

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 example, 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^{10} 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.

Molarity methylene blue	Time of exposure	[Phage] developed after adding exposed cells to phage [Phage] ₀ of mixture = 2×10^8 P.U./ml.	[Bacteria] by plate count
9.1×10^{-7} M	<i>min.</i> 0 (Control)	1.22×10^9 P.U./ml.	7.0×10^9 /ml.
9.1×10^{-7} M	1.0	1.5×10^9 P.U./ml.	6.2×10^9 /ml.
9.1×10^{-7} M	5.0	1.4×10^9 P.U./ml.	6.2×10^9 /ml.
9.1×10^{-7} M	10.0	1.32×10^9 P.U./ml.	6.4×10^9 /ml.
9.1×10^{-7} M	15.0	1.25×10^9 P.U./ml.	5.6×10^9 /ml.
9.1×10^{-7} M	30.0	4.8×10^8 P.U./ml.	5.8×10^9 /ml.
9.1×10^{-7} M	45.0	3.6×10^8 P.U./ml.	7.2×10^9 /ml.
9.1×10^{-7} M	60.0	2.2×10^8 P.U./ml.	5.7×10^9 /ml.

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 9.1×10^{-7} M methylene blue contains about 5×10^{14} molecules. The cell concentration used in the precursor inactivation experiments is 5×10^9 bacteria/ml. Therefore there are available for each cell only 1×10^5 molecules of dye assuming that all of it is taken up by the bacteria. If this ratio of 1×10^5 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

5×10^9 activated bacteria/ml. in 9.1×10^{-7} M methylene blue kept in dark. After intervals noted, placed in light 5 min. and tested for [precursor] and [bacteria]			1×10^{10} activated bacteria/ml. exposed to light. At intervals 1 ml. samples mixed with 1 ml. methylene blue to 9.1×10^{-7} M. Kept in dark 5 min. and tested for [precursor] and [bacteria]		
Period exposed to methylene blue in dark	[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1×10^9 units per ml.)	[Bacteria]	Period exposed to light (no methylene blue)	[Phage] after adding 4 ml. cell suspension to 1 ml. phage (1×10^9 units per ml.)	[Bacteria]
<i>min.</i>			<i>min.</i>		
5	2.4×10^9	5.6×10^9	10	2.1×10^9	6.4×10^9
15	2.6×10^9		30	2.1×10^9	6.1×10^9
30	6.6×10^8	5.9×10^9	60	1.7×10^9	6.8×10^9
45	4.2×10^8				
60	2.5×10^8	4.6×10^9			

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 5×10^9 activated bacteria per ml. and 9.1×10^{-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 1×10^{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 9.1×10^{-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 combination 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 1×10^6 molecules of dye/bacterium inactivates 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.

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