THE PREPARATION, PURIFICATION, AND RECOVERY OF *STREPTOMYCES GRISEUS* PHAGE

by

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Typed by Vera Evenson

To my husband, Kenny, for his love and understanding.

To my parents for their continued interest and guidance.

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INTRODUCTION

The problem of the chemical and physical nature of bacteriophage can often be approached only when the phage are separated from their immediate environment. Since they arise from the infection and subsequent lysis of susceptible bacterial hosts, the resulting environment contains bacterial lysis products such as proteins, particulate cellular debris, and nucleoproteins. The medium in which lysis occurred also adds to this extraneous material. Because much of the contaminating material in the lysate is both chemically and physically similar to the phage, the problem of separating out the phage becomes an imposing one. Methods must be designed to accomplish this separation while still preserving the integrity of the phage itself. This last point becomes a serious one since phage are often easily inactivated. Since purified preparations must evidence a relative absence of non-phage material, they must be subjected to purity tests.

Very little is known about the chemical or physical nature of bacteriophage infecting members of the genus Streptomyces. Even less is known about the origin of the protein and nucleic acid material of these phages. An investigation into these problems necessitates developing a method of purifying the phage. For these reasons the study to be presented was initiated with the following objectives:

1. To develop a suitable procedure for obtaining large quantities of phage material.
2. To evaluate and adopt appropriate procedures for purifying the phage, keeping in mind the importance of maintaining its biological activity.
3. To evaluate the purity and investigate the chemical nature of the purified phage.

HISTORICAL

A considerable number of bacteriophages have been purified in recent years and from these preparations much has been learned of their chemical, biological, and physical properties. The first successful attempt to purify a bacteriophage was in 1933 by Schlesinger (22, p. 6-12) who concentrated coliphage WLL by high speed centrifugation. Although present methods to determine purity were not available then, Schlesinger's results compare remarkably well with recent preparations. Since this time numerous reports have been made of purified coliphages as well as some preparations of purified staphylococcal, salmonella, and megaterium phages. There is one report to date of a purification procedure carried out with an actinophage, 530-p infecting Streptomyces griseus (23, p. 333-338).

A prerequisite to any phage purification procedure is the initial possession of material containing a high concentration of phage. Ordinary broth lysates containing more than 1×10^{10} phage particles per ml are unusual, especially when prepared on a large scale. Generally definite steps must be taken to adjust conditions of cell

growth and lysis in order to obtain high phage titers. Phages which have a short latent period are usually obtained in high concentrations by infecting a broth culture with massive doses of the phage. Lysis, which is usually complete within a few hours, releases the phage particles into the medium.

Fraser (8, p. 117) designed an apparatus expressly to obtain high titer lysates on a large scale. Under conditions of vigorous aeration, lysates of phage T3 were obtained with more than 10^{11} particles per ml. Wyatt and Cohen (29, p. 775) also increased the aeration of the lysing culture by rotating the growth flask in such a manner that an enriched medium was constantly layered on its inner surface. Titers of between 5×10^{11} and 1×10^{12} virus particles per ml were obtainable by this method for several of the T-series coliphages. Adequate aeration brought about by vigorous shaking allowed Herriott and Barlow (13, p. 24) to obtain large amounts of T2, titering $2\text{-}5 \times 10^{11}$ phage per ml.

After lysate material with adequate phage concentrations is obtained, it is important to control environmental factors so that loss of biological activity be kept at a minimum. This gains particular significance in a phage purification procedure where yield of biological

activity is a prime index of concentration and purification.

Phages are generally stable in their own lysates and if stored at low temperatures, the infective titer will generally fall only slightly with increasing time. The low temperature storage of lysates has an additional advantage of decreasing the growth of phage-resistant host bacteria or contaminants.

Bacteriophages are generally stable over the pH range of 5 to 8 and this can sometimes be extended to pH 4 to 10 if low storage temperatures are used. Curves showing pH stability as a function of pH have been given for purified preparations of coliphage T6 by Putnam, Kozloff, and Neil (21, p. 311). The pH range of stability of T2 bacteriophage extends from pH 5 to 9 as shown by Sharp, et al. (24, p. 262). Shang-Chi Hsu, et al. (23, p. 335) report that the actinophage 530-p is active biologically between pH 4.5 and 10.

Purification methods themselves must be carefully chosen for each particular phage system in order to avoid inclusion of a step which unnecessarily reduces the infectivity of the preparation. High speed centrifugation, which has been used extensively for purification of phage, has been reported to cause irreversible inactivation of

some phages (15, p. 250; 18, p. 259). Beard (3, p. 56) has also suggested that repeated packing may physically damage the virus particles. This damage may be caused by separation of phage heads and tails or by an actual rupture of the particle itself with a subsequent leakage of nucleoprotein (19, p. 190). For these reasons several purification procedures have been outlined which substitute high speed centrifugation with other physical or chemical methods. The procedure proposed in this thesis for the purification of Streptomyces griseus phage 514-3 has no high speed centrifugation step because large losses in titer were encountered upon attempted resuspension of phage pellets.

As with proteins such as enzymes, phage are often rapidly inactivated when subjected to the unbalanced forces existing at gas-liquid or liquid-liquid interfaces. This surface denaturation can be prevented by the presence of enough protein to saturate the interface and prevent access of the phage to the surface (2, p. 442). However, when the purification procedure is designed to eliminate extraneous protein the phage becomes increasingly vulnerable. Care must be exercised in handling purified preparations so that this surface inactivation is held to a minimum.

The choice of chemical purification procedures is influenced by phage susceptibility to inactivation by some chemical agents. While undiluted glycerol and ethanol cause rapid loss of infectivity in phages, cold aqueous solutions of these solvents do not inactivate most phages, and in fact 30% ethanol has been used by at least one group of workers as a purification treatment with T6 (21, p. 307). Other solvents such as acetone, isobutanol, and chloroform have been used with success in the purification of staphylococcal bacteriophage K (15, p. 252). Thymol and chloroform exhibit no harmful effects on phage systems so far reported and in fact have been used as an aid in preserving phage preparations (28, p. 431). Most phages are stable to quite high concentrations of neutral salts such as ammonium sulfate and magnesium sulfate, both of which have been used to precipitate phage (18, p. 260).

Having developed a method for obtaining high titered phage material and having determined at least in general the physical and chemical conditions of stability of a particular phage system, workers may utilize several possible purification methods. Many physical, chemical, and biological aids for purification are reported in the literature, centrifugation being the method of choice in

a majority of cases. The initial clarification of a crude lysate to remove bacterial debris is usually accomplished by low speed centrifugation or filtration through Mandler candles or filter aid materials such as celite (13, p. 19; 18, p. 260; 21, p. 305).

Following the initial clarification, the lysate material is usually concentrated by centrifugation and resuspension of the phage. Many workers using a variety of coliphages have employed the centrifuge procedure and have frequently extended it to a differential centrifugation sequence to obtain purified preparations (14, p. 244; 20, p. 179).

Since most of the T phages precipitate immediately upon acidification to about pH 4.0 but undergo inactivation quite slowly, isoelectric precipitation has been used to concentrate phage preparations by Herriott and Barlow (13, p. 20) and Putnam, Kozloff, and Neil (21, p. 309). The phage were precipitated in the cold by the addition of acid to a pH of 4.0, the precipitate dissolved at pH 6.5 and then further purified by differential centrifugation.

A customary method of protein fractionation, salting out by the use of neutral salts, has been applied to precipitation of phage by a number of workers. Northrop (17, p. 337) first used ammonium sulfate to precipitate

staphylococcal phage. He later used both ammonium sulfate and magnesium sulfate to precipitate megaterium phage (18, p. 260). Sinsheimer (25, p. 38) used ammonium sulfate both for initial concentration of a coliphage lysate and for later fractionation of the preparation, although considerable loss of infective phage was incurred. Cavallo and Schramm (4, p. 580) included ammonium sulfate precipitation in their purification of a temperate staphylococcal phage with good recovery of titer. Ammonium sulfate precipitates coliphage T6 quantitatively but with loss of activity under the conditions described by Putnam, Kozloff, and Neil (21, p. 307). Ammonium sulfate fractionation has also lent itself to the purification of actinophage 530-p (23, p. 335) with no appreciable loss of activity indicated.

Alcohol can be employed successfully for fractionating an already concentrated phage preparation as evidenced by Putnam, Kozloff, and Neil (21, p. 307) who used graded dialysis against buffered ethanol solutions as a ready means of precipitating the phage T6. In purifying staphylococcal phage K, Hotchin (15, p. 251) found acetone effective as a precipitating agent in which the phage was remarkably stable.

Reversible adsorption of bacteriophage to inert materials has proved a very successful method of

concentrating and purifying certain phages. Wahl and Monceaux (28, p. 433) have demonstrated the adsorption of bacteriophage to a calcium phosphate precipitate at pH's below 6.3 with subsequent elution of the phage in phosphate buffer. In this work calcium phosphate at pH's above 6.3 was used to adsorb inert proteins from the lysate as an aid in purification. Cavallo and Schramm (4, p. 580) have applied this same technique to remove extraneous proteins from a partially purified preparation of staphylococcal phage. Adsorption and subsequent elution from ion exchangers has recently become another method of virus purification in which viruses function as anions in the exchange relationship. Hoyer, et al. (16, p. 860-862) have purified several viruses by using cellulose anion exchangers DEAE-SF and ECTEOLA-SF as adsorbents. Creaser and Taussig (7, p. 203) employed ECTEOLA cellulose for bacterial virus purification by using both batch adsorption and elution techniques and column chromatography for finer separations of phage from contaminating proteins and nucleic acids. Another application of cellulose adsorbent DEAE has been reported by Hall, Maclean, and Tessman (11, p. 192) who used it to purify phage ϕX174 in preparation for electron microscopy.

Enzymatic digestion represents an additional aid

to purification of bacteriophage. Treatment with deoxyribonuclease has frequently been included in purification procedures for the purpose of eliminating bacterial deoxyribonucleic acid or that arising from ruptured phage (13, p. 20; 19, p. 190). It was noted that the viscosity of preparations was reduced by DNAse treatment with no effect on the phage titer. Ribonuclease and trypsin are also effective purification aids, trypsin being used extensively to digest contaminating proteins without affecting the activity of the virus under the conditions described (18, p. 260; 23, p. 334).

Appropriate criteria of purity must be applied to any purified phage preparation to give validity to any claims concerning its properties. It has been emphasized that one criterion alone is not sufficient evidence for purity and a combination of different ones is desirable.

Of primary importance in purity demonstration is some indication of homogeneous biological characteristics. These might include plaque size and morphology, activity on known sensitive bacteria, conformity to the established latent period, and other additional properties peculiar to a given phage strain.

Infectivity, which is defined as the weight of virus per plaque-forming unit, is a very useful indicator of gross impurities or aggregates and inactive particles

in a phage preparation. The weight may be expressed in terms of grams of nitrogen, grams of phosphorous, or grams of DNA per phage (19, p. 192). The constancy of infectivity per unit of turbidity at 400 millimicrons and infectivity per unit of absorbancy at 260 millimicrons was used as a criterion of homogeneity of purified T2 phage by Herriott and Barlow (13, p. 20).

Direct examination in the electron microscope of phage preparations is a qualitative method of detecting aggregates as well as contaminating particles and this evidence is often included to substantiate purity claims (11, p. 192; 15, p. 251; 16, p. 860; 21, p. 316; 25, p. 38).

A most probable impurity in phage preparations is bacterial debris which should react antigenically with antibacterial antibodies. This immunological criterion of purity was used by Cohen and Argoblast (5, p. 610) as well as Herriott and Barlow (13, p. 20).

Another means of purity evaluation makes use of the analytical centrifuge to detect impurities with a slower rate of sedimentation than the virus. Phages investigated in the analytical ultracentrifuge have generally given a single sharp sedimenting boundary indicating a high degree of homogeneity (4, p. 582; 20, p. 181; 24, p. 263; 25, p. 39).

Various workers have subjected purified phage preparations to electrophoretic study to detect impurities which have a different mobility in the electric field (19, p. 198). Cavallo and Schramm (4, p. 581) utilized the Tiselius apparatus to separate slower-moving impurities from the virus, thus attaining an additional degree of purity.

Because nucleic acid of bacteriophage absorbs ultraviolet light intensely, this property can be used advantageously to indicate the degree of purity in a preparation. The absorption spectrum of coliphage T6 has been examined by Putnam, Kozloff, and Neil (21, p. 309) who observed a steady increase in light absorption as the wave length was decreased from 1000 to about 300 millimicrons. Below 300 millimicrons the optical density rises sharply with a slight hump at 280 millimicrons owing to absorption by aromatic amino acids, a sharp peak at 260 and a minimum at about 240 millimicrons. The maximum at 260 and the 240 minimum are characteristic of the absorption of purines and pyrimidines and are good indicators of impurities. Sinsheimer (25, p. 39) has recently reported the ultraviolet absorption spectrum of a purified preparation of bacteriophage in which a ratio of the absorption at 260 millimicrons to that at 280 millimicrons is 1.5, a value which he uses as a sensitive

indicator of impurities.

The constancy of the analytical composition of phage preparations may be used to determine the general extent of purity. An abnormally high content of nitrogen or phosphorous would be presumptive evidence for impurities from contaminating proteins or perhaps phosphorous-containing nucleic acids. Adams (1, p. 87) and Putnam (19, p. 219) have summarized the elementary analyses of several groups of phages and from these data it can be concluded that the phages reported so far have approximately 12-14% nitrogen, 3-5% phosphorous, and 40-50% carbon on a dry weight basis. Analyses for other elements are generally lacking. There seems to be general agreement that phage are about one-half protein and one-half nucleic acid of the deoxyribose type. Phosphorous and nitrogen contents per infective particle have frequently been reported and their ratio is a good measure of the presence of inactive phage particles which are physically present but biologically unaccounted for (1, p. 94).

Phage purification, pioneered by Schlesinger in 1933, has paralleled the general progress of phage research since that time. It has developed a highly precise methodology combining physical, chemical, and biological aids to purification and purity demonstration.

EXPERIMENTAL METHODS

Methods have been developed in this study for obtaining large quantities of high titer phage material with which to work. Additional methods were developed or existing ones modified to suit the particular requirements of the phage system in order to arrive at a purified preparation of this phage. Several types of determinations were made to assess the purity of the preparation. Since a great share of the work in this study centered around the methodology, this aspect will be treated in considerable detail.

Phage-host system

Streptomyces griseus (strain 3475 Waksman) and phage 514-3 were used throughout this study. The nature and reproduction characteristics of this bacteriophage have been reported by Gilmour, Noller, and Watkins (9, p. 186-192) and by Gilmour and Ingalsbe (10, p. 193-196).

One general type of medium was used both for producing spores and for obtaining phage lysates. It consisted of nutrient broth supplemented with 0.5% glucose and a trace of yeast extract. When solid media were necessary, agar was added in the desired amounts. Media were adjusted if necessary to pH 6.8-7.0 and autoclaved 20-30 minutes at 121°C, depending on the volume dispensed per container.

Stocks of S. griseus were maintained in sterile soil contained in 125 ml Erlenmyer cotton-stoppered flasks. The larger surface-to-volume ratio of these flasks as compared to tubes allowed better aeration and consequently more abundant and consistent sporulation. The production of spores was initiated by streaking generous amounts of soil containing the organism onto bottle slants of the nutrient medium with 2% agar added. Following a four-day incubation period at 32°C, the dense spore growth was scraped from the agar surface into nutrient broth. This suspension was then filtered through sterile gauze pads and diluted with broth to a turbidity calibrated to yield a concentration of about 5×10^8 spores per ml. Accurate spore concentration determinations were carried out by standard plate count procedures. Purity checks were made on each spore lot by cultural and microscopical examinations. To prevent germination, spore suspensions were always stored at 2-4°C.

Phage stocks consisted of Seitz-filtered broth lysates which were accurately titered and maintained under refrigeration. Under these conditions, titer losses were incurred in time, but the drop was always gradual.

Phage assays were made according to the agar layer method described by Adams (1, p. 450), using 0.5 ml of

the spore suspension as inoculum. Plaques were easily discernible after 24 hours of incubation at 32°C. Phage titer values were determined by averaging the results from quadruplicate plates at one dilution. In particularly important determinations, two dilution sequences were carried out and quadruplicate plates made from each, the results of which were averaged.

Production of high titer lysates

Apparatus It was discovered that constant temperature and aeration of lysing cultures was vital to the production of high titer phage lysates. It also became necessary to produce these lysates in amounts of 1 to 5 liters but with the additional requirement of rigid sterility conditions. For these reasons a simple apparatus was developed which allowed temperature and aeration control, was easily handled from a sterility standpoint, and was adjustable to volumes of 0.5 to 6 liters. The apparatus is shown in Figure 1. One liter Erlenmyer flasks capable of holding 500 ml of broth were fitted with sintered-glass spargers which were extended well into the medium. The top end of the sparger was fitted to a cotton air filter designed to sterilize air passing into the flask. A screw-clamp fitted at the end of the air filter controlled the amount of air entering the

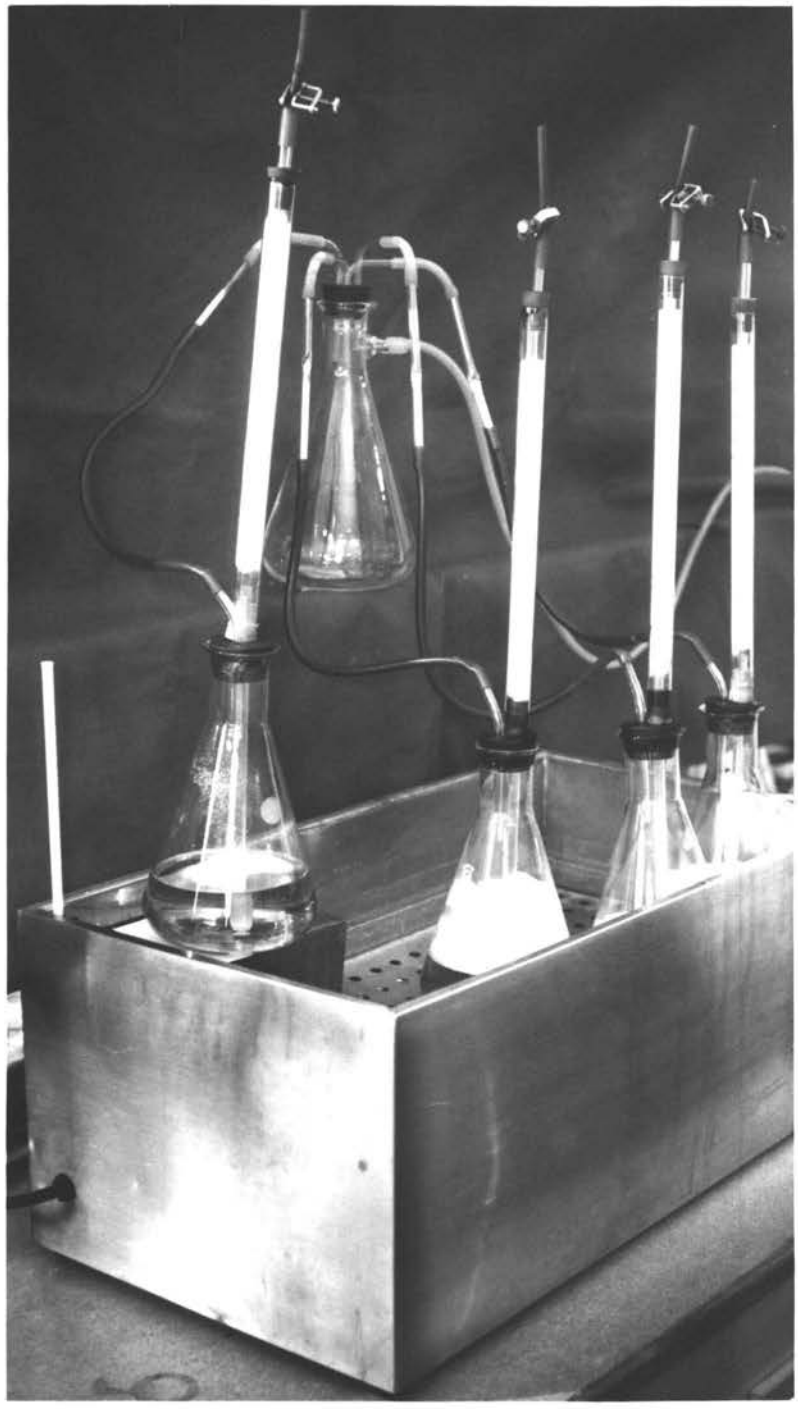

Figure 1. Apparatus for the production of phage lysates.

flask. Air was pulled through the filter into the medium by way of the sparger and out through an outlet connected to a water aspirator. Three groups of four such flasks were hooked to one aspirator by means of rubber tubing. The temperature was controlled by standing the flasks in water baths adjusted to the desired temperature.

Ratio-temperature adjustment From preliminary experimentation, it was soon found that a procedure which would consistently yield high titer lysates of more than 1 x 10^{10} phage particles per ml was dependent upon the factors of temperature, aeration, and phage/spore input ratio. Temperature controls were made as described above, with three water baths which could be set at 27°C, 32°C, and 37°C. Aeration was controlled both by the water aspirator (5 inches of mercury) and by the screw-clamps on the air inlet but was usually kept at a maximum with resulting vigorous bubbling.

Input phage/spore ratios were adjusted by varying the amount of phage stock which was added to a constant amount of 25 ml of the diluted spore suspension in the 500 ml of medium. Accurate phage titers and spore counts were predetermined on the stocks used as inocula. Ratios used for the lysate production usually favored the spores with phage/spore ratios varying from 1:50 to 1:1 being

used. Excessive foaming was controlled by the use of Dow Corning spray Antifoam B added to the media before autoclaving. Complete lysis occurred between 15 and 20 hours.

pH stability

In order to investigate possible purification methods, it was considered essential to know the general range of pH stability of the phage. Nutrient broth was adjusted to a pH of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 by the addition of either 1N HCl or 1N NaOH. 9.9 ml of the chilled broth at each pH were dispensed into tubes to which was added 0.1 ml of a titered stock phage suspension. The tubes, held at 2-4°C throughout the experiment, were sampled for phage activity after 1 hour and again after 24 hours. Percentages of the input phage which survived were calculated from these titers.

Purification steps

The purification scheme finally accepted as suitable for this particular phage consisted of an initial clarification of the lysate by filtration, a two-step enzymatic digestion, an adsorption process with calcium phosphate, two precipitations with ammonium sulfate, dialysis, and lyophilization. Each step was carried out in the cold

(2-4°C) and was preceded and ended with a phage assay. While the scheme in its entirety is presented diagramatically in Figure 2, the procedure for each step will be considered individually.

Clarification Although the lysate production was designed to yield completely clear lysates, it was considered essential to remove any remaining cells and cellular debris. Filtration was chosen in preference to centrifugation because of the speed, ease of handling large volumes, and the lack of possible physical damage to the phage. Crude lysates were clarified by filtering with suction through Büchner funnels equipped with Whatman #3 filter paper covered by a 1 gram pad of analytical-grade celite filter aid.

Deoxyribonuclease digestion To digest cellular DNA and that DNA possibly arising from ruptured phage particles, 1 microgram per ml of crystalline deoxyribonuclease was added to the clarified lysate after it was first adjusted to pH 6.5. Magnesium sulfate, added to a final concentration of 0.003 M, served as a co-factor for the enzyme. The digestion was allowed to act for 30 minutes at room temperature.

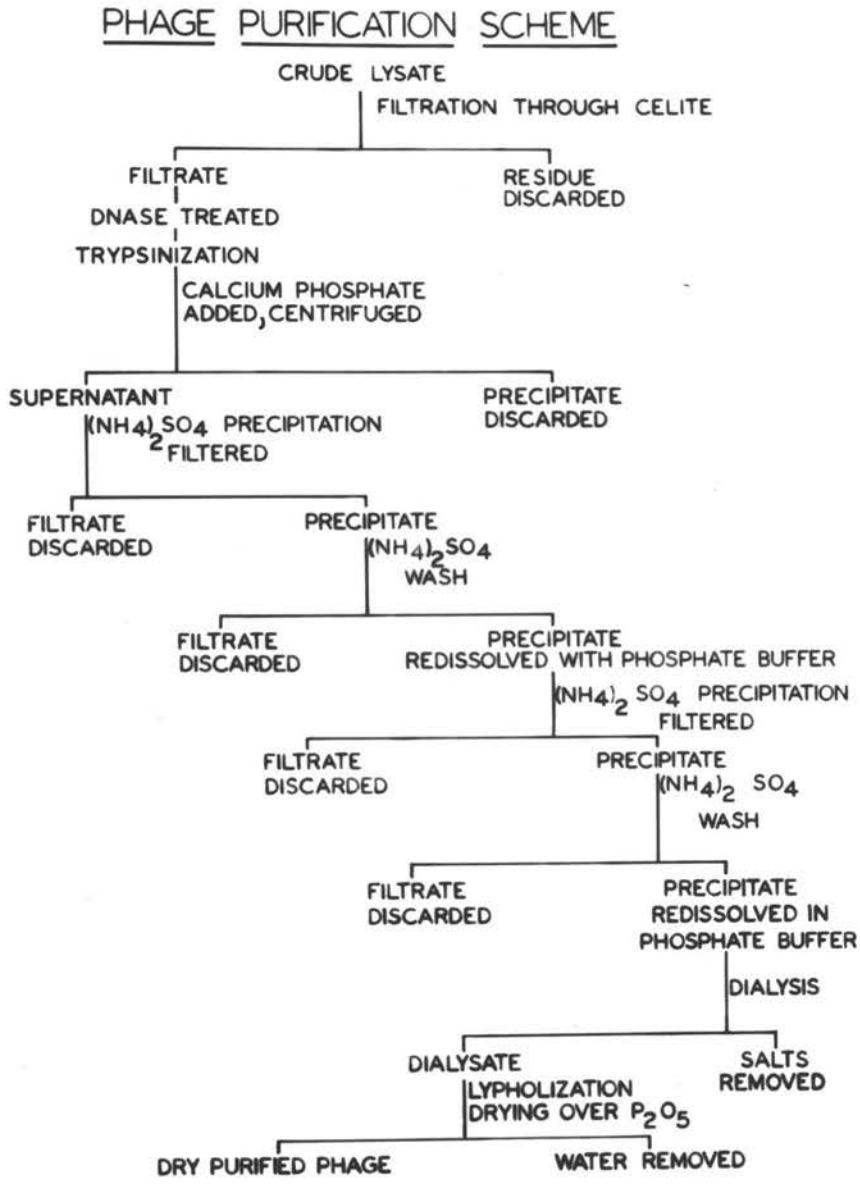

Figure 2. Flow diagram of purification scheme.

Trypsinization Trypsin was found to successfully digest extraneous cellular and medium proteins without harm to the phage if conditions were controlled properly. After adjusting the preparation to pH 8.0 with 5N NaOH, twice crystallized trypsin was added to a concentration of 1 microgram per ml. Digestion was allowed to procede for 1 hour at 37°C although initially several time periods up to 4 hours were tried as was a 48-hour digestion at refrigerator temperatures.

Adsorption of proteins by calcium phosphate Freshly precipitated calcium phosphate was effective in removing non-phage proteins and colored broth components remaining in the trypsinized preparation. The trypsinized preparation was immediately cooled to below 10°C in ice water and to it was added 170 ml of a cold, buffered concentrated phosphate solution which consisted of 79.1 grams of Na_2HPO_4 and 4.5 grams of KH_2PO_4. Then 370 ml of cold 0.22 M $CaCl_2$ solution were added and the resulting dense precipitate kept in suspension by gentle swirling in an ice bath. A small sample was quickly centrifuged and the pH of the supernate checked. If the pH was above 6.3, small additional amounts of $CaCl_2$ were added to the original mixture to lower it to pH 6.3, above which no phage adsorbs to the precipitate. The calcium phosphate

remained suspended in the preparation for 10 minutes at pH 6.3, and then was removed by a short low-speed centrifugation. The resulting supernate was maintained in an ice bath.

Ammonium sulfate precipitation A salting out procedure using ammonium sulfate served to concentrate the phage preparation and with proper selection of salt concentration accomplished fractionation of the phage from non-phage protein.

Initial experiments to determine the proper ammonium sulfate concentration for protein fractionation resulted in a drop in phage titer of 50% or more. Since it was soon discovered that time of exposure to the ammonium sulfate was the determining factor in the recoveries from this step, an experiment to determine the minimum time for phage precipitation after the addition of the salt was necessary. To each of 5 batches of 200 ml of lysate were added 56.5 grams of finely-ground solid ammonium sulfate to yield a concentration of 40% of saturation (at 0°C). The flasks were gently mixed to dissolve the salt and then allowed to stand for varying intervals of time before separating the precipitate by filtration. This involved using a Büchner funnel under suction with Whatman #3 filter paper and a 1 gram pad of analytical celite

as a filter aid. This step was considered superior to centrifuging out the precipitate because of speed of operation and ease of redissolving the precipitate. The precipitate was dissolved on the filter paper and washed through it by 5 successive 10 ml aliquots of cold M/15 phosphate buffer at pH 8.0.

After it was found that 5 minutes was sufficient for phage precipitation, almost complete phage recoveries could be obtained by this method. A more complete study of the minimum ammonium sulfate concentration required for precipitation involved the above procedure but with varying amounts of ammonium sulfate being used to bring its concentration to 25%, 30%, 35%, and 40% of saturation at 0°C.

In an actual purification run it was necessary to divide the preparation into several 200 ml batches and to perform separate precipitations on each as described above, using the 5 minute time limit. An additional step to wash the precipitate for removal of soluble impurities was carried out by washing the pad holding the precipitate with 5 successive 10 ml aliquots of a 45% saturated ammonium sulfate solution. The washings, which did not remove the phage but contained considerable color, were discarded. The phage was resuspended as before with buffer. The time of phage exposure to the

concentrated ammonium sulfate was carefully controlled so that the precipitation, washing, and resuspension never exceeded 10 minutes.

A second ammonium sulfate precipitation, carried out in an identical manner on the redissolved precipitate from the initial precipitation, served to further fractionate and concentrate the preparation. In this step in which only one batch precipitation was necessary, the precipitate was filtered, washed, and resuspended as before.

Dialysis Dialysis experiments were carried out using cellophane dialysis tubing in the cold (2-4°C). Periods up to 24 hours and a variety of phosphate buffers at different molarities as well as distilled water were used. Constant agitation was accomplished by a magnetic stirrer and frequent changes of buffer or water were made. Since excessive titer losses were always a result of dialysis, an attempt to check the time factor involved in the inactivation required filling three dialysis bags with 5 ml each of a phage preparation and dialyzing against M/15 phosphate buffer at pH 8.0 in the cold. At 2, 4, and 6 hours a bag was removed and sampled for phage activity.

A dialysis step was considered necessary to remove

salts and small molecular weight impurities from the second redissolved ammonium sulfate precipitate, in order that preparations for electron microscopy, dry weight determinations, and elemental analysis might be made. This was done in the same manner as before against 4 liters of distilled water for 3 days with several changes of water.

Lyophilization The dialyzed preparation was quickly frozen in an ethanol-dry ice mixture. It was frozen in a thin layer on the inside of a 250 ml round-bottom flask. This was then dried under vacuum. The freeze-dried preparation was stored in a vacuum desiccator over P_2O_5 and weighed after 24 hours.

Criteria of Purity

Several methods were employed to evaluate both the stepwise removal of contaminating material and the relative absence of it in the final preparation.

Specific infectivity Since a great part of the contaminating material in a phage lysate prepared in the aforementioned manner is protein, an expression showing the relationship between the phage activity and total protein would be revealing. Such an expression has been made for each step of the purification. Nitrogen content is a good index of protein content and has been used to

advantage in this aspect of the study.

Protein nitrogen analyses were made on aliquots of various preparations by precipitating the proteins with trichloroacetic acid, washing the precipitate, digesting it with acid and then determining the ammonium nitrogen by using a Nessler determination. To 1-10 ml samples containing up to 80 micrograms of protein nitrogen was added 10 ml of cold 5% trichloroacetic acid solution. The suspension of the resulting precipitate was allowed to stand about 30 minutes at room temperature, was centrifuged in a Servall angle-head centrifuge for 5 minutes, and washed at least twice with 5% trichloroacetic acid. The final supernatant was checked for ammonia by a spot plate Nessler determination and if a positive test was evident, the precipitate was washed again. The precipitate was then digested about 12 hours with 2 ml of a 2N sulfuric acid digestion mixture (27, p. 238) in a sand bath at approximately 400°C.

Color development using Nesslers reagent was then done as suggested by Umbreit (27, p. 238). After allowing the color to develop for 15 minutes, readings were made on a Bausch and Lomb spectrophotometer at 490 millimicrons. Reagent blanks and standards were always run with the unknown samples. It was found very convenient to precipitate, centrifuge, wash the precipitate, digest

it and develop the color all in the same thick-walled Servall glass centrifuge tube. Loss of sample was minimized since no transfers were necessary.

Each step of the purification procedure was evaluated with respect to its part in removing extraneous protein. By relating the phage activity to the protein nitrogen following each part of the procedure, a specific infectivity determination was calculated. It is expressed as numbers of phage particles per micrograms of protein nitrogen.

Phosphorous, nitrogen analysis of final preparation

Total nitrogens on accurately weighed samples of the final dry preparations were carried out by the aforementioned Nesslerization methods.

Total phosphorous determinations on an accurately weighed sample of the dry purified phage were made according to the Fiske-SubbaRow method (12, p. 635). Reagent blanks and phosphorous standards were digested with the unknown samples.

A calculation of micrograms of phosphorous or nitrogen per number of phage particles is often useful to detect gross impurities or an excessive amount of inactive phage particles. The nitrogen and phosphorous determinations were related to phage titers on the final preparation

before dialysis to give such values.

A property often reported of purified phage is its percentage of nitrogen and phosphorous. These values were determined by dividing the sample weight by the weight of phosphorous or nitrogen.

Particle weight A figure expressing the weight in grams per phage particle can be calculated from the known weight of the dried preparation and its corresponding phage titer. As before, this titer was taken on the liquid sample before dialysis.

Ultraviolet absorption spectrum A spectral analysis in the ultraviolet range was made on the final dialyzed preparations diluted 1:2 with water. A Beckman Model DU spectrophotometer was employed in the range from 230 to 340 millimicrons.

Electron microscopy Preparations used for the electron microscopy were dialyzed, diluted with water 1:25 and spotted on grids covered with collodion films. For the purified phage the grids used were obtained from the Bitterroot Specialty Company, Hamilton, Montana, and are positively charged to minimize aggregation of the preparation on the grid. Lysate samples underwent an initial high speed centrifugation and resuspension before

being spotted on ordinary collodion-covered grids. The preparations were all air dried over P_2O_5 overnight and then shadowed with a palladium-platinum alloy at an angle of 32°. The preparations were viewed in an RCA electron microscope and micrographs taken at an original magnification of approximately 19,000 times.

EXPERIMENTAL RESULTS

By developing new methods or modifying existing ones, procedures were developed to produce and purify measurable amounts of this actinophage. This particular phage is extremely sensitive to environmental conditions and mechanical damage. The continual threat of inactivation governed the choice of purification procedures to a large extent. The purified phage preparations were subjected to purity evaluation. Properties of the purified phage served as a basis of comparison with the purity and properties of other phages.

High titer lysates

The use of the apparatus pictured in Figure 1 allowed consistent production of as much as 6 liters of high titer lysate (1-5 x 10^{10} phage/ml). Controlling factors were temperature and phage/spore input.

Temperature effect As might be expected, the general temperature range optimum for S. griseus is most favorable for phage replication. This is indicated in the data from Table 1. While very little cellular growth occurred at 37°C, both 27°C and 32°C brought about abundant cell growth. The effect of temperature is quite striking on the phage titer yields. Essentially no phage

Table 1

EFFECT OF TEMPERATURE AND PHAGE/SPORE RATIO ON PHAGE YIELD

Temperature	Phage/spore ratio	Time of complete lysis in hours	Titer phage/ml	Grams dry weight in cell control (no phage)
27°C	1:50	18	3.4×10^{10}	0.91
	1:10	16	9.2×10^{9}	
	1:1	14	4.0×10^{9}	
32°C	1:50	18	1.1×10^{10}	1.2
	1:10	16	6.0×10^{9}	
	1:1	14	1.8×10^{9}	
37°C	1:50	not lysed in 24 hrs	----	0.03
	1:10	18	1.0×10^{8}	
	1:1	18	1.0×10^{8}	

reproduction occurred at the highest temperature while the lowest temperature yielded the highest titers. After spore germination, S. griseus exhibits the branching mycelial type of growth characteristic of the genus. Even though one segment of the mycelium is infected with phage, the other parts are able to continue growing normally. When the infected parts burst and release more phage, they will go on to infect more mycelium. This recycling is directly responsible for the production of high phage yields. A condition which favors the cells to such an extent that extremely abundant cell growth results might never allow complete lysis to occur. The possibility of this happening is even more evident when one considers that the latent period of the phage is about 2 hours. From these considerations it is not difficult to understand why a slightly sub-optimal temperature could actually increase phage yield.

Ratio effect The same interrelationship of cell growth and recycling of phage infection is important in the phage/spore input. The spore state of this organism is the only stage in which it can be accurately quantitated in terms of "single" cells. It was soon recognized that phage infection should be initiated at this single cell stage in order to give significance to a host-phage

input ratio. Phage are unable to infect the spores but if phage are added to the spores they will adsorb to and infect the newly germinated mycelium which results. A phage input greatly in excess of the number of spores would insure infection of all the newly germinated spores, but after one latent period and burst of phage very few cells would remain to support further phage replication. Titers produced from such a procedure would be dependent on a single burst of the phage. If the burst size is not exceptionally high, as is the case with this particular phage, resulting titers would not be high.

From these considerations it is evident that a low phage/spore ratio would allow a continual recycling of adsorptions and bursts with a gradual build-up in titer until all cellular material is lysed. The data in Table 1 indicate that these observations can be substantiated experimentally.

pH stability

There is quite a broad range of pH values to which the phage may be subjected with little or no permanent damage. This range is shown in Figure 3 which also indicates the pH at which the phage is permanently inactivated. Below pH 4 the phage is almost completely inactivated perhaps because of precipitation at its iso-electric

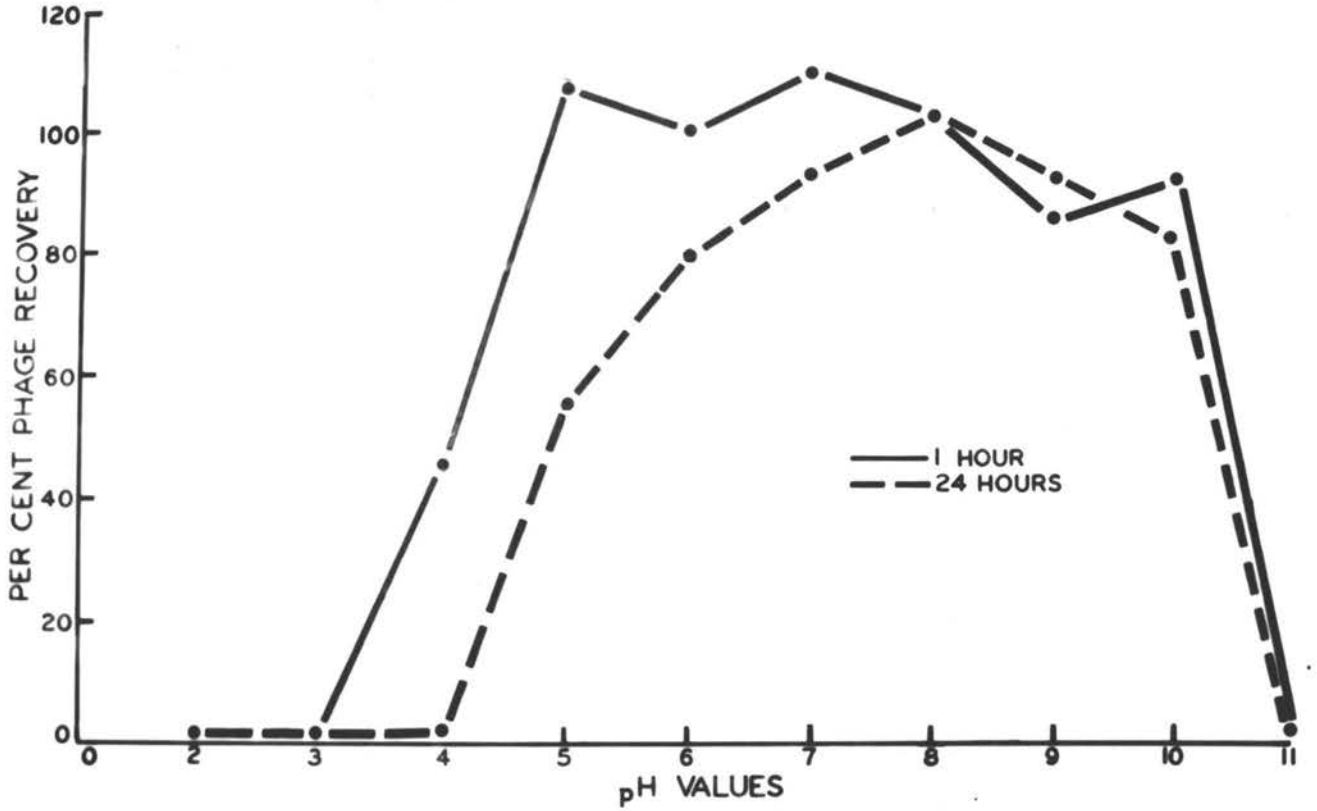

Figure 3. pH stability curves for phage in nutrient broth at 2-4 °C.

point. These findings are compatible with those reported by Shang-Chi Hsu, et al (23, p. 338) for actinophage 530-p in regard to pH stability.

Purification--Individual Steps

The inclusion of each individual step in the final purification scheme was considered from two viewpoints: effect of procedure on the biological activity of the phage and its usefulness as a remover of contamination. The results concerning these two points will be considered for each step separately and are summarized later in the text.

Clarification This initial step served to remove from the crude lysate cellular debris resulting from lysis as well as a few remaining whole cells. Initial protein nitrogen determinations showed that this amounted to a removal of up to 8% of the total protein nitrogen. A slight titer drop usually accompanied the filtration, but since celite itself does not adsorb phage under these conditions, the drop is due to removal of infected cells and cell debris which would yield a plaque if plated on S. griseus.

DNAse treatment Deoxyribonucleic acid, a possible contaminating material in a phage lysate, can be effectively removed by the enzyme DNAse. The only measurement of the activity of the enzyme that was made was the observation of lack of viscosity of the preparations after treatment. Concentrated preparations that were not treated with DNAse were extremely viscous, probably indicating a large amount of DNA present. Protein nitrogen drops were always associated with the DNAse treatment; this might mean that some DNA ordinarily is brought down with the protein when it is precipitated by trichloroacetic acid.

Trypsinization A review of the literature does not indicate any digestion of phage or loss of titer by the action of trypsin. However, this particular actinophage is very sensitive to the enzyme over a broad temperature range if the exposure time is much beyond one hour. The data from Table 2 attest to this observation. At 37°C as much as 60% of the original protein nitrogen may be digested without a decrease in phage titer. On this basis, the trypsinization step became a very valuable purity aid.

Table 2

EFFECT OF TRYPSIN ON PHAGE ACTIVITY AS INFLUENCED BY TIME AND TEMPERATURE

Temperature	Time of exposure in hours	Phage titer percent decrease	Protein nitrogen percent decrease
2-4°C	24	33.7	37.4
	48	83.7	----
37°C	1	1.0	60.7
	2	17.2	78.4
	3	31.7	87.4

Adsorption of proteins by calcium phosphate Calcium phosphate, precipitated in the phage lysate above pH 6.3, adsorbed up to 75% of the non-phage proteins present. Small amounts of phage were sometimes lost by this step, probably by trapping of the phage by the precipitate rather than by adsorption. It can be shown that the phage does not adsorb to calcium phosphate above pH 6.3. Between pH 5.3 and 6.3, however, all of the phage will adsorb to the calcium phosphate. This additional step was not included in the purification because the elution step as outlined by Wahl and Monceaux (28, p. 434) was very inefficient.

Ammonium sulfate precipitation This phage can be quantitatively precipitated by a high concentration of ammonium sulfate. The amount of ammonium sulfate necessary to do this was initially determined and the results of these experiments are shown in Table 3. It was evident that phage activity could be permanently lost by long exposure to ammonium sulfate or to even short time exposures at very high concentrations. In reference again to Table 3, it may be noted that the concentrations of ammonium sulfate above 40% saturation tended to inactivate the phage even though all of the phage was precipitated. The concentrations below 40% did not

Table 3

AMMONIUM SULFATE CONCENTRATION EFFECTING QUANTITATIVE PRECIPITATION OF THE PHAGE

Final percent of saturation of $(NH_4)_2SO_4$ *	Percent of original phage recovered	
	In redissolved precipitate	In filtrate
25	66.0	17.1
30	82.2	7.9
35	81.0	2.9
40	96.6	0.4
50	44.0	< 0.1
75	56.0	< 0.1
100	38.8	< 0.1

* calculated from percent saturation at $0^{\circ}C$

consistently precipitate all of the phage, as can be seen by noting the titers remaining in the filtrate. Thus 40% of saturation was selected as the concentration which quantitatively precipitated the phage without precipitating undue amounts of additional proteins or causing excess phage inactivation.

The information in Table 4 supplements the material in Table 3 to confirm the previous observation that the ammonium sulfate inactivation of phage is affected by time and concentration. An exposure time of 5 minutes allowed almost complete recovery of the phage.

The ammonium sulfate step functioned as a fractionation method to remove proteins. Because it also served to concentrate the volume of the preparation, many non-protein impurities also were eliminated. Depending on the preparation, as much as 60% of the non-phage protein present could be removed in this manner. The ammonium sulfate precipitates were washed with a solution of ammonium sulfate which was calculated not to dissolve the precipitate but to merely remove impurities clinging to the filter pad and the precipitate. The discarded washings were always yellow, indicating some pigmented compounds were also removed.

Table 4

EFFECT OF TIME OF EXPOSURE TO 40% SATURATED AMMONIUM SULFATE ON PHAGE ACTIVITY

Time of exposure to $(NH_4)_2SO_4$ before removing precipitated phage	Percent of original phage recovered	
	In redissolved precipitate	In filtrate
5 minutes	96.5	< 0.1
15 minutes	76.7	< 0.1
30 minutes	61.3	< 0.1
1 hour	56.9	< 0.1
2 hours	39.6	< 0.1

Dialysis A loss of phage activity always resulted from dialysis. This loss ranged from 50 to 95% of the total input phage. The titer loss was irreversible and proceded with time. This latter factor is established by the data in Table 5.

Since phage are relatively large particles and would not be expected to pass through a semi-permeable membrane, the loss in titer reflects a destruction of biological activity of the phage while it remains in the dialysis bag. The possibility of oxidation damage, which seems remote when one considers that the phage are produced and stored under aerated conditions, was considered and checked by adding reducing agents to the buffer used in the dialysis. No increase in recoveries was detectable. Many different buffers as well as distilled water used for dialysis always yielded the same poor recoveries.

In the purification procedure the final redissolved ammonium sulfate precipitate was dialyzed until no more ammonium ion remained in the bag; a negative Nessler spot test was used as an index of complete salt removal. Since the phage titrations on a dialyzed preparation would definitely be in error, the titer before dialysis was used for some of the calculations.

Table 5

EFFECT OF DIALYSIS ON PHAGE ACTIVITY

Time dialyzed	Percent of original phage recovered
2 hours	35.3
4 hours	5.2
6 hours	1.0

Lyophilization The lyophilization was not considered a purification step since only water was removed. It served the purpose of getting the phage into a solid form for observation, dry weight determinations, and elemental analyses. The final yield of dry, slightly brownish purified phage was 20.4 milligrams. This resulted from the purification of one liter of lysate and compares favorably with yields reported for similar preparations of other phages.

A summary of representative calibration data for each individual purification step is given in Table 6. The recoveries are expressed as percent of previous step, thus each step is considered individually.

Final purification

Using the information gathered from initial calibration work on each purification step, several complete experiments were carried out combining all of the procedures into one integrated process. To minimize dangers of titer loss and bacterial contamination, the whole experiment was done in the course of one day, with the exception of dialysis and lyophilization. Typical results of such an experiment are shown in Table 7. Infectivity data are given in terms of phage particles per microgram

Table 6

SUMMARY OF PHAGE TITER AND PROTEIN NITROGEN RECOVERIES IN INDIVIDUAL PURIFICATION STEPS

Purification steps	Range in recoveries expressed as percent of previous step	
	Phage titer	Protein nitrogen
Celite clarification	92-100	92-97
DNAse treatment	95-100	88-95
Trypsinization	92-100	32-60
$Ca_3(PO_4)_2$ adsorption	85-95	25-45
$(NH_4)_2SO_4$ precipitation # 1	70-85	53-75
$(NH_4)_2SO_4$ precipitation # 2	65-70	65-70
OVERALL YIELD	30-60%	2-8%

Table 7

PHAGE AND PROTEIN NITROGEN RECOVERIES FROM PURIFICATION EXPERIMENT # 9

Purification step	Volume in ml	PHAGE		PROTEIN NITROGEN		INFECTIVITY
		Phage per ml	Percent of original	Micro-grams per ml	Percent of original	Phage/micro-gram protein nitrogen $\times 10^9$
Clarification	1000	1.14×10^{10}	100.0	17.0	100.0	0.671
DNAse treatment	1000	1.13×10^{10}	99.1	14.8	87.1	0.764
Trypsinization	1000	1.11×10^{10}	97.4	5.50	32.4	2.02
$Ca_3(PO_4)_2$ adsorption	1360	7.63×10^9	91.1	1.79	10.5	4.26
$(NH_4)_2SO_4$ precipitation # 1	210	3.93×10^{10}	72.4	4.50	5.56	8.73
$(NH_4)_2SO_4$ precipitation # 2	50	9.67×10^{10}	42.4	13.3	3.99	7.30

FINAL YIELD: 20.4 milligrams dry weight

of protein nitrogen. The continued increase in these infectivity values indicates a gradual removal of non-phage protein while the phage concentrations remains constant or decreases somewhat. The infectivity increases by a factor of 13 down through the first ammonium sulfate precipitation. For the next step this value is somewhat lower, undoubtedly a reflection of the lowered phage yield. It is very unlikely that this reduced titer was due to incomplete precipitation or a similar actual physical loss of phage, but it is probably caused by inactivation by ammonium sulfate.

Elemental analysis

Nitrogen, phosphorous, and dry weight determinations on the purified phage resulted in the data given in the first part of Table 8. For comparison purposes, the same type of determinations are listed for representative purified phages reported in the literature. Some properties of the phage under study do not conform to values reported for other phages, notably members of the T series. This is neither surprising nor necessarily regretable since the physical and chemical make-up of phages might be expected to vary considerably, especially between non-related phages.

Table 8

SOME PROPERTIES OF PURIFIED PHAGES

Phage	Micrograms per 10^{11} phage		Percent dry weight		Particle dry weight grams $\times 10^{-16}$	Reference
	Nitrogen	Phosphorous	Nitrogen	Phosphorous		
S. griseus phage 514-3	38.1	7.4	9.0	1.6	42	this thesis
T2	----	----	11.8	3.7	----	6, p. 513
T2	10-20	3.5	13.5	4.8	10	14, p. 256
T2	8-10	2.7	16.0	5.2	5	13, p. 22
T5	36	10	----	---	----	26, p. 145
T6	9-18	----	13.4	---	7.5	21, p. 306
T7	6	1.5	----	---	4	20, p. 179
Staph phage	20	6.7	14.3	4.8	18	17, p. 335
Megaterium phage	17	----	----	---	----	18, p. 260
Actinophage 530-p	22	----	19.0	---	11.5	23, p. 338
ø X174	----	----	----	2.9	----	25, p. 37

The nitrogen and phosphorous percentages of 9.0 and 1.6 respectively are somewhat lower than corresponding values for other phages. The nitrogen percentage is especially at variance with the only other available data on actinophage, the 19% nitrogen reported by Shang-Chi Hsu et al. (23, p. 338).

The ratio between nitrogen and phosphorous content of the purified phage under study is about 5.6 in contrast to a lower ratio of between 3 and 4 for other phages.

A comparison of the particle weights reveals for this phage a weight considerably higher than those reported for other bacteriophages. This weight of course will vary considerably due to the size variation of phages, both in head diameter and tail length. The weight is calculated per active phage particle. Any inactive phage will add weight to the preparation but not be considered in the enumeration. This is very likely the cause of the high value of 42×10^{-16} grams for phage 514-3. Reference to Table 7 will show that this certainly could be the case since only 42% of the original phage remained in the final preparation.

Ultraviolet absorption properties

A typical curve resulting from ultraviolet light absorption studies made on purified preparations of the

phage is given in Figure 4. The characteristic minimum around 240 millimicrons and the maximum at approximately 260 millimicrons indicate the presence of nucleic acids. Although considerable absorption occurs at 280 millimicrons, it is not extensive enough to yield a peak at this wavelength. The ratio of the absorbancy at 260 to that at 280 millimicrons is 1.33, indicating no gross protein contamination which would absorb at 280 millimicrons.

Electron micrographs

A qualitative indication of the purity which can be obtained with this purification procedure may be seen in Figure 5. Electron micrographs of the original lysate material are included to demonstrate the tremendous amount of contaminating material present initially. That most of this contamination has been removed is evident from a study of parts A and B of Figure 5. Further it should be mentioned that the phage in the final preparations appear homogeneous with respect to size and morphology. Almost all of the phage in the preparation were complete with tails; however, many free tails are also present. The separation of tails from heads is probably a major source of inactivation so easily incurred with

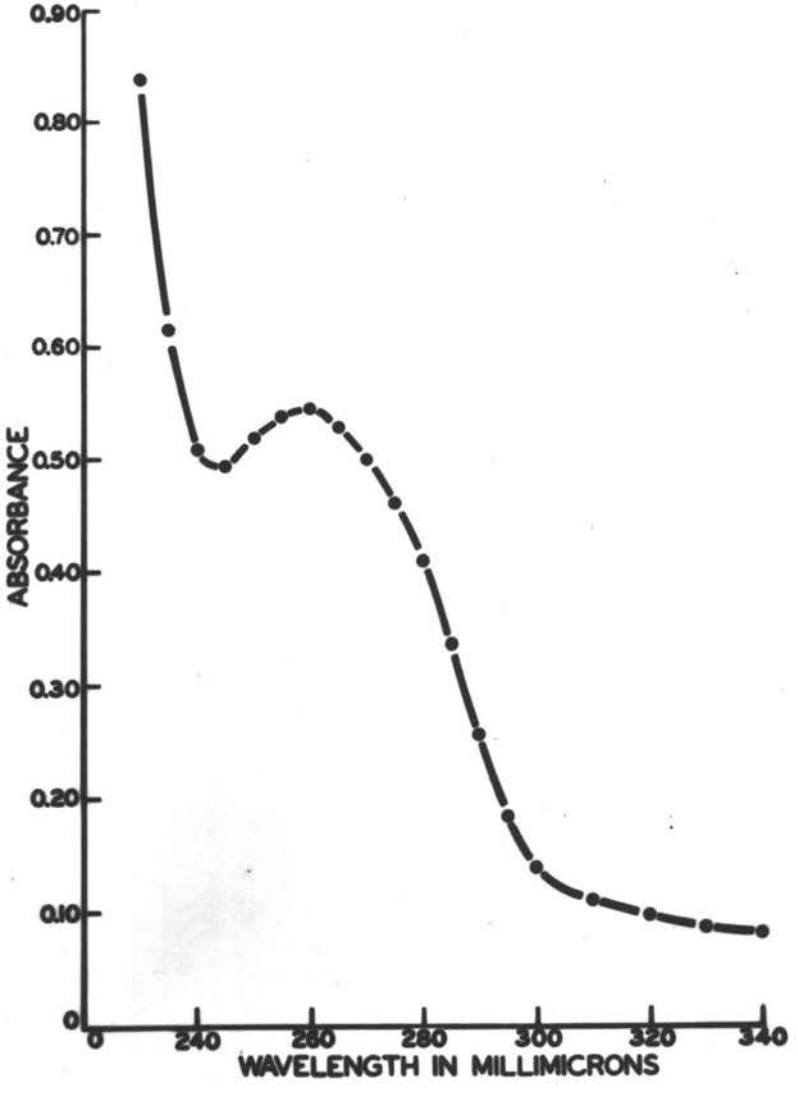

Figure 4. Ultraviolet absorption spectrum of purified phage.

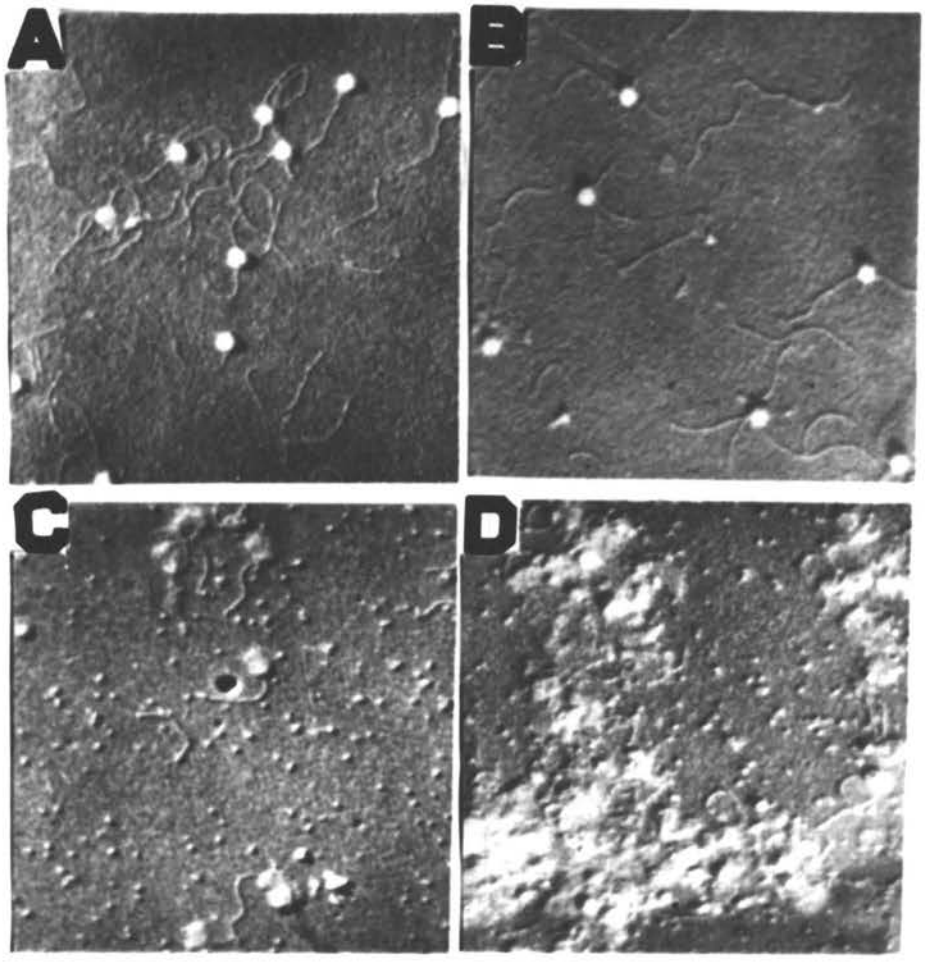

Figure 5. Electron micrographs of purified phage preparations (A and B) and original lysate (C and D).

this phage. This breakage is not surprising when one notes that the tail is 350 to 360 millimicrons long compared to a head diameter of about 100 millimicrons (9, p. 189).

DISCUSSION

A method has been presented for the production and purification of measurable amounts of Streptomyces griseus phage 514-3. The emphasis in the initial phases of this work was on obtaining high titer phage lysates. This emphasis was justified as can readily been seen when one considers that the higher the initial phage titer, the more actual purified phage will result. A titer of at least 1×10^9 phage/ml is not difficult to obtain with this phage-host system; in fact, almost any lysate will titer in this range. Hard to obtain consistently, however, is a titer of over 1×10^{10} phage/ml. The key to the production of lysates in this high titer range is control of temperature, aeration, phage/spore ratios, and any other factors affecting the physiological state of the host organism. The final yield of over 20 milligrams of purified phage reported in this work resulted from the purification of one liter of lysate titering over 1×10^{10} phage/ml. If an original lysate titer of only 1×10^9 had been used, only about one-tenth the yield or about 2 milligrams would have resulted. This amount would have been both too small to quantitatively purify and too little to use for analysis later. From these considerations, it is not difficult to understand the importance of starting

with material of a high titer.

The methods chosen to purify the phage were those that best satisfied the requirements of removing contaminating material while still preserving the biological activity of the phage. This latter point was actually the most exacting and the most unpredictable. This particular phage is unusually susceptible to inactivation. As pointed out earlier, part of this susceptibility may be due to the ease of mechanical injury to the phage itself. The elongated tail is apparently easily separated from the head, as was evidenced from the electron micrographs in Figure 5. This factor dictated the choice of purification methods to a large degree; no method involving extensive shaking, stirring, or packing of the phage was used. Even methods which appeared on the surface not to be harmful physically to the phage were unacceptable. Such a method was adsorption onto inert materials such as Dowex, ECTEOLA, and DEAE ion exchangers and onto calcium phosphate. By proper adjustment of conditions, adsorption could be brought about; but no satisfactory elution was ever achieved from any of the adsorbents mentioned. Dialysis, a commonly used procedure in phage purification, for some unexplainable reason also brought about great reductions in phage recoveries.

An explanation for these observations might be that the phage is actually quite different from other phages reported. This difference might be due to an unusual protein make-up. Perhaps the area of attachment to the host cell is easily denatured or altered in some way to make it non-infective. The fact that adsorption on inert materials is practically irreversible might further substantiate this speculation. The phage is sensitive to trypsin, a fact which has not been reported for other phages. This observation also attests to the unique nature of phage 514-3 protein.

Indications that phage 514-3 might be unusual in chemical make-up have also been presented. Preliminary results indicate a low percentage of both nitrogen and phosphorous content. This might lead one to believe that there is some moiety in the phage besides protein and nucleic acid, both of which should give a nitrogen content of greater than 9.0%. Perhaps the protein contained in the phage has an extremely low nitrogen content. The low phosphorous content might lead one to believe that the nucleic acid content of the phage is considerably less than one-half of the total weight; phages are generally considered to be about one-half DNA. These considerations require further investigation into the nature of the phage. More analyses on purified

preparations are necessary before any concrete assessment of the phage's chemical make-up can be made.

An answer to the imposing question, "How pure is this phage preparation?," must be attempted. From all indications the preparations finally obtained from this method were of a degree of purity comparable with reports of other pure preparations. To be sure, the preparations resulting from this work were never subjected to some of the more elaborate purity-evaluating methods such as the use of the analytical centrifuge or an electrophoresis apparatus.

The low nitrogen and phosphorous contents of the dried preparation certainly do not indicate a high degree of contamination from proteins or nucleic acids. There is the chance, however, that some contaminant not containing much nitrogen or phosphorous is present.

Another good indication that the preparation is relatively pure is the ultraviolet absorption spectrum obtained. Extensive contamination with nucleic acids would have been indicated by an increased absorption at 260 millimicrons, while gross protein impurities would have caused an absorption peak at 280 millimicrons. Other indications of purity such as amount of phosphorous or nitrogen per infectious unit or dry weight per particle

are subject to the error caused by the presence of inactive phage particles. This again points up the hazards which may be encountered when working with such an easily inactivated phage.

The continued observation that this phage reacts to environmental conditions, both physical and chemical, in a manner different from most other phages is an even greater incentive to develop a method of purifying it. Only then can the apparent uniqueness of this phage be investigated.

SUMMARY

Suitable methods have been developed for the preparation and purification of Streptomyces griseus phage 514-3. The resulting purified phage was analyzed chemically and was subjected to purity assessment. Pertinent results may be summarized as follows:

1. An apparatus was designed and proper conditions developed for the consistent production of phage lysates titering between 1 and 5×10^{10} phage/ml.
2. A purification sequence was used which included an initial celite clarification, enzymatic digestion using DNAse and trypsin, removal of proteins with calcium phosphate, two successive precipitations with ammonium sulfate, dialysis, and finally lyophilization. The effectiveness of each step was evaluated by a specific infectivity determination.
3. A final yield of 20.4 milligrams of dry phage was obtained. It contained 9.0% nitrogen and 1.6% phosphorous with a particle weight of 42×10^{-16} grams.
4. Ultraviolet absorption studies as well as electron micrographs revealed a considerable degree of purity had been achieved.

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