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Photodynamic Action of Proflavine on Coliphage T3

I. Kinetics of Inactivation

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The photodynamic inactivation of coliphage T3 was studied over a wide range of concentrations of the dye proflavine. With 2×10^7 phage/ml, two modes of inactivation were observed. Between 0.25 and 12 to 13 μg/ml, inactivation was biphasic. There was an initial first-order inactivation (Rx1) which became temporally associated with an apparently multiorder process (Rx2) at higher light doses. Dye concentrations above 12 to 13 μg/ml showed only two-target inactivation curves (Rx3), except at high dye concentrations where processes kinetically identical to Rx1 and Rx2 reappeared. Rx2 showed a normal rectangular hyperbolic saturation curve but Rx1 and Rx3 appeared to saturate prematurely. The saturation behavior of Rx1 and Rx2 was independent of phage concentration, but Rx3 was lost at phage titers above 2×10^7/ml. No dark inactivation was seen with Rx1 and Rx2 subsequent to a period of illumination. With Rx3, an exponential dark inactivation was seen for at least 1 hr after a period of illumination. The dye-phage system equilibrated immediately, at any temperature, at proflavine concentrations where Rx1 and Rx2 occurred. With Rx3, prolonged equilibration times were necessary. Moreover, there was a temperature effect. The rate of inactivation at equilibrium was temperature-dependent, whereas the initial rate at which equilibrium was approached was essentially temperature-independent.

Photodynamic action may be defined as sensitization of a biological system to inactivation by visible light through the action of certain dyes. It is generally held that inactivation results from alterations of nucleic acids rather than alterations of protein (6, 9, 10, 11, 12). Since dye must be bound to the nucleic acid to mediate inactivation (5), permeability of the biological system becomes very important. Certain viruses, notably the T-even coliphage, have impermeable heads (2). This report describes the photodynamic effect of the acridine dye, proflavine (3,7-diaminoacridine), on a permeable coliphage, T3.

Three distinct reactions are described which differ in various kinetic parameters. Evidence is presented that a long-lived radical is associated with one of the processes. Studies of the cysteine sensitivity of the reactions and the nature of the damages caused to the T3 chromosome by photodynamic action with proflavine are presented in the accompanying papers (13, 14).

MATERIALS AND METHODS

Bacteria. Overnight cultures of *Escherichia coli* B were routinely used as plating bacteria.

Phage. Coliphage T3 was used throughout the investigation. It was obtained from the American Type Culture Collection.

Media. Illumination medium (1.5% phosphate buffer) was composed of 4.5 g of KH_2PO_4, 10.4 g of Na_2HPO_4, 3.0 ml of gelatin (1%), and 2.5 ml of $MgSO_4$ (10%) per liter (pH 7.14 $\pm$ 0.05). Bacterial growth medium was 3xD (3). Dilution medium was "D" medium (one-third strength 3xD). Top and bottom agar were prepared as is customary (1).

Dye. Proflavine sulfate (Abbott Co., Chicago, Ill.) stocks, 1.6×10^{-4} M in proflavine, were used. In 1.5% phosphate buffer, the molar absorbancy at 445 nm was 3×10^4 for dye concentrations below 5×10^{-5} M. In 25% (v/v) aqueous ethanol, a molar absorbancy of 4×10^4 was obtained, in agreement with published observations (8). Absorbancy readings were made with a Beckman DU spectrophotometer (model 2400).

Preparation and purification of viruses. Plate washings of T3 were prepared on nutrient agar plates (1). Preparation and purification of high-titer phage stocks involved the usual methods.

Illumination. Phage and dye were diluted in 1.5% phosphate buffer to give the desired concentration of each. The source of light was a 125-v, 100-w Champion frosted incandescent bulb backed by a reflector and alimented through a constant-voltage regulator. Four-milliliter samples of phage suspensions were illuminated in an open petri dish placed 23 cm from the bulb. Under these conditions, the level of illumination at the solution was 750 ft-c. In most experiments, the dishes were swirled by a black-surfaced

gyratory shaker (New Brunswick Scientific Co., model G-33) at 88 oscillations/min. Samples were removed at set intervals, diluted into "D" medium, and stored on ice until plated. Illumination and plating were conducted in a photographic dark room, with either a 60-w or 100-w G.E. yellow incandescent bulb as "safe light." The temperature of the room was generally 12 C, and control experiments revealed no differences in rates of inactivation for temperatures as high as 25 C, in agreement with published data (4, 12). For plating, the temperature of the room was raised to 20 to 22 C.

Effect of temperature of and length of time of preincubation of phage with dye. Dye solutions and phage suspensions were pre-equilibrated at a given temperature and then mixed. For experiments at 0 and 2 C, preincubation was conducted in an ice bath and a constant temperature refrigerated room, respectively. In experiments at 12 and 18.5 C, preincubation was done in the dark room. At intervals, 4-ml samples were removed, illuminated for 10 min, diluted, and plated.

RESULTS

Experimental error. The figures and tables represent typical results of at least five experiments each. Unless otherwise stated, all numbers are accurate to ±20%. Most of this error is due to the plating error of T3 (±15%).

Controls. No detectable inactivation of T3 occurred when phage were illuminated in the absence of dye, incubated with dye in the dark, or left unilluminated except by the room safelight in the presence or absence of dye.

Effect of duration of and temperature of preincubation of phage with dye. T3 was used in these experiments because the phage-dye system is reported to equilibrate immediately and there is no temperature effect (9). Under the conditions employed here, such was the case only for proflavine concentrations equal to or less than 13 μg/ml.

When the dye concentration exceeded 13 μg/ml, equilibration was not immediate and there was a temperature effect. As shown in Fig. 1, an increase in temperature of preincubation did not affect the initial rate at which equilibrium is approached, but it did affect the rate of inactivation at equilibrium quite drastically. The rate of inactivation at equilibrium was almost 100 times greater when preincubation was at 2 C than at 0 C. Although equilibration was reached at 0 and 2 C, at 18.5 C, the system apparently did not equilibrate during the course of the experiment. At 18.5 C, there was also a slow exponential inactivation in the dark, giving about 70% kill within 18 hr.

Survival curves. In the relationship between dye concentration and mode of inactivation, two types of response were observed with T3 titers equal to or less than 2×10^7/ml.

The first type of response was seen at dye concentrations equal to or less than 12.1 μg/ml. These inactivation curves were biphasic (Fig. 2a). There was an initial reaction which was first-order with time and is designated as Rx1. At higher doses of light, Rx1 became temporally associated with an apparently higher-order reaction which is labeled Rx2.

At the lowest dye concentration examined (0.25 μg/ml), Rx2 did not begin until 70 to 80 min of illumination (ca. 4% survival). The time of appearance of Rx2 decreased quite rapidly with increasing proflavine concentration until, at dye concentrations between 1.1 and 12.1 μg/ml, Rx2 appeared always at 8 min (at about 20 to 25% survival).

To estimate the rate of Rx2, it was assumed that Rx1 continues at the same exponential rate. Presumed inactivations due to the extrapolation of Rx1 were therefore subtracted from the total inactivation. With this assumption, Rx2 has a long shoulder and then is exponential as far as the re-

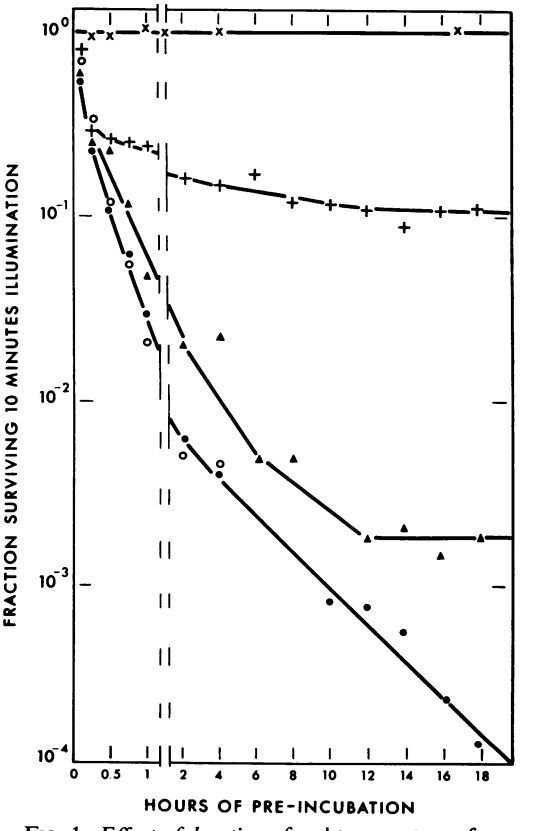

FIG. 1. *Effect of duration of and temperature of preincubation of dye with phage. Symbols: ×, 13.0 μg of dye per ml at any temperature; +, 17.1 μg of dye per ml at 0 C; ▲, 17.1 μg of dye per ml at 2 C; ○, 17.1 μg of dye per ml at 12 C; ●, 17.1 μg of dye per ml at 18.5 C.*

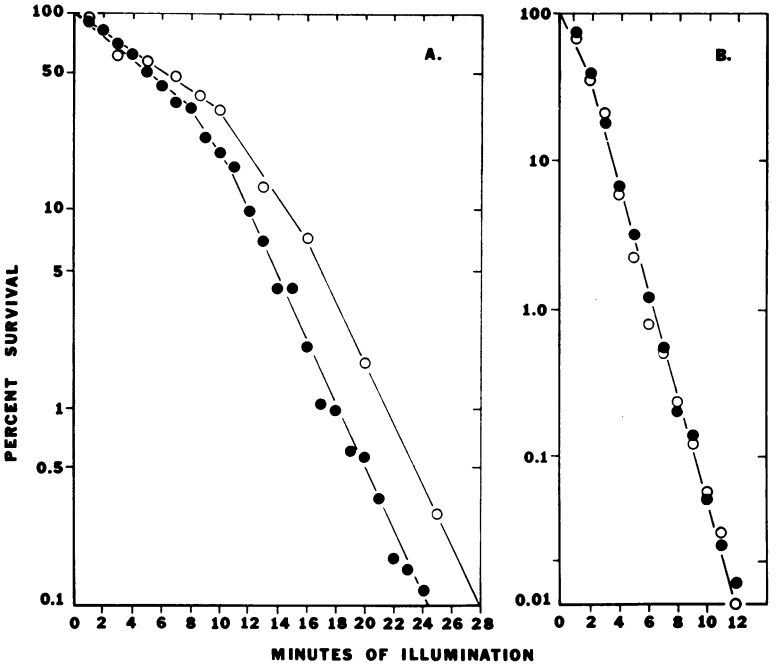

FIG. 2. *Types of kinetics observed with T3 illuminated in the presence of different concentrations of proflavine. (A) Proflavine at 8.5 μg/ml and 2 × 10¹¹ T3/ml (●) or 2 × 10¹¹ T3/ml (○). (B) Proflavine at 17.1 μg/ml and 2 × 10⁶ T3/ml (○) or 2 × 10⁷ T3/ml (●). Pre-incubation times for (A) and (B) are 5 min and 17 hr, respectively, at 2 C.*

action has been followed, suggesting multitarget kinetics. However, the extrapolation number of Rx2 was not constant and varied from 4 (0.8 μg/ml) to 40 (saturating concentrations of dye).

At concentrations above 12 to 13 μg of dye per ml (Fig. 2b), only two-target inactivation curves were observed (Rx3).

Saturation curves. Figure 3 shows the relationship between slopes of the survival curves and dye concentration, with 2 × 10⁷ phage/ml. Rx1 became zero-order with respect to dye concentration between 2 and 3 μg of dye per ml, and Rx2 reached its maximum rate between 3 and 4 μg/ml. Rx3 began above 12 to 13 μg/ml and reached a maximum rate at 17.1 μg/ml.

Double reciprocal plots of the saturation data (not shown) reveal that Rx1 and Rx3 saturated prematurely; that is, their saturation curves do not define complete rectangular hyperbolae.

Table 1 shows saturation data with four phage concentrations. Obviously, the fluctuations in behavior of Rx1 and Rx2 are minor compared to the wide range of phage titers employed.

Rx3 disappeared at a phage concentration somewhere between 2 × 10⁷ and 2 × 10⁸ phage/ml. With phage titers of 2 × 10⁸/ml and 2 × 10¹¹ to 4 × 10¹¹/ml, runs were made with dye concentrations as high as 285 μg/ml, but Rx3 was still absent despite the fact that Rx1 and Rx2 saturated at the normal dye concentration, with the higher phage titers. At phage concentrations where Rx3 does occur, this reaction was present over a comparatively narrow dye concentration range. This has not yet been studied in detail but, at 2 × 10⁷ T3/ml and some dye concentration between 25 and 125 μg/ml, Rx3 disappeared and Rx1 and Rx2 reappeared, as indicated by resumption of their typical kinetic behavior and by the effects of cysteine (13).

Postillumination dark inactivation. With Rx1 and Rx2, no dark inactivation was observed subsequent to a period of illumination. On the other hand, Rx3 did exhibit dark inactivation. Figure 4 demonstrates that an exponential inactivation continued for at least 1 hr in the dark when phage and 17.1 μg of dye per ml were illuminated for 1 or 5 min. A fully equilibrated system is necessary for maximum dark inactivation.

The two examples of dark inactivation shown in Fig. 4 are typical with respect to rate and exponential character, but it should be mentioned that the exponential rate did not appear to increase monotonically with time of the light process. The effect of the much slower dark inactivation on the rate of the light reaction is negligible at all times.

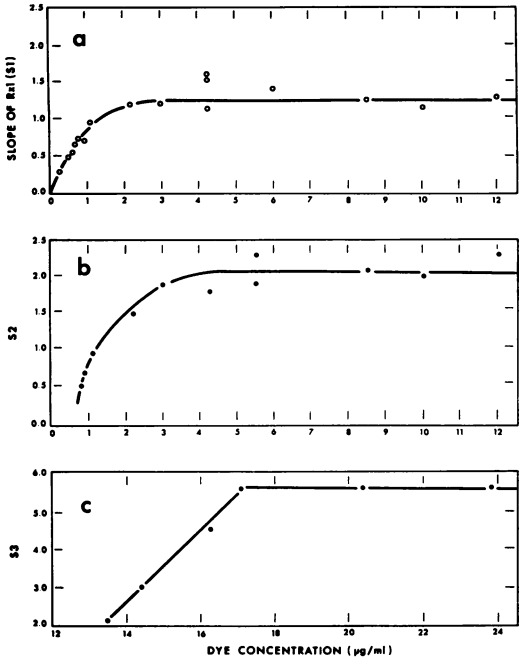

FIG. 3. *Saturation curves with 2 × 10⁷ phage/ml. (a) Rx1 (slope designated S1). (b) Rx2 (slope designated S2). Under the standard conditions (2 × 10⁷ T3/ml; 750 ft-c of light) employed, Rx2 does not occur at very low dye concentrations until high doses of light have been administered; S2 is small and quite irreproducible. Therefore, the points below 0.8 μg/ml have been omitted. A few experiments have been done with a 9× increase in light intensity, and extrapolation of these slopes to the standard light intensity indicates that the saturation curve is a smooth rectangular hyperbola with its origin at zero. (c) Rx3 (slope designated S3).*

Diluting the sample to a final dye concentration below 13 μg/ml terminated the dark inactivation.

DISCUSSION

Preincubation of phage with dye. Dye must be bound to the DNA molecule for photodynamic action to occur (6). Consequently, the alterations in inactivation rates observed in these experiments should reflect changes in the amount of bound dye (Fig. 1). Dye binding to the DNA of intact phage requires at least two, presumably, reversible steps: (i) penetration of the dye molecules into the phage head and (ii) the actual binding of the dye to the deoxyribonucleic acid (DNA). The prolonged equilibration times which are necessary at proflavine concentrations where Rx3 occurs suggest that one or both of these steps is slow. Why this is restricted to Rx3 is, as yet, conjectural.

Since the rate of inactivation itself is temperature-independent, the observed temperature effect must be associated with dye binding. The maximum amount of dye which can be bound seemingly increases with temperature because the rate of inactivation at equilibrium increases, whereas the initial rate at which equilibrium is approached is little affected.

TABLE 1. *Effect of phage titer on saturation points and observed maximum velocities of Rx1, Rx2, and Rx3*

Phage titer (per ml)	Saturation point (μg/ml)			Observed maximum velocities (per min)		
	Rx1	Rx2	Rx3	Rx1	Rx2	Rx3
2×10^6	0.75	0.75	18.5	0.85	0.91	4.7
2×10^7	2.50	3.50	17.1	1.26	2.05	5.7
2×10^8	—[a]	—[a]	—[b]	1.21	1.09	—[b]
2×10^{11}	3.00	5.00	—[b]	0.99	1.10	—[b]

[a] Not tested.
[b] Rx3 does not occur at these phage titers.

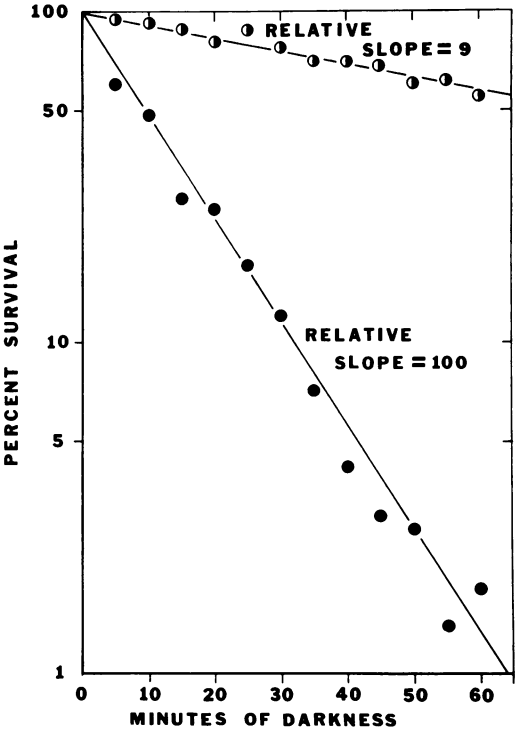

FIG. 4. *Dark inactivation of phage T3 after a period of illumination in the presence of 17.1 μg of proflavine per ml. Symbols: ○, dark inactivation after 1 min of illumination; ●, dark inactivation after 5 min of illumination. Relative slopes are based on the rate of dark inactivation after 5 min of illumination. The percentages surviving T3 after 1 and 5 min of illumination are 85 and 5, respectively.*

This is opposite to the well-known temperature effect seen with viruses that have impermeable heads (e.g., the T-even coliphage). With T2, the rate at which equilibrium is approached changes (7), suggesting that increasing temperature allows far more rapid dye penetration or binding, or both, rather than an increase in maximum amount of dye which can be bound.

Survival curves. Rx1 is first-order with respect to time (Fig. 2). Work described in an accompanying paper (14) and published data (5) imply that its mechanism involves cleavage of bases from nucleotide residues. Such a mechanism would result in exponential inactivation if the probability of a hit at any of the bases involved is constant with time or if the bases concerned form a uniform gradient of sensitivities. Neither of these possibilities can be eliminated.

Rx2 cannot be a true multitarget process since its extrapolation number is not constant. This suggests that shoulder length and rate of exponential killing are not strictly related. For example, the length of the shoulder is constant at 8 min for any dye concentration equal to or greater than 1.1 μg/ml, but the rate of exponential killing increases from 0.94 to 2.05 per min (at 2×10^7 T3/ml).

It seems best to propose that Rx2 consists of at least two sequential reactions. An early reaction, of unknown order, produces nonlethal damage. This reaction probably saturates around 1.1 μg/ml since no further decrease in shoulder length is seen beyond this dye concentration. A subsequent, exponential reaction, Rx2, converts this nonlethal damage into lethal damage; that is, Rx2 does not operate on normal nucleotides but on nucleotides that have been altered in some manner.

Rx3 demonstrates two-target kinetics. The most likely explanation is that this process involves some damage that must occur on both strands of the DNA molecule, with the two strands behaving as independent targets.

Saturation curves. Consistent with a mechanism involving an equilibrium between dye and binding site, rather than a stoichiometric titration of binding sites by dye, is the relative invariance of the saturation curves with changes in phage concentration (Fig. 3, Table 1). However, there are some anomalies which should be brought out but which do not alter this conclusion. (i) The saturation curves of Rx1 and Rx3 are incomplete hyperbolae, which indicates that these reactions saturate prematurely. (ii) Rx3 occurs only at low phage concentrations and, even then, only within a restricted range of dye concentrations. These anomalies indicate complex interactions between the dye molecules concerned with Rx1 and Rx3.

Dark inactivation. Rx3 appears to have at least one long-lived radical associated with it, since inactivation continues in the dark after a period of illumination (Fig. 4). Because a fully equilibrated system is necessary for maximum dark inactivation, bound dye is probably responsible.

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