A NOVEL EFFECT OF ACTINOMYCIN D IN PREVENTING BACTERIOPHAGE T4 MATURATION IN ESCHERICHIA COLI

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The potent effect of actinomycin D on the growth of susceptible species of bacteria is generally attributed to the ability of this agent to inhibit the synthesis of DNA-directed RNA (messenger RNA) (Hurwitz et al., 1962). Although there is no direct unequivocal evidence that the growth inhibition caused by this antibiotic in vivo is in fact due to this mechanism of action, neither is there evidence that seriously challenges this hypothesis. It is the purpose of this communication to demonstrate for the first time an in vivo inhibitory action of actinomycin D that is not explicable on the basis of inhibition of messenger RNA synthesis.

Nakata et al. (1961) have reported that actinomycin, in concentrations ranging from 1 to 100 μg per ml, inhibited the formation of phage T2r in cells of E. coli B but did not prevent the accumulation of normal amounts of DNA in the infected cells. Since the effect of the antibiotic on RNA and protein synthesis under these conditions was not reported, and since E. coli itself is not normally susceptible to actinomycin, these results were both unexpected and very difficult to interpret.

During the course of an investigation into the effects of actinomycin D on phage T4 infection of cells of E. coli that had been rendered susceptible to this antibiotic (Leive, 1965) (hereinafter to be referred to as sensitized cells), we have obtained data that both confirm and

significantly amplify the findings of Nakata et al. In particular we have been able to demonstrate clearly that under certain circumstances actinomycin D can specifically block phage maturation without detectably affecting messenger RNA formation.

Materials and Methods--All of the infection experiments to be described employed as the recipient bacterium E. coli CR34 (a polyauxotroph of K12 requiring for growth threonine, leucine, thiamine, and thymine), originally obtained from Dr. A. D. Kaiser, and phage T4d8, a thymineless mutant of phage T4D originally isolated and given to us by Dr. I. Tessman (Simon and Tessman, 1963). Standard phage techniques were employed, as described by Adams (1959). Actinomycin D was the gift of the Merck, Sharp and Dohme Research Laboratory. Radioisotopically labelled compounds were obtained from the New England Nuclear Corporation and included: Thymine-methyl-^{3}H (specific activity 10.4 C/mmole), uracil-2-^{14}C (specific activity 30 mC/mmole), uracil-^{3}H (specific activity 2.5 C/mmole), L-leucine-^{14}C (U.L.) (specific activity 220 mC/mmole), and algal protein hydrolysate-^{14}C (specific activity 1.6 mC/mg). The standard liquid culture medium contained per liter: 5.9 g Na_2HPO_4, 2.65 g KH_2PO_4, 7.5 g glucose, 0.49 g NaCl, 0.97 g NH_4Cl, 0.19 g $MgSO_4 \cdot 7H_2O$, 1 g casamino acids, 0.1 g each of L-threonine, L-leucine, and thiamine hydrochloride, 50 μmoles of thymine, and 50 mg of L--tryptophan. Tryptone broth and its agar derivatives were prepared as previously described (Kaiser and Hogness, 1960). The incorporation of radioactive precursors into trichloroacetic acid (TCA)-insoluble material was determined in phage-infected cells by adding to chilled 1.0 ml aliquots of culture 0.5 ml of a carrier solution containing 500 μg salmon sperm DNA, 5 μmoles each of thymine and uracil, and 100 μmoles of NaN_3, followed by ice-cold TCA to a final concentration of 10%. The resulting precipitates were then prepared for counting in a liquid scintillation spectrometer by methods previously described (Weissbach

and Korn, 1964). Sensitization of cells of E. coli CR34 to actinomycin D was achieved by a modification of the technique of Leive (1965): Exponentially growing cells were harvested, washed twice with cold 0.05 M Tris-chloride buffer, pH 8.0, suspended in the same buffer at a concentration of 2-3 x 10^9/ml, and gently agitated for 2 minutes at 37° in the presence of 2 x 10^{-4} M ethylenediaminetetraacetate. The cells were then diluted 10-fold into fresh liquid medium to begin the desired experiment.

Results--We have been able to confirm the findings of Nakata et al. The addition of actinomycin D, at concentrations of 10 to 100 μg per ml, to T4-infected, non-sensitized cells of E. coli CR34 within the first 3 to 5 minutes after infection leads to 80 to 95% inhibition of phage yield. At these high concentrations of antibiotic there is no significant inhibition of RNA, DNA, or protein synthesis in the infected cells (Table I). However, the addition of actinomycin to these cells at later times after infection is without effect on phage production.

These results are difficult to evaluate because of the known in-

TABLE I. Effect of actinomycin D on RNA, DNA, and protein synthesis in T4-infected, non-sensitized cells of CR34

Actinomycin added at 0 minutes (25 μg/ml)*	Radioactive precursor added at 0 minutes	Incorporation measured at 30 minutes (cpm/ml)
+	Uracil-^{3}H	9,991
-		10,664
+	Thymine-^{3}H	39,129
-		37,694
+	Leucine-^{14}C and algal protein hydrolysate-^{14}C	12,491
-		14,040

Exponentially growing cells of CR34 were harvested, resuspended in fresh medium (2-3 x 10^8 cells per ml), and infected with phage T4 at an input multiplicity of about 6 to 1. Extent of incorporation was measured as described in "Methods." Specific activities of precursors in medium were: Uracil, about 10 μC/μmole; thymine, about 20 μC/μmole; leucine, about 0.2 μC/μmole. Algal protein hydrolysate, 0.2 μC per ml of medium.

* The phage titer in the actinomycin-treated lysate was 13% of that in the control lysate.

sensitivity of E. coli to actinomycin and because of the very sharply delimited time period, immediately following infection, during which the antibiotic was effective. We therefore decided to explore this phenomenon in sensitized cells of E. coli that have been shown to respond to actinomycin in the same manner as do normally sensitive bacteria (Leive, 1965 and unpublished data).

We first demonstrated that sensitized cells of E. coli CR34 support T4 phage production in a manner identical to that observed in non-sensitized cells of this strain. Under our experimental conditions, infection of sensitized CR34 with phage T4 at an input multiplicity of 5-7:1 leads to brisk lysis beginning at about the 35th minute and average burst sizes ranging from 50-80 plaque-forming particles (PFU) per infected cell. The kinetics of intracellular infectious phage formation in sensitized CR34 are identical to those observed in non-sensitized cells.

Preliminary experiments in this laboratory had indicated: (1) In T4-infected, sensitized cells of CR34 RNA synthesis (presumably almost all of which is messenger RNA (Brenner et al., 1961; Nomura et al., 1960, 1962)) ceases by about the 20th minute. (2) Incorporation of ^{14}C-uracil into RNA is inhibited 95 to 100% by the addition of actinomycin D (final concentration 10 μg/ml) to infected, sensitized cells of CR34 at any time during the latent period. However, from the 10th minute of infection on, the addition of actinomycin does not inhibit DNA synthesis (measured by the incorporation of ^{3}H-thymine); and from the 20th minute on, actinomycin does not inhibit protein synthesis (measured by the incorporation of ^{14}C-leucine). (3) The addition of actinomycin to the infected cells from the 12th minute on does not result in detectable decay into acid-soluble material of RNA synthesized during the first 8 to 10 minutes of the latent period. It therefore appeared that from the 20th minute of infection on, actinomycin D had

TABLE II. Actinomycin inhibition of RNA, DNA, and protein synthesis in T4-infected, sensitized cells of CR34

Actinomycin added at 0 minutes (10 μg/ml)	Radioactive precursor added at 0 minutes	Incorporation measured at 30 minutes (cpm/ml)
+	Uracil-^{14}C	50
-		8,350
+	Thymine-^{3}H	200
-		42,800
+	Leucine-^{14}C and algal protein hydrolysate-^{14}C	0
-		36,429

Sensitized cells of CR34 (2-3 x 10^8 cells/ml) were infected with T4 (input multiplicity about 6 to 1) at 0 minutes. Extent of incorporation was measured as described in "Methods." Specific activities of precursors in medium were: Uracil, about 4 μC/μmole; thymine, about 40 μC/μmole; leucine, about 0.5 μC/μmole. Algal protein hydrolysate, 0.5 μC per ml of medium.

TABLE III. Effect of addition of actinomycin D at 20 minutes on macromolecular synthesis in T4-infected cells of E. coli CR34

Actinomycin D added at 20 minutes after infection (final concentration 10 μg/ml)	Radioactive precursor added at (minutes after infection)	Incorporation measured at 30 minutes after infection (cpm/ml)
+	Uracil-^{14}C, 3 minutes	8,800
-		8,950
+	Uracil-^{14}C, 20 minutes	400
-		550
+	Thymine-^{3}H, 3 minutes	90,670
-		92,340
+	Thymine-^{3}H, 21 minutes	74,100
-		74,050
+	Leucine-^{14}C and algal protein hydrolysate-^{14}C, 3 minutes	39,500
-		35,500
+	Leucine-^{14}C and algal protein hydrolysate-^{14}C, 21 minutes	12,500
-		11,000

Sensitized cells of CR34 (2-3 x 10^8 cells/ml) were infected with T4 (input multiplicity about 6 to 1) at 0 minutes. Radioactive precursors were added at the times indicated and the extent of incorporation measured as described in "Methods." Specific activities of precursors in medium were as described in Table II.

no measurable effect on RNA, DNA, or protein metabolism in the infected cells. Table II illustrates that the addition of actinomycin (final concentration 10 μg per ml) at 0 minutes to T4-infected, sensitized cells of CR34 leads to complete inhibition of RNA, DNA, and protein synthesis. Table III contains data demonstrating that the addition of antibiotic at 20 minutes is without apparent effect on RNA, DNA, and protein metabolism in the infected cells.

In contrast to these observations, however, the addition of antibiotic at a final concentration of 10 μg per ml at any time during the latent period was found to produce an immediate, complete inhibition of infectious phage formation (Table IV). Thus the final titer of plaque-forming particles in the lysate prepared from the culture that had received actinomycin D at any given time during the latent period was essentially identical to the intracellular phage titer demonstrable at the time of addition of actinomycin. The possibility that the antibiotic was in some way deleterious to mature phage particles was

TABLE IV. Effect of addition of actinomycin D at various times during latent period on phage formation

Time of addition of actinomycin (10 μg/ml) (mins. after infection)	Intracellular phage titer at time of addition of actinomycin (PFU/infected cell)	Final phage titer (PFU/infected cell)
(Control-- no actinomycin)	---	(62)
5	<0.05	<0.05
10	0.67	0.52
20	10.5	9.5
25	24.0	23.0
30	39.0	38.0

Sensitized cells of CR34 (about 2 x 10^8 per ml) were infected with T4 (input multiplicity about 6 to 1) at 0 minutes. Intracellular phage were determined in aliquots of culture that were chilled and shaken with chloroform at the times indicated.

excluded by the observation that addition of antibiotic at a concentration of 10 μg per ml to purified T4 phage caused no loss of viability. Insensitivity of mature phage to actinomycin had previously been shown for T2r by Nakata et al. (1961).

More striking evidence suggesting a difference in the effects of actinomycin D on phage production on the one hand, and on macromolecular synthesis on the other, was obtained in experiments in which very low concentrations of antibiotic were added to T4-infected, sensitized cells of CR34 at 3 minutes after the initiation of infection. The inability of these low concentrations of actinomycin D to inhibit RNA, DNA, or protein synthesis in infected cells of CR34 is demonstrated in Table V. In marked contrast is the potent inhibitory effect of low levels of antibiotic, added at 3 minutes, with respect to the production of phage progeny (Table VI). It should be noted that in all of the experiments presented in Table VI, the number of infected cells present at 8 minutes was identical to that in the control culture, thus eliminating the possibilities that actinomycin at these low concentrations was exerting a differential effect on the infected cell population, or was partially interfering with the initial penetration steps of T4 infection.

TABLE V. Effect of early addition of actinomycin D at very low concentration on macromolecular synthesis in T4-infected cells of CR34

Actinomycin D added at 3 minutes after infection (Final concentration 0.10 μg/ml)	Radioactive precursor added at 4 minutes after infection	Incorporation measured at 22 minutes after infection (cpm/ml)
+	Uracil-^{14}C	8,852
-		8,941
+	Thymine-^{3}H	5,215
-		5,015
+	Leucine-^{14}C and algal protein hydrolysate-^{14}C	4,235
-		4,671

Experimental conditions were as described in the legend for Table II.

TABLE VI. Inhibition of T4 formation by low concentrations of actinomycin D added 3 minutes after infection

Concentration of actinomycin D added at 3 min. after infection (μg/ml)	Phage titer in lysate as percent of control
0.25	<0.1
0.10	2.0
0.05	7.5
0.025	20.0
0.012	47.5

Discussion--The data presented in this communication demonstrate that under at least two different experimental conditions actinomycin D can block the development of T4 phage progeny in sensitized cells of E. coli without measurably interfering with the synthesis of RNA, DNA, or protein. We have further been able to extend the original observation of Nakata et al. and show that actinomycin interference with T-phage production in non-sensitized cells of E. coli also does not involve the inhibition of macromolecular syntheses.

Thus it has been possible for the first time to document a clear inhibitory effect of actinomycin D in vivo that appears not to result from inhibition of messenger RNA synthesis. It is of interest that the action of actinomycin under our experimental conditions seems to be similar to that heretofore uniquely attributed to proflavine (DeMars, 1955; Kellenberger, 1961). This similarity is intriguing when one considers the close structural resemblance between the acridine moiety of proflavine and the phenoxazinone moiety of actinomycin. Studies are currently in progress in this laboratory to attempt to define more precisely exactly how actinomycin interferes with phage production and to determine the structural features of the actinomycin molecule that are required for this in vivo effect.

At the present time the mechanism by which actinomycin inhibits phage formation is unknown. It may be due to binding of the antibiotic to DNA, thereby interfering with packaging; or, alternatively, it may reflect inhibition of a uniquely sensitive cistron required for phage assemblage.

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