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AN ABSTRACT OF THE THESIS OF Shirley Phillips Schnider for the
Master of Science in Biology presented May 23, 1969.

Title: The Biological Properties of Pseudomonas aeruginosa
Bacteriophage 7V.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

Earl Fisher, Jr., Chairman

W. Herman Taylor

John W. Myers

The present study was undertaken to define the standard conditions for growth of bacteriophage 7V on its host Pseudomonas aeruginosa strain PS-7 and to determine the factors which affect the quantity and quality of plaques in the plaque count assay. Observations from the single-step growth experiment and single-burst experiment are also included.

Plaque count assays were performed under various environmental conditions. Conditions were selected as "standard" if they yielded: 1) relative maximum number of infective centers per ml of stock 7V phage, 2) clear, haloed plaques at least 2.0 mm in diameter, and 3) reproducible assays limited only by the sampling error.

These conditions are:

1. fresh NBYE or NBYE agar for the growth medium
2. NBYE or buffered salts solution for diluent
3. physiologically young cells in the log phase between $1-5 \times 10^8$ bacteria/ml
4. stock and diluted stock suspensions stored at refrigerator temperatures.

Adsorption rate experiments which measured both unadsorbed phage and infective centers were performed in minimal media, minimal media supplemented with organic and ionic cofactors, and complete media. Although overnight lysates of PS-7 in minimal media produced a high titer of phage, the rate of adsorption of phage 7V in PS-7 was extremely slow in minimal media. Addition of tryptophan caused a decrease in free phage without a corresponding increase in infective centers. Casamino acids plus tryptophan caused an increase in the velocity of the adsorption reaction which was less than the rate of adsorption of phage 7V to its host PS-7 in NBYE. In NBYE 90 percent of the initial phage were adsorbed in 5 minutes, but the recovery of phage as free phage or infective centers was not equal to the input of phage.

These results suggest that this system requires a cofactor, organic, ionic, or both, in order that adsorption of phage 7V to its host PS-7 proceed at a maximum rate. And it further suggests that the incidence of abortive infection in this system is high. In this particular system under standard conditions it appears that the size of the plaques is controlled mainly by environmental factors, while the relative number of plaques is a characteristic of the system.

THE BIOLOGICAL PROPERTIES OF
PSEUDOMONAS AERUGINOSA BACTERIOPHAGE 7V

by

SHIRLEY PHILLIPS SCHNIDER

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
1969

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May 23, 1969

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INTRODUCTION

In 1953 Lwoff asked, "whether or not the characters, temperate and virulent, are correlated with specific properties other than lysogenization" (Lwoff, 1953). A virulent phage which differs from a temperate phage by one, or a few mutational steps, must also differ from this phage in the structure of at most a few cistrons and, therefore, in some recognizable morphological or biochemical characteristics.

Ten years later Feary (1963) reported the isolation of a bacteriophage, designated 7V, which he postulated arose as a spontaneous mutation, or series of mutations, of a temperate phage lysogenic for strain PS-7 of Pseudomonas aeruginosa. The temperate phage which gave rise to phage 7V was not isolated, probably because of the lack of sensitive indicator strains. On agar plates seeded with PS-7, phage 7V produces clear, haloed plaques with no bacterial growth in the center, indicating that phage 7V is a virulent mutant unable to lysogenize its host PS-7 (Stent, 1963). In addition to plaque morphology, phage 7V also resembles the supervirulent T-2 phage in size, morphology, and chemical composition (Feary, 1963).

Coliphage T-2 is not lysogenic. Upon entry into the bacterial cell, it causes disintegration and dispersal of the bacterial DNA within the first few minutes (Stent, 1963). Other virulent mutants of K12 λ have low probabilities of incorporation as prophage into the bacterial genome. K12 λ c, for instance, allows bacterial growth after infection and is virulent because it fails to produce repressor.

(Stent, 1963).

Phage 7V may resemble the supervirulent coliphage T-2 in its means of perpetuation; or, although physically similar to T-2, it may resemble the moderate virulent K12 λ + phage. Should phage 7V prove to be a supervirulent mutant, it would be a means of testing the generality of the models proposed on the basis of work done with the coliphage, and it may provide the means of determining virulence characteristics which have eluded study in the coliphage systems.

Since this is a new system, before any investigation can be undertaken a standard set of conditions must first be defined. The present study is an attempt to determine the essential qualities of the plaque count assay leading to: 1) the maximum number of infective centers, 2) large, clear haloed plaques, and 3) a reproducible assay limited only by the sampling error.

MATERIALS AND METHODS

Bacterial Culture. Pseudomonas aeruginosa strain PS-7 isolated from an ocular infection and maintained by serial transfer on trypticase soy blood agar was provided by Dr. Earl Fisher, Jr. It was grown in Difco nutrient broth supplemented with .3% Difco yeast extract, hereafter called NBYE; or the chemically defined media, M-9 with 1% glucose, described in Adams (1959). Agar plates were made by adding 1.5% Difco agar to the liquid media. In some experiments Difco Pseudomonas F agar or Pseudomonas P agar was substituted. The plates containing approximately 30 ml of agar media per plate were incubated at room temperature until all surface moisture had evaporated. Cultures of P. aeruginosa were always incubated at 37° C with active aeration.

Bacteriophage 7V Stock. A sample (0.1 ml) of an 18 hour culture of PS-7 was added to 50 ml of NBYE in a 125-ml erlenmeyer flask and the culture was incubated until the bacterial density reached $2-4 \times 10^8$ bacteria per ml. A previously prepared and accurately titered stock phage 7V was added to obtain a ratio of one phage for every 400 to 500 bacteria. The flask was mixed and incubated at 37° C with aeration for 5 minutes. Two ml of this phage-bacterial suspension was then added to 100 ml of NBYE in an 500-ml erlenmeyer flask warmed to 37° C. The mixture was incubated for 6 hours (Hook et al., 1946 and Flaks, 1959). A drop of chloroform was added and the suspension was vigorously mixed and then centrifuged at 16,300 X g for 10 minutes to remove debris. The lysate was then passed through a corning sintered glass filter to

remove the remaining particulate matter. Each step of the purification was assayed for plaque forming units by the soft agar overlay plaque count method, attributed to Gratia, and described in Adams (1959). The soft agar was prepared by adding 7 grams of Difco purified agar to 1 liter of distilled water. Another phage 7V stock was prepared as described using the M-9 defined media in the place of the NBYE media.

Assay of PS-7. A sample of a young broth culture of PS-7 growing in NBYE at 37° C with aeration was removed and an aliquot placed immediately in an ice bath. The turbidity of the culture was determined in a Bausch and Lomb spectronic 20 at 540 mμ wavelength. Samples of 0.1 ml each of several consecutive dilutions of the aliquot in the ice bath were mixed with 2.5 ml of soft agar and poured over the surface of an agar plate. Three plates per dilution were made. The plates were incubated at 37° C for 48 hours. The viable cell count and the optical density were correlated (Figure 1) and thereafter, under this standard set of cultural conditions, only turbidity measurements were made and the viable cell numbers estimated from the standard curve (Meynell and Meynell, 1965).

Preparation and Assay of Antiphage Sara. An antigen to elicit rabbit antiphage antibodies was prepared by further concentration and purification of the phage 7V stock prepared in NBYE. The filtered lysate was centrifuged at 54,000 X g for 90 minutes in a Spinco model L-50, washed twice in saline, and brought to a final concentration of 1.66×10^{10} plaque forming units per ml in saline. Three rabbits were injected intravenously with 0.5 cc. daily for 5 days, rested for 10 days, and then given a booster dose of 1.0 cc. daily for 2 days. One

Figure 1. Growth curve of *Pseudomonas aeruginosa* Ps-7 correlated with absorbance.

week later the animals were test bled. The serum assay described in Adams (1959) revealed a K value of 115 for rabbit # 1, 445 for rabbit #2, and 760 for rabbit #3; so the rabbits were bled out. Figure 2 is a graph of the kinetics of neutralization of phage 7V by two different dilutions of rabbit #3 antiphage serum. The graph shows that the equation $p/p_0 = e^{-kt/d}$ (where p/p_0 is the fraction of surviving phage, t the time, d the reciprocal of the dilution, and k is the rate constant) holds for the inactivation of 90 percent of the initial phage present, and that the value of K is dependent on the concentration of the serum. K increases as the dilution increases. A 1:800 dilution of rabbit #3 antiphage serum will inactivate about 99 percent of a sample of unadsorbed phage in 5 minutes.

Growth Studies. The assay of unadsorbed phage and the determination of infective centers were described in Adams (1959). It was determined from adsorption experiments with PS-7 grown in NBYE that 90 percent of a sample of phage 7V will be adsorbed in 5 minutes. The single-step growth curve and the single burst experiment were performed according to the protocol described in Adams (1959).

Determination of cofactor requirements in the adsorption of phage 7V to PS-7. Cofactors assayed were L-tryptophan $10\mu\text{g/ml}$, Difco vitamin free caseamino acids 1 mg/ml , 0.001 M calcium ions, $.085\text{ M}$ sodium ions. The cofactor to be assayed was incubated for 5 minutes at 37°C with an accurately titrated quantity of phage 7V diluted in M-9 with 1% glucose. The phage-cofactor mixture was then added to a known quantity of PS-7 grown in minimal media to a concentration of $1-5 \times 10^8$. The multiplicity of infection was .1 or less. This suspension of phage-

Figure 2. Kinetics of neutralization of bacteriophage 7V by two different dilutions of rabbit #3 anti-7V serum. 1:100 dilution of rabbit #3 anti-7V serum (●), 1:1000 dilution of rabbit #3 anti-7V serum (○).

cofactor and bacteria was incubated for 5 minutes at 37° C with active aeration and a sample then was removed and diluted 1:100 in minimal media. The sample was centrifuged at 4340 X g for 5 minutes. The number of infective centers in the pellet and the number of unadsorbed phage in the supernatant fluid were assayed by the plaque count method on M-9 with 1% glucose agar and M9YE agar. The entire procedure was completed in less than 23 minutes.

RESULTS

Definition of Standard Conditions. An accurate, reproducible plaque assay is the basis of all phage work. Repeated assays of phage 7V stock should give the same titer within the value statistically expected from the total number of plaques counted. Early in this investigation the observation was made that both quantity and quality of plaques produced was not constant. This study defines a standard set of conditions that produce: 1) a relative maximum of infective centers per ml of stock 7V phage, 2) clear, haloed plaques at least 2.0 mm in diameter, and 3) reproducible assays limited only by the sampling error.

Bronfenbrenner and Korb (1923) using a phage virulent for Shigella dysenteriae found that increased agar concentration reduced the number of plaques in the plaque count assay. Ellis and Delbruck (1948) found that increased agar concentration did not change the number of plaques but it did reduce the size of the plaques. It is evident from the data in Table I that both plaque numbers and plaque size were significantly decreased as the agar concentration increased due to loss of water.

As Bronfenbrenner and Korb postulated, the reduction in plaque forming units is probably due to insufficient supply of water to the host; but a reduction in plaque number and size as the NBYE agar plates aged could also be due to the breakdown of some of the essential bacterial metabolites.

Hershey, Kalmanson and Bronfenbrenner (1944) found that the

TABLE I
EFFECT OF AGE OF NBYE PLATES ON PLAQUE
NUMBER AND PLAQUE SIZE

	Age of Agar Plates		
	24 hours	1 week	2 weeks
Plaque count	107	94	72
($\times 10^8$)	89	93	56
	<u>90</u>	<u>96</u>	<u>57</u>
Average plaque count	95	94	62
($\times 10^8$)			
Average plaque size	2.0mm	1.2mm	<0.5mm

efficiency of plating varies with the concentration of electrolyte added to the agar. The ions of sodium, potassium, magnesium, chloride and phosphate are present in high concentration in the yeast extract (Meynell and Meynell, 1965). Therefore it is unlikely that they are present in limiting concentrations for phage multiplication. Calcium ions have been shown to affect many systems (Adams, 1959), and it may be present in limiting concentrations if this system has a specific calcium ion requirement. First it was necessary to determine the effect of varying concentrations of calcium ions on PS-7. The factors that affect the bacterium will obviously affect the multiplication of the phage and lysis of the host. Table II shows the results of an experiment designed to determine the effect of varying concentrations of calcium ion on PS-7 and on the number of plaque forming units per ml of stock 7V phage.

At a concentration of 10^{-2} M calcium ion reduces the number of viable bacteria per ml to approximately one-half. However, the number of plaque forming units is not significantly reduced at this concentration, but the plaque size is reduced. It appears from this data that if there is a specific calcium ion requirement by this system, the requirement is supplied in adequate amounts in .3% yeast extract because the efficiency of plating is not increased by the addition of calcium in the tested concentrations. If the multiplicity of infection is .1 or less, half the bacteria can be affected without changing the plaque count. The reduction in plaque size in 10^{-2} M Ca^{++} further indicates that it is the bacterial metabolism which is affected by high concentrations of Ca^{++} which in turn affects the burst size.

TABLE II

THE EFFECT OF VARYING CONCENTRATIONS OF CALCIUM IONS
ON THE NUMBER OF VIABLE BACTERIA PER ML
AND PLAQUE FORMING UNITS PER ML

Additions to .7% Soft Agar	Number of Viable Bacteria/ml X 10^8	Number of PFU/ml X 10^{10}	Average Plaque Size
No calcium	3.55 3.40 <u>3.24</u> 3.40	1.56 1.46 <u>1.64</u> 1.55	1.8mm
10^{-5} M Ca^{++}	3.50 3.30 <u>3.90</u> 3.60	1.56 1.47 <u>1.37</u> 1.47	1.7mm
10^{-4} M Ca^{++}	3.73 3.37 <u>3.05</u> 3.38	1.64 1.52 <u>1.51</u> 1.56	1.6mm
10^{-3} M Ca^{++}	3.33 2.64 <u>4.10</u> 3.36	1.52 1.42 <u>1.27</u> 1.40	1.6mm
10^{-2} M Ca^{++}	1.56 1.28 <u>1.54</u> 1.46	1.43 1.35 <u>1.30</u> 1.38	0.5mm
10^{-1} M Ca^{++}	0	0	0

In order to determine if calcium ions act as a competitive inhibitor of magnesium at concentrations of 10^{-2} M and higher, this same experiment was repeated using magnesium ion in the place of calcium ion. The results too were the same. At a concentration of 10^{-2} M magnesium ion reduced the number of viable bacteria per ml to approximately one-half, while the number of plaque forming units was not significantly reduced at this concentration.

The results of an experiment to determine the effects of temperature and dilution on the number of plaque forming units per ml are summarized in Table III.

TABLE III

THE EFFECT OF TEMPERATURE AND DILUTION ON THE PLAQUE FORMING ABILITY OF A STOCK 7V PHAGE IN NBYE

Temperature and Time	Undiluted Stock 7V Phage/ml	Stock 7V Phage, Diluted 10^{-7}
Refrigerated for 24 hours	121	117
	124	126
	125	124
	123×10^8 /ml	122×10^8 /ml
Room temperature for 24 hours	154	46
	175	41
	160	39
	163×10^7 /ml	42×10^8 /ml

Reproducible plaque count assays could be obtained from a single tube, if the diluted stock 7V phage was refrigerated immediately after dilution. Two weeks after the initial experiment, reproducible plaque count assays were obtained from the refrigerated diluted stock 7V phage.

When diluted stock 7V phage was held at room temperature or 37° C overnight, there was some inactivation of the phage.

Studies of the Adsorption of Phage 7V to PS-7. Phage adsorption can be described by the equation $-dp/dt = KBP$ where P is the number of phage particles remaining after a time t, B the concentration of bacterial cells in the adsorption mixture, and K is the adsorption rate constant expressed in ml per minute (Adams, 1959). This equation states that the rate of adsorption (the slope of the curve) is dependent upon the concentrations of bacteria and bacteriophage in the reaction mixture, and that the free phage decreases with the time allowed for adsorption. The adsorption of 95 percent of the phage population obey the kinetics of this reaction (Adams, 1959).

The kinetics of the adsorption of phage 7V to its host PS-7 are shown in Figure 3, a graph of a typical adsorption experiment in which unadsorbed phage were measured.

If PS-7 is assumed to have more than one adsorption site like the coliphage T-2 (Watson, 1950) and if the multiplicity of infection is low (in this case .2), the bacterial concentration can be considered constant. The reaction becomes a pseudo-first order reaction. The logarithm of the fraction of unadsorbed phage versus time should then give a straight line which will not flatten out because the bacterial surface cannot become saturated. As infection proceeds, the bacterial cell may be changed in such a way that its velocity constant is no longer the same and, therefore, as shown in figure 3, the velocity constant would be constantly changing. Figure 3 may also be the illustration of adsorption of phage to its host PS-7 by two distinct

Figure 3. Kinetics of the adsorption of phage 7V to its host Ps-7.

populations of phage with different adsorption rates. The data in Table IV points out the fact that the equation, $-dp/dt = KBP$, does not take into account biological properties of phage or bacteria such as the physiological state of the bacterial cells.

TABLE IV
KINETICS OF THE ADSORPTION OF BACTERIOPHAGE 7V TO ITS
HOST PS-7 AT VARIOUS DENSITIES OF BACTERIA
AND BACTERIOPHAGE

	Exp. #1	Exp. #2	Exp. #3
Phage concentration	$8.0 \times 10^6/\text{ml}$	$8.0 \times 10^6/\text{ml}$	$9.5 \times 10^6/\text{ml}$
Bacterial conc.	$6.25 \times 10^8/\text{ml}$	$1.5 \times 10^7/\text{ml}$	$1.7 \times 10^8/\text{ml}$
Percent initial phage adsorbed in 5 minutes	88.10%	89.75%	96.78%
Adsorption Rate ($-dp/dt$)	-1.41×10^6	-1.43×10^6	-1.79×10^6
K ml/min	$.68 \times 10^{-9}$	3.034×10^{-9}	3.06×10^{-9}

In the first two experiments, the concentration of phage is constant but the bacterial density is higher in Exp. #2; so the rate of adsorption in Experiment 1 should be greater than the rate in Experiment 2. But it is not. Schlesinger (Adams, 1959) showed that the adsorption rate of phage to living bacteria is 2.6 times faster than to heat killed bacteria. Since the PS-7 cells were in the stationary phase in Experiment 1, this may account for the reduced adsorption rate. The rate constant, K, reveals that for cells in the early logarithmic phase, the velocity of adsorption proceeds at a rate 38 times greater than for

cells in the stationary phase.

Under optimal conditions the adsorption of phage 7V to its host proceeds at a rapid rate. Ninety percent of the initial phage are adsorbed in 5 minutes.

The optimal conditions for adsorption depend not only on the condition of the bacterium, both physically and biochemically, but also on environmental factors. Puck, Garen and Cline (1951) concluded from their study of the effect of salt on coliphage adsorption that the first step in phage adsorption is the establishment of electrostatic bonds between phage and bacterium. The data in Table V shows the results of an experiment designed to determine the effect of .001 M calcium chloride and .085 M sodium chloride (ionic strength .003 and .085 respectively) on the adsorption rates of phage 7V to its host PS-7. The velocity constant, K, is used to characterize the adsorption rates.

TABLE V
EFFECT ON ADSORPTION RATE CONSTANT OF
CALCIUM AND SODIUM IONS

Additions to NBYE	Adsorption Rate Constant K in ml/min
None	3.06×10^{-9}
0.085 M NaCl	1.05×10^{-9}
0.001 M CaCl_2	1.00×10^{-9}

It is evident from the table that the optimal salt concentration for phage 7V adsorption is found in the NBYE because phage 7V is

adsorbed with maximal velocity to its host PS-7 in NBYE. The addition of .085 M sodium chloride or .001 M calcium chloride reduced the adsorption rate constant indicating that adsorption rate is a function of ionic concentration. Since the velocity constant is the same for both sodium and calcium ions, the action of the ions must be non-specific interference with the electrostatic bonds between phage and bacterium.

A comparison of the plating efficiency of phage 7V under various conditions revealed that when the plating bacteria were grown in minimal media, the number of plaques formed on NBYE + .5% NaCl agar was higher than the number of plaques formed on minimal agar (Table VI). To rule out the possibility that the plating bacteria grown in minimal media were so poorly nourished that they were unable to support the multiplication of adsorbed phage on minimal agar, plating bacteria were grown in NBYE + .5% NaCl to a concentration of $1-5 \times 10^8$ bacteria per ml, washed twice in minimal media, and resuspended to their original volume in minimal media. It is apparent from Table VI that the effect of the minimal media is not to inhibit the production of phage because of poor nutrition of the bacterial host.

Anderson (1945) working with the T-even phages of E. coli demonstrated that in chemically defined media the rate of adsorption of T-4 mutants to their host was extremely slow compared to their rate of adsorption in a complete media consisting of amino acids, purines, pyrimidines and vitamins. He further demonstrated that the addition of tryptophan to T-4r¹ in synthetic media or the addition of tryptophan + calcium ion to T-4r² in synthetic media increased the rate of adsorption

TABLE VI
COMPARISON OF PLAQUE COUNT ASSAY ON COMPLETE
AND CHEMICALLY DEFINED MEDIA

Growth Media for Strain PS-7	Minimal Agar Plates	NBYE .5% NaCl Agar Plates
Liquid minimal media	7.2 X 10 ⁹ 7.0 X 10 ⁹ <hr/> 7.1 X 10 ⁹	9.9 X 10 ⁹ 8.7 X 10 ⁹ 11.2 X 10 ⁹ <hr/> 9.9 X 10 ⁹
average plaque size	3.3mm	2.8mm
NBYE .5% NaCl	1.11 X 10 ¹⁰ 1.12 X 10 ¹⁰ 1.07 X 10 ¹⁰ <hr/> 1.08 X 10 ¹⁰	1.13 X 10 ¹⁰ 1.14 X 10 ¹⁰ 1.11 X 10 ¹⁰ <hr/> 1.13 X 10 ¹⁰
average plaque size	2.5mm	.55 to 3.15mm
NBYE .5% NaCl washed and resuspended in minimal media	6.5 X 10 ⁹ 3.7 X 10 ⁹ 4.3 X 10 ⁹ <hr/> 4.8 X 10 ⁹	8.0 X 10 ⁹ 6.9 X 10 ⁹ 6.5 X 10 ⁹ <hr/> 7.1 X 10 ⁹
average plaque size	3.3mm	2.8mm

to that observed in the complete media.

Table VII shows the results of experiments designed to determine the effect of cofactors on the adsorption of phage 7V in chemically defined minimal media.

There must be some factor present in NBYE, but absent in the minimal media, which increases the velocity of the adsorption reaction. This could be an organic cofactor or it could be a specific ion requirement or both. The addition of casamino acids and tryptophan increases somewhat the velocity of the reaction, but the maximum velocity observed in NBYE is not attained. The addition of tryptophan or tryptophan + calcium ions has no effect on the velocity of the adsorption reaction in minimal media as determined by the measurement of infective centers. Measurement of unadsorbed phage in these systems indicates that adsorption of phage to PS-7 is taking place. Tryptophan or tryptophan + calcium cause a disappearance of free phage with no corresponding increase in infective centers. Calcium ions, .001 M, alone do not produce this effect suggesting that tryptophan alone is responsible for the apparent increase in the velocity of the reaction. It seems that the phage is either injecting its DNA into the environment or after injecting its DNA into the host, the host behaves as if non-infected (phage disappear), or the host is killed—all examples of abortive infection (Lwoff, 1953).

Support for this view is the observation that under optimal conditions in NBYE, the input of phage is never equal to the number recovered as free phage plus infective centers. Further this difference is greater than can be accounted for by sampling error.

TABLE VII

EFFECT OF COFACTORS ON THE ADSORPTION RATE OF PHAGE 7V TO
ITS HOST PS-7 IN MINIMAL MEDIA

Cofactor Added to Minimal Media	Recovery of 7V		Input of 7V	Supernatant	Pellet	Total	Adsorption in 5 min Based on Infective Centers	Adsorption in 5 min Based on Free Phage	X ml/min
	Input of 7V	Supernatant							
None	6.7 X 10 ⁵	7.3 X 10 ⁵	4.8 X 10 ⁴	1.17 X 10 ⁶	7.15%	0	---	---	---
	9.5 X 10 ⁵	1.13 X 10 ⁶	4 X 10 ⁴	1.17 X 10 ⁶	4.21%	0	---	---	---
10µg Tryptophan/ml	9.5 X 10 ⁵	6.9 X 10 ⁵	5.5 X 10 ⁴	7.45 X 10 ⁵	5.78%	27.37%	.02 X 10 ⁻⁹		
10µg Tryptophan/ml .001 M Ca++	9.5 X 10 ⁵	6.3 X 10 ⁵	4 X 10 ⁴	6.7 X 10 ⁵	4.21%	33.69%	.14 X 10 ⁻⁹		
.001 M Ca++	9.5 X 10 ⁵	9.8 X 10 ⁵	5.5 X 10 ⁴	10.3 X 10 ⁵	5.78%	0	---		
.1% Caseinino Acids + 10µg Tryptophan/ml	6.7 X 10 ⁵	5.1 X 10 ⁵	1.37 X 10 ⁵	6.47 X 10 ⁵	20.44%	23.89	.26 X 10 ⁻⁹		
MBYE	9.5 X 10 ⁵	5.64 X 10 ⁴	---	---	---	94.07	3.06 X 10 ⁻⁹		
MBYE + .085% NaCl	9.5 X 10 ⁵	3.63 X 10 ⁵	3.94 X 10 ⁵	7.57 X 10 ⁵	41.47%	61.8	1.05 X 10 ⁻⁹		

These same results might also be observed if more than one phage had adsorbed to a bacterial cell. Since the multiplicity of infection was less than 0.002, only 8 percent of the bacterial population could have been multiply infected.

Since tryptophan was in contact with PS-7 for only a short time prior to measurements for free phage or infective centers, this helped to eliminate the possibility that tryptophan was metabolized to a product which inhibited phage adsorption (Delbruck, 1948).

It seems likely that these cofactors are necessary for adsorption and not for multiplication, since overnight lysates of PS-7 yield a high titer of phage 7V.

Determination of growth characteristics. Figure 4 is a typical one-step growth curve obtained following infection of PS-7 with phage 7V in NBYE; multiplicity of infection was 0.003. The latent period is 23 minutes and the rise period 10 minutes. Burst size determined in this manner averaged 7. This is quite low; an experiment to determine the burst size more accurately revealed an average burst size of 36.

Figure 4. Single-step growth curve with phage 7V on its host PS-7 in NBYE.

DISCUSSION

Since this is a new bacterium-bacteriophage system, a standard set of growth conditions must be defined. In the literature, "standard conditions" means that experiments are carried out using log-phase bacteria growing in nutrient broth plus 0.1 M sodium chloride at 37° C. These conditions imply recognition of the importance of nutrition and physiological state of the bacterial cell to the production of the maximum number of plaque forming units from a given stock phage. These conditions also indicate the importance of the ionic environment which is the most crucial factor in phage adsorption (Adams, 1959).

Ions can be required in all three stages of phage growth. Puck et al. (1951) postulated that the first step in phage adsorption is the establishment of electrostatic bonds. The requirement for ions in this reaction would be nonspecific. Dalbruck (1948) demonstrated that T-4 coliphage specifically requires calcium ions for adsorption. Adams (1959) has compiled a list of ions essential for multiplication of several bacteriophage but not their hosts. This study indicates that for the phage 7V-PS-7 system both specific and nonspecific ion requirements are best met in NBYE. No specific ion requirements could be demonstrated.

Standard conditions for phage 7V and its host PS-7 are defined as:

1. fresh NBYE or NBYE agar used for the growth medium
2. NBYE or buffered salts solution used for the diluent
3. physiologically young cells in the log-phase between $1-5 \times 10^8$ bacteria per ml
4. stock and diluted stock stored at refrigerator temperature.

Under standard conditions, the number of plaques produced should be a function of the number of phage adsorbed, if the multiplicity of infection is less than one. However, the number of unadsorbed phage plus the number of infective centers was shown to be less than the input of phage. That unrecoverable phage did not produce lysogenic clones was shown by the observation that clear, haloed plaques were consistently formed. The nature of the abortive infection was not investigated, but it would be worthwhile to determine the fate of the phage DNA since many dispositions are possible.

The size of the plaque produced under optimal conditions is a function of the velocity of adsorption as well as the burst size and latent period. Delbruck (1940) described plaque formation as a race between the multiplication of phage and the growth of the bacteria. Even in minimal media where the adsorption rate was shown to be extremely slow, the phage would have time to multiply before most of the bacteria reached the stationary phase. Under standard conditions the latent period is constant at 23 minutes and the average burst size is also constant although small. Therefore, the size of the plaques produced appears to be controlled by environmental conditions, while the relative number of plaques produced is a characteristic of the phage-bacterium system.

Finally it should be noted that when the plaque size varies from 2.5mm to less than 0.5mm on a crowded plate, many of the smaller plaques may be missed. Therefore, the quantity of plaques may remain relatively constant but appear to change drastically because of the quality of the plaques.

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