JOURNAL OF VIROLOGY, Mar. 1971, p. 319-322
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Vol. 7, No. 3
Printed in U.S.A.

Photodynamic Action of Proflavine on Coliphage T3

II. Protection by L-Cysteine

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Received for publication 14 October 1970

Three kinetically different reactions (Rx1, Rx2, and Rx3) have been distinguished in the photoinactivation of phage T3 in the presence of the dye proflavine. The response of these reactions to the presence of the radical trap L-cysteine has been examined. At dye concentrations equal to or less than 2.2 μg/ml, Rx1 was composed of at least two parallel first-order reactions, one cysteine-insensitive (Rx1A) and one cysteine-inhibited (Rx1B). Rx2 was completely cysteine-insensitive (Rx2A). The cysteine sensitivity of these reactions changed abruptly at dye concentrations above 2.2 μg/ml. Rx1A and Rx1B now operated in tandem, rather than simultaneously, with Rx1B being confined to the first 1 min at most. Rx2, on the other hand, was completely cysteine-inhibited (Rx2B). Rx3 was inhibited roughly 75 to 80% by saturating concentrations of cysteine regardless of the time of addition of cysteine. The dark inactivation associated with Rx3 was inhibited roughly 85% whether the radical trap was added during the light or dark regimes. Changes of initial phage titer did not alter the cysteine sensitivity of a reaction.

The obligate requirement of photodynamic action for molecular oxygen (2, 5) has prompted the suggestion that oxidizing radicals are involved (1). If such is the case, the process should be inhibited by an oxidizing-radical scavenger. The use of L-cysteine in this role has led to the discovery of a complex series of events in the photodynamic inactivation of coliphage T3.

MATERIALS AND METHODS

Phage and dye were illuminated for the desired period of time, and then crystalline L-(+)-cysteine·H_2O (Coleman, Matheson, and Bell, Cincinnati, Ohio) was added to give the desired concentration of cysteine. Routinely, 0.025 M cysteine was used. This is at least twice the lowest concentration of cysteine giving maximum response at any dye concentration. All other methods are as described in the previous paper (3).

Controls. No detectable inactivation occurred when T3 was illuminated with cysteine alone or incubated with cysteine in the presence or absence of dye in the dark.

RESULTS

Effect of 0.025 M cysteine at dye concentrations equal to or less than 2.2 μg/ml. At these dye concentrations (Fig. 1 and 2; Table 1), the presence of cysteine from time zero resulted in a 50 to 60% reduction in the slope of Rx1 (S1) but no reduction in the slope of Rx2 (S2). With Rx1, the kinetics were exponential in either case. It can be hypothesized that Rx1, here, consists of at least two parallel first-order reactions, one cysteine-insensitive (Rx1A) and the other cysteine-inhibited (Rx1B). Such a hypothesis predicts that no matter when cysteine is added, S1 should be reduced by the same relative amount. As can be seen in Table 1, this prediction was realized.

Effect of 0.025 M cysteine at dye concentrations between 3 and 8.5 μg/ml. In the range of dye concentrations between 3 and 8.5 μg/ml, the presence of cysteine from time zero caused a marked change in the kinetics (Fig. 3; Table 2). In general, these curves were comprised of a shoulder region, followed by a brief transition period of nonexponential kinetics which was succeeded by exponential inactivation. Within reasonable error, the slope of the exponential portion equalled S1 of the unprotected process.

These kinetics suggest that Rx1A and Rx1B have become dissociated and that the latter is restricted to the early portion of Rx1. Since the final slope of the protected process was equal to S1 of the unprotected process, it is necessary to hypothesize that the cysteine-insensitive reaction (Rx1A) is capable of driving Rx1 at its normal rate, but elimination of the cysteine-inhibited reaction (Rx1B) disrupts some rapid, early step required for the initiation of Rx1.

Experiments were conducted at 8.5 μg of dye

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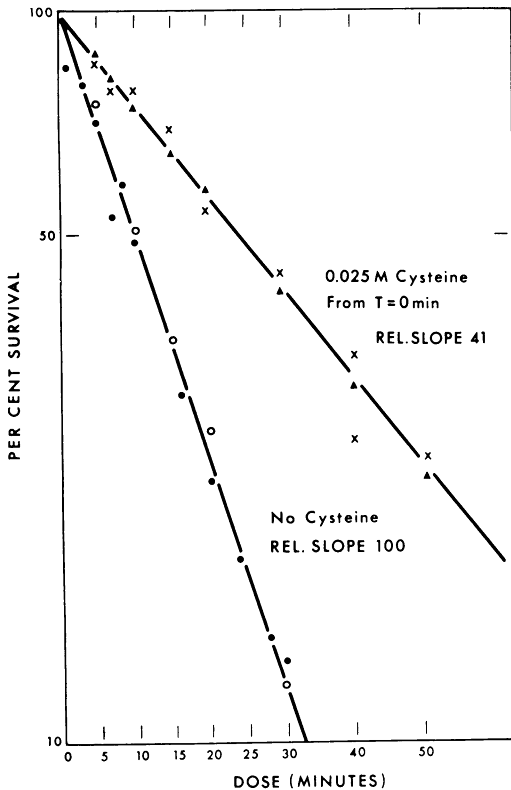

FIG. 1. *Effect of 0.025 M cysteine, added at time zero, on the kinetics of inactivation at 0.25 μg of dye per ml. Only the first 50 min of illumination is shown.*

per ml to determine how much time is required for Rx1 to become refractile to the presence of cysteine. As shown in Fig. 3 and Table 2, if Rx1 was allowed to proceed for as little as 1 min, it became completely indifferent to the presence of cysteine.

Rx2 was inhibited no matter when cysteine was added. This will be designated Rx2B to distinguish it from the cysteine-insensitive Rx2A which was observed at dye concentrations equal to or less than 2.2 μg/ml.

Effect of 0.025 M cysteine at 10 and 12.1 μg of dye per ml. When cysteine was present from zero time, the shoulder noted in the previous section disappeared. The inactivation curves now consisted of a short period of nonexponential inactivation, followed by exponential kinetics with a slope equal to S1 of the unprotected process. Rx2 remained completely cysteine-sensitive.

Effect of cysteine on Rx3. Protection was observable with cysteine concentrations as low as 2.5×10^{-4} and it maximized at 6×10^{-3} M with 75 to 80% inhibition (Fig. 4 and 5). With 0.025 M cysteine, the amount of protection was the same, regardless of the time of addition of the radical scavenger. It should be mentioned that the rate

Symbols: ●, no cysteine, phage at 2×10^{7}/ml; ○, no cysteine, phage at 2×10^{11}/ml; ▲, cysteine, phage at 2×10^{7} ml; ×, cysteine, phage at 2×10^{11} ml. Relative slopes are based on the rate of inactivation in the absence of L-cysteine.

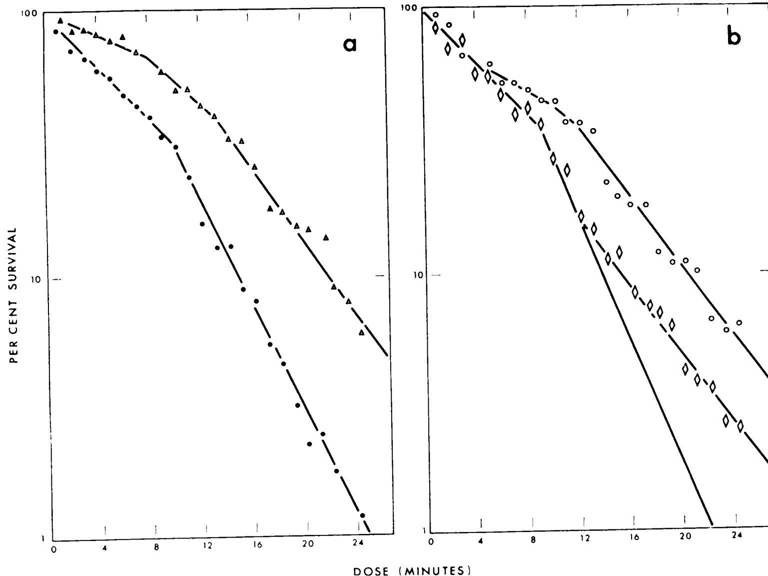

FIG. 2. *Effect of 0.025 M cysteine on the kinetics of inactivation at 1.1 μg of proflavine per ml. (a) Symbols: ●, no cysteine; △, cysteine added before illumination. (b) Symbols: solid line, no cysteine, same as Fig. 2a; ○, cysteine added after 5 min of illumination; ◇, cysteine added after 12 min of illumination.*

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TABLE 1. *Effect of time of addition of cysteine on the rates of inactivation of T3 in the presence of 1.1 μg of proflavine per ml*

Time of illumination before cysteine added (min)	Relative rate of Rx1	Relative rate of Rx2
No cysteine	100	100
0	42	101
1	40	85
5	47	96
12	43[a]	93

[a] Average relative rate of Rx1 in the presence of cysteine from time 0, 1, and 5 min.

TABLE 2. *Effect of time of addition of cysteine on the rates of inactivation of T3 in the presence of 8.5 μg of proflavine per ml*

Time of illumination before cysteine added (min)	Relative terminal slope of cysteine-protected process[a]
0	109
1	98
5	120
12	105
15	110

[a] Based on S1 of unprotected process.

of inactivation continued at the unprotected rate for roughly 1 min after the addition of cysteine.

The dark inactivation associated with Rx3 was about as cysteine-sensitive as the light reaction. The amount of protection was the same whether cysteine was added before illumination or after a period of dark inactivation (Fig. 6).

DISCUSSION

The response of Rx3 to the presence of L-cysteine is straight-forward. At saturating concentrations of cysteine, Rx3 is inhibited roughly 75 to 80%, no matter when the radical trap is added during the time course of the reaction. This suggests that no change occurs in the radical mechanism.

About the same amount of protection is observed with the dark inactivation associated with Rx3. The degree of protection is the same whether cysteine is added during the light regime or after a period of dark inactivation. These data suggest that (i) the radicals involved with the light and dark inactivations of Rx3 are the same and (ii) these radicals are formed in the light and their life times extend into the dark phase.

The dye concentration-dependent responses of Rx1 and Rx2 to the presence of L-cysteine are difficult to interpret exactly. As pointed out in the previous paper (3), Rx1 saturates prematurely at 2.2 μg of proflavine per ml, the same dye concentration at which the cysteine sensitivity of Rx1 and Rx2 changes abruptly. Obviously, major differences in the chemical behavior exist between dye molecules which mediate Rx1 and Rx2 above and below 2.2 μg/ml.

There are many possible models which could account, at least qualitatively, for these observations. Suffice it to say for the present, the various models can be grouped into two major categories. Either a change in reactivity of the radicals occurs with respect to cysteine and the target molecule or a change occurs in the position at which the radicals are generated relative to cysteine and the target molecule. Also, a reaction involving a direct energy transfer from excited dye molecules to the target molecule would presumably be cysteine-noninhibitable.

The kinetics of Rx1 and Rx2 does not change with altering cysteine sensitivity so it is doubtful that changes in the targets or lethality of the damages are involved. Indeed, as is shown in the suc-

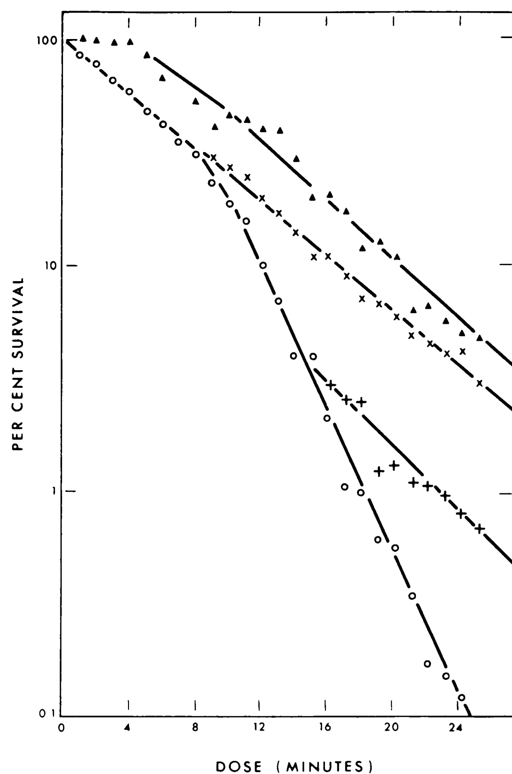

FIG. 3. *Effect of 0.025 M cysteine on the kinetics of inactivation at 8.5 μg of proflavine per ml. Symbols:* ○, *no cysteine;* ▲, *cysteine added before illumination;* ×, *cysteine added after 1 min of illumination;* +, *cysteine added after 15 min of illumination.*

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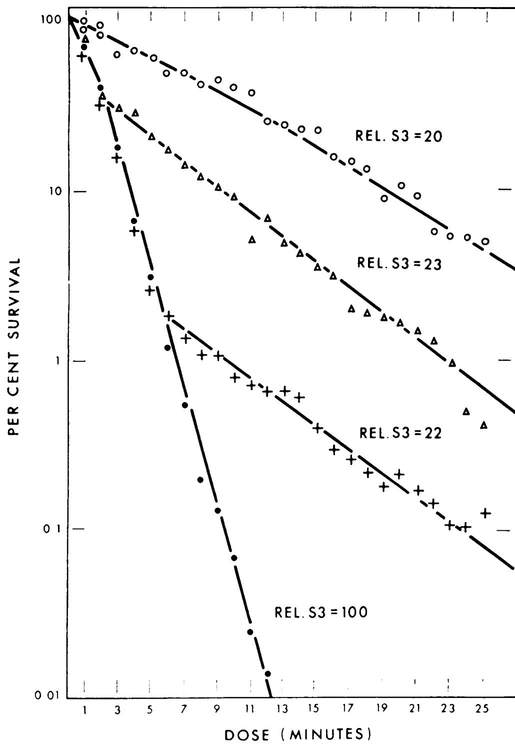

FIG. 4. *Effect of 0.025* M *cysteine on the kinetics of inactivation of 17.1 μg of proflavine per ml. Symbols:* ●, *no cysteine;* ○, *cysteine added before illumination;* △, *cysteine added after 1 min of illumination;* +, *cysteine added after 5 min of illumination. Relative slopes are based on the rate of inactivation in the absence of* L*-cysteine.*

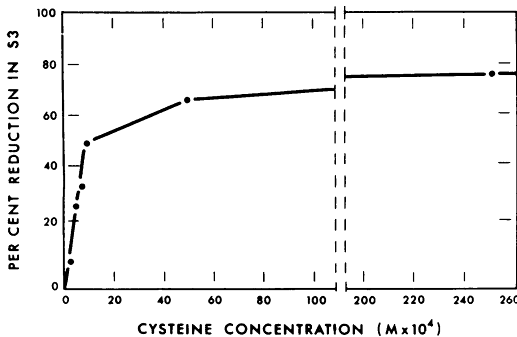

FIG. 5. *Per cent reduction of the rate of inactivation of T3 exposed to light and 17.1 μg of dye per ml as a function of cysteine concentration.*

ceeding paper (4), the lethal damage of Rx1 and the amount of this damage required to kill a phage appear to remain the same despite the different

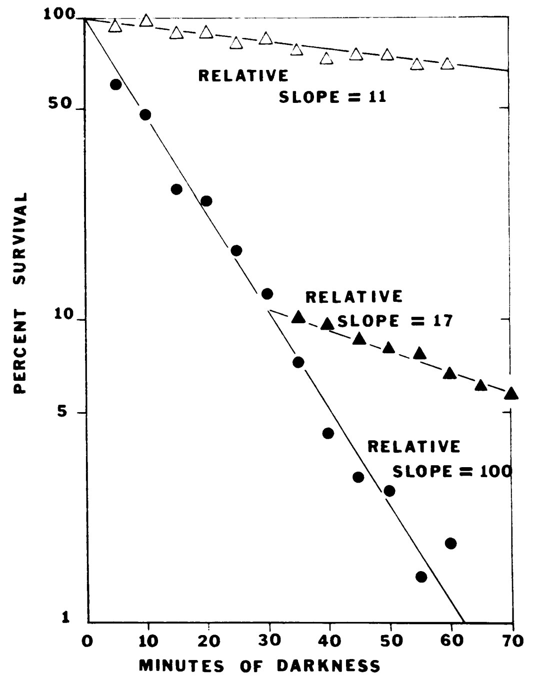

FIG. 6. *Effect of 0.025* M *cysteine on the dark inactivation associated with Rx3. Symbols:* ●, *light extinguished after 5 min of illumination, no cysteine added;* △, *cysteine added before illumination, light extinguished after 5 min of illumination, and cysteine added after 31 min of dark inactivation. Relative slopes are based on the rate of dark inactivation without cysteine present.*

cysteine sensitivity of Rx1 at different dye concentrations.

ACKNOWLEDGMENTS

This investigation was supported by Microbiology Training Grant 5T1-GM503-09 (to H. W.) from the National Institute of General Medical Sciences and Public Health Service grant R01-CA02772 (D. F.) from the National Cancer Institute.

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