ultraviolet for at least as long as 10 min. Since the photosensitizing effect

of as little as 5 μ g 8-methoxypsoralen added to 3×10^8 strain B cells, with 60 sec irradiation, could be readily detected (figure 3), the hypothetical concentration would have to be considerably below this amount of furocoumarin.

It seems more likely that the normal lethal effect of long wave length ultraviolet irradiation is the result of reactions other than sensitization by endogenous furocoumarins. The most striking point of difference between such irradiation in the absence and presence of the test compound is the temperature coefficients of the two processes. In the normal irradiation system on *E. coli*, without 8-methoxypsoralen, the temperature coefficient was reported by Hollaender (1943) to be 1.7 to 2.2, over the range 15 to 35 C. In the photosensitizing system, with the compound, the temperature coefficients over the range 10 to 45 C were 0.67 for strain B, and 0.44 for B/r. It was suggested earlier in this report that these fractional coefficients might be due to competing reactions, concerned perhaps with absorption or metabolic processes, whose coefficients were above 1.0. If this were actually the case, the temperature coefficient of the photosensitization process itself might be actually close to 1.0, the theoretical coefficient for purely photochemical reactions. From the coefficients obtained by Hollaender, it is apparent that these postulated reactions either do not operate in normal irradiation without furocoumarin, or else so reinforce the photochemical action of the irradiation as to superimpose their temperature coefficients on the overall lethal process.

The characteristics of the photosensitization of bacteria to long wave length ultraviolet are also sharply distinct from the characteristics of photosensitization by methylene blue to visible light. The most important distinction lies in the different responses to the presence of molecular oxygen. The photosensitizing effect of methylene blue, in its marked augmentation by oxygen, is clearly defined as a type of photodynamic action. The photosensitization effect of 8-methoxypsoralen, on the other hand, cannot be so classified by present definition of this term, since it is certainly not augmented by oxygen and may even be somewhat repressed in the presence of this gas. It differs also from methylene blue photosensitization in having an over-all temperature coefficient below 1.0, and in manifesting much lower sensitivity to changes in environmental

pH. The two photosensitizations are alike in one regard: they are both more effective on gram-positive organisms than on gram-negative ones, although they share this property with so many other noxious agents that no close kinship between the two is thereby conferred.

All three radiation processes, long wave length ultraviolet, with and without furocoumarin, and visible light with methylene blue, exhibit similar killing rate curves. These curves are nonlinear on semilogarithmic plots, and show a greater or lesser threshold effect. Kinetics of this sort are suggestive of multiple, cumulative hits required for inactivation of the cellular unit that is measured by viability counts. With methylene blue, there is considerable evidence that the mode of action involves primarily the destruction of aromatic amino acids, free or within proteins (Fowlks, 1959), and if this is in reality the mode of action within the cell, one might well expect cumulative hits to be necessary. The mode of action of long wave length ultraviolet irradiation, and its counterpart in the presence of furocoumarin, remain to be revealed, but one might predict from the kinetics of the two processes a similar type of cytoplasmic target.

SUMMARY

Photosensitization by the furocoumarin 8-methoxypsoralen of *Staphylococcus aureus* and *Escherichia coli* strains B and B/r results in markedly increased lethal action of long wave length ultraviolet radiation. The shape of the survival curves suggests a multiple event process.

8-Methoxypsoralen photosensitization differs from methylene blue photodynamic action with visible light in its lack of stimulation by the presence of molecular oxygen, and its relative insensitivity to environmental pH.

Stepwise increases in the temperature of irradiation from 0 to 45 C result in proportional diminution of the lethal effect of 8-methoxypsoralen photosensitization. This results in a Q_{10} of 0.67 for strain B, and of 0.44 for strain B/r, over the range from 10 to 45 C. These temperature coefficients are markedly variant from those of methylene blue photodynamic action (1.6) and of long wave length ultraviolet irradiation without furocoumarin (1.7 to 2.2).

Action spectrum studies do not clearly implicate 8-methoxypsoralen as the chromogen in this system nor is there any direct evidence of a cell

constituent:8-methoxypsoralen complex with an absorption maximum in the long wave length ultraviolet region.

The susceptibility of bacterial cells to the photosensitization varies with the age of the culture. Strain B is more susceptible in the logarithmic phase of growth, strain B/r in the lag phase. Outgrowth of 8-methoxypsoralen photosensitized cells is dependent on the media used prior and subsequent to exposure. Cells grown on complex medium throughout exhibit better survival than do cells grown on a synthetic medium at either step. Outgrowth on this synthetic medium after exposure is improved by addition of the components of the complex medium.

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