Light-Induced Lysis and Carotenogenesis in *Myxococcus xanthus*¹

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**ABSTRACT**

BURCHARD, ROBERT P. (University of Minnesota, Minneapolis), AND MARTIN DWORKIN. Light-induced lysis and carotenogenesis in *Myxococcus xanthus*. J. Bacteriol. 91:535–545. 1966.—Myxococcus xanthus, grown vegetatively in the light, developed an orange carotenoid after the cells entered stationary phase of growth; pigment content increased with age. Cells grown in the dark did not develop carotenoid and could be photo lysed by relatively low-intensity light only during stationary phase; rate of photolysis increased with age. Photolysis adhered to the reciprocity law, was temperature-independent and oxygen-dependent, and required the presence of nonspecific, monovalent cations; it was inhibited by one of several divalent cations. Logarithmic-phase cells were photosensitized by 100,000 × g pellet preparations of sonic-treated stationary-phase cells grown in the light and dark. A porphyrin with a Soret band at 408 mμ was isolated from photosensitive cells; logarithmic-phase cells contained about 3/4 of the amount of porphyrin of stationary-phase cells. The purified material had spectral and chemical properties of protoporphyrin IX and photosensitized logarithmic-phase cells. Its spectrum was similar to the action spectrum for photolysis. We concluded that protoporphyrin IX is the natural endogenous photosensitizer. Carotenogenesis was stimulated by light in the blue-violet region of the visible spectrum and was inhibited by diphenylamine, resulting in photosensitivity of the cells. Photoprotection by carotenoid was lost in the cold. A mutant which synthesized carotenoid in the light and dark was photosensitive only after growth in diphenylamine. The ecological significance of these phenomena is discussed.

The observation which led to this investigation was that colonies of *Myxococcus xanthus* FB on agar, incubated for 7 to 8 days in the dark and then inadvertently exposed to light, lysed from their centers outward to their peripheries. Preliminary attempts to attribute this phenomenon to induction of a prophage by visible light were unsuccessful. Further investigation of the phenomenon revealed it to be the result of photolysis of physiologically old cells grown in the dark. It was also observed that colonies developing in the light produced orange pigment, probably carotenoid, and were insensitive to light.

Blum (2) has described several photodynamic diseases of man and higher animals. Among the lower protista, Corynebacterium poinsinii (14), Sarcina lutea (21), Mycobacterium marinum (19), and Halobacterium salinarium (6) can be photo sensitized by exogenous dyes when deprived of their carotenoids. Sistrom et. al. (25) studied photodynamic killing of a carotenoidless mutant of *Rhodopseudomonas spheroides* and concluded that endogenous bacteriochlorophyll was the photosensitizer. Among chemotrophic bacteria demonstrated to be photosensitive, neither isolation nor identification of a photosensitizer has been reported. As indicated above, carotenoid-containing microorganisms are capable of being photosensitized when deprived of their pigment. Griffiths et al. (13) proposed that the primary function of carotenoids is a photoprotective one.

*Myxococcus xanthus* is an inhabitant of the soil; this plus its ability to move over a semisolid substrate would expose the organism to alternating light and dark conditions. As indicated above, light


has marked effects on \textit{M. xanthus}. The role of light as an inducer of lysis is considered in this paper specifically in terms of the photolytic process, conditions required for photosensitivity, and the location and identity of the photosensitizer. In addition, some data pertaining to light-induced carotenogenesis are presented.

**MATERIALS AND METHODS**

\textit{Organism.} \textit{M. xanthus} FB (8), a dispersed-growing myxobacterium, routinely dissociated into yellow and tan colonies. Ultraviolet irradiation of \textit{FB} and repeated selection of a tan variant eventually gave rise to a homogenous nondissociating form (\textit{FB}_d). \textit{FB}_d, a yellow variant, was selected as a spontaneous rare mutant of \textit{FB}. Both \textit{FB}_d and \textit{FB}_r synthesized carotenoid pigments only in response to visible light (see Results). \textit{FB}_r gave rise spontaneously to another stable variant, \textit{FB}_r ct, which synthesized carotenoids in the dark as well as in the light.

\textit{Cultivation of vegetative cells.} Cells were cultured in CT-1 liquid medium [2\% Casitone (Difco), 10^{-3} M K\textsubscript{2}HPO\textsubscript{4}-KH\textsubscript{2}PO\textsubscript{4} (pH 7.6), and 1.85 \times 10^{-4} M MgSO\textsubscript{4}; this differs slightly from CT (8), in that the MgSO\textsubscript{4} concentration was halved] on a rotary or reciprocating shaker at 30 or 22°C, respectively. Cultures were incubated in the light under fluorescent lamps (GE, cool white, 15 W) at 500 to 1,000 ft-c, as measured by a Photovolt Universal photometer, model 200. Cultures incubated in the dark were wrapped in aluminum foil.

\textit{Age of cells.} Logarithmic-phase cells were generally sampled at a culture density of 2 \times 10^{10} to 9 \times 10^{10} per milliliter. When stationary-phase cells were used, the culture was generally at a concentration of 1.2 \times 10^{10} to 2.5 \times 10^{10} cells per milliliter.

\textit{Enumeration of cells.} Cell counts were carried out with a Petroff-Hauser counting chamber and a Zeiss phase-contrast microscope. Dilution of cells, when required, was carried out in deionized water. The spread-plate technique on CT-1 agar (incubation at 30°C for 5 to 6 days) was used for viable counts.

\textit{Cultivation of phototropically carotenoidless cells.} Carotenoid synthesis was inhibited by addition of 10^{-4} M diphenylamine (11) to the growth medium. Culture flasks were wrapped in red, gelatin film, transmitting light primarily above 590 m\AA. This quality light normally induced some carotenogenesis; red light did not photodecompose diphenylamine (14).

\textit{Extraction of pigments.} The bacteria were sediments the centrifugation of the culture medium at 10,000 \times g. Wet pellets were suspended in acetone. The suspension was centrifuged, leaving most of the pigment in the supernatant fraction.

\textit{Preparation of cell-free extracts.} Large quantities of cells were harvested by centrifugation, washed twice in 0.02 M phosphate buffer (pH 7), and resuspended in a small but workable volume of buffer (all steps were carried out at 4°C). The washed cell suspension was sonic-treated for 1.5 min in a 20-kc ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd.). The resulting preparation of ruptured cells was centrifuged at 10,000 \times g for 15 min to eliminate the few whole cells and large debris. The supernatant fraction was centrifuged in a model 40 head in a Spincoco L-2 centrifuge at 4°C for 75 min at 100,000 \times g. The pellet was then resuspended in phosphate buffer (0.02 M, pH 7).

\textit{Isolation of porphyrin.} A variation on the method of Bogorad and Granick (3) was employed for extraction of porphyrins. Approximately 1.3 \times 10^{10} cells (about 1 g, dry weight) were harvested, washed once in deionized water, and extracted twice with 75 ml of glacial acetic acid-concentrated hydrochloric acid (98:2). The preparation was centrifuged at 10,000 \times g, and the supernatant fractions were pooled and adjusted to pH 3.5 with concentrated ammonium hydroxide. Approximately 0.5 volume of water was added, and the pigment was then extracted into ether. The ether was extracted with 0.1 and 1.0 N HCl in sequence, and the 0.1 N extract was discarded.

\textit{Procedure for general photolysis experiments.} Cell suspensions were placed in screw-capped, glass containers to a depth of 1 to 2 mm, and were exposed to cool white, 15-W fluorescent lamps (1,000 to 1,500 ft-c) on a reciprocating shaker at 22°C. When higher-intensity light was required, a 75- or 500-W GE reflector spotlight was used. The latter required the use of a fan and the interposition of a glass dish of water between the cells and the light source to prevent overheating of the suspension. Under these conditions, the 500-W lamp supplied approximately 18,000 ft-c, and the temperature of the cell suspension did not rise above 30°C.

\textit{Preliminary action spectrum.} Light-sensitive cells were placed in screw-capped, square, tablet bottles. Colored filters (gelatin films, Klett-Summerson colorimeter filters, or a Kodak Wratten 18A filter) were attached to the upper face of the bottles. The remaining exposed glass was covered with aluminum foil.

\textit{Spectroscopy of cells and subcellular fractions.} Difference spectra of 100,000 \times g pellets and other fractions were recorded according to the method of Dworkin and Niederpruem (9). Most of the difference spectra and pigment extracts were scanned with a Beckman DK-2A spectrophotometer.

\textit{RESULTS}

\textit{Growth in light and dark.} \textit{M. xanthus} \textit{FB}_d was cultivated in the light and dark; there was no detectable difference in growth rate and yield of the organisms under either growth condition.

\textit{Observations on photolysing cells.} Vegetative cells examined by phase-contrast microscopy were long, slender, flexible, optically dense rods. After exposure of photosensitive cells grown in the dark to light, microscopically visible changes began to occur. The cells became vacuolar or granulated, and distorted. Opacity of an individual cell decreased until the cell appeared as a ghost. Finally, all structural integrity was lost. With the appearance of visible alterations, the cells were no longer viable. Figure 1 indicates the total and viable counts of cultures incubated...
in the light and in the dark. The Petroff-Hausser counts of visually unaltered cells correlate well with viable count and were used as a parameter for viability.

Appearance of photosensitivity. To determine at what stage of growth the dark cells developed photosensitivity, a number of flasks were inoculated with equal amounts of logarithmic-phase cells and incubated at room temperature. For the entire experiment one flask was incubated in the dark, and one in the light. Each of the other flasks was illuminated for a 12-hr period and incubated in the dark for the remainder of the experiment. Data plotted in Fig. 2 indicate that photolysis occurred in cultures exposed to light for the first time at 36 hr or later. The logarithmic phase of growth ended at approximately 24 hr; carotenoid appeared in the culture exposed continuously to light and in that exposed during the 24- to 36-hr period.

To determine the relationship between age of cells in stationary phase and rate of photolysis, FBt cells grown in the dark were exposed to light at various times after the onset of the stationary phase (Fig. 3). As the cells aged, the lag in the survival curve became shorter, the rate of lysis increased, and the percent survivors decreased sharply.

Characteristics of photolysis. Photolysis generally follows a triphasic curve. When the logarithm of the number of the surviving cells was plotted against time of exposure to light, a lag occurred before cell death was detectable. This lag was followed by two phases of exponential death, the second steeper than the first. With higher light intensity or more sensitive cells, or both, we were unable to detect the lag phase or
one phase of exponential death, or both. The photolytic process adhered to the Bunsen-Roscoe reciprocity law; i.e., it was independent of light intensity over the range tested (300 to 660 ft-c). It was also independent of temperature (between 4 and 22 C; Fig. 9).

To determine whether photolysis is oxygen-dependent, as is characteristic of all photodynamic processes (2), photosensitive cells were placed in Thunberg tubes, which were evacuated and flushed 10 times with N2. The cells were then exposed to light in a N2 atmosphere; they did not photolyse, whereas control cells in air did.

*Role of cations in photolysis.* Photolysis is nonspecifically dependent on the presence of monovalent cations (Table 1). No difference in the rate of photolysis was detected with K+, Na+, and Li+

To determine whether light and monovalent-cation dependence are separable, photosensitive FB1 cells suspended in water were exposed to light for the same length of time required for a 100-fold drop in cell number when a water plus K+ suspension of cells was irradiated. The irradiated cell suspension was then divided into two parts; one was placed in the dark and one was placed in the light. K+ was added to both, resulting in an almost immediate decrease in the optical density of both suspensions and distortion and vacuolization of the cells; no lysis occurred. Control cells incubated in water in the dark lysed upon exposure to light and K+; K+ added in the dark resulted in no detectable change.

The lytic process was inhibited in the presence of one of several divalent cations, MgCl2, BaCl2, or CaCl2 at 10^-2 m were equally effective. Cells grown in CT (containing twice the MgSO4 concentration as CT-1) into the stationary phase were not photosensitive in the growth medium or in water plus K+.

*Location and identification of the photosensitizer.* To identify the photosensitizing moiety of stationary-phase cells of *M. xanthus*, a preliminary action spectrum for photolysis was carried out with a series of filters (detailed description in Burchard, Ph.D. Thesis, Univ. Minnesota, 1965). The data led to the suggestion by Burchard and Dworkin (Bacteriol. Proc., p. 92, 1965) that reduced, endogenous cytochromes were the photosensitizers.

To obtain a more precise action spectrum, the Argonne National Laboratory biological spectrograph was employed. The data (5) yielded an action spectrum clearly resembling the generalized absorption spectrum of porphyrins (16).

If stationary-phase cells of *M. xanthus* contain a photosensitizer not present in logarithmic-phase cells, it should be possible to photosensitize the latter by adding this material. It was initially observed that light-insensitive, exponentially growing cells were, themselves, photolysed when added to the light to a photolysed culture of stationary-phase cells grown in the dark. Furthermore, the sensitizing moiety was released by physical disruption of sensitive cells, as was demonstrated by addition of sonic-treated preparations to logarithmic-phase cells. Photosensitive cells were sonic-treated and centrifuged at 10,000 X g, and the supernatant fraction was centrifuged at 100,000 X g. Most of the photosensitizing material was localized in the 100,000 X g pellet, suggesting that it may be bound to the wall-membrane complex.

An experiment was carried out to determine whether stationary-phase cells grown in the light, as well as logarithmic-phase cells, could be photosensitized exogenously and whether photosensitizer was present in sonic-treated preparations of logarithmic phase and light-grown stationary-phase cells, as well as in preparations from cells grown in the dark. The data (Table 2) indicate that the stationary-phase cells grown in the light are insensitive to exogenous, as well as endogenous, photosensitizer. That these cells do have a photosensitizer was confirmed by the ability of a sonic-treated cell preparation to photosensitize logarithmic-phase cells. A detectable amount of photosensitizer was not released when logarithmic-phase cells were disrupted.

Chemical reduction of 100,000 X g pellet preparations of photosensitive cells with Na2S2O4 did not significantly enhance their photosensitizing activity, nor did oxidation with air or K3Fe(CN)6 destroy the activity. Also, ammonium sulfate fractions of 100,000 X g supernatant and pellet preparations demonstrated no relationship between the ability to photosensitize and the height of 425-mu peaks in difference spectra.
Both experiments suggested that the photosensitizing moiety was not a reduced cytochrome(s).

An experiment was carried out in which FB₁ cells grown in the dark were sampled at various times during the stationary phase. Each sample was adjusted to contain essentially equal numbers of cells and was assayed for: rate of endogenous photolysis (Fig. 3), rate of exogenous photosensitization of logarithmic-phase cells by 10,000 × g supernatant fractions of sonic-treated cells (Fig. 4), difference spectrum of 10,000 × g supernatant fraction (Fig. 5), and spectrum of 10,000 × g supernatant fraction (Fig. 6). A 500-ml culture of FB₁ was sampled seven times from onset of the stationary phase (zero-time) to 31 hr into the stationary phase.

Figure 3 demonstrates increasing rates of endogenously sensitized photolysis with age during the stationary phase.

Figure 4 provides evidence that, as the endogenous photosensitivity of the cells increased, the ability of cell-free preparations exogenously to photosensitize insensitive cells also increased.

The 425-mu peaks of difference spectra of the 10,000 × g supernatant fractions are depicted in Fig. 5. There is little detectable difference in height of the peaks, except at 31.3 hr, indicating no correlation between absorption at 425 mu and increasing photosensitivity (Table 3).

Figure 6 demonstrates that the amount of pigment absorbing at 410 mu increased markedly during the stationary phase. Lesser peaks appear at 507, 542, 579, and 633 mu. There is a relatively constant ratio of the height of the 410-mu peak to the slope of the first phase of decline of logarithmic-phase cells exogenously photosensitized

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**Table 2. Photosensitization of Myxococcus xanthus FB₁ logarithmic-phase cells by particulate fractions of sonic-treated preparations of dark- and light-grown stationary-phase cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sonic-treated prepn</th>
<th>Incubation in light*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Logarithmic</td>
<td>—</td>
<td>No lysis</td>
</tr>
<tr>
<td>Stationary, dark</td>
<td>—</td>
<td>Lysis</td>
</tr>
<tr>
<td>Stationary, light</td>
<td>—</td>
<td>No lysis</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>Logarithmic cells</td>
<td>No lysis</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>Stationary, dark</td>
<td>Lysis</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>Stationary, light</td>
<td>Lysis</td>
</tr>
<tr>
<td>Stationary, light</td>
<td>Logarithmic cells</td>
<td>No lysis</td>
</tr>
<tr>
<td>Stationary, light</td>
<td>Stationary, dark</td>
<td>No lysis</td>
</tr>
<tr>
<td>Stationary, light</td>
<td>Stationary, light</td>
<td>No lysis</td>
</tr>
</tbody>
</table>

* Incubation in the dark consistently produced no lysis.
3. TABLE at different of dark-grown was tion the by 10,000 times Variation in spectrum action was made of spectrum cells. in Fig. a Slp 2.90 0.09 1.65 Stimmle hase supernatant 6. FIG. 7. Spectrum of protoporphyrin IX from stationary-phase Myxococcus xanthus FBt cells (solvent: HCl).

The 408-mu Soret band and smaller peaks at 556 and 602 mu were similar to the spectrum of protoporphyrin IX. Spectroscopy of a dioxane solution demonstrated peaks at 405, 503, 537, 575, and 630 mu, which were identical with those of protoporphyrin IX. Additional techniques (24) were used to confirm the identification. The pigment fluoresced red when excited with ultraviolet light. Upon fractionation with butanol-10% NaOH, the butanol phase fluoresced, indicating that the porphyrin contains two carboxyl groups. The pigment was not extractable from ethyl acetate with 1% HCl, but it was extracted with 5% HCl, giving an approximate HCl number consistent with the properties of protoporphyrin IX.

Equal quantities of light-insensitive logarithmic-phase cells and light-sensitive stationary-phase cells of FBt were extracted for protoporphyrin IX. A small amount of the pigment was found in logarithmic-phase cells; approximately 16 times this amount was found in stationary-phase cells. To determine whether the extracted protoporphyrin IX could photosensitize logarithmic-phase cells, 0.2 ml of a solution containing approximately 1 ug/ml and brought to pH 7.8 with NH4OH was added to 0.3 ml of logarithmic-phase FBt cells in CT-1 at an approximate concentration of 4 x 10^6 cells per milliliter. The data are presented in Table 4. The isolated protoporphyrin IX photosensitized logarithmic phase cells, and an authentic crystalline sample of protoporphyrin IX also acted as a photosensitizer.

Spectroscopy of the isolated protoporphyrin IX at pH 7.8 demonstrated no characteristic peaks, nor did the solution fluoresce. Reacidifica-

by 10,000 x g supernatant preparations (Table 3). Variation in the value of the ratio may reflect the manner in which the rate of photosensitiza-
tion was calculated.

The spectrum of these preparations and the action spectrum (5) are similar to the absorption spectrum of porphyrins. Therefore, an attempt was made to isolate porphyrin(s) from photosensitive cells. The 1.0 N HCl extract contained a pigment with an absorption spectrum depicted in Fig. 7.

**TABLE 3. Correlation of spectral peaks of 10,000 x g supernatant fractions of dark-grown Myxococcus xanthus FBt cells with rates of exogenous photosensitization of logarithmic-phase FBt cells by these fractions**

<table>
<thead>
<tr>
<th>Sample time</th>
<th>I Slope of first phase of log decline (Fig. 4)</th>
<th>Optical density</th>
<th>Column II/1</th>
<th>Column III/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>hr</td>
<td>II At 425 mu, reduced (Fig. 5)</td>
<td>III At 410 mu (Fig. 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.59</td>
<td>0.035</td>
<td>0.29</td>
<td>0.059 0.49</td>
</tr>
<tr>
<td>1.5</td>
<td>0.42</td>
<td>0.035</td>
<td>0.28</td>
<td>0.083 0.67</td>
</tr>
<tr>
<td>3.5</td>
<td>0.70</td>
<td>0.025</td>
<td>0.34</td>
<td>0.036 0.49</td>
</tr>
<tr>
<td>5.8</td>
<td>0.65</td>
<td>0.03</td>
<td>0.42</td>
<td>0.046 0.65</td>
</tr>
<tr>
<td>8.3</td>
<td>1.06</td>
<td>0.025</td>
<td>0.61</td>
<td>0.024 0.58</td>
</tr>
<tr>
<td>19.3</td>
<td>1.65</td>
<td>0.03</td>
<td>0.72</td>
<td>0.018 0.44</td>
</tr>
<tr>
<td>31.3</td>
<td>2.90</td>
<td>0.09</td>
<td>0.98</td>
<td>0.031 0.34</td>
</tr>
</tbody>
</table>

**TABLE 4. Photosensitization of logarithmic-phase Myxococcus xanthus FBt cells by protoporphyrin IX extracted from stationary-phase FBt cells**

<table>
<thead>
<tr>
<th>Porphyrin IX</th>
<th>Incubation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Light</td>
<td>Lysis</td>
</tr>
<tr>
<td>+</td>
<td>Dark</td>
<td>No lysis</td>
</tr>
<tr>
<td>-</td>
<td>Light</td>
<td>No lysis</td>
</tr>
<tr>
<td>-</td>
<td>Dark</td>
<td>No lysis</td>
</tr>
</tbody>
</table>

FIG. 6. Spectra of 10,000 x g supernatant fractions of dark-grown Myxococcus xanthus FBt cells sampled at different times after onset of the stationary phase.
tion resulted in the return of the characteristic behavior.

Pigment characterization. The predominant pigment in acetone extracts of stationary-phase FBt cells grown in the light was a carotenoid with absorption maxima at 455, 477, and 510 μm. FBt extracts had additional peaks at 360, 373, and 405 μm; FBt extracts demonstrated absorption in this region, but distinct peaks were rarely detectable.

Extracts of stationary-phase FBt cells grown in the dark demonstrated absorption maxima at 361, 379, 403, 504, 537, 579, and 633 μm. FBt extracts had almost identical maxima. However, the relative heights of the peaks varied. The yellow color of stationary-phase FBt cells grown in the dark was probably due to its high content of pigment absorbing at 379 μm.

Appearance of pigment. Pigmentation, as well as photosensitivity, also appeared after termination of the logarithmic phase. Figure 8 shows the contents per cell of pigments absorbing at 379 and 477 μm after different lengths of incubation (acetone extracts of wet cell pellets). The point at which logarithmic growth terminated is indicated. No significant amount of either pigment was present until the onset of the stationary phase, when a sharp rise in pigment synthesis occurred. There was no detectable carotenoid synthesis at 477 μm in either strain when grown in the dark. Other experiments demonstrated that a pigment absorbing at 403 μm, also appearing during the stationary phase, developed to the same level per cell in light and dark cultures.

Fruiting-body pigmentation. Myxococci are often identified by the color of their fruiting bodies. It was of interest to examine the location of pigments in these multicellular structures. Greene and Leadbetter (Bacteriol. Proc., p. 34, 1962) reported that M. xanthus microysts contain smaller amounts of saturated carotenoids and larger amounts of less-saturated (colored) carotenoids than do vegetative cells. Strain FBt, because of its higher pigment content, was used for fruiting-body experiments, as described by Dworkin (8). The pigmentation of fruiting bodies incubated in light or dark was qualitatively the same as that found in the respective stationary-phase vegetative cells grown in light or dark. Sonic treatment of a suspension of these fruiting bodies, containing vegetative cells as well as microysts, released the pigment into the 5,900 × g supernatant fraction; unpigmented microysts resistant to sonic treatment were separated from the suspension by centrifugation. Therefore, essentially all of the 477-μm pigment in light-incubated, and 379-μm pigment in dark-incubated,

![Fig. 8. Development of pigment in Myxococcus xanthus FBt and FBt during light and dark growth. Symbols: ○, dark-grown, 379 μm; △, dark-grown, 477 μm; ○, light-grown, 379 μm; △, light-grown, 477 μm.](http://jb.asm.org/)

6- to 7-day-old fruiting bodies is associated with vegetative cells.

Photoinduction of carotenogenesis. Incubation of cells in various combinations of light and dark periods indicated that illumination was not required throughout the period of carotenogenesis. Furthermore, photoinduction of carotenogenesis appeared to be independent of light intensity over a range of 11 to 165 ft-c of light passing through yellow gelatin filters. This suggested that photoinduction sites were saturated at a low light intensity.

A crude action spectrum for carotenogenesis was carried out with filters described by Burchard (Ph.D. Thesis, Univ. Minnesota, 1965). In determining the radiant power actually reaching the cells at any one wavelength or in any band, both the spectrum of emission of the lamp and the broad spectrum of transmission of the respective filters had to be considered. The data suggested that light in a band between 390 and 425 μm and in the green region of the visible spectrum stimulated carotenogenesis.

Carotenoid function. It was of interest to determine whether the function of the carotenoid in M. xanthus is a photoprotective one as it is in other microorganisms containing these pigments.
It was indicated above that carotenoid absorbing at 477 m\(\mu\) appeared in light-grown cells immediately prior to the time of appearance of photosensitivity in dark-grown cells. To determine whether there was a correlation between appearance of photosensitivity in dark-grown cells and appearance of carotenoid in light-grown cells, two FB\(_1\) cultures were inoculated with equivalent amounts of exponentially growing FB\(_1\) cells and incubated in light and dark. At intervals, samples were withdrawn from the light-grown culture for assay of carotenoid content (477-\(m\mu\) absorption) and from the dark-grown culture for determination of the rate of photolysis, calculated as the slope of the curve during the first exponential phase of decline (\(\Delta\) log of total cell number/\(\Delta\) time). The data are presented in Table 5. Any variation from the relatively constant ratio of rate of photolysis of dark-grown cells to carotenoid content of light-grown cells could not be correlated with age.

Further evidence regarding the photoprotective role of carotenoids arose from experiments in which diphenylamine was used to inhibit synthesis of unsaturated carotenoids. Dark-grown stationary-phase FB\(_1\) cells were light-sensitive with or without diphenylamine in the growth medium. Cells grown in white and red light in the absence of diphenylamine developed 477-\(m\mu\) carotenoid and were found to be insensitive to light. Cells grown in red light in the presence of 10\(^{-4}\) M diphenylamine developed only 10\% of the carotenoid found in cells grown in white light without diphenylamine; the former were photolyzed by exposure to white light.

FB\(_{1}\)-dc, the mutant which synthesizes carotenoid during the stationary phase in both light and dark, was insensitive to light unless carotenoid synthesis was inhibited by growth in diphenylamine.

Dworkin (7) and Mathews (20) demonstrated a loss of photo-protection when carotenoid-containing *Rhodopseudomonas spheroides* and a *Mycobacterium* sp. were illuminated in the cold. A similar experiment was carried out with stationary-phase FB\(_1\) cells grown in the light (Fig. 9). It was found, by use of a high-intensity light source (approximately 18,000 ft-c), that carotenoid-containing cells photolyzed relatively rapidly at 4 C. Dark-grown cells photolyzed extremely rapidly at the same temperature. Photolysis of the light-grown cells also occurred at 22 C, albeit at a considerably lower rate than at 4 C.

**DISCUSSION**

The photolysis of dark-grown stationary-phase cells of *M. xanthus* is a typical photodynamic process. It is purely photochemical, involving no diffusional or enzymatic steps, as demonstrated by adherence of the process to the reciprocity law and by its temperature independence.

Burchard and Dworkin (Bacteriol. Proc., p. 92, 1965) suggested that endogenous reduced cytochromes are the photosensitizers of stationary-phase *M. xanthus* cells. The suggestion was based on a crude photolysis action spectrum, which resembled the difference spectrum of cytochromes of *M. xanthus* (9), and on the location of the photosensitizer in a particular fraction of the cells. Further experiments attempting to increase and decrease photosensitization in 100,000 \(\times\) g pellet preparations of photosensitive...
cells by chemical reduction and oxidation, respectively, were inconclusive. If reduced cytochromes were the photosensitizing moieties, a decrease in the height of the 425-μm Soret peak of difference spectra would be expected with increasing age and photosensitivity. The data in Table 3 do not support this suggestion. However, there is the possibility that in the preparation of the fractions cytochromes are oxidized to the same level by O₂.

A refined photolysis action spectrum, isolation of protoporphyrin IX from photosensitive cells, and photosensitization of light-insensitive cells by protoporphyrin IX have since demonstrated that protoporphyrin IX is the photosensitizing moiety in stationary-phase M. xanthus cells. Furthermore, the photosensitization of logarithmic-phase cells is correlated with the presence of relatively small quantities of protoporphyrin IX.

Blum (2) suggested that endogenous protoporphyrin photosensitizes red blood cells. Rudzinska and Granick (23) found that old cultures of *Tetrahymena geleii* produce protoporphyrin after growth in the dark. Upon exposure to light, cells containing the pigment either lysed or the pigment was released into the medium. Granick (12) identified, as protoporphyrin IX, a pigment which accumulates in an X-ray induced mutant of *Chlorella* after the termination of logarithmic phase. Lascelles (15) discussed microorganisms which form considerable amounts of free porphyrin when grown under conditions limiting synthesis of heme enzymes. Thus, protoporphyrin has been implicated as an endogenous photosensitizer; furthermore, in some organisms free porphyrins appear in quantity only during the stationary phase or at times of biosynthetic limitation. We suggest that protoporphyrin IX in *M. xanthus* may be a precursor or breakdown product of particle-bound cytochromes, and its appearance may reflect a biosynthetic alteration resulting from respiratory changes occurring in the stationary phase.

Any molecule lacking significant light absorption and unable to fluoresce would not be expected to photosensitize (2). The loss of fluorescence and typical absorption spectrum of isolated protoporphyrin IX in the physiological pH range, probably due to colloid aggregation, suggests that association with the wall-membrane complex of the cell may be necessary for the characteristic absorption and fluorescence properties. An alteration of the absorption spectrum of chlorophyll upon isolation has been reported by Wassink et al. (27). To account for the observed exogenous photosensitization by protoporphyrin IX, it is suggested that absorption of the molecules to the wall-membrane complex of viable cells simulates its in vivo state in stationary-phase cells, resulting in the reappearance of the characteristic absorption spectrum, its ability to fluoresce, and its photosensitizing ability.

Our results indicate that the site of photodamage is the cell wall-membrane complex. The facts, that 100,000 × g pellet fractions of sonic-treated photosensitive cells can photolyse insensitive cells and that morphological changes occurring during cell photolysis indicate alteration of the limiting membranes of the cells, suggest that the photosensitizer is bound to the wall-membrane complex. Also, the carotenoid must be closely associated with the photosensitizer to serve in a photoprotective role; the pigment is apparently located in the wall-membrane complex (18).

It appears that divalent cations maintain a wall-membrane macromolecular configuration similar to that present in a medium of low ionic strength, possibly by forming salt bridges between amino acid carboxyl groups as was suggested by Brown (4) for a marine pseudomonad. This configuration may be such that immediate contact of structural components with bound photosensitizer is prevented. Monovalent cations in the absence of divalent cations may alter the configuration of membrane-bound structural components, placing them in apposition to photosensitizing moiety and subjecting them to photooxidation.

Further study is required regarding two observations. (i) Visible photodamage appears to be divisible into light-dependent and monovalent cation-dependent phases. It may be that light, in the absence of monovalent cation, may cause damage which is not visibly manifested. (ii) Cells grown in CT, with twice the MgSO₄ concentration as in CT-1, are not photolyzed. This would suggest an alteration in the wall-membrane complex. McQuillan (17) has reviewed several studies which indicate that Mg²⁺ functions in maintaining the integrity of protoplast membranes and preventing penicillin lysis in gram-positive and gram-negative microorganisms. The fact that cells do photolysed in CT-1, containing 1.85 × 10⁻³ M MgSO₄, suggests that the photoprotective Mg²⁺ may be chelated by medium constituents.

The spectra of acetone-soluble pigments of *M. xanthus* FB₁ and FB₂, correlates well with that of *Myxococcus* pigments described by Greene and Leadbetter (Bacteriol. Proc., p. 34, 1962; p. 54, 1963; p. 17, 1964). The orange carotenoid in light-grown cells appears to be Greene and Leadbetter's P-476 or P-478. The predominant pigment(s) of dark-grown cells (maxima at 361 and 379 μm) may be identical to Greene and Lead-
better's P-355 and P-378. These authors (Bacteriol. Proc., p. 17, 1964) suggested that P-378 is a precursor for the light-induced P-478. The 403-nm peak in acetone extracts of wet pellets of stationary-phase cells is identical to the 410-nm peak predominant in aqueous suspensions of cell-free preparations; we suggest that it is the Soret band of protoporphyrin IX.

As in other carotenoid-containing microorganisms the pigment(s) in *M. xanthus* are photoinduced, but only at a time immediately prior to the appearance of photosensitivity in dark-grown cells. The increase in carotenoid content in the light parallels increased photosensitivity in the dark, suggesting a finely controlled regulation of the amount of photoprotective pigment.

From a crude action spectrum we would suggest that the photoreceptor for carotenogenesis could also be protoporphyrin IX. A sublethal amount of the porphyrin is present in light-insensitive cells; relatively little light in contrast to that required for photolysis is required for carotenogenesis. The initial photinduction is probably followed by light-independent dark reactions, as suggested for *Mycobacterium* carotenogenesis (22).

It has been proposed that carotenoids function as a substrate for photooxidation by the excited photosensitizer in various microorganisms; they may then be cycled back to the reduced form by an enzymatic mechanism (1, 7, 10, 20, 26). Evidence for the same function of carotenoid in *M. xanthus* has been presented in the form of an experiment which demonstrates photo-sensitization of carotenoid-containing cells at high light intensity at 4 C. It is suggested that the unsaturated carotenoid absorbing at 477 nm is interposed between the photosensitizer and the photo-oxidizable structural component of the cell wall-membrane complex; the carotenoid may then serve as an alternative substrate for photooxidation. A reductase serves to cycle the oxidized carotenoid to the reduced state. This activity is inhibited at low temperature. Upon exposure to light, carotenoid-containing cells incubated at 4 C or dark-grown stationary-phase cells deplete their oxidizable pigment or have none, respectively; they then photolyse.

Exposure to light may have provided selective pressure for the development of carotenoids in *M. xanthus* and their role in photoprotection. However, presence of photosensitizer is only manifested in stationary-phase cells. In the ecological niche of *Mycococcus*, is it likely that the organism ever enters the stationary phase? In batch cultures, the stationary phase of growth is usually a consequence of the depletion of an essential component for growth or of the accumulation of toxic end products. The ability of the organism to move over a solid surface in its natural environment and its ability to form fruiting bodies and microcysts under conditions of nutrient depletion suggest that it need never encounter either of these situations in the vegetative cell state. When, then, does photosensitivity appear in nature? Also, the question arises as to whether the organism encounters the ionic conditions required for manifestation of photolysis in its environment. The ecological significance of this photosensitivity is, therefore, not clear. Yet, assuming that the primary role of carotenoids is a photoprotective one, the organism's ability to synthesize carotenoid(s) would indicate that it encounters conditions in which it is potentially photosensitizable.

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