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# EFFECT OF CALCIUM ION ON THE LYTIC CYCLE OF BACTERIOPHAGE 41C

EDWARD F. LANDRY

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EFFECT OF Ca$^{++}$ ON THE LYTIC CYCLE OF

BACTERIOPHAGE 41c

by

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ABSTRACT


EFFECT OF $Ca^{++}$ ON THE LYTIC CYCLE OF

BACTERIOPHAGE 41c


by


EDWARD F. LANDRY


The lytic cycle of Bacillus subtilis phage 41c was
found to be dependent upon the presence of at least 10 mM
$Ca^{++}$. In the absence of this ion, the plaquing efficiency of
the virus was reduced to less than 0.1 and equimolar concen-
trations of other divalent ions failed to enhance this effi-
ciency.

Adsorption studies indicated that phage attachment
was $Ca^{++}$-dependent; however, the requirement could be met
with $Ca^{++}$ concentrations ranging from 10 mM to 0.1 mM.
Although adsorption was efficient at reduced $Ca^{++}$ levels,
successful infection could only be established with 10 mM
$Ca^{++}$. At suboptimal ionic levels, a loss of infected centers
occurred within one min after infection and penetration
studies indicated that an irreversible inhibition of injec-
tion occurred under these conditions with fewer than 10% of
the infected complexes surviving. Consistently, the surviving
population (which completed penetration) failed to exhibit a

normal one step growth curve, implicating a post-penetration $Ca^{++}$ requirement.  When infected complexes were exposed to 10 mM $Ca^{++}$ for 2.5 min and diluted into media lacking this ion, few infectious virus developed confirming this supposition. On the other hand, dilution of infected cells into $Ca^{++}$- deficient medium six min into infection had no apparent effect and the burst appeared normal.  Thus, these results confirmed the assumption that an early stage of intracellular develop- ment was $Ca^{++}$-dependent.  Additional confirmation of intra- cellular involvement was obtained with spermine.  The poly- amine could substitute for $Ca^{++}$ in the adsorption and penetration  phases of 41c; however, spermine failed to permit intracellular phage development.

Analysis of transcriptional events in the presence and absence of $Ca^{++}$ by RNA-DNA hybridization indicated that messenger RNA production was similar at both ionic levels. Similar concentrations of phage coat protein appeared to be synthesized in both systems.  However, phage DNA synthesis was somewhat reduced in the absence of $Ca^{++}$.  These data indicated that $Ca^{++}$ might be required for DNA synthesis or more likely,  the assembly of virus into infectious units. In any event, this ion plays a multiple role in 41c infec- tion, affecting both extracellular and intracellular events.

# INTRODUCTION

Bacteriophage 41c is a double-stranded deoxyribo-nucleic acid (DNA) containing virus of <u>Bacillus</u> <u>subtilis</u> 168 (Zsigray, Miss and Landman, 1973). Morphologically, it consists of a head 50 nm in diameter and a non-contractile tail approximately 140 nm long and 10 nm wide. The phage appears to be unique in at least one phase of its replication process since infection is sensitive to treatment with deoxyribonuclease (DNase).

Additional studies with 41c indicated that the infectious process required $Ca^{++}$ at an optimal concentration of 10 mM. Although the literature establishes that a number of bacteriophage require divalent cations for successful infection, the stage affected varies depending on the particular system studied. Most phage systems require the ion at one specific stage; however, in a few cases, two or three-stage requirements have been observed.

In the present study, experimentation was undertaken to determine: 1) at what stage(s) in the lytic cycle of 41c $Ca^{++}$ exerts its effect(s); and 2) whether or not other ions substitute effectively for $Ca^{++}$ in the infectious process of this virus.

REVIEW OF THE LITERATURE

## The Effects of Ions on the Adsorption
## of Bacteriophage to Host Cells

Adsorption of phage to its appropriate host cell represents the initial stage in the lytic cycle. Adsorption is characterized by a two-step process involving random collision between the tail of the phage particle and its appropriate receptor site on the surface of the bacterial cell (Zarybnicky, 1969; Garen and Puck, 1951). The rate at which virus attach to receptors is dependent upon the concentration of the host cells and the concentration of the virus employed (Adams, 1959). Additionally, attachment can be influenced by the pH and ionic environment of the medium.

Phage attachment is electrostatic in nature and is initially reversible. In some systems, as in T1, monovalent as well as divalent ions can meet the ionic requirement for proper electrostatic interaction between phage and bacteria (Garen and Puck, 1951; Puck, Garen and Cline, 1951). In other instances specific divalent cations are required. In all cases, the ions act to neutralize the electrostatic repulsive forces created by critical chemical groupings on the surfaces of a negatively charged host and a negatively charged virus (Stent, 1963; Puck, 1953). Following this initial phase, an irreversible temperature dependent stage occurs insuring a permanent attachment of the virus to the host

cell. Although a variety of phages have been reported to require ions for adsorption, the attachment properties of each are essentially the same except for ion specificity and the concentration required (see Appendix I).

## The Effect of Divalent Ions on the Penetration
## of Viral Nucleic Acid

### General Aspects

During phage penetration viral nucleic acid is released from the protein coat and, in most cases, is the only component of the virus particle which enters the host cell (Hershey and Chase, 1952). With complex viruses, such as T-even coliphage, contraction of the tail sheath forces the tail through an opening in the cell wall followed by release of the nucleic acid in the region of the periplasmic space (Hausmann, 1973; Simon and Anderson, 1967) where entry occurs via a transformation-like process. In simple phage which lack a contractile tail, the process is not as defined and the penetration process is substantially slower.

The entry of the viral genome is a diverse process. Injection may be triggered by the hydrolysis of ATP located in the sheath of the virus (Kozloff and Lute, 1960) or alternatively, nucleic acid release might result from repulsive forces between phage nucleic acid and coat protein (Zarybnicky, 1969). In addition, the DNA might be injected with the same genes entering the host first as is the case

with T5 (Lanni, 1968), SP82G (McAllister, 1970) and T7 (Pao and Speyer, 1973). On the other hand, other viruses, such as $\lambda$ show an equal possibility of the left or right-hand end of the molecule being injected first (Sharp, Donta and Freifelder, 1971).

### The Effect of Divalent Cations on the Penetration of DNA-Containing Bacteriophage

A number of bacteriophage require divalent cations for successful entry of the viral genome. In some of the typing phage of Staphylococcus, ions are not only required for the attachment of the virus to host cells but are also needed for penetration of nucleic acid into the cell (Roundtree, 1955). Roundtree noted that addition of 0.1% ethylenediaminotetraacetic acid (EDTA) to $Ca^{++}$-supplemented-infected cells resulted in a drastic loss of infected centers when the chelator was added within the first few min of infection. Viral inactivation values of 90% occurred when EDTA was added at 2 or 3 min post-infection; however, addition of the chelator 10 min after infection did not influence the survival of the infected centers. Inactivation appeared irreversible since removal of EDTA by dilution failed to increase the number of infectious centers. Roundtree concluded that EDTA removed the $Ca^{++}$ normally required for an early stage following adsorption, presumably penetration. $Mg^{++}$ could substitute for $Ca^{++}$ whereas $Sr^{++}$ was generally non-effective.

A similar pattern of multi-step ion involvement was found in $\emptyset$U-4 of B. stearothermophilus (Shafia and Thompson, 1964). $Ca^{++}$ was required for the adsorption of the virus; however, if low levels of citrate were added between 1 and 5 min after infection, less than 0.5% of the adsorbed phage survived as infectious centers. Partial restoration was achieved if 10 mM $Ca^{++}$ was supplied during this period. These data suggested that the penetration process was strictly dependent upon $Ca^{++}$.

Similarly, the ionic requirement of PL-1 of Lactobacillus casei was established (Watanabe and Takesue, 1972). $Ca^{++}$, at an optimal concentration of 10 mM, was necessary for successful infection. Since equal amounts of unadsorbed viruses could be found in the presence or absence of this ion, $Ca^{++}$ was not involved with adsorption. If virus attachment occurred in $Ca^{++}$-supplemented media and shortly thereafter transferred to a growth tube lacking $Ca^{++}$, a normal intracellular development and burst occurred. If, however, the initial phase occurred in the absence of $Ca^{++}$, a marked decrease in the number of infectious centers was noted. This implied that a $Ca^{++}$-dependent stage occurred shortly after adsorption but prior to intracellular growth, presumably penetration.

The penetration process of PL-1 was studied using $^{32}P$-labelled phage. In $Ca^{++}$-supplemented or non-supplemented media, 95% of the viable phage and 20% of the radioactivity adsorbed to the host within 3 min at 37 C. On further incu-

bation for 30 min at 37 C in the presence of $Ca^{++}$, 54% of the adsorbed virus survived as infected centers and 17% of the radioactivity was cell-associated. The subjection of such complexes to blending at 20 C for 5 min resulted in 24% of the cell-associated $^{32}P$ being released into the supernatant fluid. In addition, greater than 90% of the infected complexes were resistant to blending. On the other hand, blending of infected complexes formed in the absence of $Ca^{++}$ resulted in the release of 83% of the cell-associated $^{32}P$ into the supernatant fluid while only 11% of the infected complexes survived treatment. From these results, the authors concluded that, in the absence of $Ca^{++}$, the genome of PL-1 failed to penetrate the cell and was released from the host by blending. In the presence of $Ca^{++}$, the penetration process was not altered and infection proceeded normally.

Loss of infected complexes in the absence of $Ca^{++}$ could not be reversed by later addition of this ion unless administered within two min following adsorption. In $Ca^{++}$-deficient infections, the viral genome, which failed to penetrate the host, was released from the head resulting in loss of infectivity. Thus, later $Ca^{++}$ additions would have no effect since viable phage was non-existent and the host cell remained viable. Other experiments indicated only $Sr^{++}$, in equimolar concentrations, could replace efficiently $Ca^{++}$ while other ions tested ($Mg^{++}$, $Mn^{++}$, $Be^{++}$, $Ba^{++}$, $Co^{++}$ and $Zn^{++}$) were not effective.

The Effect of Divalent Cations on the Penetration

of Single-Stranded RNA Bacteriophage

The lytic cycle of single-stranded RNA bacteriophage, including MS2, R17 and f2, has been shown to be dependent on divalent cations. In general, infection is initiated by the virus adsorbing to the sides of the F-pili produced by male strains of E. coli(Brinton, Gemski and Carnahan, 1964). Attachment is not ion specific but depends on an ionic strength of 100 mM. Following irreversible adsorption, the virus enters a temperature and energy-dependent eclipse period (Danziger and Paranchych, 1970b) prior to penetration. At this time, the viral genome becomes accessible to degradation by RNase (Paranchych, Krahn and Bradley, 1970).

In R17 infections, a steady decline in the number of infectious centers occurred in the absence of $Mg^{++}$ with over 90% of the complexes being inactivated (Paranchych, 1966). A comparison of the data obtained with 5 mM $Mg^{++}$ indicated that no decline in infectious centers occurred during infection. Additional experiments employing labelled virus eliminated an adsorption requirement. In addition, the temporary RNase sensitive period occurred in the presence or absence of $Mg^{++}$ indicating the $Mg^{++}$-dependent period occurred beyond eclipse.

Penetration studies employing $^{32}P$-labelled virus was studied next. In $Mg^{++}$-supplemented media, the majority of the radioactivity became cell-associated on incubation for

30 min at 37 C. Subsequent washings released some label; however, 60% remained irreversibly cell bound, presumably in an intracellular state. In $Mg^{++}$-deficient media, the same quantity of label initially became cell-associated; however, 40% of the label was eluted gradually with incubation. On washing, the remaining 60% of the cell-associated label was released from the host indicating that the RNA failed to penetrate in the absence of $Mg^{++}$. Paranchych (1966) concluded that penetration was the only step that was dependent upon $Mg^{++}$ since addition of EDTA 10 min into infection had no effect on survival. In addition, the penetration process was found to be irreversible since addition of $Mg^{++}$ 2.5 min after initiation of infection resulted in only 42% of the complexes surviving while only 8% survival was noted when $Mg^{++}$ was added at 10 min. $Ca^{++}$, $Ba^{++}$ and $Sr^{++}$ in equimolar concentrations were as effective as $Mg^{++}$ in promoting penetration while $Zn^{++}$, $Co^{++}$, $Mn^{++}$ and $Ni^{++}$ could not meet the ionic demand.

Loeb and Zinder (1961) observed f2 abortive infections in the absence of 2 mM $Ca^{++}$. $Ca^{++}$ was required for some stage other than attachment or the eclipse phase since both events occurred when 25 mM EDTA was added 1 min prior to infection (Silverman and Valentine, 1969). Penetration was implicated since, in the absence of $Ca^{++}$, the labelled RNA remained associated with the phage protein coat. The genome of a small fraction of the viral population was found associated with the pilus but failed to become cell-associated

in the absence of $Ca^{++}$. It was postulated that $Ca^{++}$ was acting
to weaken bonds between the RNA and the protein coat either at
the phage level with the weakening of bonds within the coat or
at the pilus level involved in RNA release. In the absence of
$Ca^{++}$, most of the virus would retain the genome and desorb
while few would manage to associate with the pilus. However,
in both cases, the genome remained sensitive to hydrolysis
by RNase.

## The Effect of Divalent Cations on Post-Penetration
Stages of Virus Replication

### General Aspects

Following penetration, the viral nucleic acid is
transcribed, replicated and translated into proteins necess-
ary for production of phage progeny. Three classes of phage
proteins were distinguished in T7 by Studier (1972). Class
I proteins synthesized during the 4th and 8th min of infec-
tion included a new phage-specific RNA polymerase and ligase.
Class II proteins were produced between 6 and 15 min of in-
fection and contained DNA polymerase, endonucleases and lyso-
zyme. Class III proteins, synthesized from the 8 min mark
until lysis, included a head maturation protein, head compo-
nents and tail components. Once accumulated, the phage
components assembled spontaneously and the cycle was completed
by the release of the phage progeny by lysis or extrusion
(Bradley and Dewar, 1967). Although divalent cations influ-
ence intracellular development of virus, little is known

concerning their mechanism of action.

Late Stage Ion Involvement in

Bacteriophage Development

Divalent ions were involved in both the adsorption and penetration of Staphylococcal typing phages 3A and 29 (Roundtree, 1955). However, the concentration of $Ca^{++}$ required for optimal phage development was substantially greater than that necessary for adsorption or penetration. Infection with phage 3A at 0.68 mM $Ca^{++}$ resulted in a burst size of 50; however, if 0.1% EDTA was added between 20 and 50 min after the initiation of infection, the burst size was reduced to 20 while readdition of $Ca^{++}$ prior to lysis resulted in a normal burst. Since phage penetration was complete prior to EDTA addition, Roundtree (1955) concluded that $Ca^{++}$ was required for an intracellular step in phage production. This requirement was specific since $Mg^{++}$ which could permit penetration failed to replace $Ca^{++}$. Similar results were obtained with phage 29.

Brodetsky and Romig (1965) studied the $Ca^{++}$ requirements of several Group I phage of B. subtilis. Although adsorption occurred in the absence of this ion, optimal phage development only occurred at 18 mM $Ca^{++}$. In SP9 infections, employing the same concentration of $Ca^{++}$, a latent period of 30 min and burst size of 87 were noted. Without $Ca^{++}$, only 4% of the infected centers survived and these complexes exhibited an increased latent period and a reduced burst size.

All related Group I phage exhibited similar effects. Although there was no particular $Ca^{++}$-dependent stage cited, the difference in burst size seems to imply a late $Ca^{++}$-dependent function in phage development. Likewise, late ionic involvement has been observed in the lytic cycles of phages GV4 of B. thuringienis (Colasito and Rogoff, 1969) and $\phi$U-4 of B. stearothermophilus (Shafia and Thompson, 1964).

Late Ion Involvement in T5 Infections

Adsorption of T5 is independent of $Ca^{++}$; however, in media lacking this ion, only 20% of the infected centers survive (Adams, 1949). In addition, a latent period of 70 min and a burst size of 10 was noted. With 1 mM $Ca^{++}$, a latent period of 55 min and a burst of 40 was exhibited. Addition of $Ca^{++}$ to complexes early in the latent period resulted in partial reversal of inactivation while later additions had decreased effects. Similarly, early transfer of $Ca^{++}$-supplemented infected cells to $Ca^{++}$-deficient media resulted in a large decrease in infected complexes while delaying transfer permitted survival (Luria and Steiner, 1954).

Cytological studies on host chromatin material after $Ca^{++}$- and non-$Ca^{++}$-supplemented T5 infections showed differences indicating some stage of phage multiplication was affected by $Ca^{++}$ (Murray and Whitfield, 1953).

The infection process of T5 was dissociated into two distinct stages (Lanni, 1960a). The first stage was a $Ca^{++}$-

independent stage involving the transfer of T5 DNA into the
host by a unique two-step mechanism (Lanni, 1968). Initially,
8% of each DNA molecule, constituting the first-step-transfer
(FST) was rapidly transferred into the host cell. This
portion of the T5 DNA contained two genes necessary for the
synthesis of proteins involved in the degradation of host
DNA (Lanni, McCorquodale and Wilson, 1964; McCorquodale and
Lanni, 1964) and for the transfer of the remaining 92% of
the DNA molecule (Lanni, 1965).

The second stage of T5 infection involved an unknown
$Ca^{++}$-dependent process which permitted stabilization of the
infected complex (Lanni, 1960b). Stabilization was propor-
tional to the concentration of $Ca^{++}$, being optimal at 1 mM
while decreasing sharply at 0.1 mM and 0.01 mM (Lanni, 1961).
Complexes failing to achieve stabilization resulted in a
loss of plaquing centers. Complexes which had completed
penetration in the absence of $Ca^{++}$ could be stabilized by
the addition of $Ca^{++}$ as late as 15 min after infection, in-
dicating that $Ca^{++}$ was necessary for a stage beyond penetra-
tion.

A number of mechanisms were proposed for the role
of $Ca^{++}$. These were: 1) $Ca^{++}$ was necessary for penetration
of unknown essential phage constituents (Lanni, 1960b); 2)
$Ca^{++}$ might be a cofactor for an enzyme involved in the
degradation of host DNA (Lanni and McCorquodale, 1963);
and 3) $Ca^{++}$ might be stabilizing the phage head until all
the DNA had penetrated the host (Lanni, 1961).

The latter hypothesis can be eliminated by recent studies of Labedan and Legault-Demare (1973). When infection with T5 was arrested after FST penetration and the phage-bacterium complexes centrifuged, the adsorbed capsids were released into the surrounding medium (Labedan, Crochet, Legault-Demare and Stevens, 1973) while the DNA, which remained associated with host, uncoiled. Penetration was completed and infected centers were then formed when protein synthesis was continued in the presence of 1 mM $Ca^{++}$ indicating the phage head does not have to be intact for completion of DNA penetration. Whatever the role of $Ca^{++}$ in T5 infection, it appears complex and has not been resolved to date.

## The Effect of $Ca^{++}$ on Cell Membrane Permeability

Transfection of E. coli by λdg DNA requires the presence of helper virus, presumably facilitating the uptake or penetration of naked DNA molecules (Kaiser and Hogness, 1960). Similarly, linear DNA of phage P2 could transfect E. coli only if related helper phage was present suggesting an alteration in wall permeability of the recipient bacteria (Mandel, 1967). This hypothesis was substantiated when cells not normally susceptible to the inhibitory action of Actinomycin D could be rendered sensitive following treatment with $Ca^{++}$ indicating a cell permeability role for this ion. Also, the role of helper virus in transfection of E. coli by P2 DNA could be eliminated by the addition of 25 to 50 mM $Ca^{++}$

(Mandel and Higa, 1970).

Cohen, Chang and Hsu (1972) found $Ca^{++}$ permitted the uptake of R factor DNA by E. coli, transforming them to multiple antibiotic resistance. Recently, transformation in E. coli was reported for the first time, requiring $Ca^{++}$ at an optimal concentration of 30 mM (Oishi and Cosloy, 1972; Cosloy and Oishi, 1973a). The investigators theorized $Ca^{++}$ might be acting either to neutralize the negative charges on the cell surface permitting DNA uptake or to partly dissoc- iate the membrane structure increasing membrane permeability (Cosloy and Oishi, 1973b). However, other agents known to increase cell wall permeability failed to promote trans- formation.

## MATERIALS AND METHODS

### Bacteriophage and Host

Bacteriophage 41c and its host, B. subtilis 168
trp⁻str$^r$, have been described (Zsigray et al., 1973).

### Preparation of Spore Stock

Freshly poured Oxoid Nutrient Agar plates were
inoculated with 0.01 ml of an exponentially growing cell
suspension of B. subtilis 168 and the plates incubated for
96 h at 37 C.  The growth was removed from the surface of
the plate with 3 ml of 0.85% saline.  The spore preparations
were washed and resuspended with 20 ml of saline and heat-
treated at 70 C for 20 min to kill vegetative forms.  Spore
suspensions were stored at 5 C in 1.0 ml aliquots and used
as inocula for all experiments.

### Media

Liquid media employed for the preparation of host
cells and for infection studies were TP (Kawakami and Landman,
1968) and TYS (Zsigray et al., 1973) broths.  The former
medium consisted of the following ingredients per liter of
deionized distilled water (pH 7.0):  glucose, 2 g; tris
(hydroxymethyl) aminomethane (pH 7.0), 2.4 g; maleic acid, 2.3
g; $(NH_4)_2SO_4$, 1 g; NaCl, 5 g; $K_2HPO_4$, 0.1 g; acid-hydrolyzed
casein (Nutritional Biochemical Corp.), 0.2 g; tryptophane,

0.02 g; $MgCl_2$, 2 mM; $MnCl_2$, 0.1 mM; $FeCl_3$, 0.01 mM. Here-
after, TP media supplemented with 10 mM $Ca^{++}$ will be desig-
nated TP-1. TP media containing 0.1 mM $Ca^{++}$ will be desig-
nated TP-2 and TP media lacking $Ca^{++}$ will be designated
TP-3.

TYS broth contained the following ingredients per
liter: tryptone, 10 g; NaCl, 10 g; yeast extract, 5 g.

Solid media employed for the cultivation of host or
for phage assay contained the same ingredients as TP and TYS
broths with the addition of 1.5% agar for basal media or 0.85%
for soft agar overlays.

For optimal growth of phage 41c, all media were
supplemented with 10 mM $Ca^{++}$. Media used solely for the
cultivation of host cells were not supplemented with $Ca^{++}$.

## Cultural Conditions of Host Cells

Spores of B. subtilis (0.01 ml) were inoculated onto
the surface of fresh TP agar and the plate incubated for 18
h at 37 C. Cells were harvested in 3 ml of TP-3 broth and
the suspension used to inoculate 50 ml of TP-3 broth to an
optical density (OD) of 0.05 (670 nm - Bausch and Lomb
Spectronic 20). The culture was aerated at 37 C until an
OD of 0.2 was obtained. Cells were centrifuged at 12,000 x
g for 10 min and resuspended to one-fourth volume with fresh
TP-3 broth. This technique consistently yielded cell popula-
tions of 1-2 x $10^8$ colony-forming units per ml (CFU/ml) and
were designated log phase cells (Zsigray et al., 1973).

## Bacteriophage Titrations

Phage 41c was assayed by a modification of the soft agar overlay method described by Adams (1959). Dilutions of phage samples were prepared in appropriate broth and 0.1 ml of the desired dilution inoculated onto corresponding media. Approximately 4 ml of the appropriate overlay, seeded with host cells (1 x $10^8$ CFU/ml), were added to the plated sample and mixed by gentle swirling. Solidified plates were inverted and incubated for 18 h at 37 C at which time they were scored for plaque-forming units/ml (PFU/ml).

## Preparation of 41c Phage Lysate

Log phase cultures in 1 liter of TP-1 broth were in-fected with 41c at a phage-bacterium ratio of 2.0. Aeration was continued at 30 C for 6 h until lysis was apparent. The lysate was cleared of intact cells and cellular debris by centrifugation at 12,000 x g for 10 min and treated for 60 min at 37 C with 10 µg/ml each of deoxyribonuclease I (DNase I, Worthington Biochemicals, Freehold, N. J.) and ribonuclease (RNase A, Worthington).

The phage preparation was filter sterilized through a 0.45 µm filter and then concentrated by centrifugration in a type 30 rotor at 66,000 x g ($R_{av}$) for 2 h in a Spinco Model L Ultracentrifuge. The opaque pellet was resuspended in 3 ml of TP-3 broth and assayed for PFU.

## Adsorption of 41c

Log phase hosts (4.5 ml), in 18 mm test tubes, were infected with 0.5 ml of 41c at a phage-bacterium ratio of 0.5 at varying $Ca^{++}$ concentrations ranging from 10 mM to 0.1 mM. A culture was also infected in the absence of $Ca^{++}$. Adsorption of 41c to its host was allowed to occur either by aeration for 5 min at 30 C or by a 30 min adsorption period at 5 C. Samples were centrifuged at 12,000 x g for 10 min and the supernatants assayed for free virus. The pellets were resuspended to the original volume with appropriate broth and assayed for PFU. A few drops of chloroform were added to the pellet and samples assayed to determine the number of uninjected phage. In addition, adsorption kinetics were studied according to the methods of Stent (1963).

## One Step Growth Curves

Log phase cells were infected with 41c at a phage-bacterium ratio of 0.5. The culture was aerated for 15 min at 30 C and centrifuged for 10 min at 12,000 x g to remove unadsorbed virus. Infected pellets were resuspended in 5 ml of appropriate broth and diluted through $10^{-7}$. The $10^{-5}$, $10^{-6}$ and $10^{-7}$ dilutions were placed on a Burrell Wrist Action Shaker and aerated at 30 C. Samples were withdrawn over a 180 min period and assayed for PFU.

## Preparation of $^{32}$P-Labelled 41c

One hundred ml of host grown to an OD of 0.2 were
centrifuged and resuspended in 100 ml of fresh TP-1 broth.
Twenty μCi/ml (final concentration) of $^{32}$P (New England
Nuclear) was added and aeration continued for 10 min at 37 C.
Phage 41c was added at an input of 2.0 and the mixture aerated
at 30 C for 180 min. The lysate was centrifuged at 12,000 x g
for 10 min and the supernatant fluids treated with DNase I
and RNase A (final concentration = 10 μg/ml each) for 30 min
at 37 C. The labelled phage was purified by filtration
through a 0.45 μm filter and concentrated by ultracentri-
fugation as described. The supernatant was decanted and the
pellet washed by allowing 10 ml of 0.15 M sodium chloride-
0.015 M sodium citrate (1 x SSC, pH 8.0) to gently flow down
the side of the tilted tube, opposite the pellet. The con-
tents were discarded and the procedure repeated 3 times.
Finally, the washed pellet was resuspended in 2 ml of 1 x SSC
(pH 8.0).

The labelled virus was further purified using a pre-
formed CsCl gradient (Kellenberger, Bolle, Boy de la Tour,
Epstein, Franklin, Jerne, Reale-Scafati, Sechaud, Bendet,
Goldstein and Lauffer, 1965) with modifications suggested
by D. M. Green (personal communication). A CsCl stock was
prepared by dissolving 20 g of CsCl in 13.2 ml of distilled
water and 1.5 ml of 0.01 M Tris (pH 7.0). Seven substocks
were prepared by adding 1.7 ml, 1.6 ml, 1.5 ml, 1.4 ml, 1.3
ml, 1.2 ml and 1.0 ml of Cs stock to 7 tubes and adjusting

the final volume to 2.0 ml with Tris buffer. The gradient

was prepared by adding, in descending concentrations, 0.5 ml

of CsCl substocks. One and one-half ml of the concentrated

labelled virus was layered on the gradient and centrifuged

in a Spinco Model L Ultracentrifuge at 73,400 x g ($R_{av}$) for

30 min at 20 C employing a SW39L rotor. The cellulose nitrate

tube was tapped and 29 fractions collected and assayed for

PFU and counts per min (CPM). Fractions showing peak radio-

logical and biological activity were pooled and dialyzed

against 2 liters of Tris buffer for 2 h at 4 C. The dialyzed-

purified-labelled phage stock was then used in penetration

studies.

## Assay for Radioactivity

The scintillation fluid used for counting radio-

active samples contained the following ingredients: Triton

X-100 (Packard), 166 ml; 2,5-diphenyloxazole, (Packard

PPO) 0.1 g; 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

(Package dimethyl POPOP), 0.1 g; deionized distilled water,

1000 ml. All ingredients were mixed for 60 min at 25 C and

filtered through Whatman no. 1 filter paper (Zsigray et al.,

1973). Samples of 1.0 ml volumes were placed in scintilla-

tion vials containing 15 ml of scintillation fluid. Counting

was done in a Packard Tri-Carb Liquid Scintillation Spectro-

meter Model 3330 at a gain setting of 7% and window settings

of 40-1000. All counts were corrected for background.

## Penetration of 41c DNA into Host Cells

Log phase cells, resuspended in TP-1 or TP-2, were infected with $^{32}$P-labelled 41c at a phage-bacterium ratio of 0.5. The culture was aerated for 15 min at 30 C and centrifuged at 18,900 x g for 2 min. The supernatant fluid was assayed for free virus and unadsorbed label while the pellet was resuspended in 5 ml of the appropriate TP broth. One ml of the unblended pellet was assayed for PFU and CPM and the remaining 4 ml placed in a Sorvall Micro Attachment and blended using the Sorvall Omni Mixer for 5 min at 115 volts at 2 C. Three ml of the blended culture were centrifuged at 18,000 x g for 2 min and the pellet resuspended to the same volume. Both supernatants and pellets were assayed for CPM and PFU.

## Protoplasting and DNase Treatment of Infected Cells

Log phase cells were infected with 41c at a multiplicity of infection (MOI) of 0.5 for 2.5 min at 30 C in the presence of 10 mM $Ca^{++}$. Infected complexes were diluted 100-fold into TP-1 or TP-3 broth and aerated at 30 C for 15 min. Chloramphenicol was added to a final concentration of 20 μg/ml, followed by the addition of 2M sucrose to a final concentration of 0.8 M. Complexes were protoplasted by treatment with lysozyme (200 μg/ml) for 30 min at 37 C. DNase I (20 μg/ml) was added to the infected protoplasts and the mixtures incubated for 15 min at 37 C. Both TP-1

and TP-3 cultures were assayed for PFU before and after DNase

treatment on TYS plates supplemented with 0.5M sucrose

(Zsigray et al., 1973). The sensitivity of uninfected pro-

toplasts to DNase treatment was studied by the formation of

L-colonies on DP Media (Miller, Zsigray and Landman, 1967).

### 41c DNA Extraction

Phage 41c DNA was extracted according to the methods

used by Mandel and Hershey (1960). Four ml of a concentrated

phage stock (5 x $10^{10}$ PFU/ml) was added to an equal volume of

redistilled, SSC-saturated phenol. The sample was gently

agitated at 4 C for 30 min and centrifuged at 12,000 x g

for 10 min. The supernatant was collected carefully using

an inverted 5 ml pipette and added to an equal volume of SSC-

phenol. The extraction was repeated 3 additional times for

15 min at 4 C. The supernatant fluid from the final extrac-

tion was dialyzed against 1 liter of 1 x SSC (pH 8.0) at 4 C

with changes at 2, 4 and 6 h followed by dialysis overnight.

The DNA was collected and stored over chloroform at 4 C. DNA

concentrations were determined spectrophotometrically at 260

nm (Bausch and Lomb Spectronic 600) or by the diphenylamine

reaction (Burton, 1956) employing calf thymus DNA as standard.

DNA used in hybridization experiments was denatured

by boiling for 10 min in a low salt buffer [1 mM NaCl; 1 mM

EDTA; 1 mM Tris (pH 8.0)] followed by rapid cooling in an

ice bath (Bolle, Epstein, Salser and Geiduschek, 1968).

## Extraction of RNA from Phage Infected Cells

Host cells were prepared by inoculating 100 ml of TP broth to an OD of 0.05 and aerating the culture at 37 C until an OD of 0.7 was achieved (approximately 4.5 h). Cells were concentrated 20-fold by centrifuging and resuspending the pellet in 5 ml of fresh TP-1 broth. This yielded a cell density of 4-5 x $10^9$ CFU/ml.

Lysates were prepared for extractions by infecting concentrated cells, resuspended in TP-1, with 41c (input of 10.0) for 2 min. Cultures were diluted 100-fold into 500 ml of either TP-1 or TP-3 and aeration continued for 20 min. The infected cells were centrifuged at 12,000 x g for 2 min at 25 C and the pellet resuspended in a final volume of 12 ml of 1 x SSC. Lysozyme was added to a final concentration of 800 µg/ml and the culture incubated for 5 min at 37 C. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1% and the mixture cleared of debris by centrifugation at 12,000 x g for 2 min. For the preparation of labelled RNA, radioactive $^{32}$P-labelled lysates were prepared by the addition of 20 µCi/ml of $^{32}$P (New England Nuclear) at the time of infection in TP-1 broth. Cultures were aerated 22 min at 30 C without dilution, centrifuged and lysed as above.

The RNA was extracted by a CsCl technique (Glisin, Crkvenjakov and Byus, 1974). Twelve ml of sample were added to 12 g of CsCl crystals and mixed until the Cs dissolved. Four ml of the sample was gently layered on a 1.2 ml cushion

of 5.7 M Cs stock (density = 1.7070 g/cm$^3$) in 3 cellulose

nitrate tubes. Tubes were mounted in a SW39L rotor and

centrifuged at 100,000 x g (R$_{av}$) for 12 h at 25 C in a Spinco

Model L Ultracentrifuge. The Cs was decanted and the tube

cut to within 1 cm of the bottom. The RNA appeared as a

small opaque pellet and was dissolved in 0.5 ml of 0.01 M

Tris (pH 7.5). The RNA was used immediately or precipitated

overnight with 2.5 volumes of 80% ethanol-0.2 M NaCl at -20 C.

Ribonucleic acid concentrations were determined by the orcinol

method (Clark, 1964) employing torula RNA as standard.

## RNA-DNA Hybridization

Competition RNA-DNA hybridization experiments were

carried out according to the methods of Bolle et al. (1968).

One tenth ml of denatured DNA (80 μg/ml) in 0.3 M NaCl-0.03 M

Na citrate (2 x SSC) was placed in 10 x 70 mm acid-washed

test tubes. Labelled RNA obtained after 25 min of infection

in the presence of Ca$^{++}$ (0.1 ml of a 51 μg/ml stock in 2 x

SSC) was added to each tube. Two tenths ml of 25.5 μg/ml

or 2000 μg/ml of RNA extracted from TP-1 or TP-3 cultures,

25 min post infection, were added to the tubes bringing the

final volume of 0.4 ml. Therefore, the final concentrations

were: denatured DNA 20 μg/ml; labelled RNA 12.5 μg/ml; un-

labelled RNA 12.5 μg or 1000 μg/ml. Tubes were capped and

the conditions for annealing were 70 C for 3 h (Milanesi and

Melgara, 1974; Milanesi and Cassani, 1972) in a Tamsen Thermo-

static Circulating Waterbath. Annealed nucleic acid samples

were then exposed to heat-treated RNase A (10 μg/ml final concentration) for 15 min at 37 C. Nuclease-treated samples were diluted to 15 ml with 0.5 M KCl-0.01 M Tris (pH 7.5) and filtered through 25 mm Schleicher and Schuell B-6 nitro-cellulose membrane filters (Nygaard and Hall, 1963; 1964) which were previously soaked for 15 min in KCl-Tris, mounted on a Millipore 3025 Sampling Manifold and washed with 10 ml of KCl-Tris buffer. Filters containing the impinged nucleic acid were washed with 80 ml of KCl-Tris and placed in scintillation vials. Ten ml of Aquasol (New England Nuclear) was added and the filters allowed to dissolve overnight at 4 C. Samples were counted in a Packard Tri Carb Liquid Scintillation Spectrometer at a gain setting of 1.7% and window settings of 40-1000. All counts were corrected for background.

RESULTS

## Effect of Ca$^{++}$ on the Plaquing

## Efficiency of Phage 41c

To demonstrate the effect of Ca$^{++}$ on the replication
of 41c, the virus was assayed on media supplemented with, or
lacking, 10 mM Ca$^{++}$ (Table 1). When undefined TYS agar
medium was employed, 83% of the phage population was inhi-
bited in the absence of Ca$^{++}$. The inhibitory effects of
this medium could be enhanced if TYS broth was initially
dialyzed for 5 h against distilled water (TYS-D), depleting
it of most contaminating ions. In three separate experiments,
the degree of inhibition in this medium was in excess of 93%.
Similar inactivation values were noted with TP media. In a
totally ion-deficient medium, virus inactivation was in
excess of 99%, however plaques appeared quite small. De-
creasing the Mg$^{++}$ concentration in TP medium to 1 mM did not
significantly affect inhibition. Since this medium was
defined and exhibited a high degree of inhibition, it was
chosen for use in the majority of experiments.

In all cases, a residual population of 41c managed
to survive in the absence of Ca$^{++}$. These survivors were
studied to determine if they could replicate independently of
Ca$^{++}$. Plaques developing on TP-3 plates were picked, placed
in broth and assayed in the presence and absence of Ca$^{++}$. Re-
sults of this experiment showed that these apparent Ca$^{++}$-

Table 1.  Effect of $Ca^{++}$ on the Plaquing Efficiency of Phage 41c.

| Exp. No. | Media | PFU/ml Recovered at | | Inhibition (%) |
|---|---|---|---|---|
| | | 10 mM $Ca^{++}$ | no $Ca^{++}$ | |
| 1 | TYS | $1.02 \times 10^9$ | $1.68 \times 10^8$ | 83.53 |
| 2 | TYS-D[a] | $1.52 \times 10^{10}$ | $1.06 \times 10^9$ | 93.10 |
| 3 | TYS-D | $1.02 \times 10^{10}$ | $1.60 \times 10^8$ | 98.43 |
| 4 | TYS-D | $8.93 \times 10^9$ | $5.80 \times 10^7$ | 99.35 |
| 5 | TP (no ions) | $2.23 \times 10^9$ | $4.80 \times 10^6$ | 99.78 |
| 6 | TP | $1.16 \times 10^9$ | $7.16 \times 10^7$ | 93.83 |
| 7 | TP | $6.06 \times 10^{10}$ | $3.36 \times 10^9$ | 94.46 |
| 8 | TP (1 mM $Mg^{++}$) | $2.23 \times 10^9$ | $7.06 \times 10^7$ | 96.83 |

[a]TYS media dialyzed 5 h against 4 liters of deionized distilled water.

independent phage did indeed require $Ca^{++}$ for replication since 95% of the population was lost in the absence of $Ca^{++}$.

## Specificity of the $Ca^{++}$ Requirement of 41c

Divalent cations are known to substitute for $Ca^{++}$ in several phage systems (Paranchych, 1966; Sinsheimer, 1968). Therefore, it was of interest to determine if similar substitutions could occur in the lytic cycle of 41c. A number of ions were tested in TYS-D agar for their ability to support 41c production. The results (Table 2) demonstrated that this requirement is specific since none of the ions tested could efficiently substitute for $Ca^{++}$ in 41c infection. The highest survival (15%) observed was in infections containing 1 mM $Mg^{++}$.

## Effect of Host Culture Conditions on 41c Recovery

The optimal conditions required for the growth of host cells used in 41c infection was studied next. Log phase cells were grown in TYS and TP-3 broths, concentrated by centrifugation, and resuspended in TP-1 broth. These preparations were infected with 41c (MOI of 0.5) at 30 C with aeration and sampled for PFU at 5 and 15 min. It can be seen from Table 3 that less than half of the input phage were recovered as infective centers when cells were prepared in TYS and infected in TP-1. However, cells grown in TP-3 and infected in TP-1 supported 41c infection efficiently since 80% of the input virus were recovered as infected centers.

Table 2.  Specificity of the $Ca^{++}$-Requirement of Phage 41c.[a]

| Ion | Concentration (mM) | PFU/ml Recovered | Survival (%) |
|---|---|---|---|
| $Ba^{++}$ | 10 | $1.07 \times 10^9$ | 10.49 |
| $Ca^{++}$ | 10 | $1.02 \times 10^{10}$ | 100 |
| $Mg^{++}$ | 10 | $1.07 \times 10^9$ | 10.49 |
| $Mg^{++}$ | 1 | $1.58 \times 10^9$ | 15.49 |
| $Mn^{++}$ | 1 | $1.25 \times 10^9$ | 12.25 |
| $Sr^{++}$ | 10 | $7.43 \times 10^8$ | 7.28 |
| None | | $1.60 \times 10^8$ | 1.57 |

[a]All platings were made on TYS-D Medium.

Table 3. Effect of Host Culture Conditions on 41c Recovery.[a]

| Exp. No. | Time of Infection (min) | Input (PFU/ml) | Infected Complexes (PFU/ml) Recovered from | |
| --- | --- | --- | --- | --- |
| | | | TYS Grown Cells | TP-3 Grown Cells |
| 1 | 5 | $4.25 \times 10^7$ | $2.25 \times 10^7$ (52.94)[b] | $3.60 \times 10^7$ (84.71) |
| | 15 | | $1.75 \times 10^7$ (41.18) | $2.80 \times 10^7$ (65.88) |
| 2 | 5 | $4.20 \times 10^7$ | $2.15 \times 10^7$ (51.19) | |
| | 15 | | $2.10 \times 10^7$ (50.00) | |
| 3 | 5 | $3.05 \times 10^7$ | | $3.40 \times 10^7$ (100) |
| | 15 | | | $3.30 \times 10^7$ (100) |
| 4 | 5 | $4.30 \times 10^7$ | $2.09 \times 10^7$ (48.60) | |
| | 15 | | $1.52 \times 10^7$ (35.35) | |
| 5 | 5 | $3.80 \times 10^7$ | | $2.86 \times 10^7$ (75.26) |
| | 15 | | | $2.90 \times 10^7$ (76.31) |

[a]Host cells grown in TYS or TP-3 and infected with 41c in TP-1 media.

[b]Value in parentheses are percent recoveries of input value.

Host cells grew equally well in the presence or absence of 10 mM $Ca^{++}$; therefore, experiments were conducted to see if the presence of $Ca^{++}$ in the culture media influenced 41c infection. Log phase cells prepared in TP-1 and TP-3 media were infected with 41c in the presence of 10 mM $Ca^{++}$ and assayed for PFU after 5 min of aeration at 30 C. Over 90% of the input virus could be recovered as infected centers under both experimental conditions indicating that the presence of $Ca^{++}$ in the growth medium had no effect on 41c infection. Therefore, log phase cells were prepared in TP-3 broth and infected in TP-1 broth in subsequent experiments.

### Effect of $Ca^{++}$ on the Adsorption of 41c

Initially, optimal adsorption conditions for 41c infection were established by infecting host with 41c (MOI of 0.5) in TP-1 broth. Following an adsorption period of either 30 min at 5 C or 5 min at 30 C with aeration, cultures were centrifuged and assayed for unadsorbed and cell-associated phage. From the data presented in Table 4, it is evident that more efficient adsorption occurred at the higher temperature with 97% of the virus adsorbing in 5 min. Assays of cell-associated phage revealed optimal recoveries were only found at 30 C while lower temperatures appeared to inactivate virus-host complexes. Therefore, conditions of aeration for 5 min at 30 C were used for adsorption in all future experiments.

Table 4. Effect of Temperature on Adsorption and Survival of Phage-Bacterium Complexes Formed at 10 mM $Ca^{++}$.

| Exp. No. | Input (PFU/ml) | Adsorption Conditions | | PFU/ml Recovered in | |
| | | Time (min) | Temperature (C) | Supernatant | Pellet |
|---|---|---|---|---|---|
| 1 | $5.90 \times 10^7$ | 5 | 30 | $1.73 \times 10^6$ (2.93)[a] | $7.50 \times 10^7$ (100) |
| | $5.90 \times 10^7$ | 30 | 5 | $1.35 \times 10^7$ (22.88) | $3.35 \times 10^7$ (56.78) |
| 2 | $5.10 \times 10^7$ | 5 | 30 | $1.11 \times 10^6$ (2.18) | $6.50 \times 10^7$ (100) |
| | $8.10 \times 10^7$ | 30 | 5 | $7.45 \times 10^6$ (9.20) | $3.35 \times 10^7$ (41.36) |

[a]Values in parentheses are percent recoveries of input value.

The effect of $Ca^{++}$ concentrations on the adsorption of 41c was investigated by infecting cultures with virus in TP media supplemented with $Ca^{++}$ at the following concentrations; 10 mM, 1 mM, 0.1 mM, none. Following aeration for 5 min at 30 C, the phage-bacterium complexes were centrifuged and the supernatant fluids assayed for unadsorbed virus. The pellets were resuspended to the original volume in the appropriate broth and assayed for PFU after continued aeration for 15 min at 30 C. At 10 mM $Ca^{++}$, less than 1% of the virus remained unadsorbed after 5 min, while 80% of the adsorbed virus were able to infect the host (Table 5). Efficient adsorption was also seen at 1 mM $Ca^{++}$ with greater than 98% of the virus becoming cell-associated. However, at this reduced level of $Ca^{++}$, 41c failed to establish successful infection since only 38% of the virus were recovered as infected complexes. Adsorption was somewhat hampered at 0.1 mM $Ca^{++}$ with 80% of the phage adsorbing after 5 min but recovery of adsorbed phage was drastically reduced with only 6% establishing infection. In the complete absence of $Ca^{++}$, only 53% of the virus adsorbed while the majority of these failed to successfully establish infection. These data implied that efficient adsorption of 41c occurred at 10 mM, 1 mM, and 0.1 mM $Ca^{++}$ while poor adsorption took place in the absence of $Ca^{++}$. However, successful infection was noted only when 10 mM $Ca^{++}$ was present. This seems to indicate that low concentrations of $Ca^{++}$ can promote adsorption, but high concentrations are necessary for a stage in infection beyond adsorption.

Table 5. Effect of $Ca^{++}$ on the Adsorption of 41c.[a]

| Exp. No. | Input[b] (PFU/ml) | PFU/ml Recovered at $Ca^{++}$ Concentration of[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 mM | | 1 mM | | 0.1 mM | | None | |
| | | Supernatant | Pellet | Supernatant | Pellet | Supernatant | Pellet | Supernatant | Pellet |
| 1 | 470 | 6.65(1.41)[c] | 300(63.83) | 5.8(1.23) | 138(29.36) | 97(20.61) | 13.3(2.83) | 215(45.74) | 6.90(1.47) |
| 2 | 415 | 2.60(0.63) | 215(51.81) | 4.0(0.96) | 132(31.81) | 51(12.29) | 15(3.61) | 220(53.01) | 7.40(1.78) |
| 3 | 270 | 2.20(0.81) | 316(100) | 2.6(0.97) | 160(59.26) | 46.3(17.15) | 30.3(11.22) | 104(38.52) | 14(5.19) |
| 4 | 280 | 1.56(0.56) | 236(84.29) | 5.96(2.13) | 94.0(33.57) | 78.3(27.96) | 17.3(6.18) | 145(51.79) | 20(7.14) |

[a]Adsorption conditions were aeration for 5 min at 30 C.

[b]Values reported are PFU/ml recovered x $10^5$.

[c]Values in parentheses represent percent recoveries of input.

The rate at which 41c adsorbed to host at 10 mM and 0.1 mM $Ca^{++}$ was studied. Five cultures of host cells were infected with 41c at 1 min intervals at input values of 4 x $10^7$ and 9 x $10^5$ PFU/ml, respectively. Immediately following the last phage addition, cultures were centrifuged and supernatants assayed for unadsorbed viruses. The fraction of unadsorbed virus was obtained by dividing the amount of free virus by the initial input of 41c.

At 10 mM $Ca^{++}$ greater than 90% of the virus adsorbed after 1 min at 30 C while 96% had adsorbed after 5 min (Fig. 1). This yielded an adsorption constant of 3.72 x $10^{-9}$ ml/min and 4.82 x $10^{-9}$ ml/min for experiments 1 and 2 respectively. Adsorption at 0.1 mM $Ca^{++}$ (Fig 2) was found to be significantly slower with less than 50% of the virus adsorbing after 1 min. However, efficient adsorption had occurred after 5 min with 70% of the virus becoming cell-associated. The adsorption constants at this concentration of $Ca^{++}$ were 1.28 x $10^{-9}$ ml/min, 1.10 x $10^{-9}$ ml/min and 1.46 x $10^{-9}$ ml/min, respectively, for 3 experimental runs.

## Survival of Phage-Bacterium Complexes
### Formed in TP-2 Medium

Since adsorption was found to be efficient at 0.1 mM $Ca^{++}$ but recovery of adsorbed virus greatly reduced, experiments were designed to establish the time period at which complexes aborted in the absence of $Ca^{++}$. Cultures were infected with 41c in TP-2 broth at an MOI of 0.5 for 5 min
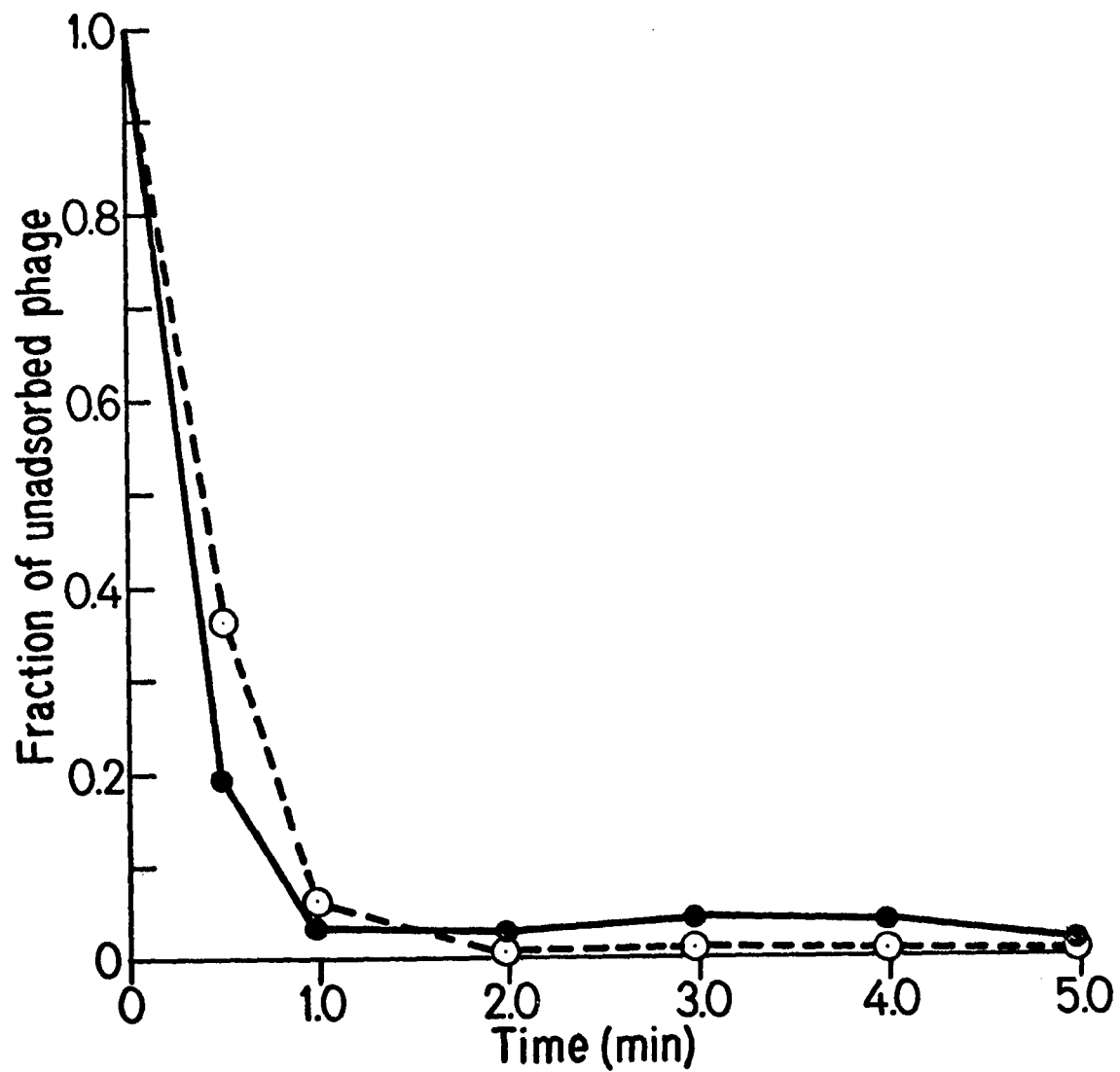
Fig. 1. Adsorption Kinetics of 41c in TP-1 Medium.
Symbols: ●, Experiment 1; ○, Experiment 2.
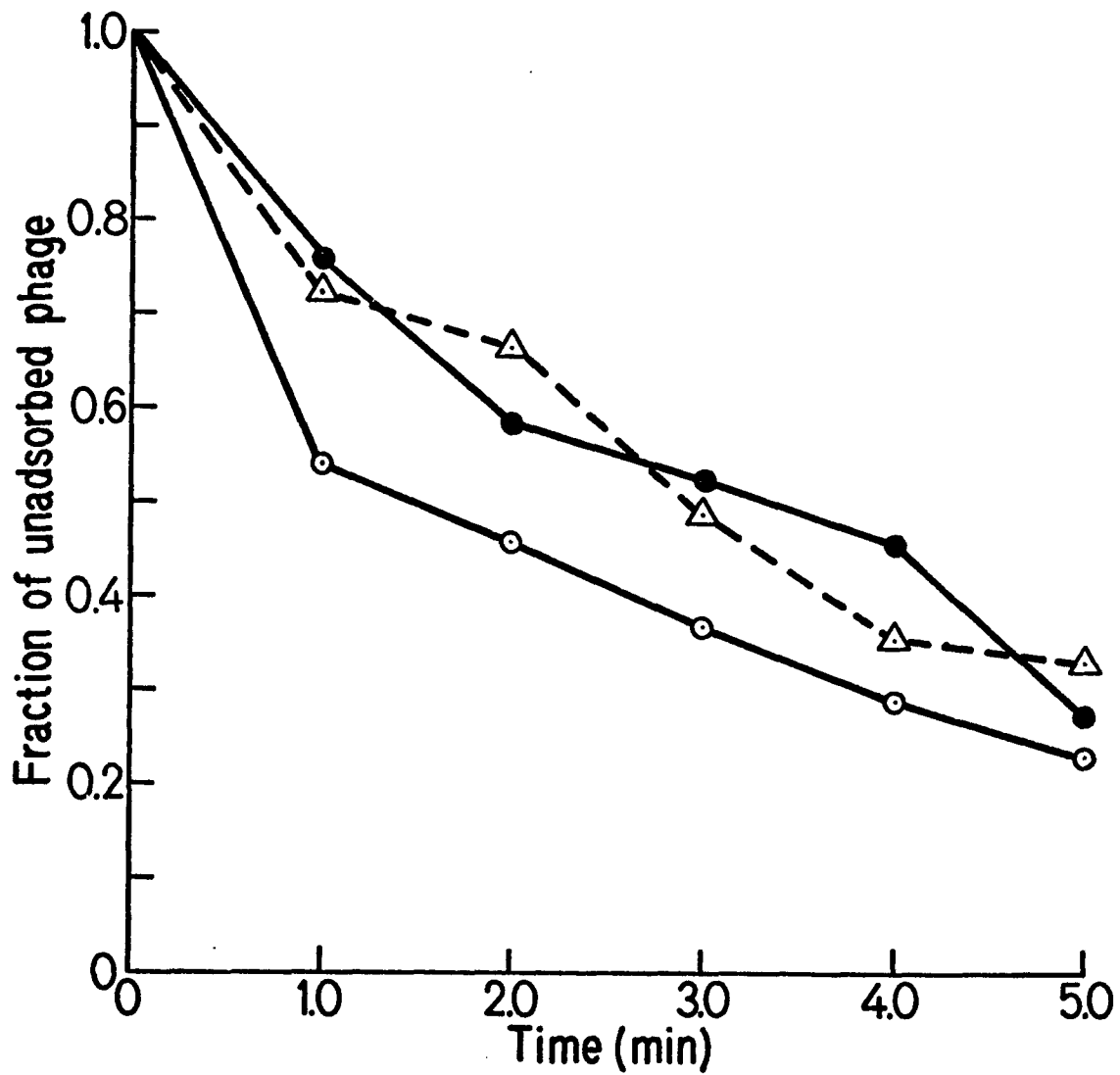
Fig. 2. Adsorption Kinetics of 41c in TP-2 Medium.
Symbols: ●, Experiment 1; △, Experiment 2; ⊙, Experiment 3.

at 30 C.  The culture was centrifuged to remove any free
virus and the pellet resuspended in TP-3 media.  Aeration
was continued and samples taken at 7, 10, 15 and 20 min.
The results (Table 6) demonstrated that fewer than 9% of the
infected cells could be recovered when $Ca^{++}$ is replaced
after 7 min of infection indicating that virus inactivation
occurred very early in the infection cycle.

To pinpoint the exact time when inactivation occurred,
cells were infected with 41c for 0.5, 1, 2, 3, 4 and 5 min
in TP-2 broth, centrifuged and assayed.  The theoretical
amount of adsorbed virus was calculated and compared to the
number of infected complexes recovered from the resuspended
pellet.  The results, seen in Table 7, show that the majority
of abortive infections occurred immediately after the first
min of phage addition since 85% of the complexes were lost
at 0.5 min.  Thus, $Ca^{++}$ is required in the early stages of
infection immediately following the adsorption of the virus
to host, presumably penetration.

## Penetration of 41c at 10 mM and 0.1 mM $Ca^{++}$

The penetration process of 41c was investigated util-
izing $^{32}$P-labelled virus.  The labelled virus was purified
initially by ultracentrifugation through a performed CsCl
gradient.  The results of such a fractionation can be seen
in Fig. 3.  Seventy percent of the input virus were recovered
from the entire gradient, banding chiefly about 29 mm from
the top of the gradient.  This band corresponded to fractions

Table 6.  Time of Inactivation of 41c Infected Complexes
in TP-2 Medium.

| Exp. No. | Time of Sampling (min) | PFU/ml Recovered of Adsorbed Virus | Recovery (%) |
|---|---|---|---|
| 1 | 7 | $3.15 \times 10^6$ | 10.60 |
|   | 10 | $2.70 \times 10^6$ | 9.10 |
|   | 15 | $2.95 \times 10^6$ | 10.00 |
|   | 20 | $3.50 \times 10^6$ | 11.80 |
| 2 | 7 | $5.75 \times 10^6$ | 6.05 |
|   | 10 | $4.55 \times 10^6$ | 4.79 |
|   | 15 | $5.25 \times 10^6$ | 5.53 |
|   | 20 | $3.45 \times 10^6$ | 3.63 |

Table 7. Time of Inactivation of 41c Infected Complexes in TP-2 Medium.

| Time of Sample (min) | PFU/ml Recovered as[a] | | | Survival of Adsorbed Phage (%) |
| | Unadsorbed Phage | Adsorbed Phage | Surviving Phage | |
| --- | --- | --- | --- | --- |
| 0.5 | 78[b](67)[c] | 6.5(22) | 2.6(3.5) | 40(15.68) |
| 1.0 | 61.5(48.5) | 23(40.5) | 1.8(1.9) | 7.83(4.69) |
| 2.0 | 56(40.5) | 28.5(48.5) | 2.05(1.75) | 7.19(3.61) |
| 3.0 | 41(33) | 43.5(56) | 2.2(2.9) | 5.06(5.18) |
| 4.0 | 34.5(25) | 50(64) | 1.75(3.2) | 3.50(5.0) |
| 5.0 | 28(20.5) | 56.5(68.5) | 1.95(4.20) | 3.45(6.13) |

[a]Values reported are PFU/ml x $10^4$.

[b]Input value for experiment 1 was 8.45 x $10^5$ PFU/ml.

[c]Values in parentheses are for experiment 2 with input value of 8.90 x $10^5$ PFU/ml.
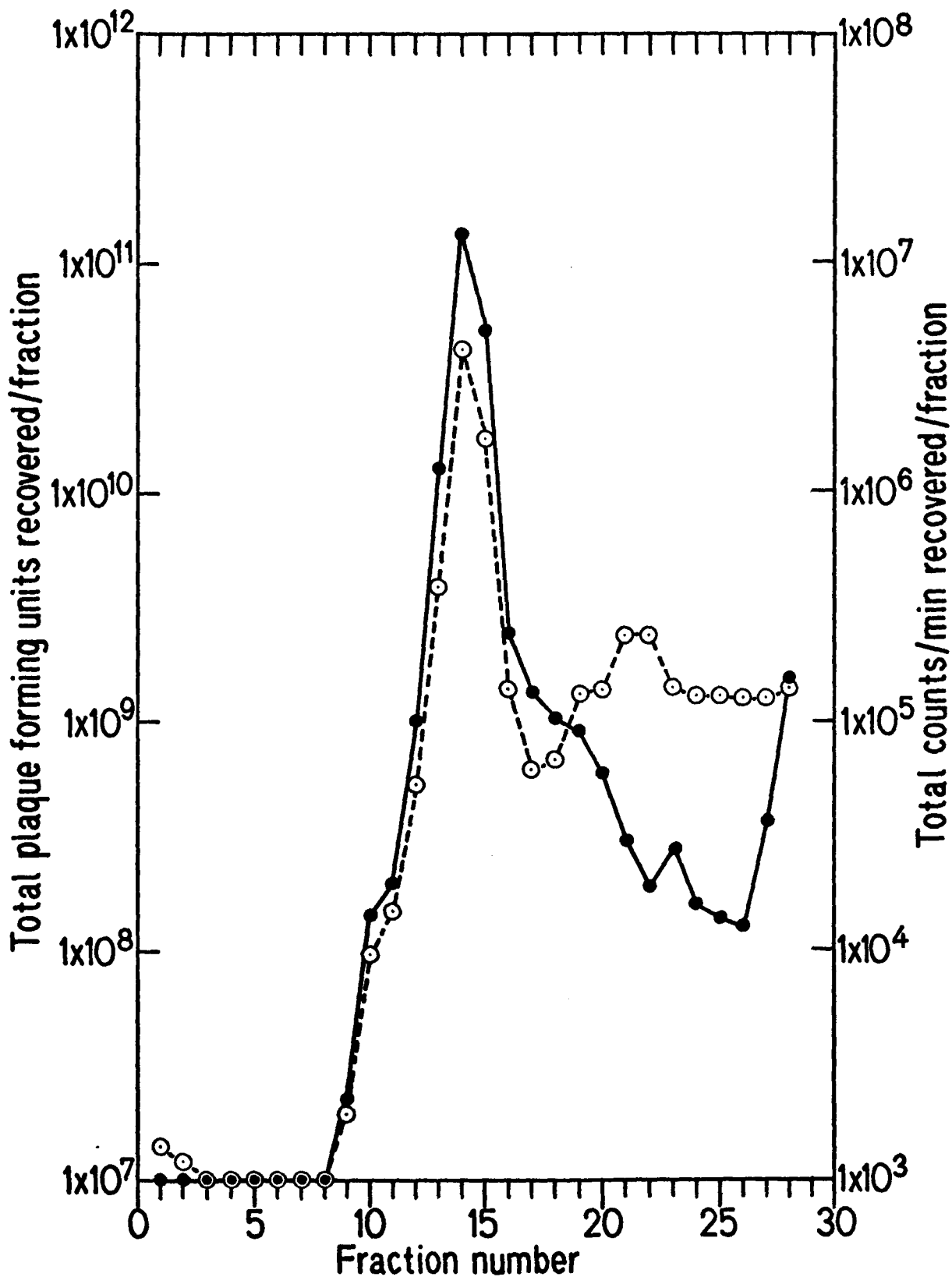
Fig. 3. CsCl Purification of $^{32}$P-Labelled 41c. Symbols: ●, Total PFU/Fraction; ⊙, Total CPM/Fraction.

14 and 15 which showed peak biological and radiological

activity and accounted for 88% of the recoverable virus.

These two fractions were pooled, dialyzed and used as stocks

for penetration studies.

Prior to determining the extent of DNA penetration at

10 mM and 0.1 mM $Ca^{++}$, it was necessary to eliminate the

possibility that blending might influence the survival of

virus and/or host. Consequently, log phase cells were blended

for 5 min at 115 volts at 2 C and samples assayed for CFU

before and after blending. In a similar manner, 5 ml of 41c

stock (5 x $10^7$ PFU/ml) were subjected to the same treatment

and assayed for PFU. The results (Table 8) demonstrated that

blending failed to have any effect on the survival of either

uninfected host cells or free virus.

Penetration of phage nucleic acid into the host can

be determined by blender treatment. During blending of phage-

host complexes, the tail of the virus is sheared releasing

most of the protein coat and any uninjected nucleic acid

from the host cell. Subsequent centrifugation will sediment

infected cells containing injected DNA while uninjected DNA

will remain in the supernatant fluids.

Experimentally, log phase cells were infected with

labelled 41c for 15 min with aeration at 30 C in TP-1 and

TP-2 media. Following centrifugation, the supernatants were

decanted and assayed for unadsorbed PFU and CPM. The pellets

were resuspended to the original volume of TP-1 and TP-3, re-

spectively and assayed for adsorbed radioactivity and biolog-

Table 8.  Survival of Phage 41c and Host Following
          Blender Treatment.

| Exp. No. | Treatment None | Blended | Host CFU/ml Recovered | Phage PFU/ml Recovered |
|---|---|---|---|---|
| 1 | + | − | $2.38 \times 10^8$ | $4.80 \times 10^7$ |
|   | − | + | $2.56 \times 10^8$ | $4.95 \times 10^7$ |
| 2 | + | − | $3.60 \times 10^8$ | $3.56 \times 10^9$ |
|   | − | + | $4.46 \times 10^8$ | $3.16 \times 10^9$ |

ical activity.  The theoretical amount of adsorbed virus was

obtained by subtracting the supernatant value from the input

value.

At 10 mM Ca$^{++}$ (Table 9) only 1% of the viruses were

found in the supernatant fluids indicating biological adsorp-

tion of 99% while 83% of the radiological activity became

cell-associated.  Adsorption of labelled virus was also

efficient at 0.1 mM Ca$^{++}$ with 95% of the virus and 71% of

the radioactivity adsorbing to host cells after 15 min

(Table 10).  Blender experiments were based on the amount

of adsorbed viruses rather than the initial input value since

100% of the label and virus did not become cell-associated.

To determine the amount of penetration completed

after 15 min of infection, 4 ml of adsorbed complexes were

blended at 115 volts for 5 min at 2 C.  The blended cultures

(3 ml) were centrifuged and the pellets resuspended in 3 ml

of TP-1 or TP-3 media.  Both supernatant fluids and pellets

were then assayed for PFU and CPM.  As controls, additional

samples were taken prior to blending.

At 10 mM Ca$^{++}$, almost all of the adsorbed complexes

were recovered as infectious centers (Table 11).  After

blending and centrifugation, less than 1% of the phage was

found in the supernatant while approximately 35% of the

adsorbed label was released.  This released label can be

attributed to a portion of the phage population which attached

but failed to inject its DNA.  On the other hand, the blended

pellet retained 83% of the complexes and corresponded to 48%

Table 9. Adsorption of $^{32}$P-Labelled 41c to Host Cells at 10 mM Ca$^{++}$.

| Exp. No. | PFU/ml Recovered from | | | CPM Recovered from | | |
|---|---|---|---|---|---|---|
| | Input | Supernatant | Pellet[a] | Input | Supernatant | Pellet |
| 1 | $3.50 \times 10^6$ (100)[b] | $5.50 \times 10^4$ (1.57) | $3.44 \times 10^6$ (98.43) | 409 (100) | 38 (9.28) | 400 (97.76) |
| 2 | $2.80 \times 10^7$ (100) | $2.95 \times 10^5$ (1.05) | $2.77 \times 10^7$ (98.95) | 4091 (100) | 251 (6.15) | 3145 (76.86) |
| 3 | $2.20 \times 10^7$ (100) | $2.45 \times 10^5$ (1.11) | $2.17 \times 10^7$ (98.89) | 4091 (100) | 231 (5.66) | 3071 (75.06) |

[a]Values reported in Pellet are theoretical amount of adsorbed virus obtained by subtracting supernatant values from the input.

[b]Values in parentheses are percent recovery of the input.

Table 10. Adsorption of $^{32}$P-Labelled 41c to Host Cells at 0.1 mM $Ca^{++}$.

| Exp. No. | PFU/ml Recovered from | | | CPM Recovered from | | |
|---|---|---|---|---|---|---|
| | Input | Supernatant | Pellet[a] | Input | Supernatant | Pellet |
| 1 | $3.50 \times 10^6$ (100)[b] | $1.80 \times 10^5$ (5.14) | $3.32 \times 10^6$ (94.86) | 409 (100) | 150 (36.66) | 335 (81.99) |
| 2 | $2.80 \times 10^7$ (100) | $4.50 \times 10^5$ (1.60) | $2.76 \times 10^7$ (98.40) | 4091 (100) | 886 (21.66) | 2968 (72.55) |
| 3 | $2.20 \times 10^7$ (100) | $1.28 \times 10^6$ (5.81) | $2.07 \times 10^7$ (94.19) | 4091 (100) | 1123 (27.46) | 2452 (59.94) |

[a]Values are theoretical amount of adsorbed virus calculated by subtracting number unadsorbed virus from the input.

[b]Values in parentheses are percent recovery of the input.

Table 11. Biological and Radiological Assays of Blended Complexes Formed with $^{32}$P-Labelled 41c: effect of 10 mM Ca$^{++}$.[a]

| Exp. No. | PFU/ml Recovered from (x10$^4$) | | | CPM Associated with | | |
|---|---|---|---|---|---|---|
| | Unblended Control | Blended Supernatant | Blended Pellet | Unblended Pellet | Blended Supernatant | Blended Pellet[b] |
| 1 | 305 (88)[c] | 1.40 (<1) | 365 (100) | 400 (100) | 150 (37) | 220 (55) |
| 2 | 2800 (100) | 5.95 (<1) | 2100 (76) | 3145 (100) | 1094 (34) | 1549 (49) |
| 3 | 2200 (100) | 6.5 (<1) | 1500 (70) | 3071 (100) | 1027 (33) | 1224 (39) |

[a]Values are based on the amount of adsorbed virus and label equalling 100% (Table 9).

[b]Maximum injectable label that can penetrate at 10 mM Ca$^{++}$.

[c]Values in parentheses are percent recoveries of adsorb PFU or CPM.

of the adsorbed label being cell-associated.  Since penetration must be complete for complexes to survive blender treatment, the pellet-associated label represented the maximum injectable $^{32}P$ needed to establish complete (or near complete) penetration.

Results of similar experiments performed with cultures supplemented with 0.1 mM $Ca^{++}$ are shown in Table 12.  The adsorption patterns obtained were comparable to those observed with 10 mM $Ca^{++}$.  However, under these reduced $Ca^{++}$ conditions, only 10% of the adsorbed complexes established successful infection before or after blender treatment.  The radiological data indicated that the majority (92%) of the label was sheared from the host cells on blending while a small percentage of the adsorbed label remained cell-associated.  To account for the labelled virus seen in the previous experiment which adsorbed but failed to penetrate, the maximum injectable label was calculated.  This value (which represents the 100% level) was obtained by multiplying the amount of adsorbed label by the average label transferred under optimal conditions, this being 48.04% (Table 11).  Using this calculation only 21% of the DNA became blender-resistant indicating that penetration of phage 41c was dependent on 10 mM $Ca^{++}$.

To determine the nature of the material released by blending, the supernatant fluids were precipitated with cold 5% trichloroacetic acid (TCA).  Because most (80%) of the label was TCA precipitable, the phage DNA was not extensively degraded and was in the form of large molecular weight components.

Table 12. Biological and Radiological Assays of Blended Complexes Formed with $^{32}$P-Labelled 41c:  effect of 0.1 mM Ca$^{++}$.

| Exp. No. | PFU/ml Recovered from $(\times 10^4)$[a] | | | | CPM Associated with | | |
| | Unblended Control | Blended Supernatant | Blended Pellet | Unblended Pellet | Maximum Injectable Label[b] | Blended Supernatant | Blended Pellet |
|---|---|---|---|---|---|---|---|
| 1 | 40 (12)[c] | 0.5 (<1) | 30 (9) | 335 | 184 (100) | 332 | 21 (11) |
| 2 | 295 (10) | 0.6 (<1) | 235 (9) | 2968 | 1462 (100) | 2439 | 378 (25) |
| 3 | 205 (10) | 2.6 (<1) | 130 (6) | 2452 | 977 (100) | 2335 | 274 (28) |

[a]Values are based on the amount of adsorbed virus and label equalling 100% (Table 10).

[b]Maximum injectable label is calculated by multiplying the absorbed label in the unblended pellet by 48.04% (average % label injecting at 10 mM Ca$^{++}$; see Table 11).

[c]Value in parentheses represent average percent recoveries of adsorbed PFU or injectable CPM.

## Irreversibility of the Penetration Block

Roundtree (1955) observed that delayed penetration could be resumed by the addition of $Ca^{++}$. Therefore, it seemed possible that addition of 10 mM $Ca^{++}$ to cultures initially infected at 0.1 mM $Ca^{++}$ might stimulate the transfer of 41c DNA remaining outside the cell. Consequently, 10 ml of log phase cells were infected with 41c at 0.1 mM $Ca^{++}$ for 15 min at 30 C. Following centrifugation, the supernatant fluid was decanted and assayed for unadsorbed PFU and CPM. The pellet was resuspended in 10 ml of TP-3 broth and assayed for adsorbed label and surviving infected complexes. The culture was then divided into two 5 ml aliquots. One portion was blended immediately and assayed while the second was supplemented with 10 mM $Ca^{++}$ and reincubated at 30 C for 15 min, blended and sampled. The biological results are presented in Table 13 while the radiological data appear in Table 14.

Adsorption and survival patterns in the unsupplemented culture were comparable to those previously observed (Tables 10 and 12). Eighty eight percent of the virus adsorbed representing 54% of the input label while 12% of the PFU and 37% of the label remained unadsorbed. Only 16% of the injectable $^{32}P$ was cell-associated after blending resulting in only 5% of the infected complexes surviving.

In cultures supplemented with 10 mM $Ca^{++}$, the amount of blender-resistant infected centers rose to 14% while the

Table 13.  Irreversibility of the Penetration Block on Delayed Addition of 10 mM $Ca^{++}$:
Biological Data.[a,b]

| Exp. No. | PFU/ml Recovered from | | | PFU/ml Recovered after 10 mM $Ca^{++}$ Addition | |
| | Unblended Control | Blended Supernatant | Blended Pellet | Blended Supernatant | Blended Pellet |
|---|---|---|---|---|---|
| 1 | 190 (3)[c] | 7 (<1) | 150 (2) | 6.8 (<1) | 180 (3) |
| 2 | 595 (12) | 55 (1) | 350 (7) | 35 (<1) | 515 (10) |
| 3 | 885 (8) | 10 (<1) | 590 (5) | 153 (1) | 3210 (28) |

[a]Cultures infected in TP-2 medium.

[b]Values reported as PFU/ml x $10^4$.

[c]Values reported in parentheses represent percent recoveries of adsorbed complexes which were 5280, 4960 and 11,200 x $10^4$ PFU/ml, respectively for 3 experimental runs.

Table 14. Irreversibility of Penetration Block on Delayed Addition of 10 mM $Ca^{++}$: Radiological Data.[a]

| Exp. No. | CPM Associated with | | | CPM Associated with after 10 mM $Ca^{++}$ Addition |
|---|---|---|---|---|
| | Centrifuged Pellet[b] | Maximum Injectable Label[c] | Blended Pellet | Blended Pellet |
| 1 | 2829 | 1359 (100)[d] | 152 (5) | 483 (17) |
| 2 | 2508 | 1205 (100) | 225 (8) | 472 (18) |
| 3 | 2237 | 1075 (100) | 200 (8) | 377 (16) |

[a]Cultures infected in TP-2 medium.

[b]These values represent cell-associated $^{32}P$; Input values were 4782, 4586 and 4540 for 3 experiments, respectively.

[c]Calculated from adsorbed label x 48.04% (see Table 9).

[d]Values in parentheses represent percent recoveries of injectable label.

amount of cell-associated label increased from 16% to 36%. This indicated that some of the phage which failed to inject at 0.1 mM $Ca^{++}$ could be stimulated to resume the injection process of addition of optimal levels of this ion. Since only slight increases in plaquing centers were noted after $Ca^{++}$ supplementation, the inhibitory effect produced at low $Ca^{++}$ levels cannot be reversed by subsequent increases in this ion.

## Entry Time of 41c DNA

To follow the course of penetration with time, complexes infected at 10 mM ion were blended 2 min after the initiation of infection. The results (Table 15) showed that penetration is apparently completed by 2 min since all the complexes were resistant to treatment.

Additional experiments utilizing DNase were performed to confirm the early entry of the nucleic acid. Host preparation were infected with 41c in TP-1 media and treated with (200 µg/ml) DNase before and 2 min after initiation of infection. The complexes were aerated for 15 min at 30 C and assayed for PFU. Penetration was effectively inhibited (Table 16) if DNase was present at zero time (Zsigray et al., 1973). However, addition of the enzyme 2 min after initiation of infection had no effect on the PFU recovered since penetration was completed prior to DNase addition.

Table 15.   Resistance of Phage-Bacterium Complexes to
            Shearing Forces 2 Min after Infection.[a]

| Exp. No.[b] | Type of Treatment | | PFU/ml Recovered | Recovery After Blending (%) |
|---|---|---|---|---|
| | Blending | $CHCl_3$ | | |
| 1 | - | - | $5.15 \times 10^7$ | |
| | - | + | $2.00 \times 10^6$ | |
| | + | - | $4.90 \times 10^7$ | 97.97 |
| | + | + | $5.21 \times 10^5$ | |
| 2 | - | - | $5.25 \times 10^7$ | 100 |
| | - | + | $1.05 \times 10^6$ | |
| | + | - | $5.95 \times 10^7$ | |
| | + | + | $1.00 \times 10^5$ | |
| 3 | - | - | $5.30 \times 10^7$ | |
| | - | + | $7.50 \times 10^5$ | 95.60 |
| | + | - | $5.05 \times 10^7$ | |
| | + | + | $5.00 \times 10^5$ | |

[a]Optimal conditions (TP-1 medium) were employed.

[b]Input for all experiments = $5.00 \times 10^7$ PFU/ml.

Table 16. Effect of DNase on Phage 41c Penetration.[a]

| Time of Treatment (min) | Type of Treatment | | PFU/ml Recovered |
|---|---|---|---|
| | None | DNase | |
| 0 | + | − | $6.43 \times 10^7$ |
| | − | + | $1.48 \times 10^7$ |
| 2 | + | − | $6.20 \times 10^7$ |
| | − | + | $6.25 \times 10^7$ |

[a]DNase final concentration was 200 μg/ml.

## One Step Growth Curve

Previous experiments had indicated that a small fraction of the virus population was capable of establishing successful infection at 0.1 mM $Ca^{++}$. Since penetration is complete in these complexes, they should exhibit a normal one step growth curve. To verify this assumption host cultures (4.5 ml) were infected with 41c (0.5 ml) at an MOI of 0.5 in TP-1 and TP-2 media for 15 min at 30 C. Cultures were centrifuged to remove any unadsorbed virus and the pellets resuspended in TP-1 and TP-3, respectively. Dilutions were made in appropriate media and the $10^{-4}$ through $10^{-7}$ dilutions aerated at 30 C and sampled over a 180 min period.

The growth curve of 41c at 10 mM $Ca^{++}$ (Fig. 4) exhibited a latent period of 50 min and a burst size of 324. On the other hand, at 0.1 mM $Ca^{++}$, a 90% reduction in the number of infected complexes was noted. Complexes completing penetration at this $Ca^{++}$ level exhibited a latent period of 70 min and a burst of 192. These results implied that $Ca^{++}$ not only affected adsorption and penetration but also influenced the intracellular replication of the virus since reduced $Ca^{++}$ levels caused an increased latent period and a reduced burst.

## Effect of EDTA on Post-Penetration 41c Infection

To investigate the cause of the delayed latent period and reduced burst observed at reduced $Ca^{++}$ levels, experiments utilizing a chelating agent were initiated. The
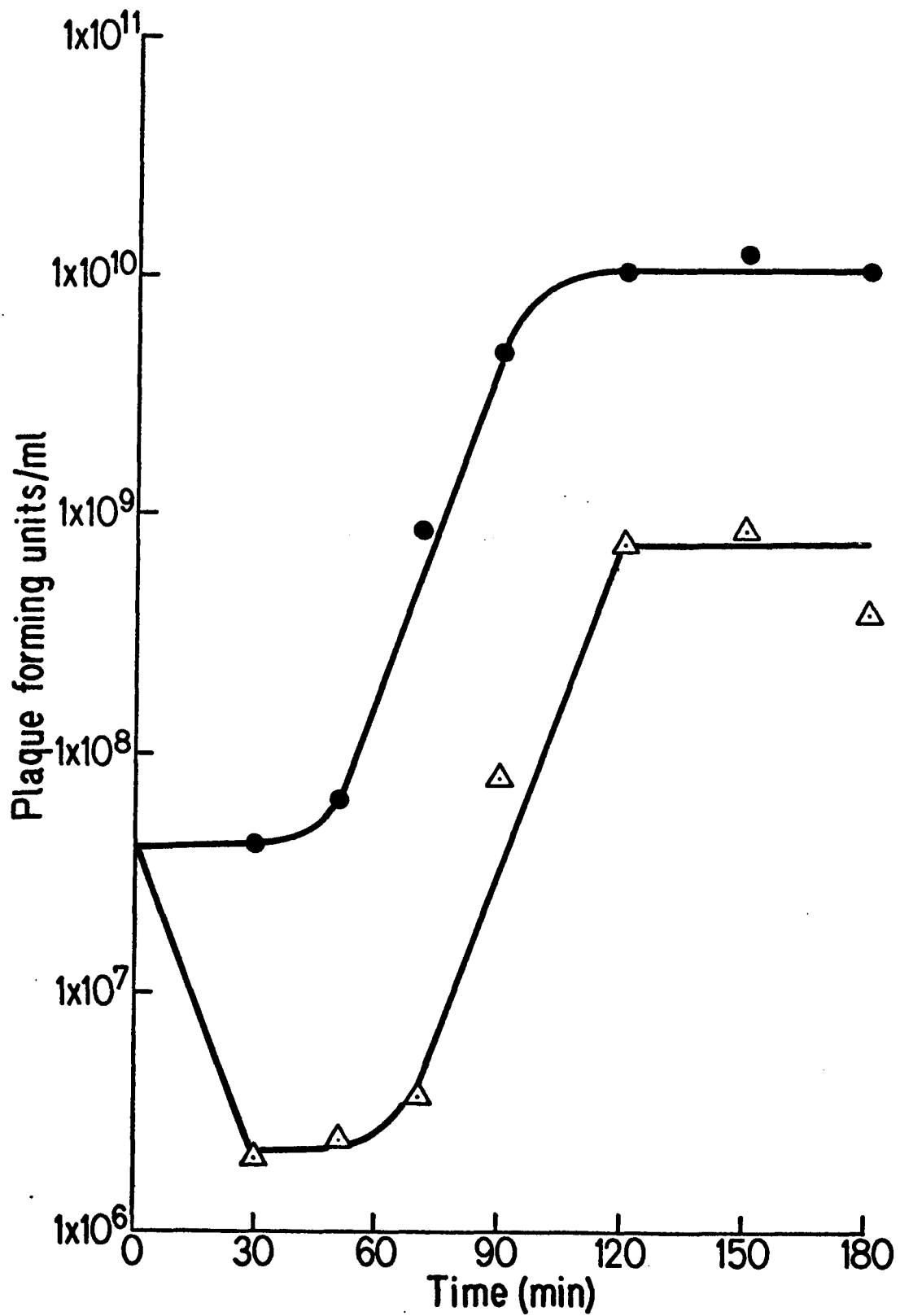
Fig. 4.   One Step Growth Curve of 41c in TP-1 and TP-2
Media.   Symbols:   ●, 10 mM $Ca^{++}$, Δ, 0.1 mM $Ca^{++}$:

addition of equimolar concentrations of EDTA to infected

complexes would act to remove $Ca^{++}$ from the system thus making

them unavailable for phage infection. By varying the time

of EDTA addition, the time of a post-penetration $Ca^{++}$ require-

ment could be determined.

A series of log phase cultures were infected with 41c

(MOI of 0.5) in TP-1 medium. The cultures were aerated for

5, 15 or 30 min at which time 10 mM EDTA was added and incu-

bation continued for 5 min before sampling. The results

(Table 17) indicated that irrespective of the time of EDTA

addition, 41c infected complexes appeared to establish

successful infections. This suggested that either $Ca^{++}$ was

not necessary for intracellular phage development following

penetration or that the post-penetration, $Ca^{++}$ requiring

stage had been completed prior to EDTA addition.

The chelator was then added to infected cells 1 min

after the initiation of infection and followed over a 180

min period. As controls, one step growth curves were also

performed at 10 mM and 0.1 mM $Ca^{++}$. Under optimal conditions,

a normal burst of 353 was noted (Fig. 5) while at 0.1 mM

$Ca^{++}$, a rapid inactivation of infectious complexes occurred

within 15 min, followed by a reduced burst of 153. In cul-

tures supplemented with EDTA 1 min after infection, various

inactivation values were obtained within 15 min with 46% and

18% of the complexes being inactivated in 2 experiments.

This inactivation and lack of burst were presumably due to

the chelation of $Ca^{++}$ necessary for the completion of pene-

Table 17.  Effect of EDTA Addition on 41c Infected
Complexes in TP-1 Medium.[a]

| Time of EDTA Addition (min) | PFU/ml Recovered from | |
| | $Ca^{++}$-Supplemented Infection | $Ca^{++}$-Supplemented Infection + EDTA |
| --- | --- | --- |
| 5 | $4.00 \times 10^7$ | $3.55 \times 10^7$ |
| 15 | $3.80 \times 10^7$ | $4.45 \times 10^7$ |
| 30 | $3.20 \times 10^7$ | $4.00 \times 10^7$ |

[a]Input = $4.15 \times 10^7$ PFU/ml.
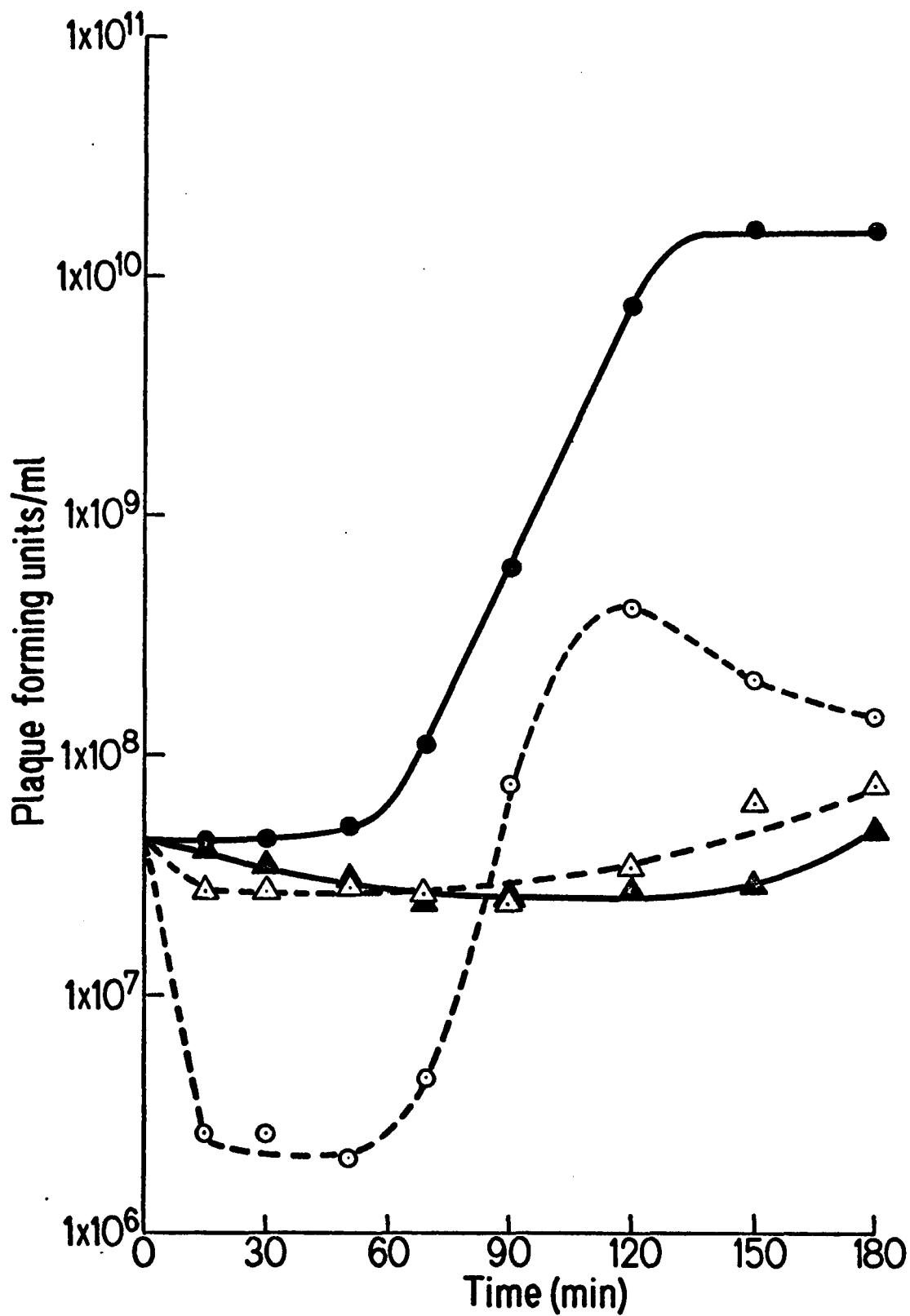
Fig. 5. Effect of Chelating Ca$^{++}$ with EDTA at 1 Min after the Initiation of Infection. Symbols: ●, 10 mM Ca$^{++}$; ⊙ 0.1 mM Ca$^{++}$; △, EDTA Experiment 1; ▲, EDTA Experiment 2.

tration in some complexes. To insure that penetration was complete, two cultures were infected with 41c in TP-1 for 5 and 10 min prior to the addition of chelator and the sample followed over a 180 min period. The 10 mM $Ca^{++}$ control showed a normal one step growth curve with a latent period of 50 min and a burst of 171 (Fig. 6). Cultures treated with EDTA at 5 and 10 min into infection exhibited a slight degree of inactivation (38%) with the surviving complexes failing to yield a burst. This appeared to indicate that $Ca^{++}$ was being removed from infection at some critical stage following the completion of penetration, thereby inhibiting intracellular phage development.

To determine the time at which EDTA addition no longer influenced the lytic cycle, six cultures were infected with 41c at 10 mM $Ca^{++}$ and aerated at 30 C. At 10 min intervals, EDTA was added and all samples assayed after 180 min. It can be seen from Table 18 that addition of the chelator before 30 min resulted in a drastic decrease in the burst size. Later additions of EDTA showed increased burst sizes but in all cases the burst was generally less than half that obtained in the $Ca^{++}$ control indicating that $Ca^{++}$ was needed throughout the entire infection cycle.

### Effect of EDTA on the Growth of Host Cells
### and on the Stability of 41c

Since addition of EDTA 60 min into the infection resulted in a reduced burst, it seemed possible that the chelator might be acting to non-specifically inhibit either
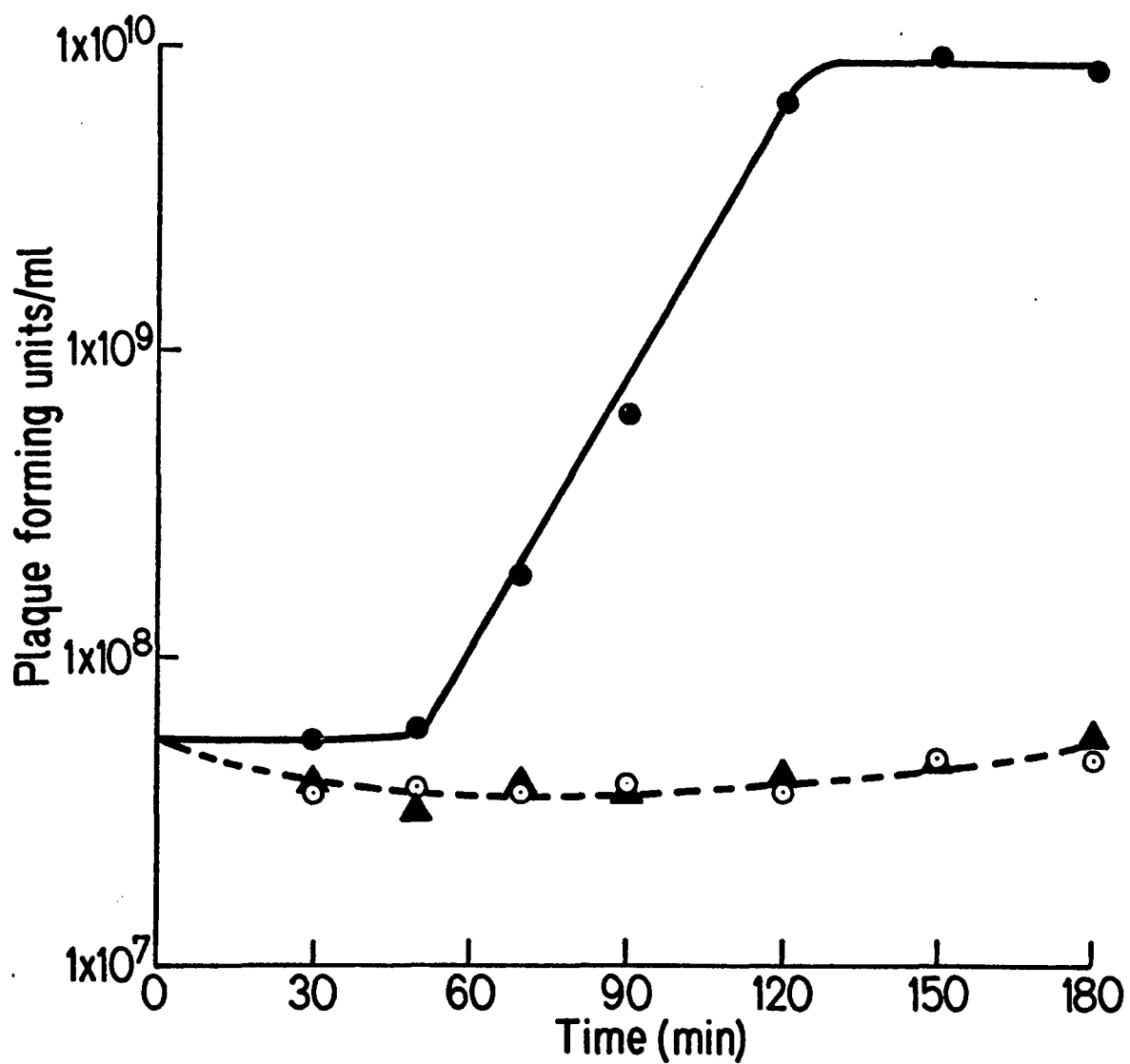
Fig. 6. Effect of Chelating $Ca^{++}$ with EDTA at 5 and 10 Min
after the Initiation of Infection. Symbols: ●, 10 mM $Ca^{++}$;
⊙, EDTA added 5 min after infection; ▲, EDTA added 10 min
after infection.

Table 18.  Effect of EDTA Addition on Burst Size of 41c.

| Exp. | Burst Size Recovered after EDTA Addition at | | | | | | Min |
| No. | 10 | 20 | 30 | 40 | 50 | 60 | No Addition |
| 1[a] | 3.95 | 3.04 | 13.60 | 88 | 63 | 177 | 232 |
| 2[b] | 3.57 | 16.93 | 33.33 | 132 | 84.65 | 86.24 | 204 |

[a]Input $5 \times 10^7$ PFU/ml.

[b]Input $9.45 \times 10^7$ PFU/ml.

the host or the intact virus. Normally, at this time in the lytic cycle, maturation is evident and the phage are in the process of being released from the host. Since these later events were not found to be ion-dependent in other systems (Watanabe and Takesue, 1972; Paranchych, 1966) the possibility of non-specific phage or host inactivation was considered.

EDTA is known to effect the growth rate of bacteria (Leive, 1965). The chelator might be acting to remove critical ions such as $Mg^{++}$ or $Fe^{++}$ which are necessary for cell function as well as phage development. To study the effect of EDTA on the growth of the host, cells were grown in TP-1 to an OD of 0.1 (670 nm) and treated with 10 mM EDTA. Aeration was continued at 37 C and growth observed over a 210 min period. Initially the growth rates observed in the EDTA-supplemented cultures were similar to that observed in the control culture (Fig. 7). However, after 60 min, cultures supplemented with EDTA exhibited a decreased growth rate. Since $Ca^{++}$ is not required for the growth of the host, the decrease in growth rate might represent chelation of $Mg^{++}$ ions necessary for cell function. However, increasing $Mg^{++}$ concentrations to double the EDTA concentration did not alter the growth pattern observed earlier. Since the initial growth rates appeared normal for the first 60 min at which time phage development is in its final stages, inhibition of phage production by EDTA was not considered to be due to inhibition of host cell metabolism.
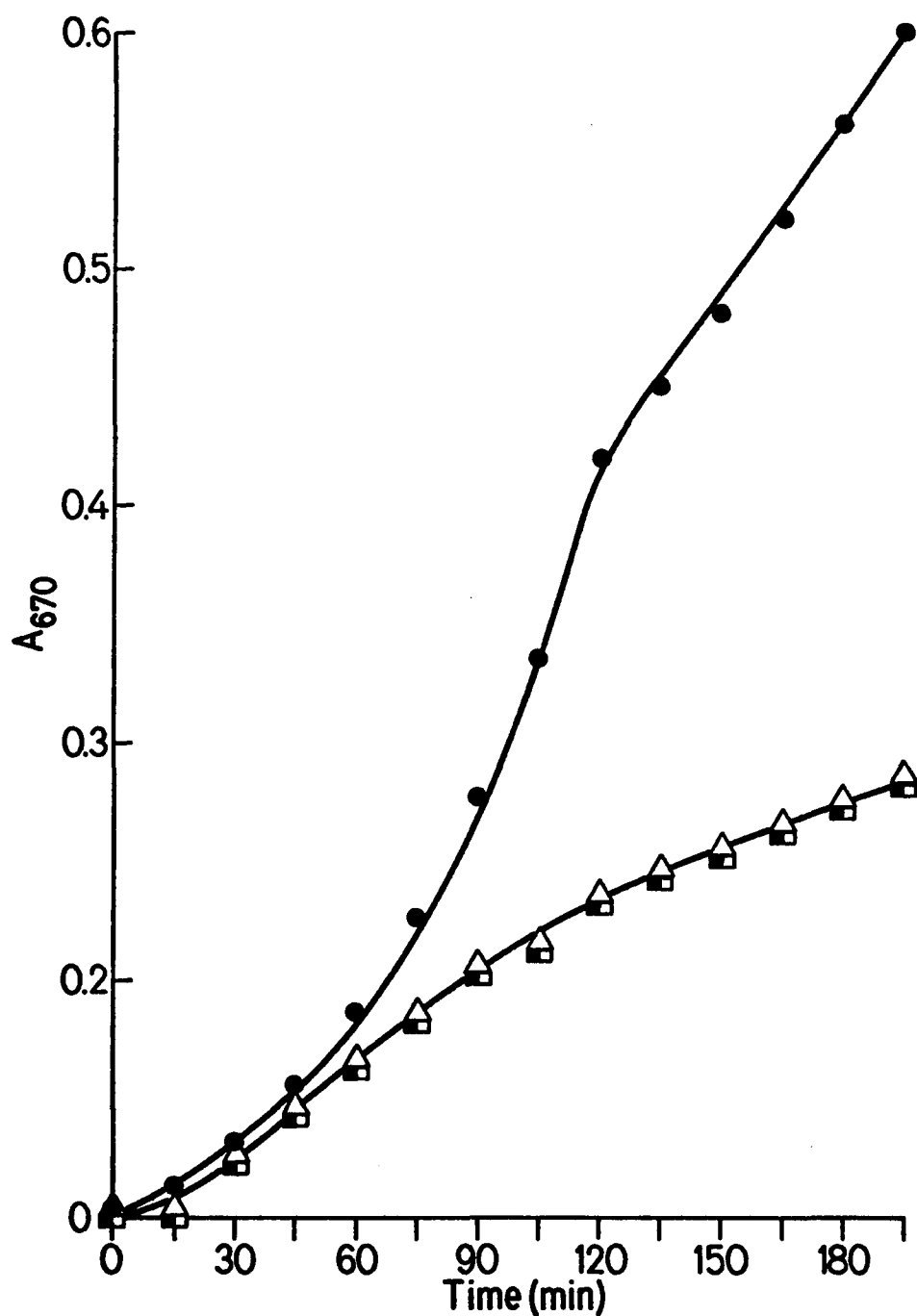
Fig. 7. Effect of EDTA on the Growth of B. subtilis 168.
Symbols: ●, 10 mM $Ca^{++}$, 2 mM $Mg^{++}$; △, 10 mM $Ca^{++}$, 2 mM $Mg^{++}$, 10 mM EDTA; ■, 10 mM $Ca^{++}$, 10 mM $Mg^{++}$, 10 mM EDTA; □, 10 mM $Ca^{++}$, 20 mM $Mg^{++}$, 10 mM EDTA.

An alternate possibility for the reduced bursts seen in EDTA infections might be inactivation of the infectious virus by the chelator. To test this possibility, 41c (5 x $10^7$ PFU/ml) suspended in TP-1 medium was exposed to 10 mM EDTA and assayed at 60 min intervals for 180 min. As controls, the stability of the virus was tested in TP-1, TP-2 and TP-3 media. The results (Table 19) indicated a significant reduction in PFU occurred in the presence of 10 mM EDTA. No significant loss of virus was apparent at 10 mM $Ca^{++}$, 0.1 mM $Ca^{++}$ or in the absence of $Ca^{++}$. Although 50% of the intact phage might be inactivated by EDTA following lysis, this observation does not account for the severe inhibition observed when EDTA is added early in the infection (Fig. 6). Thus, it appears the chelator is removing ions necessary for intracellular phage development.

## Identification of a Third $Ca^{++}$-Dependent Stage

Because a high percentage of virus was inactivated at 10 mM EDTA, an alternate method was sought to verify the existence of a post-penetration, $Ca^{++}$-dependent stage. A possible alternative might be to initiate infection in TP-1 medium for 2 min (allowing penetration to be completed) followed by dilution into TP-3 medium. If a third $Ca^{++}$-dependent stage existed, a 100-fold dilution should result in inhibition. When such an experiment was performed and the infected cultures sampled 30 min after dilution, no differences in recoveries could be noted between the $Ca^{++}$

Table 19. Stability of 41c in 10 mM EDTA.

| Exp. No. | Time (min) | PFU/ml Recovered from | | | |
|---|---|---|---|---|---|
| | | TP-1 Medium | TP-2 Medium | TP-3 Medium | TP-1 + 10 mM EDTA |
| 1 | 60 | $4.85 \times 10^7$ | $5.55 \times 10^7$ | $5.00 \times 10^7$ | $4.05 \times 10^7$ |
| | 120 | $3.85 \times 10^7$ | $5.50 \times 10^7$ | $4.44 \times 10^7$ | $3.15 \times 10^7$ |
| | 180 | $4.65 \times 10^7$ | $4.70 \times 10^7$ | $6.30 \times 10^7$ | $2.55 \times 10^7$ |
| 2 | 60 | $4.95 \times 10^7$ | $6.10 \times 10^7$ | $5.70 \times 10^7$ | $2.60 \times 10^7$ |
| | 120 | $3.65 \times 10^7$ | $4.90 \times 10^7$ | $4.45 \times 10^7$ | $1.80 \times 10^7$ |
| | 180 | $4.20 \times 10^7$ | $3.00 \times 10^7$ | $4.30 \times 10^7$ | $2.10 \times 10^7$ |

control cultures and the diluted samples (Table 20). The lack of inactivation at low $Ca^{++}$ levels seems to indicate that the events following penetration were independent of $Ca^{++}$.

Although complexes diluted in $Ca^{++}$-free media showed no inactivation after 30 min, it was possible that such complexes would fail to produce a burst in the absence of $Ca^{++}$. Therefore, cells infected with 41c in TP-1 medium, were diluted into $Ca^{++}$-free medium at 1, 3.5 and 6 min after initiation of infection and sampled over a 180 min period. In TP-1 medium, a latent period of 50 min and a burst of 252 were observed (Fig. 8). Cultures diluted at 1 min showed some virus inactivation presumably due to lack of penetration in the absence of $Ca^{++}$. However, the surviving complexes were only able to give rise to a burst of 33. Complexes diluted at 3.5 min displayed no immediate inactivation on dilution; however, these complexes failed to exhibit a normal one step growth curve and released only 26 viral particles per infected cell. When the time of dilution was delayed until 6 min after the initiation of infection, a normal one step growth curve was observed resulting in a burst size of 238. These data indicated that there is an intracellular $Ca^{++}$-dependent process occurring between 1 min and 6 min after the onset of infection.

Although intracellular development was affected, it was not known whether all complexes were exhibiting a low-level production and release of virus or if the majority

Table 20.   Survival of 41c Infected Complexes Diluted
            100-Fold into TP-3 Medium 2 Min Post-Infection.

| Exp. No. | Input (PFU/ml) | PFU/ml Recovered after 100-Fold Dilution into | |
| --- | --- | --- | --- |
| | | TP-1 Medium | TP-3 Medium |
| 1 | $2.10 \times 10^7$ | $2.72 \times 10^7$ | $2.54 \times 10^7$ |
| 2 | $3.95 \times 10^7$ | $3.85 \times 10^7$ | $3.80 \times 10^7$ |
| 3 | $3.95 \times 10^7$ | $3.40 \times 10^7$ | $4.05 \times 10^7$ |
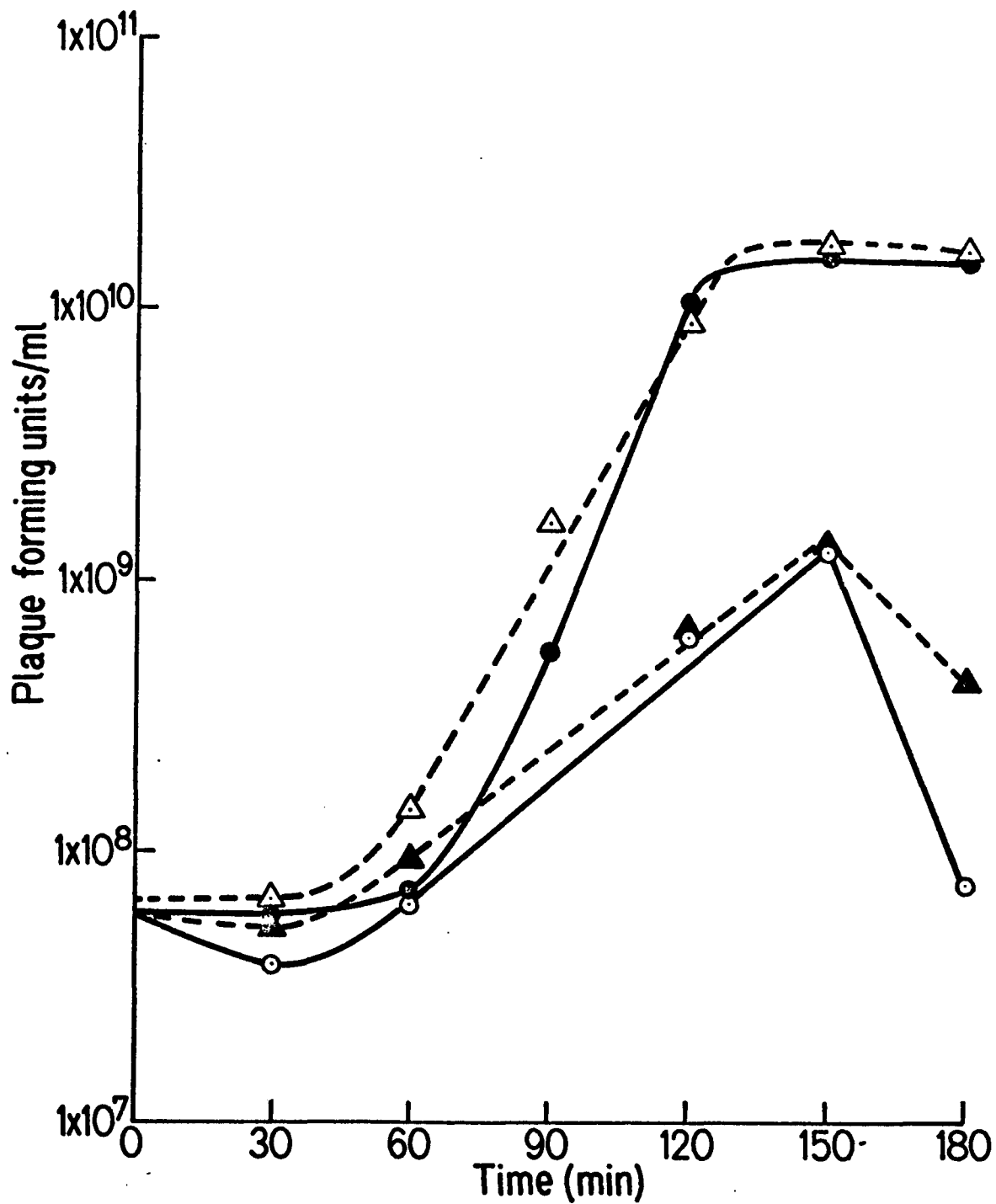
Fig. 8.  Effect of Dilution of Ca$^{++}$-Supplemented Infected Complexes at 1, 3.5 and 6 Min into TP-3 Medium.  Symbols: ●, 10 mM Ca$^{++}$;  ⊙, 1 min diluted complexes; ▲, 3.5 min diluted complexes, Δ, 6 min diluted complexes.

of the complexes were completely inhibited at this intra-
cellular phase.  In such a case, a complete lytic cycle
produced by a small fraction (10%) of the infected complexes
would appear erroneously as a reduced burst.  This hypothe-
sis was tested by the use of phage specific antiserum.  If
all infected cells were producing low-level lysis, the anti-
serum would inactivate rapidly the released phage.  Alter-
nately, if only a few infected cells were releasing a normal
burst, the released virus would be neutralized while the
majority of the infected complexes would survive antibody
treatment.  Consequently, log phase cultures were infected
with 41c in TP-1 medium and diluted 100-fold into TP-1 or
TP-3 medium 2.5 min after infection.  Additional dilutions
were made in media supplemented with antiserum.  All cul-
tures were aerated at 30 C and assayed over a 210 min period.
The $Ca^{++}$ control yielded a burst of approximately 1000 while
antiserum treatment rapidly inactivated the virus as they
were being released (Fig. 9).  TP-3 diluted cultures exhib-
ited a burst size of 150 with antiserum treatment effectively
neutralizing the released phage.  This indicated that all
infected cells were apparently allowing low-level phage pro-
duction rather than complete inhibition of phage development.

Effect of Protoplasting and DNase Treatment

on TP-3 Diluted Complexes

Since the post-penetration $Ca^{++}$ requirement occurs
between 1 min and 6 min after the initiation of infection,
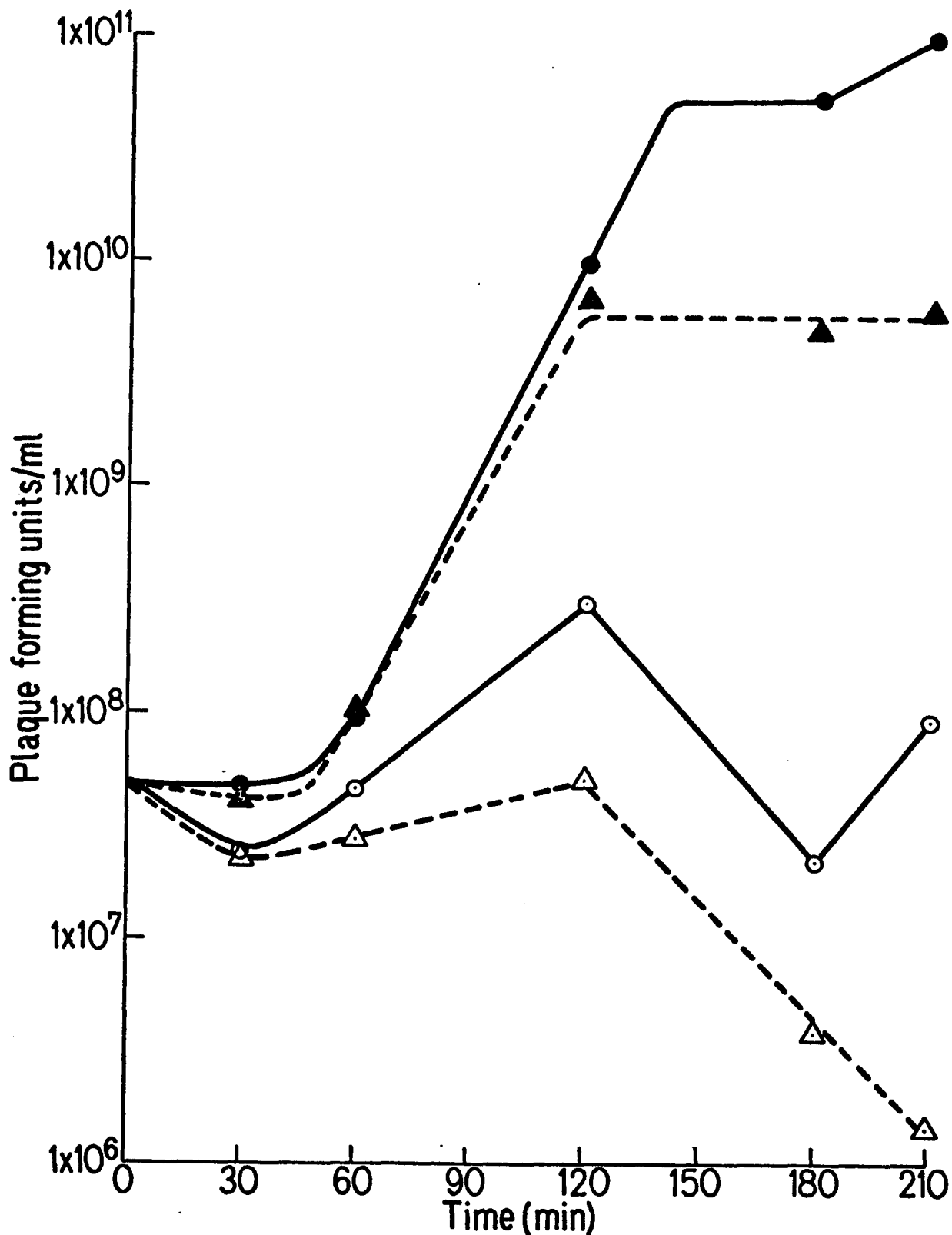
Fig. 9. The Effect of Phage Antibody on the One Step Growth
Curve of Phage 41c Following Dilution into TP-1 and TP-3.
Symbols: ●, TP-1 diluted culture; ⊙, TP-1 diluted culture
+ antibody; ▲, TP-3 diluted culture; △, TP-3 diluted culture
+ antibody.

the event must occur shortly after the completion of penetration to a blender-resistant stage. Penetration can be a two-stage process with the DNA initially being injected into the periplasmic space between the wall and membrane (Simon and Anderson, 1967). At this step the complexes would be resistant to blender treatment but the viral genome still extracycloplasmic. The second stage involves the transport of the nucleic acid across the membrane into the cell proper. It is possible that in 41c penetration, this latter stage might require $Ca^{++}$ and the removal of this ion early in infection traps the DNA between the wall and membrane. If such a model were existing in 41c infection, the infection could be terminated by enzymatic removal of the cell wall releasing the trapped phage DNA or by treatment of such complexes with DNase.

To insure that protoplasting and DNase treatment would not non-specifically inactivate the host cell, resulting in aborted infections, the following experiments were performed. Five ml of log phase cells in TP-1 medium supplemented with 0.8M sucrose were treated with 200 μg/ml of lysozyme for 30 min at 37 C. When greater than 90% of the population were converted to protoplasts (as judged by microscopic examination) samples were treated with 20 μg/ml DNase for 15 min at 37 C. All samples were diluted in TP-sucrose broth and assayed for viable L-colonies on DP medium (Miller et al., 1967).

The results (Table 21) indicated that 2 x $10^8$ protoplasts arose from 1 x $10^8$ CFU indicating that host cells were probably diplobacilli. Treatment of such protoplasts with

Table 21.   Effect of DNase Treatment on B. subtilis
168 trp⁻str$^r$ Protoplasts.

| Type of Treatment | | |
| --- | --- | --- |
| Protoplasting | DNase | CFU/ml Recovered |
| - | - | $1.05 \times 10^8$ |
| + | - | $2.04 \times 10^8$ |
| + | + | $2.23 \times 10^8$ |
| + | + | $2.25 \times 10^8$ [a] |

[a]These protoplasts were diluted 100-fold prior to DNase treatment.

DNase had no effect on viability, therefore any decrease in
the number of infected cells after such treatment would be
due to release of periplasmic bound phage DNA.

To determine if the membrane transport of the viral
genome was blocked in the absence of $Ca^{++}$, log phase cells
were infected with 41c in TP-1 and diluted 100-fold in TP-1
or TP-3 after 2.5 min. Aeration was continued for 10 min
and the tonicity of the culture adjusted to 0.8M with 2M
sucrose. In addition, the culture was supplemented with 20
µg/ml chloramphenicol to halt phage development. The com-
plexes were protoplasted by treatment with 200 µg/ml lysozyme
at 32 C for 30 min. Once protoplasted DNase (20 µg/ml) was
added and the culture reincubated for 15 min. Samples were
diluted in TP-sucrose broth and assayed for PFU on TYS-
sucrose plates (Zsigray et al., 1973). The recovery of in-
fected complexes in TP-1 medium remained constant throughout
the course of the experiment (Table 22). Similarly, non-$Ca^{++}$
infected cells showed no loss of PFU on protoplasting and
DNase treatment indicating membrane transport of the viral
genome had been completed.

Effect of $Ca^{++}$ on the Production of Phage RNA

From the foregoing, it appeared that $Ca^{++}$ was required
for a stage in phage development occurring shortly after com-
pletion of intracellular penetration. Since transcription
of the viral genome is one of the earliest events following
penetration, $Ca^{++}$ might be required at this level. In such

Table 22. Survival of DNase-Treated Infected Protoplasts Diluted 1:100 into TP-3 Two Min Post-Infection.[a]

| Exp. No. | Type of Treatment | | PFU/ml Recovered from | |
| --- | --- | --- | --- | --- |
| | Protoplasting | DNase (20 µg/ml) | $Ca^{++}$ Control | $Ca^{++}$-Deficient Sample |
| 1 | - | - | $8.65 \times 10^6$ | $1.14 \times 10^7$ |
| | + | - | $7.95 \times 10^6$ | $9.15 \times 10^6$ |
| | + | + | $9.40 \times 10^6$ | $6.85 \times 10^6$ |
| 2 | - | - | $9.65 \times 10^6$ | $9.30 \times 10^6$ |
| | + | - | $1.00 \times 10^7$ | $1.11 \times 10^7$ |
| | + | + | $8.15 \times 10^6$ | $1.31 \times 10^7$ |

[a]Input = $1 \times 10^7$ PFU/ml.

a system, normal levels of phage messenger RNA (mRNA) might be transcribed at 10 mM $Ca^{++}$ while little or no transcription might occur in the absence of $Ca^{++}$.

The amount of transcription can be measured by RNA-DNA hybridization techniques. When $^{32}P$-labelled phage RNA is mixed with denatured 41c DNA under appropriate conditions, the labelled RNA will anneal to the DNA forming a RNA-DNA complex. Such hybrids can be retained on specially treated filters while unannealed RNA and single-stranded DNA will pass through the filters. If unlabelled homologous RNA is added to a mixture of labelled RNA and denatured DNA, the homologous species will compete for binding sites on the DNA molecule. As the concentration of unlabelled RNA is increased, the chance of this cold RNA binding to the DNA also increases, preventing the labelled RNA from annealing to these same sites. This results in a drop in the specific activity of the hybrid due to less label being retained on filters.

If unlabelled non-homologous RNA is mixed with $^{32}P$-labelled RNA and denatured DNA, no competition is observed since non-homologous species will not compete for the complementary sites on the DNA molecules. Therefore, the same amount of label should be associated with the hybrid regardless of the concentration of the challenge RNA.

Following this reasoning, $^{32}P$-labelled RNA extracted from infected cells in TP-1 medium was challenged with increasing concentrations of non-labelled RNA extracted from cells infected in TP-3 medium. By comparing the amount of

label retained on filters at low and high concentrations of the unlabelled species, the degree of homology can be determined.

For this technique, RNA had to be extracted from a sizable population of infected cells. Since the concentration of infected cells following 1:100 dilution in TP-3 medium was approximately 5 x $10^5$ infected complexes/ml, the population had to be increased. Therefore, log phase cells were grown to an OD of 0.7 and concentrated 20-fold by centrifugation yielding a bacterial density of 5 x $10^9$ CFU/ml. Concentrated cells (0.9 ml) were infected with 41c (0.1 ml) at an MOI of 1.0 in TP-1 medium. After aeration at 30 C for 2.5 min, cultures were diluted 100-fold into TP-1 or TP-3 media and aeration continued for 180 min. The results (Table 23) indicated that increasing the concentration of infected cells did not influence the appearance of the post-penetration $Ca^{++}$ requirement since complexes without $Ca^{++}$ failed to produce a significant burst. Therefore, this method was utilized for preparation of infected complexes for RNA extraction.

Three types of RNA were required for RNA-DNA hybridization experiments: $^{32}$P-labelled and unlabelled RNA from $Ca^{++}$-supplemented infected cultures; unlabelled RNA from $Ca^{++}$-deficient infected cultures. Labelled RNA was extracted from cells infected with 41c (MOI of 10) in TP-1 medium for 25 min. For the preparation of unlabelled homologous RNA cells were infected with 41c in TP-1 media for 2.5 min and diluted 1:100 into TP-1 for 22 min. For $Ca^{++}$-deficient RNA,

Table 23.   Survival of 41c Infected Complexes at 5.00 x $10^9$
            CFU/ml after 100-Fold Dilution to TP-1 or TP-3
            Medium.

| Time (Min) | PFU/ml Recovered after 100-Fold Dilution into | |
|---|---|---|
| | TP-1 Medium | TP-3 Medium |
| 60 | $1.30 \times 10^7$ | $1.40 \times 10^7$ |
| 120 | $1.06 \times 10^9$ | $9.50 \times 10^6$ |
| 180 | $3.95 \times 10^9$ | $1.50 \times 10^7$ |

infected complexes were initiated in TP-1 medium for 2.5 min then diluted 100-fold in TP-3 medium. Approximately 1 mg of RNA was recovered per extraction. Analysis of the RNA by the Burton method indicated that no significant DNA was present in extracted samples.

### Competition Hybridization Experiments

When labelled RNA was mixed with denatured DNA in the absence of competing RNA, 3.45% of the label hybridized to the phage DNA, representing the maximum hybridization efficiency (Table 24). In the presence of low concentrations of homologous RNA, little label was displaced from the hybrid. However, increasing the concentration to 1000 μg/ml resulted in 69% of the label being removed from the hybrid.

No significant label was displaced from the RNA-DNA hybrid in the presence of 12.5 μg/ml of RNA extracted from infected complexes formed with 0.1 mM $Ca^{++}$. When the concentration of this RNA was increased to 1000 μg/ml, 76% of the labelled homologous RNA species could be displaced from the hybrid indicating that transcription was occurring under suboptimal $Ca^{++}$ conditions.

### Effect of $Ca^{++}$ on Coat Protein Production

To determine the extent of translation in the absence of $Ca^{++}$, the amount of phage protein synthesized during infection was measured using serum blocking. $Ca^{++}$-supplemented and non-supplemented lysates were prepared. One tenth ml of a

Table 24. Competition Between RNA Obtained from $Ca^{++}$-Supplemented and Non-Supplemented Infection.[a]

| Sample No. | 41c DNA Concentration (μg/ml) | RNA Concentration (μg/ml) Extracted from | | | CPM Retained on Filter | CPM Retained on Filter (%) |
|---|---|---|---|---|---|---|
| | | $^{32}$P-Labelled Complexes in TP-1 Medium | Unlabelled Complexes in TP-1 Medium | Unlabelled Complexes in TP-3 Medium | | |
| 1 | 20 | 12.5 | | | 398.8 | 100[b] |
| 2 | 20 | 12.5 | 12.5 | | 421.9 | 105.79 |
| 3 | 20 | 12.5 | 1000 | | 123.7 | 31.02 |
| 4 | 20 | 12.5 | | 12.5 | 383.3 | 96.11 |
| 5 | 20 | 12.5 | | 1000 | 99.3 | 24.90 |

[a]Maximum counts retained on filters in the absence of denatured DNA was 33 CPM.

[b]Maximum hybridization efficiency 3.45%; Value calculated by dividing the counts retained on the filter in the absence of competing RNA (398.8) by the amount of input label (11,561).

1:50,000 dilution of phage 41c antiserum was added to each of two tubes containing 0.9 ml of adjusted phage stocks (Table 25). The samples were incubated at 37 C for 5 min and the amount of neutralization determined. One tenth ml of a known phage stock (6.6 x 10$^5$ PFU/ml) was then added to each culture and incubation continued for 5 min. Samples were then assayed for PFU to obtain the degree of inactivation of newly added virus. The results are shown in Table 25. On the average, 55% of the phage produced in the presence of Ca$^{++}$ were init- ially inactivated when exposed to phage antiserum while 47% of the phage produced in the absence of Ca$^{++}$ were neutralized. When known amounts of virus were readded to the mixtures inactivation values of only 29 and 11%, respectively, were obtained indicating that phage protein was being synthesized in the two systems.

Effect of Ca$^{++}$ on DNA Replication

Since Ca$^{++}$ had no effect on the production of phage mRNA or its translation, the influence of this ion on DNA replication was studied next. Ca$^{++}$-supplemented and non- supplemented lysates were prepared by growing 400 ml of host cells to an OD of 0.2 at 37 C. The culture was centrifuged and resuspended in 9 ml of TP-1 media and infected with 41c at a phage-bacterium ratio of 2.0. The complexes were aerated for 2.5 min at 30 C and diluted 100-fold into 500 ml of TP-1 and TP-3 medium. Aeration was continued for 3 h at 30 C and the lysates filtered, treated with nucleases and concentrated

Table 25. Serum Blocking Activities of 41c Lysates Prepared in $Ca^{++}$-Supplemented and Non-Supplemented Media.

| Exp. No. | $Ca^{++}$ Added | Input (PFU/ml) | PFU/ml Recovered | | Neutralization (%) | |
| | | | After Initial Antibody Treatment | After Readdition of $6.6 \times 10^5$ PFU/ml to Antibody Mixture | After Initial Antibody Treatment[a] | After Readdition of 41c Stock[b] |
|---|---|---|---|---|---|---|
| 1 | + | $4.14 \times 10^6$ | $1.88 \times 10^6$ | $1.68 \times 10^6$ | 54.59 | 33.90 |
| | − | $2.32 \times 10^5$ | $9.10 \times 10^4$ | $5.90 \times 10^5$ | 60.78 | 21.33 |
| 2 | + | $4.44 \times 10^6$ | $1.70 \times 10^6$ | $1.73 \times 10^6$ | 61.71 | 26.69 |
| | − | $2.32 \times 10^5$ | $1.30 \times 10^5$ | $6.90 \times 10^5$ | 43.97 | 12.66 |
| 3 | + | $3.65 \times 10^6$ | $1.82 \times 10^6$ | $1.79 \times 10^6$ | 50.16 | 27.53 |
| | − | $2.42 \times 10^5$ | $1.54 \times 10^5$ | $8.35 \times 10^5$ | 36.37 | 0.00 |

[a]Percent neutralization equals PFU/ml recovered after initial treatment divided by input value subtracted from 100%.

[b]Percent neutralization equals PFU/ml recovered after readdition of known phage stock divided by PFU/ml recovered after initial treatment + readded stock ($6.6 \times 10^5$ PFU/ml) subtracted from 100%.

as previously described. The DNA concentration of both ly-
sates was measured with intact virus by the diphenylamine
method (Burton, 1956). Such an analysis indicated that lysate
prepared with $Ca^{++}$ contained 690 µg/ml per 4.3 x $10^{11}$ PFU.
The other lysate contained 7.0 x $10^{10}$ PFU/ml and the diphenyl-
amine reaction indicated a DNA concentration of 377 µg/ml,
this being 55% of the concentration observed in the control.
Thus, reduced DNA synthesis was only partially responsible
for the decreased burst observed in $Ca^{++}$-deficient cultures.

## Effect of Spermine on 41c Replication

Previous studies demonstrated that equimolar concen-
trations of divalent cations failed to substitute for $Ca^{++}$
in 41c infection. However, it was not known whether or not
other cationic substances, such as polyamines, could replace
the $Ca^{++}$ requirements of 41c. Therefore, studies were
initiated to determine if one such substance spermine tetra-
hydrochloride (Cal Biochem, La Jolla, Ca.) could permit
successful 41c infection.

Log phase cells were infected with 41c at 10 mM $Ca^{++}$
or 10 mM spermine for 15 min at 30 C. Following centrifugation
the supernatants were assayed for unadsorbed virus while the
pellets were resuspended in appropriate broth and reincubated
for 15 min. The results (Table 26) indicated that 89% of the
adsorbed complexes formed in the presence of polyamines could
be recovered when compared to the $Ca^{++}$-supplemented control.
Since this recovery was significantly higher than that ob-

Table 26.    Recovery of 41c Infected Complexes at 10 mM
            Spermine.

| Conditions of Infection | PFU/ml Recovered as | |
|---|---|---|
| | Unadsorbed Virus | Infected Centers |
| 10 mM Ca$^{++}$ | $1.38 \times 10^6$ | $5.25 \times 10^7$ |
| 10 mM Spermine | $6.10 \times 10^6$ | $4.10 \times 10^7$ |

served with other divalent cations, additional studies were performed to determine the nature of spermine in 41c infection.

Effect of Spermine on the Adsorption of 41c

To determine if spermine could permit efficient adsorption of virus, cultures were infected with 41c at 10 mM and 0.1 mM polyamine concentrations for 5 min at 30 C. The phage-bacterium mixtures were centrifuged and the supernatants and pellets assayed for PFU. A similar adsorption pattern was shared by $Ca^{++}$ and spermine (Table 27) since at 10 mM concentrations of the polyamine, 82% of the virus adsorbed within 5 min. Reducing the concentration to 0.1 mM still permitted 72% of the phage to adsorb. As seen in $Ca^{++}$ infection, assays of the adsorbed complexes indicated that the infection process required 10 mM concentrations of spermine for optimal survival while few complexes (3%) could establish successful infection at 0.1 mM concentrations.

Effect of Spermine on the Penetration of 41c

To establish a correlation between $Ca^{++}$-dependent penetration and spermine-associated transfer, log phase cells were infected with $^{32}P$-labelled 41c for 15 min at 30 C with 10 mM or 0.1 mM polyamine. The cultures were centrifuged and the supernatant fluids assayed for unadsorbed PFU and CPM. The pellets were resuspended in the appropriate broths and assayed for adsorbed biological and radiological activity.

Table 27. Effect of Spermine on Adsorption of 41c.

| Exp. No. | Input (PFU/ml) | PFU/ml Recovered at Spermine Concentrations of | | | |
| | | 10 mM | | 0.1 mM | |
| | | Supernatant Fluid | Pellet | Supernatant Fluid | Pellet |
|---|---|---|---|---|---|
| 1 | $4.90 \times 10^7$ | $1.21 \times 10^7$ (24.69)[a] | $3.00 \times 10^7$ (61.22) | $1.95 \times 10^7$ (39.80) | $1.75 \times 10^6$ (3.57) |
| 2 | $4.45 \times 10^7$ | $5.30 \times 10^6$ (11.91) | $2.05 \times 10^7$ (46.06) | $8.05 \times 10^6$ (18.08) | $6.15 \times 10^5$ (1.38) |

[a]Values in parentheses are percent recoveries of input.

With 10 mM spermine, adsorption was 88% efficient after 15
min incubation while 58% of the label became cell-associated
(Table 28). At reduced concentrations of cation, adsorption
was less efficient with 56% of the phage and 40% of the label
becoming cell-associated (Table 29).

At 10 mM spermine, 56% of the adsorbed complexes were
recovered before and after blender treatment, corresponding
to the transfer of 57% of the label to a blender-resistant
state (Table 30). The non-transferable label (31%) released
on blender treatment presumably represented defective phage.

At reduced polyamine concentration, less than half
of the adsorbed complexes survived blender treatment (Table
31). Since only 57% of the adsorbed label was transferred
under optimal conditions, the maximum injectable label was
calculated by multiplying the adsorbed label by 57%. Based
on these calculations, it was found that 54% of the inject-
able DNA could penetrate the cell in the presence of 0.1 mM
polyamine. These data indicated that a similar penetration
mechanism existed in $Ca^{++}$- and spermine-supplemented infec-
tions.

Since the two systems appeared closely related, the
rate of DNA penetration with polyamine was studied next using
phage specific antiserum to inhibit penetration. Log phase
cultures were infected with 41c at 10 mM polyamine and a
portion of the sample treated with antiserum at 5, 10, 15
and 20 min followed by aeration for 5 min at 37 C. Maximum
establishment of antiserum-resistant infected complexes was

Table 28. Adsorption of $^{32}$P-Labelled 41c at 10 mM Spermine.

| Exp. No. | PFU/ml Recovered from | | | CPM Recovered from | | |
|---|---|---|---|---|---|---|
| | Input | Supernatant | Pellet[a] | Input | Supernatant | Pellet |
| 1 | $1.85 \times 10^7$ (100)[b] | $4.10 \times 10^6$ (23) | $1.44 \times 10^7$ (77) | 2203 (100) | 639 (29) | 1129 (51) |
| 2 | $2.50 \times 10^7$ (100) | $1.81 \times 10^6$ (8) | $2.32 \times 10^7$ (92) | 2520 (100) | 451 (18) | 1473 (58) |
| 3 | $3.65 \times 10^7$ (100) | $1.80 \times 10^6$ (5) | $3.47 \times 10^7$ (95) | 2322 (100) | 393 (17) | 1475 (64) |

[a]Values reported are theoretical amounts of adsorbed virus obtained by subtracting the value in the supernatant from the input.

[b]Values in parentheses are percent recoveries of input.

Table 29.  Adsorption of $^{32}$P-Labelled 41c at 0.1 mM Spermine.

| Exp.<br>No. | PFU/ml Recovered from | | | CPM Recovered from | | |
|---|---|---|---|---|---|---|
| | Input | Supernatant | Pellet[a] | Input | Supernatant | Pellet |
| 1 | $1.85 \times 10^7$ (100)[b] | $8.75 \times 10^6$ (47) | $9.75 \times 10^6$ (53) | 3393 (100) | 1897 (56) | 1225 (36) |
| 2 | $2.50 \times 10^7$ (100) | $1.03 \times 10^7$ (41) | $1.47 \times 10^7$ (59) | 3386 (100) | 1554 (46) | 1503 (44) |

[a]Values reported are theoretical amounts of adsorbed virus obtained by subtracting the value in the supernatant from the input.

[b]Values in parentheses are percent recoveries of input.

Table 30.  Biological and Radiological Assays of Blended Complexes Formed with $^{32}$P-Labelled 41c:  effect of 10 mM Spermine.

| Exp. No. | PFU/ml Recovered from (x $10^4$) | | | CPM Associated with | | |
| | Unblended Control | Blended Supernatant | Blended Pellet | Unblended Pellet | Blended Supernatant | Blended Pellet[b] |
| --- | --- | --- | --- | --- | --- | --- |
| 1 | 1150 (80)[c] | 8.05 (<1) | 1010 (70) | 1129 (100) | 412 (36) | 638 (57) |
| 2 | 1370 (59) | 5.75 (<1) | 1290 (56) | 1473 (100) | 497 (34) | 731 (50) |
| 3 | 1800 (52) | 6.80 (<1) | 1500 (43) | 1475 (100) | 363 (25) | 977 (66) |

[a]Values based on the amount of adsorbed virus and label and equals 100% (Table 27).

[b]Maximum injectable label at 10 mM Spermine.

[c]Values in parentheses represents percent recoveries of adsorbed.

Table 31. Biological and Radiological Assays of Blended Complexes Formed with $^{32}$P-Labelled 41c: effect of 0.1 mM Spermine.

| | PFU/ml Recovered from (x $10^4$)[a] | | | | CPM Associated with | | |
|---|---|---|---|---|---|---|---|
| Exp. No. | Unblended Control | Blended Supernatant | Blended Pellet | Unblended Pellet | Maximum Injectable Label[b] | Blended Supernatant | Blended Pellet |
| 1 | 490 (50)[c] | 17 (<1) | 385 (40) | 1225 | 693 (100) | 781 | 329 (47) |
| 2 | 615 (42) | 8 (<1) | 475 (32) | 1503 | 746 (100) | 764 | 463 (62) |

[a]Values are based on amount of virus adsorbed (Table 28).

[b]Maximum injectable label is calculated by multiplying absorbed label found in unblended pellet by 57% (Table 29) which is average label injectable at 10 mM Spermine.

[c]Values in parentheses are percent recoveries of adsorbed.

not seen until 15 min indicating that the injection process was substantially slower than $Ca^{++}$-facilitated penetration (Table 32).

### Effect of Spermine on Post-Penetration
### Stages of 41c Infection

After it appeared that the requirement of $Ca^{++}$ in phage adsorption and penetration could be replaced with equimolar concentrations of spermine, experiments were initiated to determine if the post-penetration $Ca^{++}$ requirement could be met by the polyamine.

Initially, a one step growth curve was performed in 10 mM spermine by infecting a log phase culture with 41c in the presence of the polyamine for 15 min at 30 C. The culture was centrifuged to remove unadsorbed virus and the pellet resuspended in spermine-supplemented media and the sample assayed over 180 min. As a control, a similar growth curve was carried out in 10 mM $Ca^{++}$. The results are shown in Fig. 10. The growth curve at 10 mM $Ca^{++}$ exhibited a latent period of 50 min and a burst of 384. However, in the presence of equimolar polyamine, the latent period of the virus was extended to 75 min and the burst reduced to 20.

Similar experiments were performed comparing infections with 10 mM and 0.1 mM polyamine. At the higher concentration of spermine, the phage exhibited a latent period of 70 min ultimately releasing 28 infectious particles per cell (Fig. 11). At reduced levels of polyamine, 90% of the virus

Table 32. Effect of Antiserum Addition on Recovery of 41c
Infected Complexes at 10 mM Spermine.[a]

| Exp. No. | Antiserum Addition (Min) | PFU/ml Recovered from | |
|---|---|---|---|
| | | Untreated Cultures | Antiserum Treated Cultures |
| 1 | 5 | $6.20 \times 10^7$ | $3.90 \times 10^7$ |
| | 10 | $5.98 \times 10^7$ | $3.76 \times 10^7$ |
| | 15 | $8.00 \times 10^7$ | $5.33 \times 10^7$ |
| | 20 | $5.80 \times 10^7$ | $6.35 \times 10^7$ |
| 2 | 5 | $4.25 \times 10^7$ | $2.80 \times 10^7$ |
| | 10 | $4.30 \times 10^7$ | $2.50 \times 10^7$ |
| | 15 | $3.35 \times 10^7$ | $3.60 \times 10^7$ |
| | 20 | $6.10 \times 10^7$ | $5.23 \times 10^7$ |

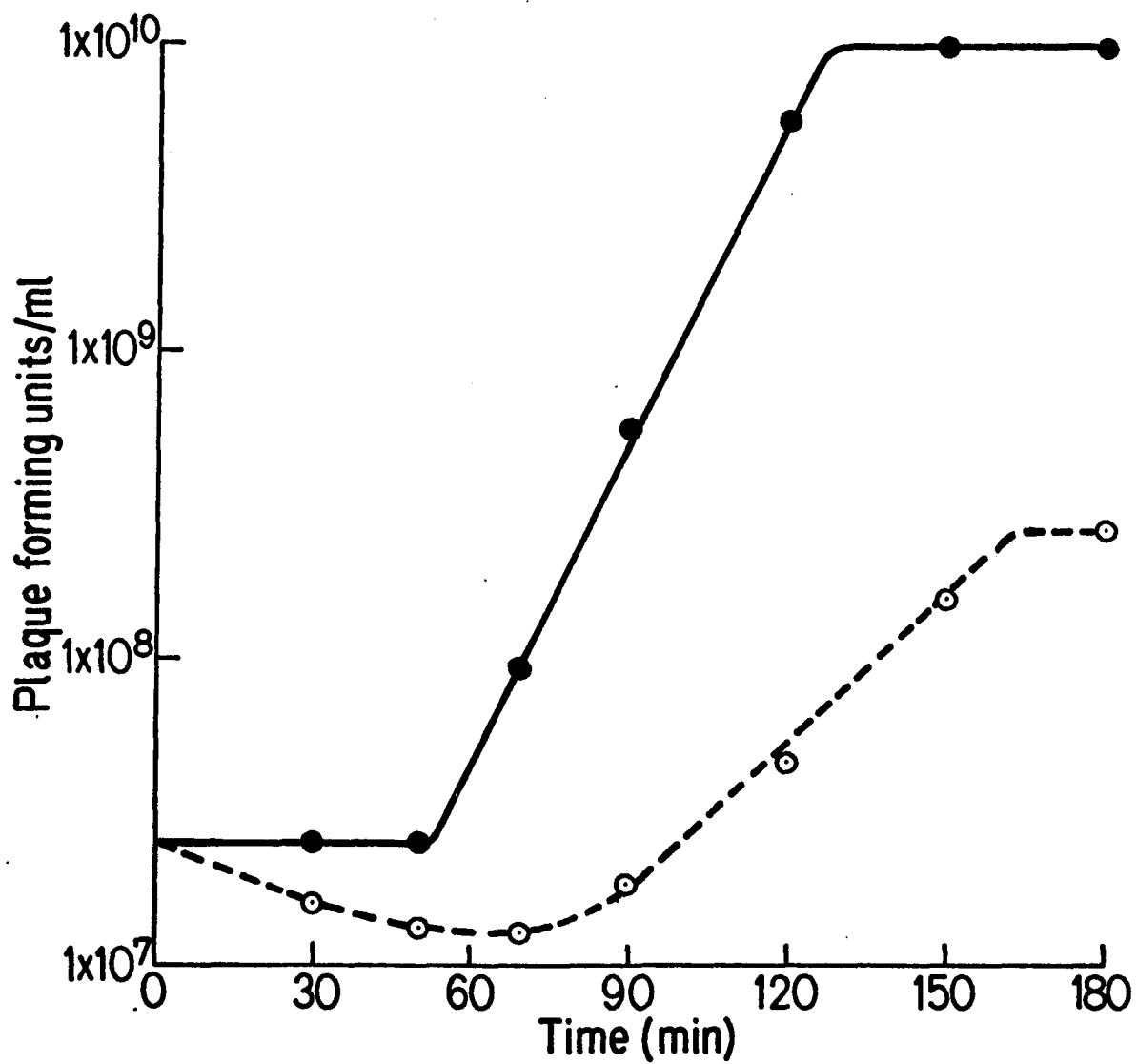[a]Input = $9.0 \times 10^7$ PFU/ml.

Fig. 10.   Growth Curve of 41c in the Presence of 10 mM Ca$^{++}$ and 10 mM Spermine.   Symbols: ● 10 mM Ca$^{++}$,  ⊙, 10 mM Spermine.
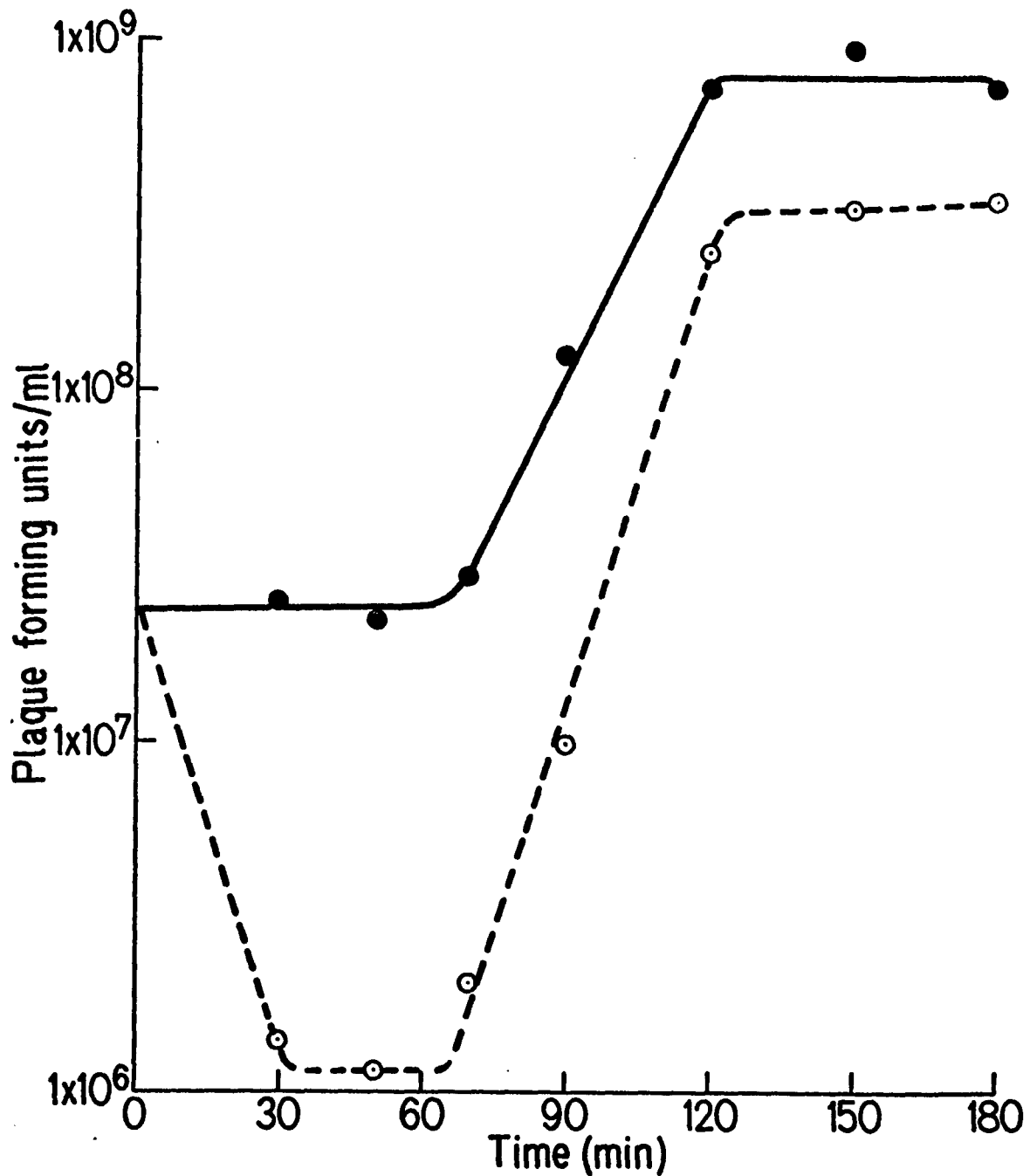
Fig. 11.   Growth Curve of 41c at 10 mM and 0.1 mM Spermine.
Symbols:  ●, 10 mM Spermine; ⊙,0.1mM Spermine.

population were inactivated within 15 min presumably due to
the lack of penetration in the absence of polyamine. Com-
plexes completing penetration had an extended latent period
of 70 min but released 239 particles per infected cell.

There appeared to be 4 possible reasons for the re-
duced burst size observed at 10 mM spermine:  1) spermine was
stabilizing the membrane of the infected cells, thereby in-
hibiting the lysis of the cells; 2) spermine was inactivating
completed phage; 3) spermine might be inhibiting host cell
metabolism; 4) spermine failed to permit post-penetration
phage development.

Polyamines, in general, are stabilizers of host cell
membranes (Bachrach, 1970) and could be acting to prevent
the release of a normal complement of infectious particles.
If this were indeed the case, artificial lysis of the infec-
ted cells by chloroform treatment should result in a normal
burst with spermine-supplemented infections. Therefore, log
phase host cells were infected with 41c at 10 mM spermine and
sampled over a 180 min period. At the time of sampling a
portion of the culture was treated with 0.5 ml of chloroform
to lyse the infected cells.  Both samples were then assayed
for PFU.  The results (Fig. 12) showed that all the viral
particles being produced in 10 mM polyamine infection were
released since artificial lysis failed to increase the amount
of PFU recovered.  Thus, it appeared only a few viral particles
were being produced in each infected cell.
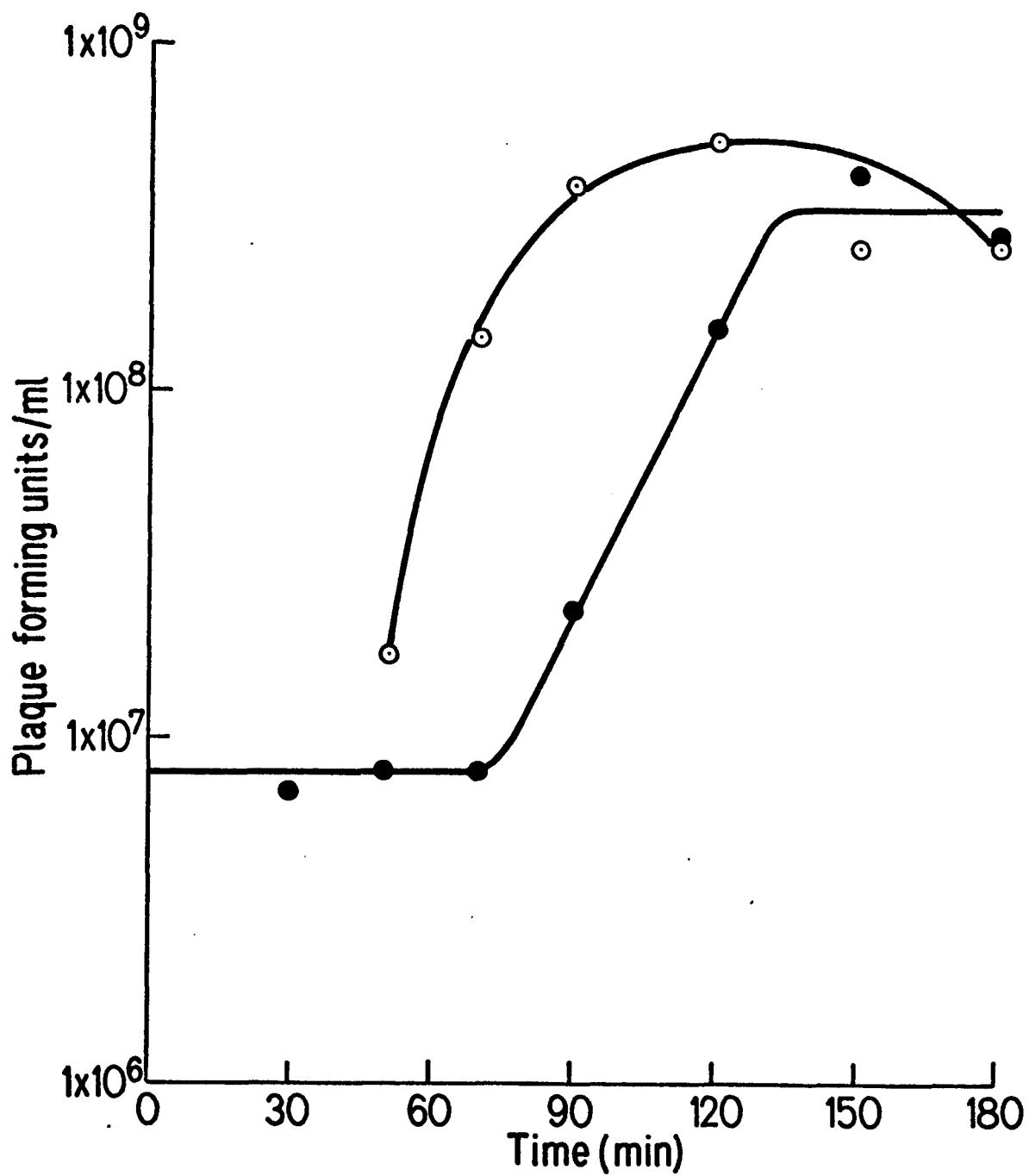
Fig. 12. Release of Completed Phage Particles by Chloroform
Lysis at 10 mM Spermine. Symbols: ●, 10 mM Spermine; ⊙,
chloroform samples.

Alternately, spermine could be inactivating the infectious virus as they were being released from the cell. When 41c was exposed to 10 mM spermine over a 3 h period no such inactivation was observed (Table 33).

Spermine might be inhibiting phage development by interferring with host cell metabolism thus slowing down the mechanisms needed for normal phage production. However, this did not prove to be the case since host cells grew equally well in the presence of 10 mM polyamine and 10 mM $Ca^{++}$ (Fig. 13). Therefore, the polyamine, while allowing adsorption and penetration, failed to replace the $Ca^{++}$ requirement necessary for some phase of intracellular development of 41c.

Table 33.  Stability of 41c in 10 mM Spermine.[a]

| Exp. No. | PFU/ml Recovered after | | |
|---|---|---|---|
| | 60 Min | 120 Min | 180 Min |
| 1 | $9.30 \times 10^7$ (93)[b] | $1.13 \times 10^8$ (100) | $9.40 \times 10^7$ (94) |
| 2 | $1.05 \times 10^8$ (100) | $1.12 \times 10^8$ (100) | $8.50 \times 10^7$ (85) |

[a]Input value was $1.00 \times 10^8$ PFU/ml.

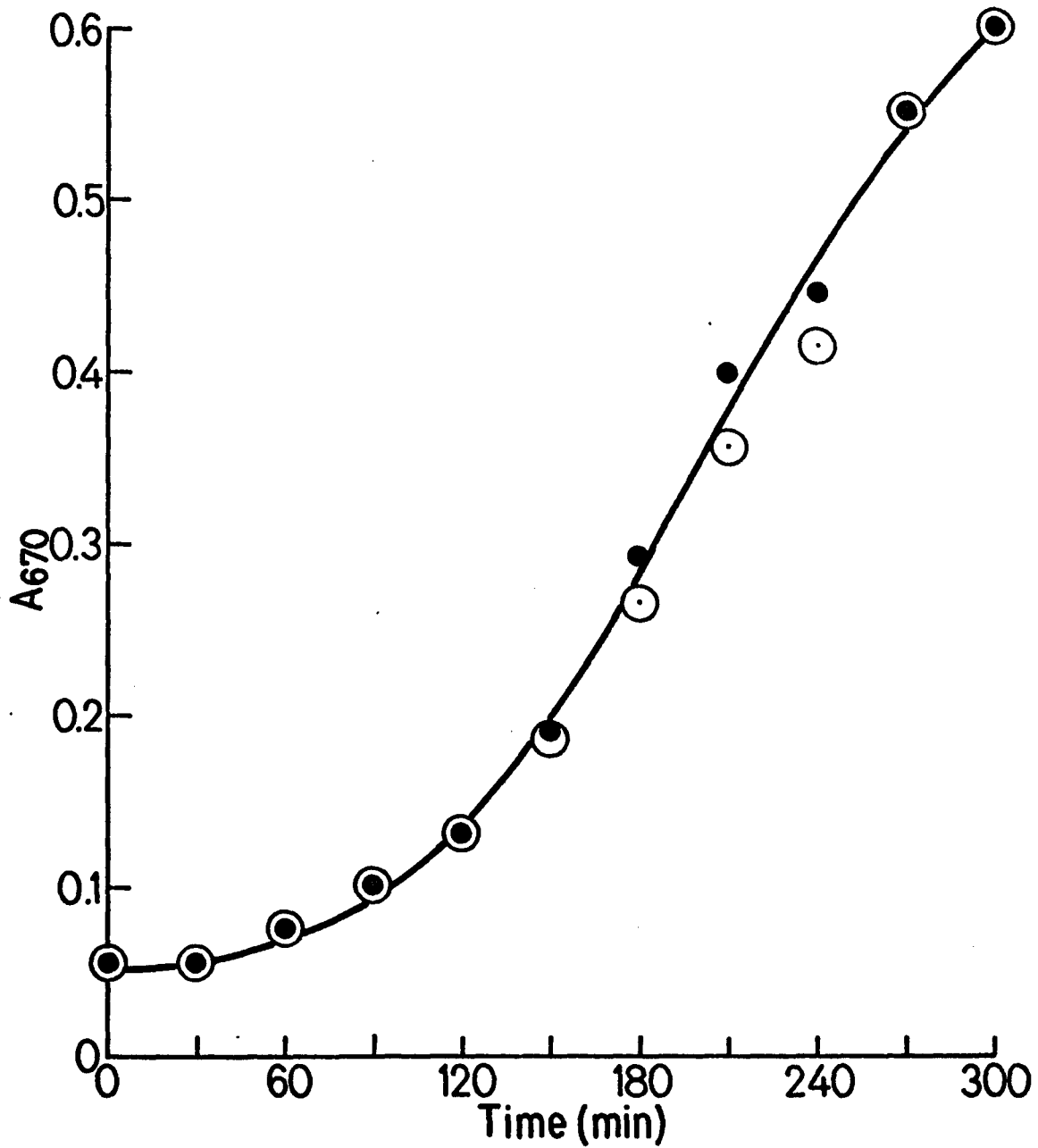[b]Values in parentheses are percent recoveries of input.

Fig. 13.  Effect of 10 mM Spermine on the Growth of B. subtilis 168.  Symbols:  ●, 10 mM $Ca^{++}$; ⊙, 10 mM Spermine.

# DISCUSSION

$Ca^{++}$ and other divalent cations are known to affect the lytic cycle of many bacteriophage. In this investigation, it was shown that a multi-stage $Ca^{++}$ requirement existed in 41c infection, involving adsorption, penetration and intracellular development. If 10 mM $Ca^{++}$ was omitted from plating media, the plaquing efficiency of this virus was less than 0.1. In all cases, a small fraction of the population survived in the absence of $Ca^{++}$, being greater in undefined media than in defined media. The survivors exhibited $Ca^{++}$-dependency on replating and were not $Ca^{++}$-independent mutants as observed by Bertani et al. (1969). Sufficient contaminating ion is apparently present in the media and glassware permitting the replication of a few infected cells. Alternately, a small percentage of cells might be displaying presporulation activities and, as a result, are concentrating $Ca^{++}$ from the media (Murrell, 1969) allowing phage replication.

Optimal conditions for infection in TP-1 media required the preparation of log phase cells in TP media. Apparently, cells grown in TYS media and infected in TP-1 media lose essential log phase characteristics necessary for successful infection (Zsigray, 1968). In addition, chilling of the host cells had an inhibitory effect on phage-bacterium complexes, possibly due to metabolic disruption of the host or the release of nucleases by cold-shocked cells.

## Role of $Ca^{++}$ in Phage Adsorption

The adsorption of the phage 41c was optimal at 10 mM $Ca^{++}$ with the majority of the viruses adsorbing within 1 min. The rate of attachment after 5 min was similar to that seen with other phages of B. subtilis (Brodetsky and Romig, 1965). Unlike other systems where adsorption occurs only at high concentrations of ion (Reese et al., 1974) efficient adsorption was observed at 1 mM and 0.1 mM $Ca^{++}$. However, adsorption of 41c was greatly reduced in the absence of $Ca^{++}$.

Although monovalent and divalent ions are known to meet the ionic demand for adsorption of some phage (Garen and Puck, 1951; Tzagoloff and Pratt, 1964) this did not prove to be the case in 41c since $Na^+$ (80 mM) was in the media and failed to permit efficient attachment. In addition, 2 mM $Mg^{++}$ could not meet the ionic requirements of this process.

The mechanism of the $Ca^{++}$-requiring adsorption process most likely involves the neutralization of the negatively charged host or virus. On the other hand, $Ca^{++}$ might cause a subtle alteration of a receptor site on the virus particle or the host cell (Stent, 1963). In both cases, the mechanism is $Ca^{++}$-specific since monovalent and other divalent ions cannot be substituted for this cation.

## The Effect of $Ca^{++}$ on Phage Penetration

A second $Ca^{++}$-dependent stage was found to exist in 41c infection since viruses (which had adsorbed) failed to

establish successful infection at reduced concentrations of $Ca^{++}$. This loss in infected centers occurred early in the lytic cycle and was attributed to lack of DNA penetration as proposed in other Bacillus phage systems (Shafia and Thompson, 1964; Brodetsky and Romig, 1965). Consequently, the penetration process was studied using $^{32}P$-labelled phage. Under optimal infecting conditions (10 mM $Ca^{++}$), all the infected complexes were resistant to blender treatment 15 min post-infection indicating that the phage components necessary for establishing successful infection were located intracellularly. However, due to a portion of the phage population which either: 1) failed to adsorb (Luria and Steiner, 1954); 2) spontaneously ejected their DNA on adsorption (Labedan and Legault-Demare, 1974); or 3) adsorbed but failed to inject (Lanni, 1960b; Paranchych, Krahn and Bradley, 1970), only 48% of the adsorbed radioactivity became cell-associated. In my studies, the amount of label adsorbed (83%) and the values obtained as maximum injectable label (48%) compare favorably with those obtained for T5 (Luria and Steiner, 1954), PL-1 (Watanabe and Takesue, 1972) and R17 (Paranchych, 1966).

Infection with 41c at 0.1 mM $Ca^{++}$ for 15 min resulted in only 8% of the infected complexes surviving blender treatment. Radiological data revealed that some (21%) label penetrated the host cell at this concentration of $Ca^{++}$. A fraction of this injected label represented viruses which had completed penetration since these complexes were blender-

resistant.  The remaining cell-associated label is presumably

due to partial injection by the majority of the population

since few intact viruses were recovered on chloroform treat-

ment.  These findings are in good agreement with those

observed in $Ca^{++}$-deficient penetration studies employing

PL-1 (Watanabe and Takesue, 1972).  In this case, only 17%

of the adsorbed label could penetrate in the absence of $Ca^{++}$,

resulting in only 10% of the infected complexes surviving.

It is not known if the low-level penetration occurring with-

out $Ca^{++}$ is due to contaminating ions in the media or pre-

sporulation concentration of $Ca^{++}$ by a small fraction of

cells.  An alternate possibility might be that $Mg^{++}$ (2 mM)

which is in the medium could be contributing to the penetra-

tion process since, in other systems, divalent cations such

as $Sr^{++}$, $Ba^{++}$ and $Mg^{++}$ could substitute for $Ca^{++}$ in DNA

penetration (Watanabe and Takesue, 1972; Paranchych, 1966;

Roundtree, 1955).

The DNA which failed to penetrate at reduced concen-

trations of $Ca^{++}$ remained in large molecular weight form and

failed to complete penetration on addition of 10 mM $Ca^{++}$.

This observation is in agreement with that observed with PL-1

of Lactobacillus where injection could not be reversed on

delayed $Ca^{++}$ addition (Watanabe and Takesue, 1972).  The

authors proposed that the genome had leaked out of the phage

head; however, in studies with T5, such extruded DNA was found

to be quickly hydrolyzed by extracellular nucleases (Labedan

and Legault-Demare, 1974). Therefore, the genome of 41c is most likely remaining within the phage head in an undegraded form.

Another possible explanation for the lack of injection might be that penetration is initiated but halted due to the lack of $Ca^{++}$. The arrested genome might be exposed to limited hydrolysis by extracellular nucleases due to a faulty phage-host attachment (Zsigray et al., 1973) breaking the continuity of the molecule. Therefore, delayed addition of $Ca^{++}$ would fail to stimulate further penetration.

Additional blending and DNase experiments indicated that penetration was complete 2 min after the initiation of infection. Earlier studies on 41c penetration indicated that transfer of the viral genome required 10 min for completion (Zsigray et al., 1973). In these studies, chilling of phage-bacterium complexes for 15 to 30 min at 4 C was employed for adsorption. Such treatment apparently slows the penetration process of the phage.

The role of $Ca^{++}$ and other divalent cations in membrane transport is not well understood. One role proposed for such ions involves the stabilization and maintenance of the secondary structure of the DNA during the penetration process (Danziger and Paranchych, 1970a). This stabilization appears to be non-specific since a number of divalent cations ($Mg^{++}$, $Ca^{++}$, $Ba^{++}$ and $Zn^{++}$) can bind to phosphate groups on the viral genome neutralizing the negative charges resulting in an increase in the temperature required for thermal denaturation

of the DNA (Eichhorn, 1962). Such a role appears to be important in the uptake of transforming (Young and Spizizen, 1962; Fox and Hotchkiss, 1951; Goodgal and Herriott, 1961) and transfecting (Sjöström, Lindberg and Philipson, 1972; Mahler, George and Grossman, 1974) DNA by competent cells.

An alternate and more specific penetration role has been proposed recently for $Ca^{++}$ in the transformation (Cosloy and Oishi, 1973b) and transfection (Mandel and Higa, 1970) of E. coli. These authors demonstrated an increased permeability in the cell membrane of $Ca^{++}$-treated cells and proposed that the altered permeability allowed the exogenous DNA to enter the cell. To date, no other ions are known to substitute for $Ca^{++}$ in this system.

It is not known which mechanism is responsible for 41c DNA penetration. Due to the apparent specificity of $Ca^{++}$ in E. coli transforming and transfecting systems, this may be the model in the 41c system. However, the role may involve a complex interaction of stabilization, neutralization and altered membrane permeability by $Ca^{++}$.

## Intracellular $Ca^{++}$ Requirement

In addition to adsorption and penetration, $Ca^{++}$ is required for an intracellular stage in 41c phage development. Such a role has been demonstrated in infections with Group I phages of B. subtilis (Brodetsky and Romig, 1965), phage A6 (Friend and Slade, 1967) and GV4 (Colasito and Rogoff, 1969).

In all cases, increased latent periods and reduced bursts were noted when infection proceeded in $Ca^{++}$-deficient media. $Ca^{++}$ was postulated to be required for a post-penetration stage; however, the nature of the requirement has never been elucidated.

With 41c, initial studies employing EDTA demonstrated that $Ca^{++}$ was needed subsequent to penetration since no burst was observed when the chelator was added 5 or 10 min post-infection. However, the exact time at which $Ca^{++}$ exerted its effect could not be determined since 41c was inactivated (to some degree) by EDTA. Similar inactivations of phage by EDTA have been reported (Roundtree, 1955; Shafia and Thompson, 1964); presumably, the chelator is disrupting the structure of the virus causing the nucleic acid to be released from the phage head (Lanni, 1960b).

The time of the post-penetration requirement was demonstrated using a dilution procedure. When injected complexes were diluted from TP-1 into TP-3 media 2.5 min after the initiation of infection, such complexes failed to give rise to a normal burst. Since penetration has already occurred prior to dilution, these results suggested that an intracellular stage must be influenced by $Ca^{++}$. However, dilution into TP-3 6 min into the lytic cycle had no effect on progeny formation.

Because transcription represents one of the initial events during intracellular replication, competition RNA-DNA hybridization experiments were conducted employing RNA ex-

tracted 25 min after the initiation of infection. Recent evidence indicated that both early and late mRNA is present throughout the latent period of B. subtilis phages having the same structural dimensions of 41c (Schachtele, DeSain and Anderson, 1973; Willis and Ennis, 1969). These small viruses, exhibit little temporal control of transcriptional events and the production of early mRNA is not repressed (Schachtele et al., 1973). Also large viruses which do exhibit temporal control of transcription retain early mRNA as late as 20 min into the infection cycle.

The hybridization efficiency (3.45%) obtained for 41c RNA-DNA complexes agrees well with those values reported in the literature for T4 (Bolle et al., 1968) and $\emptyset$29 (Schachtele et al., 1973). The labelled RNA unable to hybridize to phage DNA in the absence of competing RNA presumably represents RNA of host origin. Evidence for this has been presented for phage systems of B. subtilis (Schachtele, DeSain, Hawley and Anderson, 1972; Milanesi and Cassani, 1972) since unmodified host RNA polymerase is responsible for viral transcription. Thus, the RNA polymerase is capable of transcribing host RNA throughout infection (Milanesi and Cassani, 1972).

From my results, transcription does not appear to be the post-penetration, $Ca^{++}$-dependent process. High concentrations of unlabelled RNA extracted from $Ca^{++}$-deficient cultures competed successfully for binding sites on the DNA molecule with labelled RNA from $Ca^{++}$-supplemented cultures. Approximately the same amount of label was displaced from the

hybrid regardless of the source of competing RNA.

While a 90% reduction in infective particles was seen in $Ca^{++}$-deficient cultures, this loss could not be attributed to a lack of translation since phage protein (as measured by serum blocking power) was synthesized in the absence of $Ca^{++}$. Neither could this reduction be attributed to reduced DNA synthesis since over 50% of the DNA was seen in non-$Ca^{++}$ lysates. Because the diphenylamine reaction was performed on pelleted viruses, maturation or packaging of the DNA into phage head had to occur. However, such an assembly must be defective since these viruses were lost as PFU.

## Effect of Spermine on the Replication of 41c

The $Ca^{++}$ requirement of 41c appeared to be specific since equimolar concentrations of $Mg^{++}$, $Ba^{++}$, $Mn^{++}$ and $Zn^{++}$ failed to replace this ion. Surprisingly, the polyamine spermine was found to substitute for $Ca^{++}$.

Polyamines are normal components of some bacteriophage (Ames, Dubin and Rosenthal, 1958; Kay, 1959). The concentration of any given polyamine appears to be dependent upon the concentration initially present in the previous host and on the permeability of the phage to divalent ions (Ames and Dubin, 1960). Permeable phage, such as T3 and P22, replace the amines with divalent ions while other phage (T2 and T4) retain their original concentration of polyamine.

In addition, the presence of exogenous polyamines are known to influence the replication of bacteriophage (Bachrach, 1970).

My initial studies indicated a large number of complexes established infection in the presence of 10 mM spermine. Therefore, adsorption, penetration and intracellular development of 41c were investigated to determine whether or not spermine acted in an analogous manner as $Ca^{++}$.

Spermine is known to inhibit the adsorption of T1, T3 and T7 (Reiter, 1963b) while the attachment of f2 (Young and Srinivasan, 1974) remains undisturbed. In this study, the polyamine stimulated the adsorption of 41c in much the same manner as $Ca^{++}$. As in the $Ca^{++}$-facilitated systems, optimal adsorption was found at 10 mM spermine while efficient but a somewhat reduced attachment was noted as 0.1 mM.

Optimal recovery of infected complexes was only seen when high concentrations of spermine were employed. As with reduced $Ca^{++}$, 0.1 mM polyamine failed to support successful infection indicating the polyamine was required subsequent to adsorption. Penetration studies revealed 10 mM spermine performed a similar function as 10 mM $Ca^{++}$ in 41c infection allowing 57% of the adsorbable $^{32}P$ to penetrate the host cell. However, although optimal penetration occurred with spermine, fewer infected complexes developed indicating that polyamine cannot totally replace the $Ca^{++}$ requirement. Using phage-specific antiserum to study penetration (Reiter, 1963a), an additional difference between the $Ca^{++}$ and spermine-facilitated

penetration process was noted since the entry of the viral genome was substantially slower in polyamine infections. Penetration data obtained with 0.1 mM polyamine demonstrated a more efficient injection process at reduced spermine concentrations than at reduced $Ca^{++}$ levels. Although injection appeared to be enhanced at reduced polyamine levels, a corresponding increase in infected centers was not observed. Again these data support the observation that an additional $Ca^{++}$ requirement was necessary for the 41c replication.

As in $Ca^{++}$-facilitated penetration, stabilization of the DNA by spermine may be the major ionic role of the polyamine. Spermine has been reported to stabilize DNA (Horacek and Cernohorsky, 1968) protecting the viral DNA from inactivation by EDTA, pyrophosphate and citrate (Van Vunakis and Herriott, 1962). Spermine stabilization also resulted in an increased thermal denaturation temperature (Mandel, 1962). Unlike the permeability role proposed for $Ca^{++}$ in DNA uptake, spermine has an opposite effect since it stabilizes the cell membrane preventing leakage of cellular components (Mager, 1959; Tabor, 1962; Grossowicz and Ariel, 1963; and Harold, 1964).

Post-penetration studies of spermine-supplemented infected complexes revealed that the action of spermine and $Ca^{++}$ on intracellular development are quite different. Growth curves at optimal spermine levels reflected greatly extended latent periods and reduced bursts indicating that spermine failed to allow intracellular phage development or alternately

inhibited the release of infectious phage. The latter mechanism has been observed in a number of polyamine supplemented phage infections. T4rII mutants were able to replicate in a non-permissive host supplemented with polyamine (Ames and Ames, 1965). Apparently, the polyamines stabilized the cell membrane preventing leakage of critical components necessary for phage development. Membrane stabilization was found to be non-specific since other cationic substances permitted T4rII replication (Garen, 1961; Brock, 1965; Buller and Astrachan, 1968). Other investigators observed that the release of completed phage particles could be prevented by incorporating spermine in the infection media (Groman, 1966; Groman and Suzuki, 1966; Gschwender and Hofschneider, 1969). However, in my studies, this did not prove to be the cause of the reduced bursts observed in spermine-supplemented infections since artificial lysis of infected cells failed to increase the number of phage.

Additional studies demonstrated that the metabolic functions of the host were not affected by spermine as reported for other Gram positive organisms (Friedman and Bachrach, 1966). In addition, spermine had no inactivating effects on the intact phage. Therefore, spermine can substitute for $Ca^{++}$ during adsorption and penetration of 41c; however, the polyamine fails to substitute for $Ca^{++}$ which is required for intracellular phage development. These data imply that the attachment and injection mechanism of the virus might not be as specific as first thought. The intracellular requirement

for $Ca^{++}$ appears to be specific; however, these data do not eliminate the possibility that spermine is prevented from entering the infected host.

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Appendix I.   Ions Required for the Adsorption of Bacteriophage.

| Phage | Host | Ion Required | Conc. | Reference |
|-------|------|--------------|-------|-----------|
| T1 | Escherichia coli B | Monovalent<br>Divalent | 100 mM<br>1 mM | Garen and Puck, 1951 |
| T4,12 | E. coli B | $Ca^{++}$ | 6.8 μM | Delbrück, 1948 |
| P2 | E. coli C | $Ca^{++}$ | 2.5 mM | Bertani, Choe and Undahl, 1969 |
| ØX174 | E. coli C | $Ca^{++}$; $Mg^{++}$ | 100 mM | Sinsheimer, 1968 |
| ST-1 | E. coli K12 | $Ca^{++}$ | 10 mM | Bowes and Dowell, 1974 |
| M13 | E. coli ($F^+$) | Monovalent<br>Divalent | 100 mM | Tzagoloff and Pratt, 1964 |
| MS2 | E. coli C ($F^+$) | $Ca^{++}$ | 2 mM | Davis and Sinsheimer, 1963 |
| R17 | E. coli K12 (Hfr) | Monovalent<br>Divalent | 100 mM | Danziger and Paranchych, 1970a |
| ØU-4 | B. stearothermophilus | $Ca^{++}$ | 10 mM | Shafia and Thompson, 1964 |
| B | Corynebacterium diphtheria | $Ca^{++}$ | 1 mM | Barksdale and Pappenheimer, 1954 |
| type I; II | Streptomyces griseus | $Ca^{++}$ | 10 mM | Perlman, Langlykke and Rothberg, 1951 |
| Bo1; Bo2a | Mycobacterium smegmatis | $Ca^{++}$ | 200 mM | Kraiss, Gelbart and Juhasz, 1973 |
| Rs1 | Rhodopseudomonas spheroides | $Ca^{++}$<br>$Mg^{++}$ | 1 mM<br>5 mM | Abeliovich and Kaplan, 1974 |

Appendix I. (Continued)

| Phage | Host | Ion Required | Conc. | Reference |
|-------|------|--------------|-------|-----------|
| 42E;<br>42B; 7 | Staphylococcus<br>aureus | $Ca^{++}$ | 0.34 mM | Roundtree, 1951 |
| 2 | Pseudomonas<br>aeruginosa | $Na^{++}$<br>$Ca^{++}$; $Mg^{++}$ | 100 mM<br>10 mM | Reese, Dimitracopoulous<br>and Bartell, 1974 |
| MVL-1 | Acholeplasma<br>laidlawii | Monovalent<br>Divalent | 80 mM | Fraser and Fleischmann, 1974 |