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This investigation was undertaken to provide a chemical description of an actinophage for Streptomyces griseus, and to determine the base composition of the phage DNA moiety. It was believed of interest to learn whether the actinophage for the filamentous S. griseus host would be different in its chemical properties from the bacteriophages. No such chemical analysis of an actinophage has been reported. Complete chemical analyses are available only for a few of the Escherichia coli phages.

A method for isolation and purification of the phage was developed, which consisted of isolation by several cycles of differential centrifugation and final purification by centrifugation in a cesium chloride density gradient. The purified phage was analyzed for phosphorus, DNA, nitrogen and protein by means of

standard analytical methods and the resulting data reported, relative to the dry weight of the purified phage. The DNA was extracted from purified phage by the phenol extraction procedure. The purine and pyrimidine base composition of the DNA was determined by standard methods for the hydrolysis of DNA and analysis for the bases by paper chromatography, and also by calculation from the thermal denaturation temperature, or melting point, of the DNA.

It was concluded that the S. griseus phage under study did not differ significantly in its chemical properties from most of the other bacteriophages that have been similarly analyzed. It was shown to consist of about 60% DNA and 40% protein. The DNA content of 60% was somewhat higher than the 50% value generally reported for phages. The analysis of the extracted DNA for purine and pyrimidine bases showed it to be composed of the four usual DNA bases. There was no evidence for the presence of an unusual base. The DNA base composition value of 53.4% guanine plus cytosine determined by chromatographic analysis agrees within experimental error with the 51.4% value calculated from the thermal denaturation temperature. The buoyant density in cesium chloride was found to be 1.55 g/cc.

# PURIFICATION AND ELEMENTARY ANALYSIS OF *STREPTOMYCES GRISEUS* PHAGE

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# PURIFICATION AND ELEMENTARY ANALYSIS OF *STREPTOMYCES GRISEUS* PHAGE

## INTRODUCTION

The chemical characterization of bacteriophage has become an integral part of virus research. Such information is being used in the development of a system for classifying viruses. In addition the chemical nature of phage represents an important feature of the study of phage synthesis.

The *Streptomyces griseus* host phage system differs from others that have been analyzed chemically because of the filamentous and mold-like growth habit of the host. This phage was isolated from soil by Gilmour and Buthala (1950). Growth characteristics of the system have been described by Gilmour, Noller and Watkins (1959). One step growth curve studies showed a latent period of 120 minutes and a burst size of 75 to 100 phages released per germinating spore. Electron microscope studies have shown it to have the tadpole shape characteristic of most bacteriophages. The head is about 95 mμ in diameter and the tail about 350 mμ long.

Few attempts have been made to purify and determine the elementary composition of actinophage. Only Kolstad and Bradly (1964) have reported chemical analyses. Bacq and Dierick

(1962) presented a purification scheme but reported no chemical analysis.

An early attempt to purify and analyze S. griseus phage was made by Evensen (1960). This preparation provided a usable phage protein fraction. However, the low phosphorus content and high nitrogen to phosphorus ratio suggested that possibly some DNA was lost by rupture of phage particles during the harsh ammonium sulfate purification treatment. The S. griseus phage has been found to be sensitive, as are other phages, to a variety of standard chemical treatments such as osmotic shock, ammonium sulfate precipitation, column chromatography on cellulose, etc.

The present study was undertaken to provide a chemical characterization of actinophage. Its objectives were (1) to devise an effective chemical purification method that would minimize loss of phage viability (2) to determine the chemical composition of the purified phage, and (3) to determine the over-all base composition and base ratios of the DNA moiety.

# HISTORICAL REVIEW

The first investigator to report on the chemical composition of bacteriophage was Max Schlesinger (1934). At that time the physical nature of bacteriophage had not been clearly established. Schlesinger believed that such agents must have the form of discrete, biologically active particles. Hetler and Bronfenbrenner (1931) thought that bacteriophage was a molecular size material, attached to colloidal particles in solution. Phage diffusion rates and ultrafiltration data, which clearly must have been in error, suggested to Hetler and Bronfenbrenner (1931) that phage activity was associated with particles of a broad spectrum of sizes, i.e. 0.6 mμ to 11.4 mμ.

However, Schlesinger (1934) recognized that Hetler and Bronfenbrenner's measurements were not reliable, and thereby suggested that other contaminating materials from the broth lysates were present as well as phage. Schlesinger's (1934) sedimentation studies, using the analytical ultracentrifuge, showed the phage particles to have a diameter of 90 mμ. The latter worker had been able to recover all the phage activity in his centrifuged pellets, and on repeated centrifugation did not lose any in the supernatant solutions. He described the turbidity and bluish opalescence characteristic of phage suspensions, and recognized

the characteristic Tyndall light scattering effect, suggesting the presence of discrete particles.

Subsequently, Schlesinger (1934) set out to isolate bacteriophage particles in weighable quantity and determine their chemical composition. His coliphage WLL was believed to be similar to the present day phage T2. He grew the phage in a synthetic medium containing only inorganic salts and sodium lactate to minimize the danger of contamination from other medium components, and described clear lysates containing $5 \times 10^{9}$ active phage units/ml. His purification scheme consisted of filtration through a fine collodion membrane to remove cell debris, followed by repeated centrifugation and resuspensions of the pellet in distilled water. Schlesinger's preparation was not chemically pure by present day criteria, yet his procedure did represent a significant technological advance.

Schlesinger (1934) dried his purified phage carefully in vacuo over $P_2O_5$ to a constant weight of 13.7 mg at $70^{\circ}C$. He related weight and phage biological activities to obtain replicate particle weights of 4.3, 4.6, and $5.0 \times 10^{-13}$ mg/particle. These values check well with the $5 \times 10^{-13}$ mg/particle reported later by Herriott and Barlow (1952) and are not greatly different from the $3.6 \times 10^{-13}$ mg/particle value recently reported by Kozloff

and Cummings (1960). Then, Schlesinger (1934) determined nitrogen, carbon, hydrogen, and phosphorus using the methods commonly used by organic chemists of that time. These data are given in Table 1. His 3.7% phosphorus and 13.4% nitrogen values were only a little lower than Herriott and Barlow's determinations (1952). Phage activity measurements were made by a dilution end point technique. Plaque counting did not come into use until later. His color reaction tests indicated that the phage was composed of the same biochemical substances as the host cells and that phage must be principally nucleoprotein. He obtained a positive Millon's protein test which did not diminish with repeated washing, and a negative Molisch test for carbohydrate. Ether extraction of the phage preparation gave a small lipid residue, containing no nitrogen or phosphorus. Staining reactions and the high phosphorus content convinced Schlesinger that his phage must contain nucleic acid. Subsequently, Schlesinger (1936) described an intense Feulgen Reaction with the same WLL phage and with a Staphylococcus phage. This reaction is generally regarded as specific for DNA. He did not observe a similar reaction with material from the host cells. Thus he presented the first critical data on the chemical nature of bacteriophage.

Other workers reported data on the purification of

Table 1. Observed chemical composition of bacteriophages (percent of dry weight)

| Phage | Phosphorus | DNA | Nitrogen | Protein | Reference |
|---|---|---|---|---|---|
| WLL (T2) | 3.7 | | 13.2 | | Schlesinger (1934) |
| T2-PC | 0.07 | | 14.0 | | Kalmanson and Bronfenbrenner (1939) |
| T2 | 4.8 | 40 | 13.5 | | Hook et al. (1946) |
| T2 | 5.0 | 42 | 13.4 | | Taylor (1946) |
| T2 | 3.7 | 37 | 11.8 | | Cohen and Anderson (1946) |
| T2 | 5.2 | 53 | 16.2 | | Herriott and Barlow (1952) |
| T2 | 3.9 | | | | Cummings and Kozloff (1960) |
| T2 | | 50 | | 40 | Adams (1959) |
| T6 | 5.0 | 42 | 13.4 | | Kozloff and Putnam (1949) |
| T7 | 3.8 | | 12.3 | | Kerby et al. (1949) |
| | 4.2 | 41 | 14.7 | 49 | Czaky et al. (1950) |
| | 5.2 | 52 | 16.2 | | Davidson and Freifelder (1962) |
| Lambda | 4.7 | | | | Kaisar and Hogness (1960) |
| Staphylococcus phage | 4.8 | | 14.4 | | Northrop (1938) |

bacteriophage using the reduction in nitrogen content per unit of viable phage as the criterion of purification. Colwell (1937) purified phage by scraping a lysogenic Escherichi coli culture into saline and centrifuging the cells. Her best preparation contained 270 μg $N/10^{11}$ infective phage units. Kliger and Olitsky prepared purified coliphage containing 1400 μg $N/10^{11}$ infective phage units. Today the generally accepted value for phage T2 is 8-10- μg $N/10^{11}$ plaque forming units.

Northrop (1938) analyzed a Staphylococcus albus phage. The phage was grown in yeast extract broth, which he said contained less than 10 μg of protein nitrogen/ml. His purification consisted of filtration through filter-cel treatment with trypsin to remove a stickly mucin-like material, precipitation with 60% saturated ammonium sulfate, treatment with charcoal to remove color, reprecipitation with 60% saturated ammonium sulfate, filtration through filter-cel, dialysis against water, and drying in vacuo. However, Northrop lost half of the phage activity with each ammonium sulfate precipitation. His final preparation contained about $5000 \times 10^{10}$ active particles /mg N or 20 μg nitrogen per $10^{11}$ active phage particles.

Northrop (1038) also believed that bacteriophage must be a nucleoprotein. His chemical data are shown in Table 1. He reported that the phage contained about 1% glucose and

that the hydrolyzed solution gave a precipitate with silver nitrate that indicated the presence of purine bases. Ultrafiltration experiments showed the particles to be about 90 mμ in diameter, and sedimentation studies gave an $S_{20}$ value of about 650. With a similar phage, Wyckoff (1939) reported that in sedimentation studies sharp boundaries were obtained and that phage activity was alwayss associated with the heavier or lower fraction. However, bands of other lighter material were also observed, indicating contamination by other cellular or medium components.

Kalmanson and Bronfenbrenner (1939) performed similar experiments with coliphage T2-PC. These investigators also cultured the phage in a synthetic medium in order to minimize the danger of contamination of the purified phage with medium components. Their purification consisted of filtering through a Berkefield filter to remove cell debris, collecting the phage on a collodion filter, washing the residue until a Nessler's test showed complete removal of ammonium ion, and drying in vacuo.

Kalmanson and Bronfenbrenner (1939) reported that their final preparation contained 1.8 μg of nitrogen/$10^{11}$ active phage particles, and that the particle weight was $6 \times 10^{-11}$ μg/phage unit. Since these values were much lower than more recent data, the possibility of procedural errors existed. When the phage was

grown in Northrop's yeast extract broth a nitrogen content of 2600 $\mu g/10^{11}$ phage units and a particle weight of 35,000 $\mu g/10^{11}$ particles was obtained. They concluded that the purification method was inadequate when the phage was grown in yeast extract broth.

Kalmanson and Bronfenbrenner (1939) analyzed chemically the dried purified phage from the lysates grown in synthetic medium (Table 1). The results correlated well enough with other reported data except that the phosphorus content was so low as to be negligible. With disregard for the evidence the other workers had reported for a nucleic acid component, they concluded that bacteriophage must be all protein.

Bacteriophage was now known to exist as discrete uniform biologically active particles made of nucleoprotein. It was the protein component which was considered important; little attention being given to the nucleic acid moiety. Tobacco mosaic virus had been crystallized, and virus activity was believed to be associated with the crystalline protein and in fact inseparable from it. The small nucleic acid component was not considered to be essential for virus biological activity. Viruses were thought of as being like a self replicating enzyme-like protein. It was believed then that some enzymes could be self replicating. Northrop's purification scheme was patterned after enzyme purification methods. The function and importance of DNA and RNA

in biology was not yet recognized.

A number of years passed before more work was carried out on the chemical composition of phage. The electron microscope became available, and much was learned about the morphology of viruses. Bacteriophages were found to be composed of unique morphological structures, presumable synthesized in an orderly fashion. Many exhibited a tail and a head containing DNA inside a protein coat. This knowledge led to a different concept of the nature of viruses as parasites; they could no longer be thought of as being enzyme-like. And it had become apparent that proteins were not self-replicating entities. Meaningful metabolic studies were begun to learn how phage replicates in a cell, and how cell metabolism changes on phage infection. The plaque counting method for measuring phage activity had come into use.

It became clear that the greatest problem in conclusively determining the chemical composition of the bacteriophage was that of obtaining phage in an entirely pure form. Some of the early workers thought that phage could be purified by differential centrifugation alone. It became apparent that lysates contained particulate cell debris matter which could not be separated by centrifugation alone, as well as other impurities which tended to

adhere to the phage. And, at first, criteria for determining purity were not clearly established. For example, early analyses of the T2 phage showed the presence of an RNA component. Those early preparations must have contained some host cell ribosomal material. Later analyses of more highly purified phage preparation demonstrated that the phage particle does not contain RNA. For several years it was believed to be true for phage as well as for bacteria that those grown in a rich broth medium had a different chemical composition from those grown in a strictly synthetic medium. This idea was abandoned as more highly purified phage preparations were obtained and analyzed.

Coliphage T2 was selected for use as the model for the study of bacteriphage growth, and became therefore, the system whose chemical composition was investigated the most thoroughly. Phages T2, T4, and T6 were closely related in morphology and in chemical characteristics. Phage T7 had also been well studied by investigators who wished to study a coliphage other than T2. Phage T7 is smaller, has only a very short tail and is serologically unrelated to the T-even phages. Also, its DNA does not contain the hydroxymethyl cytosine component which is characteristic of the T-even phages.

Hooke et al (1946) investigated the chemical nature of

coliphage T2. However, their preparation was not entirely pure. They reported RNA and lipid components which must have been impurities. Their purification method included only an initial filtration through a Mandler candle, one cycle of differential centrifugation, dialysis against water, and dry-in vacuo. Pertinent results are listed in Table 1. These investigators reported positive color reactions for protein, pentose, deoxypentose, and for a hexose, presumably glucose.

Sedimentation studies carried out with phage T2 by Hooke et al. (1946), using the analytical ultracentrifuge showed, along with slow moving bands of impurities, two faster moving bands with $S_{20}$ values of about 700 and 1000. Both the latter two fractions were associated with phage activity. The slower moving band was the more prominent in an NaCl solution containing no calcium, while the faster moving fraction became the more predominant one when calcium was added. This double boundary phenomenon has been explained only recently by Kozloff and Cummings (1960) as resulting from a change in form of the head which they believed to be a result of configurational changes in the head protein. They found by electron microscope measurements that the slower moving form had dimensions of 123 mμ x 85 mμ, and the faster one 106 mμ x 86 mμ, along with a more

defined polyhedral shape. They observed that low temperatures and the presence of calcium tended to maintain the phage heads in the shorter, faster moving form.

Taylor (1946) reported analysis of a T2 phage preparation which was supposedly more refined. He carried out three cycles of differential centrifugation in place of only one. His data are given in Table 1. He also reported RNA and lipid components which he believed to be part of the phage. He also analyzed phage grown in both broth and synthetic medium and reported small differences in composition.

Cohen and Anderson (1946) presented data on another carefully prepared T2 phage product and also provided chemical data. Their data for the preparation are shown in Table 1. The purification included an initial filtration through celite, several cycles of differential centrifugation, and dialysis against distilled water. These workers claimed that they did not find an RNA component. They also stated that the amount of deoxypentose measured by the diphenylamine reaction, which is specific for deoxypentose, accounted for all the pentose measured by the orcinol and the Seliwanoff reactions. In addition, Cohen and Anderson (1946) observed a color reaction with the orcinol-sulfuric acid reagent which must have arisen from the glucose now known to be a component of phage T2.

Kozloff and Putnam (1940) reported on the purification and analysis of phage T6, which was known to closely relate to phage T2. They gave more attention to the purity of their final product. The purification was effected by an initial filtration several cycles of differential centrifugation, and dialysis against distilled water. Pertinent chemical results are given in Table 1. These workers found their preparation to be homogenous when subjected to electrophoresis and sedimentation procedures. The possible presence of an RNA component was carefully checked by separation of RNA from DNA by the Schmidt-Thannhauser procedure and analyzing the RNA fraction for phosphorus. The amount of RNA phosphorus was not sufficiently high to establish the presence of RNA in a material rich in DNA. Also, they analyzed phage grown in broth and in a strictly synthetic medium and found no difference in composition of the phage. These investigators observed the double boundary phenomenon during ultracentrifugation studies. They concluded that some of the particles were heavier because of adhering free DNA from ruptured phage. They had noticed that purified phage did sometimes have some free DNA associated with it.

Cohen and Arbogast (1950) provided analyses for phages, T2, T4, and T6. These workers suspected that phage purified

by differential centrifugation might contain some host cell particulate material. They used the absence of a serological reaction with host cell antibody as the criterion of purity. They observed that the preparations containing non-DNA phosphorus also contained host cell antigens. Their data for DNA content relative to units of phage plaque forming activity are given in Table 2.

Herriott and Barlow (1952) reported a chemical analysis of phage T2 that has been the most reliable. Their results are given in Table 1. These investigators employed a more complete purification method. Their scheme included an initial filtration with celite, precipitation of the phage at pH 4.0, treatment with DN Ase, and several cycles of differential centrifugation. Presumably, the product was of higher purity than the preparations described by other workers. No evidence of measurable RNA or lipid was found. All of the phosphorus was accounted for as DNA phosphorous. The DNA content obtained by colorimetric analyses with diphenylamine and DNA calculated from the phosphorus content (assuming DNA to be 10% phosphorus) were identical. Cummings and Kozloff (1960) have reported a phosphorus content of 3.9%, which is lower than Herriott and Barlow's value of 5.2%. However, Cummings and Kozloff (1960) did not describe methods used to obtain their data.

Volkin and Astrachan (1956) further investigated the question of whether phage T2 contains an RNA fraction. They purified the phage by the procedure described by Herriott and Barlow (1952). There was only a trace of RNA in some of the preparations, and the RNA that was identified was shown to be of the same base composition as the RNA of the host cells. It was now demonstrated conclusively that the phage T2 particle does not contain an RNA component.

Kerby et al. (1949) carried out an analysis for phage T7. Chemical purification was attained by initial filtration using celite and several cycles of differential centrifugation. The chemical results are listed in Tables 1 and 2. These workers expressed nitrogen content relative to units of phage activity as well as to dry weight. They also reported a particle weight of 40 $\mu g/10/^{11}$ plaque forming units. The data expressed in terms of phage plaque forming units appeared higher than similar data reported by others. It was probably that the purified phage suspensions contained inactive phage particles. Their sedimentation studies did not reveal the double boundary phenomenon that had been observed with phage T2.

Csaky et al. (1950) reported on the chemical analysis for phage T7. Their results are listed in Table 1. They prepared

Table 2. Observed chemical composition of bacteriophages in $\mu g/1 \times 10^{11}$ plaque forming units

| Phage | Phosphorus | DNA | Nitrogen | Protein | Reference |
|---|---|---|---|---|---|
| T2 | | | 10.0 | | Hook et al. (1946) |
| T2 | | 34 | | | Cohen & Arbogast (1950) |
| T2 | 4.6 | | | | Labaw (1951) |
| T2 | 2.3 | | | | Stent & Fuerst (1955) |
| T2 | | 33 | | 33 | Creasser & Taussig (1957) |
| T4 | | 23 | | | Cohen & Arbogast (1950) |
| T6 | | 34 | | | Cohen & Arbogast (1950) |
| T6 | 3.3 | | | | Labaw (1953) |
| T6 | 3.95 | | | | Kozloff and Putnam (1949) |
| T1 | 2.1 | | | | Labaw (1951) |
| T1 | 1.8 | | | | Labaw (1953) |
| T1 | 0.7 | | | | Stent & Fuerst (1955) |
| T1 | | 12 | | 24 | Creasser and Taussig (1957) |
| T3 | 1.7 | | | | Labaw (1951) |
| T3 | 0.9 | | | | Stent and Fuerst (1955) |
| T7 | | | 20.0 | | Kerby et al. (1949) |
| T7 | 1.7 | | 5.3 | | Putnam et al. (1950) |
| T7 | 1.7 | | | | Labaw (1951) |
| T7 | 1.3 | | | | Labaw (1953) |
| T7 | 0.9 | | | | Stent and Fuerst (1955) |
| T7 | 0.82 | 6.25 | | | Lunan and Sinsheimer (1956) |
| T5 | 10.0 | | 36.0 | | Smith and Wyatt (1951) |
| T5 | 4.0 | | | | Labaw (1951) |
| T5 | 3.9 | | | | Labaw (1953) |
| T5 | 1.8 | | | | Stent and Fuerst (1955) |
| MSP8[1] | 1.47 | 14.5 | 4.0 | | Kolstad & Bradley (1964) |
| Phage G[2] | 0.69 | | 2.16 | | Murphy and Phillipson |

Table 2 (Continued)

[1]An actinophage for *Streptomyces venezuelae*
[2]A phage for *Bacillus megaterium*

their phage by an initial filtration, several cycles of differential centrifugation, and dialysis against distilled water. The product was probably not altogether pure since they claimed to have observed an RNA fraction.

Lunan and Sinsheimer (1956) performed a similar purification procedure with phage T7, but included a DNAse treatment following the dialysis against water. They reported phosphorus and DNA contents, which are given in Table 2. However, these workers could account for only 71% of the phosphorus as DNA phosphorus. The DNA content as determined by the diphenylamine reaction was only 71% of that calculated from the phosphorus content.

Davidson and Freifelder (1962) claimed to have accounted for all the phosphorus as DNA phosphorus. However, they did not discuss the pertinent chemical procedures. Their primary interest in phage T7 was in the latter's use as a source of monodisperse phage DNA for physical studies. These workers calculated that the phage was about 50%-55% DNA and reported a nitrogen to phosphorus ratio of 3.1. These data implied 5.0% to 5.5% total phosphorus and 15.5% to 17.5% total nitrogen. These values were somewhat higher than those reported earlier, indicating that more highly purified preparation was used for the

analyses. The purification procedure involved an initial filtration through celite, several cycles of differential centrifugation, and banding in a cesium chloride density gradient.

Smith and Wyatt (1951) reported nitrogen and phosphorus data for phage T5 relative to phage activity. A few other investigators have reported similar data expressed on the basis of viable phage. Labaw (1951, 1953) analyzed several phages for phosphorus in connection with studies on phosphorus metabolism obtained during phage synthesis. Hershey, Dixon and Chase (1953) reported a phosphorus content for phage T2 in conjunction with a metabolic study. Stent and Fuerst (1955) gave phosphorus values for several phages that were obtained from the measurement of incorporated radioactive phosphorus. Creasser and Taussig (1957) reported DNA and protein contents for phages T1 and T2. All of these data are listed in Table 2.

Murphy and Phillipson (1962) have presented a purification and analysis of Bacillus megaterium phage G. The purification scheme included differential centrifugation, filtration through filter-cel, and column chromatography on DEAE cellulose. Their values for nitrogen and phosphorus relative to phage activity are given in Table 2. As might be expected, the values proved to be lower than those reported for phage T2, since T2 is

2.6 times larger than the B megaterium phage.

Kolstad and Bradley (1964) studied the purification of Streptomyces venezuelae phage MSP by a two phase partion system using sodium dextran sulfate and polyethylene glycol, differential centrifugation, and treatment with RNAse and DNAse. This method included concentration of the phage by dialysis of a dilute suspension against polyethylene glycol, and purification by DEAE cellulose column chromatography. Their results for nitrogen, phosphorus, and DNA relative to phage activity are given in Table 2.

An aforementioned survey of the literature revealed that only with the coliphages had the chemical composition been carefully determined and reported relative the dry weight of the purified phage preparation. In recent years there has been more interest in determining the relative amounts of the purine and pyrimidine bases in phage DNA. Smith and Wyatt (1951) reported the first DNA base composition analyses. They described analyses of DNA for phages T2 and T 5, and a gypsy moth virus. They hydrolyzed the DNA in perchloric acid, separated the bases chemically by paper chromatography, eluted the bases from the paper, and detected these by measuring UV absorption. In any double stranded DNA, guanine is paired with cytosine and

adenine with thymine according to Watson and Crick's classic model of DNA structure. All known DNA is double stranded except for that of a few small viruses. The molar amounts of guanine and cytosine and of thymine a double stranded DNA must therefore be equal (G + C value).

Wyatt and Cohen (1953) first reported identification of the unusual base hydroxymethylcytosine which replaces cytosine in the DNA on the T-even coliphages. One other instance of the occurrance of an unusual pyrimidine base in DNA has been reported. Takahashi and Marmur (1963) found 5-hydroxymethyluracil to replace thymine in the DNA of Bacillus subtilis phage PBS2. Such replacement of one of the usual bases with a different one has not been observed for any biological species other than these two bacteriophages.

In recent years methods have been developed for determining base composition of DNA expressed in %G + C by measuring the thermal denaturation temperature or the buoyant density in cesium chloride. These methods have been considerably less tedious and time consuming than Wyatt's classic chemical separation by paper chromatography.

The thermal denaturation temperature, which is commonly referred to as the melting point or $T_m$ value, is the temperature at which the hydrogen bonds between the two strands of the DNA

are broken. The $T_m$ value is determined by observing the temperature at which the sharp increase of about 30% in the optical density of the DNA occurs. Marmur and Doty (1962) have developed a convenient quantitative relationship by which %G + C can be calculated from the melting point. In addition, Schildkraut, Marmur, and Doty (1962) have developed a convenient method for calculating the %G + C from the phage bouyant density value.

The DNA base composition reported for a number of bacteriophages and for some of their bacterial hosts are summarized in Tables 3 and 4. Examination of these data suggest that there was not a direct relationship between the DNA base compositions of the virulent phages and those of their host bacteria.

Some biologists have been interested in relating DNA base compositions to species differentiation and groupings among species. Among the simpler forms of life such as viruses and bacteria, DNA base composition values have varied widely, from 25% to 75% guanine plus cytosine. It has been currently of interest to consider whether determination of DNA base compositions of bacteriophages might aid in the development of a system for classifying them, and whether phages of similar DNA base ratios might be related. There is not yet sufficient DNA base composition data for enough bacteriophages for a conclusion

Table 3. DNA base composition of some Escherichia coli bacteriophages[1]

| Phage | mo l% Guanine | Cytosine | Adenine | Thymine | % G C | Reference |
|---|---|---|---|---|---|---|
| T2[2] | 18.3 | 17.0 | 32.4 | 32.4 | 35.3 | Wyatt and Cohen (1953) |
| T4[2] | 18.0 | 16.3 | 32.2 | 33.5 | 34.3 | Wyatt and Cohen (1953) |
| T6[2] | 17.7 | 16.6 | 22.5 | 33.4 | 34.3 | Wyatt and Cohen (1953) |
| C16[2] | 18.0 | 19.0 | 31.0 | 32.0 | 37.0 | Jesaitis (1959) |
| T1 | 23.0 | 25.0 | 27.0 | 25.0 | 48.0 | Creasser and Taussig (1957) |
| T3 | 23.5 | 26.3 | 22.8 | 27.8 | 49.8 | Knight (1954) |
| T7 | 24.6 | 22.8 | 25.7 | 26.9 | 47.4 | Volkin, Astrachan, & Countryman (1958) |
| Lambda | 24.4 | 24.2 | 25.6 | 25.6 | 49.0 | Kaisar and Hogness (1960) |

[1] The % G + C for the Escherichia coli host is 50%

[2] Hydroxymethyl cytosine replaces cytosine

Table 4. DNA base composition for selected bacteriophage systems

| Host and Phage | mol % | | | | % G+C | Reference |
|---|---|---|---|---|---|---|
| | Guanine | Cytosine | Adenine | Thymine | | |
| Condrococcus columnaris[1] | | | | | | |
| Myxobacteriophage C2 | 23 | 18.5 | 28.8 | 30.3 | 41.0 | Kingsbury (1964) |
| Streptococcus[2] diacetylactis | | | | | | |
| Phage acili | | | | | 35.7 | Henning (1966) |
| Streptococcus group D | | | | | | |
| Phage P3 | | | | | 30.0 | Brock, Johnson & |
| Phage P9 | | | | | 39.0 | Deville (1965) |
| Micrococcus lysodiekticus[3] | | | | | | |
| Phage N1 | 31.9 | 37.2 | 18.0 | 13.0 | 69.1 | Sculetti and |
| Phage N6 | 30.6 | 39.1 | 16.0 | 14.6 | 69.7 | Naylor (1959) |
| Bacillus subtilis[4] | | | | | | |
| Phage SP8[5] | | | | | 43.1 | Kallen, Simon & Marmur (1962) |
| Phage $\phi$ e[5] | | | | | 40.3 | Roscoe & Tucker (1964) |
| Phage SP10[5] | 21.2 | 22.0 | 29.9 | 27.1 | 43.2 | Okuba et al. (1963) |
| Phage PBS2 | 14.5 | 13.8 | 36.4 | 35.4 | 27.8 | Takahashi & Marmur (1961) |

Table 4. (Continued)

| Host and Phage | mol % | | | | | |
|---|---|---|---|---|---|---|
| | Guanine | Cytosine | Adenine | Thymine | % G + C | Reference |
| Bacillus megaterium Phage alpha | 19.7 | 21.6 | 28.8 | 30.5 | 41.3 | Aurisicchio et al. (1960) |
| Bacillus steareothermophilis[6] Phage Tp-84 | | | | | 41.7 | Saunders and Campbell (1966) |
| Brucella species series of 11 phages | | | | | 45.5[7] 46.7 | Calderone & Pickett (1965) |
| Mycobacterium jucho phage choremis | 33.4 | 33.4 | 17.2 | 15.9 | 66.9 | Tokunaga, Mizughi, Murohashi (1963) |
| Pseudomonas aeruginase[8] Phage 2 | 27.5 | 27.0 | 22.5 | 23.0 | 54.5 | Grogan and Johnson (1965) |

[1] % G + C for host is 43%
[2] % G + C for host is 28.6%
[3] % G + C for host is 75.6%
[4] % G + C for host is 42%
[5] 5-hydroxymethyl uracil replaces thymine
[6] % G + C for host is 50.5%
[7] % G + C for all the 11 phage were within this range
[8] % G + C for host is 66.1%

to be drawn. However, a study of the data summarized in Tables 3 and 4 might suggest some groups of phages similar in other characteristics that are similar in DNA base ratios as well. While DNA base compositions have been reported for several Streptomyces species, such information has not yet been reported for a phage for a Streptomyces host.

# MATERIALS AND METHODS

## The Host-phage System

The phage used was actinophage strain 514-3 isolated from soil by Gilmour and Buthala (1950). The growth characteristics have been described by Gilmour, Noller, and Watkins (1959).

The phage was assayed by the soft agar overlay technique described by Adams (1959). The medium used was glucose nutrient agar containing 0.5% glucose, 0.5% peptone, 0.3% beef extract, and 0.01% yeast extract. The soft agar contained 0.65% agar; the base layer 1.7%. Three ml of soft agar at 50°C were mixed with 0.2 ml of spore suspension and 0.1 ml of phage suspension, and the mixture poured over 20 ml of base agar. The plates were incubated 24 hours at 30°C before the plaques were counted.

High titer stock suspensions of the phage were prepared from confluently lysed soft agar overlay plates. After 15-18 hours incubation at 30°C the soft agar layer was scraped off into a 0.25% peptone solution, shaken ten minutes on a platform shaker, centrifuged to remove agar, and sterilized by filtration through a Seitz filter.

The host used was Streptomyces griseus strain 3475, the

original Waksman streptomycin producer. The stock culture was maintained in sterilized soil. Soil inoculum was streaked on slants of a semi-synthetic medium consisting of 1% glycerol, 0.1% asparagine, 0.1% glucose, 0.1% $K_2HPO_4$, and 1.7% agar. These were incubated for two days at 30°C and five days at room temperature. Then the thick white spores on the surface of the slant were scraped off into 0.25% peptone. Finally, the spore suspension was filtered through a 30 layer gauze pad and diluted with 0.25% peptone to a standard optical density corresponding to $6 \times 10^8$ spores/ml, as determined by plate counts in a glucose nutrient agar.

Preparation of High Titer Lysates

Lysates containing $1 \times 10^{10}$ phage plaque forming units/ml (PFU) could be obtained easily, and lysates containing 5 to 10 x $10^{10}$ PFU/ml could be prepared routinely, after an intuitive acquaintance with the system had been gained. The broth medium contained 1% peptone, 0.2% glucose, 0.1% yeast extract, and two sprays of Dow Corning antifoam A. Sterile $CaCl_2$ solution to give a 0.001 - 0.002M concentration was added after sterilization. The calcium was found to be essential for S. griseus phage replication.

Lysates were grown at 30°C in liter quantities in two liter flasks, equipped with sintered glass dispersion tubes for aeration. Air was drawn in via a column of sterile cotton, using a water faucet pump to obtain the necessary vacuum. Lysates could also be prepared on a platform shaker incubator without aeration, but much better phage titers were obtained with aeration.

## Phage Purification

A number of preliminary centrifugation and pellet resuspension procedures were tested prior to the emergence of the final integrated purification scheme. The complete step-wise procedure is given below whereas the procedure for individual steps is given along with results. This organization was adopted because of the numerous tests carried out before the final scheme was adopted.

The phage was purified by three cycles of differential centrifugation, treatment with DNAse and RNAse, and banding in a CsCl density gradient. The lysate was first centrifuged at 7000 x g for an hour to sediment cell debris, then filtered through a Whatman #3 filter paper. The filtrate was centrifuged at 30,000 x g for 2.5 hours to sediment the phage. The pellets were resuspended in 1-2% glycerol containing 0.01M $CaCl_2$ and $MgCl_2$.

The suspension was then centrifuged at 6000 x g for five to ten minutes to remove particulate cell debris. The supernatant suspension was now incubated with DNAse and RNAse at a concentration of 5-10 μg/ml of each enzyme at room temperature for one hour, and then centrifuged at 30,000 x g for two hours. This final pellet was resuspended in a minimum amount of the glycerol solution, and centrifuged at 6000 x g. The concentrated supernatant phage suspension was mixed with an equal volume of saturated CsCl solution so that the density of the mixture was 1.46 g/cc. The mixture was then centrifuged at 30,000 rpm in a SW39 swinging bucket rotor for 20 hours in a Spinco model L-2 ultracentrifuge. A sharp band containing the phage formed a little below the middle of the tube, and one or two diffuse bands of contaminating protein material appeared near the surface. The phage band was separated from the others by use of a tube slicer, and the suspension dialyzed against distilled water or against 0.01M tris buffer at pH 7.2 containing 0.01M $CaCl_2$ and $MgCl_2$.

Phosphorus Analysis

The method of Berenblum and Chain as modified by Martin and Doty (1949) was used. The ammonium phosphomolybdate complex was extracted into a 1:1 mixture of isobutanol and

benzene and then reduced with $SnCl_2$. The usable range for the method was from 2 to 16 μg of phosphorus.

The standard phosphorus solution was prepared from $KH_2PO_4$ that had been recrystallized three times, dried at 110°C, and stored in a desiccator over concentrated $H_2SO_4$. The solution was prepared at a concentration of 1 mg/ml of phosphorus and stabilized by the addition of a few drops of $KMNO_4$ solution. A working standard was prepared fresh each day by diluting it 1:100 (W. A. Pons, Jr., M. F. Stansbury, and C. L. Hoffpauir; 1953).

The purified phage in water solution was wet ashed in 10N $H_2SO_4$ at 145°C for 30 minutes (Manometric Techniques 3rd. ed. 1957). If the solution was still colored a small drop of 30% $H_2O_2$ was added and heating repeated. This treatment was repeated until the solution was colorless. An excess of $H_2O_2$ must be avoided because it interferes with formation of the ammonium phosphomolybdate complex. The ashed solution was diluted with 1-2 mls of water, heated in boiling water to decompose pyrophosphates, and then neutralized with 5N NaOH. Five ml of a 1:1 mixture of benzene and isobutanol were followed by 0.7 to 0.8 ml of 10% ammonium molybdate reagent. The solutions were mixed by shaking on a vortex mixter 20 to 30 seconds. Then three ml were taken from the organic layer and diluted with five ml

of acid alcohol reagent. The blue color was developed by the addition of 0.5 ml of diluted $SnCl_2$ reagent, and the samples allowed to stand for 1 or 2 hours to obtain maximum color development. Optical density was measured at 625 mμ using a Bausch and Lomb spectronic 20 colorimeter.

Reagents were prepared as follows: 1. 10% ammonium molybdate; 10 g $(NH_4)_6MoO_{24}$ were dissolved in 40 ml 10N $H_2SO_4$, then diluted to 100 ml with water; 2. Acid alcohol; 95% ethanol was made 3.2% v/v in conc. $H_2SO_4$ (This reagent must be prepared fresh each day); 3. $SnCl_2$ reagent; 20 g of $SnCl_2$ were dissolved in 10 ml of conc. HCl. The diluted solution was prepared by diluting it, 1:200 with 1 ON $H_2SO_4$. The diluted $SnCl_2$ solution must be prepared immediately before use.

Colorimetric Analysis for DNA

The method of Burton (1956) using the reaction of diphenylamine with the deoxyribose of DNA was used. The standard used was a Sigma chemical company high quality calf thymus DNA preparation. A stock standard solution was prepared by dissolving the DNA at a concentration of 0.4 mg/ml in 5 mM NaOH. Working standards were prepared by mixing a measured volume with an equal volume of 1N $HClO_4$ and heating at 70°C for 15

minutes. Standardization was based on phosphorus content, as determined by analyzing the stock for phosphorus. The concentration range of the analysis was from 2 to 20 μg of DNA phosphorus. The results are reported as DNA-P delivered by the diphenylamine reaction. Calculation of the amount of DNA was based on the assumption that DNA is 10% phosphorus.

The phage DNA analysis was carried out on aliquots of purified phage in water solution. The aliquots were diluted to 2 ml and made 0.5N $HClO_4$, then mixed with double the volume of the diphenylamine reagent solution. The blue color was developed by incubation at 32°C for 16-20 hours. Optical density at 660 mμ was measured using a Bausch and Lomb spectronic 20 colorimeter.

The diphenylamine reagent was recrystallized twice from 75% ethanol. The solution was prepared by dissolving 1.5 g in 100 ml redistilled glacial acetic acid, then adding 1.5 ml conc. $H_2SO_4$. One-tenth ml of acetaldehyde solution containing 16 mg/ml was added for each 20 ml of reagent. The acetaldehyde must be added immediately before use.

Nitrogen Determination

A micro-Kjehldahl method for the determination of organic

nitrogen described by Ballantine (1957) was used. Organic nitrogen was converted to $NH_4HSO_4$ by digestion with $H_2SO_4$ and $H_2O_2$. Ammonia was released by addition of strong alkali, steam di stilled into a boric acid solution, and titrated with standard $KH10_4$. The concentration for which the method was used is 100 µg to 1000 µg of nitrogen. An $(NH_4)_2SO_4$ solution was used as the standard.

Aliquots of purified phage in water solution containing from one to ten mg of protein were digested in micro-kjeldahl flasks with one ml $H_2SO_4$ until dense white fumes of $SO_3$ appeared. The flasks were cooled. Two drops of $H_2O_2$ were added carefully, one at a time, and the mixture heated again for five minutes. This treatment was repeated until the mixture was colorless. The digest was then transferred to a micro-Kjehdahl glass steam distillation apparatus and made alkaline by the addition of 20 ml of 10% NaOH. The ammonia was steam distilled into a receiving flask containing five ml of 4% boric acid and a few drops of indicator. The indicator was a mixture of five parts of 0.2% bromcresol green and one part 0.02% methyl red in 95% ethanol. The mixture was titrated with standard 0.01M $KH10_4$ solution. Each ml of the standard $KH10_4$ solution was equivalent to

0.14008 mg of nitrogen.

Protein Determination

A colorimetric assay for protein was done by the method of Lowry (1951). The final blue color is a result of a biuret reaction of copper with protein in alkaline solution and of the reduction of the phosphomolybdate-phosphotungstate reagent which is the essential ingredient of the Folin-Ciocalteau phenol reagent. The method described was used for 40 μg or less of protein. The analysis was standardized using a solution of bovine serum albumin.

Reagent A was 2% $Na_2CO_3$ in 0.10 NaOH and served to buffer the reaction mixture at pH 10, and reagent B was 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium or potassium tartrate. Reagent C was 50 parts of A mixed with one part of B. This solution was prepared fresh each day. The Folin-Ciocalteau phenol reagent was obtained commercially from the Fisher Scientific Company.

Aliquots of purified phage in water solution containing less than 40 μg of protein in 0.5 ml were prepared. Two and one-half of reagent C were added. The sample was mixed well and let stand at room temperature for at least ten minutes. One tenth of Folin reagent was added and the solution mixed very rapidly,

since the reagent was stable for only a short time at the highly alkaline pH of the reaction mixture. After 30 minutes or longer the optical density at 725 mμ was measured in a Beckman model B spectrophotometer.

Extraction of DNA

A modification of the phenol extraction method described by Foldes and Trautner (1964) was used. The purified phage was placed in 0.01M tris buffer at pH 7.2. An equal volume of 0.2M NaCl in 0.01M tris buffer pH 7.2, containing 20 mg/ml of sodium lauryl sulfate was added. An equal volume of freshly distilled phenol, saturated with 0.01M tris buffer pH 8.5 was added, and the mixture shaken gently for ten minutes in a water bath at 50°-60°C. The mixture was then centrifuged in a clinical centrifuge to separate the layers. The viscous aqueous layer, containing the DNA, was removed with a pipet. The protein remained at the interface. The extraction was repeated twice. The DNA fibers were collected by winding on a stirring rod, and then redissolved in diluted saline citrate (0.015M NaCl in 0.0015M trisodium citrate at pH 7.0). Since DNA dissolved very slowly it was necessary to let it stand in the cold overnight. The precipitation was repeated twice. For optical density measurements

the solution was brought to the standard saline citrate concentration of 0.15M NaCl and 0.015M trisodium citrate by adding 1.5M NaCl in 0.15M trisodium citrate at pH 7.0. DNA to be used for paper chromatography was air dried on the stirring rod.

## Determination of DNA Base Composition

The DNA was hydrolyzed in 88% formic acid as described by Wyatt (1951). Two to three mg of air dried DNA were mixed with 0.5 to 1.0 ml of 88% formic acid and the tube carefully sealed. The hydrolysis was carried out in an oven at 120°C for 15 hours. The tube was opened carefully to allow escape of gases produced by decomposition of formic acid. The formic acid was evaporated at 75°C under reduced pressure, and the residue taken up in 50 λ of 1N HCl.

The base composition was determined by separating the purines and pyrimidines by two dimensional paper chromatography as described by Wyatt (1955). The bases were eluted from the paper into 0.1N HCl, and the respective optical densities measured at the wave length of maximum ultraviolet absorption, as described by Bendich (1957).

The Whatman #1 paper was first acid washed to reduce the background of UV absorbing materials by letting 2N HCl run

over the paper for a day or two in the chromatography tank. An all glass tank designed for descending chromatography was used.

Ten λ to 15 λ of the DNA hydrolysate solution were spotted on one corner of the paper. A mixture of 68 parts isopropanol, 16.7 parts conc. HCl, and 15.3 parts water was the first solvent. It moved about 30 cm in about 15 hours. The second solvent was a mixture of 100 parts n-butanol, 18 parts water, and three parts conc. $NH_4OH$. The latter solvent ran off the edge of the paper at about 40 cm in about 15 hours. The compounds were located by shining an ultraviolet mineralight lamp on the paper. Squares of paper of constant size were cut from the paper and shredded into screw capped tubes. Similar squares were cut from appropriate locations on the paper for use as blanks. Five ml of 0.1N HCl were added to each and the tubes shaken for 24 to 36 hours. Then the acid solution was decanted from the shredded paper, and shreds of cellulose removed by centrifugation at 10,000 x g for 30 minutes. Optical densities of the supernatant solutions were then measured using a Cary model 11 recording spectrophotometer. The solutions similarly eluted from appropriate blank areas on the paper were used to balance the instrument's base line.

Extinction coefficients used were $13.1 \times 10^3$ at 262 mμ for adenine, $10.4 \times 10^3$ at 275 mμ for cytosine, $11.4 \times 10^3$ at

248 mμ for guanine (General Biochemicals Company technical bulletin AD-12), and $7.9 \times 10^3$ at 265 mμ for thymine (Sigma Chemical Company 1964 price list). Molar concentrations were calculated using the relationship; molar concentration = O. D. at maximum / extinction coefficient.

## DNA Melting Point Determination

The thermal denaturation temperature, or melting point, of the DNA was determined by observing the temperature at which the optical density at 260 mμ increased sharply. The DNA was dissolved in standard saline citrate (0.15M NaCl in 0.015M trisodium citrate at pH 7.0), as described by Mamur and Doty (1962). The concentration of the solution was adjusted so that the optical density was 0.2 at 260 mμ.

The measurement was made using a Gilford model 2000 multiple sample absorbance recorder equipped with a HAAKE temperature control bath, a thermocouple for temperature recording, an automatic sample changer, and the Beckman DU spectrophotometer, as the monochromator. The recorder measured optical density and temperature simultaneously, and its chart provided the correlation between them. The melting point was the temperature at the midpoint where the largest

increase in optical density occurred.

Measurement of Buoyant Density

Buoyant density was measured by density gradient centrifugation in CsCl as described by Weigle and Meselson (1959). Lambda phage, whose buoyant density was known to be 1.51, was used as the reference standard. Two and one half ml of saturated CsCl solution were mixed with 2.5 ml of S. griseus phage suspension containing approximately $1 \times 10^8$ PFU and 0.02 ml of the lambda phage suspension containing approximately $1 \times 10^8$ PFU in a 5 ml cellulose nitrate centrifuge tube. The mixture was centrifuged 20 hours at 29,000 rpm in a SW39 swinging bucket rotor in a Spinco model L-2 ultracentrifuge.

Fractions of four drops each were collected in a series of small tubes from a small hole in the bottom of the centrifuge tube. Alternate tubes contained nutrient broth, and these fractions were used for phage activity measurement. The portions in the other tubes were used for density measurement. Density was calculated from the refractive index, which was measured using a Bausch & Lomb abbé refractometer. The calculation was made using the relation $\sigma^{20} = 10.860\ N^{25}_{D} - 13,500$, in which $\sigma$ is density and N is refractive index for the

sodium D line. Density of the solution and phage activity were plotted against fraction number. The bouyant density of the phage was taken as the density of the portion of the solution containing the phage activity.

# RESULTS

## Preparation of High Titer Lysate

The preparation of high titer lysates often constitutes a serious preliminary problem in any phage purification work. The S. griseus host phage system produces progeny at a rather low growth level; thereby dictating that a procedure be developed for the production of high titer lysates. To obtain maximum phage yield with this filamentous host system requires mainly that the host and phage be mixed so that a maximum of phage-growth recycling occurs. The method for attaining the latter aim was determined through experience and observation of many lysates.

A good yield could be obtained by adding 25 ml of a S. griseus spore suspension containing $3 \times 10^8$/ml of spores to 500 ml of glucose nutrient broth. The spores were incubated at 32°C with aereation for about seven hours. Then stock phage suspension was added to give a phage-spore ratio of 1:50. This method, while successful, required a large amount of spore suspension. The preparation of spore suspensions in such quantity was time consuming. Equally good yields were obtained by starting with vegetative growth rather than spores. Vegetative growth was obtained by incubating 1 ml of a stock

spore suspension ($3 \times 10^8$ spores / ml) in 100 ml of glucose nutrient broth, with shaking, for 24 hours at 30°C. Forty ml of the vegetative cells in glucose nutrient broth were added to one liter of fresh glucose nutrient broth and incubated at 32°, with aeration, for 5.25 to 5.5 hours. Finally 1 ml of stock phage ($4 \times 10^4$ PFU/ml) was added to the large flask. This latter method was adopted for preparation of phage in quantity.

Lysis occurred 12 to 16 hours after the addition of phage. Experience showed that if the host was allowed to grow for a longer period of time prior to the addition of phage, lysis did not occur. Old cultures showed little or no tendency to undergo lysis. On the other hand, if sufficient vegetative cell mass was not present the host-phage mixture would not undergo sufficient phage replication to produce a maximum yield. In this regard, control of the amount of host growth seemed more important than the amount of phage added. The length of time the host culture was allowed to grow before phage addition proved to be the important control factor.

Lysates containing 2 to $3 \times 10^{10}$ PFU/ml were obtained and lysates containing 5 to $10 \times 10^{10}$ PFU/ml could be obtained under maximum conditions.

## Development of the Purification Method

It was desired to develop a method that would not completely inactivate the phage. For chemical work it is mandatory to have a preparation containing relatively undamaged phage particles. Excessively harsh treatment often results in the loss, not only of a physical structure (the tail), but also of a chemical component when the phage particle is ruptured. From early experience it had been thought that the phage under study was inactivated by pelleting via high speed centrifugation. It was now decided to re-examine this idea.

### Centrifugation and Resuspension of Pellets

A portion of lysate was filtered through celite on Whatman #3 paper and centrifuged at 6000 x g to remove cell debris. Then the filtrate was centrifuged at 22,000 x g for three hours to sediment the phage. The pellet was first resuspended in the glucose nutrient broth used for culturing the host, because it had been observed that the phage under study did not show a significant reduction in titer when preserved in the same broth medium. On resuspension of four different pellets, 77%, 75%, 96% and 100% of phage activity was recovered. It was evident

that centrifugation of phage activity per se did not inactivate the phage. However, a greater recovery of phage activity was obtained when the resuspending phage was handled with care. The cooled pellets were allowed to resuspend slowly on standing. To avoid mechanical damage to the concentrated suspension, the pellets were maintained at 5°C and were not stirred mechanically or shaken vigorously.

Unfortunately, a phage suspension in glucose nutrient broth could not be used for chemical analysis. It was necessary to find a suspending solution containing no nitrogen or phosphorus that would solubilize the pellets and also maintain phage biological activity. The S. griseus phage did tend to be more sensitive to many commonly used chemicals. However, a number of pellets were resuspended in various test solutions and the amount of phage activity remaining was measured. The results are given in Table 5. The phage solubilized very readily in sucrose but lost over 50% of its original activity. At first, gelatin appeared to be effective, though clearly a suspension in gelatin could not be used for subsequent phage nitrogen determinations. Sodium chloride (0.15 M) with calcium and magnesium added appeared to be usable. One percent mannitol, glycerol and sorbitol plus 0.05 M $CaCl_2$ and $MgCl_2$ appeared

Table 5. Resuspension of centrifuged phage pellets

| Resuspension Solution | Percent of input phage activity recovered |
|---|---|
| Nutrient broth | 77, 75, 96, 100 |
| 0.15 M NaCl 0.015 M in trisodium citrate pH 7.0[1] | 0 |
| 0.15 M NaCl 0.015 M in trisodium citrate pH 8.0[1] | 0 |
| 0.067 M phosphate buffer pH 8.0[1] | 0 |
| 0.15 M NaCl[1] | 10 |
| 0.15 M NaCl 0.001 M in $CaCl_2$ and $MgCl_2$ | 85 |
| 0.01 M Tris buffer pH 8.0 | 40 |
| 0.01 M Tris buffer pH 8.0 0.001 M in $CaCl_2$ and $MgCl_2$ | 85 |
| 0.4% methyl cellulose | 47 |
| 0.1% sucrose 0.001 M in $MgCl_2$ | 39 |
| 0.4% sucrose[2] | 17 |
| 0.4% sucrose 0.001 M in $MgCl_2$ | 45 |
| 1.0% sucrose[2] | 20 |
| 5.0% sucrose[2] | 43 |
| 0.4% sucrose and 0.2% gelatin[2] | 70 |
| 0.1% gelatin | 132 |
| 0.1% gelatin[3] | 120 |
| 1.0% gelatin[3] | 100 |
| 1.0% gelatin 0.001 M in MgCl2[3] | 128 |
| 1.0% peptone 0.0005 M in $CaCl_2$ and $MgCl_2$[4] | 111 |
| 0.2% starch 0.005 M in $CaCl_2$ and $MgCl_2$ | 88 |
| 1.0% starch 0.005 M in $CaCl_2$ and $MgCl_2$ | 61 |
| 0.2% mannitol 0.005 M in $CaCl_2$ and $MgCl_2$ | 58 |
| 1.0% mannitol 0.0005 M in $CaCl_2$ and $MgCl_2$[4] | 115 |
| 1.0% sorbitol 0.005 M in $CaCl_2$ and $MgCl_2$[4] | 100 |
| 1.0% glycerol 0.005 M in $CaCl_2$ and $MgCl_2$[4] | 100 |

[1] Resuspended only with difficulty
[2] Retitered 2.5 days later: greatly diminished
[3] Titer undiminished 12 days later
[4] Titer undiminished 5 days later

promising.

It was observed that the tris buffer and NaCl solution would solubilize the pellets and maintain the viability of the phage only if calcium and magnesium were added. It was not determined which of the latter cations were required. Also, sodium citrate and phosphate buffer, which tended to complex calcium and magnesium, would not solubilize the phage pellets. This observation was consistent with the fact that calcium was known to be required for S. griseus phage replication.

## Differential Centrifugation Experiments

It was now decided to test selected pellet resuspension solutions on the basis of a more complete purification schedule. Phage viability, and in some instances chemical analysis were used as criteria of purification efficiency. Several experiments involving differential centrifugation and resuspension were carried out. These consisted of several cycles of alternating low speed centrifugation at 6000 x g to remove particulate cell debris, and high speed centrifugation at 30,000 x g for two to three hours to sediment the phage. There was some particulate cell debris in the pellet even when the original suspension had appeared clear. These experiments also included treatment

of the phage suspension with enzymes to degrade protein (trypsin) or nucleic acid impurities (DNAse, RNAse). From 2 to 10 μg of the respective enzyme were used per ml of phage suspension. Treatment with DNAse and RNAse was carried out for 30 to 60 minutes at room temperature; with trypsin for 60 minutes at 37° C.

The values given in Table 6 represent percent of phage activity in the starting lysate that was recovered following each treatment step. The plaque counts from which these values were calculated were done in triplicate. The first two experiments consisted of two cycles of differential centrifugation. The first phage pellet was resuspended in 0.01 M tris buffer to which $CaCl_2$ and $MgCl_2$ were added to give a molar concentration of 0.001 M. In experiment one the resuspended phage, in tris buffer, was treated with DNAse and trypsin, whereas in experiment two the enzyme treatments were omitted. It was observed that phage activity recovered after the second high speed centrifugation was about 50% less when the enzyme treatment was used. It was considered possible that the above enzymes damaged the phage, so that it was more easily inactivated by the centrifugation procedure. However, many phages are susceptible to trypsin. Early work with the S. griseus phage had shown that it could

Table 6. Purification of S. griseus phage and loss of phage biological activity

| Purification Procedure | % Recovery of input phage | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| Raw lysate; centrifugation at 6000 x g | 100% | 100% |
| Centrifugation at 30,000 x g<br>Pellets resuspended in 0.01 M Tris buffer + Ca and Mg, pH 7.0<br>Centrifugation at 6000 x g | 80% | 82% |
| DNAse treatment | 60% | |
| Trypsin treatment | 69% | |
| Centrifugation at 30,000 x g<br>Pellets resuspended in 0.15 M NaCl + Ca and Mg<br>Centrifugation at 6000 x g | 29% | 60% |
| Analysis of final suspension | | |
| Nitrogen | 31.5 µg/$10^{11}$ PFU | |
| Phosphorus | 10.8 µg/$10^{11}$ PFU | 10 µg/$10^{11}$ PFU |
| Ratio Nitrogen/phosphorus | 2.9 | |
| UV spectrum O.D. ratio 260/280 | 1.48 | 1.55 |

be inactivated by trypsin on prolonged exposure. DNAse had not been observed to damage the test phage. In subsequent experiments with this phage the DNAse treatment did not diminish the phage titer. However, treatment with DNAse was considered vital in subsequent purification procedures.

As shown in Table 6, the final phage suspension was also analyzed for phosphorus and nitrogen, to evaluate the extent of purification. The results are given relative to phage activity of the suspension. In a few of the experiments an ultraviolet absorption spectrum was obtained for the final phage suspension. The 260/280 UV ratios of 1.48 to 1.55 were indicative of relatively pure phage preparation. However, the nitrogen and phosphorus values appeared high, attesting either to impurities or excessive loss in viability.

Further experiments were carried out using three cycles of differential centrifugation with 0.15 M NaCl with 001M and $CaCl_2$ and $MgCl_2$ as the suspending solution. The filtered lysate was treated with DNAse and with RNAse before the first high speed centrifugation. The results are given in Table 7. Half of the phage activity was lost during each of the first two centrifugation steps, and 90% after the final sedimentation. The nitrogen and phosphorus content in $\mu g/10^{11}$ than for the first two, while

Table 7. Purification of S. griseus phage with N Cl as the pellet resuspension solution

| Purification Procedure | Chemical Analysis | | | |
|---|---|---|---|---|
| | Nitrogen μg/10" PFU | Phosphorus μg/10" PFU | Ratio N/P | % Input PFU Recovered |
| Raw lysate; filtration on celite Centrifugation at 6000 x g | 4050 | | | 100% |
| RNAse, DNAse treatment | | | | 100% |
| Trypsin treatment | | | | 100% |
| Centrifugation at 30,000 x g Resuspended in 0.15 M NaCl, Ca and Mg Centrifugation at 6000 x g | 42.0 | 12.6 | 3.3 | 40% |
| Centrifugation at 30,000 x g Resuspended in 0.15 M NaCl, Ca and Mg Centrifugation at 6000 x g (14% loss) | 32.4 | 13.7 | 2.4 | 22% |
| Centrifugation at 30,000 x g Resuspended in 0.15 M N Cl, Ca and Mg Centrifugation at 6000 x g (8% loss) | 227 | 91 | 2.5 | 2% |

Table 8. Purification of the phage with gelatin as the pellet resuspension solution

| Purification Procedure | Chemical Analysis | | | |
|---|---|---|---|---|
| | Nitrogen μg/$10^{11}$ PFU | Phosphorus μg/$10^{11}$ PFU | Ratio N/P | % of input Recovered |
| Raw lysate filtration on celite | 29,000 | | | 100% |
| Centrifugation at 6000 x g | | | | |
| Centrifugation at 30,000 x g | | | | |
| Res., 1% gelatin, $Ca^{++}$ + Mg | | | | |
| Centrifugation, 6000 x g (14% loss) | | | | 56% |
| Treatment with DNAse and RNAse | | | | 66% |
| Centrifugation at 30,000 x g | | | | |
| Res., 1% gelatin, $Ca^{++}$ + $Mg^{++}$ | | | | |
| Centrifugation, 6000 x g (16% loss) | | | | 49% |
| Res., 0.15 M NaCl, $Ca^{++}$ + $Mg^{++}$ | | | | |
| Centrifugation at 6000 x g | 8.05 | 3.13 | 2.53 | |
| Centrifugation at 30,000 x g | | | | |
| (from gelatin suspension) | | | | |
| Res., 1% gelatin, $Ca^{++}$ + $Mg^{++}$ | | | | 45% |
| Centrifugation at 6000 x g | 9.9 | 3.5 | 2.69 | |
| Centrifugation, 30,000 x g | | | | |
| (from gelatin suspension) | | | | |
| Res., 0.15 M NaCl, $Ca^{++}$ + $Mg^{++}$ | | | | |
| Centrifugation, 6000 x g (11% loss) | 8.2 | 3.1 | 2.60 | 34% |

the nitrogen to phosphorus ratio remained the same, irrespective of the centrifugation step. This latter observation suggested that the final suspension must have contained a large proportion of inactivated phage. The particular treatment responsible for the eventual inactivation of the phage was difficult to localize. Thus this procedure was considered unsuitable for obtaining a usable phage preparation.

A similar sequence of experiments was done, consisting of four cycles of differential centrifugation, to learn whether elimination of the trypsin treatment and use of a less harsh suspending solution would result in a greater recovery of phage activity. One percent gelatin with 0.002 M $CaCl_2$ and $MgCl_2$ was used to resuspend the pellets after each of the first three centrifugation steps. However, as shown in Table 8, NaCl with $CaCl_2$ and $MgCl_2$ was used to resuspend the final pellet so as to eliminate nitrogen from the preparation. At the completion of the second and third centrifugation steps, separate aliquots of phage were taken up in the NaCl solution for the phosphorus and nitrogen determinations.

The data in Table 8 disclosed that each low speed centrifugation cycle effected a 10%-20% decrease in phage activity. After the first high speed centrifugation step followed by resuspension

in gelatin and sedimentation of the phage at 6000 x g, there occurred a 44% reduction in phage activity. Subsequent centrifugation and suspension treatments did not materially decrease phage activity. The nitrogen and phosphorus content expressed in $\mu g/10^{11}$ PFU did not change appreciably during the last three centrifugation steps. Since the raw lysate and final pellet nitrogen values represented comparable specific activities it was clear that a high degree of phage purification had been attained. However, it was discovered that at a pH as low as 6.0 or 6.5, coprecipitation of phage with gelatin occurred. The effect was much less at pH 8.0. A precipitate actually could be seen when the gelatin solution and phage suspension were mixed. This effect could explain the loss observed at the first centrifugation in the experiment described above. The phenomenon was not examined further.

A final sequence of centrifugation and resuspension experiments were designed, consisting of three cycles of differential centrifugation, using 1-2% glycerol containing 0.005M $CaCl_2$ and $MgCl_2$ as the suspending solution. The results are given in Tables 9 and 10. In two of the experiments the low speed centrifugation to remove cell debris was carried out for five minutes, (Table 9), and in the other two for 30 minutes (Table 10). The five minute centrifugation was effective and resulted in less

Table 9. Percent phage plaque forming units recovered during phage purification.

| | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | % Recovery of input PFU | PFU lost with cell debris | % Recovery of input PFU | PFU lost with cell debris |
| Raw lysate<br>Centrifugation at 6000 x g<br>Filtration through #3 Whatman paper | 100% | | 100% | |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 5 minutes<br>Treatment with DNAse and RNAse | 86% | 3% | 107% | 3% |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 5 minutes | 116% | 1% | 121% | 1% |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 5 minutes | 82% | 1% | 88% | 1% |

Table 10. Percent phage plaque forming units[1] recovered during phage purification

| Purification Procedure | % Recoveries | | | |
|---|---|---|---|---|
| | % Recovery of input PFU | % loss in[1] cell debris | % of raw lysate titer PFU | % lost in cell debris |
| Raw lysate<br>Centrifugation at 6000 x g<br>Filtration through #3 Whatman paper | 100% | | 1002% | |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 30 minutes<br>Treatment with DNAse and RNAse | 70% | 14% | 92% | 32% |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 30 minutes | 47% | 9% | 1% | 11% |
| Centrifugation at 30,000 x g<br>Resuspended in 1% glycerol with Ca and Mg<br>Centrifuged at 6000 x g 30 minutes | 17% | 18% | 44% | 15% |

[1] Percent of plaque forming units in the suspension immediately before centrifugation

loss of phage activity in the cell debris fraction. The latter shorter time low-speed centrifugation procedure was then used for routine purification of phage for chemical analysis. A 10-20% error was encountered even though the plaque counts were done in duplicate. The greater than 100% values observed in the experiments summarized in Table 9 can probably be attributed to variation in the phage viability measurement.

Centrifugation in Cesium Chloride

It is not possible to purify bacteriophage by differential centrifugation alone; the centrifuged phage preparations contain particulate cell debris material that sediments and resuspends along with the phage. Density gradient centrifugation is an effective, commonly used technique for the final purification of bacteriophage preparations.

The centrifuged S. griseus phage preparation was subjected to density gradient centrifugation in cesium chloride, glycerol, and sucrose. Considerable loss of phage viability occurred on centrifugation with each of these three compounds. However, centrifugation in CsCl was selected because banding of the phage from other contaminating materials was more distinct.

Three clearly separated bands were seen in the CsCl tubes. All remaining phage activity was found in a sharp band a little below the middle of the tube. There was a pellet of particulate material at the bottom of the tube. There were two or three diffuse bands of lighter material near the surface. The narrow center band, containing pure phage, was separated from the others using a tube slicer.

It was necessary to dialyze CsCl before the chemical analysis could be performed. Preparations to be used for nitrogen, protein, phosphorus, DNA-phosphorus and dry weight determinations were dialyzed against distilled water. As results were to be reported relative to dry weight, it was necessary to remove all material other than the purified phage. The phage did tend to rupture in distilled water. The dialyzed preparations contained a gelatinous mass of material, suggesting spilled out DNA and protein. Aliquots of this suspension were used for nitrogen, phosphorus, DNA analysis, and for determinations of the dry weight to which the results were related. Preparations to be used for UV spectrum of the phage or for extraction of DNA were dialyzed against 0.01 M Tris buffer plus 0.0005 M $CaCl_2$ and $MgCl_2$.

It is essential to be sure that the produce analyzed is

indeed pure phage, that it does not contain material from host cell debris. A principal criterion of purity for the preparation is that all of the phage material forms a clear sharp band during cesium chloride density gradient centrifugation. This observation implies that the preparation consisted of particles of equal density. Free protein is of a much lower bouyant density, free DNA much higher. Centrifugation in such a density gradient is a sensitive means of separating such materials by differences in density.

The final overall purification scheme adopted as a result of the described experiments is shown in Figure 1. Phage preparations obtained by the given step-wise protocol were now used for elementary analysis of the purified phage. The consistency of the latter values for replicate, separately processed purified phage preparations stand as valuable criteria of phage purity. The four primary determinations used were nitrogen, phosphorus, DNA and protein. The results agree within the expected experimental error for the methods used. Also, absence of uracil in the phage as shown by DNA chromatography indicates the absence of contamination from host cell RNA.

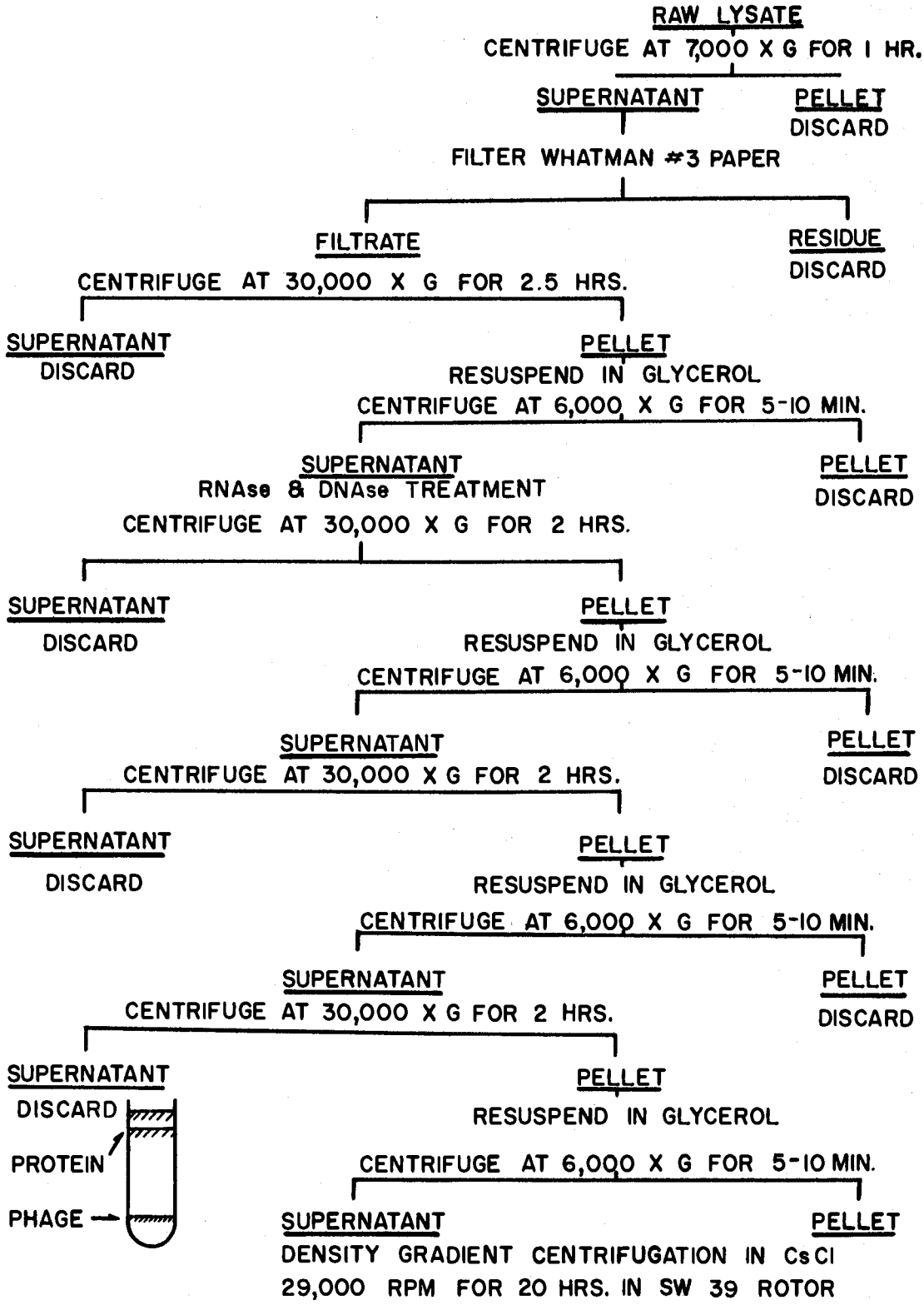


FIGURE 1 SCHEME FOR CHEMICAL PURIFICATION OF S. griseus PHAGE

## Chemical Composition

Total nitrogen, protein, total phosphorus and DNA bound phosphorus were determined as described under materials and methods. The results have been given as percent by weight of dried phage. Pertinent data are given in Table 11.

Three separately processed phage preparations were analyzed. The nitrogen and protein values were averages of duplicate or triplicate determinations; total phosphorus and DNA phosphorus values were averages of six separate determinations.

### Phosphorus and DNA

The average value for total inorganic phosphorus as determined by the method of Martin and Doty (1949) was 5.9 percent. As is evident in Table 11, the latter chemical procedure gave excellent duplication of the phosphorus data. Prior to use of diphenylamine as an indirect method for DNA phosphorus analysis, a standard curve had been prepared which equated phosphorus and DNA content. The observed phosphorus values depicted in Table 11, determined by the diphenylamine method, are lower than was obtained by direct analysis for total inorganic phosphorus. However, both sets of data showed reasonable agreement. The direct phosphorus analysis

Table 11. Chemical Composition of S. griseus phage (percent of dry weight)

| Chemical Determination | Purified Phage | | | |
|---|---|---|---|---|
| | Prep A | Prep B | Prep C | Average |
| Dry wt. | 1.42 mg/ml | 1.27 mg/ml | 1.75 mg/ml | |
| Phosphorus (Martin and Doty 1949) | 84 g/ml<br>5.9% | 76 g/ml<br>6.0% | 99 g/ml<br>5.7% | 5.9% |
| DNA* | 59% | 60% | 57% | 59% |
| DNA phosphorus by diphenylamine reaction | 71 g/ml<br>5.0% | 65 g/ml<br>5.1% | 89 g/ml<br>5.1% | 5.1% |
| DNA* | 50% | 51% | 51% | 51% |
| Nitrogen by Kjehldahl method | 213 g/ml<br>15.0% | 212 g/ml<br>16.7% | 276 g/ml<br>15.8% | 15.8% |
| Protein by Lowry's method | 724 g/ml<br>51% | 562 g/ml<br>44% | 816 g/ml<br>47% | 47% |
| N/P ratio | 2.54 | 2.76 | 2.75 | 2.68 |

*Calculated from phosphorus content assuming DNA is 10% phosphorus

was taken to be the more reliable measure of the amount of DNA.

The phage DNA content was now calculated on the basis of the total phosphorus level. Deoxyribose nucleic acid was assumed to contain 10% phosphorus. The direct phosphorus analysis and average value of 5.9% phosphorus gave an average DNA value of 59.0% DNA, whereas the diphenylamine phosphorus content of 5.1% indicated an average DNA value of 51%. It was evident therefore that the purified S. griseus phage contained approximately 59% DNA.

Nitrogen and Protein

The total nitrogen data shown in Table 11 showed excellent replication among the test phage preparations. The average total nitrogen value of 15.8% represented a realistic figure for such a preparation. The Lowry protein data did show some variation with the individual test phage preparations. However, it was evident that the average protein value falls in the range of 44-45%. Thus, if we use the DNA value of 59% and a protein figure of 45%, it becomes possible to obtain a realistic picture of the total percentage leve of these two important constituents of the phage.

## The Nitrogen to Phosphorus Ratio

The average value obtained for the ratio of the amounts of nitrogen and phosphorus in the purified phage was 2.68. The ratios obtained for the three different preparations analyzed (Table 11) were in agreement. The N/P ratio of 2.68 is an expected one for a material composed only of nucleic acid and protein in which the nucleic acid component is present in somewhat greater amount. The ratio obtained is consistent with the values of 3.0 to 3.1 that can be calculated from the data generally reported for the coliphages (Table 1).

The N/P ratio can be regarded as an indication of the relative amounts of protein and nucleic acid in a material composed of those substances alone. The nitrogen content alone cannot be taken as a measure of the amount of protein in a material rich in nucleic acid because the nucleic acid also contains nitrogen.

## Chemical Data Related to Phage Activity

Many investigators have reported chemical data for bacteriophages relative to number of phage particles as well as in terms of dry weight. The chemical data for phosphorus, DNA, nitrogen, and protein, along with dry weight for the particle,

were calculated on the basis of $\mu g/x\ 10^{11}$ phage particles. The results are presented in Table 12. The number of phage particles was determined by the standard plaque counting procedure described under methods. The PFU value used for the calculations was the value for the beginning lysate corrected for loss of phage during differential centrifugation.

This method of presenting the chemical data is regarded as being less significant, because of the errors inherent in the measurement of the number of viable phage particles. A summary of chemical data for other phages reported in terms of phage PFU is given in Table 2. It is quite evident that the data reported by the different investigators using PFU units are considerable less consistent than results reported as percent of dry weight. This observation is not unexpected. The measurement of number of viable phage particles by plaque counting as used during the course of this investigation was seen to be subject to much variation, not all of which was completely understood. Also, a sizable error was introduced, if a large proportion of the phage particles have been inactivated and do not form plaques. In this case, the phage particles were not measured by the plaque count, while their components were measured by the chemical analysis.

Table 12. Chemical composition of the S. griseus phage in μg per $1 \times 10^{11}$ plaque forming units of phage

| Chemical test | Phage prep. A | Phage prep C | Average |
|---|---|---|---|
| Dry wt. of particle | 31.8 | 35.2 | 33.5 |
| Phosphorus | 1.9 | 2.1 | 2.0 |
| DNA | 19 | 21 | 20 |
| Nitrogen | 4.8 | 5.8 | 5.3 |
| Protein | 16.1 | 17.1 | 16.6 |

The S. griseus phage, according to measurements by electron microscopy, can be considered to be roughly the same mass as phage T2. The head diameter of the S. griseus phage is about 95 mμ, and the dimensions of the head of phage T2 are about 85 mμ x 105 mμ. The particle weight value of 33.5 μg/1 x $10^{11}$ PFU are roughly in agreement with the 38 μg/ 1 x $10^{11}$ PFU reported by Cummings and Kozloff (1960) for phage T2. The values of 2 μg phosphorus/ 1 x $10^{11}$ PFU and 5.3 μg nitrogen/1 x $10^{11}$ PFU are also reasonably consistent with those reported for phage T2 (Table 2).

## DNA Base Composition

### By Chromatographic Determination of Bases

The deoxyribose nucleic acid component of the phage was extracted and individual bases isolated as outlined under methods. Determinations were made in quadruplicate as shown in Table 12. The observed molar concentrations of guanine, cytosine and adenine, thymine are equivalent as is predictable for a double stranded DNA. The indicated G+C percent of 53.4, based on actual chemical determination of the bases, is representative of other phage systems (Tables 3 and 4).

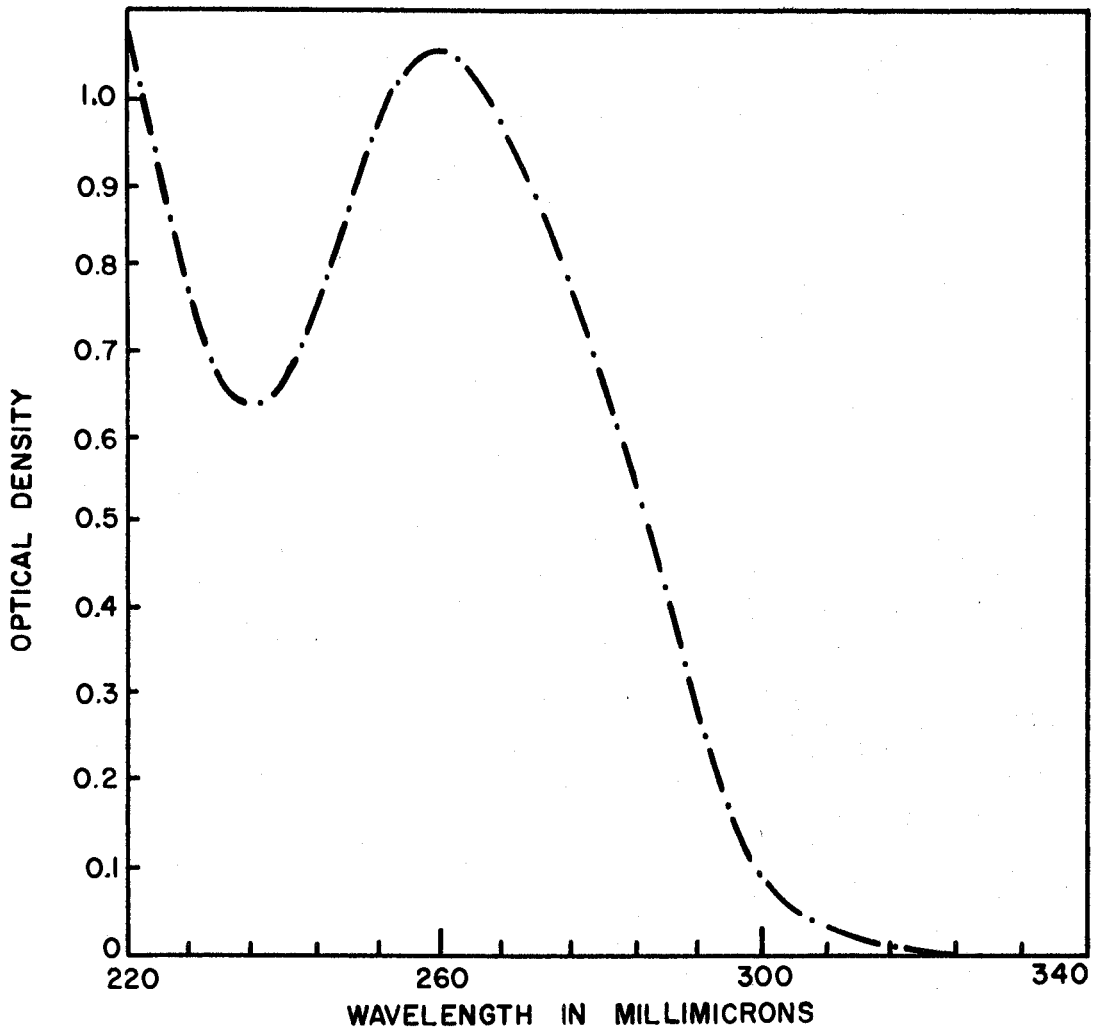


FIGURE 2 ULTRAVIOLET ABSORPTION SPECTRUM OF S. griseus PURIFIED PHAGE

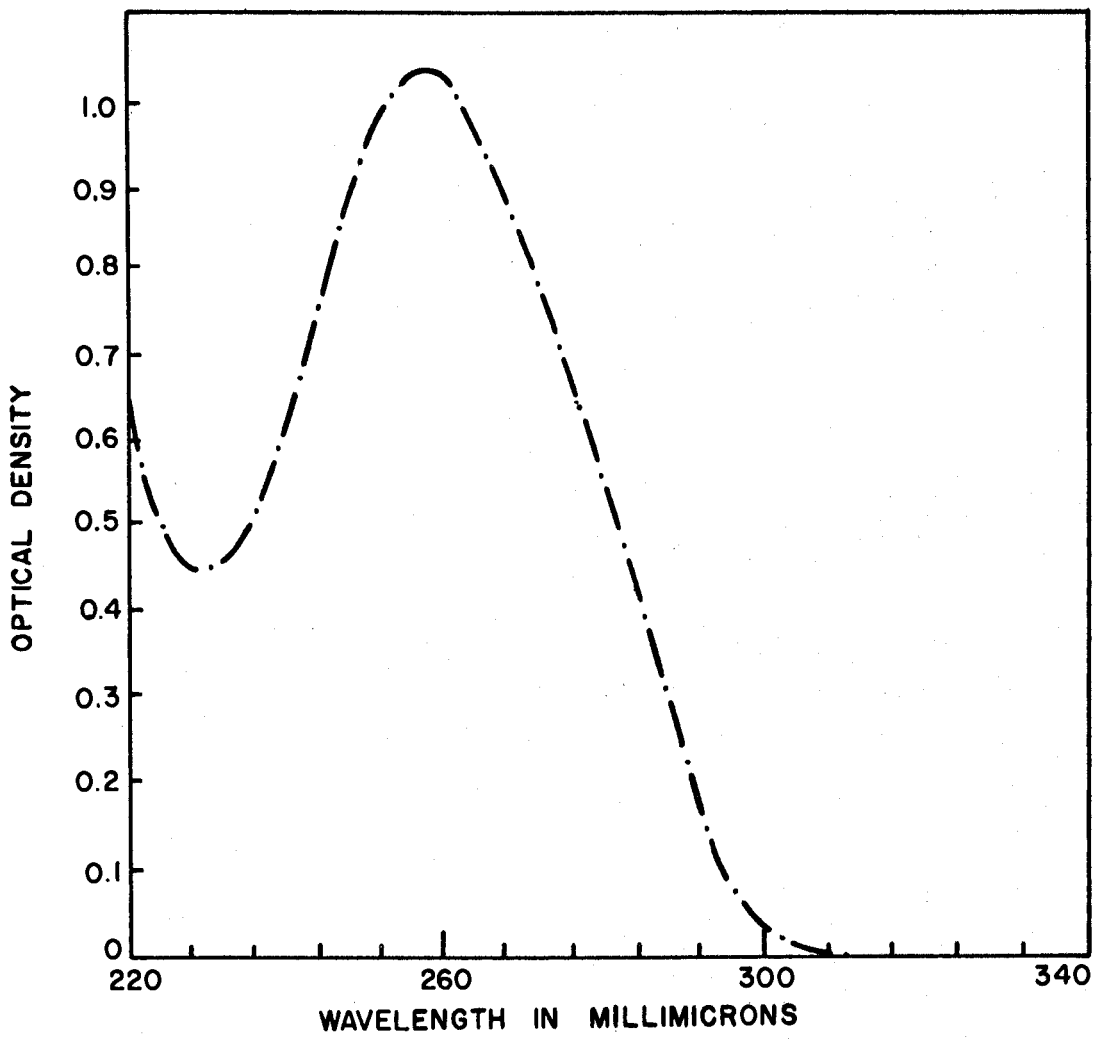


FIGURE 3 ULTRAVIOLET ABSORPTION SPECTRUM OF PURIFIED *S. griseus* PHAGE DNA

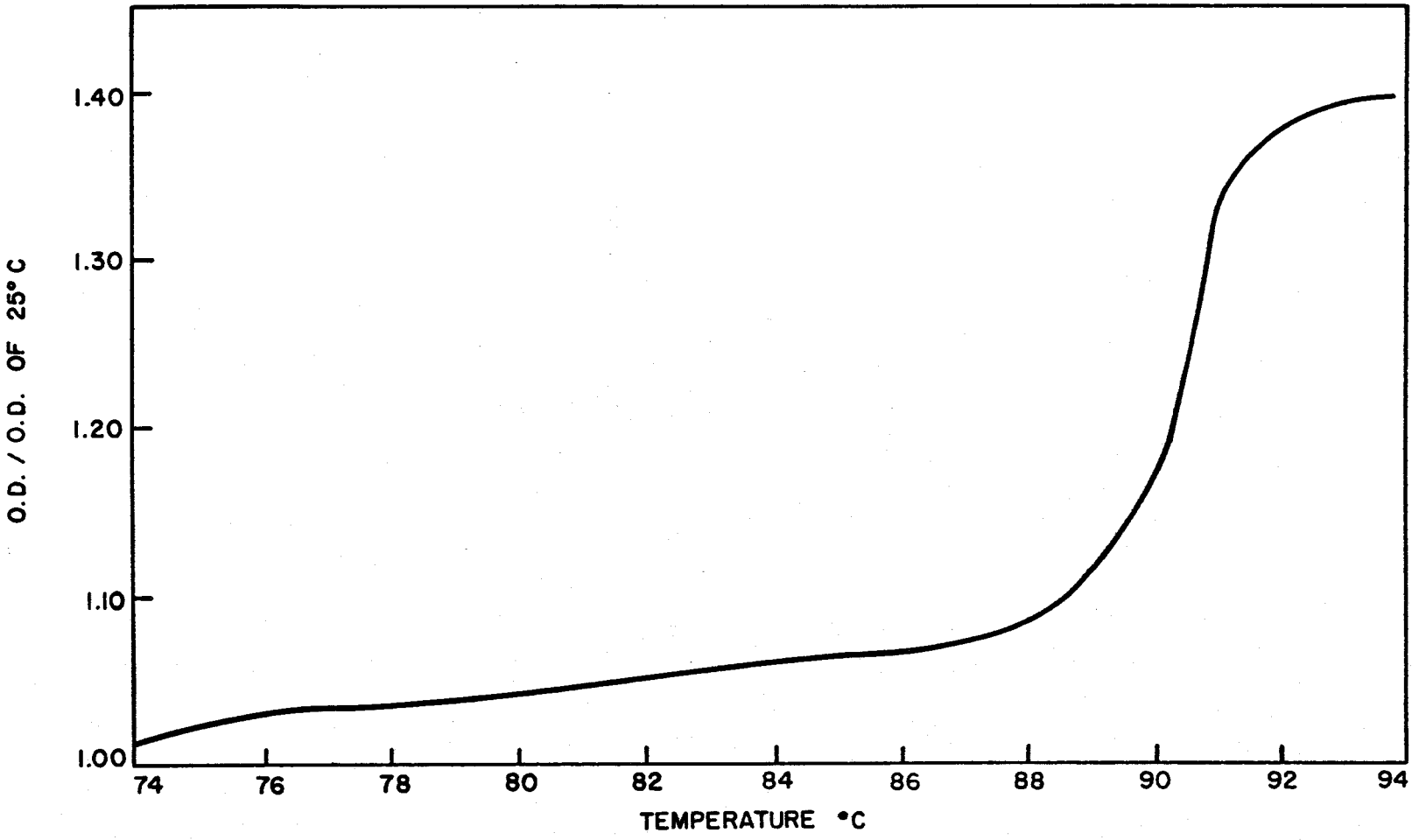


FIGURE 4 S. griseus PHAGE DNA THERMAL DENATURATION CURVE

By DNA Melting Point

The melting point of the extracted phage DNA was determined as described under Methods. The instrument was offset to record 10 at 25°C. The optical density reading of the chart was set to give a range of 0 to 0.25. Chart readings for optical density were corrected for the thermal expansion of water at elevated temperatures.

The ratio of optical density at the elevated temperature to the optical density at 25°C was plotted against temperature. This curve is shown in Figure 4. The optical density reading at the beginning of the melting range at 89.8°C was 0.123. A correction of 1.032 for thermal expansion made the former 0.127. The optical density at the close of the melting range at 91.4 was 0.375. The thermal expansion correction factor changed the latter optical density to 0.388. The midpoint between an optical density of 0.127 and 0.388 is 0.258 which corresponds to a temperature of 90.4°C. Thus, 90.4°C represents the melting point of the phage DNA. Subsequently, the percent guanine and cytosine was calculated by the Martin and Doty formula:

Table 13. Base composition of the DNA of the S. griseus phage

| Base | Aliquot of extracted phage DNA | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | | |
| | µm/ml | mol % | µm/ml | mol % | µm/ml | mol % | µm/ml | mol % | Ave. % |
| Guanine | 0.136 | 26.6 | 0.106 | 26.3 | 0.054 | 25.5 | 0.067 | 26.8 | 26.3 |
| Cytosine | 0.141 | 27.5 | 0.109 | 27.1 | 0.058 | 27.3 | 0.066 | 26.4 | 27.1 |
| Adenine | 0.118 | 23.0 | 0.093 | 23.1 | 0.049 | 23.1 | 0.057 | 22.8 | 23.0 |
| Thymine | 0.117 | 22.9 | 0.095 | 23.6 | 0.051 | 24.1 | 0.060 | 24.0 | 23.6 |
| % G ÷ C | 54.1 | | 53.4 | | 52.8 | | 53.2 | | 53.4 |

$$\% \text{ G C} = \frac{\text{Tm} - 69.3}{0.4}$$

$$= \frac{90.4 - 69.3}{0.41} = 51.5\% \text{ G+C}$$

## Ultraviolet Spectra

The optical density ratio for the UV absorption at 60 mμ and 280 mμ is regarded as a partial criterion of phage purity. For the most part, such ratios fall between 1.5 and 1.6. A representative UV absorption for the whole phage under study is shown in Figure 3. The maximum absorption peak was observed at 259 mμ and a minimum at 235 mμ, as the characteristic of nucleoprotein. The ratio of the optical densities at 260 mμ and 280 mμ was 1.63. A similar ultraviolet absorption spectrum was run on the extracted phage DNA. This curve is depicted in Figure 3. Maximum UV absorption occurred at 257 mμ with the minimum value at 235 mμ, as is characteristic of DNA. The ratio of 1.87, for the optical densities at 260 mμ and 280 mμ is indicative of the removal of the phage protein coat.

## Phage Buoyant Density

Bouyant density in CsCl was measured as described under materials and methods. The density of 1.51 g/cc determined for lambda phage was used as the reference standard and proved

to check well with the known value of 1.508 g/cc (Weigle and Meselson, 1959). The density of the S. griseus proved to be 1.55 g/cc. The latter value appears to be the highest yet reported for any phage system.

# DISCUSSION

The determination of the chemical composition of an actinophage for Streptomyces griseus showed that it is composed of about 60% DNA and about 40% protein. The 55%-65% DNA content is somewhat higher than that of other bacteriophages that have been similarly analyzed. Coliphages T2, T7, and lambda, and Bacillus megaterium phage G have been reported to contain from 45% to 55% DNA. Kolstad and Bradley (1964) have estimated actinophage MSP8 for Streptomyces venezuelae to be 57% DNA. They did not, however, report a direct chemical analysis for DNA. The small bacteriophages that have been analyzed contain smaller relative amounts of DNA. Phage alpha for Bacillus megaterium is 35% DNA, coliphage x 174, whose DNA is single stranded, is 25% DNA, and the male specific coliphage fd is only 12% DNA.

Direct determination of the relative amounts of DNA and protein in bacteriophage is not easily done with precision. Values reported by different investigators for the chemical composition of the coliphages do not agree exactly, as can be seen from Table 1. The most precise measure of DNA content is by the phosphorus analysis and appropriate calculations. Certainly, the determination of phosphate in a properly prepared sample

can be done with precision. Calculation from the molecular weights of the components of DNA shows that 10% of its weight is phosphorus. A few analyses of DNA for phosphorus have been reported which give phosphorus contents of less than 10%. However, it is practically impossible to prepare DNA that is quantitively free from protein or bound water.

The phosphorus content of 5.9% is higher than that for other bacteriophages that have been analyzed (Table 1). Calculation, assuming that DNA is 10% phosphorus, gives a DNA value of 59%, which is higher than the values reported for other phages (Table 1). The 50-51% value obtained by the colorimetric diphenylamine method analysis for DNA was lower than the value obtained by calculation from the phosphorus content. The analysis by the diphenylamine method is believed to be the less reliable. In this instance, it is believed that the DNA may have been incompletely hydrolyzed and the reaction with deoxyribose therefore incomplete.

The ratio of nitrogen content to phosphorus of 2.7 is lower than the 3.0 to 3.1 value generally reported for the other phages that have been similarly analyzed. Calculations, using the N/P ratio of 2.7, assuming DNA to be 10% phosphorus and DNA and protein both 16% nitrogen (Davidson and Freifelder, 1962),

suggest that the phage is about 60% DNA. Also, the UV spectrum, with the O. D. 260-280 mμ ratio of 1.63 is consistent with a higher DNA content.

The buoyant density in cesium chloride of 1.55 g/cc may be the highest value yet reported for a bacteriophage. Buoyant densities of phage T2 and lambda, which have been reported to contain about 50% DNA, have been reported as 1.505 g/cc (Cummings, 1960) and 1.508 g/cc (Weigle, Meselson, and Paigen, 1959), respectively. The buoyant density in cesium chloride of protein is about 1.3 g/cc, and that of DNA about 1.7 g/cc. Buoyant density of 1.50 g/cc for a phage then suggests that it must contain about 50% DNA and 50% protein. Calculations then suggest that S. griseus phage, with a buoyant density of 1.55 g/cc would be about 60% DNA and 40% protein.

The nitrogen content of 16% is in agreement with the most reliable values reported for phages T2 and T7 (Table 1). It is also in agreement with the usually accepted 16% nitrogen value for proteins. DNA also contains nitrogen. The 16% nitrogen value obtained by analysis of bacteriophage suggests that DNA contains about 16% nitrogen that can be measured by the Kjehdahl procedure. It is therefore clearly not possible to use the nitrogen analysis as a measure of protein content.

Protein in the phage was measured directly by the colorimetric phenol reagent method of Lowry and values of 44% to 51% were obtained. However, the analysis was standardized using bovine serum albumin and it is known that this method gives somewhat different results for different proteins of different amino acid composition. It can here be considered an estimate of relative amount of protein, but not as a precise measure.

These results suggest that the S. griseus phage contains no biochemical materials other than the usual DNA and protein components typical of other bacteriophages. While the density and the DNA contents are higher than those reported for the other phages, the difference is not great enough to suggest s significant difference in the character of the phage particle.

Analysis of the purified phage DNA for purine and pyrimidine bases showed the presence of the four bases commonly found in DNA. There was no indication of the presence of an unusual base such as the hydroxymethylcytosine found in the T-even coliphages or the 5-hydroxymethyluracil found in a series of Bacillus subtilis phages. The base composition of the DNA determined by chemical analysis by chromatography of 53.4% agrees within experimental error with the 51.5% value calculated from melting point. This observation suggests the absence of

an unusual component bonded to the bases in such a way as to affect the hydrogen bonding between the two strands of the DNA, as is true for B. subtilis phage PBS2. For phage PBS2, the DNA melting point is lower, and the bouyant density of the DNA higher than is predicted from the base composition. This anomolous behavior is believed to be a result of the bonding of glucose to one of the bases (Takahasi and Marmur, 1963). Phage PBS2 is also one of the group in which thymine is replaced by 5-hydroxymethyluracil. The DNA of the T-even phages in which DNA cytosine is replaced by hydroxymethelcytosine, contains glucose also. While the bouyant density in cesium chloride is greater than predicted by base composition, the melting point is not changed by the bonding of glucose to the hydroxyl of the hydroxymethylcytosine.

The molar amounts of adenine, thymine and of guanine, cytosine were equivalent within experimental error, as might be expected for any double stranded DNA. The sharp melting point curve is a characteristic of a double stranded DNA. These observations indicate that the DNA must be in the usual double stranded form. These observations do not suggest that the DNA of S. griseus phage differs in its properties from the DNA of most other bacteriophages.

The DNA base composition of the host bacterium, Streptomyces griseus, was not determined. However, it has been determined and reported by other investigators to be 71%-74% (Belozersky and Spirin, 1958), (Sueoka, 1961),( Erikson and Szybalski, 1964) and (Frantali, Hill, and Silvestri, 1965). DNA from the various living forms found in nature varies in base composition from 25% to 75%. The 53.4% G+C found for S. griseus phage DNA falls at about the middle of that range; the 74% G+C reported for the Streptomyces griseus host is at the very high end of the range.

It was concluded that the actinophage for S. griseus, the filamentous Streptomyces griseus host does not significantly differ in its chemical properties from most of the other bacteriophages that have been analyzed. The chemical composition of 60% DNA and 40% protein was close to the chemical composition values reported for most other phages. The DNA base composition of 53.4% fell in about the middle of the range of % G+C values observed for bacteria and viruses. Uniquely, however, the rather high bouyant density of 1.55 g/cc may prove to be a characteristic of all actinophage preparations.

# SUMMARY

The results obtained from this investigation of the chemical properties of the actinophage for Streptomyces griseus are summarized as follows:

1. A method for the isolation and chemical purification of the phage was developed.

2. The chemical composition of the phage was found to be 60% DNA and 40% protein.

3. The base composition of the phage DNA was found to be 53.4% guanine plus cytosine.

4. The buoyant density of the phage in cesium chloride was found to be 1.55 g/cc.

# BIBLIOGRAPHY

1. Adams, Mark H. 1959. Bacteriophages. New York, Interscience 592p.

2. Aurissiccho, S. et al. 1960. Physico-chemical and morphological aspects of temperate phage α particles. Nuovo Cimento, Supplement 18:1183-1188.

3. Bacq, Claire Michelle, and L. Dierickx. 1962. Purification de l'actinophage. Biochimica et Biophysica Acta 61:19-28.

4. Ballantine, Robert. 1957. Determination of nitrogen and ammonia. In: Methods in enzymology, ed. by Sidney P. Colowick and Nathan W. Kaplan. Vol. 3 New York, Academic Press. p. 984-995.

5. Belozersky, A. M. and A. S. Spirin. 1958. A correlation between the composition of deoxyribonucleic and ribonucleic acids. Nature 182:111-112

6. Bendich, Aaron. 1957. Methods for characterization of nucleic acids by base composition. In: Methods in enzymology, ed. by Sidney P. Colowick and Nathan W. Kaplan. Vol. 3. New York, Academic Press. p. 715-723.

7. Brock, Thomas D., Roy M. Johnson and W. B. DeVille. 1965. Physical and chemical properties of a bacterial virus as related to its inhibition by streptomycin. Virology 25:439-453.

8. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction. Biochemical Journal 62: 315-323.

9. Calderone, J. G. and M. J. Pickett. 1965. Characterization of Brucellaphages. Journal of General Microbiology 39:1-10.

10. Cohen, Seymour S. and Thomas F. Anderson. 1946. Chemical studies on host virus interactions: The effect of bacteriophage adsorption on the multiplication of its host E. coli. Journal of Experimental Medicine 84: 511-523.

11. Cohen, Seymour S. and Rachael Arbogast. 1950. Chemical studies on virus host interactions: Immunochemical studies on the purity of concentrates of various bacterial viruses prepared by differential centrifugation procedures. Journal of Experimental Medicine 91:607-618.

12. Colwell, Charlotte A. 1937. Purified bacteriophage from lysogenic cultures. Proceedings of the Society for Experimental Biology and Medicine 36:100-103.

13. Cordes, S., H. T. Epstein and J. Marmur. 1961. Some properties of the DNA of phage alpha. Nature 191: 1097

14. Creasser, F. H. and A. Taussig. 1957. The purification and chromatography of bacteriophages on anion exchange cellulose. Virology 4:200-208.

15. Cummings, Donald J. 1963. Anomalous hydrodynamic behavior of bacteriophage T2. Virology 21:575-579.

16. Csaky, T. Z. et al. 1950. Chemical analysis of the T7 bacteriophage of E. coli. Journal of Biological Chemistry 185:311-321.

17. Davidson, Peter F. and David Freifelder. 1962. The physical properties of T7 bacteriophage. Journal of Molecular Biology 5:635-642.

18. Erickson, R. L. and Waclaw Szbalski. 1964. The $CsSO_4$ equilibrium density gradient and its application to the study of T-even phage DNA; glycosylation and replication. Virology 22:111-124.

19. Evensen, Vera. 1960. The preparation, purification, and recovery of Streptomyces griseus phage. Master's thesis. Corvallis, Oregon State University. 64 numb. leaves.

20. Foldes, J. and T. A. Trautner. 1964. Infectious DNA from a newly isolated B. subtilis phage. Zeitschrift fur Vererbungslehre 95:57-65.

21. Frontali, Clara, L. R. Hill and S. G. Silvestri. 1965. The base composition of deoxyribonucleic acids of Streptomyces. Journal of General Microbiology 38:543-250.

22. Gilmour, C. M. and D. Buthala. 1950. The isolation and study of actinophage from soil. (Abstract). Bacteriological Proceedings 50:17.

23. Gilmour, C. M., E. C. Noller and B. Watkins. 1959. Studies on Streptomyces griseus phage. I. Growth characteristics of the Streptomyces phage host system. Journal of Bacteriology 78:186-192.

24. Grogan, James B. and Emmet J. Johnson. 1965. Nucleic acid composition of a Pseudomonas aeruginosa phage. Virology 24:235-236.

25. Henning, Dave. 1966. Unpublished research on a Streptococcus diacetilactis host bacteriophage system. Corvallis, Oregon State University, Dept. of Microbiology.

26. Herriott, Roger M., and James Barlow. 1952. Preparation, purification and properties of E. coli virus T2. Journal of General Physiology 36:17-28.

27. Hershey, A. D., June Dixon, and Martha Chase. 1953. Nucleic acid economy in bacteria infected with bacteriophage T2. Journal of General Physiology 36:777-789.

28. Hetler, D. M. and J. Bronfenbrenner. 1931. Detachment of bacteriophage from its carrier particles. Journal of General Physiology 14:547-562.

29. Jesaitis, Margeris. 1959. The chemical composition of the nucleic acid of C16 phage. (Abstract). Bacteriological Proceedings 59:45.

30. Kaiser, A. D. and David S. Hogness. 1960. The transformation of Escherichia coli with deoxyribonucleic acid isolated from bacteriophage λdg. Journal of Molecular Biology 2:392-415.

31. Kallen, R. G., M. Simon and J. Marmur. 1962. Occurrence of a new pyrimidine base replacing thymine in a bacteriophage DNA; 5-hydroxymethyluracil. Journal of Molecular Biology 5:248-250.

32. Kalmanson, G. and J. Bronfenbrenner. 1939. Studies on the purification of bacteriophage. Journal of General Physiology 23:203-228.

33. Kingsbury, David Thomas. 1964. Isolation and characterization of a bacteriophage infecting the myxobacterium Chondrococcus columnaris. Master's thesis. Seattle, University of Washington. 67 numb. leaves.

34. Knight, C. A. 1954. The chemical constitution of viruses. Advances in Virus Research 2:153-170.

35. Kolstad, R. A. and S. G. Bradley. 1964. Purification of Streptomyces venezuelae phage. Journal of Bacteriology 87:1157-1161.

36. Labaw, Louis. 1951. The origin of phosphorus in Escherichia coli bacteriophages. Journal of Bacteriology 62:164-173.

37. Labaw, Louis. 1953. The origin of phosphorus in the T1, T5, T6, and T7 bacteriophages of Escherichia coli. Journal of Bacteriology 66:29-436.

38. Lowry, Oliver H. et al. 1951. Protein measurement with the phenol reagent. Journal of Biological Chemistry 193:265-275.

39. Lunan, Kenneth O. and Robert L. Sinsheimer. 1956. A study of the nucleic acid of bacteriophage T7. Virology 2:455-462.

40. Marmur, J. and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. Journal of Molecular Biology 5:109-118.

41. Martin, James G. and D. M. Doty. 1949. Determination of inorganic phosphate, modificiation of the isobutyl alcohol procedure. Analytical Chemistry 21:965-967.

42. Murphy, James S. and Lennart, Phillipson. 1962. Purification of B. megaterium phage G. Journal of General Physiology 45 (2) :155-168.

43. Northrop, John H. 1938. Concentration and purification of bacteriophage. Journal of General Physiology 21:335-355.

44. Okuba, S. et al. 1963. Separation of the transforming and viral deoxyribonucleic acids of a transducing phage for B. subtilis. Proceedings of the National Academy of Sciences 50:679-686.

45. Pons, W. A., M. F. Stansbury and C. L. Hoffpauir. 1953. An analytical system for determining phosphorus compounds in plant materials. Journal of the Association of Official Agricultural Chemists 36:492-503.

46. Roscoe, R. H. and R. G. Tucker. 1964. The biosynthesis of a pyrimidine replacing thymine in bacteriophage DNA. Biochemical and Biophysical Research Communications 16:106-110.

47. Saunders, Grady F. and L. Leon Campbell. 1966. Characterization of a thermophilic bacteriophage for Bacillus steareothermophilis. Journal of Bacteriology 91:340-348.

48. Scaletti, J. V. and H. B. Naylor. 1959. Characterization of purines and pyrimidines of deoxypentosenucleic acids of Micrococcus lysodeikticus and bacteriophage. Journal of Bacteriology 78:422-426.

49. Schildkraut, Carl J., Julius Marmur and Paul Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. Journal of Molecular Biology 4:430-443.

50. Schlesinger, Max. 1934. Auf der Frage der chemische Zusemmensetzung des Bacteriophagen. Biochemische Zeitschrift 273:306-311.

51. Schlesinger, M. 1936. The feulgen reaction of the bacteriophage substance. Nature 138. 508-509.

52. Smith, J. D. and G. R. Wyatt, 1951. The composition of some microbial deoxypentose nucleic acids. Biochemical Journal 49:144-142.

53. Stent, Gunther and Clarence Fuerst. 1955. Inactivation of bacteriophages by decay of incorporated radioactive phosphorus. Journal of General Physiology 38:441-458.

54. Storck, Roger. 1966. Nucleotide composition of nucleic acids of fungi. II. Deoxyribonucleic acids. Journal of Bacteriology 91:227-230.

55. Sueoka, Noburu. 1961. Variation and heterogeneity of the base compositions of deoxyribonucleic acids; a compilation of old and new data. Journal of Molecular Biology 3:31-40.

56. Taylor, A. E. et al. Isolation of the T2 bacteriophage of E. coli. Journal of Biological Chemistry 165:241-248.

57. Tokunaga, Tohru, Yasuo Mizuguchi and Toyoko Murahashi. 1963. DNA base composition of a mycobacteriophage. Journal of Bacteriology 86:603-609.

58. Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1957. Manometric techniques. 3d ed. Minneapolis, Burgess. 338p

59. Volkin, Elliott and L. Astrachen. 1956. The absence of RNA in bacteriophage T2r+. Virology 2:594-598.

60. Volkin, Elliott, L. Astrachen and Joan L. Countryman. 1958. Metabolism of RNA phosphorus in Escherichia coli infected with bacteriophage T7. Virology 6:545-555.

61. Weigle, J., Matthew Meselson and Kenneth Paigen. 1959. Density alterations associated with transducing ability in the bacteriophage lambda. Journal of Molecular Biology 1:379-386.

62. Wyatt, G. R. 1951. Purine and pyrimidine composition of DNA. Biochemical Journal 48:584-590.

63. Wyatt, G. R. 1955. Separation of nucleic acid compounds by chromatography on filter paper. In: The Nucleic acids, ed. by E. Chargaff and J. N. Davidson. Vol. 1. New York, Academic Press. p. 243-265.

64. Wyatt, G. R. and S. S. Cohen. 1952. A new pyrimidine base from a bacteriophage nucleic acid. Nature 170:1072-1073.

65. Wyatt, G. R. and S. S. Chen. 1953. The bases of the nucleic acids of some bacterial and animal viruses. Biochemical Journal 55:774-782.

66. Wyckoff, Ralph G. 1938. An ultracentrifugal analysis of concentrated Staphylococcus bacteriophage preparations. Journal of General Physiology 21:367-373.