Differential Effect of Polyamines on T4 Morphogenesis¹

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The 5-fluorouracil (5 FU) technique for the phenotypic reversion of amber mutants was used to demonstrate that under certain circumstances, in the presence of putrescine or spermidine, early mutants have an enhanced response to 5 FU, whereas late mutants have a delayed response. Bacteria infected by T4D wild-type bacteriophage did not produce phage in the presence of high putrescine concentrations. Pulse treatments with putrescine showed that the production of lysozyme depends on a putrescine-sensitive process that begins immediately after infection at 26 C and ends at 36 min or even later. The addition of putrescine at any time during the critical period between 0 and 36 min led to a corresponding delay in lysozyme synthesis after the inhibitor was removed. Intracellular phage maturation was delayed by the addition of 100 μ moles of putrescine per ml. Early enzymes were not affected by the diamine, but the level of phage deoxyribonucleic acid was considerably decreased by the inhibitor. The putrescine-sensitive process that affects the timing of maturation is suggested to be the natural process controlling the T4 "clock."

There seem to be two major groups of genes in phage T4, those related to phage deoxyribonucleic acid (DNA) synthesis (early functions of growth) and those involving maturation functions (late functions). The early proteins are detectable immediately after infection (10, 16). The late proteins are not detectable until 7 to 10 min after infection at 37 C (7, 9). The work of Wieberg et al. (16), Watanabe (15), and Sekiguchi and Cohen (12) suggests that, whereas early functions can be expressed in the absence of DNA synthesis, late functions cannot.

The specific leakage of polyamines from T4-infected cells immediately after infection (8; Sarid and Shalit, *unpublished data*) suggests that perhaps the intracellular polyamine concentration must be reduced below a critical level before late genes can express themselves.

Polyamines are normal constituents of bacterial cells (14), they occur along with Mg^{++} ions in bacteriophage particles in amounts sufficient to neutralize about half the viral DNA, and they are known to be injected into the bacteria along with the viral DNA (1, 2).

To explore the possible causal relationship between late protein synthesis and the leakage

of polyamines, polyamines were added to the medium of phage-infected bacteria. We used conditional lethal mutants of phage T4D (7), which appear in well-separated regions along the phage chromosome. Edlin (6) has shown that the base analogue 5-fluorouracil (5FU) phenotypically reverses some amber mutants. Furthermore, it has been shown by this author that the 5FU response of phage amber mutants provides a measure of specific phage ribonucleic acid (RNA) synthesis *in vivo*.

In the present communication, we used the 5FU technique to study the effect of polyamines on the time-dependent response of early and late amber mutants. Furthermore, we studied the effect of polyamine on DNA synthesis and on the synthesis of early and late enzymes.

MATERIALS AND METHODS

Phage strains. T4D (wild type) was grown on *Escherichia coli* in M9 synthetic medium. After purification by differential centrifugation, amber mutants, amB16, amB20, amB17, amN134, amBL292, amN91, amN122, amN55, amN82, and amN130 were grown on *E. coli* CR63 (7).

Bacterial strains. *E. coli* B, *E. coli* G (λ), and *E. coli* CR63 were used.

Media. The broth used contained 1 liter of water, 10 g of tryptone (Difco), and 5 g of NaCl. EHA bottom-agar contained 1 liter of water, 1.3 g of glucose, 2 g of sodium citrate, 8 g of NaCl, 13 g of tryptone, and 14 g of agar (Difco). EHA top agar contained

¹ A preliminary account of this work has been previously published (13).

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1 liter of water, 3 g of glucose, 2 g of sodium citrate, 8 g of NaCl, 10 g of tryptone, and 6 g of agar.

Shelf M9 was composed of 1 liter of water, 1 g of NH_4Cl , 3 g of KH_2PO_4 , and 7 g of Na_2HPO_4 .

M9 medium. After autoclaving shelf M9, it was supplemented with 1 g of glucose, 10^{-3} M MgSO_4 , 10^{-4} M CaCl_2 , 10^{-6} M FeCl_3 , 10^{-2} M NaCl; 0.05% Vitamin Free Casamino Acids (Nutritional Biochemicals Corp., Cleveland, Ohio) and 0.002% L-tryptophan were added before adsorption of phage T4 to host cells.

Tris-glucose medium. After autoclaving 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.2), it was supplemented in the same way as M9 medium with the addition of phosphate buffer (pH 7.2, 5×10^{-3} M).

Chemicals. Uracil and deoxyadenosine were purchased from Nutritional Biochemicals Corp. Spermidine $\cdot 3\text{HCl}$, putrescine $\cdot 2\text{HCl}$, and thymidine were obtained from Calbiochem, Los Angeles, Calif. (Solutions of spermidine and putrescine were adjusted to pH 7.0 with NaOH.) Egg white lysozyme (twice crystallized, salt-free) was obtained from Worthington Biochemical Corp., Freehold, N.J. Chloramphenicol was purchased from Abic, Ramat Gan, Israel. 5FU was generously donated by R. Duschinsky of Hoffmann-La Roche Inc., Nutley, N.J. The following radioisotopically labeled compounds were obtained from New England Nuclear Corp., Boston, Mass.: ^3H -uridine (specific activity, 1.44 mc/mmol), ^{14}C -thymine (specific activity, 40.2 mc/mmol), and ^{14}C -phenylalanine (specific activity, 333 mc/mmol).

Assay of radioactive precursor incorporation. The incorporation of radioactive precursors into trichloroacetic acid-insoluble material was determined in phage-infected cells by adding 1.0-ml portions of culture to ice-cold trichloroacetic acid to a final concentration of 5%; the mixtures were then chilled in ice for 15 min. The resulting precipitates were then filtered through 25-mm membrane filters (Schleicher & Schuell Co., Keene, N.H.) and washed by 10 ml of chilled 6% trichloroacetic acid. Filters were dried overnight at 37 C and were counted in a liquid scintillation spectrophotometer (model 3324; Packard Instrument Co., Inc., Downers Grove, Ill.).

Lysozyme assay. Lysozyme assays were performed with supernatant fluids obtained from cells disrupted by three cycles of freezing in dry ice-acetone and quick thawing, followed by centrifugation at $8,000 \times g$ for 20 min. (Identical results were obtained with cells disrupted by 4-min sonic treatment followed by centrifugation). The rest of the procedure was done as suggested by George Streisinger and Kai-Keung Mark, Institute of Molecular Biology, University of Oregon. Lyophilized *E. coli* B cells were used as substrate. Enzyme was assayed with 400 μg per ml of cell suspension in 0.05 M Tris buffer, pH 7.2 (prepared 24 hr before use and kept in the cold) in a total volume of 1 ml. The enzyme was assayed at 26 C for 15 min to detect very low activities. Lysozyme activity is expressed as change in optical density at 350 $m\mu$, and converted into enzyme units, assuming 0.1 μg per ml of egg white lysozyme is 1 unit. Standard curves of egg white lysozyme were run under conditions identi-

cal to those used for phage enzyme assay. In case the enzyme was too concentrated, it was diluted in broth to a level where enzyme activity was proportional to enzyme concentration.

Assay of 5' hydroxyl polynucleotide kinase. This assay was done according to Richardson (11) and Novogrodsky and Hurwitz (Federation. Proc. 24:602, 1965). Reaction mixtures (0.5 ml) containing 10.6 $m\mu\text{moles}$ of ^{32}P -guanosine triphosphate (GTP; 2.0×10^6 counts per min per μmole) prepared according to Avron (3), 30 μmoles of potassium succinate buffer (pH 6.0), 4 μmoles of 2-mercaptoethanol, 4 μmoles of MgCl_2 , 0.5 μmoles of 2',3'-uridine monophosphate (UMP), and 0.5 ml of deoxyribonuclease-treated extract (2 mg of protein per ml) were incubated for 30 min at 37 C. Controls in which extract or UMP were omitted were simultaneously run. Reactions were halted by the addition of 1.5 ml of 2 N HCl, and the mixtures were heated at 100 C for 15 min. After chilling in ice, 0.05 ml of a 30% Norit suspension was added, and the suspension was filtered through a membrane filter and washed with three portions of 6% cold trichloroacetic acid. The membrane filters (Millipore Corp., Bedford, Mass.) containing the Norit were dried, covered with paper, and counted in a gas-flow counter.

Deoxyribonuclease treatment. To 0.5 ml of extract, 40 μg of deoxyribonuclease and 1 mmole of MgCl_2 were added, and the mixture was incubated for 30 min at 37 C.

Assay of β -glucosyl transferase activity. Reaction mixtures (0.2 ml) containing 0.01 ml of 1 M Tris acetate buffer (pH 7.5), 0.1 ml of MgCl_2 (0.1 M), 0.01 ml of ^{14}C -uridine diphosphoglucose (UDPG; 5 $\mu\text{C}/\text{ml}$), 0.01 ml of T2 DNA (5 μg), and 0.1 ml of extract (300 μg of protein per ml) were incubated for 15 min at 37 C. The reaction was chilled, and 0.02 of 1% albumin and 3 ml of 6% trichloroacetic acid were added. The mixtures were kept in ice for 30 min and were then filtered through a membrane filter, washed with cold 6% trichloroacetic acid, dried, and counted.

Lysine mixture. Stock solution contained Tris (1 M, pH 7.8), ethylenediaminetetraacetic acid (EDTA; 0.2 M, pH 8), and egg white lysozyme (10 mg/ml). One-tenth of EDTA and 0.05 ml of egg white lysozyme solutions were added to 2.4 ml of Tris buffer; 0.05 ml of this mixture was added for each milliliter of lysate (after which chloroform was added to the lysate).

The basic procedure for rescuing infected cells by 5FU (10 $\mu\text{g}/\text{ml}$) was the same as previously described by Edlin (6).

RESULTS

Response of amber mutants to 5FU in the presence of putrescine or spermidine. Ten amber mutants of phage T4 were studied for phenotypic reversion in the nonpermissive host in the presence of 5FU (10 $\mu\text{g}/\text{ml}$) and putrescine (125 $\mu\text{moles}/\text{ml}$). These mutants consisted of early and late mutations in different regions of the genome (Table 1). The phenotypic reversion of early amber mutants by 5FU is enhanced by 125

TABLE 1. Effect of putrescine on phenotypic reversion of T4 amber mutants by 5FU^a

Gene no.	Mutant	Function missing	Percentage of maximal rescue in the presence of 125 μ moles of putrescine per ml
7	Amb16	Tail protein	75
14	Amb20	Late	50
23	Amb17	Head protein	35
33	AmN134	Maturation defective	130
37	AmN91	Tail fibers	25
42	AmN122, AmN55	Deoxycytidine monophosphate hydroxymethylase DNA polymerase	150
44	AmN82	DNA synthesis	150
46	AmN130	DNA synthesis	150
55	AmBL292	Maturation defective	120

^a *Escherichia coli* G (λ) cells at a concentration of 10^9 cells per ml in Tris-glucose medium were infected by T4D mutants at a multiplicity of infection of 0.1 to 0.2, in the presence of 2×10^{-3} M KCN. After 5 min at 37 C for adsorption and 5 min in antiserum ($K = 3$), infected cells were diluted 20-fold into chilled Tris buffer (0.1 M, pH 7.2), centrifuged, and resuspended in the original volume of chilled Tris buffer (0.1 M, pH 7.2). At time-zero *E. coli* G (γ) infected cells were diluted into Tris-glucose medium plus 5FU (10 μ g/ml), thymidine (20 μ g/ml), and putrescine (125 μ moles/ml). A control was run in parallel without putrescine. Growth tubes were aerated at 26 C. Samples were taken at the time indicated, diluted into broth supplemented by 20 μ g of thymidine and 40 μ g of uracil per ml. Infected centers were assayed on EHA plates supplemented with uracil (40 μ g/ml) and seeded with *E. coli* CR63 cells. The maximal number of infected cells rescued by 5FU in the presence of putrescine is expressed in the table as percentage of the maximal number of infected cells rescued by 5FU in the putrescine-free control (100%).

μ mole of putrescine per ml. On the other hand, late amber mutants show a decreased rescue by 5FU in the presence of putrescine.

The kinetics of response of an early and a late mutant to 5FU in the presence of 100 μ moles of putrescine per ml is shown in Fig. 1. The early mutant showed a rapid response to 5FU, which was increased by the addition of putrescine under the experimental conditions. The late mutant exhibited a slower response, which was slowed down even more by the addition of putrescine. Higher putrescine concentrations, i.e., 250 μ moles/ml, inhibited the phenotypic reversion of early amber mutants too.

The difference between early and late mutants can also be demonstrated by the addition of spermidine to the 5FU-containing growth tube. Figure 2 demonstrates the kinetics of response to 5FU in the presence of spermidine. Spermidine was as effective as putrescine (per amine residue) in decreasing the rate of late amber rescue and in enhancing the rescue of early amber mutants.

Inhibition of the maturation of T4 wild type by putrescine. The effect of various concentrations of putrescine on intracellular T4 wild-type phage maturation is shown in Fig. 3. Putrescine at a concentration of 100 μ moles/ml had a depressing effect on the appearance of mature phage particles. Phage yield in the putrescine-treated lysate was 25% of that in the control lysate when the

complexes were incubated with 100 μ moles of putrescine per ml. Putrescine at 125 μ moles/ml lowered the phage yield to 1% of that in the control lysate. Lysis of infected cells with the lysing mixture described in Materials and Methods gave the same phage yield. Thus, phage yield was not simply reflecting intracellular lysozyme synthesis. In a one-step growth curve without the use of chloroform, or lysing mixture, a 34-min delay in lysis of the cells and an 80% inhibition of phage yield was observed in the presence of 125 μ moles of putrescine per ml. Putrescine also prevented intracellular phage maturation in *E. coli* G (λ) infected complexes (Fig. 4). *E. coli* B and *E. coli* G (λ) grew actively in the presence of 125 μ moles of putrescine per ml. Free T4Dr⁺ phage was not inactivated when exposed to 125 μ moles of putrescine for 120 min at 26 C. Thus, the inhibitory effect of polyamines at high concentrations is specific to infected complexes.

Inhibition of lysozyme synthesis by putrescine. Lysozyme synthesis in infected complexes seems to be very sensitive to the presence of putrescine in the growth tube (Fig. 5). Putrescine at 100 μ moles/ml caused a 95% inhibition of enzyme synthesis, and at 125 μ moles/ml showed a 99.5% inhibition.

Beginning of putrescine-sensitive period for phage maturation. Figure 6 shows the effects of putrescine pulses which began at time-zero, and which ended at 5, 10, 15, 20, 25, 30, 40, 50, 60,

FIG. 1. Response of amber mutants to 5 FU in the presence of putrescine. *Escherichia coli* G (λ) cells were infected by T4D amber mutants as described by Edlin (6). At time-zero of the experiment, *E. coli* G (λ) infected cells were diluted into Tris-glucose medium plus 5 FU (10 μ g/ml), thymidine (20 μ g/ml), and putrescine (100 μ moles/ml). A putrescine-free control tube and a 5 FU-free control tube were run in parallel. Growth tubes were aerated at 26 C. Samples were withdrawn at the time indicated on the abscissa, and were diluted into broth supplemented by uracil (40 μ g/ml) and thymidine (20 μ g/ml). Infected centers were assayed by plating on EHA plates supplemented by uracil (40 μ g/ml) and seeded with *E. coli* CR63 cells. The infected cells yielding phage are expressed in the graph as fractions of the maximal number of infected cells yielding phage in the untreated control (1.0); 1.0 is equal to 10% of the infected bacteria.

70, and 80 min after infection with T4 wild type. The pulses were ended by a 10-fold dilution of the complexes into putrescine-free medium. The curve clearly shows that even a short pulse of 5 min decreased final phage yield. Thus, it is demonstrated that putrescine penetrates the cells as soon as it is added to the medium. Longer pulses of putrescine led to lower phage yields. There was a gradual decrease in phage yield with

pulse length. Comparison of Fig. 6 with the control curve of Fig. 3 shows that putrescine is very effective in stopping phage development. Assuming that the complexes renew their development immediately upon removal of putrescine, one could predict the curve marked as "expected" in Fig. 6. This experiment shows that the putrescine-sensitive period for phage development begins very early after infection, close to time-zero. Figure 7 shows an analogous experiment in which lysozyme was assayed instead of phage yield. Also, in this experiment it is shown that the putrescine-sensitive period begins immediately after infection.

End of putrescine-sensitive period. The effect of time of putrescine addition on the level of lysozyme synthesis was studied by subjecting T4 *E. coli* complexes to pulses of putrescine

FIG. 2. Response of amber mutants to 5 FU in the presence of spermidine. *Escherichia coli* G (λ) cells were infected by T4D amber mutants. At time-zero, *E. coli* (λ) infected cells were diluted into M9 medium plus 5 FU (10 μ g/ml), thymidine (20 μ g/ml), and spermidine (80 μ moles/ml). A control was run in parallel without spermidine. Experimental details are given in the legend of Fig. 1.

FIG. 3. Inhibition of maturation by putrescine. *Escherichia coli* B grown to a titer of 1.5×10^8 cells per ml in M9 medium were concentrated 10-fold and infected with T4D wild-type phage at a multiplicity of 4. The adsorption tube contained $2 \times 10^{-3} M$ KCN. After 5 min for adsorption at 37 C and 5 min of anti-serum ($K = 3$) treatment, the infected bacteria were diluted 10,000-fold into three parallel M9 medium growth tubes at 26 C (time-zero). Putrescine (62.5 and 100 μ moles/ml) was added to two growth tubes at time-zero; the third growth tube was the putrescine-free control. At the time shown on the abscissa, samples were shaken with chloroform and assayed to determine the final yield of phage per infected bacterium.

starting at various times during the latent period and ending after 100 min, whereupon quick freezing in dry ice-acetone was performed (Fig. 8). By 20 min after infection (26 C), the sensitivity of lysozyme synthesis to putrescine decreased, and by 36 min after infection enzyme synthesis was insensitive to putrescine. Putrescine added later than 36 min after infection had no inhibitory effect on the lysozyme activity of the infected complexes. A similar curve was found when the final phage yields per infected cell were assayed in an experiment similar to that described in the legend of Fig. 8. It is noteworthy that a different curve was found when 250 μ moles of putrescine

per ml was used in the above experiment. In this case, the end of the putrescine-sensitive period was postponed as long as 50 min after infection (26 C).

Reversibility of putrescine inhibition. Table 2 shows the results of an experiment in which T4-*E. coli* complexes were subjected to 30-min putrescine pulses at different times during the latent period. The pulses were ended by fourfold dilution into putrescine-free medium. In every pulse tested, the enzyme activity found was similar to the activity found at 50 min in the control curve of Fig. 5. This enzyme level could be expected, assuming that enzyme synthesis is resumed immediately upon putrescine removal. The total enzyme activity revealed would then be the sum of activities before putrescine addition and after putrescine removal. As in all cases described in Table 2, there were 50 min of putrescine-free activity, and all the pulses led to about equal enzyme activities. It is obvious from Table 2 that the inhibition of lysozyme synthesis was fully reversible by putrescine removal.

FIG. 4. Effect of putrescine on intracellular phage maturation. Log-phase *Escherichia coli* G (λ) cells at a titer of 10^8 cells per ml in Tris-glucose medium were infected with T4D wild-type phage at a multiplicity of 3 to 4. Experimental details are given in the legend of Fig. 3.

FIG. 5. Effect of various putrescine concentrations on lysozyme synthesis. *Escherichia coli* B cells at a titer of 2×10^9 cells per ml in M9 medium were infected with T4D wild-type phage at a multiplicity of 20 for 2 min at 26°C. The suspension was then diluted 10-fold into M9 growth tubes at 26°C (zero-time). Three growth tubes contained 62.5, 100, and 125 μ moles of putrescine per ml. The fourth tube was the putrescine-free control. At the time shown on the abscissa, 1-ml portions were withdrawn from the growth tubes, 0.1 ml of 10-fold concentrated broth was added, and the tubes were quickly frozen in dry ice-acetone. Enzyme activity was determined as described in Materials and Methods. Putrescine does not interfere with lysozyme assay.

Effect of putrescine on early enzyme synthesis. In view of the significant inhibition of phage maturation and of phage-induced lysozyme synthesis by putrescine, it seemed important to test whether early enzymes are also inhibited by the natural diamine. Two early enzymes were chosen as representatives of early functions. Table 3 shows the assay of 5 hydroxyl polynucleotide kinase in the presence of putrescine. Synthesis of this enzyme was not affected by the presence of putrescine in the medium. The assay of β -glucosyl transferase synthesis in the presence of putrescine is shown in Fig. 9. The synthesis of this early enzyme was enhanced in the presence of putrescine. This finding is in contradistinction to the observed inhibition evoked by putrescine on the late enzyme-lysozyme.

Effect of putrescine on DNA, RNA, and protein synthesis. Since the synthesis of early enzymes

seemed to be enhanced or not affected by putrescine, it was of interest to determine whether DNA synthesis, which is the product of early enzymes, would be affected.

Figure 10 shows the incorporation of 14 C-thymine into acid-precipitable material by T4-*E. coli* complexes in the presence or absence of putrescine. DNA synthesis in the presence of 100 μ moles of putrescine per ml was decreased initially, and finally reached about 15% of the level of the putrescine-free control. This finding is of major importance for the understanding of the inhibition of phage maturation exerted by putrescine, and the significance of this result is discussed below.

At very early times after infection, when DNA replication had not yet started, a decrease in the rate of RNA synthesis was observed. Figure 11 shows the incorporation of 3 H-uridine into acid-precipitable material by T4-*E. coli* complexes in the presence and absence of putrescine. This figure, which magnifies the first few minutes after infection shown in Fig. 10, reveals a depression of RNA synthesis in the presence of putrescine. When chloramphenicol (100 μ g/ml) was present from time-zero in the 3 H-uridine incorporation experiment (Fig. 11), exactly the same rates of RNA synthesis were observed. It seems that neither protein synthesis nor messenger

FIG. 6. Effect of putrescine treatment on phage yield. The infected bacteria were diluted 10,000-fold into two parallel M9 medium growth tubes. These tubes were incubated at 26°C (time-zero). Putrescine (125 μ moles/ml) was added to one of the growth tubes at time-zero. At the time shown on the abscissa, samples were diluted 10-fold from the putrescine growth tube into putrescine-free M9 medium and incubated up to 100 min at 26°C. The putrescine-free dilutions were then shaken with chloroform and assayed to determine the final yield of phage per infected bacterium. The expected points were calculated from the control curve (see Fig. 3), assuming a delay in phage maturation as long as the putrescine pulse, and resumption of phage maturation as soon as putrescine is diluted out of the growth medium.

FIG. 7. Beginning of putrescine-sensitive period for lysozyme synthesis. At time-zero of infection, 125 μ moles of putrescine per ml was added to the infected bacteria. At the time indicated, samples were diluted fourfold from the putrescine growth tube into putrescine-free M9 medium and incubated up to 80 min at 26 C. The putrescinee-free dilutions were then supplemented by broth and quickly frozen in dry ice-acetone. Enzyme activity was determined as described in Materials and Methods. Symbols: $\bullet$, experimental points; $\circ$, expected points as calculated from the control curve (see Fig. 5), assuming an inhibition of lysozyme synthesis as long as the putrescine pulse, and resumption of synthesis as soon as putrescine is diluted out of the growth medium.

RNA (mRNA) translation has any effect whatsoever on the decreased rate of mRNA synthesis in the presence of putrescine. Net protein synthesis in phage-infected complexes was hardly affected by putrescine (Fig. 12). In the presence of 100 μ moles of putrescine per ml, protein synthesis reached a level of 75% that of the putrescine-free control. Thus, the dramatic inhibition of lysozyme synthesis in the presence of putres-

TABLE 2. Reversibility of putrescine inhibition^a

Putrescine pulse (min at 26 C)	Lysozyme activity ([E] units)		
	In the presence of putrescine	Putrescine removed at end of pulse	Expected from control curve
2-32	4	238	228
7-37	4	238	228
12-42	4.1	250	228
17-47	4.8	215	228

^a *Escherichia coli* B, 7×10^8 cells per ml, was infected by T4Dr⁺ (multiplicity of infection = 20) in M9 medium. After 2 min at 26 C for adsorption, putrescine (125 μ moles/ml) was added to the first tube. The rest of the tubes were shaken in a bath at 26 C, and putrescine (125 μ moles/ml) was added at the time indicated. At the end of a 30-min pulse at 26 C, a fourfold dilution into putrescine-free M9 medium ended putrescine activity. Tubes were further shaken at 26 C up to 80 min, and then were frozen in dry ice-acetone in the presence of broth to preserve the enzyme. The sum of enzyme activities in the various tubes before putrescine addition and after putrescine removal was equal to 50 min in all cases. The expected enzyme activity at 50 min after infection was determined by a putrescine-free control growth tube run in parallel to the experimental tubes.

FIG. 8. Inhibition of lysozyme synthesis by putrescine. Log-phase *Escherichia coli* B cells at a titer of 4×10^8 cells per ml in M9 medium were infected with T4D wild-type phage at a multiplicity of 10. After 2 min at 26 C for adsorption, the infected bacteria were divided in 1-ml samples and incubated at 26 C in a shaking bath. ($\bullet$) Treatment with putrescine (125 μ moles/ml) began at the times shown on the abscissa and ended at 100 min (26 C) by quick freezing in dry ice-acetone. A putrescine-free control growth tube was run in parallel. ($\blacktriangle$) Samples were withdrawn from the control tube at the time indicated, and were frozen in dry ice-acetone to determine enzyme activity at the time of putrescine addition. Lysozyme activity is expressed as fraction of enzyme activity found in the putrescine-free medium.

TABLE 3. 5'-Hydroxyl polynucleotide kinase in cell extracts prepared 15 min after infection with T4^a

Extract	Counts per min	Relative activity
<i>Escherichia coli</i>	120	0.03
T4-infected	3,400	1.0
T4-infected + 100 μ moles of putrescine per ml	3,210	0.91

^a *Escherichia coli* B was grown in 300 ml of M9 medium containing Casamino Acids to a cell density of 5×10^8 cells per ml. The culture was divided into 100-ml samples. Samples 2 and 3 were infected with T4D wild type at a multiplicity of infection of 20 for 2 min at 25 C without aeration. At this time, 100 μ moles of putrescine per ml was added to sample 3, and the samples were transferred to 37 C and aerated for 15 min. The cells were collected by centrifugation and resuspended in 2 ml of M9 medium. The suspension was frozen. After thawing, the suspension was sonically treated for 5 min at 0 C (Branson sonicator). The supernatant fluid (2 mg of protein per ml) contained the enzymatic activity. Assay of 5 hydroxyl polynucleotide kinase was done as described in Materials and Methods. Kinase activity is expressed as the amount of radioactivity found on the Norit-containing membrane filters.

FIG. 9. Kinetics of synthesis of β -glucosyl transferase in T4 infected cells. *Escherichia coli* B was grown in Tris-glucose medium to a density of 2×10^8 cells per ml. The cells were collected by centrifugation and resuspended in Tris-glucose medium. Three parallel cultures of 18 ml of *E. coli* B (6.6×10^8 cells per ml) were kept at 25 C for 5 min. Tryptophan was added (0.2 ml of a 0.2% solution), and cultures 2 and 3 were infected with T4D wild type (multiplicity of infection = 14) for 2 min at 25 C. At this time, 100 μ moles of putrescine per ml was added to culture 3, and the samples were transferred to 37 C and aerated. At various times after infection, 2-ml portions were withdrawn into cold tubes and frozen in dry ice-acetone. The cultures were frozen and thawed for three cycles, followed by centrifugation. The supernatant fluid contained the transferase activity, and no activity was left in the debris. Transferase activity is expressed as the amount of radioactivity found in the acid-insoluble fraction. Putrescine does not interfere with β -glucosyl transferase assay.

FIG. 10. Effect of putrescine on DNA synthesis in T4Dr⁺-infected cells of *Escherichia coli* B. *E. coli* B cells were grown in Tris-glucose medium, supplemented with 0.5 mg of thymine per ml. Cells were washed by centrifugation in Tris-glucose medium and resuspended in this medium at a concentration of 2×10^9 . Cells were infected with T4D wild-type phage for 2 min at 26 C; they were then diluted 10-fold into two parallel Tris-glucose growth tubes supplemented with 250 mg of deoxyadenosine per ml, in order to enhance thymine uptake (4), and were aerated at 26 C (time-zero). The first growth tube (+) contained ¹⁴C-thymine (0.1 μ C/ml) and putrescine (100 μ moles/ml). The second growth tube (●) was the putrescine-free control. Extent of incorporation was measured as described in Materials and Methods.

FIG. 11. Effect of putrescine on RNA synthesis in T4D⁺-infected cells of *Escherichia coli* B. *E. coli* B cells were grown in M9 medium supplemented with 0.5 μ g of uridine per ml. Cells were washed in shelf M9 by centrifugation, and were resuspended in M9 medium at a concentration of 8×10^8 per ml. Infection was carried out for 2 min at 26 C. At time-zero, infected cells were diluted 10-fold into four parallel M9 medium growth tubes containing ³H-uridine (0.05 μ C/ml) and putrescine (62.5, 100, and 125 μ moles/ml). The fourth growth tube was the putrescine-free control. Growth tubes were aerated at 26 C. Extent of incorporation was measured as described in Materials and Methods.

FIG. 12. Effect of putrescine on ^{14}C -phenylalanine incorporation in T4Dr^+ -infected cells of *Escherichia coli*. *B. E. coli* B cells were grown in M9 medium. Cells ($2.6 \times 10^9/\text{ml}$) were infected with T4D wild-type phage as described in the legend of Fig. 11. Infected cells were then diluted 10-fold (time-zero) into four parallel M9 medium growth tubes aerated at 26 C. Growth tubes contained $0.06 \mu\text{C}/\text{ml}$ of ^{14}C phenylalanine and 62.5, 100, or 125 μmoles of putrescine per ml; the fourth tube was the putrescine-free control. Extent of incorporation was measured as described in Materials and Methods.

cine is not reflected in net protein synthesis. The inhibition of late protein synthesis in the presence of the diamine is probably compensated by the over-production of early enzymes.

DISCUSSION

The inhibitory effect of polyamines on phage yield was suggested by Ferro-Luzzi Ames and Ames (8). The external putrescine concentration required to exert inhibition of phage maturation seems very high in their experiments, as in ours.

Our experiments reported herein demonstrate the differential effect of polyamines on sequential protein synthesis in phage-infected cells. The data suggest that putrescine does not inhibit the mRNA translation mechanism. If an inhibitor affects transcription but has no effect on translation, protein should be synthesized in the presence of the inhibitor as long as mRNA synthesized before addition of the inhibitor remains functional. Our data (Fig. 8) demonstrate the synthesis of lysozyme in the presence of putrescine, when putrescine was added late in the latent period. Phage maturation and phage-induced lysozyme synthesis were inhibited 95 to 99%, in accord with the decreased phenotypic rescue

of late amber mutants by 5FU in the presence of polyamines. Under the same experimental conditions, DNA was significantly inhibited. In earlier work on this system, Wieberg et al. (16) showed that phage mutants which fail to make DNA do not make any late proteins, whereas many of the earlier proteins continue to be synthesized for a longer time than with wild-type phage. Ultraviolet light irradiation, which also appears to interfere with DNA replication, inhibits the appearance of late proteins (5, 12, 15). We would suggest that in the present work the inhibition of late protein synthesis is also linked to the inhibition of DNA synthesis, and that the latter may be due to cross-linking of the DNA by putrescine. It has been shown that putrescine can inhibit mature phage production and lysozyme synthesis when added immediately after infection and as late as 20 min after infection. This suggests that there is a putrescine-sensitive process which extends over the 20-min interval. Studies reported in an accompanying paper (12a) show that selective gene transcription occurs in the presence of putrescine in phage T4 infection.

Since putrescine inhibition is completely reversible as soon as the inhibitor is removed, the regulation of the sequential events in phage-infected cells (the "clock") seems to operate like a stop watch. It stops working as soon as putrescine is added (during the sensitive period), and it resumes its activity as soon as putrescine is removed.

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