1953

Role of calcium and related ions in proliferation of lactic streptococcus bacteriophage

Norman N. Potter
Iowa State College

ROLE OF CALCIUM AND RELATED IONS IN PROLIFERATION OF LACTIC STREPTOCOCCUS BACTERIOPHAGE

by

Norman N. Potter

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Dairy Bacteriology

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State College

1953

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

INTRODUCTION

The ion requirements of lactic streptococcus bacteriophage have received relatively little study. Calcium ion has been shown necessary for multiplication of several strains of this virus while it was not required for growth of host bacteria. However, the mode of action of this ion is obscure. Calcium action in other bacteriophage systems has been investigated by various workers, but disagreement as to its function is to be found in the literature. This may be due largely to differences in the processes by which viruses are synthesized.

Several techniques are available for studying the bacteriophage proliferation cycle. Use of methods that determine such characteristics of virus multiplication as rate of adsorption to host cells, length of time from adsorption to liberation of new virus, period over which virus is liberated, and number of virus particles produced per infected cell, has proven extremely valuable in studying action of several stimulatory as well as virucidal agents. Using such methods, the present investigation has been concerned with the role of calcium and related ions in proliferation of lactic streptococcus bacteriophage.

STATEMENT OF PROBLEM

The purposes of this work have been:

A. To study certain characteristics of the calcium requirement for bacteriophage proliferation.

B. To analyze calcium action.

C. To study the effects and mode of action of other cations alone and in relation to calcium.

REVIEW OF LITERATURE

This review shall be concerned with (1) the more recently held views on the mechanics of bacteriophage multiplication, and (2) the effects of calcium and other ions on various bacteriophage-host systems. Familiarity with this material is prerequisite to the design and interpretation of experiments directed at elucidating the mode of action of calcium and certain related ions.

Multiplication of Bacteriophage

The following generalized picture may serve to illustrate the sequence of events accompanying bacteriophage (and more generally virus) production. In suitable media free bacteriophage particles coming into contact with susceptible bacteria are adsorbed and held to the cell surface by attractive forces. Following adsorption, certain processes result in the invasion of the cell by the bacteriophage, the invading bacteriophage particle losing its identity. The virus-host complex now goes through a period during which the metabolism of the host apparently is directed to the production of new bacteriophage or components thereof. During this time no

bacteriophage is liberated. Rather suddenly bursting of the host cell occurs and bacteriophage particles in varying numbers are freed. These may attach to other host cells and the process be repeated. This is an over-simplification and an enormous amount of work has been directed at more thorough understanding of the processes involved. Some of these studies will now be considered.

Adsorption

The term "adsorption" has come to have different meanings among virologists, largely because of the difficulty of determining the boundary between physical attachment of the virus and chemical invasion of the host. The most extensive studies of adsorption, as well as the other phases of bacteriophage multiplication, have been conducted with the bacteriophages of the T series (Delbrück, 1946) active against Escherichia coli strain B. The term adsorption as it is used by workers studying this system of viruses refers to "the occurrence of an irreversible specific union between virus particles and host" (Delbrück, 1946, p.13). In this thesis the term adsorption shall refer to the initial attachment of virus to cell surface and be distinguished, where possible,

from the process of invasion by which the adsorbed virus effects an entry into the host cell.

Several workers have shown that under given conditions the adsorption rate is proportional to the cell and bacteriophage concentrations and that the fraction of bacteriophage particles remaining free decreases exponentially with time (Krueger, 1931; Schlesinger, 1932; Delbrück, 1940a). Although adsorption may be very rapid, several factors are known to affect its rate. Delbrück (1940a) has shown that adsorption rate depends on the physiological state of the bacterial host. He offered two simple explanations for this. First, the size of a bacterium changes considerably depending on its phase of growth; an increased cell surface should lead to an increase in the adsorption rate onto a given number of bacteria. Second, for motile bacteria, adsorption will be more rapid when the cells are actively motile than when motility is slowed down by adverse physiological conditions. He found that adsorption rate was more than sixty times greater under optimum than poor conditions.

Certain organic materials and inorganic ions have been found to affect adsorption; these have been termed "adsorption cofactors." Coliphage T4 and T6 have been shown by Anderson (1945b, 1948) to require L-tryptophan

for adsorption. Phenylalanine, diiodotyrosine, tyrosine and several synthetic amino acids having aromatic groups were somewhat effective, but D-tryptophan appeared to be inactive. It was concluded that L-tryptophan activated the bacterial virus rather than the host cell, for the fractions of virus adsorbed varied with the conditions under which the virus had been exposed to its cofactor. Activation approached maximum at a concentration of 2 μg. L-tryptophan per ml., a temperature of 35° C, a pH of near 8, and about 2 minutes exposure of virus to cofactor. Activation was reversible by dilution of activated virus in cofactor-free medium. These characteristics suggested to Anderson that activation of the virus was not unlike the combination of substrate molecules with enzymes. Mutations of bacteriophage with respect to cofactor requirements were shown to occur by Delbrück (1948), and he described three mutant types of coliphage T^4 as follows: (1) a type requiring no adsorption cofactor; (2) a type requiring tryptophan or similar substances; and (3) a type requiring tryptophan or similar substances and in addition requiring calcium ions. Indole and to a lesser extent skatole inhibited adsorption of the mutants requiring tryptophan. Ionic cofactors were reported necessary for the infectivity of a variety of bacteriophages acting on

Escherichia, Salmonella, Eberthella, and Shigella organisms by Hershey et al. (1944). Univalent cations were most effective. For a bacteriophage active on E. coli, both rate of adsorption and a subsequent step leading to virus multiplication were influenced more by sodium and ammonium than potassium or lithium ions at equivalent concentrations. The anions were not a factor.

Considerable disagreement exists as to the nature of the adsorptive process. With the coliphages of the T series it is accepted that bacteriophages are adsorbed only by strains of the host capable of supporting their multiplication (Anderson, 1949; Benzer et al., 1950). This specificity has led some workers to suggest that the adsorption process is analogous to the union between antigen and antibody in which steric fitting of specific complementary structures is believed to occur. Such structures have been termed "receptor spots" or "receptors" and a great deal of evidence points to their existence (Hirst, 1950; Burnet, 1951); this will be discussed in connection with invasion. If receptor spots are responsible for specific adsorption, it is difficult to imagine these as restricted areas of the cell surface, for Schlesinger (1932) and others have shown that under favorable conditions almost every collision between

bacteriophage and host results in adsorption. Such first order kinetics requires that reaction be possible at any point of contact between the reacting bodies. Anderson (1949) suggests that this condition could be met if projecting elements such as "mucoid chains" existed at the surface of one or both of the colliding bodies. In contrast, Puck et al. (1951) propose that the initial binding of the virus to the host cell is an electrostatic one, determined by the presence of an appropriate configuration of ionic charges on the two bodies. They point out this theory is consistant with (1) the extremely rapid reaction rate between virus and host cell under optimum conditions; (2) employing coliphages of the T series they could adjust the rate of adsorption in chemically defined media to any value between zero and the maximum theoretically possible rate by control of the ionic constitution of the medium alone; (3) for a fairly large group of positive ions strongly influencing adsorption, the only specificity exhibited was associated with total ionic charge; and (4) adsorption of virus to polar inorganic substrates, such as glass filters, could be accomplished, and required the same ionic constituents as did union between virus and its specific host; in both cases the virus could be eluted by changing the composition of the medium so as to reverse the direction of the ionic equilibrium involved. These workers propose that high

collision efficiency can be explained by electrostatic forces tending to orient the complementary surfaces during their approach in the course of collision and even where only a fraction of the reactive groups on the surfaces came together, the strong binding forces would hold the bodies together long enough for more favorable adjustment of position to take place. They suggest adsorption specificity is the result of ions attaching to specific sites on virus and probably cell. To determine specific chemical groupings responsible for the bond formation constituting primary attachment, Tolmach and Puck (1952) treated virus and host cells with reagents which block various reactive groups and then studied the adsorption characteristics of the modified forms. Virus labelled with P^{32} permitted assay of adsorbed and free virus, although virus was inactivated by certain of the reagents. Treatment of cells with carboxyl-blocking reagents suppressed their ability to bind T2, while strong acid, an oxidizing agent, and reagents blocking amino and sulfhydryl groups had no effect. In the case of T1 virus, cells lost most of their binding ability when treated with amino-blocking reagents. Similar study with treated T2 virus suggested that amino and carboxyl groups of the virus surface also take part in the attachment. These findings were in

agreement with adsorption properties at various hydrogen ion concentrations, maximum adsorption occurring at pH's consistent with the pK values of carboxyl and amino or substituted amino groups.

Invasion

Benzer et al. (1950, p.121) indicate that while "the specific combination of phage with its receptor spot does not harm the bacterium," during the phase designated as invasion, "The bacterium is killed in the sense that it fails to proliferate and cannot be made to do so by any known means." It is suggested by Anderson (1949, p.80) that adsorption may "prepare the way in some specific manner for the virus to encounter and utilize its intracellular requirements." Several workers have reported that, following the initial contact, a host-virus complex results from which virus can no longer be eluted. Caren and Fuck (1951) present evidence that the reaction responsible for this is of enzymatic nature, being temperature dependent while the initial adsorption is not. Considerable work has been done on this phase with the influenza group of viruses; from this has come much of the knowledge on receptor spots. Hirst (1941) and McClelland and Hare (1941) discovered that when influenza virus was mixed with red blood cells the latter

agglutinated. This was found due to attachment of cells through mutually adsorbed virus. This phenomenon has been studied intensively and is discussed by Burnet (1951). After a period of 37° C, agglutinated cells are again dispersed and adsorbed virus is found to be eluted, no multiplication of the virus having occurred. Such cells can no longer be agglutinated by the same strain of virus, while the eluted virus retains ability to agglutinate additional fresh cells. The inagglutinable cells may, however, be agglutinated by a different virus strain. This was found due to destruction by the virus of a surface component of the cells which has been designated receptor material. That this destruction is enzymatic has come to be accepted (Hirst, 1950). It now is recognized that mumps, Newcastle disease, influenza, and certain other viruses, and in addition filtrates of some bacteria, can destroy receptor material. Receptor material has been isolated from many types of cells, including bacterial cells susceptible to bacteriophage attack (Benzer et al., 1950). Burnet (1951), his co-workers, and others have been interested in the chemical nature of receptor material and the process of its degradation. The receptor material of the red blood cells is a mucopolysaccharide which, when combined with influenza virus or a soluble enzyme of the cholera vibrio,

is split to yield dialysable nitrogenous material. The "receptor destroying enzyme" of cholera vibrio has been purified by Ada and French (1950). It is completely inactive in the absence of calcium ion, is active over a fairly wide pH range with an optimum at pH 6.2, and is destroyed by 30 minutes heating to 55° C. Burnet (1951, p.124) hypothesizes that in relation to influenza viruses this enzyme "in all essentials is identical with an enzyme built into the surface structure of the virus particle."

A parallelism exists between this work and certain studies with the bacteriophages. Several reports have indicated a relationship between bacterial polysaccharides, bacteriophages, and the carbohydrase lysozyme. Wollman and Wollman (1938) have reported that egg white lysozyme released bacteriophage from association with a lysogenic strain of Bacillus megatherium. White (1937) has reported similar findings with a lysogenic strain of Vibrio cholerae. Pirie (1940) has found that egg white lysozyme hydrolyzed a carbohydrate present in B. megatherium and that in doing this could release bacteriophage from its union with heat-killed bacteria. Following such treatment the bacteria could no longer adsorb bacteriophage. Anderson (1945a) has been able to separate an active

lysin from coliphage T2 following disintegration of the virus by sonic vibration. This lysin, not sedimentable at 32,000 r.p.m. for 1 hour, rapidly lysed ultraviolet irradiated cells of E. coli. It seemed to possess some of the properties of the enzyme lysozyme, the irradiated cells being as sensitive to lysozyme as the classical test organism Micrococcus lysodeikticus. The virus lysin exhibited greater specificity, however, having no visible effect on the latter organism. The implications of the above studies are not entirely clear, although it seems probable that invasion of the host is mediated by enzyme action. Destruction of receptor material has been suggested as a means by which virus effects entry into the host cell but proof of this is lacking (Hirst, 1950).

Electron microscope studies by Luria et al. (1943) on the coliphages reveal that in cases of multiple infection many adsorbed virus particles do not penetrate the host. This has been used by Delbrück (1945a) to explain the "mutual exclusion" phenomenon (Delbrück and Luria, 1942) in which simultaneous infection of a bacterium with two or more dissimilar viruses results in growth of only one of these with lysis of the host after a latent period characteristic of the bacteriophage that grows. The "penetration hypothesis" of Delbrück (1945a, p.166) suggests that "entrance of the first virus

makes the cell wall impermiable to other virus particles, just as the fertilization of an egg by one spermatozoon makes the egg membrane impermiable to other spermatoza." Another theory to explain mutual exclusion will be considered in relation to intracellular multiplication.

Loss of ability of the host to multiply was indicated as a primary effect of virus invasion. In addition, Cohen and Anderson (1946) noted that the rate of oxygen consumption and the respiratory quotient of inhibited coliform bacteria remained at values observed just before infection. This suggested that the original respiratory enzymes were unaffected by invasion but that continued synthesis of these had stopped. Monod and Wollman (1947) showed that at this stage infected strains of E. coli were unable to synthesize adaptive enzymes for the utilization of lactose. These changes, perhaps, preface intracellular multiplication.

Intracellular multiplication

A period follows invasion during which no increase in virus is demonstrable by the ordinary methods. This latent period differs in length depending on the host-virus system and other factors. For various coliphages its duration is 13 to 40 minutes (Benzer et al., 1950), for a lactic streptococcus bacteriophage it is somewhat

over 30 minutes (Cherry and Watson, 1949), while for a number of animal viruses it is several hours (Henle et al., 1947; Ginsberg and Horsfall, 1949b; Ginsberg and Horsfall, 1951). The most dynamic changes in the host-virus complex occur during this interval and it is here that knowledge is most incomplete as evidenced by the several hypotheses (Horsfall, 1949) for the accompanying synthesis.

The studies of Luria et al. (1943) indicate that multiplication must occur inside of the cell wall. Several investigations have shown that the invading particle breaks up and is not recovered among the viral progeny (Delbrück and Luria, 1942; Putnam and Kozloff, 1950; Doermann, 1951). Cohen and Anderson (1946) have reported on the chemical composition of coliphage T2. This virus contains 37 per cent desoxyribonucleic acid (DNA), and 3.7 per cent phosphorus, all of the phosphorus existing in the DNA. No ribonucleic acid (RNA) was detected. These observations have been confirmed and extended by the biochemical studies of Cohen (1948a) which showed that although normal E. coli cells synthesize more RNA than DNA, after infection phosphate is channeled into formation of only the DNA characteristic of the virus. In infected cells protein synthesis was apparent from the beginning of infection; DNA synthesis began 7 to

10 minutes later. The rates of synthesis of protein and DNA were constant regardless of the number of virus particles formed within the cell. This was interpreted as indicating that the virus does not contain the enzymes for DNA or protein synthesis and that new enzymes are not synthesized; therefore, "the metabolic equipment which synthesizes the most complex virus components are the enzymes of Escherichia coli B, presumably according to the new models supplied by the infecting virus particles" (p.291). Cohen (1948b) found that host RNA nucleotides were not precursors of virus DNA and that about 80 per cent of the phosphorylated virus components was synthesized from P^{32} labeled phosphorus derived from a synthetic medium after infection. Purine and pyrimidine synthesis in infected host cells was followed by Cohen and Arbogast (1950b) by measuring the ultraviolet absorption of the bacterial suspension. Purine and pyrimidine synthesis paralleled DNA synthesis but preceeded it slightly. From these several studies Cohen and Arbogast (1950a, p.619) concluded that virus synthesis "occurs in stages, protein being formed first, followed by purine and pyrimidine synthesis, succeeded by the formation of protein-bound DNA, and finally intact virus particles." In addition to confirming that some

80 per cent of the virus phosphorus came from the medium after infection (Kozloff and Putnam, 1950), Kozloff et al. (1951) found that about 80 per cent of the N^{15} labeled nitrogen of coliphage T6 was derived from the medium following infection. Bacterial nitrogen, contributing the remainder appeared in bacteriophage nucleic acid and protein, some bacterial DNA being transferred to each new virus particle. These findings have been interpreted as indicating that only a small fraction of total bacterial protoplasm is converted to bacteriophage (Kozloff and Putnam, 1950). The fraction of P^{32} labeled phosphorus of invading bacteriophage particles appearing in the newly formed virus progeny was found by Putnam and Kozloff (1950) to be about 22 to 42 per cent. Maaløe and Watson (1951) extended this finding by carrying coliphage $T2r^{+}$ labeled with P^{32} through two successive cycles of reproduction in unlabeled bacteria. In both cycles the parent bacteriophage particles contributed about 30 per cent of their phosphorus to the progeny bacteriophage. The phosphorus could not be accounted for in specific parts of the DNA of the progeny.

Several investigators have attempted to study the intracellular process through the use of metabolic inhibitors of known biochemical action. One of the early studies showing the dependence of bacteriophage production on certain fundamental cellular reactions

was made by Spizizen (1943). When cells of E. coli were suspended in low concentrations of glycine anhydride, their growth was stopped but they could support considerable bacteriophage production. Under such conditions phosphorylated compounds known to be important in cell respiration, such as yeast nucleic acid, α-glycerophosphoric acid, glucose-6-phosphate, adenosine triphosphate, coenzyme I, and adenylic acid stimulated virus production. Certain 4-carbon dicarboxylic acids and α-ketoglutarate also were effective. The stimulation by ferrous, ferric, manganous, and magnesium ions was considered possibly due to activation of enzymes of the cells. The respiratory poisons cyanide, iodoacetate, and arsenite inhibited bacteriophage production. This was interpreted as indicating that the cytochrome system probably was important in the production of this virus. Para-aminobenzoic acid was considered an essential metabolite in the synthesis of virus, as this compound stimulated virus production, while addition of several sulfonamide compounds inhibited it. Fitzgerald and Lee (1946) found several acridine compounds inhibited bacteriophage multiplication in infected E. coli cells at concentrations below the bacteriostatic level. The drugs did not inactivate free bacteriophage or prevent cell infection.

The effect could be counteracted by RNA, suggesting the action was directed at some cellular mechanism involving nucleic acid. The acridine compound proflavin appears to block a late step in the production of active coliphage (Foster, 1948) since removal of the drug by dilution in the later part of the latent period allows bacteriophage liberation without any appreciable delay. These compounds interfere with the multiplication of a large group of unrelated viruses including those of feline pneumonitis, meningopneumonitis, influenza A and B, mumps, vaccinia, and psittacosis-lymphogranuloma (Eaton, Cheever, and Levenson, 1951). Cohen and Fowler (1947) found 5-methyl tryptophan inhibits synthesis of the coliphage T2 by competing with tryptophan metabolism. By adding the inhibitor prior to infection and at different stages of the latent period and then removing the inhibition by further addition of tryptophan, the authors were able to conclude that tryptophan was required within the first minutes of infection and again at 12 minutes into the latent period. Wooley and Murphy (1949) have shown that desoxypyridoxine, an analogue of pyridoxine with specific antivitamin activity in animals, inhibits multiplication of coliphage T2 without materially affecting the growth of the host cell. This effect could be counteracted by pyridoxine, indicating that this vitamin is necessary

for multiplication of the bacteriophage. Since glucose would not reverse the inhibition but glucose-6-phosphate or pyruvate would, it was suggested that possibly desoxypyridoxine interfered in some way with glucose utilization. The inhibition also could be reversed by several short-chain fatty acids and to a limited extent by lactic, malic, fumaric, or succinic acids. Thompson et al. (1950) demonstrated that five purine derivatives inhibited multiplication of the vaccinia virus in chick embryonic tissues. The effect of 2,6-diaminopurine could be reversed by adenine, adenylic and guanylic acids, yeast nucleic acid, diphosphopyridine nucleotide, and hypoxanthine. Several natural amino acids reduce the rate of multiplication of influenza and mumps virus (Eaton, Magasanik, Perry, and Karibian, 1951); most active were the basic amino acids arginine, lysine, and ornithine. L-methionine was shown necessary for synthesis of a strain of influenza Type A virus by Ackermann (1951). Synthesis was prevented by methoxinine and ethionine. The literature contains reports of many such investigations.

Attempts have been made to determine at what stage of the latent period bacteriophage particles begin to appear. Using ultraviolet light to inactivate coliphage T2 during intracellular growth, Luria and Latarjet (1947) found that a multiple-hit curve first was obtained between 7 and 12 minutes after infection. This was interpreted

as indicating that in the case of this bacteriophage the number of particles begins to increase at this period. Doermann (1948,1951), by inducing premature lysis of infected bacteria with sonic vibration or a combination of chemical agents and a bacteriophage capable of producing "lysis from without" (Delbrück 1940b), found that particles of coliphage capable of forming plaques were not produced until the second half of the latent period. The number of particles then produced was a linear function of time to a maximum. Delbrück (1945b) showed that under suitable conditions this maximum for a phage active against E. coli ranged from 20 to over 1000. The wide variation in virus yield from individual cells could not be accounted for on the basis of variation in size of the bacteria alone.

In closing this section mention should be made of reports in which more than one virus type proliferates on the same host cell and the interpretation that these have received. The mutual exclusion phenomenon (Delbrück and Luria, 1942) and the "depressor effect" (Delbrück, 1945a), in which the excluded virus reduces the yield of the virus that grows, have suggested to several workers a competition for an essential substrate or enzyme system of the host. Common as is such interference of growth of one virus by a closely related type, not every virus pair

exhibits this. Syverton and Berry (1947) present cytological evidence that individual epithelial cells of the rabbit's cornea can be infected simultaneously with vaccinia and B viruses or vaccinia and herpes viruses. Similarly Anderson (1942) has shown dual infection of single cells of chick embryos with different combinations of viruses such as fowlpox and herpes simplex, fowlpox and laryngotracheitis, and herpes simplex and rabies viruses. Dual infection of cells with bacteriophages has been reported by Hershey (1946), Delbrück and Bailey (1946), and others. This has led to the hypothesis (Hershey, 1946; Ginsberg and Horsfall, 1949a; and others) that when two viruses attack a single cell they do so by entering into the intracellular mechanisms by different metabolic pathways. A similar conclusion was reached by Benzer (1952) for the coliphages T2r and T7 independantly attacking their host. Using changes in resistance to ultraviolet light of the virus-host complex as an index to the developmental process, the author observed that the survival curves of T2r were markedly different from those of T7. This was interpreted as evidence for differences in the mode of action of closely related bacteriophages.

Effects of Calcium and Other Ions on Bacteriophage-Organism Systems

The work of Hershey et al. (1944) indicating that inorganic salts influence rate of adsorption and a subsequent step leading to multiplication of a variety of bacteriophages has been mentioned, as has the theory of Puck et al. (1951) that the initial reversible attachment of virus to host is electrostatic. Garen and Puck (1951) have shown that following the initial attachment, an enzymatically controlled reaction, associated with establishment of an undissociable virus-host complex, is promoted by various cations. Zinc ion prevents this reaction with coliphage T1, presumably by being bound to specific sites on the host cell in competition with the binding of required cations such as calcium, magnesium, and sodium. The importance of the proper ionic balance for virus multiplication is further illustrated by the finding of Spizizen et al. (1951), that in a synthetic medium sulfhydryl compounds inhibit growth of coliphage T2r^{+}. This effect could be overcome by the ionic buffering agent ethylenediaminotetraacetic acid and by manganous or magnesium ions. The inhibition is believed confined to the multiplication or release of the virus, since the sulfhydryl compounds had no effect on host cells, free virus, or virus adsorption. A

stabilizing effect of cations on bacteriophages active against E. coli has been reported by Adams (1949a). When coliphage T5 was subjected to heat, its rate of inactivation was greatly decreased in the presence of 10^{-3} M calcium, magnesium, barium, strontium, manganous, cobaltous, nickel, zinc, cadmium, or cupric ions. This was thought due to the formation of virus-ion complexes.

Of the electrolytes reported to influence bacteriophage multiplication, calcium ion has been shown to have special significance. It is essential or stimulatory to the proliferation of bacteriophages attacking organisms of such genera as Shigella (Stassano and de Beaufort, 1925; Bordet and Renaux, 1928; Wahl, 1946), Escherichia (Wahl, 1946; Delbrück, 1948; Puck, 1949; Adams, 1949b; Kay, 1952), Staphylococcus (Rountree, 1947,1951; Smith, 1948), Streptococcus (Shew, 1949; Reiter, 1949; Cherry and Watson, 1949; Collins et al., 1950; Potter and Nelson, 1952a,1952b), Salmonella (Kay and Fildes, 1950) and Streptomyces (Perlman et al., 1951). The optimum calcium concentrations for bacteriophage proliferation vary in different reports, largely due to the use of growth media of varied composition and different host-virus combinations. Supplementation of calcium-deficient media of known composition with about 10^{-4} to 10^{-3} M calcium ion has permitted rapid growth of coliphage (Adams, 1949b;

Kay, 1952) and bacteriophages attacking lactic streptococci (Potter and Nelson, 1952b). That considerable quantitative differences in calcium requirement exist among closely related strains of bacteriophage is shown by the work of Burnet (1933), Adams (1949b), Potter and Nelson (1952b) and others. Wahl (1946), Rountree (1951) and Adams (1949a) have indicated that the requirement for calcium may be associated with the bacteriophage rather than the bacterial host attacked.

The mode of action of calcium is not at all clear and there is evidence that it may be different in certain systems. Thus, Rountree (1947) found that citrate prevented multiplication of bacteriophage but did not inhibit adsorption on living or dead cells. Puck (1949) and Adams (1949b) reported that, while calcium was required for multiplication, bacteriophages adsorbed on their respective host cells and stopped bacterial multiplication whether this ion was present or not. The latter author indicated that the absence of calcium seemed to block bacteriophage development at a very early stage. The slight effect of calcium on adsorption also could not explain the requirement for this ion in the study of Potter and Nelson (1952b); these workers indicated that the effect of calcium was on rate of

proliferation rather than a limitation of the amount of bacteriophage that could be produced from a given amount of calcium ion present in the medium. Kay (1952) concluded that calcium functioned in intracellular multiplication, and that for maximum virus increase it was required during the major part of the latent period. In contrast to these reports, Delbrück (1948) demonstrated an adsorption cofactor role for calcium in the case of a mutant type of coliphage T^4. This bacteriophage also required tryptophan for adsorption and the requirement for calcium could not be filled by magnesium or other ions. Cherry and Watson (1949) indicated that maximum adsorption of bacteriophage occurred at a calcium concentration optimum for lysis. Rountree (1951) found that calcium was required for adsorption of at least some of the bacteriophages she studied. The possibility that calcium may be an essential structural material for some bacteriophages also has been suggested (Cohen, 1949).

EXPERIMENTAL METHODS

Preparation of Media and Ion Solutions

The preparation of the calcium-deficient medium employed throughout this investigation has been reported by Potter and Nelson (1952b). Its composition is given in Table 1. This medium supports continued growth of all lactic streptococcus cultures studied. None of the bacteriophage strains investigated increased in numbers in the presence of actively growing host bacteria unless this medium was supplemented with calcium or certain other ions. Best results were obtained when this medium was freshly prepared weekly and stored in the dark at 2 to 3° C.

Eugonagar, minus the sulfite, (Baltimore Biological Laboratories) was used in bacteriophage plaque plates and for bacterial plate counts. It was prepared from the individual ingredients, cystine first being brought into solution by adding N NaOH dropwise to this material in a small volume of distilled water. Solution of all ingredients was facilitated by the use of flowing steam. The medium was adjusted electrometrically to pH 6.0 or 6.6, dispensed, and autoclaved at 15 pounds for 15 minutes. It was stored at 2 to 3° C.

Table 1

Composition of the calcium-deficient medium

Bacto vitamin-free casamino acids	6 g.
Glucose	20 g.
Sodium acetate	10 g.
L-Cystine	0.1 g.
Adenine	0.01 g.
Guanine	0.01 g.
Uracil	0.01 g.
Thiamine HCl	0.0001 g.
Calcium pantothenate	0.0001 g.
Pyridoxine HCl	0.0002 g.
Riboflavin	0.0002 g.
p-Aminobenzoic acid	0.0001 g.
Niacin	0.0001 g.
Biotin	0.0000004 g.
K_2HPO_4	0.5 g.
KH_2PO_4	0.5 g.
$MgSO_4.7H_2O$	0.2 g.
NaCl	0.01 g.
$FeSO_4.7H_2O$	0.01 g.
$MnSO_4.7H_2O$*	0.01 g.
Xanthine	0.005 g.
Glutamine	0.1 g.
Asparagine	0.1 g.
Tween 80**	2 g.
Sodium thioglycolate	0.1 g.
Distilled water	1000 ml.

* This and all ingredients above it are supplied in the form of 37.5 g. tryptophane assay medium, dehydrated (Difco Laboratories).

** A polyoxyethylene derivative of sorbitan monooleate.

Stock solutions of calcium were prepared by dissolving analytical grade calcium chloride in distilled water and autoclaving at 15 pounds for 15 minutes. Stock solutions of strontium, barium, magnesium, manganous, nickel, cobaltous, cadmium, chromic, aluminum, lithium, potassium, and sodium ions were prepared from their reagent grade chlorides. Solutions of zinc, cupric, ferrous, and ferric ions were prepared from their reagent grade sulfates. In the case of solutions of cobaltous, ferrous, and zinc ions showing alteration during autoclaving, concentrated stock solutions were not sterilized when shown to contain no viable organisms. Dilutions of all ions were made with sterile distilled water.

Preparation, Maintenance, and Designation of Bacterial Cultures and Bacteriophage Suspensions

The bacteriophage-organism combinations were obtained from the collection maintained in the Dairy Bacteriology Laboratories at Iowa State College. Cultures of lactic streptococci (Streptococcus lactis, Streptococcus cremoris, and intermediate forms) were propagated and carried in litmus milk and calcium-deficient medium. They were incubated at 32° C for approximately 15 hours, and when not in use were stored at 2 to 3° C. To insure a physiologically active culture, the organisms were

transferred daily for a week before being used, and when in actual use were transferred daily. Experiments in calcium-deficient medium were made only with cells that had been transferred in this medium for a week or more, as cells from milk require a period of adaptation for good growth and in addition would carry milk constituents into the experimental system. On two occasions in the course of this investigation, bacterial host ML1, after transfer for several months in the calcium-deficient medium, acquired reduced ability to support bacteriophage proliferation. By repropagating this culture from milk and transferring it for about a week in the calcium-deficient medium before use a fresh sub-culture with the original properties was obtained. Fresh bacteriophage suspensions were prepared by adding 1 ml. of an active susceptible lactic streptococcus culture and 0.5 ml. of bacteriophage-containing material to 150 ml. of sterile skim milk and incubating at 32° C for 8 to 10 hours. Approximately 5.5 ml. of sterile 10 per cent lactic acid then were added to coagulate the milk protein and the mixture filtered through sterile coarse filter paper. The filtrate recovered was passed through a Selas microporus porcelain filter of #03 porosity. One ml. of this bacteria-free filtrate containing the freshly propagated bacteriophage particles was added to 100 ml.

of calcium-deficient medium. This resulted in a slower rate of inactivation than when the bacteriophages were carried in their acidified whey filtrates or when suspended in milk. Bacteriophage suspensions were stored at 2 to 3° C.

In previous reports of work from these laboratories, strains of bacteriophage were designated with the letters F or PF followed by a number; thus, bacteriophage strains F68, PF11, etc. Bacterial hosts were designated with numbers, or letter and number combinations, for example, hosts 122-1 and H1-10. In the present investigation several bacteriophages were employed which can attack more than one bacterial host. It was found that the virus progeny resulting from growth of a given bacteriophage on these various hosts differed in certain respects. To indicate that they were different, bacteriophage produced by propagation of strain F68 on host 122-1, for example, was designated bacteriophage F68(122-1), while bacteriophage from propagation of strain F68 on host IP5 was designated F68(IP5). By this scheme, bacteriophage F68(IP5) in combination with one of its susceptible hosts, 146-1, was referred to as bacteriophage-host combination F68(IP5)/146-1. The bacteriophage preparation resulting from growth of F68(IP5) on 146-1 was designated bacteriophage F68(146-1).

Measurement of Bacteriophage and Bacterial Concentrations

Bacteriophage was enumerated by the double-layer plaque plate technique of Potter and Nelson (1952a) except that eugonagar, minus the sulfite, adjusted to pH 6.0 was used for both layers and 1.0 per cent $CaCl_2.2H_2O$ was the cell diluent. Bacteriophage titer refers to concentration of plaque-forming particles per ml. In some of the tables plaque counts were adjusted to two significant figures to facilitate comparison of data. Bacterial plate counts were made with eugonagar, minus the sulfite, adjusted to pH 6.6. Plates were incubated 48 hours at 32° C and bacterial counts reported are the average from duplicate plates. In one experiment counts of bacteria had to be made without introducing calcium into the enumeration system. This was done by the limiting dilution technique. One or 0.1 ml. quantities of successive 100-fold distilled water dilutions of bacteria were inoculated into each of four tubes containing 6 ml. of calcium-deficient medium and tubes were incubated 48 hours at 32° C. Most probable number of organisms was calculated from probability tables (Buchanan and Fulmer, 1928).

Determination of Calcium Requirement for Mass Lysis

A series of test tubes containing 4.5 ml. of calcium-deficient medium plus 0.5 ml. of various concentrations of calcium ion was inoculated with one drop of a 15 hour culture of susceptible bacteria and one drop of a bacteriophage suspension of known concentration. Following approximately 15 hours incubation at 32° C, tubes were observed for mass lysis.

One-Step Growth Technique

One-step growth curves were obtained by modifying slightly the technique of Delbrück and Luria (1942). Host bacteria were grown in calcium-deficient medium in a thermostatically-controlled water bath at 32± 0.1° C for 4.0 to 4.5 hours, depending on the culture. The physiological state and size of inoculum were controlled so as to reproduce closely the cell count for a given period of incubation; this was about 5×10^7 cells per ml. Unless otherwise stated, 4 ml. of a culture then were mixed with 1 ml. of the ion solution under study and 5 ml. of homologous bacteriophage diluted in calcium-deficient medium and at the same temperature to give a ratio of virus to cells of about 1:20. In all cases the adsorption time was 5 minutes. The "multiplicity of infection," which is the number of virus particles

adsorbed per bacterium, actually was less than the ratios indicate, for in most cases only about 50 per cent adsorption took place. Under these conditions practically no bacterium or short chain of bacteria will be infected by more than one virus particle. Following the adsorption period, the mixture was diluted 10^4-fold in calcium-deficient medium at the same temperature to prevent further adsorption and to reduce the virus concentration to a countable number (about 20 to 200 plaques per plate). After incubation for 30 minutes, an additional 10-fold dilution in the same medium was made to prevent readsorption following the burst and to retain a countable number of particles. These dilutions effectively reduced to an inactive level ions introduced into the adsorption mixture. In cases where it was desired to retain these ions in the system, they were included in the diluents. The total time of incubation in the 32° C water bath following adsorption was 70 to 90 minutes. Three ml. samples were removed from the mixture immediately following the adsorption period (zero time) and at 5 minute intervals throughout the critical portion of the curves (in one type of experiment at 10 minute intervals) and titered for virus without further dilution. Bacterial plate counts were made at zero and 70 to 90 minutes. In all figures

bacterial and plaque counts are plotted as log of count per ml.

Single Infected Cell Technique

The single infected cell method of Burnet (1929) was employed, with slight modification, to determine the effect of various ions on burst size (virus yield per cell) and the fraction of infected cells bursting. For these studies adsorption mixtures containing the ion under investigation were prepared exactly as in one-step growth experiments. Following the 5 minute adsorption period, a mixture was diluted with calcium-deficient medium to a point at which less than one infected bacterium was present in 3 ml. Three ml. samples of this mixture were dispensed into each of a series of test tubes, incubated in a water bath at 32° C for 70 minutes, and placed in an ice bath to keep further changes at a minimum. The undiluted contents of these tubes were titered for virus. Under these conditions virus content of most tubes is the result of bursting of single cells. In estimating average burst size by this method, truer (lower) values were obtained if a correction for the probability of getting more than one infected bacterium per tube is made. This correction is easily applied in cases where a constant fraction of infected cells bursts.

In data to follow, the fraction of infected cells bursting varies with the ionic composition of the medium and for this reason no simple correction is available.

Bacteriophage Adsorption (Attachment) Measurement

Adsorption mixtures were prepared as for one-step growth and single infected cell methods. A control mixture contained the bacteriophage and ion under study in calcium-deficient medium but no bacterial cells. Following 5 minute adsorption time, mixtures were dispensed in 4 ml. quantities into screw cap test tubes. Per cent adsorption was determined by titering the virus in the supernatants after centrifuging the mixtures 10 minutes at 3,000 r.p.m. in an International centrifuge with a cat. no. 813 angle head to sediment bacteriophage particles adsorbed to cells.

Ion Screening Methods

To determine ability of ions other than calcium to support bacteriophage proliferation in calcium-deficient medium, 0.5 ml. of various concentrations of the ions under study plus 4.5 ml. of calcium-deficient medium were inoculated with 1 drop of a 15 hour culture of susceptible bacteria and 1 drop of a bacteriophage suspension of known concentration. Following 15 hours

incubation at 32° C tubes were titered for virus except in some cases where growth was indicated by mass lysis. Turbidity development in a parallel series of tubes containing no bacteriophage indicated the effect of the ions on bacterial growth.

The effects of various ion-calcium mixtures on rate of bacteriophage proliferation was studied in similar manner except that tubes containing cells and virus in the ion-supplemented medium were titered for virus after 3 or 4 hours incubation, depending on the host-virus combination. During the period required for titration, tubes were held in an ice bath to minimize titer increases in the developing systems. Following additional incubation to 15 hours, tubes were observed for mass lysis and effects of ions on bacterial growth.

EXPERIMENTAL RESULTS

Some Characteristics of the Calcium Requirement for Bacteriophage Proliferation

Before starting investigation on the mode of action of calcium in the lactic streptococcus bacteriophage system, study of certain characteristics of the calcium requirement was undertaken for the information this might contribute to the above problem. Furthermore, it has been suggested that calcium requirement may be of value for identification of bacteriophages active against lactic streptococci as well as other bacteriophage strains. It was of interest, therefore, to determine whether calcium requirements are characteristic of the bacteriophage or of the organism with which the bacteriophage is in association, and in addition, to what extent calcium requirement for a given bacteriophage-host combination is constant.

Calcium requirements of a bacteriophage propagated against various bacterial hosts

In these experiments calcium requirement refers to the minimum calcium ion concentration required for mass lysis of a host culture. Using mass lysis as an indication

of calcium requirement for bacteriophage proliferation limits the interpretation that may be made from the data. While mass lysis occurs only after appreciable bacteriophage numbers have been produced, observation following 15 hours incubation tells nothing of the proliferation rate in cases where mass lysis occurred. Several experiments have shown that bacteriophage titer is much the same in systems having undergone mass lysis, regardless of calcium concentration, and that progressively lower bacteriophage numbers are produced with decreasing calcium level in systems not lysed. Effect of calcium concentration on rate of bacteriophage proliferation is considered in the section on analysis of calcium action.

Where several susceptible host cultures for a given bacteriophage were available, test cultures were selected for diversity according to their bacteriophage sensitivity patterns. The concentrations of bacteriophage particles (determined against host cultures used in their production) and susceptible cells per ml. in tubes for determination of calcium requirement, were about 10^3 and 10^6, respectively. Table 2 gives the calcium requirements for mass lysis of various bacterial hosts susceptible to a given bacteriophage strain. Calcium requirements of four of the five bacteriophage strains studied were influenced by the organism against which these bacterio-

Table 2

Calcium requirements for mass lysis* of various hosts susceptible to a particular bacteriophage

Bacteriophage/host combination	Concentration of calcium ($\times 10^{-4}$ M)										
	0	1	2	3	4	5	6	8	10	12	35
F68(IP5)/IP5	-	-	+	+	+	+	+	+	+	+	+
" /122-1	-	-	-	-	+	+	+	+	+	+	+
" /122-2	-	-	-	+	+	+	+	+	+	+	+
" /146-1	-	-	-	-	-	-	±	+	+	+	+
F69(ML1)/ML1	-	-	-	+	+	+	+	+	+	+	+
" /H1-1	-	-	-	-	-	-	+	+	+	+	+
" /H1-6	-	-	-	-	+	+	+	+	+	+	+
" /H1-11	-	-	-	+	+	+	+	+	+	+	+
F4(H1-1)/H1-1	-	-	+	+	+	+	+	+	+	+	+
" /H1-6	-	-	+	+	+	+	+	+	+	+	+
" /H1-9	-	-	-	+	+	+	+	+	+	+	+
" /H1-11	-	-	+	+	+	+	+	+	+	+	+

	Concentration of calcium ($\times 10^{-4}$ M)										
	0	2	4	6	8	10	12	14	16	20	35
F57(799)/799	-	-	-	-	-	-	+	+	+	+	+
" /E8-1	-	-	-	-	-	-	-	-	-	+	+
F63(E8-2)/E8-2	-	-	-	-	+	+	+	+	+	+	+
" /E8-1	-	-	-	-	-	-	-	-	+	+	+

* Mass lysis indicated by +.

phages were propagated. Thus, with bacteriophage F68(IP5), 2×10^{-4} M calcium ion was the minimum requirement for mass lysis of bacterial host IP5 while about four times as much calcium was required for mass lysis of host 146-1. Similarly about twice as much calcium was required for mass lysis of bacterial host H1-1, by bacteriophage F69(ML1), as was required for mass lysis of host ML1. Differences beyond probable experimental error also occurred for bacteriophages F57(799) and F63(E8-2). No significant differences in calcium requirement for growth on susceptible hosts were shown by bacteriophage F4(H1-1). Repeated trails with the various bacteriophage-organism combinations showed that the calcium requirements in Table 2 could be reproduced, in the majority of cases, with a precision of $\pm 1 \times 10^{-4}$ M calcium ion if the procedure was followed exactly. In studies of this kind, lack of reproducibility was found most commonly due to aging of the calcium-deficient medium and, as appears in a subsequent table, to differences in concentration of suspensions of a given bacteriophage.

Calcium requirements of various bacteriophages propagated against the same bacterial host

Where several bacteriophage strains attacking the

same bacterial host were available, test bacteriophages were selected for diversity by the criteria of heat resistance, host range, and antigenic property (Wilkowske, 1949). Bacteriophages were titered on their susceptible hosts, 122-1, H1-1, or E8-1, and diluted to give a concentration of 2×10^3 bacteriophage particles per ml. in tubes for determination of calcium requirement. Concentration of susceptible cells in these tubes was about 10^6 per ml. Table 3 shows that for the bacteriophage-organism combinations studied, the calcium requirement for mass lysis of a particular bacterial host is influenced by the bacteriophage carrying out the lysis. The greatest differences in calcium requirement appeared among the bacteriophages attacking host 122-1; however, reproducible differences also were obtained for the bacteriophages of hosts H1-1 and E8-1. Certain bacteriophage-host combinations appear in both Table 3 and Table 2. A comparison of calcium requirements of these in the two tables reveals an appreciable discrepancy in the case of combination F69(ML1)/H1-1, its calcium requirement being three times as great in Table 2. Subsequent experiments will show that this discrepancy can be explained logically. From the data of Tables 2 and 3 it appears that the calcium requirement for mass lysis is not associated with the bacteriophage strain alone or solely with the

Table 3

Calcium requirements for mass lysis of a particular bacterial host by various bacteriophage strains

Bacteriophage/host combination	Concentration of calcium ($\times 10^{-4}$ M)										
	0	1	2	3	4	5	6	8	10	12	35
F60(HP) /122-1	-	+	+	+	+	+	+	+	+	+	+
PF11(H1-10)/ "	-	-	-	-	-	-	+	+	+	+	+
F68(IP5) / "	-	-	±	+	+	+	+	+	+	+	+
F44(146-1) / "	-	-	-	-	-	-	-	-	-	-	+
F4(H1-1) /H1-1	-	-	-	+	+	+	+	+	+	+	+
PF14(H1-5) / "	-	-	+	+	+	+	+	+	+	+	+
F60(HP) / "	-	+	+	+	+	+	+	+	+	+	+
F69(ML1) / "	-	-	+	+	+	+	+	+	+	+	+

	Concentration of calcium ($\times 10^{-4}$ M)										
	0	2	4	6	8	10	12	14	16	20	35
F57(799) /E8-1	-	-	-	-	-	-	-	-	-	±	+
F65(FH8) / "	-	-	-	-	-	-	-	-	-	±	+
F63(E8-2) / "	-	-	-	-	-	-	-	±	+	+	+
F25(M1) / "	-	-	-	-	-	-	-	-	-	-	+

strain of host organism, but rather, is characteristic of the bacteriophage-host combination.

Effects of age and concentration of bacteriophage suspensions on calcium requirement

In the course of this work several instances were observed in which calcium requirement of a bacteriophage-host combination shifted. These shifts always were in the direction of an increased calcium requirement and were most common where several weeks had elapsed between repeated determinations. As a bacteriophage suspension ages its titer decreases and possibly other changes take place. This suggested investigating to what extent number of bacteriophage particles as well as age of these particles affected calcium requirement. In Table 4, the three bacteriophage suspensions designated "old" were close to two months old. The suspensions designated "fresh" were prepared from their respective old suspensions about 24 hours before being used in the present experiment. The concentration of host cells was about 10^6 per ml. in all cases. Table 4 shows that regardless of age, the calcium requirements of the three bacteriophage strains were appreciably influenced by concentration of bacteriophage particles. In all cases the greater the bacteriophage concentration the smaller

Table 4

Calcium requirements for mass lysis by suspensions of bacteriophage differing in age and concentration

Bacteriophage/host combination	Bacteriophage titer	Concentration of calcium ($\times 10^{-4}$ M) 0	1	2	3	4	5	6	8	10	12	35
F69(ML1)/ ML1 "fresh"	3×10^{3}	-	+	+	+	+	+	+	+	+	+	+
	3×10^{2}	-	-	-	±	+	+	+	+	+	+	+
	3×10^{1}	-	-	-	-	+	+	+	+	+	+	+
	3×10^{0}	-	-	-	-	±	+	+	+	+	+	+
F69(ML1)/ ML1 "old"	3×10^{3}	-	-	-	+	+	+	+	+	+	+	+
	3×10^{2}	-	-	-	-	+	+	+	+	+	+	+
	3×10^{1}	-	-	-	-	-	+	+	+	+	+	+
	3×10^{0}	-	-	-	-	-	-	-	+	+	+	+
F68(IP5)/ IP5 "fresh"	3×10^{3}	-	+	+	+	+	+	+	+	+	+	+
	3×10^{2}	-	-	+	+	+	+	+	+	+	+	+
	3×10^{1}	-	-	+	+	+	+	+	+	+	+	+
	3×10^{0}	-	-	±	+	+	+	+	+	+	+	+
F68(IP5)/ IP5 "old"	3×10^{3}	-	+	+	+	+	+	+	+	+	+	+
	3×10^{2}	-	-	+	+	+	+	+	+	+	+	+
	3×10^{1}	-	-	+	+	+	+	+	+	+	+	+
	3×10^{0}	-	-	-	+	+	+	+	+	+	+	+

		Concentration of calcium ($\times 10^{-4}$ M) 4	6	8	10	12	14	16	20	35
F57(799)/ 799 "fresh"	25×10^{4}	-	-	+	+	+	+	+	+	+
	25×10^{3}	-	-	-	+	+	+	+	+	+
	25×10^{2}	-	-	-	-	+	+	+	+	+
	25×10^{1}	-	-	-	-	-	±	+	+	+
F57(799)/ 799 "old"	25×10^{3}	-	-	+	+	+	+	+	+	+
	25×10^{2}	-	-	-	-	+	+	+	+	+
	25×10^{1}	-	-	-	-	-	±	+	+	+
	25×10^{0}	-	-	-	-	-	-	-	-	+

was the minimum calcium concentration for mass lysis. In the case of old and fresh suspensions of bacteriophage-host combination F69(ML1)/ML1, for example, about a 3 or 4-fold greater calcium concentration was required for mass lysis at a bacteriophage titer of $3x10^{9}$ per ml. than at a titer 1000-fold higher. A second effect, much less marked than that of bacteriophage concentration and associated with age of the bacteriophage suspension, also may exist in the case of bacteriophage-host combination F69(ML1)/ML1. At each level of bacteriophage concentration the calcium requirement of the fresh bacteriophage suspension was slightly less than that of the old suspension. This effect is not evident, however, with the other two bacteriophage-host combinations. These results indicate that for reproducibility of calcium requirements, bacteriophage concentration must be the same in each of a series of determinations. Further, they indicate that, for a comparison of the calcium requirements of various bacteriophages acting on the same host to be meaningful, these bacteriophages must be adjusted to the same concentration against the one host. This was done in obtaining the data of Table 3.

Quantitative activity differences of a bacteriophage against various susceptible bacterial hosts

During the course of selection of bacteriophage

strains and susceptible bacterial hosts used in these studies, there were indications that a difference might exist in the titer of a particular bacteriophage measured against various susceptible hosts. For example, certain bacteriophages observed to be active against a series of bacterial hosts, no longer attacked one or more of these hosts when the bacteriophage suspension had aged several months. When such a bacteriophage was repropagated, the fresh suspension frequently regained its lost activity. To test the possibility that this behavior was associated with differences in titer of a bacteriophage on various hosts, old preparations of each of three bacteriophage strains were plated for plaques using various susceptible host cultures as bacteriophage substrate. Aliquots of a given bacteriophage suspension plated on the various hosts were identical. The results of this experiment are given in Table 5. Bacteriophage F68(IP5) produced about 500 times as many plaques on host IP5 as on hosts 122-1 or 122-2 and about 1000 times as many plaques on host IP5 as on host 146-1. Bacteriophage F69(ML1) showed differences of about equal magnitude on the test organisms used with it. Only slight differences in the titer of bacteriophage F57(799) on its two test hosts occurred. Some variations in plaque size were noted when one bacteriophage acted on

Table 5

Activity of bacteriophage suspensions (old preparations) against various susceptible host bacteria

Bacteriophage	Titer and plaque size* on bacterial hosts: IP5	122-1	122-2	146-1
F68(IP5)	26×10^5 2.1 mm.	56×10^2 2.3 mm.	69×10^2 2.0 mm.	21×10^2 1.4 mm.
	ML1	H1-1	H1-6	H1-11
F69(ML1)	10×10^6 2.5 mm.	44×10^2 1.6 mm.	12×10^3 2.7 mm.	29×10^4 1.6 mm.
	799	E8-1		
F57(799)	245×10^4 3.8 mm.	187×10^4 2.3 mm.		

* Plaque size is the average diameter of twenty plaques.

several hosts; these variations were not related to differences in titer.

The question arose whether these differences in titer were the result of aging of the bacteriophage suspensions or whether they also were characteristic of freshly propagated bacteriophage. The data of Table 6, using approximately 12 hour old bacteriophage propagations show that differences in titer of a bacteriophage on various hosts occur in fresh, as well as old bacteriophage suspensions. Except for higher titers of the fresh preparations on all hosts, these results are in excellent agreement with those of Table 5. The differences in plaque size of a bacteriophage on a given host in the two experiments probably reflect minor variations in the enumeration procedure on the two trials. Fairly good agreement between relative size of plaques of a bacteriophage on its susceptible hosts was obtained in the two experiments. In these experiments the three bacteriophage strains employed gave their highest titers against the bacterial hosts on which they were propagated.

This raised the question of what effect propagation of a bacteriophage on various susceptible hosts would have on titer production of virus progeny. To study this, bacteriophage F68(IP5) was propagated against

Table 6

Activity of freshly prepared bacteriophage suspensions against various susceptible host bacteria

Bacteriophage	Titer and plaque size on bacterial hosts: IP5	122-1	122-2	146-1
F68(IP5)	22×10^8 1.9 mm.	28×10^5 1.9 mm.	57×10^5 1.9 mm.	60×10^4 1.0 mm.
	ML1	H1-1	H1-6	H1-11
F69(ML1)	39×10^8 2.0 mm.	24×10^5 1.0 mm.	41×10^5 1.4 mm.	50×10^7 0.8 mm.
	799	E8-1		
F57(799)	76×10^7 3.9 mm.	50×10^7 3.0 mm.		

bacterial hosts IP5, 122-1, 122-2, and 146-1, giving four fresh preparations of related bacteriophage. Each of these was plated for plaques against each of the four susceptible strains of bacteria and the results are given in Table 7. These show that the titer pattern of bacteriophage F68(IP5) changes when it is propagated on different host bacteria. To this extent, at least, bacteriophage F68(IP5) itself is changed by propagation on various hosts. Bacteriophages F68(IP5), F68(122-1), and F68(122-2) each gave the greatest number of plaques when plated on the parent host. Bacteriophage F68(146-1), however, gave a somewhat greater titer on hosts 122-1 and 122-2 than on its parent host 146-1.

To determine the effect of additional propagations of bacteriophage on titer patterns, bacteriophages F68(IP5), F68(122-1), F68(122-2), and F68(146-1) were carried through several propagations on hosts IP5, 122-1, 122-2, and 146-1, respectively. For these propagations the bacteriophages first were plated for plaques on the above hosts and bacteriophage picked from an isolated plaque was diluted in distilled water to give a concentration of approximately 10^2 particles per ml. in propagation bottles. This low concentration of bacteriophage permitted many generations of bacteriophage to occur during propagation. The freshly prepared suspen-

Table 7

Activity of related bacteriophage progeny against various susceptible host bacteria

Bacteriophage	Titer and plaque size on bacterial hosts: IP5	122-1	122-2	146-1
F68(IP5)	22×10^8 1.9 mm.	28×10^5 1.9 mm.	57×10^5 1.9 mm.	60×10^4 1.0 mm.
F68(122-1)	51×10^5 2.3 mm.	42×10^8 2.2 mm.	30×10^8 2.6 mm.	50×10^7 2.1 mm.
F68(122-2)	70×10^4 1.9 mm.	25×10^8 2.1 mm.	29×10^8 2.1 mm.	90×10^6 1.6 mm.
F68(146-1)	40×10^6 2.0 mm.	59×10^8 1.8 mm.	46×10^8 2.0 mm.	33×10^8 1.6 mm.

sions of bacteriophages F68(IP5), F68(122-1), F68(122-1), and F68(146-1) each was plated for plaques on the four susceptible bacterial hosts and the titer patterns of the bacteriophages noted. From the plate containing plaques produced by bacteriophage F68(IP5) on host IP5, bacteriophage-containing material was picked and diluted as above and used to make a second propagation against host IP5. Similarly, bacteriophage-containing material from plaques of F68(122-1), F68(122-2), and F68(146-1) on hosts 122-1, 122-2, and 146-1, respectively, was used to make subsequent propagations against hosts 122-1, 122-2, and 146-1, respectively. A total of four successive propagations of bacteriophages F68(IP5), F68(122-1), and F68(146-1) and two of bacteriophage F68(122-2) were made. Throughout these propagations the titer patterns of each of the bacteriophages against the four susceptible hosts remained remarkably constant. It was concluded that the titer pattern against various bacterial hosts was rapidly established by propagation of a bacteriophage on a given bacterial host; subsequent propagations on the same host failed to alter it.

The production of a bacteriophage suspension with different titers against various bacterial hosts from bacteriophage-containing material of a single plaque, indicates that these titers may arise from the growth of

an individual bacteriophage particle. To determine if this is the case, a preparation of bacteriophage F68(IP5), for example, was diluted as far as to eliminate titer against all but host IP5. This was then plated for plaques on IP5, and from the plate with the smallest number of plaques three were picked. This material was diluted and used to make three propagations of the bacteriophage on host IP5. In the sense that titer for hosts other than IP5 was diluted out, and material from isolated plaques represents the growth from individual particles of bacteriophage, the three resultant bacteriophage preparations represented the growth from three individual particles of bacteriophage F68(IP5). These preparations were plated for titer against the four bacterial hosts of bacteriophage F68(IP5). The results of this experiment are presented in Table 8. They show that the three bacteriophage preparations gave different titers on the various hosts, and that the patterns of these titers were essentially identical. Data obtained for bacteriophage F68(146-1) by the same procedure also are presented in the table. Results with both bacteriophages are similar.

An investigation of the kind outlined for bacteriophage F68(IP5) also was made with another bacteriophage strain to determine whether these differential titer

Table 8

Activity of individual bacteriophage particle propagations against various susceptible host bacteria

Bacteriophage preparation		Titer on bacterial hosts: IP5	122-1	122-2	146-1
F68(IP5)	A	178×10^7	26×10^5	49×10^5	5×10^5
"	B	82×10^7	22×10^5	31×10^5	6×10^5
"	C	121×10^7	20×10^5	36×10^5	5×10^5
F68(146-1)	A	50×10^6	54×10^8	39×10^8	30×10^8
"	B	21×10^6	47×10^8	52×10^8	38×10^8
"	C	12×10^6	21×10^8	19×10^8	22×10^8

characteristics were general or peculiar to bacteriophages of strain F68. The results of this study are summarized in Table 9, plus brief mention of certain observations. These data were obtained using the same experimental design as for the data of Table 8 except for employing bacteriophages F69(ML1) and F69(H1-1) and their susceptible hosts. These show that preparations of bacteriophage F69(ML1) possessed a markedly greater titer against susceptible bacterial host ML1 than against host H1-1. Similarly preparations of F69(H1-1) were more active against host H1-1 than against host ML1. In the case of both F69(ML1) and F69(H1-1) the different titers appear to arise from the multiplication of individual bacteriophage particles. Other experiments indicated that, as was the case with bacteriophages of strain F68, titer pattern was established rapidly by propagation of a bacteriophage on a given bacterial host. Subsequent propagations on the same host did not change this pattern.

Titer patterns may affect the apparent calcium requirements for bacteriophage growth. This is illustrated by the work with a given bacteriophage propagated against various susceptible hosts (Table 2). While data in Table 2 were obtained using identical bacteriophage aliquots against the various hosts, the titer pattern

Table 9

Repeated trial on activity of individual particle propagations of another bacteriophage strain against susceptible host bacteria

Bacteriophage preparation		Titer on bacterial hosts: ML1	Titer on bacterial hosts: Hl-1
F69(ML1)	A	38×10^8	106×10^4
"	B	29×10^8	95×10^4
"	C	36×10^8	104×10^4
F69(Hl-1)	A	196×10^2	60×10^8
"	B	40×10^2	17×10^8
"	C	$< 1 \times 10^4$	49×10^8

studies show that at least with bacteriophages F68(IP5) and F69(ML1) the aliquots did not possess identical bacteriophage concentration against the various hosts employed. In the case of bacteriophage F69(ML1), for example, bacteriophage concentration was at least 1000 times greater against host ML1 than against host H1-1. It is seen in Table 4 that such difference in concentration may have an appreciable effect on calcium requirement. This could explain the discrepancy, pointed out earlier, in the calcium requirement for mass lysis of host H1-1. In Table 2, bacteriophage F69(ML1) required 6×10^{-4} M calcium to lyse this host while in Table 3 it required only 2×10^{-4} M. However, in the experiment of Table 3 the concentration of bacteriophage F69(ML1) against H1-1 was adjusted to 2×10^{3} particles per ml., while in the experiment of Table 2, the aliquot of this bacteriophage had a concentration against H1-1 very much below this. The different calcium requirements of a bacteriophage for growth on various hosts cannot be attributed entirely to such concentration effects. Experiments in which aliquots of a bacteriophage were adjusted to contain the same bacteriophage concentration against various hosts still showed calcium requirement differences on these hosts. These differences, however, were minimized in several instances by such adjustment.

For example, bacteriophage F63(E8-2) required 8×10^{-4} M calcium for lysis of host E8-2 and 16×10^{-4} M for lysis of host E8-1 in Table 2. Adjustment to 2×10^3 particles per ml. of bacteriophage F63(E8-2) against both of these hosts resulted in a requirement of 10×10^{-4} M calcium for lysis of host E8-2 and 14×10^{-4} M for lysis of host E8-1.

Attempts to lower bacteriophage requirement for calcium

As in the determination of calcium requirements for mass lysis, series of tubes were set up containing bacteriophage, susceptible host cells, and calcium-deficient medium supplemented with various calcium concentrations. Following incubation of such a series, bacteriophage titers in tubes showing mass lysis are much the same regardless of calcium concentration, while titers in tubes not lysed decrease with decreasing calcium level. In this study, material was employed from the tube in which the calcium concentration was just below that required for mass lysis. This broth was passed, unacidified, through a Selas microporus porcelain filter of #03 porosity, titered for bacteriophage, and, following appropriate dilution, used to inoculate a second series of tubes. From the tube of this series just below the calcium concentration required for mass lysis, similarly treated material was

used to inoculate a third series of tubes, and so on. Thus, utilizing as inoculum bacteriophage produced in the presence of sub-optimum calcium concentration, a selection (or training) of bacteriophage with lowered calcium requirement was thought possible. Using each of the bacteriophages F69(H1-1), F63(E8-1), and F57(799), in combination with hosts H1-1, E8-1, and 799, respectively, ten successive propagations were made. The results obtained with combination F69(H1-1)/H1-1 are presented in Table 10. No apparent change in calcium requirement of this bacteriophage occurred as a result of these propagations. While the calcium requirements of bacteriophages F63(E8-1) and F57(799) were slightly less stable throughout the propagations, these bacteriophages similarly showed no lowering of their calcium requirements.

Another experiment was performed in which bacteriophage inoculum was obtained from the first tube of a series showing mass lysis and from tubes containing progressively lower calcium concentrations. Bacteriophage-organism combination F57(799)/799 was employed in this trial. Propagation of bacteriophage in the presence of 14, 12, 10, 8, 6, and 4×10^{-4} M calcium, resulted in titers, after filtration, of 180×10^7, 177×10^7, 23×10^7, 37×10^5, $<1 \times 10^3$, and $<1 \times 10^3$ particles per ml., respectively. Material from these tubes was diluted

Table 10

Calcium requirement of bacteriophage F69(H1-1) following repeated propagation on host H1-1 in a medium containing sub-optimum concentrations of calcium

Propagation number	Initial bacteriophage titer	Calcium conc. ($\times 10^{-4}$ M) for mass lysis					Bacteriophage titer in last unlysed tube*
		1	2	3	4	5	
1	22×10^2	-	-	+	+	+	60×10^6
2	20×10^2	-	-	-	+	+	81×10^5
3	20×10^2	-	-	-	+	+	40×10^6
4	23×10^2	-	-	±	+	+	19×10^5
5	19×10^2	-	-	±	+	+	15×10^5
6	20×10^2	-	-	+	+	+	80×10^6
7	20×10^2	-	-	±	+	+	26×10^5
8	20×10^2	-	-	-	+	+	89×10^5
9	18×10^2	-	-	-	+	+	13×10^6
10	20×10^2	-	-	-	+	+	-

* Last unlysed tube for propagation 1 contains 2×10^{-4} M calcium; for propagation 4 it also contains 2×10^{-4} M calcium.

so as to give an initial bacteriophage titer of 12×10^2 particles per ml. when inoculated into series of tubes for the determination of calcium requirement. Material with titers of $<1 \times 10^3$ was inoculated undiluted. Except for apparent absence of bacteriophage in the undiluted material, bacteriophages produced in the presence of 14, 12, 10, and 8×10^{-4} M calcium all required the same concentration of calcium, namely 12×10^{-4} M, for mass lysis of host 799.

Propagation of bacteriophage on different hosts and its effect upon calcium requirement of virus progeny

With regard to the production of titer patterns, it was shown that bacteriophage may be changed by propagation on various hosts. A study was made to determine if calcium requirement could be changed in this way. Four bacteriophage strains which had in common ability to attack bacterial host 122-1 and four more which attacked host ES-1 were selected. All eight of these bacteriophages were freshly propagated on their respective parent hosts (hosts other than 122-1, or ES-1) before being used in this experiment. The resulting bacteriophages then were propagated against either host 122-1 or ES-1. Thus, 16 fresh bacteriophage suspensions were obtained. These are listed in Table 11.

Table 11

Calcium requirements of related bacteriophages produced by propagation on different bacterial hosts

Bacteriophage	Calcium conc. ($\times 10^{-4}$ M) required for mass lysis of host 122-1										
	0	1	2	3	4	5	6	8	10	12	35
F60(HP)	-	+	+	+	+	+	+	+	+	+	+
F60(122-1)	-	+	+	+	+	+	+	+	+	+	+
PF11(H1-10)	-	-	-	-	-	-	-	+	+	+	+
PF11(122-1)	-	-	-	-	-	-	±	+	+	+	+
F68(IP5)	-	-	±	+	+	+	+	+	+	+	+
F68(122-1)	-	-	+	+	+	+	+	+	+	+	+
F44(146-1)	-	-	-	-	-	-	-	-	-	-	+
F44(122-1)	-	-	-	-	-	-	±	+	+	+	+

	Calcium conc. ($\times 10^{-4}$ M) required for mass lysis of host E8-1										
	0	2	4	6	8	10	12	14	16	20	35
F57(799)	-	-	-	-	-	-	-	-	-	+	+
F57(E8-1)	-	-	-	-	-	-	-	-	±	+	+
F65(FH8)	-	-	-	-	-	-	-	-	-	+	+
F65(E8-1)	-	-	-	+	+	+	+	+	+	+	+
F63(E8-2)	-	-	-	-	-	-	-	-	+	+	+
F63(E8-1)	-	-	-	-	-	-	-	±	+	+	+
F25(M1)	-	-	-	-	-	-	-	-	-	-	+
F25(E8-1)	-	-	-	-	-	-	-	+	+	+	+

The designation F60(HP) corresponds to the suspension from propagating bacteriophage strain F60 on its parent bacterial host HP; the designation F60(122-1) represents the suspension from propagating F60(HP) on susceptible host 122-1, and so on. The eight bacteriophage suspensions active against host 122-1 possessed different titers on this organism and so were adjusted to give a common concentration of 2×10^3 particles per ml. in calcium-containing tubes. The suspensions with activity for host E8-1 were treated similarly. This adjustment of bacteriophage material eliminated the concentration effect (Table 4) on calcium requirement. In Table 11, the calcium requirements of the progeny of three bacteriophage strains are seen to differ depending on the host used in their propagation. Thus, bacteriophage of preparation F44(146-1) required $>12 \times 10^{-4}$ M calcium for mass lysis of host 122-1, while bacteriophage of preparation F44(122-1) required between 6 and 8×10^{-4} M; bacteriophage F65(FH8) required 20×10^{-4} M calcium for mass lysis of host E8-1, while bacteriophage F65(E8-1) required only 6×10^{-4} M; and finally, bacteriophage F25(M1) needed $>20 \times 10^{-4}$ M calcium for mass lysis of host E8-1, while bacteriophage F25(E8-1) required 14×10^{-4} M. The calcium requirements of the progeny of the other five bacteriophage strains were not affected

in this way. At this point the preparations of bacteriophage F65(FH8) and F65(E8-1) were lost and so calcium requirement determinations were repeated with the remaining 14 preparations. These confirmed the results presented in Table 11.

Calcium requirements of bacteriophage produced on hosts growing in different media

Several reports indicate that much of the material of the bacteriophage particle is synthesized from medium constituents following infection of the host cell. The two media in which host bacteria were infected with bacteriophage in this experiment were sterile skim milk and calcium-deficient medium supplemented with 35×10^{-4} M calcium. Bacteriophages F57(E8-1), F63(E8-1), and F25(E8-1) were propagated against host E8-1 in each of these media and bacteriophage suspensions were prepared in the usual way, except for not acidifying the material in the calcium-deficient medium. The six filtrates were titered and diluted to give a concentration of 2×10^3 bacteriophage particles per ml. against host E8-1 in tubes for the determination of calcium requirement. The calcium requirements of the bacteriophages produced in the two media are given in Table 12. These media had little effect on calcium requirements of the bacteriophages.

Table 12

Calcium requirements of bacteriophage propagated in different media

Bacteriophage	Medium	Calcium conc. (x10-4 M) required for mass lysis of host E8-1						
		8	10	12	14	16	20	35
F57(E8-1)	milk	-	-	-	-	+	+	+
"	c.-d.*	-	-	-	-	-	+	+
F63(E8-1)	milk	-	-	-	+	+	+	+
"	c.-d.	-	-	-	+	+	+	+
F25(E8-1)	milk	-	-	-	±	+	+	+
"	c.-d.	-	-	-	+	+	+	+

* Calcium-deficient medium supplemented with 35x10-4 M calcium.

Analysis of Calcium Action

Investigations on lactic streptococcus, as well as other bacteriophage types, have indicated that calcium action cannot be explained by the effect of this ion on rate of host cell growth and usually not by its influence on bacteriophage adsorption. Beyond this point information is limited. In this study almost all experiments were performed in similar detail using the two bacteriophage-organism combinations F69(ML1)/ML1 and F63(ES-2)/ES-2. These were selected on the basis of production of readily countable plaques and of differences between the bacteriophages, including differences in amount of calcium required for rapid bacteriophage growth. Much of the data obtained with both combinations are presented to indicate the extent of differences observed.

Calcium concentration and rate of bacteriophage multiplication

Calcium-deficient medium supplemented with calcium in concentrations from 0 to 32×10^{-4} M was inoculated with approximately 4×10^{4} particles of bacteriophage F69(ML1) and 10^{6} host cells per ml. and incubated at 32° C. Titers of bacteriophage were made at hourly intervals. The results of this experiment are presented in Figure 1.

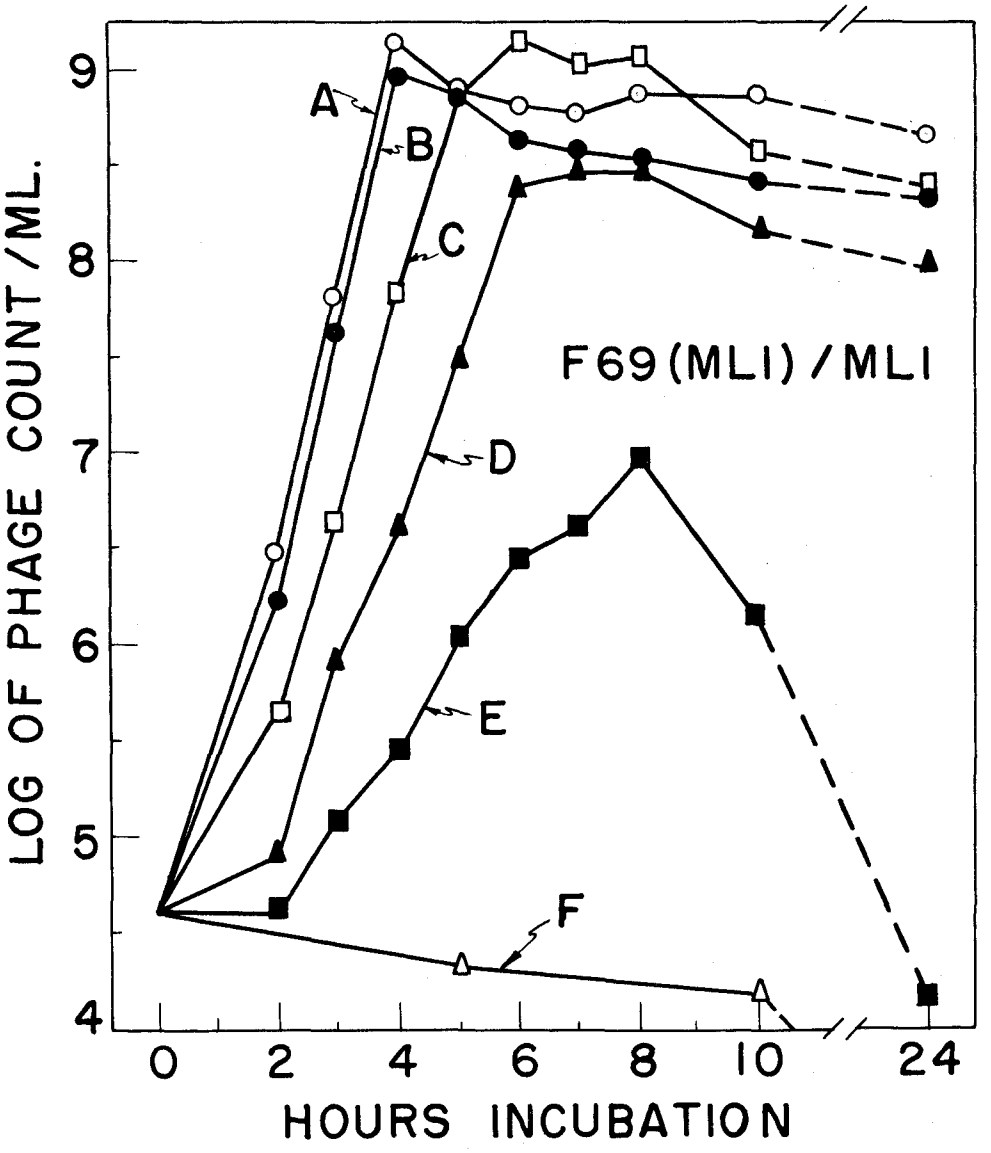

Figure 1. Effect of calcium concentration on bacteriophage proliferation.

($A=32\times10^{-4}$ M Ca^{++}, B=16, C=8, D=4, E=2, and F=0)

No bacteriophage proliferation occurred in unsupplemented medium (curve F); titer in this medium decreased with time and dropped to 6×10^2 plaque-forming particles per ml. in 24 hours, a value too low to be shown upon the graph. This decrease probably is due in part to adsorption of bacteriophage on dead cells and cell debris of the aging culture, resulting in inactivation. Rate of proliferation was least with 2×10^{-4} M calcium (curve E); at this concentration mass lysis did not occur, bacteriophage increase stopped at about 8 hours, possibly due to aging bacteria becoming unsuitable bacteriophage substrate, and bacteriophage titer began to decrease. With 4, 8, 16, and 32×10^{-4} M calcium (curves D, C, B and A), rate of proliferation was related to concentration, and mass lysis occurred after 10, 7, 5, and 4 hours, respectively. Maximum bacteriophage concentrations, in the case of curves D, C, and B, were reached some time before the appearance of mass lysis. Calcium levels above 4×10^{-4} M had little effect upon the final bacteriophage yield.

Very similar results were obtained using bacteriophage-organism combination F63(E8-2)/E8-2. No bacteriophage proliferation occurred in unsupplemented medium. In the presence of 6×10^{-4} M calcium ion, bacteriophage titer increased only about three-fold over a period of

7 hours and then decreased. Using 12, 24, and 48×10^{-4} M calcium, rate of proliferation was related to concentration and mass lysis occurred after 6.5, 5.5, and 4.5 hours, respectively. Maximum bacteriophage titer occurred close to the time of mass lysis and final bacteriophage yield was much the same with these levels, reaching a titer of approximately 10^9 plaque-forming particles per ml.

Removal of calcium by oxalate addition

Preliminary investigation showed that at a concentration of 34×10^{-4} M, sodium oxalate had no demonstrable effect on rate of growth of bacterial host E8-2 but prevented proliferation of bacteriophage F63(E8-2) in medium containing 34×10^{-4} M calcium and otherwise satisfactory. Oxalate in slight excess of calcium (48×10^{-4} M) was no more effective in preventing bacteriophage proliferation than 34×10^{-4} M, but decreased slightly rate of host bacterial growth. To study the effect of calcium removal on bacteriophage growth, calcium-deficient medium supplemented with 34×10^{-4} M calcium was inoculated with approximately 10^6 host cells and 10^3 particles of bacteriophage F63(E8-2) per ml. Aliquots of this system received 34×10^{-4} M sodium oxalate at different times during their incubation period. No oxalate was included in one control and in another it

was added to the medium before inoculation with cells and bacteriophage. In Figure 2 it is seen that oxalate added to the medium before cells and bacteriophage (curve E) prevented bacteriophage proliferation, while additions to the system in which bacteriophage was increasing stopped this increase. It will be noted, however, that oxalate addition after 1, 2, or 4 hours (curves D, C, and B) did not stop increase immediately but permitted it to continue for somewhat less than an additional hour. A repeated trial, titering for bacteriophage at 10 minute intervals, showed that this bacteriophage increase lasted for at least 40 minutes. These results suggested that calcium requirement may be confined to the early stages of the bacteriophage growth process, for if this was the case, bacteriophage-host complexes which had advanced beyond this stage in the presence of calcium would be expected to complete the synthesis and liberation of bacteriophage although this ion was removed. These results could be explained differently if cells growing in the medium before oxalate addition imbibed calcium sufficient for bacteriophage growth and this was not removed by the oxalate. Subsequent experiments indicate that this probably is not the case.

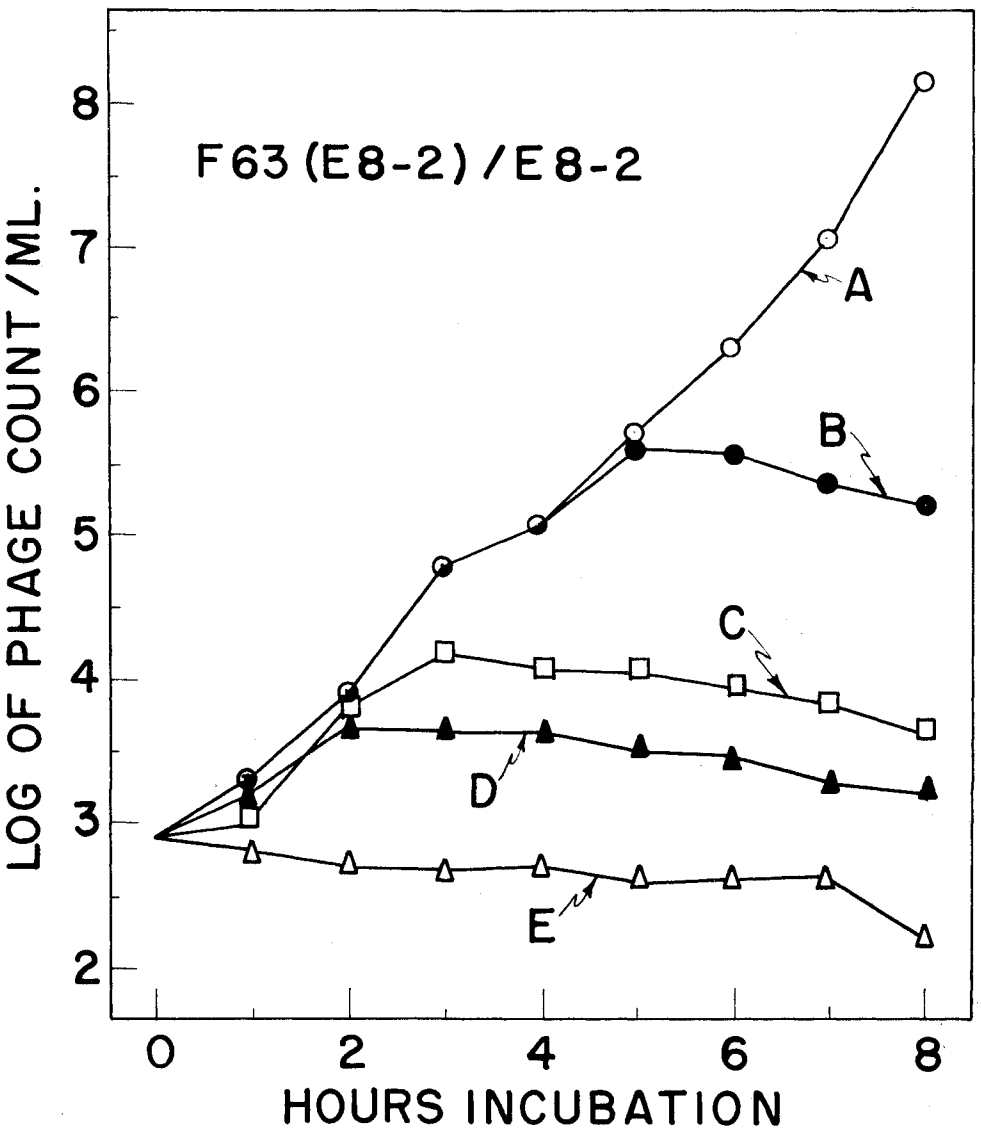

Figure 2. Effect of calcium removal by an equivalent concentration of oxalate on bacteriophage proliferation.

(A=no oxalate added, B=oxalate added at 4 hr., C=2 hr., D=1 hr., and E=0 hr.)

Determination of time during which calcium is required

Experiments of the type presented in Figures 1 and 2 tell little of the effects of calcium on the different phases of the bacteriophage increase cycle. These may be studied by the one-step growth technique adding and removing calcium after various intervals. Using bacteriophage-organism combination F69(ML1)/ML1, early one-step growth experiments showed that when calcium was present in the 5 minute adsorption period and then removed at zero time by the 10^4-fold dilution used to prevent subsequent adsorption and reduce infective centers to a countable number, completion of the bacteriophage increase cycle was not prevented. The quantity of bacteriophage produced under these conditions was the same as when calcium was present in the adsorption mixture and throughout the experiment by virtue of being present in the dilution medium. Figure 3 shows the one-step growth curves of such an experiment using 6×10^{-4} M calcium ion. The curves with calcium removed following the adsorption period (curve A) and present throughout (curve B) are essentially identical, having the same latent period, rise period (interval during which bacteriophage is liberated), and step size (increase in bacteriophage titer from latent period to end of the rise period). Latent period and rise period may be read

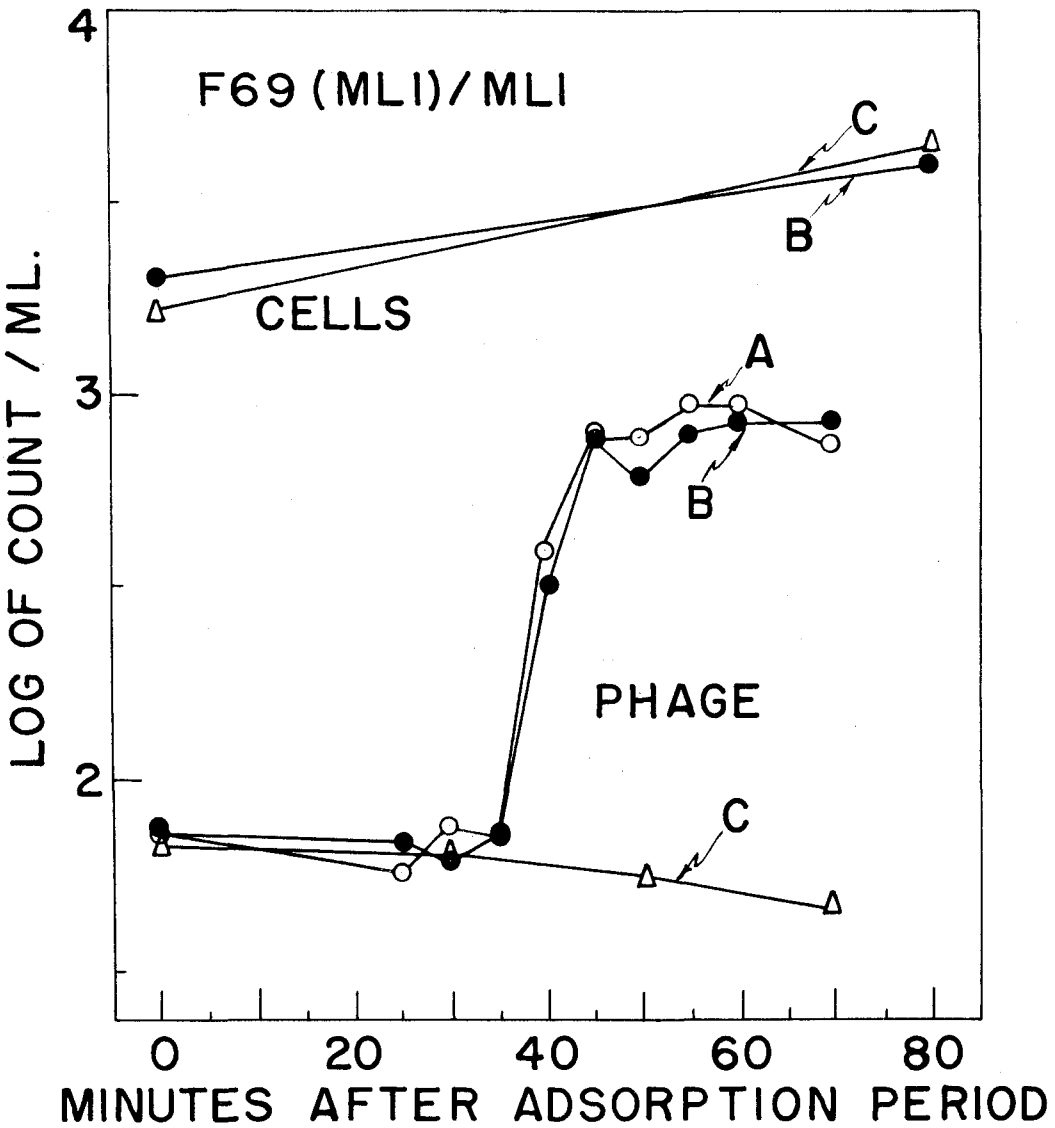

Figure 3. Effect of calcium present throughout experiment and removed following adsorption period on bacteriophage one-step growth curve.

(A=calcium removed by 10^4-fold dilution following adsorption, B=present throughout, C=no calcium)

directly from the figure and are about 35 and 10 minutes, respectively, in this experiment. It must be emphasized that because 5 minutes are allowed for adsorption before the zero time titer is determined, and since latent period read from the figure begins at zero time, true length of the latent period is somewhat longer than the figure indicates for some of the virus-host complexes entered the latent period in the early moments of the 5 minute adsorption period. Similar consideration of interval allowed for adsorption when reading length of latent period from figures applies in subsequent experiments. Step size should be thought of in connection with the per cent adsorption and fraction of adsorbed bacteriophage particles that invade their hosts, lest it suggest a falsely low production of bacteriophage per invaded cell. In this experiment about half the titer during the latent period is contributed by unadsorbed bacteriophage particles which will not multiply. With no calcium present in the control (curve C), bacteriophage titer did not increase. The slight difference in growth rate of cells with no calcium and with calcium present throughout the experiment is not significant, being within the accuracy of the experimental method. This discrepancy is not seen in subsequent trials. Very similar results were obtained with bacteriophage-organism

combination F63(ES-2)/ES-2 using 48×10^{-4} M calcium in the 5 minute adsorption period or throughout the experiment. This may be seen from curves A and B of Figure 8.

These results indicate that calcium functions within the first 5 minutes of bacteriophage-organism interaction and thus agree with the inference made from the oxalate experiments.

This type of experiment is open to the same criticism as the oxalate work, in that possibly calcium sufficient to support bacteriophage increase may not be removed from the system by the 10^4-fold dilution but remain associated with the cell or bacteriophage. To study this point calcium was added to growing host bacteria or to bacteriophage inoculum. It was washed from the bacterial cells by centrifugation and resuspension in a volume of calcium-free medium equivalent to 10^4-fold dilution. Removal from bacteriophage was by 10^2-fold dilution in calcium-free medium, as greater dilution would have reduced bacteriophage titer below the level used in one-step growth experiments. Ability of such cells or bacteriophage to give bacteriophage increase in a calcium-free system otherwise satisfactory was determined, using approximately the same concentration of cells and bacteriophage as in the type experiment of Figure 3. As

a control, bacterial cells centrifuged and washed in the same way were used with calcium in the adsorption mixture to check ability of such treated cells to support bacteriophage increase. Another control contained untreated cells and no calcium. Results of such an experiment with bacteriophage-organism combination F63(ES-2)/ES-2 and using 48×10^{-4} M calcium are presented in Figure 4. In the control with treated cells and calcium during adsorption (curve A), bacteriophage proliferation occurred with the normal growth curve for this combination had the cells not been washed and centrifuged. The control containing untreated cells and no calcium during the adsorption period (curve D) gave no bacteriophage increase. Bacteriophage proliferation did not occur in the calcium-free systems containing either treated cells (curve C) or treated bacteriophage (curve B), showing that these did not retain sufficient calcium following dilution to support bacteriophage proliferation in one-step growth experiments. Repeating the same experiment with bacteriophage-organism combination F69(ML1)/ML1 using 32×10^{-4} M calcium, very similar results were obtained. This not only supports the conclusion that calcium is required in the first 5 minutes of bacteriophage-organism interaction, but indicates that neither cells nor bacteriophage bind irreversibly

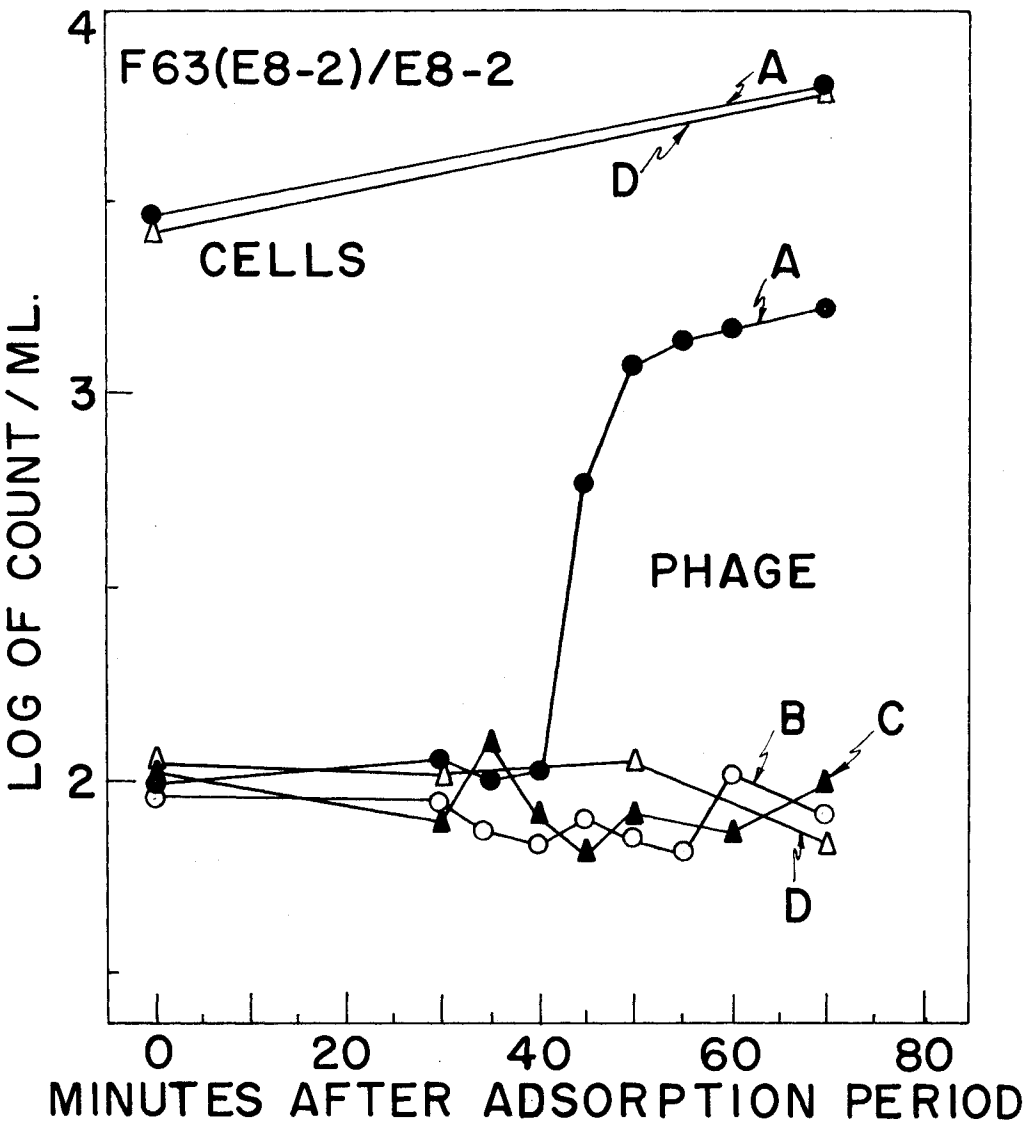

Figure 4. Efficiency of calcium removal from cells and bacteriophage by the dilution process.

(A=calcium not removed, B=removed from bacteriophage, C=removed from cells, D=no calcium present)

sufficient calcium to satisfy the calcium requirement. The possibility that the organism-bacteriophage combination may bind a greater and more adequate amount of calcium for bacteriophage proliferation than would either component alone is not removed.

Effects of calcium concentration on characteristics of the bacteriophage growth curve

The bacteriophage proliferation rate already has been shown to be related to calcium concentration. This may be due to an effect of calcium concentration on such growth characteristics as latent period, rise period, or step size. Which of these portions of the growth curve is affected was investigated by the one-step growth technique, with calcium at various concentrations included in the experiments for just the 5 minute adsorption period. Calcium concentrations studied were the same as in the long-time rate experiment of Figure 1. In determinations of this kind, where several adsorption mixtures must be prepared and subsequently diluted and titered, running overtime with any mixture is avoided by staggering addition of bacteriophage to these by 60 seconds. The effects of calcium concentrations from 0 to 32×10^{-4} M on the growth curve of bacteriophage F69(ML1) against bacterial host ML1 are presented in

Figure 5. No bacteriophage increase occurred without added calcium (curve F). With 2 or 4×10^{-4} M calcium in the adsorption mixture (curves E and D), the latent period was longest. With concentrations from 2 to 16×10^{-4} M calcium (curves E, D, C, and B), step size was related to concentration (the slightly greater step size with 16 than 8×10^{-4} M calcium was consistent on repeated trials). Above a concentration of 16×10^{-4} M calcium (curve A) step size did not increase. Except at 2×10^{-4} M calcium, concentration had little effect upon rise period. Calcium concentration apparently had no definite effect upon stability of newly formed bacteriophage. Rate of cell growth with 32×10^{-4} M and no calcium in the adsorption mixture was the same, indicating no demonstrable effect on cell growth.

Results of the same experiment with bacteriophage-organism combination F63(ES-2)/ES-2 and concentrations of calcium from 0 to 48×10^{-4} M in the 5 minute adsorption period are given in Figure 6. Bacteriophage proliferation did not occur with 0 or 6×10^{-4} M calcium in the system (curves E and D). With 12×10^{-4} M calcium and higher concentrations (curves C, B, and A), latent period and rise period were little influenced, but step size was influenced by calcium concentration. Calcium concentration had no definite effect on stability of newly formed

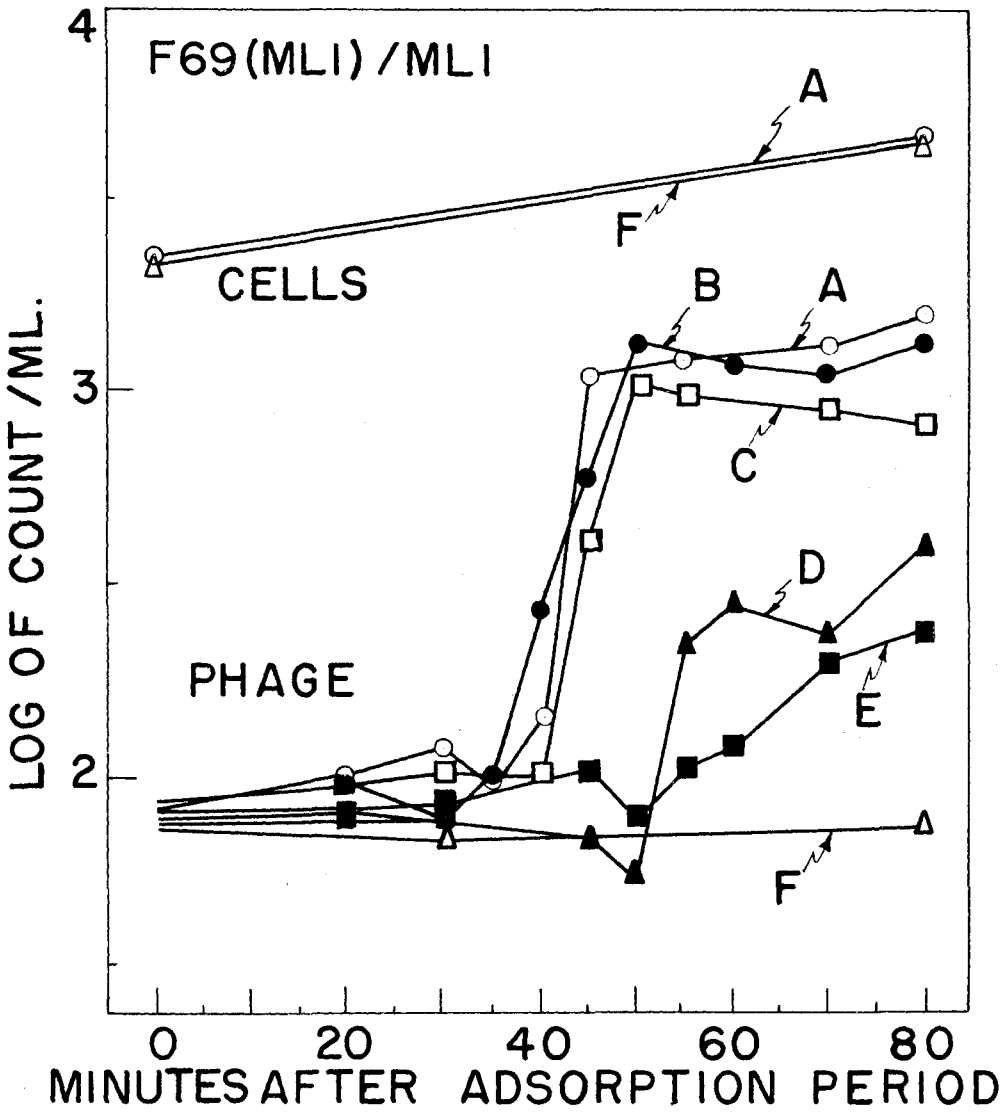

Figure 5. Effect of calcium concentration present during adsorption period on one-step growth curves of bacteriophage F69(ML1).

($A=32\times10^{-4}$ M Ca^{++}, B=16, C=8, D=4, E=2, and F=0)

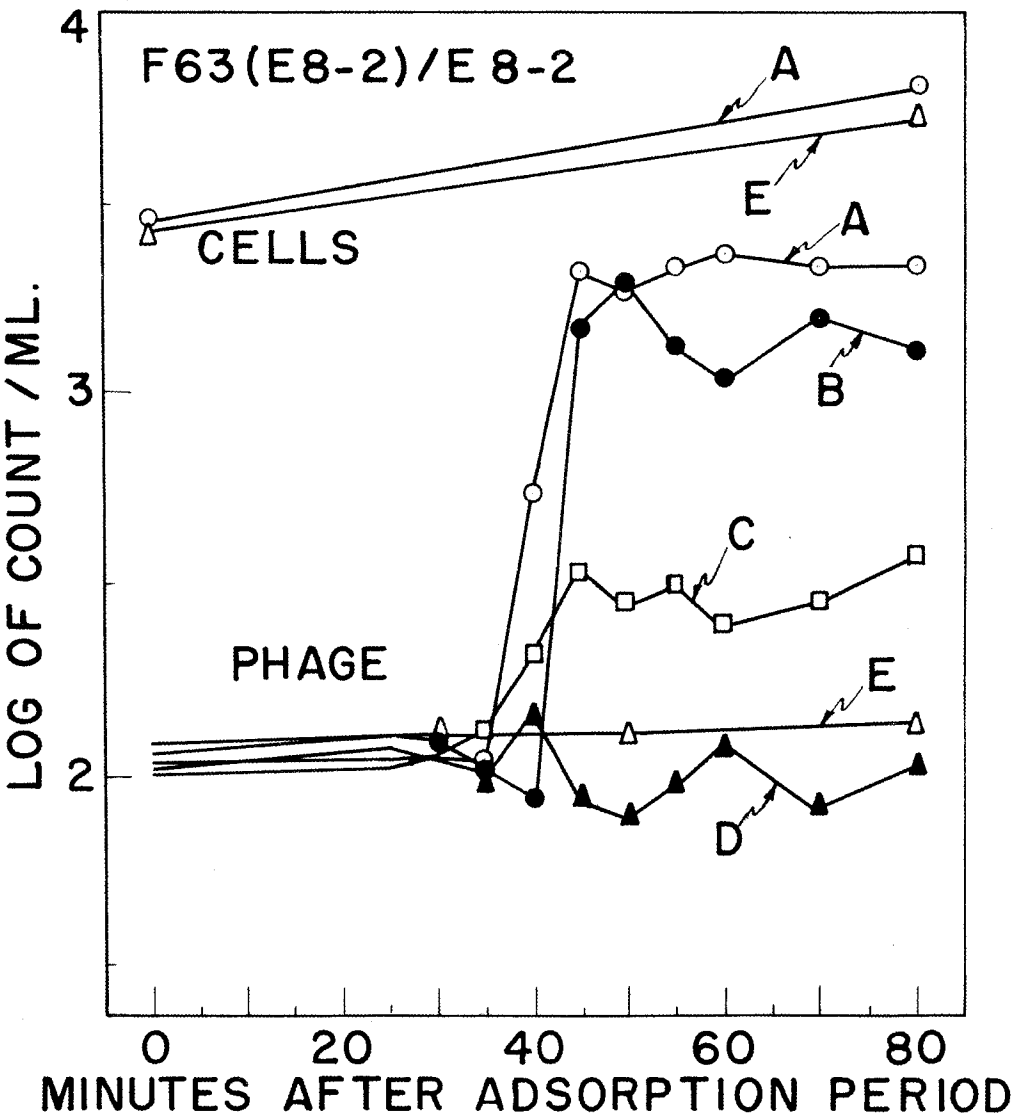

Figure 6. Effect of calcium concentration present during adsorption period on one-step growth curves of bacteriophage F63(E8-2).

(A=4.8×10^{-4} M Ca^{++}, B=24, C=12, D=6, and E=0)

bacteriophage and probably none on rate of cell growth. From these and similar experiments, it was concluded that the major effect of calcium is not upon the length of the intracellular process prior to bacteriophage liberation (latent period) or upon the period over which bacteriophage is liberated (rise period) but is on the step size.

Effects of calcium concentration on bacteriophage adsorption, burst size, and burst frequency

It was implied earlier that step size is influenced by extent of bacteriophage adsorption and by both the number of adsorbed particles that result in cell bursts and the size of these bursts. The effects of calcium concentration on these were studied.

Per cent adsorption (attachment) occurring in adsorption mixtures prepared and treated exactly as in one-step growth experiments is shown in Table 13. Calcium concentrations with bacteriophage-organism combination F69(ML1)/ML1 and F63(E8-2)/E8-2 were the same as were used with these combinations in the experiments of Figures 5 and 6. In the case of both bacteriophage-organism combinations, calcium caused only slightly increased adsorption at the higher concentrations, the effect being greater with bacteriophage F63(E8-2); this was not great

Table 13

Adsorption of bacteriophages F69(ML1) and F63(ES-2) on their hosts with various concentrations of calcium in the adsorption mixtures

Trial		Calcium conc. ($x10^{-4}$ M) in adsorption mixtures of bacteriophage-organism combination F69(ML1)/ML1*						
		0	2	4	8	16	32	Control
A	Titer**	$88x10^4$	$88x10^4$	$81x10^4$	$89x10^4$	$92x10^4$	$71x10^4$	$195x10^4$
	% Adsorption	55	55	58	54	53	64	-
B	Titer	$71x10^4$	$74x10^4$	$58x10^4$	$54x10^4$	$54x10^4$	$59x10^4$	$156x10^4$
	% Adsorption	54	53	63	65	65	62	-

		Calcium conc. ($x10^{-4}$ M) in adsorption mixtures of bacteriophage-organism combination F63(ES-2)/ES-2***					
		0	6	12	24	48	Control
A	Titer	$80x10^4$	$67x10^4$	$75x10^4$	$72x10^4$	$60x10^4$	$125x10^4$
	% Adsorption	36	46	40	42	52	-
B	Titer	$58x10^4$	$56x10^4$	$47x10^4$	$49x10^4$	$45x10^4$	$86x10^4$
	% Adsorption	33	35	45	43	48	-

* Control contains $32x10^{-4}$ M calcium and no bacterial cells; other adsorption mixtures of trials A and B with F69(ML1)/ML1 contain $39x10^6$ cells per ml.

** Titer of plaque-forming particles in supernatant of adsorption mixture.

*** Control contains $48x10^{-4}$ M calcium and no bacterial cells; other adsorption mixtures of trials A and B with F63(ES-2)/ES-2 contain $30x10^6$ cells per ml.

enough to account for the influence of calcium on step size. With no calcium present in adsorption mixtures, a condition under which no bacteriophage proliferation occurs, considerable numbers of particles were adsorbed.

The influence of calcium concentration on burst frequency and burst size was investigated with the single infected cell technique. Prior to dilution, adsorption mixtures were prepared and treated as in the one-step growth experiments, and the concentrations of calcium studied were the same as in the experiments of Figures 5 and 6 and Table 13. Using this system the following supposition was made: If calcium is required for adsorbed bacteriophage to penetrate its host cell, or for some orientation of reactive groups of bacteriophage and host leading to penetration (all of which shall be referred to as invasion), then any calcium level permitting invasion should give the same burst size, and as the calcium concentration is increased a greater proportion of bursts should occur; on the other hand, if calcium functions in intracellular multiplication of the bacteriophage following invasion, then the burst size should be influenced by calcium concentration and burst frequency need not be affected. The results of a single infected cell experiment with bacteriophage-organism combination F69(ML1)/ML1, in which 15 aliquots from each diluted

mixture were plated for bursts, are given in Table 14. In these mixtures the calcium concentration present during the adsorption period affected the number of bursts but had little influence upon average burst size. Thus, with 32 and 16×10^{-4} M calcium, about twice as many bursts occurred as with 8×10^{-4} M calcium and about six times as many as with 4×10^{-4} M calcium. No bursts appeared in the aliquots of mixtures which had 0 and 2×10^{-4} M calcium during adsorption. More truly representative results appear in Table 15, which summarizes the data from five separate experiments of this type using bacteriophage-organism combination F69(ML1)/ML1 and two experiments using combination F63(ES-2)/ES-2. The wide range in burst sizes of individual cells is characteristic of results obtained with the single infected cell technique, and is of the order reported for other bacteriophage-organism systems. It is believed due to physiological differences in cells of the culture rather than variation in size of the cells. With both combinations the frequency of bursts increases with increasing calcium concentration, while average burst size appears unaffected. This conclusion is in no way invalidated by the variation in individual burst sizes. Quantitatively these results are in good agreement with the step sizes corresponding to the various calcium concentrations as shown in Figures

Table 14

Effect of calcium concentration in adsorption mixtures on frequency and size of bursts of bacteriophage F69(ML1)

Ca^{++} conc. ($\times 10^{-4}$ M)	Distribution and size of bursts among aliquots: A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	No. of bursts	Average burst size
0	-*	1**	1	1	1	-	-	2	-	1	-	1	1	-	1	0	-
2	1	1	-	2	1	-	-	1	-	-	1	1	3	1	1	0	-
4	-	1	1	-	-	-	1	2	-	70	1	1	-	1	-	1	70
8	63	1	150	-	39	-	-	1	-	2	1	-	1	-	-	3	84
16	1	87	1	1	51	69	22	58	-	1	2	-	105	20	-	7	59
32	-	-	114	1	-	2	108	39	1	-	99	1	1	102	73	6	89

* No plaque-forming particle in this aliquot.
** This plaque represents an unadsorbed bacteriophage particle or an adsorbed particle that did not cause a burst.

Table 15

Relationship between calcium concentration in adsorption mixtures and frequency and size of bacteriophage bursts

Ca^{++} conc. ($x10^{-4}$ M)	Size* of bursts of bacteriophage F69(ML1) in 72 aliquots	No. of bursts	Burst size Av.	Median
0	-	0	-	-
2	-	0	-	-
4	25,70,138.	3	78	70
8	32,33,33,39,42,42,58,63, 70,74,87,105,127,132,150.	15	73	63
16	15,20,22,27,34,39,42,48, 50,51,57,57,58,68,69,69, 87,105,121,129,133,136, 180.	23	70	57
32	12,25,35,36,39,49,51,58, 62,67,73,74,75,99,102, 108,114,114,115,117,145, 151,172,177,187.	25**	90	75
	Size of bursts of bacteriophage F63(ES-2) in 30 aliquots			
0	-	0	-	-
6	-	0	-	-
12	45,65,87,87.	4	71	76
24	30,52,56,61,63,88,148, 168,238.	9	100	63
48	48,60,62,73,76,92,97, 109,118,126.	10	86	84

* Bursts are arranged in order of increasing size.
** These bursts occurred in only 60 aliquots of this mixture.

5 and 6. Taking as an example bacteriophage-organism combination F69(ML1)/ML1 in Figure 5, the step size with 32 and 16×10^{-4} M calcium is much the same, with 8×10^{-4} M it is slightly smaller, and with 4×10^{-4} M appreciably smaller. The number of bursts with these calcium concentrations, in Table 15, were 25 (this would have been somewhat greater if 72 aliquots were titered), 23, 15, and 3, respectively. No bursts occurred with 0 or 2×10^{-4} M calcium; had a larger number of aliquots been titered a few bursts with the latter calcium concentration might have been expected. These results suggest that calcium may function in the process of bacteriophage invasion.

Studies on bacteriophage invasion

One-step growth experiments were run in which adsorption occurred in the absence of calcium. Adsorption mixtures then were diluted in the usual way except that the diluents contained calcium. Controls with (1) calcium in the adsorption mixtures but not in the diluents, (2) calcium in adsorption mixtures and diluents, and (3) no calcium also were run. If calcium functioned in invasion, it was suspected that adding calcium to systems in which bacteriophages had been adsorbed on host cells in the absence of calcium would permit the bacteriophage particles to invade and multiply. The results of this experiment

with bacteriophage-organism combination F69(ML1)/ML1 and using 16×10^{-4} M calcium are given in Figure 7. With no calcium in the system (curve D) no bacteriophage increase occurred. With calcium present only during the 5 minute adsorption period (curve A) or throughout the experiment (curve B) the same growth curve was obtained, confirming earlier findings. Adding calcium to a system in which bacteriophages were adsorbed in its absence (curve C), resulted in invasion and multiplication of this bacteriophage. The latent period was slightly prolonged and the step size somewhat smaller in this case as compared with controls having calcium present during the adsorption period. The longer latent period can be explained if it is assumed that the invasive process requires some time and did not progress until calcium was added following the adsorption period. The smaller step size (partly due to a lower starting bacteriophage titer in this mixture and possibly to slightly reduced adsorption in absence of calcium) suggests that some invasiveness may be lost with adsorption in absence of calcium. In this type experiment, bacteriophage adsorption subsequent to calcium addition is prevented by the 10^4-fold dilution following the initial adsorption period; bacteriophage multiplication following calcium addition thus provides evidence by a method other than centrifugation that

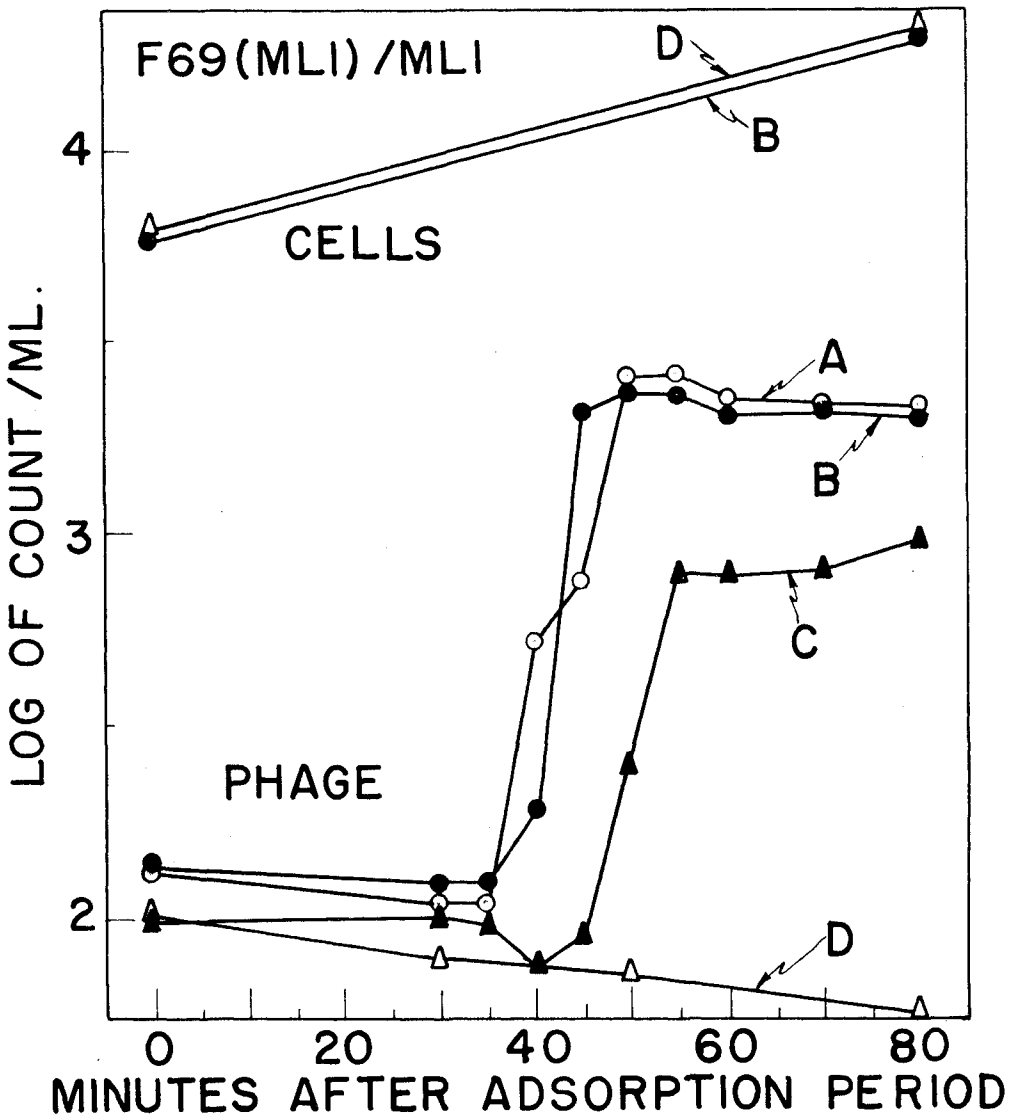

Figure 7. Effect of calcium addition on proliferation of bacteriophage F69(ML1) adsorbed in absence of calcium.

(A=calcium present during adsorption, B=present throughout experiment, C=added following adsorption in its absence, D=no calcium)

bacteriophage adsorption occurs in the absence of calcium.

Very similar results were obtained by this experiment using bacteriophage-organism combination F63(ES-2)/ ES-2 and 4.8×10^{-4} M calcium. Figure 8 shows that bacteriophage of this combination adsorbed in absence of calcium also invaded and multiplied on its host following calcium addition (curve C). Some loss of invasiveness and possibly slight prolongation of the latent period occurred under these conditions. Such findings further suggest that calcium functions in the process of bacteriophage invasion.

One way loss of invasiveness in the above experiments could occur would be through some killing of bacterial cells by bacteriophage adsorbed but unable to initiate increase in the absence of calcium. This point was studied by comparing rate of growth of bacterial cells in calcium-deficient medium with and without bacteriophage particles adsorbed to them. Bacterial counts could not be made with the usual plating medium for this contains calcium and other ions and would result in lysis of some cells in the enumeration system. Therefore, the limiting dilution method, using calcium-deficient medium in growth tubes was employed, and the most probable number of organisms calculated from probability tables. Lactic

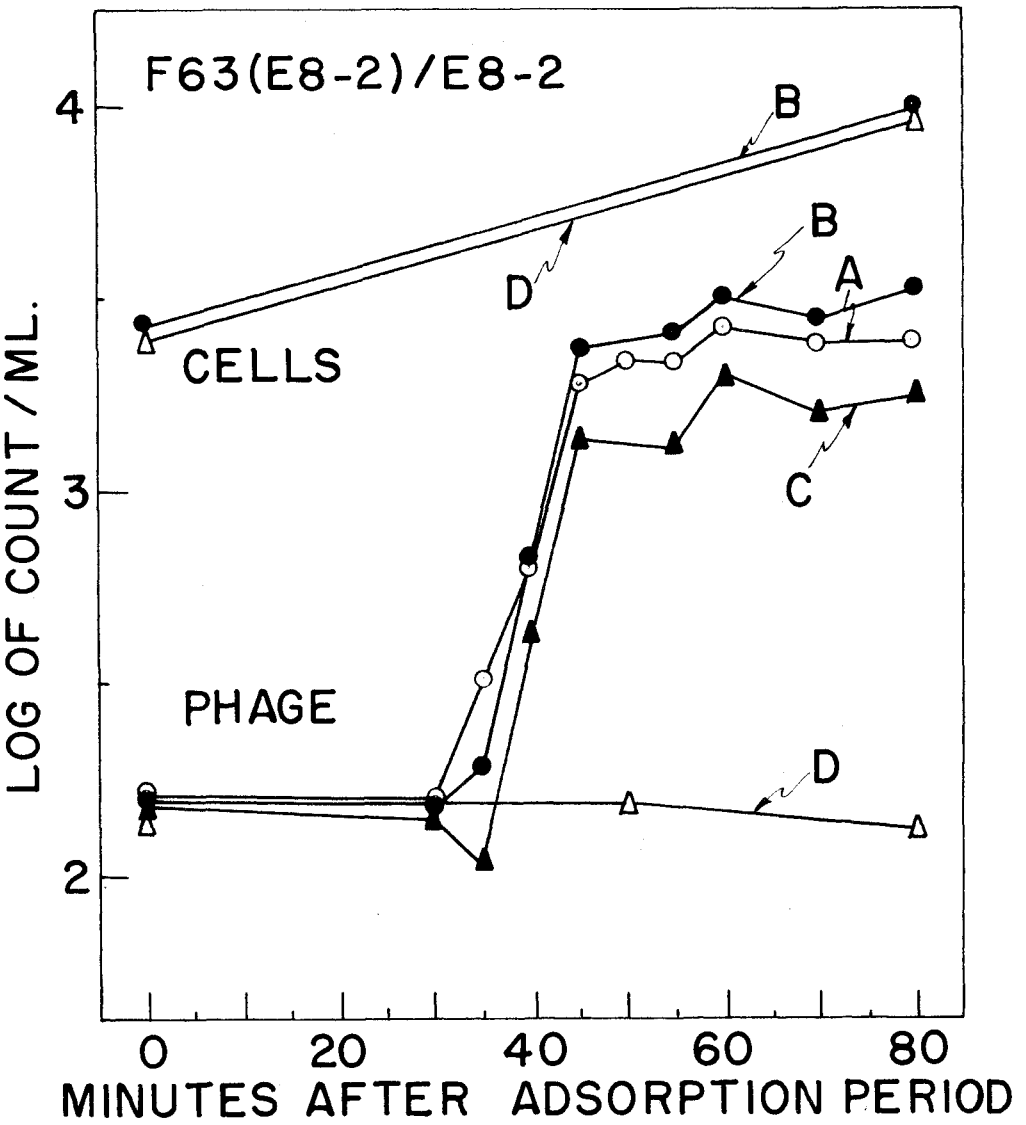

Figure 8. Effect of calcium addition on proliferation of bacteriophage F63(E8-2) adsorbed in absence of calcium.

(A=calcium present during adsorption, B=present throughout experiment, C=added following adsorption in its absence, D=no calcium)

streptococci form pairs and short chains of cells; unless concentration of bacteriophage particles is greatly in excess of the apparent cell count in systems in which adsorption is to occur, some cells will not have bacteriophage particles adsorbed to them. Such cells in a chain would be expected to grow in the enumeration system and probably mask a killing effect that might occur by bacteriophage adsorbed to adjacent cells of the chain. In this study, concentration of bacteriophage particles was about 1000-fold greater than host cells where adsorption occurred. Cells were grown in calcium-deficient medium at 32° C for 90 minutes and cell counts determined before bacteriophage was added. At this point cell suspensions were divided into two equal volumes and bacteriophage particles added to one. Following bacteriophage addition the systems were incubated at 32° C, and number of cells determined after 0.5, 1, 2, and 4 hours. Data of Table 16 indicate that neither bacteriophage F69(ML1) nor F63(ES-2) kills host cells to which they are adsorbed in absence of calcium. Under these conditions a much greater ratio of virus to cells, and a longer period in which killing of host cells could occur is provided than in the invasion experiments of Figures 7 and 8. This suggests that the loss of invasiveness following adsorption in absence of calcium in the

Table 16

Effect of bacteriophage adsorbed to host cells in absence of calcium on cell growth in the calcium-deficient medium

	No. of cells* after incubation periods (hours) of:						Titer of bacteriophage F69(ML1)	
	0	0.5	1	2	4	20	0 hour	4 hour
				ML1				
Cells alone	5.0×10^2	11.5×10^2	11.5×10^2	3.5×10^3	11.5×10^3	turbid	-	-
Cells plus phage F69(ML1)	5.0×10^2	6.0×10^2	3.5×10^2	11.5×10^2	2.5×10^4	turbid	63×10^4	62×10^4
							Titer of bacteriophage F63(E8-2)	
				E8-2				
Cells alone	11.5×10^2	9.5×10^2	11.5×10^2	5.5×10^3	11.5×10^3	turbid	-	-
Cells plus phage F63(E8-2)	11.5×10^2	11.5×10^2	16.5×10^2	2.5×10^3	6.0×10^4	turbid	12×10^5	12×10^5

* No. of cells is most probable number calculated from probability tables (Buchanan and Fulmer, 1928), using four tubes per dilution.

invasion experiments was not due to killing of host cells.

Studies within the initial minutes of bacteriophage-host interaction

Effects of calcium present in the mixture for various fractions of the 5 minute adsorption period were investigated by the one-step growth technique, using both bacteriophage-organism combinations. Calcium was added to adsorption mixtures at the same time as bacteriophage and cells, and also after bacteriophage and cell suspensions had been combined for 150, 225, and 262 seconds. Preparation of mixtures was staggered to permit dilution of all mixtures after the same time interval. Under these conditions mixtures contained calcium for 300, 150, 75, and 38 seconds. Results of this experiment using bacteriophage-organism combination F63(ES-2)/ES-2 and 48×10^{-4} M calcium are given in Figure 9. These show that calcium present for as little as 38 seconds (curve D) resulted in appreciable bacteriophage increase. Somewhat greater step sizes occurred when calcium was present for longer periods. The length of period during which calcium was present had little effect on the latent period or rise period in this experiment. Very similar results were obtained using bacteriophage-

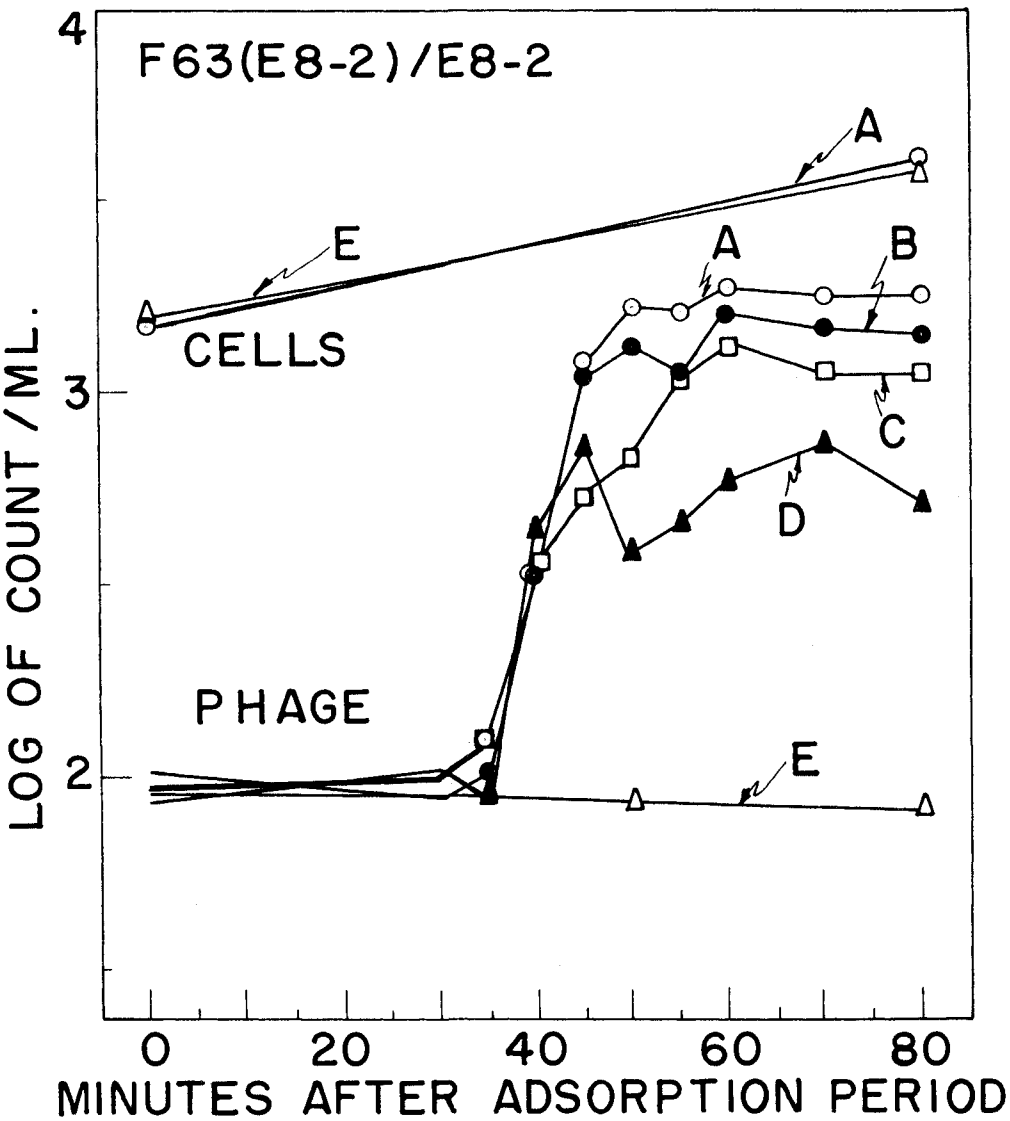

Figure 9. Effects of calcium present for various fractions of the 5 minute adsorption period.

(A=present for 300 sec., B=150 sec., C=75 sec., D=38 sec., E=no calcium)

organism combination F69(ML1)/ML1 and 16×10^{-4} M calcium; with this combination presence of calcium for 38 seconds gave a step size more nearly equal to those obtained with calcium present for longer periods.

These experiments show that calcium exerts its effect when present for as little as 38 seconds; however, because in these cases calcium was added after bacteriophage and host were together 262 seconds, the data tell little about how early in the 5 minute period calcium may function. To determine this point adsorption mixtures containing bacteriophage F69(ML1), its host bacteria, and 16×10^{-4} M calcium were diluted after 38 seconds (Figure 10, curve C). Mixtures in which bacteriophage, cells, and calcium were together for 5 minutes (curve A), and in which calcium was added for 38 seconds to cells and bacteriophage already combined 262 seconds (curve B) were used as controls. Figure 10 shows that with bacteriophage, cells, and calcium together for 38 seconds bacteriophage increase followed, although step size was appreciably smaller than in calcium-containing controls in which bacteriophage and cells were together for a longer time. This small step size probably is due largely to reduced bacteriophage adsorption in the short adsorption period. In the case of curve B, for example, adsorption had proceeded for 262 seconds before calcium was added and

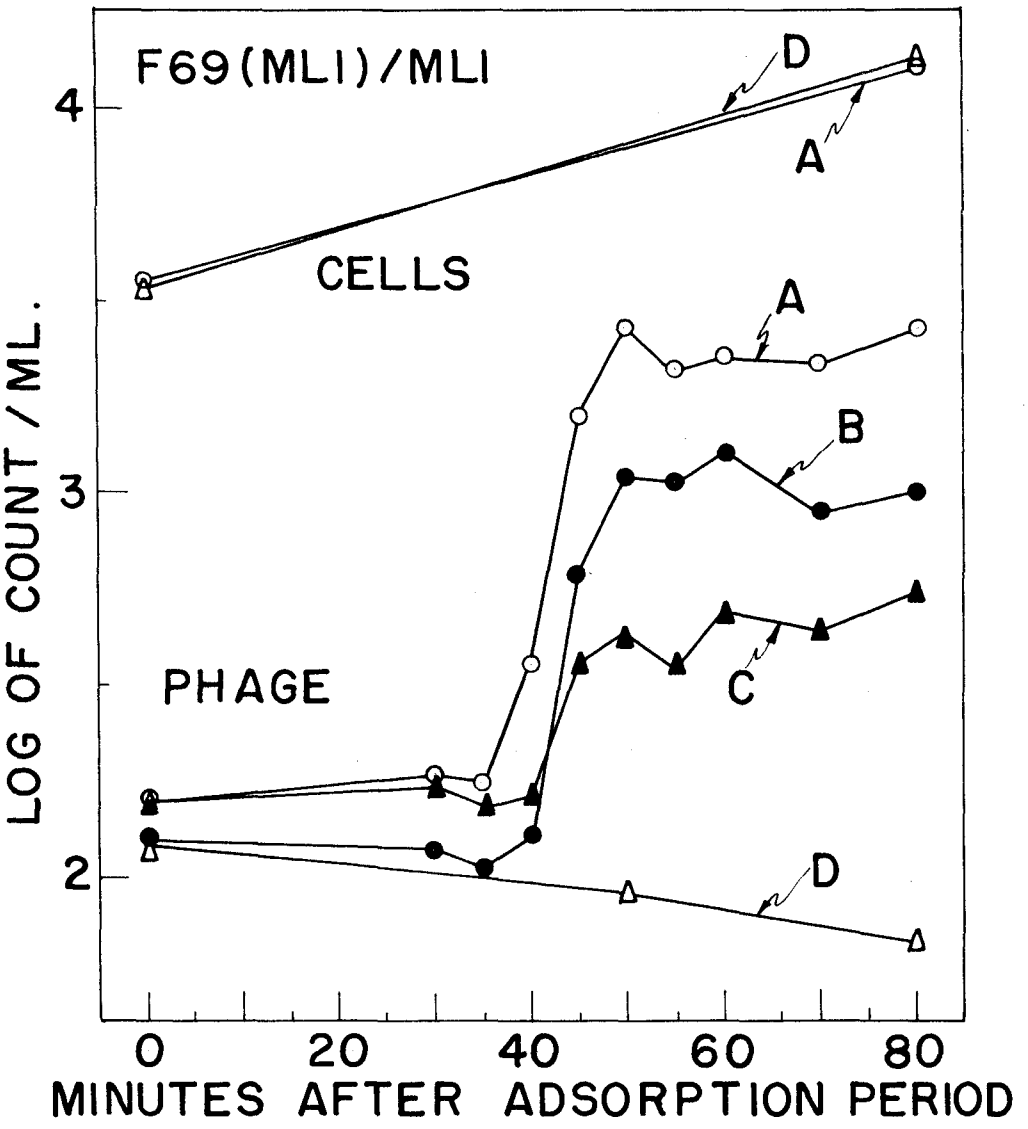

Figure 10. Proliferation of bacteriophage with calcium removed after initial 38 seconds of bacteriophage-host interaction.

(A=calcium present during 300 sec. of adsorption, B=cells and phage together 262 sec. and calcium added for additional 38 sec., C=cells, phage, and calcium together 38 sec., D=no calcium)

so a greater number of bacteriophage particles could invade and multiply when calcium was present during the subsequent 38 seconds. The slightly longer latent period of curve C than curve A would be expected because at zero time bacteriophage of curve C should be 262 seconds behind that of curve A in its development. The longer latent period of curve B than curve A could be explained if invasion required some time and could not proceed until calcium was supplied, as pointed out earlier. Similar conclusions may be drawn from the data in Figure 11 which were obtained by the same experimental procedure except for using bacteriophage-organism combination F63(ES-2)/ES-2, 48×10^{-4} M calcium, and studying calcium present for the first 75 seconds of the adsorption period (curve C). These experiments indicate that calcium functions within the very early stages (in the case of combination F69(ML1)/ML1 within the first 38 seconds) of bacteriophage-host interaction. This would be consistent with a role in bacteriophage invasion.

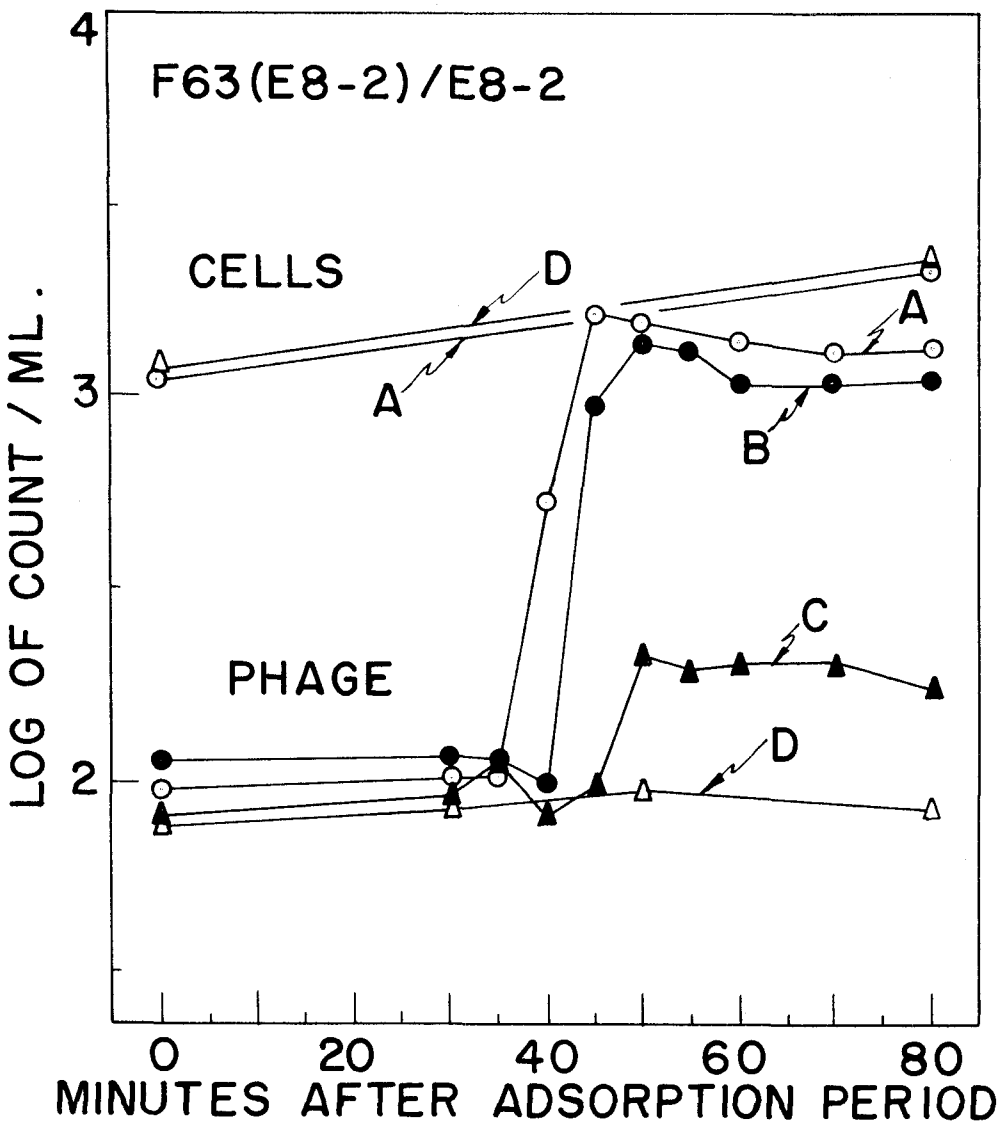

Figure 11. Proliferation of bacteriophage with calcium removed after initial 75 seconds of bacteriophage-host interaction.

(A=calcium present during 300 sec. of adsorption, B=cells and phage together 225 sec. and calcium added for additional 75 sec., C=cells, phage, and calcium together 75 sec., D=no calcium)

Investigations with Other Cations

Ability of different ions to replace calcium in the lactic streptococus bacteriophage system has received little study. Still less is known of the effects of supplementary cations on the calcium requirement of this bacteriophage. These problems have been investigated in the following experiments. Where ions other than calcium were found to influence bacteriophage proliferation, their mode of action has been studied.

Screening of ions other than calcium for ability to support bacteriophage proliferation in the calcium-deficient medium

Tubes of calcium-deficient medium supplemented with various ions in concentrations of 3, 15, and 75×10^{-4} M were inoculated with approximately 10^4 particles of bacteriophage F69(NL1) and 10^6 cells of host NL1 per ml. Following incubation for 15 hours at 32° C, tubes were observed for mass lysis and titered for bacteriophage. Control tubes, containing no bacteriophage, indicated the effects of the ions on the medium and on bacterial cell growth. The results of this experiment are seen in Table 17. Several of the ions produced precipitates with components of the medium and some were toxic to

Table 17

Proliferation of bacteriophage F69(ML1) on host ML1 in the calcium-deficient medium supplemented with various ions

Ion supplement	Bacteriophage titer* produced with ion conc. ($\times 10^{-4}$ M) of: 3	15	75
None	2×10^3		
Ca^{++}		15×10^7 ML**	
Mg^{++}	$<1\times10^3$	$<1\times10^3$	$<1\times10^3$
Ba^{++}	$<1\times10^2$	67×10^2	200×10^5 SP
Mn^{++}	$<1\times10^3$	7×10^7 ML	45×10^7 ML, SP
None	$<1\times10^2$		
Ca^{++}		7×10^7 ML	
Ni^{++}	$<1\times10^3$	$<1\times10^3$ ST	$<1\times10^3$ T
Zn^{++}	$<1\times10^3$	3×10^3 T	$<1\times10^3$ P, T
Co^{++}	$<1\times10^3$	$<1\times10^3$	$<1\times10^3$ ST
Fe^{++}	$<1\times10^3$	$<1\times10^3$ P, ST	$<1\times10^3$ P, T
Al^{+++}	$<1\times10^3$	$<1\times10^3$ P, ST	$<1\times10^3$ P, T
None	$<1\times10^2$		
Ca^{++}		10×10^7 ML	
Li^{+}	$<1\times10^3$	$<1\times10^3$	$<1\times10^3$
Cu^{++}	$<1\times10^3$ T	$<1\times10^3$ T	$<1\times10^3$ T
Cr^{+++}	$<1\times10^3$	$<1\times10^3$ P, ST	$<1\times10^3$ P, T
None	$<1\times10^2$		
Ca^{++}		10×10^7 ML	
Sr^{++}	$<1\times10^3$	5×10^7 ML	17×10^7 ML
Cd^{++}	$<1\times10^3$ T	24×10^3 T	62×10^3 P, T

* Titer was determined after 15 hr.; initial titer approx. 10^4.

** ML= mass lysis; SP= slight precipitate, P= precipitate; ST= slightly toxic to bacterial cells, T= toxic to cells.

cell growth at the higher concentrations. In cases where bacteriophage increase did not occur, titer was appreciably lower at 15 hours than at the start of the experiment. This is in agreement with earlier findings when low levels of calcium were studied (Figure 1, curves E and F). In the present case, part of this reduction in bacteriophage titer with certain ions probably is due to virucidal activity. This is indicated, for example, by comparison of the zero and 4 hour titers in presence of ferric and chromic ions in a later study (Table 22). Presence of strontium, barium, or manganous ion permitted bacteriophage increase in the otherwise unsatisfactory system. At a concentration of 15×10^{-4} M, strontium and manganous ions were more effective than barium ion and supported only slightly less bacteriophage increase than calcium. The greater bacteriophage titer obtained with 15 and 75×10^{-4} M cadmium than with other non-stimulatory ions was further investigated with bacteriophage F69(ML1) and four other bacteriophage strains. In no case was the 15 hour bacteriophage titer greater than the initial titer. Cadmium in the concentrations studied in Table 17 is extremely toxic to bacterial cells. Bacteriophage titer when not increasing, decreases more rapidly in the presence of bacterial growth than in its absence. Possibly the effect of cadmium is due to elimination of bacterial growth.

This experiment was repeated with bacteriophage-organism combination F57(799)/799; results are given in Table 18. These are similar to results in Table 17. Of the 13 cations studied in addition to calcium, only strontium and barium supported bacteriophage increase; manganous ion in this case appeared ineffective. While strontium gave greater bacteriophage increase than barium at equimolar concentration, neither of these ions was as effective as calcium in equimolar concentration. In this screening technique, ions were studied in a range of concentration similar to that in which calcium is found most effective. Within this range several of the ions exhibit varying degrees of toxicity toward host cells and probably bacteriophage particles. Many of these ions, at non-toxic levels, were investigated again in combination with calcium (Tables 22 and 23). Except for the special cases of magnesium and cobaltous ions, as shown in these later studies, none gave evidence of supporting bacteriophage increase in the calcium-deficient medium.

The influence of strontium, barium, and manganous ions on multiplication of five additional bacteriophage strains, using the same experimental procedure as in the experiments of Tables 17 and 18, is seen in Table 19. In cases where bacteriophage proliferation was indicated by

Table 18

Proliferation of bacteriophage F57(799) on host 799 in the calcium-deficient medium supplemented with various ions

Ion supplement	Bacteriophage titer* produced with conc. ($\times 10^{-4}$ M) of: 3	15	75
None	2×10^3		
Ca^{++}		318×10^7 ML**	
Mg^{++}	3×10^3	1×10^3	$<1\times10^3$
Ba^{++}	5×10^3	2×10^3	7×10^3 SP
Mn^{++}	2×10^3	3×10^3	10×10^3 SP
None	3×10^2		
Ca^{++}		205×10^7 ML	
Ni^{++}	2×10^3	7×10^3 ST	$<1\times10^3$ T
Zn^{++}	$<1\times10^3$	9×10^3 T	$<1\times10^3$ P,T
Co^{++}	2×10^3	1×10^3	13×10^3 ST
Fe^{++}	$<1\times10^3$	$<1\times10^3$ P	$<1\times10^3$ P,T
Al^{+++}	2×10^3	13×10^3 P,ST	$<1\times10^3$ P,T
None	9×10^2		
Ca^{++}		242×10^7 ML	
Li^{+}	2×10^3	2×10^3	2×10^3
Cu^{++}	2×10^3 T	$<1\times10^3$ T	$<1\times10^3$ T
Cr^{+++}	$<1\times10^3$	$<1\times10^3$ P,ST	$<1\times10^3$ P,T
None	$<1\times10^2$		
Ca^{++}		220×10^7 ML	
Sr^{++}	$<1\times10^3$	2×10^3	240×10^7 ML
Cd^{++}	$<1\times10^3$ T	21×10^3 T	18×10^3 P,T

* Titer was determined after 15 hr.; initial titer approx. 10^4.

** ML= mass lysis; SP= slight precipitate, P= precipitate; ST= slightly toxic to bacterial cells, T= toxic to cells.

Table 19

Proliferation of various bacteriophages on their respective hosts in the calcium-deficient medium supplemented with calcium, strontium, barium and manganous ions

Bacteriophage/host combination and ion supplement	Bacteriophage titer* produced with conc. ($\times 10^{-4}$ M) of: 3	15	75
PF14(H1-5)/H1-5			
None	$<1\times10^{2}$		
Ca^{++}	ML**	ML	ML
Sr^{++}	$<1\times10^{3}$	ML	ML
Ba^{++}	$<1\times10^{3}$	ML	ML, SP
Mn^{++}	118×10^{3}	ML	ML, SP
F63(E8-2)/E8-2			
None	$<1\times10^{2}$		
Ca^{++}	1×10^{3}	ML	ML
Sr^{++}	$<1\times10^{3}$	$<1\times10^{3}$	ML
Ba^{++}	$<1\times10^{3}$	$<1\times10^{3}$	31×10^{5} SP
Mn^{++}	$<1\times10^{3}$	2×10^{3}	3×10^{3} SP, ST
F44(146-1)/146-1			
None	$<1\times10^{2}$		
Ca^{++}	$<1\times10^{3}$	ML	ML
Sr^{++}	$<1\times10^{3}$	$<1\times10^{3}$	ML
Ba^{++}	$<1\times10^{3}$	$<1\times10^{3}$	ML, SP
Mn^{++}	$<1\times10^{3}$	99×10^{3}	26×10^{5} SP, ST
PF11(H1-10)/H1-10			
None	$<1\times10^{2}$		
Ca^{++}	$<1\times10^{3}$	ML	ML
Sr^{++}	$<1\times10^{3}$	$<1\times10^{3}$	ML
Ba^{++}	$<1\times10^{3}$	$<1\times10^{3}$	86×10^{5} SP
Mn^{++}	$<1\times10^{3}$	3×10^{3} ST	5×10^{3} T, SP
F4(H1-1)/H1-1			
None	8×10^{3}		
Ca^{++}	ML	ML	ML
Sr^{++}	9×10^{3}	ML	ML
Ba^{++}	6×10^{3}	34×10^{5}	ML, SP
Mn^{++}	63×10^{3}	ML	50×10^{5} ST, SP

*Titer was determined after 15 hr.; initial titer approx. 10^{4}.

**ML= mass lysis (titer $>10^{7}$); SP= slight precipitate; ST= slightly toxic to bacterial cells.

mass lysis, titers were not determined, experience showing counts to be above 10^7 per ml. Strontium and barium ions replaced calcium in supporting multiplication of all five bacteriophages. Manganous ion was effective in the case of three bacteriophages. At equimolar concentrations, however, these ions supported less multiplication of each of the five bacteriophage strains than did calcium. The relative efficiencies of the various ions were not the same for the different bacteriophages. Further, in the case of bacteriophages F44(146-1) and F4(H1-1), manganous ion supported greater proliferation than barium at a concentration of 15×10^{-4} M, but at 75×10^{-4} M, barium was more effective than manganous ion. At least part of such variation possibly can be explained by slight toxicity of manganous ion at the higher concentrations for several of the bacterial hosts. These experiments indicate that ability to replace calcium as a proliferation requirement with strontium, barium, and perhaps to a lesser extent manganous ions, may be quite general among strains of lactic streptococcus bacteriophage.

Analysis of strontium, barium, and manganous ion action

The calcium-deficient medium, unsupplemented with calcium, does not support bacteriophage proliferation. Proliferation in this medium supplemented with strontium,

barium, or manganous ions suggests that these ions may have the same function as calcium. This was investigated using the same methods as in the analysis of calcium action and the two bacteriophage-organism combinations F69(ML1)/ML1 and F63(ES-2)/ES-2. In the studies with bacteriophage F69(ML1) the concentration of strontium, barium, and manganous ions was 32×10^{-4} M; with bacteriophage F63(ES-2) it was 48×10^{-4} M. These concentrations of calcium give near-optimum increases with the bacteriophages, and so their use provides one means for comparing the relative action of the four ions. In interpreting data from subsequent experiments, it must be remembered that these concentrations are not necessarily optimum for strontium, barium or manganous ions. Optimum concentrations of these were not determined.

In preliminary trials to observe the influence of strontium, barium, and manganous ions on one-step growth curves, the ions functioned when present in the adsorption mixtures for just the 5 minute adsorption period, as was the case with calcium. In subsequent one-step growth and single infected cell experiments, these ions were removed from the experimental systems by 10^4-fold or, in the single infected cell experiments, by still greater dilution of adsorption mixtures with unsupplemented calcium-deficient medium. One-step growth

curves of bacteriophage F69(ML1) obtained with 32×10^{-4} M calcium, strontium, barium, and manganous ions in the adsorption mixtures are seen in Figure 12. These curves appear to differ significantly only in step size. This was greatest with calcium (curve A), somewhat smaller with strontium (curve B), still smaller with manganous (curve C), and smallest with barium (curve D) in the mixtures. The relative bacteriophage increases occurring with the various ions in this experiment were in much the same order as produced with equimolar concentrations of these ions in the long-time experiment of Table 17. No bacteriophage increase occurred with distilled water added to the adsorption mixture in place of an ion solution (curve E). Differences in step size could not be explained by a differential effect of the various ions on rate of bacterial cell growth. This experiment was repeated with bacteriophage F63(ES-2), using the various ions at a concentration of 48×10^{-4} M. Results are presented in Figure 13. The most significant differences in the growth curves again appear to be in step size. The slight differences in latent period with the various ions were not consistant on repeated trial. Calcium gave the greatest step size (curve A), followed by strontium and manganous ions (curves B and C). At this concentration, barium appears to have permitted slight bacteriophage

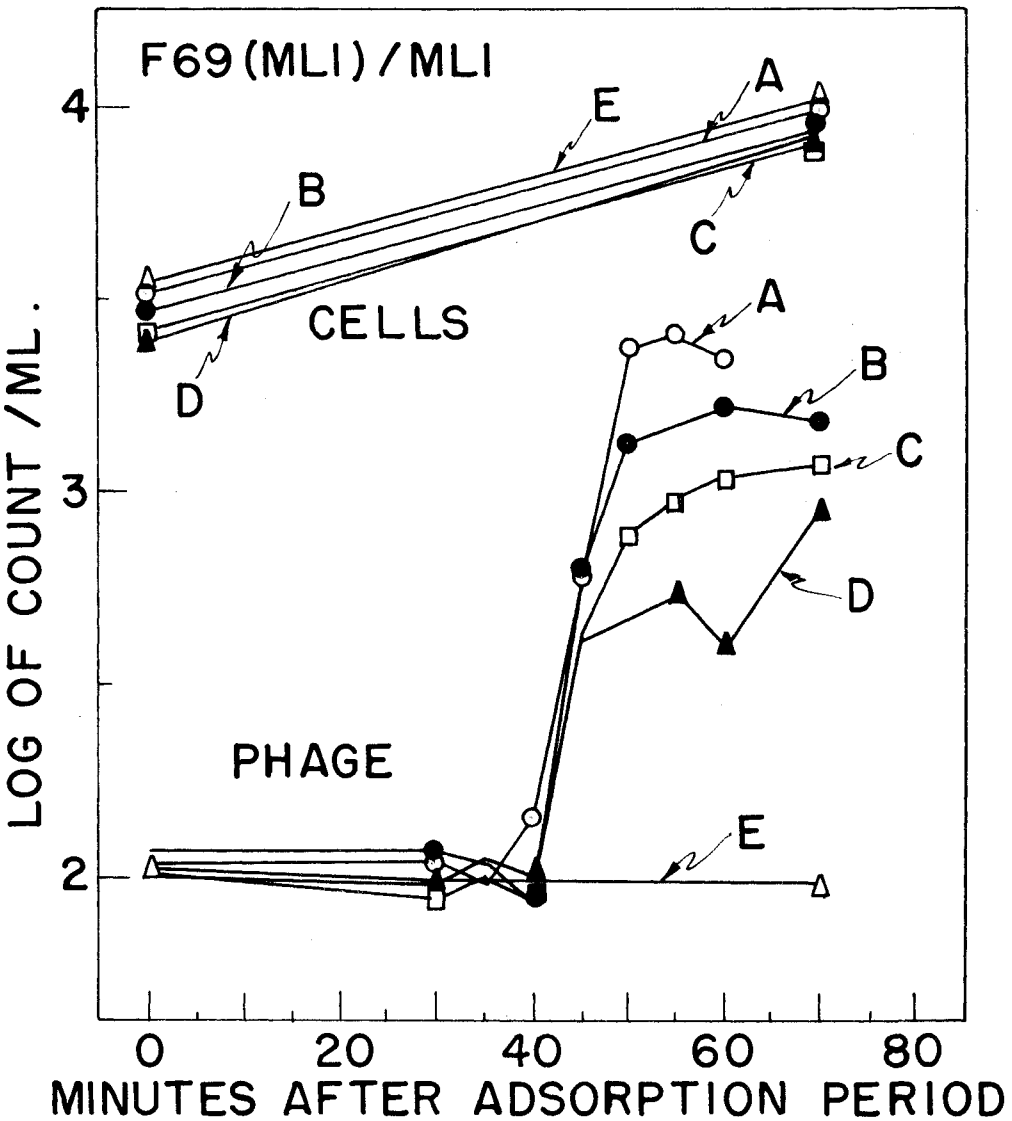

Figure 12. Effect of various ions present during adsorption period on one-step growth curves of bacteriophage F69(MLI).

(A=Ca^{++}, B=Sr^{++}, C=Mn^{++}, D=Ba^{++}, and E=none; all ions 32×10^{-4} M)

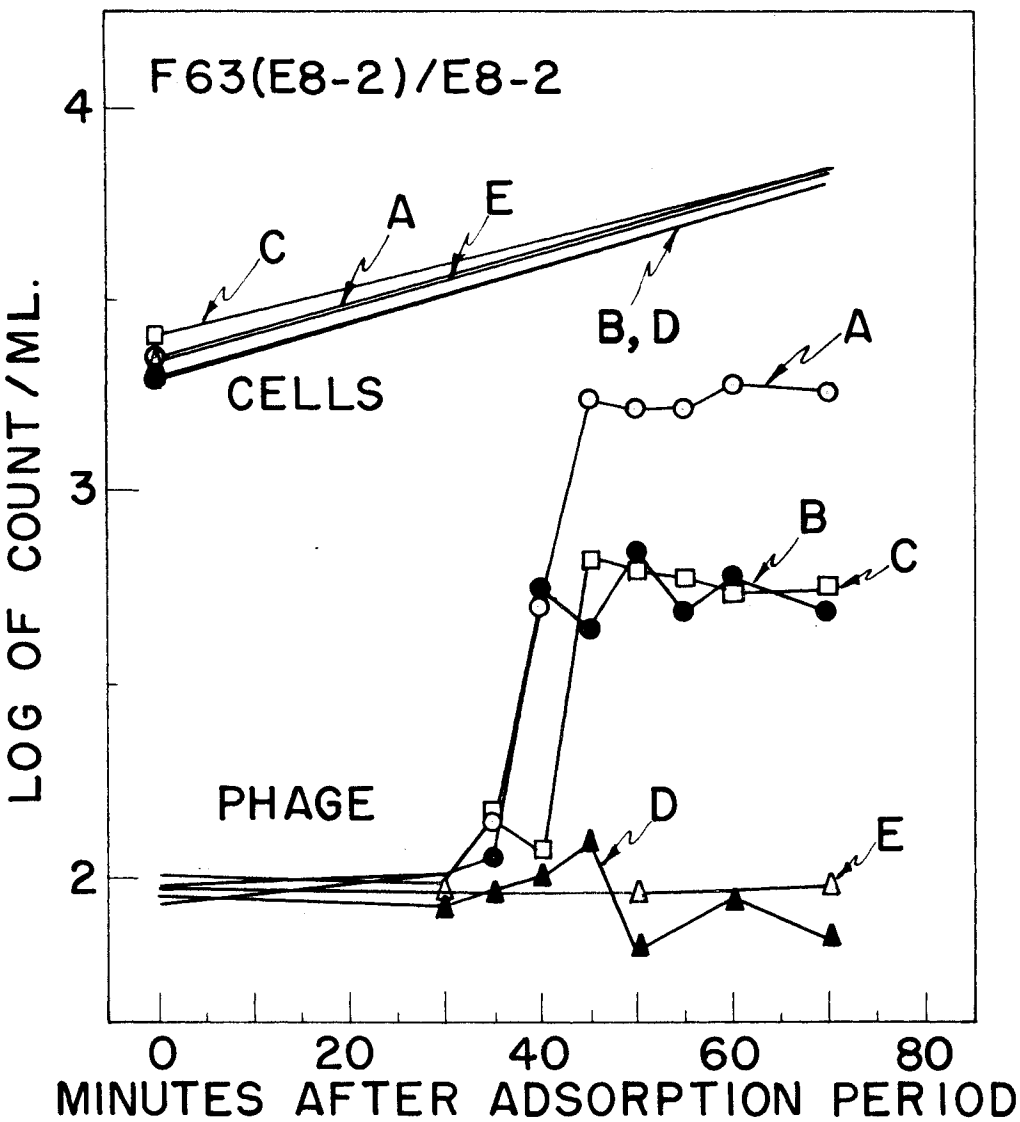

Figure 13. Effect of various ions present during adsorption period on one-step growth curves of bacteriophage F63(E8-2).

(A=Ca^{++}, B=Sr^{++}, C=Mn^{++}, D=Ba^{++}, and E=none; all ions 48×10^{-4} M)

increase to commence (curve D), but no net increase was produced by the end of the experiment. No bacteriophage increase occurred in unsupplemented medium (curve E). Differences in step size could not be explained by a differential effect of the ions on rate of bacterial cell growth. In this experiment 48×10^{-4} M manganous ion supported increase of bacteriophage F63(ES-2) while in the experiment of Table 19, manganous ion appeared ineffective with this bacteriophage. This may be explainable on the basis of concentration. While 15×10^{-4} M manganous ion in Table 19 was probably not great enough to support noticeable bacteriophage increase, 75×10^{-4} M is in the range that is slightly toxic to bacterial host ES-2. With the exception of manganous ion, the relative efficiencies of the ions to support proliferation of bacteriophage F63(ES-2) are the same in Table 19 and the present experiment.

Step size may be influenced by the extent of bacteriophage adsorption, the number of adsorbed particles that result in cell bursts, and the size of these bursts, as was pointed out in the study of calcium action, where the effect of concentration also was principally on step size. The influence of the various ions on bacteriophage adsorption was studied using adsorption mixtures prepared as in the one-step growth experiments of Figures 12 and 13.

Concentrations of the ions were the same as in these experiments. Data in Table 20 show that the various ions apparently have no significant differential effect upon adsorption of either bacteriophage F69(HL1) or F63(ES-2).

Influence of the ions on size and frequency of cell bursts was studied with the single infected cell technique. Adsorption mixtures were prepared as in the one-step growth and adsorption experiments and contained the same ion concentrations as in these studies. Results from two experiments with bacteriophage F69(HL1) and one with bacteriophage F63(ES-2) are presented in Table 21. With both bacteriophages the ion present in the adsorption mixture influenced the frequency of bursts. The number of bursts in each case was greatest with calcium, followed by strontium or manganous ion, and smallest with barium ion. Barium gave no bursts with bacteriophage F63(ES-2). This is in the same order as bacteriophage increase measured by step size with the various ions (Figures 12 and 13). No bursts occurred in the controls containing no ion supplement. Values for average and median burst size with calcium, in the case of both bacteriophages, probably are falsely large. This is because concentration of adsorbed particles of both bacteriophages in diluted mixtures were somewhat too

Table 20

Adsorption of bacteriophages F69(ML1) and F63(E8-2) on their respective hosts with various ions in the adsorption mixtures

Trial		Ions in adsorption mixtures: Ca^{++}	Sr^{++}	Mn^{++}	Ba^{++}	None	Control
		F69(ML1)/ML1*; ion conc.=$32x10^{-4}$ M					
A	Titer**	$121x10^4$	$101x10^4$	$124x10^4$	$122x10^4$	$115x10^4$	$196x10^4$
	% Adsorption	38	48	37	38	41	-
B	Titer	$122x10^4$	$117x10^4$	$101x10^4$	$116x10^4$	$96x10^4$	$194x10^4$
	% Adsorption	37	40	48	40	50	-
		F63(E8-2)/E8-2***; ion conc.=$48x10^{-4}$ M					
A	Titer	$112x10^4$	$110x10^4$	$114x10^4$	$116x10^4$	$116x10^4$	$185x10^4$
	% Adsorption	39	41	38	37	37	-
B	Titer	$114x10^4$	$97x10^4$	$110x10^4$	$106x10^4$	-	$184x10^4$
	% Adsorption	38	47	40	42	-	-

* Control contains H_2O in place of ion supplement and no bacterial cells; other adsorption mixtures of trials A and B with F69(ML1)/ML1 contain $33x10^6$ cells per ml.

** Titer of plaque-forming particles in supernatant of adsorption mixture.

*** Control contains H_2O in place of ion supplement and no bacterial cells; other adsorption mixtures of trials A and B with F63(E8-2)/E8-2 contain $24x10^6$ cells per ml.

Table 21

Relationship between various ions in adsorption mixtures and frequency and size of bacteriophage bursts

Ion supplement (32×10^{-4} M)	Size* of bursts of bacteriophage F69(ML1) in 30 aliquots	No. of bursts	Burst size Av.	Median
Ca^{++}	11,18,18,33,33,42,57,78, 88,90,97,102,110,121,142, 150,206,210,232,252,263, 276,292.	23	127	102
Sr^{++}	45,47,51,65,74,76,89,90, 95,96,116,201.	12	87	82
Mn^{++}	18,21,49,53,54,65,66,93, 110,140,192,226.	12	91	66
Ba^{++}	23,27,30,38,39,42,52,85.	8	42	38
None	-	0	-	-

Ion supplement (48×10^{-4} M)	Size of bursts of bacteriophage F63(E8-2) in 15 aliquots			
Ca^{++}	59,60,67,72,79,90,93,117, 123,129,132,156.	12	98	92
Sr^{++}	24,89,94.	3	69	89
Mn^{++}	51,63,78,81.	4	68	70
Ba^{++}	-	0	-	-
None	-	0	-	-

* Bursts are arranged in order of increasing size.

high, permitting some of the 3 ml. aliquots to contain more than one cell-bacteriophage complex. With either bacteriophage the probable distribution of aliquots containing more than one adsorbed virus particle would be the same regardless of the ion present in adsorption mixtures. However, opportunity for bursts to occur being greatest in the case of calcium, more multiple bursts would be expected among aliquots with this ion. Except for a possible depressing effect of barium on burst size of bacteriophage F69(ML1), principal influence of the various ions appears to be on frequency of bursts rather than burst size. Both the order and magnitude of this effect indicate that differences in step size with the various ions (Figures 12 and 13) may be explained on the basis of burst frequency. This suggests, as in the study on calcium action, that strontium, barium, and manganous ions function principally in the process of bacteriophage invasion.

Ability of these ions to support bacteriophage increase when added to systems in which bacteriophages were adsorbed on host cells in absence of the ions was studied. One-step growth curves in Figure 14 were obtained by the usual procedure, except that titers were determined at 10 minute intervals. Also, ions in controls (open circle curves) were present during the 5 minute

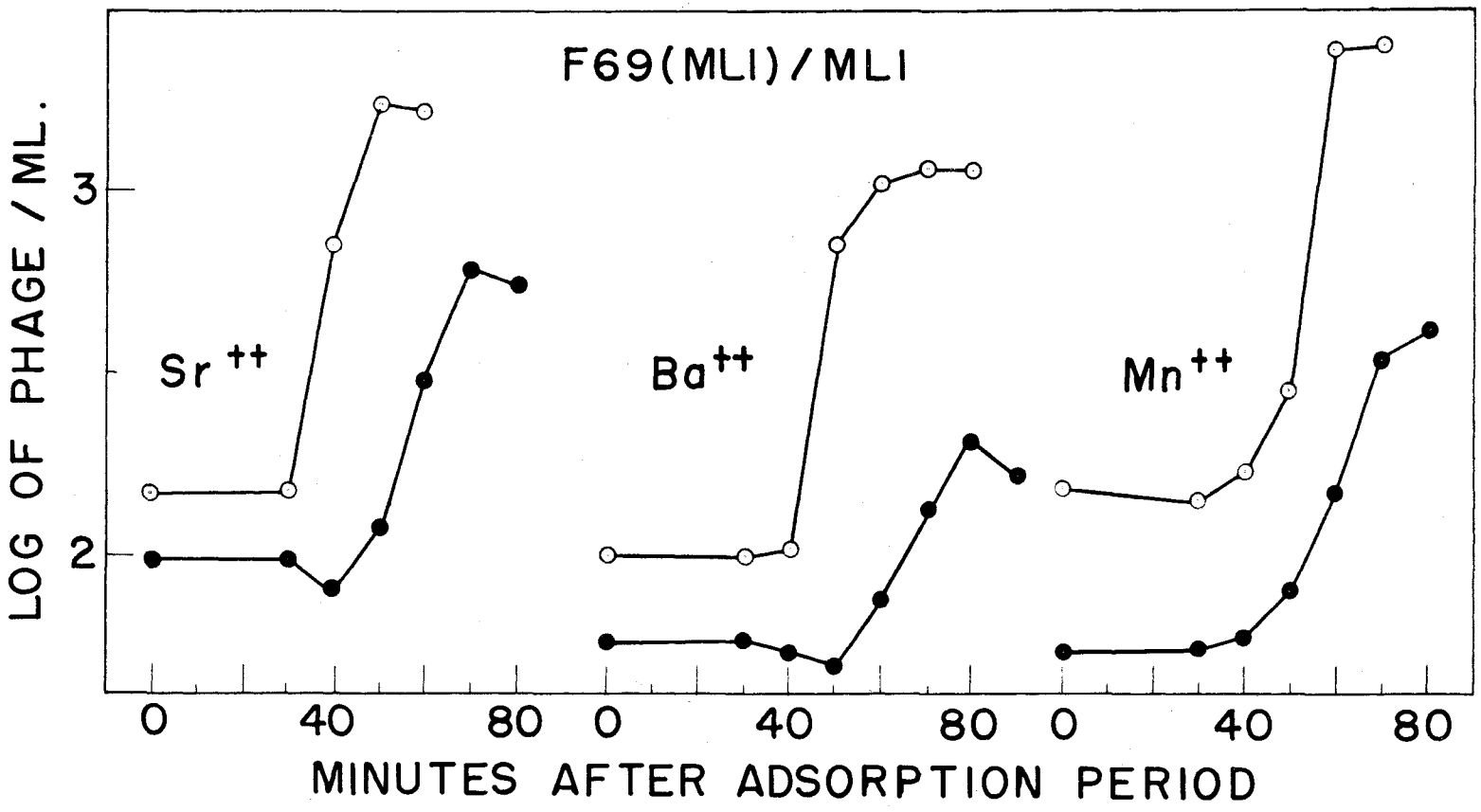

Figure 14. Effect of ion addition on proliferation of bacteriophage adsorbed in absence of strontium, barium or manganese.

(Open circles=ion present during adsorption and throughout experiment. Closed circles=ion added following adsorption in its absence and present throughout remainder of experiment. All ions 32×10^{-4} M.)

adsorption period and throughout the experiment, being included in the medium used to dilute the systems at zero time. Curves based on closed circles were obtained without the ions in adsorption mixtures, but added following the adsorption period with the dilution medium, and present for the remainder of the experiment. Concentration of the ions was 32×10^{-4} M. Control curves with ions present throughout the experiment are very similar to corresponding curves in Figure 12 in which the ions were present only during the adsorption period. Bacteriophage F69(ML1) adsorbed in absence of strontium, barium, or manganous ions, increased in titer when these ions were supplied, presumably because invasion then could occur. As in the case with calcium (Figures 7 and 8), some loss of invasiveness occurred under these conditions with each of the ions, as indicated by the smaller step size. Also the latent periods were slightly longer than when the ions were included in adsorption mixtures (with the possible exception of manganous ion, although on repeated trial a longer latent period was obtained with this ion). The longer latent periods could be explained if invasion was proceeding in ion-containing adsorption mixtures, but could not commence in mixtures without the ions until 5 minutes later when the ions were added. Loss of invasiveness of bacteriophage adsorbed in absence of the

ions is further indicated by the lower zero time titers of the closed circle curves as compared with the control curves. While with a given ion both systems contained the same concentration of bacteriophage particles during the latent period, fewer of these produced plaques in satisfactory enumeration medium when adsorbed in the absence of the ion. These growth curve characteristics (except for difference in the latent period with manganous ion) were consistent on repeated trial.

The experiments of Figures 12, 13, and 14 and Tables 20 and 21, interpreted in the same manner as in the study on calcium action, strongly suggest that the function of strontium, barium, and manganous ions is similar to that of calcium, and that these ions are involved in the process of bacteriophage invasion.

Influence of mixing calcium with other ions on bacteriophage proliferation in the calcium-deficient medium

Barium and manganous ions are much less effective than calcium, in supporting bacteriophage increase, when present in the medium in concentrations below those employed in the preceeding mechanism studies. From their mode of action, it appears that they may function at the same "site" as does calcium. Thus it was thought that barium or manganous ion, combined with calcium in low

concentration, might possibly inhibit utilization of calcium somewhat, by competitively occupying sites otherwise available to calcium. This was studied by the one-step growth technique using 12×10^{-4} M calcium combined with 12×10^{-4} M barium or manganous ion in adsorption mixtures and removed from the experimental system following the 5 minute adsorption period by dilution. Adsorption mixtures containing 12×10^{-4} M calcium, barium or manganous ions alone were used as controls. This ion concentration was chosen because 12×10^{-4} M calcium does not support maximum bacteriophage increase, and if barium or manganous ions combined with calcium were not inhibitory, but rather supplemented calcium, this possibly could be seen by an increased step size compared with that of calcium alone. The results of this experiment with bacteriophage F63(E8-2) are given in Figure 15. Bacteriophage increase was appreciable with calcium alone in the adsorption mixture (curve A), but only very slight with this concentration of barium (curve C) or manganous ion (curve B). Barium or manganous ion combined with calcium (curves E and D) had no demonstrable inhibitory effect on bacteriophage increase as indicated by bacteriophage growth curves. The combination of calcium and manganous ions may have given slightly greater bacteriophage increase than calcium

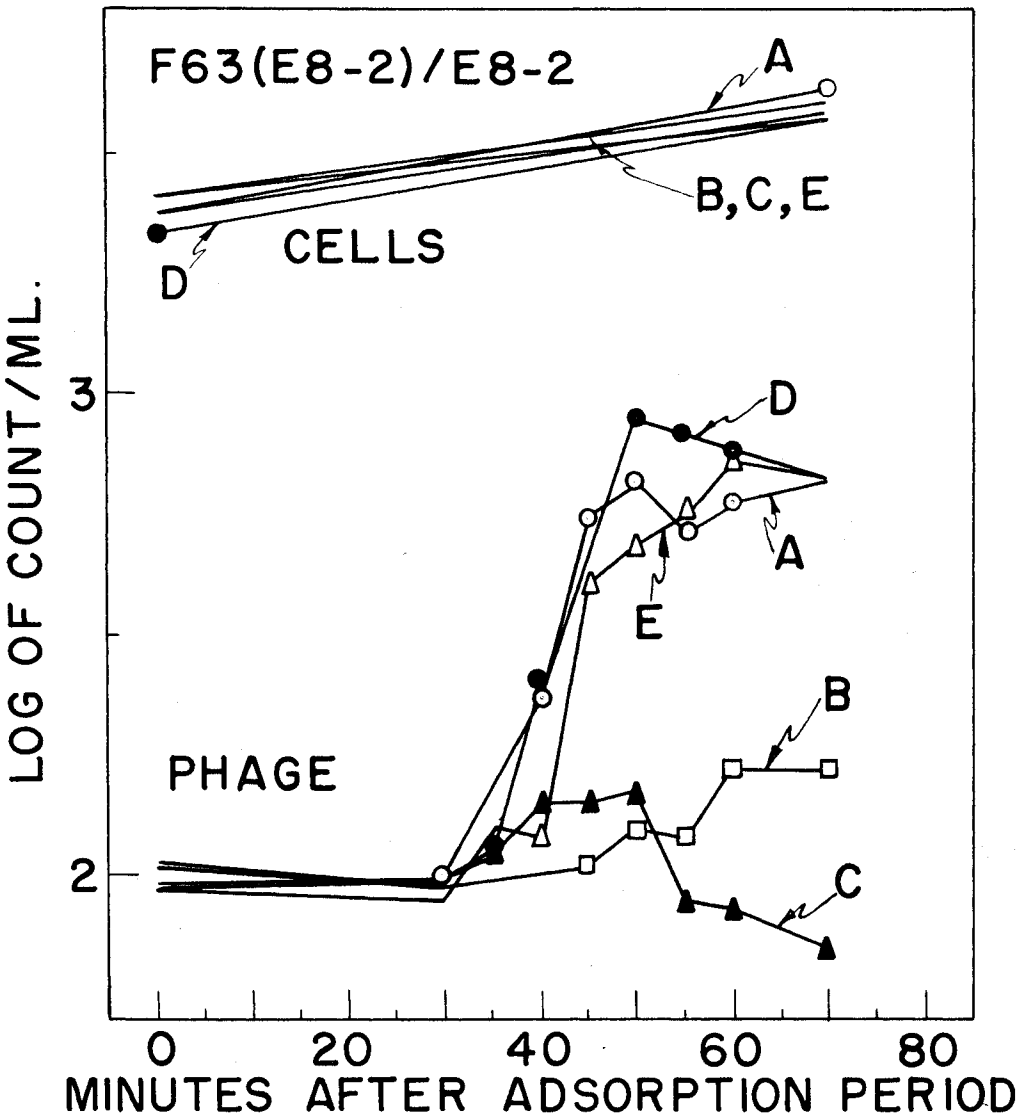

Figure 15. Effect of ion combinations present during adsorption period on bacteriophage proliferation.

(A=Ca^{++}, B=Mn^{++}, C=Ba^{++}, D=Ca^{++} plus Mn^{++}, and E=Ca^{++} plus Ba^{++}; all ions 12×10^{-4} M)

alone. This experiment was repeated with bacteriophage F69(ML1) using a combination of 16×10^{-4} M concentrations of calcium and barium ions in the adsorption mixture. While bacteriophage increase in the control containing 16×10^{-4} M barium alone was appreciably less than with this concentration of calcium alone, no inhibitory or supplementary effect of barium in combination with calcium was evident.

The calcium-deficient medium is not free of ions. Those present in appreciable concentration may be seen in Table 1. Modifying this medium with additional quantities of these as well as other ions might be expected to influence rate of bacteriophage proliferation with a given calcium supplement. Tubes of calcium-deficient medium, containing calcium alone or calcium plus other ions, were inoculated with approximately 10^5 bacteriophage particles and 10^6 host bacterial cells per ml. Titration for bacteriophage was made at the start of the experiment and after incubation at 32° C for 4 hours, in the case of bacteriophage-organism combination F69(ML1)/ML1, and for 3 hours using bacteriophage-organism combination F63(E8-2)/E8-2. Bacteriophage was multiplying rapidly at the time titers were to be determined, and so the tubes were immersed in an ice bath during the titration period to keep changes at a minimum. This

was satisfactory if the temperature was maintained below 5° C and no more tubes than could be titered within about 30 minutes were included in any one experiment. Tubes were again observed for mass lysis after 15 hours incubation at 32° C. Effects of the ion combinations on the medium and on cell growth were observed in parallel series of tubes containing no bacteriophage. In these experiments calcium concentration was maintained constant and the concentrations of different ions in combination with calcium were varied. Representative results with bacteriophage F69(ML1) are given in Table 22. As in the ion-screening studies of Tables 17 and 18, several of the ions formed precipitates with medium constituents and were toxic to bacterial growth at the higher concentrations. Comparison of zero and 4 hour titers shows that several of the ions combined in higher concentrations with calcium were toxic to bacteriophage, causing a decrease in titer. Interpretation of the data, with reference to influence of the ions combined with calcium on bacteriophage proliferation rate, must take these toxicities into account. Comparison of 4 hour titers with calcium alone and with calcium plus other ions indicates that, with the possible exception of potassium, none of the ions in the concentrations studied were noticeably antagonistic to calcium action in a manner that could not

Table 22

Proliferation of bacteriophage F69(ML1) on host ML1 in the calcium-deficient medium supplemented with calcium plus other ions

Ion supplement (units of conc. x10^{-4} M)	Bacteriophage titer 0 hour	Bacteriophage titer 4 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},5	82x10^3	46x10^5	+	-
Ca^{++},5 plus Al^{+++},45	"	23x10^3	-	T,P**
" " 15	"	3x10^5	-	T,P
" " 5	"	9x10^5	+	ST,P
" " 2	"	37x10^5	+	-
" " 1	"	51x10^5	+	-
" " 0.5	"	39x10^5	+	-
" " 0.2	"	62x10^5	+	-
" " 0.1	"	35x10^5	+	-
Ca^{++},5	99x10^3	88x10^5	+	-
Ca^{++},5 plus Fe^{+++},45	"	<1x10^3	-	T,P
" " 15	"	56x10^3	-	T,P
" " 5	"	8x10^5	+	ST,P
" " 2	"	26x10^5	+	-
" " 1	"	53x10^5	+	-
" " 0.5	"	36x10^5	+	-
" " 0.2	"	86x10^5	+	-
" " 0.1	"	62x10^5	+	-
Ca^{++},5	82x10^3	46x10^5	+	-
Ca^{++},5 plus Cr^{+++},45	"	<1x10^3	-	T,P
" " 15	"	1x10^3	-	T,P
" " 5	"	44x10^3	-	-,P
" " 2	"	63x10^3	-	-
" " 1	"	23x10^3	+	-
" " 0.5	"	38x10^5	+	-
" " 0.2	"	79x10^5	+	-
" " 0.1	"	46x10^5	+	-

Table 22 (continued)

Ion supplement (units of conc. x10^{-4} M)	Bacteriophage titer 0 hour	Bacteriophage titer 4 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},5	82x10^3	46x10^5	+	-
Ca^{++},5 plus Cu^{++},1	"	-	-	T
" " 0.5	"	6x10^5	+	ST
" " 0.2	"	15x10^5	+	-
" " 0.1	"	37x10^5	+	-
" " 0.05	"	67x10^5	+	-
" " 0.02	"	55x10^5	+	-
" " 0.01	"	52x10^5	+	-
Ca^{++},5	82x10^3	46x10^5	+	-
Ca^{++},5 plus Cd^{++},1	"	-	-	T
" " 0.5	"	20x10^3	-	ST
" " 0.2	"	110x10^3	+	-
" " 0.1	"	18x10^5	+	-
" " 0.05	"	40x10^5	+	-
" " 0.02	"	22x10^5	+	-
" " 0.01	"	43x10^5	+	-
Ca^{++},5	82x10^3	46x10^5	+	-
Ca^{++},5 plus Zn^{++},10	"	6x10^3	-	T
" " 5	"	30x10^3	-	-
" " 2	"	12x10^5	+	-
" " 1	"	22x10^5	+	-
" " 0.5	"	40x10^5	+	-
" " 0.2	"	85x10^5	+	-
" " 0.1	"	51x10^5	+	-
" " 0.05	"	46x10^5	+	-
" " 0.02	"	60x10^5	+	-
" " 0.01	"	62x10^5	+	-

Table 22 (continued)

Ion supplement (units of conc. x10^{-4} M)	Bacteriophage titer 0 hour	Bacteriophage titer 4 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},5	75x10^3	62x10^5	+	-
Ca^{++},5 plus Ni^{++},15	"	<1x10^5	-	T
" " 5	"	86x10^5	+	-
" " 2	"	96x10^5	+	-
" " 1	"	68x10^5	+	-
" " 0.5	"	65x10^5	+	-
" " 0.2	"	-	+	-
" " 0.1	"	51x10^5	+	-
Ca^{++},5	75x10^3	53x10^5	+	-
Ca^{++},5 plus Co^{++},45	"	15x10^5	+	-
" " 15	"	192x10^5	+	-
" " 5	"	247x10^5	+	-
" " 2	"	158x10^5	+	-
" " 1	"	131x10^5	+	-
" " 0.5	"	74x10^5	+	-
" " 0.2	"	54x10^5	+	-
" " 0.1	"	48x10^5	+	-
Ca^{++},5	78x10^3	86x10^5	+	-
Ca^{++},5 plus Mg^{++},360	"	556x10^5	+	-
" " 180	"	540x10^5	+	-
" " 90	"	564x10^5	+	-
" " 45	"	530x10^5	+	-
" " 15	"	440x10^5	+	-
" " 5	"	131x10^5	+	-
" " 2	"	82x10^5	+	-
" " 1	"	83x10^5	+	-
" " 0.5	"	-	+	-
" " 0.2	"	59x10^5	+	-
" " 0.1	"	83x10^5	+	-

Table 22 (continued)

Ion supplement (units of conc. x10^{-4} M)	Bacteriophage titer 0 hour	Bacteriophage titer 4 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},5	78x10^3	92x10^5	+	-
Ca^{++},5 plus Na^+,2000	"	2x10^3	-	ST
" " 1000	"	5x10^5	-	ST
" " 500	"	25x10^5	+	-
" " 200	"	68x10^5	+	-
" " 100	"	75x10^5	+	-
" " 50	"	84x10^5	+	-
" " 20	"	75x10^5	+	-
" " 10	"	87x10^5	+	-
" " 5	"	80x10^5	+	-
" " 2	"	109x10^5	+	-
" " 1	"	93x10^5	+	-
Ca^{++},5	75x10^3	74x10^5	+	-
Ca^{++},5 plus K^+,1000	"	4x10^5	-	-
" " 500	"	6x10^5	-	-
" " 200	"	31x10^5	+	-
" " 100	"	51x10^5	+	-
" " 50	"	63x10^5	+	-
" " 20	"	71x10^5	+	-
" " 10	"	77x10^5	+	-
" " 5	"	77x10^5	+	-
" " 2	"	72x10^5	+	-
" " 1	"	61x10^5	+	-

* Observation following 15 hours incubation.
** T= toxic to bacterial cells; ST= slightly toxic to cells; P= precipitate.

be explained by toxicity to either virus or host. In the case of zinc, for example, while a concentration of 2×10^{-4} M in combination with calcium did not appear toxic to cell growth on the basis of developed turbidity, more careful study on influence of certain ion combinations on cell growth (Table 24), showed that the slightly lower 4 hour bacteriophage titer in Table 22 produced with this concentration of zinc as compared with calcium alone, could be explained by a reduced rate of bacterial growth. The possible effect of potassium was not observed except at exceptionally high concentrations. While 500×10^{-4} M potassium appeared not to affect bacterial growth rate in Table 24, such a concentration would be expected to influence electrostatic and other properties of virus and host. In contrast, magnesium or cobaltous ions combined with calcium, resulted in more rapid bacteriophage proliferation than calcium alone in the concentration used. This was true at several concentrations of cobalt or magnesium. Maximum effect with magnesium and cobaltous ions occurred at concentrations of about 90 and 5×10^{-4} M, respectively. Slight inhibitory action appeared with 45×10^{-4} M cobalt.

Several of the ions in Table 22 were studied again with bacteriophage F63(E8-2) (Table 23). Magnesium and cobaltous ions combined with calcium again produced more

Table 23

Proliferation of bacteriophage F63(E8-2) on host E8-2 in the calcium-deficient medium supplemented with calcium plus other ions

Ion supplement (units of conc. x10^{-4} M)	Bacteriophage titer 0 hour	Bacteriophage titer 3 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},15	118x10^3	97x10^5	+	-
Ca^{++},15 plus Al^{+++},10	"	4x10^5	-	ST,P**
" " 3	"	43x10^5	+	P
" " 1	"	61x10^5	+	-
" " 0.3	"	106x10^5	+	-
" " 0.1	"	-	+	-
Ca^{++},15	118x10^3	85x10^5	+	-
Ca^{++},15 plus Zn^{++},10	"	9x10^3	-	T
" " 3	"	<1x10^5	-	T
" " 1	"	4x10^5	+	-
" " 0.3	"	87x10^5	+	-
" " 0.1	"	81x10^5	+	-
" " 0.03	"	97x10^5	+	-
Ca^{++},15	118x10^3	125x10^5	+	-
Ca^{++},15 plus Ni^{++},10	"	26x10^5	-	ST
" " 3	"	51x10^5	+	-
" " 1	"	127x10^5	+	-
" " 0.3	"	108x10^5	+	-
" " 0.1	"	111x10^5	+	-

Table 23 (continued)

Ion supplement (units of conc. x10^{-4} M)	Bacterio-phage titer 0 hour	3 hour	Mass lysis*	Effect on cells and medium*
Ca^{++},15	118x10^3	105x10^5	+	-
Ca^{++},15 plus Co^{++},45	"	8x10^5	+	-
" " 15	"	100x10^5	+	-
" " 5	"	167x10^5	+	-
" " 2	"	214x10^5	+	-
" " 1	"	178x10^5	+	-
" " 0.5	"	150x10^5	+	-
Ca^{++},15	118x10^3	114x10^5	+	-
Ca^{++},15 plus Mg^{++},90	"	1138x10^5	+	-
" " 45	"	652x10^5	+	-
" " 15	"	444x10^5	+	-
" " 5	"	262x10^5	+	-
" " 2	"	184x10^5	+	-
Ca^{++},15	118x10^3	100x10^5	+	-
Ca^{++},15 plus K^{+},1000	"	4x10^5	+	-
" " 300	"	51x10^5	+	-
" " 100	"	109x10^5	+	-
" " 30	"	112x10^5	+	-
" " 10	"	108x10^5	+	-

* Observation following 15 hours incubation.
** ST= slightly toxic to bacterial cells; T= toxic to cells; P= precipitate.

rapid bacteriophage proliferation than calcium alone at a concentration of 15×10^{-4} M. Effective concentrations of cobalt and magnesium were in the same range as with bacteriophage F69(ML1), although in this case the calcium concentration employed was three-fold greater. The other ions tested were without stimulatory effect and caused inhibition in some cases.

To determine if the stimulatory effect of magnesium and cobaltous ions could be explained by an influence on rate of host bacterial growth, calcium-deficient medium supplemented with calcium plus these ions or calcium alone was inoculated with hosts ML1 and E8-2 and incubated at 32° C for 5 hours. Bacterial counts were made at hourly intervals. Table 24 shows that rates of growth of these bacterial hosts in calcium-containing medium are much the same whether or not magnesium or cobaltous ions are present. The influence of potassium and zinc ions has already been mentioned.

In the ion screening studies of Tables 17 and 18, supplementation of calcium-deficient medium with magnesium or cobaltous ions alone failed to produce increase in titer of bacteriophages F69(ML1) or F57(799). This was again studied with bacteriophages F69(ML1) and F63(E8-2), using the same procedure as in the experiments of Tables 22 and 23 and determining titers after 3 and 5 hours. Concentrations of magnesium and cobaltous ions

Table 24

Growth of bacterial hosts ML1 and E8-2 in the calcium-deficient medium supplemented with calcium plus other ions

Ion supplement (units of conc. x10^{-4} M)	Cell counts after incubation periods (hours) of: 0	1	2	3	4	5
			ML1			
Ca^{++},5	$126x10^4$	$136x10^4$	$43x10^5$	$108x10^5$	$38x10^6$	$50x10^6$
Ca^{++},5 plus Mg^{++},90	$140x10^4$	$157x10^4$	$67x10^5$	$138x10^5$	$38x10^6$	$66x10^6$
" Co^{++},5	$124x10^4$	$159x10^4$	$46x10^5$	$116x10^5$	$40x10^6$	$51x10^6$
" K^+,500	$132x10^4$	$146x10^4$	$46x10^5$	$112x10^5$	$40x10^6$	$38x10^6$
" Zn^{++},2	$130x10^4$	$136x10^4$	$26x10^5$	$59x10^5$	$18x10^6$	$26x10^6$
			E8-2			
Ca^{++},15	$114x10^4$	$194x10^4$	$54x10^5$	$96x10^5$	$32x10^6$	$56x10^6$
Ca^{++},15 plus Mg^{++},90	$118x10^4$	$199x10^4$	$39x10^5$	$124x10^5$	$32x10^6$	$53x10^6$
" Co^{++},2	$116x10^4$	$182x10^4$	$47x10^5$	$112x10^5$	$25x10^6$	$54x10^6$
" K^+,1000	$126x10^4$	$182x10^4$	$46x10^5$	$126x10^5$	$38x10^6$	$36x10^6$
" Zn^{++},1	$125x10^4$	$173x10^4$	$30x10^5$	$70x10^5$	$14x10^6$	$24x10^6$

were the same as produced maximum effect when combined with calcium in these experiments. Results in Table 25 show that both test bacteriophages decrease slightly in titer, within a period of 5 hours, when calcium-deficient medium is supplemented with cobaltous ion alone. Similarly, magnesium alone supports no increase in titer of bacteriophage F63(ES-2). Bacteriophage F69(ML1) increased slightly in titer in 5 hours in medium containing 90×10^{-4} M magnesium ion, but the rate of increase was not great enough to produce mass lysis in a period of 15 hours. Failure to observe this slight effect of magnesium in the experiment of Table 17 may have been due to the slightly lower magnesium concentration employed, or to decrease in titer following a slow rise, resulting in no net increase at 15 hours. Results in Tables 22, 23, and 25, indicate that while magnesium and cobaltous ions increase rate of bacteriophage proliferation when combined with calcium, they are ineffective (with the possible exception of a slight effect of magnesium ion in the case of bacteriophage F69(ML1)) when present in absence of calcium. A subsequent experiment (Table 27) where magnesium is studied in concentration of 15×10^{-4} M, shows that at this concentration magnesium alone produces no increase in titer of bacteriophage F69(ML1) as indicated by 10 hour determination, but

Table 25

Proliferation of bacteriophages F69(ML1) and F63(ES-2) on their respective hosts in the calcium-deficient medium supplemented with calcium, magnesium, or cobaltous ions

Ion supplement (units of conc. x10^{-4} M)	Titer of bacteriophage at: 0 hour	3 hour	5 hour	Mass lysis*
		F69(ML1)		
Ca^{++},5	54x10^3	1x10^5	46x10^5	+
Mg^{++},90	62x10^3	54x10^3	10x10^5	-
Co^{++},5	68x10^3	8x10^3	1x10^3	-
		F63(ES-2)		
Ca^{++},15	50x10^3	46x10^5	>1000x10^5	+
Mg^{++},90	36x10^3	20x10^3	10x10^3	-
Co^{++},2	48x10^3	41x10^3	15x10^3	-

* Observation following 15 hours incubation; mass lysis indicated by +.

increases bacteriophage proliferation when the ion is present in combination with calcium.

Further studies on the action of magnesium and cobaltous ions

One-step growth experiments were run to determine the effects of magnesium and cobaltous ions in combination with calcium on characteristics of the bacteriophage growth curve. Preliminary trials indicated that much if not all of the effect of these ions was obtained when they were present for just the 5 minute adsorption period. Results of an experiment with bacteriophage F63(E8-2) are given in Figure 16. Control curve D was obtained with 18×10^{-4} M calcium present in the adsorption mixture and removed by dilution with calcium-deficient medium at zero time. Curves A and B were obtained with 18×10^{-4} M calcium plus 36×10^{-4} M magnesium ions in adsorption mixtures. In the case of curve B these ions were removed by dilution following the 5 minute adsorption period while for curve A they were present throughout the experiment, being included in the dilution medium. Curve C was obtained with 18×10^{-4} M calcium plus 2×10^{-4} M cobaltous ion in the adsorption mixture and diluted out at zero time. Calcium in 18×10^{-4} M concentration was employed in this experiment, as lower

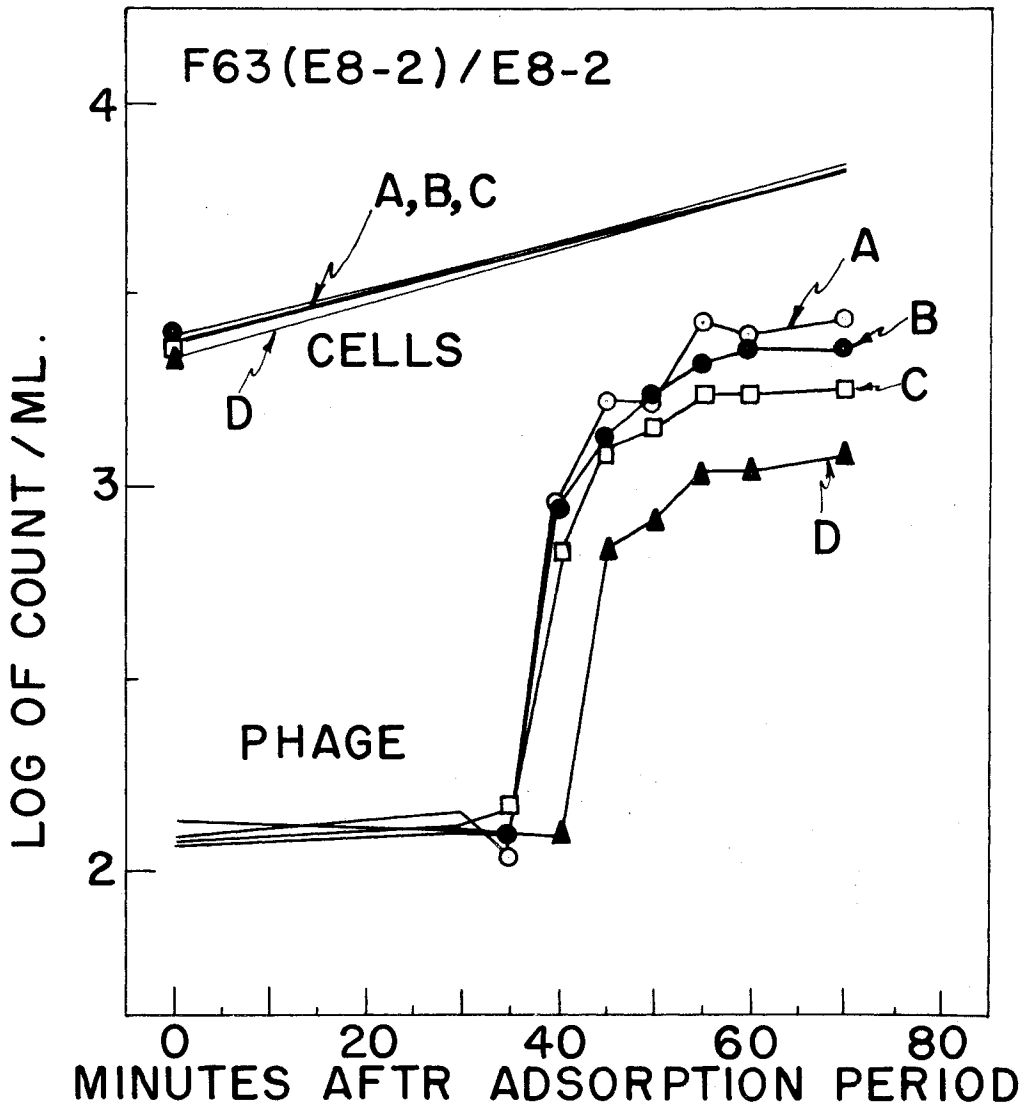

Figure 16. Effect of magnesium and cobaltous ions combined with calcium on bacteriophage proliferation.

(A=18×10^{-4} M Ca^{++} plus 36×10^{-4} M Mg^{++} present throughout experiment; B=18×10^{-4} M Ca^{++} plus 36×10^{-4} M Mg^{++}; C=18×10^{-4} M Ca^{++} plus 2×10^{-4} M Co^{++}; and D=18×10^{-4} M Ca^{++} present only during adsorption period.)

concentrations give but poor proliferation of this bacteriophage, while somewhat higher levels of calcium would be expected to support maximum proliferation and so possibly prevent a supplementary effect of magnesium or cobaltous ions from becoming apparent. Concentration of cobaltous ion is near-optimum, concentration of magnesium ion is somewhat below optimum (Table 23). The 5 minute longer latent period of curve D may not be significant, as it did not occur in repeated trials with bacteriophage F69(ML1). Rise periods of the various curves appear the same. Step size with cobaltous and magnesium ions in the adsorption mixtures were greater than with calcium alone, magnesium producing possibly a slightly greater effect than cobaltous ion. The very small difference between curves A and B indicates that most if not all of the magnesium effect was produced within the first 5 minutes of bacteriophage-host association. The curve for cobaltous ion present throughout the experiment is not presented because of error in zero-time dilution. Using bacteriophage F69(ML1), one-step growth curves were obtained with 8×10^{-4} M calcium and this concentration of calcium plus 16×10^{-4} M magnesium or 8×10^{-4} M cobaltous ion in adsorption mixtures. At these concentrations magnesium and cobaltous ions produced very slight effect on step size and no apparent effect on

latent or rise periods.

Adsorption mixtures containing the same ion supplements as in one-step growth experiments were prepared and the extent of adsorption occurring in 5 minutes determined. In this experiment, controls containing no bacterial cells were prepared for each of the ion supplements. Bacteriophage titers of supernatants following centrifugation were very similar regardless of the ion supplement, indicating no apparent activating influence of magnesium or cobaltous ions on free bacteriophage particles. Table 26 shows that the stimulatory effect of magnesium and cobaltous ions is not due to influence upon adsorption.

Single infected cell experiments were run with bacteriophage F63(E8-2) to determine if the effect of magnesium and cobaltous ions could be explained by an increased burst size or burst frequency. These experiments were carried out as in previous single infected cell studies. Adsorption mixtures contained 18×10^{-4} M calcium, 18×10^{-4} M calcium plus 36×10^{-4} M magnesium, or 18×10^{-4} M calcium plus 2×10^{-4} M cobaltous ion. Summarizing the results of 4 trials representing 90 aliquots of each mixture, average burst sizes with calcium alone, calcium plus magnesium, and calcium plus cobaltous ions were 55, 56, and 62, respectively; number

Table 26

Adsorption of bacteriophages F69(ML1) and F63(E8-2) on their hosts with calcium plus magensium or cobaltous ions in the adsorption mixtures

	Ion conc. (in units $x10^{-4}$ M) used with F69(ML1)/ML1					
	Adsorption mixtures*			Controls**		
	Ca^{++},8	Ca^{++},8 + Mg^{++},16	Ca^{++},8 + Co^{++},8	Ca^{++},8	Ca^{++},8 + Mg^{++},16	Ca^{++},8 + Co^{++},8
Titer***	$98x10^4$	$106x10^4$	$100x10^4$	$200x10^4$	$189x10^4$	$188x10^4$
% Adsorption	49	45	48	-	-	-

	Ion conc. (in units $x10^{-4}$ M) used with F63(E8-2)/E8-2					
	Adsorption mixtures*			Controls**		
	Ca^{++},18	Ca^{++},18 + Mg^{++},36	Ca^{++},18 + Co^{++},2	Ca^{++},18	Ca^{++},18 + Mg^{++},36	Ca^{++},18 + Co^{++},2
Titer	$132x10^4$	$128x10^4$	$136x10^4$	$227x10^4$	$225x10^4$	$236x10^4$
% Adsorption	42	44	41	-	-	-

* Adsorption mixtures of both combinations contained approx. $25x10^6$ cells per ml.

** Control value used in calculation of adsorption is average of the three controls as ions had no differential effect on free phage in these.

*** Titer of plaque forming particles in supernatant of adsorption and control mixtures.

of bursts were 26, 26, and 21, respectively. Values for average burst sizes with the various ion supplements possibly are more reliable than frequency of bursts, for while the former were quite consistent in the 4 trials, the relative number of bursts with a given ion supplement was not. In two of the above trials an adsorption mixture containing 18×10^{-4} M calcium plus 36×10^{-4} M magnesium plus 2×10^{-4} M cobaltous ions was included. Results with these suggested that magnesium and cobaltous ions might be affecting frequency of bursts rather than burst size, for in both trials the trinary ion supplement gave a greater number of bursts than calcium alone or calcium plus either magnesium or cobaltous ions. The average burst sizes with the various ion supplements, however, were quite similar. The difficulty in attempting to determine the mode of action of magnesium and cobaltous ions by one-step growth and single infected cell experiments arises from the fact that their influence on bacteriophage proliferation is relatively slight when measured in terms of a single growth cycle. The appreciable stimulatory effects of these ions as seen in Tables 22 and 23, on the other hand, represent several successive growth cycles. Whether magnesium and cobaltous ions influence frequency or size of bursts remains to be determined definitely.

Up to this point, all experiments involving magnesium and cobaltous ions were carried out in the presence of minimum calcium concentration. The extent to which magnesium and cobaltous ions influenced the calcium concentration required for rapid bacteriophage growth was studied. The procedure employed was similar to that used in the early part of this investigation for determination of calcium requirements of bacteriophage-organism combinations. Tubes of calcium-deficient medium supplemented with various concentrations of calcium plus magnesium and/or cobaltous ions were inoculated with a known concentration of bacteriophage particles and approximately 10^6 host bacterial cells per ml. and incubated at 32° C for 10 hours. Tubes were observed during the incubation period and at 10 hours for occurrence of mass lysis. In two experiments selected tubes were titered at 10 hours for a quantitative measure of bacteriophage increase. Titration at 10 hours rather than at a later time was chosen because in cases where mass lysis did not occur, bacteriophage titer could be expected to decrease with time following initial rise. Preliminary trial showed that calcium concentration required for mass lysis was appreciably reduced when magnesium or cobaltous ions were present in the system. Further, certain combinations of these ions reduced the

time required for mass lysis as compared with sub-optimum levels of calcium alone. In the case of bacteriophage-organism combination F69(ML1)/ML1, for example, calcium requirement for mass lysis was reduced by magnesium ion in concentrations from 5 to 45×10^{-4} M. Reduction occurred with 1 and 5×10^{-4} M cobaltous ion and further reduction did not occur with a concentration of 15×10^{-4} M. Mixtures of magnesium and cobaltous ions were more effective than these ions alone. Results of such experiments in which titers of selected tubes were made are seen in Tables 27 and 28. Bacteriophage F69(ML1) (Table 27) required 6×10^{-4} M calcium ion for mass lysis with magnesium and cobaltous ions absent. This was reduced to 3×10^{-4} M in presence of either 5×10^{-4} M cobaltous or 15×10^{-4} M magnesium ions. Still greater reduction occurred when both ions, in these concentrations, were present. Similar effects of the ions on calcium requirement for mass lysis with bacteriophage-organism combination F63(ES-2)/ES-2 are seen in Table 28. These occurred at different ion concentrations than in Table 27. Bacteriophage titers in these tables show that bacteriophage increases were much the same with each ion mixture permitting mass lysis.

Whether magnesium and cobaltous ions function through a sparing type action for calcium or by increasing

Table 27

Proliferation of bacteriophage F69(ML1) on host ML1 in the calcium-deficient medium containing mixtures of calcium, magnesium, and cobaltous ions

Supplementary ions (units of conc. $x10^{-4}$ M)	Mass lysis and titers* with supplementary ions plus the following conc. ($x10^{-4}$ M) of calcium:						
	0	1	2	3	4	5	6
None	- $<1x10^2$	-	-	-	-	- $15x10^7$	+ $78x10^7$
Co^{++},5	- $<1x10^2$	-	- $72x10^4$	+ $5x10^7$	+	+	+
Mg^{++},15	- $<1x10^2$	-	- $45x10^6$	+ $15x10^7$	+	+	+
Co^{++},1 + Mg^{++},5	- $<1x10^2$	-	-	- $12x10^7$	+ $20x10^7$	+	+
Co^{++},5 + Mg^{++},15	- $<1x10^2$	- $2x10^4$	+ $13x10^7$	+	+	+	+

* Mass lysis observation and titers were made after 10 hours incubation; initial bacteriophage titer and cell count were $5x10^2$ and approx. 10^6 per ml., respectively. Mass lysis is indicated by +.

Table 28

Proliferation of bacteriophage F63(E8-2) on host E8-2 in the calcium-deficient medium containing mixtures of calcium, magnesium, and cobaltous ions

Supplementary ions (units of conc. x10^{-4} M)	Mass lysis and titers* with supplementary ions plus the following conc. (x10^{-4} M) of calcium:								
	0	4	6	8	10	12	14	16	18
None	- $1x10^2$	-	-	-	-	-	- $89x10^6$	± $17x10^8$	+
Co^{++},2	- $1x10^2$	-	-	-	-	- $13x10^7$	+ $36x10^8$	+	+
Mg^{++},30	- $2x10^2$	-	-	-	- $63x10^7$	+ $64x10^8$	+	+	+
Mg^{++},90	- $1x10^2$	- $14x10^6$	+ $29x10^8$	+	+	+	+	+	+
Co^{++},2 + Mg^{++},90	- $<1x10^2$	- $97x10^6$	+ $56x10^7$	+	+	+	+	+	+

* Mass lysis observation and titers were made after 10 hours incubation; initial bacteriophage titer and cell count were $56x10^2$ and approx. 10^6 per ml., respectively. Mass lysis is indicated by +.

the physiological capacity of host-virus complexes to produce bacteriophage is not apparent from the foregoing experiments. In no case was the influence of magnesium and cobaltous ions added to a system containing optimum calcium concentration studied. Calcium-deficient medium was supplemented with 32×10^{-4} M calcium, which appears optimum for bacteriophage F69(ML1) (Figure 1), and with this concentration of calcium plus (1) 15×10^{-4} M magnesium, (2) 5×10^{-4} M cobaltous, and (3) 15×10^{-4} M magnesium plus 5×10^{-4} M cobaltous ions. A control contained 52×10^{-4} M calcium ion. Tubes of medium containing these ions were inoculated with approximately 2×10^{4} particles of bacteriophage F69(ML1) and 10^{6} host cells per ml. and incubated 10 hours at 32° C. Bacteriophage titers were made at 2 hour intervals. Table 29 shows that proliferation rate and final bacteriophage yield were much the same with 32 and 52×10^{-4} M calcium, indicating that 32×10^{-4} M calcium supports maximum bacteriophage increase. No noticeable increase in proliferation rate or final yield occurred with magnesium, cobaltous, or both of these ions added to systems containing 32×10^{-4} M calcium (the slightly greater titer with calcium plus magnesium at 4 and 6 hours did not occur on repeated trial). This indicates that magnesium and cobaltous ions, in presence of sub-optimum calcium concentration, function through a sparing type action for calcium.

Table 29

Proliferation of bacteriophage F69(ML1) on host ML1 in calcium-deficient medium containing magnesium and cobaltous ions plus optimum calcium concentration

Ion supplement (units of conc. $x10^{-4}$ M)	Bacteriophage counts after an incubation period (hours) of:					
	0	2	4	6	8	10
Ca^{++},32	$22x10^3$	$101x10^4$	$104x10^6$	$20x10^7$	$23x10^7$	$28x10^7$
Ca^{++},32 + Mg^{++},15	"	$108x10^4$	$185x10^6$	$48x10^7$	$20x10^7$	$19x10^7$
Ca^{++},32 + Co^{++},5	"	$86x10^4$	$85x10^6$	$18x10^7$	$18x10^7$	$18x10^7$
Ca^{++},32 + Mg^{++},15 + Co^{++},5	"	$80x10^4$	$82x10^6$	$14x10^7$	$14x10^7$	$12x10^7$
Ca^{++},52	"	$94x10^4$	$82x10^6$	$25x10^7$	$34x10^7$	$30x10^7$

DISCUSSION

The calcium-deficient medium used in this investigation supported continued growth of all lactic streptococcus cultures studied, but increase in numbers of bacteriophages on actively growing host bacteria occurred in no case unless the medium was supplemented with calcium or certain related ions. However this medium, although deficient in calcium, is not ion-free, and data from experiments carried out in it must be interpreted with this in mind. The ions known to be present in the medium are seen in Table 1; trace amounts of other ions undoubtedly also are present. The ion requirements and the other complexities of the medium required for growth of lactic streptococci preclude the satisfactory use of ion-depleted media in an investigation of the present kind.

In the calcium-deficient medium, rate of bacteriophage proliferation was related to calcium concentration (Figure 1); all of the experiments on mechanism of ion action lead to the conclusion that this is due to a dependence of bacteriophage on calcium for "invasion" of host cells. Exactly what the nature of the invasive process is remains to be established. The term invasion

as used here means penetration by attached bacteriophage or orientation of reactive groups of bacteriophage and host leading to penetration.

Calcium ion appears to occupy a unique position in the lactic streptococcus bacteriophage system. Strontium, barium, and manganous ions may replace calcium, but at equimolar concentration are appreciably less effective in satisfying the requirement for bacteriophage proliferation (Figures 12 and 13). The mode of action of these ions is the same as that of calcium. Magnesium and cobaltous ions influence bacteriophage proliferation, but apparently only through a sparing action for calcium, in which, combined with sub-optimum levels of calcium, they substitute for part of the calcium requirement for optimum bacteriophage increase (Tables 27 and 28). This is surprising because, with the possible exception of high concentrations of magnesium ion, in the case of bacteriophage F69(ML1), these ions by themselves cannot account for an increased multiplication (Table 25). A large group of other cations failed to replace calcium as a requirement for proliferation in the calcium-deficient medium or to increase bacteriophage proliferation when combined with a minimal concentration of calcium.

A function of calcium in bacteriophage invasion is

suggested by the following observations. The requirement for calcium by lactic streptococcus bacteriophages clearly cannot be explained by an effect of this ion on rate of host bacterial growth (Collins et al., 1950; Figures 3-11 and others), on stability of free bacteriophage (Figures 5 and 6), or by the slight influence of this ion on rate of bacteriophage adsorption (attachment) to host cells (Tables 13 and 19). Further, calcium functions in the very early stages of the bacteriophage growth cycle (Figures 2, 3, 8, 10 and 11). This early requirement for calcium agrees with the findings of Adams (1949b) and Kay (1952), who investigated coliphage. The relation between calcium concentration and rate of bacteriophage proliferation was studied further by one-step growth curves and single infected cell experiments. If calcium functioned principally in some stage of the intracellular multiplication process, then the greater bacteriophage proliferation rate with increased calcium concentration would be expected to be associated with either a shortened latent period or an increased burst size at the higher levels of calcium. Figures 5 and 6 and Table 15 show no such effects. Similarly, if calcium functioned in the process of virus liberation following intracellular multiplication, a shortened rise period or increased burst size might occur with increased calcium concentration.

This, too, is not seen in Figures 5 and 6 and Table 15. On the other hand, if calcium were concerned in the process of invasion, then concentration would be expected to have little effect on the characteristics of the bacteriophage growth cycle as such but rather to influence the number of these cycles that could occur. Table 15 shows that calcium concentration determines the proportion of attached bacteriophage particles that result in cell bursts with liberation of bacteriophage.

There is evidence in the literature that adsorption of bacteriophage may be of more than one type. Thus, Hershey et al. (1944) noted that while attachment of a coliphage to host cells was increased by 3 or 4-fold in the presence of salt-containing medium as compared with "salt-free" medium, infectivity of the virus under former conditions was about 10,000 times greater. The work of Puck et al. (1951) and Garen and Puck (1951) suggests that possibly the findings of Hershey et al. (1944) were due to a second ion-dependent reaction leading to virus growth, rather than to a different type of virus attachment. In the present study, bacteriophage adsorbed in absence of calcium invaded and multiplied when this ion was supplied (Figures 7 and 8). This indicates that if adsorption (attachment) was different in absence and presence of calcium, adsorption of the former type could

be changed to the infectious kind by calcium addition. More likely what is being termed invasion is analogous to the step following primary attachment which was studied by Puck and co-workers. While calcium addition permits invasion and multiplication of bacteriophage adsorbed in absence of this ion, these conditions result in a slight prolongation of the latent period and slight loss of invasiveness (Figures 7 and 8). The latent period, measured from the end of the interval allowed for adsorption, would be expected to be longer in the case of calcium addition following the adsorption period as compared with calcium inclusion in the adsorption mixture, were calcium required for the growth cycle to progress beyond the attachment stage. With calcium present in the adsorption mixture, invasion would get underway during the adsorption period. The reduction of invasiveness, while also observed by other workers under somewhat similar conditions, is more difficult to explain. Adams (1949b) noted that a delay in the addition of calcium to an otherwise satisfactory coliphage-host system resulted in a loss in the number of infective centers. Foster (1948) and others also have found that interference with bacteriophage synthesis resulted in loss of plaque-forming ability of virus-host complexes. In the present work such reduction in plaque-forming

ability is quite marked when bacteriophage and host are present together in the absence of calcium for several hours (Figure 1); in this case loss in plaque-forming ability may be due to inactivation of bacteriophage through attachment to dying cells. The slight reduction of invasiveness in the experiments of Figures 7 and 8 does not appear due to some killing of bacterial cells by bacteriophage adsorbed but unable to initiate increase in the absence of calcium (Table 16). Lack of such killing action might be expected if calcium were necessary for invasion and this step had not occurred, for Benzer et al. (1950) point out that attachment of coliphage to host cells does not kill the cells unless invasion occurs. A possible explanation for the slight loss of invasiveness may be associated with the nature of the bacteriophage-host complex formed in presence and absence of calcium. The work of Rountree (1951), Garen and Puck (1951) and others, indicates enzymatic reactions occur very soon after virus attachment. Garen and Puck (1951) suggest that initial virus attachment may be to the same enzyme molecule of the host that functions in the enzymatic reaction. Edney (1949) presents evidence that the requirement of calcium ion by the "receptor destroying enzyme" of cholera vibrio, presumably identical with a surface enzyme of influenza virus, is for the primary

enzyme-substrate union. If in the present study calcium functions in invasion, and serves to link enzyme and substrate, then some loss of invasiveness might be explainable were reactive groups of virus and host combined "improperly" in the absence of calcium, and not completely reoriented on subsequent addition of this ion. This is highly speculative, as is the whole problem of enzyme-substrate union in bacteriophage invasion. Adams (1949a) has shown that coliphage T5 is more stable to heat in the presence of bivalent cations and suggests this is due to the formation of virus-ion complexes. Rountree (1951) and Garen and Puck (1951) suggest that both virus and host bind ions from the medium. In the present system, while virus and host probably bind calcium to some degree, this ion appears not be to held in an irreversible manner by virus or cells in quantity sufficient to satisfy the requirement for proliferation of the virus (Figure 4). A similar conclusion was reached by Beumer and Beumer-Jochmans (1951) working with dysenteric bacteriophage. This requirement is satisfied, however, when calcium is supplied to adsorbing or adsorbed virus for as little as 36 seconds (Figures 9 and 10). This indicates that if calcium is required for the invasive process, penetration or steps leading to it are completed very rapidly.

While this work was in progress, Kay (1952) reported

on the effects of calcium on multiplication of coliphage T5st. Several observations were in agreement with those presented here. In his studies calcium functioned when supplied to the virus-host complex for as little as one minute. Virus-host complexes formed in absence of calcium produced bacteriophage when calcium was supplied. However, virus increase was related to the fraction of the latent period calcium was present, and maximum increase occurred only when calcium was present throughout the major part. These and other considerations led Kay to conclude that the calcium requirement of coliphage T5st was for intracellular multiplication of the virus. Results of the present study do not indicate a similar function for calcium in the case of lactic streptococcus bacteriophages.

Ability of strontium, barium, or manganous ions to replace calcium and inability of several other ions (Tables 17 and 18) to function in this respect indicates that the ion requirements for multiplication of lactic streptococcus bacteriophage are somewhat specific. Calcium, strontium, and barium supported proliferation of all seven bacteriophages studied (Tables 17, 18, and 19), and manganous ion functioned in the case of most of these, suggesting that ion requirements of various strains of this bacteriophage are quite similar. Kay

(1952) noted that strontium replaced the need for calcium for multiplication of a coliphage, but barium and manganese were inactive. In the present study aluminum, ferric, chromic, cupric, cadmium, zinc, nickel, and cobaltous ions exhibited varying degrees of toxicity toward virus or host cells at concentrations required for strontium, barium, or manganous ions to be effective. Were some of these ions non-toxic in the concentrations at which calcium and related ions are effective, possibly they could perform a similar function in bacteriophage proliferation. Toxicity for various host bacteria of manganous ion, at concentrations which would be required for virus increase, possibly is responsible for its inability to support proliferation of all of the bacteriophages in Table 19. The series of experiments indicating that strontium, barium, and manganous ions function in the process of bacteriophage invasion in a manner similar to calcium was carried out with these ions at equimolar rather than individual optimum concentrations. This should not affect interpretation.

An antagonism between sodium and calcium ions in the multiplication of coliphage has been reported by Hershey *et al.* (1944). Additions of calcium to a medium poor in salts were reported to depress both size and number of plaques produced from a bacteriophage inoculum.

In presence of optimal sodium this did not occur. Garen and Puck (1951) found that zinc prevented the enzymatic reaction following primary attachment of coliphage T1 to its bacterial host. They suggested this was due to zinc ions combining with elements of the cell surface in competition with such ions as calcium, magnesium and sodium, which promote this reaction. In the present investigation, neither sodium, zinc, nor any of the eleven other cations studied in combination with calcium (with the possible exception of potassium in very high concentration) appeared antagonistic to calcium action in a manner that could not be explained by toxicity either to virus or host (Figure 15 and Tables 22 and 23).

The stimulatory effect of magnesium and cobaltous ions combined with calcium of sub-optimum concentration again emphasizes the importance of calcium for proliferation of lactic streptococcus bacteriophage. Except as a substitution for part of the calcium requirement, these ions appear to be non-functional in the present system. The close association of their action with that of calcium is indicated by their lack of influence, when combined with calcium, upon bacteriophage adsorption (Table 26), and rate of host bacterial growth (Table 24). They appear to exert their effect within the early minutes of virus-host association. Combined with calcium

they appear to influence step size rather than latent or rise periods. They do not appear to function by increasing the physiological capacity of bacteriophage-host complexes to produce virus, for combined with optimum concentration of calcium (Table 29) they had no noticeable stimulatory effect. In relatively high concentration magnesium ion does support very slight multiplication of bacteriophage F69(ML1) in calcium-deficient medium (Table 25). Similar observations were made by Collins et al. (1950) with two other bacteriophages of this group, using a somewhat different medium. This does not appear to explain the stimulatory effect on multiplication of bacteriophage F69(ML1) also observed when low concentrations of magnesium are combined with calcium (Table 27). Bacteriophage F69(ML1) has a low calcium requirement for proliferation on host ML1. The calcium-deficient medium probably is not completely free of calcium. The possibility exists that the slight proliferation of bacteriophage F69(ML1) in calcium-deficient medium supplemented with a high concentration of magnesium ion alone is due to combined action of magnesium and traces of calcium already present in the medium. Experiments on magnesium and cobaltous ion action tell little of the nature of the apparent sparing action of these for calcium. While in presence of sub-

optimum calcium concentrations they appear to substitute for part of the calcium requirement, the data fail to explain their inability to function in absence of calcium. Contrary to the influence of cobaltous ion in the present system, this ion recently has been found inhibitory for multiplication of influenza virus in embryonated eggs (Schmidt and Rasmussen, 1952).

Garen and Puck (1951) present evidence that cells of E. coli bind ions which perform a function in an enzymatic reaction following bacteriophage attachment. This would seem to indicate an important role of the host cell in determining the types and concentrations of ions that would promote rapid proliferation of adsorbed bacteriophage. In the case of the lactic streptococcus bacteriophage system the host cell is important in determining the ion requirements for virus increase. While various bacteriophage strains attacking the same host strain have different calcium requirements (Table 3), the calcium requirement of a given bacteriophage propagated against different bacterial hosts also varies (Table 2). This latter condition is in contract to the suggestion by Adams (1949a) investigating coliphages, and by Rountree (1951) studying staphylococcus bacteriophages. These investigators felt that the calcium requirement is associated with the bacteriophage

rather than the bacterial host. While these workers observed calcium requirements of various bacteriophages active against the same host they did not study the case of the same bacteriophage on different hosts. For the lactic streptococcus bacteriophage system the calcium requirement appears to be characteristic not of the bacteriophage or host cell alone, but rather of the virus-host combination. This puts a serious limitation on use of calcium requirement for identification of lactic streptococcus bacteriophage strains. While the minimum calcium ion concentration required by a bacteriophage for mass lysis of a host culture can be determined with an accuracy of $\pm$ 1×10^{-4} M, where several susceptible hosts of a given bacteriophage exist, this information is meaningless unless the particular host is specified.

In the early experiments of the present investigation, mass lysis of a host culture was used to indicate calcium requirement for bacteriophage proliferation. Occurrence of mass lysis in these experiments represented bacteriophage proliferation at a rate great enough to overgrow the host culture before the culture reached a stage in which bacteria became unsuitable bacteriophage substrate. At certain levels of calcium below that required for mass lysis, bacteriophage proliferation occurs at a reduced rate (Figure 1); above the minimum

calcium concentration for mass lysis, proliferation rate increases with concentration to a limiting rate characteristic of the host's ability to synthesize virus under the experimental conditions. Occurrence of mass lysis within a 15 hour period, as was used to indicate calcium requirement in the early experiments, limits the interpretation that may be made from these data, for it tells little of bacteriophage proliferation rate.

Bacteriophage proliferation rate probably can explain the relationship between calcium requirement for mass lysis and concentration of the bacteriophage (Table 4). The same bacteriophage required greater amounts of calcium for mass lysis when dilute than when used as concentrated inoculum. Rate of proliferation is related to calcium concentration. A heavy bacteriophage inoculum would be expected to overgrow the host culture more rapidly than a light inoculum with a given calcium level, and should require less calcium than a light inoculum to reach numbers great enough to cause mass lysis before the bacteria become physiologically old and unsuitable as a bacteriophage proliferation substrate. The influence of various hosts on the calcium requirement for rapid proliferation of a given bacteriophage probably is related to some degree to the effect of bacteriophage concentration on calcium requirement, for the titer of a

bacteriophage preparation may be markedly different against various susceptible hosts (Tables 5-9). Differences in titers of a bacteriophage as determined against various susceptible hosts, however, cannot alone explain the influence of host on calcium requirement, for when bacteriophage preparations were diluted to have the same titer against various bacterial hosts, differences in calcium requirement for growth on these hosts, although reduced, still remained.

Quite apart from any influence on calcium requirement, differences in quantitative susceptibility of various bacterial hosts to a given bacteriophage, as indicated by the different titers produced against these hosts, appear to be quite significant. If a bacteriophage preparation has 1000 times greater titer for one host than another, it would be expected that upon aging, titer of the preparation might so decrease that activity against the less susceptible host eventually would be lost, assuming the same rate of inactivation with time for the two activities. This would result in a change in the apparent host range of the bacteriophage. This was much the situation that led to recognition of the titer pattern phenomenon at the beginning of the present investigation. While host range has been a useful criterion in classifying lactic streptococcus bacterio-

phages, it would appear that under certain conditions its value may be limited. Recognition of titer patterns can be important in the design and interpretation of certain quantitative experiments. If, for example, one was studying growth of a bacteriophage on various hosts and wished to start with the same bacteriophage concentration against each host, simply inoculating the various host cultures with equal aliquots of the bacteriophage preparation would not accomplish this in many cases. In titering for bacteriophage increase on these several hosts, very false conclusions might be drawn if titrations were made using a single bacterial host as indicator.

As in the case of calcium requirement, it was of interest to obtain information on the constancy of the titer pattern exhibited by a bacteriophage. This has led to some interesting findings not entirely unrelated to the question of the constancy of the calcium requirements. The titer patterns apparently arising from the growth of single bacteriophage particles (Tables 8 and 9) change when the bacteriophage is propagated on different hosts (Tables 7 and 9). This provides evidence that the bacteriophage itself is changed by such propagation. The change comes about rapidly, the new titer pattern appearing after the first propagation on the new host and remaining unaltered by successive propagations on this

host. This indicates that the bacterial host on which a bacteriophage is multiplied influences the nature of the virus progeny. Recently Luria and Human (1952) have reached a similar conclusion with bacteriophages of the T system propagated against mutant forms of E. coli strain B. These findings may be extremely significant from a genetic as well as epidemiological standpoint. The problem of host-induced virus change, including shifts in titer patterns, is receiving further study in these laboratories at the present time. One result of this work has been demonstration of a widening of bacteriophage host range by selection of the bacterial host used in bacteriophage propagation (Lawton, 1953). Shifts in titer patterns are not the only changes that may occur following bacteriophage propagation on different hosts. Calcium requirements of bacteriophages also were changed in this way (Table 11). This further limits the value of calcium requirement for identifying strains of lactic streptococcus bacteriophage.

The studies on calcium requirements of bacteriophages liberated from hosts grown in different media, and produced from hosts growing in medium containing sub-optimum calcium concentrations (Tables 12 and 10), were designed to indicate further the constancy of the calcium requirement; however, the data from these

experiments are not conclusive. A large fraction of bacteriophage nitrogen and phosphorus is derived from the medium following infection (Cohen, 1948b; Kozloff et al., 1951). This suggests the possibility of an effect of propagating medium on bacteriophage calcium requirement. However, the calcium-deficient medium and milk used in this study may be very similar with regard to the bacteriophage precursor materials they contain. Although attempts to reduce calcium requirements by selecting and propagating bacteriophages produced in presence of sub-optimum levels of calcium failed, this does not eliminate the possibility that under conditions imposing greater "selection pressure," adaptation of bacteriophages to different calcium requirements might occur.

The present investigation fails to explain why different bacteriophage strains attacking the same host, or a given bacteriophage acting on various host strains, should require different concentrations of calcium for rapid proliferation. It is not unlikely that differences in calcium requirement, as well as differences in concentrations of strontium, barium, or manganous ions for rapid virus increase (Table 19) are due to slight differences in reactive groups of the bacterial cell surfaces as well as of the virus particles. If such

reactive groups differ, this is not limited to strain level. A non-homogeneity with respect to ion concentrations necessary for invasion is apparent among host-virus complexes of a given combination of organism and bacteriophage. In the presence of low concentrations of calcium only a fraction of the virus-host complexes produce new virus (Table 15). As the ion concentration is increased, the proportion of the adsorbed bacteriophage particles which will proliferate is increased. Why some hosts are invaded while others in the same system are not is only conjectural at the present time. At calcium concentrations able to support sub-optimum virus increase, the number of calcium ions is enormously in excess of the number of virus-host complexes. Concentration of these ions is such that they would be expected to be associated with numerous elements of the surfaces of both host and virus. Yet doubling the concentration of calcium from 4 to 8×10^{-4} M in the case of bacteriophage-organism combination F69(ML1)/ML1, for example, has a marked effect on invasion of adsorbed virus. Possibly large numbers of calcium ions must activate a broad area of virus or host before invasion can occur. Perhaps calcium affects the physical properties of the cell membranes, making them more "permeable" to virus over a narrow range of concentration

slightly above 10^{-4} M. Differences in virus-host complexes of a given population may reflect physiological variation among individual cells with respect to these membranes. Conceivably what appears to be differences between virus-host complexes of a population may not be physiological, but rather due to probability limitations. This could come about if many calcium ions were required to saturate some mosaic of reactive groups of either virus or host before invasion could occur, and chance collisions of calcium ions with virus or cell surface determined whether fully effective saturation was reached. These and other problems related to bacteriophage invasion might be discussed in less nebulous terms were more known about the cytology and physico-chemical nature of the bacterial surface.

The question of why a relatively small group of ions should be effective in the lactic streptococcus bacteriophage system is not answered by the present investigation. Strontium and barium ions are closely related to calcium with respect to the type of chemical reactions which they undergo. Manganous ion is not so related. Magnesium and cobaltous ions influence bacteriophage proliferation, but apparently only when combined with calcium (combination of these ions with strontium, barium, or manganous ions were not studied). The unique position of calcium ion

in the present system must be explained ultimately on the basis of structure of the atoms of this metal. Kay (1952) suggests that activity of calcium, strontium and magnesium ions, and inactivity of several other divalent cations in supporting multiplication of a coliphage, may be explained in terms of similar ionic dimensions and electrostatic configurations of calcium, strontium, and magnesium. This may be an oversimplification in the case of the present system, where manganous and cobaltous ions possess different electrostatic configurations than calcium but are not without activity, and barium, whose inactivity Kay attributed to the large size of this ion, is effective.

SUMMARY AND CONCLUSIONS

1. The calcium-deficient medium supported continued growth of all lactic streptococcus cultures studied, but increase in numbers of bacteriophages on actively growing host bacteria occurred in no case unless the medium was supplemented with calcium or certain related ions.

2. Rate of bacteriophage proliferation on host bacteria in this medium was related to concentration of calcium added, up to a limiting calcium level.

3. A given bacteriophage required greater amounts of calcium for rapid proliferation, as indicated by mass lysis, when dilute than when used as concentrated inoculum. This probably is due to greater ability of concentrated inoculua to overgrow the host culture, at a given calcium concentration, before the bacteria become unsuitable as bacteriophage substrate.

4. The calcium level required for rapid bacteriophage proliferation is associated not with the bacteriophage or host bacterial strain alone, but is characteristic of the bacteriophage-organism combination. While various bacteriophages attacking the same host have different calcium requirements, the same bacteriophage requires different amounts of calcium for rapid proliferation on different susceptible hosts.

5. The influence of bacterial host on bacteriophage calcium requirement may be related to the effect of bacteriophage concentration on calcium requirement, for the titer of a bacteriophage preparation may be markedly different against various susceptible hosts. Such titer differences on various hosts, alone will not explain the influence of host on calcium requirement; probably differences in the nature of the cell surface are important here.

6. Titer pattern of a bacteriophage on various hosts changes when the bacteriophage is propagated on different hosts. The change is rapid and following the first propagation on the new host subsequent propagations fail to alter titer pattern of the virus progeny. Such host-induced virus change may be extremely significant from a genetic as well as epidemiological standpoint.

7. Propagation of a bacteriophage on various susceptible hosts may result in virus progeny with different calcium requirements. This is another form of host-induced virus change.

8. Proliferation of bacteriophage on a host growing in different media, or in medium containing various levels of calcium, failed to influence the calcium requirement of virus progeny in the limited studies that were undertaken.

9. The requirement for calcium by lactic streptococcus bacteriophage cannot be explained by an effect of this ion on rate of host bacterial growth, stability of free bacteriophage particles, or by the slight influence of this ion on rate of bacteriophage adsorption (attachment) to host cells.

10. Calcium functions in the very early stages of the bacteriophage growth cycle, apparently within the first minute of virus-host association.

11. Using one-step growth curves and also single infected cell techniques, the relation between calcium concentrations from 2×10^{-4} M to 48×10^{-4} M and rate of bacteriophage proliferation, does not appear due to effect upon latent period, rise period, or burst size. It is explainable on the basis of influence of calcium concentration on frequency of cell bursts. This has been interpreted as indicating a function of calcium in the process of bacteriophage invasion. The nature of bacteriophage growth curves obtained when calcium was added to systems containing bacteriophage adsorbed to cells in absence of this ion, the lack of a killing action on cells by bacteriophage adsorbed in absence of calcium, and the early requirement for this ion support this conclusion.

12. Neither virus nor host suspended in calcium-

containing medium, appears to bind irreversibly sufficient calcium to satisfy the requirement for proliferation of the virus when these are combined in calcium-deficient medium.

13. In the present system, strontium, barium, or manganous ion may replace calcium as a requirement for bacteriophage proliferation. However, at equimolar concentration, these ions were less effective than calcium. Magnesium, nickel, cobaltous, zinc, cupric, cadmium, ferrous, aluminum, chromic, and lithium ions failed to replace calcium in the calcium-free medium.

14. Ability of calcium, strontium, and barium to support proliferation of seven bacteriophages studied, and manganous ion to function in the case of most of these, indicates that ion requirements of various strains of lactic streptococcus bacteriophage may be quite similar.

15. Strontium, barium, and manganous ions apparently function in the same manner as calcium.

16. None of thirteen cations studied in combination with calcium (with the possible exception of potassium in very high concentration) appeared antagonistic to calcium action in a manner that could not be explained by direct toxicity either to virus or host.

17. In contrast, magnesium or cobaltous ions combined with calcium, resulted in more rapid bacterio-

phage proliferation than calcium alone at a minimal concentration. These ions appear ineffective in absence of calcium.

18. Studies on the function of magnesium and cobaltous ions fail to explain their action through an effect upon rate of host bacterial growth, bacteriophage adsorption, length of latent or rise periods, or burst size. They do not increase the physiological capacity of bacteriophage-host complexes to produce virus when combined with optimum concentration of calcium. As in the case of calcium, they appear to exert their effect within the early minutes of virus-host association. Their action appears closely associated with that of calcium, and they perhaps function by substituting for part of the calcium requirement in presence of sub-optimum levels of this ion.

BIBLIOGRAPHY

Ackermann, W. W. 1951 The role of l-methionine in virus propagation. J. Exp. Med., 93:337-343.

Ada, G. L. and French, E. L. 1950 Purification of the receptor destroying enzyme of V. cholerae. Australian J. Sci., 13:82.

Adams, M. H. 1949a The stability of bacterial viruses in solutions of salts. J. Gen. Physiol., 32: 579-594.

Adams, M. H. 1949b The calcium requirement of coliphage T5. J. Immunol., 62:505-516.

Anderson, K. 1942 Dual virus infection of single cells. Am. J. Path., 18:577-583.

Anderson, T. F. 1945a On a bacteriolytic substance associated with a purified bacterial virus. J. Cellular Comp. Physiol., 25:1-13.

Anderson, T. F. 1945b The role of tryptophane in the adsorption of two bacterial viruses on their host, E. coli. J. Cellular Comp. Physiol., 25:17-26.

Anderson, T. F. 1948 The activation of bacterial virus T4 by L-tryptophan. J. Bact., 55:637-649.

Anderson, T. F. 1949 On the mechanism of adsorption of bacteriophages on host cells. In Miles, A. A. and Pirie, N. W. The nature of the bacterial surface. p. 76-95. Charles C. Thomas, Springfield, Ill.

Benzer, S. 1952 Resistance to ultraviolet light as an index to the reproduction of bacteriophage. J. Bact. 63:59-72.

Benzer, S., Delbrück, M., Dulbecco, R., Hudson, W., Stent, G. S., Waston, J. D., Weidel, W., Weigle, J. J. and Wollman, E. L. 1950 A syllabus on procedures, facts, and interpretations in phage. In Delbrück, M. Viruses 1950. p. 100-147. Calif. Inst. Tech., Pasadena, Calif.

Beumer, J. and Beumer, Jochmans, M. P. Les exigences en
1951 calcium du phage de Lisbonne. Ann. inst.
Pasteur, 81:489-506.

Bordet, J. and Renaux, E. L'autolyse microbienne trans-
1928 missible ou le bacteriophage. Ann. inst.
Pasteur, 42:1283-1335.

Buchanan, R. E. and Fulmer, E. I. Physiology and bio-
1928 chemistry of bacteria. Vol. I, p. 11.
Williams and Wilkins Co., Baltimore, Md.

Burnet, F. M. A method of study of bacteriophage multi-
1929 plication in broth. Brit. J. Path.,
10:109-114.

Burnet, F. M. The classification of dysentery-coli
1933 bacteriophages. III. A correlation of the
serological classification with certain
biochemical tests. J. Path. Bact.,
37:179-184.

Burnet, F. M. Some biological implications of studies
1951 on influenza viruses. I. The process of
infection by the virus. Bull. Johns Hopkins
Hosp., 88:119-137.

Cherry, W. B. and Watson, D. W. The streptococcus lactis
1949 host-virus system. II. Characteristics of
virus growth and the effect of electrolytes
on virus adsorption. J. Bact., 58:611-620.

Cohen, S. S. The synthesis of bacterial viruses. I.
1948a The synthesis of nucleic acid and protein in
Escherichia coli B infected with T2r+ bacterio-
phage. J. Biol. Chem., 174:281-293.

Cohen, S. S. The synthesis of bacterial viruses. II.
1948b The origin of the phosphorus found in the
desoxyribonucleic acids of the T2 and T4
bacteriophages. J. Biol. Chem., 174:295-303.

Cohen, S. S. Growth requirements of bacterial viruses.
1949 Bact. Rev., 13:1-24.

Cohen, S. S. and Anderson, T. F. Chemical studies on
1946 host-virus interactions. I. The effect of bacteriophage adsorption on the multiplication of its host, Escherichia coli B. With an appendix giving some data on the composition of the bacteriophage, T2. J. Exp. Med., 84:511-523.

Cohen, S. S. and Arbogast, R. Chemical studies in host-
1950a virus interactions. VII. A comparison of some properties of three mutant pairs of bacterial viruses, T2r+ and T2r, T4r+ and T4r, T6r+ and T6r. J. Exp. Med., 91:619-636.

Cohen, S. S. and Arbogast, R. Chemical studies in host-virus
1950b interactions. VIII. The mutual reactivation of T2r+ virus inactivated by ultraviolet light and the synthesis of desoxyribose nucleic acid. J. Exp. Med., 91:637-650.

Cohen, S. S. and Fowler, C. B. Chemical studies on host-
1947 virus interactions. III. Tryptophane requirements in the stages of virus multiplication in the Escherichia coli-T2 bacteriophage system. J. Exp. Med., 85:771-784.

Collins, E. B., Nelson, F. E. and Parmelee, C. E. The
1950 relation of calcium to proliferation of lactic streptococcus bacteriophage. J. Bact., 60:533-542.

Delbrück, M. Adsorption of bacteriophages under various
1940a physiological conditions of the host. J. Gen. Physiol., 23:631-642.

Delbrück, M. The growth of bacteriophage and lysis of
1940b the host. J. Gen. Physiol., 23:643-660.

Delbrück, M. Interference between bacterial viruses.
1945a III. The mutual exclusion effect and the depressor effect. J. Bact., 50:151-170.

Delbrück, M. The burst size distribution in the growth
1945b of bacterial viruses. J. Bact., 50:131-135.

Delbrück, M. Bacterial viruses or bacteriophages. Biol.
1946 Rev. Cambridge Phil. Soc., 21:30-40.

Delbrück, M. Biochemical mutants of bacterial viruses.
1948 J. Bact., 56:1-16.

Delbrück, M. and Bailey, W. T., Jr. Induced mutations
1946 in bacterial viruses. Cold Spring Harbor Symposia Quant. Biol., 11:33-37.

Delbrück, M. and Luria, S. E. Interference between
1942 bacterial viruses. I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth. Arch. Biochem., 1:111-141.

Doermann, A. H. Intracellular growth of bacteriophage.
1948 Carnegie Inst. Wash. Yearbook, 47:176-182.

Doermann, A. H. Intracellular phage growth as studied
1951 by premature lysis. Federation Proc., 10:591-594.

Eaton, M. D., Cheever, F. S. and Levenson, C. G. Further
1951 observations of the effect of acridines on the growth of viruses. J. Immunol., 66:463-476.

Eaton, M. D., Magasanik, B., Perry, M. E. and Karibian,
1951 D. Inhibition of influenza and mumps viruses in tissue culture by basic amino acids. Proc. Soc. Exp. Biol. Med., 77:505-508.

Edney, M. The influence of calcium ion on the reactions
1949 of the V. cholerae enzyme RDE and influenza virus with specific mucopolysaccharides. Australian J. Exp. Biol. Med. Sci., 27:253-264.

Fitzgerald, R. J. and Lee, M. E. Studies on bacterial
1946 viruses. II. Observations on the mode of action of acridines in inhibiting lysis of virus-infected bacteria. J. Immunol., 52:127-135.

Foster, R. A. C. An analysis of the action of proflavine
1948 on bacteriophage growth. J. Bact., 56:795-809.

Garen, A. and Puck, T. T. The first two steps of the
1951 invasion of host cells by bacterial viruses. II. J. Exp. Med., 94:177-189.

Ginsberg, H. S. and Horsfall, F. L., Jr. Concurrent
1949a infection with influenza virus and mumps virus or pneumonia virus of mice(PVM) as bearing on the inhibition of virus multiplication by bacterial polysaccharides. J. Exp. Med., 89:37-52.

Ginsberg, H. S. and Horsfall, F. L., Jr. A resistant
1949b variant of mumps virus. Multiplication of the varient in the presence of inhibitory quantities of Friedländer bacillus polysaccharide. J. Exp. Med., 90:393-407.

Ginsberg, H. S. and Horsfall, F. L., Jr. Characteristics
1951 of the multiplication cycle of pneumonia virus of mice (PVM). J. Exp. Med., 93:151-160.

Henle, W., Henle, G. and Rosenberg, E. B. The demonstra-
1947 tion of one-step growth curves of influenza viruses through the blocking effect of irradiated virus on further infection. J. Exp. Med., 86:423-437.

Hershey, A. D. Mutation of bacteriophage with respect
1946 to type of plaque. Genetics, 31:620-640.

Hershey, A. D., Kalmanson, G. M. and Bronfenbrenner, J.
1944 Coordinate effects of electrolyte and antibody on the infectivity of bacteriophage. J. Immunol., 48:221-239.

Hirst, G. K. The agglutination of red cells by allantoic
1941 fluid of chick embryos infected with influenza virus. Science, 94:22-23.

Hirst, G. K. Hemagglutination as applied to the study
1950 of virus infection. In Delbrück, M. Viruses 1950. p. 44-51. Calif. Inst. Tech., Pasadena, Calif.

Horsfall, F. L., Jr. Viral multiplication. Federation
1949 Proc., 8:518-522.

Kay, D. The effect of divalent metals on the multiplica-
1952 tion of coli bacteriophage T5st. Brit. J. Exp. Path., 33:228-235.

Kay, D. and Fildes, P. The calcium requirement of a
1950 typhoid bacteriophage. Brit. J. Exp. Path., 31:338-348.

Kozloff, L. M., Knowlton, K., Putnam, F. W. and Evans, E. A., Jr. 1951 Biochemical studies of virus reproduction. V. The origin of bacteriophage nitrogen. J. Biol. Chem., 188:101-116.

Kozloff, L. M. and Putnam, F. W. 1950 Biochemical studies of virus reproduction. III. The origin of virus phosphorus in the Escherichia coli T6 bacteriophage system. J. Biol. Chem., 182:229-242.

Krueger, A. P. 1931 The sorption of bacteriophage by living and dead susceptible bacteria. I. Equilibrium conditions. J. Gen. Physiol., 14:493-516.

Lawton, W. 1953 [Personal communication.] Iowa State College. Ames, Iowa.

Luria, S. E., Delbrück, M. and Anderson, T. F. 1943 Electron microscope studies of bacterial viruses. J. Bact., 46:57-77.

Luria, S. E. and Human, M. L. 1952 A nonhereditary, host-induced variation of bacterial viruses. J. Bact., 64:557-569.

Luria, S. E. and Latarjet, R. 1947 Ultraviolet irradiation of bacteriophage during intracellular growth. J. Bact., 53:149-163.

Maaløe, O. and Watson, J. D. 1951 The transfer of radioactive phosphorus from parental to progeny phage. Proc. Nat. Acad. Sci. U. S., 37:507-513.

McClelland, L. and Hare, R. 1941 The adsorption of influenza virus by red cells and a new in vitro method of measuring antibodies for influenza virus. Can. J. Pub. Health, 32:530-538.

Monod, J. and Wollman, E. 1947 L'inhibition de la croissance et de l'adaptation enzymatique chez les bacteries infectees par le bacteriophage. Ann. inst. Pasteur, 73:937-957.

Perlman, D., Langlykke, A. F. and Rothberg, H. D., Jr. 1951 Observations on the chemical inhibition of Streptomyces griseus bacteriophage multiplication. J. Bact., 61:135-143.

Pirie, A. 1940 The effect of lysozyme on the union between a phage and the susceptible Bacillus megatherium. Brit. J. Exp. Path., 21:125-132.

Potter, N. N. and Nelson, F. E. 1952a Effects of calcium on proliferation of lactic streptococcus bacteriophage. I. Studies on plaque formation with a modified plating technique. J. Bact., 64:105-111.

Potter, N. N. and Nelson, F. E. 1952b Effects of calcium on proliferation of lactic streptococcus bacteriophage. II. Studies of optimum concentrations in a partially defined medium. J. Bact., 64:113-119.

Puck, T. T. 1949 A reversible transformation of T1 bacteriophage. J. Bact., 57:647-655.

Puck, T. T., Garen, A. and Cline, J. 1951 The mechanism of virus attachment to host cells. I. The role of ions in the primary reaction. J. Exp. Med., 93:65-88.

Putnam, F. W. and Kozloff, L. M. 1950 Biochemical studies of virus reproduction. IV. The fate of the infecting virus particle. J. Biol. Chem., 182:243-250.

Reiter, B. 1949 Lysogenic strains of lactic streptococci. Nature, 164:667-668.

Rountree, P. M. 1947 Staphylococcal bacteriophages. II. Bacteriophage adsorption by staphylococci. Australian J. Exp. Biol. Med. Sci., 25:203-212.

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

Schlesinger, M. 1932 Quantitative Untersuchungen über die Bindungsgeschwindigkeit und die Sättigung. Berechnung der Teilchengrösze des Bakteriophagen aus deren Ergebnissen. Z. Hyg. Infektionskrankh., 114:149-160.

Schmidt, J. R. A. and Rasmussen, A. F., Jr. Inhibitory
1952 effect of cobaltous ions on multiplication of influenza virus. Proc. Soc. Exp. Biol. Med., 81:244-246.

Shew, D. I. Effect of calcium on the development of
1949 streptococcal bacteriophages. Nature, 164: 492-493.

Smith, W. H. Calcium-deficient media; their effect on
1948 bacteriophage action. Nature, 161:397.

Spizizen, J. Biochemical studies on the production of
1943 virus reproduction. II. Studies on the influence of compounds of metabolic significance on the multiplication of bacteriophage. J. Infectious Diseases, 73:222-228.

Spizizen, J., Hampil, B. and Kenney, J. C. Biochemical
1951 studies on the phenomenon of virus reproduction. IV. The inhibition of coliphage T2r+ multiplication by sulfhydryl compounds and its prevention by ethylenediaminotetraacetic acid and metabolic ions. J. Bact., 62:331-336.

Stassano, H. and deBeaufort, A. D. Action du citrate de
1925 soude sur le principe lytique transmissible. Compt. rend. soc. biol., 93:1380-1382.

Syverton, J. T. and Berry, G. P. Multiple virus infection
1947 of single host cells. J. Exp. Med., 86:145-151.

Thompson, R. L., Price, M. L., Minton, S. A., Jr.,
1950 Elion, G. B. and Hitchings, G. H. Effects of purine derivatives and analogues on multiplication of the vaccinia virus. J. Immunol., 65:529-534.

Tolmach, L. J. and Puck, T. T. The mechanism of virus
1952 attachment to host cells. III. J. Am. Chem. Soc., 74:5551-5553.

Wahl, R. Influence de la composition du milieu sur la
1946 bacteriophage. Ann. inst. Pasteur, 72:73-80.

White, P. B. Lysogenic strains of V. cholerae and the
1937 influence of lysozyme on cholera phage activity. J. Path. Bact., 44:276-278.

Wilkowske, H. H. 1949 Characterization of bacteriophage active against lactic streptococci. Unpublished Ph.D. thesis, Iowa State College Library, Ames, Iowa.

Wollman, E. and Wollman, E. 1938 Recherches sur le phenomene de Twort-D'Herelle. Bacteriophagie ou autolyse heredo-contagieuse. Ann. inst. Pasteur, 60:13-57.

Wooley, J. G. and Murphy, M. K. 1949 Metabolic studies on T2 Escherichia coli bacteriophage. I. A study of desoxypyridoxine inhibition and its reversal. J. Biol. Chem., 178:869-875.

ACKNOWLEDGMENTS

The author wishes to express sincere appreciation to Dr. F. E. Nelson for his patient guidance and valuable suggestions throughout the course of this investigation, and for his help in the preparation of the manuscript.

Thanks are extended to other members of the Dairy Industry staff for their friendly counsel.

The author is grateful to the National Institutes of Health for the financial aid which helped make this investigation possible.