Arch. Microbiol. 124, 249–254 (1980)

Archives of
Microbiology

Requirement for Calcium Ions in Mycobacteriophage I3 DNA Injection and Propagation

V. Nagaraja and K. P. Gopinathan*

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore – 560012, India

Abstract. Ca^{2+} ions are absolutely necessary for the propagation of mycobacteriophage I3 in synthetic medium. These ions are required for successful infection of the host and during the entire span of the intracellular development of the phage. A direct assay of the phage DNA injection using ^{32}[P] labelled phage, shows that Ca^{2+} ions are necessary for the injection process. The injection itself is a slow process and takes 15 min to complete at 37° C. The bacteria infected in presence of Ca^{2+} tend to abort if the ions are subsequently withdrawn from the growth medium. The effect of calcium withdrawal is maximally felt during the early part of the latent period; however, later supplementation of Ca^{2+} ions salvage phage production and the mature phage progeny appear after a delayed interval, proportional to the time of addition of Ca^{2+}.

Key words: Calcium ions – Phage propagation – Phage DNA injection – Mycobacteriophage I3.

Extensive investigations have been carried out on the nutritional requirements for the propagation of bacteriophages (Adams, 1959). Many phages required divalent cations, mainly Ca^{2+} or Mg^{2+} for adsorption to host cell surface. There are also occasional instances of organic cofactor requirements, like tryptophan for phage T4 (Delbrük, 1948). Tryptophan alters the structure of phage T4 tail fibres in such a way as to promote the attachment to the host cell surface (Kellenberger et al., 1965). While the requirement for Ca^{2+} in successful phage infection has been known for a long time, the exact nature of its involvement has not been elucidated. However, the available evidences indicate that it is involved in the penetration of the phage into the host (Luria and Steiner, 1954; Potter and Nelson, 1953), or some time during the late stages of phage development (Rountree, 1955).

In our attempts to propagate the mycobacteriophage I3, we have found that the phage did not multiply in synthetic minimal medium although reasonably high titers were obtained in nutrient broth or nutrient agar plates. Recently, however, we have developed a minimal medium for successful phage growth. Ca^{2+} ion was found to be absolutely necessary for the propagation of phage I3 in this medium as well as other minimal media in which we could not grow the phage earlier. We have investigated the steps of the phage I3 growth which require this cation. The results presented in this communication show that Ca^{2+} is required for phage DNA injection into the host as well as for the maturation of the phage.

Materials and Methods

A clear plaque mutant (C5) of the mycobacteriophage I3 (Gopinathan et al., 1978) was used in these investigations. The host strain used was *Mycobacterium smegmatis* SN2.

Bacto-agar, yeast extract and Bacto-tryptone were from Difco Laboratories, Detroit, Michigan, USA. Peptone and meat extract were from Centron Research Laboratories, Bombay, India. Tween-80 (polyoxyethylene sorbitan monooleate), ethylene-glycol-bis-tetraacetic acid (EGTA), cesium chloride, Tris, and DNase I were from Sigma Chemical Co., St. Louis, Missouri, USA. ^{32}[P]-orthophosphate (carrier free) was obtained from Bhabha Atomic Research Centre, Bombay, India.

Bacteria were routinely grown in the synthetic medium of Youmans and Karlson (1947). The medium contained per litre, 5 g asparagine, 5.9 g potassium dihydrogen phosphate, 0.5 g potassium sulfate, 0.6 g magnesium carbonate, 1.5 g citric acid and 20 ml glycerol; 0.3% (v/v) Tween-80 was also included to give dispersed growth.

Abbreviations: moi = multiplicity of infection; PFU = plaque forming units; EGTA = ethylene-glycol-bis (β-aminoethyl ether) N,N'-tetraacetic acid

* To whom offprint requests should be sent

0302-8933/80/0124/0249/$01.20

Preparation of ^{32}[P]-Labelled Phage and DNA

1 μCi/ml of ^{32}[P]-orthophosphate was added to synthetic medium from which KH_2PO_4 was omitted. Bacteria were grown to early exponential phase (14 h, 5×10^7 cells/ml) and then phage was added at a multiplicity of infection (moi) of 5, along with $CaCl_2$ to a final concentration of 1 mM. After a further incubation of 12 h at 37° C a few drops of $CHCl_3$ were added and the lysates were prepared by centrifugation at 7,000 rev/min for 20 min in a Sorvall GSA 3 rotor. The phage was purified by several steps of low and high speed centrifugations, DNase digestion and banding on a cesium chloride density step-gradient. The phage band was collected and used directly for DNA injection experiments. Dialysis resulted in significant loss of viability of the phage.

^{32}[P]-Phage DNA Injection Assay

The assay was carried out by the method of Newbold and Sinsheimer (1970) as adapted by Gadagkar and Gopinathan (1978). After adsorption with ^{32}[P] labelled phage, the infected cells (containing the injected and uninjected phages) as well as the uninfected cells were collected and washed several times with sodium tetraborate (pH 9.1) containing 0.3% (v/v) Tween-80. After 4 to 5 washings, the subsequent washings did not remove any radioactivity. The cells were then collected on millipore filter and the radioactivity was determined in a Beckman LS-100 liquid scintillation spectrometer.

Results

The yield of phage particles in the synthetic medium is given in Table 1. While the growth was extremely poor in the unsupplemented synthetic medium (Burst size < 1), high titers were obtained when calcium salts were added. The addition of $MgCl_2$ improved the phage yield over the control, but was not as effective as Ca^{2+}. With K^+ or NH_4^+ supplementation, the yields were lower than input phages.

The effect of varying concentration of Ca^{2+} on phage production is given in Fig. 1. The minimal optimum concentration of Ca^{2+} was 100 μg/ml (0.68 mM) and the phage yields were in the same range up to 400 μg/ml $CaCl_2$.

Since the synthetic medium employed contains citrate (used as a growth factor in addition to the carbon source, glycerol) which is a known chelator for Ca^{2+} and Mg^{2+}, the growth and phage yield were examined in the same medium devoid of citrate, and containing Mg^{2+}. Even in this medium, Ca^{2+} supplementation (0.68 mM) enhanced phage production significantly (phage titer, in absence of Ca^{2+}, 1.5×10^8/ml and in presence of Ca^{2+}, 5×10^9/ml).

The effect of Ca^{2+} on phage production at varying moi was studied and the results are presented in Table 2. The number of infected bacteria as well as the total phage yield were much higher in presence of Ca^{2+}, at every moi. At very low moi, even in presence of Ca^{2+}, the phage production (output/input) was lower than 1; the low burst sizes may be due to reinfection of the bacterial population. At any time, the number of bacteria infected in the absence of Ca^{2+} was only 10–50% of the number in presence of Ca^{2+}. The differences were however, most pronounced at low moi. The narrowing differences at higher moi is presumably due to the Ca^{2+} carried by the inoculating phage. For instance, at moi 10, there was 10 μg/ml (0.068 mM) final concentration of Ca^{2+} carried over by the infecting phage, and this corresponds to nearly 10–20% of the optimum Ca^{2+} requirements (cf: Fig. 1).

Table 1. Effect of salts on phage production

Supplement	Concentration (mM)	Titer (PFU/ml)
1. Control (none)	–	1.5×10^7
2. $CaCl_2$	0.68	6×10^9
3. $CaSO_4$	0.58	3×10^9
4. $Ca(NO_3)_2$	0.42	5×10^9
5. $MgCl_2$	0.50	5×10^8
6. KCl	1.34	7×10^6
7. NH_4Cl	1.87	4×10^6

Various salts were added to the synthetic minimal medium at 100 μg/ml concentration and early exponential phase cells were infected with phage at a moi = 1. After 12 h, the lysates were titrated on the indicator bacterium, *Mycobacterium smegmatis* SN 2

In order to see whether Ca^{2+} was needed during adsorption or propagation of the phage, an experiment was designed to specifically remove Ca^{2+} at each of these stages by using EGTA. The results are presented in Table 3.

The number of infected bacteria were reduced by 40% when adsorption was carried out in the absence of Ca^{2+} and in the presence of EGTA. The effect was most drastic if Ca^{2+} was absent during both adsorption and propagation. If Ca^{2+} was removed during propagation, after carrying out the adsorption in presence of Ca^{2+} (line 2 in Table 3), there was a substantial decrease in the net phage yield as well as the burst size. When the infected bacteria formed in the absence of Ca^{2+} were suspended in Ca^{2+} containing medium and propagated, there was greater reduction in the yield and burst sizes.

In this experiment, the number of infected bacteria recorded may be an overestimate because the technique does not differentiate between a host cell to which the phage is attached (but DNA is not injected) and a cell into which DNA injection has taken place. Most, if not all of the bacteria infected in the absence of Ca^{2+} would belong to the former group, as Ca^{2+} is necessary for the injection of phage DNA to the host (see the later section). However, both of them would behave like true infective centers on plating out on the assay plates, since they contain Ca^{2+}. In the liquid medium used for

Table 2. Effect of varying moi on phage infection in presence and absence of Ca^{2+}

Moi	Absence of Ca^{2+}			Presence of Ca^{2+}		
	Infected bacteria/ml	Phage titer	Burst size	Infected bacteria/ml	Phage titer	Burst size
0.1	7.5×10^5	2.5×10^5	0.33	8.4×10^6	7.0×10^6	0.83
0.2	2.0×10^6	5.0×10^5	0.25	1.2×10^7	2.0×10^7	1.67
0.5	4.0×10^6	3.0×10^6	0.75	1.9×10^7	8.0×10^8	42
1.0	7.5×10^6	1.6×10^7	2.1	2.2×10^7	2.0×10^9	91
2.0	1.8×10^7	5.5×10^7	3.0	4.3×10^7	5.2×10^9	121
5.0	5.0×10^7	7.5×10^8	15	1.0×10^8	5.4×10^9	54
10.0	7.0×10^7	1.2×10^9	17	1.3×10^8	5.0×10^9	38

Early exponential phase cells of *M. smegmatis* were infected with phage I3 at various moi (0.1 to 10) in presence or absence of 1 mM $CaCl_2$. After adsorption for 20 min, at 37° C, the cells were collected by centrifugation. The unadsorbed phages and infected bacteria were determined by titrating against indicator bacteria. The infected bacteria were resuspended in fresh medium with or without calcium and incubated for 5 h at 37° C (one cycle of growth). The progeny phage titers were also determined (PFU/ml)

Table 3. Effect of Ca^{2+} during different stages of phage propagation

Presence of Ca^{2+} during		Infected bacteria	Progeny titer	Burst size	Unadsorbed phages
Adsorption	Propagation				
+	+	2.1×10^7	1.0×10^9	48	4.8×10^7
+	−	2.4×10^7	4.6×10^8	20	4.8×10^7
−	+	1.4×10^7	5.8×10^7	4	5.3×10^7
−	−	1.3×10^7	1.9×10^6	<1	5.6×10^7

M. smegmatis cells growing at early exponential phase were infected with I3 at an moi = 1 in presence or absence of 1 mM $CaCl_2$. To ensure complete removal of calcium, EGTA was added in excess (final concentration 1 mM). After allowing for adsorption for 20 min at 37° C, the cells were collected by centrifugation (4K, 10 min) and resuspended in medium, containing 1 mM $CaCl_2$ or 1 mM EGTA. After 5 h incubation at 37° C, the lysates were prepared and titrations were carried out as before

Table 4. Requirements for phage DNA injection

No. Treatment	CPM of ^{32}[P] DNA injected
1. None (control)	188
2. 2 mM Ca^{2+}, 37° C	1,865
3. 2 mM Ca^{2+}, 20° C	850
4. 2 mM Ca^{2+}, 0° C	506
5. Tween-80 (0.3% v/v)	195
6. Tween-80 (0.3% v/v) + 2 mM Ca^{2+}	946

^{32}[P]labelled phage was added to 1 ml suspension of cells (10^8 cells/ml synthetic medium) in presence of the various components and incubated at the indicated temperatures. After 30 min incubation, the cells were collected by centrifugation at 4,000 rev/min for 10 min and processed as described under "Materials and Methods"

propagation, on the other hand, it is likely that some of these infective centers belonging to the former category would have aborted in course of time, and hence the apparent reduction in burst size. This in turn, may imply that Ca^{2+} did not have any effect on propagation of phage but was only reflected as a consequence of its effect on DNA injection. In other words, once DNA injection was complete, Ca^{2+} probably did not have any effect; this aspect was therefore, studied in greater detail.

To determine the point at which Ca^{2+} was acting at the early stages of phage infection, phage DNA injection was assayed in the presence and absence of calcium. The results are presented in Table 4.

DNA injection was temperature dependent and required Ca^{2+}. Maximal DNA injection was observed at 37° C in presence of 1−2 mM Ca^{2+}. Tween-80 completely inhibited DNA injection and this inhibition was relieved to a great extent by Ca^{2+} ions.

The kinetics of DNA injection by ^{32}[P] labelled phages are presented in Fig. 2. The injection process was completed in 15 min at 37° C, in presence of Ca^{2+}. Addition of chloramphenicol (100 μg/ml) during DNA injection or pretreatment of cells with the antibiotic did not influence the extent of DNA injection. Hence, the injection process was not dependent on fresh protein synthesis.

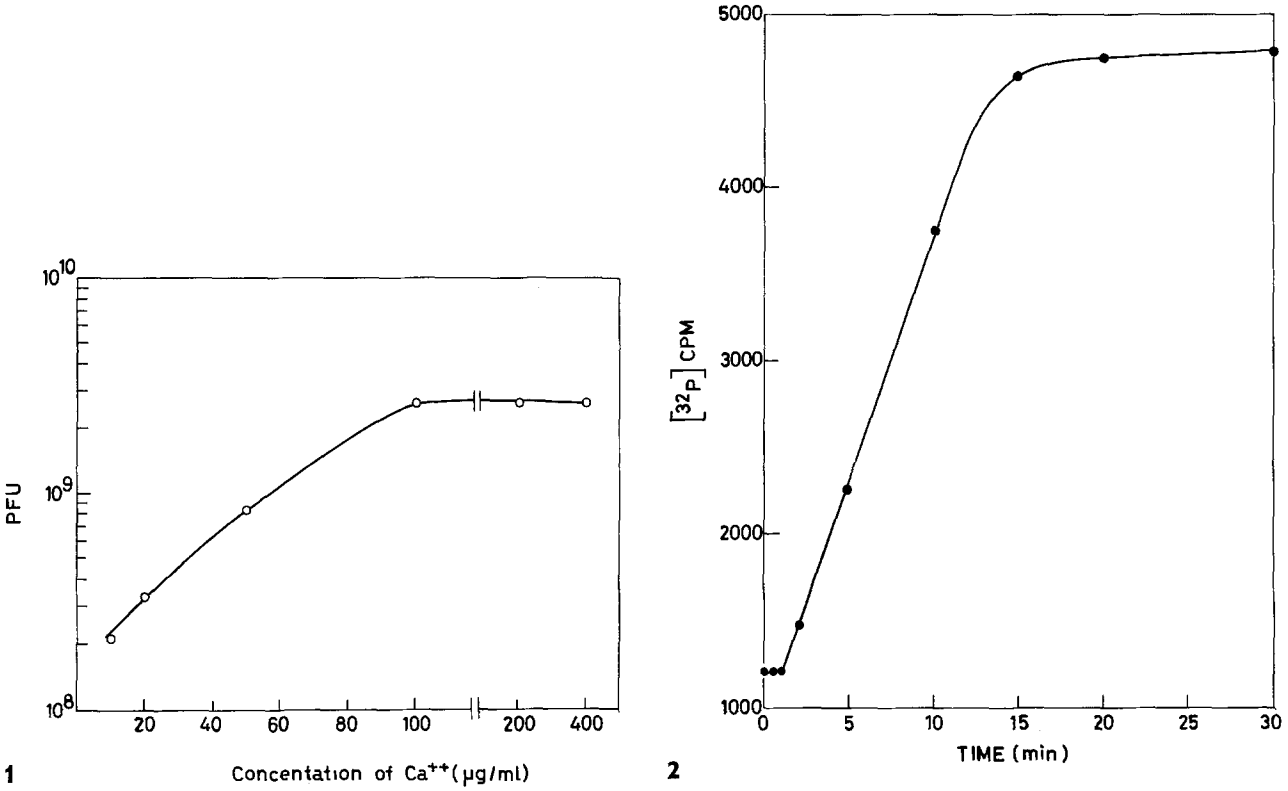


Fig. 1. Effect of calcium ions in phage production. Various amounts of $CaCl_2$ were added to the synthetic growth medium (cell population 5×10^7/ml) at the time of phage infection (moi = 1). The cultures were incubated at 37° C with aeration for 12 h. The lysates were clarified by centrifugation at $8{,}000 \times g$ for 10 min, and titrated on indicator bacteria, *Mycobacterium smegmatis* SN 2. ($CaCl_2$, 100 µg/ml = 0.68 mM)

Fig. 2. Kinetics of phage I3 DNA injection. ^{32}[P]labelled phage was added to 1 ml cell suspension (10^8 cells/ml) in presence of 1 mM $CaCl_2$, and incubated at 37° C. At various times, the injection process was stopped by adding ice cold borate buffer containing 0.5 % Tween-80 and 0.5 mM EGTA

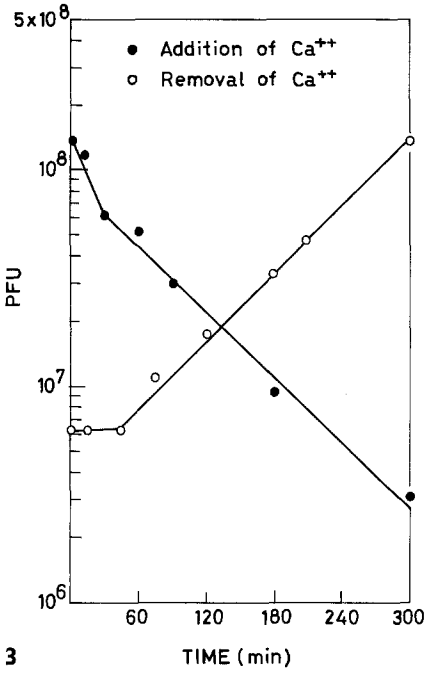
Role of Ca^{2+} in Phage Propagation

To evaluate the role of Ca^{2+} in phage I3 propagation, 2 types of experiments were carried out and the results are presented in Fig. 3.

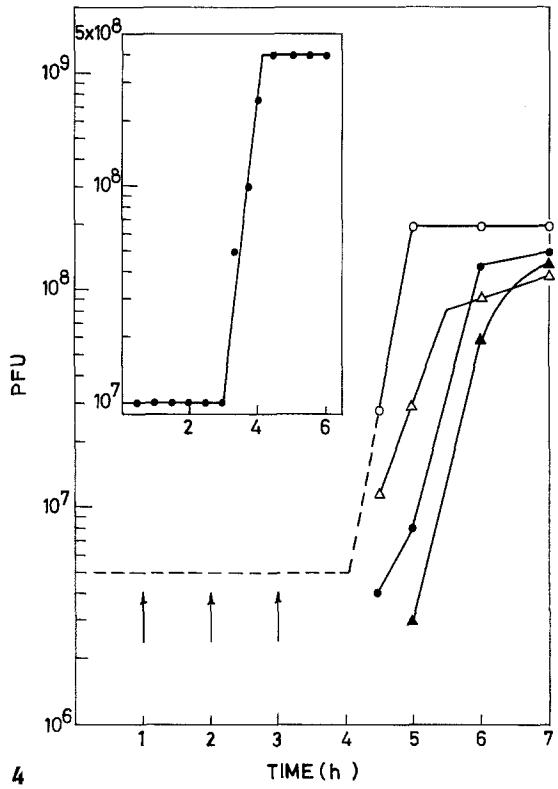
Bacteria were infected in presence of Ca^{2+}. In one set of experiments, they were then suspended in Ca^{2+} free medium and Ca^{2+} was added at various times as shown. Alternatively, the infected bacteria were suspended in a medium containing Ca^{2+}, and excess EGTA was added at noted times to remove Ca^{2+} from the system. These cells were incubated for a total time of 5 h from the time of suspending infected bacteria to complete the life cycle. The results from both these sets of experiments were nutually complementary. The samples which contained Ca^{2+} from zero time gave the highest yields, with a gradual reduction in titers as the addition of the ion was delayed. The effect was most pronounced when Ca^{2+} was withheld in the initial periods, but the continued presence of Ca^{2+} appeared to be necessary throughout the latent period, for the best phage yields. Even when EGTA was added to remove Ca^{2+} at 210 min (after the completion of latent period and beginning of rise period), the phage production was diminished. EGTA itself did not have any effect on mature phages. The above results suggested that Ca^{2+} may have some effect even on a late process.

On the other hand, if the phages needed 5 h to complete the life cycle from the time of Ca^{2+} addition (provided this ion is required at the very early stages of phage development after DNA injection), the observed reduction in titers may be only superficial, since they were not able to complete the life cycle. This explanation may be valid because the total time of incubation was only 5 h in the above experiment. The situation was verified by the experiment presented in Fig. 4. The one-step growth curve of the phage 13 is also provided within this figure, for ready comparison.

The latent period and rise period were 180–200 min and 45–60 min for the C mutant of phage I3, in the synthetic medium, containing Ca^{2+} (0.68 mM). The average burst size was 40. It is evident that the delaying in the addition of Ca^{2+} delays the onset of rise period further, and hence the decrease in titers at 4.5–



3



4

Fig. 3. Requirement of Ca^{2+} in phage production. *M. smegmatis* cells in the early exponential phase were infected with phage I3 at an moi = 1, in presence of 1 mM $CaCl_2$. The mixture was incubated at 37° C for 20 min to complete phage DNA injection. In one set of experiments, the cells were centrifuged and suspended in fresh medium containing $CaCl_2$ (1 mM). At various times, EGTA was added to a final concentration of 2 mM to chelate the Ca^{2+} and the incubation continued for a total length of 5 h. A few drops of $CHCl_3$ were added to complete the lysis and the clarified lysates were titrated on indicator bacteria. In the other experiments, Ca^{2+} was removed from the infected cells by incubating for 5 min with 2 mM EGTA, centrifuging and resuspending in medium lacking Ca^{2+}. At various times, $CaCl_2$ was added back to a final concentration 1 mM. Incubation was continued for a total of 5 h at 37° C and the phage titers were determined as in the previous case. o——o Time of addition of EGTA; ●——● time of addition of Ca^{2+}

Fig. 4. Continuous requirement of Ca^{2+} during intracellular development of phage. Phage infection was carried out in presence of Ca^{2+} (1 mM) at 37° C for 20 min and EGTA was added (2 mM final concentration). After 5 min at 37° C the infected bacteria were collected by centrifugation, suspended in Ca^{2+} free medium and incubated at 37° C. At various times, $CaCl_2$ (1 mM) was added to different samples. Samples were removed periodically after 4.5 h and the phage titers were determined. Time of addition of Ca^{2+}: o——o, 0 min; △——△, 60 min; ●——●, 120 min; ▲——▲, 180 min. The inset curve is the one step growth curve of the phage in the same medium, containing Ca^{2+}

5 h. If sufficient time was allowed for completion of lysis, the observed reduction in titers disappeared and eventually, the same final titers were attained. In this set of experiments, however, there was increase in the total duration of latent period even in the case of control (Ca^{2+} added at zero time); this is probably a consequence of the treatment of the infected cells with EGTA.

Discussion

The results presented show that Ca^{2+} ions are essential for successful propagation of the mycobacteriophage I3 in synthetic medium. We were interested in developing a minimal medium for radioactive labelling of phage to be used for characterization of various structural components, but most of our initial attempts to grow the phage had failed. Subsequently we found that the medium of Youmans and Karlson (1947) which supports the growth of the host *Mycobacterium smegmatis* can also be used for the propagation of phage I3, if supplemented with Ca^{2+}. The host itself does not show any specific requirement for this ion.

Sunder Raj and Ramakrishnan (1970) have reported earlier that phage I3 does not require Ca^{2+} or Mg^{2+} for growth. The conclusions were, however, based on their studies employing nutrient broth or Luria broth for phage propagation and now we have evidence that they contain sufficient quantities of Ca^{2+} carried by the other constituents (data not presented).

Requirements for divalent cations for propagation of many phages have been known for a long time

(Adams, 1959) and in most instances they are confined to the early part of the phage infection process, presumably the injection. In our studies we have separately tested the involvement of Ca^{2+} at all stages of the phage propagation.

In *M. smegmatis*-I3 system, DNA injection is a slow process and is dependent on temperature and presence of Ca^{2+} ions. A slow injection process observed in the case of phage T5 has been attributed to the requirement for fresh protein synthesis to complete the injection process (Lanni, 1965, 1968). Such a requirement is not seen in the case of phage I3 DNA injection, since it is independent of the presence of chloramphenicol. Tween-80, the surface active detergent used routinely for the dispersed growth of Mycobacterium interferes with phage propagation (Gadagkar and Gopinathan, 1978). It inhibits DNA injection and this inhibition is reversed by the presence of Ca^{2+} ions. Furthermore, Ca^{2+} also stimulates the uptake of isolated phage I3 DNA (unpublished observations). Hence we conclude that the role of these ions is to modify cell surface and thereby facilitate DNA injection, apart from any direct action on phage particles.

It is evident that Ca^{2+} is needed both for the injection of phage DNA into the host, and during the entire latent period. A delay in the addition of Ca^{2+} delays the onset of the appearance of mature phage particles. The Ca^{2+} depletion effect was most pronounced when the ion was withdrawn in the early part of the latent period. Hence, Ca^{2+} is obviously involved in some biochemical processes essential for the development of phage. The nature of Ca^{2+} requirement in several biochemical reactions is being established in various laboratories, and processes such as protein phosphorylation and gene activation in eukaryotic cells are coming into prominence as the targets of Ca^{2+} action (Greengard, 1978). Presently, we are investigating the biochemical nature of Ca^{2+} involvement in phage I3 development.

References

Adams, M. H.: Bacteriophages. New York: Interscience 1959

Delbrük, M.: Biochemical mutants of bacterial viruses. J. Bacteriol. **56**, 1–13 (1948)

Gadagkar, R. R., Gopinathan, K. P.: Inhibition of DNA injection from mycobacteriophage I3 by Tween-80. Virology **91**, 487–489 (1978)

Gopinathan, K. P., Saroja, D., Gadagkar, R. R., Ramakrishnan, T.: Control of lysogeny in mycobacteria. In: Genetics of actinomycetales (E. Freerksen, I. Tarnok, J. H. Thumim, eds.), pp. 237–242. Stuttgart-New York: G. Fischer 1978

Greengard, P.: Phosphorylated proteins as physiological effectors. Science **199**, 146–152 (1978)

Kellenberger, E., Bolle, A., Boy De La Tour, E., Epstein, R. H., Franklin, N. C., Jerne, N. K., Reale-Scafati, A., Sechaud, J., Bendet, I., Goldstein, D., Lauffer, M. A.: Functions and properties related to the tail fibres of bacteriophage T4. Virology **26**, 419–440 (1965)

Lanni, Y. T.: DNA transfer from bacteriophage T5 to host cells: Dependance on intercurrent protein synthesis. Proc. Nat. Acad. Sci. USA **53**, 969–973 (1965)

Lanni, Y. T.: First-step-transfer deoxyribonucleic acid of bacteriophage T5. Bacteriol. Rev. **32**, 227–242 (1968)

Luria, S. E., Steiner, D. L.: Role of calcium in the penetration of bacteriophage T5 into its host. J. Bacteriol. **67**, 635–639 (1954)

Newbold, J. E., Sinsheimer, R. L.: The process of infection with bacteriophage ∅ ×174. XXXII: Early steps in the infection process: Attachment, eclipse and DNA penetration. J. Mol. Biol. **49**, 49–66 (1970)

Potter, N. N., Nelson, F. E.: Role of Ca^{2+} and related ions in proliferation of lactic Streptococcus bacteriophage. J. Bacteriol. **66**, 508–516 (1953)

Rountree, P. M.: The role of divalent cations in the multiplication of Staphylococcal bacteriophages. J. Gen. Microbiol. **12**, 275–287 (1955)

Sunder Raj, C. V., Ramarkrishnan, T.: Transduction in *Mycobacterium smegmatis*. Nature (Lond.) **228**, 280–281 (1970)

Youmans, G. P., Karlson, A. G.: Streptomycin sensitivity of tubercle bacilli: Studies on recently isolated tubercle bacilli and development of resistance to streptomycin in vivo. Amer. Rev. Tuberc. Pul. Dis. **55**, 529–534 (1947)

Received April 10, 1979