Effect of Spermine on Lysis and Reproduction by Bacteriophages ϕ X174, λ , and f_2

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ABSTRACT

GROMAN, NEAL B. (University of Washington, Seattle), AND GRACE SUZUKI. Effect of spermine on lysis and reproduction by bacteriophages ϕ X174, λ , and f_2 . *J. Bacteriol.* 92:1735-1740. 1966.—A test was made of the hypothesis that lysis by all bacteriophages shares as a common and critical step an alteration in the osmotic stability of the infected cell. This was done by examining the effect of spermine on lysis. Spermine is one of a number of compounds which can stabilize spheroplasts and protoplasts to lysis in distilled water. Spermine stabilized both ϕ X174- and f_2 -infected cells at concentrations ranging from 2×10^{-3} to 4×10^{-2} M, but failed to stabilize λ -infected cells at concentrations up to 8×10^{-2} M. Stabilization was reflected both in optical density measurements and in the retention of mature phage in structures sedimentable at low speeds. At optimal concentration, over 90% of the phage was retained in these structures. These data suggest that the mechanism of lysis by ϕ X174 and f_2 differs sharply from that caused by λ , and other observations suggest that there are differences in the lytic process of ϕ X174 and f_2 as well. Spermine also displayed a differential effect on phage reproduction. The reproduction of λ and f_2 was inhibited by spermine, though the data do indicate that maturation occurs in its presence. The reproduction of ϕ X174 was enhanced by spermine.

Most questions concerning the mechanism of lysis by bacteriophage remain unanswered at present. The lysozyme-like enzymes produced during some infections undoubtedly play a role. Nevertheless, attempts to demonstrate similar enzymes in some lysing systems have failed (3, 5). The general question remains: are the lytic processes as distinct as the phages themselves or do they have some common underlying feature?

It was previously suggested that a change in permeability brought about by phage action might be the feature common to all phage-induced lysis (1). It is known that certain polyamines stabilize bacterial protoplasts and spheroplasts (2, 4). Since both gram-positive and gram-negative organisms are affected, it can be tentatively concluded that the stabilizing effect is due at least in part to the interaction of these compounds with the cytoplasmic membrane. If phage lysis involved a change in the osmotic stability of the infected cell owing to some alteration of the membrane, then polyamines might prevent lysis by stabilizing the membrane. Furthermore, if lysis involved a common alteration of the membrane by all phages, then polyamines might prevent lysis in all cases. With this in mind, the effect of spermine

on lysis by phages representative of three major groups was examined. The phages studied included ϕ X174, containing single-stranded deoxyribonucleic acid (DNA), phage λ c₈₀, containing double-stranded DNA, and phage f_2 , containing single-stranded ribonucleic acid.

MATERIALS AND METHODS

Bacteria and phages. *Escherichia coli* strain C and phage ϕ X174 were obtained from R. L. Sinsheimer. An F⁺ derivative of *E. coli* C was made in this laboratory. Phages λ c₈₀, a c₁ mutant, and f_2 were obtained from V. C. Bode and N. D. Zinder, respectively. Phage stocks of ϕ X174 and λ c₈₀ were grown on *E. coli* C, and f_2 was grown on the F⁺ derivative. The same strains were used as indicators for phage assays.

Media and materials. In all experiments, cells were grown and infected in LB medium or some variation of the medium. LB medium contained 1% tryptone (Difco), 1% NaCl, 0.5% yeast extract, and 0.1% glucose, at a final pH of 7.4. The variations in this medium are noted below and in the Results. Dilutions were made in LB (–NaCl) medium. Phage indicator strains were grown in Trypticase (BBL)-soy-yeast extract medium to 1.5×10^8 cells per milliliter, centrifuged, and suspended in LB medium containing 0.002 M CaCl₂. All phages were assayed in the same agar overlay system, consisting of base plates (1.5%

agar) and overlay (0.7% agar) of tryptone medium containing 1% tryptone, 0.5% NaCl, and 1 mg of thiamine-HCl per liter (pH 7).

Spermine·4HCl was obtained from Calbiochem, Los Angeles, Calif., and Sigma Chemical Co., St. Louis, Mo. It was prepared as a 1 M stock solution in distilled water or broth medium every 1 to 2 weeks. Lysozyme (Calbiochem) was prepared daily in distilled water as a stock solution containing 10,000 $\mu\text{g}/\text{ml}$.

Methods. All cultures were aerated in a reciprocal shaking water bath (160 1.5-inch strokes per minute). Incubation was at 37 C. Optical densities were measured in a Klett-Summerson colorimeter with a no. 54 filter.

Phage was released from infected cells by the chloroform-lysozyme method. Four or five drops of chloroform were added per milliliter of sample. The suspension was agitated vigorously on a Vortex mixer. Lysozyme was then added to a concentration of 100 $\mu\text{g}/\text{ml}$; the suspension was agitated again and then incubated for 40 min at room temperature with occasional mixing. The lysed cell suspension was centrifuged at $2,500$ to $3,500 \times g$ for 10 min, and the supernatant fluid was assayed.

To determine free and intracellular phage, 2 ml of an infected culture was centrifuged at $3,500 \times g$ for 10 min at room temperature. Free phage was determined in the supernatant liquid, and intracellular phage in the sediment. The sediment was resuspended in LB medium, diluted 1:100, and phage was released by the chloroform-lysozyme method. Total phage was assayed on an uncentrifuged sample diluted 1:100 in LB medium and treated with chloroform-lysozyme.

RESULTS

Effect of spermine on infection with $\phi X174$. Virtually complete stabilization of optical density was achieved by 10^{-2} M spermine (Fig. 1) even when it was added during the lytic period. The optical density of uninfected cultures increased at similar rates in the absence or presence of spermine. In concert with the stabilization of optical density, spermine prevented lysis of the infected cells. Approximately 80% of the phage was retained in spermine-treated cells or structures which sedimented at low speed (Table 1). Only small amounts of the phage produced in the (no spermine) control were retained. These values varied from experiment to experiment, but they were usually less than 20% of the total yield. Total phage yield was increased significantly by the addition of spermine. A maximal 2.5-fold increase occurred when spermine was added 10 to 15 min after infection, and some increase was observed even with the 25-min addition.

There was increasing stabilization of optical density as the spermine concentration increased (Fig. 2). The highest concentration added at 10 min was as effective as that observed previously with the zero-time addition (Fig. 1).

FIG. 1. Effect of time of spermine addition on lysis by $\phi X174$. Cells were infected at a multiplicity of about 10, and spermine (10^{-2} M) was added at the times indicated. LB medium was used.

TABLE 1. Effect of time of spermine addition on $\phi X174$ yield and distribution^a

Determination	Expt no.	Time of spermine addition (min)				No spermine
		0	10	15	25	
Relative yield (%)	1	90	80	100 ^b	63	40
	2	78	100 ^c	73	65	36
Intracellular phage (% of total)	1	78	83	90	79	17
	2	79	76	67	71	0

^a Cells were grown in LB medium to about 5×10^8 per milliliter, and MgSO_4 (10^{-3} M) was added. Phage was added at a multiplicity of about 10, and shaking was resumed. Spermine (10^{-2} M) was added at various times, and 90 min after infection the cultures were assayed for total, free, and intracellular phage.

^b Represents 3×10^{11} particles per milliliter.

^c Represents 3.7×10^{11} particles per milliliter.

Lysis was prevented and phage was retained intracellularly with increasing efficiency as the spermine concentration increased. Retention at 10^{-3} M was not significantly different from that of the control, but there was virtually complete retention at 2×10^{-2} M and above (Table 2). There was some tendency for higher concentrations of spermine to give better yields of phage, though

FIG. 2. Effect of spermine concentration on lysis by ϕ X174. Cells were infected at a multiplicity of about 10, and spermine was added 10 min after infection. LB medium was used.

the differences were relatively minor between 5×10^{-3} and 4×10^{-2} M. The control yields were exceeded at all concentrations, but 10^{-3} M appears to be borderline for this effect.

Infection with λ c₈₀. Phage λ c₈₀ was prepared by passage in *E. coli* C in LB medium from which glucose was omitted. It was observed that phage produced in LB medium, i.e., with glucose, produced poor lysis regardless of the medium in which lysis took place. For the time being, there is no explanation for the difference between phage produced in the presence and in the absence of glucose. The selection of a phage variant in glucose has not been excluded.

The effect of spermine on optical density is given in Fig. 3. The effects seen with 10^{-2} M were as great as those observed at 8×10^{-2} M spermine, the maximal concentration tested. With the exception of the zero-time addition, the results suggested that at most spermine stabilized the cells only slightly. However, it is evident (Tables 3 and 4) that, under all conditions, most of the phage produced appeared as free phage, and hence little stabilization actually occurred. Although some phage was retained in sedimentable structures, the direct assay of the sediment never yielded more than 5% of the total phage. This discrepancy is unexplained, although it reinforces the conclusion that the release of λ c₈₀ phage is complete in the presence of spermine regardless of when it is added.

TABLE 2. Effect of spermine concentration on ϕ X174 yield and distribution^a

Determination	Expt no.	Spermine (molarity $\times 10^2$)					No spermine
		0.1	0.5	1	2	4	
Relative yield (%)	1	83	74	100 ^b	89	94	43
	2	46	76	73	91	100 ^c	35
Intracellular phage (% of total)	1	45	54	80	87	94	40
	2	47	64	80	91	95	54

^a Same procedure as in Table 1, except that the various spermine concentrations were added 10 min after infection.

^b Represents 3.5×10^{11} particles per milliliter.

^c Represents 3.7×10^{11} particles per milliliter.

FIG. 3. Effect of time of addition of spermine on lysis by λ c₈₀. Cells were infected at a multiplicity of 5 to 6, and spermine was added at the times indicated. LB (—glucose) medium was used.

The data on phage yield (Table 3) show that 10^{-2} M spermine markedly inhibited λ c₈₀ reproduction, the time of addition determining the degree of inhibition. Addition at zero-time abolished phage reproduction, and hence lysis, thus explaining the optical density stabilization; 50% inhibition was usually observed even when addition was delayed to 40 min after infection. At 40 min, lysis is about to begin or has already begun, and about 20% of the total phage yield has already matured. Thus, it is clear that maturation can proceed in the presence of spermine, but that some earlier process, e.g., the synthesis of phage components, is blocked. Table 4 shows that the degree of inhibition was relatively constant between 5×10^{-3} and 2×10^{-2} M when spermine was added 30 min after infection. However, inhibition was not observed at higher concentrations. It should be noted that spermine stabiliza-

TABLE 3. *Effect of time of spermine addition on λc_{50} yield and distribution^a*

Determination	Expt no.	Time of spermine addition (min)				No spermine
		0	15	30	40	
Relative yield (%)	1	2	29	99	100 ^b	98
	2	<1	17	56	100 ^c	78
	3	—	17	48	—	100 ^d
Free phage (% of total)	1	43	80	91	100	94
	2	100	97	76	72	100
	3	—	64	100	—	100

^a Cells were grown in LB (glucose) medium to about 5×10^8 per milliliter, and $MgSO_4$ (10^{-3} M) was added. Phage was added at a multiplicity of 5 to 6, and shaking was resumed. Spermine (10^{-2} M) was added at various times, and 90 min after infection the cultures were assayed for total, free, and intracellular phage.

^b Represents 7×10^9 particles per milliliter.

^c Represents 1.8×10^{10} particles per milliliter.

^d Represents 1.9×10^{10} particles per milliliter.

TABLE 4. *Effect of spermine concentration on λc_{50} yield and distribution^a*

Determination	Expt no.	Spermine (molarity $\times 10^2$)					No spermine
		0.5	1	2	4	8	
Relative yield (%)	1	51	48	58	—	—	100 ^b
	2	44	51	67	—	—	100 ^c
	3	—	32	45	86	83	100 ^d
	4	—	42	61	64	100 ^e	73
Free phage (% of total)	1	76	100	65	—	—	100
	2	100	100	62	—	—	89
	3	—	100	100	84	100	76
	4	—	79	75	100	100	100

^a Same procedure as in Table 3, except that spermine at various concentrations was added 30 min after infection, at which time 3% of the total phage had already matured.

^b Represents 1.9×10^{10} particles per milliliter.

^c Represents 1.8×10^{10} particles per milliliter.

^d Represents 2.9×10^{10} particles per milliliter.

^e Represents 3.3×10^{10} particles per milliliter.

tion of $\phi X174$ -infected cells was tested under the same conditions used for λc_{50} . The important observation was that the degree of stabilization of cells to lysis measured by optical density and phage retention was the same as that observed in LB medium.

Infection with f_2 . It was observed in preliminary experiments that f_2 lysed late log-phase or early stationary-phase cells more readily than rapidly growing cells, and lysed better in the absence of NaCl. These observations defined the

method of growing and infecting cells described in Table 5.

As seen in Fig. 4, spermine at 2×10^{-2} M completely prevented or interrupted lysis by f_2 at all times after which a marked increase in optical density occurred. Over the range of concentrations tested (Tables 5 and 6), spermine almost completely prevented the release of phage. There was, however, no exact correlation between optical density stabilization and phage retention, since the sharpest rise in optical density (Fig. 4, 45-min addition) was accompanied by the least amount of stabilization (Table 5).

TABLE 5. *Effect of time of spermine addition on f_2 phage yield and distribution^a*

Determination	Expt no.	Time of spermine addition (min)					No spermine
		0	10	20	30	45	
Relative yield (%)	1	1	18	43	61	96	100 ^b
	2	1	40	38	68	100 ^c	88
Intracellular phage (% of total)	1	>99	99	98	96	77	56
	2	>99	98	96	94	80	45

^a Cells were grown in LB (—NaCl) medium to about 10^9 per milliliter, centrifuged, and resuspended in fresh medium to 5×10^8 per milliliter. $MgSO_4$ (10^{-3} M) was added. Phage was added at a multiplicity of about 10, and shaking was resumed. Spermine (2×10^{-2} M) was added at various times, and at 90 min after infection the cultures were assayed for total, free, and intracellular phage.

^b Represents 2.3×10^{11} particles per milliliter.

^c Represents 2.5×10^{11} particles per milliliter.

FIG. 4. *Effect of time of spermine addition on lysis by f_2 phage. Cells were infected at a multiplicity of about 10, and spermine (2×10^{-2} M) was added at the times indicated. The medium was LB (—NaCl).*

TABLE 6. *Effect of spermine concentration on f₂ phage yield and distribution^a*

Determination	Expt no.	Spermine (molarity $\times 10^2$)					No spermine
		0.1	0.2	0.5	1	2	
Relative yield (%)	1	43	29	26	32	50	100 ^b
	2	42	31	28	18	36	100 ^c
Intracellular phage (% of total)	1	73	91	99	99	98	13
	2	76	85	99	98	98	26

^a Same procedure as in Table 5, except that spermine at various concentrations was added 20 min after infection, at which time 3% of the total phage had already matured.

^b Represents 3×10^{11} particles per milliliter.

^c Represents 3.1×10^{11} particles per milliliter.

Tables 5 and 6 show that spermine markedly inhibited *f₂* reproduction. The degree of inhibition was roughly related to the time of addition, but was constant over the range of concentrations tested. Significant inhibition was observed even when spermine was added as late as 30 min after infection, which was just before lysis began in the control. At this time, the cells contained about 10% of their final yield of mature phage. As with λc_{50} , maturation apparently proceeded in the presence of spermine.

Cell growth and reproduction in spermine. When growth of *E. coli* C in the presence of spermine (10^{-8} to 5×10^{-2} M) was measured by optical density, it appeared that the growth rate was slower in the presence of spermine. This decrease in rate is probably more apparent than real, since it was observed that cells clump extensively in the presence of spermine but do not clump in its absence in LB (–NaCl). To determine whether cell division accompanies growth in 10^{-2} M spermine, viable counts were made. Samples were mixed vigorously in a Vortex mixer, in the hope of breaking up clumps. Despite the technical problem of clumping, the data showed that the division rate was the same whether or not spermine was present. Therefore, *E. coli* C does grow and reproduce in the presence of spermine.

Phage-inactivation studies. The stability of phages $\phi X174$, λc_{50} , and *f₂* was determined under conditions identical to those employed in the experiments. This included a 90-min period of incubation in the appropriate medium with shaking at 37 C, and 40 min in the assay medium under standard assay conditions at room temperature. The titer of both $\phi X174$ and λc_{50} decreased 25% and that of *f₂* decreased approximately 15%. There was no significant difference between phages incubated in the presence of spermine and in its absence.

DISCUSSION

The present study was undertaken to determine whether the lytic process of phages $\phi X174$, λc_{50} , and *f₂* share a common denominator. It was postulated that, in lysis by all phages, an alteration of the permeability barrier of the cell is a common and critical step. The hypothesis predicted that if polyamine stabilized lysing cells in one infection it would do so in all of them. The data presented failed to support this hypothesis. Most dramatically, they showed that cells infected by λc_{50} were not stabilized by spermine, whereas those infected with $\phi X174$ and *f₂* were. Thus, the damage suffered by λ -infected cells is more profound than that suffered in the other infections. There are also indications that the lytic mechanisms of $\phi X174$ and *f₂* differ substantially. Phage $\phi X174$ lysed *E. coli* C in LB or LB (–NaCl) medium, whereas lysis by *f₂* took place only in LB (–NaCl). It appears that *f₂* lysed older, less active cells more effectively than log-phase cells, whereas $\phi X174$ lysed cells in both phases of growth.

The logical suspect for the more destructive character of lysis by λc_{50} is the phage-induced enzyme endolysin, which attacks the mucopeptide layer of the cell wall. Assuming endolysin was responsible for lysis, it would follow from the results that the cell membrane was disrupted at the same time as the mucopeptide layer, thus preventing spermine from stabilizing the cells. However, we have observed that spheroplasts of *E. coli* K-12, produced by use of endolysin in place of lysozyme, are stabilized by spermine to lysis in distilled water (Anderson and Groman, unpublished data). Therefore, cells exposed to endolysin obviously retain considerable structural integrity. Additional evidence that destruction of the mucopeptide layer does not preclude stabilization by spermine is the observation that spheroplasts are formed by $\phi X174$ -infected cells in the presence of spermine. It seems unlikely, therefore, that endolysin action can account for the destructive nature of λc_{50} lysis.

Another finding of interest is the differential action of spermine on the reproduction of the three phages. The data suggest that the reproductive processes of λc_{50} and *f₂* share a spermine-sensitive step, one not displayed by $\phi X174$. On the basis of their nucleic acid composition and structure, this combination of sensitive phages is the most unlikely pair to share a common step. It would have seemed more logical if both DNA phages ($\phi X174$ and λc_{50}) or both phages with single-stranded nucleic acid ($\phi X174$ and *f₂*) behaved similarly. However, more detailed knowl-

edge of the cycle and of the mode of action of spermine is required before one can rule out a common step for λc_{50} and f_2 . There is the possibility that the differential action of spermine on the three phages and *E. coli* reflects differences in the permeability of the cell to spermine during growth or during infection. Thus, uninfected cells and $\phi X174$ -infected cells may be less permeable and hence are not susceptible to its action. If permeability differences are involved, however, it is paradoxical that the reproduction of λc_{50} and f_2 was sensitive to spermine, but that only cells infected with f_2 phage were stabilized by spermine.

In $\phi X174$ and f_2 infections, spermine permitted retention of phage in structures that sedimented at low speeds. These experiments were performed at high cell density. Therefore, it is possible that phage was released, readsorbed, and finally released again during the lysing procedure. Phage retention would then reflect enhanced readsorption rather than cell stabilization. To test this hypothesis, $\phi X174$ antibody was added to a $\phi X174$ -infected culture. Whereas most of the phage was neutralized in the control lacking spermine, virtually all the phage was detected in the sediment

of the spermine-treated culture. This argues that spermine does actually cause phage to be retained intracellularly.

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