ISOLATION, AND CHARACTERIZATION OF A BACTERIOPHAGE

ACTIVE AGAINST PSEUDOMONAS ACIDOVORANS

by

ANDREW MAITLAND BOLESLAW KROPINSKI

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ABSTRACT

A phage was isolated from sewage which was active against
the non-fluorescent Pseudomonad, P. acidovorans. It was given the
designation ØW-14. This is the first phage reported to be active
against P. acidovorans.

ØW-14 was larger than previously reported Pseudomonas phages.
It had an icosahedral head some 90 mµ in diameter and a contractile
tail 140 mµ long and 20 mµ in diameter. The phage tended to form
large aggregates.

ØW-14 formed haloed plaques on three strains of the host organism,
but mutated at a high frequency $(4 - 9 \times 10^{-4})$ to a clear plaque
type when grown on P. acidovorans #14. The haloed plaque form was
considered to be the wild type and was given the designation ØW-14a+. The
clear plaque mutant appeared stable and was given the designation ØW-14a.

Adsorption of ØW-14a+ was biphasic, with 35% of the phage
population having a decreased adsorption capacity. The K value was
1.9×10^{-9} ml/min. More normal kinetics were observed with ØW-14a
and with ØW-14a+ in low-salt broth. The K value for ØW-14a was 4.2
$\times 10^{-9}$.

The burst size for ØW-14a+ obtained from one-step growth experi-
ments was 300, with a latent period of 67 minutes. ØW-14a had a 50%
higher burst size but the same latent period. The average burst size
was markedly affected by the physiological age of the bacterial culture,

and by the multiplicity of infection.

Sensitivity of the phage to heat, pH, sonication and ultra-violet light was investigated. At least 70% of the lethal effects of UV irradiation could be reversed by photoreactivation. Thermal inactivation kinetics of ØW-14a+ in broth were biphasic at 55 C and 60 C, the ΔH* being 75,700 calories/mole. ØW-14a appeared to be more thermo-labile.

Nucleic acid isolated from ØW-14a+ was double-stranded DNA. There was a significant discrepancy between the moles % GC calculated from buoyant density determinations (1.666 g/cc^3 -6% GC) and Tm determinations (98.4 C - 71.9% GC). The DNA was unusual also in that it yielded five bases upon hydrolysis. The fifth base was not one of those commonly found in DNA. It has not been identified. The base composition of the DNA as determined by chromatographic separation and quantitation of the bases was: Adenine, 21.8 moles %; Guanine, 28.2 moles %; Thymine, 11.1 moles %; Cytosine, 26.6 moles %; Unknown, 12.3 moles %; based upon the relationship:

$$\frac{[\ A + G\ ]}{[\ T + C + Unknown\ ]} = 1$$

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Dedicated to:

SHARON AND MARY CATHERINE

INTRODUCTION

The phages of the phytopathogenic Pseudomonads have received a great amount of attention due to the considerable economic importance of their host organisms. Phages have been isolated which are active against: P. phaseolicola (Katznelson and Sutton, 1951); P. morsprunorum, P. viridilivida (Crosse and Garrett, 1963); P. atrofaciens, P. pisi, P. coronafaciens (Sutton and Katznelson, 1953); P. tabaci, P. angulata (Fulton, 1950); P. syringae (Baigent, de Vay, and Starr, 1963); P. solanacearum (Matsui, 1952). With the exception of the latter, all the other reports were primarily concerned with the isolation and host-range properties of the phages as a means of classifying this confusing group of organisms. Matsui has concerned himself with the physiology and genetics of a P. solanacearum phage, SP$_1$ (Matsui, 1952; Matsui, 1953 a, b).

The phages of P. aeruginosa (P. pyocyanea) have been studied in great detail, but again, with some exceptions, the purpose has been taxonomic, since this organism is associated with a number of clinical manifestations. Some of the best studied P. aeruginosa phages have been: F1, F2, and F3 (Alföldi, 1956; Lovas, Egyessy and Alföldi, 1957); p8 and P2 (Jacob, 1952); phage 2 (Grogan and Johnson, 1964 a, b,); a group of phages from a lysogenic strain (Feary, Fisher and Fisher, 1964). Phages against another human pathogenic Pseudomonad - P. pseudomallei - were isolated from stagnant waters in Vietnam by Lederc and Sureau (1956).

The most thoroughly investigated group of Pseudomonas phages –

Table 1. Properties of various Pseudomonas phages – Physiology of Growth

Phage	Host bacterium	Temp.	Latent period	Rise period	Burst size	Reference
PX4	P. aeruginosa 2	25 C·	52 min		103	1
PX2	P. aeruginosa 1	37	85		162	1
PX3	P. aeruginosa 2	37	35		88	1
PX7	P. aeruginosa R629	37	50		55	1
CB3	P. aeruginosa 1c	37	45		75	1
7v	P. aeruginosa Ps-7	37	23	9 min	230	2
1	P. aeruginosa	37	45–50		100	3
2	P. aeruginosa	37	37	15	111	4
K	P. aeruginosa (Kitasato strain)		35	50	130	5
Seven	P. aeruginosa		35–40		40–100	6
p8	P. pyocyanea 13	37	55–65		40–60*	7
p2	P. pyocyanea 13	37	25–30		50–70*	7
F1	P. pyocyanea 3R	37	30	48	150–250	8
F2	P. pyocyanea 3R	37	15	21	30–60	8
F3	P. pyocyanea 3R	37	20	22	120–180	8
PX4	P. fluorescens 14	25	60		23	1
PX10	P. fluorescens 22	25	35		80	1

Phage	Host bacterium	Temp.	Latent period	Rise period	Burst size	Reference
PX12	P. fluorescens 35	25	40		50	1
PX1	P. putida A.3.12	25	35		106	1
gh-1	P. putida A.3.12	33	21	34	103	9
PX14	P. geniculata 4	25	30	15	120	10
Pg8	P. atrofaciens		55-60	15	2.1	11
SP_1	P. solanacearum S		95	15	130	12
SP_1h_1**	P. solanacearum B19		120	25	70	12

* determined using minimal-glucose medium

** a host-range mutant of SP_1

1. Olsen, Metcalf and Todd (1968).

2. Feary, Fisher and Fisher (1964).

3. van den Ende, Don, Elford, Challice, Dawson, and Hotchin (1952).

4. Grogan and Johnson (1964 b).

5. Takeya, Mori, Ueda, and Toda (1959).

6. O'Callaghan and Grogan (1967).

7. Jacob (1952).

8. Alföldi (1956).

9. Lee and Boezi (1966).

10. Olsen (1967).

11. Sutton (1966).

12. Matsui (1953 b).

isolated against P. aeruginosa, P. fluorescens, P. putida and P. geniculata - is that reported by Olsen, Metcalf and Todd (1968). In this study, the physiology of growth, sensitivity to citrate, pH and osmotic shock, as well as the phage morphology and the GC content of the DNA were studied. Phages active against P. fluorescens and P. putida had been isolated previously by Klinge (1959) and Lee and Boezi (1966). In this latter paper, the properties of phage gh-1 were studied in depth.

Until the present research only two phages had been isolated against nonfluorescent, nonphytopathogenic, Pseudomonads - P. fragi (Roberts and Doetsch, 1966) and P. stutzeri (Espejo and Canelo, 1968). The latter phage is quite unique in that the phage coat contains an appreciable amount of lipoidal material.

The original intention of this research was to isolate a phage against a nonfluorescent Pseudomonad - P. acidovorans. In particular a temperate transducing phage was desired since the genetics of this group of organisms has received little attention. Although several phages active against P. acidovorans were isolated, all were lytic phages. This thesis describes the properties of one of them - ØW-14.

Tables I and II summarize the properties of various Pseudomonas phages, and is based upon a search of the literature up to the end of 1968.

Table II. Properties of various Pseudomonas phages - Nucleic Acids

Phage	Host Bacterium	Nucleic Acid	%GC	Method	Reference
2	P. aeruginosa	ds DNA*	54.7	b,c**	1
SD1	P. aeruginosa	ds DNA	53.8	a,c	2
PX2	P. aeruginosa	ds DNA	68.2	a	3
PX3	P. aeruginosa	ds DNA	45.0	a	3
PX7	P. aeruginosa	ds DNA	54.6	a	3
CB3	P. aeruginosa	ds DNA	60.4	a	3
B3	P. aeruginosa	ds DNA			4
D3	P. aeruginosa	ds DNA			4
E79	P. aeruginosa	ds DNA			4
F116	P. aeruginosa	ds DNA			4
Pf	P. aeruginosa	ss DNA			5
PP7	P. aeruginosa	ss RNA			6
7s	P. aeruginosa	ss RNA	50.4***	c	7
PX4	P. fluorescens	ds DNA	44.4	a	3
PX10	P. fluorescens	ds DNA	53.0	a	3
PX12	P. fluorescens	ds DNA	55.8	a	3
Pf	P. putida	ds DNA	62.0	a,b	8
PX1	P. putida	ds DNA	52.5	a	3
gh-1	P. putida	ds DNA	57.0	a,b,c	9
PM2	P. stutzeri	ds DNA	43.0	a,b	10
PX14	P. geniculata	ds DNA	53.8	a	3

* ds (double-stranded); ss (single-stranded); RNA (ribonucleic acid); DNA (deoxyribonucleic acid)

** a (determined from Tm); b(determined from buoyant density); c (determined by quantitative chromatography after acid hydrolysis)

*** Adenine (23.8 moles%); Guanine (24.6 moles%); Cytosine (25.8 moles%); Uracil (25.8 moles%)

1. Grogan and Johnson (1964 a).

2. Shargool and Townsend (1966).

3. Olsen, Metcalf and Todd (1968).

4. Davidson, Freifelder and Holloway (1964).

5. Takeya and Amako (1966).

6. Bradley (1966).

7. Feary, Fisher and Fisher (1963).

8. Niblack and Gunsalus (1965).

9. Lee and Boezi (1966).

10. Espejo and Canelo (1968).

MATERIALS AND METHODS

I. Organisms

The sources of the organisms used are listed in Table III. The organisms were maintained in standard minimal base stabs with 0.5% yeast extract (Stanier, Palleroni and Doudoroff, 1966) at 4 C, and were transferred every two months.

II. Media

Modified Luria broth was used throughout as the medium for bacterial and phage growth, and as a diluent. The composition was, in g per litre: tryptone (Difco), 10; yeast extract (Difco), 5; NaCl, 5; mannitol, 1. The medium was made-up in distilled water, adjusted to pH 6.5, and sterilized by autoclaving. Bacteriological agar (Difco) was added to a final concentration of 1% (w/v) for plating medium, while 0.6% agar was added to the overlays used in titring phage.

Luria broth without added NaCl was used for the adsorption studies – Luria broth (-NaCl) – and had the following composition, in g per litre: tryptone, 1; yeast extract, 0.5; mannitol, 2.

The minimal medium contained, in g per litre: Na_2HPO_4, 5.3; KH_2PO_4, 2.3; NH_4Cl, 1.0; $MgSO_4$ (added after autoclaving), 0.3; mannitol, 4.0. The pH was adjusted to pH 7.0 prior to autoclaving.

Table III: <u>Sources of organisms</u>

Organism	Strain	Source
P. acidovorans	#14; #29; #114; #146	Dr. R. Stanier, Dept. of Bacteriology, University of California, Berkeley.
	AK-11	Norleucine enrichment
	15666	Dr. E. F. Lessel,
	15667	Curator of Bacteria,
	15668	American Type Culture Collection.
P. testosteroni	#78; #138	Dr. R. Stanier
	11996	Dr. E. F. Lessel
P. mucidolens	ATCC 4687	Culture Collection, Dept. of Microbiology, University of British Columbia, Vancouver.
P. putrefaciens	Hammer	Dept. of Microbiology (U.B.C.)
P. ovalis	ATCC 950	Dept. of Microbiology (U.B.C.)
P. taetrolens	ATCC 4683	Dept. of Microbiology (U.B.C.)
P. synxantha	ATCC 796	Dept. of Microbiology (U.B.C.)
P. convexa	ATCC 795	Dept. of Microbiology (U.B.C.)
P. aeruginosa	ATCC 9027	Dept. of Microbiology (U.B.C.)
P. aeruginosa	ATCC 9721	Dept. of Microbiology (U.B.C.)
P. fragi	ATCC 4975	Dept. of Microbiology (U.B.C.)
E. coli K12	3000	Dr. R.A.J. Warren, Dept. of Microbiology, University of British Columbia Vancouver.

<u>Strain</u>

<u>Organism</u>   <u>Source</u>

<u>E</u>. <u>coli</u>   Dr. R.A.J. Warren

Appendix 1. Characteristics of Organisms of the <u>P</u>. <u>acidovorans</u> Group

"The acidovorans group consists of non-pigmented, nutritionally versatile aerobic pseudomonads which share a distinctive nutritional spectrum and certain unique metabolic properties. All strains are multitrichous." (Stanier, Palleroni and Doudoroff, 1966).

Table 41. *Acidovorans group. The group characters of greatest differential value in the recognition of the acidovorans group, based on the analysis of 26 strains*

Characters	Number of positive strains	Ideal phenotype
1. Poly-β-hydroxybutyrate as cellular reserve material	26	+
Utilization of		
2. L-arabinose	0	−
3. D-Glucose	0	−
4. D-Galactose	0	−
5. 2-Ketogluconate	0	−
6. Saccharate	25	+
7. Pelargonate	0	−
8. Adipate, pimelate, suberate, azelate, sebacate	26	+
9. Glycollate	24	+
10. Laevulinate	26	+
11. Itaconate	26	+
12. *m*-Hydroxybenzoate	25	+
13. Norleucine	25	+
14. Putrescine	0	−

Table 42. *Acidovorans group. Number of strains of different species or groups of species of aerobic pseudomonads conforming to the selected fourteen characters (Table 41) which define the ideal phenotype of the acidovorans group*

	No. of characters of 'ideal' phenotype														
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
Acidovorans group	24	.	1	1	.	..	.	.	.	.	.	.	.	.	.
Fluorescent group	.	.	.	.	.	.	.	.	1	24	31	44	48	27	.
Pseudomallei group	.	.	.	.	.	..	.	.	.	1	23	10	7	.	.
P. multivorans	.	.	.	.	.	.	.	.	18	6	.	.	.	.	.
P. stutzeri	.	.	.	.	.	.	.	1	8	8	.	.	.	.	.
P. maltophilia	.	.	.	.	.	.	.	.	.	23	.	.	.	.	.
Alcaligenes group	.	.	.	.	.	.	.	.	4	2	1	.	.	.	.
P. lemoignei	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.

III. Isolation of Phage and General Properties

1. Isolation of phage

Activated sludge or raw sewage from the Greater Vancouver
Sewage Processing Plant (Iona Island) was "clarified" by centri-
fugation at 20,000 x g for 5 min. A 10 ml sample of the supernatant
was added to 10 ml of double-strength Luria broth, and the mixture
inoculated with about two drops of an overnight culture of P. acidovorans
#14. The flask was incubated under static conditions at 30 C for 48
hr. A few drops of chloroform were added to the flask, which was then
shaken vigorously. The suspension was centrifuged at 6000 x g for
10 min. to remove bacterial cells and debris, and the supernatant
was carefully decanted and stored over several drops of chloroform.

The enrichment was diluted and plated with P. acidovorans #14
by the overlay method (Adams, 1959). A single plaque was picked
from one of the plates with a sterile wire and suspended in Luria
broth. The phage was purified by repeated single plaque picks.

Subsequently, a great number of phages were isolated from
raw sewage collected from the same source using various other
Pseudomonas species as host organisms.

2. Plaque morphology

The typical plaque morphology was determined using the overlay method with thick (40 ml medium) Luria agar plates which had been dried previously at 37 C for several hours (Hershey, and Rotman, 1949).

3. Host range

The host ranges of the phages were determined by spot testing a high titre lysate (ca. 1 x 10^{12} pfu/ml) on overlays containing the various organisms, and incubating the plates overnight at 30 C or 37 C.

4. Relative efficiency of plating

A pure line of phage was diluted appropriately and eight equal volumes were plated with the various host organisms. After an overnight incubation period the plaque counts were tabulated, and the means and standard deviations calculated.

5. Production of a high titre lysate

A number of 500 ml erlenmyer flasks each containing 150-200 ml of Luria broth were inoculated with 10 ml of an overnight culture

of P. acidovorans #29. The flasks were incubated at 30 C and

250 rpm in a Metabolyte model G77 shaker water-bath (New Brunswick

Scientific Co., New Brunswick, N.J.) until the cultures reached

an optical density at 650 mμ of 1.5-2.0. This was equivalent to

10-13 x 10^8 cells/ml. Phage ØW-14 was added to give a multiplicity

of approximately 1, and incubation was continued for a further 6 hr.

Using this method it was possible to routinely obtain lysates with

titres of 1-3 x 10^{11} pfu/ml.

6. Phage purification

Phage lysates were freed of whole cells and debris by centri-

fugation at 20,000 x g for 5 min. The turbid supernatant fluid was

carefully decanted and centrifuged at 14,000 rpm for 4 hr. using

the A-28 rotor (approximate capacity 2000 ml) in an International

Model B-60 preparative ultracentrifuge (International Equipment

Co., Needham Heights, Mass.). The clear supernatant, containing

less than 2% of the total plaque forming units, was removed by

suction and discarded. The phage pellet was allowed to resuspend

in 0.05M Tris (hydroxymethyl)aminomethane-HCl- 0.01 M citric acid -

0.005 M NaCl buffer pH 8.1 at 4 C for several days. The highly

turbid suspension was digested with DN'ase I (Worthington Biochemical

Corp., Freehold, N.J.) and RN'ase A (Schwartz Bioresearch Inc.,

Orangeburg, N.Y.), both at 10 μg/ml, for 1 hr at 37 C. The suspension

was chilled to 4 C and ice-cold 95% ethanol added dropwise to a final

concentration of 16%. After 2 hr. the suspension was centrifuged

at 6000 x g for 10 min., and the supernatant removed and stored.

7. Plaque morphology mutants

A total of twenty discrete a+-type plaques (turbid plaque type)

were picked from overlay plates into small tubes containing 1 ml of

sterile Luria broth. The suspensions were mixed vigorously and

stored at 4 C for 1-2 days. The tube contents were then diluted

and plated using $\underline{P}$. $\underline{acidovorans}$ #14 as the host. On the plates con-

taining the lowest dilutions (10^{-1} and 10^{-2}) of the phage the a+-type

plaques formed a confluent lawn on which it was possible to distinguish

clear areas (a-type plaques). All distinct, large, clear plaques

were totalled to give the minimum value of the mutational frequency,

while the total of all possible clear plaques gave the maximum

value.

8. Electron microscopy

Partially dried preparations of ØW-14 were stained with 2%

phosphotungstic acid, pH 7.2. The grid was allowed to air dry before

being examined with a Phillips EM-200 electron microscope at an

operating voltage of 60 KV. Magnifications, before printing, ranged

from 15,000 to 27,800 X.

IV. Kinetics of Adsorption

An exponentially growing culture of P. acidovorans #29 was diluted with fresh, ice-cold, Luria broth to a density of 1 x 10^8 cells/ml. Ten millilitres of this cell suspension were added to a 125 ml erlenmyer flask which was then placed in a shaker water-bath. In all experiments, unless stated otherwise, the temperature was maintained at 30 C, and the speed at 250 rpm.

After a five minute period for temperature equilibration, 0.1 ml of phage suspension, suitably diluted, was added to the flask to give an initial multiplicity of infection (moi) of 0.01. At one minute intervals thereafter, 0.1 ml samples were removed from the adsorption flask to 9.9 ml of ice-cold Luria broth contained in large broth tubes (2.2 x 20 cm) stored in an ice bucket. These were mixed vigorously using a Vortex Junior Mixer (Scientific Industries Inc., Queens Village, N.Y.).

At the termination of the experiment, 5 ml samples from each broth tube were centrifuged in the cold at 20,000 x g for 5 min to sediment bacterial cells. The supernatants were then appropriately diluted and plated with P. acidovorans #29 as the host organism.

In the case of experiments run to show the effect of sodium chloride on adsorption, a culture of P. acidovorans grown in normal Luria broth was diluted into Luria broth (-NaCl). The phage lysate was similarly diluted.

The adsorption rate constants were calculated using the equation
derived by Schlesinger (1932):

$$K = \frac{2.3 \times \log_{10} Po/Pt}{N \times T}$$

where $\underline{K}$ is the velocity constant for cell attachment; $\underline{Po}$ is the number
of plaque forming units at zero time; $\underline{Pt}$ is the number unadsorbed at
time $\underline{T}$; and, $\underline{N}$ is the concentration of bacteria in cells/ml.

V. Intracellular Phage Development

1. One-step growth experiment

A 0.3 ml volume of an overnight culture of $\underline{P.}$ $\underline{acidovorans}$ #29
was added to 10 ml of fresh Luria broth in a 125 ml erlenmyer
flask. This cell suspension was incubated with aeration at 30 C
for 3 hr. The log phase cells (approximate density 1×10^9 cells/ml)
were diluted to a density of 1×10^8 cells/ml with fresh Luria
broth. A 10 ml sample of this suspension was transferred to a fresh
125 ml erlenmyer flask which was incubated in a shaker bath.
After 5 min., 0.1 ml of an appropriately diluted phage suspension
was added to give a moi of 0.01. After 7 min the contents of the
adsorption flask were diluted into tubes of Luria broth in a static
water-bath as follows: 1/100 (tube 1), 1/1,000 (tube A) and
1/10,000 (tube B). 0.1 ml samples were plated at regular intervals

from tubes A and B (the burst tube). The number of unadsorbed phage was determined after 8 min by centrifuging a 3 ml sample from tube A at 4 C for 5 min. at 20,000 x g and plating triplicate 0.2 ml samples of the supernatant.

2. Effect of culture age on the burst size

Eight 125 ml erlenmyer flasks each containing 10 ml of Luria broth were inoculated with an overnight culture of P. acidovorans #29 to give an initial cell density of 1.2 x 10^8 cells/ml. The flasks were incubated in a shaker water-bath. At hourly intervals, the contents of the flasks were diluted to give 1 x 10^8 cells/ml and a modified one step growth experiment was performed using the diluted culture.

Quadruplicate samples were plated from tube A at 10 min., and from tube B at 150 min. Also, four samples were plated to determine the number of free phage particles after the adsorption period. The means of the plaque counts from these three sources were used in the determination of the average burst size.

VI. Lysis Inhibition

1. Turbidimetric method

An overnight culture of P. acidovorans #29 was used to inoculate three 125 ml sidearm flasks each containing 10 ml of medium. After approximately one hour's incubation, ØW-14a+ was added to one flask at a moi of 5, the second flask was inoculated with ØW-14a at the same moi, while the third was maintained as an uninfected control. Every thirty minutes turbidity readings on the three flasks were made using a Klett Summerson Photoelectric colorimeter (Klett Mfg. Co., N.Y.) equipped with a 540 filter.

2. One-step growth method

To 10 ml of log phase cells (1 x 10^8 cells/ml) phage was added at a moi of 5. After a 10 min period for adsorption, more phage was added, again at a moi of 5. After a further 10 min, the flask contents were diluted and plated in the manner outlined for the one-step growth experiment.

VII. Thermal Inactivation

Large broth tubes (2.2 x 20 cm) containing 10 ml of Luria broth were placed in deep water-baths at 50, 55, 60 and 65 C. After a 10 min. period for temperature equilibration, a small volume of phage suspension, generally less than 0.2 ml, was added to each tube to give 1.5-3.5 x 10^3 pfu/ml (except the tube at 65 C, in which the initial

titre was 2×10^4 pfu/ml). The tubes were mixed vigorously and re-

placed immediately in their appropriate water-baths. At regular

intervals, 0.5 ml samples were removed to small test tubes chilled in

an ice-bucket. At the end of the experiment samples were plated.

The rate constants for the thermal inactivation of ØW-14 were

calculated using the following equation (Pollard, 1953):

$$-k_\Delta = \frac{\log_e Nt/No}{T}$$

where k_Δ is the rate constant; $\underline{No}$ the number of plaque forming units

at zero time; and $\underline{Nt}$ the number of plaque forming units at any time $\underline{T}$.

The rate constants are expressed in reciprocal minutes (min^{-1}).

In the calculation of the activation energies, the following

equation was employed (Neilands and Stumpf, 1958):

$$E = \frac{\log_{10} \frac{k_\Delta 2}{k_\Delta 1} (2.3 \times R \times T_1 T_2)}{T_2 - T_1}$$

where $\underline{E}$ is the Arrhenius energy of activation; $k_\Delta 1$ and $k_\Delta 2$ are the

rate constants for thermal inactivation at temperatures T_1 and T_2,

respectively; and $\underline{R}$ is the universal gas constant.

Because the heat of activation ($\Delta H*$) differs by about 600 calories

from the Arrhenius energy of activation, the following corrective

equation was applied (Dixon and Webb, 1964):

$$\Delta H^* = E - RT$$

VIII. Sonic Sensitivity of Phages

Quadruplicate 2.8 ml samples of phages S13, T1 and $\emptyset$W-14a+, each containing 1×10^6 pfu/ml, were subjected to thirty second bursts of acoustic energy from a Biosonic II (Bronwill Scientific, Rochester, N.Y.) equipped with a needle probe. The frequency of ultrasonic output of this instrument is 20 Kcps $\pm$ 400 cps. A setting of 70 was used throughout, being equivalent to 87.5 Watts.

The samples were chilled in ice-water prior to and during sonication to reduce heat denaturation. Then they were diluted and 0.1 ml samples were plated on the appropriate hosts: E. coli B for S13 and T1, and P. acidovorans #29 for $\emptyset$W-14a+.

IX. pH Inactivation

Luria broth was adjusted to pH 2-12 by the addition of 1M HCl or 1M NaOH, and 4.5 ml amounts were added to test tubes in an ice-water bath. After 15 min., 0.5 ml samples of phage preparations, suitably diluted with physiological saline to 3×10^4 pfu/ml, were added to each tube and the tubes shaken vigorously. After 30 min. incubation, 0.1 ml samples were plated from each tube.

X. Sensitivity to Ultraviolet Light and Photoreactivation

1. UV inactivation

A mixture of coliphage T1 and ØW-14a+, each at a titre of 1 x 10^6 pfu/ml, was prepared using Luria broth as the diluent. One millilitre samples of the phage suspension were placed in disposable plastic Petri dishes (Millipore Filter Corp., Bedford, Mass.) of 2.5 cm diameter and irradiated at a distance of 50 cm with a General Electric 15 Watt germicidal lamp (principle wavelength at 2575 A). The samples were stirred continuously during the irradiation period with a magnetic stir-bar and stirrer. The irradiated samples were then diluted and plated under yellow, nonphotoreactivating, light. P. acidovorans #29 was used as the host for ØW-14a+ and E. coli B was used for T1. The plates were incubated at 30 C and 37 C, for ØW-14a+ and T1 respectively, in light-proof boxes.

The rate constants were calculated from the linear region of the graphed results using the following equation (Pollard, 1953):

$$-k_{uv} = \frac{\log_e Nt/No}{T}$$

where k_{uv} is the rate constant for ultraviolet light inactivation; No is the number of pfu/ml at the "beginning" of the linear region

of inactivation; and, $\underline{Nt}$ is the number of pfu/ml remaining after $\underline{T}$ seconds irradiation.

2. Photoreactivation

In order to obtain photoreactivation, the UV-irradiated phage was plated under white light, and the uninverted plates were incubated 12 inches from a lamp fitted with twin Westinghouse 15 Watt Cool White or General Electric 15 Watt Daylight fluorescent bulbs (principle wavelength approximately 5700 A in both cases). Irradiation was continued overnight at the appropriate incubation temperatures.

The photoreactivable sector (Dulbecco, 1950) was calculated using the following equation:

$$P_s = 1 - \frac{k_p}{k_{uv}}$$

where $\underline{P_s}$ is the proportion of the phage photoreactivated; $\underline{k_p}$ is the rate of UV-inactivation of the phage as measured by incubation in the light; and, $\underline{k_{uv}}$ is the rate constant for UV-inactivation as measured by incubation in the dark.

XI. <u>Phage Resistant Mutants and the Carrier State</u>

1. Isolation of resistant strains

Sufficient ØW-14a was added to 10 ml of a log phase culture

of <u>P</u>. <u>acidovorans</u> #29 (1 x 10^8 cells/ml) to give a moi of 5. The

mixture was incubated for 10 min. at 30 C, following which 0.1

ml samples were spread on Luria agar plates.

The colonies which appeared on the plates after incubation

at 30 C for 48 hr were picked with sterile tooth picks to overlay

plates containing a high concentration (5 x 10^9 pfu/overlay) of

ØW-14a. These plates were incubated overnight at 30 C. Those

colonies which grew best were transferred to tubes of tryptone

broth and regrown with aeration at 30 C. These broth cultures

were serially transferred four times to dilute out any residual

ØW-14a.

The cultures were then tested for their resistance to ØW-14a+

and ØW-14a. The presence of an intracellular form of the phage

was checked for by spot testing the cultures on overlays containing

<u>P</u>. <u>acidovorans</u> #29. The resistant, non-phage carrying cultures were

purified by several cycles of spreading and single colony selection.

Because the continuence of the carrier state depends upon phage

reinfection of sensitive cells, those cultures confirmed as being

phage infected were purified by streaking-out on overlays containing

ØW-14a. The colonies which arose were inoculated into tubes of Luria broth and transferred serially as described above.

2. Microscopy

a. Motility

The motility of the cultures was determined by the hanging drop method using log phase cells and a magnification of 400 x.

b. Phase contrast microscopy

The samples were prepared essentially as described by Shaw (1968), and photographed with a Zeiss microscope using Adox KB14 film (Adox Fotowerke, Frankfurt/Main).

3. Confirmation of the carrier state

"Carrier" cultures can be differentiated from lysogenic cultures by the fact that in the former case only a portion of the cells actually carry the phage genome, while in the latter case, all the cells are "infected". To show which state existed in the phage infected cells of P. acidovorans, log phase cells of a "carrier" culture were harvested by centrifugation at 4 C, and the cell

pellet washed three times with an equal volume of ice-cold Luria

broth. The washed and resuspended cell suspension was diluted to

10^{-6}, and three 1.0 ml samples were plated by the Standard Plate

Count Method (Standard Methods, 1960). Three 0.1 ml samples were

plated from each of the 10^{-3}, 10^{-4} and 10^{-5} dilutions by the overlay

method with P. acidovorans #29. After appropriate incubation periods

the total numbers of plaques and colonies were tabulated.

XII. Recombination

An overnight culture of P. acidovorans #29 was inoculated into fresh

Luria broth and regrown to mid-long phase. This culture was diluted to give

a density of 1 x 10^{8} cells/ml, and 5 ml of this suspension was transferred

to a sterile, screw-capped tube. Phages ØW-14a+ and ØW-14h were

added at a moi of 5 for each. After 10 min incubation on a tube roller,

the cells were harvested by centrifugation and the supernatant dis-

carded. The cell pellet was resuspended in 5 ml of fresh Luria broth

and incubation was continued for a further two hours. A few drops of

chloroform were then added to the tube, which was shaken vigorously

for a minute. The tube contents were centrifuged to remove bacterial

cells and debris, and the supernatant was diluted and plated by the overlay

method on P. acidovorans #29 and on a mixed indicator composed of the

latter organism and P. acidovorans #29-5. After overnight incubation

at 30 C the recombinants were identified and tabulated.

XIII. <u>Physical and Chemical Properties of ØW-14 DNA</u>

1. Isolation of DNA

a. Perchlorate method (Freifelder, 1966)

A 7.5 M solution of sodium perchlorate in 0.001 M EDTA
was adjusted to pH 7-9 by the dropwise addition of 1N NaOH,
and 50 ml of it was added to 113 ml of a purified phage suspension
(10^{12} pfu/ml) with stirring. The final concentration of
perchlorate was approximately 2.3 M. The solution was stirred
slowly at room temperature for 30 min., following which the
precipitated material was removed by centrifugation.

The nucleic acid was precipitated from the supernatant by
the addition of two volumes of 95% ethanol, and the fibrous
material collect on a magnetic stir-bar. This material was
washed once with 66% ethanol and then dissolved in 0.1 x SSC
(0.015 M NaCl - 0.0015 M Na$_3$citrate buffer pH 7.0). After
several days stirring at 4 C, the insoluble material was
removed by centrifugation and the nucleic acid purified further
by the isopropanol precipitation method of Marmur (1961).

b. Trypsin-phenol method

A partially purified preparation of ØW-14 (10^{13} pfu/ml)

was digested with 0.1 mg/ml trypsin 1-300 (Nutritional Bio-
chemicals Corp., Cleveland, Ohio) in the presence of 10^{-3} M
$CaCl_2$ for 8 hr at 37 C. Then the mixture was kept at 4 C for
several days. The highly viscous solution, containing less
than 1% of the total pfu input, was deproteinized by shaking
with buffer-saturated phenol. The DNA was precipitated by
the addition of two volumes of 95% ethanol, redissolved in
SCC (0.15 M NaCl - 0.015 M Na_3citrate buffer pH 7.0), and
purified further by the isopropanol precipitation method
(Marmur, 1961).

c. Isolation of P. acidovorans DNA

P. acidovorans DNA was purified by a modification of the
sodium dodecyl sulfate-phenol method (Mandel, 1966) from 17.8
g, wet weight, of cells. After the initial deproteinization with
phenol, the aqueous phase was deproteinized six consecutive
times by shaking slowly at 4 C with chloroform - isoamyl alcohol
(24; 1, v/v). The DNA was precipitated from the aqueous layer
with two volumes of absolute ethanol and dissolved in SSC
buffer. After removing residual phenol by extracting several
times with 0.1 volume of ether, the solution was treated with
ribonuclease (DN'ase free), 50 µg/ml, for 30 min. at 37 C,
after which the deproteinizing step was repeated once. The

DNA was purified further by precipitating twice with iso-propanol (Marmur, 1961).

2. General properties of the phage nucleic acid

a. UV spectrum

The UV spectrum of ∅W-14 DNA dissolved in 0.1 x SSC was obtained using a Unicam SP.800B Spectrophotometer (Unicam Instruments Ltd., Cambridge, England).

b. Calculation of DNA concentration

DNA concentrations were estimated using an extinction co-efficient of 20 cm^2/mg based upon the optical density at 260 mμ (Lee and Boezi, 1966).

c. Colorimetric assays

The diphenylamine test (Ashwell, 1957) was used to determine the presence of DNA, while the orcinol assay (Ashwell, 1957) was used for RNA determinations.

d. Enzyme sensitivity

Phage DNA was adjusted to a concentration of 50 μg/ml in SSC. Three tubes were set-up each containing 0.84 ml of DNA and 0.05 ml of SSC-0.018 M $MgCl_2$ buffer pH 7.0. To the first tube was added 0.01 ml of DN'ase; to the second 0.01 ml of RN'ase; and, to the third 0.01 ml of SSC. In each case the final enzyme concentration was 10 μg/ml. The tubes were incubated at 37 C for 30 min., then chilled on ice. To each tube was added 0.1 ml of 30% $HClO_4$ to precipitate undegraded nucleic acid. After a further 15 min. the tubes were centrifuged and the OD 260 of the supernatants determined.

3. Melting temperatures

A Beckman Model DUR recording quartz spectrophotometer (Beckman Industries Inc., Fullerton, Calif.) equipped with a Gilford 2000 multiple sample adsorbance recorder (Gilford Instrument Laboratories Inc., Oberlin, Ohio) was the basic unit used in the determination of the melting temperatures (Tm). A thermostatically controlled waterbath with pump (Haake constant temperature circulator, Model F) was used to heat and circulate the ethylene glycol to the inner thermospacers flaking the cuvette chamber. The rate of heating of the fluid was automatically controlled by a variable speed

motor and controller (Gerald K. Heller Co., Las Vegas, Nevada).

Prior to the Tm determination the phage DNA was dialyzed over-
night at 4 C against 300 volumes of 0.1 x SSC. The dialyzed DNA
solutions were diluted in 0.1 x SSC to obtain samples with 0.4 -
0.6 OD 260 units per millilitre. Three millilitre samples of the
solutions were transferred to glass-stoppered quartz cuvettes of
1 cm light path. These were then placed in the cuvette chamber
along with a control cuvette containing 0.1 x SSC buffer.

The temperature was raised rapidly to approximately 50 C, then
the regulator was adjusted to give a rate of increase of 1 C every
4 min.

4. Buoyant density

a. General method

The method used was essentially that of Mandel, Schildkraut
and Marmur (1968), using a Beckman Model E analytical ultra-
centrifuge (Beckman Instruments Inc., Palo Alto, Calif.) at
44,000 rpm for 22 hr at 20 C. E. coli DNA (Worthington) was
used as the density marker and it was assumed that its density
was 1.710 g/cc^3 (Mandel et al., 1968).

Kodak Commercial Film (Eastman Kodak Co., Rochester, N.Y.)
was used for the UV photography, and the negatives were developed

using D-11 developer. The films were scanned using a Joyce-Loebl double-beam recording microdensitometer.

The buoyant density of the phage DNA was determined using the following equation (Mandel et al., 1968):

$$\rho = 1.710 - 0.0089 \ (r^2 - r_o^2) \ g/cc^3$$

where r and r_o are the peak distances from the centre of rotation of the phage DNA and the E. coli marker, respectively.

The GC content of the phage DNA was calculated using the formula of Schildkraut, Marmur and Doty (1962):

$$GC = \frac{\rho - 1.660}{0.098}$$

b. Preparation of denatured phage DNA for buoyant density analysis.

i. Alkaline denaturation. A small sample of ∅W-14 DNA in DNA in SSC was adjusted to pH 12.5 by the dropwise addition of 1N NaOH. After standing at 22 C for 30 min., the solution was diluted by the addition of 0.02 M Tris-HCl buffer pH 8.5, and adjusted to pH 8.5 by the careful addition of 0.2 M HCl. This solution was diluted further with buffer to a concentration of 50 μg/ml.

ii. Thermal denaturation. Phage DNA in SSC was diluted 1/10 into distilled water, and a small sample heated at 100 C for 10 min. The solution was rapidly chilled on ice, following which it was diluted to 50 μg/ml with 0.02 M Tris-HCl buffer pH 8.5.

5. Chemical composition of the phage DNA.

a. Hydrolysis

i. Hydrolysis to free bases. Phage DNA in SSC was precipitated from solution by the addition of three volumes of 95% ethanol. The precipitate was washed with anhydrous acetone and dried. A 1 mg sample was placed in a quartz-glass hydrolysis tube (9 mm external diameter; 6 mm internal diameter), sealed at one end, and 0.71 ml of 88% formic acid was added. After freezing the contents of the tube in a mixture of dry-ice and acetone, the tube was sealed under reduced pressure. Hydrolysis was carried out for 45 min at 175 C, with the hydrolysis tube inside a cast-iron guard tube in case of explosion.

After cooling to room temperature, the contents of the tube were again frozen in a mixture of dry-ice and acetone. Gentle heating of the end of the tube was used to permit the controlled release of the internal pressure.

The tube contents were evaporated to dryness and the residue was dissolved in 50 μl of 20% isopropanol - 0.1 N HCl.

ii. <u>Hydrolysis to free saccharides</u>. The DNA was prepared
for hydrolysis as described above. A 1 mg sample of the DNA was placed
in a hydrolysis vial together with 1 ml of 1 N HCl. The vial was sealed
under reduced pressure, and hydrolysis was carried out at 100 C for
90 min.

After cooling, the contents of the vial were trans-
ferred to a centrifuge tube. To this was added an equal volume of a
slurry of acid-washed charcoal (Norit A). The mixture was swirled gently
for 5 min. at room temperature, then centrifuged at low speed. The
supernatant was removed carefully with a Pasteur pipette and evaporated
to dryness. The residue was dissolved in 100 μl of 10% isopropanol.

iii. <u>Nature of the attachment site of the saccharide compo-</u>
<u>nent of $\emptyset$W-14 DNA</u>. The method of Alegria and Kahan (1968) was used to
determine whether or not the saccharide component of $\emptyset$W-14 DNA was
covalently bonded to the DNA. The phage DNA (214 μg) in SSC was pre-
cipitated by the addition of an equal volume of ice-cold 10% trichloro-
acetic acid (TCA). After chilling the acidified solution at 0 C for
10 min., the precipitated material was collected by centrifugation.
The supernatant was carefully decanted and extracted with ethyl ether
to remove TCA. The pellet was resuspended in a volume of water equal
to that of the supernatant, and the presence of carbohydrate in the
two fractions was assayed for by the Anthrone method (Spiro, 1966).

b. Paper chromatography

i. <u>Separation of bases</u>. The descending method of paper
chromatography on Whatman #1 paper was used to separate the free bases.
Three solvent systems were employed: (a) <u>t</u> - butanol - 88% formic acid -
water (16:1:4, v/v) (Roberts, 1961); (b) <u>n</u> - butanol - methanol - water -
concentrated ammonium hydroxide (60:20:20:1, v/v) (Randerath, 1965);
(c) <u>iso</u>-propanol - concentrated hydrochloric acid - water (65:17:18,
v/v) (Bendich, 1957). After irrigation of the paper the chromatogram was
dried and the bases were located by their fluorescence in shortwavelength
(ca. 254 mμ) UV-light.

ii. <u>Separation of saccharides</u>. Two solvent systems were
used to separate sugars on Whatman #1 paper: (a) ethyl acetate - pyridine -
water (120:50:40, v/v); (b) <u>iso</u>-propanol - water (160:40, v/v) (Smith,
1960). The sugars were located by developing the chromatogram with either
silver nitrate reagent or aniline-diphenylamine reagent (Smith, 1960).

c. Quantitative estimation of ØW-14 DNA base composition

Phage DNA (500 μg) was hydrolyzed as described above (5.a.i.).
The yellow residue remaining after evaporation of the formic acid
was redissolved in 100 μl of 10% <u>iso</u>-propanol - 0.1 N HCl. The
total hydrolysate was applied to a sheet of Whatman #1 chromato-
graphy paper. Separation of the bases was accomplished by using
solvent system C. After time sufficient to ensure the best possible

separation of the bases, the chromatogram was removed from the tank and dried. The areas showing fluorescence were cut out as well as corresponding areas from an unspotted area of the chromatogram. The latter areas served as the controls.

The fluorescent areas and the controls were eluted with 0.1 N HCl, with the exception of thymine, in which case 0.01 N HCl was used. Elution was carried out in a humidified container using the second method described by Heppel (1968). The eluates were adjusted to a uniform volume and spectra were obtained for the bases in acidic and alkaline solutions.

The concentrations of the individual bases were calculated from their molar extinction coefficients in acidic solution: Adenine 13.1×10^3 at 265.5 mμ; Cytosine 10.0×10^3 at 276 mμ; Guanine 11.4×10^3 at 248.5 mμ; Thymine 7.89×10^3 at 264.5 mμ (Besch and Goldwyn, 1966).

RESULTS AND DISCUSSION

Section I. Isolation and General Properties of the Phage

1. Isolation

Various Pseudomonas phages were isolated by enrichment culture

from raw sewage or activated sludge (Table IV). At present, only

the phage active against P. acidovorans #14 has been investigated

in any depth.

The original enrichment contained only one plaque type: a

small, discrete, clear plaque. However, when the original enrich-

ment was retitred after approximately one month at 4 C, it was noted

that the number of clear plaque formers had decreased markedly,

and the presence of a few haloed plaques was observed. The latter

were chosen for further study and given the name ∅W-14. Since it

later transpired that the two phages were related the characteristics

of both were investigated. Lytic agents were also identified upon

Mitomycin C induction (0.05 μg/ml Mitomycin C in Luria broth) of

P. acidovorans and P. testosteroni cultures. Also some examples

of cross-reactivity between these two groups of organisms was

obtained with the induced cultures. The nature of these agents,

whether phage or bacteriocin, was not further investigated.

Table IV. Previously unreported phages isolated from raw Vancouver sewage.

Organism	Phage isolated
P. acidovorans #29	+
P. acidovorans #14	+
P. acidovorans 15666	+
P. acidovorans 15667	+
P. testosteroni #78	+
P. testosteroni #138	+
P. testosteroni 11996	+
P. mucidolens 4687	+
P. putrefaciens (Hammer)	+
P. convexa 795	+
P. synxantha 796	+
P. taetrolens 4683	+
P. ovalis 950	+

2. Plaque morphology

Five distinct types of plaques were observed: (a) a^+-type: small plaque, approximately 2 mm in diameter, with a very small clear centre and a fairly turbid halo; the halo appeared faintly ringed. (b) a-type: plaque approximately 1.5 mm in diameter with a wide central clear zone and a very small indistinct halo. (c) h-type: a spontaneously arising host range mutant of a which produced the same type of plaque. (d) t-type: the same size as $\underline{a}^+$, with a very small clear central zone bordered by a single distinct clear ring and a turbid halo. (e) sc-type: approximately the same size as $\underline{a}^+$, but with a larger clear zone, surrounded by a fairly turbid halo; the halo sometimes appeared as faint, concentric, rings. Fig. 1 shows the difference between the a^+ and a- type plaques.
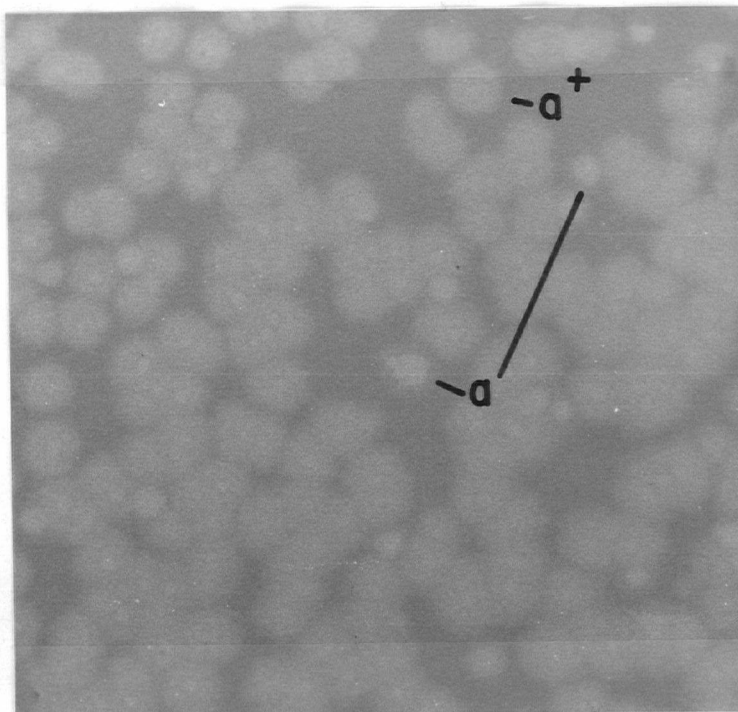
Table V: Host-range of ØW-14a+ and derivatives.

		Lysis by:	
Organism	ØW-14a+	ØW-14a	ØW-14h
P. acidovorans #29	+	+	+
P. acidovorans #14	+	+	-
P. acidovorans #114	-	-	-
P. acidovorans #146	-	-	-
P. acidovorans AK-11	+	+	-
P. acidovorans 15666	-	-	-
P. acidovorans 15667	-	-	-
P. acidovorans 15668	+	+	nd*
P. testosteroni #78	-	nd	+
P. testosteroni #138	-	nd	-
P. testosteroni 11996	-	nd	-
P. aeruginosa 9027	-	nd	nd
P. aeruginosa 9721	-	nd	nd
P. ovalis 950	-	nd	nd
P. fragi 4975	-	nd	nd
P. convexa 795	-	nd	nd
E. coli k12	-	nd	nd
E. coli B	-	nd	nd

* nd - not tested

3. Host range

ØW-14 showed a limited host range, lysing only three of the

seven different strains of P. acidovorans tested and none of the

related organism, P. testosteroni. The clear plaque mutant, ØW-14a,

had an identical host range, and the plaques on the three hosts

were always haloed for a+ and clear for a. The host range mutant,

ØW-14h, did not lyse P. acidovorans #14 nor P. acidovorans AK-11 -

hosts for the parent strain - but did lyse P. acidovorans #29

and P. testosteroni #78 (Table V). It is interesting to note

that ØW-14h did not lyse P. testosteroni 11996, since P. testosteroni 78

and the former are supposedly identical organisms (Stanier,

Palleroni and Doudoroff, 1966).

4. Relative efficiency of plating

The determination of the relative efficiency of plating (REP) on

various host strains appeared to be dependent to some extent, upon

the strain of P. acidovorans used to prepare the phage stock (Table VI).

For a given phage stock the highest titres were always obtained

when the phages were titred on P. acidovorans #29. The plaques

formed on strain AK-11 were small and indistinct, so that the REP

could not be determined accurately with this strain.

Table VI: Relative efficiency of plating of ØW-14a+ and ØW-14a

Phage

Growing host	ØW-14a+		ØW-14a	
	Plating hosts		Plating hosts	
	P. acidovorans #14	P. acidovorans #29	P. acidovorans #14	P. acidovorans #29
P. acidovorans #14	3.3×10^{-1}	1	5.0×10^{-1}	1
P. acidovorans #29	5.0×10^{-1}	1	3.3×10^{-1}	1

5. Phage purification

Various methods were used in an attempt to purify ∅W-14. These included ammonium sulfate precipitation, DEAE-cellulose column chromatography, adsorption chromatography on magnesium pyrophosphate gel (Schito, 1966), and differential centrifugation. The first three of these methods gave very poor recoveries of pfu. Differential centrifugation proved most useful because of its simplicity and the good recoveries obtained, but it was not without its pitfalls since the phage particles suspended in buffer were almost quantitatively sedimented in 10 min. at 6000 x g. Lee and Boezi (1966) found it necessary to centrifuge at 16,000 x g for 2 hr. to sediment P. putida phage gh-1.

This phenomenon was investigated further in an attempt to obtain disperse phage suspensions. The results of a number of experiments are presented in Table VII. It was shown subsequently, that the pH of the buffer, in the range of 6.5 to 8.1, had no effect on the sedimentation properties of the phage particles.

The addition of ethanol to a final concentration of 16% resulted in the greatest increase in nonsedimentable plaque forming units. Such preparations still exhibited a high degree of light scattering. Under the electron microscope, a great number of phage aggregates were observed, but very little bacterial debris was present.

The inactivation of phage in lysates is caused usually by the adsorption of the particles to fragments of cell walls. However, particles may be inactivated by interaction with soluble components of the host cell.

Burnet and Freeman (1937) observed that crude bacterial polysaccharide preparations would inhibit homologous phage. However, inactivation did not depend necessarily upon a stereospecific relationship between the two entities since Ashenburg et al, (1940) found that saline suspensions of Aerobacter capsular polysaccharide, as well as starch, glycogen and gum arabic, were as effective as the homologous capsular polysaccharide in inhibiting plaque formation by a Klebsiella pneumonia phage. A relationship between these substances and the inactivation by aggregation of ØW-14 does not appear likely since P. acidovorans does not store intracellular polysaccharide (Stanier, Palleroni and Dourdoroff, 1966). The existence of extracellular slime or capsular polysaccharide in this organism has not been examined. Cell wall lipopolysaccharides can inactivate coliphages T3, T4 and T7, and the Felix 0-1 phage of Salmonella minnesota. Inactivation in the latter case appeared to be due to adsorption followed by tail contraction and phage DNA ejection (Lindberg, 1967). Lipopolysaccharide isolated from P. acidovorans by the phenol-water method (Osborn, 1966) appeared not to inactivate ØW-14.

Proteinaceous materials, other than antiphage and normal sera,

Table VII: Aggregation phenomena of partially purified suspensions

of ØW-14.

Additions		% pfu nonsedimentable
None		11
Distilled water	2:10 (v/v)	13
Distilled water	9:1 (v/v)	68
95% ethanol	1:10 (v/v)	55
95% ethanol	2:10 (v/v)	82
95% ethanol	3:10 (v/v)	72
10^{-2}M EDTA pH 7	1:10 (v/v)	11
0.05M Tris pH 7.4	9:1 (v/v)	26
0.05M KPO_4 pH 7.4	9:1 (v/v)	27

Phage ØW-14, suspended in 0.05M Tris(HCl)-0.01M Citric acid-
0.005M NaCl buffer pH 8.1, at a titre of 1.1 x 10^{12} pfu/ml
was treated at 4 C as outlined above. After 1 hr suspensions
were centrifuged at 6000 x g for 10 min., and the pfu remaining
in the supernatant were titred.

can have antiphage activity. Brinton, Buzzell and Lauffer (1954)

found that partially purified preparations of E. coli B/6 filaments

(pili) markedly inhibited the plaque forming ability of coliphage

T2, T4, T5 and T6. This possibility is ruled out in the present

case since no foreign particulate matter appeared to be responsible

for the aggregation of ØW-14 (Fig. 8) Das and Marshall (1967)

showed that casein inhibited the ability of a S. aureus phage to

form plaques. Though casein was not present, some similarities

between their observations and the present results are apparent.

With both S. aureus phage and ØW-14a, an increase in the titre of

the preparations was observed when the pH was adjusted to 4-5,

suggesting that the adsorptive agent had an isoelectric point in this

region, and that its neutralization released the bound phage.

However, this does not explain the results with ØW-14a+ (see Section

IX). The fact that ethanol dissociates the larger aggregates also

suggests an ionic interaction, since it is known that this solvent

lowers the dielectric constant of solutions.

Examples of unexplained spontaneous aggregation are to be

found in the literature. Putnam (1951) observed that particles of

T6 tended to aggregate and settle out if their concentration was

greater than about 4×10^{12} pfu/ml, while more dilute suspensions

were disperse. It was observed with ØW-14 that dilution of the phage

1/10 into buffer had little effect on the number of plaque forming

units which remained nonsedimentable. Lanni (1958) observed that

Table VIII: Quantitative distribution of plaque morphology mutants in individual ∅W-14a+ plaques.

Clone #	Total PFU (x 10^{-6})	Min. (x 10^{-3})	Max.	Mutation frequency (x 10^{4}) Min.	Max.
1	130	57	270	4.38	20.77
2	37	17	55	4.59	14.86
3	267	220	560	8.24	20.97
4	399	44	117	1.10	2.93
5	243	42	103	1.73	4.24
6	94	102	155	10.85	16.49
7	50	10	34	2.00	6.80
8	318	71	135	2.23	4.25
9	448	145	590	3.24	13.17
10	221	62	160	2.81	7.96
11	308	115	159	3.73	5.16
12	39	15	33	3.85	8.46
13	64	10	38	1.56	5.94
14	78	23	55	2.95	7.05
15	20	1	11	0.50	5.50
16	119	73	147	6.15	12.35
17	48	74	104	15.42	21.67
18	55	24	38	4.36	6.91
19	29	3	16	1.03	5.52
20	80	41	87	5.13	10.88
Means:	152.4	57.5	143.4	3.77	9.41

when purified suspensions of T5 in buffer were diluted into nutrient

broth and stored at 4 C the plaque count decreased 80-90% in

several weeks. This titre was restored to the starting value by

heating the suspension at 44 C for 10 min.

6. Plaque morphology mutants

When grown on P. acidovorans #14, ∅W-14a+ mutated at a high

frequency to the clear plaque forming type (Table VIII; Fig. 2).

If the clear areas were picked and plated out, clear and haloed

plaques were obtained in approximately equal numbers. The

minimal estimate of the mutation frequency was 3.8 x 10^{-4}; the

maximum estimate was 9.4 x 10^{-4}. In the case of two clones,

frequencies of 2.2 x 10^{-3} were observed. When P. acidovorans #29

was used as the host the mutation was not observed. Because of

this mutation and the higher burst size of the a-type phage

(see Section III), great difficulty was encountered in preparing

a high titre lysate of predominantly the a+-type phage when

P. acidovorans #14 was used as the host.

The spontaneous mutation of a turbid to a clear plaque type

is a quite common occurrence, having been seen with a number of

phages from different sources. Phage 7 m of P. aeruginosa mutates

to a clear plaque type at a frequency of 10^{-4} to 10^{-5} (Feary,

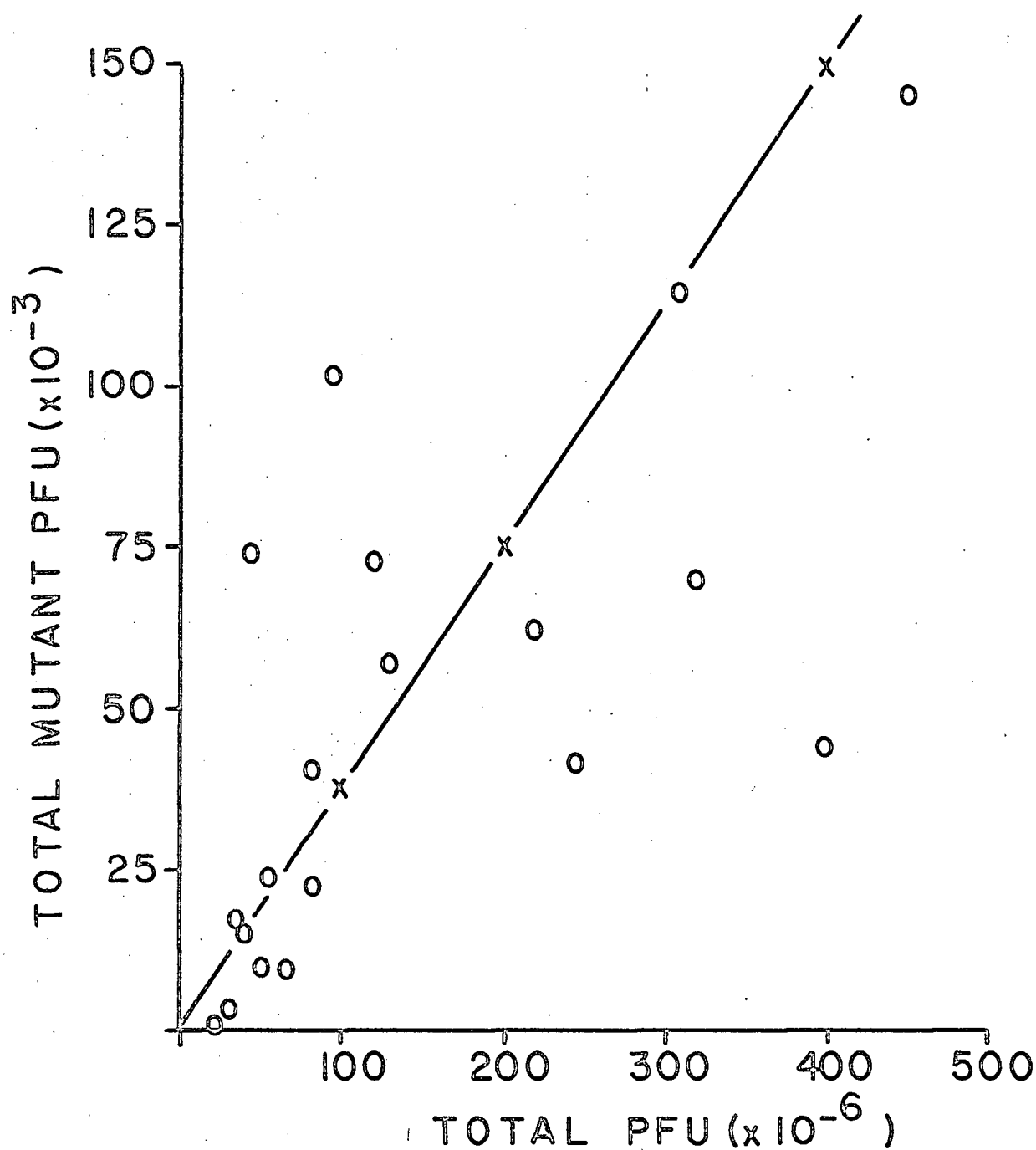Fisher and Fisher, 1964). For coliphage T2H, the observed

Fig. 2. Graphical representation of the occurrence of clear plaque
mutants as a function of the total number of plaque forming
units in individual plaques.
0 - individual experimental results.
x - x - plot of the mean.

frequency was approximately 10^{-3} (Hershey, 1946). <u>Hydrogenomonas</u>

<u>facilis</u> phage ØHF shows this type of mutation (Pootjes, 1964),

and Murphy (1954) reported a high frequency of mutation of the

turbid wild type phage plaque type of <u>Bacillus</u> <u>megatherium</u> (sic)

to a clear plaque type. Thus the values obtained for ØW-14

fall within the general range reported for this type of mutation.

The exactness of these results is in doubt since difficulty was

encountered in distinguishing the clear plaques on lawns of the

haloed plaque type. In addition, the differences in adsorption

kinetics (see Section II) and burst sizes (see Section III) between

the two phage types would influence the results. The only accurate,

but highly laborious, means of determing the true mutation

frequency is by the single burst method (Luria, 1951).

The a-type of the phage appeared to be more stable than the

a+-type since reversion was rarely, if ever, observed in this type.

7. Electron microscopy

Phage ØW-14 had a regular icosahedral head 87 mμ in diameter.

The particle had a tail which is about 20 mμ in diameter and 140 mμ

long. Subunits could be seen in the tails of some of the particles

(Fig. 3). In some cases, the tail appeared to be constricted

where it joined the head. The baseplate was approximately 50 mμ

in diameter, and appeared to carry tail pins (Fig. 4) about

Fig. 3. <u>P</u>. <u>acidovorans</u> phage ØW-14, PTA, x 245,200, scale 100 mµ

Fig. 3.

Fig. 4.   ØW-14 tail detail,
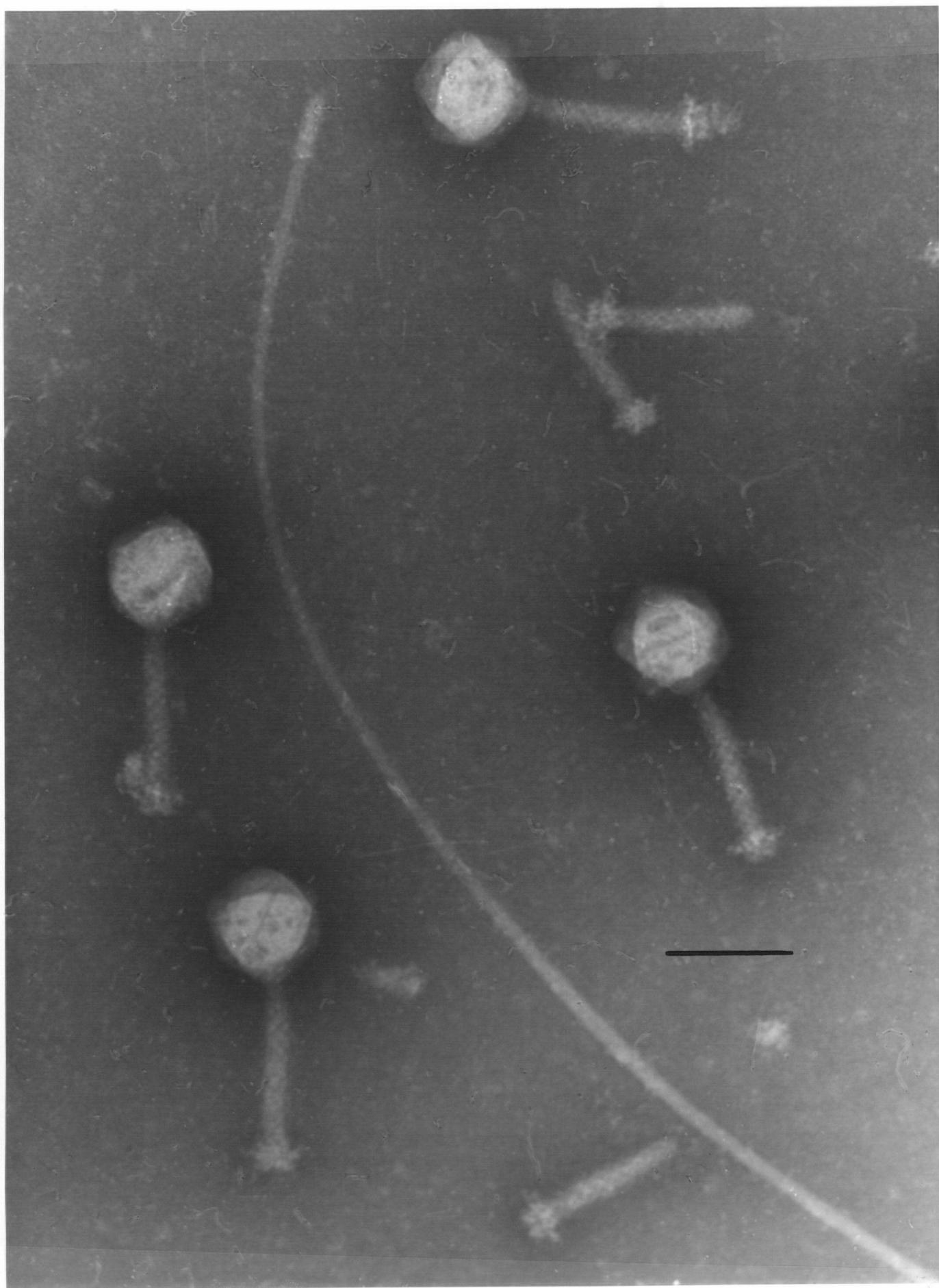   and empty heads,
          PTA, x 367,500,
          scale 100 mμ.
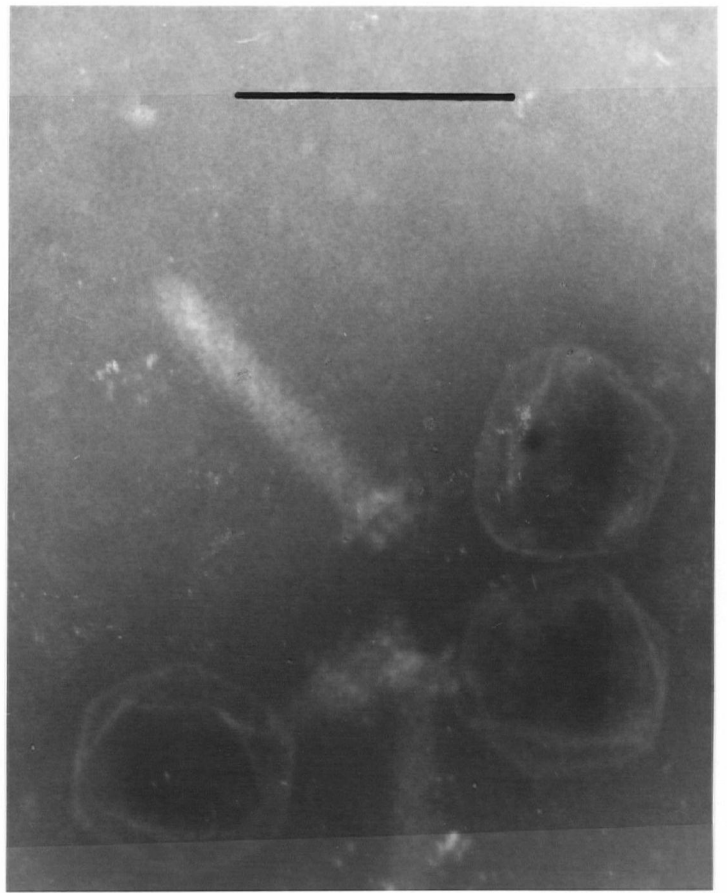

Fig. 5.
       ØW-14 showing
       contracted sheath,
       and exposed core,
       PTA.
       x 254,800
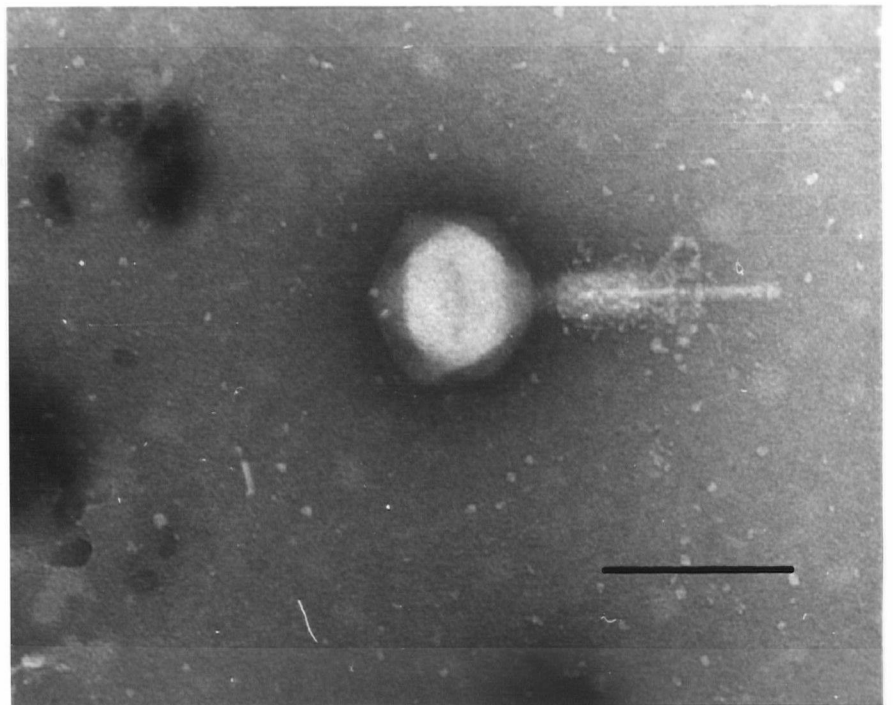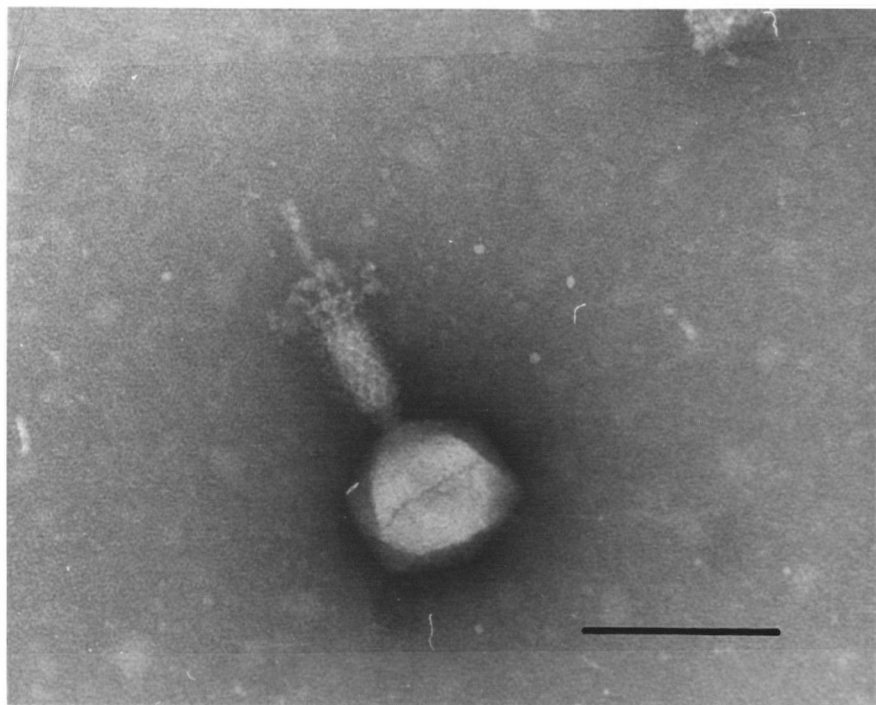   scale 100 mμ

Fig. 6.
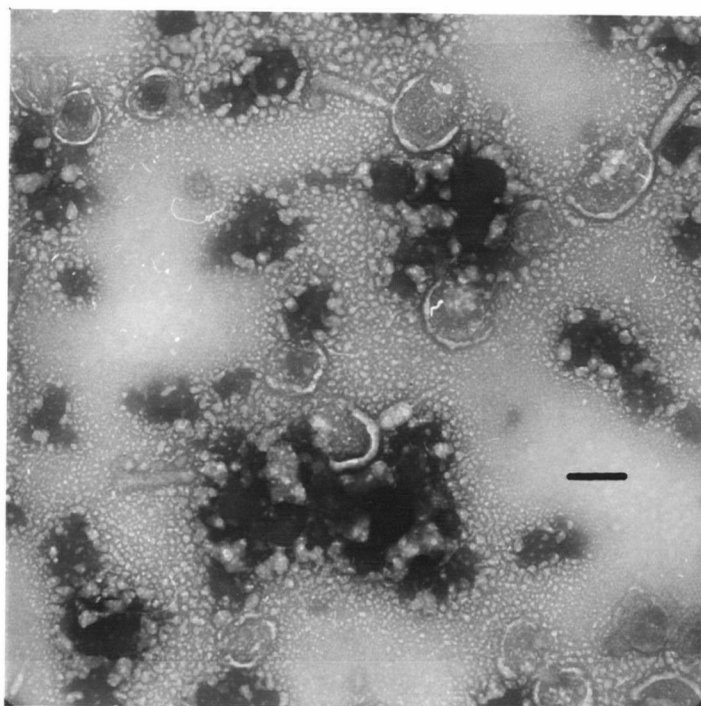 x 256,300
  scale 100 mμ
  PTA

Fig. 7.
  Effect of 0.1 M
  NaClO4 on integrity
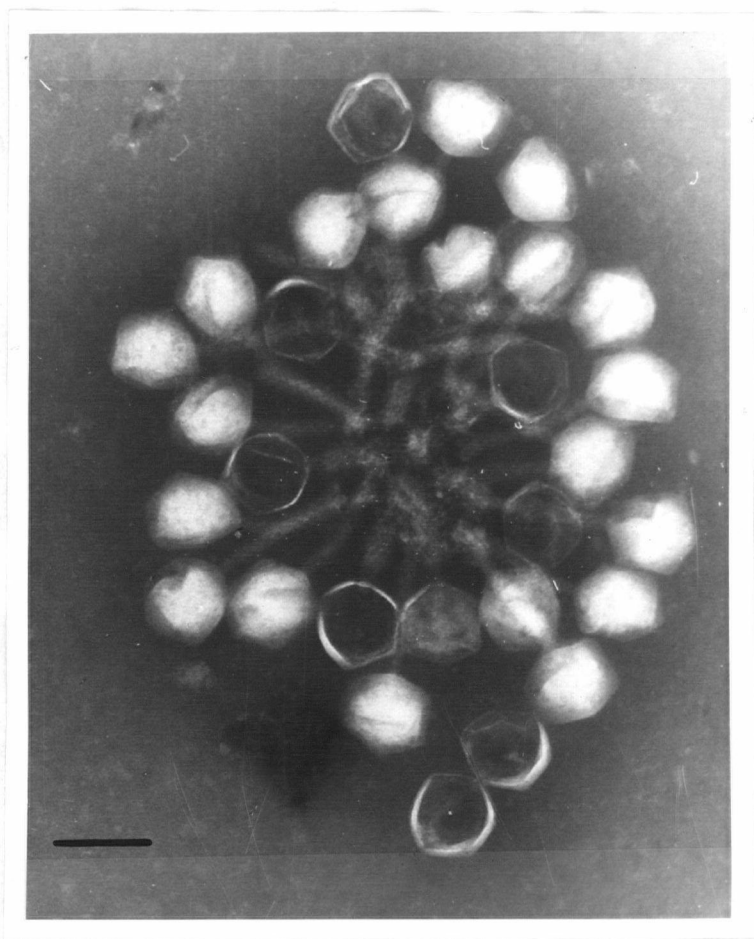 of ØW-14, PTA,
  x 82,800
  scale 100 mμ.

Fig. 8. ØW-14 aggregate, PTA, x 133, 100, scale 100 mμ.

4 x 8 mμ. A number of empty heads were observed which had
retained to a considerable extent the angular nature of the
complete head (Fig. 4.). A few complete phage particles showing
empty heads were also observed in the preparation. A great many
free tails 130 mμ in length were seen in the electron micrographs.
In order to demonstrate the presence of a contractile tail sheath,
sodium perchlorate, buffered to pH 7-9, was added to a concentra-
tion of 0.025 M (Freifelder, 1966). At this low concentration,
a number of phage particles with contracted sheaths were observed
(Fig. 5; Fig. 6). Some of these contracted particles seemed to
have a collar at the juncture of the head and tail. The con-
tracted sheath (25 x 40 mμ) exposed the tail core – a slender
projection 7 x 130 mμ. A skirt of projections, originating in
the region of the former base plate, fanned out around the
exposed core.

At higher concentrations of sodium perchlorate (>0.1 M) the
phage head ruptured (Fig. 7) and the viscosity of the medium
increased.

Phage ØW-14 showed a marked tendency to aggregate (Fig. 8).
No bacterial debris appeared to be present to account for this
aggregation.

Phage ØW-14 falls into Bradley's morphological classification
group A, which contains all phages having contractile tails
(Bradley, 1968). Morphologically it resembles other Pseudomonas

phages, but it is considerably larger than those previously

reported, the heads of which range from 50 - 60 mμ in diameter

(Lee and Boezi, 1966; Bradley, 1967; Olsen et al., 1968).

Aggregation of phage particles has been reported previously, but

in most cases it appears to involve adsorption to pieces of

cellular debris. The considerable number of aggregates and the

number of phage particles involved per aggregate could explain

the unusually high light scattering seen with purified prepara-

tions of ØW-14.

Section II. Kinetics of Adsorption

The results of the adsorption experiments are presented in Fig.

9. The concentration of unadsorbed phage decreased exponentially

with first order kinetics. Adsorption was biphasic in each case.

In the case of ØW-14a+, the time required for adsorption of 50% of

the phage to the host cells was approximately 3.9 min. This corres-

ponded to an adsorption constant (K value) of 1.9×10^{-9} ml/min.

The rate of adsorption decreased markedly (K of 3×10^{-10} ml/min.)

or appeared to stop after only 60% (range 49-72%) of the phage had

adsorbed. At 0 C, the rates of adsorption were almost the same as at

30 C, and again adsorption was biphasic.

In attempt to find the affect of NaCl concentration on the

adsorption rate, without resorting to the use of buffers - as all

previous experiments had used Luria broth - a complex medium contain-
ing a low concentration of NaCl was prepared. The concentration of
NaCl was calculated to be approximately 2×10^{-3} M. Using this medium,
the rate of adsorption increased 1.6 fold, 50% of the phage binding to
the cells in 2.2 min. The adsorption rate constant was 3.0×10^{-9}
ml/min. The kinetics of adsorption appeared to be "normal", with a
decrease in the rate occurring after 93% of the free phage had been
irreversibly bound.

In the case of the spontaneously occurring mutant, $\emptyset$W-14a,
the rate of adsorption was 2.2 fold faster than $\emptyset$W-14a+, with 50%
of the phage being adsorbed in 1.7 min., giving a K value of 4.2×10^{-9} ml/min. As was the case with the wild-type phage in low-salt
broth, the change in the rate of adsorption occurred after approxi-
mately 94% of the free phage had bound to the Pseudomonas cells. It
appeared that the adsorption of $\emptyset$W-14a was unaffected by the NaCl
concentration of the medium, or at least not to the same extent as
was the case with $\emptyset$W-14a+.

Because the adsorption stage of the one-step growth experiment
is usually carried out with the phage-cell mixture under static
conditions, the rate of adsorption was examined under these conditions.
If, after 1 min. incubation of the phage and cells at 250 rpm, the
speed of rotation was reduced to 50 rpm, the subsequent rate of
adsorption of $\emptyset$W-14a+ was markedly depressed. The time required for
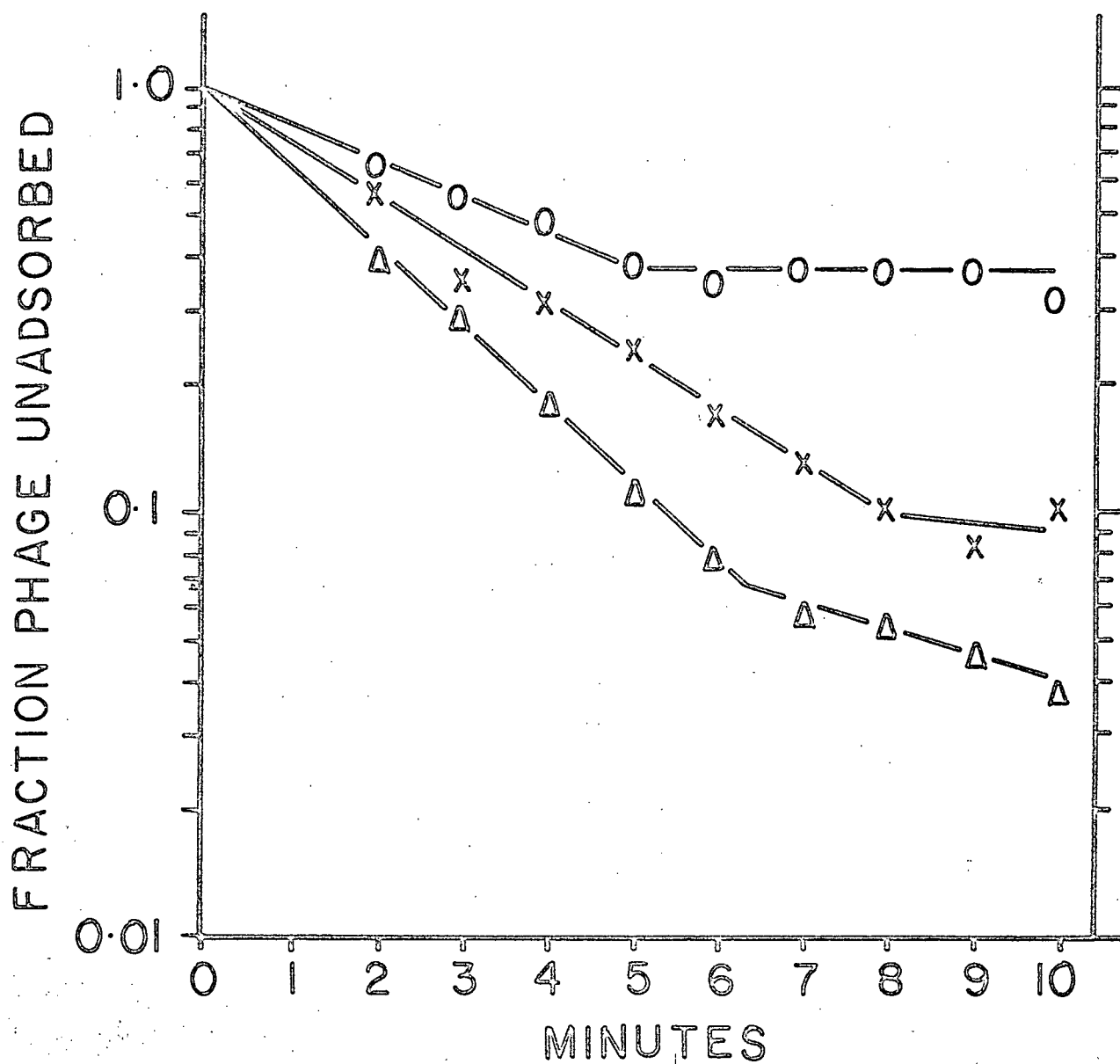50% adsorption of the phage was increased to 0.2 min., equivalent to a

Fig. 9. Adsorption of ØW-14a+ and ØW-14a to P. acidovorans #29
Log phase cells at 1 x 10^8 per millilitre were infected
with phage at a moi of 0.01. 0 - 0, adsorption of ØW-14a+;
Δ - Δ, adsorption of ØW-14a; X - X, adsorption of ØW-14a+
in low-salt Luria broth.

velocity constant for cell attachment of 7.3×10^{-10} ml/min. As a result of this, the classical one-step growth experiment (Ellis and Delbruch, 1939) was modified as outlined in Materials and Methods (V).

Two possible explanations of the unusual adsorption kinetics of ØW-14a+ are:

a. Sagik inhibition phenomenon.

Sagik (1954) noted that coliphage T2 was present in freshly prepared lysates of E. coli B in an inhibited state. Standing for a period of days in the cold or dilution into distilled water brought about a great increase in the titre of the lysate. Concomitant with this rise were the appearance of regular plaque morphology and normalization of the heat incativation and adsorption kinetics. In fresh lysates which contained both inhibited and normal phage particles, the adsorption rates were very slow; even after an extended period for adsorption, 43% of the total phage remained unadsorbed.

It is unlikely that my results are due to particle aggregation because (a) no rise in titre was observed when ØW-14a+ was diluted into or dialyzed against 0.005 M NaCl, and (b) no activation, or delay, was observed in the heat inactivation studies with this phage. Similarly, if inhibition was to account for the peculiar results the nature of the inhibitory substance must be such that it binds to the phage preventing its easy access to the cellular attachment

sites, but does not bind to the adsorptive organelles of the phage. It is possible with this theory to explain the salt effects by the elution of the inhibitor off the phage particles.

b. Phenotypic and genotypic mutants.

Schlesinger (1932), Delbruck (1940 a) and Garen (1954) all demonstrated a degree of heterogeneity, with respect to adsorptive properties, in phage populations. The percentage of phage particles in a lysate with decreased adsorptive capacity is very low, usually being less than 1% of the total. However, there are some notable exceptions. In lysates of P. aeruginosa phage 7v some 20% of the particles had a decreased capacity to absorb (Feary, Fisher and Fisher, 1964), and extrapolation of the data of Schade and Adler (1967) with $\emptyset$ χ indicates that a large fraction (25%) adsorbed to the host at a slow rate. Three explanations are possible to explain our results with respect to the mutation theory: (a) a proportion of the phage, for an unknown reason, adsorbs at a lower rate, (b) for a proportion of the phage the salt concentration is too high for optimal adsorption, and (c) the increased NaCl concentration is such that it causes the reversible binding of a proportion of the phage to the cells. These possibilities could be investigated further following an enrichment for the mutant using the method of Delbruck (1940).

The finding that the a+ form of the phage adsorbed at a slower

rate than the a form has a number of precedents in the literature.
Minamishima et al. (1968) presented evidence that the r+ form of
their fibrous P. aeruginosa phage Pf2 was adsorbed at a 1.5 fold
slower rate than the r mutant. Cohen and Arbogast (1950) showed
that coliphage T4r+ adsorbed very slowly to E. coli B in mineral
medium supplemented with limiting (0.2 μg/ml) tryptophan, while
T4r adsorbed rapidly. At high concentrations of the adsorption
cofactor, the adsorption of the two phages became almost identical.
As an optimal concentration of NaCl is required for T1 adsorption
(Puck, Garen and Cline, 1951) it is possible that the mutant and
wild-type phages of P. acidovorans differ in the optimal salt
concentrations required for adsorption.

Section III. One-step Growth Experiment

In order to determine the latent period and the average burst
size of phage ØW-14, one-step growth experiments were carried out with,
in most cases, P. acidovorans #29.

The latent period (Fig. 10) was between 61 and 66 min. This was
followed by a gradual rise (rise period) in the phage titre for a
further 37-45 min. The burst size computed by dividing the total
number of phage particles released by the number of infected cells
was found to be, on the basis of six different experiments, 300.
The range of burst sizes, using mid-log phase cells, was 214-470.
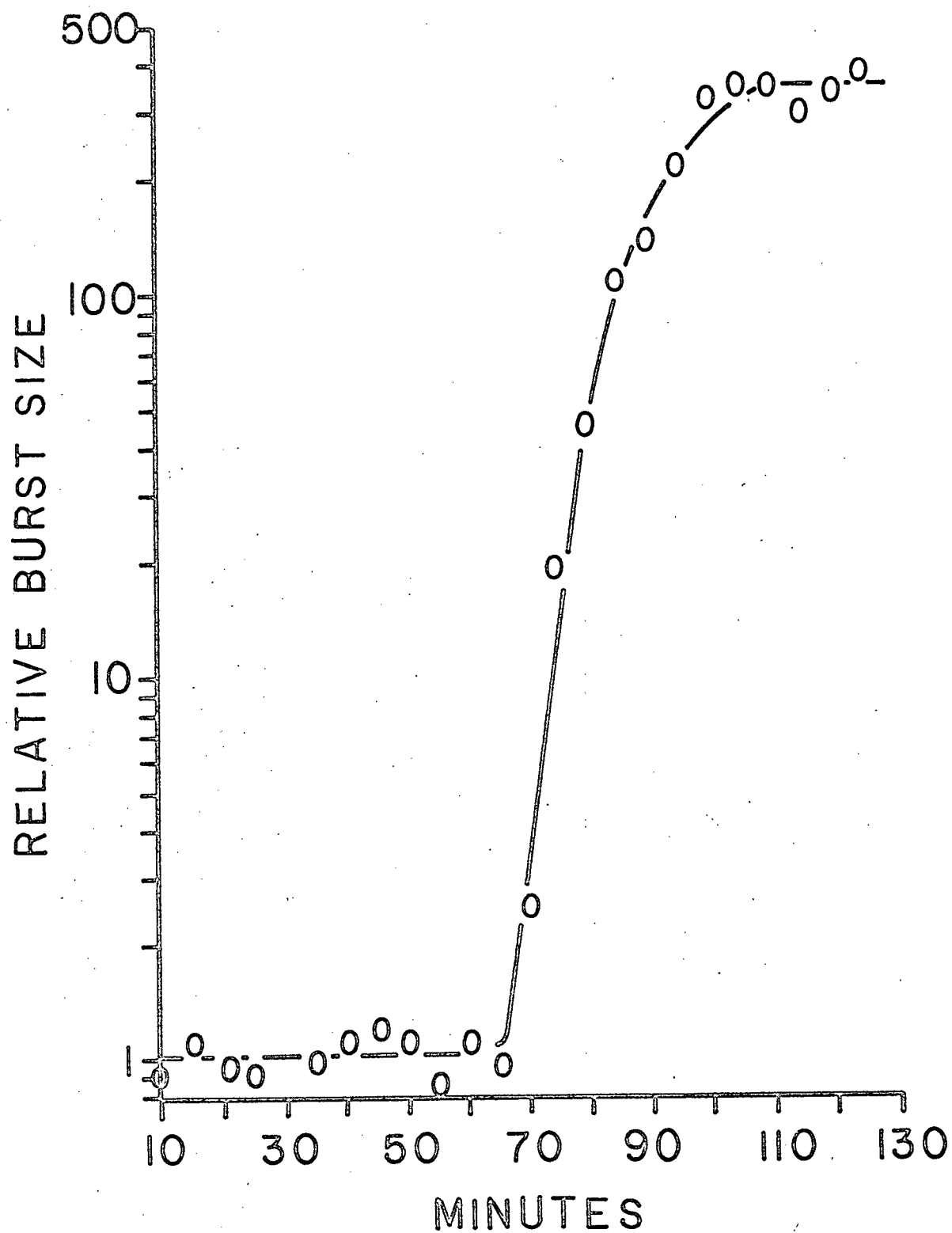
Fig. 10. One-step growth curve of ØW-14a+ and P. acidovorans #29.

The burst size was markedly dependent upon the age of the culture
(Fig. 11). The burst sizes were found to range from 30 for stationary
phase cells to about 600 for late log phase cells. As the cells entered
the log phase of growth, the average burst size increased rapidly to a
maximum of late log phase and then decreased as the cells entered
stationary phase. The same type of dependence curve was also observed
when P. acidovorans #14 was used as the host.

The adsorption rate constant for P. acidovorans #14 was very low,
so that estimations of the burst size could be obtained only by passing
the infected culture through a 0.45 μ millipore membrane (Millipore
Filter Corp., Bedford, Mass.) and washing the cells free of unadsorbed
phage prior to running the one-step growth experiments.

In the case of the mutant, ØW-14a, the latent period and the rise
period were the same as those of the wild type, but the burst size was
about 50% greater.

Attempts to lyse infected cells prematurely with chloroform or
lysozyme were unsuccessful.

ØW-14 is distinguished from the other Pseudomonas phages by its
long latent and rise periods, and the high burst size (Table I).

The burst yield as a function of the culture age of the host cells
has been investigated previously. Delbruck (1940 b) working with
E. coli and its phage compared rapidly dividing cells with a 24 hr.
aerated culture. The one-step growth experiment results indicated an
increase in the length of the latent and rise periods, and a decrease
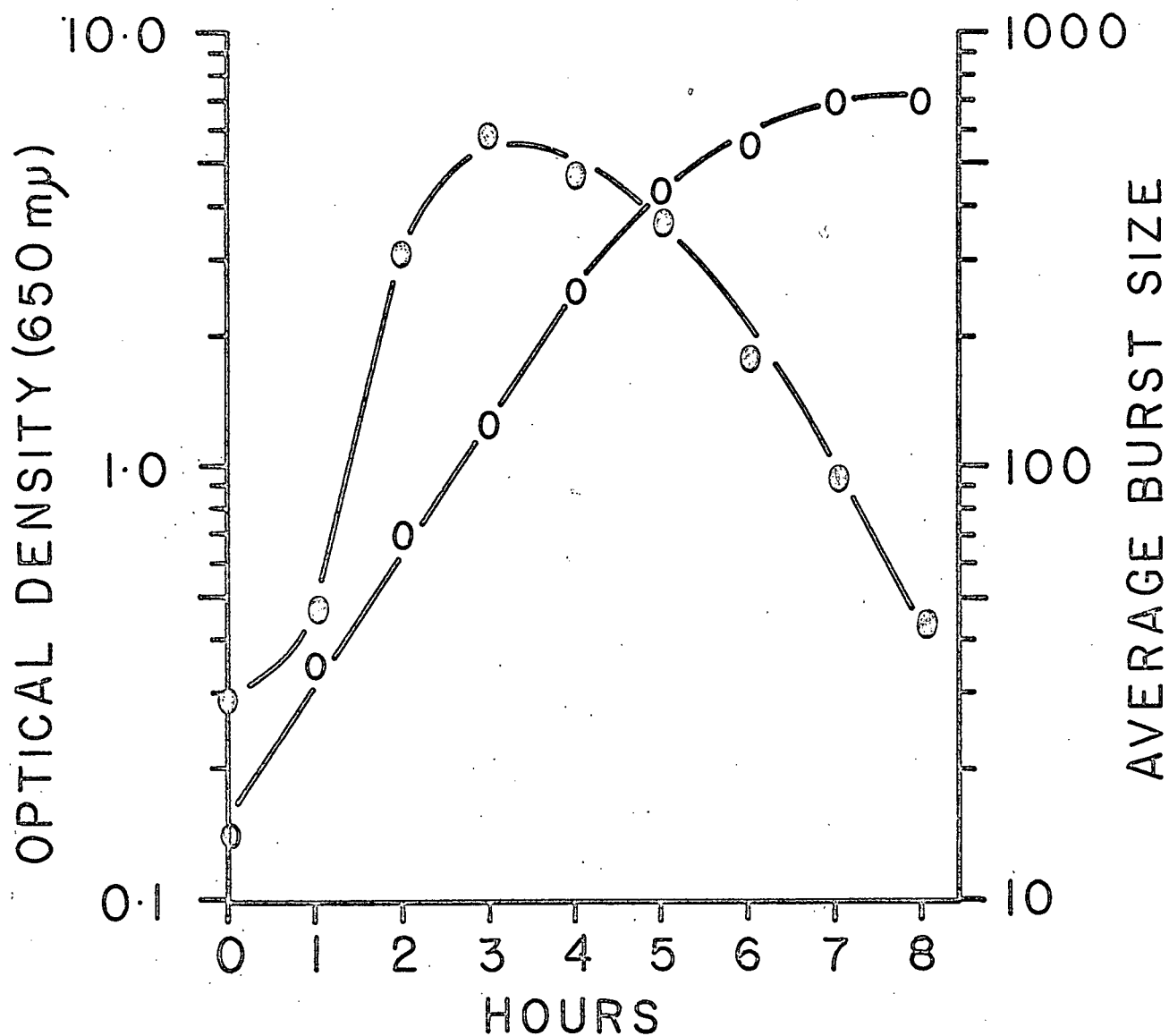in the burst size for stationary phase cells. Heden (1951) using

Fig. 11. Effect of cell age on phage development as measured by the
one-step growth method. 0 - 0, growth of P. acidovorans #29
at 30 C measured in OD 650 units/ml. θ - θ, average burst
size from cell samples taken at specified times and infected
with ØW-14a+.

phage T2 and E. coli B, compared phage release from infected cells in the lag and log phases of growth. Though he noted considerable and reproducible variation in the average burst sizes, the latent period was of constant length. His results unexpectedly showed the highest phage yield at the transition from lag to log phase growth, which was followed by a rapid decrease during logarithmic growth. These results are difficult to compare with those of the other workers since the method used for cultivation of the host was radically different. Baer and Krueger (1952), using Bacillus mycoides N phage, found little difference in phage production by lag or log phase cells. From a search of the literature it appears that the present publication is the only investigation of the burst from lag through log to stationary phase cells. This work was considerably simplified by the longer division cycle of the organism (approximately 60 min. in Luria broth at 30 C with adequate aeration) and the considerably modified one-step growth experiment method.

The higher burst size of the mutant ØW-14a appears to have few parellels in the literature, and may be due to the more complete lysis of the infected cells rather than any actual difference in the growth cycle of the phage (see Section IV). Cohen and Arbogast (1950) observed that T4r+ had a higher burst size than T4r.

Section IV. <u>Lysis Inhibition</u>

Thirty minutes after infection of a culture of <u>P</u>. <u>acidovorans</u> #29 with ØW-14a, the turbidity of the culture decreased markedly and then remained constant for the remainder of the experiment (Fig. 12). Following infection of the same strain with ØW-14a+, there was a slight decrease in the turbidity of the culture, but then there was a gradual increase in turbidity during the next 3 hr. This increase in turbidity appeared to be caused by growth of the organism rather than to disaggregation (Fig. 12). Thus a culture infected with ØW-14a+ appears to be lysis inhibited.

Lysis inhibition is characterized by an increase in the length of the latent period and an increase in the burst size following superinfection by an r+-type phage of a culture primarily infected with an r+ phage. This has been shown with the T even coliphages by Doermann (1948), Rutberg and Rutberg (1965) and Bode (1967). Attempts at demonstrating lysis inhibition by phage superinfection of <u>P</u>. <u>acidovorans</u> #29 were unsuccessful, though the burst size was usually considerably depressed over that of the control. In the case of these experiments the control was run simultaneously with a multiplicity of infection of 0.01. This depression of the average burst size by the use of a high moi affected the wild-type and its mutant, ØW-14a, similarly.

The inability to demonstrate lysis inhibition with ØW-14a+ may have been due to the fact that the a+ ----→ a mutation is not
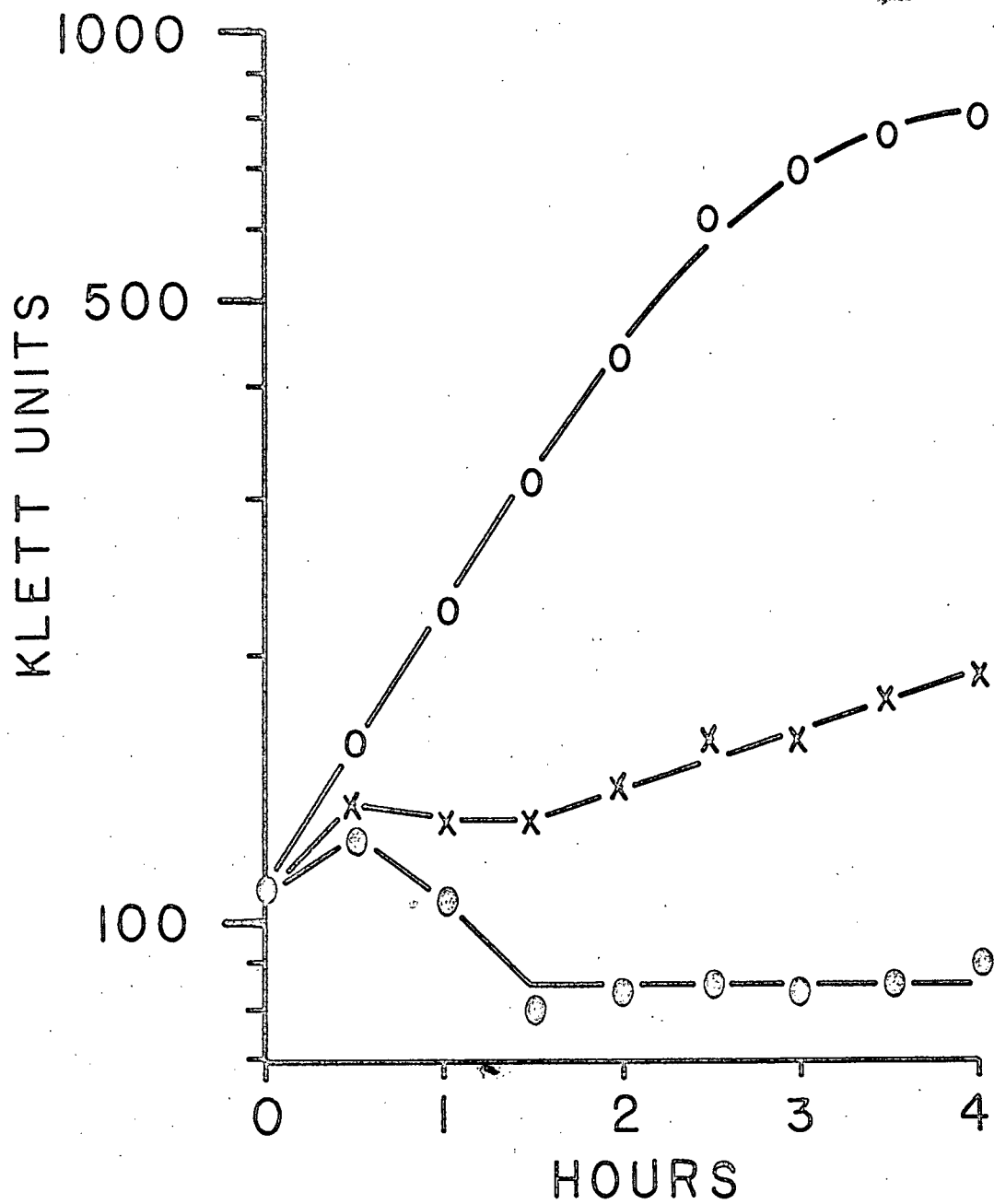
Fig. 12. Growth of P. acidovorans #29 infected at zero time with

ØW-14a+ (X - X) or ØW-14 a (0 - 0); Uninfected control (0 - 0).

analagous to the r+ ---→ r mutation seen in the T even coliphages,
or to the conditions used not being conducive to the production of
this state.

The unusual decrease in the average burst size warrants further
discussion. It is believed generally that the burst size is independent
of the multiplicity of infections. However, a high multiplicity of
infection has been shown either to increase the burst size or to decrease
it. Delbruck and Luria (1942) showed that the multiple infection of
E. coli B with coliphages α and β resulted in a 45-100% increase in
the burst size. The possibility of lysis inhibition was ruled-out
since the latent period was unaffected. Price (1950) showed that the
burst size of a phage in Staphylococcus muscae cultures varied
directly with the increase in the multiplicity of infection. Cells of
Shigella sonnei gave smaller bursts following infection with a large
excess of Coliphages T4 or T7 (Barry and Goebel, 1951). These results
and those obtained with P. acidovorans and ØW-14 can not be explained
at present.

Section V. Thermal Inactivation

ØW-14a+ was essentially stable at 50 C in Luria broth, but for
every 5 rise in temperature between 55 C and 65 C, there was an
approximate six fold increase in the rate of inactivation. The rate
constants were 0.033 min.$^{-1}$, 0.18 min.$^{-1}$, and 1.06 min.$^{-1}$ at 55 C,

60 C, and 65 C respectively. These values corresponded to half-lives

ranging from 21 to 0.65 min.

At 55 C and 60 C inactivation was biphasic (Fig. 13). Approxi-

mately 35% of the phage particles appeared to have some degree of

resistance. This fraction appeared to be stable at 55 C but slowly

inactivated at 60 C, the rate constant being 0.032 min.$^{-1}$.

The clear plaque mutant, $\emptyset$W-14a, showed biphasic inactivation at

55 C but not at 60 C.

Using the Arrhenius plot of the thermal inactivation of $\emptyset$W-14a+

(Fig. 14) the heat of activation (ΔH*) was calculated to be 75,700

calories/mole. $\emptyset$W-14a was found to be slightly more thermo-labile

the ΔH* being approximately 62,500 calories/mole.

Biphasic thermal inactivation kinetics, though quite common

in the animal viruses, have not been observed often in bacteriophages.

Exceptions, with the percentage of the population having differing

thermal stability in parentheses, are some of the Neisseria phages

(2-10%; Phelps, 1967); Bacillus cereus phages α, β, and γ (4.9%;

Meynell, 1962); P. aeruginosa phage 7m (29%; Feary, Fisher and Fisher,

1964); and, $\emptyset$W-14a+ (35%; present research). Adams (1953), working

with coliphage T5, found a fraction of the phage preparation was stable

after heating for several hours in buffered saline at 50 C. The

majority of the phage in this fraction were phenotypically resistant,

rather than genotypically resistant, since their progeny exhibited

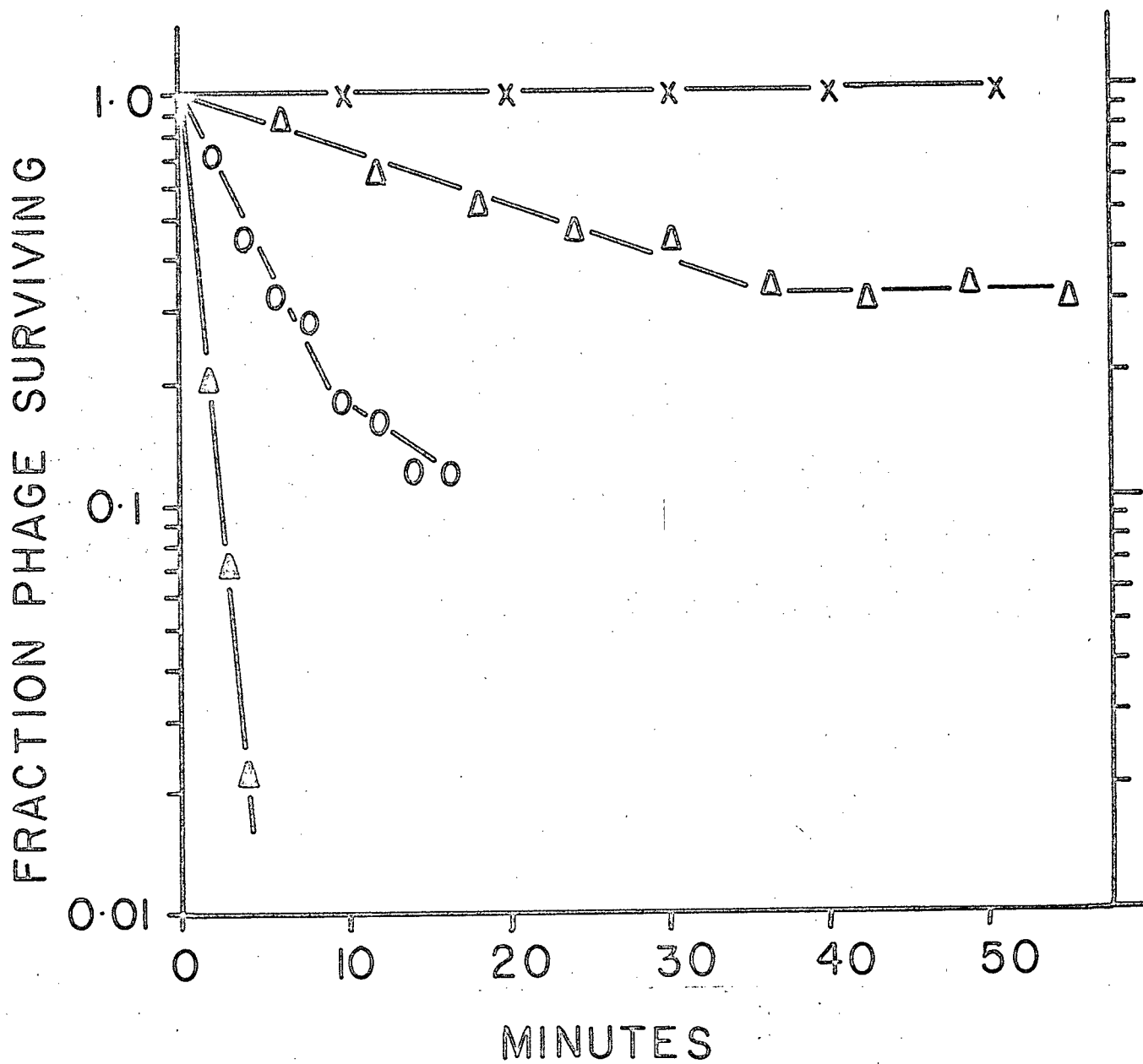the same thermal stability as the wild type. In the case of T5,

Fig. 13.   Thermal inactivation curves of ∅W-14a+ in Luria broth.
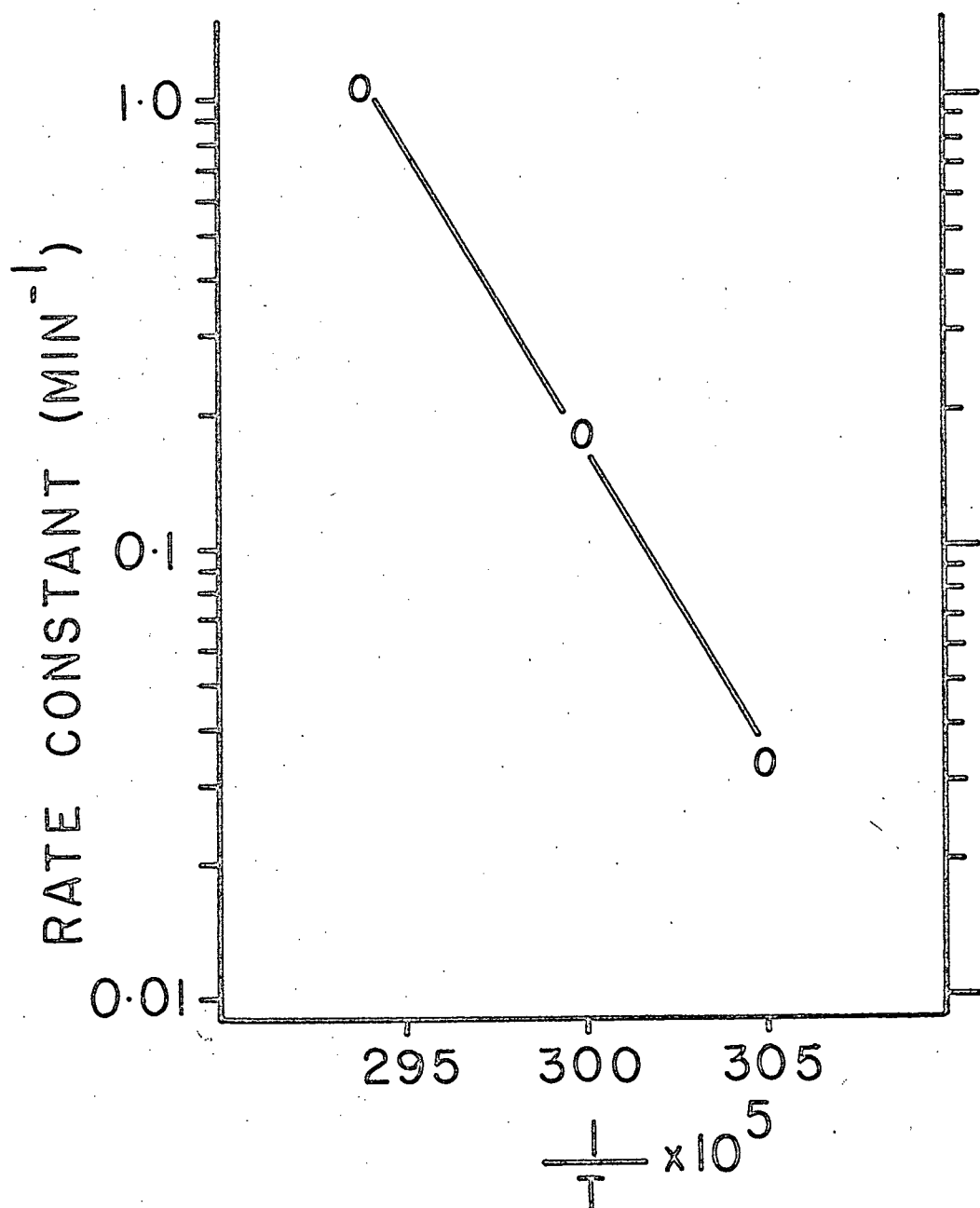X - X, 50 C; Δ - Δ, 55 C; 0 - 0, 60 C; ▲ - ▲, 65 C.

Fig. 14. Arrhenius plot of the thermal inactivation of ØW-14a+

0.1% of the population were phenotypically resistant, while the
incidence of genotypic resistance was of the order of 10^{-7} to 10^{-8}.

It was most probable, therefore, that the heat resistant ØW-14a+
particles were phenotypically rather than genotypically resistant.
It would be of interest to investigate the adsorption kinetics of this
resistant fraction since an almost identical fraction of the phage
showed differing adsorption and heat inactivation kinetics.

The activation energy calculated for this phage is similar to
those obtained for Bacillus megaterium phage M-1 (76,000), E. coli
phage T2 (71,700), and Streptococcus lactis phage 122-4 (76,000)
(Pollard, 1953).

Section VI. Sonic Sensitivity of Phages

The sonic stability of phages is mainly of theoretical interest,
though sonication has been used to study the intracellular development
of an RNA-containing phage (Paranchych and Graham, 1962).

The three phages tested were inactivated exponentially by sonic
irradiation (Fig. 15). Coliphage T1 and P. acidovorans phage ØW-14a+
showed quite similar inactivation rates, with k_s values of 1.45 min.$^{-1}$
for T1 and 1.74 min.$^{-1}$ for ØW-14a+. Phage S13 was far more stable
to sonic irradiation than either of the other phages and gave a k_s
value of 0.12 min.$^{-1}$.

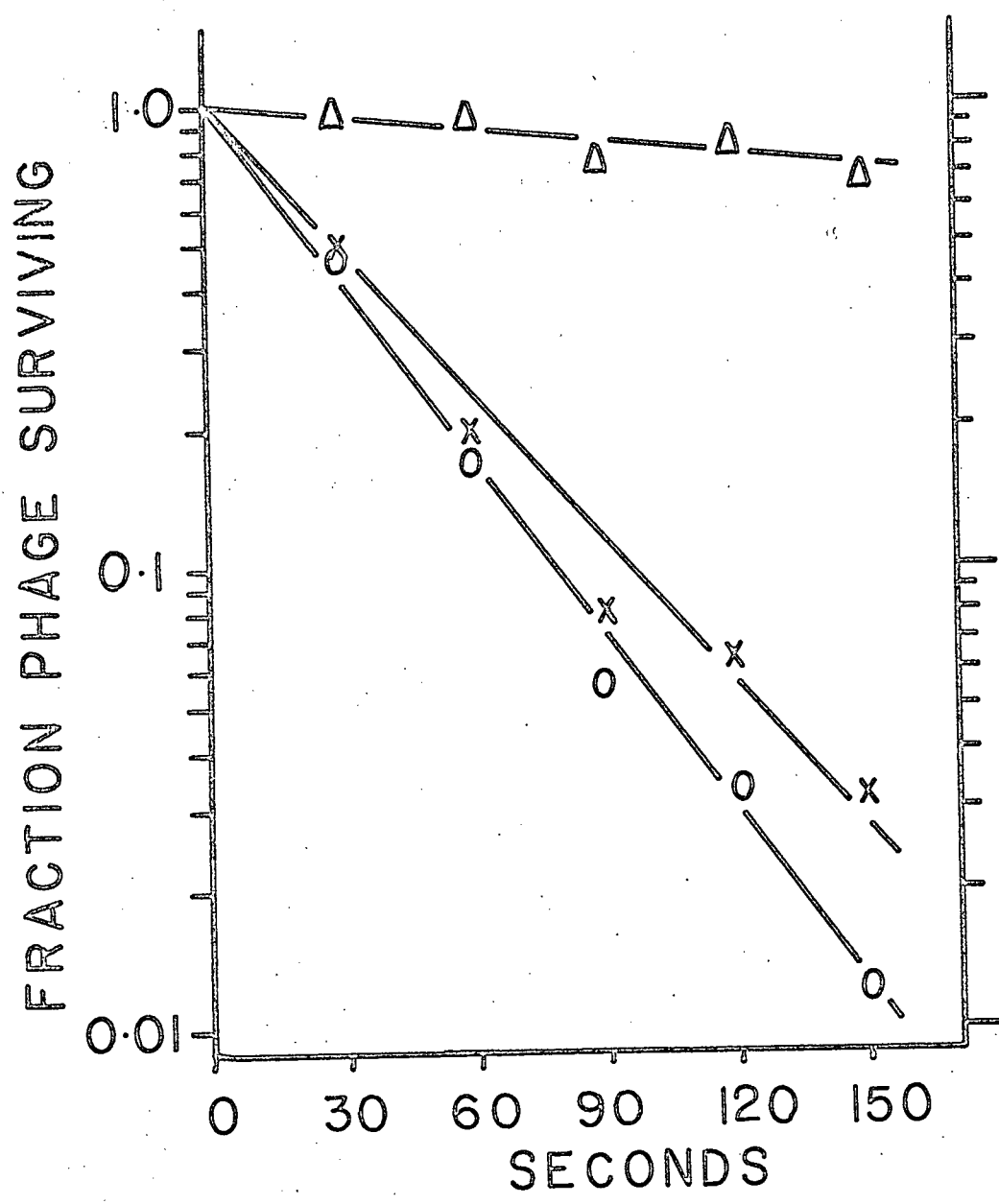The few reports which exist in the literature concerning the sonic

Fig. 15. Sonic sensitivity of coliphage S13, T1 and ØW-14a+:
(Δ --- Δ), S13; (X --- X),T1; (0 --- 0), ØW-14a+.

inactivation of bacteriophages indicate that the small, compact viruses are far more stable than the larger ones (Anderson et al., 1948). The spherical single-stranded DNA-containing phages ØX174 and S13 (Minamishima et al., 1968; present research) and the small RNA-containing phages (Paranchych and Graham, 1962) are particularly stable; on the other hand, the long fibrous phage Pf1 of P. aeruginosa is highly labile (Minamishima et al., 1968). A general relationship exists between the virus volume and the rate of sonic inactivation (Pollard, 1953), which in the case of this study would indicate that T1 and ØW-14 are quite similar in size. This was not borne out by electron microscopy which showed that ØW-14 possessed a heat 87 mμ in diameter, while it is known that the head diameter of T1 is 50 mμ (Williams and Frazer, 1953).

Section VII. pH Inactivation

A normal inactivation curve was obtained for the wild type phage, ØW-14a+ (Fig. 16). However, an anomalous inactivation curve was obtained for ØW-14 (Fig. 16): the phage appeared to be activated by incubation at pH 4-5. Approximately 1.6 fold more plaque forming units were found in the preparation at pH 4-5 than were present in the preparation at pH 6-9. Similar results were obtained with the host range mutant ØW-14h.

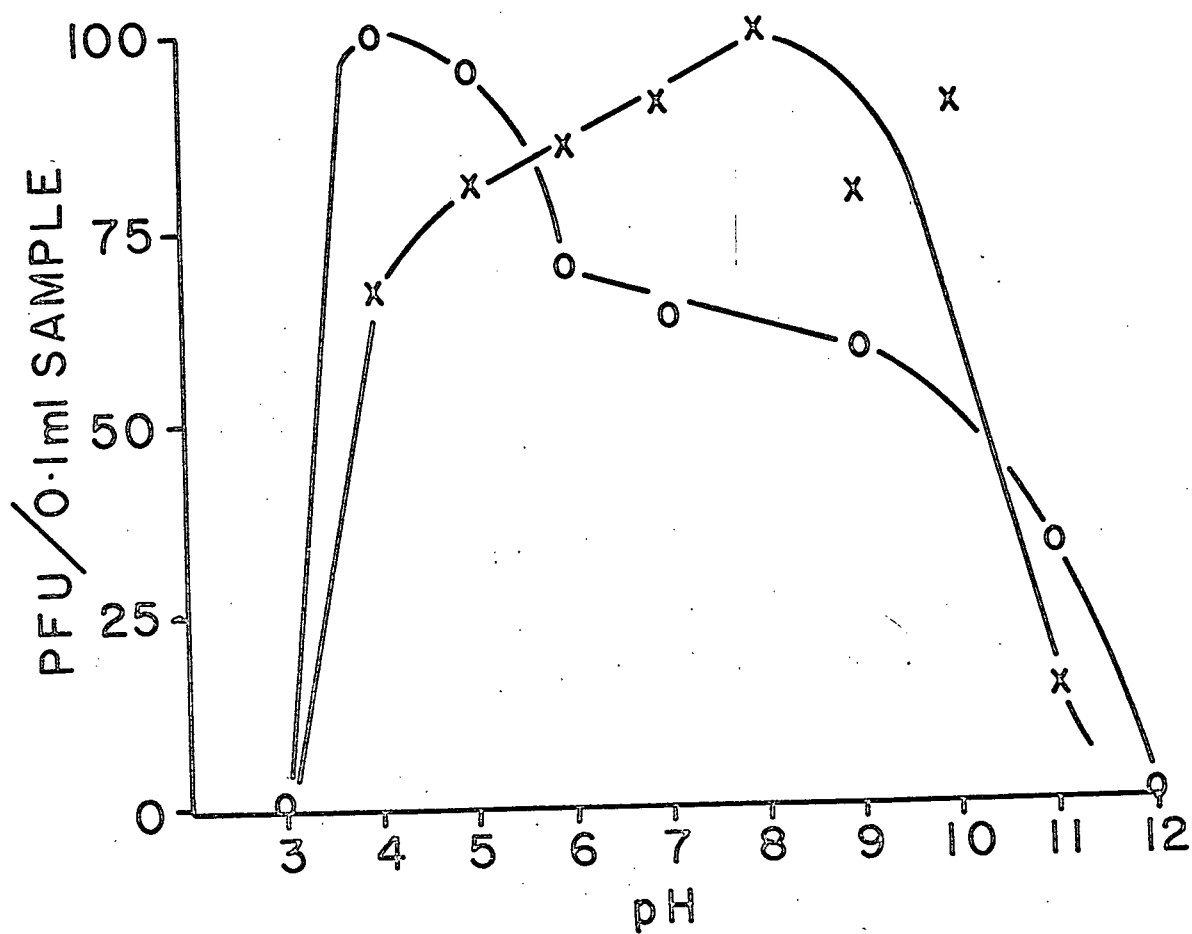The results of the pH inactivation experiment further distinguished

Fig. 16. pH inactivation of ØW-14a+ (X --- X) and ØW-14a (0 --- 0).

the mutant phage from the wild type.

Section VIII. Sensitivity to Ultraviolet Light and Photoreactivation

Multihit kinetics were observed for the UV-inactivation of $\emptyset$W-14a+ and coliphage Tl (Fig. 17). Extrapolation of the exponential regions of the curves to the ordinate gave values 2.4 and 1.6 times higher than the initial titre of the two phages. The rate constants (k_{uv}) calculated from the exponential regions of the curves were 0.60 min.$^{-1}$ for Tl, and 4.35 min.$^{-1}$ for $\emptyset$W-14a+. Therefore, $\emptyset$W-14a+ was 7 times as sensitive as Tl to UV-irradiation.

The lethal effects of ultraviolet light were reversed to a considerable extent by irradiation of the overlay plates with white light. Photoreactivable sectors of 0.35 and 0.71 were calculated for Tl and $\emptyset$W-14a+, respectively.

Prior to this research, the ultraviolet sensitivity of a number of Pseudomonas aeruginosa phages had been reported (Benzer and Jacob, 1953; Jacob, 1952; Matsui, 1952; Holloway and Monk, 1959; Holloway et al., 1962). The irradiation death curves of $\emptyset$W-14a+ closely resembled those obtained for the T-even phages (Harm, 1959) in comparison to those obtained for Tl (Dulbecco, 1950; present research, Fig. 17). The UV sensitivity of $\emptyset$W-14 is of special interest because the main lethal effect of such irradiation is the formation of thymine dimers (Wacker, 1963), and this phage contains a novel base partially replacing thymine

Fig. 17. UV inactivation and photoreactivation of coliphage T1
          and ØW-14a+. ØW-14a+ was irradiated with UV light and
          incubated in the dark (⊙ --- ⊙) or in the light
          (0 --- 0). T1 was irradiated and incubated in the
          dark (Δ --- ▲); in the light (Δ --- Δ).

Fig. 17.
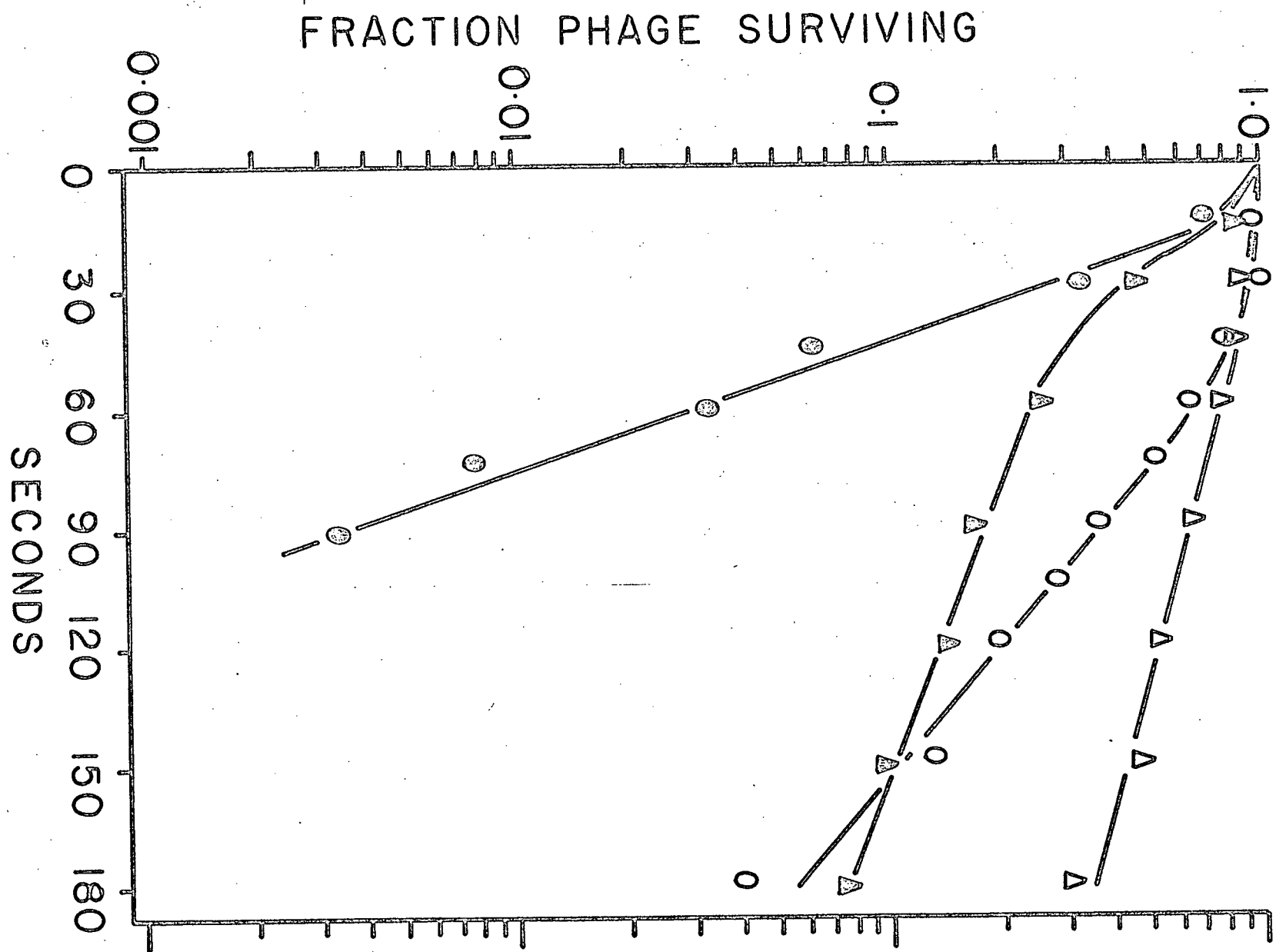
(see Section IX).

P. acidovorans appears to contain a photoreactivating enzyme
system since a major increase in the titre of the irradiated phage was
obtained by incubation of the phage infected cells in white light.
It appears that the majority (71%) of the lethal effects of UV light
can be reversed by photoreactivation. The value obtained for the
photoreactivable sector of T1 did not agree with the value of 0.68
obtained by Dulbecco (1950). The reason for this discrepancy was
not apparent.

Section IX. Phage Resistant Mutants and the Carrier State

1. Phage resistant mutants

Surprisingly, selection for phage resistant strains of P.
acidovorans appeared in some cases to select for strains with
alterations in cell division and/or motility. The phage resistant
mutants fell into six distinguishable groups with respect to cell
morphology at various temperatures and to motility. They fell into
two groups with respect to their sensitivity to ØW-14h. In all,
nine groups of mutants were classified (Table IX).

Photomicrographs of the wild type and mutant 29-20 are
presented in Fig. 18, Fig. 19. When mutant "snakes" prepared at
30 C were transferred to fresh broth at 22 C, they divided to

produce cells of normal size. In many cases these apparently
normal cells were nonmotile.

Filament formation in bacteria can be induced by a variety
of physical and chemical agents, amongst them antibiotics (Hunt
and Pittillo, 1968), D-amino acids (Grula and Grula, 1962), heavy
metal ions (Rosenberg et al., 1967), UV irradiation (Deering,
1958), X-rays (Adler and Hardigree, 1964), increased hydrostatic
pressure (Zobell and Cobet, 1964) and extremes of temperature
(Hoffman and Frank, 1963; Terry, Gaffar and Sagers, 1966; Shaw,
1968). At elevated temperatures, i.e. those near the maximum
temperature for growth, E. coli, Clostridium acidiurici, and
P. acidovorans tended to elongate and form filaments (commonly
called "snakes"), presumably because of an inhibition of cell
division. If the temperature of incubation of the culture is
decreased, normal cells are segmented off. The fact that six
of our phage resistant mutants isolated isolated during the course of
this research formed filaments at a temperature at which the wild
type cells were "normal" was of considerable interest, as was the
observation that four of them were nonmotile at 22 C.

Golub and Orlova (1968) compared the growth of an phage release
from lysogenic cultures of E. coli C at 37 C and 45 C. Lysogeniza-
tion of E. coli C with a mutant of phage 299 rendered the culture
more thermosensitive in that it formed filaments at the higher
temperature while E. coli C and E. coli C(299) were unaffected.

Table IX.   Characteristics of Some Phage Resistant Mutants of
P. acidovorans #29.

| Mutant # | Pattern of Phage Resistance | | | Growth at: | | |
| | Lysis by: | | | 22 C | 30 C | 37 C |
	ØW-14a	ØW-14a+	ØW-14h			
29-1	−	−	+	NnM*	SnM	SnM
29-5	−	−	+		N M	SnM
29-6	−	−	+		N M	SnM
29-7	−	−	+		N M	SnM
29-8	−	−	+		N M	SnM
29-11	−	−	−	N M	S M	SnM
29-12	−	−	−		N M	SnM
29-15	−	−	−		N M	SnM
29-16	−	−	−		NnM	SnM
29-18	−	−	+	NnM	SnM	SnM
29-20	−	−	−	NnM	SnM	SnM
29-21	−	−	−	N M	SnM	SnM
29-22s	−	−	−		N M	PNG
29-221	−	−	−		N M	SnM
29-23	−	−	+		N M	PNG
29-24	−	−	−	NnM	SnM	SnM
Wild type	+	+	+	N M	N M	SnM

* N (normal cell morphology); S (snake-like cell morphology);
M (motile); nM (nonmotile); PNG (poor or no growth)

Fig. 18 a.
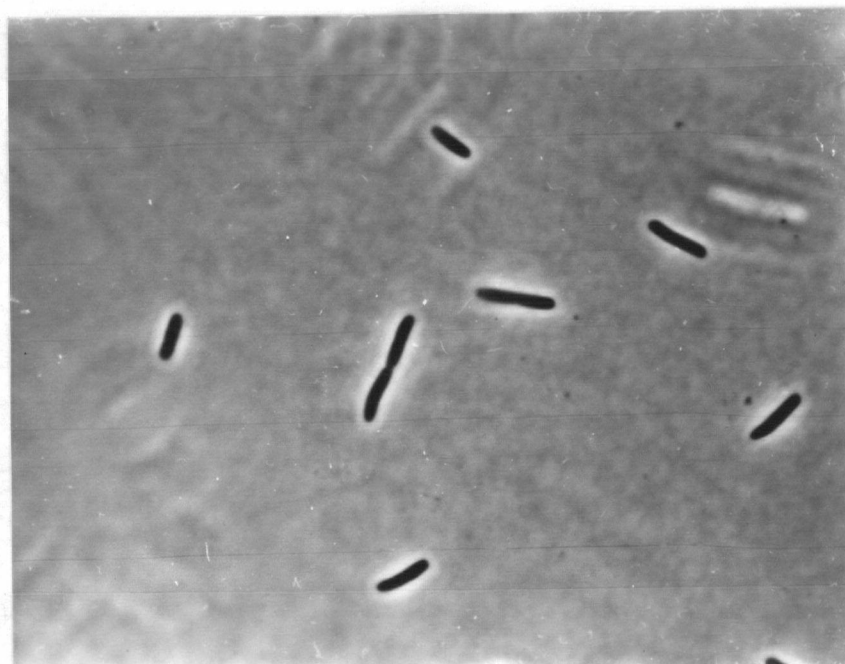
P. acidovorans #29 grown at 30 C, x 4,400
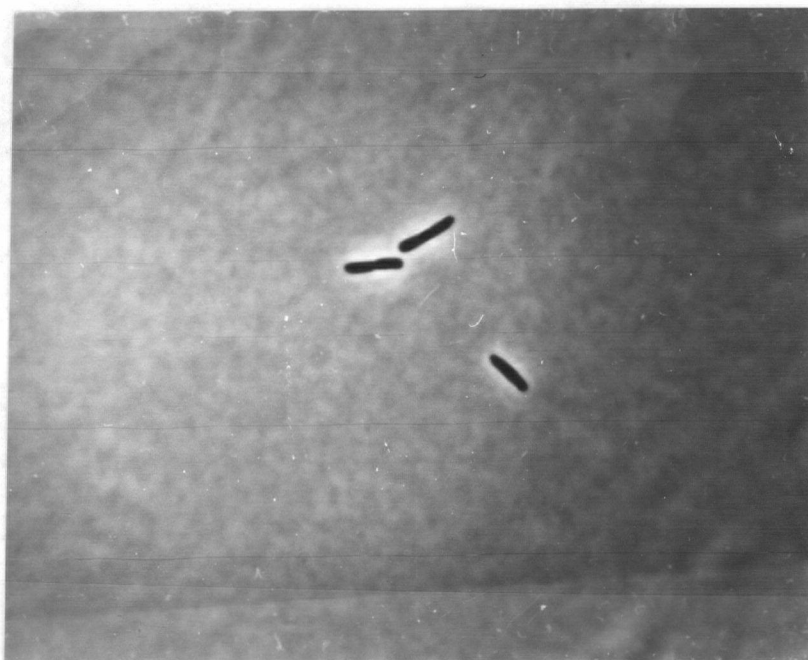

Fig. 18 b.
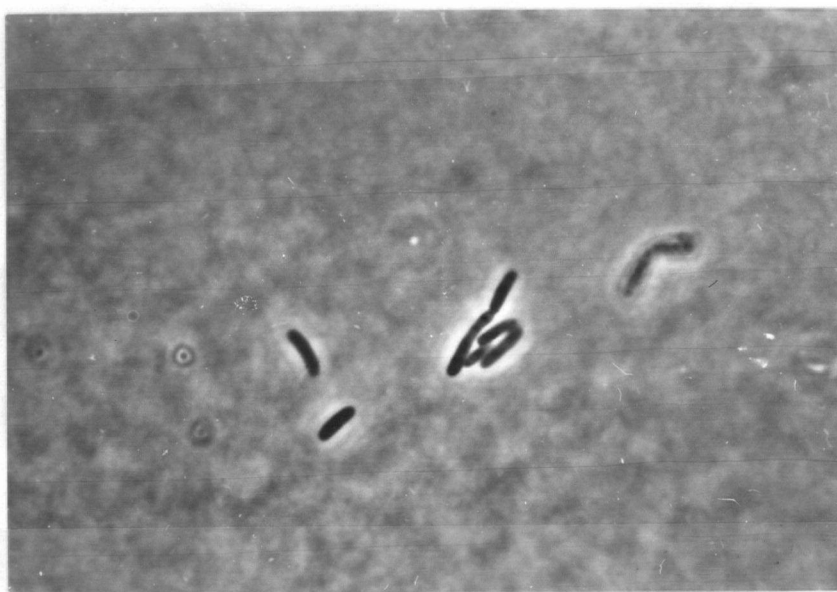
P. acidovorans #29 grown at 18 C, x 4,400

Fig. 19 a.

P. acidovorans #29-20 grown at 30 C, x 4,400

Fig. 19 b.

P. acidovorans #29-20 grown at 18 C x 4,400

2. Carrier state

Attempts at the purification of the phage infected cultures
by plating them on Luria agar plates failed in that all of the
colonies which arose on the plates were phage deficient. The
existence of cells previously infected with phage was indicated
by a number of small viscous colonies which appeared on some of
the plates. Kawakami and Landman (1968) used minimal medium
supplemented with 0.01% casein hydrolysate and sodium succinate
(0.48M) or sucrose (0.8M) to stabilize carrier cells of B. subtilis.
Supplementation of minimal medium with 70 ml of Luria broth
(per litre) did not stabilize P. acidovorans carrier cells, neither
did the addition of sodium succinate (0.48M), sucrose (0.8M) or
glycerol (5%, w/v). Streaking the cultures on overlays containing
ØW-14 appeared to be the only means of purifying these strains,
though it had obvious disadvantages.

When washed, phage infected cells were plated to determine
the total number of viable cells and the number of phage infected
cells, a discrepancy was always noted. The percentage of infected
cells in the cultures varied from 0.2 to 37% of the total cell
number, a fact which confirmed the suspected carrier state.
Carrier cultures containing a lower percentage of infected cells
appear to be unstable, as the phage is readily lost on transfer
in broth. One example of a hyper-carrier culture was found but

this was highly unstable and "grew" in a partially lysed condition.

The carrier cultures grew at rates comparable with that of P. acidovorans #29, from which they were derived. Phage was released only during the logarithmic phase of growth. Either of the two phages ØW-14a+ or ØW-14a, could enter into a carrier state and be released from infected cultures.

The carrier state is a phage-bacterium relationship intermediate between lysis and lysogeny, hence the synonyms "pseudo-lysogeny" (Lwoff, 1953) and "lasting semitemperate complex" (Frazer, 1957). This relationship, which will be described in greater detail later, occurs in many bacteria e.g.: E. coli B with phage T3 #40 (Frazer, 1957); E. coli K12 with phage T7^{sh} (Li, Barksdale, and Garmise, 1961); E. coli C3000 with phage HR (Hsu, 1968); Brucella abortus 544A phage P19 (Jones, McDuff and Wilson, 1962); Proteus mirabilis with phage 57 (Coetzee and Hawtrey, 1962); Shigella dysenteriae cultures with phage T7 (Li et al., 1961); Salmonella typhimurium LT22 with phage PLT22 (Li et al., 1961). Nor is this phenomenon restricted to Gram-negative organisms since carrier conditions have been reported in B. subtilis infected with SP13 (Romig and Brodetsky, 1961) and SP-10 (Kawakami and Landman, 1968).

When plated, carrier cells segregate at a high frequency into normal, phage sensitive, cells and into carrier cells capable of producing phage. This is different from the lysogenic state, in which the frequency of segregation of nonlysogenic cells is low.

In addition, the phage genome in a carrier cell exists as a plasmid rather than as an integrated prophage (Takahashi, 1964); and, carrier cultures grown in the presence of antiphage serum are rapidly converted to a phage sensitive state, indicating that phage reinfection is necessary for the maintenance of this state.

The experiments conducted with P. acidovorans and phage ØW-14 suggested the occurrence of a carrier state in this system. Development of this aspect of the problem depends upon a suitable solid medium being found to stabilize the phage infected cells.

Section X. Recombination

Only one previous case exists for recombinational experiments being run on Pseudomonas phages (Egan and Holloway, 1961). In the case of the present research, the preliminary experiments indicate that it was possible to carry out the following cross: a+h+ x ah ----→ a+h+, ah, a+h, and ah+. Using mixed indicators it was possible to identify the a+h recombinant, but ah+ resembled a+h+ too closely to be distinguished. The former recombinant appeared at a frequency of approximately 6%.

Section XI. Phage ØW-14 DNA

1. General properties

The purified nucleic acid extracted from ∅W-14 formed a viscous solution when dissolved in SSC. The spectrum of this solution exhibited a λ max. at 258 mμ and a λ min. at 232 mμ when diluted into 0.1 x SSC. Upon the addition of NaOH to 0.25 M, the λ max. shifted to 263 mμ and the λ min. to 237 mμ and the chromicity at 260 mμ increased by almost 30 percent. The spectral ratios 1: 0.46: 0.51 (260 mμ: 230 mμ: 280 mμ) agreed well with Marmur"s (1961) value of 1: 0.450: 0.515 for pure DNA.

The nucleic acid was diphenylamine positive and orcinol negative. It was sensitive to DN'ase digestion but resistant to RN'ase. Therefore, the nucleic acid isolated from ∅W-14 was DNA and not RNA.

2. Melting temperatures

The Tm values in 0.1 x SSC for the DNA preparations from P. acidovorans #14 and from ∅W-14 were 80.7 and 83.0 C, respectively.

As the Tm of DNA is 15.4 C lower in 0.1 x SSC than in SSC the following equation was used to calculate the moles percent guanine plus cytosine in the phage nucleic acid (Mandel and Marmur, 1968):

$$\text{Moles \% GC} = 2.44\,(Tm - 53.9)$$

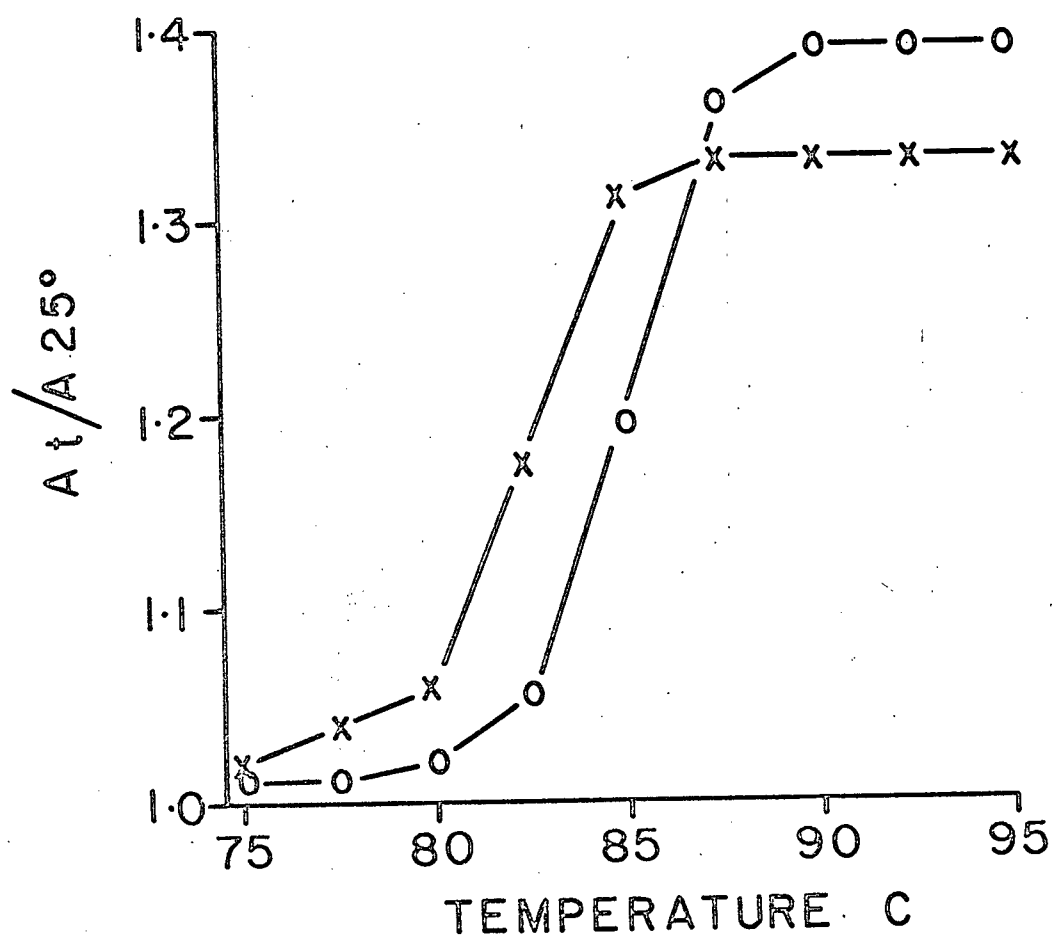The GC content of the host DNA was calculated to be 65.4% and that of the phage DNA 71.9%.

Fig. 20. Melting profiles of ØW-14 DNA (0 --- 0) and P. acidovorans #14 DNA (X --- X) in 0.1 x SSC.

Upon heating the hyperchromic shift at 260 mµ for the host

DNA was 31%, that for ØW-14 DNA 37%.

3. Buoyant density

The density of the phage DNA, on the basis of three determina-

tions using two different preparations was 1.666 g/cc^3, a value

which corresponded to about 6% GC. The density of heat-denatured

phage DNA was 1.681 g/cc^3. Alkaline denaturation increased the

density to 1.680 g/cc^2. There was no indication of multiple

density bands in the denatured DNA preparations (see Fig. 21).

P. acidovorans #14 DNA banded at a density of 1.723 g/cc^3

(Fig. 21). This value corresponds well to the literature values

of 1.724 g/cc^3 (Colwell, Citarella and Ryman, 1965) and 1.7255

(Mandel, 1965) for this DNA.

Though the density of the phage DNA was unusually low, the

DNA behaved normally on denaturation by heat or alkali. A density

increase of 15 mg/cc^3 agreed well with those values obtained upon

the denaturation of E. coli DNA - 15 mg/cc^3 - (Lee and Boezi, 1966).

The lack of multiple density peaks upon denaturation indicated that

the material was homogeneous with respect to its buoyant density,

i.e. each strand of the double helix was of similar density.
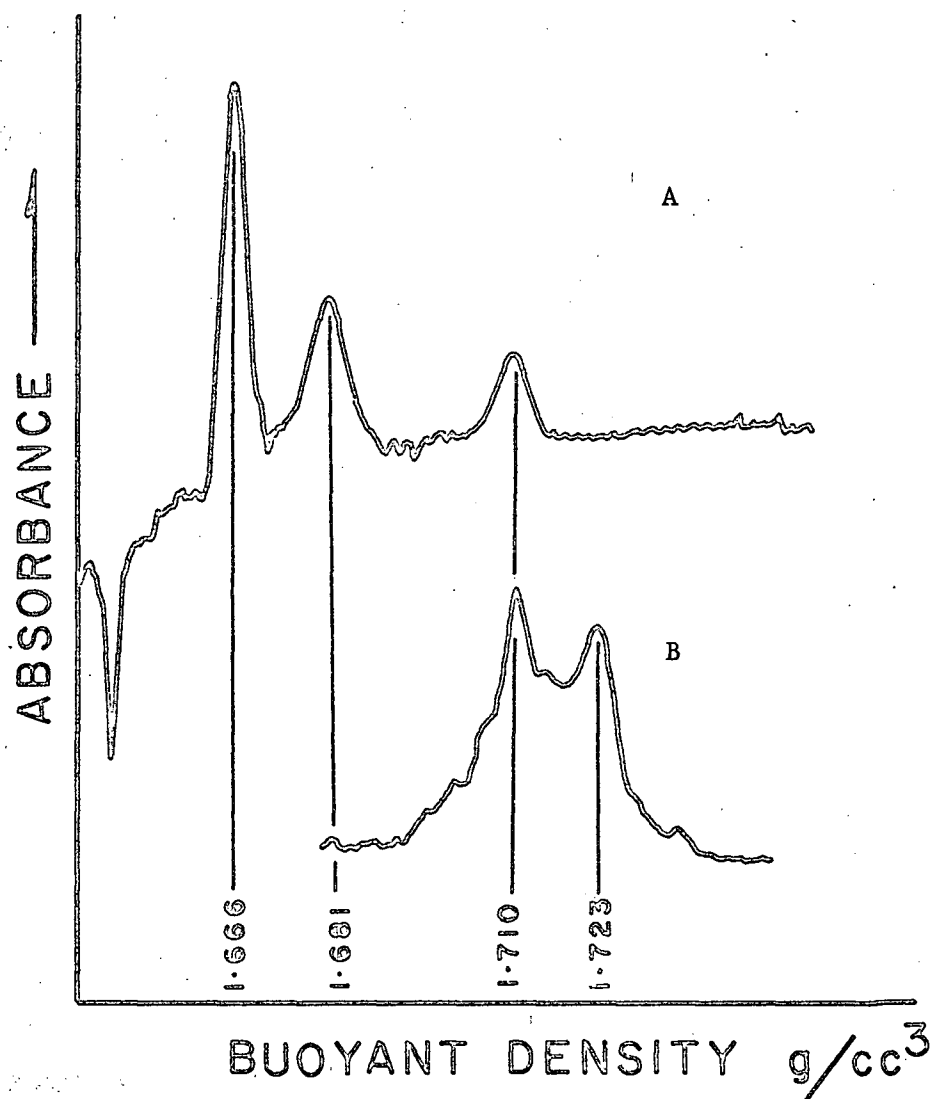
Fig. 21.   Microdensitometer scans of CsCl density gradients.
A.   ØW-14 DNA (p 1.666), denatured ØW-14 DNA (p 1681),
and E. coli DNA marker (p 1.710).
B.   E. coli DNA marker (p 1.710) and P. acidovorans #14
DNA (p 1.723).

Samples were centrifuged using Beckman Model E analytical
ultracentrifuge at 44,000 rpm. for 22 hr. prior to photography
using the UV optics.

4. Chemical composition of ØW-14 DNA

Hydrolysis of the phage DNA with formic acid liberated five

bases. Four of the bases were identified by paper chromatography

(Table X b) and their spectral properties in acidic and basic

solution as adenine, guanine, cytosine and thymine (Table XI).

The fifth base, which migrated slower than guanine in the

isopropanol-HCl-water solvent system of Bendich (1957), had the

spectral properties described in Table XII and Fig. 22. This

base was present in the formic acid hydrolysates and in a hydro-

chloric acid digest but not in a perchloric acid hydrolysate.

Further evidence for the existence of another base was suggested by

the fact that $[A + G] / [T + C] \neq 1$. The concentration of thymine

in three different experiments was always about one-half the

concentration of adenine. The exact nature of the other base

was not clear since its spectrum did not correspond to any of the

common minor bases found in the literature.

The ØW-14 DNA preparations also gave a very strong anthrone

reaction, the coloured product of which had a λ max. at 624 mµ,

and a spectrum identical to that of the reaction product obtained

with glucose. A number of saccharides were separated by paper

chromatography following acid hydrolysis of the DNA. However,

if the DNA was precipitated with TCA and then hydrolyzed, no

saccharides could be detected in the hydrolysate by paper chroma-

tography.

Table X (a).   Paper Chromatographic Separation of Purine and
                Pyrimidine Bases.

Standards	Solvent System			Fluorescence*
	A	B	C	
	(Rf x 100)			
Adenine	55	43	30	pu
Guanine	37s**	11	20s	bl
Cytosine	50	34	44	pu
5-Hydroxymethylcytosine (HMC)	47	23	45	pu
Thymine	74	56	71	pu
5-Hydroxymethyluracil (HMU)	55	32	59	pu
Uracil	64	40	61	pu

   *   fluorescence of the spots on the paper under UV
        light: pu (purple) and bl (blue)

  **   s (streaked)

Table X (b).   Separation of the Bases in a ØW-14 DNA Hydrolysate

		(Rf x 100) and Fluorescence			
(i)	Solvent System A	38s (bl)	51 (pu)		
	Identification	Guanine	Cytosine		
(ii)	Solvent System B	13 (bl)	34 (pu)	42 (pu)	56 (pu)
	Identification	Guanine	Cytosine	Adenine	Thymine
(iii)	Solvent System C	21 (bl)	31 (pu)	44 (pu)	71 (pu)
	Identification	Guanine	Adenine	Cytosine or HMC	Thymine

Table XI. Spectra Properties of the Bases Isolated from ∅W-14 DNA.

Base*	pH**	λmax.(mμ)	λmin.(mμ)	Absorbance Ratios	
				250/260	280/260
Adenine	1	263(262)***	229.5(229)	0.76	0.41
	13	269(269)	238(237)	0.58	0.62
Cytosine	1	276(275)	239(238.5)	0.47	1.57
	13	282(281.5)	250.5(250.5)	0.67	2.20
Guanine	1	249(248)	224(224)	1.31	0.78
	13	275(274)	240.5(239.5).	0.84	1.24
Thymine	2	262.5(264.5)	233.5(233)	0.73	0.50
	12	289(291)	244(244)	0.79	1.12

* tentatively identified by paper chromatography

** pH 1 (0.10 N HCl); pH 2 (0.01 N HCl); pH 12 (0.01 N NaOH); and, pH 13 (0.1 N NaOH)

*** values in parentheses are from the literature

Table XII. Spectral Properties of a Novel Base Found in ØW-14 DNA

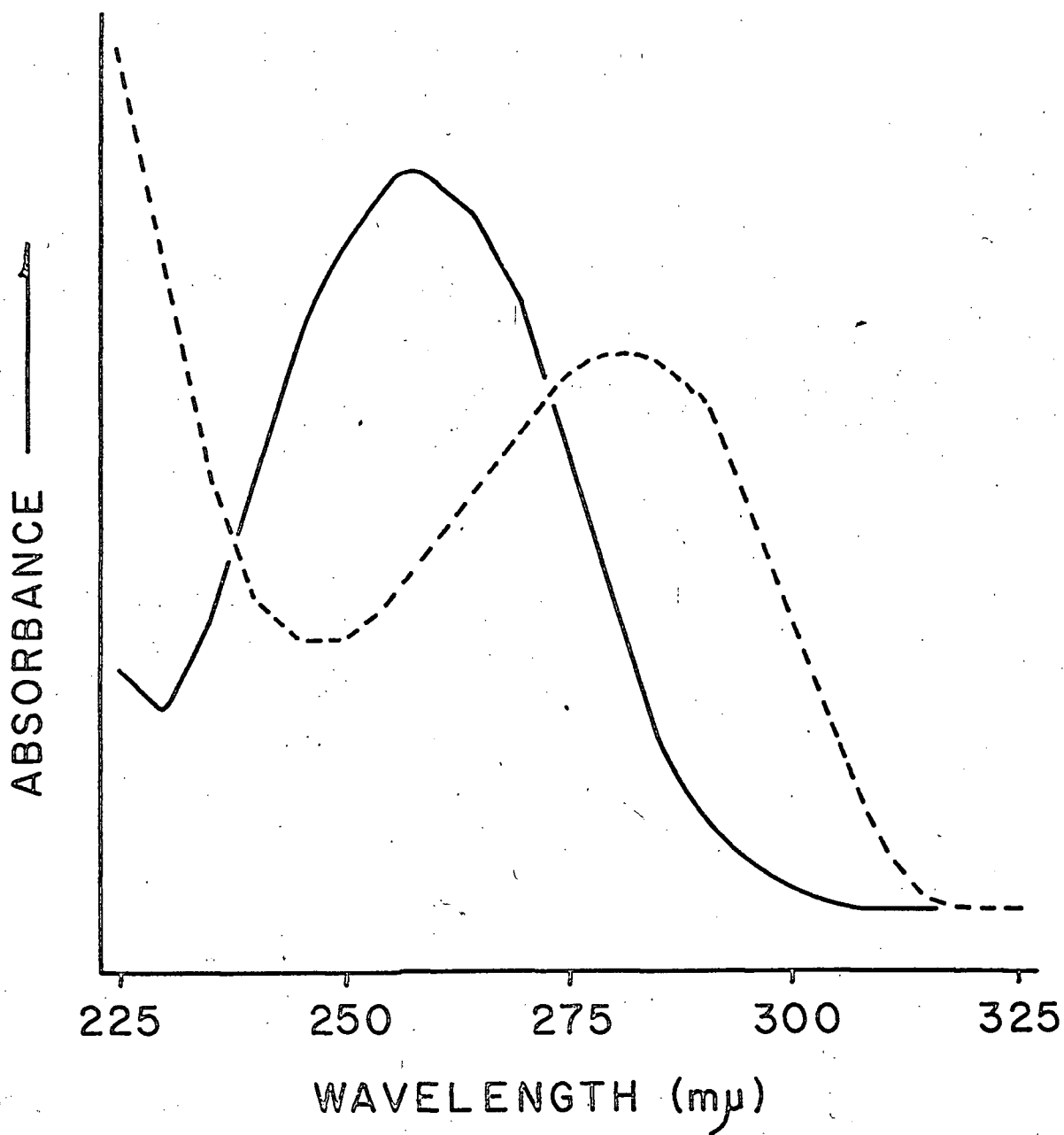| pH	λmax.(mμ)	λmin.(mμ)	Absorbance Ratios			Relative Absorbance
			250/260	280/260	290/260	E257mμ/E281mμ
1	257	228.5	0.91	0.47	0.18	
						0.80
13	281	246.5	0.80	1.49	1.39	

Fig. 22. Spectra of the Novel base in ØW-14 DNA. ---
spectrum in 0.1 N NaOH; _____
spectrum in 0.1 N HCl.

The possibility that this material, though not covalently bonded to the phage DNA, might have affected the buoyant density of the DNA was ruled out in the following manner. The TCA precipitate was dissolved in 0.2 M Tris-HCl buffer pH 8.5 with gentle heating, and subjected to Cs Cl density gradient analysis. Two predominant bands were formed corresponding to the double-stranded and denatured forms of the phage DNA.

The presence of unusual bases found previously in DNA tends to alter the physical properties of the DNA from some phages. The presence of 5-hydroxymethylcytosine in the DNA of the T even coliphages, and the glucosylation of this base results in a decrease in the expected density but has little effect on the Tm (Szybalski, 1968). A number of B. subtilis phages contain either uracil (phage PBS2-Takahashi and Marmur, 1963) or 5-hydroxymethyl-uracil (phage SP8-Kallen, Simon and Marmur, 1962) the presence of which markedly increases the buoyant density and decreases the Tm. The artificial replacement of 66% of the thymine residues in T3 DNA by 5-ethyluracil residues was shown to lower the Tm by 4.5 C (Pietrzkowska and Shugar, 1967). The presence of a fifth base in bacteriophage DNA appears to be rare. In Bacillus phage Vx the presence of a fifth base, a pyrimidine, was suspected since the ratio of adenine to thymine was 1: 0.22. However, no unusual base was observed in hydrolysates of the DNA and it was concluded that the fifth base was either acid labile or non-UV

Table XIII.   Base Composition of ØW-14 DNA as Determined by Three Methods.

Buoyant Density		Thermal		Spectro-chemical		
$\rho(g/cc^3)$	%GC	Tm	%GC	Base	Moles %	%GC
1.666	6	98.4 C	71.9	Adenine	21.8	54.8
				Cytosine	26.6	
				Guanine	28.2	
				Thymine	11.1	
				Unknown	12.3*	

* based on the relationship:

$$\frac{[A + G]}{[T + C + Unknown]} = 1$$

adsorbing (Ikeda et al., 1965). Pons (1967) reported that the

DNA from _Serratia marcescens_ phage 7 contained five bases. In

this case, the fifth base substituted for a deficiency of

guanine residues. It was isolated and its spectral properties

listed but it was not identified. The unknown pyrimidine base

isolated from ØW-14 DNA, though present in a fairly low concen-

tration, appears to have a marked effect on the buoyant density and

the Tm of the DNA (Table XIII). Its presence poses a number of

interesting problems, not the least of which are its structure,

biosynthesis, and what controls are employed to regulate its

incorporation into DNA in the presence of thymine.

GENERAL DISCUSSION

Further research on ∅W-14 by way of extending or explaining the present results could include the following items:

The development of methods for the preparation of non-aggregated phage particles would enable analysis of highly purified preparations of the phage. This is necessary to allow further physical characterization of the phage.

A thorough investigation of the adsorption of ∅W-14a+ and ∅W-14a using inorganic buffers rather than Luria broth as the diluent should be carried out. The effect of temperature and of various additions, such as salts and proteinaceous materials, could then be studied.

A further attempt to demonstrate lysis inhibition in this phage system, and an electron microscopic study of intracellular phage multiplication in singly and multiply infected cells, which might supply some explanation for the lower burst sizes from multiply infected cells.

An attempt to obtain lytic transduction of genetic markers with ∅W-14 using the method of Gunsalus et al (1967). Though the biochemical capabilities of P. acidovorans have been investigated in detail (Stanier, Palleroni, and Doudoroff, 1966) the genetics of this important group have received no attention.

A more detailed study of the phage resistant mutants, including an electron microscopic study of the filaments to determine whether or

not they are septate, and a search for a possible explanation for the unusual temperature sensitive mutant strains.

A detailed investigation of the phage DNA and the difference between the wild-type and ØW-14a. This would include: buoyant density in CsCl; sedimentational analysis; analysis for single-stranded breaks; viscometry; and chemical analysis - total P, deoxyribose, and bases. It would also entail the identification and spectral properties of the unusual base.

An attempt at obtaining transfection with ØW-14 DNA, and competent B. subtilis cells or P. acidovorans spheroplasts.

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