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Some Physical and Chemical Properties of SALMONELLA POTSDAM Bacteriophages

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SOME PHYSICAL AND CHEMICAL PROPERTIES OF SALMONELLA POTSDAM BACTERIOPHAGES

by

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A Thesis in Partial Fulfillment

of the Requirements for the Degree

Master of Science in the Field of

Microbiology

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IN MEMORY OF MY FATHER

維公元一九六四年五月廿一日 女煥英承畢業美洲魯馬連達大學之際謹作簡誌以追念 先父

顯考 諱 東昌 字 世儉 生於農曆民國三年十二月初五日廣東台山
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公少有大志勤學而敏事為鄉黨重視及成長之年擬學醫東洋蒙 祖考逝難志願未得申而迫於就業存君子之志遵聖賢之道立己而立人祖先之餘蔭非公之鴻鵠恭兄而友弟盡情而不傷理勤奮而自立專力於事業歷盡各艱苦至承先啟後積歷年心得達惟惜運籌然公不自滿進而至立人假東方中學以化育後進而不遺餘力公之所樂也見有志之士必盡力鼓勵親友有困者必盡力幫助教子女之道嚴勵而有方求百練成鋼然百慮一身致過勞而殞公也仰無愧於天俯無愧於人子女學未成未克於孝養雖涕泣終日無補之憾矣女未克侍殮謹具短文以慰在天之靈

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INTRODUCTION

The lytic action of bacteriophage, more commonly known as phage, was first observed by Twort in 1915. The name bacteriophage was given by d'Herelle in 1917 to the agent responsible for lysis. While the ultimate origin of phage is still uncertain, lysogenic strains of bacteria are generally acceded to be the natural source of these viruses. A lysogenic bacterium possesses and transmits to its progeny the capacity to produce phage. It was early found that lysogenic bacteria do not contain vegetative phage particles (Burnet and McKie, 1929, Wollman and Wollman, 1936, Gratie, 1936) but carry the information needed for their production in the form of non-infective units, to which the name prophage was applied (Lwoff and Gutmann, 1950). The prophages of some phages have been found to occupy specific loci on the bacterial chromosome and to behave in bacterial crosses as bacterial genes (Jacob and Wollman, 1961). Lysogenic bacteria can produce phage spontaneously at a frequency of about 1×10^{-6} resulting in liberation of infective phage particles. When one of these phages comes in contact with a bacterial cell that is sensitive to lysis, the phage becomes adsorbed to the cell, injects its nucleic acid, multiplies within the cell and eventually causes lysis with the liberation of many new phage particles. Lysogenic bacteria may occasionally produce mutants which are non-lysogenic. A non-lysogenic bacterium may be relysogenized by reinfection with phage.

Burnet (1932) was the first to work with lysogenic strains of bacteria. He classified the phages that were isolated from lysogenic strains of various members of the Salmonella into different groups based on plaque morphology. He further subdivided these groups into subgroups by serology and sensitivity to different physical and chemical agents. He found also that there were more lysogenic strains than non-lysogenic ones in S. enteritidis and S. paratyphi C and he concluded that lysogenicity was a "normal" condition in the Salmonella group. He also demonstrated the possibility of double lysogenicity, in which more than one phage could be isolated from a single lysogenic strain.

Boyd (1950) worked with S. typhimurium. He confirmed Burnet's statement that the majority of strains are lysogenic, that some multiply and that the phages can be placed into different groups on the basis of a number of different criteria.

Williams Smith (1951) demonstrated lysogenicity in S. thompson, S. enteritidis, S. dublin, S. pullorum and S. typhimurium. Atkinson and Geytenbeek (1953) showed a high percentage of lysogenicity in S. adelaide, Atkinson et al. (1952) found that all strains of S. bovis morbificans tested, were lysogenic, although only few strains of S. waycross appeared to be lysogenic (Atkinson and Carter, 1953)

Atkinson and Bullas (1956a, 1956b) and Bullas (1957) demonstrated the possibility of subdividing the phages from lysogenic strains of S. bovis-morbificans, S. muenchen, S. enteritidis, S. blegdam, S. derby

and S. potsdam into subgroups according to their heat sensitivity. Thus Group A phages were resistant to exposure to a temperature of 80°C for 30 minutes while Group B will not survive this temperature. Moreover, Group A phages were serologically similar if isolated from members within the same Kauffman-White grouping. This group lysed only those bacteria with specific somatic antigens and similar temperate type plaques were produced on indicator strains. Group B phages were serologically heterogeneous and their lytic effect was not directed towards any specific antigen. Plaque morphologies varied. The phages had a wider host range.

In recent years studies on the nucleic acids have grown rapidly, due in part to improved techniques. Techniques such as ion-exchange chromatography (Cohn, 1955), paper chromatography (Wyatt, 1955), electrophoresis (Smith, 1955), and color reactions (Dische, 1955), have been employed to investigate the nature of the nucleic acids.

Of the bacteriophages, only a few phages have been investigated for nucleic acid analysis. These include the T phages and phages λvir, Salmonella phages A1 and P22, (Sinsheimer, 1960). Each of these phages contains normal, double-helical deoxyribonucleic acid of DNA. In T2, T4 and T6, moreover, 5-hydroxymethylcytosine replaces the more regular cytosine. Phage f2 was found to be a ribonucleic acid or RNA phage (Loeb and Zinder, 1961) and so was MS2 (Sinsheimer

and Strauss, 1963). Phage 7s isolated from a lysogenic strain of Pseudomonas aeruginosa was also found to be an RNA phage (Feary et al., 1963). Phage ϕ x 174 (Sinsheimer, 1959) and phage ϕ R (Kay, 1962) were found to be different from other phages investigated, for although their nucleic acid was definitely DNA, all information indicated that it occurred only as a single strand rather than in the more regular double strand.

The object of the work reported here is to investigate some of the properties of lysogenic phages, to analyze their nucleic acids, and to compare their analysis with the nucleic acids of their host and of the propagating strains.

The phages chosen for the study were three phages isolated from different strains of Salmonella potsdam, representing both the A and the B groups and originally isolated and grouped by Bullas (1957). These phages were called P3/2, P4/2 and P9a/2 and were readily available (the first number represents the lysogenic culture from which the phage was derived and the second number, the propagating strain). Since the original grouping of these phages was based on heat resistance, the kinetics of heat inactivation was especially studied. Other investigations included the kinetics of inactivation of each phage by ultraviolet (UV) light and its morphology by electron microscopy.

MATERIALS AND METHODS

A. Origin and Maintenance of Strains

The strains of Salmonella potsdam 2, 3, 4 and 9 were obtained from Dr. L. R. Bullas who had obtained these strains from the University of Adelaide, Adelaide, Australia. They are designated here as P2, P3, P4 and P9 respectively.

The cultures were subcultured into nutrient agar stabs. After overnight incubation at 37°C, the strains were stored at room temperature. These cultures were used as stock cultures while a second set of cultures was made in the same way. The latter was used as a working set from which subcultures were made. Both sets had been subcultured into fresh agar stock stabs every 12 months.

B. Media

For bacterial growth, Todd Hewitt broth and Fraser's medium were used. Phage buffer and ammonium acetate solution were employed for phage storage and dilution. L-agar was used in both culture slants and agar plates for phage assays. Nutrient agar was used for bacterial culture and top layer agar was used in phage assays. All media were autoclaved at 15 lbs. pressure for 20-30 minutes before using. The following table gives specifications for all media employed.

Medium	Ingredients in grams/liter (except where indicated)
Todd Hewitt Broth	
Todd Hewitt broth powder (BBL)	30.0
Fraser's medium (Fraser and Jerrel, 1953)	
Anhydrous KH_2PO_4	4.5
NH_4Cl	3.0
$MgSO_4 \cdot 7H_2O$	0.3
1M $CaCl_2$	0.3 ml
Glycerine	24 ml
Casamino acid	15.0
1% gelatin solution	3.0 ml
Anhydrous Na_2HPO_4	10.5
Phage buffer (Bowen, 1953)	
Anhydrous KH_2PO_4	3.0
Anhydrous Na_2HPO_4	3.5
NaCl	2.0
$MgSO_4 \cdot 7H_2O$	0.1
1% gelatin	5 ml
Ammonium acetate	
Ammonium acetate Titrate with NH_4OH to pH 7.2	7.08
L-agar (Lennox, 1955)	
Bactotryptone	10
Yeast extract	5
NaCl	5
Glucose	1
Powder agar	10
1N NaOH	1.5 ml
Nutrient agar (Difco)	
Nutrient broth powder	8
NaCl	5
Agar powder	15

Top agar (Difco)

Nutrient broth powder	8
NaCl	5
Agar powder	7

C. Isolation of Phages

Broth cultures of bacteria were made by inoculating Todd Hewitt broth with P2, P3, P4 and P9 and incubating overnight at 37°C. The phages were isolated from appropriate lysogenic strains by a method which will be illustrated as follows with the isolation of phage P3/2.

The method is called the patch-spot method. A nichrome loop 4 mm in diameter was used to transfer one loopful of P2 onto an L-agar plate, making a circular area about 1.5 cm in diameter, called a "patch." The culture was allowed to dry into the agar. Then P3 was inoculated with the same loop into the center of the patch, in a smaller circular area called a "spot", which was also allowed to dry into the medium. The plate was then incubated overnight. Lysis of bacteria was observed within the area of the spot.

The spot was then removed by cutting out the agar with the nichrome loope and placing it into a thick-walled test tube, containing 1.0 ml of Todd Hewitt broth. The tubes were shaken vigorously to emulsify the broth from the agar surface and then centrifuged to clarify the supernatant fluid extract called the "crude phage" containing P3/2.

The phage extract was assayed by the standard agar-layer technique (Adams, 1950), using tenfold dilutions to 10^{-4}. After overnight incubation, plaques were formed on each plate which were examined on the Spencer colony counter.

In order to be sure of a pure phage preparation, a single plaque picking technique was followed. A single plaque was touched with the tip of a sterile inoculating wire and inoculated into 10 ml of Todd Hewitt broth which had been previously inoculated with one loopful of P2, and incubated for 2 hours. Culture inoculated with phage was incubated for an additional four to five hours and then centrifuged to clarify. The supernatant fluid thus contained phage propagated from one single plaque.

P4/2 and P9a/2 were obtained in like manner except that P4 and P9 were used for the spot respectively. The chief difficulty in isolation of P9a/2 was the fact that P9 is lysogenic for two phages, P9a/2 and P9c/2. However, since these two phages give morphologically distinct plaques, phage P9a/2 was easily isolated by picking a phage that fitted the original description given by Bullas (1957).

D. Tests for Purity of Phages

There were three tests which were used to assure the purity of the preparation and to verify the identification of the phage isolated.

1. The Host Range Test

A "patch-spot" test method was used. A loopful of the culture to be tested for lysis by a phage was inoculated onto the surface of a dried L-agar plate and allowed to dry into the agar. This area was called the "patch." Then a loopful of the phage was inoculated into the center of the plate and also allowed to dry. The plates were then incubated at $37^{o}C$ and the pattern of lysis observed. Incubation was generally overnight but it was possible to detect lysis after a period of six to eight hours incubation.

Each of the phages was tested for lysis against strains P2, P4, P9 and P10, since these strains give a sufficiently different pattern of lysis with the three phage P3/2, P4/2 and P9a/2 (Bullas, 1957).

2. Antiserum Neutralization Test

Antisera against P3/2, P4/2 and P9a/2 prepared by Dr. L. R. Bullas in 1956 were obtained from the University of Adelaide by courtesy of Dr. Nancy Atkinson. Volumes of 0.1 ml of antiserum diluted 1/10, 1/20, 1/40, 1/80, 1/160 and 1/320 in Todd Hewitt broth were dispensed into small test tubes. To each tube was then added 0.5 ml of a dilution of the appropriate phage. Two controls were included for each test -- a phage control tube which contained 0.5 ml of broth plus 0.5 ml of phage solution and a serum control which contained 0.5 ml of 1/10 dilution of antiserum plus 0.5 ml of broth.

Tubes were shaken well, then incubated in a water-bath at $40^{o}C$ for one hour. Each tube was tested for lysis by the patch test method

with P2 as indicator culture. Plates were incubated overnight and observed for lysis.

Phage P4/2 was tested against the P4/2 antiserum only but phage P3/2 and P9a/2 were tested against antisera prepared against both of these phages. If the phages isolated here were identical to the phages originally described, they should be neutralized by the appropriate phage antisera.

3. Heat Sensitivity Test

Phages P3/2, P4/2 and P9a/2 were diluted in phage buffer to the concentration of 10^6/ml. A volume of 1 ml of each phage was placed in a small test tube and all of these tubes were put in a water-bath at temperatures of 55°C, 60°C, 70°C, 75°C and 80°C for 30 minutes. At intervals of 10 minutes, 0.1 ml samples were assayed for plaque forming units.

The temperature at which complete inactivation of lytic activity occurred was recorded.

E. Preparation of Phages in Large Volumes

A 10 ml overnight culture of P2 was prepared in broth. Phage lysates prepared previously were used. A four liter bottle containing two liters of Fraser's medium was fitted with a rubber stopper with three holes. One hole was for the introduction of a sintered glass aeration stem and another was for a short glass tube which had a diameter large enough for the entrance of a 5 ml pipette. This tube was sealed with a

rubber stopper when not in use. The third hole was for a right-angled small glass tube which served as an air-vent tube to equalize the pressure within the bottle. The aeration tube and vent tube were each plugged with cotton to prevent contamination. The rubber stopper was tied to the neck of the bottle. The whole apparatus was autoclaved for sterilization. A schematic drawing of the apparatus is shown in Figure 1.

The 10 ml of overnight culture of P2 was inoculated into the Fraser's medium, and bubbling with air commenced. Bacterial concentration was checked by a Petroff-Hausser bacterial counting chamber on the stage of a phase contrast microscope. It took about five hours for the bacterial concentration to reach 2×10^9/ml. A calculated volume of phage was then inoculated into the bacterial culture by use of the short tube. The amount of phage used was calculated to give a multiplicity of infectivity of 0.1. This particular figure was used in order that only about one-tenth of the bacterial population would become infected by the added phage, so that at the first burst of infected bacteria, all the remaining bacteria would be infected by fresh, active phage particles. The first burst appeared thirty minutes after the inoculation. The culture was then seen to foam. A rubber tubing connected the vent tube to a sterile glass bottle so that any excess of foam might pass over and be collected. Debris of lysed bacteria was seen at the neck of the bottle. Maximum foaming occurred at about three hours after inoculation. Good aeration was necessary for lysis of the maximum number of cells and yet it had to be kept at a certain level to avoid too much foam.

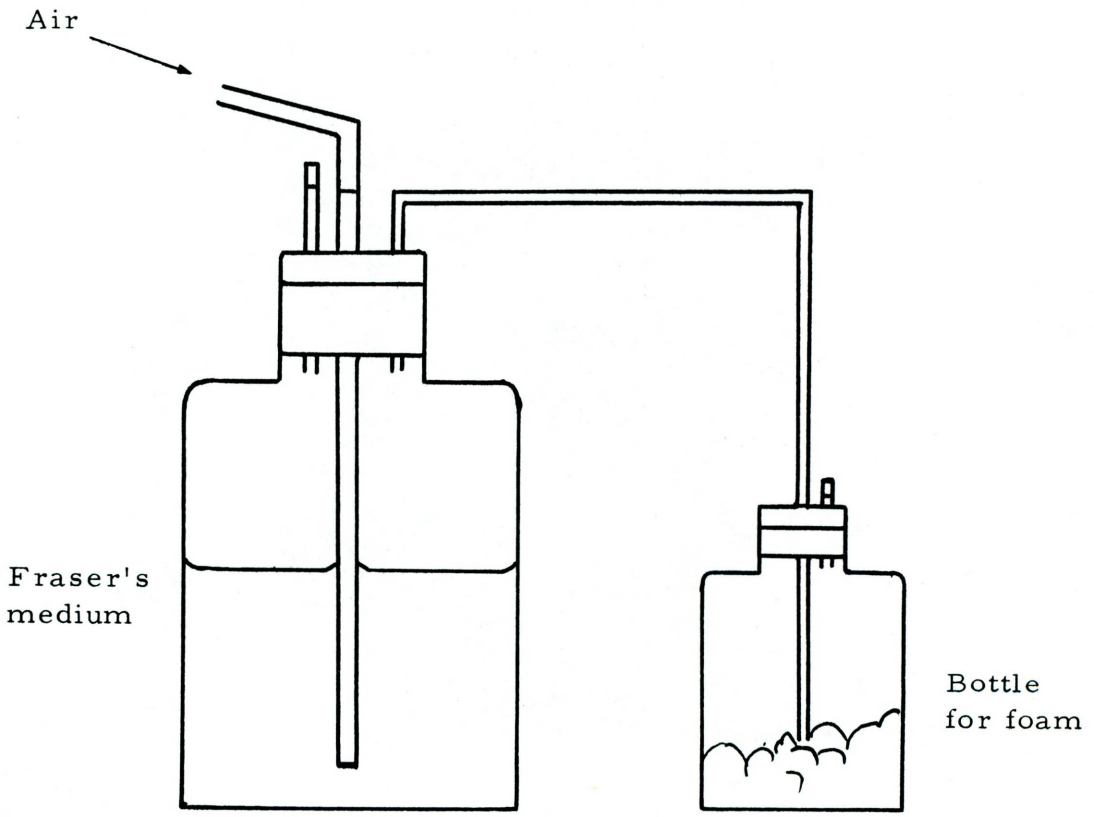

Figure 1. Phage Propagation Apparatus

About five to six hours after inoculation, maximum lysis was completed. The lysate was assayed for plaque forming particles by the agar layer method.

F. Concentration and Purification of the Phages

The method used was a modification of Lunan and Sinsheimer (1956). Enzymatic digestion and differential centrifugation was used to purify the supernatants. To the lysates was added $MgCl_2$ to furnish an Mg^{++} concentration of 0.005 M. Then deoxyribonuclease (DNase) and ribonuclease (RNase) (each obtained from Nutritional Biochemicals) were added to give a concentration of 2 μg per ml respectively. After sitting at room temperature for two hours, the mixture was centrifuged in a Servall RC-1 refrigerated centrifuge at 5900g (g is for acceleration due to gravity) for five minutes. The supernatant fluid contained very few bacterial cells when observed in the Petroff-Hausser counter but contained the same amount of phage as the non-centrifuged extract. The supernatant fluid was then centrifuged at 41700g ($2^{\circ}C$) for two hours in a Servall SS-2 centrifuge. The precipitated buttons of material which contained the phage particles were resuspended in ammonium acetate or phage buffer. It was necessary to subject the suspension to oscillation by a Raytheon 10 kilocycle sonic oscillator for five seconds in order to produce a homogenous suspension. This suspension was again sedimented in the Servall RC-1 centrifuge

at 5900g for five minutes to eliminate any remaining bacteria and suspended impurities.

The final supernatant fluid was the purified phage preparation. Each of the phages P3/2, P4/2 and P9a/2 was prepared in this way as were the phages used in all tests. This concentrated stock phage suspension was kept in screw-cap bottles at 2°C in the refrigerator.

G. Kinetics of Heat Inactivation

From the first series of heat inactivation experiments, section D-3, it was possible to determine temperature at which the reduction in viable phage particles relative to time would best be measured. Temperatures used for phage P3/2 were 61°C, 65°C and 68°C; for phage 4/2 were 68°C, 70°C and 75°C and for P9a/2, 60°C, 62°C and 65°C. Volumes of 1.0 ml of the phages in phage buffer, with an initial titer of about 10^6/ml were placed in 10 ml test tubes. The tubes were then placed in a water-bath at the appropriate temperature, and the phage content assayed at 10 minute intervals by the agar layer method, with P2 as indicator strain.

The logarithm of percent of survival was then plotted showing the decrease of viable phage particles with increasing time at each temperature. From the results, the Arrhenius constants of heat inactivation for each phage were calculated. (Krueger, 1931-1932)

The formulae for the velocity constant, K, and Arrhenius constant μ are as follows:

$K = 2.303/t \times \log P_o/P_o-P_i$
t = time
P_o = initial phage/ml
P_i = inactivated phage/ml at time t
μ = -4.85 x slope (of Arrhenius plot)

H. Ultraviolet Light Inactivation of P3/2, P4/2 and P9a/2

The ultraviolet (UV) light source was a General Electric germicidal lamp exciting UV light of predominantly wave length 2537Å with an energy of approximately 16 ergs per square millimeter per second. The light was placed 50 cm above a table and was turned on 30 minutes before irradiation of phage.

A volume of 10 ml of phage diluted to a titer of about 1×10^6/ml in phage buffer, was placed in a sterile Petri dish, which was rotated by hand throughout the irradiation. A 0.1 ml sample was assayed for phage at intervals of 10 seconds throughout the 120 second irradiation period. From the results, the first order velocity constants of inactivation were calculated using the formulae.

$k = 2.303/t \times \log P_o/P_o-P_i$, k = velocity constant
P_o = initial phage/ml
P_i = inactivated phage/ml at time t
P_o-P_i = survival phage

The average value of k was the mean of the values of k obtained after 50 seconds and 100 seconds irradiation.

I. Preparation of Specimens for Electron Microscopic Observation

A 0.1% solution of parlodion in amyl acetate was used to prepare films on 200 mesh copper grids. Phage of as high a titer as available

was sprayed onto the grids. At first, phage in buffer was used but because of the salt crystals that formed on the film, the phages were later suspended in 0.1 ml ammonium acetate solution. For shadowed preparations, an alloy of 80% platinum and 20% palladium was used in a Model SC-3 high vacuum evaporator. The electron microscope used was a Bendix-Akashi Tronscope 80.

In order to have a stronger film on the grid, it is necessary to reinforce the grid with carbon in the vacuum evaporator prior to deposition of phage.

Phages were also observed by the "negative staining" method with phosphotungstic acid (Brenner and Horne, 1959), modified by Winocour (1963). Equal mixtures of phage in ammonium acetate and 4% phosphotungstic acid plus 0.4% sucrose at pH 7 were sprayed onto grids which were placed in the electron microscope as soon as possible after preparation.

Grids with E. coli phage T2 were prepared in a similar way as a control. Photographs were taken of suitable areas showing phages.

J. Nucleic Acid Tests

For some of these tests an hydrolyzate of whole phage was necessary. One part of 2.5 M perchloric acid was added to nine parts of phage suspension in a small test tube, which was placed in an ice bath for 30 minutes. The tube was shaken every few minutes. The hydrolyzate was centrifuged at 4340g for 10 minutes. The supernatant fluid

was discarded and a volume of deionized distilled water equal to the original suspension was added to resuspend the pellet. Oscillation by the Raytheon 10 kilocycle oscillator was needed to homogenize the suspension. This hydrolyzed solution was used in the tests for RNA, DNA and phosphorus.

1. Drury's Test for RNA

Drury's orcinol test for RNA was used (1948). The orcinol reagent was made by dissolving 0.1 gram of reagent grade ferric alum in 100 ml of concentrated reagent grade HCl. Before use, 1 gram of orcinol was added to every 100 ml of the solution. A standard solution of yeast RNA containing 400 µgm/ml had been prepared. Volumes of 25 λ and 50 λ of this standardized RNA solution were added to 1.475 and 1.450 ml of deionized distilled water. A blank control tube containing 1.5 ml of distilled water alone was also prepared. The phage sample tube consisted of 1.5 ml of phage hydrolyzate. To each tube, 1.5 ml of the orcinol reagent was added. All tubes were then heated in a boiling water bath for 30 minutes and cooled to room temperature. The UV absorbance of the tubes was read in the Beckman DU spectrometer at a wave length of 680 mµ and at a variety of other wave lengths from 400-750 mµ.

2. Burton's Test for DNA

Burton's modification of the Diche diphenylamine test for DNA was used (Burton, 1956). The diphenylamine reagent was prepared as

follows: 15 grams of steam-distilled diphenylamine was added to 100 ml of redistilled glacial acetic acid plus 1.5 ml concentrated H_2SO_4. Just before using, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added to each 20 ml of the reagent used.

Volumes of 25 λ and 50 λ of previously prepared standard calf thymus DNA solution were added to 975 λ and 950 λ of distilled water respectively. The blank control tube contained 1 ml of distilled water. One ml of phage hydrolyzate was used in the test. A volume of 2 ml of the freshly prepared reagent was added to each tube (standard, blank and sample), and the tubes were then incubated at 30°C in a water-bath for 16-20 hours. Low speed centrifugation was used to clarify the solutions. The absorbance of each of the solutions was determined in the Beckman DU spectrophotometer at a wave length of 600 mμ.

3. Acridine Orange Test for the Configuration of DNA

This test may be used to distinguish between single stranded DNA double stranded DNA or RNA (Mayor, 1961). The acridine orange reagent was prepared by adding 0.01 gram of acridine orange to 100 ml of 0.2 M ammonium acetate buffer at pH 4.6. The buffer was prepared by adjusting 0.2 M ammonium acetate solution to pH 4.6 with 0.2 M acetic acid. One drop of the whole phage in phage buffer was placed onto a clean slide and dried at room temperature. To fix, sufficient acetone was poured onto the slide to cover it for 10 minutes, then discarded. Sufficient ammonium acetate buffer was added to cover the slide for one minute and also discarded. The acridine orange reagent

was added to cover the slide for four minutes. The stained slide was rinsed with ammonium acetate buffer. When the slide had dried, it was covered with one drop of 10% glycerine in saline and a cover slip. It was observed by fluorescence microscopy. Phage T2, being a known double stranded DNA phage, was similarly prepared and used as a control.

4. Phosphorus Determination

The method of Russel and Allen (1940) was closely followed. A standard reference tube was prepared by adding 0.4 ml of a standard solution of phosphorus, (concentration 20 mg/ml), to 0.1 ml of deionized distilled water in a micro-Kjeldahl tube. The blank control tube contained 0.5 ml of deionized distilled water only. A volume of 0.5 ml of the phage hydrolyzate was added to each of four different tubes. To each tube was then added 2.0 ml of 60% $HClO_4$ and a new, reagent grade boiling chip was dropped in. The tube contents were digested for 15 minutes in a micro-Kjeldahl digestion apparatus. Each solution was then transferred to a 10 ml volumetric flask and the tubes were rinsed twice with deionized distilled water. Two ml of a 5% solution of ascorbic acid were added to each flask, followed by 1.0 ml of 2.5% solution of ammonium molybdate in 0.05 N H_2SO_4. The volume was adjusted to 10 ml with deionized distilled water. Each tube was then placed in a water-bath at 60°C for 20 minutes. The absorbance at 800 mμ was measured in the Beckman DU spectrophotometer.

K. DNA Base Analysis by Paper Chromatography

The method used was developed originally by Smith and Wyatt (1951), and is still in current use.

1. Bacterial DNA Extraction and Hydrolysis

Bacteria were grown on the surface of nutrient agar in large flat-sided bottles for 48 hours. The cells were harvested with 0.9% (w/v) saline solution. Bacterial suspensions were spun in a refrigerated centrifuge at 4540g for 10 minutes and washed once with saline and twice with 90% (v/v) aqueous ethanol. Cells were then resuspended in ether for 30 minutes and suspensions again centrifuged. Cells were placed in the incubator at 37°C overnight to dry.

To the dried cells was added 10 ml of 1 N NaOH and the mixture placed at 37°C for 16-20 hours. This procedure lyses the cells and completely hydrolyzes the RNA but does not hydrolyze the DNA (although it was depolymerized somewhat). Insoluble material was removed by centrifugation. The supernatant fluid which contained the DNA was adjusted to pH 4 with acetic acid. An equal volume of 95% ethanol was added and the precipitate collected by centrifugation. The precipitate was redissolved in 4 ml of 1 N NaOH. An equal volume of a mixture which contained eight parts of chloroform to one part octanol was added (Sevag, 1938). The DNA solution and octanol-chloroform mixture was shaken by hand for one minute. Following centrifugation, the clear supernatant solution was decanted from the octanol-chloroform

gel which contained the protein and was adjusted to pH 4 with acetic acid. An equal volume of 95% ethanol was added to precipitate the DNA which was collected by centrifugation.

To the wet DNA in a small test tube was added 1.0 ml of 75% perchloric acid, and the tube was placed in a boiling water-bath at 100°C for one hour. A volume of 0.25 ml of distilled water was added to the hydrolyzate in order to allow good spread of the spots on the chromatographic paper and to avoid charring of the paper with subsequent loss of the spot.

The perchloric acid in the hydrolyzate was removed in some extracts by precipitation with 12 N KOH. This procedure did not cause any detectable or significant alteration in relative base values.

2. Phage DNA Extraction and Hydrolysis

Ten ml of phage in buffer were lyophilized. The dried powder was then hydrolyzed with 72% perchloric acid and the DNA extracted and hydrolyzed by the same method described for dried bacterial cells.

3. Paper Chromatographic Analysis of DNA Hydrolyzates

Spots containing 50 λ volumes of hydrolyzate were placed on Whatman No. 1 chromatographic paper. The following solvent systems were used for chromatography.

Solvent 1: Isopropanol 170 parts, HCl 41 parts, H_2O 39 parts. (Wyatt, 1951).

Solvent 2: Isopropanol seven parts, H_2O three parts, NH_3 in vapor phase. (Dunn and Smith, 1957).

Separation of the bases was by the descending chromatographic method. Development in the first solvent was about 24 hours followed by air drying at room temperature. The second development with solvent 2, at right angles to the first, was about 10-12 hours. Paper was again air dried.

UV absorbing spots were located with a low pressure Mineralight, Model SL 2537, manufactured by Ultra-Violet Products Inc. The position of the spots was marked with a lead pencil, and a control, non-UV absorbing spot was similarly marked. On a single chromatogram, all spots were marked to be of equal size. Rf values (ratio of distance from the origin to spot and origin to solvent front) were read for all UV absorbing spots. Spots containing the bases and the blank spot were cut from the chromatogram, cut into a comb-like shape, and placed into test tubes containing 5 ml of 0.1 N HCl. Tubes were vigorously shaken for one hour to elute the bases and the chromatogram paper was removed.

The absorbance of the eluted bases was read in the Beckman DU spectrophotometer using the blank extract as control. For the first few chromatograms, complete spectra between 240 mμ and 300 mμ were taken to verify identification of the bases from the Rf values. Thereafter, absorbance at the peak wave length and at either 290 mμ or 300 mμ, where appropriate, were read.

The differential extinction method described by Bendich (1957) was used to calculate the amounts of each base in the eluate. The values

were then converted to micromoles which were in turn converted to moles/100 moles of total bases extracted from the DNA.

Constants used in the calculation were:

Molecular weights of purine and pyrimidine bases:

Guanine - 151
Adenine - 135
Cytosine - 111
Thymine - 126

Δ Values: - the difference between maximum absorbance and absorbance at 290 mμ or 300 mμ. (Bendict, 1957).

Guanine (249 mμ and 290 mμ) - .475
Adenine (263 mμ and 290 mμ) - .900
Cytosine (276 mμ and 300 mμ) - .863
Thymine (265 mμ and 290 mμ) - .549

Calculation of amount of base in the 5 ml of 0.1 N HCl:

= total amount of base in 5 ml
= 5 x 10 X
X = reading for unknown

From the calculated amounts of each base, the molar percentage of each base was then determined; the ratios A/T, G/C and A+T/G+C (Spirin, 1960) and the amount of each base relative to adenine (Kay, 1962) were calculated.

RESULTS

A. Verification of Identification of the Phages

From the lysogenic strains of P3, P4, and P9, with use of the indicator strain P2, three phages designated P3/2, P4/2, and P9a/2 respectively, were isolated. There were three possible ways that the identity of each of these phages to the phages originally described as isolated from these lysogenic strains could be verified.

1. The Host Range Test

The results given by the patch-spot test using phages P3/2, P4/2 and P9a/2 tested on strains P2, P4, P9 and P10 are shown in Table I. These results agree with those of Bullas (1957), and thus verify the identity of each phage.

TABLE 1

HOST RANGE TESTS FOR PHAGES P3/2, P4/2 and P9a/2

Bacterial Strains	Phages		
	P3/2	P4/2	P9a/2
S. potsdam 2	+++	+++	+++
S. potsdam 4	+++	-	+++
S. potsdam 9	++	-	-
S. potsdam 10	++	+	-

\- = no lysis
\+ = few plaques
++ = many plaques
+++ = semi-confluent to confluent lysis

2. Serological Test

The original, Adelaide antisera of P3/2, P4/2, P9a/2 were titrated against the phages prepared here in this laboratory. The results are shown in Table 2; they agree with the original results and thus also verify the identity of each of the phages.

TABLE 2

ANTISERUM NEUTRALIZATION TESTS OF PHAGES P3/2, P4/2 and P9a/2

Phage	Antiserum against phage	Final antiserum dilution						
		C	1/10	1/20	1/40	1/80	1/160	1/320
P3/2	P3/2	-	+	+	+	+	±	-
	P9a/2	-	-	-	-	-	-	-
P4/2	P4/2	-	+	+	+	+	+	+
P9a/2	P3/2	-	±	-	-	-	-	-
	P9a/2	-	+	+	+	±	±	±

+ = neutralization
+ = partial neutralization
- = no neutralization
C = phage control tube

3. Heat Sensitivity Test

Results of the heat sensitivity tests are shown in Table 3.

TABLE 3

HEAT SENSITIVITY TESTS FOR PHAGES P3/2, P4/2 and P9a/2

Temperature in ^{o}C	Phage		
	P3/2	P4/2	P9a/2
55	+	+	+
60	+	+	±
65	+	+	-
70	±	+	-
75	-	±	-
80	-	-	-
control (not heated)	+	+	+

+ = complete lysis; no inactivation
± = reduced degrees of lysis; partial inactivation
- = no lysis; thus inactivated

The results here show that phage P3/2 is inactivated at a temperature which is five degrees higher than that originally found to inactivate this phage. The greater heat resistance of phage P3/2 may be, however, a feature of the different medium in which the phage is suspended from the medium used originally. It is well known that the suspending medium may profoundly affect phage properties. This explanation is supported by the results of the other two tests - the phage isolated here gives the same host range and is serologically identical with the phage originally described isolated from lysogenic strain P3. Since there

was also evidence that P3 was lysogenic for one phage only (comparison of "crude phage" and purified phage host ranges) the phage P3/2 isolated here is taken to be identical with the phage originally described, isolated from this strain, in spite of the difference in heat sensitivity. The verification of the identity of phages P4/2 and P9a/2 with the phages originally described isolated from lysogenic strains P4 and P9 is supported by the two tests of host range and serology, and each phage is inactivated at a temperature close to that originally described for the particular phage.

B. Growth and Propagation of Phages

Each of the three phages propagated to high titers in Fraser's medium when aerated. Representative titers of plaque forming units for each of the phages were 3×10^{10}/ml for P3/2, 7.5×10^{11}/ml for P4/2 and 7×10^{10}/ml for P9a/2. These values were obtained by assaying the crude phage lysates for plaque forming units immediately after propagation. The method of propagation used was therefore satisfactory to obtain initially high titers of each of the phages, prior to any treatment for concentration and purification.

C. Purification of the Phages

Various methods of treatment were first tested with one of the phages, P4/2, in order to test the effects of such treatments on plaque forming ability. These methods included treatment with the enzymes

DNase and RNase, centrifugation at various speeds for varying times, and filtration. The effects of these treatments on the plaque forming properties of P4/2 are shown in Table 4.

TABLE 4

EFFECT OF VARIOUS TREATMENTS ON THE PLAQUE FORMING UNITS OF A PREPARATION OF P4/2 PROPAGATED IN FRASER MEDIUM

Treatment	Initial titer phage/ml	Resulting titer phage/ml
No treatment	7.5×10^{11}	7.5×10^{11}
2 μgm DNase/ml and 0.005 M $MgCl_2$ Room temperature 2 hours	7.5×10^{11}	7.5×10^{11}
2 μg RNase/ml Room temperature 2 hours	7.5×10^{11}	7.5×10^{11}
Centrifugation at 3020g for 20 minutes	7.5×10^{11}	3.0×10^{11}
Centrifugation at 5900g for 15 minutes	7.5×10^{11}	2.0×10^{11}
Centrifugation at 5900g for 5 minutes	7.5×10^{11}	7.5×10^{11}
Filtration with Whatman No. 1 Filter paper	7.5×10^{11}	1.3×10^{11}

From these results it may be seen that treatment with both DNase and RNase did not reduce the plaque forming titer. Centrifugation at 5900g for five minutes similarly did not affect the titer but centrifugation for 15 and 20 minutes reduced the titer appreciably. Filtration through Whatman No. 1 filter paper (to remove gross impurities) also reduced the titer appreciably.

Phage preparations with a titer between 10^{12}/ml and 10^{13}/ml were regularly obtained with phage P4/2 after the purification and concentration procedure. However, greatly reduced titers were regularly obtained with phages P3/2 and P9a/2 with final titers between 10^{7} to 10^{9} only being obtained.

By electron microscopy, many empty phages or "ghosts" were observed with both of these phages. The method of concentration therefore resulted in the loss of a considerable proportion of the plaque forming units of phages P3/2 and P9a/2. This difficulty of obtaining high titer phage stocks after enzyme treatment and centrifugation has been a problem with other phages (Lunan and Sinsheimer, 1956). The preparations of P3/2 and P9a/2 obtained were used in the heat inactivation and ultraviolet inactivation studies but were considered to be unsatisfactory for chemical analysis.

Since the only contaminating material in the preparation of P4/2 appeared to be flagella and portions of cell wall of the bacteria, which are predominantly protein (Weibull, 1958) (Work, 1957) and therefore

would not contribute to the nucleic acid content, the preparation of P4/2 was considered sufficiently pure for analysis of the nucleic acid of this phage.

D. Some Comparative Physical Properties of Phages P3/2, P4/2 and P9a/2

1. Heat Inactivation

The phage P3/2 was heated at the three temperatures of 61°C, 65°C and 68°C for various times and the numbers of plaque forming units assayed. The results are shown in Table 5 and illustrated in Figure 2, in which the logs of percent surviving plaque forming units are plotted against time of heat treatment. It is clear that heat inactivation is exponential with time. The table also includes, for each treatment, the calculated velocity constants, which are referred to in the succeeding section.

Phage P4/2 was heated at 68°C, 70°C and 75°C and samples were assayed at varying time intervals. The results are shown in Table 6. The results of log percent of surviving plaque forming units against time are plotted in Figure 3.

TABLE 5

TITRATION OF PLAQUE FORMING UNITS OF PHAGE P3/2, HEATED IN PHAGE BUFFER, AT 61°C, 65°C and 68°C FOR VARIOUS TIMES

Initial titer	Temperature in °C	Time of exposure in minutes	Survival titer phage/ml	Percent of survival	Velocity constant k in minutes^{-1}
3.3×10^7	61	10	3.0×10^7	90.91	0.013
		20	2.5×10^7	75.76	0.013
		30	2.2×10^7	66.67	0.013
		40	1.8×10^7	54.54	0.014
		50	1.6×10^7	48.48	0.013
		60	1.3×10^7	39.39	0.013
5.0×10^7	65	10	2.8×10^7	56.00	0.059
		20	1.7×10^7	34.00	0.055
		30	8.2×10^6	16.40	0.058
		40	5.0×10^6	10.00	0.057
		50	2.7×10^6	5.40	0.058
		60	1.6×10^6	3.20	0.056
2.4×10^7	68	5	1.2×10^7	50.00	0.13
		10	6.3×10^6	26.25	0.13
		15	3.2×10^6	13.33	0.13
		20	1.7×10^6	7.08	0.13
		25	7.8×10^5	3.25	0.13
		30	3.9×10^5	1.63	0.13

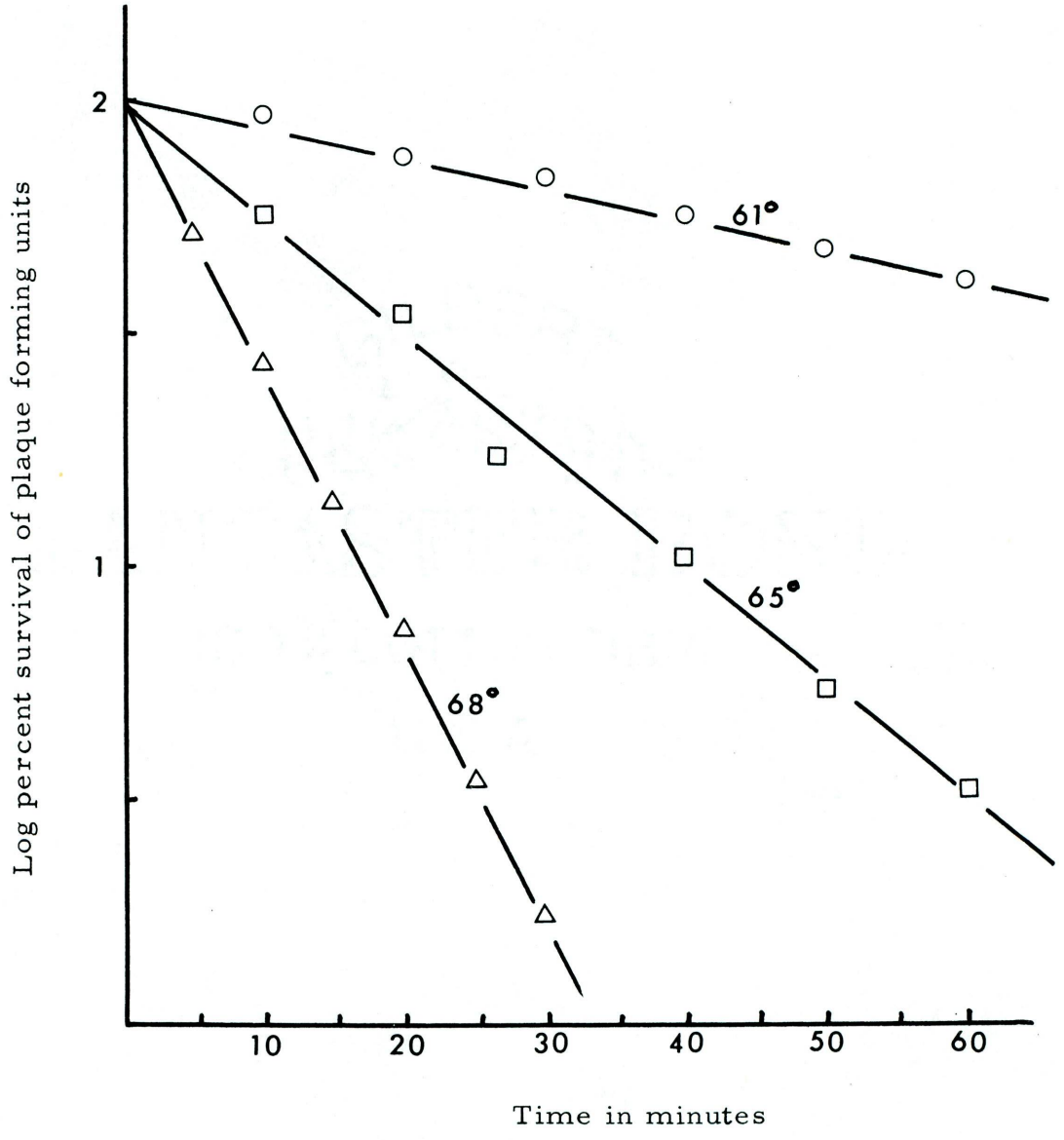

Figure 2. Heat Inactivation of Phages P3/2 at 61°C, 65°C and 68°C

TABLE 6

TITRATION OF PLAQUE FORMING UNITS OF PHAGE P4/2, HEATED IN PHAGE BUFFER, AT 68°C, 70°C and 75°C FOR VARIOUS TIMES

Initial titer	Temperature tested in °C	Time of exposure in minutes	Survival titer phage/ml	Percent of survival	Velocity constant k in minutes^{-1}
2.6×10^{12}	68	10	8.9×10^{11}	34.23	0.086
		20	4.2×10^{11}	16.15	0.085
		30	1.8×10^{11}	6.92	0.085
		40	9.6×10^{10}	3.69	0.086
		50	3.6×10^{10}	1.38	0.086
		60	9.9×10^{9}	0.38	0.086
10×10^{10}	70	10	2.5×10^{10}	25.00	0.15
		20	4.8×10^{9}	4.80	0.15
		30	1.6×10^{9}	1.60	0.14
		40	3.9×10^{8}	0.39	0.14
		50	1.0×10^{8}	0.10	0.17
		60	3.0×10^{7}	0.03	0.13
2.6×10^{12}	75	5	1.2×10^{10}	0.46	1.02

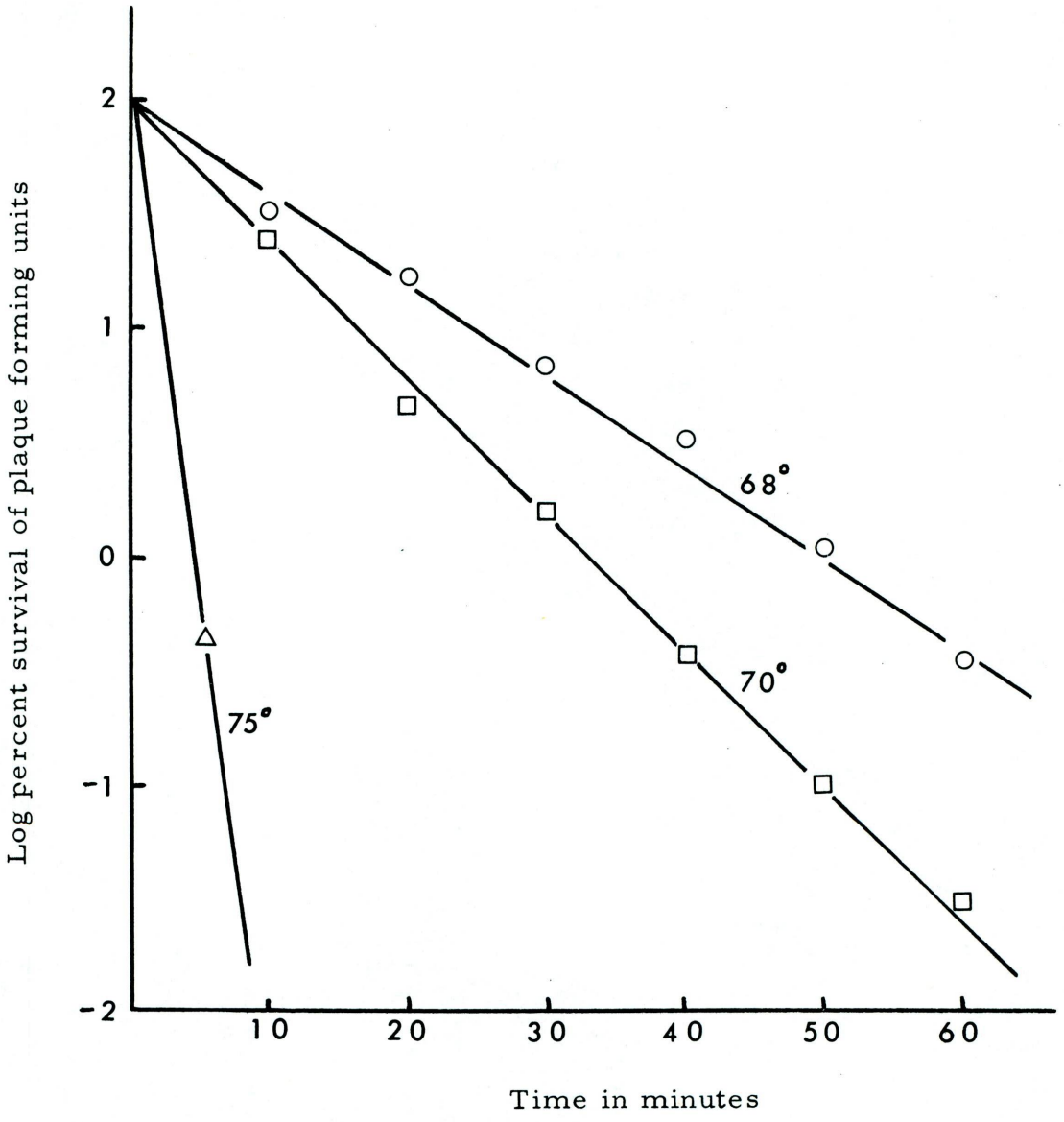

Figure 3. Heat Inactivation of P4/2 at 68°C, 70°C and 75°C

Reduction in titer of phage P4/2 is also exponential with time for each of these temperatures.

Phage P9a/2 was heated at temperatures of 60°C, 62°C and 65°C and samples removed at different times for assay of surviving plaque forming units.

The results are shown in Table 7. The results of log percent survival of plaque forming units against time are shown in Figure 4. Inactivation of P9a/2 is also shown to be exponential for each of the three temperatures.

The results of heat inactivation of the three phages were compared. The percent survivals of each of the phages at each of the test temperatures when heated for 20 and 30 minutes respectively are shown in Table 8. The log percent survival plotted against temperature of inactivation for both of these times is shown in Figure 5. This figure clearly shows that each of the three phages has a different range of temperature sensitivity which is a definite distinguishing property of that particular phage.

2. Velocity Constant and Arrhenius Plots

As shown in the above paragraph, the heat inactivation of the three phages at different temperatures proceeded logarithmically with time. The reaction might thus conform to the general mass law and might be expressed by the following equation (Krueger, 1931-1932).

TABLE 7

TITRATION OF PHAGE FORMING UNITS OF PHAGE P9a/2, HEATED IN PHAGE BUFFER, AT 60°C, 62°C and 65°C FOR VARIOUS TIMES

Initial titer	Temperature tested in °C	Time of exposure in minutes	Survival titer phage/ml	Percent of survival	Velocity constant k in minutes^{-1}
5.3×10^6	60	10	3.2×10^6	60.38	0.050
		20	1.8×10^6	33.96	0.054
		30	9.0×10^5	16.98	0.058
		40	6.6×10^5	12.45	0.052
		50	3.9×10^5	7.36	0.053
		60	2.5×10^5	4.72	0.054
2.0×10^8	62	10	8.2×10^7	41.00	0.088
		20	3.5×10^7	17.50	0.086
		30	1.3×10^7	6.50	0.089
		40	6.5×10^6	3.25	0.084
		50	2.5×10^6	1.25	0.087
		60	1.1×10^6	0.55	0.085
2.0×10^8	65	5	8.0×10^7	40.00	0.17
		10	3.5×10^7	17.50	0.17
		15	1.4×10^7	7.00	0.15
		20	6.0×10^6	3.00	0.17
		25	2.4×10^6	1.20	0.17
		30	1.0×10^6	0.50	0.17

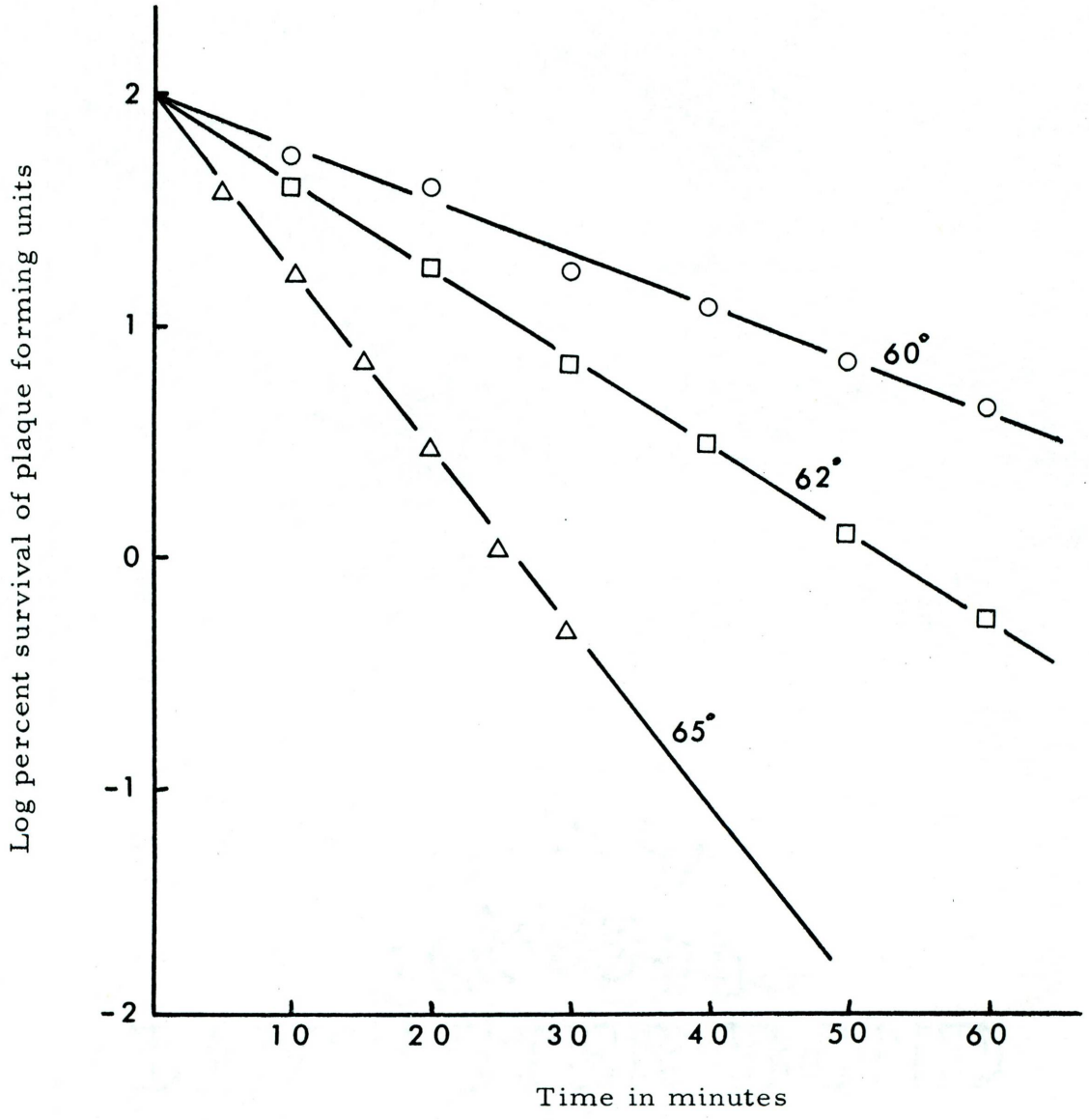

Figure 4. Heat Inactivation of P9a/2 at 60°C, 62°C and 65°C

TABLE 8

COMPARISON OF HEAT RESISTANCES OF PHAGES P3/2, P4/2 AND P9a/2 WHEN HEATED FOR 20 AND 30 MINUTES AT VARIOUS TEMPERATURES

Phage	Temperature in °C	Time of inactivation in minutes	Percent survival	Log percent survival
P3/2	61	20	75.8	1.88
	65		34.0	1.53
	68		7.08	0.85
	61	30	66.7	1.82
	65		16.4	1.21
	68		1.63	0.21
P4/2	68	20	16.2	1.2
	70		4.8	0.68
	75		-	-
	68	30	7.0	0.85
	70		1.60	0.20
	75		-	-
P9a/2	60	20	33.9	1.53
	62		17.5	1.24
	65		3.0	0.48
	60	30	17.0	1.23
	62		6.5	0.81
	65		0.5	-0.30

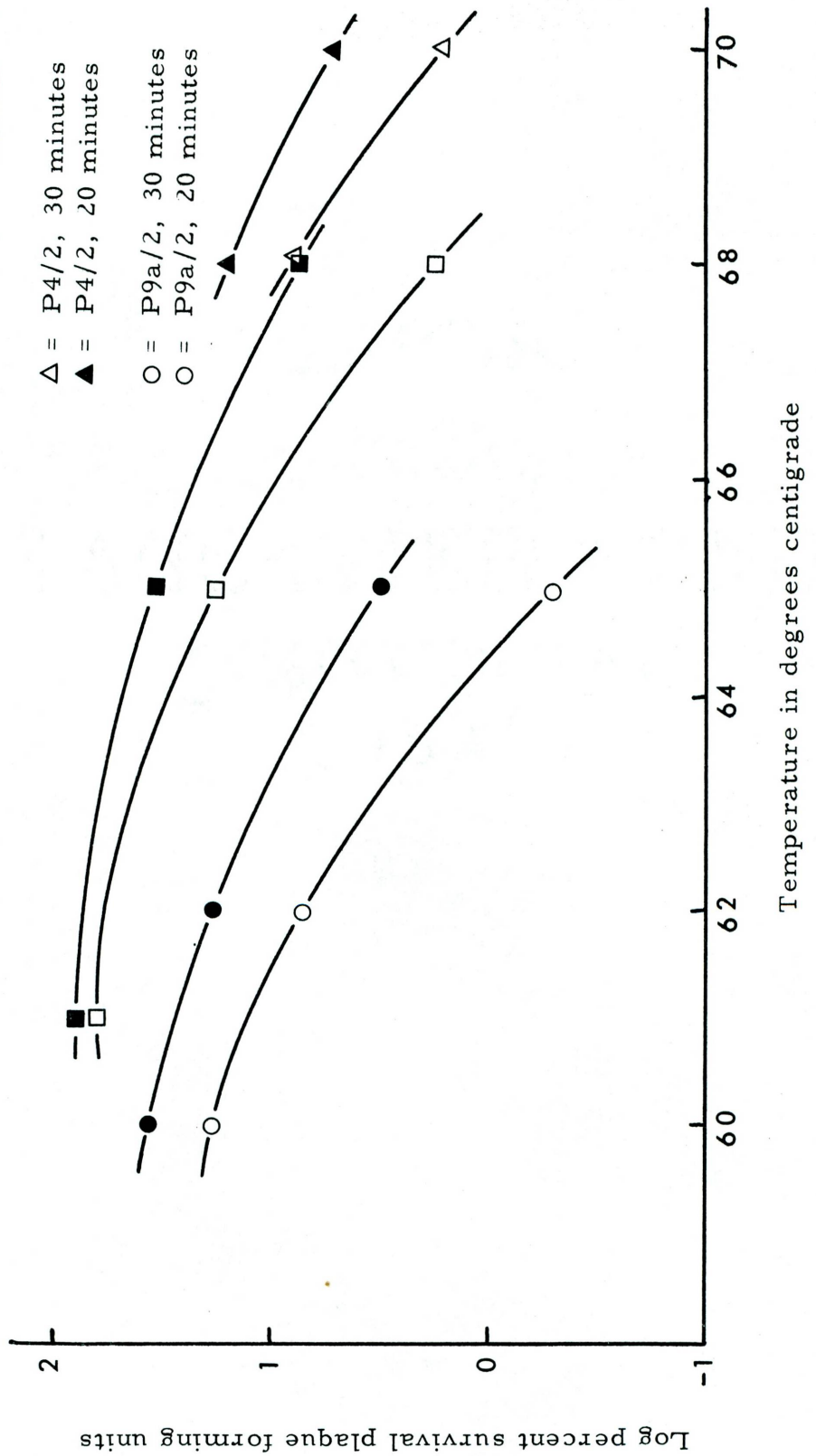

Figure 5. Comparison of Heat Resistance Curves of Phages P3/2, P4/2 and P9a/2 when Heated for 20 and 30 Minutes at Various Temperatures

$k = 2.303/t \times \log P_o/P_o-P_i$
P_o = initial phage/ml
P_i = inactivated phage/ml at time t
P_o-P_i = survival phage

From this equation, the velocity constant, k, was calculated and an average value of k obtained for each temperature used. The velocity constant, k, for each phage at the different test temperatures are recorded in Table 9.

TABLE 9

ARRHENIUS PLOTS AND ARRHENIUS CONSTANTS OF THE PHAGES

Phage	Average velocity constant k min^{-1}	Log k	Temperature in ^oC	Absolute temperature (T)	$10^3/T$	Arrhenius constant
P3/2	0.013	-1.887	61	334	2.993	73,280
	0.057	-1.224	65	338	2.958	
	0.130	-0.886	68	341	2.932	
P4/2	0.086	-1.065	68	341	2.931	84,630
	0.150	-0.824	70	343	2.917	
	1.2	0.010	75	348	2.867	
P9a/2	0.053	-1.275	60	333	3.003	56,334
	0.086	-1.075	62	335	2.985	
	0.17	-0.769	65	338	2.958	

The log of the average value of k plotted against the reciprocal of absolute temperature should form a straight line (Krueger, 1931- 1932). The constant of the line may be expressed by μ, the critical thermal increment or the Arrhenius constant. For the three phages, log k values were therefore found and plotted against the reciprocals of the absolute temperature T, where $T = T_1 + t$.

T_1 - 273

t - the test temperature in degree Centigrade

The Arrhenius plots for each of the phages P3/2, P4/2 and P9a/2 are shown in Figure 6. The data used for the plots are shown in Table 9. The three points for each phage fall quite satisfactorily on straight lines, as expected.

The thermal increment, μ, is expressed by μ = slope x -4.58. The Arrhenius constant, μ, for each of the three phages are also shown in Table 9. Phage P4/2 has the highest value of μ 84,630, while P3/2 and P9a/2 are lower with values of 73,280 and 56,334, respectively.

3. Ultraviolet Light Inactivation

Phage suspended in phage buffer were irradiated from 10 to 120 seconds at 10 second increments, and the plaque forming units assayed. The results are shown in Table 10. The logarithm of the percent of surviving plaque forming units was plotted against time of inactivation. The phage titer declined exponentially for each phage with increasing UV dosage.

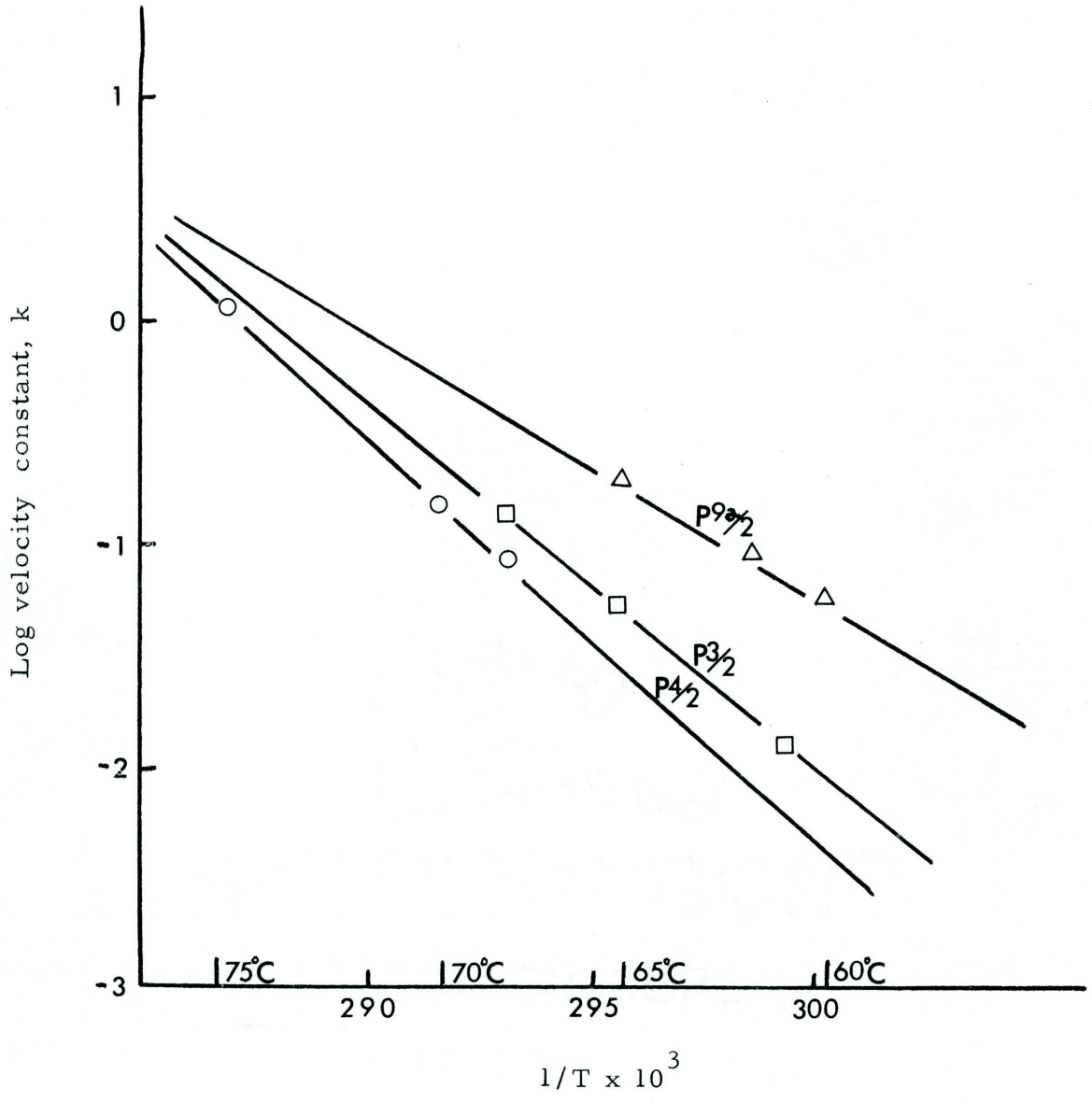

Figure 6. Plots of Logarithms of Velocity Constants for Heat Inactivation of Phages at Various Temperatures Against the Reciprocal of the Absolute Temperature.

TABLE 10

ULTRAVIOLET LIGHT INACTIVATION

Phage	Initial titer phage/ml	UV light dose in seconds	Survival titer phage/ml	Percent survival
P3/2	3×10^7	10	2.5×10^7	83.33
		20	1.8×10^7	60.00
		30	1.7×10^7	56.67
		40	1.1×10^7	36.67
		50	8.5×10^6	28.33
		60	7.2×10^6	24.00
		70	5.4×10^6	18.00
		80	4.2×10^6	14.00
		90	3.3×10^6	11.00
		100	2.5×10^6	8.33
		110	2.0×10^6	6.67
		120	1.8×10^6	6.00
P4/2	10×10^{10}	10	7.3×10^{10}	73.00
		20	5.6×10^{10}	56.00
		30	4.3×10^{10}	43.00
		40	3.2×10^{10}	32.00
		50	2.5×10^{10}	25.00
		60	1.8×10^{10}	18.00
		70	1.4×10^{10}	14.00
		80	9.0×10^9	9.00
		90	8.0×10^9	8.00
		100	6.1×10^9	6.10
		110	4.5×10^9	4.50
		120	3.6×10^9	3.60
P9a/2	5.3×10^6	10	4.3×10^6	81.13
		20	3.5×10^6	66.04
		30	2.9×10^6	54.72
		40	2.5×10^6	47.17
		50	1.9×10^6	35.85
		60	1.5×10^6	28.30
		70	1.3×10^6	24.53
		80	9.9×10^5	18.68
		90	8.2×10^5	15.47
		100	7.4×10^5	13.96
		110	5.6×10^5	10.57
		120	4.7×10^5	8.87

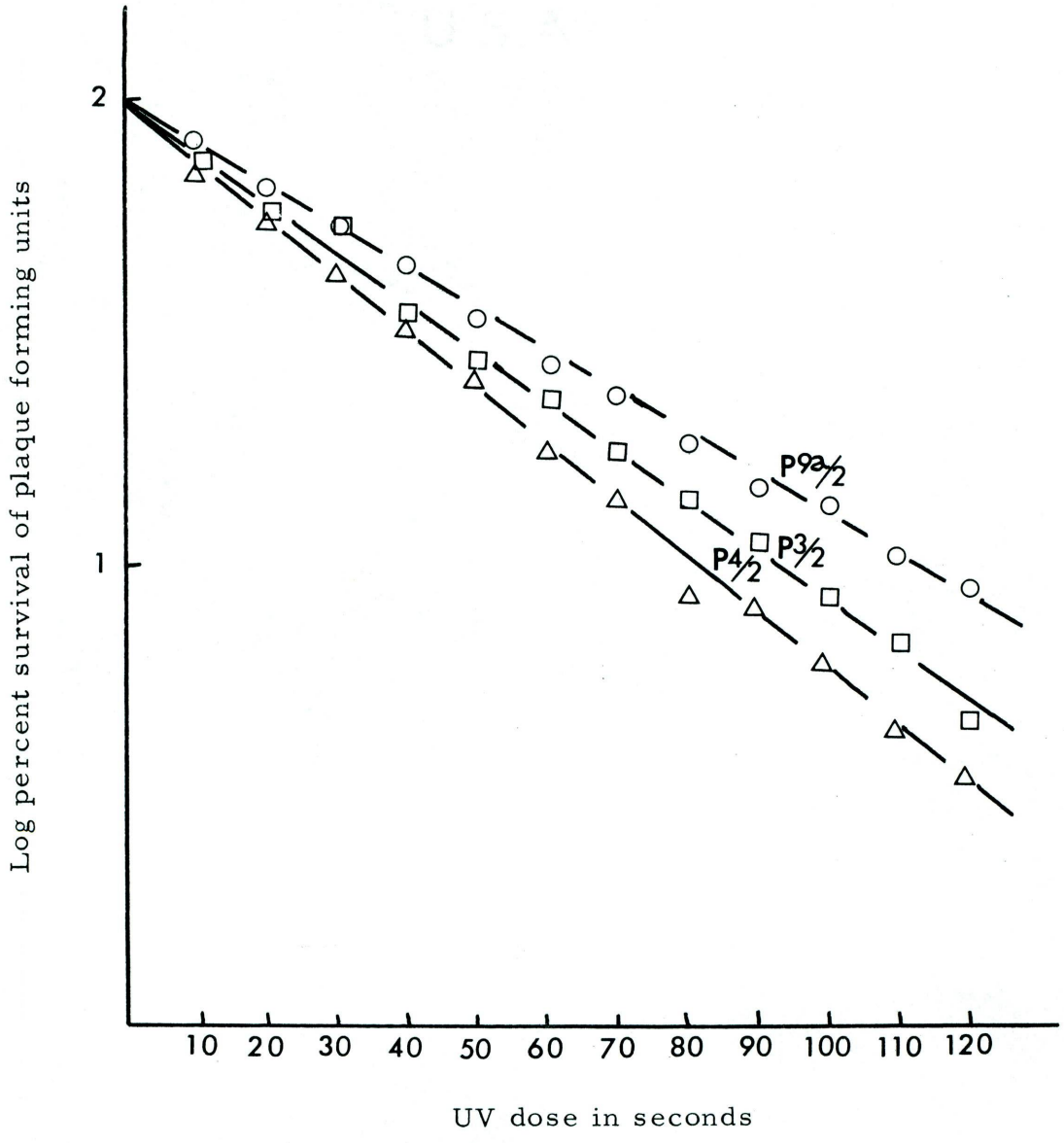

Figure 7. Ultraviolet Light Inactivation Curves of Phages P3/2, P4/2 and P9a/2

Phage P9a/2 showed least sensitivity to UV inactivation; phage P4/2 showed greatest sensitivity and phage P3/2 had an intermediate degree of sensitivity.

The first order velocity constant, k, of inactivation by UV light are shown in Table 11. Results showed that k for P3/2 was 0.025, for P4/2, 0.028 and for P9a/2, 0.020, min^{-1}.

TABLE 11

FIRST ORDER VELOCITY CONSTANTS OF INACTIVATION BY UV LIGHT OF PHAGES P3/2, P4/2 and P9a/2

Phage	Time UV light inactivation in seconds	Sampling velocity constant k in min^{-1}	Average velocity constant k in min^{-1}
P3/2	50	0.025	0.025
	100	0.025	
P4/2	50	0.028	0.028
	100	0.028	
P9a/2	50	0.020	0.020
	100	0.020	

$k = 2.303/t \times \log P_o/P_o-P_i$

4. Morphology by Electron Microscopy

Results of electron microscopy at first were disappointing and were attributed to the following factors:

a. The film was too thin.

b. The preparations contained much cell debris and the phage were difficult to detect.

c. Flagella pieces, especially in the preparation of phage P3/2 and P9a/2 hindered observation.

d. The negative stain method did not come out as expected.

It was, therefore, decided to concentrate on the morphology of one phage only, and the phage chosen was P4/2, since this phage was present in highest, viable concentration. A new phage P4/2 preparation was made. The same method of concentration and purification was used except that filtration through Whatman No. 1 filtrate filter paper was done before high speed centrifugation. Final resuspension was in 0.1 M ammonium acetate at pH 7.2. The preparations so obtained were much clearer and little or no debris due to chemical crystals was observed.

Electron micrographs of P4/2 both by shadowed and by negative staining are shown in Figures 8 and 9. From the electron micrographs the dimension of the head of P4/2 is approximately 59 mμ x 51 mμ with a tail of about 25 mμ in length and 10 mμ in width. This calculation is based on the known diameter of the latex particle, 0.514 μ, used as an internal standard in the preparation. The particle measured 7 cm or 7×10^4 μ in the micrograph.

The shadowed preparations suggest the structure of a dodecahedron, 12 faces with five sides to a face. In the electron micrograph of the negatively stained preparations, the empty phage P4/2 "ghost" shows the outline of hexagons. This observation seems to agree with the above suggestion. The tail is visible if the phage is properly oriented.

A diagram of the suggested morphology of phage P4/2 is shown in Figure 9.

The non-phage material in the phage preparations consisted of broken threads of flagella of apparently similar weight to the phage so that they are sedimented at the same rate, and round, cup-like fragments of what appeared to be cell wall material. Many of these latter were still attached to the phage by the tail and may therefore represent the receptor sites of the phage.

E. Some Chemical Properties of Phage P4/2

1. The Orcinol Test

Results of the orcinol test for RNA were as follows. The standards gave an absorbance reading of 0.104 for an amount of 25 λ and 0.204 for 50 λ. The spectra reading of the phage sample between 400 and 750 mμ could not be compared with those of the standards. Therefore, the phage appeared to be free of RNA.

2. The Diphenylamine Test

The results of the diphenylamine test for DNA are shown in Table 12. Each sample shows considerable absorbance and thus provides evidence that phage P4/2 is a DNA-containing phage. From the viable count of the phage and the volume of phage preparation used, the calculated weight of DNA per phage particle was 3.2×10^{-17} grams. The manner of calculation to obtain the value is shown as follows:

Figure 8:

a. Four phage particles attached to a cup-shaped fragment of cell wall. Tails are evident on two of the particles.

b. Phage ghost in upper left corner showing tail. Another ghost in lower right corner.

c. Phage ghost attached by tail to cell wall fragment.

d. Free phage ghost.

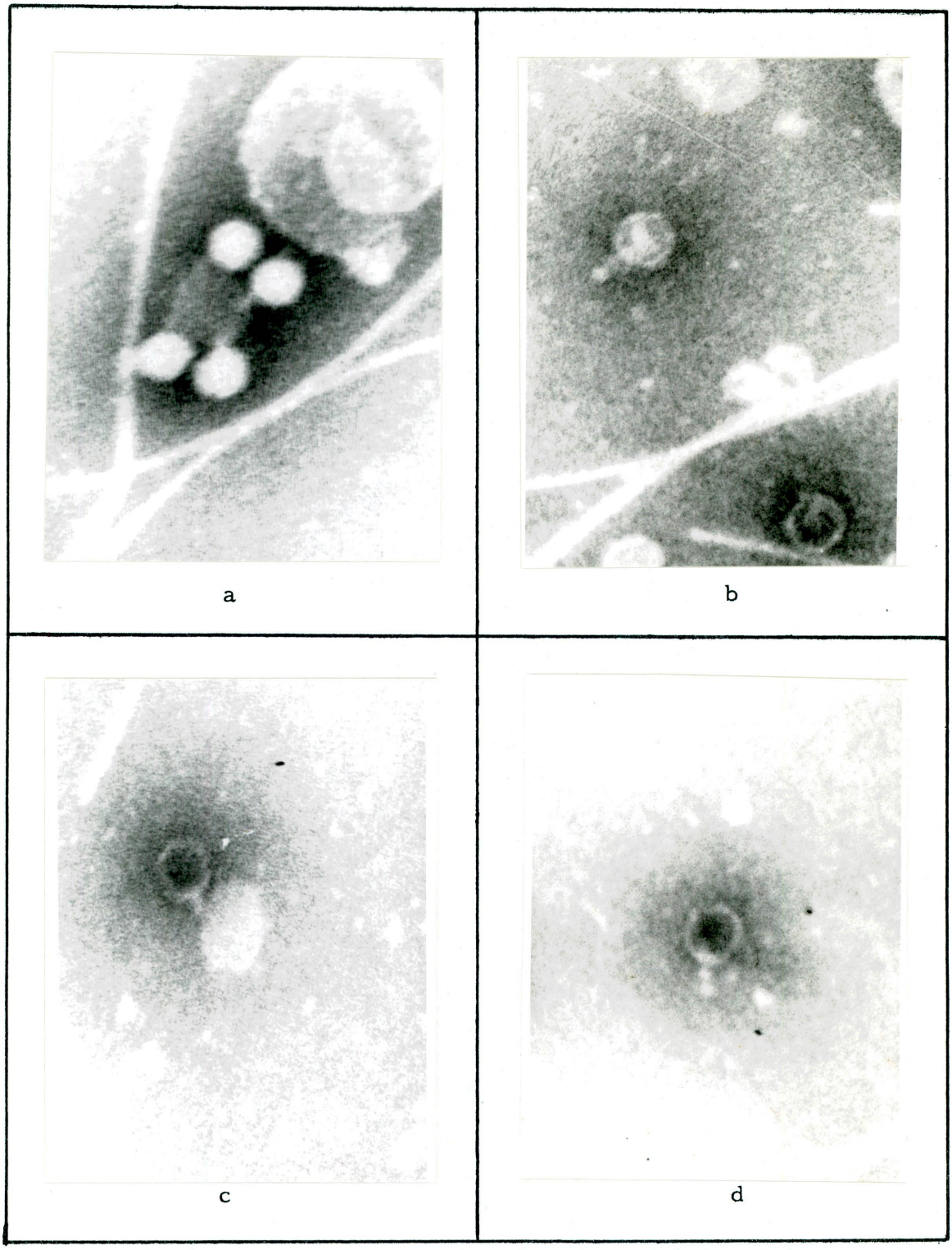

Figure 8. Electron Micrographs of Phage P4/2, Prepared by the Negative Stain Method With 4% Phosphotungstate and 0.4% Sucrose at pH 7. Magnification Approximately 175,000X.

Figure 9:

a. Two phage particles in shadow of latex particle. Definite hexagonal shape but tail not evident.

b. Shadowed phage particle showing some surface structure.

c. Diagrammatic representation of P4/2.

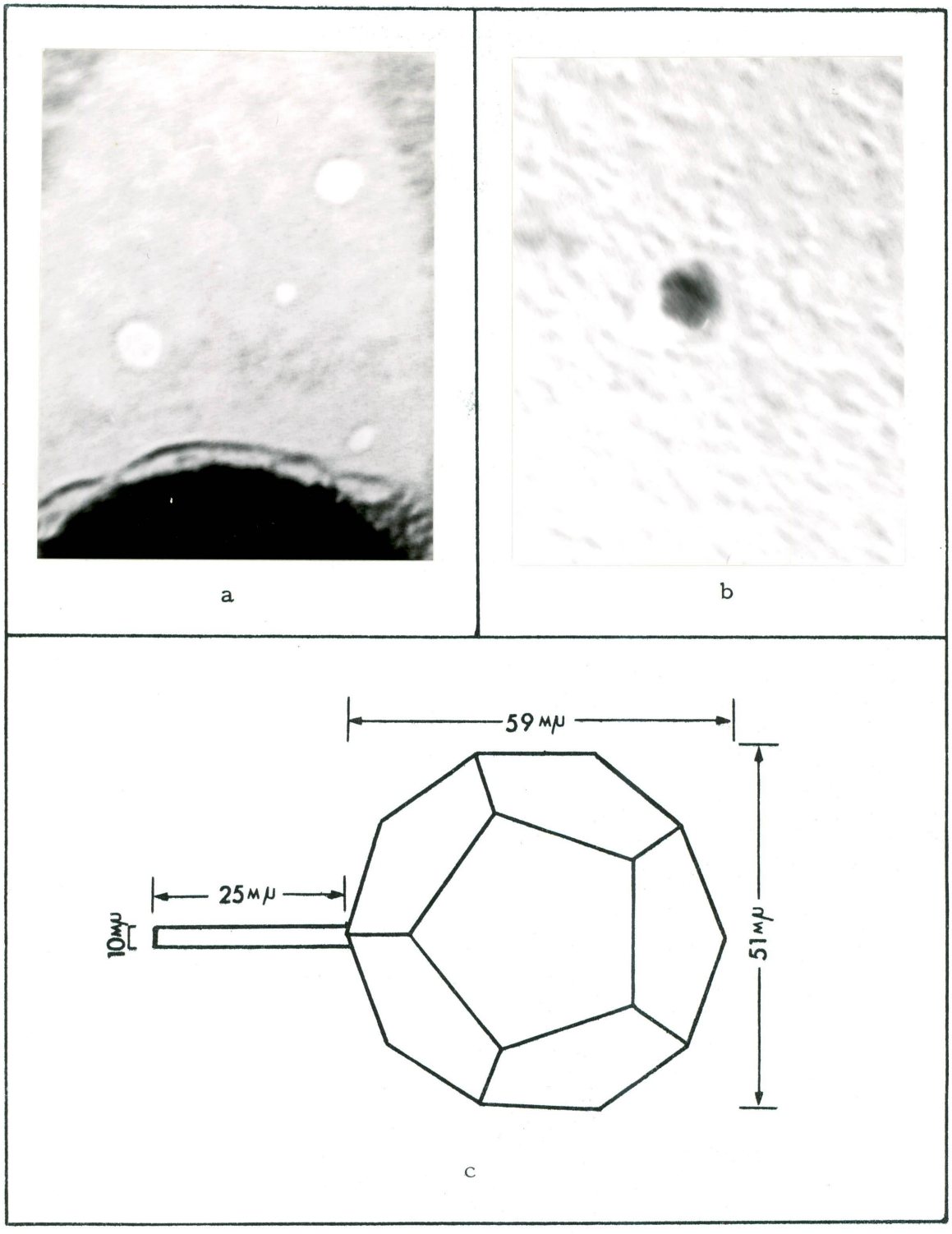

Figure 9. Electron Micrographs of Phage P4/2 Shadowed with Platinum and Palladium and a Diagrammatic Representation of Phage P4/2. Magnification Approximately 140,000X.

If Cu = sample concentration
Au = sample A 600
As = standard A 600
Ms = amount of DNA in standard
= 15.625 μg
Vu = sample volume
Cu = Ms/As x Au/Vu
= 15.625/0.10 x 1.07/1
= 166 μg/ml
phage titer = 5.2×10^{12}/ml
DNA/phage = $166/5.2 \times 10^{12}$ μg
= 3.2×10^{-11} μg
= 3.2×10^{-17} g

TABLE 12

DNA CONCENTRATION OF P4/2

	Absorbance reading from Beckman DU Spectrophotometer	Amount of DNA
Standard$_1$ 50λ	0.190	31.25 μgm/ml
Standard$_2$ 25λ	0.110	15.625 μgm/ml
Sample$_1$	1.05	3.2×10^{-11} μgm/phage
Sample$_2$	1.09	3.2×10^{-11} μgm/phage
Sample$_3$	1.08	3.2×10^{-11} μgm/phage
Sample$_4$	1.09	3.2×10^{-11} μgm/phage

3. The Acridine Orange Test

Since P4/2 is probably a DNA containing phage, it was desirable to carry out the acridine orange test which would differentiate between single stranded and double stranded DNA. The T2 preparation was used as a control since it is a known double stranded DNA phage. The

test showed that T2 appeared yellow, the appearance expected for double stranded DNA, but P4/2 appeared orange-red, the result expected for single stranded DNA or RNA. Since it was previously shown that phage P4/2 contained no RNA, the test strongly suggests that phage P4/2 contains DNA which is single stranded.

4. Phosphorus Determination

An analysis of the phosphorus content of phage P4/2 was attempted. The phage was resuspended in 0.1 M ammonium acetate. The results were calculated as follows:

Cu = sample concentration
Au = sample A 800
As = standard A 800
Ms = amount of phosphorus in standard
= 8 μg
Vu = sample volume
Cu = Ms/As x Au/Vu
= 8/0.572 x 0.404/0.5
= 11.3 μg/ml
phage titer = 3×10^{11}/ml
P/phage = $11.3/3.0 \times 10^{11}$
= 3.4×10^{-11} μg

This value is 100% of the DNA content and is 10 times greater than expected. The exact reasons for the result are unknown, although the known presence in the phage preparation of bacterial material undoubtedly contributed to the elevated value. A bacterial debris-free preparation should therefore be obtained in order to derive a concise value of the phosphorus content.

F. DNA Base Ratio Analysis of the Propagating Strain P2, the Lysogenic Strains P3, P4, P9, and Phage P4/2

1. The position of UV absorbing spots for hydrolyzates of both bacteria and phage was as shown in Figure 9. From published results preliminary identification of the spots was as indicated (Wyatt, 1951) (Dunn and Smith, 1957). Complete spectra of each base, eluted from one chromatogram are shown in Figure 10 and thus verify their identification. Peak absorption values were 249 mμ for guanine, 263 mμ for adenine, 265 mμ for thymine and 276 mμ for cytosine.

2. Average Rf values with the range for each of the bases from all extracts are shown in Table 13.

TABLE 13

COMPARISON OF Rf VALUES FROM KNOWN NUCLEIC ACID BASES WITH THOSE OF BACTERIAL AND VIRAL NUCLEIC ACIDS

	Solvent I			Solvent II		
	Rf value*	Rf value	Range	Rf value†	Rf value	Range
Guanine	0.25	0.21	0.18-0.30	0.43	0.40	0.34-0.46
Adenine	0.36	0.29	0.25-0.42	0.60	0.59	0.52-0.64
Cytosine	0.47	0.43	0.37-0.53	0.61	0.62	0.60-0.68
Thymine	0.77	0.73	0.60-0.85	0.70	0.73	0.66-0.80

*Wyatt, 1951
†Dunn and Smith, 1957

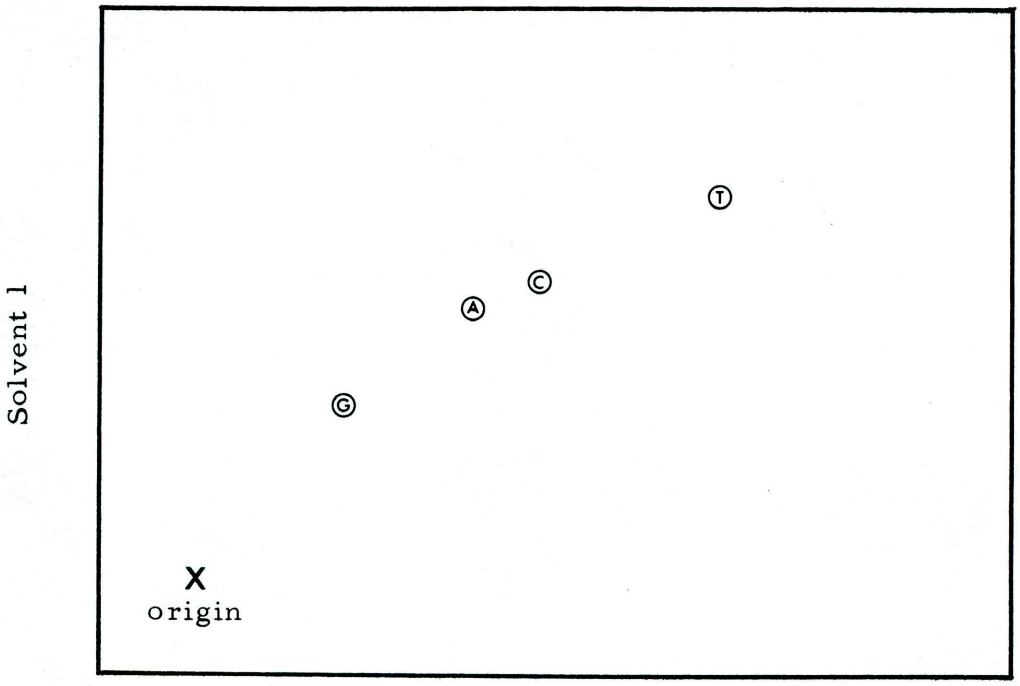

Figure 10. The Positions of Nucleic Acid Derivatives on Two-dimensional Chromatogram Run on Whatman No. 1 Paper by Descending Technique.

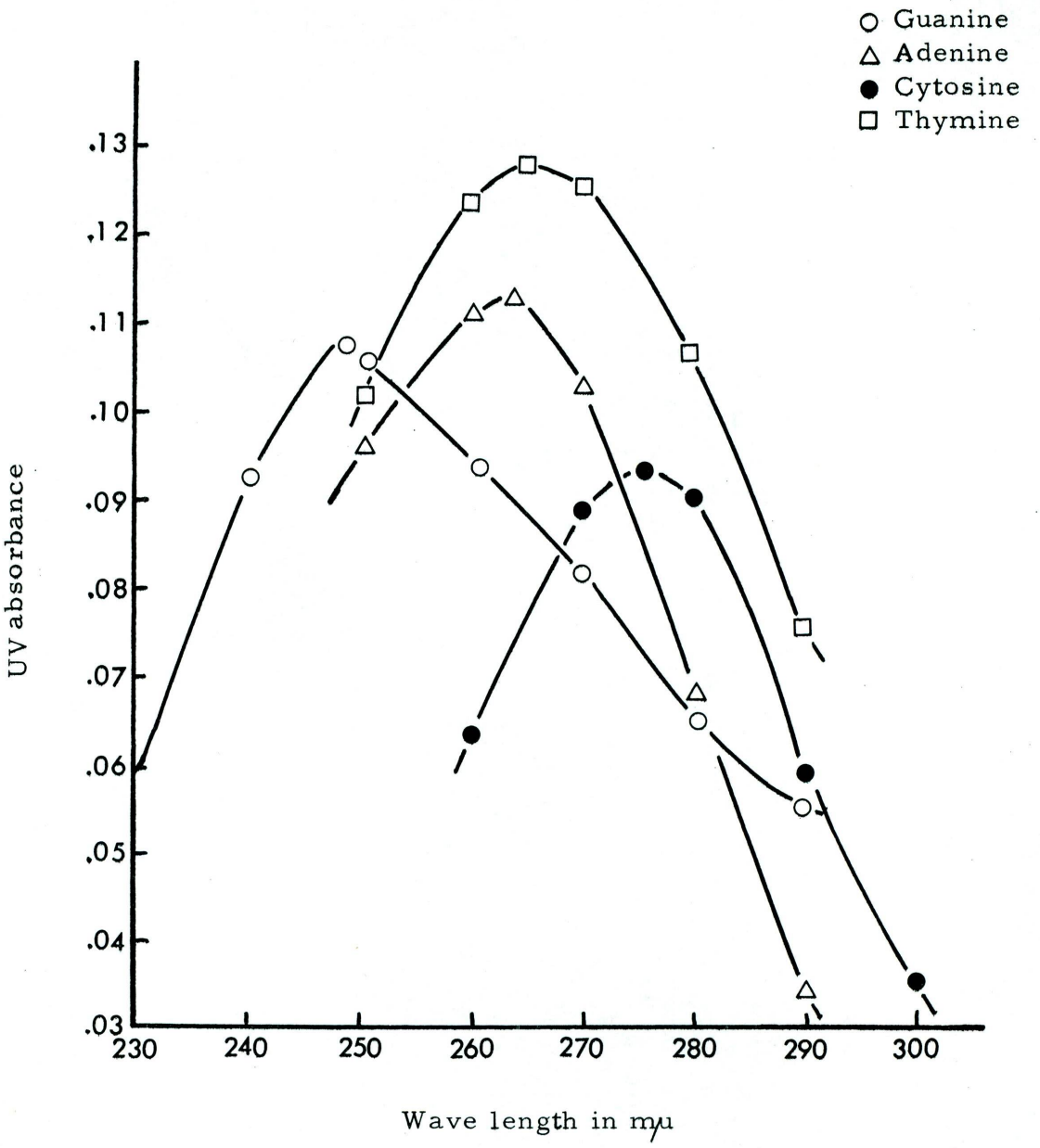

Figure 11. UV Absorbance Curves of Guanine, Adenine, Cytosine and Thymine in P2.

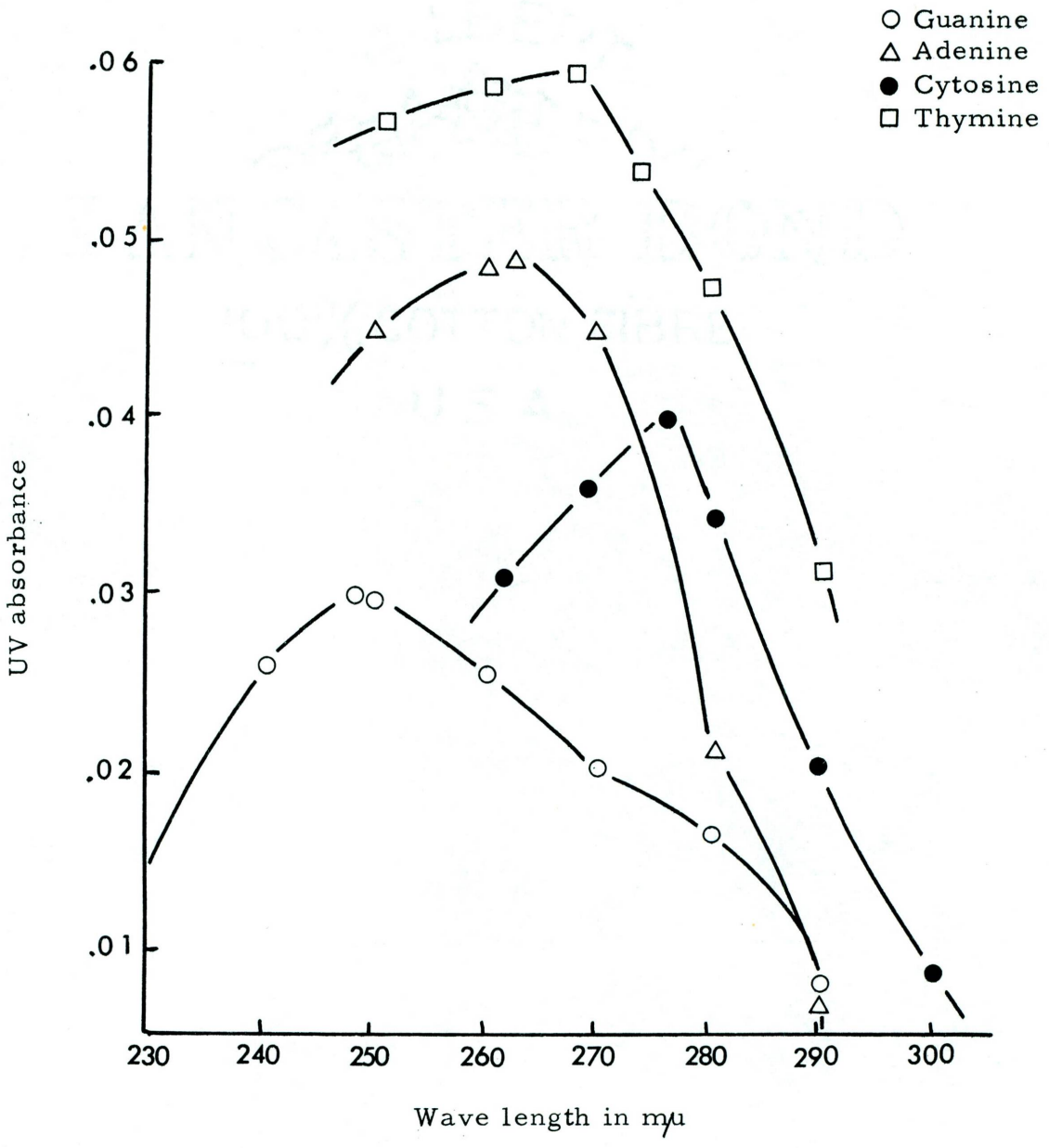

Figure 12. UV Absorbance Curves of Guanine, Adenine, Cytosine and Thymine in P4/2.

The spectrophotometric data of the eluted bases from the chromatograms of hydrolyzates of cells and phage are shown in Tables 14 to 23. Each table records the value obtained from four to six different chromatograms from each separated sample. Results with the two samples of hydrolyzate of P2 are shown in Tables 14 and 15, of samples P3 in Tables 16 and 17, of P4 in Tables 18 and 19, of P9 in Tables 20 and 21 and of phage P4/2 in Tables 22 and 23. Each of these tables includes: (1) the UV absorbance reading at the peak and at 290 mμ for guanine, adenine, thymine and at 300 mμ for cytosine (2) the calculated amounts of each base, (3) the percentage (moles per 100 moles or M/100M) of each base relative to the total bases; (4) the ratios adenine/thymine, guanine/cytosine and adenine + thymine/guanine + cytosine.

The average percentage of each base in each sample, the various ratios and the amount of each base relative to adenine of P2, P3, P4, P9 and phage P4/2 are shown in Table 24.

In Table 25 are presented the total average molar ratios of each base with standard deviation, the total average ratio and average amounts of each base relative to adenine of P2, P3, P4, P9 and phage P4/2.

Example of calculations:

Data: E_{max} Guanine = 0.117
E_{290} Guanine = 0.037

$$\Delta_x = 0.117 - 0.037$$

$$= 0.080$$

$$\gamma \text{ in } 5 \text{ ml} = 5 \times 10 \times \Delta_x / \Delta_s$$

$$= 5 \times 10 \times 0.08/0.475$$

$$= 0.842$$

$$\text{mM} = \gamma \text{ /MW Guanine}$$

$$= 0.842/151$$

$$= 0.0557 \text{ mM}$$

The results obtained from each of the two samples for the bacterial cultures and of phage P4/2 are very similar (Table 24).

In Table 25, the molar ratios of the bases for each of the bacteria including the propagating, non-lysogenic strain P2 and the lysogenic strain P3, P4, and P9, do not significantly vary. The molar ratios of adenine to thymine and of guanine to cytosine are also similar. For P2, the average A/T and G/C ratios are 0.87 and 0.97 respectively, for P3, 0.90 and 0.95, for P4 0.86 and 0.91 and for P9, 0.93 and 0.91. The average A+T/G+C ratios for P2 is 0.92, for P3, 0.97, for P4, 0.96 and for P9, 0.92.

Although the molar ratios of the bases adenine-thymine and guanine-cytosine are not exactly complementary, the difference probably only reflects experimental deviation since the nucleic acid in bacterial cells is well known to be double stranded.

TABLE 14

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P2 (SAMPLE 1)

Bases	λ_{max}	$\frac{290}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.117	0.037	8.42	0.0558	25.6	0.876	0.988	0.962	1.13
Adenine	0.117	-0.003	6.67	0.0494	22.7				1.00
Cytosine	0.117	0.009	6.26	0.0564	25.9				1.14
Thymine	0.123	0.045	7.10	0.0564	25.9				1.14
Guanine	0.111	0.034	8.11	0.0537	25.3	0.892	0.962	0.949	1.11
Adenine	0.116	-0.002	6.56	0.0486	22.9				1.00
Cytosine	0.115	0.009	6.14	0.0553	26.1				1.13
Thymine	0.120	0.044	6.92	0.0549	25.9				1.13
Guanine	0.113	0.035	8.21	0.0544	26.0	0.810	0.977	0.901	1.22
Adenine	0.114	0.006	6.00	0.0444	21.2				1.00
Cytosine	0.106	-0.001	6.29	0.0558	26.6				1.25
Thymine	0.120	0.044	6.92	0.0549	26.2				1.24
Guanine	0.125	0.044	8.53	0.0565	25.8	0.890	0.984	0.923	1.14
Adenine	0.124	0.004	6.67	0.0494	22.6				1.00
Cytosine	0.125	0.015	6.37	0.0574	26.2				1.15
Thymine	0.120	0.043	7.01	0.0557	25.4				1.12
Guanine	0.115	0.033	8.63	0.0572	25.9	0.905	0.989	0.921	1.14
Adenine	0.120	-0.002	6.78	0.0502	22.7				1.00
Cytosine	0.110	-0.001	6.43	0.0579	26.2				1.15
Thymine	0.120	0.043	7.01	0.0557	25.2				1.11
Guanine	0.120	0.040	8.42	0.0558	25.6	0.864	0.973	0.926	1.15
Adenine	0.117	-0.001	6.55	0.0486	22.3				1.00
Cytosine	0.124	0.014	6.37	0.0574	26.3				1.18
Thymine	0.125	0.047	7.10	0.0564	25.8				1.15

TABLE 15

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P2 (SAMPLE 2)

Bases	λ_{max}	$\frac{\lambda_{290}}{\lambda_{max}}$ (300)	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.267	0.083	19.36	0.128	25.8	0.908	0.977	0.913	1.14
Adenine	0.284	0.009	15.28	0.113	22.7				1.00
Cytosine	0.280	0.027	14.65	0.132	26.4				1.16
Thymine	0.265	0.093	15.66	0.124	25.0				1.10
Guanine	0.257	0.071	19.57	0.130	25.4	0.875	0.947	0.915	1.14
Adenine	0.277	0.000	15.38	0.114	22.3				1.00
Cytosine	0.266	0.004	15.18	0.137	26.8				1.20
Thymine	0.260	0.080	16.39	0.130	25.5				1.14
Guanine	0.278	0.092	19.58	0.130	25.5	0.864	0.958	0.919	1.15
Adenine	0.280	0.005	15.29	0.113	22.2				1.00
Cytosine	0.275	0.015	15.06	0.136	26.6				1.20
Thymine	0.257	0.076	16.48	0.131	25.7				1.14
Guanine	0.275	9.090	19.47	0.129	25.4	0.868	0.954	0.919	1.14
Adenine	0.274	0.000	15.27	0.113	22.2				1.00
Cytosine	0.279	0.020	15.00	0.135	26.6				1.20
Thymine	0.272	0.092	16.39	0.130	25.6				1.15
Guanine	0.285	0.099	19.58	0.130	25.6	0.864	0.962	0.918	1.15
Adenine	0.281	0.007	15.23	0.113	22.2				1.00
Cytosine	0.282	0.024	14.94	0.135	26.6				1.20
Thymine	0.300	0.120	16.39	0.130	25.6				1.15
Guanine	0.280	0.094	19.58	0.130	25.7	0.853	0.973	0.902	1.17
Adenine	0.275	0.004	15.07	0.112	22.0				1.00
Cytosine	0.273	0.017	14.83	0.134	26.4				1.20
Thymine	0.265	0.085	16.39	0.130	25.8				1.17

TABLE 16

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P3 (SAMPLE 1)

Bases	λ_{max}	λ_{290} (300)	Y	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.247	0.089	16.63	0.110	23.7	0.900	0.888	0.956	1.01
Adenine	0.281	0.017	14.68	0.109	23.5				1.00
Cytosine	0.254	0.016	13.79	0.124	26.7				1.14
Thymine	0.239	0.072	15.21	0.121	26.1				1.11
Guanine	0.238	0.073	17.36	0.115	24.7	0.937	0.946	0.968	1.04
Adenine	0.267	0.000	14.87	0.110	23.8				1.00
Cytosine	0.242	0.010	13.44	0.121	26.1				1.10
Thymine	0.226	0.063	14.84	0.118	25.4				1.07
Guanine	0.235	0.074	16.94	0.112	24.2	0.900	0.927	0.986	1.03
Adenine	0.268	0.003	14.72	0.109	23.5				1.00
Cytosine	0.239	0.007	13.44	0.121	26.1				1.11
Thymine	0.235	0.068	15.21	0.121	26.1				1.11
Guanine	0.240	0.081	16.74	0.111	24.1	0.933	0.903	0.967	1.02
Adenine	0.270	0.006	14.67	0.109	23.7				1.00
Cytosine	0.240	0.005	13.61	0.123	26.7				1.13
Thymine	0.224	0.062	14.75	0.117	25.4				1.07
Guanine	0.244	0.081	17.15	0.114	24.3	0.889	0.920	0.970	1.05
Adenine	0.265	0.000	14.72	0.109	23.2				1.00
Cytosine	0.245	0.008	13.73	0.124	26.4				1.14
Thymine	0.231	0.062	15.39	0.122	26.0				1.12
Guanine	0.240	0.072	17.69	0.117	24.7	0.858	0.969	0.992	1.15
Adenine	0.270	0.005	14.72	0.109	23.0				1.00
Cytosine	0.239	0.007	13.34	0.121	25.5				1.11
Thymine	0.234	0.058	16.03	0.127	26.8				1.17

TABLE 17

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P3 (SAMPLE 2)

Bases	λ_{max}	$\frac{\lambda_{290}}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.254	0.088	17.47	0.116	24.7	0.895	0.935	0.957	1.07
Adenine	0.274	0.012	14.57	0.108	23.1				1.00
Cytosine	0.259	0.021	13.78	0.124	26.4				1.14
Thymine	0.278	0.110	15.30	0.121	25.8				1.23
Guanine	0.237	0.071	17.47	0.116	24.7	0.936	0.921	0.939	1.06
Adenine	0.257	0.010	14.85	0.110	23.4				1.00
Cytosine	0.244	0.004	13.90	0.125	26.8				1.15
Thymine	0.230	0.068	14.75	0.117	25.0				1.07
Guanine	0.243	0.079	17.26	0.114	24.7	0.875	0.918	0.938	1.09
Adenine	0.257	0.005	14.00	0.104	22.6				1.00
Cytosine	0.244	0.007	13.73	0.124	26.9				1.19
Thymine	0.239	0.075	14.93	0.119	25.8				1.14
Guanine	0.250	0.084	17.47	0.116	24.8	0.914	0.946	0.960	1.06
Adenine	0.271	0.007	14.67	0.109	23.4				1.00
Cytosine	0.251	0.017	13.55	0.122	26.2				1.12
Thymine	0.236	0.071	15.02	0.119	25.6				1.09
Guanine	0.248	0.080	17.69	0.117	24.4	0.845	0.957	0.998	1.07
Adenine	0.270	0.002	14.89	0.110	22.9				1.00
Cytosine	0.246	0.012	13.55	0.122	25.5				1.11
Thymine	0.256	0.076	16.40	0.130	27.1				1.18
Guanine	0.262	0.090	18.11	0.120	26.8	0.902	1.180	0.982	1.12
Adenine	0.265	0.004	14.51	0.107	23.9				1.00
Cytosine	0.252	0.056	11.36	0.102	22.7				0.95
Thymine	0.244	0.080	14.93	0.119	26.5				1.11

TABLE 18

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P4 (SAMPLE 1)

Bases	λ_{max}	$\frac{\lambda_{290}}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+G}$	Amount of base rel. to adenine
Guanine	0.320	0.114	21.68	0.144	24.5	0.858	0.901	0.945	1.10
Adenine	0.320	0.002	17.66	0.131	22.3				1.00
Cytosine	0.338	0.032	17.73	0.160	27.2				1.22
Thymine	0.287	0.075	19.32	0.153	26.0				1.17
Guanine	0.305	0.092	22.42	0.148	25.0	0.850	0.929	0.927	1.13
Adenine	0.315	-0.004	17.72	0.131	22.1				1.00
Cytosine	0.340	0.035	17.67	0.159	26.9				1.22
Thymine	0.285	0.072	19.41	0.154	26.0				1.25
Guanine	0.310	0.101	22.00	0.146	24.8	0.857	0.918	0.929	1.12
Adenine	0.325	0.007	17.66	0.131	22.2				1.00
Cytosine	0.341	0.036	17.67	0.159	27.0				1.22
Thymine	0.271	0.060	19.22	0.153	25.9				1.17
Guanine	0.292	0.091	22.16	0.140	24.2	0.837	0.881	0.934	1.10
Adenine	0.293	-0.016	17.17	0.127	22.0				1.00
Cytosine	0.329	0.024	17.67	0.159	27.5				1.25
Thymine	0.260	0.050	19.12	0.152	26.3				1.20
Guanine	0.305	0.098	21.80	0.144	24.5	0.875	0.904	0.938	1.08
Adenine	0.329	0.005	18.00	0.133	22.6				1.00
Cytosine	0.342	0.035	17.79	0.160	27.1				1.20
Thymine	0.265	0.055	19.12	0.152	25.8				1.14
Guanine	0.298	0.090	21.90	0.145	24.2	0.884	0.900	0.942	1.06
Adenine	0.339	0.007	18.44	0.137	22.8				1.00
Cytosine	0.345	0.032	18.13	0.163	27.2				1.19
Thymine	0.307	0.093	19.49	0.155	25.8				1.13

TABLE 19

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P4 (SAMPLE 2)

Bases	λ_{max}	$\frac{\lambda_{290}}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{G+C}$	Amount of base rel. to adenine
Guanine	0.271	0.081	20.00	0.132	24.1	0.839	0.906	0.972	1.07
Adenine	0.299	-0.001	16.66	0.123	22.5				1.00
Cytosine	0.298	0.018	16.22	0.146	26.6				1.18
Thymine	0.290	0.087	18.52	0.147	26.8				1.19
Guanine	0.274	0.091	19.26	0.128	24.0	0.866	0.880	0.971	1.06
Adenine	0.280	-0.011	16.18	0.120	22.6				1.00
Cytosine	0.270	-0.002	15.75	0.142	26.7				1.18
Thymine	0.265	0.070	17.75	0.141	26.6				1.16
Guanine	0.281	0.095	19.57	0.130	24.0	0.872	0.905	0982	1.04
Adenine	0.314	0.015	16.61	0.123	23.1				1.00
Cytosine	0.294	0.020	15.87	0.143	26.5				1.15
Thymine	0.286	0.088	18.03	0.143	26.5				1.15
Guanine	0.285	0.094	20.10	0.133	24.4	0.845	0.917	0.958	1.09
Adenine	0.300	0.003	16.50	0.122	22.4				1.00
Cytosine	0.295	0.016	16.16	0.146	26.6				1.19
Thymine	0.285	0.085	18.21	0.145	26.5				1.18
Guanine	0.275	0.090	19.47	0.129	24.1	0.850	0.913	0.982	1.07
Adenine	0.280	-0.015	16.38	0.121	22.6				1.00
Cytosine	0.262	-0.008	15.64	0.141	26.4				1.17
Thymine	0.274	0.075	18.12	0.144	26.9				1.19
Guanine	0.280	0.088	20.21	0.134	24.4	0.839	0.921	0.966	1.09
Adenine	0.300	0.000	16.67	0.123	22.4				1.00
Cytosine	0.290	0.010	16.22	0.146	26.5				1.18
Thymine	0.297	0.094	18.49	0.147	26.7				1.19

TABLE 20

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P9 (SAMPLE 1)

Bases	λ_{max}	$\frac{\lambda_{290}}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{G+C}$	Amount of base rel. to adenine
Guanine	0.360	0.117	25.57	0.169	25.0	0.900	0.919	0.916	1.11
Adenine	0.372	0.000	20.66	0.153	22.6				1.00
Cytosine	0.372	0.021	20.33	0.183	27.2				1.20
Thymine	0.305	0.070	21.40	0.170	25.2				1.12
Guanine	0.350	0.114	24.84	0.165	24.5	0.887	0.900	0.934	1.08
Adenine	0.378	0.005	20.72	0.153	22.7				1.00
Cytosine	0.370	0.019	20.32	0.183	27.2				1.20
Thymine	0.309	0.071	21.68	0.172	25.6				1.13
Guanine	0.361	0.116	25.79	0.171	25.3	0.904	0.940	0.933	1.11
Adenine	0.381	0.008	20.72	0.153	22.7				1.00
Cytosine	0.367	0.018	20.22	0.182	26.9				1.19
Thymine	0.297	0.062	21.40	0.170	25.1				1.11
Guanine	0.364	0.119	25.79	0.171	25.2	0.915	0.920	0.903	1.11
Adenine	0.376	0.002	20.77	0.154	22.4				1.00
Cytosine	0.375	0.018	20.68	0.186	27.4				1.21
Thymine	0.306	0.073	21.22	0.168	24.8				1.09
Guanine	0.356	0.116	25.26	0.167	24.8	0.905	0.918	0.930	1.08
Adenine	0.388	0.012	20.88	0.155	22.9				1.00
Cytosine	0.371	0.021	20.27	0.183	27.0				1.18
Thymine	0.309	0.074	21.41	0.170	25.3				1.11
Guanine	0.356	0.117	25.15	0.167	25.2	0.916	0.936	0.937	1.10
Adenine	0.377	0.008	20.50	0.152	22.9				1.00
Cytosine	0.351	0.010	19.75	0.178	26.9				1.17
Thymine	0.311	0.082	20.86	0.166	25.0				1.09

TABLE 21

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF P9 (SAMPLE 2)

Bases	λ_{max}	$\frac{\lambda_{290}}{(300)}$	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.337	0.112	23.68	0.157	25.0	0.936	0.932	0.930	1.08
Adenine	0.366	0.011	19.72	0.146	23.3				1.00
Cytosine	0.340	0.018	18.65	0.168	26.8				1.15
Thymine	0.300	0.084	19.68	0.156	24.9				1.07
Guanine	0.314	0.104	22.11	0.146	24.2	0.971	0.864	0.913	1.03
Adenine	0.366	0.019	19.27	0.143	23.5				1.00
Cytosine	0.341	0.017	18.77	0.169	28.0				1.19
Thymine	0.281	0.078	18.49	0.147	24.2				1.03
Guanine	0.317	0.104	22.42	0.148	24.5	0.947	0.866	0.896	1.07
Adenine	0.346	0.007	18.83	0.139	23.0				1.00
Cytosine	0.340	0.012	19.00	0.171	28.3				1.23
Thymine	0.277	0.074	18.49	0.147	24.3				1.06
Guanine	0.315	0.100	22.64	0.150	25.4	0.967	0.927	0.894	1.09
Adenine	0.340	0.007	18.50	0.137	23.2				1.00
Cytosine	0.320	0.010	17.96	0.162	27.4				1.18
Thymine	0.279	0.084	17.76	0.141	24.0				1.04
Guanine	0.316	0.101	22.64	0.150	24.6	0.899	0.921	0.951	1.06
Adenine	0.344	0.001	19.06	0.141	23.1				1.00
Cytosine	0.324	0.011	18.13	0.163	26.7				1.16
Thymine	0.294	0.077	19.76	0.157	25.7				1.13
Guanine	0.329	0.112	22.84	0.154	24.8	0.979	0.895	0.904	1.06
Adenine	0.345	0.012	18.50	0.137	23.5				1.00
Cytosine	0.327	0.017	18.02	0.162	27.7				1.18
Thymine	0.271	0.077	17.66	0.140	24.0				1.02

TABLE 22

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF PHAGE P4/2 (SAMPLE 1).

Bases	λ_{max}	λ_{290} (300)	γ	mM	$\frac{10}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.250	0.087	17.15	0.114	26.1	0.70	1.19	1.08	1.22
Adenine	0.266	0.041	12.50	0.093	21.4				1.00
Cytosine	0.225	0.043	10.54	0.095	21.9				1.03
Thymine	0.276	0.092	16.76	0.133	30.6				1.43
Guanine	0.255	0.095	16.84	0.112	25.9	0.71	1.14	1.07	1.21
Adenine	0.250	0.025	12.50	0.093	21.5				1.00
Cytosine	0.213	0.027	10.77	0.097	22.5				1.05
Thymine	0.255	0.075	16.39	0.130	30.2				1.41
Guanine	0.242	0.076	17.47	0.116	26.7	0.70	1.21	1.05	1.27
Adenine	0.250	0.029	12.28	0.091	21.1				1.00
Cytosine	0.205	0.023	10.54	0.095	21.9				1.04
Thymine	0.260	0.080	16.39	0.130	30.1				1.43
Guanine	0.216	0.075	14.84	0.098	26.0	0.72	1.33	1.19	1.14
Adenine	0.235	0.027	11.56	0.086	22.8				1.00
Cytosine	0.158	-0.003	9.32	0.074	19.6				0.86
Thymine	0.265	0.100	15.03	0.119	31.6				1.39

TABLE 23

SPECTROPHOTOMETRIC ANALYSIS OF BASES OF HYDROLYZATE OF PHAGE P4/2 (SAMPLE 2)

Bases	λ_{max}	λ_{290} (300)	γ	mM	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{C+T}$	Amount of base rel. to adenine
Guanine	0.052	0.019	3.48	.0230	25.1	0.64	1.16	1.15	1.19
Adenine	0.045	-0.002	2.61	.0193	21.1				1.00
Cytosine	0.042	0.004	2.20	.0198	21.6				1.02
Thymine	0.107	0.066	3.74	.0296	32.8				1.55
Guanine	0.034	0.005	3.05	.0202	25.6	0.67	1.14	1.08	1.23
Adenine	0.039	-0.001	2.22	.0164	20.8				1.00
Cytosine	0.039	0.005	1.97	.0177	22.4				1.08
Thymine	0.099	0.065	3.10	.0246	31.2				1.50
Guanine	0.037	0.007	3.16	.0209	25.6	0.72	1.14	1.08	1.18
Adenine	0.048	0.005	2.39	.0177	21.7				1.00
Cytosine	0.040	0.005	2.03	.0183	22.4				1.03
Thymine	0.097	0.063	3.10	.0246	30.2				1.39
Guanine	0.037	0.007	3.16	.0209	25.6	0.70	1.19	1.11	1.19
Adenine	0.037	-0.006	2.39	.0177	21.6				1.00
Cytosine	0.037	0.003	1.97	.0177	21.6				1.00
Thymine	0.097	0.062	3.19	.0253	31.0				1.44
Guanine	0.037	0.007	3.16	.0209	26.1	0.76	1.21	1.10	1.15
Adenine	0.037	-0.007	2.44	.0181	22.6				1.00
Cytosine	0.039	0.006	1.91	.0172	21.5				0.95
Thymine	0.099	0.006	3.01	.0239	29.8				1.32
Guanine	0.040	0.014	2.74	.0181	24.9	0.73	1.15	1.15	1.10
Adenine	0.029	-0.011	2.22	.0164	22.6				1.00
Cytosine	0.028	-0.002	1.74	.0157	21.6				0.96
Thymine	0.091	0.060	2.82	.0224	30.8				1.37

TABLE 24

AVERAGE BASE RATIO OF THE TWO SAMPLES OF HYDROLYZATES OF P2, P3, P4, P9 and P4/2

Hydrolyzate	Base	$\frac{M}{100M}$	A/T	G/C	$\frac{A+T}{G+C}$	Amount of base rel. to adenine
Sample 1	Guanine	25.7	0.873	0.979	0.930	1.15
	Adenine	22.4				1.00
P2	Cytosine	26.2				1.17
	Thymine	25.7				1.15
Sample 2	Guanine	25.6	0.872	0.961	0.914	1.15
	Adenine	22.2				1.00
	Cytosine	26.6				1.19
	Thymine	25.5				1.15
Sample 1	Guanine	24.3	0.903	0.926	0.973	1.10
	Adenine	23.5				1.00
P3	Cytosine	26.3				1.12
	Thymine	26.0				1.11
Sample 2	Guanine	25.0	0.895	0.976	0.962	1.08
	Adenine	23.2				1.00
	Cytosine	25.8				1.11
	Thymine	26.0				1.14
Sample 1	Guanine	24.5	0.860	0.906	0.938	1.10
	Adenine	22.3				1.00
P4	Cytosine	27.2				1.22
	Thymine	26.0				1.18
Sample 2	Guanine	24.2	0.852	0.907	0.972	1.07
	Adenine	22.6				1.00
	Cytosine	26.6				1.18
	Thymine	26.7				1.18

TABLE 24 (continued)

Sample 1	Guanine	25.0	0.905	0.922	0.926	1.10
	Adenine	22.7				1.00
P9	Cytosine	27.1				1.19
	Thymine	25.2				1.11
Sample 2	Guanine	24.8	0.949	0.901	0.915	1.07
	Adenine	23.3				1.00
	Cytosine	27.4				1.18
	Thymine	24.5				1.06
Sample 1	Guanine	26.2	0.71	1.21	1.10	1.21
	Adenine	21.7				1.00
P4/2	Cytosine	21.5				1.00
	Thymine	30.6				1.42
Sample 2	Guanine	25.5	0.70	1.17	1.11	1.17
	Adenine	21.7				1.00
	Cytosine	21.9				1.00
	Thymine	31.0				1.43

TABLE 25

AVERAGE BASE RATIO ANALYSES OF NUCLEIC ACID, DERIVED FROM P2, P3, P4, P9 and P4/2, WITH STANDARD DEVIATION

	Molar ratios							Amount of base relative to adenine			
	Adenine	Guanine	Thymine	Cytosine	A/T	G/C	$\frac{A+T}{G+C}$	A	G	T	C
P2	22.3±.10	25.6±.01	25.6±.08	26.4±.09	0.87	0.97	0.92	1.00	1.15	1.15	1.18
P3	23.4±.06	24.6±.06	26.0±.18	26.1±.30	0.90	0.95	0.97	1.00	1.09	1.13	1.12
P4	22.5±.09	24.4±.09	26.4±.10	26.9±.10	0.86	0.91	0.96	1.00	1.09	1.18	1.20
P9	23.0±.10	24.9±.10	24.9±1.0	27.3±.10	0.93	0.91	0.92	1.00	1.09	1.09	1.19
P4/2	21.7±.28	25.8±.14	30.9±.40	21.8±.30	0.71	1.21	1.11	1.00	1.19	1.43	1.00

The corresponding values obtained for phage P4/2 do, however, differ significantly from those obtained from the bacterial cultures. There was no evidence of complementariness between the bases adenine and thymine, and guanine and cytosine. The A/T ratio was 0.71, the G/C ratio was 1.21, and the A+T/G+C ratio was 1.11. There was relatively much greater guanine and thymine relative to adenine than in DNA of the bacterial cultures. The results with phage P4/2 support the earlier results which suggest that the nucleic acid in the phage occurs as a single strand.

In order to investigate the significance in the different values in molar base ratios obtained with phage P4/2 and the bacterial cells, a Chi square test was used. There are approximately equal molar amounts of each of the four bases in the DNA from the cells. This is in agreement with published results for other Salmonella types (Spirin, 1960). Thus, the Chi square value was calculated from the results in Table 25 for both cells and phage on the hypothesis that each base contributed 25% of the total base content. The values of Chi square and the probability of agreement with the hypothesis are shown in Table 26. The probability of agreement for each of the cell preparation is very great -- between 90% and 99%, whereas the probability of agreement with the hydrolyzate of the phage DNA is much lower -- between 50% and 75%. The base ratios of phage DNA are, therefore, significantly different from the base ratios of the bacterial DNA.

TABLE 26

CHI SQUARE VALUES AND PROBABILITY THAT MOLAR RATIOS OF EACH OF THE FOUR BASES IN THE DNA OF P2, P3, P4, P9 AND PHAGE P4/2 ARE EQUIVALENT

	Chi square values	P
P2	0.40	$< 0.95 > 0.90$
P3	0.20	$< 0.99 > 0.975$
P4	0.48	$< 0.95 > 0.90$
P9	0.37	$< 0.95 > 0.90$
P4/2	2.26	$< 0.75 > 0.50$

DISCUSSION

The main purpose of this paper was to compare some physical and chemical properties of the temperate phages isolated from lysogenic strains of Salmonella potsdam; and to relate the nucleic acid composition of the phages to the nucleic acid of the lysogenic strains from which they were derived and to the nucleic acid of the propagating strain on which they were grown.

The choice of P3/2, P4/2 and P9a/2 was based on a grouping for which temperature resistance was the main criterion of division. Phage P4/2 belongs to Group **A**, being resistant (in broth) to temperatures up to 80^{o}C and P3/2 and P9a/2 are in Group B, sensitive to lower temperatures.

By the host range and serological tests, these three phages were shown to be identical with those which were originally isolated by Bullas (1957); but by the heat sensitivity test, both P4/2 and P9a/2 showed resistance to lower temperatures. This result was, however, most probably due to the fact that the phages were suspended in a buffer solution, whereas the original phages had been tested in broth. P3/2 in buffer solution was more heat resistant than in broth, while phages P4/2 and P9a/2 were slightly less heat resistant in buffer than in broth. Environmental conditions play an important part in phage stability. Lark and Adams (1953) showed that phages in broth were much more stable at elevated temperatures than in chemically defined

media. The unusual finding that phage P3/2 was more stable in buffer than in broth warrants further investigation.

The method used to propagate the phages was satisfactory, although many difficulties were encountered in their purification. Enzymatic treatment (with DNase and RNase) and low speed centrifugation proved to be satisfactory for eliminating the extracellular DNA and RNA and the majority of bacterial cells without a lowering of the viable titer, for each phage. High speed centrifugation at 41700g was suitable for P4/2 but too high for P3/2 and P9a/2. By electron microscopic observation it was seen that many phage particles of these latter phages had lost their DNA content and thus appeared as phage "ghost" cells. Bacterial impurities such as flagella pieces and cup-shaped cell wall fragments were also present in each phage preparation. The flagella pieces were probably of similar weight to the phage particles themselves and thus accompanied the phage in the centrifugation. Many of the cup-shaped cell wall pieces were still attached to the bacteriophage particles and thus probably represent phage receptor sites. Loss of viability as a result of purification procedures has been encountered with many other phages (Lunan and Sinsheimer, 1956). It would be necessary to modify the procedure for purification of phages P3/2 and P9a/2. Differential centrifugation in a cesium chloride gradient would probably be satisfactory but would yield only small amounts of phages.

The impurities, mostly protein, in the P4/2 preparation did not hinder the analysis of the nucleic acid. But for P3/2 and P9a/2, the low titers made analysis of the nucleic acid impractical.

The kinetics of heat inactivation experiments demonstrated that the rate of inactivation at selected temperatures was characteristic for each phage. Thus heat resistance was a valid criterion for classifying different phages.

The Arrhenius constants for each phage were of the order of magnitude of those determined for heat denaturation of protein and for heat inactivation of enzymes and bacterial toxin (Adams, 1949). From Table 9, P4/2 has the highest Arrhenius constant μ of 84,630, while P9a/2 has the lowest of 56,334 and P3/2 has an intermediate value of 73,280.

At 60°C and by reference to Figure 6, it can be seen that the rate of inactivation in buffer of P3/2 is about four times the inactivation of P4/2 at the sample temperature and that the rate of inactivation of P9a/2 is about 30 times that of P4/2.

Krueger (1931-1932) concluded in his experiments on antistaphylococcus bacteriophage that the high value of μ indicated that the carrier particles possessed a high temperature co-efficient for heat denaturation, and that the change produced in them resulted in inactivation of attached "lytic substance."

Lark and Adams (1953) confirmed that a high Arrhenius constant was associated with the denaturation of vital protein in the phage

particles. Furthermore, it was possible that heat induced changes in nucleic acids might be analogous to protein denaturation and involve the breaking of hydrogen bond linkages and result in a loss of specific configuration. Adams also demonstrated that heating phage T5 resulted in loss of nucleic acid from the phage. The difference in Arrhenius constants for each of the S. potsdam phages might indicate that different proteins necessary for attachment to the cell had been denatured or rendered non-functional. Heat might also cause loss of the nucleic acid with resultant loss in activity of the phage particles.

The UV light inactivation experiments showed that P9a/2 was most resistant while P4/2 was most sensitive and P3/2 had an intermediate sensitivity. The kinetics of inactivation were of the first order with no detectable slight initial lag as detected by Adams (1952) for some E. coli viruses.

Phage T7 with a diameter of 47 mμ was found to contain 6.25×10^{-17} g DNA/phage particle (Williams and Fraser, 1953). Phage P4/2 with a diameter of about 59 mμ was estimated to contain 3.2×10^{-17} g DNA/phage particle. Thus phage P4/2 contains about half the DNA content of phage T7. Mayor (1961) did the acridine orange test on ϕx 174 which reflected a flame-red color by fluorescence microscopy. Since P4/2 revealed the same characteristic color, it is possible that P4/2 also contains single-strand DNA. The fact that this phage contains half the amount of DNA of a double stranded phage

of similar size is supporting evidence that the DNA from phage P4/2 may be single stranded.

With reference to the base analysis, the results shown in Table 26 indicate that there is no appreciable difference in the molar ratios of the DNA bases in the four strains of bacteria. The DNA of P4/2 is surprisingly non-conventional for the purines and pyrimidines are not complementary as is necessary for a double helix. Thus there is added evidence that the DNA of phage P4/2 is single stranded.

Up to the present time, the only known single-stranded DNA phages are ϕ x 174, the serologically related phage S13 (Zahler, 1958), and phage ϕR. However, each of these is a virulent phage, not isolated from lysogenic bacteria. For comparison, the amount of various bases, relative to adenine of phages ϕ x 174, ϕR and P4/2 are presented in Table 27 (Kay, 1962).

TABLE 27

Phage	Adenine	Guanine	Cytosine	Thymine
ϕR	1.00	1.03	0.83	1.41
ϕ174	1.00	1.06	0.82	1.31
P4/2	1.00	1.19	1.00	1.43

The base content of the nucleic acid of phage P4/2 is thus quite dissimilar to the base content of the nucleic acid of the lysogenic

strain from which it was derived, P4, the propagating strain upon which it was grown, P2, and the other lysogenic strains, P3 and P9. There was no appreciable difference in the base ratios of the DNA of the four different strains of bacteria, however.

Up to the present time, only three temperate phages λvir, S. A1 and P22 have been analyzed for DNA base composition (Sinsheimer, 1960). Each of these phages had DNA in which the purines and pyrimidines were complementary. Stent (1958) and Bertani (1958) suggested that the DNA of temperate phages possessed some genetic homology fo the DNA of their host cells and that they were expected to contain the usual DNA found in the host. This suggestion, however, was not borne out in the results presented here with phage P4/2.

The electron micrograph of the shadowed preparation of P4/2 revealed that it is very likely that P4/2 has the shape of a dodecahedron. By the negative staining method, additional information on the structure P4/2 was obtained. A tail was also observed. With a diameter of approximately 59 mμ, the size of phage P4/2 is close to the size of the E. coli phages T3 and T7, whose diameters have been measured at 47 mμ. Each of these phages also has a tail 15 mμ long and 10 mμ wide.

The single stranded phage, ϕ x 174 was shown by Sinsheimer to have the shape of a icosahedron, with a diameter of 30 mμ but with no evidence of a tail. Bradley (1962) isolated a number of

similar small phages from sewage which also were possibly single stranded, but these were not chemically analyzed.

Phage P4/2 may be the first temperate phage, isolated from a lysogenic strain, which shows evidence of containing single stranded DNA. This result could be confirmed by additional data obtained from experiments such as nucleotide analysis, molecular weight determination, and density by cesium chloride gradient centrifugation.

SUMMARY AND CONCLUSIONS

Three temperate bacteriophages P3/2, P4/2 and P9a/2 were isolated from the lysogenic strains S. potsdam 3, 4 and 9, and propagated on S. potsdam 2. The phage preparations were each purified by differential centrifugation.

Heat inactivation of phages in buffer solution revealed that P4/2 was the most heat resistant, P3/2 was intermediate and P9a/2 was the most sensitive. The Arrhenius constants of each phage were determined; for P3/2 it was 73,280; for P4/2, 84,630, and for P9a/2, 56,334.

The ultraviolet light inactivation experiments showed that P4/2 was the most sensitive; P3/2 was intermediate while P9a/2 was the most resistant. The inactivation constants were 0.025 for P3/2; 0.028 for P4/2; 0.020 for P9a/2.

Results of a number of tests indicated that phage P4/2 possessed an unusual structure with reference to its nucleic acid.
no RNA was detected.

The acridine orange test suggested a single stranded configuration of DNA.

The molar ratios of thymine and adenine were not equivalent, nor were the molar ratios of guanine and cytosine.

The phage DNA did not resemble in molar ratio of bases either the DNA of the lysogenic culture from which it was isolated, or the

culture upon which it was propagated. Thus, the evidence strongly suggested that phage P4/2 contained single stranded DNA. The base ratios of the lysogenic and non-lysogenic bacteria were identical.

By electron microscopy, P4/2 was revealed as a small phage with a diameter of approximately 59 mμ, probably a dodecahedron and with a short but definite tail about 25 mμ long and 10 mμ wide.

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SOME PHYSICAL AND CHEMICAL PROPERTIES OF SALMONELLA POTSDAM BACTERIOPHAGES

by

Norma W. Lee

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ABSTRACT

The phages isolated from different lysogenic strains of Salmonella may be divided into different groups on the basis of their heat resistance. In Group A are phages resistant to a temperature of 80°C and in Group B phages that are killed at this temperature. Three temperate bacteriophages P3/2, P4/2 and P9a/2 isolated from lysogenic strains of S. potsdam were investigated. Phage P4/2 is in Group A and phages P3/2 and P9a/2 in Group B.

The kinetics of heat inactivation and of ultraviolet light inactivation of each of these phages were compared. Base ratio analysis of the nucleic acid by the method of paper chromatography of each of the bacterial strains and phage P4/2 was also done. Phage P4/2 was examined by electron microscopy.

Heat inactivation of phages in buffer solution revealed that P4/2 was most heat resistant, P3/2 was intermediate and P9a/2 was most sensitive. The Arrhenius constant of each phage were determined; for P3/2 it was 73,280; for P4/2, 84,630; and for P9a/2, 56,334.

The ultraviolet light inactivation experiments showed that P4/2 was most sensitive; P3/2 was intermediate while P9a/2 was most resistant. The inactivation constants were 0.025 for P3/2; 0.028 for P4/2; 0.020 for P9a/2.

Results of a number of tests indicated that phage P4/2 possessed an unusual structure with reference to its nucleic acid.

The diphenyalamine test showed that the nucleic acid was DNA; no RNA was detected.

The acridine orange test suggested a single stranded configuration of DNA.

The molar contents of thymine and adenine were not equivalent, nor were the molar contents of guanine and cytosine.

The phage DNA did not resemble in molar ratio of bases either the DNA of the lysogenic culture from which it was isolated, or the culture upon which it was propagated. Thus the evidence strongly suggested that phage P4/2 contains single stranded DNA. The base ratios of the lysogenic and non-lysogenic bacteria were identical.

By electron microscopy, P4/2 was revealed as a small phage with a diameter of approximately 59 mμ, probably a dodecahedron and with a short but definite tail about 25 mμ long and 10 mμ wide.