THE PHOTODYNAMIC INACTIVATION OF PHAGE PRECURSOR
BY METHYLENE BLUE*

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It has been shown that phage-susceptible staphylococci grown under
optimal conditions in oxygenated broth and subsequently brought to the
resting state by storage in Locke's solution at 5° C., have the property
of rapidly producing a marked increase in [phage] upon addition to phage
(1, 2). This effect, confirmed by Northrop (3), has been ascribed to the
presence in the activated staphylococci of a phage precursor which when
brought into contact with phage is autocatalytically transformed into
phage (4). The intracellular precursor is more labile than either the cells
which produce it or the phage into which it is converted (5, 6). For exam-
ple, activated staphylococci can be made to lose their phage-augmenting
capacity by the following procedures: (1) treatment with anti-staphylococcal
serum; (2) heating at 45° C. for 20 minutes; (3) exposure to 1 × 10^{-4} M
iodoacetic acid for 30 minutes. In all three cases the phage precursor
content of the cells is destroyed before any measurable number of cell
deaths has occurred as determined by plate counts. The data we present
here indicate that similar inactivation of intracellular precursor can be
obtained through the photodynamic action of methylene blue.

Suspensions of activated staphylococci were prepared as described in an earlier paper
(4). The organisms were concentrated by centrifugation and were resuspended in
Locke's solution of pH 7.0 to give a density of 1 × 10^{8} bacteria/ml. 1.0 ml. of activated
cell suspension was added to 1.0 ml. of chemically pure methylene blue of appropriate
concentration dissolved in Locke's solution. The mixtures were exposed to strong light
of 4000–8000 Å emanating from a 500 watt cold crash lamp, 120 volts, type C, at a
distance of 36 cm. from the end of the filament. During exposure the preparations were
maintained at 5° C. and at intervals samples were removed for determination of viable
cell content and for detection of residual phage precursor. For the latter purpose
aliquots were diluted to contain 5 × 10^{8} bacteria/ml and 4.0 ml. of each sample were
mixed with 1.0 ml. of phage containing 1 × 10^{9} activity units/ml. The phage-cell
mixtures were kept at 5° C. for 10 minutes to permit interaction of the residual precursor

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and the added phage. After this interval dilutions were made for titration by the activity method (7), using three dilution checks for each unknown.

Experimental controls included the following: (1) Duplicates of each mixture kept in the dark at 5°C. (2) Experiments to detect:

(a) Any possible effect on the titration system of the small concentrations of methylene blue present in the final dilutions of each unknown.

(b) Lethal action of the concentrations of methylene blue used on activated staphylococci.

(c) Possible inactivating effect of methylene blue on phage during the 10 minute interval each mixture was kept.

(d) The effect of the light alone on the precursor content and viability of activated cells.

Table I

Photodynamic Inactivation of Intracellular Phage Precursor by Methylene Blue

Activated staphylococci in Locke's solution containing 9.1 × 10^{-7} M methylene blue exposed to strong light. Samples at intervals tested for residual phage precursor and for [bacteria]. Data represent averages of five consecutive experiments.

<table>
<thead>
<tr>
<th>Molarity methylene blue</th>
<th>Time of exposure (min.)</th>
<th>Phage developed after adding exposed cells to phage</th>
<th>Bacteria by plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>0</td>
<td>1.22 × 10^5 P.U./ml.</td>
<td>7.0 × 10^9/ml.</td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>1.0</td>
<td>1.5 × 10^5 P.U./ml.</td>
<td>6.2 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>5.0</td>
<td>1.4 × 10^5 P.U./ml.</td>
<td>6.2 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>10.0</td>
<td>1.32 × 10^5 P.U./ml.</td>
<td>6.4 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>15.0</td>
<td>1.25 × 10^5 P.U./ml.</td>
<td>5.6 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>30.0</td>
<td>4.8 × 10^5 P.U./ml.</td>
<td>5.8 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>45.0</td>
<td>3.6 × 10^5 P.U./ml.</td>
<td>7.2 × 10^9/ml.</td>
</tr>
<tr>
<td>9.1 × 10^{-7} M</td>
<td>60.0</td>
<td>2.2 × 10^5 P.U./ml.</td>
<td>5.7 × 10^9/ml.</td>
</tr>
</tbody>
</table>

The control experiments were completely negative; that is, the concentration of methylene blue employed had no direct action on phage, no killing action on the bacteria, no demonstrable effect on the titration system, nor did the mixtures kept in the dark show any inactivation of precursor. Further, exposure of activated cells to the light source did not result in precursor inactivation.

It is evident from the data summarized in Table I that 9.0 × 10^{-7} M methylene blue will inactivate the phage precursor in a suspension of activated staphylococci containing 5 × 10^9 bacteria/ml. exposed to light of 4000-8000 Å without producing cell death. With [bacteria] held constant this inactivating effect of methylene blue on phage precursor can be demon-
strated only over a very narrow range of methylene blue concentrations for relatively slight increases in [methylene blue] begin to cause cell death. If [methylene blue] is reduced appreciably the inactivating effect is lost.

1.0 ml. of $9.1 \times 10^{-7} \text{M}$ methylene blue contains about $5 \times 10^{14}$ molecules. The cell concentration used in the precursor inactivation experiments is $5 \times 10^9$ bacteria/ml. Therefore there are available for each cell only $1 \times 10^5$ molecules of dye assuming that all of it is taken up by the bacteria. If this ratio of $1 \times 10^6$ molecules of methylene blue/bacterium is maintained when [bacteria] and [methylene blue] are varied, the photodynamic inactivation of phage precursor occurs over a fairly wide concentration range of reactants without causing cell death.

### TABLE II

**Experiments to Determine the Reaction Responsible for the Lag Phase of Photodynamic Inactivation of Phage Precursor by Methylene Blue**

<table>
<thead>
<tr>
<th>Period exposed to methylene blue in dark min.</th>
<th>[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1 $\times 10^9$ units per ml.)</th>
<th>[Bacteria]</th>
<th>Period exposed to light (no methylene blue) min.</th>
<th>[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1 $\times 10^9$ units per ml.)</th>
<th>[Bacteria]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$2.4 \times 10^9$</td>
<td>$5.6 \times 10^8$</td>
<td>10</td>
<td>$2.1 \times 10^9$</td>
<td>$6.4 \times 10^9$</td>
</tr>
<tr>
<td>15</td>
<td>$2.6 \times 10^9$</td>
<td>$5.9 \times 10^8$</td>
<td>30</td>
<td>$2.1 \times 10^9$</td>
<td>$6.1 \times 10^9$</td>
</tr>
<tr>
<td>30</td>
<td>$6.6 \times 10^9$</td>
<td>$4.2 \times 10^8$</td>
<td>30</td>
<td>$1.7 \times 10^9$</td>
<td>$6.8 \times 10^9$</td>
</tr>
<tr>
<td>45</td>
<td>$4.2 \times 10^9$</td>
<td>$4.6 \times 10^8$</td>
<td>60</td>
<td>$1.7 \times 10^9$</td>
<td>$6.8 \times 10^9$</td>
</tr>
</tbody>
</table>

It will be noted (Table I) that the first 15 minutes’ exposure of activated bacteria to methylene blue in the light does not bring about any detectable reduction in the [precursor]. To determine the mechanism involved in this lag phase of the inactivation curve, activated cell suspensions were treated as follows:

1. A mixture containing $5 \times 10^8$ activated bacteria per ml. and $9.1 \times 10^{-7}$ M methylene blue was placed in the dark and samples were removed from it at short intervals. Each aliquot was placed in the light for 5 minutes, then diluted 1:10 in Locke’s solution and tested for: (a) precursor content, and (b) [bacteria].

2. A suspension containing $1 \times 10^{10}$ activated bacteria/ml. was exposed to the light source for periods up to 1 hour. 1 ml. samples were added to 1 ml. of $9.1 \times 10^{-7}$ M methylene blue and the mixtures were kept in the dark
for 5 minutes before diluting 1:10 in Locke's solution and testing for: (a)
precursor content, and (b) [bacteria].

The experimental results (Table II) indicate that the lag phase represents
time consumed in a reaction taking place between the cells and methylene
blue. Whether the limiting factor is diffusion into the cell, or a combina-
tion of diffusion and chemical action, the presence or absence of light seems
to have no influence on the rate. When the primary reaction between the
activated cells and methylene blue has gone to completion a relatively brief
exposure to light will result in complete inactivation of the intracellular
phage precursor.

SUMMARY AND CONCLUSIONS

Methylene blue added to suspensions of activated staphylococci in
amounts sufficient to furnish $1 \times 10^4$ molecules of dye/bacterium inacti-
vates the phage precursor content of the cells without causing cell death
when the mixtures are exposed to strong light of 4000–8000 Å. There is a
lag phase of approximately 15 minutes in the photodynamic inactivation
of phage precursor by methylene blue. This delay seems to be due to a
primary reaction between the cell and methylene blue after the completion
of which exposure to light brings about the inactivation of precursor quite
promptly.

REFERENCES

   1939, 40, 573.