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CALCIUM ION REQUIREMENT FOR PROLIFERATION OF BACTERIOPHAGE $\phi\mu$-4

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ABSTRACT

SHAFIA, FRED (University of Nebraska, Lincoln), AND T. L. THOMPSON. Calcium ion requirement for proliferation of bacteriophage $\phi\mu$-4. J. Bacteriol. **88**:293-296. 1964.—Divalent ions are essential for proliferation of phage $\phi\mu$-4. Small amounts of citrate interfere with efficient adsorption of phage to the host cells. Penetration of phage material into the cell is strictly dependent on divalent ions and is inhibited by low levels of citrate. Inhibition of infection can be partially reversed, in early latent period, by calcium ions. Synthesis of new phage particles is also dependent on divalent ions. Addition of citrate to infected cell suspensions significantly reduced the number of phage progeny produced. Chelates such as phosphate and citrate rapidly inactivated the free phage particles at 65 C. Chelate inactivation of phage is not reversible; however, it can be prevented to some degree by calcium ions.

Calcium ion requirement for adsorption of bacteriophage has been observed in several phage-cell systems (Garen and Puck, 1951; Rountree, 1951; Barksdale and Pappenheimer, 1954). Luria and Steiner (1954) noted that calcium ions are required for penetration of phage T_5 into the host cell. Lanni (1960) reported on calcium-dependent and calcium-independent phases of phage T_5 invasion. Rountree (1947), Delbruck (1948), and Adams (1949) reported on calcium requirement for synthesis of the bacteriophages they studied.

We reported that plaque formation of phage $\phi\mu$-4 is calcium-dependent and is inhibited by citrate (Shafia and Thompson, 1964). This investigation was undertaken to determine the effects of calcium and citrate on adsorption, penetration, and synthesis of phage $\phi\mu$-4.

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MATERIALS AND METHODS

Bacterium and phage. Methods for culture of the host organism *Bacillus stearothermophilus* NU-10 and bacteriophage $\phi\mu$-4 were described previously (Shafia and Thompson, 1964).

Media. The basic dilute medium employed in these studies contained 0.1% Trypticase (BBL), 0.015% yeast extract, and 0.005% dextrose. The broth was supplemented with either calcium chloride or sodium citrate (final concentrations, 0.01 and 0.003 M, respectively), depending on the particular phase of study.

Infected cell and phage assays. Host cells exposed to phage particles were collected on Millipore (type HA, pore diameter 0.45 μ) filters, rinsed to remove free phage, and assayed for plaque-forming infected cells (PFIC). Procedure for PFIC determination was similar to the phage assay method described previously (Shafia and Thompson, 1964), except that dilutions of the infected cells were substituted for phage samples.

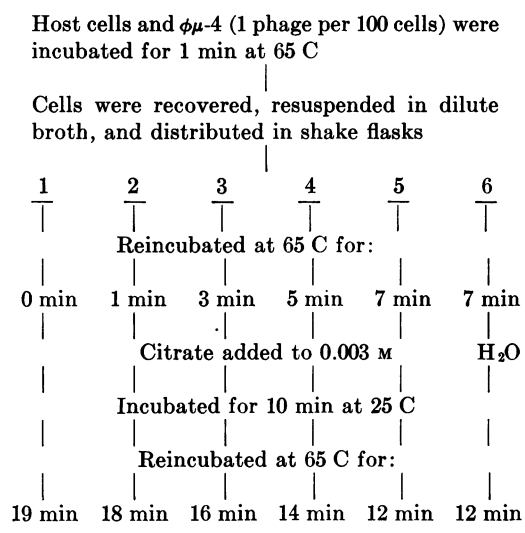
Cell preparation. Exponentially growing host cells were sedimented and resuspended in the dilute broth. The cell population of the suspension was standardized turbidimetrically to a bacterial density of approximately 10^7 cells per ml. The cell suspension thus prepared was used in these studies.

Citrate on adsorption. Host cells in citrate broth were incubated in shake flasks for 15 min at 65 C. Phage particles were introduced to a multiplicity of one phage per 100 cells, and incubation was continued. Prior to addition of phage, chloramphenicol (final concentration, 100 μg/ml) was added to the suspension to eliminate interference from newly formed phage particles. Control systems were treated similarly, except that calcium-containing broth was used instead of citrate broth.

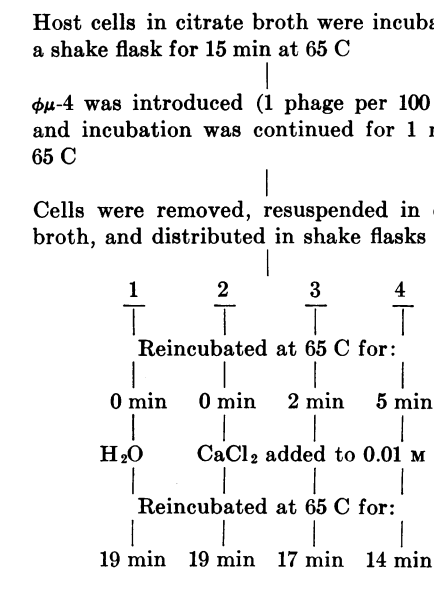
Citrate on infection. The procedure was similar to citrate treatment for adsorption, except that

chloramphenicol was omitted, and the phage-cell system was incubated for 15 min at 65 C. After the incubation, the cells were recovered for infected-cell assay.

Citrate sensitivity period. Duration of citrate sensitivity in penetration and synthesis of phage was determined by the procedures given in the flow diagrams 1 and 2.



Flow diagram 1



Flow diagram 2

Referring to flow diagrams 1 and 2, after the final incubation each suspension was cooled to 5 C and diluted in broth, and a portion of each was assayed for infected-cell determination. To determine the effect of citrate on phage synthesis, a portion of each suspension was diluted to contain approximately ten infected cells per ml according to the following procedure. Flow diagram 1: suspensions 1 through 5 were diluted in citrate broth and suspension 6 in calcium-containing broth. Flow diagram 2: suspension 1 was diluted in citrate broth and suspensions 2 through 4 in calcium-containing broth. The preparations were reincubated, in shake flasks, for 45 min at 65 C for lysis to occur.

Phage sensitivity to chelates. Dilutions (100-fold) of a phage stock (2.77×10^7 phage particles per ml) in 0.1 M phosphate buffer (pH 7.2) and 0.1 M sodium citrate solution (pH 7.2) were incubated in shake flasks for 15 min at 65 C. To a similar set of phage dilutions, calcium chloride was added, prior to incubation, to a final concentration of 0.1 M. At intervals, the titer of active phage in each suspension was determined.

RESULTS AND DISCUSSION

It was determined that neither the activity of free phage nor the growth rate of the host organism was markedly affected by 0.003 M sodium citrate.

The adsorption studies carried out in citrate broth demonstrated partial inhibition of this process. Attempts to use stronger chelates, such as ethylenediaminetetraacetate (EDTA), for complete inhibition of adsorption were unsuccessful owing to extreme sensitivity of phage φμ-4 to EDTA at a pH range suitable for optimal chelating action.

The rate of adsorption of φμ-4 in citrate broth was similar to that of the control for the initial 15 min, at which time 70 to 90% of the phage particles irreversibly adsorbed. After the first 15 min, however, the rate of adsorption in citrate broth was greatly reduced (Fig. 1). The observed effect could have two interpretations: either alteration in host cells as the result of exposure to citrate or depletion of some factor in the medium. To test the former, host cells which were in contact with phage for 15 min were challenged with a second dose of phage particles. The pattern of adsorption was similar to that of earlier observations: 83% of the initial phage dose adsorbed in citrate broth, in contrast to 89% in the control system. However, only 65% of the challenged

dose adsorbed in citrate broth in contrast to 84% of the challenged control (Fig. 2). To determine whether depletion of some factor in the medium affected adsorption, host cells were incubated in citrate broth for 45 min prior to addition of phage. It was found that cells treated with citrate for 45 min adsorbed 71% of the phage particles during the initial 15 min of incubation in contrast to 84% in systems treated with citrate for 15 min. Therefore, prolonged preincubation of cells in citrate further reduced the adsorption rate of phage $\phi\mu$-4.

Adsorption of phage in citrate broth does not necessarily indicate infection of the host cell. The number of cells which were successfully infected in citrate broth comprised about 0.5% of the infected cells observed in the control system (Table 1). The remaining 99.5% of the cells which had adsorbed phage particles were abortively infected in citrate and failed to produce phage progeny. The abortive infection of cells resulted in the loss of both the infecting phage and the infected cell. Presumably, once the adsorption process was initiated the phage particles irreversibly attached to the host cells, but in citrate the invasion of the cell was interrupted. Luria and Steiner (1954) observed a similar phenomenon in working with T_5. They noted that adsorption of phage was not followed by penetration of deoxyribonucleic acid (DNA) in the absence of divalent ions.

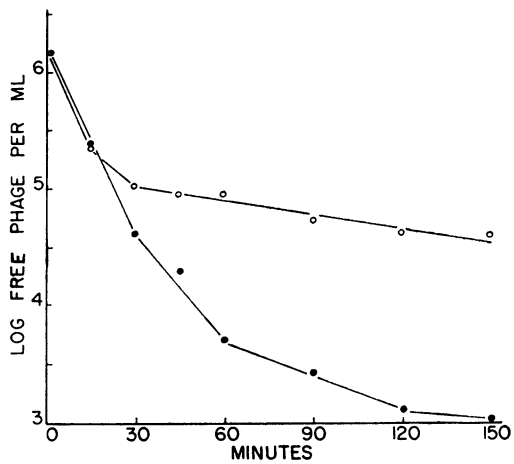


FIG. 1. *Effect of citrate on adsorption of $\phi\mu$-4. Sodium citrate-containing system* (○). *Citrate-free control* (●).

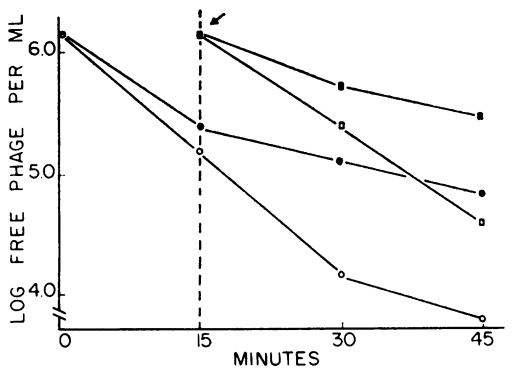


FIG. 2. *Adsorption pattern of phage $\phi\mu$-4 in citrate broth. Sodium citrate-containing systems* (●, ■). *Citrate-free controls* (○, □). *Challenge dose introduced* (↙).

TABLE 1. *Effect of citrate on infection of host cells by phage $\phi\mu$-4*

Expt	Ca^{++} broth (0.01 M $CaCl_2$)	Citrate broth	
		0.0015 M*	0.003 M
	PFIC/ml	*PFIC/ml*	*PFIC/ml*
1	7.44×10^5	—	3.80×10^3
2	7.04×10^5	1.09×10^4	1.62×10^3
3	7.89×10^5	—	4.75×10^3

* Indicates citrate concentration.

TABLE 2. *Inactivation of phage $\phi\mu$-4 in phosphate and citrate at 65 C*

Medium	Active phage/ml[a]	Final pH
Phosphate buffer[b]	2.1×10^3	7.2
Phosphate buffer + Ca^{++}[c]	1.5×10^4	7.2
Sodium citrate[d]	7.1×10^3	7.2
Sodium citrate + Ca^{++}[c]	1.6×10^5	7.3
Dilute broth	2.8×10^5	7.1
Dilute broth + Ca^{++}[c]	2.7×10^5	7.1

[a] Phage particles recovered after 15 min of incubation.
[b] Potassium salts, 0.1 M.
[c] $CaCl_2$, 0.1 M.
[d] Concentration, 0.1 M.

The concentration of citrate was important in determining the number of successfully infected cells produced. A comparison of systems containing different concentrations of citrate (Table 1) shows that a 50% decrease in the level of citrate increased the number of PFIC produced by approximately fourfold.

Addition of citrate, 1 to 5 min after the initiation of adsorption, greatly affected the number of PFIC produced. However, the inhibitory effect of citrate was much greater when introduced 15 min prior to addition of phage. Citrate also decreased the phage yield from each successfully

infected cell. In systems where citrate was added 1 min after the initiation of phage adsorption, each infected cell produced 75 phage particles. In citrate-free systems under similar conditions, 175 particles were released from each infected cell. Partial reversal of citrate effect on infection was possible by addition of calcium 1 to 6 min after adsorption initiation, being most effective when introduced immediately after phage addition. Similarly, phage yield from infected cells in citrate was increased when calcium was introduced in the early latent period. It is, therefore, concluded that divalent ions are required during early phases of penetration and synthesis of phage $\phi\mu$-4.

Normally, either adsorption of phage (Garen and Puck, 1951; Barksdale and Pappenheimer, 1954) or its penetration-synthesis (Perlman, Langlykke, and Rothberg, 1951; Kay, 1952; Potter and Nelson, 1953) requires divalent ions. In $\phi\mu$-4 proliferation, however, all phases are dependent on divalent ions. The amount of calcium or magnesium required for adsorption was rather small and probably was essential as a metal ion in the enzymatic process of adsorption (Thompson and Shafia, 1962).

Free phage particles were rapidly inactivated in phosphate or citrate solutions at 65 C. Calcium ions offered some protection to particles in citrate and to a lesser degree to particles in phosphate (Table 2). The affinity of phage sites for chelates is apparent by inactivation of phage particles in phosphate buffer at 25 C. The mechanism of phage $\phi\mu$-4 inactivation by citrate has not been studied; however, it could be similar to the mode of inactivation of T_5 in citrate, which is release of DNA from phage particles (Lark and Adams, 1953).

Divalent ions apparently play a dual role in the latent period of this phage. Aside from being required for penetration, they (calcium or magnesium) also control phage synthesis mechanisms. Since free phage is irreversibly inactivated by chelates, and divalent ions are required for its synthesis, it is speculated that calcium or magnesium ions are intimately associated with the phage surface.

ACKNOWLEDGMENTS

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LITERATURE CITED

ADAMS, M. H. 1949. The calcium requirement of coli phage T5. J. Immunol. **62**:505–516.

BARKSDALE, W. L., AND A. M. PAPPENHEIMER, JR. 1954. Phage-host relationship in nontoxigenic and toxigenic diphtheria bacilli. J. Bacteriol. **67**:220–232.

DELBRUCK, M. 1948. Biochemical mutants of bacterial viruses. J. Bacteriol. **56**:1–16.

GAREN, A., AND T. T. PUCK. 1951. The first two steps of the invasion of host cells by bacterial viruses. J. Exptl. Med. **94**:177–189.

KAY, D. 1952. The effect of divalent metals on the multiplication of coli bacteriophage T5st. Brit. J. Exptl. Pathol. **33**:228–235.

LANNI, Y. T. 1960. Invasion by bacteriophage T5. II. Dissociation of calcium-independent and calcium-dependent processes. Virology **10**:514–529.

LARK, K. G., AND M. H. ADAMS. 1953. The stability of phages as a function of the ionic environment. Cold Spring Harbor Symp. Quant. Biol. **18**:171–183.

LURIA, S. E., AND D. L. STEINER. 1954. The role of calcium in the penetration of bacteriophage T5 into its host. J. Bacteriol. **67**:635–639.

PERLMAN, D., A. F. LANGLYKKE, AND H. D. ROTHBERG. 1951. Observations on the chemical inhibition of *Streptomyces griseus* bacteriophage multiplication. J. Bacteriol. **61**:135–143.

POTTER, N. N., AND F. E. NELSON. 1953. Role of calcium and related ions in proliferation of lactic streptococcus bacteriophage. J. Bacteriol. **66**:508–516.

ROUNTREE, P. M. 1947. Staphylococcal bacteriophages. II. Bacteriophage adsorption by staphylococci. Australian J. Exptl. Biol. Med. Sci. **25**:203–212.

ROUNTREE, P. M. 1951. The role of certain electrolytes in the adsorption of staphylococcal bacteriophages. J. Gen. Microbiol. **5**:673–680.

SHAFIA, F., AND T. L. THOMPSON. 1964. Isolation and preliminary characterization of bacteriophage $\Phi\mu$-4. J. Bacteriol. **87**:999–1002.

THOMPSON, T. L., AND F. SHAFIA. 1962. Energy requirement for adsorption of bacteriophage $\Phi\mu$-4. Biochem. Biophys. Res. Commun. **8**:467–470.