

Effect of Calcium Ions on the Infection of *Bacillus subtilis* by Bacteriophage SF 6

By H. Y. STEENSMA AND J. BLOK*

Laboratory of Microbiology, Delft University of Technology, Delft, The Netherlands

(Accepted 23 August 1978)

SUMMARY

Infection of *Bacillus subtilis* 168Wt by SF 6 resulted in a rapid reduction in the number of phages. This could be counteracted by the addition of calcium, barium or strontium ions. At the optimum concentration of 7.5×10^{-2} M, the number of p.f.u. remained constant until lysis began. Although cultures of another host, *B. subtilis* 31 try⁻ his⁻, at the end of the logarithmic growth phase produced a substance which inactivated free phages, this was not the major cause of the reduction in the numbers of p.f.u. during infection experiments at low Ca²⁺ concentrations. The diminution of the number of p.f.u. was therefore attributed to the fact that at least one of the steps of the lytic cycle was calcium dependent. Adsorption of SF 6 was equally effective in media containing high or low concentrations of calcium ions. Infection experiments with phages whose DNA had been labelled radioactively revealed that, at high concentrations of calcium ions, the label remained associated with the host cells until lysis commenced. At low concentrations, however, a dissociation between phage DNA and the host was found, although adsorption took place at a normal rate. From these experiments we concluded that a high concentration of calcium ions was required for the penetration of phage DNA. Similar experiments with phages whose protein coat had been labelled showed the same results, indicating that desorption of the inactivated phages occurred. Both electron microscopy and column chromatography with hydroxyapatite showed that a considerable fraction of the inactivated phages had ejected their DNA into the medium. A hypothesis explaining these results is presented.

INTRODUCTION

SF 6, a *Bacillus subtilis* phage isolated from garden soil (Steensma *et al.* 1974), was killed by its own host, as indicated by the decrease in p.f.u. during one step growth experiments. Initial tests showed that high concentrations of CaCl₂ counteracted this effect. Many other bacteriophages are also known to require divalent cations for productive infection (Adams, 1959). Optimum concentrations ranged from 3×10^{-4} M to 1.8×10^{-2} M and exceeded those necessary for growth of the hosts (Brodetsky & Romig, 1965; Snipes *et al.* 1974). Divalent ions have been reported to play a role in each step of the lytic cycle, i.e. phage adsorption (Garen & Puck, 1951; Reese *et al.* 1974), penetration of nucleic acid (Paranchych, 1966; Watanabe & Takesue, 1972) and intracellular phage development (Snipes *et al.* 1974). Phages with a non-contractile tail often require divalent cations for the penetration of

* Present address: Laboratory for Electron Microscopy, University of Leiden, Leiden, The Netherlands.

nucleic acid, although little is known about this mechanism. We have investigated the effect of calcium ions on the stability of SF 6 and on the various steps leading to phage production, in order to localize the calcium dependent step or steps.

METHODS

Organisms. Phage SF 6 was isolated from garden soil (Steensma *et al.* 1974). The bacterial strains most frequently used in this study are listed in Table 1. All strains, except *B. subtilis* 168/SF 6, were obtained from the bacterial collection of the Delft Laboratory of Microbiology. *B. subtilis* 168/SF 6 was isolated by plating spores of *B. subtilis* 168Wt on D.A.L. plates with an excess of SF 6 in the top layer. Single colonies were purified by repeatedly plating on peptone agar and tested for their resistance to SF 6 infection.

Media. Peptone water (PW) contained 10 g peptone and 5 g NaCl per litre of tap water, pH 7.3. Peptone agar (PA) and soft peptone agar (SPA) consisted of PW, solidified with 2% and 1% agar (Difco) respectively. CaCl₂ and other salts were added after sterilization. RS contained 1 g (NH₄)₂SO₄, 0.175 g K₂HPO₄, 0.02 g MnCl₂.4H₂O, 0.1 g MgSO₄.7H₂O, 0.5 g tri-sodium citrate.2H₂O and 3 g tris per litre of demineralized water, pH 7.2. After sterilization, 0.5% separately sterilized glucose was added. RSY was the same as RS except that after sterilization, 0.1% yeast extract (Difco), 20 µg/ml L-histidine and 20 µg/ml L-methionine had been added. RSC contained 0.8 g NH₄Cl, 0.175 g K₂HPO₄, 0.02 g MnCl₂.4H₂O, 0.16 g MgCl₂.6H₂O, 0.5 g tri-sodium citrate.2H₂O and 3 g tris per litre of demineralized water, pH 7.2. After sterilization 0.5% of separately sterilized glucose was added. Tris buffer consisted of 1.2 g tris, 0.5 g MgCl₂ and 22 g KCl per litre of demineralized water, pH 7.5.

Preparation and purification of SF 6 suspensions. *B. subtilis* 168Wt was grown in 1 l of PW at 37 °C to a cell concentration of 10⁹/ml, or until the absorbance had risen by 250 Klett units, usually after 5 to 6 h. The cells were centrifuged and resuspended in the same volume of pre-warmed PW to which CaCl₂ (7.5 × 10⁻² M) and a drop of PPG (polypropylene glycol 2000, Baker) were added. After 45 min, SF 6 was added to give a m.o.i. of 1 to 5. Lysis generally occurred after 90 min. Cells and cell debris were then removed by centrifugation. The phages were concentrated by precipitation with 10% PEG (polyethylene glycol 6000, Baker; Yamamoto *et al.* 1970), followed by CsCl centrifugation. The collected phage fractions were dialysed against tris buffer. Purified phage suspensions contained approx. 10¹³ p.f.u./ml.

When the DNA of the phages was to be labelled, *B. subtilis* AHM was used as the host. The medium employed was RSY supplemented with 10 µg/ml adenine for cell growth and 20 µCi/ml 8-³H-adenine (22 Ci/mmol, Radiochemical Centre, Amersham) during infection with SF 6. Cell and cell debris were removed from the lysate by centrifugation followed by incubation with 50 µg/ml DNase and RNase (Nutritional Biochemicals Corporation) for 30 min at 37 °C. After dialysis against tris buffer, further purification was performed by column chromatography, using hydroxyapatite (Bio-Gel HTP, Bio Rad Laboratories) and Sepharose 4B (Pharmacia).

Phage proteins were labelled by growing *B. subtilis* 168Wt in RSC medium supplemented with 40 µCi/ml ³⁵SO₄²⁻ (Radiochemical Centre) until the absorbance had risen by 120 Klett units. CaCl₂ was added to a concentration of 7.5 × 10⁻² M, and then SF 6 at a m.o.i. of 1 to 5. Purification of the centrifuged lysate consisted of dialysis against tris buffer and column chromatography using Sepharose 4B.

Phage assay. The optimum conditions for the plating of SF 6 have been described elsewhere (Steensma, 1977). To 2 ml SPA (45 °C), supplemented with 10⁻² M-CaCl₂, were added

0.25 ml of a suspension of spores of *B. subtilis* 168Wt, containing at least 10^{10} spores/ml, and 0.25 ml of an appropriate phage dilution. This mixture was poured on to a warm PA plate, which had previously been dried for 45 min at 65 °C. The plates were incubated at 37 °C for 6 to 24 h.

Radioactivity measurements. Samples of 20 to 1000 μ l in polyethylene ^3H or glass (^{35}S) counting bottles were mixed with 12 ml of a Triton-toluene scintillation fluid, which was prepared by dissolving 5 g PPO (2,5-diphenyloxazole, Merck) and 50 mg DMP [2,2'-*p*-phenylen-bis-(4-methyl-5-phenyloxazole), Merck] in 1 l toluene, to which 0.5 l Triton X-100 (BDH Chemicals Ltd) was added. The samples were counted in a Nuclear Chicago Mark II Scintillation Counter. Corrections for quenching were made by means of the channels ratio method.

Infection experiments. Host cells, grown in the appropriate medium to the desired Klett density, were centrifuged and resuspended in pre-warmed fresh medium. Incubation was continued for 30 to 45 min before SF 6 was added at a m.o.i. of 0.01 to 0.1. The number of free phages was determined by centrifuging 0.5 to 1 ml samples for 30 to 60 s in a Jobling Microcentrifuge and plating of the supernatant.

Inactivation of free phages. Cells of logarithmic growing cultures were removed from the culture fluid by centrifugation or membrane filtration. Phages were added to the supernatant and incubated at 37 °C. Samples were taken at 0, 15 and 30 min and assayed for phages.

Electron microscopy. Preparations for the electron microscope were made by placing a drop of phage suspension on 200 or 400 mesh copper grids coated with Formvar. After 30 s the grids were rinsed with distilled water and negatively stained with a 2% uranyl oxalate solution (Mellema *et al.* 1967). The preparations were examined in a Philips EM 201.

The determination of the e.o.p. of SF 6, using latex spheres as a reference, has been described elsewhere (Steensma, 1977).

DNA preparation. SF 6 DNA was isolated and purified as described for PBS X by Steensma & Sondermeijer (1977).

Transfection. Cells of the recipient, *B. subtilis* 31 *try*⁻ *his*⁻, were made competent according to the method of Spizizen (1959) and incubated with 200 $\mu\text{g}/\text{ml}$ of SF 6 DNA for 50 min.

Spot tests. A 0.05 ml sample of a purified SF 6 suspension, containing 10^{11} p.f.u./ml, was spotted on to a D.A.L. plate with the test organism in the top layer. Clear spots in the bacterial lawn after incubation were considered positive.

RESULTS

Characterization of SF 6

SF 6 is a virulent phage with an icosahedral head, 53 nm in diam., and a flexible, non-contractile tail, 165 nm long and 10 nm wide. The tail terminates in a small base plate and occasionally a tail fibre, approx. 35 nm long and 4 nm wide, has been observed. In Fig. 1 examples of these structures, resembling those of *Escherichia coli* phage λ , are shown.

SF 6 has a buoyant density in CsCl of 1.565 g/ml. The nucleic acid of the phage consists of double-stranded DNA with a mol.wt. of 1.5×10^7 and a GC content of 43%. The latter value has been derived from both the melting point and buoyant density and was the same as that for the DNA of *B. subtilis* 168Wt, the most frequently used host, and of *Bacillus funicularius*. SF 6 formed clear plaques on all the strains listed in Table 1, except *B. subtilis* 168/SF 6. Neither plaques nor positive spot tests were obtained on *B. subtilis* W23 str^r and 23 other *Bacillus* strains belonging to the species *B. lentus*, *B. pulvifaciens*, *B. aminovorans* and the majority of the 22 species of group I, quoted in Bergey's Manual of Determinative

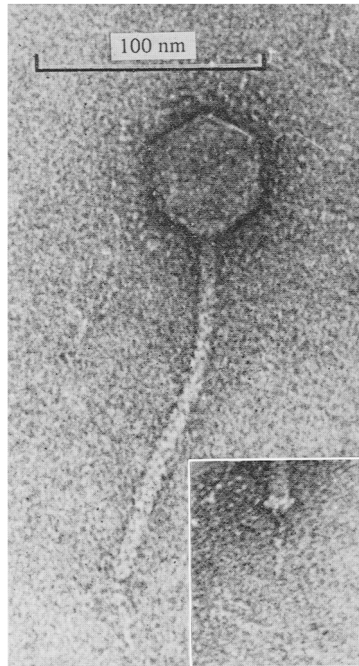


Fig. 1. Bacteriophage SF 6 stained with 2 % uranyl oxalate.

Table 1. *Bacterial strains*

Strain	Source	Remarks
<i>Bacillus subtilis</i> 168Wt	LMD 69.3	
<i>B. subtilis</i> 31 try ⁻ his ⁻	LMD 69.5	Tryptophane and histidine deficient mutant of 168Wt
<i>B. subtilis</i> AHM	LMD 69.6	Adenine, histidine and methionine deficient mutant of 168Wt
<i>B. subtilis</i> Marburg	LMD 70.64	
<i>B. subtilis</i> var. <i>aterrimus</i>	LMD 48.28	
<i>B. palustris</i>	LMD 38.4	
<i>B. funicularius</i>	LMD 25.3	
<i>B. subtilis</i> 168/SF6	New isolate	SF 6 resistant mutant of 168Wt

Bacteriology (Gibson & Gordon, 1974). Under optimum conditions, i.e. in PW containing 7.5×10^{-2} M-CaCl₂ at 37 °C, we have found an average burst size of 230 and a latent period of 30 min.

Stability of SF 6

When SF 6 was added to *B. subtilis* 168Wt or another host (Table 1), the number of p.f.u. decreased rapidly. In PW cultures of *B. subtilis* 168Wt, 98 % of the initial number of p.f.u. was usually lost in 10 min. In RS this loss amounted to 80 %. Occasionally a small burst, 40 to 50 min after infection, could be detected; an example is presented in Fig. 2. This effect was not due to the instability of the free phages. Control experiments showed that SF 6 alone was completely stable in both media during periods exceeding the duration of the infection experiments described and at higher temperatures. For instance, no drop in phage titre was observed during incubation in PW at 45 °C for 90 min.

The addition of calcium ions to the medium prior to infection diminished the decrease of

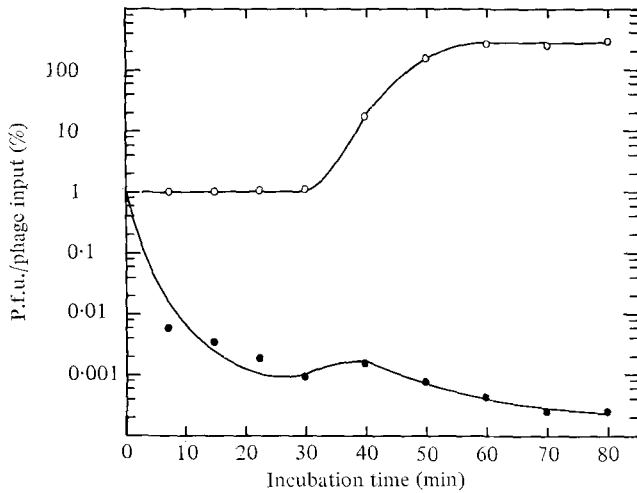


Fig. 2

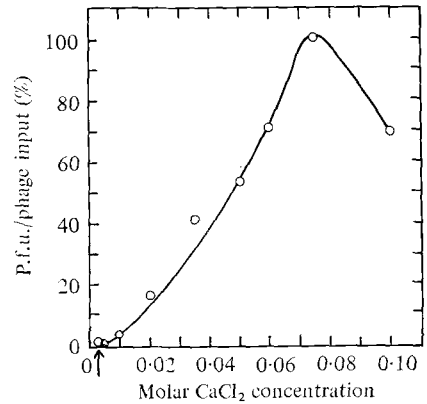


Fig. 3

Fig. 2. Infection by SF 6 at high and low calcium concentrations. *B. subtilis* 168Wt was infected with SF 6 at a m.o.i. of 0.05 in PW with 7.5×10^{-2} M-CaCl₂ (○—○) and in PW (●—●).

Fig. 3. Effect of CaCl₂ concentration on the recovery of phage input. Logarithmically growing host cells were suspended in PW with various Ca²⁺ concentrations and infected with SF 6 at a m.o.i. of 0.1. Samples were assayed for p.f.u. at 0 and 30 min. The arrow indicates the concentration of Ca²⁺ present in PW.

p.f.u. and led to a burst. The PW used contained 2.5×10^{-3} M-Ca²⁺, as determined by atomic adsorption analysis. In order to determine the optimum calcium concentration for the stabilization of SF 6 in the presence of its hosts, infection experiments were performed in PW with various calcium chloride additions. Samples taken immediately after phage infection (0 min) and at the end of the latent period (30 min) were assayed for p.f.u. The effect of the calcium chloride concentration on the recovery of the phages is illustrated in Fig. 3. Complete survival required a final concentration of 7.5×10^{-2} M; a concentration which also resulted in normal phage production, as shown in Fig. 2.

Direct action of CaCl₂

Since the calcium concentration required to obtain normal infection is high compared to most other phage-host systems, we investigated whether the effect of CaCl₂ was due to an element contaminating the CaCl₂. This was tested by infection experiments in which KCl, MnCl₂, NiSO₄, CoCl₂, FeSO₄, FeCl₃, H₃BO₃, Al₂(SO₄)₃ and Na₂MoO₄ were added to PW in concentrations of 10^{-3} and 10^{-4} M. These values were chosen because lower concentrations would not exceed those already present in PW and because contaminating elements were not expected to occur in higher concentrations in the CaCl₂ used. None of the ions promoted phage production, indicating that the effect of CaCl₂ could not be ascribed to a contamination by any of the elements tested.

When calcium chloride of different make and purity was used in infection experiments, the same optimum concentration of 7.5×10^{-2} M was necessary for complete survival of SF 6. Moreover, when five other divalent cations, Mg²⁺, Sr²⁺, Ba²⁺, Zn²⁺ and Mn²⁺ were tested, Sr²⁺ and Ba²⁺ were found to be equally effective at the same optimum concentration as Ca²⁺. Mg²⁺ did promote phage production but a total recovery of phage input was not

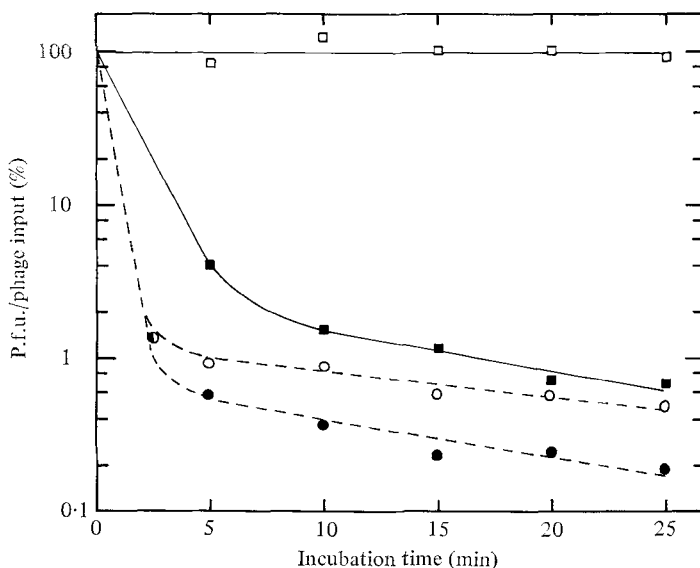


Fig. 4. Influence of calcium ions on the adsorption of SF 6. Cells of *B. subtilis* 168Wt, suspended in PW or PW with 7.5×10^{-2} M- CaCl_2 were infected with SF 6 at a m.o.i. of 0.01 to 0.05. Samples were assayed for p.f.u. (□—□, PW with 7.5×10^{-2} M- CaCl_2 ; ■—■, PW) and the number of free phages (○---○, PW with 7.5×10^{-2} M- CaCl_2 ; ●---●, PW).

achieved. Although Zn^{2+} stabilized the number of p.f.u. to a large extent, phage production did not occur. Mn^{2+} had no influence on the stability of SF 6 in the presence of its host.

As none of the elements tested in low concentrations had any effect on the stability and burst of SF 6 and as the various batches of CaCl_2 , and also the BaCl_2 and SrCl_2 , required the same optimum concentration for normal phage production, we regarded it as highly unlikely that an element contaminating the CaCl_2 was responsible for the stabilizing effect.

Various chemicals, e.g. chelating agents and polyamines (Shafia & Thomson, 1964; Reiter, 1963) are known to interfere with phage infection, without a direct effect on the phages. Phosphate was found to prevent the formation of plaques by SF 6 (H. Y. Steensma & J. Blok, unpublished data). PW might thus contain an inhibitory substance which is removed in the precipitate frequently occurring on addition of CaCl_2 , or which is inactivated by high concentrations of calcium ions. Infection experiments in RS, a minimal medium of which all components were found to be devoid of inhibitory activity, however, revealed the same requirement for calcium ions as in PW. The presence of an inhibitory substance in peptone was therefore excluded.

From the preceding data it may be concluded that high concentrations of calcium ions were required for normal productive infection by SF 6. We have therefore studied the influence of the concentration of calcium ions on the adsorption of the phages, the penetration of the phage DNA and the intracellular phage development in order to localize the calcium dependent steps.

Adsorption

Infection of a host strain by SF 6 at a low calcium concentration resulted in a rapid decrease of infective centres. This could be the result of inactivation of free phages, as suggested by the almost identical decrease of the total phage count and free phages. This is illustrated in Fig. 4. Incubation of SF 6 in the supernatant of a logarithmically growing culture of *B. subtilis* 168Wt revealed that after 30 min, only 25% of the phages were inacti-

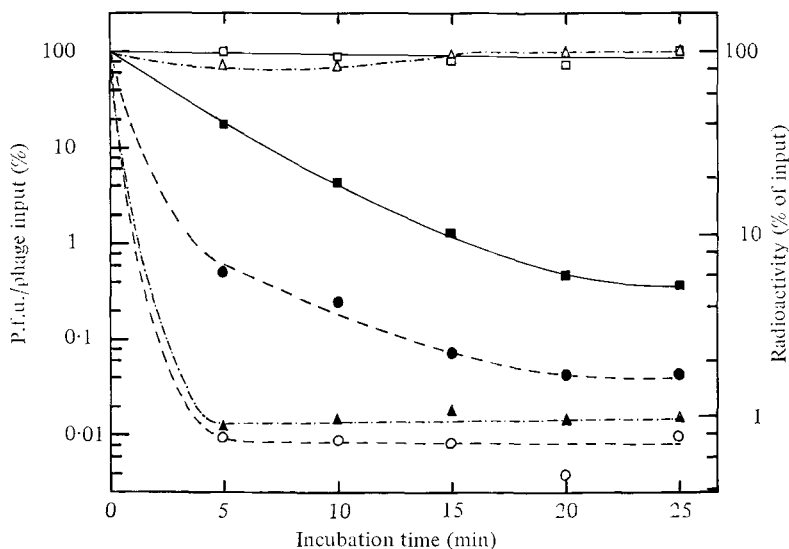


Fig. 5. Infection of *B. subtilis* 168Wt with tritium labelled SF 6. Logarithmically growing cells, suspended in PW or PW with 7.5×10^{-2} M-CaCl₂, were infected with ³H-SF 6 at a m.o.i. of 0.05. Samples, taken at 5 min intervals, were assayed for p.f.u. (□—□, PW with CaCl₂; ■—■, PW), free phages (○—○, PW with CaCl₂; ●—●, PW) and the amount of radioactivity associated with host cells (△—△, PW with CaCl₂; ▲—▲, PW). The 100 % value of radioactivity corresponds to approx. 7000 d/min/ml.

vated, considerably less than during infection experiments. The phages were less stable, however, in the supernatant of logarithmically growing cultures of *B. subtilis* 31 try⁻ his⁻. The rate of inactivation was dependent on the growth phase of the culture, reaching a maximum of 80% inactivation in 30 min at the end of the logarithmic growth phase. This decrease was reduced to about 30% by the addition of calcium ions (H. Y. Steensma & J. Blok, unpublished results). As more than 98% of the p.f.u. was lost in 10 min during infection experiments, inactivation of free phages by a substance excreted by the host cells was considered of minor importance and the decrease in the number of free phages in infection experiments was therefore regarded as due mainly to adsorption of the phages on to the host cells. From this it could also be deduced that adsorption did not require high calcium concentrations as in both PW and PW with 7.5×10^{-2} M-CaCl₂, the disappearance of free phages, and hence their adsorption, took place with almost the same efficiency. An example is shown in Fig. 4. The necessity of irreversible adsorption for phage inactivation at low calcium concentrations was confirmed by the following experiment. An SF 6 resistant mutant of *B. subtilis* 168Wt, blocked in a step leading to irreversible adsorption, was isolated. During infection experiments with cells of this mutant, which we called *B. subtilis* 168/SF 6, as the host, the number of free phages decreased to 10% in 10 min, slightly less than with other hosts. When the cells and adsorbed phages were removed by centrifugation and resuspended in fresh medium, desorption of the phages took place until equilibrium was reached. No reduction in phage titre was observed, however, indicating that reversible binding of the phages was not sufficient for inactivation of SF 6. Inactivation, therefore, must occur in one of the succeeding steps.

DNA penetration

DNA penetration is the next step in the infection process and little is known of the mechanism employed by phages with a non-contractile tail, like SF 6. Since both DNA and

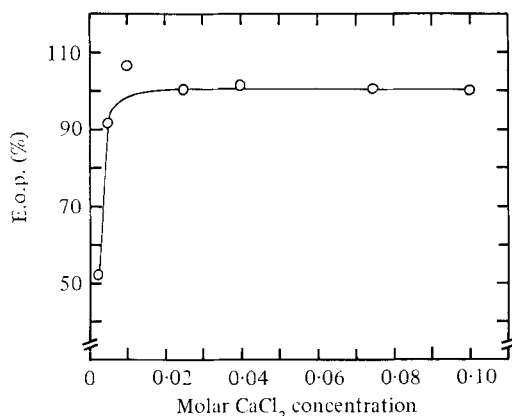


Fig. 6. Effect of CaCl₂ concentration on the e.o.p. The values are the means of four experiments.

teichoic acid, a major part of the cell wall of *B. subtilis*, contain negatively charged phosphate groups, calcium ions might be necessary to diminish repulsive forces during a stage of penetration at which a close contact between DNA and the cell wall of the host exists. To study the penetration process, infection experiments were performed with phages whose DNA had been labelled by means of tritiated adenine. Samples of infected cultures of *B. subtilis* 168Wt in PW and PW with 7.5×10^{-2} M-Ca²⁺ taken at various time intervals were assayed for p.f.u., free phage and radioactivity associated with the host cells. Fig. 5 shows that, at low Ca²⁺ concentrations, the radioactive label rapidly appeared in a form not associated with the cells, although phage adsorption took place at the normal rate. In cultures supplemented with 7.5×10^{-2} M-CaCl₂, however, the label remained associated with the host cells until lysis commenced (not shown). This meant that, at low calcium concentrations, desorption of the phages in a biologically inactive state took place, or that, while the transfer of the phage DNA was triggered, penetration into the host cell was blocked, thus leading to the release of nucleic acid into the surrounding medium. To distinguish between these possibilities, similar experiments were performed using phages whose protein coat had been labelled with ³⁵S. The partition of the sulphur label showed the same pattern as that obtained with the labelled DNA. From the above results, we concluded that inactivation of SF 6 at low Ca²⁺ concentration was due to desorption of the phages in an inactive state. An indication of whether the inactivated phages occurred as a protein-DNA complex or in a dissociated form was obtained from an infection experiment in which a mixture of the ³⁵S- and ³H-labelled phages was employed. The supernatant of this culture was run through a hydroxyapatite column. The majority of the ³⁵S-labelled protein was eluted at a low phosphate concentration, whereas most of the ³H-labelled DNA was retained. This indicated that many phages were dissociated. Additional support was obtained from the electron microscopical observation of many phage ghosts in cultures infected with SF 6 at a low calcium concentration.

Intracellular phage development

It has been shown that high Ca²⁺ concentrations were required for the penetration of phage DNA into the host. This did not exclude the possibility that calcium also played a role in intracellular phage development. However, when cells of *B. subtilis* 168Wt were infected with SF 6 in PW with 7.5×10^{-2} M-CaCl₂ and diluted 100-fold into PW after 5 min, a normal one step growth curve was obtained. Furthermore, the observation that transfection

with SF 6 DNA did not depend on the presence of calcium ions in high concentrations led to the conclusion that these were not required for intracellular phage development.

Efficiency of plating of SF 6

Since calcium ions were found to play an important role in the infection process of SF 6 in liquid medium, a correlation between the plating efficiency of the phages and the calcium concentration in the plating medium could be expected. A suspension of SF 6 was therefore enumerated using D.A.L. plates supplemented with various amounts of CaCl_2 . The phage counts reached a maximum value at a calcium concentration of 10^{-2} M or higher, as is illustrated in Fig. 6. Electron microscopical determinations, using PA and SPA with a calcium concentration of 7.5×10^{-2} M, showed that the e.o.p. at the plateau level of the phage counts was 100%.

DISCUSSION

The calcium concentration needed for maximum stability and production of SF 6 was found to be 7.5×10^{-2} M. Other bacteriophages with a non-contractile tail, including *E. coli* phage T5 (Luria & Steiner, 1954), are known to require the presence of calcium or other divalent cations in concentrations exceeding those necessary for good growth of the host cells. To our knowledge no concentrations higher than 1.8×10^{-2} M have been reported and therefore SF 6 seems to be unique in this respect. Infection of *E. coli* by T5 requires calcium ions for DNA penetration (Luria & Steiner, 1954; Lanni, 1960). SF 6 also has this requirement, but at low concentrations both phage DNA and protein dissociate from the host separately, in contrast to T5. Another remarkable feature of SF 6 is the inactivation of the phage by a substance excreted in the medium by *B. subtilis* 31 $\text{try}^- \text{his}^-$. Since the inactivation of free and adsorbed phages at low calcium concentrations resulted in the ejection of DNA into the medium, it is likely that the mechanism of both inactivations have aspects in common. An explanation is offered by the following hypothesis for which we assumed that the mechanism of DNA penetration is similar to that for *E. coli* phage λ as proposed by Mackay & Bode (1976). This comprised three steps: after a lag, irreversible adsorption is followed by triggering of the DNA release from the phage head and entry of the genome into the host cell. The triggering is temperature dependent and may be due to an alteration of the tail structure, starting at the distal end. In our opinion, high calcium concentrations were required for the firm attachment of SF 6 to its receptor. After triggering of the DNA injection, low calcium concentrations would result in a rupture of the bond between the phage tail and receptor by shearing, by repulsive forces between the DNA and the teichoic acid in the cell wall, by a recoil reaction caused by the initiation of the DNA movement or by a combination of these factors. In the presence of high calcium concentrations, the bond between the phage tail and bacterial receptor is sufficiently strong, thus allowing normal penetration. Inactivation of free phages in the supernatant of *B. subtilis* 31 $\text{try}^- \text{his}^-$ cultures was, according to our hypothesis, caused by the release of phage receptors or a substance originating from the phage receptors involved in the mechanism of triggering. This inactivation was only partially counteracted by calcium ions, implying that the stabilization of free phages differed from that of adsorbed phages. A similar stabilization by calcium ions against inactivation of *Staphylococcus* phages by cell wall extracts has been reported (Beige & Seltmann, 1978).

As a consequence of this hypothesis, conditions with diminished shearing stress would result in an improvement of SF 6 infection at low Ca^{2+} concentrations. This was established to some extent, when cultures were not shaken after infection and by using solid medium where the optimum calcium concentrations amounted to 10^{-2} M (e.o.p. experiments).

Although the hypothesis presented here is supported by our own and other results, many questions still remain, the most intriguing being why only Ca^{2+} , Ba^{2+} , Sr^{2+} and, to a smaller extent, Mg^{2+} , are active, whether the mechanism proposed for λ is also valid for SF 6, and the nature of the substance causing inactivation of free phages.

We thank Mr Bart de Rooter for the determination of the buoyant densities and sedimentation coefficients and Lesley A. Robertson, M.I.Biol., for reading the manuscript.

REFERENCES

- ADAMS, M. H. (1959). In *Bacteriophages*, p. 235. New York: Interscience Publishers, Inc.
- BEIGE, U. & SELTMANN, G. (1978). Wirksamkeit von Mureinpräparationen im Phagenhemmtest-Einfluss von Ca^{++} -Ionen und EDTA. *Zeitschrift für Allgemeine Mikrobiologie* **18**, 27–32.
- BRODETSKY, A. M. & ROMIG, W. R. (1965). Characterization of *Bacillus subtilis* bacteriophages. *Journal of Bacteriology* **90**, 1655–1663.
- GAREN, A. & PUCK, T. P. (1951). The first two steps of the invasion of host cells by bacterial viruses. II. *Journal of Experimental Medicine* **94**, 177–189.
- GIBSON, T. & GORDON, R. E. (1974). In *Bergey's Manual of Determinative Bacteriology*, pp. 529–550. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: The Williams & Wilkins Company.
- LANNI, Y. T. (1960). Invasion by bacteriophage T5. II. Dissociation of calcium-independent and calcium-dependent processes. *Virology* **10**, 514–529.
- LURIA, S. E. & STEINER, D. L. (1954). The role of calcium in the penetration of bacteriophage T 5 into its host. *Journal of Bacteriology* **67**, 635–639.
- MACKAY, D. J. & BODE, V. C. (1976). Events in lambda injection between phage adsorption and DNA entry. *Virology* **72**, 154–166.
- MELLEMA, J. E., VAN BRUGGEN, E. F. J. & GRUBER, M. (1967). Uranyl oxalate as a negative stain for electron microscopy of proteins. *Biochimica et Biophysica Acta* **140**, 180–182.
- PARANCHYCH, W. (1966). Stages in phage R 17 infection: the role of divalent cations. *Virology* **28**, 90–99.
- REESE, J. F., DIMITRACOPOULOS, G. & BARTELL, P. F. (1974). Factors influencing the adsorption of bacteriophage 2 to cells of *Pseudomonas aeruginosa*. *Journal of Virology* **13**, 22–27.
- REITER, H. (1963). The location of the inhibitory action of KCN and several polyamines on bacteriophage replication. *Virology* **21**, 636–641.
- SHAFIA, F. & THOMSON, T. L. (1964). Calcium ion requirement for proliferation of bacteriophage $\phi\mu$ -4. *Journal of Bacteriology* **88**, 293–296.
- SNIPES, W., CUPP, J., SANDS, J. A., KEITH, A. & DAVIS, A. (1974). Calcium requirement for assembly of the lipid-containing bacteriophage PM 2. *Biochimica et Biophysica Acta* **339**, 311–322.
- SPIZIZEN, J. (1959). Genetic activity of deoxyribonucleic acid in the reconstitution of biosynthetic pathways. *Federation Proceedings* **18**, 957–965.
- STEENSMA, H. Y. (1977). The efficiency of plating (E.O.P.) of the bacteriophages SF 6, SF 7, ϕ 29, PBS-I, GA-I, T 3 and T 4. *Annali di Microbiologia* **27**, 29–35.
- STEENSMA, H. Y., HAVELAAR, A. H., LEYDENS, H. M. & WIKÉN, T. O. (1974). Bacteriophages of *Bacillus subtilis*: explanation of the influence of the number of indicator bacteria on the efficiency of plating (E.O.P.). *Annali di Microbiologia* **24**, 11–19.
- STEENSMA, H. Y. & SONDERMEIJER, P. J. A. (1977). A counting method for determination of the burst size of defective phages from *Bacillus subtilis*. *Antonie van Leeuwenhoek* **43**, 305–316.
- WATANABE, K. & TAKESUE, S. (1972). The requirement for calcium in infection with *Lactobacillus* phage. *Journal of General Virology* **17**, 19–30.
- YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORN, L. & TREIBER, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**, 734–744.

(Received 4 May 1978)