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ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE INFECTIOUS FOR PSEUDOMONAS AERUGINOSA

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ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE INFECTIOUS FOR PSEUDOMONAS AERUGINOSA

APPROVED BY

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Bacteriophage which infect Pseudomonas aeruginosa are morphologically similar to those which infect the coliform bacteria. Bradley (1967) reports that there are six general morphological types of bacteriophage and that five of these have been reported as infectous for the pseudomonads. The phage may have long tails with or without sheaths. Those lacking sheaths may have various structures such as fibers, knobs or cross shaped appendages on the tail. The tails may be straight or curved. Some of the short tailed phage also have complex fibrous structures. Phage lacking tails have also been reported. These are ribonucleic acid containing phage which attach to bacterial pili. Tailless phage infecting Escherichia coli are male specific while those infecting the pseudomonads have not been found to be sex specific. Filamentuous phage have been reported among those infecting both the coliform and pseudomonad bacteria.

No genetic studies of phage infectious in the pseudomonads have been reported. As a preliminary step toward such studies a collection of five strains of Pseudomonas aeruginosa and bacteriophage infectious in them has been made. The bacteria have been identified as Ps. aeruginosa on the basis of criteria described by Stanier (1966) and in Bergey's Mannual (1956).

Many strains of Ps aeruginosa demonstrate an autolytic phenomenon. This autolytic phenomenon is the spontaneous lysing of some of the bacteria within a colony or streak of bacteria. The result is a plaque which resembles the plaques formed by phage. However the autolytic plaques thus far studied have failed to yield phage.

Some Pseudomonads also produce pyocins, which are bacteriocins specific to the pseudomonads. Bacteriocins resemble phage in that they produce lysis of infected bacteria, exhibit host range characteristics similar to but not identical with those of phage. Unlike phage the lysis of infected bacteria by bacteriocins fails to yield progeny. Phage and the pyocins have been used in typing strains of Ps aeruginosa (Stanier, 1966, Sutler, 1963, and Darrell and Wahaba, 1964).

Pigments produced by the pseudomonads are also useful in their characterization. Ps aeruginosa produces a blue pigment, pyocyanin, which is useful in identifying this species. It is soluble in chloroform and in water. It is

easily separated from the fluorescins which are not soluble in chloroform. In water which is acid the color changes from blue to various shades of red or brown. When both the fluorescins and pyocyanin are present the medium becomes green.

The phage used in this project have been characterized in respect to their plaque morphology, viron morphology, serological relationships, growth characteristics, resistance to various chemical and physical agents, nucleic acid type and the melting point and base ratios of their nucleic acids.

Phage which had characteristics of interest to a geneticist were selected for more detailed study. Some of these characteristics include the stability of lysates on storage and the uniformity of plaque morphology. Other interesting characteristics recognized in the course of the program will be presented in this dissertation, along with comparisons of these phage with those described in the literature.

CHAPTER II

MATERIALS AND METHODS

Isolation of Pseudomonas aeruginosa Strains

One strain of Ps aeruginosa was obtained from the culture collection of the Department of Botany and Microbiology at the University of Oklahoma, Norman, Oklahoma. Three additional strains were isolated from raw sewage. The sewage was obtained from the municipal sewage plant at Norman, Oklahoma. The method used was that of Ringen et al. (1952). A fifth strain was obtained from a clinical specimen. The strains have been designated as P1 for the culture collection strain, 4,5, and X for the sewage isolates and H for the one isolated from the clinical specimen.

Culture Media

Difco nutrient broth and Difco nutrient hard agar (1.5%) and Difco nutrient soft agar (0.7%) supplemented with 0.75% NaCl were used to cultivate both the bacteria and the phage. Because the bacteria and phage are to be used in future genetic studies their ability to grow in defined media was also investigated. The medium selected was that of

Vogel and Bonner (1956) herein referred to as Vogel's medium.

Some media were chosen to characterize the newly isolated bacterial strains. These media are listed in Table 1. Kings media A and B are used to enhance the production of pyocyanin or flourescin, respectively (King, 1954). Pyocyanin is believed to be produced only by Ps aeruginosa. The fluorescins are produced by several members of the genus (Bergey's manual , 1956). Ps putida and Ps fluorescens and Ps aeruginosa produce fluorescent pigments.

Lipase production was determined on Sierra's medium which permits the growth of the pseudomonads whether or not they are able to hydrolyze the test lipid. A positive test is indicated by the formation of a white precipitate in the region of growth (Sierra, 1957). These lipolytic enzymes are able to diffuse through the medium so readily that only one strain could be streaked on a plate.

Wensinck et al. (1967) investigated the influence of nutritional conditions on the autolytic phenomenon. They used three media to study the relationship to tryptophan metabolism. One of these, the glycerol ammonium chloride medium is supposed to enhance this phenomenon, thus making it more accessible to investigation. This medium was used to test the various strains for autolysis. A positive result was indicated by pocking of the streak of bacterial growth followed by a brown discoloration and irridescence on the

surface of the growth. These characteristics were compared to those developing on nutrient agar cultures. At the time this phenomenon was considered to be of interest for a possible genetic study.

The presence of proteolytic enzymes was investigated using Frazier's gelatin agar and litmus milk. The litmus milk also was used as an indicator of changes in pH during the development of a culture (Frazier 1926, cited in Skerman 1959).

The ability of Ps aeruginosa to grow at high (42 C) temperatures but not at 4 C is used to distinguish this species from Ps putida and Ps fluorescens (Bergey 1959, Stanier 1966). Growth at 4 C and at 31 C, 37 C and 42 C was tested using broth cultures. The growth was measured by means of increasing absorbancy at 420 mμ, measured with a Bausch and Lomb spectronic 20 colorimeter. Observations were continued until an absorbance of 1.0 was attained, or for seven days in those cases in which no increase in absorbancy was observed.

Ps aeruginosa is characteristically resistant to many antibiotics (Stanier 1966, Doi 1968). The capacity of strains to grow in the presence of several antibiotics was tested by inoculating nutrient agar plates with a washed overnight culture of the test strain. The plates were incubated for 90 min at 31 C and then Difco sensi disks were placed over the surface of the plate. Incubation at 31 C

was continued for two days. The plates were observed at least three times during this period. A positive test was indicated by clearing around the disk.

Unless otherwise indicated all of the cultures described above were incubated at 31 C.

Growth Studies

The ability of these bacteria to grow under various conditions of temperature and aeration was determined. This information is needed to determine the times of cultivation required to obtain the desired numbers of bacteria for particular experiments. The number of phage per bacterium must be known in order to interpret the results of adsorption studies and one step growth studies of the different phage. This information is also needed in the planning of experiments designed to obtain mutants and to determine the percentage of survivors of any chemical or physical treatments. The rapid growth of Ps aeruginosa at 37 C made this temperature undesirable for the preparation of overnight cultures. Growth in nutrient broth at 31 C was found to be the most satisfactory means of obtaining such cultures. Because this species is aerobic, it is necessary to incubate broth cultures on a sloping surface in order to permit exchange of gases. These overnight cultures were centrifuged and resuspended in a smaller volume of broth, usually 1/5 the original. This preparation was then used as the inoculum for cultures made throughout the day.

It was kept at room temperature. When the titer of the culture at a particular time had to be known the culture was started at an absorbancy of 0.05-0.09. Shake cultures incubated at 37 C were grown in the New Brunswick gyrotory shaker incubator. The time of incubation differed for the various strains used. Strain P1 was incubated 2-3 hours and strain H for three to three and one half hours to obtain titers of approximately 10^8 cells per ml. Increasing absorbancy could not be used as an indication of the number of cells/ml due to production of slime which also scattered light. Growth curves by colony counts made on soft agar overlay plates were used to determine the desirable incubation times Adams (1959).

Isolation of Bacteriophage

The bacteriophage used in these studies were isolated from raw sewage by the method described in Adams (1959). Sewage was obtained from the municipal sewage plant at Norman, Oklahoma. Phage from more than fifty plaques were isolated from bacterial strain P1. Of these only one had the stability on storage and consistancy of plaque type desirable for genetic investigations, and was obtainable in high titers by the normal laboratory procedures. This phage was designated as VI. Other phage isolated from strain P1 have been kept and were subjected to some of the tests applied to VI. Nine phage were isolated from strain H. Of these, two proved to be consistant in plaque morphology and

obtainable in high titers. They are not as stable on storage as VI but are stable enough to be used, provided fresh lysates are prepared at about weekly intervals. These phage have been designated 2H and 3H.

Morphology of the Phage

An estimate of the size of the virons was obtained by passing a centrifuged lysate through a 10 mμ Milipore filter. The size of the virons was also estimated by the measurement of electron micrographs. These electron micrographs were prepared in the Samuel Roberts Nobel Laboratory of Electron Science Microscopy. Grids were prepared from lysates grown on nutrient soft agar plates by the overlay technic, or from broth cultures of the phage. Enough phage were added to the soft agar to yield confluent lysis. The plates were incubated overnight at 31 C. The lysate was collected in nutrient broth and centrifuged, and the supernatant was recentrifuged and decanted. The grids were prepared by placing a drop of this lysate on the grid, allowing it to adsorb for one minute and then negative staining it for 35 sec. The stain was sodium phosphotungstic acid (1%) in 0.4% sucrose.

Phage Antisera

Specific antisera were prepared using each of the three phage as antigens. Crude centrifuged lysates were prepared each week for use as the antigen. The method used

is that described in Adams (1959). The sera were stored in the refrigerator, and were used to determine the serological relationships among the phage, the adsorption rate of the phage to their host cells, and in the performance of one step growth experiments.

Differentiation among the Phage

Phage were selected early in the isolation procedure on a basis of differences in plaque morphology. Further differentiation was made on the basis of differences in the host range and efficiency of plating(eop) characteristics. Efficiency of plating is described as the number of viable phage particles which can be detected using a particular bacterial indicator strain. It is a characteristic of both the phage and the host bacterium. The phage selected by these methods were then tested for their serological relationships.

The host range of the phage was determined by the cross streak method. Overnight broth cultures of the five strains of Ps aeruginosa were centrifuged and resuspended in nutrient broth. Two strains were streaked on each nutrient agar plate. The three plates were incubated at 31 C for a short time (usually 90 min.) and then broth lysates of the phage were streaked across each of the bacterial strains. A streak of nutrient broth was used as a control. The plates were incubated at 31 C and observed a few hours after streaking and again after 18-24 hours of incubation. Areas of

clearing greater than those made by the broth control were considered as positive. When plaques were observed in the cross streak and not along the entire length of the streak they were also considered as positive. These results were then checked and further screened by an efficiency of plating experiment using only those phage which appeared to be distinct strains.

The eop screening test was made by picking plaques of the phage into broth and plating loopfulls by the soft agar overlay technique (Adams 1959). The results of these tests are therefore only roughly quantitative. They did permit a comparison of the ability of the phage to grow in the various bacterial strains and allowed a comparison of the plaque morphology in each strain. Experiments more appropriately called eop were made using phages VI, 2H and 3H. In these experiments broth lysates were assayed using each of the bacterial strains as the lawn.

The serological relationships between the phage were determined by performing experiments in which the inactivation of phage by the several different antisera were compared (serum inactivation experiments). The technique used was that described in Adams (1959). Each of the three antisera was used to inactivate each of the three phage.

The serum inactivation experiment was performed at 37 C. A known number of phage were diluted into a known volume of serum. Samples were taken at intervals and diluted

to stop the action of the serum. The sample was then plated with appropriate indicator cells to determine the percentage of survivors. The data may also be used to calculate the phage inactivation velocity constant (K) of each serum. In either case the K value or the percent survival is used to indicate the serological relationships between phages. These values are also used to determine the dilution of the serum and the time needed to inactivate a certain number of phage. This information is needed in adsorption studies and one step growth experiments.

Adsorption of Phage to Bacteria

The rate at which phage is adsorbed to the host cells was determined by the method described in Adams (1959). Log phase bacterial cultures were prepared as previously described. The phage were diluted to a concentration of about 5×10^7 plaque forming units (pfu) per ml. The homologous antiserum should be diluted so that about 98% of the free phage would be inactivated within five minutes. The greatest dilution of serum which will inactivate this number of phage is used so that a minimum of dilution will stop the activity of the serum. At zero time (t_0), 0.1 ml of phage are added to 0.9 ml of cells at 37 C. At intervals samples are diluted into the serum and incubated at 37 C for 5 min. They are then further diluted to stop the serum from inactivating any phage liberated by bursts. Samples of these

infective centers are plated using log phase cells as the lawn. The antisera are effective only against unadsorbed phage.

One Step Growth Studies

One step growth experiments are made to determine the length of time from the mixing of the phage and host cells to the first liberation of new phage (the latent period), the length of time from the end of the latent period to the plateau (the rise period), and the average number of phage liberated by a bacterium (the burst size). The procedure for performing this experiment is described in Adams (1959). Log phase cells and diluted phage are mixed as they are in an adsorption experiment. After time has been allowed for about 90% of the phage to adsorb, a 0.1 ml sample is diluted into 0.9 ml of specific antiserum. Time is allowed for the inactivation of 98% of the free phage. The mixture is then diluted to stop inactivation by the serum. The dilution tube is further diluted so that the first growth tube contains about one thousand pfu/ml and the second growth tube contains about ten pfu/ml. Samples of the two tubes are plated at intervals until the end of the rise period. The plating method is the soft agar overlay technique. The plates were incubated at 31 C. Both the input phage and the log phase cells must be assayed at the start of the experiment. The titers of phage and bacteria are used to determine the ratio of phage to bacteria at time t_0. Samples must be

taken before the end of the latent period and through the rise period for sufficient time to be certain that no more bursts will take place.

Adsorption experiments indicated that even at high phage to cell ratios the adsorption of these phage is not as rapid or complete as it is in case of the T phages. Because the number of infectious centers is small the procedure was modified to obtain more satisfactory counts on plates made during the latent period. The 1/10 dilution made of the serum tube was omitted and 1 ml volumes rather than 0.1 ml were plated. However this made possible the inactivation of phage liberated by bursts taking place in the first growth tube. An attempt was made to design a control experiment which would provide a correction for this "error." The control was a serum inactivation determination made with the same concentration of phage used in the one step growth experiment. The procedure followed the one step growth study dilution protocol and samples were taken from the equivalent of the first growth tube at the same times as were used in the one step growth study. In the adsorption tube cells were replaced by broth.

The apparently small burst sizes and the failure of cultures of phage to clear seemed inconsistent with the high titers of phage obtained. It seemed possible that the phage released during the first bursts might in some way be more efficient in infecting host cells than the phage which had

been stored. This could be due to the presence of some enzyme or enzymes in the lysate which were capable of attacking bacterial components. This might make the cell more susceptible to attack by phage. Two experiments were designed to test this hypothesis.

In the first experiment a one step growth study was made. At the end of the experiment a sample of the first growth tube was added to a fresh volume of log phase cells. An adsorption experiment was made using this mixture.

The second experimental design also involved an initial one step growth study. In this design at the end of the rise period the contents of the first growth tube were centrifuged and the supernatant fluid passed through a 45μ Millipore filter. The filtrate was then passed through a ten mμ Millipore filter. This filtrate should be free of both cellular debris and phage. A sample of uninfected broth and this filtrate were frozen and stored. An adsorption experiment was made as follows. A log phase bacterial culture was prepared as usual. Two one ml samples of the culture were centrifuged and decanted. The broth and filtrate preparations were thawed. One ml of each was added to the pads of centrifuged cells. 0.1 ml was removed and the cells assayed. The remaining material was then used in the usual adsorption experiment. The results of the two experiments were compared to determine whether or not the filtrate permitted a more efficient adsorption. Phage 3H

was used for this experiment, and for the one step growth study modifications.

Observations of Phage Adsorption Electron Microscopy

The adsorption of phage 3H to H cells was further investigated by means of electron microscopy. Three experimental designs were followed.

In the first the usual adsorption experiment was performed. The grids were prepared at the end of the serum inactivation period. Because the number of bacteria in the adsorption tube is small and there is a 1/10 dilution of the adsorption mixture into the serum, too few bacteria were found on the grids.

The second design was used to obtain a higher concentration of bacteria in the adsorption tube. Two ml volumes of the log phase strain H were centrifuged and decanted. Phage were added to one pad and broth to the other and the pads resuspended by means of a Vortex mixer. Samples of the bacteria plus phage mixture were diluted into specific antiserum at various times and after inactivation grids were prepared from the contents of the serum tube. An assay of the broth plus bacteria tube was made so that the phage to bacteria ratio could be determined. It will be recalled that the sera were prepared from crude lysates. Two observations were made from this material. From the electron micrographs the antiserum is seen to contain substances

which alter the appearance of the slime and other material which is extruded by the bacteria. Secondly the time required for the inactivation step permitted the events leading to phage production to continue.

In the third experimental design the serum inactivation step was omitted. Samples were taken as rapidly as possible. The first sample was taken as soon as the mixing was completed.

The staining procedures used in these experiments were those previously described. Therefore in each case there was a one minute period after the sample was taken before the stain was added to the grid. It has been assumed that the staining procedure would stop further adsorption and the events which follow it.

Inactivation of Phage by Chemical and Physical Agents

The ability of phage to survive exposure to various physical and chemical agents was investigated. During the isolation, cultivation and purification of phage, dilution for assay and preparation for chemical analysis a variety of chemical agents are used (Brownell et al. 1967). The survival of the phage in the presence of some of these agents was investigated. The agents used are listed in the left hand column of Table 14. Unless otherwise indicated the incubation temperature was 31 C for two hours. The phage were diluted prior to the test so that there were

approximately 10^8 pfu/ml. The agents, except where otherwise indicated, were made up in distilled water and the phage were diluted in distilled water. The control was a water suspension of the phage at the same initial concentration as the experimental tubes. The exceptions indicated were the 54 C and 56 C temperature inactivation tests which were incubated for 15 min. Inactivation at 31 C was compared to a broth control. 8 M urea was prepared in two buffers.

Phage were frozen at -40 C and were freeze dried at this temperature. These preparations were stored in a refrigerator freezer. One week after they were treated they were thawed and sampled for assay. They were refrozen and stored. Over a period of eight months the frozen stocks were thawed and tested for the presence of viable phage.

The ability of bacteria grown in Vogel's medium to produce phage and the ability of these phage to survive on storage was investigated. A growth curve for P1 and for H cells in Vogel's medium was prepared. Three carbon sources were used. Log phase cultures were prepared using these data as a guide. These cultures were inoculated with the three phage. Incubation was at 37 C shaken. Incubation was continued until the tubes cleared or until uninfected cultures would have reached the growth plateau. The lysates were then centrifuged to remove cellular debris. The supernatants were refrigerated. The phage were assayed using

log phase broth cultures of strains P1 or H. They were reassayed after seven months of storage.

Because the phage had survived treatment with 8 M urea a killing curve was made using time increments of 30, 60, 90 and 120 min exposure. In the duplicate experiment a sample was also taken at 150 min.

Nucleic Acids

The nucleic acid type of the phage was determined using Burtons modification of the diphenylamine test (1955 and 1956) to detect deoxyribonucleic acid and the orcinol test was used to detect ribonucleic acid (Clark 1964). Phage were prepared for these tests by centrifuging lysates to remove bacterial debris, washing the phage with saline solution to remove soluble bacterial products and resuspending the pad of phage in saline solution. Washing was accomplished by centrifugation in the Beckman model L2 ultracentrifuge using the 40.3 rotor, for three hours at 44,718 X g. The whole virons were used in these tests. In Burtons modification of the diphenylamine test the tubes are incubated at 31 C for about 17 hours. All three of the phage proved to be DNA phage.

Once the phage were identified as DNA containing phage experiments were designed to extract the nuclei acid in quantities sufficient for the determination of the base composition by the thermal denaturation technique (Marmur and Doty 1962).

Phage were collected for extraction by ammonium sulfate precipitation. Five liters of phage lysate were centrifuged to remove cellular debris. Then one liter volumes were placed in a large beaker on the pyro-magnestirer. 700 grams of $(NH_4)_2SO_4$ were added in small increments. Mixing was continued until no increase in the volume of precipitate was observed. The material was collected by centrifugation for 30 min at 5,860 X g in the Sorval superspeed RC-2 automatic refrigerated centrifuge or in the Serval refrigerated centrifuge using the GSA head. The precipitate had two forms, a foamy crust which was concentrated but not sedimented by centrifugation, and a more readily sedimented fraction. Both of these fractions contained high titers of phage. They were combined, resuspended in distilled water and dialyzed against distilled water in the cold. The dialysis was continued for 24 hours with at least three changes of the distilled water. The phage was then concentrated by submerging the dialysis sac in polyethylene glycol (mw 1,300-1,600) until the volume was reduced to from one-half to one-third the original volume. Five ml portions were then centrifuged for three hours in the 40.3 rotor of the Beckman L2 ultracentrifuge at 44,718 X g. The supernatant was discarded and the phage resuspended in 0.1 M phosphate buffer at pH 7. The suspension was treated with 0.2% RNAase and 0.2% DNAase at 37 C for 30 min. The tubes were recentrifuged, decanted and

resuspended in phosphate buffer. They were then treated with 0.05% pronase at 37 C for 30 min. The purified phage were then placed in the freezer.

Extraction of phage nucleic acid.--Two methods were used to extract the phage nucleic acid, the micromethod described in Cantoni and Davies (1966) and the method of Freifelder (1965).

The method of Cantoni and Davies is a micro phenol extraction procedure using only 0.5 ml of phage. The phage was treated in a centrifuge tube. After treatment with 80% phenol the tube was centrifuged and the phenol and denatured protein removed from the bottom of the tube with a Pasteur pipette. The extraction was repeated twice, the phenol layer removed and the DNA collected by ethanol precipitation. The remaining phenol was removed by repeated ethanol precipitations. The DNA was resuspended in saline citrate buffer after each precipitation. Of the three phage only VI DNA could be spindled from ethanol. When the spindled DNA was subjected to isopropanol precipitation it could not be respindled. (Marmur, 1961 method of extracting bacterial DNA). Because difficulty was experienced in separating the denatured protein from the DNA the procedure was modified. The top (DNA) layer was transferred to another centrifuge tube after the first extraction step. A second volume of phenol was added to the first tube and a small amount of phosphate buffer added. The aqueous layer from

this extraction was added to that from the first. The phenol extraction of this aqueous fraction was repeated two times. The DNA was precipitated from cold ethanol and resuspended in saline citrate buffer until no odor of phenol could be detected. After the last ethanol precipitation the DNA was resuspended in acetate buffer (0.015 M NaCl and 0.0015 M Na Acetate μ=0.0165). The products were subjected to the diphenylamine test (Burton 1955 and 1956). The DNA was stored in the freezer.

The technique of Freifelder (1965) was also modified. One ml of 5 M $NaClO_4$ prepared in distilled water was placed in the bottom of a centrifuge tube, 0.5 ml of 30% sucrose in phosphate buffer was gently layered on top and the tube was filled to the top with the purified phage suspension. The tubes were centrifuged in the SW39 rotor of the Beckman L2 ultracentrifuge for three hours at 47,286 X g. The fluid from the bottom of the tube was collected in a volume approximating the volume of phage added, and the pad was separately collected in saline citrate buffer after the upper layer of fluid had been removed. All three fractions were chilled and the nucleic acid extracted with cold ethanol. No material precipitated from the top layer. Both the pad and fluid taken from the bottom of the tube yielded DNA. There was a large amount of grey material, presumably denatured protein, in suspension of the pad. This was removed by repeated alcohol precipitations. Of the three phage

treated only the DNA of 3H could be spindled. It could only be spindled during the first precipitation. The possibility that the buffer might be breaking the strand into fragments was considered. Small samples of the 3H DNA were resuspended in phosphate buffer or acetate buffer and the DNA precipitated with cold ethanol. None of the products could be spindled. The DNA of each method was subjected to the diphenylamine test. Those showing the strongest reaction were selected for melting point determinations. All of the DNA preparations were precipitated by ethanol and resuspended in acetate buffer for the melting point determination.

Extraction of bacterial deoxyribonucleic acid.--In order to have a basis for comparison of the viral melting points with those of known sources two samples of bacterial DNA were prepared. E. coli was kindly provided by Prof. John Lancaster, Ps aeruginosa was the strain P1 obtained from the department of Botany and Microbiology culture collection at the University of Oklahoma, Norman, Oklahoma. The extraction procedure was that of Marmur (1961) as modified by Mandel (1966).

Bacteria were grown to log phase in five liters of nutrient broth on the New Brunswick Gyrotory incubator shaker at 37 C. The bacteria were concentrated by centrifugation in either the Serval refrigerated centrifuge or in the Sorval superspeed RC2 automatic refrigerated centrifuge at 5,860 X g for 30 min. E. coli was washed by centrifugation

with 0.15 M NaCl plus 0.1 M EDTA. Ps aeruginosa is lysed by EDTA (Eagon 1965, Tabor 1961). Therefore these cells were washed in 0.15 M NaCl. The DNA was extracted from the cells with sodium lauryl sulfate followed by phenol (Mandels modification)-chloroform-isoamyl alcohol treatment to denature the protein. The DNA was then collected by spindling from cold ethyl alcohol. The spindled DNA was dissolved in saline citrate buffer (0.15 M NaCl plus 0.015 M trisodium citrate). The DNA was treated with 0.2% RNAase, which had been heated to inactivate any contaminating DNAase, for 30 min at 37 C. The DNA was then reprecipitated from isopropyl alcohol to remove polysaccharides and any remaining RNA. The DNA from a second isopropanol precipitation was washed in a few changes of 70-90% ethanol to remove the salts and then resuspended in acetate buffer. These preparations were stored in the freezer.

The determination of thermal denaturation points.-- The standard curve of thermal denaturation points (Tm) against the percent guanine plus cytosine (GC) was prepared using the two bacterial sources and calf thymus DNA obtained from Calbiochem, Los Angeles. The GC values of these deoxyribonucleic acids were obtained from the literature (Marmur and Doty 1962, Mandel 1966).

The DNA samples were tested three at a time using the Gilford model 2000 multiple sample absorbance recorder. The DNA samples were diluted in acetate buffer (0.015 M

NaCl+0.0015 M Na acetate μ=0.0165) so that the absorbancies were sufficiently different that they would be well spaced on the recorder. A reservoir of paraffin oil was heated by means of an electric hot plate and circulated by means of a centrifugal pump (Easy Industries). The instrument was calibrated using the acetate buffer as the blank. The absorbancy of the unheated cuvettes was recorded and then the temperature of the oil bath raised rapidly to 50-60 C. After this the temperature was raised slowly until denaturation was complete or until it reached 100 C on the Gilford chart.

The data were converted to relative absorbancies and plotted against temperature. The Tm was then determined. The Tm of the known DNA samples were then plotted against their GC %. The Tm of the viral DNAs was then used to determine their GC %. Protein content was estimated by comparing absorbancies at 280 mμ and 260 mμ.

CHAPTER III

RESULTS

Characterization of Bacterial Strains

The results of the studies made to identify the newly isolated strains of Pseudomonas aeruginosa and to characterize them are presented in Table 1. Strain H was not included in many of the tests because it had been identified at the time it was received. The results are those which are to be expected on the basis of data presented by Stanier (1966) and from descriptions in Bergey's Mannual (1956).

The results of the antibiotic resistance experiments are recorded in Table 2. Ps fluorescens and Ps putida are included for comparison. Strains of Ps aeruginosa show a marked resistance to antibiotics. These strains show a wide range of resistance and vary in the degree of resistance to the drugs (Doi et al. 1968). The results reported here also indicate that the age of the inoculum may influence the degree of resistance to the drugs. Doi et al. (1968) have isolated enzymes from cultures of Ps aeruginosa which are capable of inactivating some of the antibiotics. One of these enzymes seems to be active against both kanamycin and

neomycin. These antibiotics have some structural similarities (Wilson et al. 1966). In these tests there is no correlation in resistance to the two antibiotics. Strain X seems to be more sensitive to the antibiotics than the other strains. Strains 4 and 5 are less resistant than P1 and strain H.

The autolytic phenomenon was apparently enhanced in the region of terramycin and chloramphenicol. The production of pyocyanin was apparently inhibited or the pigment was bleached in the presence of chloromycetin and terramycin. Fedorko (1967) observed increased autolysis in the presence of chloramphenicol, tetracycline and sulfioxazole.

Growth Studies

The growth of stationary cultures of strains P1, H and 5 at 31 C and in aerated cultures at 37 C is recorded in Graphs 1, 2 and 3. The shape of the curve at each of the temperatures is similar for each of the three strains. However growth rates seem to differ. The results are somewhat confusing because the production of slime, sediment and pelicle materials as well as viable cells contribute to the changes in absorbance at 420 mμ. Slime is also produced in the aerated cultures. When an attempt was made to compare the increase in absorbancy at 420 mμ with viable counts the results were inconsistent.

Growth as indicated by increase in viable count of aerated nutrient broth cultures of strains P1 and H grown at 37 C is recorded in Table 3. The results of two experiments

are included. They indicate the variation in the numbers of bacteria at time t_0 even though the absorbancy was the same. In some experiments made under presumably similar conditions small clumps of bacteria formed during growth. These clumps were not disrupted by shaking or by dilution into warm nutrient broth. The addition of NaCl to the culture medium may have corrected this tendency. Clumping has not occurred in cultures of the more frequently used strains P1 and H for many months. It is observed in the less frequently used strain 5.

The ability of strains P1 and H to grow in Vogel's medium is illustrated in Table 4. Again growth was recorded as an increase in absorbancy at 420 mμ. It may be seen that the growth in glucose and glycerol is similar in both strains but that growth with acetate as the carbon source is quite slow. So slow in fact that it is difficult to detect the transition to the log phase of the growth curve. The apparent differences between the two strains may be real or reflect the differences in the starting titer.

Characterization of the Phage

Plaque morphology of the phage.--At each step in the purification procedure the plaques isolated were designated by a number or letter. The combinations of numbers and letters used to designate the phage strains are the last two or three in the longer series. Phage VI grown in strain P1 (VI.P1) has a small, usually clear plaque. The margins may

be surrounded by a grey crusty ring. This is especially true when the phage have been exposed to homologous antiserum or to some of the chemicals used in the phage inactivation experiments. The occurrence of this phenomenon is not very consistent.

Phage 2H and 3H have similar plaques when grown in strain H. The size of the plaque varies but is usually small with a halo of variable width. Bacterial strain H produces a large amount of slime. The variations in the plaque size may reflect the production of this opaque material in amounts great enough to cover some of the plaques entirely and partly cover others. The time at which plaques appear also varies considerably even on different areas of the same plate. Some plaques are countable after five or six hours of incubation at 31 C. Others will appear on further incubation when some counted at six hours will no longer be detectable.

Morphology of the virons.--Phage VI has a hexagonal head, a narrow neck region and a long tail. The tail is shaped somewhat like a baseball bat. Phage 2H has a very short tail. It has been difficult to obtain good photographs of this viron even with high-titer lysates. The phage seem to be damaged during the preparation of the grids. This phage also has a hexagonal head (Fig. 1--1).

Phage 3H is very similar to 2H but is less easily damaged during the preparation of the electron microscopic

grids. Occasionally the micrographs reveal some virons which seem to have longer tails than the majority. The significance of these individuals has not yet been investigated (Fig. 1--3 and 4).

Phage gl-clear has a hexagonal head and a tail with a sheath. The neck region is quite narrow. Some structural characteristics are observable on the sheath. It will be of interest to examine shadowed preparations of this viron. A band is seen near the bottom of the sheath and baseplate and fibers may be seen in some preparations. There are striations along the length of the extended sheath. Examination of the heads suggests that these virons may actually be ghosts (Fig. 2--3).

Phage 15x resembles phage 2H and 3H. In some cases these phage appear to attach to a flagellum (Fig. 1--2 and 2a).

Phage 15zc has a hexagonal head. The tail lacks a sheath. The tails seem to thicken distally to the head but not to the extent observed in VI (Fig. 2--6).

Phage 3b2 also has a hexagonal head and long sheathless tail. Tail fibers extending at right angles to the tail may be seen (Fig. 2--5).

The dimensions of these phage are recorded in Table 5. Although phage 15x resembles phages 2H and 3H, it is somewhat larger. There are also differences in the size of the phage which have sheathless tails. It is interesting

that the tail of phage gl-clear resembles a structure found in a preparation of H cells infected with a short tailed phage. Gl-clear does not grow in strain H. There was no opportunity for the H cell preparation to become contaminated with the gl-clear phage. It is suggested that the structure observed in the H cell preparation is a pyocin. The tail like structure observed in preparations of P1 cells infected with phage gl-clear is smaller than that seen in H cell preparations. Estimations of the sizes of the tail of gl-clear and the headless tail from a gl-clear preparation and the particle suspected of being a pyocin are as follows:

gl-clear: tail 860 Å x 90-190 Å (Fig. 2--3)

P1 and gl-clear headless tail: 380 Å x 180 Å (Fig. 2--2)

H 340 or 600 Å x 170 Å (Fig. 2--1)

The structure seen in the H cell preparations varies in length but not width. This may represent normal variations in the size of the structure. However it has been suggested that the pyocins may be defective or incomplete (Bradley 1967) phage. The tail structure observed in the gl-clear preparation may be a phage subunit which was not organized into a complete viron. Perhaps it was not complete at the time of phage maturation, since it is smaller than the tail of a completed viron.

Host Range Studies

The results of the host range experiments made by the cross streaking technique are recorded in Table 6. The

stock culture strain P1 was used to isolate all of the phage, listed in the left hand column of the table, except 2H and 3H. Comparison of these results with those obtained in eop experiments reveal some discrepancies. One reason a positive host range test may differ from the eop test of the same phage and host is the autolytic phenomenon. Some of the cross streak tests had to be repeated several times because the uninnoculated bacteria contained autolytic plaques. Phage strain gl will not grow in bacterial strain H yet there were plaques in the bacterial streak. The tests recorded as doubtful represent those in which clearing was incomplete or there was evidence of overgrowth.

Eop tests made by the loop dilution technique were used to compare plaque morphology on the different strains as well as their ability to permit the development of phage progeny. The results of these experiments are recorded in Tables 7 and 8. Strain gl failed to lyse bacterial strains 4 and 5 in the cross streak tests yet did produce plaques in the loop dilution experiments. More inconsistencies are to be seen in tests made with phage VI. Although there was no evidence of growth in strains 5 and X when the cross streak technique was used, low titers of phage were observed in the eop tests.

Strain gl seems to undergo a host modification in P1 cultures. As each stock of gl is regrown in P1 the titer of the lysate decreases. If the low titer phage are grown

in another strain such as 4, 5 or X and these lysates used to initiate a P1 lysate (gl.P1) a high titer gl.P1 stock is obtained.

Clear plaques of phage Q1 may be defective phage or may have been the product of the autolytic phenomenon. They could not be propagated. Clear plaques of phage Q2 have a host range similar to the parent stock but with a somewhat different plaque morphology in strain 4. Phage g1 produces both clear and turbid plaques. The clear plaques are less subject to the host modification than the turbid but they yield a mixed plaque morphology even after several serial transfers in strain P1.

Attempts to obtain stable clear or turbid lines of these three phage have failed. It is possible that the phage are initiating the release of defective phage from the host strains. These might be able to form sterile plaques. Olsen (1968) reports a consistent variation in the plaque morphology of Ps aeruginosa phage.

Serological Studies

The K values of the three specific antiphage sera are recorded in Table 9. Although these are quite low they do fall within the range described by Adams (1959) for the T phages 1 and 5.

The serological relationships among the phage are illustrated in Table 10. Anti VI antiserum was used to test the relationships of phage isolated in the bacterial strain

P1. No serological relationship was observed between phage VI and phages Q, 15x, 15zc. A weak relationship may exist with phage g1. Because crude phage lysates were used as the antigen for the preparation of the antisera there are antibodies in the sera against some components of the host. These may have had an influence on the adsorption of the phage to the host cells, and might lead to false assumptions of relationships among the phage.

Experiments in which anti VI antiserum was used to inactivate phage prepared in H cells indicates a possible relationship with 2H and 3H. There is some additional evidence when the anti 2H antiserum is used to inactivate phage VI. However conflicting data are obtained when the anti 3H antiserum is used. It is possible that there are some antigens in common to all three phages but that they were not effective in stimulating antibody production in the case of phage 3H.

The increase in titer in the case of anti 3H antiserum used with phage VI is of interest. All three of the antisera contained the products of hemolysis. To be certain that these products were not responsible for the weaker inactivations of nonhomologous phage a nonhomologous antiserum which also contained products of hemolysis was obtained. It was used in serum inactivation experiments with each of the three phage. In every case anti UCI antiserum led to a survival of more than 100%. This suggests that there is a

component of these sera which increases the adsorption of the phage to the host cell. No experiments have been made with a nonhomologous antiserum which does not contain the products of hemolysis. Therefore it is uncertain whether the substance is present in the normal serum, hemolyzed serum or in sera of rabbits exposed to phage.

Adsorption Studies

The results of experiments made to determine the rate of adsorption of phage to bacteria are recorded in Tables 11 and 12. The data in Table 11 presents the number of infectious centers at various times after infection expressed as the percent of the phage added to bacteria at time t_0. The organization of the data in this way is of value in planning one step growth experiments. In such experiments it is necessary to know how many infectious centers will be present at a particular phase of the experiment.

Some of the adsorption rate data were used to determine the adsorption velocity constants. These constants are recorded in Table 12. The formula for determining these (K) constants is: $K = 2.3/(B)(t) \log Po/P$
B is the concentration of bacteria at time t_0, Po is the concentration of phage of t_0 and P is the number of infectious centers at time t. Comparison of the K values calculated at various times may be of value in deciding the most advantageous time to stop the adsorption in a one step growth experiment. Although adsorption may continue up to

the time of the first bursts the rate of adsorption may decrease so that little advantage is gained by postponing dilution into antiserum. In the adsorption of phage VI to P1 there seems to be two phases: an early rapid adsorption and then a later one between 20 and 30 min.

Increasing the ratio of phage to bacteria at time t_0 does not greatly increase the number of phage which adsorb to their hosts. However when the phage infecting strain H are considered increasing this ratio may actually increase the numbers of infective centers formed when phage 3H is adsorbing to H cells, or to 5 cells. These phage adsorb more efficiently to strain 5 even at very low phage:cell ratios. The end of the latent period in this system is at about 25 min. Thus both the decrease in the number of pfu in the case of 3H.5 at 0.02 and the increase at 0.03 multiplicity may reflect the events which follow the release of phage progeny. In the one case phage may be adsorbing to new host cells, and in the other a burst may have added to the free phage population which then were inactivated by dilution into anti phage antiserum. These data will be reconsidered in connection with the one step growth experiments.

Electron micrographs of phage 3H adsorbing to strain H have also been examined. Some of these photographs are presented in Figs. 3, 4 and 5. In Fig. 3--1 the cells which have not been treated in any way other than the preparation

of the grid are illustrated. In Fig. 3--1a the bacteria and phage sampled as soon after mixing as possible are represented. Because the sample is adsorbed to the grid for one minute before the addition of the stain it could have been possible for the adsorption to continue. The phage at this time would be reversibly adsorbed and addition of the stain would dislodge them. Fig. 3--2 indicates that by the time a sample could be taken 40 sec after mixing, phage were in the region of the cell wall and the tails were in the direction which would permit attachment if it had not already taken place. Fig. 3--3 shows that the attachment of the phage to the cell wall has indeed taken place. It is probable that this attachment is reversible. Fig. 3--4 shows some of the phage tails attached to the bacterial cell wall. In Fig. 3--5 and 5a the condition of bacteria 4 min after mixing of a 30./1 ratio of phage and bacteria is illustrated. The damage to the cell wall is so extensive that in some cases the cell degenerates completely. Fig. 4--1 demonstrates that the phage at this time, 4 min, have not yet injected their DNA. This is given some support from the absence of phage on the bacterium illustrated in Fig. 4--2.

At 9 min after mixing of a 1/10 ratio of phage and bacteria there is still no evidence that the phage have injected their nucleic acid. It is probable however that one of the phage not illustrated did inject. The appearance of the cell wall in this picture is quite different from those

made of higher ratio mixtures. The damage to the cell wall at the sight of attachment seems less extensive. The tails of the phage may be seen penetrating the wall through a comparatively narrow area. In some cases the cell membrane seems withdrawn from the wall. However this is not related to the presence of phage in all cases.

It would be difficult to distinguish between the events which cause the degeneration seen in Figs. 3--5a and 4--4 by examining the photographs. The sample used to prepare the grid illustrated in Fig. 4--4 was from a 1/10 ratio mixture incubated for 30 min. The latent period for 3H.H is 25 min thus this sample probably represents the burst and phage release.

Fig. 5--1 was prepared from a 1/10 phage/bacteria mixture which had been incubated for six minutes before it was diluted into serum. It is suggested that the serum reacted with material extruded by the bacterium and made it more electron dense, thus "staining" it. This staining by specific serum helped to make the slime layer and the dissolution of it by phage more accessible to examination (Fig. 4--3). The presence of free phage in Fig. 5--2 is probably due to a release of adsorbed phage on the grid during treatment with stain. It is too long before the start of the rise to suggest this is due to a burst. The picture is intended to illustrate the presence of bacteria which

have not been attacked by phage, even though free phage are present in the region of the cells.

In Fig. 5--3, the tail like objects which appear to be associated with the phage heads are too long and too thick to arise from the short tail of phage 3H. There are however some phage heads which are attached by tails to the cell wall. These are longer and perhaps more slender than the tail of the unattached phage. Because they are attached it is not possible to measure them accurately. Fig. 5--4 indicates that at time t_{20} the degeneration of some bacteria continues to take place due to the lysis from without phenomenon, even though the ratio is only 3/1, and that apparently not all the bacteria become infected.

One Step Growth Experiments

Graphs 4 through 7 represent the results of the one step growth experiments.

The growth of phage VI in strain P1 (VI.P1) was found to have a latent period of 39-42 min, a rise lasting 21-26 min and a burst size of 13-23 when the ratio of phage to bacteria was between 0.05 and 0.21 (Graph 4).

The growth of phage 2H in strain H (2H.H) was found to have a latent period of 24-29 min, a rise of 12-17 min and a burst size of 18-66 when the phage to bacteria ratio was between 0.04 and 0.01 (Graph 5).

The growth of phage 3H in H cells (3H.H) was found to have a latent period of 25 min, a rise of about 12 min

and a burst size of 39 when the phage to bacteria ratio was 0.045. When this same phage was grown in strain 5 (3H.5) the latent period was 25 min, the rise period 13 min and the burst size was 48 when the ratio of phage to bacteria was 0.04. Thus the two experiments may be considered comparable (Graph 6, curve A and Graph 7). Examination of curve B of Graph 6 illustrates the experiment made to increase the number of infective centers present in the first growth tube during the latent period. The increase was obtained. However the value of the experiment is limited by the inability to develop a correction factor which could be applied to the data obtained during the rise period. Thus the burst size can not be more accurately determined by this method than by the conventional test.

The results of the experiments made using the phage released in the first growth tube are presented in Table 13. These data do indicate an increased efficiency of adsorption. However they represent the results of a single experiment.

The Effects of Chemical and Physical Agents on the Phage

The effects of the various chemical and physical agents on the survival of these phage are recorded in Table 14. In several experiments the treated phage have a survival of more than 100%. These phage tend to clump and it is likely that these higher titers are the result of the separation of the members of the clumps. An alternate

possibility is that treatment increased the ability of the phage to adsorb to host bacteria, so that more virons were counted.

Most of the data represent the average of two experiments. An exception was the treatment of phage 2H and 3H with b-mercaptoethanol.

Phage 2H and 3H have a similar but not identical susceptibility to the agents used. Phage VI is more resistant to low pH than the phage from strain H. Toluene has occasionally been used to preserve phage stocks: chloroform is more frequently used for this purpose. Some chloroform sensitive phage have been isolated (Cota-Robles et al. 1968). The two phage isolated in H cells seem to have a slight sensitivity to these solvents. They differ in the degree of their sensitivity to these agents and to alcohol as well as to incubation at 4 C and 56 C.

Phage prepared in Vogel's medium yielded high titers which proved to be stable on storage. The titers are slightly lower than those obtained from nutrient broth cultures. Unlike the broth lysates, these phage lost little of their titer on storage for several months. The stocks were kept on the same shelf of the cold room. The phage prepared in Vogel's medium with acetate as the carbon source yielded slightly lower titers of the phage isolated in strain H than when glucose or glycerol were the carbon sources.

There was no loss of titer from the phage quickly frozen and stored frozen. Lyophilization did not completely inactivate the phage so treated. Viable cultures were obtained from a tube one week after it was freeze dried.

Because all three phage had been found quite resistant to 8 M urea, a killing curve was prepared using phage VI. Up to time t_{90} min the survival exceeded 100%. In one experiment a sample taken at 120 min had a survival of 100% while in a second experiment the survival was only 76%. In one experiment there was a sample taken at 150 min. In this experiment the survival was again 100%. However phage VI was strongly inactivated by 8 M urea prepared in either pH 7 or pH 9 glycine-sodium glycinate u=0.05 buffers. The buffers alone were not inhibitory to the phage (Table 14).

Determination of Thermal Denaturation of Phage DNA

The DNA from phage VI prepared by the micromethod of Cantoni and Davies denatured over a narrow temperature range. It should be recalled that this preparation could be spindled in the first purification step. It was precipitated by centrifugation from cold ethanol out of citrate buffer in the subsequent purification steps. The thermal denaturation profile of DNA from VI prepared by Freifelders method had a broader temperature range. The Tm for the DNA propanol preparation by the micromethod was 86.5 which indicates a

guanine plus cytosine (GC) value of 64%. That for the Freifelders method was 80 or 54.5% (Graph 9).

The DNA of phage 2H prepared by the micro method denatures irregularly between 60 and 85 C and then regularly to 90.5 C. From these temperature ranges three Tm and their GC values may be derived. One of these is that for the entire profile the other two for the two major sections. If the irregular and regular portions of the profile are combined their Tm is 90 and GC 70%. The fraction which denatured first has a Tm of 74 C and GC of 44% the second part of the profile has a Tm of 92 C and GC of 74%. When Freifelders technique was used to prepare 2H DNA the product had a two mode profile. Unfortunately only the first phase is of value because the denaturation was incomplete at 100 C, the limit of the chart on the Gilford. This GC was 70% (Graph 11).

The DNA prepared from phage 3H by the Cantoni and Davies technique denatured rapidly between 72 and 82 C, slowly to 91 and rapidly to 93 C. If the first two phases are combined they have a Tm of 80.5 C and GC of 54%, the second phase has a Tm of 92 C and GC of 74% the Tm of the entire profile is 81 C and GC 56%. When Freifelders technique was used to prepare the DNA there was only a slight break in the profile. This is of interest because the DNA extracted by this method could be spindled in the first purification step but not in any subsequent precipitation. The denaturation

began at 74 C. The break occurred at 77 C and was completed at 80 C. The Tm value of the first fraction was 75.5 C and the GC content was 46.5%; that of the second 78.5 C and GC 52% and the profile as a whole 76 C and 48% GC (Graph 10).

The ratio of absorbancy at 280 and 260 mμ for the standard DNA preparations and for the viral DNA prepared by Freifelder's method are as follows:

P1 0.79; E. coli 0.87; calf thymus 0.55

VI 0.70; 2H.H 0.7; 3H 0.7 - 0.79.

It seems apparent that the preparations made for this project had a higher protein content than the commercially prepared calf thymus. The origin of this protein is viral.

CHAPTER IV

DISCUSSION

The resistance of the strains of Pseudomonas aeruginosa to antibiotics was anticipated. The unusual sensitivity of strain X to these drugs is therefore of interest. Doi et al. (1968) have been able to extract some enzymes from strains of Pseudomonas aeruginosa which are capable of inactivating the antibiotics streptomycin, kanamycin and neomycin. The enzyme which inactivates neomycin is apparently the same as the one which inactivates kanamycin. An examination of the structural formulas of these two drugs shows that they do have similarities. They both contain deoxystreptamine (Wilson et al., 1966).

Examination of the data in Table 2 indicates that the resistance of strains used in these studies must have a different pattern than those studied by Doi. A study of the genetics and enzyme kinetics of the resistance to kanamycin would be of interest. Comparison of the kinetics of this enzyme in an in vitro situation might prove of value. It is possible that the neomycin might be unable to induce enzyme synthesis but be inactivated by the enzyme produced in

response to kanamycin. Kanamycin is a smaller molecule, and the induction might fail in the case of neomycin because of steric hindrance or a requirement for the bond between two of the three components of kanamycin. The strains investigated by Doi may be induced in a different way. The genetics of the resistance might be examined by means of the transduction technique. Strain X would make a good recipient and strain P1 and one of the other three as donor strains would provide the material of a preliminary test, because P1 is only slightly sensitive to neomycin and thus may produce some of the enzyme in response to this drug.

Another interesting program would be an investigation of the mechanism of the inhibition or bleaching of pyocyanin by chloromycetin and megamycin. Like litmus it is an indicator of pH. It is also an oxidation-reduction mediator. In the metabolism of carbohydrates, there is increased CO_2 formation from substrates metabolized via the phosphogluconate pathway (Landau et al. 1963). Thus it may be a very simple matter to investigate. As may be seen from Table 2 the strains which have this bleaching phenomenon are resistant to these drugs. It might be possible to extract an enzyme from cells which would, either alone or in an in vitro system with the phosphogluconate pathway intermediates, cause this bleaching.

Fedorko reported (1967) a relationship between the autoplaque phenomenon and increased irridescence, and the

presence of chloramphenicol, tetracycline and sulfisoxazole. Wensick (1967) believed there is a relationship between the autoplaque phenomenon and tryptophan metabolism. It would be interesting to determine whether or not these drugs have an effect on the metabolism of tryptophan in Ps aeruginosa.

The variation in plaque morphology in those phage strains not consistent enough for use in genetic studies might be related to differences in their ability to produce enzymes attacking the bacterial slime. Another possibility is the release of defective phage from the host cells. These possibilities are accessible to investigation. Elucidation of the cause might permit development of assay systems making these virus useful in genetic study.

The pyocin-like structure in the 3H.H preparation suggests that this strain may carry pyocins which could be induced. The preparation obtained could be used for a comparison with the results of spotting cultures of the bacteria on lawns of the various strains. The activity of these pyocins might elucidate the perplexing inconsistencies in the host range studies.

Phage

The antisera prepared against the three phage had K values within the range described by Adams for the T phage 1 and 5. Feary also reported low K values for the phage he studied. However in the one case he reported the value was

increased from 48 to 46,000 by a second immunization four months after the first bleeding. An improved antiserum might permit a shorter inhibition time. This would permit more samples to be taken during the latent period of a one step growth experiment. The accuracy of the estimate of the burst size would be increased.

Because of the poor adsorption of the phage to their host and because many phage seem to attach to a single bacterium, even at low ratios of phage to bacteria, there are uninfected bacteria in the first and second growth tubes. Some of the Ps aeruginosa phage are able to attach to bacteria without giving rise to an infective center (Feary 1964). This suggests that not all the cells in a population are subject to infection by the phage. Mead (1956) was able to extract a substance from sensitive mutants of resistant strains of bacteria which was able to inhibit the infection by two of three phage but enhanced infection of both sensitive and resistant strains by the third phage. This substance was not present in the growth filtrate of the bacteria. The experiment made using the filtrate of the first growth tube to increase the adsorption of phage 3H to strain H suggests that substances were present which did increase adsorption of this virus.

Examination of Fig. 1--4 shows that some of the 3H phage have long tails, while others are quite short. The phage which seem to have attached and penetrated the bacterial

cell wall also have long tails. It is suggested that it is the long tailed form which are capable of infecting the bacteria. If the short tailed phage are incomplete, then it is possible that a substance released from the bacteria, either as a result of lysis from without or from phage production might make it possible for these phage to adsorb effectively to the uninfected bacteria.

D. Cox (personal communication) suggests an alternate possibility. If the tail is coiled in the phage which are capable of effective adsorption then it is the short tailed form which is infectious. If the uncoiling of the tail is an irreversible event those phage which are unattached and long tailed are not infectious. This could explain the low adsorption rate and the failure of the k of adsorption to increase with time. The theory could be tested by preparing phage and separating the two types by means of a density gradient. The ability of each of the fractions to infect bacteria could then be investigated by the conventional adsorption experiment. Thin sections prepared from the adsorption experiment could be examined by electronmicroscopy and comparisons made of the penetration of the tails in each fraction.

The electron micrographs of 3H adsorbing to strain H indicate the phage are able to make a depression in the bacterial cell wall. This suggests that the damage is not limited to the area of tail penetration. The wall remaining

between the degenerated areas appears to be swollen. Distinct layers may be observed in the wall structure. An enzyme extract of the phage might make the wall structure more accessible to study, both from a chemical and cytological approach (Fig. 3--3, 4, 5 and Fig. 4--1, 2 and 4). In Fig. 3--4 and a 3 min incubation at a 3/1 phage/cell ratio the tails are seen to be penetrating the wall, but the wall is not yet depressed. In Fig. 4--3 after 9 min of adsorption at a 0.1 ratio the phage on the surface of the bacteria are in a depression in the surface of the cell (Figs. 3--5 and 5--3).

Bayer (1968) describes the attachment of E coli phage as taking place only at sites where there are protoplasmic bridges between the membrane and the cell wall. Bayer used plasmolyzed cells for these experiments. There is no evidence that this is the case in the 3H.H system unless the structures interpreted as tails are the protoplasmic ducts described by him. The membranes of the cells seem to be drawn or pushed back from the cell wall in the areas of attachment.

The fact that the nonhomologous antiserum increased the adsorption of 3H to H suggests that some component of this serum aids in adsorption. The presence of iron in the serum suggests that this might be the factor. However some other inorganic component such as calcium could also be present, or some cofactor for an enzyme might be present in

the serum. Further investigation should be made. This investigation should include a serum which does not contain the products of hemolyzed red blood cells. Addition of $CaCl_2$ and $FeCl_2$ to the nutrient broth are other tests which should be made. The results of these tests would indicate the advisability of performing experiments which would involve the use of more complex substances.

Comparison of the latent periods, burst size and the GC value of these phage with those described by Grogen (1964 and 1964a) and Olsen et al. (1968) indicate that these are not identical with any of theirs although there are some similarities.

Phage VI most resembles Grogan's phage 2 and Olsen's PX3, but in each case there are differences. VI differs from PX3 in the morphology of the viron. PX3 is a short tailed phage, VI is long tailed. PX3 has a similar latent period (1p) but the burst size is larger than that of VI. VI also resembles phage 2 in respect to the 1p but has a lower burst size. The differences in burst size may reflect a different eop in the P1 cells compared to the phage bacterial system used by Grogan. It would be interesting to compare the burst size of VI in the bacterial strains used by these authors.

Resistance to different conditions of chemical and physical stress are hard to compare due to the differences in experimental techniques. Phage VI seems to be more resistant to high temperature than PX3 but may be less

resistant than phage 2. The Pseudomonas phage reported in the literature seem to have a common resistance to high pH. They differ in their sensitivity to low pH. Phage VI is resistant to pH as low as 4.

The GC value of VI prepared by Freifelder's technique but not by the micro method resembled phage 2, but was much higher than that of PX3.

Olsen used a phenol extract of the phage in saline citrate buffer and compared his data with those of Marmur and Doty (1961). Grogan used a chloroform methanol extraction and perchloric acid purification and denaturation procedure prior to chromatographic analysis of the bases. He also used density gradient analysis. Thus the methods of Olsen are more like those reported in this work.

Phage 2H was not like any of those reported in these papers in respect to the lp, burst size or GC. The GC is higher than any of those reported.

Phage 3H is unlike any of those reported in these two papers in respect to the lp and burst size. The GC is within the range of those reported but is not like any one phage.

None of these authors mentions observing the kinds of breaks in the Tm profile observed with 2H and 3H. There are at least three possible explanations for these breaks. One is that there is more than one molecule of DNA in these phage. Another is that there is one molecule but with

regions of greater GC content. The third is that the DNA is contaminated by protein, either from the phage coat or normally present with the DNA. All three possibilities could be investigated. Density gradient centrifugation should distinguish between the first two and a chemical test for the presence of protein should indicate the third.

CHAPTER V

SUMMARY

Phage infectious for Pseudomonas aeruginosa have been isolated from sewage obtained from the municipal sewage plant at Norman, Oklahoma. Three of the phage were selected for characterization on the basis of their stability on storage, the consistency of their plaque morphology, and their host range. They have been examined in respect to their serological relationships, adsorption, one step growth curves, the type of nucleic acid and the base ratio of their nucleic acid as well as their stability under treatment with various chemical and physical agents.

They have been compared as carefully as differences in experimental technique permit with other Pseudomonas phage reported in the literature (Feary, 1964; Grogan, 1964 and Olsen, 1968). They have been found to be similar but not identical with those reported in the literature. The phage reported here are of particular interest in respect to their adsorption and base ratio characteristics.

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Table 1

Characterization of the Pseudomonas aeruginosa Strains

	Strain			
Test	P1	4	5	X
Pyocyanin[1]	+	+	+	+
Fluorescein[1]	+	+	+	+
Gelatinase[2]	+	0	0	+
Lipase[3]				
Tween 40	+	+	+	+
Tween 60	+	+	+	+
Tween 80	+	+	+	+
Change in pH[4]	alkaline	alkaline	alkaline	alkaline
Caseinase[4]	liquification	liquification	liquification	liquification
Growth:				
4 C	0	0	0	0
31 C	+	+	+	+
37 C	+	+	+	+
42 C	+	+	+	+
Autolysis[5]	+	+	+	+

[+]Positive test; 0 negative test.

[1]King's media A and B (1954).

[2]Frazier's Gelatin.

[3]Sieras medium (1957) Tweens are fatty acid esters of a sorbitan derivative: 40 palmitic, 60 stearic, 80 oleic.

[4]Litmus milk.

[5]Wensinck's medium (1967).

Table 2

Drug Sensitivity of the *Pseudomonas* Strains

Drug	conc. in meg.	Strains of *Pseudomanas*						
		Ps putida	*Ps fluo-rescens*	*Ps aeruginosa* P1	4	5	X	H
Aureomycin	5	S	S	R	S1	S1	S	R
	10	Sxx	S	R	S1	S1	S	S1
	30	Sxx	S	R	S1	S1	S	S1
Chloromycetin	30	Sx	S	R*	R	R	S	R
Erythromycin	2	S1x	Rxx	R	--	--	--	--
	15	S1x	Rxx	R	R	R	S	R
Kanamycin	5	Sx	S1	R	R	R	S	R
	30	S	Sxx	R	R	R	S	R
Megamycin	1	R	R	R	R	R	S	R
Neomycin	30	Sxx	S	S1	S	S	S	S
Novobiocin	30	R	R	R	S1	R	S	R
Penicillin	2 units	R	R	R	R	R	S	R
	10 units	R	R	R	R	R	S	R
Polymyxin	10	S1xx	Sxx	S	S	S	S1	S
Terramycin	5	R	Sxx	R*	R	S	S	S1-R
	10	Sxx	S	R	R	R	S	S1-R
	30	S	Sx	R	S1	S1	S	R
Streptomycin	10	--	--	--	S	S	S	R

S: Sensitive.

S1: Slightly Sensitive.

R: Resistant.

--: Not done.

*: Bleaching of pyocyanin production of irridescence and plaques.

x: 48 hour cultures less sensitive.

xx: 48 hour cultures more sensitive.

Table 3

The Growth of Strains P1 and H at 37 C with Aeration

Time in Hours	The bacteria grown from an absorbancy of 0.05 at 420 mμ	
	P1 /ml x 10^7	H /ml x 10^7
0	5.1	1.7
	2	3.4
1	2.8	3
	2.7	1.97
2	2.8	1.47
	7.5	2.18
3	65	4
	59	9.8
4	80	29
	110	62

Table 4

The Growth of Strains P1 and H at 37 C with Aeration, in Vogel's Medium with Three Different Carbon Sources (Growth is recorded as increase in adsorbancy at 420 mμ)

Time in Hours	P1 Glucose	P1 Glycerol	P1 Acetate	H Glucose	H Glycerol	H Acetate
0	0.08	0.09	0.07	0.05	0.05	0.06
2	0.2	0.23	0.05	0.09	0.1	0.05
3 1/2	0.68	--	0.07	0.12	0.28	0.06
4 1/2	greater than 1.0	0.99	0.11	0.1	0.25	0.14
5 1/2		1.0	0.17	0.13	0.23	0.18
8			0.31	0.34	0.34	0.17

Table 5

Dimensions of the Bacteriophage

	Dimensions in Angstroms				
	Head		Tail		
Phage	Length	Width	Length	Width	Total Length
15x	440		150	150	590
15 zc	540	310	980	100	1520
gl clear	460		860	*90-190	1390
3b 2	520		1040	130	1940
VI	380		970		1460**
2H	340		110	100—	
3H	390		100-390	100	

*The smaller number represents the contracted sheath, the larger the tail with the sheath uncontracted.

**The knob has the dimensions 660 x 280.

Table 6

Host Range of the Bacteriophage

	Strain of *Pseudomonas aeruginosa*				
Phage	P1	4	5	X	H
VI	+	+	0	0	0
15 x	+	+	+	+	+
15 zc	+	+	+	+	?
gl	+	0	0	plaques	?
Ql	+	+	+	?	+
3b	+	-	-	-	0
2H	+	+	0	+	+
3H	+	+	+	+	+

+: a clear area in the intersection of the cross streaks.

?: reaction doubtful.

-: experiment not done.

0: no clearing in the intersection of the cross streaks.

Table 7

Efficiency of Plating

Bacterial Strains	Phage		
	VI	2H	3H
P1	8.5×10^{10}	1.5×10^{5}	1.2×10^{5}
4	1×10^{9}	2×10^{9}	8×10^{9}
5	1×10^{6}	1.3×10^{10}	1×10^{10}
X	1×10^{8}	2×10^{10}	1×10^{10}
H	0	1×10^{10}	3×10^{10}

Table 8

Plaque Morphology and Host Range

Phage Strain	Bacterial Strain	
	P1	4
G1[1]	Mixed clear and turbid	A faint halo plaque is small
3 b	Mixed some have clear centers others a central colony	No plaques
Q1	Mixed very large clear with or without central colony with or without a halo	Center of plaque clear irregular halo
Q1 clear	No plaques	No plaques
Q2	Mixed morphology	Tiny very clear plaques
Q2 clear	Mixed morphology	Small turbid plaques

[1]G1 cannot be regrown in P1 cells but the clear plaques from g1 will grow in P1 and yield a mixed morphology.

Table 8--Continued

Bacterial Strain		
5	X	H
Small very clear plaque no halo	Mixed clear and turbid plaques	No plaques
No plaques	No plaques	Autolytic effect
Center of plaque very clear no halo	Plaques very clear, large	Small very turbid plaques
No plaques	No plaques	No plaques
Mixed but most are small clear plaques	Clear plaques	Probably autolytic
Mixed some have a central colony others a halo	All are very clear	No plaques

Table 9

K Values of the Homologous Phage and Antisera

Phage and Serum	Dilution of Serum	Inactivation Time	K Value
VI	1/500	10 min	100
2H	1/100	10 min	32-44
3H	1/100	10 min	31-40

Table 10

Serological Relatedness of the Phage
(% survival of t_0 titer)

Antiserum	Dilution	Phage	% Survival	Relatedness
VI[1]	1/100	VI[3]	0.66	homologous
		VI[4]	0.33	homologous
		gl clear	87.00	doubtful
		Q	100	not related
		15 X	100	not related
		15 zc	100	not related
VI[2]	1/500	VI	13	homologous
		2H	47	not related
		3H	30	not related
2H[2]	1/100	VI	58.0	not related
		2H	4.0	homologous
		3H	1.0	closely related
3H[2]	1/100	VI	210.0	not related
		2H	2.5	closely related
		3H	0.48	homologous

[1]Incubated 5 min at 37 C.

[2]Incubated 10 min at 37 C.

[3]Prepared in nutrient broth.

[4]Prepared in Vogel's medium.

Table 11

The Percentage of Adsorption of Pseudomonas Phage to its Host Bacterium

Time in Minutes	Ratio VI Phage to P1 cells		Ratio 2H Phage to H cells		Ratio 3H Phage to H cells		Ratio 3H Phage to 5 cells	
	0.07	0.13	0.12	0.38	0.1	1.0	0.02	0.03
2-3	5.8	60.0	1.6	0.38			8.3	23.0
5-6			2.1	6.9	0.17	4.0		
7	56.9	60.0	2.1	5.1				
8-9					2.0	1.8	9.4	45.0
10-11		86	1.9			2.0		
12-13	60.0		2	4.8	1.4		10.7	51.0
14-15			1.9					
17-18		45			5.7		32	75
19-20	86		2.8	10.3				100
25	97	80	4.8		7.9		24	630
27				29.7				
30	100	86			32		29	
35		110						

Table 12

Adsorption Velocity Constants of *Pseudomonas* Phage to its Host

Phage	Bacterium	Ratio	Time min.	K in ml/min
VI	P1	0.07	2	1.4×10^{-10}
			25	8.5×10^{-10}
		0.13	2	2.4×10^{-9}
			25	4×10^{-10}
2H	H	0.1	2	1.3×10^{-7}
			20	7×10^{-9}
		0.38	2	3.7×10^{-8}
			10	3×10^{-9}
3H	H	0.1	6	2.8×10^{-9}
			17	7.4×10^{-10}
		1.0	5	3.1×10^{-8}
			10	8.4×10^{-9}
	5	0.02	3	3.1×10^{-9}
			12	6.1×10^{-9}
		0.03	3	7.7×10^{-9}
			12	9.0×10^{-10}

Table 13

The Effect of the Growth Supernatant on Adsorption of the Homologous Phage*

Time in min.	% Adsorption	
	Supernatant	Broth
3	80	30
9	100	76
12	90	40
15	81	75
17	--	120

--: not done.

*: the phage was 3H.H.

Table 14

The Effects of some Chemical and Physical Agents on Phage VI 2H and 3H

Agent		Phage VI	Phage 1H	Phage 3H
		Percent Survival		
Toluene 10%		28.	120.	48.
Chloroform 10%		63.	88.	240.
Peroxide 0.3%		59.	8.	37.
Ethanol 10%		72.	55.	130.
Thymol 0.05%		690.	59.	48.
Mercapto-Ethanol 1.5 M		44.	0.	0.
0.15 M		38.	0.	0.
8 M Urea pH 7		77.	65.	58.
8 M Urea pH 9		0	0.	0
8 M Urea pH 10		0	9.	0
Buffers pH				
Acetate 3.4		100.	0.	0.
Acetate 4.9		92.	0.23	0.14
Acetate 5.0		160.	0.58	0.93
Acetate 5.6		130.	9.7	15.
Glycine 9.0		200.	140.	200.
Glycine 9.5		97.	100.	200.
Glycine 10.0		150.	95.	67.
Temperature	4 C	93.	58.	100.
	31 C	270.	51.	77.
	46 C	79.	130.	180.
15 Min	54 C	130.	58.	94.
15 Min	56 C	60.	5.4	34.
Vogel's Medium	Date			
		Titer		
Glucose	Jan	1.5×10^9	3.8×10^{10}	7.3×10^{10}
	July	9.8×10^8	3.5×10^{10}	3.7×10^{10}
Glycerol	Jan	5×10^8	3.7×10^9	1×10^9
	July	1×10^9	1×10^{10}	3×10^9
Acetate	Jan	1×10^9	3×10^9	2.6×10^9
	July	1.2×10^9	1.8×10^9	1.3×10^9

Figure 1

1 Phage V1. X 329,400.

2 Phage 15x. X 67,200.

3a Phage 15x. X 154,800.

3 Phage 2H. X 199,200.

4 Phage 3H. X 116,400.

5 Phage on debris, phage are probably ghosts. X 155,450.

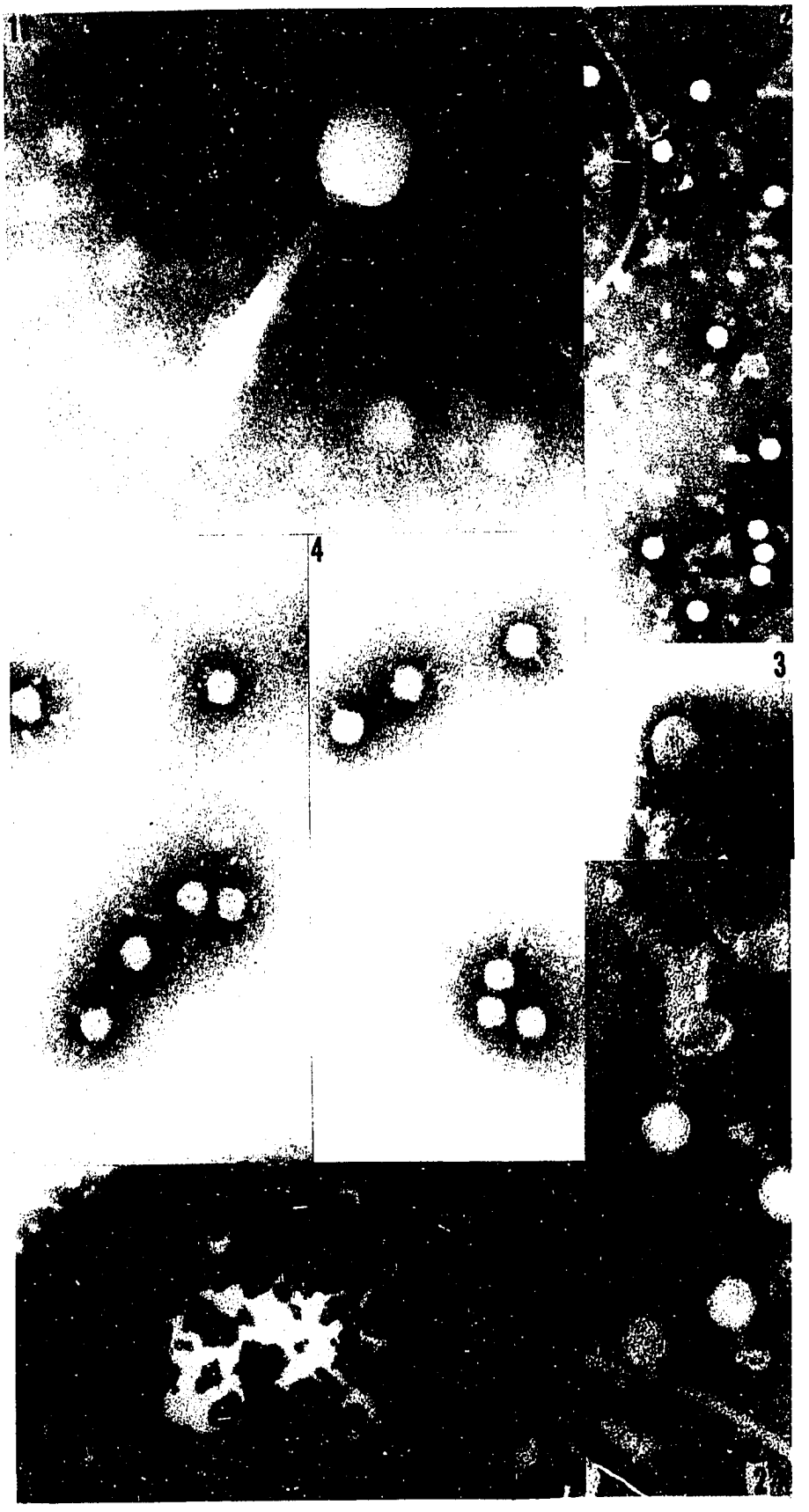

1
4
3
2

Figure 2

1 Probable pyocin from strain H. X 199,200.

2 gl clear tail structure or pyocin from strain P1. X 329,400.

3 Phage gl clear, note the resemblence to the structures in 1 and 2. X 256,200.

4 Phage 2H with strain H. X 44,625.

5 Phage 3b2 careful examination for the presence of tail fibers extending laterally to the long axis of the tail. X 88,200.

6 Phage 15zc. X 116,400.

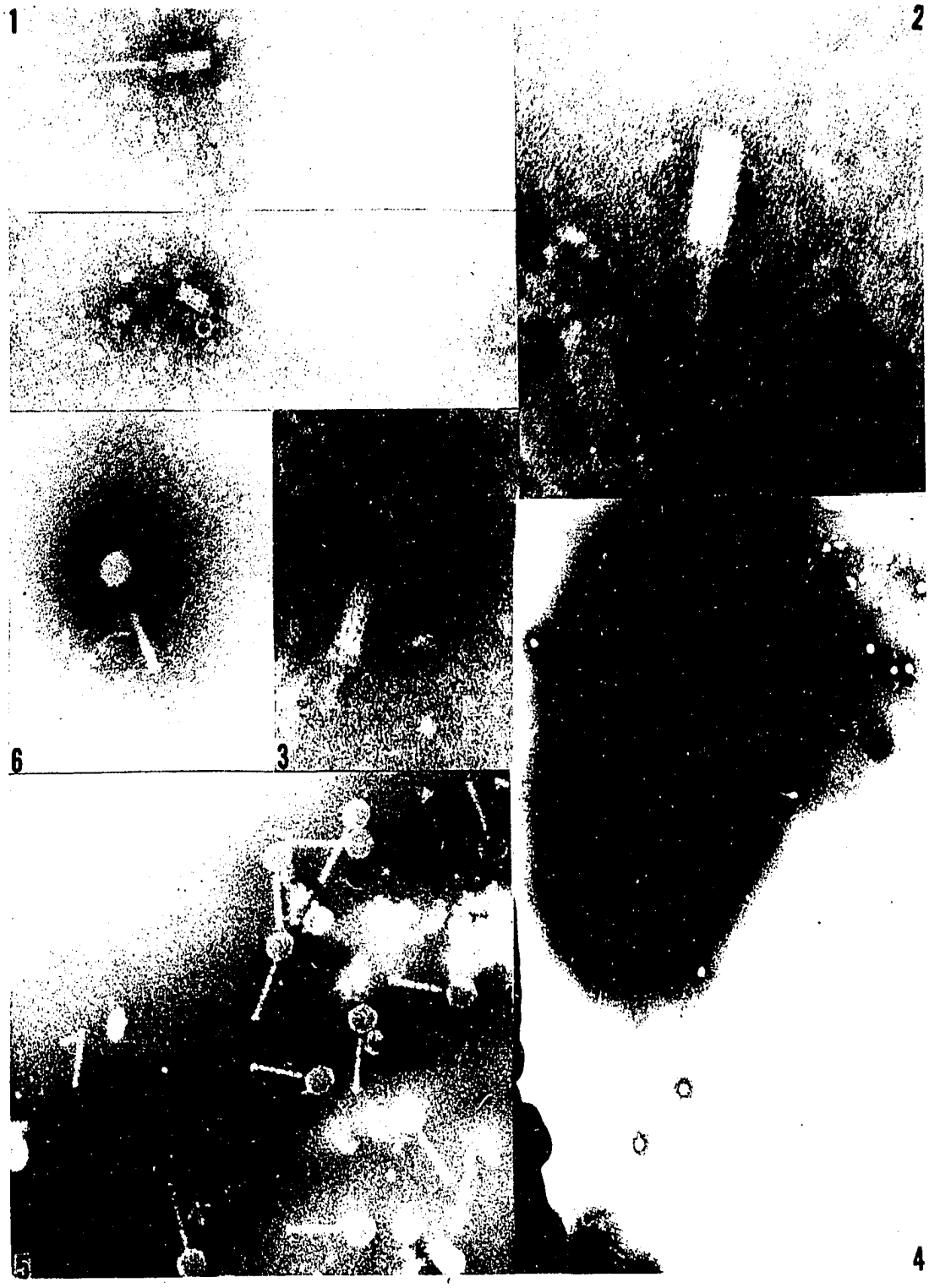

1
2
6
3
5
4

Figure 3

1 Uninfected strain H of Pseudomonas aeruginosa. X 22,880.

1a Strain H of Ps aeruginosa with phage 2H at time t_0 The ratio of phage to bacteria was 3/1. X 38,250.

2 Strain H of Ps aeruginosa with phage 3H at time t 40 sec This preparation was treated with serum at time 40 sec the ratio of phage to bacteria was 30/1. X 51,000.

3 Strain H of Ps aeruginosa with phage 3H at time t 1 min, no serum was used in this experiment, the ratio of phage to bacteria was 3/1. Phage at this time may be attached. X 77,400.

4 Strain H of Ps aeruginosa with phage 3H at time t 3 min, no serum was used in this experiment, the ratio of phage to bacteria was 3/1. There is some evidence that phage tails have attacked the cell wall. X 88,200.

5 Strain H of Ps aeruginosa with phage 3H at time t 4 min, serum was used in this experiment, the ratio of phage to bacterium was 30.1. Extensive damage to the cell wall has taken place. X 132,800.

5a Strain H of Ps aeruginosa with phage 3H at time t 4 min, serum was used in this experiment, the ratio of phage to bacteria was 30/1. Lysis from without is taking place. X 19,500.

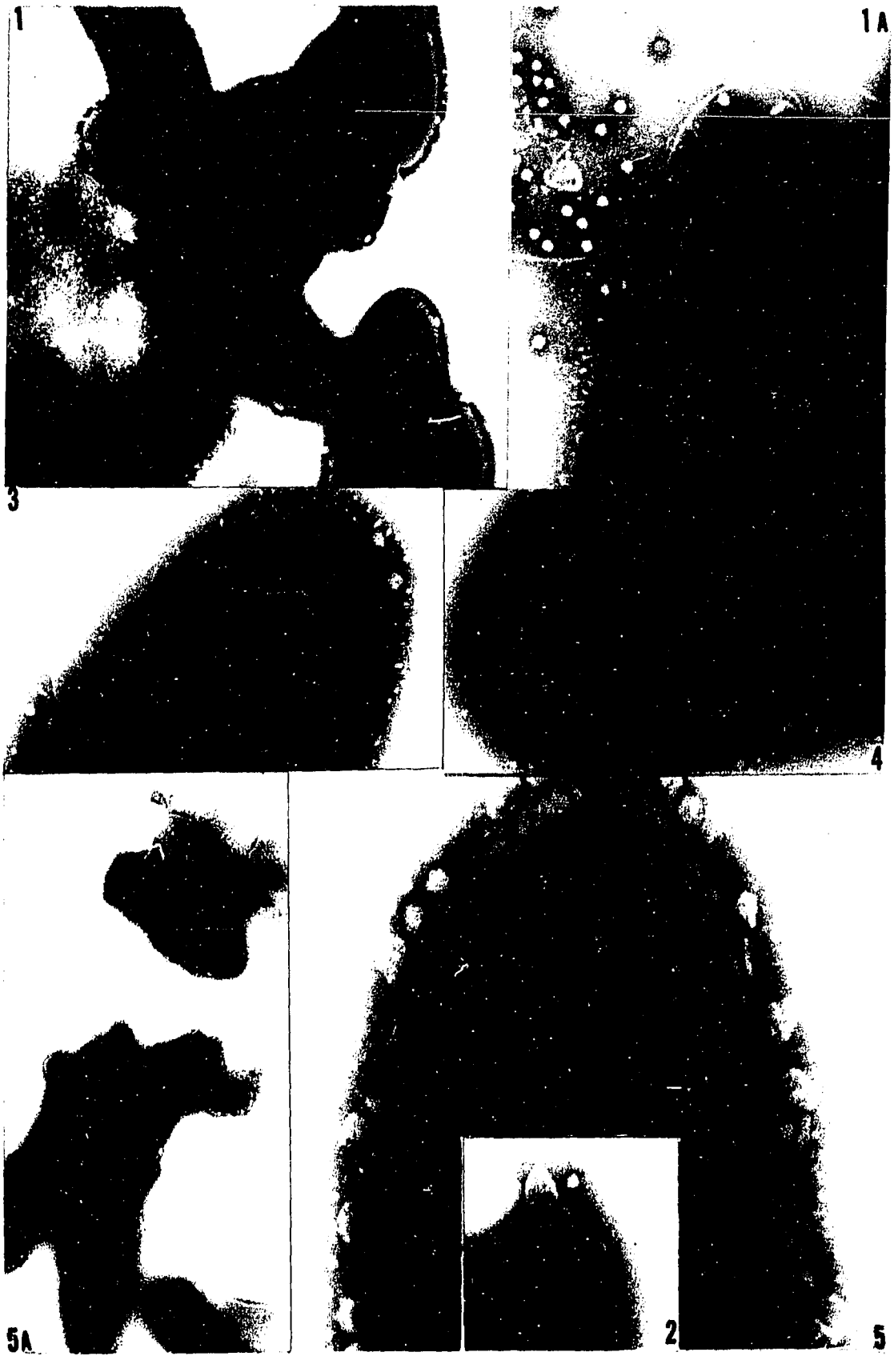

1
1A
3
4
5A
2
5

Figure 4

1 Strain H of Pseudomonas aeruginosa with 3H phage at time t 4 min, this preparation had received serum treatment, the ratio of phage to bacteria was 30/1. X 58,800.

2 Strain H of Ps aeruginosa with phage 3H at time t 4 min this preparation received serum treatment, the ratio of phage to bacteria was 30/1. X 129,600.

3 Strain H of Ps aeruginosa with phage 3H at time t 9 min the preparation had received serum treatment, the ratio of phage to bacteria was 0.1. There is evidence that the tail of the phage has penetrated the wall and that in some cases the membrane has separated from the wall area. X 132,800.

4 Strain H of Ps aeruginosa with phage 3H at time t 30 min, the preparation received serum treatment, the ratio of phage to bacteria was 0.1. The bacteria are degenerating, at this time the breakdown of the bacterium could be due to the burst. X 28,350.


1
2
3
4

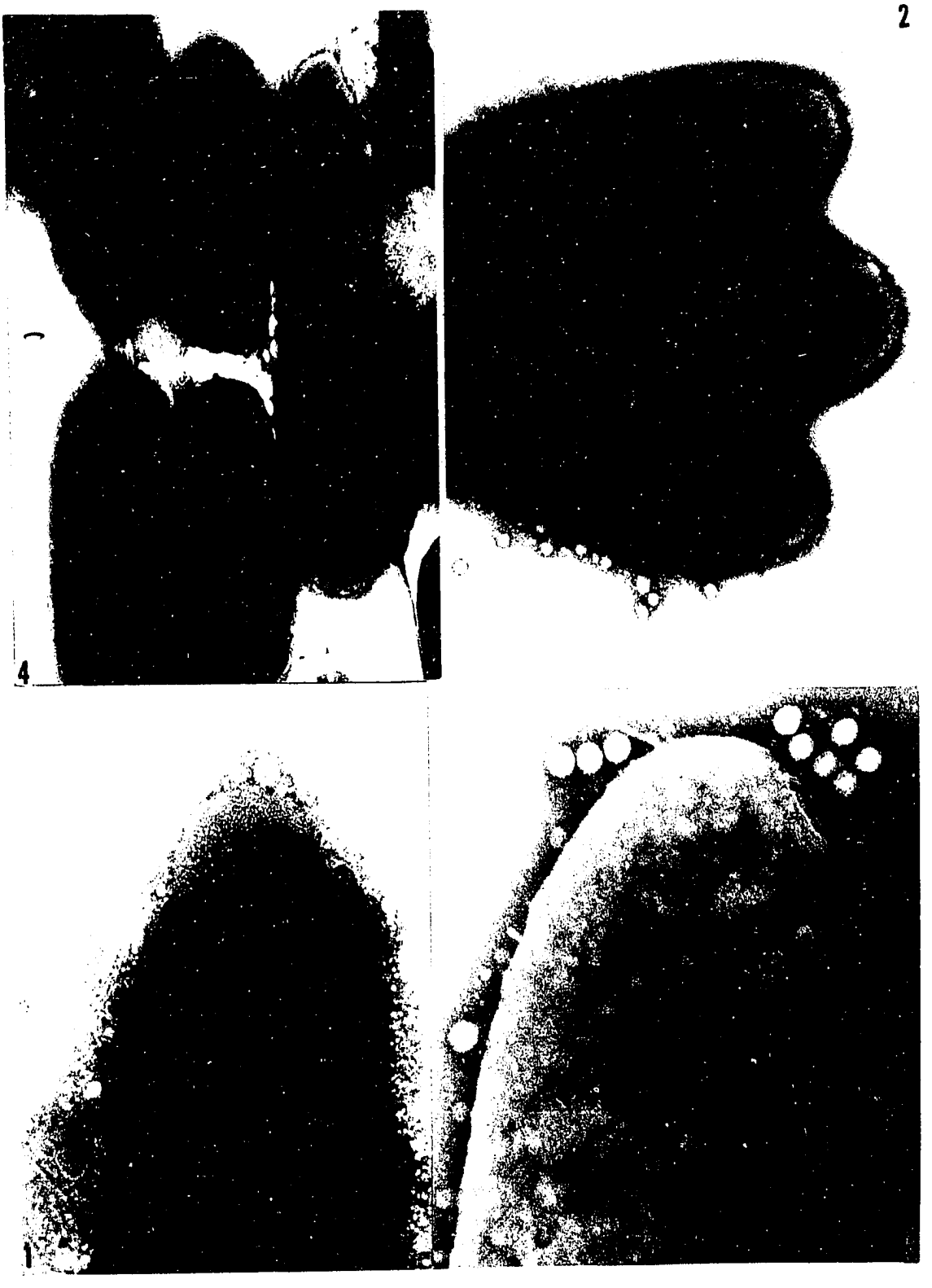
Figure 5

1 Strain H of Pseudomonas aeruginosa with phage 3H at time t 6 min, serum was used in this experiment. The ratio of phage to bacteria was 0.1. It is suggested that it "stained" bacterial exudates. X 51,450.

2 Strain H of Ps aeruginosa with phage 3H at time t 10 min, serum was used in this experiment, the ratio of phage to bacteria was 30/1. The phage not attached to the bacteria must have been liberated during the preparation of the grid. X 44,800.

3 Strain H of Ps aeruginosa with phage 3H at time t 15 min, no serum was used in this experiment, the ratio of phage to bacteria was 3/1. The length of the tails is of interest some tail like objects may be debris. X 103,600.

4 Strain H of Ps aeruginosa with phage 3H at time t 20 min, no serum was used in this experiment, the ratio of phage to bacteria was 3/1. Both intact and degenerating cells are present, at this time degeneration is probably due to cell wall damage rather than to bursts. X 34,000.


2
4
1

Graph 1

The growth of Pseudomonas aeruginosa strain P1.

Curve a: Growth at 37 C with shaking.

Curve b: Growth at 31 C without shaking.

Graph 2

The growth of Pseudomonas aeruginosa strain H.

Curve a: Growth at 37 C with shaking.

Curve b: Growth at 31 C without shaking.

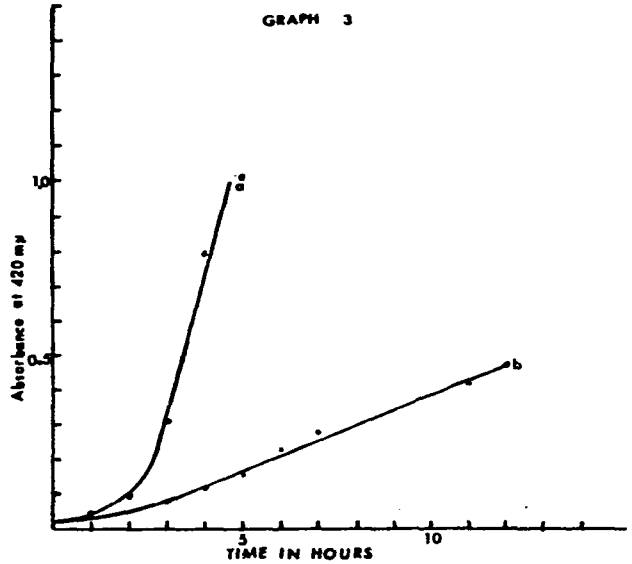
Graph 3

The growth of Pseudomonas aeruginosa strain 5.

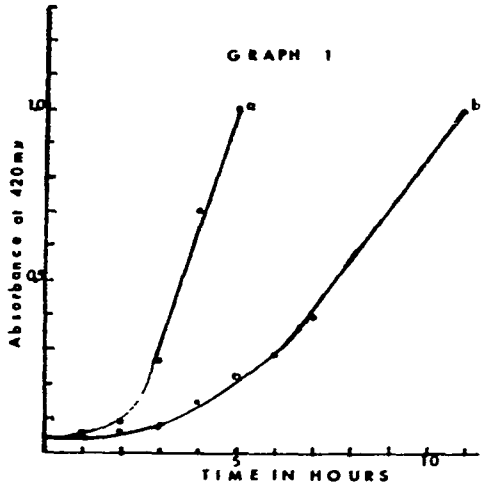
Curve a: Growth at 37 C with shaking.

Curve b: Growth at 31 C without shaking.

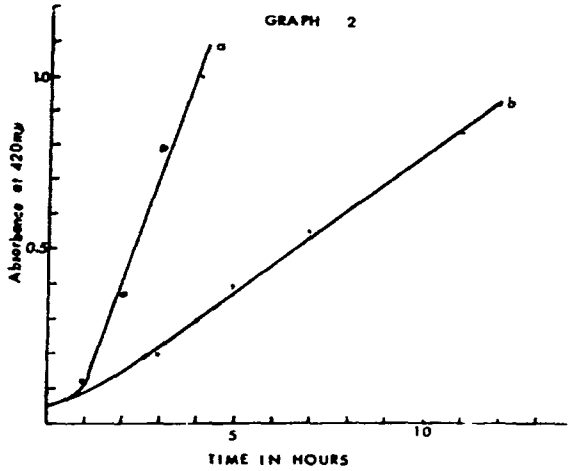
GRAPH 3



GRAPH 1



GRAPH 2

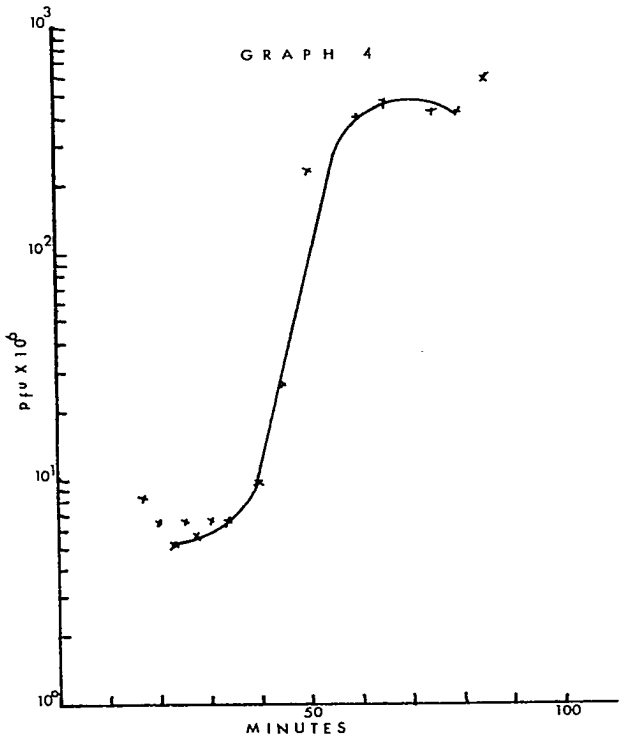


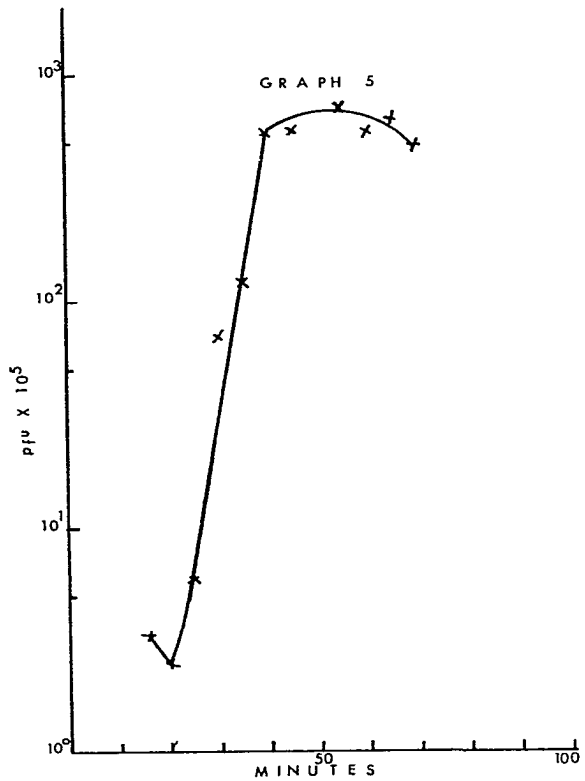
Graph 4

One step growth curve of bacteriophage VI grown in *Pseudomonas aeruginosa* strain P1.

Graph 5

One step growth curve of bacteriophage 2H grown in *Pseudomonas aeruginosa* strain H.


GRAPH 4
10^3
10^2
10^1
10^0
pfu X 10^6
50
100
MINUTES


GRAPH 5
10^3
10^2
10^1
10^0
pfu X 10^5
50
100
MINUTES

Graph 6

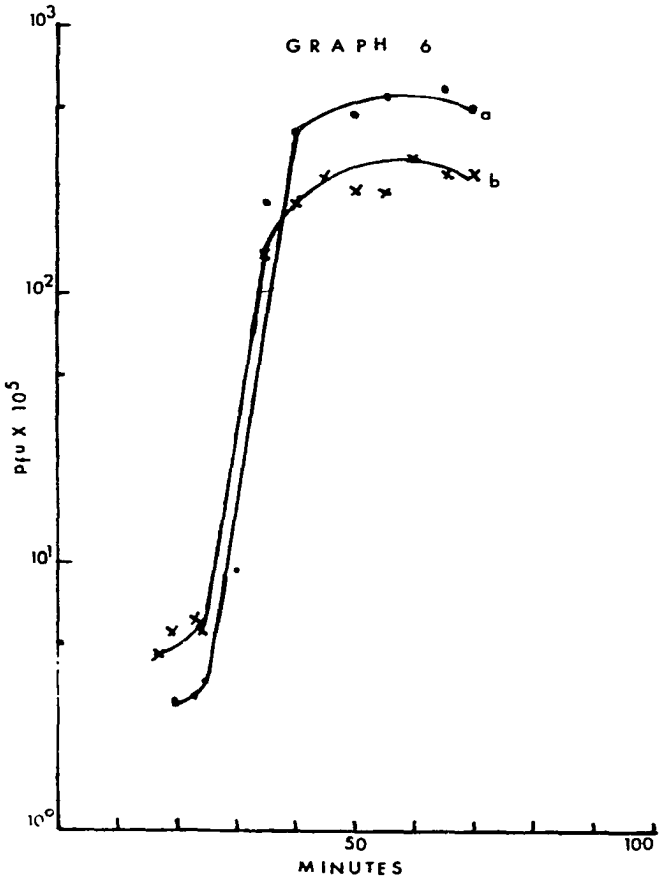
One step growth curve of bacteriophage 3H grown in *Pseudomonas aeruginosa* strain H.

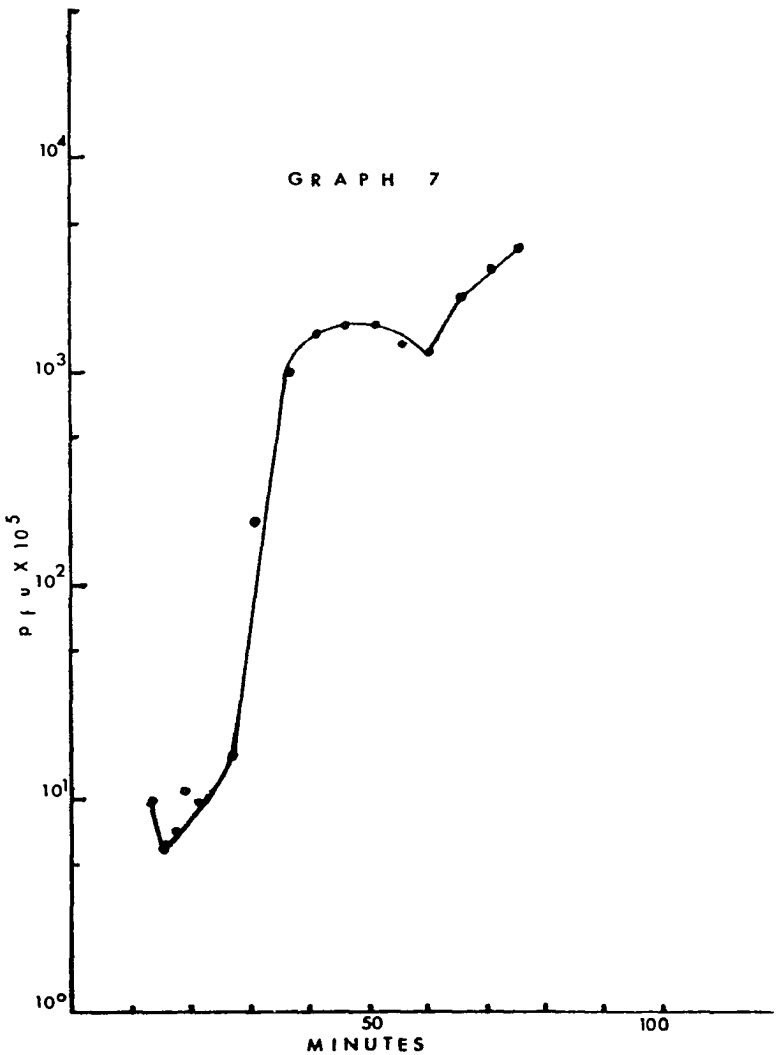
Curve a: The typical experiment.

Curve b: Modified to increase the number of infective centers during the latent period.

Graph 7

One step growth curve of bacteriophage 3H grown in *Pseudomonas aeruginosa* strain 5.


GRAPH 6
10^3
10^2
10^1
10^0
pfu X 10^5
a
b
50
100
MINUTES


GRAPH 7
10^4
10^3
10^2
10^1
10^0
pfu X 10^5
50
100
MINUTES

Graph 8

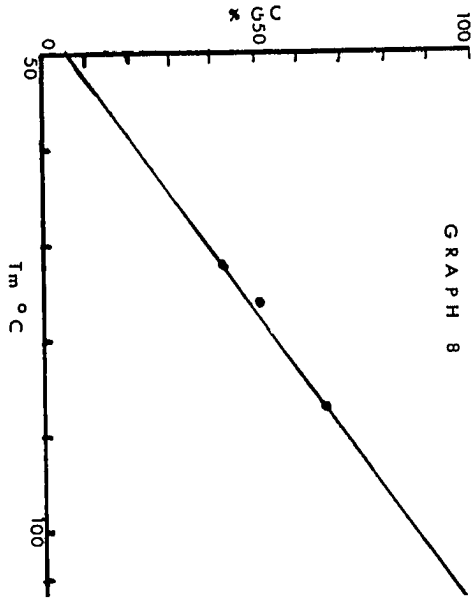
The standard curve of Tm against the percent of guanine plus cytosine in deoxyribose nucleic acids. The sources of nucleic acid are Escherichia coli, Pseudomonas aeruginosa and calf thymus.

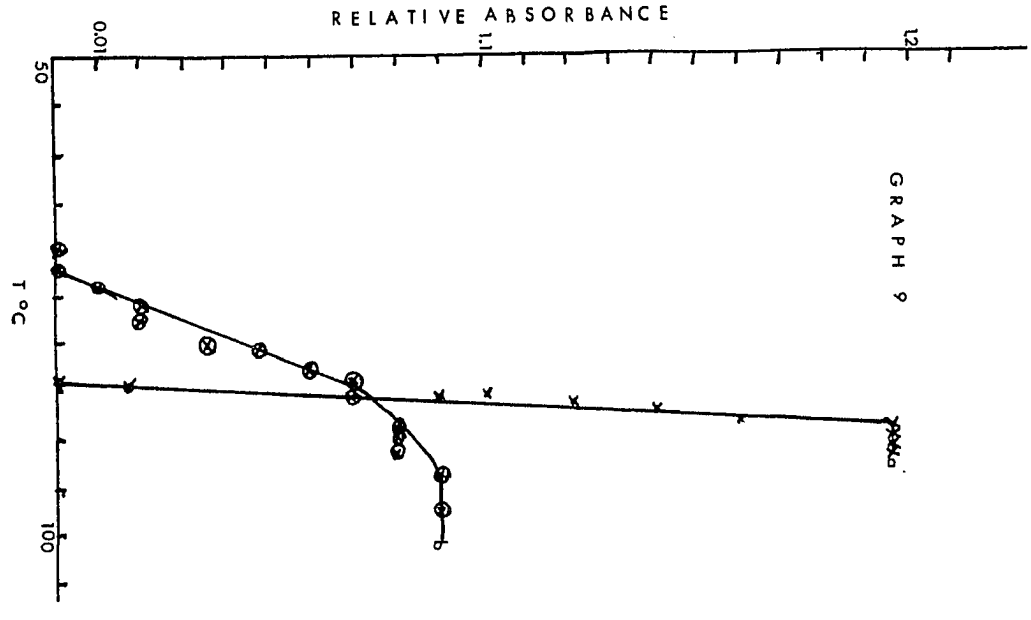
Graph 9

The thermal denaturation profile of the nucleic acid from bacteriophage VI.

Curve a: The nucleic acid was extracted by the phenol micro method.

Curve b: The nucleic acid was extracted by the modification of Freifelder's technic.


% C 550
0
50
100
Tm °C
100
GRAPH 8


RELATIVE ABSORBANCE
0.01
1.1
1.2
50
T °C
100
GRAPH 9

Graph 10

The thermal denaturation profile of the nucleic acid of bacteriophage 3H.

Curve a: The nucleic acid was extracted by the phenol-micro technic.

Curve b: The nucleic acid was extracted by the modification of Freifelders technic.

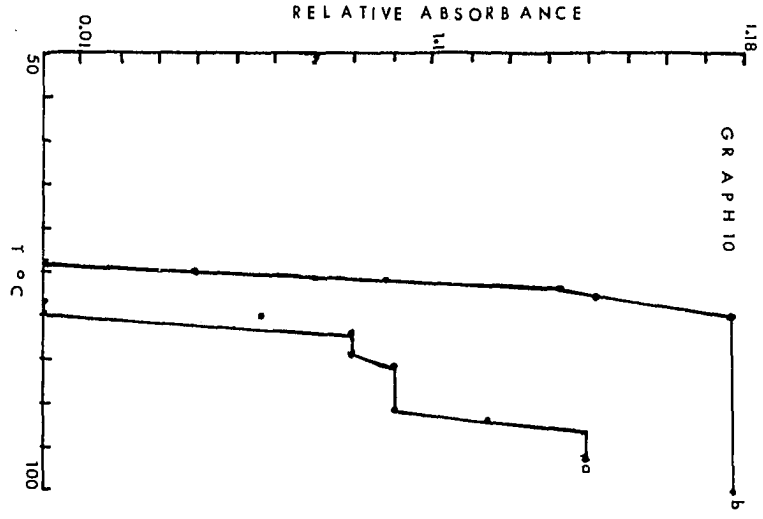
Graph 11

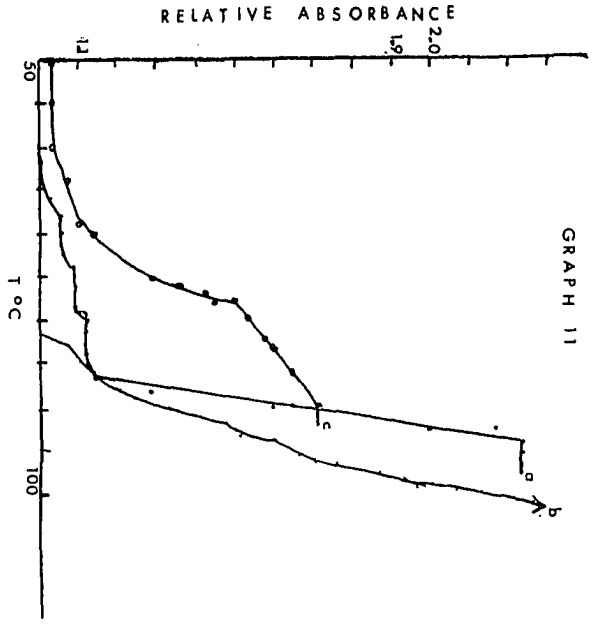
The thermal denaturation profile of the nucleic acid of bacteriophage 2H.

Curve a: The nucleic acid was extracted by the phenol-micro technic.

Curve b: The nucleic acid was extracted by the modified Freifelder's technic.

Curve c: The nucleic acid was extracted by the modified Freifelder's technic but re-suspended in the saline citrate buffer, it was diluted in the same buffer as the other nucleic acid preparation at the time the denaturation experiment was made.


RELATIVE ABSORBANCE
0.01
1.1
1.18
50
T °C
100
a
b
GRAPH 10


RELATIVE ABSORBANCE
1.1
1.6
2.0
50
T °C
100
a
b
c
GRAPH 11