FACTORS INFLUENCING THE MULTIPLICATION OF A PHAGE SPECIFIC FOR *STREPTOMYCES GRISEUS*

By

EDWIN L. HURD

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Oklahoma Agricultural and Mechanical College

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THESIS AND ABSTRACT APPROVED:

L. M. Ehrenberg
Head of the Department

Campbell P. Gilmour
Thesis Adviser

T. L. Johnson
Faculty Representative

W. G. M. Cutler
Dean of the Graduate School

278005

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INTRODUCTION

Recently a new phage specific for Streptomyces griseus was isolated during the course of the streptomycin fermentation (5). As might be expected a phage infestation of this type presented an acute industrial problem. For a considerable period of time, the source of the phage contamination remained unknown. Gilmour and Buthala (13), however, reported that phages active against various Streptomyces species were carried into the streptomycin plants on dust particles, originating in the soil. These workers also isolated and tentatively identified several of these new phages specific for the Streptomyces group.

The question of control of these Streptomyces phages opens up two main avenues of investigation. The first, involves the selection of phage resistant strains of Streptomyces griseus by way of the gradual exposure of the culture to the phage. This method provides only a temporary cure and does not ensure long time protection. The second avenue of control of phage infection involves an understanding of the factors influencing phage infection and multiplication. It is evident, that once such knowledge is available, a procedure might be utilized so as to prevent the initial phage infection.

The present study has been undertaken with two primary objectives in mind: 1. to study the fundamental nature of the phage with special emphasis on the influence of environmental factors on phage multiplication and 2. to effect a method of control of such phage infestations by means of a specific inhibitor of phage growth.

HISTORICAL

Twort and d'Herelle (11) in the years 1915 and 1916 respectively, reported on the occurrence of a lytic agent active against bacterial broth cultures. d'Herelle, however, was the first worker who demonstrated that the lytic agent multiplies in the presence of the host cells and produces plaques on solid agar bacterial cultures. This phenomenon of a ultramicroscopic agent capable of multiplication has been termed bacteriophage (specific for bacteria). As might be expected many of these phages have been found for a variety of bacterial species. Extensive studies have been made on the fundamental nature and properties of these lytic agents. The following review of literature presents information relative to the present study.

One of the properties of bacteriophage, that distinguishes it from autolytic agents, is multiplication. Autolytic agents dissolve the bacterial cell, whereas a phage is adsorbed into the cell. The phage particle multiplies within the host cell with an ultimate burst and disruption of the cell wall to release large numbers of phage particles into the surrounding medium. Delbruck (10) used the one step growth curve as a method to measure burst intervals. The one step growth curve is essentially the multiplication of the phage within a host cell. A small number of phage particles is added to a suspension containing bacteria in high concentrations. Within a few minutes each phage particle attaches itself to a bacterium. The mixture is then diluted with a large volume of broth, in order to have the bacteria in low concentrations, so that after the first burst a long time elapses before reinfection. This

unique method of demonstrating multiplication of phage by bursts, has been used to other advantages in studying properties of phage. Through the use of burst times it has been shown that bacteria in their lag phase will generally produce less phage than bacteria growing in their logarithmic phase of growth (17, 12). In addition the effect of temperature on phage multiplication has been used in correspondence with average burst time. As demonstrated by Ellis (12), a low temperature of 16.6° C. influences the latent period of the phage particle and not the final burst size. The same burst size will be produced at 16.6° C. as that produced at 37° C., but the final titer produced takes 6 times longer at the lower temperature (12).

Anderson (3) found that temperature influences the size of plaques produced by Escherichia coli. At 37° C. distinct plaques were formed, but at 14° C. no plaques were observed. The explanation of the temperature effect on plaque formation involves the co-factor concentration, in which the bacterial cells at 14° C. are unable to provide co-factors for the initial activation of the phage. Therefore, on agar plates at 14° C. the bacteria are resistant to the phage. In consequence it has been found that there are certain co-factors needed for phage multiplication in the host cells. Some of these co-factors found with Escherichia coli phages are tryptophan, isoleucine and Ca^{++}. These factors are required for adsorption of the phage and subsequent multiplication. Certain other substances, iodoacetate, fluoride and azide have been found to prevent the multiplication of bacteriophage. These substances all inhibit the formation of adenosinetriphosphate. In this respect it has suggested that energy-rich phosphate is needed in the formation of

bacteriophage (18, 2).

The H-ion concentration of the medium has been found to effect the lysis of cells by phage. Cherry (6) observed that Streptococcus lactis cells were lysed by the homologous phage at pH 6.0, 7.0 and 8.0 but below 5.0 lysis did not occur. In a similar way, cells of Clostridium madisonii were lysed by the specific phage between pH 5.0 and 7.6 but did not undergo lysis below 5.1 (14). In the latter instance, neutralization of the excess acidity brought about immediate lysis of the test culture.

Electrolytes have been found that both stimulate and inhibit virus adsorption and cellular lysis. The multiplication of Streptococcus lactis phage has been shown to be stimulated by potassium phosphate, potassium chloride, sodium chloride, calcium chloride, magnesium sulfate and sodium acetate (7, 22). Similarly, sodium chloride resulted in a five to tenfold increase in the amount of phage produced when Staphylococcus phage and the susceptible organism were suspended in 0.25 M NaCl (21). However, Adams (1) reported that the seven bacterial viruses of the T group, active against Escherichia coli, are more rapidly inactivated by heat when suspended in 0.1 N solutions of sodium salts. The rate of inactivation of phage T 5 in 0.1 N NaCl can be greatly decreased by the inclusion of divalent cations such as Ca, Mg, Ba, Sr, Mn, Co, Ni, Zn, Cd and Cu. For this reason Adams (1) proposed that the increase in stability of phage T 5 in the presence of various cations is the result of a complex formed between the phage and the cation.

In 1946 Bennett (5) discovered a new phage in streptomycin fermentation beers. This phage as later revealed by Saudek (20), Reilly (19),

Woodruff (23), Koerber (15), and Perlman (16) lysed Streptomyces griseus cells and was given the name actinophage. The phage is of particular interest because of its inhibition of Streptomyces griseus, the streptomycin producing organism. Although, relatively little data has been published on this phage, the available experimental data shows that it has many properties similar to bacteriophage (20). In this respect Woodruff (23) demonstrated the particulate nature of the lytic agent. The close resemblance of this phage to strains of Escherichia coli bacteriophage was shown by electron micrographs. Further studies by Woodruff (23) using the chromium shadowing technique, indicated a surprising diversity of structure of the phage particles. In general all particles showed a long, relatively thick but bent tail of approximately 0.015 by 0.15 microns. The majority of the heads appeared symmetrically spherical, 0.05 microns in diameter. Many particles were composed of two distinct bodies with a few appearing to be similar to tetrads. In more recent work by Koerber (15) it has been shown that there are two distinct types of phage that will attack Streptomyces griseus preventing the production of streptomycin. These types can be distinguished by host specificity, plaque morphology and size.

The Streptomyces griseus phage passes through a bacterial filter, and may be propagated in the presence of young growing cultures of Streptomyces griseus. It is also interesting that phage-sensitive cultures of Streptomyces griseus give rise to strains which are resistant to the action of the phage (20, 19). Even though, burst times have not been reported, lysis has been observed 6 hours after inoculating Streptomyces griseus cells with the phage (23). Shortly thereafter,

the cultures lyse completely and only occasionally does a resistant colony grow out.

Reilly (19) found that the optimum temperature for Streptomyces griseus phage multiplication was 28° C. and that phage multiplication did not occur at 37° C. and above. However, Woodruff (23) demonstrated that this type of phage was destroyed if held at 80° for 15 minutes. Approximately 0.5% of phage remained after holding at 60° C. for 15 mimutes. Reilly (19) reported that the phage may be stored at 6° C. without loss of activity, but at 28° C. and above it is greatly inactivated.

The best method for control of the phage in streptomycin fermentations, at the present time, consists of culturing a resistant host culture. In addition some progress has been made in the use of chemical inhibitors. Sodium citrate, sodium oxalate, sodium phytate, sodium hydroxyisobutyrate, sodium tartrate and sodium gluconate have been found to inhibit the phage in the agar plate test (16). Of this group sodium gluconate and sodium tartrate were unsatisfactory in the fermentation test while the other compounds gave only a temporary inhibition of phage activity. It would seem that these inhibitors do not present the answer to the practical problem of phage infection. A number of organic acids have been tested, among these were ethylenediamine tetraacetic acids, including glutamic and aspartic, and straight chain and branched chain fatty acids (16). Many of this group were effective in the agar plate test but either failed to inhibit bacteriophage multiplication or limited streptomycin production in the fermentation test. Acriflavine, a filtrate from an unidentified bacterium has been tested against actinophage,

but Streptomyces griseus was inhibited in concentrations which were destructive to the actinophage (23).

EXPERIMENTAL METHODS

A streptomycin producing strain of Streptomyces griseus No. 3475 obtained from S. A. Waksman was used throughout the study. In a previous investigation this culture permitted multiplication of phage obtained from a streptomycin fermentation (19). The phage employed in the present study was isolated from soil and has been designated by the number 514-3 (13). This phage among other active filtrates had previously been tested against a large number of unknown Streptomyces strains and was known to lyse culture 3475.

A. Media Studies

The type of medium employed often determines the rate and extent of lysis of a susceptible organism by phage. For the purposes of the present study, it was decided to investigate two types of media: 1. a complex organic medium and 2. a strictly synthetic medium.

The basal organic medium contained the following nutrients: meat extract - 3 gm., bacto-peptone - 5 gm., and distilled water - 1000 ml. This broth was standardized at pH 7.8 by means of a Beckman Model H-2 pH meter. The ingredients added to the basal medium are described in Table 1. The resulting eight media, were transferred in 50 ml. aliquots to 6 oz. bottles and autoclaved for 20 minutes at 15 pounds pressure. Then 0.5 ml. of Streptomyces griseus cells were transferred to each medium and incubated for 24 hours at 28° C. Subsequently one-tenth ml. of standard phage was added to each of the eight media. A second inoculated set of the media did not receive the phage treatment. After the addition of phage the media were incubated for 24 hours at 28° C. Phage activity

TABLE 1.

Description of the organic media

Medium Number	Organic media*
1 . .	: Basal Medium (bacto-peptone - 0.5%, meat extract - 0.3%)
2 . .	: Basal Medium + 0.01% yeast extract.
3 . .	: Basal Medium + 0.5% NaCl.
4 . .	: Basal Medium + 0.01% yeast extract + 0.5% NaCl.
5 . .	: Basal Medium + 1% glucose.
6 . .	: Basal Medium + 1% glucose + 0.5% NaCl.
7 . .	: Basal Medium + 0.01% yeast extract + 1% glucose.
8 . .	: Basal Medium + 0.01% yeast extract + 0.5% NaCl + 1% glucose

*pH - 7.8

was measured by rate and extent of lysis of the culture cells.

The synthetic media used are described in Table 2. The same procedure was used in testing phage activity in these media as was described for the above organic media.

B. Phage Assay

1. Inoculum

The size of phage inoculum, to be used for the demonstration of lysis, was varied so as to note any difference in the degree of cell lysis over a 48 hour period. Twenty four hour old cultures of Streptomyces griseus, in 50 ml. alequots of nutrient broth, were prepared. One culture received 0.1 ml. of standard phage, a second culture 0.5 ml. and a third culture 1.0 ml. of phage. A fourth culture was not exposed to the phage and was regarded as the control. All four cultures were incubated for 48 hours at 28° C., after which the extent of lysis was observed.

Streptomyces griseus cultures tend to form hard matted colonies on nutrient agar plates. In consequence, it was decided to vary the amount of spores added to the nutrient agar to observe the effect on plaque counts. From a 10^4 dilution of standard phage, 1 ml. was pipetted into tubes containing 15 ml. of nutrient agar. Then one set of tubes received 0.1 ml. of culture spores, a second set 0.2 ml. and a third set 0.5 ml. of the culture inoculum. Plaque counts were made for the respective inoculum treatments after an incubation period of 48 hours at 28° C.

2. Standard assay

Various methods have been used to determine the titer of a specific phage preparation. Generally these procedures are based on the principle

TABLE 2.

Description of the synthetic media

Medium Number	Synthetic medium
1 . .	Asparagine 0.1%, Glucose 1%, K_2HPO_4 0.1%.
2 . .	Peptone 0.1%, Glucose 1%, K_2HPO_4 0.1%.
3 . .	K_2HPO_4 0.1%, Glucose 1%, Amino acids (10 γ/ml) - Alanine. Tryptophane, Proline, Arginine, Leucine, Isoleucine, Serine, Tryosine, Methiomine, Valine, Hydroxyproline, Phenyalonine, Lysine Hydrochloride, Aspartic acid, Histidine, Cysteine, Glutamic acid.

that a single point of phage infection grows in the presence of the host cells to give a clear zone of lysis. Such a zone is called a plaque. The count of these plaques at a particular dilution, supplies data relative to the potency or titer of the phage. The activity of the phage under study was determined by the mentioned plaque technique and also by observing the test culture for lysis. Five-tenth ml. of submerged culture cells were added to a 50 ml. aliquot of the basal medium, and the culture incubated for 24 hours at 28° C. At the close of the incubation period 0.1 ml. of standard phage was added to the culture and the phage and cells were again incubated for 48 hours at 28° C. The initial activity of the phage was determined by observations on lysis of the cells. After lysis was noted, 1 ml. of the lysed culture was diluted by serial transfer in sterile basal medium from 10^1 to 10^6. One ml. of each dilution was then added to 15 ml. of nutrient agar along with 0.1 ml. of a fresh spore suspension of the host culture, (3 day old spores were used and suspended in basal medium). The tubes were thoroughly shaken and poured into standard size petri dishes. These were incubated at 28° C. for 48 hours, at which time plaque counts were made. The above method for making plaque counts will be used through out the study and is referred to as the standard procedure. However, in the case of the preparation of standard phage inoculum, the lysed culture was first passed through a Seitz filter, size L 3.

C. Phage Type Differentiation

Since plaque size has been used as a basis for the separation of phage types, it was decided to follow this principal in studying the homologous nature of phage 514-3. An attempt was made to pick different

size plaques that would on subsequent reproduction, show essentially similar plaque types.

Isolated plaques of pin point, small and large size were removed from nutrient agar plates by inserting a sterile needle in the center of each plaque. The portion of plaque removed was inoculated into a 24 hour old culture of Streptomyces griseus cells. The culture-phage mixture was incubated for 48 hours at 28° C., at which time lysis was observed. Plaques were produced from the lysed culture by the described standard procedure.

D. Phage Multiplication and Burst Time

The characteristic of transmissibility and multiplication differentiate phage from various autolytic agents. In consequence a newly isolated phage must be able to pass through a bacteriological filter and the filtrate be capable of demonstrating multiplication in the presence of the original host culture.

Transmissibility was demonstrated by subjecting the standard phage to the host cells and preparing a Seitz filtrate from the lysed culture. If the phage is truly transmissible the latter filtrate should be capable of causing lysis of the test culture.

Multiplication of phage was effected by taking one-tenth ml. of the standard phage which was added to a 24 hour old Streptomyces griseus culture. After thoroughly mixing the phage and culture for 2 to 3 minutes, a standard plaque count was run at zero time. Following the zero time reading, plaque counts were carried out at regular time intervals ranging from 0 to 48 hours.

E. Influence of Selected Factors on Phage Multiplication

1. Temperature

In preliminary experiments a temperature of 55° C. was found to inhibit plaque formation. In consequence, it was decided to investigate the influence of temperature on phage itself and on phage in the presence of the host culture.

The experiment was organized along two distinct lines. First, the influence of temperature on phage alone was investigated. For this test 10 ml. portions of standard phage were pipetted into sterile test tubes and heated for ten minutes at 30°, 40°, 50°, 60°, 70°, 75°, 80°, 90° and 100° C. respectively. After the heat treatment, a standard plaque count was carried out for each temperature level.

Secondly, the influence of temperature on the phage plus host cells was demonstrated. One-tenth ml. of standard phage was added to several 24 hour old cultures of Streptomyces griseus. These were incubated in water baths at 5°, 22°, 28°, 35°, 45°, 50° and 55° C. respectively. After 48 hours of incubation the phage titer for each temperature level was determined by the standard plaque count procedure.

2. H-ion Concentration

As with temperature the pH of a medium often influences the multiplication of phage in susceptible cultures. For this reason, it was decided to explore the range in pH, which might prove desirable or prove inhibitory for normal phage multiplication.

Cultures of Streptomyces griseus that had been incubated in nutrient broth for 24 hours were adjusted to various pH levels ranging from pH 4.75 to pH 9.15 by means of a Beckman Model H-2 pH meter. One-tenth ml. of

standard phage was then added to each culture and these incubated for 48 hours at 28° C. The usual procedure for making plaque counts was used to check the effect of pH on the action of the phage.

3. Influence of Salts

Limiting Concentration

It will be remembered that sodium chloride in the basal organic medium, inhibited phage multiplication. The present experiment was designed with the idea of testing this inhibitory effect of NaCl along with other salts. The possibility of using a salt as an agent for the control of Streptomyces phage also gave impetus to this study.

In order to determine the range in tolerance of the phage to salt, NaCl was added to nutrient broth in concentrations of 0% to 0.7%. Each set of medium was inoculated with 0.5 ml. of Streptomyces griseus cells, and incubated for 24 hours at 28° C. Then 0.1 ml. of phage was added to each culture. The cultures were incubated for 48 hours at 28° C., at which time the phage titer was determined by the standard plaque count. In addition, the influence of NaCl, $NaCo_3$, KCl, and $MgCl_2$, was investigated in the previously described synthetic medium (Table 2). These salts were added to nutrient broth in 0%, 0.2%, 0.3%, 0.4% and 0.5% concentrations respectively. Each salt medium was inoculated with 0.5 ml. of Streptomyces griseus cells and incubated for 24 hours at 28° C. Following growth, 0.1 ml. of standard phage was added to each medium and the cultures incubated for 48 hours at 28° C. Standard plaque counts were then carried out for each salt concentration.

Mode of Action

The manner by which NaCl effects the inhibition of phage activity

lies open to conjecture. It is probable, however, that NaCl acts to either inactivate the phage itself or perhaps to influence the chemical nature of the host cells. A third alternative might involve both the phage and cells together. In the latter instance the NaCl would show its inhibitory effect when the salt, phage and cells were present at one and the same time. In order to study the way by which NaCl effects the inhibition of phage activity the following plan of procedure was adopted.

The influence of NaCl on the phage particle itself was determined by adding a 5 ml. aliquot of standard phage to each of two tubes. One aliquot of phage received 0.5% NaCl. A second aliquot was not treated with salt. Both the salt treated and non-treated quantities of phage were incubated for 24 hours at 28° C. Then 0.1 ml. of each phage sample was added to a 24 hour old culture of Streptomyces griseus in nutrient broth and observations on lysis noted.

Whether Streptomyces griseus cells grown in a salt medium, then removed and washed free of adhering salt, are more resistant to the action of phage, remains open to conjecture. If such cells proved to be phage resistant, then the inhibition of phage actively by NaCl might be attributed to a cellular influence and not to a salt effect on the phage particle itself. In order to test this hypothesis, NaCl was added to 50 ml. of nutrient broth so as to give a final concentration of 0.5%. A second 50 ml. quantity of broth did not receive NaCl. Both the NaCl treated and non-treated broth were inoculated with Streptomyces griseus cells and incubated for 24 hours at 28° C. At the close of the latter period, the cells from both treated and non-treated broths were centrifuged

and washed in distilled water. The washed cells were then resuspended in salt free nutrient broth and 0.1 ml. of standard phage added to the salt treated and non-treated cells. The extent of resulting lysis was used to measure phage activity.

A final experiment was undertaken involving cells, phage and NaCl. One-tenth ml. of the phage sample which had been submitted to 0.5% NaCl was added to the NaCl treated culture cells. In addition one-tenth of the phage sample which did not receive NaCl was added to the culture which had been grown in the NaCl free medium. Phage activity was measured by the extent of lysis of Streptomyces griseus.

EXPERIMENTAL RESULTS AND DISCUSSION

A. Media Studies

Reference to the data recorded in Table 3 discloses that the inclusion of 0.5 percent sodium chloride in the organic basal medium, prevents the lysis of *Streptomyces griseus* cells by phage 514-3. In the case of media 1, 2, 5 and 7, where sodium chloride was omitted from the media, extensive lysis was observed. It is immediately significant that the rather pronounced inhibition of phage activity by sodium chloride, may provide a partial answer to the problem of control of such phage infestations. This conclusion becomes more consolidated when it is realized that sodium chloride as well as exerting the observed inhibitory effect on the phage also is known to stimulate the production of streptomycin. Such a compound would appear to represent the ideal control agent.

It is interesting that glucose neither stimulated nor inhibited phage activity. This result might be expected since any inhibitory effect of glucose is often attributed to the presence of acids, resulting from the utilization of this sugar. In the present instance, the pH trend was quite high in the alkaline range (7.8 - 8.0).

After a careful survey of the above data, it was decided to omit sodium chloride and glucose from the organic basal medium. In consequence, the organic medium selected for use in further work was the described basal medium plus 0.01 percent yeast extract.

As is shown in Table 4, the synthetic medium permitted lysis of the host culture equally well in medium 2 and 3. It is evident that Bacto-peptone or the complement of amino acids are required. The medium which

TABLE 3.

The extent of lysis of Streptomyces griseus in various organic media

Medium Number	Observed lysis
1	+++
2	+++
3	-
4	-
5	+++
6	-
7	+++
8	-

\+ - slight lysis
++ - moderate lysis
+++ - extensive lysis

TABLE 4.

The extent of lysis of Streptomyces griseus in various synthetic media

Medium Number	Observed lysis
1	-
2	+++
3	+++

contained asparagine as the sole source of nitrogen did not allow lysis of the test culture. Thus, it may be concluded that a complement of amino acids, acting either singly or in various combinations, are essential for normal lysis and phage multiplication. At this juncture in the study, the identity of these essential amino compounds are not known. However, when the synthetic type medium was used, care was taken to include the necessary complement of amino acids. This was accomplished by including the seventeen amino acids listed in Table 2.

B. Phage Assay

1. Inoculum

The results obtained using various sizes of inoculum indicated that a phage inoculum of 1.0 ml., 0.5 ml. and 0.1 ml. did not appreciably influence the resulting phage activity. In each instance, the extent of lysis at the end of 48 hours, was essentially similar. It might be expected that a phage inoculum of 1.0 ml. would produce a greater titer than 0.1 of a ml. The above conflicting result may be explained on the basis of the time factor involved. Unrecorded data, has shown that with the larger phage inoculum, multiplication ceases in a far shorter time than with the smaller inoculum. Thus, when the experiment is carried over a 48 hour period, there is sufficient time for equalization of the titer.

The amount of *Streptomyces griseus* spores, used in the plating for plaque counts does not appear to influence the resulting count. An inoculum of 0.5 ml., 0.2 ml. and 0.1 ml. of spores, produced an approximately similar plaque count. For this reason the smaller spore inoculum of 0.1 ml. was used in subsequent work.

2. Standard Assay

The titer of the standard phage was found to be 1.2×10^5 particles per ml. A standard stock of this phage was kept in nutrient broth at 5° C. The titer of standard phage was maintained at approximately 1.2×10^5 particles per ml. However, some variation in the titer of the phage was observed during the course of the investigation.

C. Phage Type Differentiation

Each size of plaque picked was active in lysing Streptomyces griseus. In addition plates made from each lysed culture reproduced all three types of original plaques picked. Therefore, it is apparent that the plaque size was not stable over the first transfer. At this juncture the phage under study does not appear to show any division of types which can be traced to plaque size. However, concomitant tests carried out by Buthala (personal communication) show that phage 514-3 is capable of lysing several Streptomyces species. This might infer that the phage is polyvalent in nature.

D. Phage Multiplication and Burst Time

A study of the individual plaque counts given in Table 5 and illustrated in Figure 1 discloses that a sharp rise in phage titer occurs at the close of the 10-12 hour period. This observation is rather significant since previous workers (19, 23) were not able to demonstrate multiplication over such a short period of time. It was implied that phages specific for the Streptomyces group required a much longer period for multiplication. In this respect Reilly (19) showed an increase in phage titer over a 48 hour period. The shorter time for the manifestation of phage multiplication

TABLE 5.

Observed multiplication of phage 514-3 as determined by plaque count

Time Hours	Dilution with resulting plaque count						
	10^1	10^2	10^3	10^4	10^5	10^6	Control
0 . . .	19	2	-	-	-	-	-
1 . . .	16	4	1	-	-	-	-
1.5 . .	15	-	-	-	-	-	-
2 . . .	27	3	-	-	-	-	-
4 . . .	17	2	-	-	-	-	-
6 . . .	21	3	-	-	-	-	-
8 . . .	3	2	-	-	-	-	-
10 . .	31	1	-	-	-	-	-
12 . .	TNC*	35	4	-	-	-	-
24 . .	TNC	300	168	7	-	-	-
28 . .	TNC	TNC	300	109	20	-	-
32 . .	TNC	TNC	300	199	1	-	-
36 . .	TNC	TNC	300	79	10	3	-
48 . .	TNC	TNC	300	46	7	1	-

*TNC designated plates which have been completely lysed by phage 514-3.

Figure 1. Observed Multiplication of Phage 514-3 as Determined by Plaque Count.

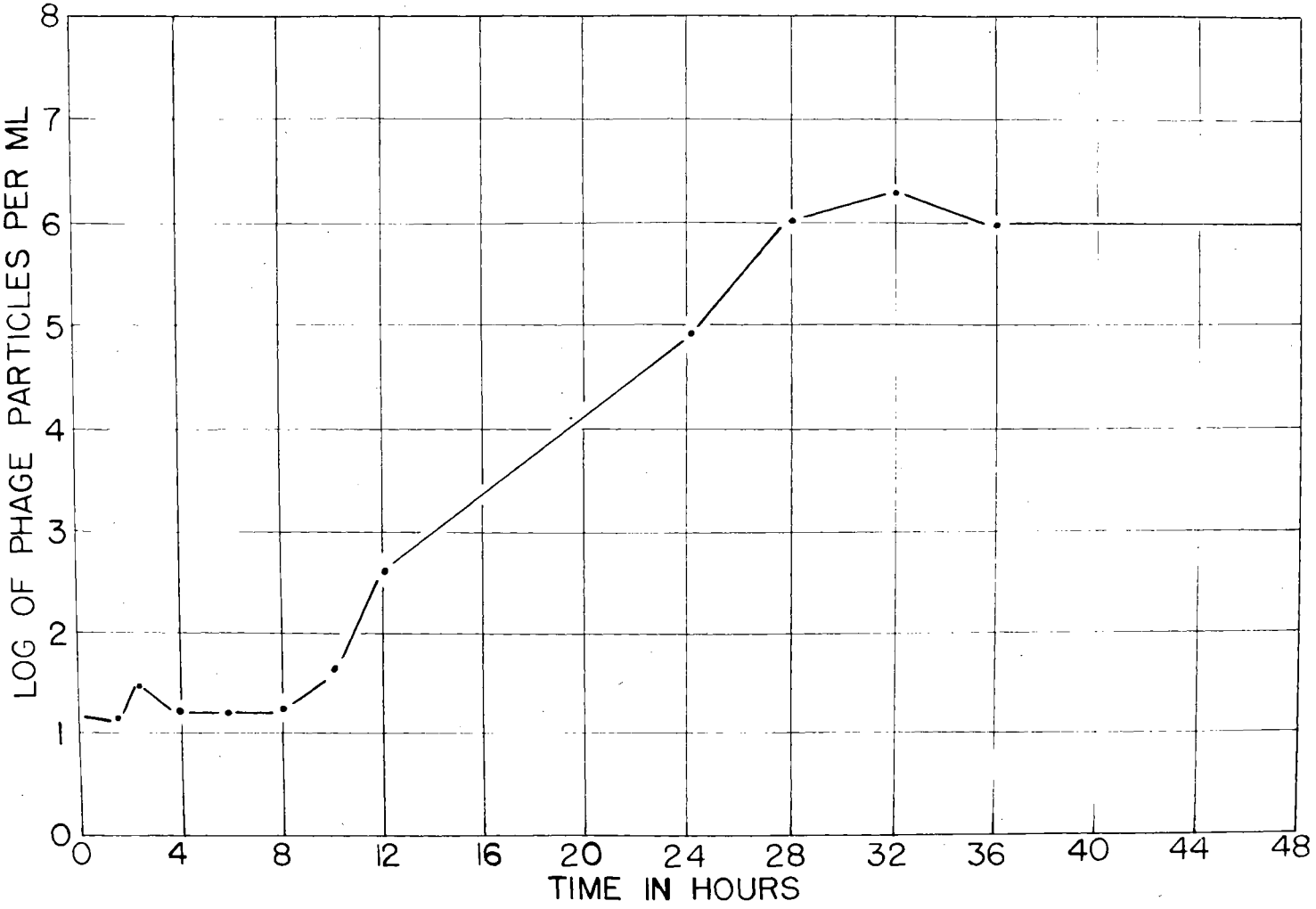
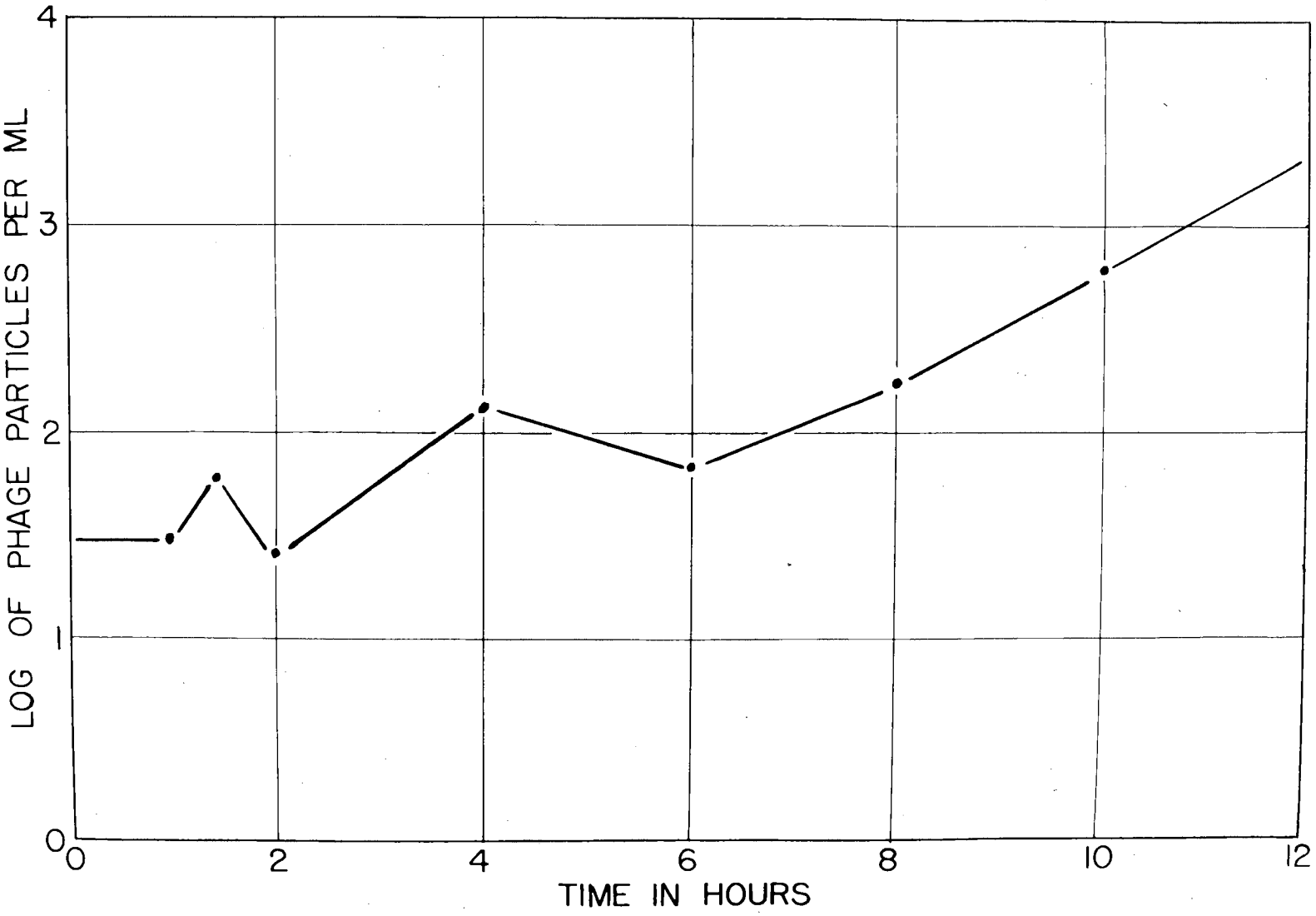

LOG OF PHAGE PARTICLES PER ML
0
1
2
3
4
5
6
7
8
0
4
8
12
16
20
24
28
32
36
40
44
48
TIME IN HOURS

TABLE 6.

Observed increase of phage particles over a twelve hour period

Time Hours	Phage particles per ml. x 10^3
0	0.30
1	0.30
1.5	0.91
2	0.20
4	1.36
6	0.60
8	1.48
10	4.80
12	20.30

Figure 2. Observed Increase of Phage Particles Over a Twelve Hour Period.


LOG OF PHAGE PARTICLES PER ML
0
1
2
3
4
0
2
4
6
8
10
12
TIME IN HOURS

reported in the present study is corroborated in the increase of phage particles at 12 hours, shown in Table 6 and Figure 2.

Further reference to Table 5 and Figure 1, shows that after the rise in phage titer at 12 hours further increases occur. It is significant that after 32 hours, multiplication of the phage appears to reach a maximum and then level off.

The determination of a burst time for phages specific for Streptomyces species presents certain difficulties. In the first place, the filamentous nature of Streptomyces cells, rules out the possibility of establishing an accurate phage-cell ratio. Therefore, in the true sense, a one step growth curve, as described by Delbruck (10) can hardly be used. However, during the course of the present study, an attempt has been made to arrive at some estimation of the time required for phage absorption, multiplication and subsequent release. The rise of titer at 12 hours has not been considered as the first significant burst. Other increases, occurring over a shorter period of time, in all probability do take place. These are often hidden by immediate readsorption. For this reason, a total plaque count was used rather than the usual free phage plaque count.

The data reported in Tables 7, 8, 9 and Figure 3 attests to the idea that shorter bursts of phage do take place, than was indicated in previous experiments. In this respect the results shown in Table 7 suggests that the burst time is less than 3 hours. It is significant, that an increase of phage particles from 4.1×10^2 to 1.4×10^3 takes place over the indicated 3 hour period. Reference to the data in Table 8 and Figure 3 would infer that the burst time lies between 100 to 120 minutes. As is shown in Table 9, no increase in phage titer has been

TABLE 7.

Increase of phage particles over a three hour period

Time Hours	Phage particles per ml. x 10^2
0	4.1
1	6.3
2	10.5
3	14.0

TABLE 8.

Observed bursts of phage appearing between one and two hours

Time Minutes	Phage particles per ml. x 10^2
0	1.7
60	2.8
65	3.3
70	4.6
75	5.8
80	2.7
85	4.2
90	6.3
95	5.9
100	35.0
105	9.3
110	8.0
115	9.1
120	21.0

Figure 3. Observed Bursts of Phage Appearing Between One and Two Hours.

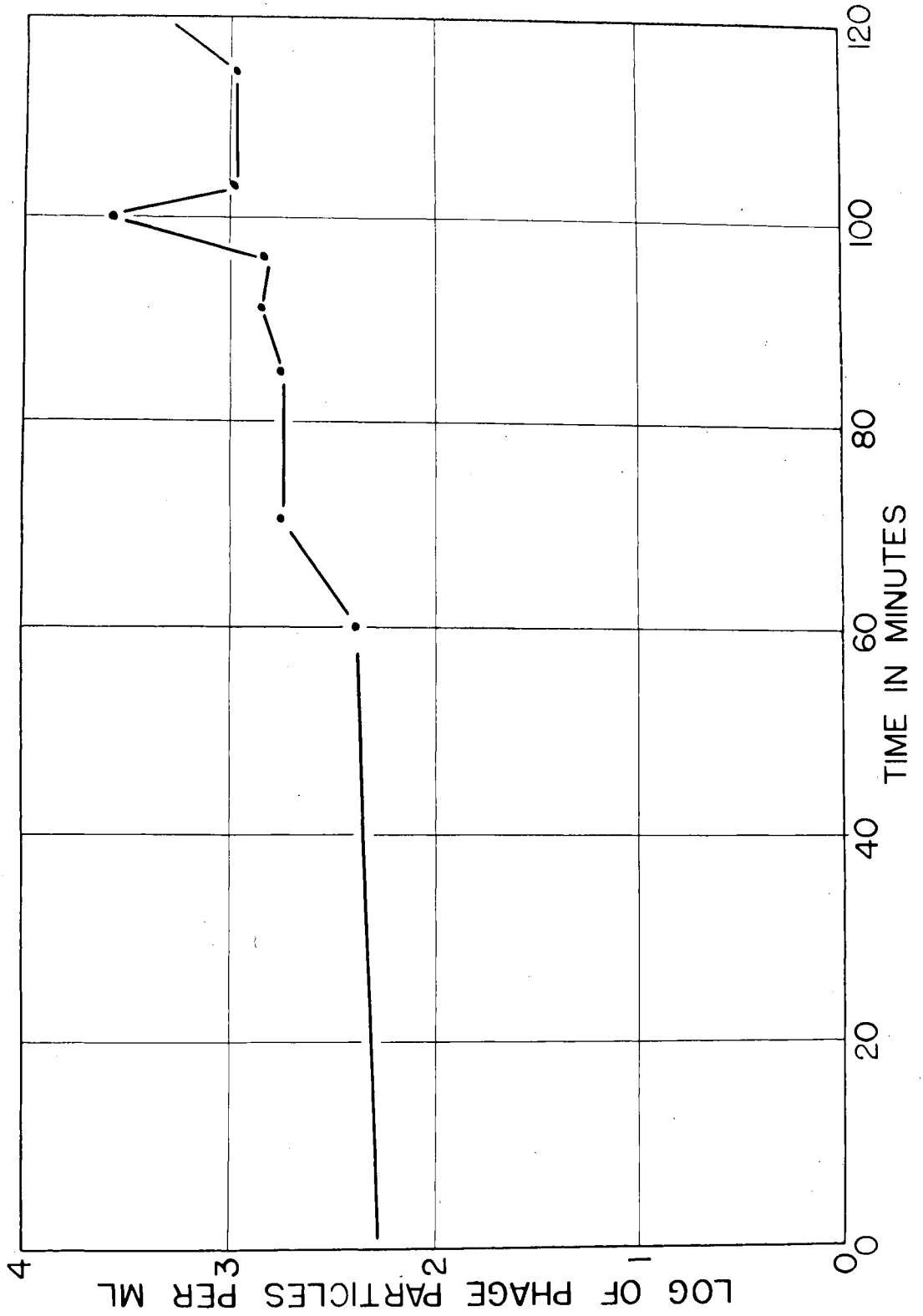

LOG OF PHAGE PARTICLES PER ML
4
3
2
1
0
0
20
40
60
80
100
120
TIME IN MINUTES

TABLE 9.

The lack of increase in phage titer over a sixty minute period

Time Minutes	Phage particles per ml. x 10^2
0	7.0
5	8.9
10	8.0
15	7.2
20	5.1
25	7.6
30	7.2
35	6.5
40	3.7
45	2.5
50	5.9
55	3.9
60	6.0

observed over a 60 minute period. This places the more probable burst time somewhere between the aforementioned 100-120 minute period.

E. Influence of Selected Factors on Phage Multiplication

1. Temperature

The heat treatments to which the phage was exposed and the resulting effect on the phage particles are listed in Table 10. It is apparent that the inactivation of the phage by heat starts at 50° C. with almost complete inhibition of the phage occurring at 60° C. No phage activity was observed with those samples which had been heated above 60° C. It may be considered from these data that the phage under study is quite sensitive to temperatures above 40° C. and is inactivated at temperatures of 70° C. and above. These findings are in general agreement with those of Woodruff (23).

The influence of the incubation temperature on the phage-cell mixture is shown in Table 11. In this case, both low and high temperatures are included. It may be observed that slight phage multiplication was evidenced at temperatures below 22° C. and above 45° C. Complete inhibition of phage multiplication occurred at 55° C. and above. The optimum temperature for phage multiplication appears to be between 28° C. and 35° C.

It is interesting that the phage multiplication process is inactivated at a lower temperature than was observed with the heat treated phage particles. The explanation for this observation may be found in the fact that the phage plus cells were subjected to the described treatments for 48 hours, whereas the phage alone, was treated for only 10 minutes.

TABLE 10.

Inactivation of phage 51^{4}-3 by heat

Temperature Degree C.	Phage particles per ml. x 10^6
25	1.40
40	1.30
50	1.03
60	0.0023
70	0.00
75	0.00
80	0.00
90	0.00
100	0.00

TABLE 11.

Influence of temperature of incubation on multiplication of phage 514-3

Temperature Degrees C.	Phage particles per ml. x 10^4
5	0.006
22	0.04
28	9.20
35	13.00
45	0.50
50	0.20
55	0.00

2. H-ion Concentration

Reference to the data in Table 12 attests to the marked influence of Hydrogen-ion concentration on the phage under study. It is evident that the phage is quite active over a broad range in pH. This point is reflected in the observation that appreciable phage multiplication takes place at pH 5.5 and pH 8.25. However, it is obvious that the optimum range for phage multiplication lies between pH 7.5 and pH 8.25 with complete inhibition occurring at pH 4.25 and pH 8.51.

3. Influence of Salt

Limiting Concentration

The inhibitory effect of sodium chloride on phage 514-3, reported in an earlier experiment is corroborated by the data shown in Table 13 and Figures 4 and 5. However, phage multiplication appeared to be enhanced by one particular concentration of sodium chloride (0.1%) in nutrient broth. Inactivation of the phage became pronounced at 0.3% sodium chloride. It will also be observed that a marked decrease in phage multiplication occurred with the increase in NaCl concentration. At 0.5% NaCl complete inhibition of phage activity is evident. In consequence it would seem that NaCl is stimulatory to phage activity in minute amounts, but inhibitory when present in larger quantities.

The data observed in Table 14 points to the marked inhibition of phage multiplication by sodium chloride in a strictly synthetic medium. Complete inactivation of the phage occurred at 0.2 percent salt concentration. In addition the observation that $NaNo_3$, KCl and $MgCl_2$ also exert an inhibitory effect would tend to give promise that the inhibitory effect

TABLE 12.

Influence of H-ion concentration on multiplication of phage 514-3

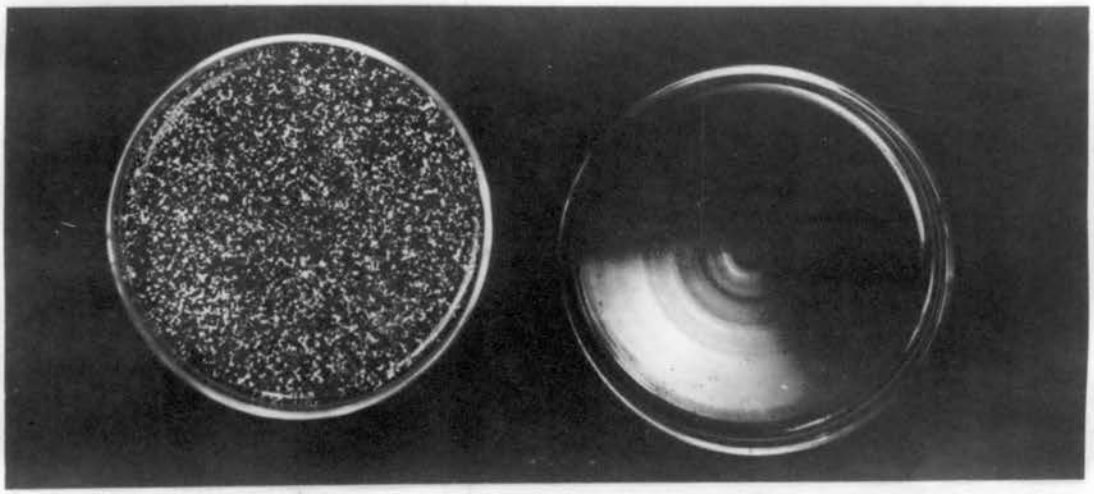
pH	Phage particles per ml. x 10^4
4.75	0.00
5.50	0.01
6.65	0.16
7.50	0.60
7.90	1.38
8.25	0.78
8.51	0.00
9.15	0.00

TABLE 13.

Inhibition of phage 514-3 multiplication by NaCl in nutrient broth

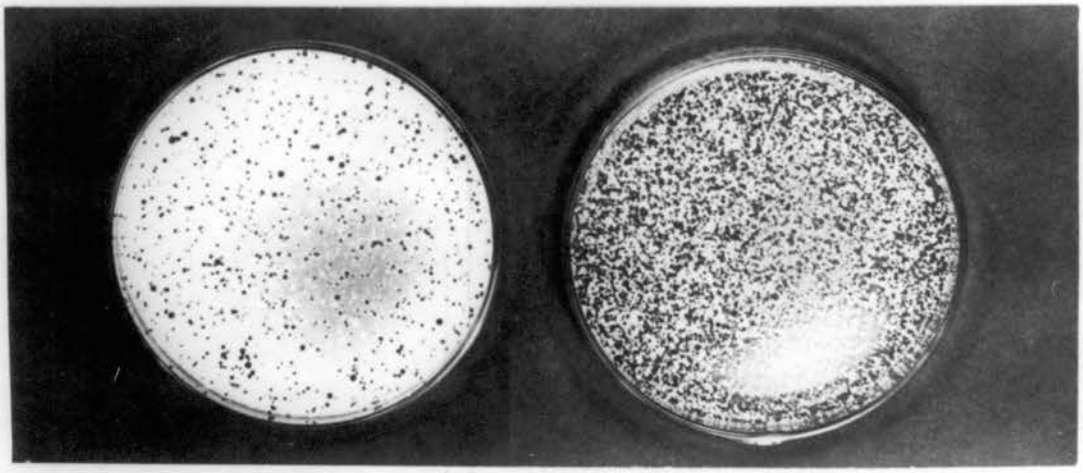
Salt Conc. (%)	Phage particles per ml. x 10^6
0	1.00
0.04.	1.25
0.08.	1.11
0.10.	1.50
0.20.	0.96
0.30.	0.06
0.40.	0.01
0.50.	0.00

Figure 4. Inhibition of Phage 514-3 Multiplication by NaCl in Nutrient Broth.



10^2 10^1

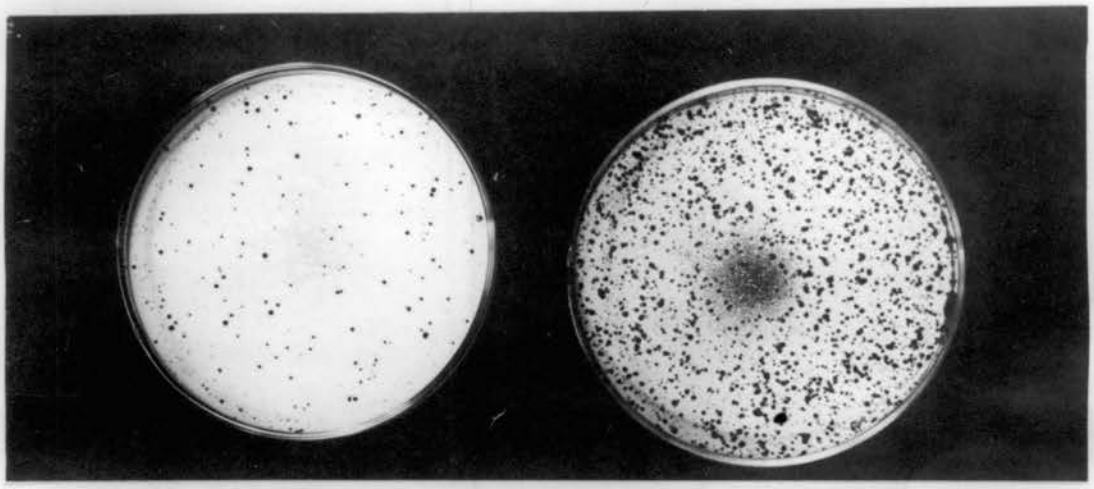
MINUS SALT



10^2 10^1

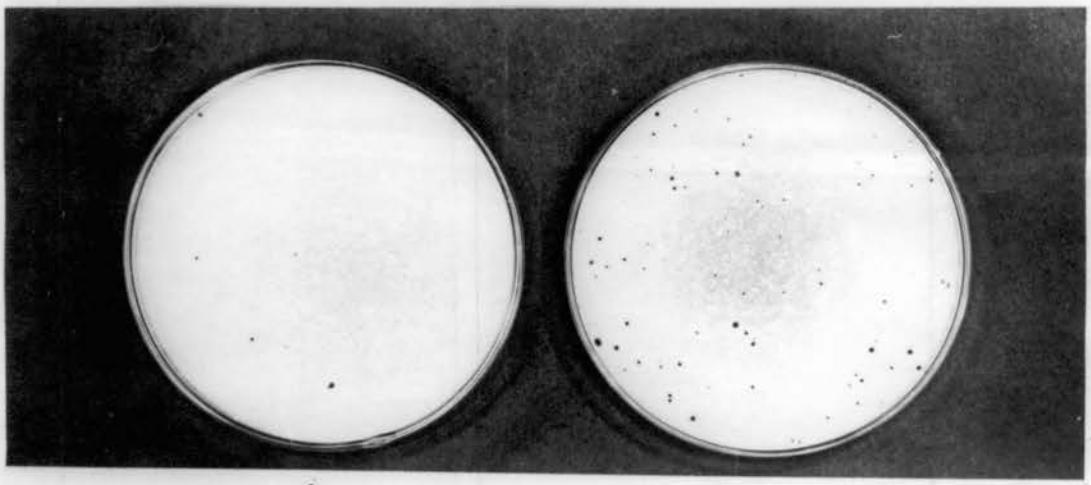
.3% SALT

Figure 5. Inhibition of Phage 514-3 Multiplication by NaCl in Nutrient Broth.



10^4 10^3

MINUS SALT



10^4 10^3

.3% SALT

TABLE 14.

Inhibition of phage 514-3 multiplication by salts in synthetic medium

Salt Conc. (%)	Phage particles per ml. x 10^6 with			
	NaCl	$NaNo_3$	KCl	$MgCl_2$
0.0	1.30	1.39	0.75	0.47
0.2	-	-	-	-
0.3	-	-	-	-
0.4	-	-	-	-
0.5	-	-	-	-

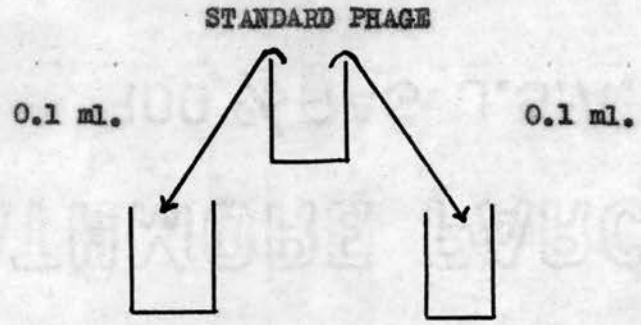
of the tested salts can be attributed to the respective cation. The role of these cations, may possibly be explained on the basis of a charge effect. If sodium through its charge (+), effects a neutralization of an assumed negative charge carried by the phage particles, or increases the zeta potential of the phage particles, then adsorption of the phage on the host cell may be decreased.

The difference between the phage inhibiting concentration of NaCl in nutrient broth and the synthetic medium may be explained on the basis of the difference in the media components. The presence of peptone and beef extract in the organic medium evidently hinders the inhibition properties of sodium chloride.

Mode of Action

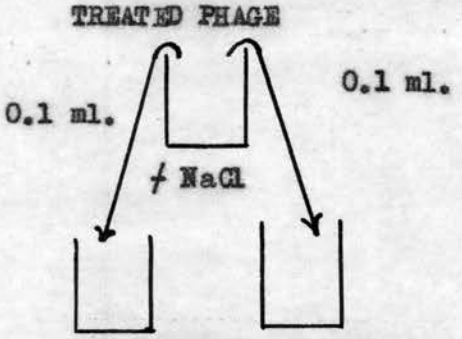
The data illustrated in Figure 6 indicates no direct effect of NaCl on the phage particle or on the Streptomyces griseus cells. Thus the salt effect is centered largely in the medium supporting the phage and cells. It is probable that the sodium chloride interferes with the initial adsorption of the phage on the host cells and thereby inhibits the general multiplication process.

Figure 6. Mode of Action of NaCl on Phage Particles and *Streptomyces griseus* Cells.



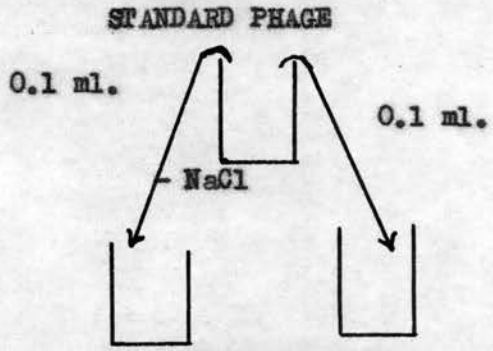
Streptomyces griseus
in nutrient broth.

+ NaCl	- NaCl
Lysis	
-	+



Streptomyces griseus
in nutrient broth

Lysis	
+	+



Streptomyces griseus grown in nutrient broth plus NaCl. Cells centrifuged, washed and resuspended in nutrient broth.

Lysis	
+	+

GENERAL DISCUSSION

An investigation of the fundamental nature of a phage specific for Streptomyces griseus and the possible discovery of an inhibitor for the control of such phages represent the chief objectives of the present study. Many factors are involved and all appear equally important.

The study of the nature of phage 514-3 revealed that multiplication occurred over a 12 hour period. It was found that earlier bursts of this phage are not easily detected because of the extensive mycelium growth exhibited by Streptomyces griseus. In all probability bursts do occur but may be immediately readsorbed by the mass of cells. Although this difficulty of detecting bursts has not been completely eliminated, yet approximate bursts were found occurring at one to two hour intervals. These bursts were consistent in their occurrence as shown by repeated experiments. However, the validity of the latter burst time is not considered to be complete.

It is evident that temperature and H-ion concentration influences the activity of phage 514-3. This phage was most active at the temperature and H-ion concentration optimum for the host culture. This result might be expected since the activity of all phages depends somewhat on the metabolism of the host culture.

The nature of the medium used for testing phage activity influences the subsequent multiplication of the phage. In this respect it was found that a specific complement of amino acids were necessary for phage multiplication. It is interesting to speculate on the role played by these nitrogen compounds. It may be that the amino acid effect is felt

by way of the cellular metabolism of the host culture or it may be directly effecting the phage particle itself. This aspect of the problem certainly deserves further attention.

In addition, sodium chloride and similar salts were found to inhibit phage multiplication. Sodium chloride may be the partial answer to the problem of phage infection in streptomycin fermentations. This type of control has merit since sodium chloride is used in low concentrations in media for the streptomycin fermentation. Moreover the use of sodium chloride would not only represent an inexpensive method of phage control but also a quick method of eliminating phage activity.

The question of the mechanism of sodium chloride inhibition presents a real problem. However, it is felt that the inhibitory effect of salt may be explained on the basis of faulty phage adsorption. Yet it must be admitted that the nature of this inhibition still deserves further consideration.

SUMMARY

1. Phage inocula of 1.0 ml., 0.5 ml. and 0.1 ml. did not influence the resulting phage activity. The extent of lysis at the end of 48 hours was essentially similar.

2. Streptomyces griseus spore inocula of 0.5 ml., 0.2 ml. and 0.1 ml., used in the plating for plaque counts did not appear to influence the resulting count.

3. The titer of standard phage 51^{4}-3 was found to be 1.2 x 10^{5} particles per ml.

4. Phage 51^{4}-3 did not appear to show any division of types which could be traced to plaque size.

5. Phage multiplication occurred, with a sharp rise in phage, at the close of a 10-12 hour period.

6. Bursts of phage 51^{4}-3 appeared to occur between a 100-120 minute period.

7. Phage particles were quite sensitive to temperatures above 40° C. with complete inactivation at temperatures of 70° C. and above.

8. Optimum temperature for phage multiplication was found to lie at 28° C. to 35° C., with complete inhibition occurring at 55° C. and above.

9. The optimum pH range for phage multiplication lies between 7.5 and 8.25 with complete inhibition occurring at pH 4.25 and pH 8.51.

10. Phage activity was inhibited in nutrient broth with 0.3% sodium chloride, complete inhibition occurred with 0.5% sodium chloride.

11. Phage activity was completely inhibited in synthetic medium with

0.2 percent NaCl, $NaNo_3$, KCl and $MgCl_2$ respectively.

12. The mode of action of sodium chloride indicated no direct effect of NaCl on the phage particle or on the Streptomyces griseus cells, but the salt effect was centered largely in the medium supporting the phage and cells.

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NAME OF AUTHOR: Edwin L. Hurd

THESIS ADVISER: Campbell M. Gilmour

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NAME OF TYPIST: Jessie A. Hurd