PHOTODYNAMIC INACTIVATION OF BACTERIOPHAGE[1]

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Received for publication January 25, 1954

The extensive literature on the photosensitizing action of dyes on biological systems has been summarized by Blum (1941). Reference should be made to this book for the general principles of photodynamic action. Previous studies on photodynamic inactivation of phages have been made by Clifton (1931) and by Perdrau and Todd (1933). Burnet (1933) found that the relative sensitivity of phage strains to photodynamic inactivation was correlated with serological grouping and hence was of taxonomic significance. The present paper is a kinetic study of the variables affecting photodynamic sensitivity.

MATERIALS AND METHODS

The phage strains and bacterial hosts used are listed in table 1 with references to the ultimate source. Routine methods of phage technology have been described by Adams (1950). The saline buffer diluent contained 0.85 per cent NaCl, 100 μg of gelatin per ml, 0.001 M $MgSO_4$; and it was buffered at pH 7.0 with the appropriate mixture of M/15 Na_2HPO_4 and M/15 KH_2PO_4. The methylene blue dye was National Aniline Certified, 84 per cent dye content, and was dissolved in the saline diluent at a concentration of one g of dye per 10 liters of solution ($1/10{,}000$). A $1/25$ dilution of this stock solution had an optical density of 0.650 in the Beckman spectrophotometer at the absorption peak of 665 mμ. The light source was a 15 watt GE fluorescent light in a Fisher illuminator with reflector. For most experiments the distance from lamp to irradiated mixture was 25 cm. All experiments were performed in a dark room illuminated only by a dim green bulb which caused no detectable inactivation of photosensitized phage.

The phage was diluted to about 10^8 particles per ml in the methylene blue solution in saline diluent. Four ml of phage solution were irradiated in a 5 cm petri dish giving a liquid depth of 4 mm. After irradiation the phage was appropriately diluted in broth for assay by the agar layer method. For light controls the phage was illuminated in saline diluent without methylene blue. For dark controls the phage in methylene blue solution was held in the dark under the same environmental conditions as in the inactivation experiments.

RESULTS

Inactivation kinetics. Phage T1 was selected for a study of the effects of environmental variables on the kinetics of phage inactivation. After an initial lag the inactivation was an exponential function of time to a survival of less than 10^{-4} of the population (figure 1). The first order velocity constant calculated from the slope of the exponential portion of the curve was used to characterize the reaction rate. The velocity constant was quite reproducible, a series of six replicate experiments giving values from 0.676 min^{-1} to 0.766 min^{-1} with a mean of 0.723 min^{-1} and standard deviation of 0.032 min^{-1} at a methylene blue concentration of $1/100{,}000$. The initial lag was somewhat variable usually falling between that of a 2 hit and a 3 hit curve. The effect of possible dark reactions on the inactivation kinetics was studied by varying the duration of dark periods which were alternated with light periods. When 2 min, 10 min, or 20 min dark periods were alternated with 2 min light periods, the inactivation rates were the same as that with uninterrupted light when calculated in terms of illumination time. The inactivation was solely a function of the total duration of light periods and independent of the duration and spacing of dark intervals. Therefore, the inactivation could be interrupted at any time for sampling by turning off the light, with no effect on the kinetics of inactivation.

Preirradiation of the methylene blue in saline

[1] Aided by a grant from the National Foundation for Infantile Paralysis, Inc. This paper is the essence of a thesis submitted by J. N. Welsh in partial fulfillment of the requirements for the degree of M.S. in the Graduate School of Arts and Sciences of New York University.

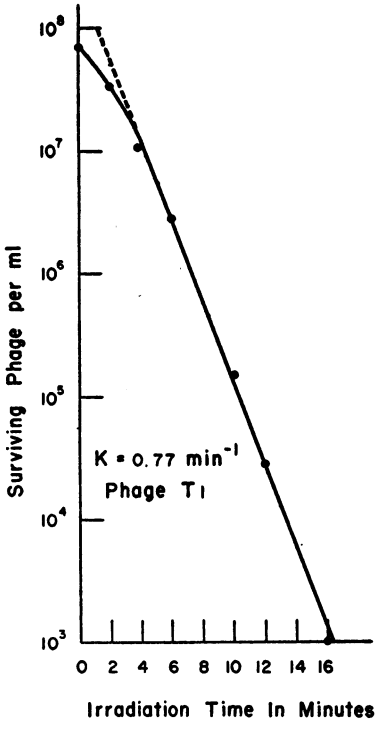

Figure 1. The photodynamic inactivation of phage T1 in 1/100,000 methylene blue at pH 7.0.

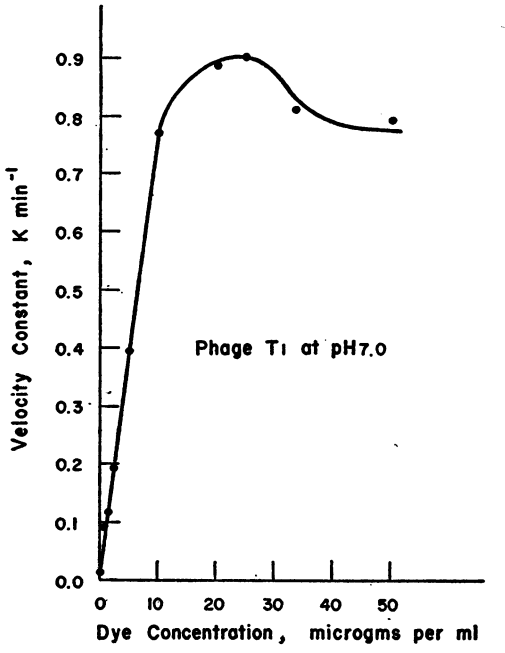

Figure 2. The rate of inactivation of phage T1 as a function of the concentration of methylene blue.

diluent for periods up to 30 min had no effect on subsequently added phage. Illumination of phage in the saline diluent without methylene blue resulted in a slow inactivation, the velocity constant being 0.009 min^{-1} as compared with 0.77 min^{-1} in the presence of methylene blue, a ratio of 1/85. This slow inactivation had been noted previously with certain phages by Wahl and Latarjet (1948) and may be due to small amounts of natural photosensitizing materials in some phage preparations. It is evident that the inactivation is negligible unless both phage and dye are present in the mixture during the period of illumination. Mixtures of phage and methylene blue are quite stable in the dark, there being a loss of only 20 per cent after 17 hours.

Effect of temperature. Inactivation experiments were carried out at 4 C in an ice bath, at a room temperature of 25 C, and in a sand bath at 42 C with the following results: k(4 C) = 0.605 min^{-1}, k(25 C) = 0.766 min^{-1}, k(42 C) = 0.851 min^{-1}. This small temperature effect corresponds to an Arrhenius constant of about 1,500 cal per mole, a value typical of photochemical reactions. Critical temperature control obviously was not required, and so kinetic experiments were run at room temperature, 23 to 25 C.

Effect of light intensity. The inactivation rate increased with increasing light intensity, indicating that the distance of the sample from the light source is a variable which must be controlled. The intensities used were well below those required to saturate the system.

Effect of dye concentration. The effect of changes in dye concentration may be seen from figure 2. At low concentrations the inactivation rate is directly proportional to dye concentration, presumably because only adsorbed dye is effective in photodynamic inactivation (Heinmets *et al.*, 1952) and the amount of dye adsorbed is proportional to its concentration. At higher concentrations the inactivation rate reaches a maximum and then decreases because the filtering effect of the dye decreases the effective light intensity.

Attempts were made to estimate the rate of dye adsorption and elution by changing the dye concentration during irradiation and determining the rate of inactivation before and after the change in dye concentration. Such experiments indicated that equilibrium between dissolved dye and adsorbed dye was reached too rapidly to be

measured by the kinetic techniques used. The photodynamic effect of methylene blue could be eliminated at any time by adequate dilution of the phage-dye mixtures.

Effect of hydrogen ion concentration. Inactivation velocity constants were determined for phage-methylene blue mixtures over a pH range of 5.4 to 9.0 by the use of appropriate mixtures of M/15 Na_2HPO_4 and M/15 KH_2PO_4 in the saline diluent. The velocity constant was a linear function of pH over this entire range as shown in figure 3. It is evident that below pH 5.2 there is no photosensitized inactivation of phage T1 by methylene blue. Increasing the pH by one unit at $1/100,000$ methylene blue is equivalent to increasing the dye concentration by 5 μg per ml at pH 7.0. The most probable explanation for this effect is that an increased pH permits an increased efficiency of adsorption of the dye to the phage by altering the surface charge of the phage particle. Phage T1 is anionic at the pH values studied (Puck and Sagik, 1953), and methylene blue is cationic.

Effect of molecular oxygen. Phage T1 in $1/100,000$ methylene blue was placed in Thunberg tubes, and these were freed of oxygen by alternate evacuation and refilling with a mixture of 95 per cent nitrogen and 5 per cent CO_2 for a total of four cycles. Upon exposure to the usual light intensity, there was about 50 per cent inactivation in 15 min after which no further inactivation occurred during the next 45 min. In control experiments in air filled Thunberg tubes, there was a survival of only 2×10^{-7} after 20 min exposure to light. It seems reasonable to assume that the small amount of inactivation under anaerobic conditions was due to a residue of oxygen which was not removed. These results are in agreement with those of Heinmets *et al.* (1952) and Oster and McLaren (1950). It is evident that molecular oxygen is essential for the photodynamic inactivation of bacteriophage, as has been found for most other photodynamic systems studied.

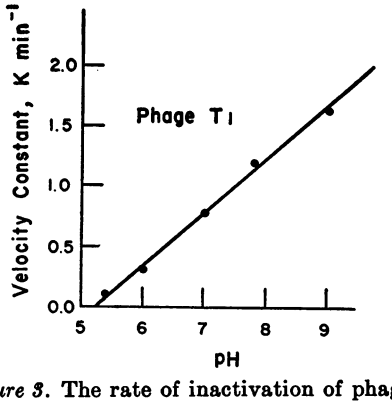

Figure 3. The rate of inactivation of phage T1 in 1/100,000 methylene blue as a function of pH.

Comparative study of different phages. Fourteen phage strains in four different serological groups were tested for their sensitivity to photodynamic inactivation. Kinetic measurements were carried out using $1/100,000$ methylene blue in saline buffer diluent at pH 7.0 and with light and dark controls as described in the methods section. In all cases the phages were inactivated in accordance with first order kinetics after a short initial lag period. The reaction kinetics remained first order as far as they were followed, usually to a survival between 10^{-3} and 10^{-5}. In each case the light control showed some inactivation, but the rate was negligibly slow in comparison with the photosensitized inactivation. There was no measurable inactivation in the dark controls during the experiments.

The results of these experiments are summarized in table 1, in which the phages are grouped according to their serological relationships. It is evident that the phages within each group are closely similar in sensitivity to inactivation, but that there is a 20-fold range between the least sensitive and most sensitive serological groups studied. These experiments are in complete agreement with those of Burnet (1933) and confirm his conclusion that this physiological property is correlated with the serological classification. Three of these phages, C16, D20, and D44, were studied also by Burnet who found the same relative sensitivities for them. It is evident from the table that photodynamic sensitivity is not correlated with phage particle size in the way that X-ray sensitivity is (Lea, 1946). Also there seems to be no correlation between photodynamic sensitivity and sensitivity to inactivation by ultraviolet, heat, pH, or sonic vibration.

The utilization of photodynamic sensitivity as a gene marker in genetic recombination studies does not seem promising because of the rather small range in sensitivities observed within each serological group. At least a twofold range in sensitivities is desirable for such experiments.

Properties of photodynamically inactivated phage particles. Studies of the properties of phage T2

TABLE 1

The rates of photodynamic inactivation of bacteriophages as correlated with serological grouping

PHAGE	HOST	REFERENCE	DIAMETER[5]	INACTIVATION VELOCITY CONSTANT
T1	*Escherichia coli*, strain B	[1]	50 mμ	0.75 min^{-1}
D20	*Escherichia coli*, strain B	[2]		1.05
T2	*Escherichia coli*, strain B	[1]	95 by 65	0.12
T4	*Escherichia coli*, strain B	[1]		0.15
T6	*Escherichia coli*, strain B	[1]		0.16
C16	*Escherichia coli*, strain B	[2]		0.14
T3	*Escherichia coli*, strain B	[1]	47	2.1
T7	*Escherichia coli*, strain B	[1]		2.0
D44	*Escherichia coli*, strain Templeton	[3]		2.7
SM I	*Serratia marcescens*, strain 3	[3]		1.9
T5	*Escherichia coli*, strain B	[1]	65	2.7
PB	*Escherichia coli*, strain Cullen	[4]		2.7
BG3	*Escherichia coli*, strain Cullen	[4]		2.7
29 alpha	*Escherichia coli*, strain Cullen	[4]		2.8

References: [1] Demerec and Fano (1945); [2] Burnet (1933); [3] Wassermann and Seligmann (1953); [4] Adams (1952); [5] phage diameters from Williams and Fraser (1953).

TABLE 2

The host killing ability of photodynamically inactivated T2 phage

	CONCENTRATION IN ADSORPTION MIXTURE	
	Initial viable bacteria	Final viable bacteria
Experiment T2 + broth + B	1.1×10^8/ml	2×10^7/ml
Control T2 + anti-T2 + B	1.1×10^8	1.1×10^8
Control T2 + broth + B/2	6.7×10^7	6.2×10^7

A T2 phage stock was inactivated photodynamically to a survival of 10^{-4}. The phage concentration in the adsorption mixtures was 2.8×10^9/ml total and 2.3×10^5/ml viable, and the adsorption period was 10 min at 25 C. The proportion of surviving bacteria was $2 \times 10^7/1.1 \times 10^8 = 0.18 = e^{-n}$, whence n = 1.7 killing phage particles adsorbed per bacterium. The killing phage particles were $1.7 \times 1.1 \times 10^8 = 1.9 \times 10^8$/ml or 7 per cent of the total phage population of 2.8×10^9/ml.

inactivated by ultraviolet light (Luria and Delbrück, 1942; Luria, 1947) and by X-rays (Watson, 1950) have indicated that the different physiological properties of this phage may be inactivated at different rates. Therefore, the rate of loss of the "host killing" property of phage T2 was determined in comparison with the rate of loss of the plaque forming property by photodynamic action. A typical experiment is described in table 2. It is evident that photodynamically inactivated phage T2 is able to kill strain B but not strain B/2, and that this host killing ability is neutralized by anti-T2 serum. The controls indicate that the bacterial killing is due to T2 phage particles and not to some other toxic substance in the preparation. Application of the Poisson formula indicates that photodynamic inactivation which reduces the plaque count from 3×10^9 per ml to 3×10^5 per ml reduces the host killing particles only to 1.8×10^8 per ml. The host killing ability is lost at about ⅓ the rate of the plaque forming ability, a result which is similar to the action of X-rays but quite different from the action of ultraviolet irradiation. Attempts to demonstrate multiplicity reactivation (Luria, 1947) and photoreactivation (Dulbecco, 1950) with photodynamically inactivated phage T2 were unsuccessful.

To determine if the host killing property resided in single phage particles, the phage-bacterium ratio was varied, and the number of surviving bacteria determined for each ratio. Application of the Poisson equation to the experi-

TABLE 3

The proportion of host killing phage particles in a photodynamically inactivated phage stock as a function of the total phage concentration in the adsorption tube

TOTAL PHAGE CONCENTRATION	SURVIVING BACTERIA	e^{-n}	n	KILLING PARTICLES	PROPORTION OF KILLERS
1.8×10^8/ml	1.2×10^8/ml	1.0	0	—	—
3.5×10^8	7.9×10^7	0.66	0.42	5.0×10^7/ml	0.14
7.0×10^8	6.3×10^7	0.52	0.65	7.8×10^7	0.11
1.5×10^9	6.8×10^7	0.56	0.58	7.0×10^7	0.05
2.9×10^9	2.7×10^7	0.22	1.5	1.8×10^8	0.06
5.7×10^9	1.8×10^7	0.15	1.9	2.2×10^8	0.04

The phage T2 stock was inactivated to a survival of 3×10^{-4} and mixed in various proportions with a constant concentration of bacteria. The initial bacterial concentration was 1.2×10^8/ml in each case, and the adsorption period was 10 min at 25 C. The proportion of surviving bacteria is e^{-n}, from which n, the mean number of killing particles adsorbed per bacterium, is calculated. The concentration of killing particles is $n \times 1.2 \times 10^8$/ml. This number divided by the total phage concentration in each adsorption tube gives the proportion of killers. If death of a bacterium can be caused by adsorption of one phage particle, the proportion of killers calculated as above should be essentially constant and independent of the phage concentration.

mental results in table 3 indicated that the adsorption of a single photodynamically inactivated phage particle was sufficient to kill the bacterial cell.

DISCUSSION

Photodynamic inactivation of bacteriophages requires the adsorption of a suitable dye to the phage particles, light of sufficient intensity and of a wavelength absorbed by the dye, and the presence of oxygen. Interference with any of these requirements will affect the inactivation rate. Riboflavin and eosin, which are photodynamically active in certain other systems (Blum, 1941), had no effect on the phages tested, perhaps because they did not adsorb to the phages. The inactivation kinetics were first order after a short initial lag. The rate was proportional to the methylene blue concentration and pH, both probably affecting adsorption of dye to phage.

The experimental results are in agreement with the conclusions of Burnet (1933) that sensitivity to photodynamic inactivation is correlated with serological grouping and is of taxonomic significance. Unfortunately there is so little variation in sensitivity within each serological group that it is improbable that this characteristic will be of value in genetic studies. However, there is at least a 20-fold range in sensitivity from one serological group to another. The relative sensitivities showed no correlation with size, morphology, or other physiological properties, yet these differences must reflect basic differences in the phage species studied which are not subject to major changes by mutation.

Because photodynamic inactivation is an oxidative process, it was thought that these differences might reflect general differences in susceptibility to inactivation by oxidizing agents. However, phage strains from all serological groups were inactivated at about the same rate by an appropriate concentration of hydrogen peroxide, regardless of their sensitivity to photodynamic action. It is evident that differences in susceptibility to oxidizing agents are not responsible for the differences in photodynamic sensitivity. The effect of dye concentration and pH on the inactivation kinetics suggests that the amount of dye adsorbed to the phage particles may be the rate determining factor. If this is so, the basic difference among the various serological groups may be in surface characteristics which affect adsorption of dye to phage particles.

SUMMARY

Kinetic studies of photodynamic inactivation of phages quantitatively confirm Burnet's conclusions that serologically related phages are similar in susceptibility to inactivation. The variation in sensitivity within a serological group is too small to be utilized as a gene marker in genetic studies. There is no evidence for the presence of resistant variants in any of the phage stocks studied. Excepting only serological

specificity, the photodynamic sensitivity does not correlate with other known properties of phages. The kinetics of inactivation and the environmental variables affecting the kinetics are in agreement with the established principles of photodynamic action.

Photodynamic action of methylene blue on phage T2 results in destruction of host killing property at ⅓ the rate of loss of plaque forming ability. Attempts to demonstrate photoreactivation and multiplicity reactivation of photodynamically inactivated phage preparations were unsuccessful. These results indicate that the damage by photodynamic action differs from that caused by ultraviolet or X-ray irradiation.

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