OBSERVATIONS ON THE MULTIPLICATION AND INHIBITION OF A PHAGE INFECTING STREPTOMYCES GRISEUS

by

ERIC CHARLES NOLLER

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APPROVED:

Redacted for Privacy

Assistant Professor of Bacteriology

In Charge of Major

Redacted for Privacy

Head of Department of Bacteriology

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

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Typed by Patricia Haag

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OBSERVATIONS ON THE MULTIPLICATION AND INHIBITION OF A PHAGE INFECTING *STREPTOMYCES GRISEUS*

INTRODUCTION

Within recent years, a phage capable of infecting *Streptomyces griseus* has been reported. Members of the genus *Streptomyces* are now used in the production of several economically important antibiotics. Phage infestations of streptomycin fermentations have occurred and present an acute industrial problem. Adherance to absolute cleanliness in plant operation and the use of phage-resistant cultures usually constitute good preventative steps. However, a more fundamental approach involves the life history of the associated host-virus system. The mode of reproduction and the influence of environmental factors represent critical information for the development of phage control measures.

Two primary objectives were adhered to in the study of this new host-virus system. 1) An investigation of the reproductive cycle of the host-phage system, and 2) a study of possible control measures for the phage in question.

Streptomyces griseus 3475, a streptomycin producing strain obtained from the collection of Dr. S. A. Waksman, was used in these studies. The phage investigated was isolated from soil.

HISTORICAL

Since Twort's discovery of a lytic factor for bacterial suspensions in 1915 (24, p.1242) and the proof of the transmissibility of this factor by d'Herelle in 1917 (7), interest has grown in the field of bacteriophage research. While the actual nature of bacteriophage is still under speculation, there is much evidence to indicate that these extremely small lytic agents are viruses similar in many respects to those infecting plants and higher animals. Bacteriophages are therefore ultramicroscopic particles capable of infecting and multiplying within a bacterial host.

The most extensively studied of the bacteriophages are the seven "T" viruses specific for Escherichia coli. These bacterial viruses have been termed coliphage and comprise a group of morphologically and serologically different phage types. The results of coliphage studies have been similar to those found for other phage-bacterium systems, and a brief review of their general characteristics will aid in an understanding of other bacteriophage systems.

Before the actual mechanism of bacteriophage multiplication was understood, it was felt that the lysis of bacterial suspensions was of an autolytic nature. Autolysis of bacterial cells does not cause the production of

a lytic principle. In 1940, Katznelson noted the lysis of cultures of Actinomyces. He could not demonstrate any transmission or multiplication of the lytic factor and concluded that host disintegration was autolytic (14, p.86). The fact that bacteriophages actually multiply within the host and are transmissible differentiates them from other lytic agents.

The cycle of bacteriophage multiplication includes adsorption of phage to the host, penetration of the cell, multiplication within the host, and finally lysis of the cell with the release of newly-formed virus.

Before multiplication can occur, it is essential that the phage become attached to the host. The rate of phage adsorption may have a marked effect on the time of lysis of the host (8, p.372). Ellis and Delbruck found that 70 per cent adsorption of phage to Esch. coli occurred in three minutes and 98 per cent adsorption occurred in ten minutes (8, p.373). The rate of adsorption remained constant until 90 per cent of the phage had become attached.

Following adsorption, the phage multiplies within the host. The time elapsed between adsorption of phage and lysis of the host is known as the latent period of the virus and represents the time required for multiplication of the phage. The latent period of T-1 bacteriophage is about 13 minutes (6, p.170). The latent period was greatly increased when the infected cells were incubated below 37°C.

These investigations were carried out using the one-step growth curve. After adsorption of the phage, the phage-cell suspension was greatly diluted with chilled medium to prevent readsorption of liberated phage by uninfected cells. In this manner, the size of individual bursts can be determined (5, p.653). Each infected cell normally liberates an average of 60 phage particles following the regular latent period. The amount of phage liberated is termed the lytic threshold of the host.

When phage-sensitive cells are challenged with small concentrations of phage, phage-resistant mutants of the host become apparent. In many instances, these resistant cultures carry a phage precursor through many generations before liberation of phage occurs (17, pp.72-77; 18, pp. 132-136). Such resistant cultures are termed lysogenic.

Bacteriophage exhibit a varied morphology. The coliphage may be single spheres or may be spheres possessing tails. The serologically similar T-2, T-4, and T-6 phages possess a tail about 145 millimicrons long and have a mean head diameter of about 80 millimicrons. T-3 and T-7 are spheres about 45 millimicrons in diameter (9, pp. 424-425). Phage infecting other members of the Eubacteriales exhibit similar morphological characteristics.

Many investigations have been made on compounds that inhibit or stimulate phage multiplication. Scribner and Krueger reported in 1937 that 0.25 M sodium chloride present

in a staphylococcus host-phage system increased phage yield five to ten-fold (22, p.11). The presence of sodium chloride apparently increased the lytic threshold of the host, since lysis was delayed by 42 minutes, and multiplication continued at the usual rate. In 1938, Krueger and Streitmann observed that the presence of M/8 sodium sulfate in a staphylococcus host-virus system prolonged the time of lysis, decreased adsorption, reduced phage multiplication to one-third of the normal rate, and increased the phage-cell ratio required to cause lysis by a factor of four (16, p.138). It was concluded that the presence of this electrolyte inhibited adsorption of the phage. It was shown by Gest in 1943 that 0.01 M magnesium chloride had no effect on the adsorption of phage by Esch. coli (10, p.138). T-2 virus requires a high sodium ion concentration and a low level of calcium ion for proper plaque formation (12, p.268). In 1949, Cherry and Watson noted that the adsorption of phage infecting Streptococcus lactis was stimulated by potassium phosphate, potassium chloride, sodium chloride, calcium chloride, magnesium sulfate, and sodium acetate in concentrations from 0.005 to 0.05 M. These compounds stimulated lysis in accord with their ability to stimulate adsorption (3, p.612). Sodium citrate prevented lysis, but it did not prevent adsorption in 0.005 M concentrations.

One of the first reports of a phage causing the lysis of a filamentous micro-organism was made in 1947 by Saudek

and Colingsworth. These workers found that stock cultures of Streptomyces griseus and streptomycin beers were infected by a phage (21, p.41). The phage was capable of passing bacterial filters and multiplication in the presence of S. griseus. A similar report was made by Smith and co-workers in the same year (23, p. 545). Further studies by Woodruff, et al (25, pp.536-541), Reilly, et al (20, pp.453-466), and Koerber, et al (15, pp.30-38) demonstrated that the lytic agent was a true phage similar to those reported for other bacteria. Reilly suggested the term actinophage for this group of viruses (20, p.451).

Latent periods reported for actinophage have been remarkably long when compared with other bacteriophage systems. Perlman and co-workers, in their studies on S. griseus bacteriophage multiplication (19, p.141), suggested that the latent period was less than nine hours and probably about six hours. Using Actinophage 514-3, isolated by Gilmour and Buthala in 1950 (11, p.17), Hurd showed a small increase in phage at 100 to 120 minutes followed by a large increase between 10 and 12 hours. While the main increase in phage occurred during the 10 to 12 hour interval, the earlier increase indicated a much shorter latent period (13, p.26). Woodruff, et al, found that the time of lysis of a suspension of germinating S. griseus spores was slightly more than six hours when phage was added with the spore inoculum (25, p.538). This observation would indicate

a much shorter latent period than six hours if phage infection did not occur until after the time required for germination of the spores.

In 1951, Perlman, et al, reported that calcium-sequestering compounds such as citrate, oxalate, and phytate inhibited actinophage multiplication without markedly decreasing streptomycin production by the host (19, p.142). Hurd reported in 1951 that 0.3 per cent sodium chloride would inhibit lysis of the host (13, p.39). Such inhibitors have been suggested as possible means of controlling actinophage infection in streptomycin beers.

EXPERIMENTAL METHODS

GROWTH CHARACTERISTICS OF *STREPTOMYCES GRISEUS* 3475

Turbidimetric studies

Media. The basal medium used throughout these studies contained 0.5 per cent glucose, 0.5 per cent peptone, 0.3 per cent beef extract, and a trace of yeast extract. Tap water was used in media preparation; the resulting nutrient broth showed a pH of 6.8 to 6.9 following autoclaving at 15 pounds pressure for twenty minutes. Three per cent agar was added when solid media was desired for the cultivation of spores, while 1.5 per cent agar was used for plating techniques.

Preparation of spores. Spores of *Streptomyces griseus* 3475 (Waksman collection) were streaked on slants of nutrient agar contained in 250 ml. prescription bottles. After 96 hours incubation at 30°C., growth and sporulation were at a maximum. Thirty ml. of nutrient broth were added aseptically, and the spores teased off into the broth with a sterile loop. Mycelial fragments and spore clumps were removed by passing the suspension with mild suction through 36 layers of 50 mesh gauze held in a Seitz filter. The resulting suspension was evenly turbid and remained so throughout serial dilution. Viable spore counts were standardized against turbidity by running standard plate counts

on dilutions of determined optical density.

Growth conditions. One hundred ml. aliquots of broth diluted spores were grown in 250 ml., 500 ml., and one liter Erlenmeyer flasks. For simplicity in making direct turbidimetric readings on the growing mycelium, spores were also grown in special cuvettes made from standardized 20 mm. Pyrex culture tubes cut to a length of ten cm. and provided with aluminum caps. Flasks and cuvettes were incubated at 30°C. on a rotary bed shaker at 240 rotations per minute. Turbidity was determined with a Beckman Model B spectrophotometer. All values were read as optical density (2 - log G) giving a direct indication of cell mass. Three ml. aliquots were removed from the flasks at intervals to standard one cm. cuvettes for turbidity readings. In the case of the special cuvettes, readings were taken directly with the aid of a test tube adaptor on the spectrophotometer. All determinations were made at 640 millimicrons light wave-length. This wave-length gave maximum differentiation between the transmission of the broth and that of the suspended spores or mycelium.

The effect of slight incubation temperature changes on the germination and growth of Streptomyces griseus spores was determined. Spores suspended in nutrient broth at pH 6.8 were grown in the cuvettes at temperature intervals of 3° ranging from 27° to 39°C. on the rotary shaker. Interval turbidity readings were made directly with the

spectrophotometer.

The effect of hydrogen-ion concentration on the germination and growth of S. griseus spores was determined by growing the spores in broth at pH 6.8, 7.1, 7.5, and 7.8. Spores were incubated in the cuvettes at 36°C. on the rotary shaker. Turbidimetric readings were made at intervals.

Photomicrographic evidence of the germination and growth of Streptomyces griseus spores

Spores were germinated and grown in cuvettes at 36°C. in nutrient broth at pH 6.8. At varying intervals, small samples were removed with a sterile loop, and smears were made on microscope slides. The smears were heat-fixed and stained with aqueous crystal violet for two minutes. Photomicrographs were made using Kodak Plus-X sheet film.

GROWTH CHARACTERISTICS OF ACTINOPHAGE 514-3

Preparation of high-titer filtrates

High-titer filtrates were obtained from confluently lysed assay plates. The stock phage filtrate was diluted in broth to contain from 10^4 to 10^5 plaque-forming units per ml. One ml. of the dilution was added to 2.5 ml. of semi-solid agar (0.75 per cent agar) to which had been added 0.1 ml. of the undiluted spore suspension. The semi-solid agar was kept liquified by placing it in a water bath at 45°C.

The mixture of semi-solid agar, spores, and phage dilution was then mixed and then layered over 20 ml. of solidified agar contained in a petri plate. After solidification of the semi-solid layer, the plates were inverted and incubated at 30°C. for 48 hours. After this time interval, complete lysis of the mycelium produced by the spores had occurred. Five ml. of broth was poured over the semi-solid layer, and this layer was minced off into the broth leaving the solid agar layer behind. After allowing the suspension of semi-solid agar in broth to stand for a period of 15 minutes, the mixture was passed through a Seitz SI filter.

Morphology of Actinophage 514-3 as revealed by electron photomicrographs

Five ml. aliquots of the high-titer phage filtrate, contained in Lusteroid tubes, were centrifuged at 21,000 times gravity in a Sorval angle-head centrifuge for three hours. After the indicated period, a definite pellet having a pale blue color had formed in the bottom of the centrifuge tube. The supernate was carefully drained off, and the pellet was resuspended in one-half ml. of distilled water. The resulting suspension was clear and exhibited a light blue opalescent effect.

The suspension was serially diluted with distilled water and screens were prepared from selected dilutions for use in the electron microscope. The screens were air dried

without washing and shadowed with 30 angstroms of chromium from an angle of 20°.

The electron microscope was standardized by photographing a shadowed collodion replica of a diffration grating ruled to 600 lines per mm. Original magnification was 16,800 diameters plus or minus five per cent.

The Multiplication of Actinophage 514-3

Adsorption of phage to the host. One ml. of spores diluted to contain about 10^8 spores per ml. was added to 99 ml. of nutrient broth contained in a 250 ml. Erlenmeyer flask. The flask was incubated at 30°C. on the rotary shaker for six hours. One ml. was then removed for plate count of spore numbers. A slide was also prepared which indicated that greater than 90 per cent of the spores had germinated and exhibited a germ tube about two microns long.

One ml. of phage filtrate containing 2.62×10^9 plaque-forming units per ml. was added to the suspension of germinated spores. Two ml. aliquots were removed to chilled 18 ml. broth dilution blanks at intervals of 5, 10, 20, 30, and 60 minutes. Five ml. aliquots of the one to ten dilution of the phage-cell mixture were centrifuged at 3000 revolutions per minute for five minutes to remove the suspended cells and adsorbed phage. One ml. aliquots of the supernate were removed and plated in triplicate at dilutions of 10^{-5} and 10^{-6}. The plating procedure for

assaying numbers of phage was the same as that used in the preparation of high-titer phage filtrates, except phage dilutions used were higher than would produce confluent lysis of the mycelium. Areas of lysis (plaques) appeared which were counted to determine the number of plaque-forming units present at a given dilution.

Multiplication of Actinophage 514-3 and lysis of the host. One hundred ml. of broth containing 2 X 10^7 spores per ml. was shaken in a 250 ml. Erlenmeyer flask at 30°C. for six hours. After the germination period, one ml. was removed for plate count of viable spores, and one ml. of phage containing 4.6 X 10^8 plaque-forming units per ml. was added. At intervals, five ml. samples were removed and centrifuged for five minutes at 3000 revolutions per minute. One ml. samples of the supernate were diluted with broth and plated to determine the increase in numbers of free phage.

After infection of the host with Actinophage, there is a period of multiplication of the virus within the host followed by lysis of the host and liberation of the newly-formed phage. Lysis of the host cells results in a clearing of the turbid suspension of mycelium and may be followed turbidimetrically.

Nine ml. of broth-diluted spores containing about 10^7 spores per ml. (optical density of 0.05 to 0.10) were placed in the cuvettes and incubated on the rotary shaker at 30°C.

until germination became evident as indicated by increase in turbidity. Phage filtrates were prepared and diluted in broth to contain 2 X 10^{10}, 2 X 10^{9}, and 2 X 10^{8} plaque-forming units per ml. After germination of the spores (four and one-half hours), one ml. of each dilution of phage was added to the tubes. All tubes were run in duplicate. Control tubes received one ml. of sterile broth. The tubes were returned to the shaker, and turbidimetric readings were made at half-hour intervals for the following five hours and at hourly intervals thereafter. Samples were taken at the same intervals, and smears were prepared and stained for photomicrographs.

Factors influencing the multiplication of Actinophage 514-3

Temperature and hydrogen-ion concentration. Experiments were run to determine the effect of incubation temperature and hydrogen-ion concentration on the multiplication of the phage. The experimental procedure exactly duplicated those experiments previously described on the effect of temperature and pH on the germination of S. griseus spores.

Inorganic ions. The effect of certain inorganic ions on the multiplication of Actinophage 514-3 was determined by the turbidimetric procedure. Nutrient broth was prepared to which a series of inorganic salts were added in a concentration of 0.05 N. The salts used were ammonium chloride,

lithium chloride, potassium chloride, sodium chloride, and magnesium chloride.

Eight ml. aliquots of the various media were added to the cuvettes, and one ml. of a spore suspension containing 10^7 spores per ml. was added. The tubes were then incubated at 36°C. on the shaker until germination was evident. At this time, one ml. of phage containing 10^9 plaque-forming units per ml. was added, and the tubes were replaced on the shaker. Growth and lysis were followed turbidimetrically.

THE LYSOGENICITY OF PHAGE-RESISTANT CULTURES OF *STREPTOMYCES GRISEUS* 3475

When confluently lysed plates were allowed to incubate for five to seven days, phage-resistant colonies of *S. griseus* appeared. About one spore per thousand produces mycelium resistant to phage attack. To demonstrate possible lysogenicity of these cultures, a colony was picked and streaked on an agar spore slant. Four serial subcultures were carried out. From the fifth generation resistant culture, spores were placed in 100 ml. of broth and transferred to a 250 ml. Erlenmeyer flask. A similar culture was prepared from susceptible spores. The flasks were incubated at 30°C. on the rotary shaker for 96 hours. Following incubation, five ml. aliquots were removed from the flasks and centrifuged at 3000 revolutions per minute for five minutes

to remove the mycelial mass.

Three sets of tubes were prepared containing ten ml. of a broth suspension of spores obtained from susceptible and resistant cultures of the host. The first set of tubes received one ml. each of the supernate from the shake culture of susceptible mycelium. The second set received one ml. each of the supernate from the shake culture of resistant mycelium, while a third set received one ml. each of high-titer phage. The tubes were incubated on the shaker at 30°C. for 24 hours after which time they were observed for lysis.

EXPERIMENTAL RESULTS

GROWTH CHARACTERISTICS OF *STREPTOMYCES GRISEUS* 3475

Turbidimetric studies

The effect of growth conditions. Before experiments could be carried out on the growth of *S. griseus* incubated in the 20 mm. cuvettes, it was necessary to determine whether or not the decreased surface-volume ratio would have any marked effect on the germination and growth of the spores. Reference to Figures 1 and 2 discloses that there is little difference in the rates and amounts of growth between flask-grown and cuvette-grown spores. Apparently the shaking of the medium was sufficient to allow maximum aeration of the medium in the cuvettes. Therefore all subsequent experiments were performed using the cuvettes.

Reference to Figure 3 indicates an optimum growth temperature of 36°C. for the spores grown in nutrient broth. For this reason, later experiments were run at the above temperature.

As shown in Figure 4, optimum growth of the spores occurs at pH 6.8. Since the pH of industrial mashes seldom falls below 6.8, the effect of higher hydrogen-ion concentrations was not studied. During the process of growth of *S. griseus*, the medium becomes progressively alkaline due to the accumulation of end-products. For this reason, the

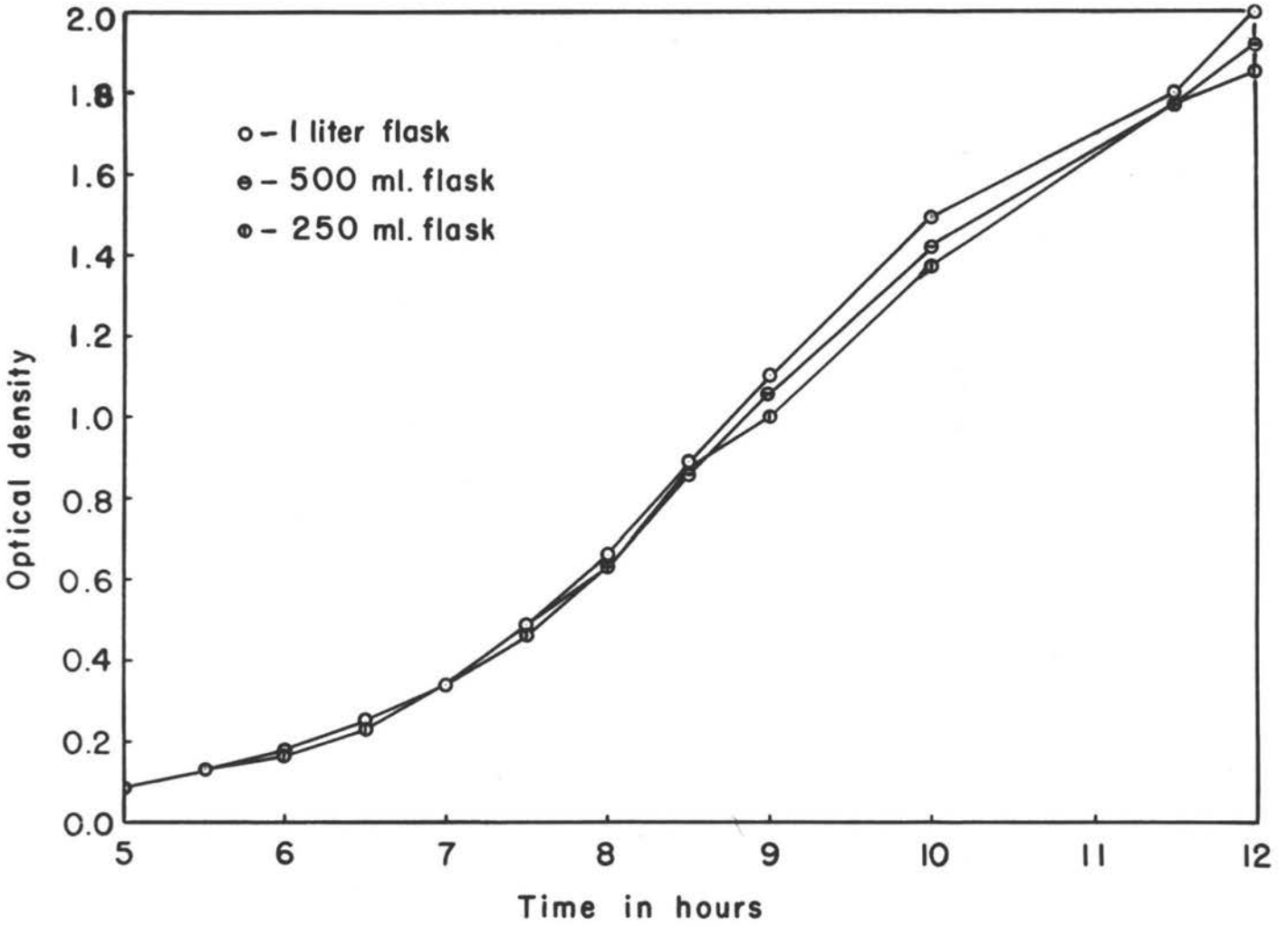

Fig. 1 The influence of surface-volume ratio on the growth of Streptomyces griseus.

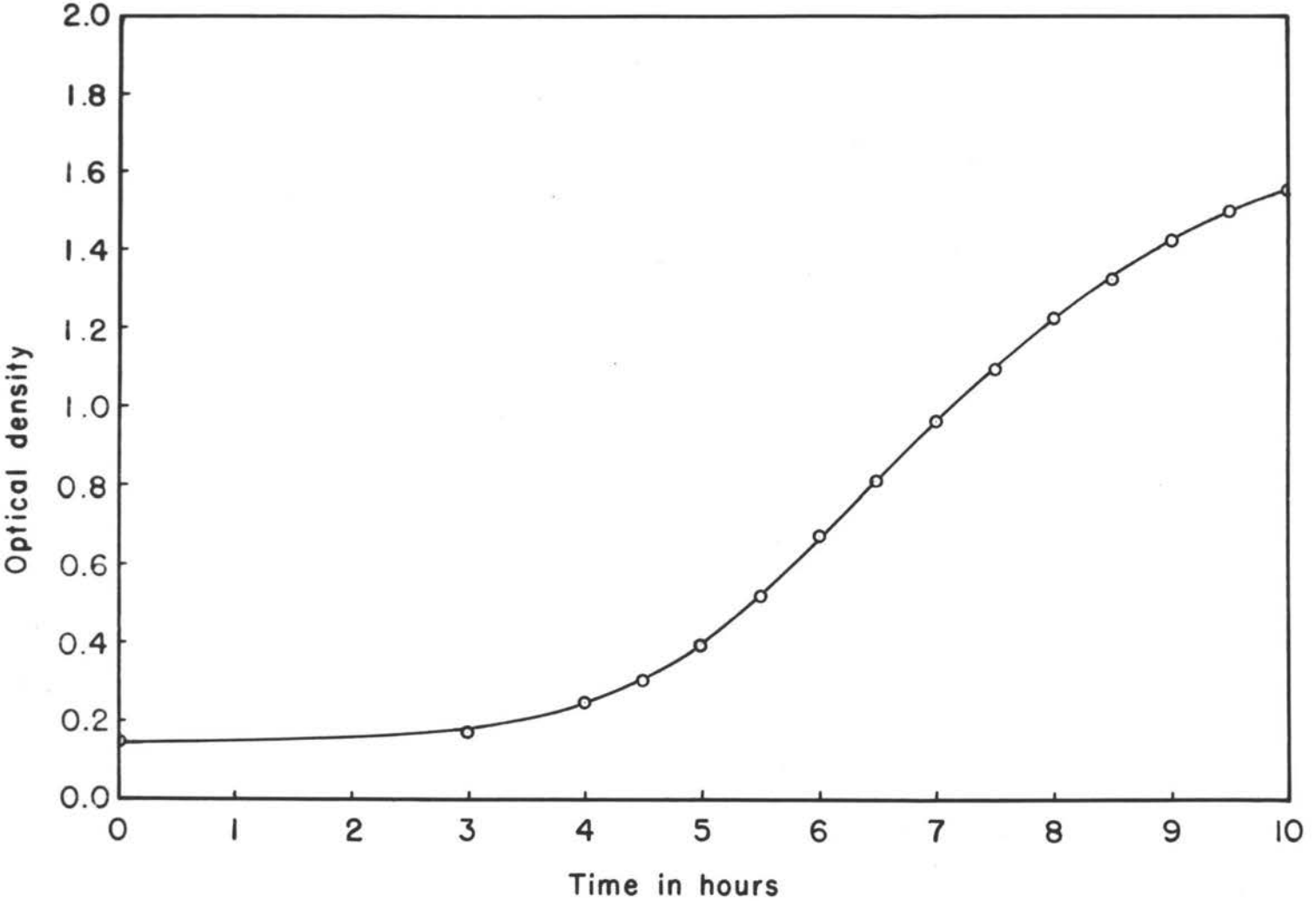

Fig. 2 Observed growth of Streptomyces griseus incubated in 20 mm. cuvettes.

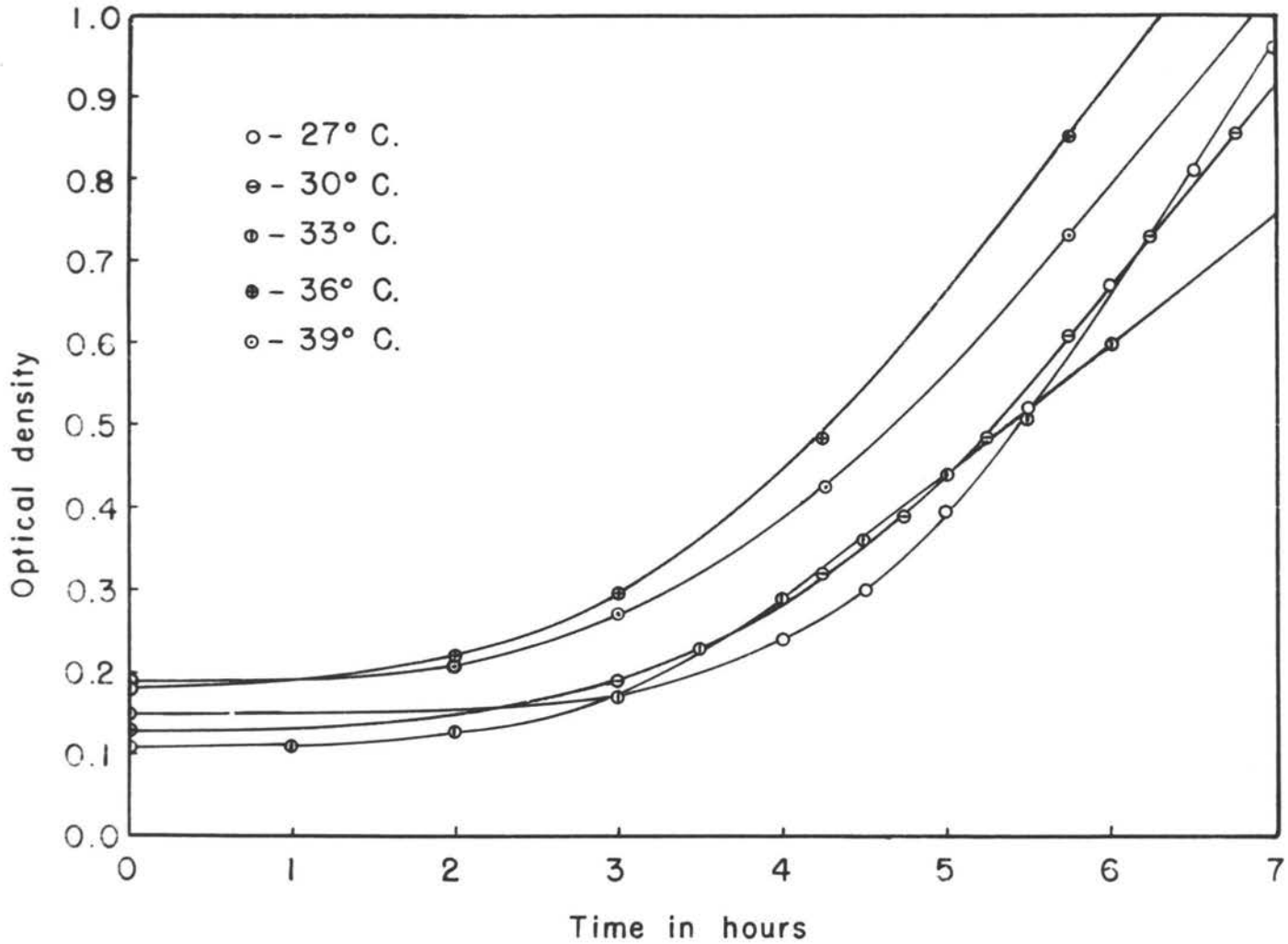

Fig. 3 The influence of incubation temperature on the growth of Streptomyces griseus.

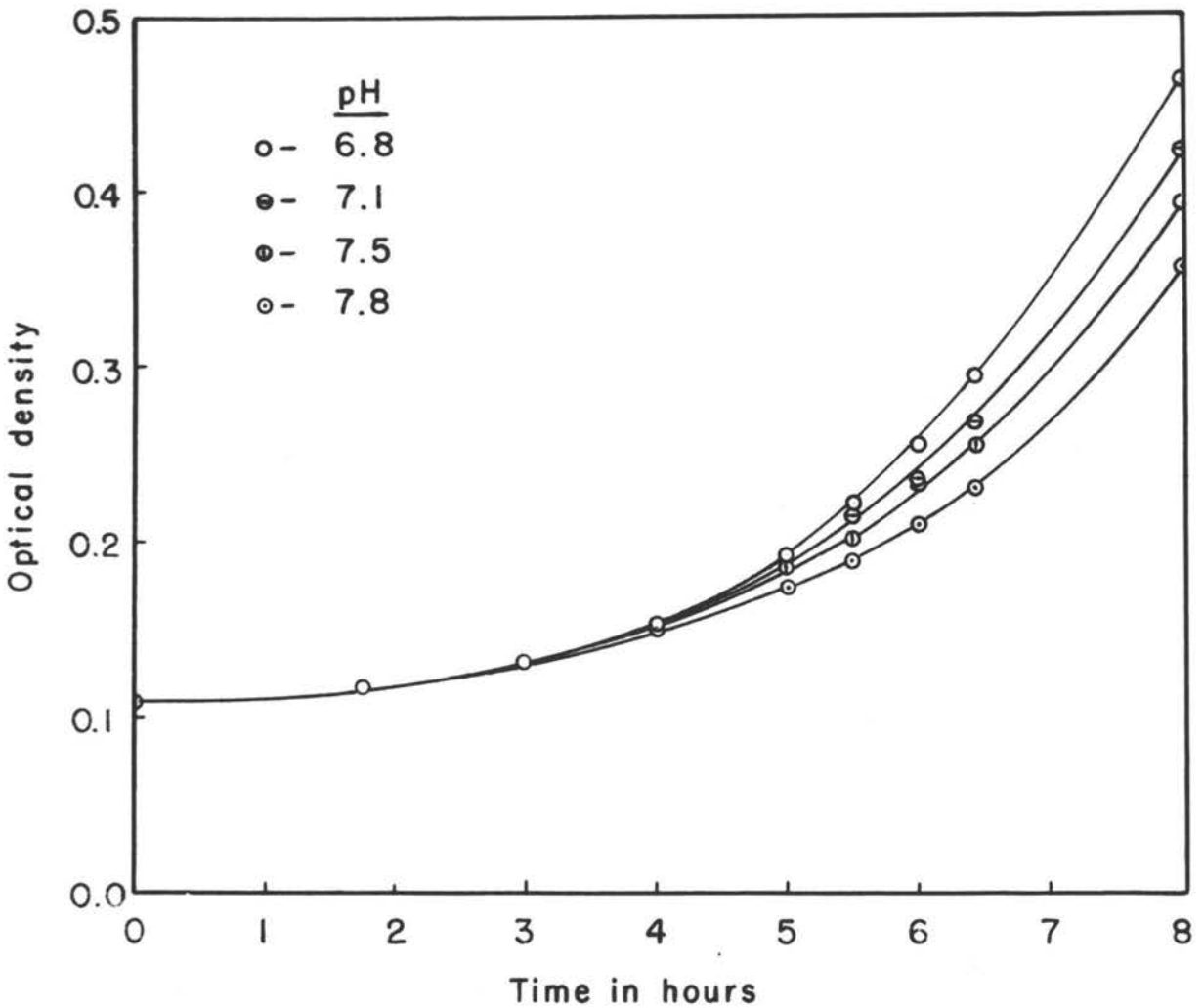

Fig. 4 The influence of hydrogen-ion concentration on the growth of Streptomyces griseus.

effect of increased pH on the germination and growth of the spores was investigated. Such alkaline media did not support growth as well as those having a hydrogen-ion concentration nearer to neutrality.

Photomicrographic evidence of the germination and growth of Streptomyces griseus spores

Reference to Plate 1 reveals that the normal germination of spores occurs after about three hours incubation at 36°C. Each spore may produce from one to four germ tubes. The germ tube grows at a rate of about five microns per hour. During the process of growth, extensive branching of the germ tube occurs producing the typical mold-like mycelium characteristic of the Streptomyces.

GROWTH CHARACTERISTICS OF ACTINOPHAGE 514-3

High-titer filtrates

Stock phage prepared by confluent lysis of the host mycelium showed an average count of 10^9 to 10^{10} plaque-forming units per ml. of filtrate. Significant decreases in titer occurred upon prolonged storage at 5°C.

Morphology of Actinophage 514-3 as revealed by electron photomicrographs

The actinophage under study has the tad-pole morphology characteristic of many other bacterial viruses (Plate 2). The phage shows an average head diameter of 95 millimicrons

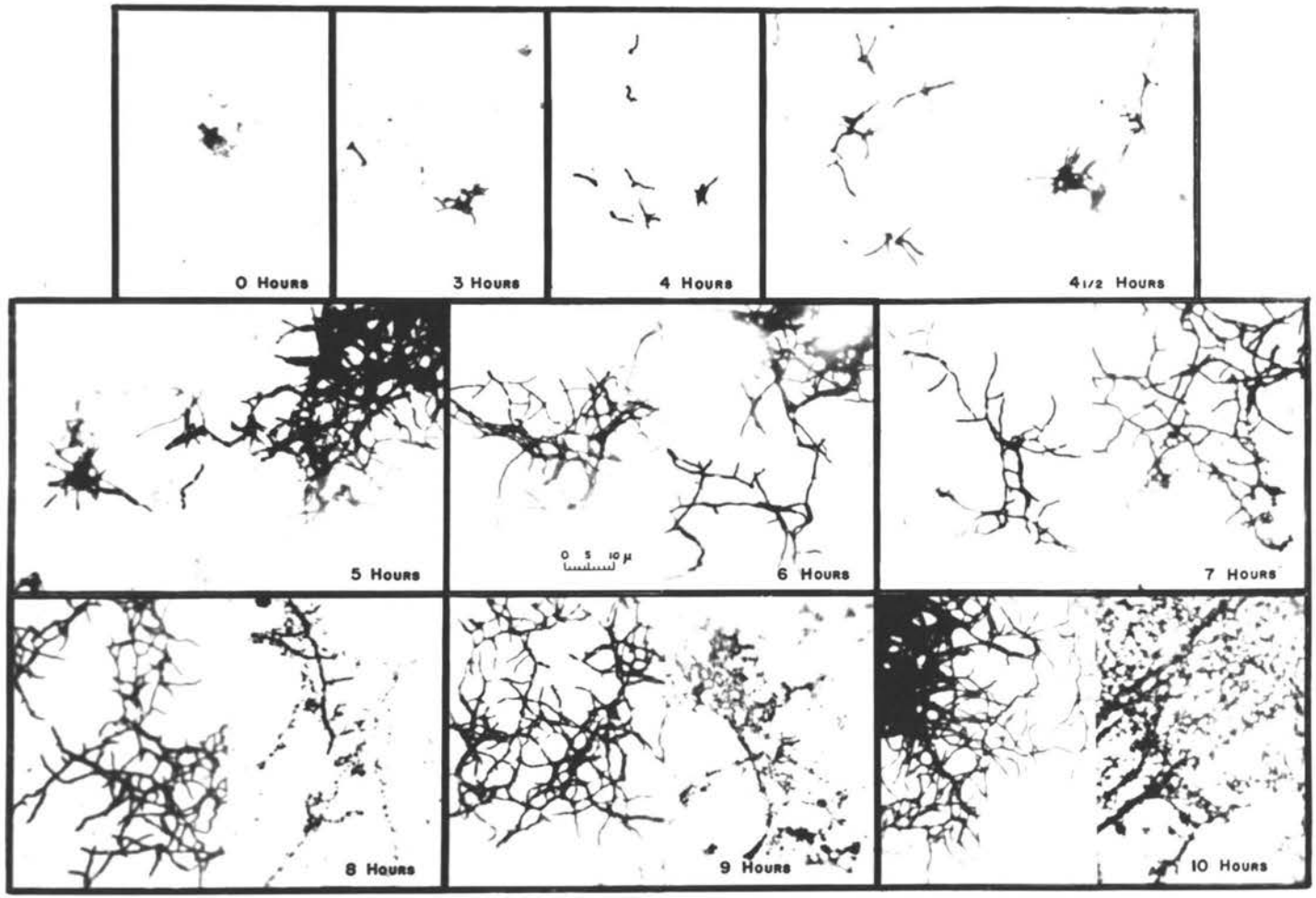

Plate 1 Comparative photomicrographs of growing and lysing mycelium of Streptomyces griseus at varying time intervals. Normal mycelium appears on the left-hand side of each group, while infected mycelium is shown on the right-hand side.

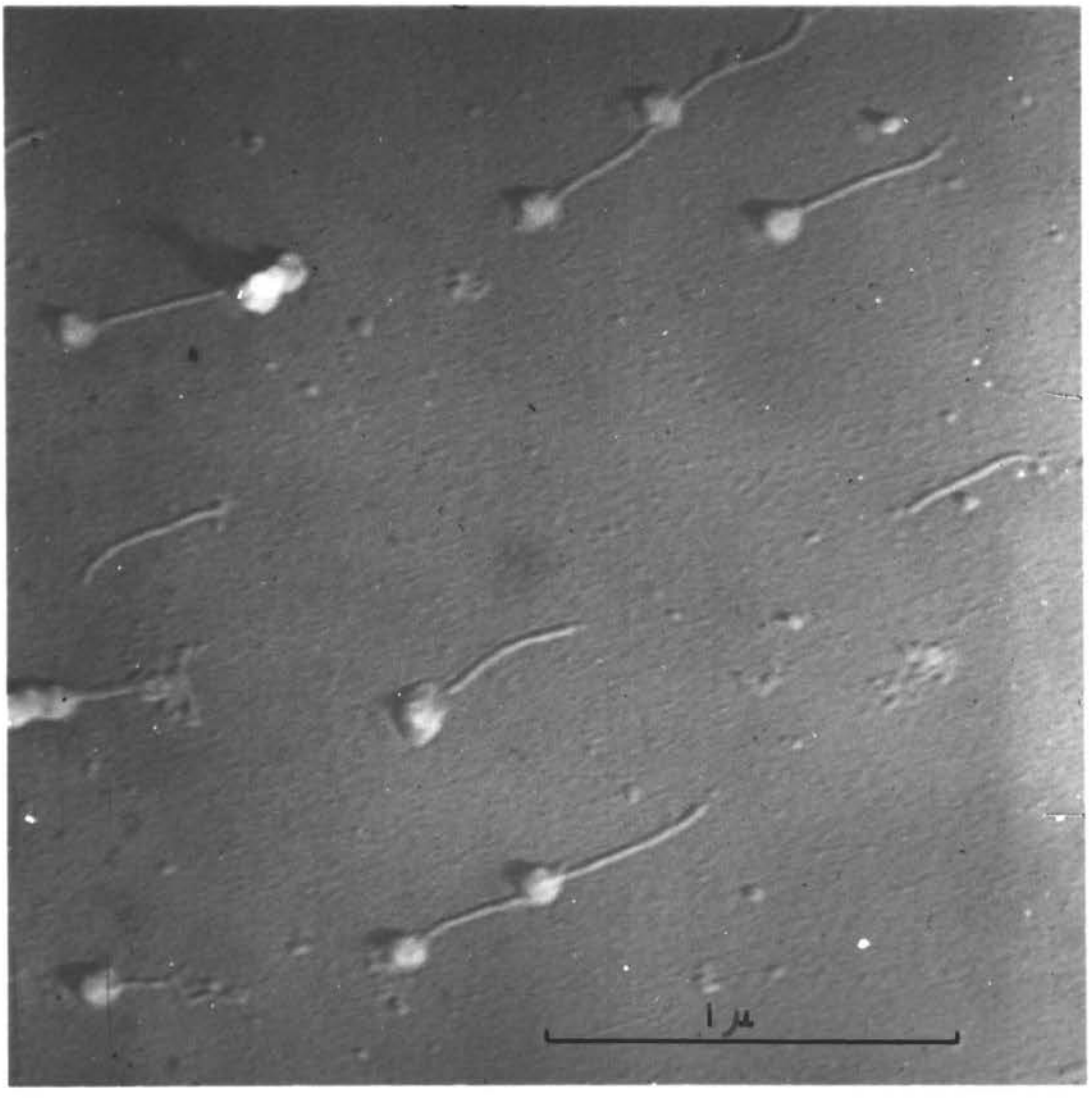

Plate 2 Electron photomicrograph of Actinophage 514-3.

and an average tail length of 360 millimicrons. The head is apparently smooth and spherical. The head and tail are not too firmly attached as is indicated by the presence of free heads and tails.

The multiplication of Actinophage 514-3

Adsorption of phage to the host. A study of Figure 5 discloses that the free phage titer drops from 2.62×10^7 to 1.87×10^7 during the initial thirty minute interval. This decrease in titer represents an adsorption of 28.6 per cent of the added phage by the host mycelium. Further adsorption was negligible in the following thirty minute period. This relatively slow rate of adsorption appears to be characteristic of the actinophage under investigation.

Multiplication of Actinophage 514-3 and lysis of the host. Reference to Figure 6 reveals that a major increase in free phage occurs between eight and nine hours. A second large increase is evident after 18 hours. Subsequent phage release resulted in complete lysis of the host mycelium. The small burst at 100 to 120 minutes reported by Hurd (11, p.17) was not observed.

Reference to Figure 7 indicates that the concentration of phage added to a given number of spores has a marked effect on the time of lysis of the host mycelium. The addition of phage in a ratio of two plaque-forming units per spore present has little effect on the growth of the

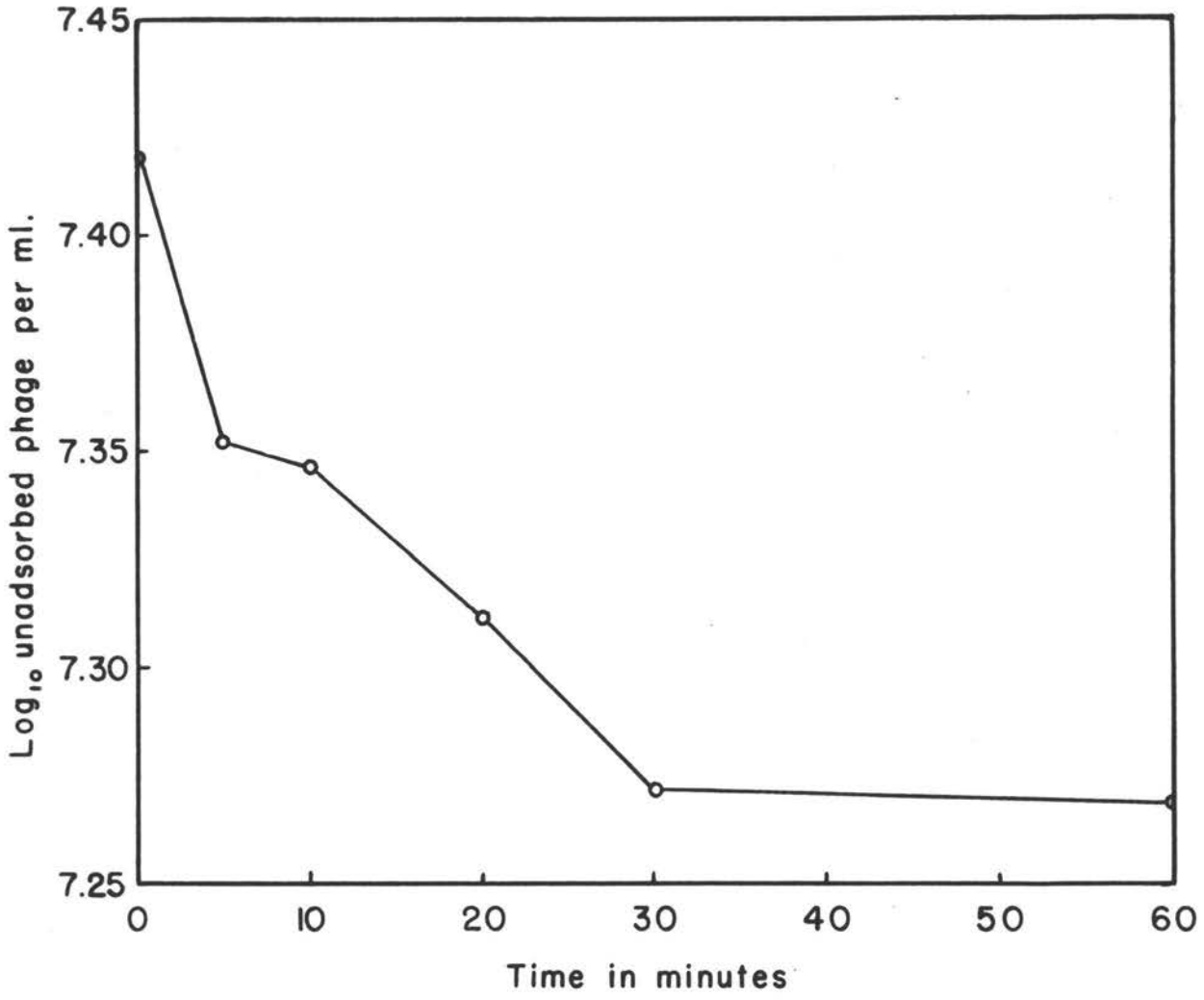

Fig. 5 The adsorption of phage by cells of Streptomyces griseus.

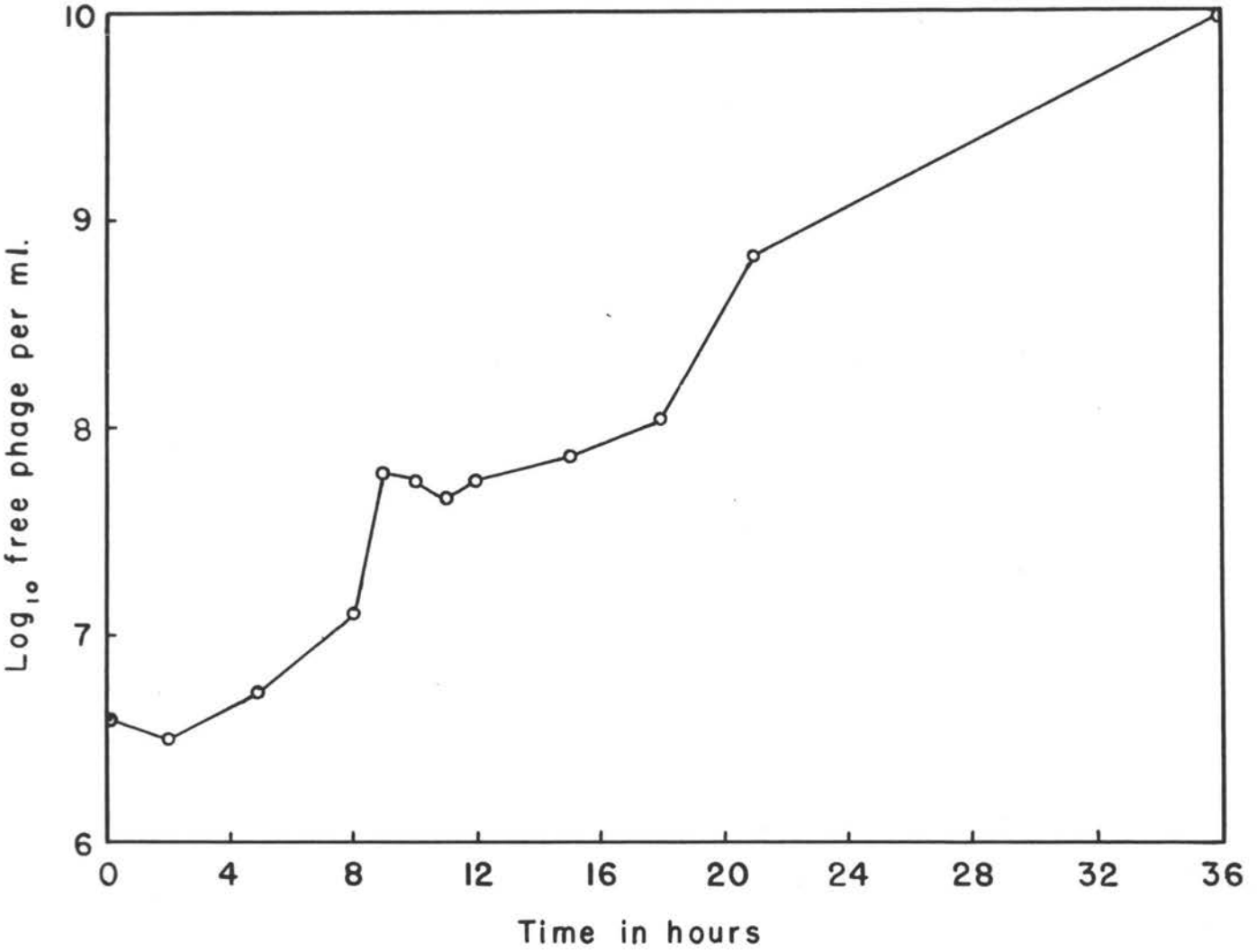

Fig. 6 Observed increase in free phage count at varying time intervals.

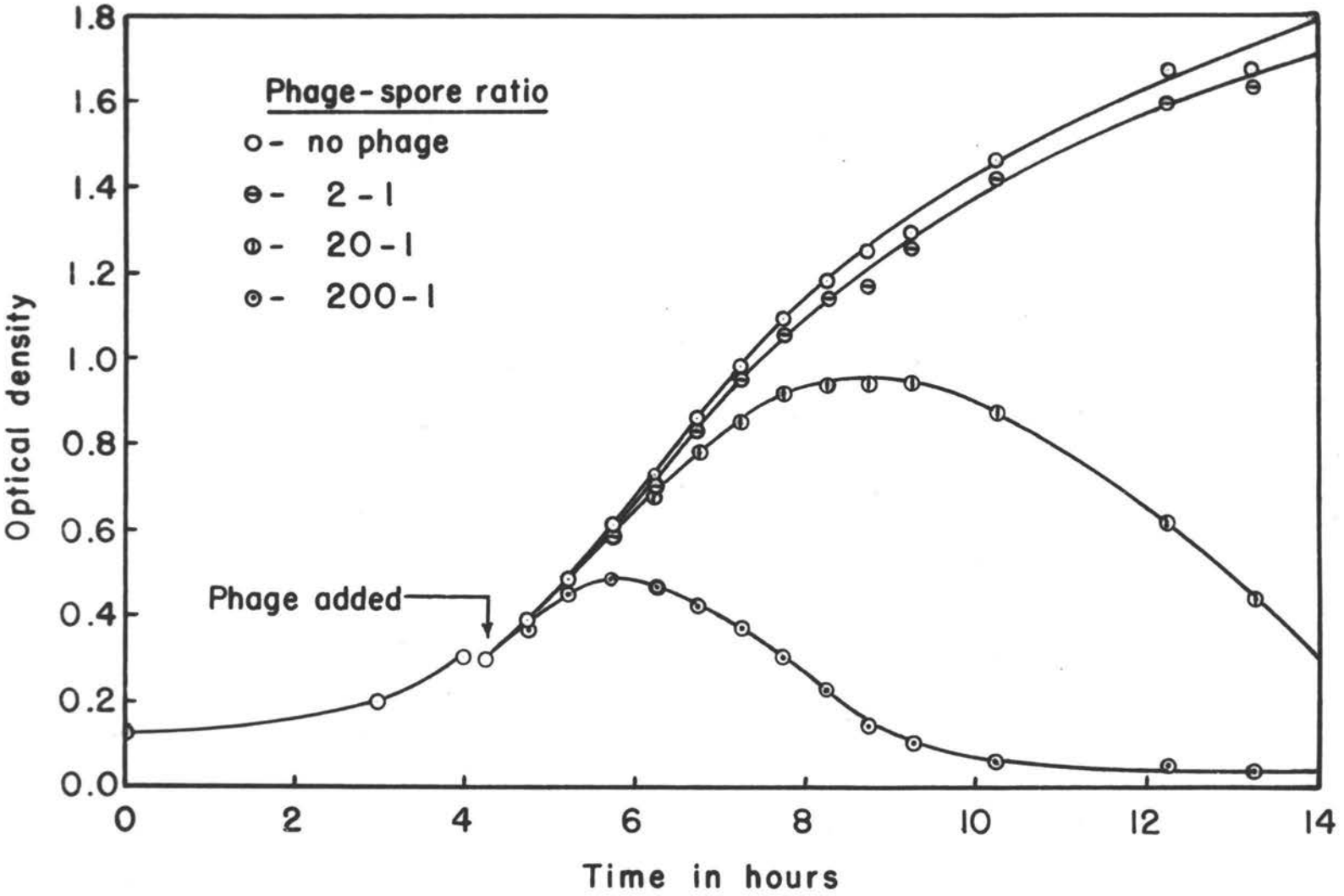

Fig. 7 Multiplication of phage followed by the time of lysis of the host mycelium.

germ tube. However, when this ratio is increased to 20 to 1, lysis of the host occurs in 4.5 hours. A phage-spore ratio of 200 to 1 causes lysis to occur in 1.5 hours. Further increase in this phage-spore ratio does not shorten the lysis time below 1.5 hours. The lysis of the host is undoubtedly caused by liberation of new bacteriophage, since a ten-fold increase in phage titer was observed following complete host lysis.

The right-hand photomicrographs shown in Plate 1 illustrate the lytic effect of phage on the host mycelium. Little difference can be seen between the infected mycelium and the normal mycelium until about three hours after the addition of phage. At this time, lysis of the mycelial tips becomes apparent. Four hours after infection, extensive lysis occurs.

Factors influencing the multiplication of Actinophage 514-3

Temperature and hydrogen-ion concentration. Optimum conditions for the multiplication of the phage were found to be incubation at 36°C. and adjustment of the medium to pH 6.8 (Tables 1 and 2). That optimal conditions for phage multiplication are similar to those for growth of the host is to be expected, since multiplication is dependent upon normal metabolism of the host.

Inorganic ions. The data presented in Table 3 indicate that ammonium chloride, sodium chloride, lithium chloride,

magnesium chloride, and potassium chloride all inhibit lysis of the host in 0.05 N concentration. Perhaps the best inhibitors of Actinophage 514-3 in the group studied are sodium chloride and potassium chloride, since these salts caused the least deviation of growth from the control. Ammonium chloride, while causing inhibition of lysis, also caused some inhibition of the host. Lithium chloride and especially magnesium chloride caused marked inhibition of host growth. The addition of phage to these latter cultures increased growth of the host though lysis was inhibited.

THE LYSOGENICITY OF PHAGE-RESISTANT CULTURES OF STREPTOMYCES GRISEUS 3475

A study of the data recorded in Table 4 attests to the lysogenicity of phage-resistant cultures. When subcultures of phage-resistant cultures of S. griseus were incubated for 96 hours at 36°C., the supernate contained phage capable of causing the lysis of the parent host. The supernate from a similar culture of the parent host contained no such lytic factor. The resistant culture is not lysed by either the new phage or the parent Actinophage 514-3.

TABLE 1

The effect of hydrogen-ion concentration on the time of host lysis.

pH	Time of lysis
6.8	2 hours 35 minutes
7.1	2 " 50 "
7.5	3 " 15 "
7.8	Greater than 5 hours

TABLE 2

The effect of incubation temperature on the time of host lysis.

Temperature	Time of lysis
30°C.	3 hours
33°C.	2 " 15 minutes
36°C.	2 "
39°C.	4 " 25 "

TABLE 3

The effect of inorganic ions on lysis of the host.

Compound (0.05 N)	Relative growth*		Time of lysis
	+ phage	- phage	
Control	57	100	2 hours 30 minutes
NH_4 Cl	84	86	No lysis
Na Cl	90	93	" "
Li Cl	83	73	" "
Mg Cl_2	74	43	" "
K Cl	91	87	" "

*Control growth without added phage or inhibitors set at 100.

TABLE 4

The effect of culture supernate on resistant and susceptible strains of *Streptomyces griseus*.

Lytic agent	Indicator strain	
	S. griseus 3475	*S. griseus* (resistant)
Actinophage 514-3	Lysis	No lysis
Culture supernate from *S. griseus* 3475	No lysis	No lysis
Culture supernate from *S. griseus* (resistant)	Lysis	No lysis

DISCUSSION

The cultivation of Streptomyces griseus mycelium yielding large numbers of viable spores for turbidimetric studies presented certain difficulties. Repeated transfer of cultures tended to cause the cultures to become asporogenous. This characteristic was pronounced when 1.5 per cent agar was used in the spore slants. By increasing the agar content to three per cent, a drier agar surface was obtained which appeared to favor sporulation. While the use of three per cent agar increased several times the number of transfers possible before the cultures became asporogenous, it was often necessary to return to soil stocks to obtain fresh sporogenous cultures.

The turbidimetric approach to a study of the S. griseus host-virus system has overcome many problems inherent in a study of a filamentous micro-organism. Since the Streptomyces grow by extension of their mycelial filaments instead of by binary fission, it would be impossible to determine increase or decrease in growth by the standard plating procedures. By starting with a viable spore suspension, the increase in turbidity of the suspension serves as a valid indication of growth. Reference to Figure 2 indicates that germination of the spores begins shortly after three hours. By five and one-half hours, the rate of mycelial growth has become constant and continues so

for about two hours. After this time, the rate of growth apparently begins to decrease. However, the fact that the optical density of the suspension decreases does not necessarily mean that growth is not continuing at a constant rate. As the mycelium develops, considerable aggregation of the suspended filaments occurs resulting in an actual decrease in optical density. Were experiments run for periods longer than ten hours, this aggregation would be cause for criticism of the turbidimetric method of determining cell growth. Since most of the experiments here reported were completed in five or six hours, the method was felt to be satisfactory.

The rate of adsorption of phage to the host mycelium indicated in Figure 5 is very slow when compared to other bacteriophage systems. As mentioned earlier, coliphage particles are almost completely adsorbed to the host in ten minutes. The reason for the slow rate of actinophage adsorption has not been explained. Perhaps certain essential adsorption co-factors are absent or are present in limited quantities. Since the rate of adsorption is slow, it is difficult to explain why a burst was not observed at 90 to 120 minutes in the experiment illustrated in Figure 6. Were the rate of adsorption as slow as has been indicated, the amount of adsorption of liberated phage should have been small and an increase in free phage easily detected. There are two observations that may explain this

paradox. The amount of phage released is apparently small. When phage was added in a phage-spore ratio of 200 to 1, an increase in phage of only ten times was found following complete lysis of the mycelium. This increase is small enough to remain undetected by the plaque-count assay method if lysis occurred over a long period. That phage is probably liberated over a long interval is indicated by the lysis curve presented in Figure 7. While the shortest lysis time indicated was 90 minutes, lysis continued to occur for five hours. This might be expected since the rate of adsorption (initiation of infection) is so slow. Only a small portion of the mycelium is infected in the first ten minutes and infection continues slowly thereafter. In this manner, infection is a continuous process and a continuous process of lysis and phage liberation must follow. While the turbidimetric approach has proven useful in the determination of an approximate latent period for Actinophage 514-3, it must be emphasized that the time when the rate of growth becomes zero does not show the exact latent period of the phage. It can only be said that the latent period must be shorter than the indicated time of host lysis. The addition of smaller phage-spore ratios of phage would be expected to prolong the time of lysis of the host, since the rate of adsorption of phage is proportional to the concentrations of phage and host.

The morphology of Actinophage 514-3 illustrated in Plate 2 is of interest. This phage is extremely large having an over-all length of about 455 millimicrons. It may be wondered how a phage approaching the dimensions of the smaller bacteria can pass a filter designed for the retention of bacteria. A possible explanation is that bacteria are retained due to the charge on the asbestos filter. The phage protein may have an isoelectric point at a pH where it possesses the same net charge as the filter. Were this the case, it could pass through the filter while bacteria would be retained.

The inhibitory effect of certain electrolytes on the lysis of the host is not yet explained. It is probably due to prevention of phage adsorption by an ionic charge effect. Of interest is the fact that the inhibition of host growth caused by lithium chloride and especially magnesium chloride can be decreased by the addition of phage. This is probably due to combination of the relatively high concentration of added phage with the electrolyte causing effective removal of the inhibitor. Were such the case, the electrolytes may have their inhibitory effect on lysis by inactivating the adsorption mechanism of the phage.

The observation that phage-resistant cultures of S. griseus may be lysogenic is of interest, since the use of phage-resistant cultures has been one of the means used in preventing phage attack in industry. Such a practice may

cause the incorporation of phage into the fermentation beers.

SUMMARY AND CONCLUSIONS

A turbidimetric method of studying the multiplication of a phage infecting Streptomyces griseus has been described. It was found that the rate of adsorption of Actinophage 514-3 is extremely slow and that its latent period is shorter than 90 minutes. Lithium chloride, potassium chloride, sodium chloride, ammonium chloride, and magnesium chloride were found to inhibit the multiplication of the phage in concentrations of 0.05 N. Sodium chloride and potassium chloride were found to be the best inhibitors in the group studied. The use of such electrolytes may serve as an effective means of control of infection of Streptomyces griseus by phage.

Actinophage 514-3 has been found to be larger than most bacterial viruses.

Evidence has been reported that indicates that phage-resistant host cultures may be lysogenic.

The factors influencing the rate of phage adsorption and the mechanism of lysis inhibition by electrolytes certainly require further investigation.

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