CHARACTERIZATION AND GENETIC STUDIES OF A BACTERIOPHAGE FOR *PSEUDOMONAS AERUGINOSA*

By

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CHAPTER I

INTRODUCTION

Bacteriophages consist of only a protein coat and a nucleic acid core of either DNA or RNA. Since they are the simplest replicating structures known, they have been extremely valuable as tools in the study of molecular genetics. Genetic studies with bacteriophages began twenty-five years ago with the discovery of recombination between mutants (Delbruck and Bailey, 1946; Hershey, 1946). The history of the development of phage genetics has been covered in specialized textbooks (Adams, 1959; Stent, 1963; Luria and Darnell, 1967), and collections of early papers (Stent, 1965; Taylor, 1965). Current work in the field has been thoroughly reviewed recently (Dove, 1968; Levine, 1969; Marvin and Hohn, 1969; Pratt, 1969; Radding, 1969; Calendar, 1970). Work specifically relevant to the present study will be described in Chapter IV.

From early mapping studies utilizing readily detectable markers such as plaque morphology and host range, the first map of three linkage groups (Hershey and Rotman, 1949) evolved into a single linkage group (Brenner, 1959). One of the plaque morphology mutants, rII, will not form plaques on Escherichia coli strain KB. Benzer (1955) was the first to recognize and exploit this characteristic which would allow detection of r^+ recombinants at a frequency as low as 10^{-8}. It was through the detailed analysis of this one gene that the nucleotide was

recognized as the smallest unit of information in DNA (Benzer, 1957; Nomura and Benzer, 1961).

While host range and rII mutants are conditional lethals in a sense, the term is generally used in reference to temperature-sensitive and suppressor-sensitive mutations. These mutants have the added advantage of being scattered throughout the genome (Epstein et al., 1963), and because of this quantitative mapping over the complete genome was possible. Mutants in essential functions are generally lethal and, therefore, are difficult to study. With the conditional lethals, it was possible to study these functions by using permissive or nonpermissive conditions. An important contribution resulting from these studies was the demonstration of nonrandom distribution of genes in phage T4. The grouping of functionally related genes has since been demonstrated in other phages (Dove, 1968; Calendar, 1970).

The rapid advances seen in bacteriophage genetics and molecular genetics were largely due to a concentrated study of a single group of closely related phages (T2, T4, and T6) whose host bacterium, E. coli, was already well studied. While a concentrated effort in a limited area can result in fantastic growth, the ability to make sound generalizations is also limited. Work with phage lambda was aided by earlier work with T4, but lambda was found to be quite different in many respects. Some of those differences have contributed greatly to molecular genetics. The ability of the lambda prophage (DNA) to coexist with the host cell by integrating into the host genome has offered a system for studying recombination (Campbell, 1962). The uneven distribution of GC pairs along the genome has allowed separations of the strands and of the shear-halves of the genome. These and other characteristics not

possessed by the T-phages may prove to be the rule rather than the exception among phages, but they are probably only evidence of a spectrum of variation.

While many other phages have been isolated and characterized morphologically (Bradley, 1967), nearly all the genetic studies have been done with phages for *E. coli*. As pointed out earlier, this was largely due to the fact that *E. coli* was being studied in many laboratories. Genetic studies of the genus *Pseudomonas* have been initiated more recently (de Ley, 1964; Stanier et al., 1966; Holloway, 1969). These studies have revealed the absence of clustering of functionally related genes. The clustering observed in *E. coli* served as the basis for the operon model of genetic control (Jacob and Monod, 1961), which could also be applied to most coliphage systems. It would be interesting to learn which type of genetic organization the phages for *Pseudomonas* have. The differences in the two bacterial genera can be rationalized by considering their very different ecological niches; however, since all phages multiply intracellularly and have restricted genetic capabilities, it would be more difficult to rationalize basic organizational differences involving efficiency of genetic control.

It is our hope that, beyond the information presented in this report, we can demonstrate the potential of this system for use in answering some of these questions, and thus encourage others to contribute to that search.

CHAPTER II

MATERIALS AND METHODS

Bacterial Cultures

The bacterial culture used in most of the experiments reported herein was *Pseudomonas aeruginosa* Holloway strain 1 (PA-1). Other cultures used, and their origins, are listed in Table I. All cultures were maintained by inoculating onto Difco nutrient agar slants, incubating at 37 C overnight and storing at 0-5 C.

The standard procedure for preparing liquid cultures was to wash a fresh nutrient agar slant (incubated overnight) with 2 ml of Difco nutrient broth and to transfer 0.02 ml of this suspension into 7 ml nutrient broth in a 20 mm culture tube. This was incubated at 37 C on a reciprocal shaker for the desired time.

Bacteriophage

The bacteriophage used throughout this study was isolated in this laboratory from the supernatant of a 48-hr nutrient broth culture of *P. aeruginosa* isolate OHC-12, using PA-1 as the indicator strain. Isolate OHC-12 was lost from the culture collection prior to the initiation of these studies. The bacteriophage was coded as Pφ-6, and this designation was used throughout to indicate the original phage as well as those derived from it. The code designation Pφ-6.0 was used to distinguish the parental phage from its mutants, which were coded as

TABLE I

ORIGINS OF BACTERIAL STRAINS

Source	Organisms			
Dr. Glenn Bulmer, Oklahoma Medical Research Center	*Pseudomonas aeruginosa* strains:			
	B-1	E-3	G-8	PHL-5
	B-2	E-4	G-9	PHL-7
	B-3	E-7	G-10	PHL-9
	B-4	E-8	G-12	PHL-10
	B-5	F-1	G-16	PHL-12
	B-6	F-5	H-4	PHL-14
	B-7	F-6	H-5	PHL-15
	B-8	F-8	H-7	SHD-3
	B-10	F-10	H-8	SHD-1293
	C-4	G-4	H-10	SHD-1341
	D-4	G-6	PHL-2	SHD-1371
	E-1	G-7	PHL-3	SHD-1375
Dr. Leon Unger, Oklahoma Medical Research Center	*Pseudomonas putida* C1-op			
Dr. Michael Doudoroff, University of California at Berkeley	*Pseudomonas testosteroni* PT-15 *Pseudomonas testosteroni* PT-16			
Dr. John Drake, University of Illinois	*Escherichia coli* B, Benzer			

Pφ-6tsn or Pφ-6cn for temperature-sensitive, or clear plaque type, respectively, with n being an arbitrary number to indicate individual isolates.

Media

Pseudomonas phage broth (PφB) contained 0.8 per cent Difco nutrient broth, 0.5 per cent Difco yeast extract, and 0.5 per cent NaCl in distilled water. The phage plating medium (PφA) contained 1.1 per cent Difco agar in PφB for the bottom layer and 0.65 per cent Difco agar in PφB for the top layer (Holloway et al., 1962).

Pseudomonas phage lactate agar (PφLA) contained 0.5 per cent NaCl, 0.1 per cent K_2HPO_4, 0.1 per cent $NH_4H_2PO_4$, 0.02 per cent $MgSO_4 \cdot 7H_2O$, 0.12 per cent sodium lactate (Fisher Scientific Co.), and either 1.1 per cent or 0.65 per cent Difco agar in distilled water (Sutter et al., 1963).

Coliphage broth (CφB) contained 0.8 per cent Difco nutrient broth and 0.5 per cent NaCl in distilled water. The phage plating medium (CφA) contained 1.1 per cent (bottom layer) or 0.65 per cent (top layer) Difco agar in CφB.

Saline soft agar (SSA) contained 0.5 per cent NaCl and 0.65 per cent Difco agar in distilled water.

Preparation and Titering of Phage Stocks

Phage stocks were prepared by a modification of the agar layer method reported by Swanstrom and Adams (1951). One drop of a liquid culture was mixed with 0.1 ml of a phage suspension (10^5 to 10^6 PFU/ml) in 3 ml of melted PφA (0.65 per cent agar) held at 47 C. This was

poured onto a plate containing 30 ml solidified PφA (1.1 per cent agar) and allowed to solidify. After 12-15 hr incubation, usually at 37 C, the phage were harvested by flooding each plate with 5 ml PφB. After 30 min, the broth was removed with a pipet, centrifuged to remove cells and cell debris, and filtered (Millipore, HA, 0.45 μm pore size).

Phage titers (PFU, plaque-forming units) were determined by the same plating technique. Preparations were diluted to give approximately 10^3 PFU per ml. PφLA or CφA was used for more easily counted plaques with SSA used for the overlay. Plaques were counted after 12-15 hr incubation.

Single-Step Growth Experiments

The protocol used for the single-step growth experiments is summarized below. Flask 1 was a 250 ml Erlenmeyer flask fitted with an 18 mm side-arm to facilitate optical density readings (Coleman, Model 6D). Flasks 2 through 5 were 50 ml Erlenmeyer flasks. Flask 1 was inoculated from an overnight liquid culture of PA-1. With approximately 8×10^7 cells per ml in flask 2, 73 per cent of the phage would be adsorbed within 5 min. The 1/2000 dilution of antiserum was calculated to inactivate 97 per cent of the free phage remaining in flask 3.

Time (min)	Flask	Procedure
-180	1	Add 0.1 ml cell suspension to 20 ml PφB
-5		Read $O.D._{540}$ of #1
-3		Make duplicate platings at 10^{-5} and 10^{-6} of #1
-1	2	Transfer 9.9 ml from #1
0		Add 0.1 ml phage suspension to #2

5	3	Transfer 1 ml from #2 into 9 ml of a 1/2000 dilution of anti-phage serum in nutrient broth
10	4	Dilute #3 10^{-2} and transfer 0.1 ml of the dilution to 9.9 ml nutrient broth
11	5	Transfer 0.1 ml from #4 into 9.9 ml nutrient broth
12-45	4)	Make duplicate platings with 0.05 ml per
30-90	5)	plate at 2-5 min intervals

Treatment of Phage with Ethyl Methanesulfonate

The method used for *in vitro* treatment of Pφ-6.0 with ethyl methanesulfonate was modified from Benzer (1961). A 0.2 ml volume of ethyl methanesulfonate was added to 9.7 ml of M-9 buffer and heated in a water bath at 37 C until dissolved (approximately 30 min was allowed). At zero time, 0.1 ml of Pφ-6.0 plate stock was added and incubation was continued at 37 C for 75 min. A 0.1 ml aliquot was removed and diluted into M-9 buffer. Dilutions were made and plated under conditions appropriate for selection of the mutants desired.

The M-9 buffer (Roberts et al., 1957) contained per liter of distilled water: 1.0 gm NH_4Cl, 6.0 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 5.0 gm NaCl and 0.1 gm $MgSO_4$.

Isolation of Temperature-Sensitive Mutants

Temperature-sensitive mutants of Pφ-6.0 were isolated by plating dilutions of ethyl methanesulfonate-treated phage in duplicate and incubating both sets of plates at 25 C. After 3 to 4 hr incubation,

one set of plates was transferred to 41 C and incubated for 12 to 15 hr. Small plaques were picked with a flamed and cooled inoculating needle, from the plates which had been incubated at 41 C. The needle was touched very lightly to the surface of the plaque, then touched to the surfaces of two plates pre-seeded with PA-1 in a soft agar overlay. Transfers were made to correspondingly numbered squares. One set of plates was incubated at 41 C, the other at 25 C. After 12-15 hr, those squares which showed lysis at 25 C, but not at 41 C, were used to inoculate a second set of duplicate plates, by transferring to corresponding squares as before. The second set of plates was incubated in the same way, and transfers were made from these to 2 ml of PφB, which was diluted, and plated for isolated plaques at 41 C and 25 C. Only those which produced no plaques at 41 C were used to make plate stocks. After plate stocks were made by the standard method, they were again tested for plaque formation at 41 C by titering at 25 C and 41 C. Those which produced plaques at 41 C were discarded.

Mapping of Temperature-Sensitive Mutants

A set of temperature-sensitive mutant stocks was made by dilution to approximately the same concentration to reduce the input variation from experiment to experiment. A 7-hr liquid culture of PA-1 was diluted to an $O.D._{540}$ of approximately 0.44, and platings were made on nutrient agar from a dilution of $1/4 \times 10^{-6}$ for colony counts. A 0.1 ml volume of each of two mutant phages was added to 3.7 ml of CφB in a 50 ml Erlenmeyer flask in a shaking water bath at 37 C. At zero time, 0.1 ml of the cell dilution was added to the phage mixture. Total

moi's (multiplicities of infection) varied from 7.9 to 10.5 at this concentration of cells.

After 10 min adsorption (approximately 63 per cent adsorbed), antiphage serum was added to a final dilution of $1/4 \times 10^{-3}$. This was calculated for inactivation of approximately 97 per cent of the free phage in 10 min. At 20 min, the mixture was diluted $1/4 \times 10^{-3}$ into 3.9 ml of nutrient broth in another 50 ml Erlenmeyer flask.

After 90 min, 5 drops of chloroform were added to each flask, and incubation was continued for an additional 10 min. The resulting lysates were diluted 10^{-2} for plating at 43 C, and 10^{-3} for plating at 37 C. Platings were made with _P. aeruginosa_ strain B-5 on CφA using SSA overlays. The plates were scored after 12-15 hr. The recombination frequency equalled twice the number of plaques at 43 C divided by the number of plaques at 37 C corrected for the difference in dilutions plated. This method allowed all six possible crosses of four mutants and the four controls to be included in a single experiment by using staggered sequences of 1-min intervals.

Lysis Inhibition

The method described by Doermann (1948) for demonstrating lysis inhibition was used with minor changes. The culture was grown in 20 ml of PφB in a 250 ml Erlenmeyer flask with an 18 mm side-arm to facilitate optical density readings. Incubation was at 37 C in a shaking water bath. When the $O.D._{540}$ reached 0.2 - 0.3, an appropriate dilution of phage was added. The $O.D._{540}$ was then read at 5-min intervals until lysis was complete. A Coleman spectrophotometer, Model 6-D, was used for all determinations of optical density.

CHAPTER III

RESULTS

Characterization of Phage Pφ-6.0

Host Range of Pφ-6.0

The host range of Pφ-6.0 was checked by spot-testing on various cultures maintained in this laboratory. Forty-eight of these had been tentatively identified as *P. aeruginosa*; 14 of these carried one or more phages to which PA-1 was sensitive. Three other species were also tested, two of which also belonged to the genus *Pseudomonas*. Table II lists the results which show that Pφ-6.0 has an intraspecies range of approximately 30 per cent of those tested and, on the basis of this limited test, it does exhibit species specificity.

Plaque Morphology

The plaques produced by Pφ-6.0 on PA-1 had clear centers surrounded by turbid halos. The halos varied in density and appearance in response to changes in the plating conditions. When plated on PφA with PA-1 at 37 C, the halos were often so dense that the plaques appeared to be small and clear without halos. Exposure to chloroform caused the halos to appear, indicating that the cells in this region were more sensitive to lysis by chloroform than were the cells in the surrounding lawn. When plated on PφA with PA-1 at 42 C, or when plated

TABLE II

SPOT TEST OF Pφ-6.0 HOST RANGE[1]

Lysis	No Lysis			No Growth
B-5	B-1	E-4	G-12	B-10
B-6	B-2	F-1	H-4	E-7
B-7	B-3	F-5	H-5	E-8
F-8	B-4	F-6	H-7	PHL-2
G-8	B-8	F-10	H-10	PHL-3
G-10	C-4	G-4	PHL-7	PHL-5
G-16	D-4	G-6	PHL-14	PHL-10
H-8	E-1	G-7	SHD-1341	PHL-12
PHL-9	E-3	G-9		PHL-15
SHD-1293	*P. testosteroni* PT-15		*P. putida* C1-op	SHD-3
SHD-1371	*P. testosteroni* PT-16		*E. coli* B, Benzer	SHD-1375

[1]*P. aeruginosa* strains unless specified otherwise.

with the *P. aeruginosa* isolate B-5 on PφA, the halos were absent or just slightly visible due to more complete lysis in the halo region.

Both PφLA and CφA were used for titering because of the reduced amount of cell growth relative to that on PφA, which made the scoring of plaques easier. PφLA, which was used throughout the characterization and clear plaque mutant studies, gave well-defined, uniform plaques whose halos (if present) were approximately half as dense as the surrounding lawn. CφA was used in the temperature-sensitive mutant studies when it was found that cell growth took much longer on PφLA at 25 C and 30 C. The halo density was intermediate between those on PφA and PφLA.

Since it did not affect plaque morphology, SSA was used for the soft agar overlays to avoid the necessity of having two different kinds of soft agar media tubes available. Its use also eliminated the problems of contamination during storage.

Factors Affecting Efficiency of Plating

To determine the optimal period of incubation for cultures of PA-1 to be used in experiments with Pφ-6, the efficiency of plating for Pφ-6 on cultures of PA-1 of different ages was determined. The initial test involved duplicate platings of Pφ-6.0, Pφ-6c1, and Pφ-6c10 with liquid (nutrient broth) cultures of PA-1 grown from a standard inoculum for 4-, 7-, and 10-hr at 37 C with aeration. Table III shows that, with the three phages tested, the 7-hr culture gave the maximum number of plaques. Since the pattern was similar for all three phages, Pφ-6.0 alone was tested with 6-, 7-, and 8-hr cultures. As shown in Table IV, the 7-hr culture again gave the maximum plating efficiency.

TABLE III

EFFICIENCY OF PLATING ON FOUR-, SEVEN-, AND TEN-HOUR CULTURES

Phage	Pφ-6.0			Pφ-6c1			Pφ-6c10		
Culture Age, Hr	4	7	10	4	7	10	4	7	10
Plaque Counts	132	182	165	71	81	71	69	85	75
	135	190	-	52	83	68	67	87	73
Totals	267	372	(330)	123	164	139	136	172	148
Efficiency	.72	1.00	.89	.75	1.00	.85	.79	1.00	.86

TABLE IV

EFFICIENCY OF PLATING ON SIX-, SEVEN-, AND EIGHT-HOUR CULTURES

Culture Age, Hr	6	7	8
Plaque Counts	340	389	345
	267	363	351
	331	350	339
	361	391	331
	330	364	342
	303	337	326
	340	347	346
	346	371	348
Totals	2618	2912	2728
Efficiency	.90	1.00	.94

Since Pφ-6.0 plaques were easier to score on B-5, it was desirable to check the efficiency of plating on B-5 relative to that on PA-1 to determine whether the two host strains could be used interchangeably. The procedure used was modified from the standard method for titering to reduce variation due to pipetting errors. For each culture, 1 ml of a 7-hr culture and 0.1 ml of phage suspension at approximately 5×10^4 PFU/ml were added to 80 ml of melted SSA at 47 C. An automatic syringe was used to distribute 3-ml volumes on solidified, dried CφA plates. Sixteen replicate plates were prepared for each culture. Half of each set was incubated at 30 C, and half at 37 C. Table V shows the results. A 2 X 2 factorial analysis of variance indicated no significant difference due to either treatment or culture. There was an indication of interaction which was significant at the 5 per cent level. A closer look at the simple effects revealed that this significant interaction could be traced to the decreased plating efficiency on B-5 at 30 C relative to that at 37 C. It could also be seen that there was no significant difference in platings with PA-1 at 30 C or 37 C, or at 37 C with PA-1 or B-5. Consequently, isolate B-5 was used only for platings at the higher temperature and only when plaque morphology was not important.

Treatment with Chloroform

Chloroform is frequently used to insure release of all mature phage from infected cells. However, some phages are sensitive to chloroform and cannot be released by this method without loss of titer.

The effect of chloroform on Pφ-6.0 was tested by the following method: Eight plates were poured with PA-1 and Pφ-6.0 using the

TABLE V

COMPARISON OF PLATING EFFICIENCIES ON HOST STRAINS PA-1 AND B-5

Host Strain	PA-1		B-5	
Temperature	30 C	37 C	30 C	37 C
Plaque Counts	180	168	167	194
	167	181	151	210
	194	180	171	168
	188	172	178	190
	185	173	167	169
	179	171	161	179
	195	174	119	172
	177	177	200	179
Totals	1465	1396	1314	1461

Treatment Totals

	PA-1	B-5	Totals
30 C	1465	1314	2779
37 C	1396	1461	2857
Totals	2861	2775	5636

Analysis of Variance

Source	df	Sum Square	Mean Square	F
Treatments	3	1879		
Host Strain	1	231	231	1.08
Temperature	1	190	190	<1
Interaction	1	1458	1458	6.82*
Error	28	5986	214	
Total	31	7865		

Treatment Comparison	df	Mean Square	F
Between Host Strains at 30 C	1	1425	6.66*
Between Host Strains at 37 C	1	239	1.12
Between Temperatures on PA-1	1	298	1.39
Between Temperatures on B-5	1	1351	6.31*

standard overlay technique for preparing phage stocks. Four of the plates were harvested in the usual way, but the other four were harvested with PφB saturated with chloroform. Following centrifugation and membrane filtration, the two plate stocks were stored at 0-5 C for 10 days before titering. Titers were determined by pouring six plates with a dilution of 5×10^9 for each plate stock. The plates were incubated overnight with half of each set at 37 C and half at 25 C.

As shown in Table VI, the chloroform treatment increased the titer of the plate stock obtained by approximately 55 per cent. The absence of a significant difference in plating efficiencies at the two temperatures is evidence that the phage proteins were not made temperature-sensitive by chloroform treatment.

Centrifugal Force Required for Sedimentation of Pφ-6

Following treatment for two hours with 30 μg/ml each RNase and DNase, 10-ml volumes of a Pφ-6 plate stock (2.6×10^{11} PFU/ml) were centrifuged at three different speeds for 60 min in an RC-2B Sorvall, using an angle head rotor. The pellets were resuspended by covering with 1-ml volumes of potassium phosphate buffer (0.05 M pH 7.2) and allowing them to stand overnight at 0-5 C. The per cent of the initial infectivity recovered at each speed is listed in Table VII along with the concentration factors for the resuspended pellets.

Adsorption Rate Constant

The adsorption rate for Pφ-6 on PA-1 was determined by measuring free phage remaining after exposure to cells for known intervals of time (Adams, 1959). Since centrifugation to remove cells and adsorbed

TABLE VI

EFFECT OF CHLOROFORM ON PHAGE Pφ-6 TITERS

Growth Temperature	25 C		37 C	
Eluant	PφB	PφB+$CHCl_3$	PφB	PφB+$CHCl_3$
Plaque Counts at 5×10^{-9}	158	226	145	259
	155	236	124	223
	168	258	154	198
Totals	481	720	423	680

Treatment Totals

	25 C	37 C	Totals
PφB	481	423	904
PφB+$CHCl_3$	720	680	1400
Totals	1201	1103	2304

Analysis of Variance

Source	df	Sum Square	Mean Square	F
Treatments	3	21,329		
Temperature	1	800	800	2.14
Eluant	1	20,501	20,501	55.00**
Interaction	1	28	28	<1
Error	8	2,983	373	
Total	11	24,312		

TABLE VII

CONCENTRATION OF PHAGE Pφ-6 BY CENTRIFUGATION

R.P.M. X 10^{-3}	R.C.F.	Fraction*	% Recovery of PFU	Concentration Factor
8	7,710	S	37	-
		P	56	5.6
10	12,100	S	26	-
		P	63	6.3
12	17,300	S	9	-
		P	78	7.8

*S is supernatant fluid, P is pellet.

phage gave unsatisfactory results, the free phage were removed from the adsorption mixture by membrane filtration of samples at given times. The zero time titer was determined from a sample filtered prior to addition of bacteria ($P_o = 1.32 \times 10^6$ PFU/ml). The concentration of cells was determined by plating dilutions on nutrient agar immediately before mixing phage and bacteria.

The adsorption mixture was incubated with aeration at 37 C in a shaking water bath. The plot of P/P_o vs. time of sampling is shown in Figure 1. The rate constant for adsorption was determined from the equation

$$K = \frac{2.3 \log P_o/P}{(B)\ (t)}$$

where B = cells/ml and t = time in min. The adsorption rate constant was calculated for the 5- and 10-min exposure times as follows:

$$K = \frac{2.3 \log 1320/127}{(1.4 \times 10^8)(5)} = 3.34 \times 10^{-9} \text{ ml/min}$$

$$K = \frac{2.3 \log 1320/15}{(1.4 \times 10^8)(10)} = 3.19 \times 10^{-9} \text{ ml/min}$$

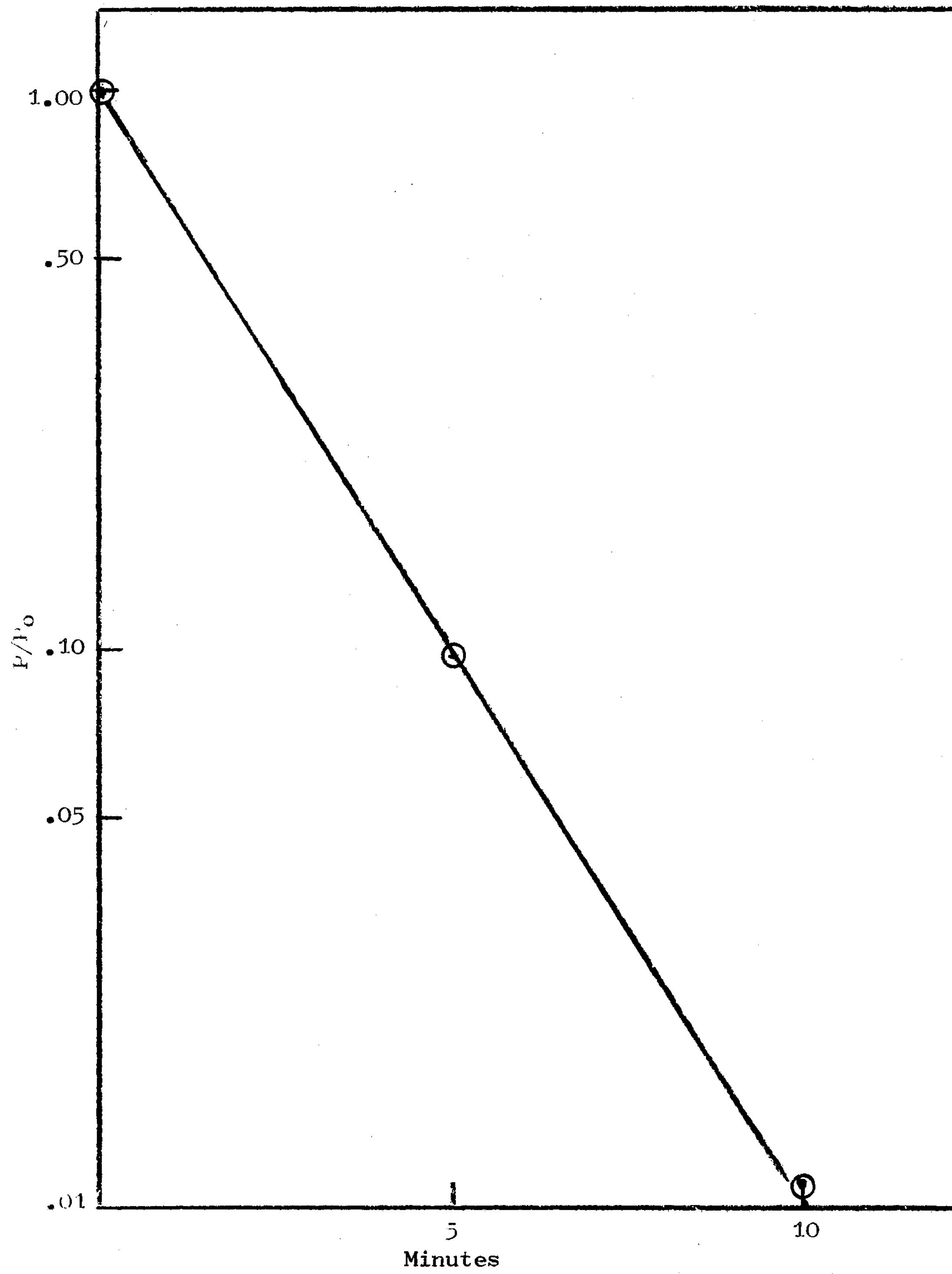
Average $K = 3.3 \times 10^{-9}$ ml/min

Rate Constant for Inactivation by Antiserum

Rabbit antiphage serum for Pφ-6 was prepared and kindly supplied by Floyd White of this laboratory. The K value was determined by the method described by Adams (1959). The serum was diluted 10^{-3} and 10^{-4} in PφB. A 0.9 ml aliquot of each dilution was placed in a 50 ml Erlenmeyer flask and prewarmed to 37 C in a shaking water bath. At

Figure 1. Adsorption Rate of Pφ-6 on PA-1

Adsorption was terminated at timed intervals by membrane filtration of samples. The filtrates were assayed to determine the unadsorbed phage titer. P/P_o is the ratio of the unadsorbed phage titer to the initial phage titer.


1.00
.50
.10
.05
.01
P/Po
5
10
Minutes

zero time, 0.1 ml of Pφ-6 at a concentration of 1.4 X 10^7 PFU/ml was added to each flask. At 2.5, 5, 10, 15, 20, and 30 min, 0.1 ml samples were removed and diluted into 9.9 ml PφB to stop the reaction. Duplicate platings were made using 0.1 ml aliquots from the dilution tubes, except for the 2.5 min sample which was also plated at an additional 10^{-1} dilution. The plates were incubated at 37 C overnight before scoring.

The K value was determined for each sample from the equation

$$P/P_o = e^{-Kdt}$$

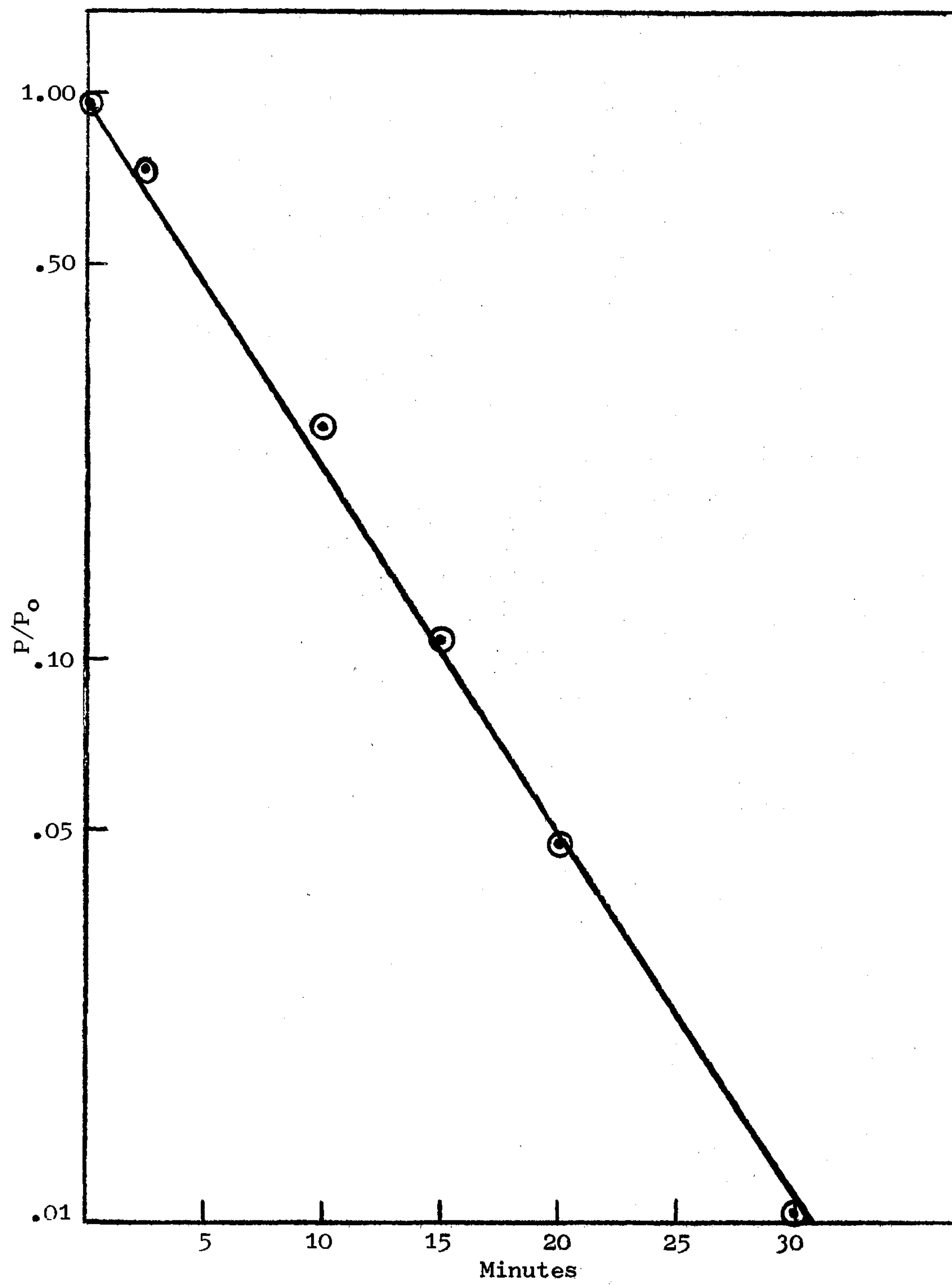
where P/P_o = the fraction of free phage remaining at time t, and d = the dilution of antiphage serum used. At a serum dilution of 10^{-3}, only the 2.5 min sample could be used, and it gave a K of 1.51 X 10^3/min. The K values from the 10^{-4} serum dilution were averaged to give K = 1.46 X 10^3/min. A plot of the inactivation, shown in Figure 2, indicates that the inactivation follows first-order reaction kinetics. This, in turn, indicates that Pφ-6 is inactivated by fixation of the antibody to a single critical site, as is the case for the T-even phages of *E. coli*, but not for all phages (Adams, 1959).

Nucleic Acid of Pφ-6

Phage Pφ-6 was stained with acridine orange according to Bradley's method (1966) for the identification of nucleic acid type by differential staining. A crude lysate was treated with DNase and RNase at concentrations of about 30 μg/ml each for 2 hr. Low speed (1000 X g) and high speed (17,300 X g) centrifugations in potassium phosphate buffer (0.05 M, pH 7.2) were used to purify the phage.

Figure 2. Inactivation of Pφ-6 With Antiserum

Anti-Pφ-6 serum was diluted 1/100 in PφB. Inactivation of phage was stopped at timed intervals by dilution of samples. Assay platings were made immediately. P/P_o is the ratio of the active phage titer to the initial phage titer.


1.00
.50
.10
.05
.01
P/P_o
5
10
15
20
25
30
Minutes

Small drops of the purified preparation were dried on microscope slides and fixed with Carnoy's fluid (1 part glacial acetic acid, 3 parts chloroform, and 6 parts ethanol) by immersion for 5 min at room temperature. The slides were washed briefly with absolute ethanol, and air-dried. They were then placed in 0.01 per cent acridine orange in buffer (6 parts 0.1 M citric acid: 4 parts 0.15 M Na_2HPO_4, pH 3.8) for 5 min. After brief rinsing in two separate baths of the same buffer at pH 3.8, they were soaked in 0.15 M Na_2HPO_4 solution for 15 min. Excess liquid was shaken off and the color was observed under an ultraviolet lamp (Mineralight UVS-n, Ultraviolet Products, San Gabriel, California). One slide was dipped continuously in and out of a one per cent molybdic acid solution in a dish under the UV lamp while observing for color changes (15 - 90 seconds for completion). Another slide was placed in a dish of 0.1 M tartaric acid and observed for color changes by UV without removal from the solution (2 -5 min for completion). By comparison with Bradley's results, as summarized in Table VIII, Pφ-6 appears to contain double-stranded DNA.

A 2.2 ml aliquot of a washed, concentrated Pφ-6 preparation was placed in a centrifuge tube, 1 ml of 5 M $NaClO_4$ was placed at the bottom of the tube, and 0.5 ml of 30 per cent sucrose was carefully layered onto the $NaClO_4$ (Freifelder, 1965). This was centrifuged at 44,000 X g for 120 min in a Spinco Model L centrifuge using a SW39L rotor. The perchlorate layer was removed with a Pasteur pipette. The diphenylamine assay (Burton, 1956) showed that it contained greaterthan 300 μg per ml of DNA. The DNA was also precipitated from the perchlorate by adding two volumes of 95 per cent ethanol and "spooling" (Marmur, 1961) onto a glass stirring rod. This was redissolved, as described by Marmur,

TABLE VIII

DIFFERENTIAL STAINING OF PHAGE NUCLEIC ACID

Nucleic Acid	Na_2HPO_4	MoO_3	Tartaric
2 - DNA*	Green	Green	Orange
2 - RNA*	Green	Green (fading)	Red
1 - DNA*	Red	Paler Green	Paler Green
1 - RNA*	Red	Paler Red	Paler Red
Pφ-6	Green	Green	Orange

*Bradley, 1966

and the final solution contained the DNA in 0.15 M NaCl, 0.015 M trisodium citrate. Hyperchromicity was demonstrated on a Cary 14 recording spectrophotometer. Heating followed by rapid cooling increased the absorption at 2600 Å from 0.133 to 0.163 (22.6 per cent increase), supporting the conclusion based on staining that the Pφ-6 nucleic acid was double-stranded.

Single-Step Growth Experiments

Several single-step growth experiments were carried out with Pφ-6 and PA-1 in PφB at 37 C. A curve showing the typical shape observed in all experiments is illustrated in Figure 3. Additional data from this and similar experiments are given in Table IX. The multiplicities of infection (moi) varied from 0.14 to 0.23, while the probability of cells being multiply infected varied from 0.01 to 0.05. In all four experiments, the latent period ended between 30 and 35 min. The burst sizes varied from 102 to 190, and appeared to be inversely related to the multiplicity of infection. With the higher multiplicities of infection used in the temperature-sensitive mutant crosses, the burst sizes varied from 89 to 282, and for the temperature-sensitive mutant controls, from 116 to 227.

Mutants of Phage Pφ-6

Types of Mutants Isolated

Eight temperature-sensitive (ts) mutants were isolated by the selection procedure previously described. One of these, Pφ-6ts7, was not used in these studies, since it did not yield sufficiently high titer stocks and artificial means of concentration would have been

Figure 3. Single-Step Growth of Pφ-6.0

Cells and phage were mixed at zero time. The phage were allowed to adsorb for 5 min at which time the phage-cell mixture was diluted into anti-Pφ-6 serum to inactivate the unadsorbed phage. At 10 min the complexes (infected cells) were diluted into growth flasks. The growth flasks were sampled at timed intervals and duplicate platings were made immediately to assay for infective centers (complexes and free phage).

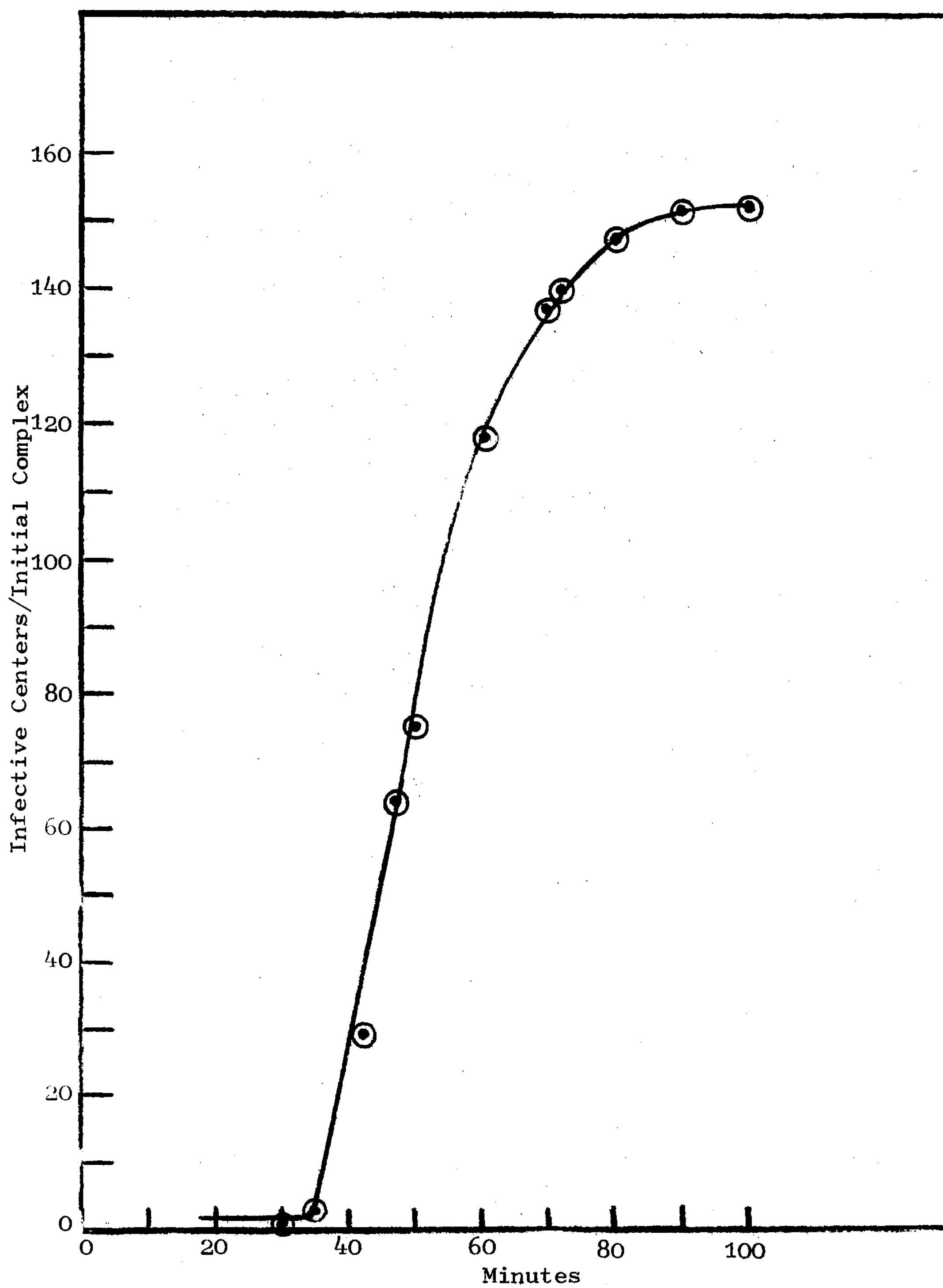

160
140
120
100
80
60
40
20
0
Infective Centers/Initial Complex
0
20
40
60
80
100
Minutes

TABLE IX

DATA FOR SINGLE-STEP GROWTH EXPERIMENTS

	Experiment Number			
	1	2	3	4
Cells/ml X 10^{-7}	3.3	4.7	8.4	15.1
M.O.I.	0.23	0.20	0.16	0.14
Phage/Cell, P(0)	0.79	0.82	0.85	0.87
Phage/Cell, P(1)	0.16	0.16	0.14	0.12
Phage/Cell, P(>1)	0.05	0.02	0.01	0.01
Burst Size	102	152	164	190

required. Although these mutants were originally isolated at a permissive temperature of 25 C and a restrictive temperature of 41 - 42 C, further studies were undertaken at the temperatures of 37 C and 43 - 44 C, respectively. The increase in restrictive temperature was necessary because of wide temperature fluctuations in the high temperature incubator; however, many platings were unacceptable due to temperature increases which restricted wild type plaque formation.

The earliest and most frequently observed morphological mutant type gave large clear plaques without halos when plated on PA-1. These plaques were of the same diameter as the halo of Pφ-6.0 plaques under the same plating conditions. When both types were present on the same plate, clear plaques were flat on edges adjacent to wild type plaques. Thirty-nine mutants of this morphological type were isolated from EMS-treated Pφ-6.0.

Other morphological mutants observed during the isolation of ts mutants included the following types: haloed at 43 C, haloed on B-5, haloed with very small, clear centers, chloroform-resistant halos, and minute plaques.

Attempts to isolate host range mutants gave inconsistent results. Further studies with this class of mutants were postponed until more information was available on resistance and lysogeny in this system.

Two of the above mutant types were selected for further investigation and for use in genetic studies. The clear plaque mutants were of particular interest, since they might be similar to either the clear plaque mutants characteristic of lysogenic systems, e.g., λ and P22, or the rapid lysis mutants of the T-even phage. The ts mutants were chosen for mapping because of the greater probability that they would be

randomly distributed over the genome and the potential for extremely high selection which would allow scoring of low frequency recombinants.

Host Range of Clear Plaque Mutants

Since plaque morphology is sensitive to variations in plating conditions, scoring of clear plaque mutant crosses would be subject to some error due to subjective evaluation. Clear plaque mutant crosses would also be less suitable for detecting low frequency recombination since both parental and recombinant plaques would appear together. These mutants would be more valuable as genetic markers if a system could be found which would allow selection against the mutants but not the wild type such as the rII mutants with *E. coli* K12(λ) or amber or ochre mutants with nonpermissive host strains.

In looking for such a system, the thirty-nine clear plaque mutants were spot-tested on the eleven strains of *P. aeruginosa* which were sensitive to Pφ-6.0 (see Table II). Nine of the mutants which appeared to differ from Pφ-6.0 in host range were checked for efficiency of plating on PA-1 and on the three isolates which appeared to give the greatest variation, B-5, B-7, and G-10. Table X shows that none of the mutant host cell combinations would allow scoring of only recombinant particles, i.e., none would provide 100 per cent exclusion of non-recombinant types.

Lysogenization Studies with Clear Plaque Mutants

Since Pφ-6 was derived from a bacterial culture by a method commonly used to isolate temperate phages, it was possible that the clear plaque mutants were comparable to the c mutants described for the

TABLE X

EOP OF C MUTANTS ON THREE HOST STRAINS RELATIVE TO PA-1

Mutant	Plaque Counts on PA-1	EOP on Cell Strain:		
		B-5	B-7	G-10
1	90	100	32	67
3	166	90	39	36
6	249	79	66	68
8	429	79	64	57
12	151	68	65	62
16	191	86	21	64
17	793	-	56	100
18	70	129	99	83
28	95	82	67	67

temperate phages λ and P22 by Kaiser (1957) and Levine (1957), respectively. This could be the case if the halos were due to lysogenization resulting in immunity during the later stages of plaque development.

Several attempts were made to isolate lysogenized cells from the halos of plaques. Those isolates which were still insensitive to Pφ-6 after a minimum of three sequential single colony isolations were tested for phage production by filtering 24-hr and 48-hr supernatants and plating these on PA-1 and by cross-streaking with PA-1. No lysis of PA-1 could be detected by either method for any of the isolates.

Of the twelve isolates tested, all had lost the ability to adsorb Pφ-6 at the wild type rate, indicating that they were probably resistant rather than immune.

Four of the isolates were tested on two different media for chemical induction of phage as described by Mayer (1969), but all gave results similar to the PA-1 control. The inducing agents tested and the results obtained are shown in Table XI. According to Mayer, induction of a prophage should result in formation of isolated plaques outside the zone of inhibition.

Two temperature-sensitive mutants, Pφ-6ts4 and Pφ-6ts6 (discussed later), were used to infect PA-1 in liquid cultures which were incubated in a shaking water bath at the restrictive temperature of 43 C. After 140 min and 300 min, respectively, 0.02 ml samples were transferred to 7 ml of fresh broth and incubated at 37 C for 12 hr. A series of transfers was made through nutrient broth, allowing growth at 37 C between transfers. After the initial inocula had been diluted by approximately 5×10^{-22}, the cells were removed by centrifugation.

TABLE XI

EFFECT OF CHEMICAL INDUCERS ON PA-1

Agent	Description of Effect
H_2O_2	Large, clear zone
HCl	Large, clear zone
NaCl	Inhibition of cell growth
Formaldehyde	Clear zone with isolated colonies
NaOH(5 N)	Clear zone
2-Aminopurine	Slight growth inhibition
5-Bromodeoxyuridine	Slight growth inhibition
Hydroxyurea	Slight growth inhibition
Nitrosoguanidine	Clear zone

Inducing agents were applied to the surface of seeded plates as crystals or as filter paper discs saturated with liquid. Effects of agents were essentially the same on PφA and PφLA. Usually there was a more pronounced effect on PφLA except with formaldehyde, which gave growth inhibition on PφLA, and a clear zone on PφA, Four cultures isolated from plaque halos were affected in the same way as PA-1 in all cases. No isolated plaques were observed in the area surrounding the inducing agent.

The supernatants were diluted and spotted on plates of PA-1 which were incubated overnight at 37 C. Both supernatants had phage titers of 10^3 to 10^4 PFU/ml.

The pellets were washed in nutrient broth and resuspended to the original volume; 0.02 ml was transferred to 7 ml of nutrient broth and incubated at 37 C for 12 hr. This procedure was repeated for both cultures. The cells harvested from the second cycle were washed, diluted, and plated with PA-1 to check for centered plaques. No plaques were found, although platings without PA-1 gave too many colonies to count.

The cultures described above were purified by streaking and picking isolated colonies, repeated through three isolations. When the pure cultures were grown in broth, their supernatants contained no phage when spot-tested on PA-1 as before.

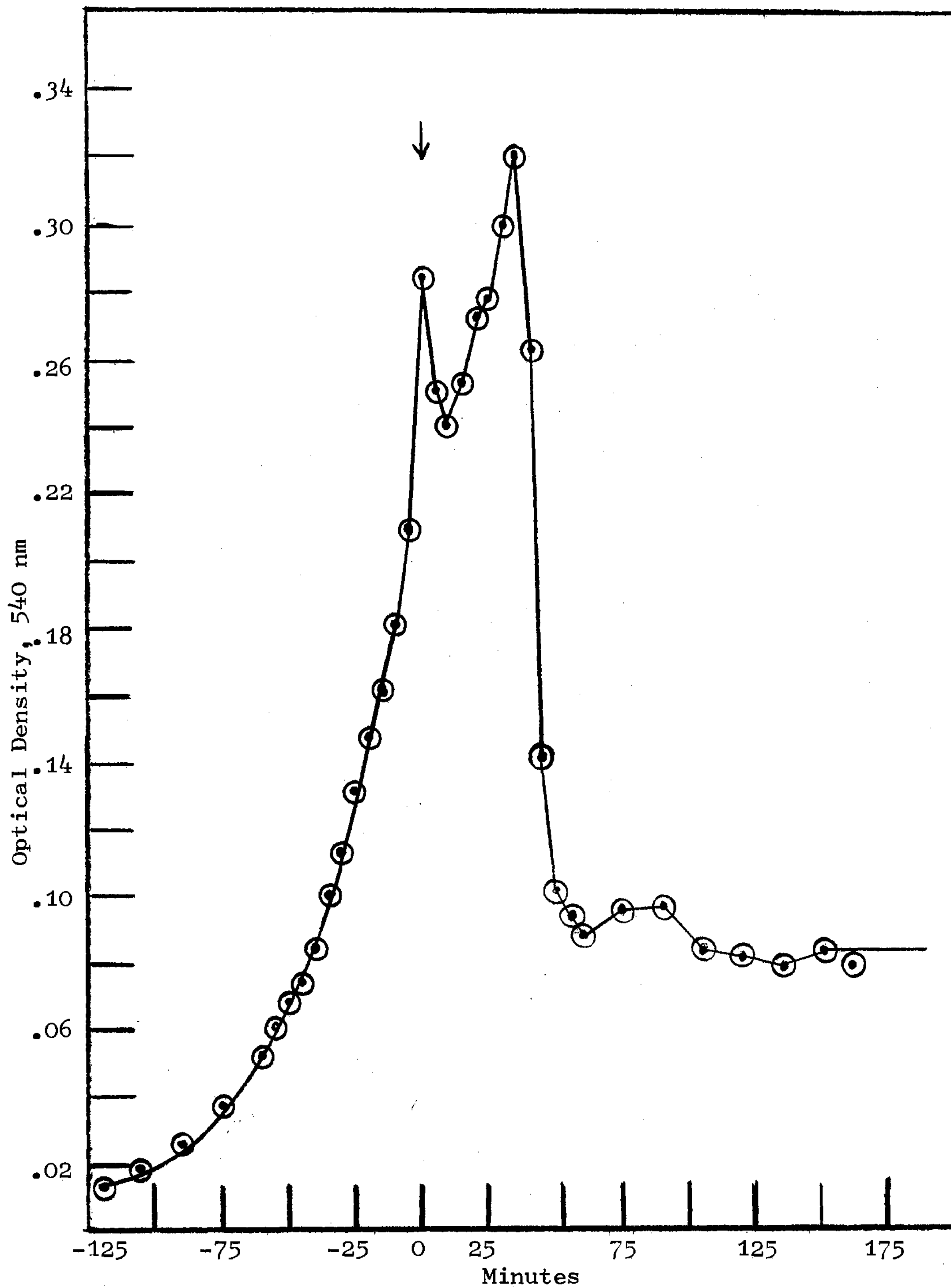
Lysis Inhibition Studies with Clear Plaque Mutants

Since the halos could not be shown to be due to lysogenization, and since the plaques resembled those of T-even phages on *Escherichia coli*, the possibility was considered that the clear plaque mutants were comparable to r mutants. Since r mutants result from the loss of ability to cause lysis inhibition, demonstration of lysis inhibition in Pφ-6.0-infected PA-1 was necessary.

Doermann's method of following the changes in turbidity of r- or r^+-infected cultures was employed (Doermann, 1948). Figure 4 shows that, with a moi of 2.2, Pφ-6.0-infected PA-1 gave a curve of lysis similar to those obtained from r-infected cultures (Doermann, 1948). Figures 5 through 7 show the results obtained when the moi was increased to 3 - 4, and secondary infections were made at 15, 25, and 30 min.

Figure 4. Turbidimetric Studies of PA-1 Following Infection With Pφ-6.0

A culture of PA-1 was grown in PφB in a side-arm flask. The optical density was read at the intervals shown. The arrow indicates the time of phage addition. The moi was 2.2.



.34
.30
.26
.22
.18
.14
.10
.06
.02
Optical Density, 540 nm
-125
-75
-25
0
25
75
125
175
Minutes

Figure 5. Turbidimetric Studies of PA-1 Following Infection With Pφ-6.0

This experiment was similar to that shown in Figure 4 except the moi was between 3 and 4 and a secondary infection was made at 15 min.

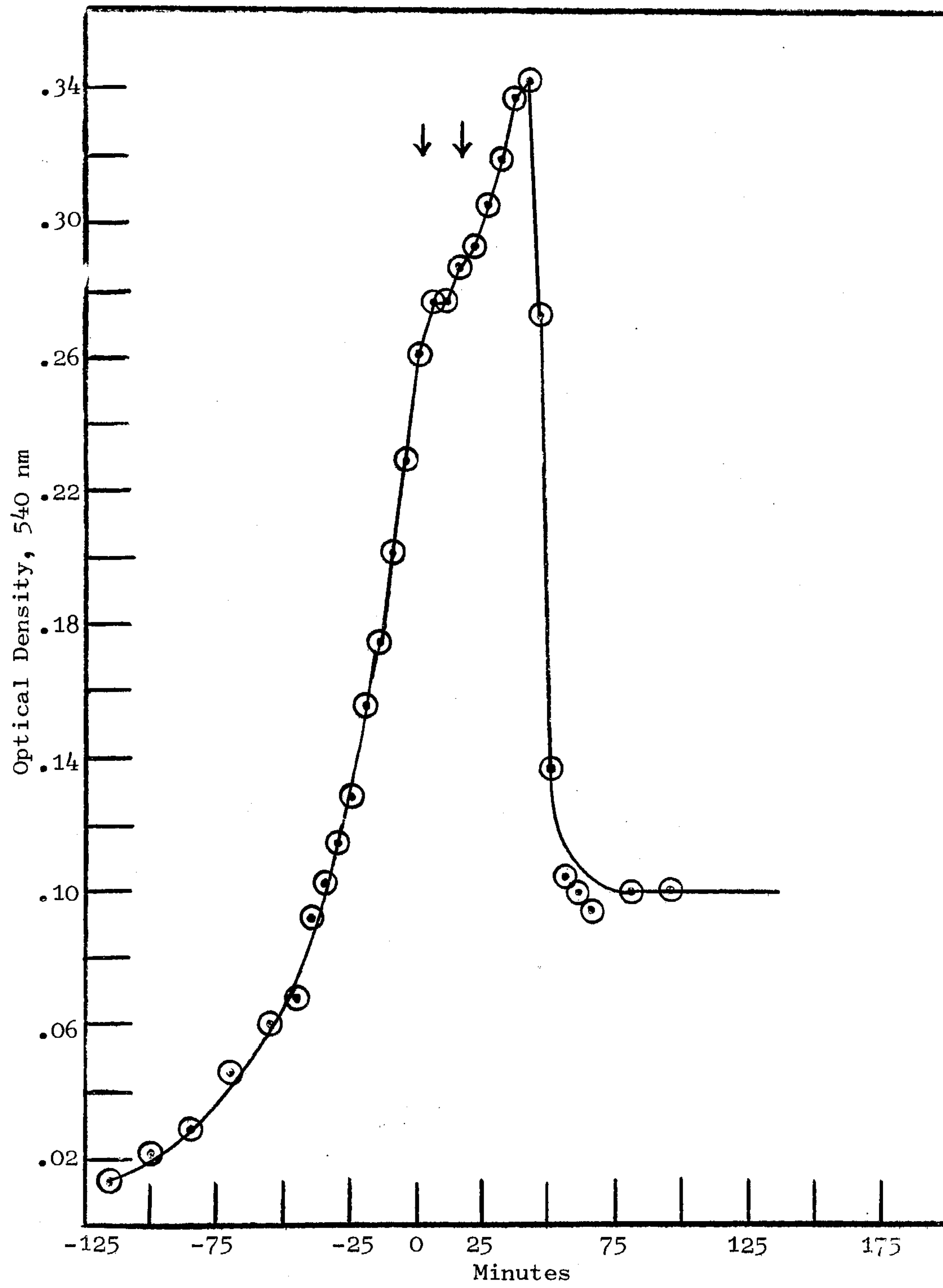
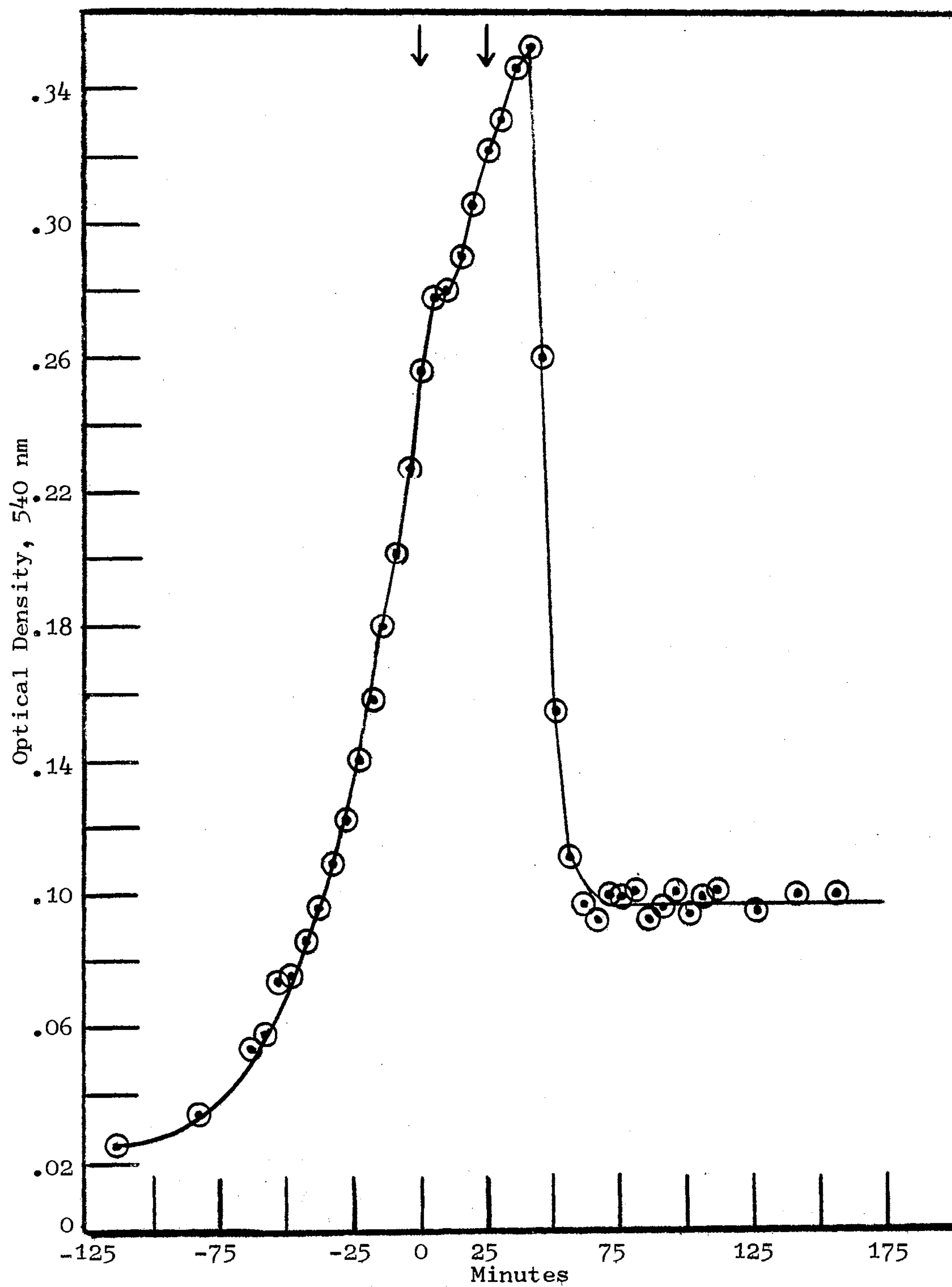

.34
.30
.26
.22
.18
.14
.10
.06
.02
Optical Density, 540 nm
-125
-75
-25
0
25
75
125
175
Minutes

Figure 6. Turbidimetric Studies of PA-1 Following Infection With Pφ-6.0

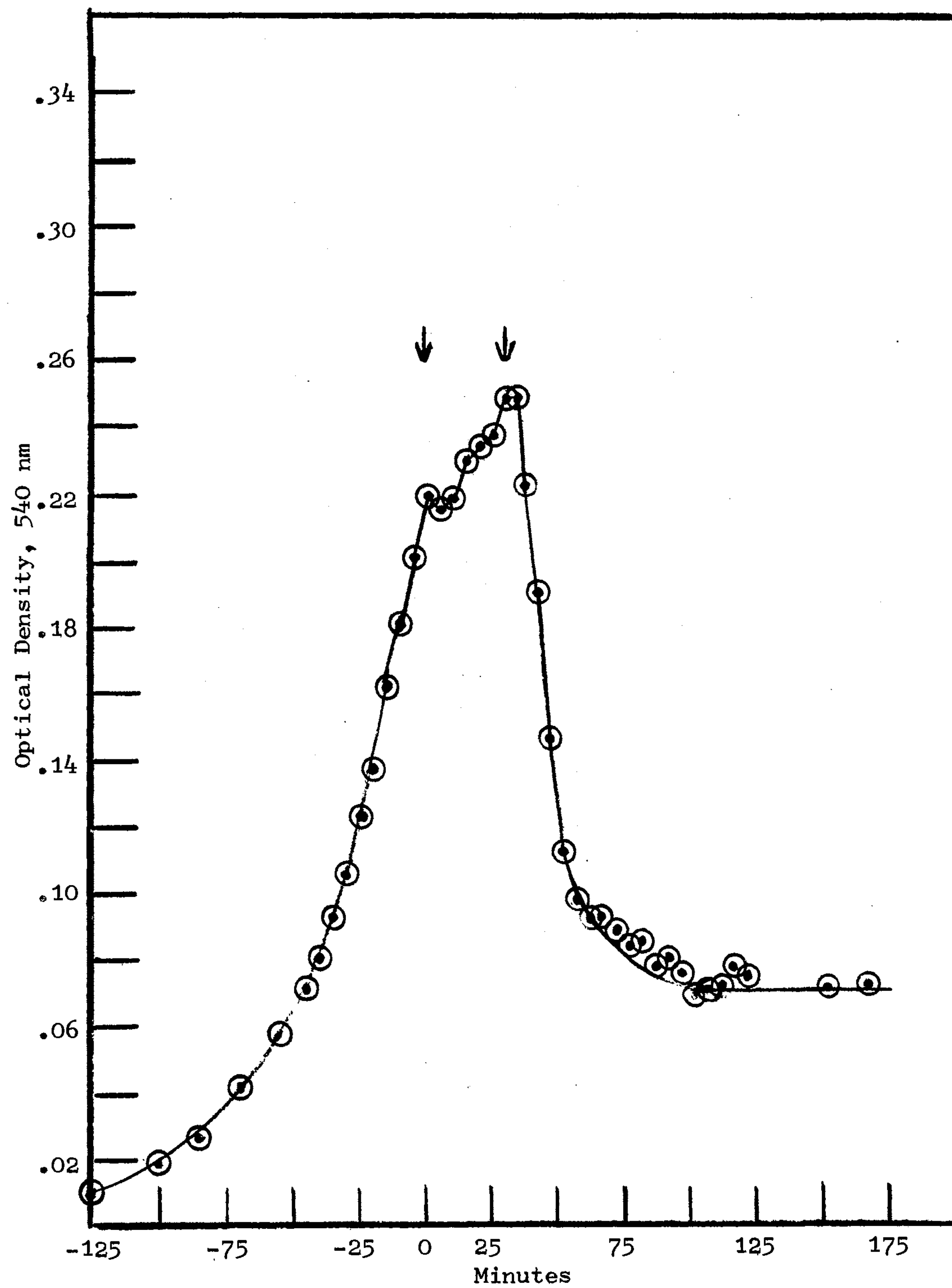
This experiment was similar to that shown in Figure 5 except the secondary infection was made at 25 min.



Optical Density, 540 nm
.34
.30
.26
.22
.18
.14
.10
.06
.02
0
-125
-75
-25
0
25
75
125
175
Minutes

Figure 7. Turbidimetric Studies of PA-1 Following Infection With Pφ-6.0

This experiment was similar to that shown in Figure 5 except the secondary infection was made at 30 min.



Optical Density, 540 nm
.34
.30
.26
.22
.18
.14
.10
.06
.02
-125
-75
-25
0
25
75
125
175
Minutes

Although lysis began 10 min later in each of these cultures, the pattern remained the same. Infection of PA-1 with Pφ-6c5 gave a pattern identical to that obtained with the Pφ-6.0-infected culture (see Figure 7). Increasing the moi to 12 with a secondary infection at 10 min had no effect on either the time or pattern of lysis when compared to the other cultures with secondary infections (see Figure 8).

Single-Step Growth of Clear Plaque Mutants

The clear plaque mutant Pφ-6c1 was used in a single-step growth experiment (see Figure 9). The procedure was the same as that used for the wild type phage. The cell concentration was 5.4×10^7/ml and the multiplicity of infection was 0.25. The burst size (109) and the length of the latent period were similar to those for Pφ-6.0. However, the shape of the curve was quite different (cf. Figure 3). Cells infected with Pφ-6c1 lysed over a much shorter time interval, as evidenced by the sharply defined break in the curve from rise to plateau. This is in contrast to the rounded, slow break observed in all experiments with the wild type phage.

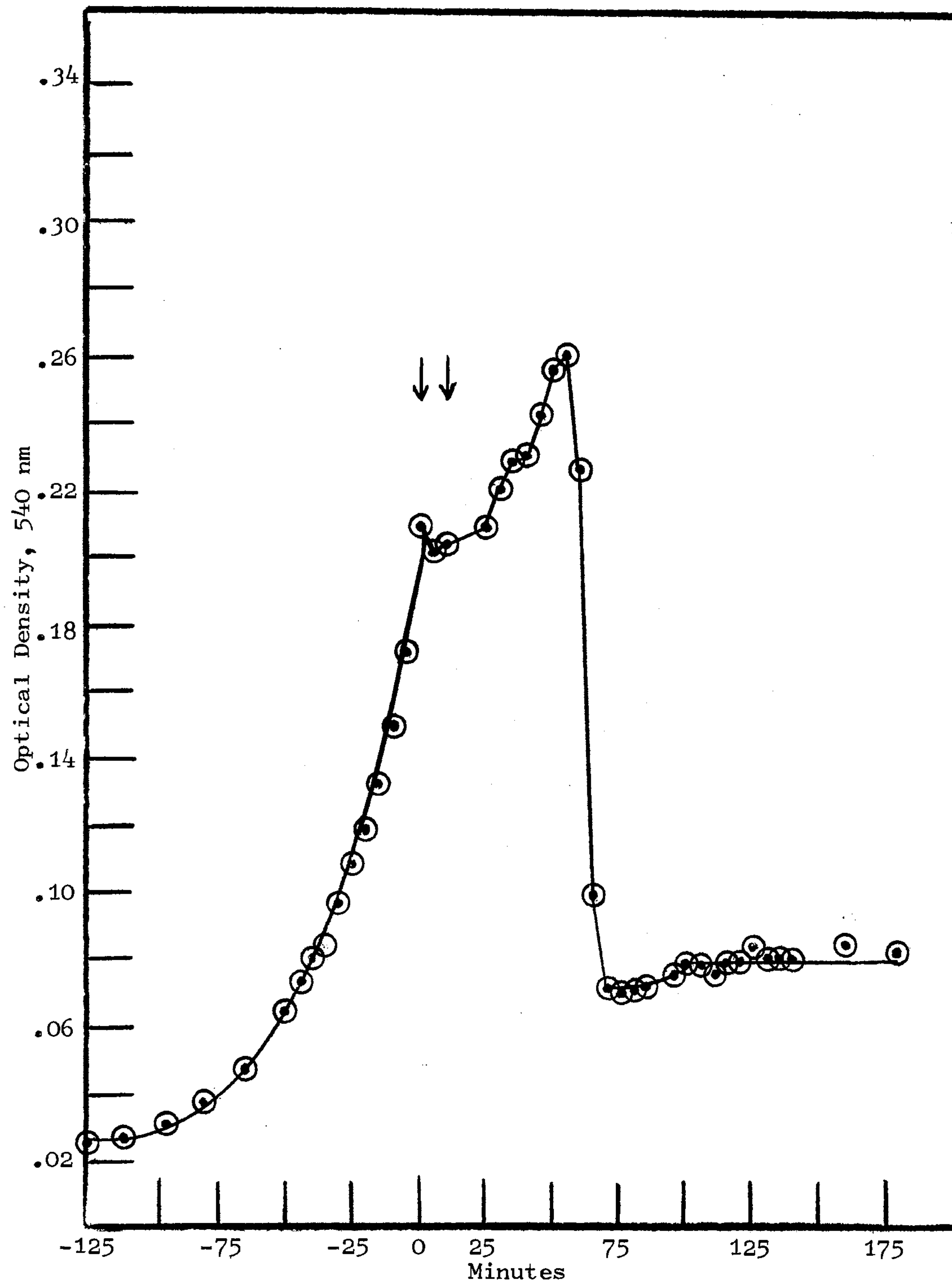
Mapping of Temperature-Sensitive Mutants

Recombination Frequencies

Since it is not feasible to determine the yield ratios of parental types from the progeny of crosses between temperature-sensitive (ts) mutants, the input ratios listed in Table XII were calculated from the titers of the set of stock dilutions of ts mutants. Only one of the combinations had a ratio which approached the limit of acceptability which was determined by Doermann and Hill (1953) to be 0.67 for T4

Figure 8. Turbidimetric Studies of PA-1 Following Infection With Pφ-6.0

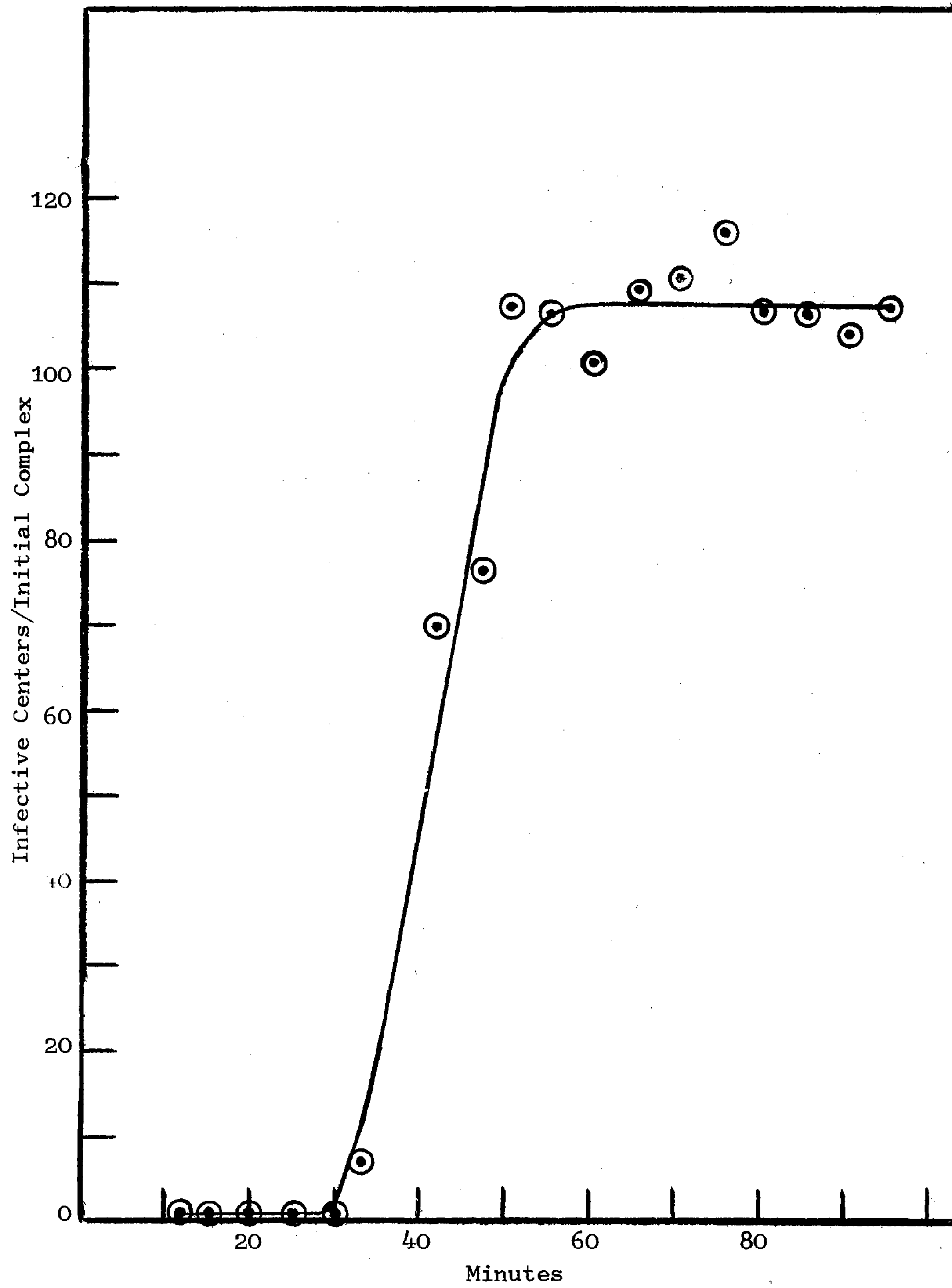
This experiment was similar to that shown in Figure 5 except the secondary infection was made at 10 min and the moi was increased to 12.



Optical Density, 540 nm
.34
.30
.26
.22
.18
.14
.10
.06
.02
-125
-75
-25
0
25
75
125
175
Minutes

Figure 9. Single-Step Growth of Pφ-6c1

Cells and phage were mixed at zero time. The phage were allowed to adsorb for 5 min at which time the phage-cell mixture was diluted into anti-Pφ-6 serum to inactivate the unadsorbed phage. At 10 min the complexes were diluted into growth flasks. The growth flasks were sampled at time intervals and duplicate platings were made immediately to assay for infective centers.



120
100
80
60
40
20
0
Infective Centers/Initial Complex
20
40
60
80
Minutes

TABLE XII

DATA FOR CROSSES OF TEMPERATURE-SENSITIVE MUTANTS

Mutant Cross a X b	Ave. of:	Input Ratio	Total Multiplicity of Input	Multiplicity of Infection			Total Plaques Scored	
				a	b	a+b	37 C	43 C
1 X 2	3	.94	14.6	4.5	4.7	9.2	1461	1115
1 X 3	1	.93	15.0	4.5	5.0	9.5	141	155
1 X 4	1	.95	13.8	4.5	4.2	8.7	150	189
1 X 5	2	.82	15.8	4.5	5.5	10.0	1021	1356
1 X 6	3	.97	14.4	4.5	4.6	9.1	1342	1229
2 X 3	1	.95	15.4	4.7	5.0	9.7	124	107
2 X 4	1	.89	14.2	4.7	4.2	8.9	143	166
2 X 5	1	.87	16.2	4.7	5.5	10.2	944	1087
2 X 6	3	.96	14.8	4.7	4.6	9.3	1587	389
3 X 4	3	.85	14.6	5.0	4.2	9.2	2407	824
3 X 5	3	.92	16.6	5.0	5.5	10.5	2468	2283
3 X 6	1	.92	15.2	5.0	4.6	9.6	136	114
3 X 8	3	.74	13.8	5.0	3.7	8.7	2239	1116
4 X 5	3	.78	15.4	4.2	5.5	9.7	1221	1218
4 X 6	1	.93	14.0	4.2	4.6	8.8	113	121
4 X 8	3	.87	12.6	4.2	3.7	7.9	1525	0
5 X 6	1	.84	16.0	5.5	4.6	10.1	982	1160
5 X 8	2	.68	14.6	5.5	3.7	9.2	1728	1151

phage crosses when input ratios were used instead of yield ratios. The multiplicities of infection were calculated on the basis of an average of 3×10^7 cells per ml, the input multiplicities, and 63 per cent adsorption within 10 min. In all the experiments used, the multiplicity of infection for each parent exceeded 3; if one had been less than 3, 5 per cent or more of the cells would not have been infected with that phage.

No correction was made for differences in plating efficiencies at 37 C and 43 C, since this would not alter either the order or the relative positions of the loci. All data used in calculating frequencies of recombination are given in Table XII, and the recombination frequencies are shown in Table XIII.

Genetic Map

Although three-factor crosses were not used for mapping the ts mutants, the loci could be ordered by sets of three using two-factor crosses. This was possible since, for any set of three loci, the recombination frequency between the outside loci was greater than that between either outside locus and the intermediate locus. To illustrate this, loci 3, 4, and 6 were ordered by recognizing that the highest recombination frequency was that between 4 and 6; therefore, the order would be 4-3-6. By this method, the data in Table XIII can be used to order the loci in the linear sequence illustrated in Figure 10. The relative positions were based on the individual frequencies between adjacent loci relative to the total of these individual frequencies.

TABLE XIII

RECOMBINATION FREQUENCIES FOR TEMPERATURE-SENSITIVE MUTANTS

ts Loci	1	2	6	3	4	8	5
1		14.3					
2	8.2		4.2				
6	8.9	2.4		29.3			
3	22.0	17.3	16.8		13.6	17.5	
4	25.2	23.2	21.4	7.8		0	33.6
8	-	-	-	10.0	0		34.7
5	27.8	27.7	26.3	23.6	19.3	19.9	

Per Cent Recombination (below diagonal)

Per Cent of Total Map Distance (above diagonal)

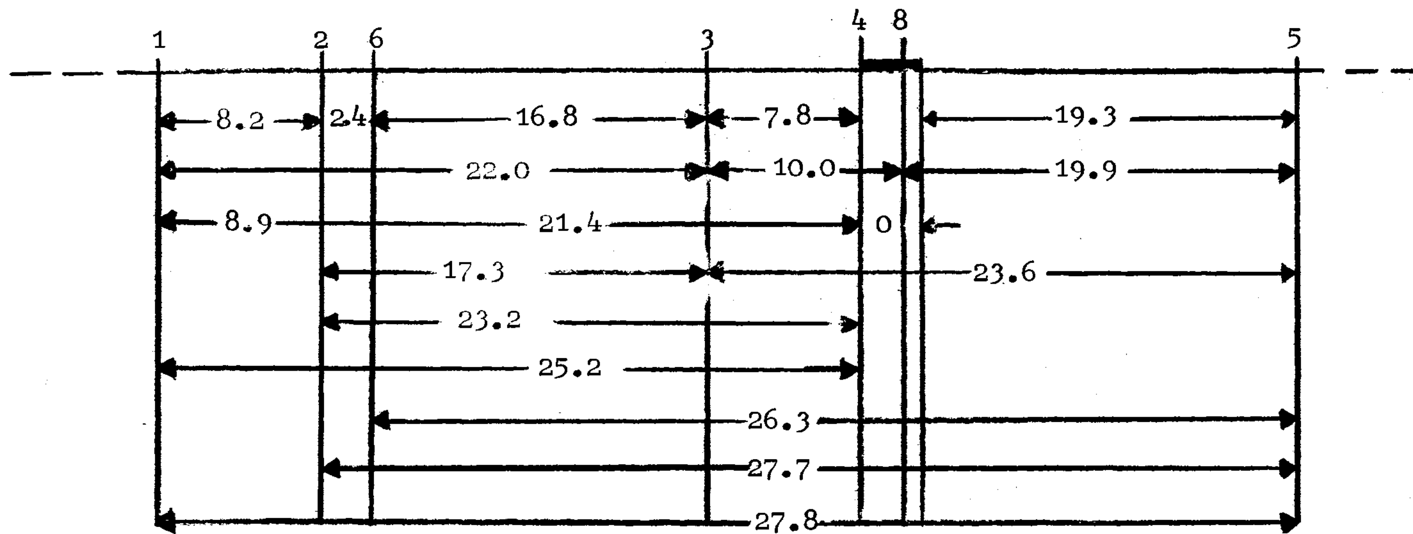


Figure 10. Genetic Map of Temperature-Sensitive Mutants of Pφ-6

Negative Interference

Statistical independence of recombination in adjacent regions was tested according to the method of Doermann and Hill (1953) using Haldane's equation:

$$R_{13} = R_{12} + R_{23} - 2R_{12}R_{23}$$

where R is the recombination frequency for the pair of loci indicated in the subscript. Table XIV shows the results of these calculations and comparisons. In every case, the observed value was less than the calculated, i.e., the occurrence of a recombinational event increased the frequency of additional recombination in that region. This is common to phage genetic systems and is referred to as negative interference, i.e., interference resulting in more recombinational events than expected.

TABLE XIV

NEGATIVE INTERFERENCE IN TEMPERATURE-SENSITIVE MUTANT CROSSES

Series of Loci Involved			Recombination Value Between Terminal Loci (R_{13})		Calculated Minus Observed
1	2	3	Calculated	Observed	
1	2	6	10.4	8.9	+1.5
2	6	3	18.8	17.3	+1.5
6	3	4	23.3	21.4	+1.9
3	4	5	25.9	23.6	+2.3
3	8	5	27.9	23.6	+4.3
5	6	1	32.9	27.8	+5.1
5	2	1	33.6	27.8	+5.8

CHAPTER IV

DISCUSSION

The biological characteristics of Pφ-6 were compared to those of other phages for *Pseudomonas* which have been studied in other laboratories (Table XV). While the normal burst size for Pφ-6 varied from 102-190, the range for HD2 was 80-174 (Grogan and Johnson, 1964) and for φW-14A$^+$, it was 214-470 (Kropinski and Warren, 1970). As can be seen in Table XV, the rate constants for adsorption varied considerably. It should be noted that O'Callaghan et al. (1969) used infected cells to determine the adsorption rate, while Feary et al. (1964), Kropinski and Warren (1970), and the studies reported herein used free phage determinations. If the level of abortive infections is quite low, the two methods should yield comparable rate constants. If only one method is to be used, the determination by free phage is preferable since it is dependent on only the adsorption rate and not on the efficiency of infection (Feary et al., 1964). It was shown in Tables III and IV that the efficiency of plating for Pφ-6 on PA-1 was affected by the age of the cell culture. The absolute efficiency of plating, i.e., plaque forming units per whole phage particle, was not determined for Pφ-6 as this would have required electron microscopy which was not available for use in this study. Neither was it reported for the other phages listed in Table XV.

While the absolute K values for antiphage sera vary within a wide

TABLE XV

SELECTED CHARACTERISTICS OF SOME PHAGES FOR PSEUDOMONAS

Host	Phage	Adsorption Rate $K \times 10^{-11}$ ml/min	Latent Period, min	Average Burst Size	Antiphage Serum K Value	Ref.*
P. aeruginosa	Pφ-6	330	30-35	152	1450	
	7v	250	23	230	4600	1
	7m	180	-	-	80	1
	7s (RNA)	4100	-	-	200	1
	PX2	-	85	162	-	2
	PX3	-	35	88	-	2
	PX7	-	50	55	-	2
	CB3	-	45	75	-	2
	352	33.0	35-40	200	36	3
	HD24	2.3	35-40	160	228	3
	HD2	14.9	35-40	100	66	3
	HD3	3.2	35-40	100	173	3
	HD7	4.1	35-40	60	391	3
	HD44	2.0	35-40	55	327	3
	HD11	5.2	60-70	60	272	3
	HD113	3.3	60-70	60	115	3
	1214	5.6	60-70	50	110	3
	HD16	1.9	60-70	45	108	3
	HD95	1.5	60-70	22	1107	3
	HD68	1.2	60-70	10	574	3
P. putida	gh-1	-	21	103	-	4
	Pf15	-	55	120	-	5
P. acidovorans	φW-14a+	190	60	300	-	6
	φW-14a	420	60	450	-	6

*1) Feary et al., 1964
2) Olsen et al., 1968
3) O'Callaghan et al., 1969
4) Lee and Boezi, 1966
5) Chakrabarty et al., 1967
6) Kropinski and Warren, 1970

range because of differences in methods of preparation, the K values were included in Table XV to indicate the relative values that have been reported. Adams (1959) listed the following ranges of K values for the T-phages of *E. coli*: T3 and T7, 500-3000; T2, T4, and T6, 200-1000; and T1 and T5, 20-100.

The nucleic acids previously reported for phages of *Pseudomonas* have included double-stranded and single-stranded DNA and single-stranded RNA. The finding of double-stranded DNA in Pφ-6 is consistent with the possibility that Pφ-6 may be capable of lysogeny.

In an attempt to identify the cause of haloed and haloless plaques of Pφ-6 on PA-1, the systems considered as models included lysogeny and clear plaque mutants (Kaiser, 1957; Levine, 1957), lysinless mutants (Chakrabarty et al., 1967), turbid and nonturbid plaques (Doermann and Hill, 1953), and lysis inhibition versus rapid lysis mutants (Doermann, 1948).

In previously described systems of lysogeny, the c^+ wild type plaques had highly turbid centers. The Pφ-6.0 plaques on PA-1 more closely resembled the c mutants. The Pφ-6.0 isolated as wild type may have been a c mutant of a temperate phage capable of lysogeny. This would agree with the results of the lysogenization studies where the frequency was found to be too low, if present, to account for the halos. Although no lysogenic derivatives of PA-1 were isolated, the experiments with the two temperature-sensitive mutants indicate that lysogeny may occur. If the mutants lysogenized at a very low frequency, they would be greatly outnumbered by resistant cells present in the liquid cultures. This was confirmed by plating dilutions of washed cells with PA-1 and

without PA-1. While there were approximately 10^3 colonies on the latter plate, there were no colonies surrounded by lysis on the former; yet, the supernatants had relatively high phage titers.

The possibility that the presence or absence of the halos was due to the presence or absence of lysin production was considered, but thought to be unlikely. While lysinless mutants have a reduced plaque size due to loss of the halo, Pφ-6 haloless mutants appear to be larger due to increased lysis in the halo region. Also, the halos were not devoid of phage, as is the case with lysin halos (Park, 1956; Bartell et al., 1966; Chakrabarty et al., 1967).

Doermann and Hill (1953) used clear (tu^+) and turbid (tu) plaque morphologies for mapping studies in which five different tu loci were identified. If the Pφ-6 system is of the same type, Pφ-6.0 would appear to have a genotype such as r tu^{ts} indicating rapid lysis and the temperature-sensitive nature of the halo. The clear mutants would then appear to be r tu^+, while those with halos at 43 C would be r tu. The absence of the halo on B-5 could be due to suppressor-sensitive tu mutants with PA-1 as the non-permissive host and B-5 as the permissive host. While the Pφ-6c isolates could be tu^+, the differences observed in their responses to different plating conditions, such as host cells or media, indicated that they were not identical. It is possible that mutations in the tu function are suppressible physiologically or genetically in several different ways such that the Pφ-6c isolates need not have the same genotype.

The inability to demonstrate lysis inhibition in liquid cultures of Pφ-6-infected PA-1 does not eliminate rapid lysis as the cause of haloless plaques. It is possible that lysis inhibition could occur on

solid media where conditions might stabilize fragile, lysis-inhibited cells which would lyse early in liquid cultures. The studies with chloroform indicated that it lysed cells on the plate which contained mature phage, on the basis of a 55 per cent increase in plate stock titer and clearing of halos of individual plaques. It was also observed that the halo condition was dominant to the haloless, as r^+ is to r, and the shape of the single-step growth curve for Pφ-6c1 was indicative of more rapid lysis.

Holloway et al. (1960) described a group (B) of phages for Pseudomonas which gave plaques with clear centers and halos, and from which clear plaque mutants were isolated. Kropinski and Warren (1970) described a phage, φW-14, for P. acidovorans which gave plaques with small, clear centers and turbid halos (designated as a^+). A mutant was described which gave smaller plaques with wide, clear centers and indistinct halos (designated as a). Their description of plaque types and turbidity changes indicated that φW-14a^+ showed lysis inhibition which contradicts their conclusion that φW-14a showed lysis inhibition. They did not include a graph of the turbidity changes, and their discussion of superinfection studies was not sufficiently detailed; therefore, no valid conclusions concerning lysis inhibition can be drawn by the reader. Minamishima et al. (1968) described a phage (Pf2) for P. aeruginosa from which they stated that rapid lysis mutants were obtained. They did not report any evidence of lysis inhibition in the r^+ phage which was lost in the r mutants, only that "the plaques formed by this mutant were larger, with more distinct colonies than the wild type (r^+). . . ."

The use of h for haloed or haloless was avoided because of its common usage to designate host range mutants. The use of r for rapid

lysis should only be used when there is clear evidence that ability to cause lysis inhibition has been lost in the mutant. Until more definitive data are obtained for Pφ-6, the haloless derivatives will be referred to as c mutants. This notation should not indicate that only one locus, or even function, is involved, but only that a phenotypic expression is being designated without regard to the number of genotypes which may yield the same apparent phenotype.

Recombinational analysis of seven temperature-sensitive mutants indicated that the map of Pφ-6 is noncircular, since loci which map progressively farther apart based on the map constructed from the smaller recombination values give progressively higher recombination values when crossed directly (see Fattig and Lanni, 1965). For Pφ-6 to have a circular genetic map, these seven loci would have to represent less than 50 per cent of the map. This is not likely since ts mutants can occur throughout the genome (Edgar and Lielausis, 1964). Only five ts mutants were required to demonstrate circularity of the map of phage P22. This was confirmed by three- and four-factor crosses involving plaque morphology markers (Gough and Levine, 1968).

The linear map of Pφ-6 had a composite length of 54 map units. This may be an underestimate since no correction was made for plating efficiency at 43 C. These crosses were all carried out in post logarithmic phase cells since these gave the highest plating efficiency. Kropinski and Warren (1970) reported a high dependence of burst size on the age of the cell culture; cells from late logarithmic phase gave a burst of 600 compared to 30 from cells in stationary phase. Burst sizes of up to 282 were obtained in crosses of Pφ-6ts mutants, compared to a maximum of 190 in single-step growth experiments. A larger burst size

should increase resolution, but should not change the ratio of recombinant to parental progeny (Hershey and Rotman, 1949).

It was noted that the map distance between loci ts3 and ts5 was longer when based on crosses with ts8 than when based on crosses with ts4. Since ts4 and ts8 yielded no recombinants when crossed, it would appear that ts4 might be a deletion with ts8 mapping within the ts4 region, near the end which is closer to ts5. Revertants of ts4 have been observed at a low frequency; therefore, it is more likely that the differences in the ts3 - ts5 distance were due to marker effects.

While genetic analysis of the c marker might answer many questions concerning this type of mutant, it was not felt that it would be justified for the present work since only a limited number of loci might be involved. This researcher did not include these mutants in the mapping studies with the ts mutants since the halo is temperature sensitive in Pφ-6.0 and recombinants at the c locus could not be detected at 43 C.

Partial maps of two other phages for Pseudomonas are available for comparison, that of D3 for *P. aeruginosa* (Eagon and Holloway, 1961), and that of Pf15 for *P. putida* (Chakrabarty et al., 1967), both of which were reportedly constructed from crosses in logarithmic phase cells. The D3 map is composed of loci for six morphological markers. The composite length was not reported, but the length over four loci gives an additive distance of 23.7 map units with one of the other two loci included in this region. The map of Pf15 is composed of two host range loci and two loci for morphological markers with a total map length of approximately 24 map units (by this author's calculations).

Analysis of the recombination frequencies for Pφ-6 showed negative interference at about the same level as was reported by Doermann and

Hill (1953) for T4 crosses. The map for D3 shows high positive and negative interference while the map for Pf15 shows high positive interference. The order of loci l_1 and h_2 in the Pf15 map was evidently based on recombination with h_1 instead of the much closer locus, mi, since recombination over the shorter interval indicates the opposite orientation.

CHAPTER V

SUMMARY AND CONCLUSIONS

In these studies, a new bacteriophage for P. aeruginosa was purified and characterized, and preliminary genetic mapping was done with temperature-sensitive mutants.

Stable, high titer, homogeneous stocks of phage Pφ-6 were easily obtained. These could be purified or concentrated by differential centrifugation. Pφ-6 was resistant to chloroform, which allowed the use of some standard techniques, e.g., killing bacteria in plaque picks, reducing bacterial contamination of stocks by treatment with chloroform before storage and lysing unlysed cells to release mature phage. By using membrane filtration instead of centrifugation to remove cells and adsorbed phages, adsorption was found to follow first order kinetics. Inactivation by anti-Pφ-6 serum was also found to be a first-order reaction. The rapid rate of adsorption observed resulted in relatively uniform plaque sizes in soft agar overlay platings. This should also give uniform input when making genetic crosses.

The single-step growth studies showed a latent period of 30 to 35 min. This allows sufficient time for various manipulations when studying the physiology of a bacteriophage. An average burst size of 152 was observed. While this is not high when compared to some systems, it will still allow studies to be made in which a 10-fold reduction in burst size is desired.

The nucleic acid of Pφ-6 was shown to be DNA by Bradley's (1966) staining technique and by the diphenylamine reaction (Burton, 1956). Both Bradley's staining technique and the demonstration of hyperchromicity indicated that it is double-stranded. Both of these conclusions are consistent with the fact that Pφ-6 originated from a lysogenic bacterium and that genetic recombination could be demonstrated.

Neither lysis inhibition nor lysogenization could be demonstrated by standard methods in repeated attempts. There was evidence that both may occur under certain conditions and this could be pursued further in studies of these specific phenomena.

Genetic recombination was demonstrated by crossing temperature-sensitive mutants. The maximum frequency of recombination was approximately 28 per cent. Negative interference was demonstrated throughout the map, indicating a positive correlation between recombinational events in adjacent regions. The mapping studies showed a single, linear linkage group which had a composite length of 54 map units.

Based on the data presented here, it is concluded that phage Pφ-6 should provide a suitable system for genetic studies comparable to those using coliphages. Genetic studies have shown clustering of the functionally related genes in both the coliphages and the host, *E. coli*. Since recent studies have shown that clustering of functionally related genes rarely occurs in *Pseudomonas*, it would be interesting to know what type of genetic organization is present in its phages. Phage Pφ-6 should prove valuable for these comparative genetic studies.

BIBLIOGRAPHY

Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.

Bartell, P. F., T. E. Orr, and G. K. H. Lam. 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. 92: 56-62.

Benzer, S. 1955. Fine structure of a genetic region in bacteriophage. Proc. Nat. Acad. Sci. U.S.A. 41: 344-354.

Benzer, S. 1957. The elementary units of hereditary. p 70-93. In W. D. McElroy and B. Glass (ed.), The Chemical Basis of Heredity. Johns Hopkins Press, Baltimore.

Benzer, S. 1961. On the topography of the genetic fine structure. Proc. Nat. Acad. Sci. U.S.A. 47: 403-416.

Bradley, D. E. 1966. The flourescent staining of bacteriophage nucleic acids. J. Gen. Microbiol. 44: 383-391.

Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. 31: 230-314.

Brenner, S. 1959. Physiological aspects of bacteriophage genetics. Advan. Virus Res. 6: 137-158.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323.

Calendar, R. 1970. The regulation of phage development. Ann. Rev. Microbiol. 24: 241-296.

Campbell, A. 1962. Episomes. Advan. Genet. 11: 101-145.

Chakrabarty, A. M., J. F. Niblack, and I. C. Gunsalus. 1967. A phage-initiated polysaccharide depolymerase in *Pseudomonas putida*. Virology 32: 532-534.

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

de Ley, J. 1964. *Pseudomonas* and related genera. Ann. Rev. Microbiol. 18: 17-41.

Doermann, A. H. 1948. Lysis and lysis inhibition with Escherichia coli bacteriophage. J. Bacteriol. 55: 257-275.

Doermann, A. H., and M. B. Hill. 1953. Genetic structure of bacteriophage T4 as described by recombination studies of factors influencing plaque morphology. Genetics 38: 79-90.

Dove, W. F. 1968. The genetics of the lambdoid phages. Ann. Rev. Genet. 2: 305-340.

Edgar, R. S., and I. Lielausis. 1964. Temperature-sensitive mutants of bacteriophage T4D: Their isolation and genetic characterization. Genetics 49: 649-662.

Egan, J. B., and B. W. Holloway. 1961. Genetic studies on lysogeny in Pseudomonas aeruginosa. Aust. J. Exp. Biol. Med. Sci. 39: 9-18.

Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. 28: 375-392.

Fattig, W. D., and F. Lanni. 1965. Mapping of temperature-sensitive mutants in bacteriophage T5. Genetics 51: 157-166.

Feary, T. W., E. Fisher, and Thelma N. Fisher. 1964. Isolation and preliminary characteristics of three bacteriophages associated with a lysogenic strain of Pseudomonas aeruginosa. J. Bacteriol. 87: 196-208.

Freifelder, D. 1965. A rapid technique for the preparation of purified bacteriophage DNA or RNA from crude lysates. Biochim. Biophys. Acta 108: 318-319.

Gough, M., and M. Levine. 1968. The circularity of the phage P22 linkage map. Genetics 58: 161-169.

Grogan, J. B., and E. J. Johnson. 1964. Phage-host relationships and physical characteristics of a Pseudomonas aeruginosa bacteriophage. J. Bacteriol. 87: 973-974.

Hershey, A. D. 1946. Spontaneous mutations in bacterial viruses. Cold Spring Harbor Symp. Quant. Biol. 11: 67-77.

Hershey, A. D., and R. Rotman. 1949. Genetic recombination between host-range and plaque-type mutants of bacteriophage in single bacterial cells. Genetics 34: 44-71.

Holloway, B. W. 1969. Genetics of Pseudomonas. Bacteriol. Rev. 33: 419-443.

Holloway, B. W., J. B. Egan, and M. Monk. 1960. Lysogeny in Pseudomonas aeruginosa. Aust. J. Exp. Biol. Med. Sci. 38: 321-330.

Holloway, B. W., M. Monk, L. Hodgins, and B. Fargie. 1962. Effects of radiation on transduction in Pseudomonas aeruginosa. Virology 18: 80-94.

Jacob, F., and J. Monod. 1961. On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol. 26: 193-209.

Kaiser, A. D. 1957. Mutations in a temperate bacteriophage affecting its ability to lysogenize Escherichia coli. Virology 3: 42-61.

Kropinski, A. M. B., and R. A. J. Warren. 1970. Isolation and properties of a Pseudomonas acidovorans bacteriophage. J. Gen. Virol. 6: 85-93.

Lee, L. F., and J. A. Boezi. 1966. Characterization of bacteriophage gh-1 for Pseudomonas putida. J. Bacteriol. 92: 1821-1827.

Levine, M. 1957. Mutations in temperate phage P22 and lysogeny in Salmonella. Virology 3: 22-41.

Levine, M. 1969. Phage morphogenesis. Ann. Rev. Genet. 3: 323-342.

Luria, S. E., and J. E. Darnell, Jr. 1967. General Virology, 2nd Edition. John Wiley and Sons, Inc., N.Y.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organism. J. Mol. Biol. 3: 208-218.

Marvin, D. A., and B. Hohn. 1969. Filamentous bacterial viruses. Bacteriol. Rev. 33: 172-209.

Mayer, V. W. 1969. Rapid plate test for evaluating phage induction capacity. Appl. Microbiol. 18: 697-698.

Minamishima, V., K. Takeya, V. Ohnishi, and K. Amako. 1968. Physio-chemical and biological properties of fibrous Pseudomonas bacteriophages. J. Virol. 2: 208-213.

Nomura, M., and S. Benzer. 1961. The nature of the "deletion" mutants in the rII region of phage T4. J. Mol. Biol. 3: 684-692.

O'Callaghan, R. J., W. O'Mara, and J. B. Grogan. 1969. Physical stability and biological and physicochemical properties of twelve Pseudomonas aeruginosa bacteriophages. Virology 37: 642-648.

Olsen, R. H., E. S. Metcalf, and J. K. Todd. 1968. Characteristics of bacteriophages attacking psychrophilic and mesophilic Pseudomonads. J. Virol. 2: 357-364.

Park, B. H. 1956. An enzyme produced by a phage-host cell system. 1. The properties of a Klebsiella phage. Virology 2: 711-718.

Pratt, D. 1969. Genetics of single-stranded DNA bacteriophages. Ann. Rev. Genet. 3: 343-362.

Radding, C. M. 1969. The genetic control of phage-induced enzymes. Ann. Rev. Genet. 3: 363-394.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten. 1957. Studies of biosynthesis in Escherichia coli, 2nd printing. Carnegie Inst. Wash. Pub. 607: 5.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43: 159-271.

Stent, G. S. 1963. Molecular Biology of Bacterial Viruses. W. H. Freeman and Company, San Francisco.

Stent, G. S. (ed.) 1965. Papers on Bacterial Viruses, 2nd ed. Little, Brown, and Company, Boston.

Sutter, V. L., V. Hurst, and J. Fennell. 1963. Semi-synthetic medium for bacteriophage typing of Pseudomonas. J. Bacteriol. 86: 1354-1356.

Swanstrom, M., and M. H. Adams. 1951. Agar layer method for production of high titer phage stocks. Proc. Soc. Exptl. Biol. Med. 78: 372-375.

Taylor, J. H. (ed.) 1965. Selected Papers on Molecular Genetics. Academic Press, New York.

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