CHARACTERIZATION OF BACTERIOPHAGE ϕh-1 FOR *PSEUDOMONAS PUTIDA*

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ABSTRACT

CHARACTERIZATION OF BACTERIOPHAGE gh-1 FOR PSEUDOMONAS PUTIDA

by Lucy Fang Lee

Bacteriophage gh-1 for Pseudomonas putida A.3.12 was isolated and purified by differential centrifugation and diethylaminoethyl (DEAE) cellulose chromatography. An electron micrograph of the phage stained with uranyl acetate revealed a regular hexagonal outline about 50 mμ across with a short, wedge-shaped tail attached at one corner of the head. The phage formed 10% as many plaques on P. putida C1S as on P. putida A.3.12, the organism used in the isolation procedure No plaques were formed on P. fluorescens (ATCC 9712) or P. aeruginosa. The latent period of the infectious cycle was 21 minutes and the average burst size was 103.

The nucleic acid component of gh-1 bacteriophage was found to be deoxyribonucleic acid (DNA) by a positive diphenylamine reaction a negative orcinol test, and by its susceptibility to deoxyribonuclease but not ribonuclease. The double-stranded character of gh-1 DNA was demonstrated by the sharpness of the rise and the extent of the hyperchromic effect in the thermal denaturation studies. Furthermore, chemical analysis of the DNA base composition showed that its mole per cent adenine (A) equaled thymine (T) and mole per cent guanine (G) equaled cytosine (C). In addition, the buoyant density of heat denatured gh-1 DNA was found to

be 0.014 g/cm^3 higher than that of its native form. This difference in buoyant density is that expected between duplex DNA and its single-stranded derivative.

The base composition of gh-1 DNA was established to be 57% GC by direct chemical analysis of its individual bases. This value agreed with that deduced from the thermal denaturation profile studies and with that calculated from the buoyant density measurement.

The buoyant density of gh-1 phage measured by cesium chloride equilibrium centrifugation was 1.45 g/cm^3, whereas that of gh-1 DNA, heat-denatured gh-1 DNA, and P. putida A.3.12 DNA was 1.716, 1.730, and 1.722 g/cm^3 respectively. The sedimentation coefficients, $S^0_{20,w}$, of gh-1 and phenol-extracted gh-1 DNA measured by the moving boundary sedimentation velocity method were 430-460 and 30.9 respectively. The molecular weight of gh-1 DNA calculated from the sedimentation coefficient was $22.6 \pm .9 \times 10^6$.

Studies of zone sedimentation of single-stranded polynucleotide chains derived from differentially labeled gh-1 and T_7 bacteriophage were performed in alkaline sucrose gradient. The results indicated that the molecular weight of the single-stranded polynucleotide chains of gh-1 DNA is proximate to that of single chains derived from T_7 DNA, and that duplex gh-1 DNA, like T_7 DNA, consists of two linear uninterrupted polynucleotide chains. Further evidence for gh-1 DNA being an intact linear duplex molecule

rather than a circular duplex molecule came from the observation that the buoyant density of heat denatured gh-1 DNA was 0.014 g/cm^3 greater than that of native DNA. Finally, the electron micrograph of gh-1 DNA clearly showed the molecule to be a linear DNA with two ends.

CHARACTERIZATION OF BACTERIOPHAGE gh-1 FOR *PSEUDOMONAS PUTIDA*

By

Lucy Fang Lee

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INTRODUCTION

One area of research which has engaged the general interest of our laboratory has been the study of RNA metabolism in uninfected and phage-infected bacteria. As one of the steps in the initiation of these studies in Pseudomonas putida, a number of bacteriophages were isolated. The present study describes the isolation and characterization of one of these bacteriophages, gh-1, for P. putida A.3.12.

Bacteriophages of the genus Pseudomonas have not been fully characterized. Excepting a few phages of P. aeruginosa (1,2,3,4,5), no phages of other species of Pseudomonas have been the subject of a detailed analysis. Some preliminary studies have been conducted by Niblack and Gunsalus (6) concerning bacteriophage Pf, the host of which is P. putida C1S. Another study dealing with the host range specificity of P. fluorescens phages (7) has also been reported.

Bacteriophage gh-1, as compared to other phages of P. putida A.3.12 isolated by us, forms a giant plaque, suggesting a small virus with a small nucleic acid molecule. It immediately received our attention. Initial cesium chloride centrifugation analysis of gh-1 lysates revealed two infectious components. We chose to study the heavier component and gave it the name gh-1. We have since found that both of these infectious components were the same phage.

Bacteriophages are classified as either deoxyribonucleic acid (DNA)-containing or ribonucleic acid (RNA)-containing, according to their nucleic acid components. Most of the phages belong to the former class. Within this class a great variation in DNA structure and chemical composition has been observed. The DNA of T-even phages, like most DNA, is double-stranded, but it differs from others in that it contains hydroxymethylcytosine instead of cytosine (8). The DNA of SP-8 phage for B. subtilis contains hydroxymethyluracil in place of thymine (9); and the DNA of PBS—1 phage for B. subtilis contains uracil in place of thymine (10). Coliphages ΦX-174 and S13 contain single-stranded DNA (11, 12). Finally, coliphages MS-2 and f_2 contain RNA as its genetic material rather than DNA (13,14).

Studies on viral DNA have revealed a great variation in the conformation of DNA molecules. The majority of phage DNA molecules studied to date have a linear double-helical structure (15,16,17). The DNA isolated from coliphage lambda is a linear duplex molecule with unpaired, single-stranded ends. The nucleotide sequences of these single-stranded regions are complementary to each other. The linear duplex molecule can form a circular structure by interation of the unpaired ends (18) The single-stranded DNA molecule of ΦX-174 is circular (19). Rodent polyoma virus contains a circular duplex in a highly twisted conformation (20,21). Other circular twisted duplex structures have been reported for human papilloma virus (22),

Shope rabbit papilloma virus (23) and simian 40 virus (24). The DNA from coliphage T_5 has recently been found to contain single-strand breaks, or gaps, in both of the duplex molecules (25).

The relationship between the sedimentation coefficient and the molecular weight of DNA molecules ($s = kM^a$) has been studied by various groups of investigators. Doty, McGill, and Rice were the first to define this relationship by use of the moving boundary sedimentation method in an analytical ultracentrifuge (26). Burgi and Hershey, on the other hand, used zone sedimentation in sucrose gradient (27), and Studier used zone sedimentation in an analytical ultracentrifuge (28). The viral DNA molecules so far studied fall within a range of molecular weights between 1.5 -130 million. T_2 and T_7 DNA, the molecular weights of which have been established as 130×10^6 and 25×10^6 respectively, have been used as standards for calibrating the molecular weights of unknown DNA molecules (29).

T_2 and T_7 have further been shown to contain one DNA molecule per phage particle (30,31,32). Current researchers assume that this is generally true of all viruses. Rubenstein and Thomas have demonstrated that the total nucleic acid content of T_2 phage equaled the total mass of the isolated T_2 DNA molecule. Further, it was found that both the T_2 particles and their isolated T_2 DNA molecules contained 130×10^6 daltons of nucleic acid (30). Similar results were obtained for T_7. Davidson and Freifelder

showed that the isolated T_7 DNA molecule has a molecular weight of 25×10^6 and is the entire nucleic acid content of the T_7 virus particle (32). Studies of a dozen or more other DNA-containing viruses have led to the general conclusion that each virus particle contains a single nucleic acid molecule (29).

Phage structure and phage nucleic acid material often define the mode of phage infection and replication. Knowledge in this regard comes mainly from the studies on T phages for E. coli. A typical T-even phage, for example, possesses a bipyramidal hexagonal prism head wherein the DNA is located (33) and a complex tail the function of which is to serve as an adsorption and injection apparatus to the host (34). The injection of viral nucleic acid material into a host cell has been well documented (35). Once the viral nucleic acid gains entrance into the host cell, it performs two principal functions. First, it programs the synthesis of enzymes used in the replication of the viral nucleic acid; second, it directs the synthesis of viral structural proteins. The end result of viral infection is, therefore, the production of many new copies of both viral nucleic acids and of the structural proteins. By some mechanism yet to be thoroughly understood, the new progeny molecules are assembled to form mature virus particles. Following the rupture of the host cell, the phage progeny are released. The yield is usually between 100 to 10,000-fold.

The objective of this report is to give a detailed description of the isolation and purification procedure for gh-1 bacteriophage and the characterization of some selected physical, chemical, and biological properties, with emphasis on the nucleic acid component.

MATERIALS AND METHODS

Organisms and Growth Medium

Pseudomonas putida A.3.12 (kindly supplied by Dr. W. A. Wood, Michigan State University) was used as the host for bacteriophage gh-1. *P. putida* A.3.12 was previously designated *P. fluorescens* (36). The organism was grown at 33°C on a gyrorotary shaker, or in a micro-fermentor, in a medium containing the following, in grams per liter: yeast extract, 5; glucose, 4; NaCl, 8; $(NH_4)_2 \cdot HPO_4$, 6; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 1; and $FeCl_3$, 0.005.

Escherichia coli B was grown at 33°C in basal C medium (37) with 0.4% glucose to serve as host for bacteriophage T_7 Coliphage T_7 used in this study was obtained from Dr. D. Schoenhard, Michigan State University. In the preparation of ^{14}C-thymidine-labeled T_7 bacteriophage, a thymine-requiring mutant of *E. coli* B (kindly supplied by Dr. R. Greenberg, University of Michigan) was used as host. The mutant was grown at 37°C in the basal C-glucose medium supplemented with 1-2 μg/ml of thymidine.

In the study of host specificity, *P. fluorescens* (ATCC 9712), *P. aeruginosa*, and *P. putida* C1S were used. *P. putida*, C1S was kindly supplied by Dr. I. C. Gunsalus, University of Illinois.

Isolation of gh-1 Bacteriophage for P. putida

Bacteriophage gh-1 was isolated from a sample taken from the aeration tank at the Waste Water Treatment Plant in East Lansing, Michigan in the summer of 1965.

The sample was centrifuged to remove debris and filtered through an ultrafine sintered-glass filter with a maximum pore size between 0.9-1.4 μ. A sample of the filtrate was added to an exponentially growing culture of P. putida A.3.12. After incubation overnight, the culture was centrifuged at 4,000 x g for 10 minutes. A sample of the supernatant fluid was tested for bacteriophage content by the agar layer technique. A well-isolated plaque of bacteriophage gh-1 was picked and replated several times to ensure genetic homogeneity.

Assay Techniques for Bacteriophage gh-1 and P. putida

Bacteriphage gh-1 was assayed by the agar layer technique of Adams (38). The technique consisted in introducing a drop of host bacteria and a sample of diluted phage, containing approximately 50 to 100 plaque-forming units (PFU), into 2.5 ml of growth medium containing 0.6% agar maintained at 45°C. This mixture was then poured onto the surface of Petri plates containing a hardened layer of 2% agar and growth medium. After being incubated at 33°C for 4 hours, the plates were read for the number of plaques formed. From this count and the dilution factor, the phage titer was calculated.

The concentration of the host organism, P. putida A.3.12, was determined by viable cell count. A sample from the last serial dilution of an exponentially growing culture at a given turbidity was spread over the surface of agar plates with a glass rod. The plates were incubated overnight at 33°C and the number of the colonies counted. From this count, the number of viable cells per milliliter was calculated. A count of 5×10^8 cells/ml gave a turbidity reading of one unit at 660 mμ in the Beckman DU Spectrophotometer. For E. coli B grown in basal C-glucose medium, 5×10^8 cells/ml gave a turbidity reading of 0.5 unit at 660 mμ.

Preparation and Assay of gh-1 Antiserum

Antiserum for gh-1 bacteriophage was prepared according to the procedure of Adams (38). For this preparation, five milliliters of the purified gh-1 containing 5×10^{10} PFU in sterile saline solution (0.8% NaCl) were injected intraperitoneally into each of two rabbits. Two injections per week were given for three weeks, followed by a collection of blood for a potency test one week after the last injection. Blood was drawn from the marginal ear vein and allowed to clot at 37°C. After storage at 5°C overnight, the blood was centrifuged to separate the serum from the red blood cells. The serum thus obtained was assayed for its ability to inactivate gh-1 bacteriophage. Since the blood showed sufficient antibody activity, one rabbit was

then bled by cardiac puncture to obtain a large quantity of serum.

The assay for gh-1 antiserum was performed in the following manner. The gh-1 antiserum was prepared for testing at dilutions of 1:100 and 1:1000. The phage stock was diluted to a titer of 10^7 PFU/ml. A sample of 0.1 ml of this phage dilution was added to 0.9 ml of diluted serum at 37°C. At specified time intervals (t = 2, 5, $7\frac{1}{2}$, 10, 15, 20, 30 minutes), 0.1 ml samples of the phage-serum mixture were withdrawn and diluted 1:100 with growth medium. Duplicate samples of 0.05 ml of this dilution were plated out by the agar layer technique. The fraction of phage remaining after various times of antiserum inactivation was calculated. A plot of the fraction of surviving phage against time on semi-log paper gave a straight line, the slope of which equaled the specific neutralization rate constant, k.

Purification of Bacteriophage gh-1

A 6- to 10-liter amount of P. putida A.3.12 was grown with vigorous aeration to a density of about 5×10^8 cells/ml. Bacteriophage at a multiplicity of about 5 were added. After 3 to 4 hours the lysed culture was centrifuged at 4,000 x g for 10 minutes to remove bacteria and cell debris. The titer of the lysates was about 2.5×10^{10} PFU/ml. The virus was collected either by centrifugation at 16,000 x g for 2 hours or by precipitation with ammonium sulfate at

50% saturation. The preparation was then suspended in buffer containing 0.05 M tris(hydroxymethyl)aminomethane (Tris) adjusted to pH 8.0 with HCl, 0.001 M $MgCl_2$, and 0.2 M NaCl. The suspension was centrifuged at low speed, and the supernatant fraction was loaded on a diethylaminoethyl (DEAE) cellulose column (3 by 17 cm) equilibrated with 0.05 M Tris chloride buffer (pH 8.0) containing 0.2 M NaCl. At this salt concentration most contaminating material is adsorbed on the column, but the phage is not. The fractions containing gh-1 were concentrated by centrifugation, and the resulting pellets were resuspended in the buffer described above. The yield of plaque-forming units was between 25 and 50%. Purified gh-1 at a concentration of 10^{12} to 10^{13} PFU/ml was stored at 5^0C.

Preparation of ^{32}P-Labeled gh-1 and ^{14}C-Thymidine-Labeled T_7

P. putida A.3.12 was grown exponentially to a cell density of 5 x 10^8 cells/ml in a medium containing 0.5% yeast extract and 0.5% tryptone. ^{32}P-phosphoric acid neutralized with NaOH (carrier free, obtained from Tracer lab, Waltham, Mass.) at 5 μc/ml was added to the growth medium. Thirty minutes later, gh-1 at the multiplicity of 2 was added. Lysis of bacteria occurred within four hours. The radioactive bacteriophages were purified according to the procedure described previously. In view of the damage to DNA structure which could be caused by the decay of the radioisotope, the purification procedure and analysis were

carried out immediately. The assay of radioactivity will be described in the later part of this section. The specific activity of gh-1 bacteriophage was found to be 1.1×10^5 cpm/A^{260}. The absorbancy readings were not corrected for the amount due to light scattering.

E. coli B thy$^-$ was grown to a density of 5×10^8 cells/ml. The culture was centrifuged, washed, and suspended at a concentration of 5×10^8 cells/ml in the growth medium minus thymidine. Radioactive [2-^{14}C] thymidine (specific activity of 30 μc/mmole, obtained from Tracerlab) at a concentration of 0.25 μc/ml was added to the culture, followed immediately by the addition of bacteriophage T_7 at a multiplicity of 0.5. The culture lysed in 4-5 hours. T_7 was purified by a series of differential centrifugations at 4,000 x g for 10 minutes followed by 16,000 x g for 2 hours. The specific activity of the purified T_7 was found to be 1.2×10^5 cpm/A^{260}.

One Step Growth Experiment

The procedure described by Adams (38) was used. The host bacteria for this experiment were grown at 33°C to a concentration of about 5×10^8 cells/ml. Bacteriophages at a multiplicity of 1 to 2 were added. After allowing 5 minutes for adsorption, the culture of the infected bacteria was diluted 1:20 into the gh-1 antiserum. The concentration of gh-1 antiserum used was sufficient to inactivate 95 to 99% of the free virus particles in 5 minutes.

After a five minute incubation period in gh-1 antiserum, the infected culture was diluted 1:500 into the first growth tube. Immediately a sample from the first growth tube was further diluted 1:100 into the second growth tube. Both infected cultures were incubated at 33°C. Every 2.5 minutes for the first 25 minutes samples were removed from the first growth tube. For the next 35 minutes, samples were taken from the second growth tube at 5-minute intervals. The samples thus obtained were assayed for plaque-forming units.

Preparation of gh-1 Bacteriophage and gh-1 DNA for Electron Microscopy

To prepare for electron microscopy, the purified phage preparation was dialyzed against 0.1 M ammonium acetate at 4°C overnight. The dialyzed phage suspension was then mixed with an equal volume of 1% (w/v) uranyl acetate solution (39). A drop of the phage suspension was transferred to a carbon-coated supporting grid and then examined under a Siemens Elmiskop 1 electron microscope at a magnification of 40,000. These experiments were performed in Dr. Hans Ris' laboratory at the University of Wisconsin. Preliminary electron microscopic studies of the virus particles were performed with the RCA EMU-2 in the Biochemistry Department at MSU and the Hitachi electron microscope in the Department of Microbiology. For such electron microscopic investigations, the virus was negatively stained with 2% (w/v)

phosphotungstic acid solution adjusted to pH 7.0 with 1 N KOH (40).

For the preparation of gh-1 DNA for electron microscopy, phenol extracted DNA was diluted to 5 μg/ml. This diluted sample was then mixed with an equal volume of 1 M ammonium acetate solution containing 0.01% (w/v) cytochrome C (41). A Teflon Petri plate was filled with a 0.1 M ammonium acetate solution. A wet microscope slide was partially immersed in the solution at an angle of about 30^0. Talcum particles were sprinkled lightly over the surface of the acetate solution in the area near the slide. A sample of 0.3 ml of the DNA-cytochrome C mixture was allowed to flow gently down the glass slide. As the DNA-cytochrome C mixture entered the acetate solution, it pushed the talcum powder away and floated as a thin film on the surface of the solution. The film was compressed lightly with two Teflon rods. A specimen for electron microscopic examination was obtained by gently touching the film with a carbon-coated grid. The grid was then dipped in ethanol and dried on filter paper. The dry specimen was shadowed with uranium at a 6^0 angle while rotated at 60 rev/min. The shadowed preparation was then examined under the Siemens Elmiskop 1 electron microscope at a magnification of about 10,000.

CsCl Equilibrium Centrifugation of Bacteriophage gh-1

Solid cesium chloride (optical grade, obtained from

Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.) was added to a sample of a purified phage preparation containing 1.2×10^{12} PFU (absorbancy, 260 mμ, of 14 units) to give an initial CsCl concentration of 1.50 g/cm^3. The suspension was centrifuged in an SW 39 rotor at 39,000 rev/min in a Spinco Model L-2 Ultracentrifuge at 7°C for 20.5 hours. Fractions were collected from the bottom of the centrifuge tube and analyzed for absorbancy at 260 mμ and for plaque-forming units. The CsCl concentration of various fractions was calculated from refractive index measurements using the equation described by Ifft, Voet, and Vinograd (42).

$$\rho^{25^0C} = 10.8601\ \eta_D^{25^0C} - 13.4974.$$

Purification of gh-1 Deoxyribonucleic Acid (DNA)

A sample of gh-1 containing 1.0×10^{12} PFU/ml (absorbancy, 260 mμ, of 14.3 units/ml) was suspended in 0.5% sodium dodecyl sulfate (pH 7.3) and then stirred with a magnetic stirring device at 5°C for 10 to 15 minutes. An equal volume of phenol was added, and the mixture was stirred for another 10 minutes. After a low-speed centrifugation the aqueous layer was removed. Phenol extraction was repeated, and the purified nucleic acid was dialyzed overnight against 0.01 M Tris chloride (pH 7.3) with 0.1 M KCl, or against SSC (0.15 M NaCl-0.015 M trisodium citrate).

For the analysis of the sedimentation velocity coefficient, DNA was prepared according to the procedure

described by Abelson and Thomas (25). The purified phage preparation was adjusted to a concentration having an absorbancy of 5-15 units/ml and placed in a glass stoppered tube. An equal volume of the freshly distilled water-saturated phenol was added and the tube was rolled in a horizontal position at 60 rev/min for 30 minutes at room temperature. The mixture was centrifuged at 4^0C for 5 minutes at 3,000 rev/min to separate the two phases. The phenol layer was removed with a Pasteur pipette. The procedure was repeated once using a 15-minute rather than a 30-minute extraction. The final aqueous layer was removed after centrifugation and dialyzed extensively with SSC buffer. The dialysis tubing was boiled in 5% sodium bicarbonate before use and washed excessively with distilled water. DNA preparations from P. putida A.3.12 and E. coli were purified as described by Armstrong and Boezi (43).

DNA concentrations were calculated from the absorbancy at 260 mμ by use of an extinction coefficient of 20 cm^2/mg. Diphenylamine reactions were carried out by the method described by Burton (44), while orcinol tests were performed according to the procedure of Mejbaum (45). Heat denaturation of gh-1 DNA was performed in 10-fold diluted SSC at 100^0C for 10 minutes, followed by quick cooling.

CsCl Equilibrium Centrifugation of DNA

CsCl equilibrium centrifugation of gh-1 DNA, heat-denatured gh-1 DNA, and P. putida A.3.12 DNA was performed

according to the procedure described by Schildkraut, Marmur, and Doty (46). The initial concentration of CsCl was 1.710 g/cm^3. E. coli DNA, the buoyant density of which was taken to be 1.710 g/cm^3, was used as the density marker. Centrifugation was performed at 25^{0}C in a Spinco Model E Analytical Ultracentrifuge for 20 hours at 44,700 rev/min. The centrifuge cell was a standard cell fitted with a 1^0 negative wedge window. Tracings from the ultraviolet absorbancy photographs were made by use of a Joyce-Loebl double-beam recording microdensitometer.

Thermal Denaturation Profile of DNA

The thermal denaturation profile of DNA was determined by measuring the absorbancy at 260 mμ at various temperatures. The buffer used in this determination was 0.01 M Tris chloride (pH 7.3) with 0.012 M KCl.

Chemical Analysis of Base Composition

A sample containing 2.5 x 10^{12} PFU of bacteriophage gh-1 was suspended in 0.1 ml of 70% perchloric acid in a small, glass-stoppered test tube. The suspension, after being heated at 100^{0}C for 1 hour with occasional agitation, was diluted to 0.5 ml with water. A black residue was removed by centrifugation. A sample of the hydrolysate was spotted on acid-washed Whatman No. 1 paper. Chromatography was performed by the descending method with isopropanol-HCl-water (65:17:18) as solvent (47). The bases

were located by use of an ultraviolet lamp. After elution from the paper with 0.1 N HCl, the isolated bases were identified, and the amount of each was determined from the ultraviolet absorption spectrum. The base composition of P. putida A.3.12 DNA was determined in a similar manner.

Moving Boundary Sedimentation Velocity Studies

The sedimentation studies of gh-1 and its DNA and T_7 DNA were carried out using the boundary sedimentation velocity method in a Spinco Model E analytical ultracentrifuge with ultraviolet optics. The centrifugations were made at 15,220 rev/min for bacteriophage gh-1 and 42,040 rev/min for DNA. The centrifuge cell was a standard cell fitted with a Kel F centerpiece. The temperatures of the runs were set variously at 18 to 22°C. After reaching the maximal speed, ultraviolet absorbancy photographs were taken for gh-1 at 4-minute intervals and for gh-1 DNA and T_7 DNA at 8-minute intervals. The ultraviolet absorbancy photographs were transcribed into a density-versus-distance plot by means of a Joyce-Loebl double-beam recording microdensitometer. The sedimentation coefficients of gh-1 and T_7 DNA were measured in 1 M NaCl at a DNA concentration of 15 to 50 μg/ml and in SSC at a concentration of 25 to 75 μg/ml for gh-1 bacteriophage. The sedimentation coefficient was corrected to standard conditions and is reported as $S_{20,w}$.

Zone Sedimentation of DNA Through Alkaline Sucrose Gradients

Sedimentation of DNA in alkaline sucrose was performed according to the procedure of Abelson and Thomas (25). A gradient of 5% to 20% sucrose (w/v) in 0.9 M-NaCl and 0.1 M-NaOH was prepared at 20°C. A sample of the radioactive bacteriophage was mixed with an equal volume of 0.2 M-Na_3PO_4 solution and allowed to stand for 10 minutes at 20°C in order to assure the complete release of DNA from the phage head. A 0.1 ml or 0.2 ml sample of the mixture containing approximately 1 to 2 μg/ml of DNA was layered onto the top of the 4.8 ml sucrose gradient. A 2-ml serological pipette was used to layer the DNA mixture gently onto the sucrose gradient. The pipette was supported mechanically and controlled with a screw-driven pipetting device. The mixture was then centrifuged at 20°C in a SW 39 swinging bucket rotor in a Model L-2 Spinco Ultracentrifuge for $2\frac{1}{2}$ to 3 hours at 35,000 rpm. At the end of the run, a hole was punched at the bottom of the centrifuge tube and seven drops per fraction were collected. The average size drop was 15.5 μl ± 0.1 μl. The radioactivity of each fraction was assayed according to the procedure described below.

Radioactivity Assaying Procedure

The general procedure for assaying radioactivity used throughout this investigation began with the addition of 250 μg of salmon sperm DNA to each sample followed by precipitation with cold 10% trichloroacetic acid (TCA). The

TCA insoluble material of each sample was collected by filtering through a nitrocellulose membrane filter (Carl Schleicher & Schuell Co., Keene, N.H.). The filter was dried at 95^{0}C for 10-20 minutes. The radioactive content was assayed in a liquid-scintillation spectrometer with a fluor containing either 0.1 g POPOP (1,4-bis-[2-(5 phenyl-oxazolyl)]-Benzene) and 4.0 g PPO (2,5-diphenyloxazole) per liter of toluene or 4.0 g BBOT (2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-Thiophene) per liter of toluene. The gain and window discriminator settings for each of the isotopes were as follows: (1) ^{3}H-gain 58% and window discriminator ratio 50-1000; (2) ^{32}P, 1.3% and 100-1000; and (3) ^{14}C, 24% and 50-800. In experiments where the ^{14}C and ^{32}P content of a sample was assayed simultaneously, an overlapping of 8% of the ^{32}P counts was found in the ^{14}C channel with less than 1% of the ^{14}C counts in the ^{32}P channel. Appropriate calculations were made to account for this fact.

RESULTS

Plaque Morphology and Host Specificity

Bacteriophage gh-1 formed a clear, smooth plaque, 4 to 6 mm in diameter, on *P. putida* A.3.12. The plaque morphology is shown in Figure 1. Bacteriophage gh-1 attacked *P. putida* C1S, forming about 10% as many plaques as on *P. putida* A.3.12. No plaques were formed on *P. fluorescens* (ATCC 9712) or on *P. aerugenosa*. Klinge (7) has made an extensive study of the host specificity of *Pseudomonas* phages. He isolated 29 different bacteriophages for *P. fluorescens* and three for *P. putida*. None of these phages lysed *P. aeruginosa*. Two *P. fluorescens* phages lysed *P. putida* and no *P. putida* phages lysed *P. fluorescens*.

An ultraviolet spectrum of purified gh-1 bacteriophage is shown in Figure 2. Only preparations of gh-1 which had been purified through the DEAE-cellulose fractionation step exhibited such a spectrum with an absorption maximum at about 260 mμ and a minimum at 240 mμ. The 260-280 mμ absorbancy ratio was 1.56 and the 260-240 mμ ratio was 1.36.

Kinetics of gh-1 Antiserum Neutralization

The kinetics of neutralization of gh-1 by antiserum is illustrated in Figure 3. The rate at which a phage preparation is neutralized by antiserum obeys the following relationship

Figure 1. Plaque morphology of gh-1 bacteriophage.

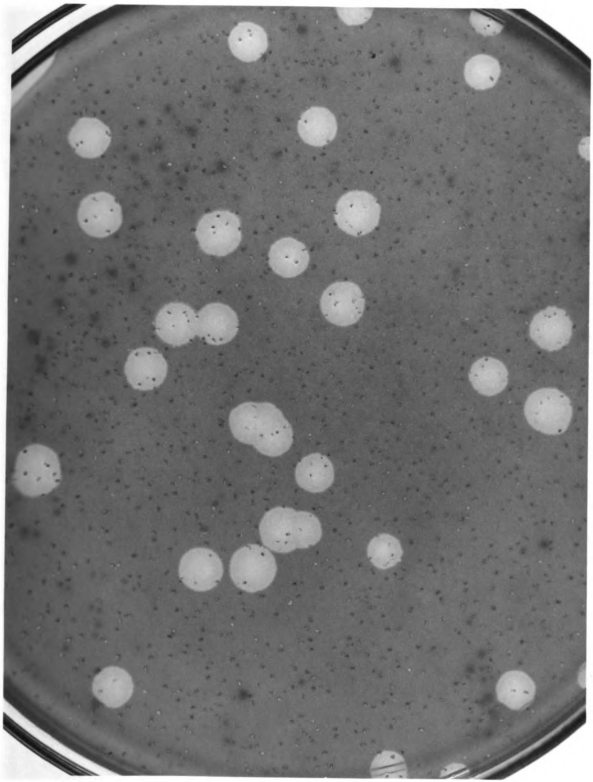


Figure 1.

Figure 2. Ultraviolet absorbancy spectrum of purified gh-1 bacteriophage.

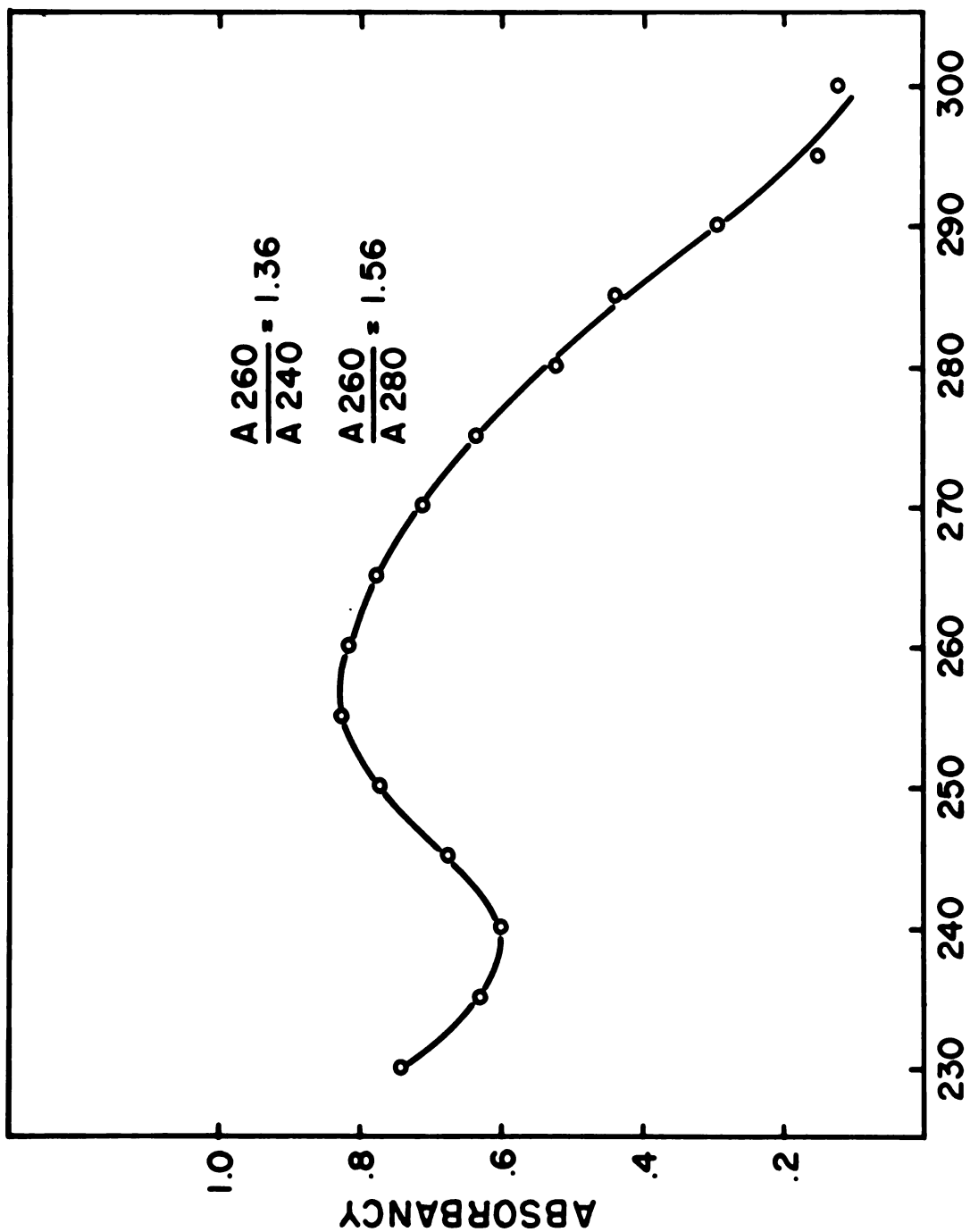

ABSORBANCY
1.0
.8
.6
.4
.2
230
240
250
260
270
280
290
300
WAVELENGTH, mμ
A 260 / A 240 = 1.36
A 260 / A 280 = 1.56

Figure 2.

Figure 3. Kinetics of neutralization of gh-1 bacteriophage by gh-1 antiserum.

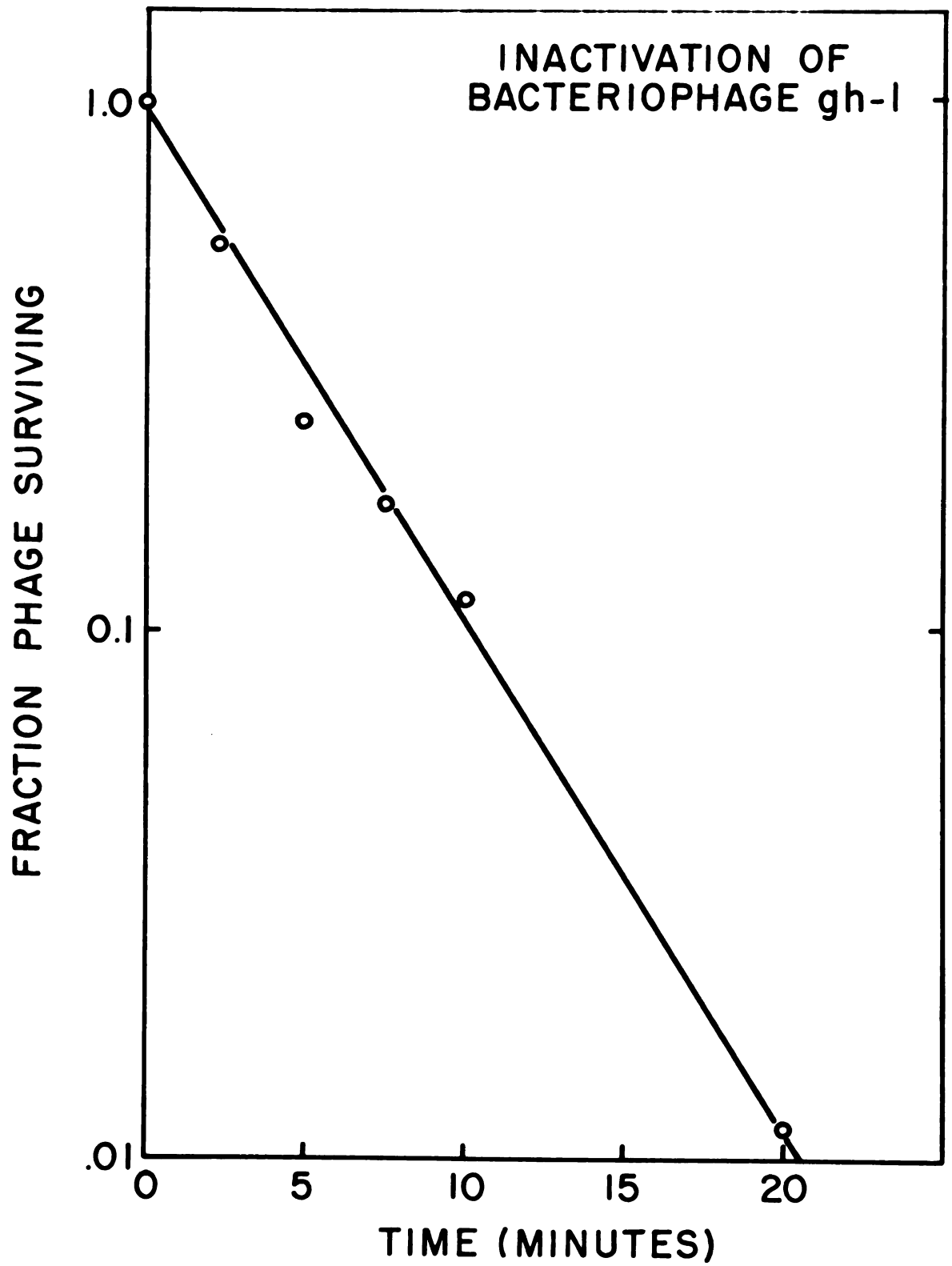

INACTIVATION OF
BACTERIOPHAGE gh-1
FRACTION PHAGE SURVIVING
1.0
0.1
.01
0
5
10
15
20
TIME (MINUTES)

Figure 3.

$$- dP/dt = kPC$$

where - dP/dt is the rate of inactivation of phage per unit time, C is the concentration of antiserum, P is the titer of phage, and k is the specific neutralization rate constant. Integrating the above equation between P_0 and P, t_0 and t, we obtain

$$\ln P_0/P = kCt$$

or

$$\log P_0/P = 0.43\ kCt$$

where P_0 is the initial phage titer at zero time (t_0), and P is the titer after t minutes of incubation with an antiserum of concentration C. Since the dilution factor D (= 1/C) of antiserum is generally known, the equation can be rewritten as

$$\log P_0/P = 0.43\ kt/D$$

The value k, the specific neutralization rate constant, can be obtained from any one measurement of inactivation by use of the above formula, or from a plot of the fraction of surviving phage against time on semi-log paper. Such a plot gives a straight line, the slope of which is k. The k determined from Figure 3 was 222. With this k value for a given antiserum it is possible to calculate the dilution of antiserum required to produce a desired amount of phage inactivation in a given time period.

One Step Growth Experiment

A one-step growth experiment was performed to determine the length of the latent period and the average burst size

of the infectious cycle. The results are given in Figure 4. The latent period, which is defined as the interval of time between adsorption of the virus to the host and lysis, was found to be about 21 minutes. The average burst size, calculated by dividing the number of plaque-forming units present after lysis by that present before lysis, was 103.

Bacteriophage Morphology

An electron micrograph of gh-1 stained with 1% uranyl acetate is presented in Figure 5. The nucleocapsid is of a regular hexagonal outline of about 50 mμ across. A short, wedge-shaped tail attached at one corner of the head can be seen. Two fibers attached to the wedge-shaped tail are visible on the bacteriophage at the center of Figure 5. The morphology of gh-1 is similar to that of coliphage T_3 described by Bradley and Key (48), that of T_7 reported by Davison and Freifelder (49), and that of bacteriophage Pf for *P. putida* C1S reported by Niblack and Gunsalus (6). The nucleocapsid of coliphage T_3, for example, has a hexagonal cross section of about 55-65 mμ and has a short wedge-shaped tail, 14 mμ long. The nucleocapsid of coliphage T_7 is about 59-65 mμ across with a small tail. Bacteriophage Pf for *P. putida* C1S has a polyhedral head of 54 mμ diameter and a 10 mμ conical tail.

CsCl Equilibrium Centrifugation of gh-1

An analysis of a purified gh-1 preparation by equilibrium

Figure 4. One step growth experiment of bacteriophage gh-1 on P. putida A.3.12.

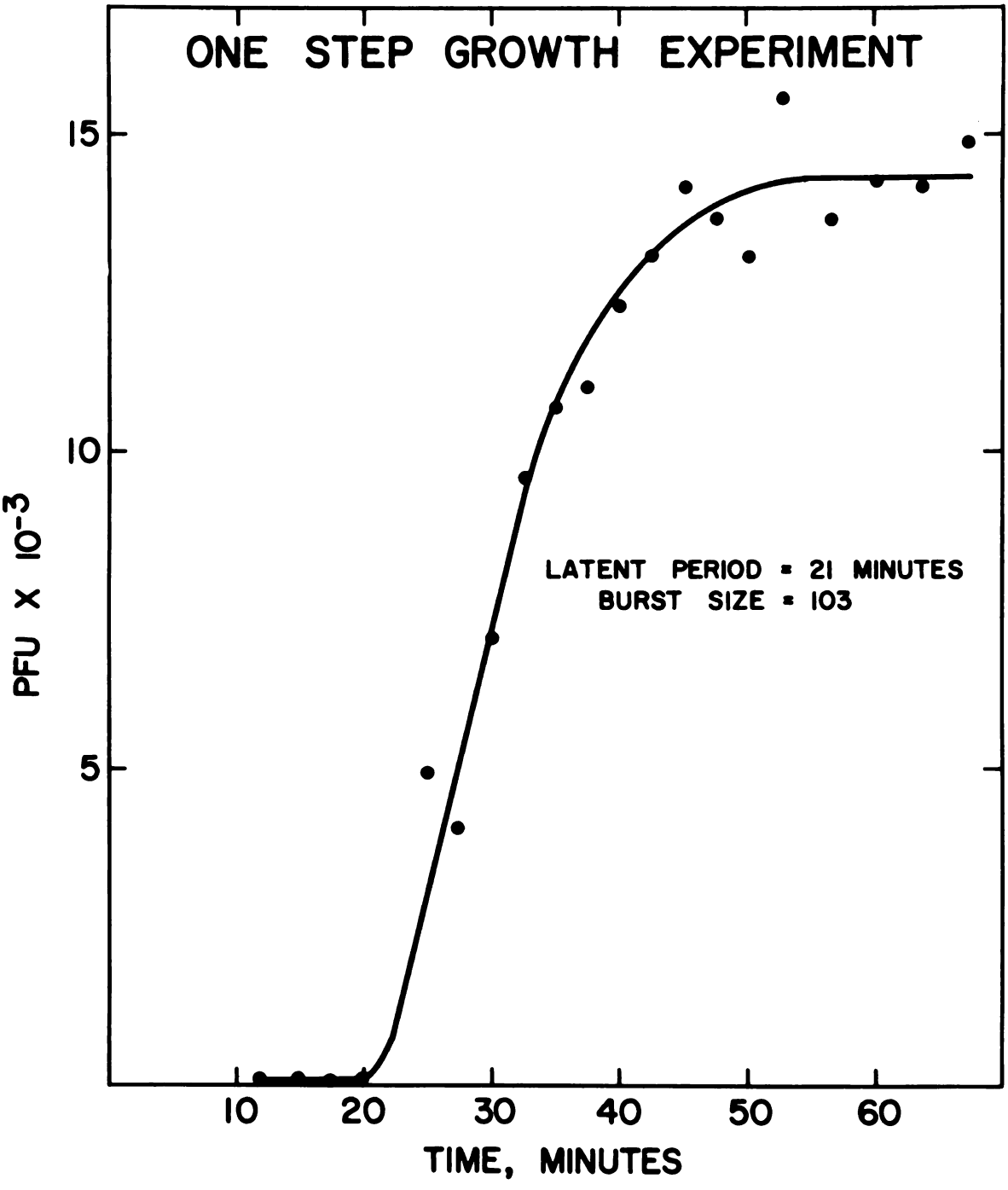

ONE STEP GROWTH EXPERIMENT
15
10
5
PFU X 10-3
LATENT PERIOD = 21 MINUTES
BURST SIZE = 103
10
20
30
40
50
60
TIME, MINUTES

Figure 4.

Figure 5. Electron micrograph of bacteriophage gh-1 negatively stained with 1% (w/v) uranyl acetate. The stained preparation was examined under a Siemens Elmiskop 1 electron microscope at instrumental magnification of 40,000.

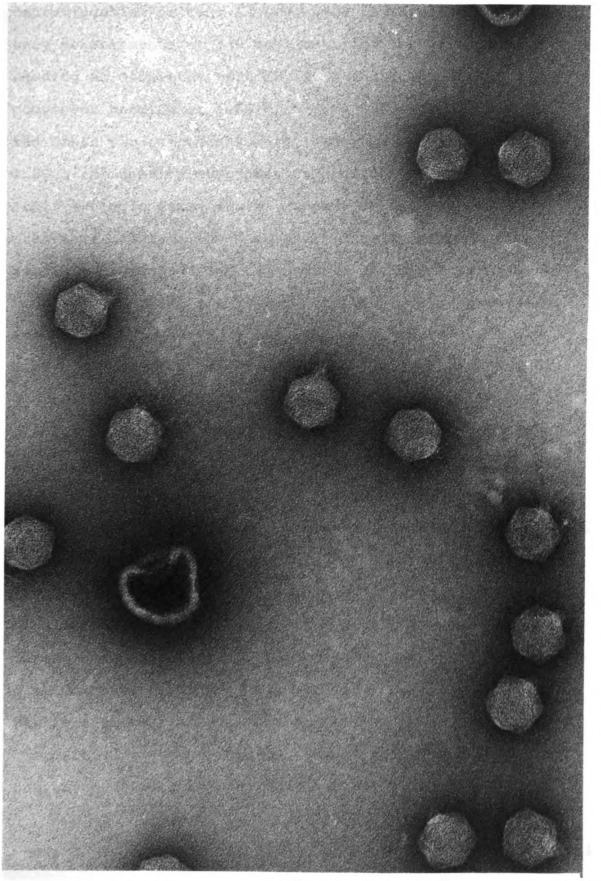


Figure 5.

centrifugation in CsCl is presented in Figure 6. Absorbancy measurements at 260 mμ identified three components banding at densities of 1.52, 1.50 and 1.45 g/cm^3. The component banding at 1.52 g/cm^3 was not infectious while the other two components were. When material from the 1.50 g/cm^3 density component was collected and rebanded in CsCl, a single component of the same density was found. When material from the 1.45 g/cm^3 density component was similarly collected and rebanded, two components of densities 1.50 and 1.45 g/cm^3 were detected in the same ratio, as shown in Figure 6. These results suggested that the 1.50 g/cm^3 density component was an artifact produced by degradation of the 1.45 g/cm^3 density component in the CsCl gradient. The 1.52 g/cm^3 density component, on the other hand, was not detected upon rebanding of either of these two lighter components in the CsCl gradient. Its identity is not known. It could be, however, defective viral particles produced during the purification steps prior to CsCl equilibrium centrifugation. The 1.45 g/cm^3 component was considered to be the intact viral particles. It was about three times as infectious per absorbancy unit as the 1.50 g/cm^3 density component. If we assume the intact viral particle to consist of only protein and DNA, the fractional composition of each component can be calculated from its buoyant density according to the equation (50)

$$1/\rho_0 = n_1/\rho_1 + n_2/\rho_2$$

where ρ_0 is the measured buoyant density of the intact viral

Figure 6. Cesium chloride equilibrium centrifugation of gh-1. Absorbancy at 260mμ (solid line) and plaque-forming units (dashed line) are plotted against fraction number. Fraction no. 1 contains material from the bottom of the centrifuge tube; fraction no. 30, from the top of the tube.

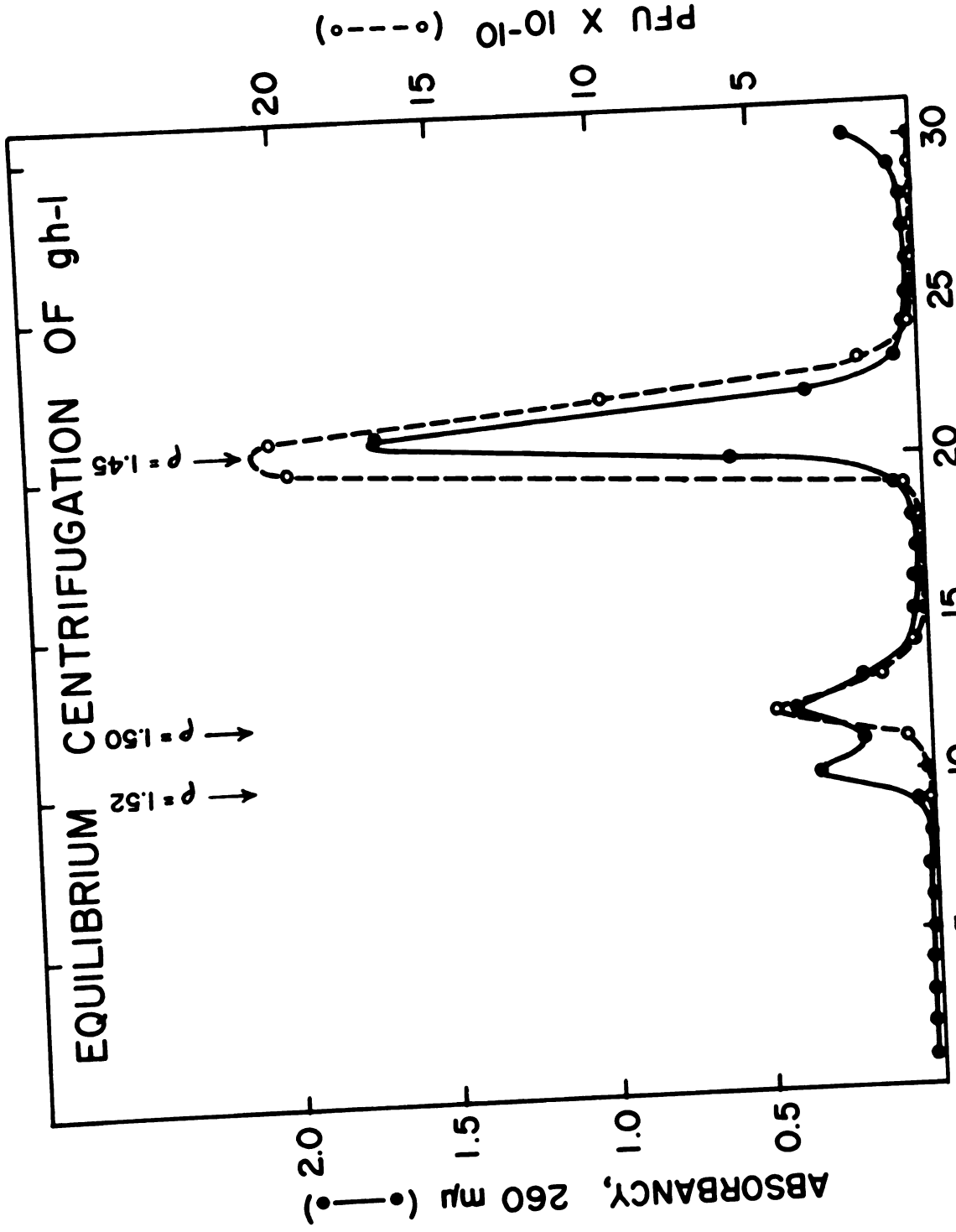

EQUILIBRIUM CENTRIFUGATION OF gh-1
ρ = 1.45
ρ = 1.50
ρ = 1.52
ABSORBANCY, 260 mμ (•—•)
PFU X 10-10 (•---•)
FRACTION NUMBER
0.5
1.0
1.5
2.0
5
10
15
20
25
30

Figure 6.

particle; ρ_1 and ρ_2 are the buoyant densities of DNA and protein and are taken to be 1.71 and 1.33 g/cm^3; n_1 and n_2 are the fractional parts of DNA and protein. Accordingly, the particle banding at ρ = 1.45 g/cm^3 was composed of 64% protein and 36% DNA.

Characterization of gh-1 Nucleic Acid as DNA

The nucleic acid of the virus was purified by phenol extraction, and shown to be DNA by a positive diphenylamine reaction, a negative orcinol test, and by its susceptability to deoxyribonuclease but not to ribonuclease. The ultraviolet spectrum of gh-1 DNA was typical of nucleic acids with a maximal absorbancy at 259 mμ, as shown in Figure 7. The ratios of absorbancies at 260 mμ to 280 mμ and 260 to 230 mμ were 1.9 and 2.2 respectively.

Electron Microscopy of gh-1 DNA

Figure 8 is a portion of an electron micrograph of gh-1 DNA prepared by the protein-monlayer technique of Kleinschmidt (41). This technique provides a direct way of examining the conformation and the contour length of DNA. As seen in Figure 8, the molecule is linear with its two ends clearly visible. This structure seems to be a single unbroken DNA molecule and represents the entire DNA content of a gh-1 virus particle. In areas not shown in Figure 8, neither circles nor twisted supercoils were observed, although many multiple-looped structures or "flowers" were

Figure 7. Ultraviolet absorbancy spectrum of gh-1 DNA.

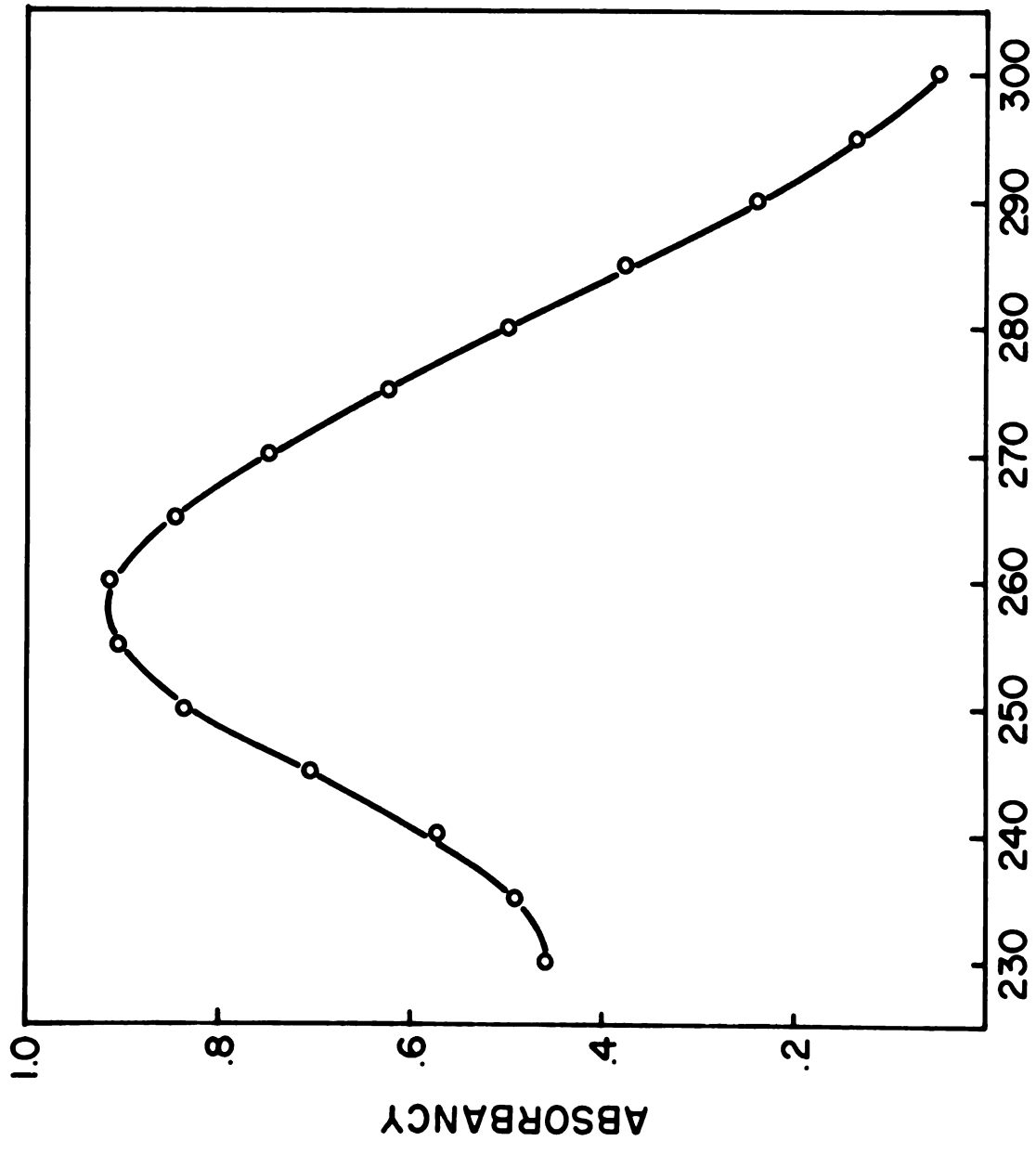

ABSORBANCY
1.0
.8
.6
.4
.2
230
240
250
260
270
280
290
300
WAVELENGTH, MμL

Figure 7.

Figure 8. Electron micrograph of purified gh-1 DNA examined under the Siemens Elmiskop 1 electron microscope at an instrumental magnification of 10,000.

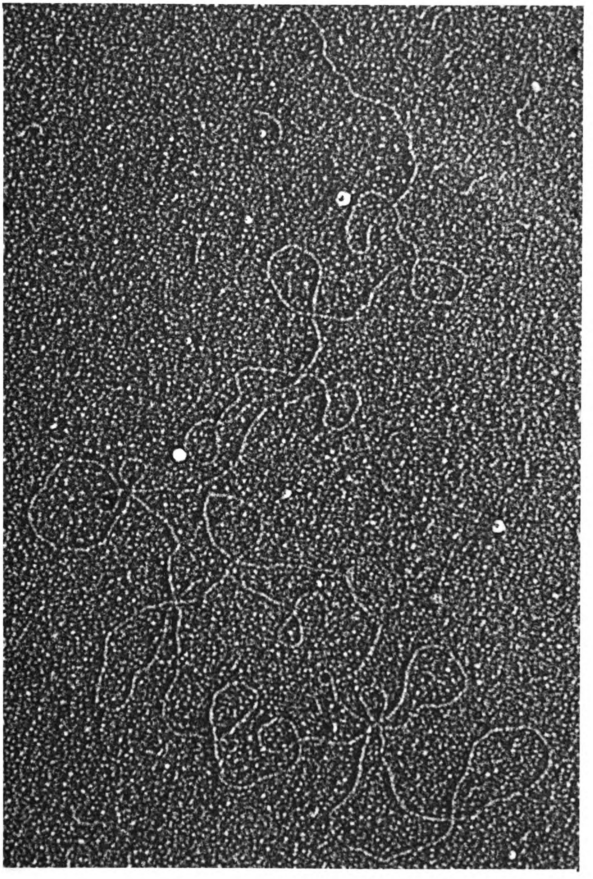


Figure 8.

present. These latter structures result when crowding of the DNA molecules occurs on the electron microscope grid.

With proper equipment the contour length of DNA can be measured. Assuming a linear density of 196 dalton/$\mathring{A}$ for DNA of the B conformation, the molecular weight of the DNA can be calculated from the length thus measured. Conversely, with known molecular weight the length of the molecule can be estimated. Since the molecular weight of gh-1 DNA has been established by sedimentation velocity studies to be about 22.6×10^6, its molecular length should be 11.5×10^4 $\mathring{A}$ or 11.5 microns. This value can be compared with the molecular length of 12.5 microns reported for T_7 DNA.

It should be mentioned here that T_7 phage has a gross morphology -- size and shape -- similar to that of gh-1 phage. Furthermore, the molecular weights of the DNA of these two phages as determined by sedimentation velocity studies were found to be close. For these reasons and for the purpose of making comparison, many of the studies in this investigation were performed on both gh-1 and T_7.

Thermal Denaturation Profile of gh-1 DNA

The thermal denaturation profile for gh-1 DNA is given in Figure 9. The absorbancy of gh-1 DNA remained constant until about 77^0C. It rose sharply thereafter and reached a maximum by 80^0C. This sharp increase in absorbancy occurred at the temperature where the DNA changed from the

Figure 9. Thermal denaturation profile of gh-1 DNA (o), *P. putida* A.3.12 DNA (Δ), and *E. coli* DNA (·).

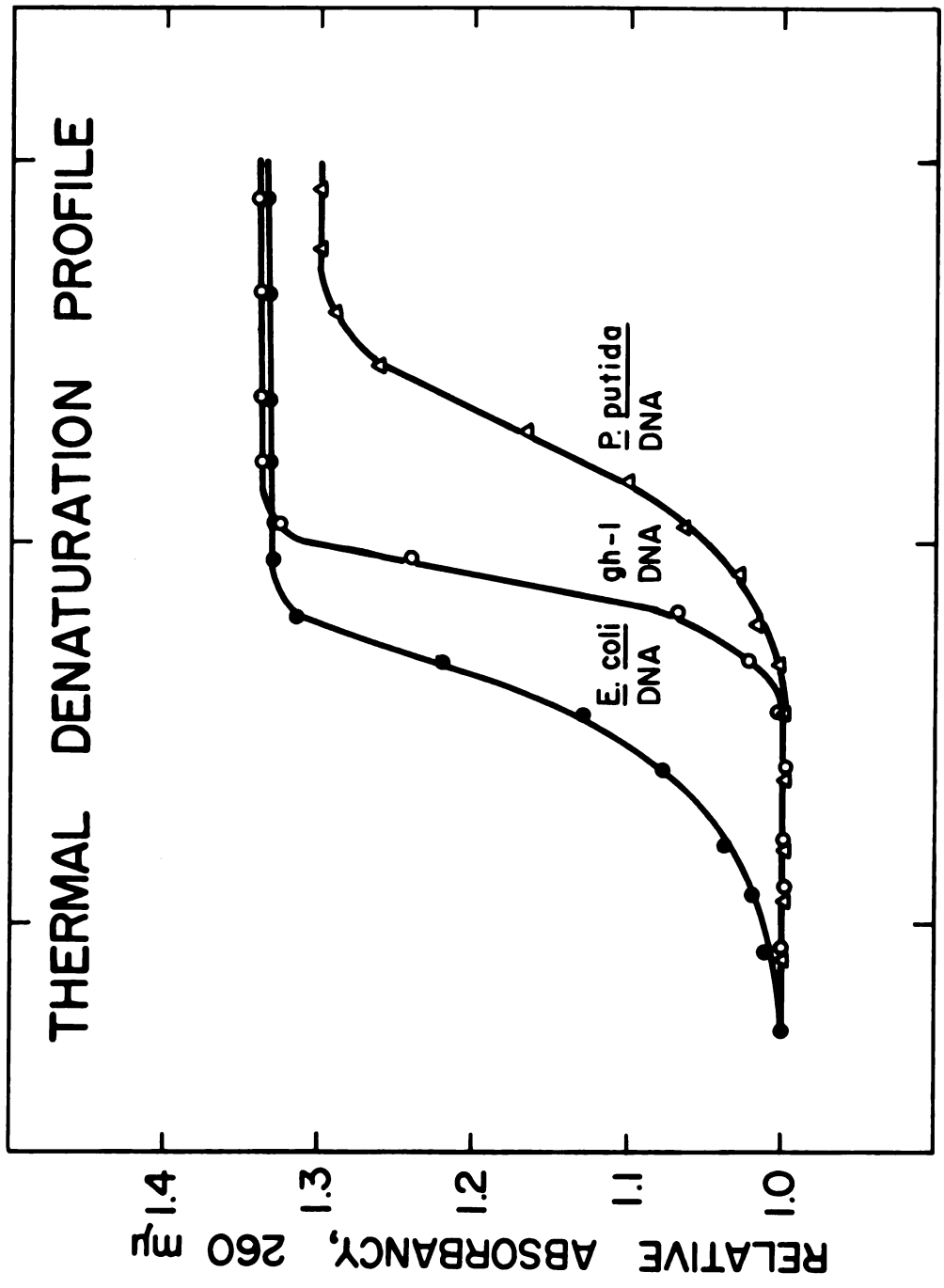

THERMAL DENATURATION PROFILE
RELATIVE ABSORBANCY, 260 mμ
1.0
1.1
1.2
1.3
1.4
70
80
90
TEMPERATURE, °C
E. coli DNA
gh-1 DNA
P. putida DNA

Figure 9.

native, double-stranded to the denatured conformation. As seen in Figure 9, the sharpness of the rise and the extent of the hyperchromic effect (change in absorbancy of 1.34) characterized gh-1 DNA as double stranded. The temperature corresponding to the midpoint of the absorbancy rise is termed the T_m (51). The T_m for gh-1 DNA was 79°C.

The thermal denaturation profiles for E. coli DNA and P. putida A.3.12 DNA are also presented in Figure 9. The T_m for the former was 76°C and that for the latter was 83.5°C. The T_m of gh-1 DNA, at 79°C, was, therefore, midway between the T_m of E. coli DNA and that of P. putida A.3.12 DNA. Since the thermal denaturation temperature is linearly related to DNA base composition (51), the base composition of gh-1 DNA should be midway between E. coli (50% GC) (46) and P. putida A.3.12 (63.4%) or 57%.

Base Composition of gh-1 DNA and P. putida A.3.12 DNA

The base compositions of gh-1 DNA and P. putida A.3.12 DNA were determined after hydrolysis in perchloric acid. The results are presented in Table I. For gh-1 DNA, the mole % adenine equaled the mole % thymine, and the mole % guanine (G) equaled the mole % cytosine (C). The base compositions of gh-1 DNA, expressed as the per cent GC, was 57.0. The base composition for P. putida A.3.12 was 63.7% GC.

Table I. Chemical analysis of DNA base compositions.

	MOLE %				% GC
	A	T	G	C	
gh-1	21.5	21.5	28.4	28.6	57.0
P. putida A.3.12	19.6	16.8	32.5	31.2	63.7
	BUOYANT DENSITY g/cm^3				% GC
gh-1	1.716				57.1
P. putida A.3.12	1.722				63.3

Buoyant Density of gh-1 DNA and P. putida A.3.12 DNA

The buoyant densities of gh-1 DNA, heat-denatured gh-1 DNA, and P. putida A.3.12 DNA were determined by equilibrium centrifugation in CsCl in an analytical ultracentrifuge. The microdensitometer tracings for these three determinations are presented in Figure 10. For all determinations, E. coli DNA, the buoyant density of which was taken to be 1.710 g/cm^3, was used as the density marker. The buoyant densities of gh-1 DNA, heat-denatured gh-1 DNA, and P. putida A.3.12 DNA were found to be 1.716, 1.730, and 1.722 g/cm^3, respectively. The buoyant density for P. putida A.3.12 DNA obtained here is in exact agreement with that obtained by M. Mandel (52).

Schildkraut, Marmur and Doty (46) have established the following equation to relate the buoyant density and % GC

$$\rho = 1.660 + 0.098\ (GC)$$

where ρ refers to buoyant density and (GC) to the mole fraction of guanine plus cytosine. The base compositions of gh-1 DNA and P. putida A.3.12 DNA, calculated from the above formula, were 57.1% GC for gh-1 DNA and 63.3% GC for P. putida A.3.12 DNA These results are in good agreement with those obtained by chemical analysis (Table I).

Sedimentation Velocity Studies of gh-1 Bacteriophage and gh-1 DNA

The sedimentation coefficient of gh-1 bacteriophage

Figure 10. Microdensitometer tracings of cesium chloride equilibrium analysis of gh-1 DNA, heat-denatured gh-1 DNA, and *P. putida* A.3 12 DNA. *E. coli* DNA with a buoyant density of 1.710 g/cm^3 was a density marker.

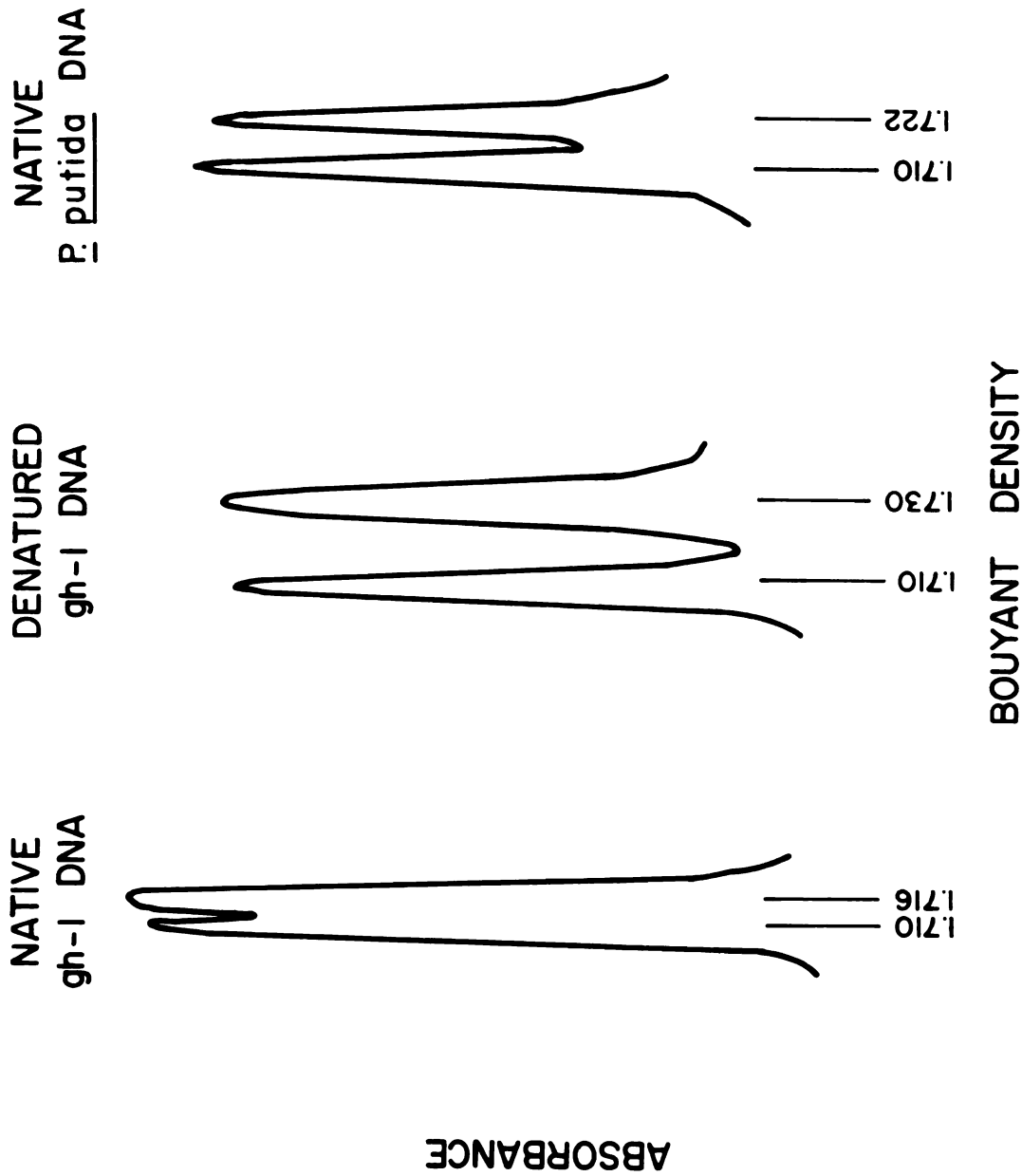

NATIVE
gh-1 DNA
1.716
1.710
DENATURED
gh-1 DNA
1.730
1.710
NATIVE
P. putida DNA
1.722
1.710
ABSORBANCE
BOUYANT DENSITY
g/cm³

Figure 10.

was determined by the moving boundary sedimentation velocity method in an analytical ultracentrifuge by use of the ultraviolet optics system. The $S^0_{20,w}$ obtained for several different preparations of bacteriophage gh-1 was between 430-460. Coliphage T_7 and Pf phage for P. putida C1S, both of which have larger molecular dimensions than gh-1 under the electron microscope, have been reported to have a $S^0_{20,w}$ of 487 ± 5 by Davison and Freifelder (49) for T_7, and 500 by Niblack and Gunsalus for phage Pf (6).

The sedimentation coefficient for gh-1 DNA was similarly determined by the moving boundary sedimentation velocity method. A series of photographs of gh-1 DNA taken at 8-minute intervals during the centrifugation in an analytical ultracentrifuge is presented in Figure 11. The distance moved at various time intervals during the centrifugation was calculated from the microdensitometer tracings of the photographs. Table II illustrates a typical sample calculation of sedimentation coefficients of gh-1 DNA and their corrections to standard conditions. The sedimentation velocity studies of gh-1 DNA were performed at various concentrations. The result of these studies is shown in Figure 12, where the reciprocal of the sedimentation coefficient was plotted against gh-1 DNA concentration in μg/ml. The $S_{20,w}$, when extrapolated to zero concentration, was 30.9 ± .4. Similar studies were carried out with coliphage T_7 DNA The $S^0_{20,w}$ obtained was 31.9. This value agrees with the published data of 32.2 ± .5 S (32).

Figure 11. Ultracentrifuge sedimentation pattern of gh-1 DNA (40 μg/ml). Exposures made at every 8 minute interval at 44,040 rev/min.

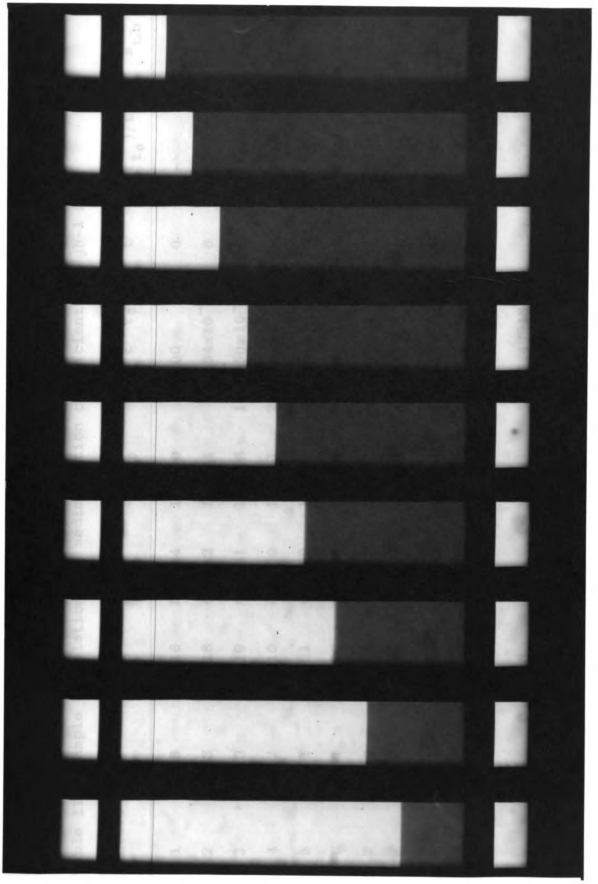


Figure 11.

Table II. Sample calculation for sedimentation coefficients of gh-1 DNA

Picture	r_p	Δr_p	r	r_t/t_{t_0}	$\log (r_t/r_{t_0})$	t	$(r_t/r_{t_0})/t$	$s_{t,b}$
1	12.3	1.3516	5.9684	1.0000	0.0000	0	--	--
2	11.3	1.2418	6.0782	1.0184	7.9184×10^{-3}	8	0.9898	19.6
3	10.3	1.1319	6.1881	1.0368	15.6950×10^{-3}	16	0.9809	19.4
4	9.2	1.0110	6.3090	1.0571	24.1161×10^{-3}	24	1.0048	19.9
5	8.1	0.8901	6.4299	1.0773	32.3367×10^{-3}	32	1.0105	20.0
6	7.1	0.7802	6.5398	1.0957	39.6917×10^{-3}	40	0.9923	19.6
7	5.8	0.6374	6.6826	1.1197	49.1017×10^{-3}	48	1.0230	20.3
8	4.5	0.4945	6.8255	1.1436	58.2741×10^{-3}	56	1.0406	20.6
9	3.1	0.3407	6.9793	1.1694	67.9631×10^{-3}	64	1.0619	21.0

RPM = 42,040 $\qquad$ $RPM^2 = 1.7674 \times 10^9$

$$\frac{3.49948}{1.7674 \times 10^9} = 1.98 \times 10^{-9} \qquad s = \frac{3.49948}{RPM^2} \times \log (r_t/r_{t_0})/t$$

Table II. (Cont.)

EXPLANATIONS FOR SYMBOLS USED IN THE SAMPLE CALCULATIONS FOR SEDIMENTATION COEFFICIENTS

r_p = distance in cm from the boundary to the outer reference hole measured directly from microdensitometer tracings of the photographs.

Δr_p = (actual distance between the boundary and outer reference) = r_p/F, where F is the magnification factor (camera lens and microdensitometer lens factors, F = 9.1).

r = (distance of the boundary from the center of the rotor) = $r_0 - \Delta r_p$, where r_0 is the distance from the center of the rotor to the outer reference. At high speed, the value is 7.32 cm.

r_t = position of the boundary from center of rotor at time = t.

r_{t_0} = position of the boundary from center of rotor at time = 0.

$s_{t,b}$ = sedimentation coefficient in Svedberg unit (10^{-13} sec) at specified temperature and solvent.

Correction of s value to standard conditions of 20^0C and water as a solvent:

$$\frac{s_{20,w}}{s_{t,b}} \simeq \frac{n_{t,w}}{n_{20,w}}$$

Figure 12. Effect of gh-1 DNA concentrations on sedimentation velocity coefficients.

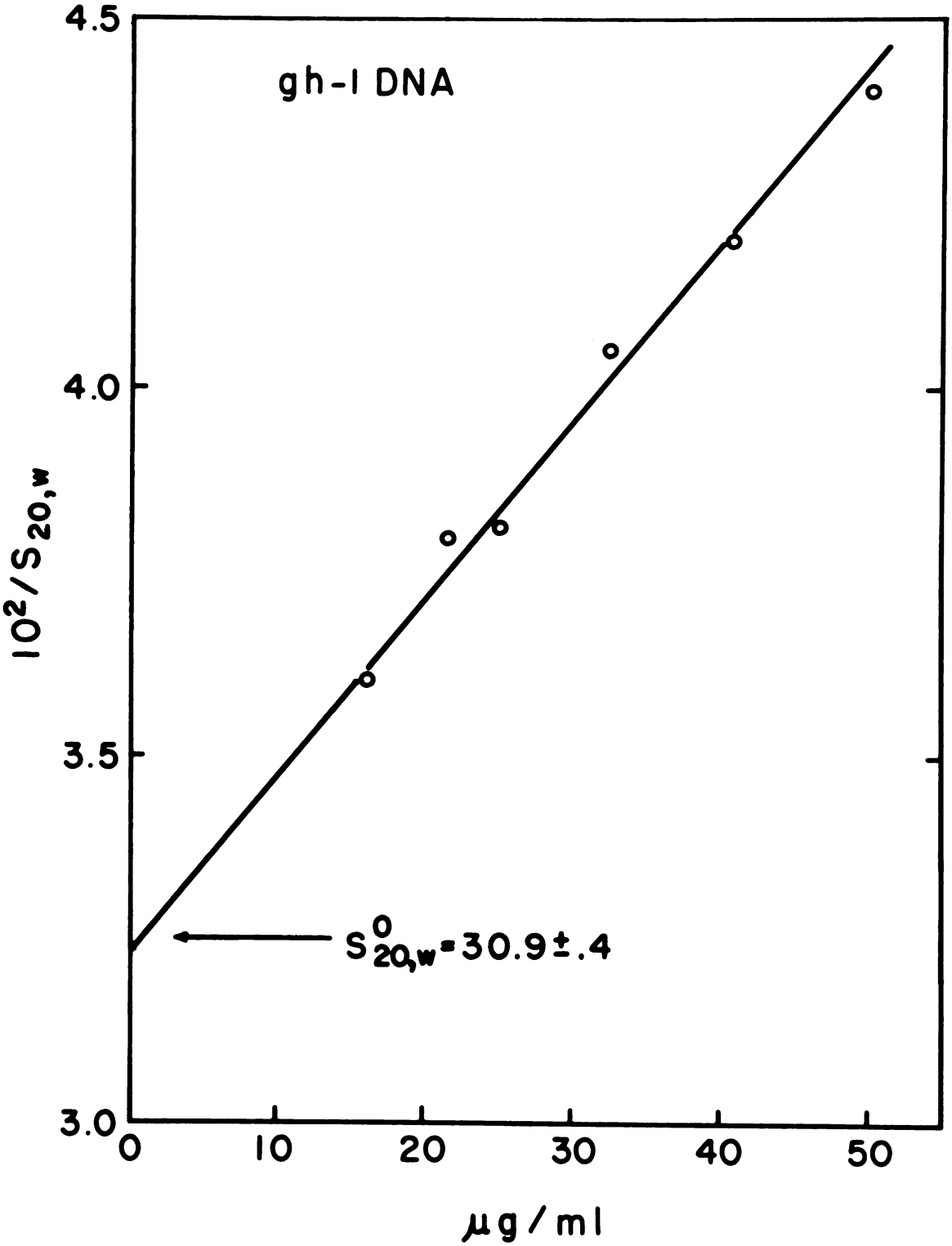


Figure 12.

From the sedimentation coefficient, it is possible to calculate the molecular weight of the DNA according to the general equation developed by Doty, McGill and Rice (26)

$$S = k\ M^{a}$$

where k and a are experimentally derived constants. Studier's evaluation of k and a leads to the following equation (28)

$$S^{0}_{20,w} = 0.0882\ M^{.346}$$

A molecular weight of 22.6×10^6 for gh-1 DNA and 24.8×10^6 for T_7 DNA was obtained by this equation. The value for T_7 DNA is in close agreement with a molecular weight of $25.5 \pm .4 \times 10^6$ (See Table III).

DNA Zone Sedimentation Studies in Alkaline Sucrose

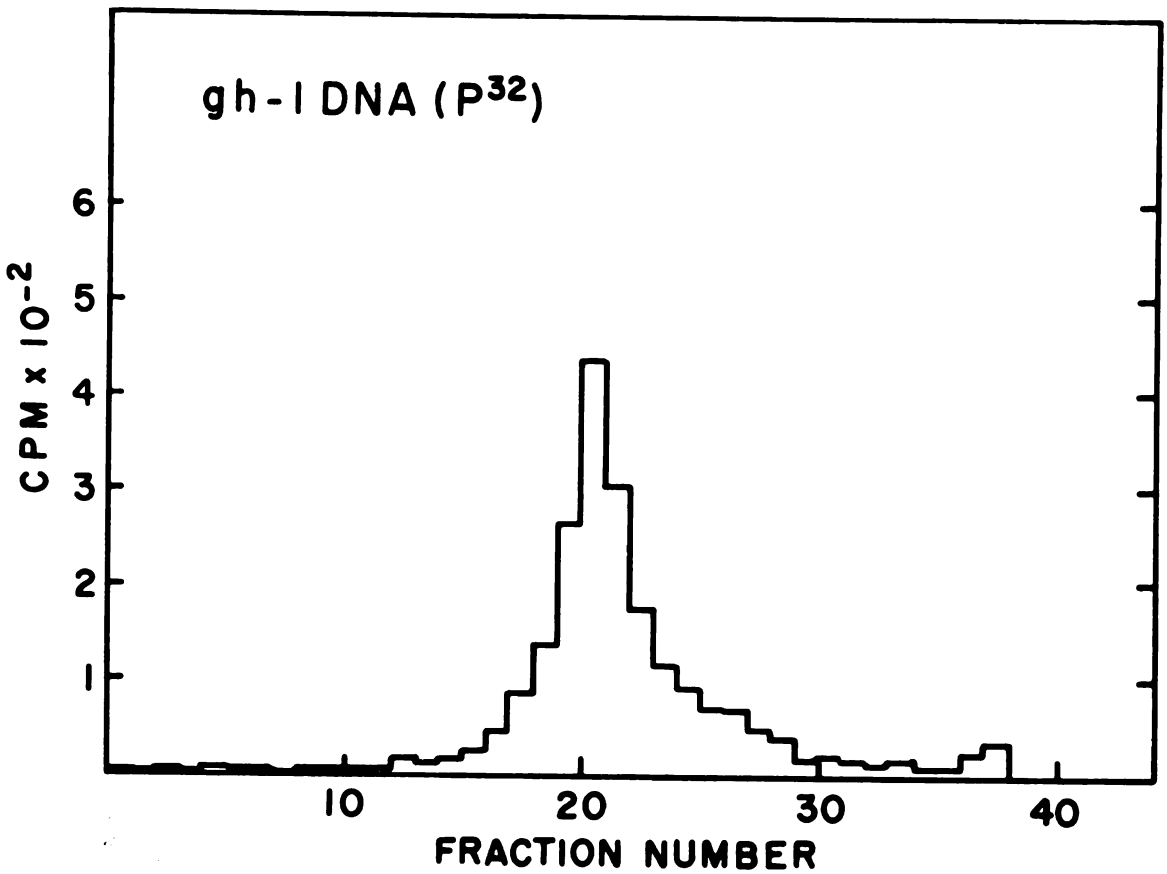
Sedimentation studies of single polynucleotide chains derived from the duplex DNA molecules were performed by the alkaline sucrose gradient technique. ^{32}P-labeled gh-1 (specific activity = 1.1×10^5 cpm/A^{260}), or ^{14}C-labeled T_7 (specific activity = 1.2×10^5 cpm/A^{260}), was mixed with an equal volume of 0.2 M Na_3PO_4. This treatment causes the release of DNA from the phage head as well as DNA denaturation. The denatured, single-stranded polynucleotide chains were then sedimented through alkaline sucrose. The sedimentation profiles for ^{14}C-labeled T_7 and ^{32}P-labeled gh-1 polynucleotide chains are shown in Figure 13. Both species of polynucleotide chains moved as a single symmetrical zone through the alkaline sucrose gradients --

Table III. Sedimentation coefficients and molecular weights of gh-1 and T_7 DNA

DNA	$S^0_{20,w}$	M
gh-1	30.9 ± .4	22.6 ± .9 x 10^6
T_7	32.2 ± .2	25.5 ± .4 x 10^6

$$S^0_{20,w} = .0882\ M^{.346}$$

Figure 13. Alkaline sucrose gradient profile of alkaline denatured gh-1 DNA and T_7 DNA.


gh-1 DNA (P^{32})
CPM x 10^{-2}
1
2
3
4
5
6
10
20
30
40
FRACTION NUMBER

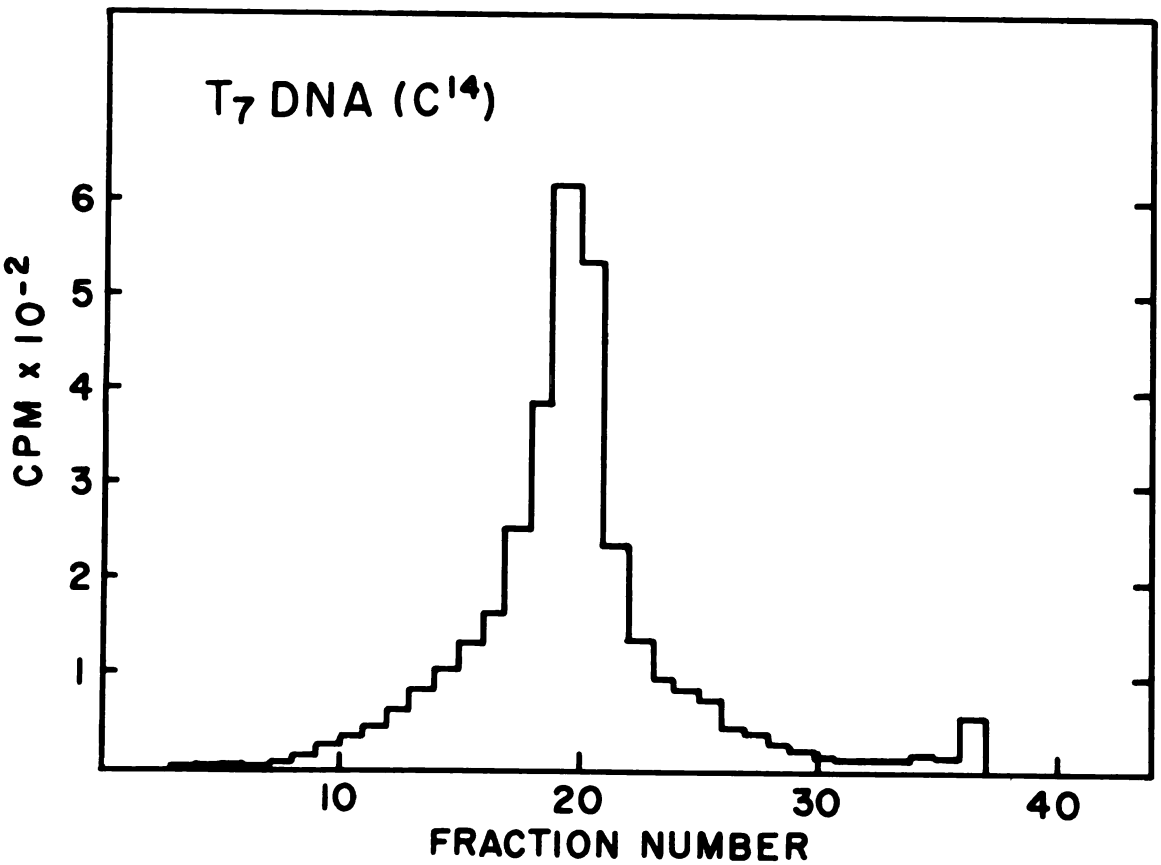

T_7 DNA (C^{14})
CPM x 10^{-2}
1
2
3
4
5
6
10
20
30
40
FRACTION NUMBER

Figure 13.

indicating that each species of DNA was composed of a homogeneous population of molecules. The distances traveled by the two species of denatured DNA were approximately the same.

Abelson and Thomas (25) derived the following equation relating the sedimentation coefficient with distance traveled in an alkaline sucrose gradient

$$S_{20,w} = 0.00662\ (r_f - r_i)/\omega^2 t$$

where $\omega^2 t$ is $(\text{rev/min})^2$ hr; $r_f - r_i$ is the distance moved in time t. From the data shown in Figure 13 and from the above equation, the $S_{20,w}$ of the single polynucleotide chains of gh-1 and T_7 were calculated to be between 43-45. Abelson and Thomas reported the sedimentation coefficient of 40-43 S for T_7 single polynucleotide chains using the alkaline sucrose gradient technique.

In order to compare directly the sedimentation behavior of both gh-1 and T_7 polynucleotide chains, an experiment was performed in which both species of polynucleotide chains were co-sedimented through an alkaline sucrose gradient. The result is shown in Figure 14. Again, both species of polynucleotide chains moved as single symmetrical zones with their peaks occurring at the same position. The skew of the T_7 profile, as compared to that of the gh-1, suggested that single chains of T_7 DNA have a slightly higher molecular weight. Better resolution of these two species of DNA was not obtained in other

Figure 14. Co-sedimentation profile of alkaline denatured gh-1 DNA and T_7 DNA in an alkaline sucrose gradient.

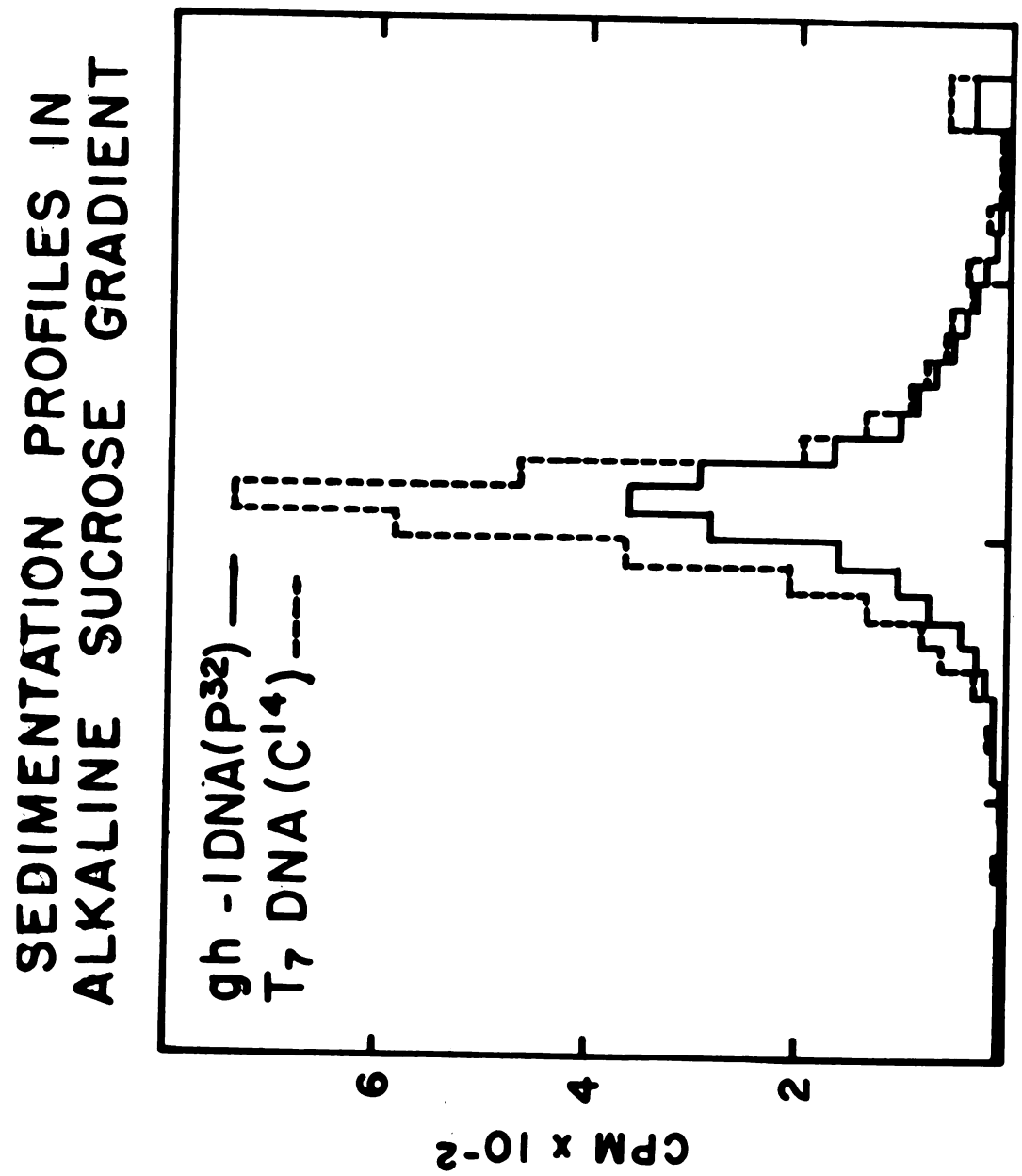

SEDIMENTATION PROFILES IN
ALKALINE SUCROSE GRADIENT
gh - 1 DNA(P32) ——
T7 DNA (C14) ----
CPM x 10-2
6
4
2
10
20
30
FRACTION NUMBER

Figure 14.

experiments in which longer centrifugation time was used and greater number of fractions were collected.

The following equation developed by Abelson and Thomas (25) relates the distances traveled to the molecular lengths for two DNA species co-sedimenting in an alkaline sucrose gradient

$$D_2/D_1 = (L_2/L_1)^{0.38}$$

Since the difference in molecular weights between gh-1 and T_7 DNA is about 10%, the difference in distances traveled by these two species calculated according to the above equation should be about 4%. The alkaline sucrose technique lacked the sensitivity necessary for resolving such a small difference. Nonetheless, from the co-sedimentation profile and the sedimentation coefficients for the single polynucleotide chains, we can conclude that the molecular weights of both gh-1 and T_7 DNA are proximate and that gh-1 DNA, like T_7 DNA, consists of two linear, uninterrupted polynucleotide chains (see discussion).

DISCUSSION

The present investigation was concerned with the characterization of gh-1 bacteriophage for Pseudomonas putida A.3.12, with emphasis on characterizing its nucleic acid component.

Since procedures generally used in the purification of coliphages proved unsatisfactory for gh-1, a different procedure was therefore developed. This consisted of a series of differential centrifugation steps, or an ammonium sulfate precipitation of gh-1 from the crude lysate, followed by DEAE-cellulose fractionation. The fractionation is dependent on NaCl concentration. The phage is not adsorbed on DEAE-cellulose equilibrated with a 0.2 M NaCl but adsorbed at lower salt concentrations and may be eluted with 0.2 M NaCl. As a result of the purification procedure, a preparation of gh-1 bacteriophage was obtained having a UV spectrum with a maximum at 260 mμ and a minimum at 240 mμ.

Electron microscopic studies of purified gh-1 bacteriophage placed it in a class of phages comparable in shape and size to coliphages T_3 and T_7 and PF phage for P. putida C1S. It was roughly hexagonal in cross section with a short wedge-shaped tail.

Equilibrium sedimentation of bacteriophage in CsCl gradients has generally been used as a step in the purification of bacteriophages and as an analytical means to measure their buoyant density. With this technique, a

single homogeneous density band has been observed for most bacteriophages. In the case of gh-1 bacteriophage, however, three components banding at densities of 1.52, 1.50 and 1.45 g/cm^3 were observed in the CsCl gradients. Only the latter two components showed, by assaying their plaque-forming units, infectivity. The ratio of infectivity for these two components was about 1:3.

Niblack, McDaniel, Killmer and Gunsalus, in their study of Pf-c phage on P. putida C1S, observed two density bands of equal infectivity in CsCl equilibrium centrifugation banding at 1.48 g/cm^3 (H) and 1.46 g/cm^3 (L). The virus preparation used in these studies had been purified by a single differential centrifugation (15 minutes at 5,000 x g, then 120 minutes at 40,000 x g) step. Upon examining the bands under the electron microscope they found the H-band to contain only intact phage and phage ghosts (phage devoid of nucleic acid) and the L-band to contain, in addition, phage-filaments and phage-spheroid aggregates. Following treatment of the material in the L-band with pronase and recentrifugation in CsCl, one band was found in the H-density region. They concluded that the aggregates in the L-band represent the association of Pf-phages with subcellular protein fragments.

In the studies of gh-1 bacteriophage, however, the virus was purified by a series of differential centrifugation followed by a DEAE-cellulose fractionation step before CsCl centrifugation analysis. Only intact phage and phage

ghosts were observed under the electron microscope with very little or no contaminating fragments visible. Finally, the infectivity per absorbancy unit of the 1.45 g/cm^3 component was three times as great as that of the 1.50 g/cm^3 component. These facts, when combined with the results of the rebanding experiments, suggested that the heavier component (1.50 g/cm^3) arose in the CsCl gradient from the degradation of the lighter component (1.45 g/cm^3)--perhaps by the loss of some phage structural protein. A loss of 20% of the phage's protein could account for the change in density from 1.45 g/cm^3 to 1.50 g/cm^3.

The nucleic acid component of the gh-1 bacteriophage was found to be DNA by a positive diphenylamine reaction, a negative orcinol test, and by its susceptibility to DNAase but not RNAase. The double-stranded character of gh-1 DNA was demonstrated by the sharpness of the rise and the extent of the hyperchromic effect in the thermal denaturation studies. Chemical analysis of the DNA base composition further showed that its mole per cent A equaled T, and G equaled C. In addition, the buoyant density of heat denatured gh-1 DNA was found to be 0.014 g/cm^3 higher than that of its native form. This difference in buoyant density is that expected between duplex DNA and single-stranded DNA, Finally, that gh-1 DNA is double stranded was indicated by its extended conformation observed under the electron microscope.

The base composition of gh-1 DNA was established to be 57.0% GC by direct chemical analysis of its individual bases. The value agreed with that deduced from the thermal denaturation profile studies and calculated from the buoyant density measurement.

The possibility of phenol-extracted gh-1 DNA being an intact circular molecular similiar to that described for polyoma DNA (53) and that for the intracellular condensed form of λ DNA (54) was considered. Such intact circular molecules can reform the native conformation during the quick cooling process after denaturation by heat. Consequently, no change in buoyant density or in sedimentation coefficient for the heat-treated material should be observed. However, the buoyant density of heat-denatured gh-1 DNA was found to be about 0.014 g/cm^3 greater than that of native DNA (see Figure 10). The sedimentation coefficient of the heat-denatured gh-1 DNA measured in M-NaCl and at neutral pH was twice that of the native form. Furthermore, alkaline denatured gh-1 DNA, when sedimented in an alkaline sucrose gradient gave a sedimentation coefficient value equivalent to that of separated single chains. Finally, the electron micrograph of gh-1 DNA clearly showed the molecule to be a linear DNA with two ends. Neither circles nor supercoils were observed. This evidence led us to conclude that phenol extracted gh-1 DNA possesses a linear conformation rather than a circular structure.

Sedimentation coefficient studies using the moving boundary sedimentation velocity method in an analytical ultracentrifuge gave a $S^0_{20,w}$ of 430-460 for gh-1 bacteriophage. Coliphage T_7, which has a similar morphology as gh-1, and which was used in many of the studies for comparative purposes, sedimented with a $S^0_{20,w}$ of 487 (49). The $S^0_{20,w}$ of gh-1 and T_7 DNA were determined in the analytical ultracentrifuge to be 30.9 and 31.9 respectively.

Once the sedimentation coefficient of the DNA is experimentally determined, it is possible to relate it to the molecular weight. The Svedberg equation relates the sedimentation coefficient to molecular weight as follows

$$M = \frac{sRT}{D(1 - \bar{v}\rho)}$$

where M and s are molecular weight and sedimentation coefficient respectively; D is the diffusion constant; R is the gas constant; T is the absolute temperature; $\bar{v}$ is the partial specific volume which is the reciprocal of buoyant density; and ρ is the density of the solvent. Since DNA molecules diffuse slowly, the determination of D is virtually impossible. An alternate method is to employ the equation derived by Scheraga and Mandelkern (55)

$$M = \left[\frac{s[\eta]^{1/3}\ \eta_0\ N\ 10^{-13}}{\beta(1 - \bar{v}\rho)}\right]$$

where $[\eta]$ is the specific viscosity in deciliters per gram; η_0 is the viscosity of the solvent; N is Avogadro's number; and β is a constant which is a function

of the shape of the molecule. The terms s, $[\eta]$, $\bar{\nu}$, and ρ are parameters determined experimentally. The value of β for T_2 DNA has been established as 2.4×10^6 (56).

Doty, McGill and Rice (26) derived the following equation to estimate molecular weight directly from sedimentation coefficient

$$s = kM^a$$

where k and a are experimentally determined constants. They measured the sedimentation coefficients of fragments of calf-thymus DNA and related them to their molecular weights determined by the light scattering technique. A log-log plot of the sedimentation coefficients against the molecular weights of the DNA fragments yielded a straight line with its slope = a and its intercept = k. Following this procedure, they obtained k = 0.063 and a = 0.37. These values are valid to use for calculating DNA molecular weights ranging up to ten million.

For DNA species of higher molecular weight, such as T_2 DNA, a determination of new values for the constants a and k was necessary. Burgi and Hershey (27) studied the sedimentation coefficient of various DNA species using zone sedimentation in sucrose gradient, and Studier (28) performed similar studies using zone sedimentation in the analytical ultracentrifuge. For the evaluation of a, Burgi and Hershey made the assumption that the DNA of T_2 and of lambda can be broken into half length pieces with molecular weight equal to half that of the whole molecules.

Using the equation $s = kM^a$ for the half and whole molecules, k was eliminated and the following relationship established

$$\frac{s_{half}}{s_{whole}} = \frac{M_{half}}{M_{whole}} = (0.5)^a$$

The constant a was determined from the ratio between the sedimentation coefficients of the half and whole molecules.

To determine the value for k, the scale of both M and s must be fixed first. This is done by selecting a DNA of known molecular weight and then determining its sedimentation coefficient. These values of a, M, and s then combined to yield an estimate of k in the Doty equation. Using this method, Burgi and Hershey have obtained values of k = 0.080 and a = 0.35 for DNA molecular weights ranging from 15.5 to 130 millions. Studier, on the other hand, using a similar approach, obtained the values of k = 0.0882 and a = 0.346. These two sets of values for k and a are, therefore, in good agreement. Studier's constants were used to calculate the molecular weights of gh-1 and T_7 DNA because the conditions under which we performed these experiments were similar to the conditions he used.

The importance of sedimentation coefficients in the determination of molecular weight is evident from the foregoing discussion. An unusually high DNA concentration dependence of sedimentation coefficient has been reported (57,58). Figure 12 illustrates this behavior for gh-1 DNA. The equation for concentration dependence is usually in the form

$$1/s = 1/s^0 + Kc$$

s^0 value can be obtained either by performing s measurements at different concentration and then extrapolating to c = 0 or by the use of an empirical equation derived by Eigner, Schilkraut and Doty (59).

$$s^0 = s(1 + 0.80\ [\eta]c)$$

or

$$1/s = 1/s^0(1 + 0.80\ [\eta]c)$$

where $[\eta]$ is the intrinsic viscosity in deciliter per gram; s^0 is the sedimentation coefficient at zero concentration; and c is the concentration of DNA in g/dl.

For gh-1 DNA, the sedimentation coefficient at different DNA concentrations was determined and extrapolated to zero concentration. The $S^0_{20,w}$ thus obtained (see Figure 12) was used to calculate molecular weight. It is important to note that the molecular weight calculated from the sedimentation coefficient at even a very low concentration ($S_{20,w}$) can be of an enormous difference from that calculated on the basis of $S^0_{20,w}$ (sedimentation coefficient at zero concentration). For example, a difference in molecular weight of 33% was found for gh-1 DNA measured at $S_{20,w}$ at 20 μg/ml, and at $S^0_{20,w}$. Eigner (29) has reported a difference of 36% for a DNA sample (molecular weight 70 millions) measured under the same conditions.

The possibility that gh-1 DNA may contain single-strand breaks in either or both of the complementary

polynucleotide chains was considered. Abelson and Thomas (25) uncovered such single-strand breaks in the T_5 DNA molecule. They found that T_5 DNA sedimented as four distinct zones in the alkaline sucrose gradients, in contrast to a single zone observed for T_2, T_4, and T_7 DNA. On the basis of the different sedimentation rates of the individual zones, they were able to calculate the various lengths of these fragments. From these facts they concluded that the duplex molecule of T_5 DNA contained one break in one of the polynucleotide chains and three breaks in the other.

T_2, T_4, or T_7 DNA in alkaline sucrose gradient sedimented as a single symmetrical zone, indicating a homogeneous population of single-strand polynucleotide chains. The sedimentation coefficient of T_7 polynucleotide chain in alkaline sucrose gradient reported by Abelson and Thomas (25) approximates that reported by Studier using the analytical zone sedimentation method. The calculated molecular weight of single polynucleotide chains of T_7, according to Studier, approximates half of that for its native duplex.

Several important facts emerged from a study of the alkaline sedimentation profiles of gh-1 and T_7 (Figure 13) and their co-sedimentation profile (Figure 14). Figure 13 shows both species of DNA move as a single symmetrical zone through the alkaline sucrose gradient, indicating that each DNA species was composed of a homogeneous population of molecules--single-stranded polynucleotide chains.

Figure 14 shows both species moved again as single symmetrical zones with their peaks occurring at approximately the same position. These facts, combined with those deduced from Figure 13, suggest that the molecular weight of gh-1 DNA is proximate to that of T_7 DNA and that gh-1 DNA, like T_7 DNA, consists of two linear, uninterrupted polynucleotide chains.

The molecular weight of gh-1 DNA was established as 22.6×10^6. A final question to be considered is whether or not the isolated DNA molecule is the entire DNA content of one phage particle. To answer this we must determine accurately the total DNA content per phage particle.

There are several techniques available for estimating the total DNA content of the phage particle. None, however, can be said to be both simple and accurate. The autoradiographic ^{32}P "star" method employed by Rubenstein, Thomas and Hershey (30) is, for example, rather tedious. In this technique ^{32}P at a high concentration was incorporated into the DNA of T_2 bacteriophage. The phage particles were then embedded in a sensitive emulson. The consequent decay of each single ^{32}P atom, i.e., β decay, produces a track in the sensitive emulsion. A number of these tracks issue from a single-point source--the viral particle--to produce a star-shaped pattern. With a given exposure time, the specific activity of the ^{32}P, and with the number of tracks emitted from a given point source (viral particle), the number of phosphorus atoms per phage

particle was calculated. Assuming that all phosphorus atoms are present in the DNA component of the phage particle, the total DNA content per phage particle can be calculated.

A simpler technique, however, seems to be the determination by chemical analysis of the amount of DNA contained in an aliquot of a purified phage preparation. This value, together with the number of plaque forming units contained in the aliquot, can be used to calculate the amount of DNA per particle. The fact that there are a number of damaged particles which do not form plaques but nevertheless contain DNA usually leads to a falsely high estimate of the DNA content per phage particle. This inaccuracy can, however, be avoided by the development of a direct counting method using the electron microscope. The method, when perfected, will give the total number of phage particles in the preparation, including both the plaque forming and the non-plaque forming units. Finally, the light-scattering technique can be used to determine the particle weight of the phage. Once the particle weight becomes known, a multiplication by the per cent weight fraction of DNA (obtained from buoyant density) gives the mass of DNA per phage particle.

For bacteriophage gh-1 we have not attempted to employ any of the above mentioned techniques. We have sought, however, to establish the DNA content per gh-1 phage particle by a comparative analysis with T_7. The nucleocapsid

for T_7 is of a hexagonal outline about 59-65 mμ across and has a small tail, whereas that for gh-1 is also of a hexagonal outline of a slightly smaller cross section of 50 mμ and with a small wedge-shaped tail. T_7 phage has its $S_{20,w}$ = 487 and its particle weight = 38 x 10^6 and contains by weight approximately 55% protein and 45% DNA. The gh-1 phage, on the other hand, is slightly smaller and its $S^0_{20,w}$ is between 430-460 and contains by weight approximately 60% protein and 40% DNA. Furthermore, purified T_7 DNA has a $S_{20,w}$ of 32.2, corresponding to a molecular weight of 25.5 x 10^6, whereas purified gh-1 DNA is slightly smaller and has a $S^0_{20,w}$ of 30.9, corresponding to a molecular weight of 22.6 x 10^6. Finally, since T_7 DNA with a molecular weight of 25 x 10^6 is established to be the entire DNA content of one T_7 phage particle, gh-1 DNA with a slightly smaller molecular weight of 22.6 x 10^6 must also be the entire DNA content of the gh-1 particle. It can thus be concluded that the gh-1, like T_7, contains one DNA molecule per phage particle.

REFERENCES

1. Davison, P. F., Freifelder, D., and Holloway, B. W., J. Mol. Biol., 8, 1 (1964).

2. Holloway, B. W., Monk, M., Hodgins, L., and Fargie, B., Virology, 18, 89 (1962).

3. Slayter, H. S., Holloway, B. W., and Hall, C. E., Ultrastruct. Res,, 11, 274 (1964).

4. Feary, T. W., Fisher, E., and Fisher, T. N., Biochem. Biophys. Res. Commun., 10, 359 (1963).

5. Feary, T. W., Fisher, E., and Fisher, T. N., Proc. Exp. Biol. and Med., 113, 426 (1963).

6. Niblack, J. F., and Gunsalus, I. C., Bact. Proc., 115 (1965).

7. Klinge, K., Arch. Mikrobiol., 34, 270 (1959).

8. Wyatt, G. R., and Cohen, S. S., Biochem. J., 55, 774 (1953).

9. Romig, W. R., and Brodetsky, A. M., J. Bacteriol., 82, 135 (1961).

10. Takahashi, I., and Marmur, J., Nature, 197, 794 (1963).

11. Sinsheimer, R. L., J. Mol. Biol., 1, 43 (1959).

12. Tessman, I., Virology, 7, 263 (1959).

13. Davis, J. E., and Sinsheimer, R. L., J. Mol. Biol., 6, 203 (1963).

14. Loeb, T., and Zinder, N. D., Proc. Natl. Acad. Sci. U.S., 47, 282 (1961).

15. Cairns, J., J. Mol. Biol., 3, 756 (1961).

16. Hershey, A. D., Burgi, E., and Ingraham, L., Biophys. J., 2, 423 (1962).

17. Thomas, C. A., Jr., and MacHattie, L. A., Proc. Natl. Acad. Sci. U.S., 52, 1297 (1964).

18. Kaiser, A. D., and Inman, R. B., J. Mol. Biol., 13, 78 (1965).

19. Fiers, W., and Sinsheimer, R. L., J. Mol. Biol., 5, 408 (1962).

20. Stoeckenius, W., Proc. Natl. Acad. Sci. U.S., 50, 737 (1963).

21. Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P., Proc. Natl. Acad. Sci. U.S., 53, 1104 (1965).

22. Crawford, L. V., J. Mol. Biol., 13, 362 (1965).

23. Kleinschmidt, A. K., Kass, S. J., Williams, R. C., and Knight, C. A., J. Mol. Biol., 13, 749 (1965).

24. Crawford, L. V., and Black, P. H., Virology, 24, 388 (1964).

25. Abelson, J., and Thomas, C. A., Jr., J. Mol. Biol., 18, 262 (1966).

26. Doty, P., McGill, B., and Rice, S., Proc. Natl. Acad. Sci. U.S., 44, 432 (1958).

27. Burgi, E., and Hershey, A. D., Biophysic J., 3, 309 (1963).

28. Studier, F. W., J. Mol. Biol., 11, 373 (1965).

29. Josse J., and Eigner, J., Ann. Rev. Biochem., 35, 789 (1966).

30. Rubenstein, I., Thomas, C. A., Jr., and Hershey, A. D., Proc. Natl. Acad. Sci. U.S., 47, 1113 (1961).

31. Davison, P. F., Freifelder, D., Hede, R., and Levinthal, C., Proc. Natl. Acad. Sci. U S., 47, 1123 (1961).

32. Davison, P. F., and Freifelder, D., J. Mol. Biol., 5, 643 (1962).

33. Williams, R. C., and Fraser, D., J. Bacteriol., 66, 458 (1953).

34. Brenner, S., Streisinger, G., Horne, R., and Champe, S., J. Mol. Biol,, 1, 281 (1959).

35. Hershey, A. D., Virology, 4, 237 (1957).

36. Stanier, R. Y., Palleroni, N. J., and Doudoroff, M., J. Gen. Microbiol., 43, 159 (1966).

37. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J., Studies of Biosynthesis in Escherichia coli, (Carnegie Institute of Washington, Publication No. 607, 1957).

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

39. Huxley, H. E., and Zubay, G., J. Biophys. Biochem. Cytol., 11, 273 (1961).

40. Brenner, S., and Horne, R. W., Biochim. Biophys. Acta, 34, 103 (1959).

41. Kleinschmidt, A., Lang, D., and Zahn, R. K., Z. Naturforsch., 166, 730 (1961).

42. Ifft, J. B., Voet, D. H., and Vinograd, J., J. Phys. Chem., 65, 1138 (1961).

43. Armstrong, R. L., and Boezi, J. A., Biochim. Biophys. Acta, 103, 60 (1965).

44. Burton, K., Biochem. J., 62, 315 (1956).

45. Mejbaum, W., Z. Physiol. Chem., 258, 117 (1939).

46. Schildkraut, C. L., Marmur, J., and Doty, P., J. Mol. Biol., 4, 430 (1962).

47. Bendich, A., Methods Enzymol., 3, 715 (1957).

48. Bradley, D. E., and Kay, D., J. Gen. Microbiol., 23, 553 (1960).

49. Davison, P. F. and Freifelder, D., J. Mol. Biol., 5, 635 (1962).

50. Huang, R. C., and Bonner, J., Proc. Natl. Acad. Sci. U.S., 54, 960 (1965).

51. Marmur, J., and Doty, P., J. Mol. Biol., 5, 109 (1962).

52. Mandel, M., J. Gen. Microbiol., 43, 273 (1966).

53. Weil, R., and Vinograd, J., Proc. Natl. Acad. Sci. U.S., 50, 730 (1963).

54. Bode, V. C., and Kaiser, A. D., J. Mol. Biol., 14, 399 (1965).

55. Scherage, H. A., and Mandelkern, L., J. Amer. Chem. Soc., 75, 179 (1953).

56. Rosenbloom, J., and Schumaker, V. N., Biochemistry, 2, 1206 (1963).

57. Crothers, D. M., and Zimm, B. H., J. Mol. Biol., 12, 525 (1965).

58. Aten, J. B. T., and Cohen, J. A., J. Mol. Biol., 12, 537 (1965).

59. Eigner, J., Schildkraut, C., and Doty, P., Biochim. Biophys. Acta, 55, 13 (1962).