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The problem investigated here is part of a larger project designed to study the nature and function of the fine structure of Streptomyces griseus phage 514-3. The aims of this portion of the project are to attempt to disrupt intact S. griseus phage by methods that reportedly disrupted T even coliphages; to select a tool to indicate the extent of the effect of the treatment; to provide an explanation of the nature of the disruption by comparing these results with those obtained for T even coliphages; and to look for structural detail using negatively stained phage preparations.

Included among the methods tested were subjecting phage to various chemical agents, to various hydrogen ion concentrations, and to the physical forces involved in osmotic shock and rapid alternate freeze thaw cycles. The reaction of the phage to the treatments was measured by comparing the extent of damage to the infectious activity of the treated phage with that of the untreated phage.

Results from these studies further indicate that S. griseus phage is unique among phages, for it is quite dissimilar from the T even coliphages. For example, because S. griseus phage does not appear to possess tail fibers or a contractile sheath, it closely resembles T1 or T5 coliphage, not the T even coliphages. Because it also has a very long, thin, nonrigid tail, it is highly unlikely that it infects its homologous host by the conventional method described for T even coliphages. Because it is activated by treatment with Tris buffer or by osmotic shock treatment with 3M $MgSO_4$ whereas T even coliphages are inactivated and because it is unaffected by osmotic shock treatments with concentrated sucrose solutions or with 3M $NaC_2H_3O_2$ whereas T even coliphages are inactivated the permeability of S. griseus phage's head membrane and hence its chemical composition must be different from that of the head membrane of T even coliphages. Finally, because PTA alone does not provide enough contrast for observation of the head membrane of S. griseus phage, this compositional difference is further substantiated.

EFFECTS OF CHEMICAL AND PHYSICAL AGENTS UPON THE STRUCTURE OF STREPTOMYCES GRISEUS PHAGE 514-3

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

EFFECTS OF CHEMICAL AND PHYSICAL AGENTS UPON THE STRUCTURE OF STREPTOMYCES GRISEUS PHAGE 514-3

INTRODUCTION

The reproductive cycle of phage may be divided into intracellular and extracellular events. In order to understand the mechanisms involved in the extracellular processes of adsorption and infection, phage substructure must be determined. Recently due to the excitement contributed by nucleic acid chemistry and microbial genetics many bits of information regarding most aspects of the intracellular phase have been inadvertently discovered.

On the other hand little is known about the substructure of extracellular phage. Yet, ironically, a program for the sole purpose of studying the extracellular phase was formulated and begun long ago. The initial objective in this program was to assay for phage substructure by subjecting phage to specific chemical and physical forces and noting the effect of these treatments upon the phage by comparing its ability to infect its homologous host with the infectious ability of untreated phage. Then any structural alterations in the treated phage were observed by electron microscopy techniques. The next step was to assign functions to each substructure with the intention of finally proposing a workable mechanism of adsorption and infection. Unfortunately it was found that the magnification and resolution of the early electron microscopes as well as techniques of specimen

preparation were inadequate to ascertain the extent of the structural alteration caused by the chemical agent.

This project's primary aim was to study the substructure of S. griseus phage by using many of the same chemical and physical agents employed in the T even coliphage studies.

HISTORICAL REVIEW

Determination of phage substructure, the first step in the study of the extracellular phase, began when researchers found that morphological alterations along with decreased infectious ability could be effected by subjecting phage to certain chemical and physical forces. For example, Cohen (1947) noticed that coliphages T2 and T4 incubated in 6M urea resulted in a dense, viscous solution. Anderson (1946) reported that the internal substance of the head membrane of T even coliphages was extracted by intense UV irradiation. Then a few years later it was reported that subjection of T even coliphages to osmotic shock treatments also elicited this extraction (Anderson, 1949).

One of the earliest opportunities to institute the second step in the study of the extracellular phase came when Herriott (1951) chemically demonstrated that osmotic shock left phage with a shell consisting mainly of protein. Hence, the function of replication was assigned to the internal substance. Further instances of this step were not encountered until much later, because methods of discerning the subtler morphological alterations were not yet developed.

Decreased infectious ability as a result of physical and chemical treatments was not limited to the T even coliphages. Staphylococcal phage K was inactivated by osmotic shock treatments (Hotchin, 1954).

Also coliphage T5 was inactivated to a certain extent by the presence of 0. 1M Na ion and to a greater extent by the presence of 0. 1M Na ion in some chelating agent such as citrate, versene (EDTA), or triphosphate. Interestingly enough, by adding 10^{-3} M divalent cations such as Ca, Ba, Sr, Mg, Mn, Co, Ni, Zn, Cd, or Cu to the Na ion solution it was possible to stabilize this effect (Lark and Adams, 1953).

During the next five years the bulk of the work published was undertaken by one group of researchers and concerned an exhaustive search for delineation and function of T even coliphage's substructure. In the first of these investigations, Kozloff and Henderson (1955) found that cyanide complexes of the zinc group metals, which include Zn, Cd, and divalent Hg, inactivated coliphage T2. Although a much longer incubation time was necessary, inactivation also was obtained when these same metals were complexed with either 0. 01M of an amino acid as glycine, glutamic acid, and leucine or 0. 2% of a protein as trypsin and chymotrypsin. However, if the metal chelating agent, versene, was added with the complexes of these metals, no inactivation occurred. Morphological evidence from electron micrographs of shadowed treated preps showed removal of the distal half of the tail at a rate almost identical to the time of inactivation of the phage.

Then Brown and Kozloff (1957) found that T4 as well as T2 could

be inactivated by metal complexes and both could be inactivated by the Kellenberger and Arber (1955) method of incubating in a solution of 3% hydrogen peroxide in 10% ethanol and by the freeze thaw cycling method of Williams and Frazer (1956). In addition, electron micrographs of these treatments showed the same distal tail alteration as first described by Kozloff and Henderson (1955).

Turning their attention to the mechanism of viral invasion, Kozloff and Lute (1957a) began to look for the presence of any or all three of the zinc group metals in the homologous host cell walls. Results of these studies showed zinc is a cell wall component present in a quantity sufficient to interact with adsorbed phage.

Subsequently, Barrington and Kozloff (1956) showed that the enzyme activity of T2 resided solely in its protein coat. Then, Brown and Kozloff (1957) found altered T2 coliphage was able to digest the cell wall of mutant E. coli B/2, and altered T4 coliphage was able to digest host cell walls without the tryptophan cofactor necessary for similar digestion by whole T4. These workers also reported that alcohol $NaHCO_3$ destroyed the cell wall digestive property of altered T2 and T4 coliphage, but not of intact phage. Thus, it was concluded that these phage particles contained an active digestive enzyme site in their tails, which was protected in the intact phage by the tip of its tail. This meant that the tip of the tail must be removed before phage enzymatic activity on the host cell wall could progress.

In another study concerning the viral invasion process, Kozloff, Lute, and Henderson (1957) decided to detect the kind of chemical bond responsible for causing alteration of the distal part of the tail. Because metal complexes do not break ordinary peptide bonds, oxygen ether or oxygen ester bonds, or phosphate ester bonds, sulfur linkages of polypeptides were suspected to be the site of attack. Now regardless whether T even coliphage was treated with papain, which has known thioesterase activity, or with either hydroxylamine or hydrazine, both of which attack thiol esters, inactivation occurred. In addition, electron micrographs of these treated phage particles showed the same kind of distal tail alteration as was obtained with cyanide complexes of the zinc group metals. Therefore, it was concluded that these metal complexes caused an alkaline hydrolysis of thiol ester bonds in the phage tail.

In the final part of this study Kozloff and Lute (1957b) found that following T2 tail alteration by zinc group cyanide complexes, phage DNA could be released at an optimum pH of 8. 75 to 9. 00 by 0. 2M concentration of compounds having a free amino group. Among the most active were glucosamine; Tris(hydroxymethyl)aminomethane buffer; and the basic amino acids, arginine, ornithine, and lysine. Of these, arginine was found to release DNA from intact phage by disrupting the head protein membrane. With the exception of Tris buffer all compounds were found in the host cell wall.

Results of these studies permitted Kozloff and his associates to propose a number of T2 coliphage substructural units including tail fibers, tail core, and tail sheath. Then by assigning certain functions to each unit, a process of phage invasion was postulated. According to his explanation the invasionary process commences when the phage adsorbs tail first to the host cell wall and the protein fibers on the distal end of the tail are partially unwrapped. Then the zinc protein in the host cell wall, rather than acting as a coenzyme, functions as a dehydrogenase to hydrolyze the thiol ester bonds which link the fibers with the tail core. This results in removing the distal end of the tail by shortening and thickening the tail sheath and in exposing the viral enzyme so it can begin its cell wall digestion. Products of these reactions or components present in the host cell wall could then trigger phage DNA release into the host cell.

According to this explanation for viral invasion, it is suggested that the tail sheath protein possesses contractile properties. From a followup study, Dukes and Kozloff (1959) actually found phosphatase activity located in the tail protein of both T2 and T4 coliphages. This activity was not believed to be due to adventitious host cell contamination, for isolated phosphatase activity of E. coli B differs markedly from those of the phage phosphatase. Further work by Kozloff and Lute (1959) revealed that this tail protein also contained contractile and enzymatic properties that are similar in many ways to actomyosin.

From these observations, postulation of the invasion process was continued. Apparently, since actomyosin is an -SH protein, removal of the tail fibers by cleavage of the thiol ester bonds expose -SH groups which trigger contraction of the remaining tail sheath protein. Furthermore, it is felt that this contraction can occur simultaneously with or immediately after the action of the phage tail enzyme on the rigid outer layer of the host cell wall. Therefore, its function may be to hold the tail enzyme in close contact with the substrate in the outer cell wall. Then after the cell wall loses its rigid structure, the contraction may force the tail core through the fragile inner cell membrane. This contraction probably also functions as a means of widening the center hole in the phage tail, so that the tail core can fall out to facilitate DNA passage into the host by unblocking the channel in the tail and by reducing the viscous drag of the molecule. (Incidentally, viscous drag is directly proportional to the length of the tube and inversely proportional to the fourth power of the diameter.)

Subjecting T2 coliphage to a series of freeze thaw cycles followed by differential centrifugation through a glycerol gradient permitted Williams and Fraser (1956) to dismember the phage into the same substructures that Kozloff and his associates reported. Although shadowed specimen preps were used in both of these studies, most of the substructural determinations performed by Kozloff's group

were done using chemical data.

The advent of microscopes with higher resolution and magnification did not solve certain problems caused by the shadowing technique such as limiting the optimum resolution of the newer electron microscopes by the granularity of the shadowing material, hiding many structural features in the shadow, and limiting the information only to size and shape and not to distribution of the chemical groups within the specimen (Beer and Zobel, 1961). To alleviate this situation Brenner and Horne (1959) devised a negative staining procedure whereby the specimen is "embedded" in an electron dense material such as phosphotungstic acid (PTA) or uranyl acetate (UA). Thus by using this method, which introduces contrast without obscuring structural detail, structures of 15A can be resolved.

To prove the effectiveness of this negative staining method, Brenner et al. (1959) examined the structure of T even coliphage. However, because they could not get effective disjoining and separation of the structural components using either the method of cyanide complexes of Kozloff's group or the Williams and Frazer freeze thaw method, they developed another procedure. All the better, for when it was shown that structures corresponding to those proposed by Kozloff could be obtained by several different methods, the existence of such entities was established beyond a doubt. In addition, more structural detail was obtained. For example, the relaxed phage tail

can be seen to consist of a hollow protein sheath built of helically arranged subunits or grooves. When the sheath is contracted these grooves run parallel to the long axis of the tail so that in cross section they give a "cog wheel" appearance. Also this sheath surrounds a tail core which contains a central hole. The tail fibers are attached to a hexagonal plate which is located at the distal end of the core.

Except for one report, almost nothing has been learned about the structure of this or any other phage head. In this report Cummings and Kozloff (1962) discovered that T2 coliphage occurs in both long and short head sizes. While adsorption can occur equally well by either head form, DNA injection appears to occur only when the phage head is in the short form. Furthermore, the ratio of long to short heads is decreased by the presence of Ca or Mg ions at 10^{-3} to 10^{-4} M levels.

The most recent type of research regarding phage structure has been to survey a variety of bacteriophages for the purpose of compiling enough information to begin a rough morphological classification. From studies of this sort by Bradley and Kay (1960), three distinct morphological groups appeared.

The members of the most complex group have cross striated contractile tail sheaths and include the much studied and discussed T even coliphages and some typhoid phages. The tails of all the phages in this group contain hollow cores which are thought to provide

rigidity to the tail. These cores are terminated by base plates. However, the individuals in this group vary as to dimensions of these structures and as to type of head shape which may be one of three kinds: bipyramidal hexagonal prisms, octahedral, or icosahedral. In addition, the presence of a collar between the base of the head and the beginning of the tail sheath of T4 coliphage reported by Daems, van de Pal, and Cohen(1961) was confirmed by electron micrographs (Bradley, 1963). However, what was referred to as a jacket surrounding the tail sheath, is now thought to be a network of fibers which run from the apices of the base plate to those of the collar. Since both collar and network of fibers have been shown in T2 coliphage, Kozloff's idea of having the fibers wound around the tail core has been discarded.

The second group is a heterogenous conglomeration of all phage with tails longer than 15 mμ for which contractile tail sheaths have not been demonstrated. (As a matter of interest, an analogous situation occurs in the Fungi Imperfecti which groups all fungi which have not been observed to have a perfect reproductive state.) However, one member, T5 coliphage, has been shown to contain cross striations on the tail when negatively stained with uranyl acetate. A second type, represented by T1 coliphage, consists of phage with polyhedral heads joined to nonrigid tails by short narrow necks. The mode of adsorption and infection of members of these two types

has not been determined. A third type includes staphylococcus and pseudomonas phages with polyhedral heads of varying sizes and shapes connected to tails of varying lengths. A narrow neck may or may not be present. Instead of terminating the tail core with a tail bob or bulb-like structure as reported by Hotchin (1954), electron micrographs divulge a polyhedral appearance. However, regardless of the shape of this structure, all three researchers agree it probably contains the specific host adsorption sites by which it can perform a tail attachment to the host. The final type in this group, represented by a streptococcus phage, is characterized by a head having no sharp angles and a tail devoid of sheath or neck.

The third group originally consisted of phages with very short wedge shaped tails attached at the corner of their polyhedral heads as in T3 and T7 coliphages and in Brucella phages, but later Bradley (1963) expanded this group to include the tailless ϕR and ϕX174 each of which consists of an icosahedral protein shell with morphological subunits attached to each apex. These subunits may play a role in the infective process.

EXPERIMENTAL METHODS

Unfortunately a major part of the essential methodology was not mentioned in the journal articles. For this reason, the procedure mechanics used in this project had to be developed in some detail as the work progressed.

Procedures concerned with ascertaining the amount and kind of cultural effect of a particular physical or chemical agent upon the S. griseus host-phage system constitute the first part of this section and those methods concerned with discovering the visual effects of the specific agent occupy the final section.

Glassware Preparation

Viruses have been shown to be very sensitive entities, for they readily respond to the minute quantities of ionic substances deposited on glassware by the regular washing treatments sufficient for most bacterial studies. Thus, before proceeding with any work on this project, special washing methods that eliminated unwanted ions were developed. These efforts resulted in a biphasic washing procedure where an initial acid wash followed by 5 tap water rinses comprised the first phase of cleaning all glassware selected for this project. The second step consisted of boiling the glassware in Sud' n detergent for 30 minutes, hand scrubbing it, rinsing it 5 times with tap water

and then 5 times with distilled water, and finally either air or oven drying the clean glassware.

Except for pipettes and Petri dishes, all glassware was sterilized in the autoclave at 121° C and 15 pounds pressure for 15 minutes. For the usual reasons pipettes and Petri dishes were dry heat sterilized in an electric oven either overnight at 110° C for a period of 10 to 12 hours or during the day at 180° C for a period of 2 hours.

Media Ingredients and Preparation

For the sake of neatness and clarity, it was decided not to scatter the recipes of the media used in this project throughout the text of this section, but instead to compile them into this subsection. Any further reference to these media beyond this subsection will be made by using their name only.

The pH of the following media was adjusted to 6.8 to 7.0. Then it was dispensed into either 8 oz prescription bottles or 250 ml Ehrlenmeyer flasks in 75 to 150 ml quantities and autoclaved at 121°C and 15 pounds pressure for 15 to 20 minutes.

Glycerol Asparagine Agar

Glycerol	10 mls
Glucose	1 gm
Asparagine	1 gm

K_2HPO_4	1 gm
Difco agar	15 gms
Distilled water to	1000 mls

Glucose Nutrient Agar

Glucose	5 gms
Peptone	5 gms
Beef extract	3 gms
Yeast extract	0.1 gm
Difco agar	15 gms
Distilled water	1000 mls

Glucose Nutrient Broth

The Difco agar was omitted from the Glucose Nutrient Agar recipe.

Semi Solid (Glucose Nutrient) Agar

The amount of Difco agar used in the Glucose Nutrient Agar recipe was reduced by one half to 7.5 gms/l of media.

Peptone Broth

Peptone	2.5 gms
Distilled water	1000 mls

Plate Counts

Plate count determinations formed an integral part of most of the procedures discussed in later parts of this methodology section. Hence, to avoid unnecessary repetition when discussing each particular procedure, the general concepts involved with spore and phage determinations will only be considered in this subsection. Generally, to obtain plate counts a 1 ml aliquot from the sample of unknown titer was serially diluted (tenfold) in test tubes containing 9 mls of peptone broth diluent until a range of 30 to 300 individuals/ml was reached. Thus, the actual titer of a spore stock suspension estimated by 18% transmission at 620 mμ to have a 2 to 5 x 10^8 spores/ml was found by first serially diluting 1 ml of the stock to the 10^{-7} dilution and then doing triplicate plates of the 10^{-6} and 10^{-7} dilutions. This was accomplished by mixing 1 ml of the appropriate dilution with about 12 mls of glucose nutrient agar in each of the six Petri plates. A 2 day incubation at 30° C was necessary before the colonies were large enough for plate counts.

The actual number of pfu/ml; that is, plaque forming units/ml; of phage stocks was determined in a similar manner. However, because of the errors inherent in dilution work and because 0.1 ml quantities instead of 1 ml quantities were titered, the two highest dilutions which were expected to contain 30 to 300 pfu/ml and 300 to

3000 pfu/ml were used. In that way errors on the high side of the expected titer were caught by the samples from the higher of the two dilution tubes and errors on the low side were caught in the lower dilution tube. Thus, the actual titer of a phage stock which usually contained 1 to 8 x 10^{10} pfu/ml was determined by first serially diluting 1 ml of the stock to the 10^{-8} dilution tube. Then triplicate plates were done on both the 10^{-7} and 10^{-8} dilutions by adding 0.2 ml of a spore stock suspension and 0.1 ml of the proper phage dilution to 3 mls of semi solid agar and then pouring this mixture over previously solidified glucose nutrient agar. These six plates were counted after a 1 day incubation at 30° C.

Host Maintenance and Cultivation

Stock cultures of Streptomyces griseus strain 3475 (Waksman) were maintained by adding 2 mls of spore suspensions to thin layers of sterilized Midwest soil buffered with $CaCO_3$ in 125 ml Ehrlenmeyer flasks. Sterilization of the uninoculated buffered soil was accomplished by autoclaving the flasks at 121° C and 15 pounds pressure for 10 hours. After inoculation the flasks were incubated at 30° C for 1 day and then at room temperature for a minimum of 13 days. Next, 2 loopsful of soil were aseptically removed and streaked onto 50 mls of glycerol asparagine agar slanted on the flat side of 8 oz prescription bottles. These inoculated slants were incubated at 30° C for 1

to 2 days and then at room temperature for 3 to 8 days until a thick powdery confluent growth was observed. At that time spores were aseptically harvested by first adding 10 mls of sterile peptone broth to each bottle culture and then scraping the growth from the slant's surface into this fluid. The spores were separated aspectically from both the mycelia and the soil clumps by passing the suspension through a sterile Seitz filter containing sterile 30 layer gauze instead of the homologous filter pads. The collecting vessel consisted of a large test tube beneath the filter apparatus and within a 100 ml suction flask. Processing 3 slants in this manner every 2 weeks provided a quantity of spore stock suspension sufficient for the requirements of this project. After removal of the filter, insertion of a snug fitting, sterile cotton plug into the tube allowed lifting of the tube with the filtered suspension from the flask. The volume of this suspension was determined while it was transferred aseptically to an 8 oz prescription bottle. A small aliquot was removed and diluted with known amounts of peptone broth until 18% transmission at 620 mμ on a spectrophotometer was obtained. Depending upon the amount of soil that passed through the filter, 18% light transmission at 620 mμ will yield a suspension titering from 2 to 5 x 10^8 spores/ml. A proportional amount of peptone broth was added to the remaining spore suspension in the 8 oz prescription bottle. The actual titer of this suspension was obtained by following the method described earlier in

the Plate Counts subsection. Also the sterility of this fresh spore stock was established by adding 1 ml of the suspension to a 250 ml Ehrlenmeyer flask containing 75 mls of glucose nutrient broth. After shaking in a 30^{o} C water bath for 1 day, the flask was removed to a stationary surface which permitted the streptomyces mycelium suspension to separate and settle to the bottom of the flask. If the preparation was sterile the top broth layer was clear, but if there was bacterial contamination present, this layer remained cloudy. The spore stock suspensions were kept refrigerated except when in use. This retarded spore germination and thus maintained spore viability at a level constant with the one established by the initial spore titer. However, after 2 weeks, refrigeration did not postpone spore germination. Hence, a stock suspension whose sterility was established could be used only for a period of 2 weeks and any suspension older than 2 weeks was discarded.

Phage Maintenance and Cultivation

Stocks of S. griseus phage 514-3, originally isolated from soil by Gilmour and Buthala (1950), were propagated by inoculating 3 mls of semi solid agar with 0. 2 ml of a spore stock suspension and 0. 1 ml of a phage suspension that possessed a titer of 1×10^{6} pfu/ml. After mixing, this inoculum was poured over a layer of solidified glucose nutrient agar in a Petri plate. Nine of these plates were

incubated at 30° C until confluent lysis occurred which varied from a period of 12 to 18 hours. Stocks requiring a shorter or longer incubation period were discarded because they invariably yielded titers of less than 1×10^{10} pfu/ml. Five mls of peptone broth was added to each confluently lysed plate and the semi solid agar layer was scraped off into a sterile 250 ml Ehrlenmeyer flask. For the purpose of releasing phage from the fragments of the semi solid agar into the peptone liquid, this flask was placed in a 30° C water bath shaker for 10 minutes. Then the contents of the flask were divided into each of 2 sterile plastic centrifuge tubes and subjected to centrifugation for 10 minutes in a small angled Servall centrifuge set at 50 pstat units. This packed most of the semi solid fragments at the bottom of the tubes. Then the supernatant was passed through a sterile Seitz filter. The collecting vessel was the same described in the Host Cultivation subsection. This filtration step was also critical, for if it took longer than 15 minutes, not only was the stock's titer less than 1×10^{10} pfu/ml, but also the plaques were of pin point size. However, if the period was less than 15 minutes a usable stock with a titer of greater than 1×10^{10} pfu/ml and with large plaques resulted. After being transferred aseptically from the receiving tube into an 8 oz prescription bottle, 1 ml of the stock was checked for sterility by adding it to 75 mls of glucose nutrient broth in a 250 ml Ehrlenmeyer flask and shaking it at 30° C for 2 days; and 1 ml

was titered according to the method described in the Plate Counts procedure subsection. When not being used, the phage stocks, like the spore suspensions, were kept in the refrigeration. However, because results from daily titers of samples from a phage stock that was taken directly from the refrigerator varied from the original titer whereas results of samples taken from a stock that was allowed to warm up to room temperature did not fluctuate, aliquots were not taken from a phage stock until it was allowed to stand at room temperature for 15 minutes.

Inactivant Preparation

Because the inactivation solutions were expressed in terms of concentration and because some were composed of organic salts while others were composed of inorganic salts, no one specific preparatory procedure could be established. Incidentally, unless otherwise stated, the concentrations of these agents cited throughout the remainder of the text of this thesis refer to their initial concentration, that is, before the phage was added.

In cases where the molarity of the inactivating solutions was greater than one molar, as occurred with all osmotic shock solutions, the solute first was divided into four parts and then each part was dissolved in 20 mls of distilled water in a 25 ml volumetric test tube. After that, the volume was brought up to 25 mls. In cases where the

molarity was less than one molar, as in the zinc cyanide solution, the entire solute was dissolved in a small amount of distilled water in a 100 ml volumetric flask and then brought to volume. Then if necessary and regardless of the solution's molarity, the hydrogen ion concentration was adjusted until the desired pH was reached. From there each solution was transferred to an 8 oz prescription bottle. Since, with the exception of sodium acetate, these solutions were composed of inorganic salts, sterility was obtained by autoclaving each solution at 121° C and 15 pounds pressure for 15 minutes.

In cases involving organic solutes where sterility by autoclaving would decompose the solution and filtration through small pore filters might remove a certain amount of the dissociated ions and/or the nondissociated molecules, almost sterile conditions were maintained by dissolving the proper amount of solute in a small amount of sterile distilled water in a sterile 100 ml volumetric flask. After dissolving the solute, the volume was brought to 100 mls. If necessary the pH was adjusted by placing the solution in a sterile beaker and exposing it for as short a time as possible to a Beckman pH meter. Then the solution was transferred to a sterile 8 oz prescription bottle. Part of this preparation was used the same day in an inactivation experiment and the rest of it was refrigerated for 1 to 2 days after which it was used in the duplication run of the experiment.

In the case of the concentrated sucrose solutions, the measure, percent by weight was used. The solute was dissolved in the proper amount of sterile distilled water in sterile 250 ml Ehrlenmeyer flasks. Aliquots from these were used the same day for an experimental run and the remaining stock solutions were refrigerated directly in their flasks for 1 to 2 days after which they were used in the duplication run of the experiment.

Finally in the case of solutions of 3% hydrogen peroxide in 10% ethanol, the measure, percent by volume was used. Because of possible decomposition problems with hydrogen peroxide, stock ethanol solutions were made by aspectically adding the proper amount to sterile distilled water in a sterile 8 oz prescription bottle. Then an aliquot of this stock sufficient for one run was removed and mixed with the proper amount of concentrated (30%) hydrogen peroxide. For use in future duplication experiments, the rest of the ethanol stock was replaced in the refrigerator.

Inactivation Procedures

Studies by previous researchers on other phage systems indicated phage could be inactivated by exposure to certain chemical and physical agents for various time intervals. However, lack of space alloted for journal articles allowed only brief explanations of the methods these investigators employed to effect inhibition. Therefore, only the general methods on solute concentration and pH of the inactivant were followed. The specific details of the following inactivation procedures were devised to allow duplication by other researchers and still encompass the general methods outlined thus far in the journals.

General Inactivation Procedure

1) Phage stock from the refrigerator was allowed to stand at room temperature for 15 minutes.

2) If necessary an aliquot of this stock was diluted with peptone broth such that 8 mls of input stock estimated for a titer of 1×10^{10} pfu/ml was obtained. This was allowed to stand in a 30° C water bath for 15 minutes.

3) The actual titer of input phage was determined by titering 1 ml according to the method described for titering phage stocks in the Plate Counts procedure subsection.

4) One ml of input phage was added to each of 5 test tubes containing 9 mls of the proposed inactivant. Unless otherwise specified, these tubes were incubated in a 30° C water bath for 1/2, 1, 2, 3, and 4 hours, respectively. A control was set up by adding 1 ml of the input phage to 9 mls of peptone broth. This control also was incubated in the 30° C water bath for 4 hours. The purpose of the 4 hour control was to see if incubation under optimal conditions was enough to cause phage inactivation. Also it acted as a base line to determine percent inactivation due to the inactivant alone and not to the combined effects of the inactivant and the inactivation procedure.

5) At the end of each incubation period 1 ml was removed from the appropriate tube and titered according to the following table.

Incubation Time of Phage Specimen	Dilutions Sampled (0. 1 ml aliquots titered)
4 hr control (untreated)	10^{-5} and 10^{-6}
1/2 hr treated	10^{-5} and 10^{-6}
1 hr treated	10^{-5} and 10^{-6}
2 hr treated	10^{-4} and 10^{-5}
3 hr treated	10^{-4} and 10^{-5}
4 hr treated	10^{-4} and 10^{-5}

Triplicate 0. 1 ml samples from each dilution were plated as described in the Plate Counts procedure subsection for stock phage preparation.

Selection of the appropriate dilutions to be titered was made by considering both the dilution factors created by the mechanics involved in performing the procedure and the assumption that the incubation agent had an adverse effect on the phage. First, since Step (4) created a dilution factor of 10^{-1}, a new estimated titer of no more than 1×10^{9} pfu/ml was expected. Now according to the concepts discussed in the Plate Counts procedure subsection, the actual titer of a phage prep estimated to contain 1×10^{10} pfu/ml was determined by plating 0. 1 ml samples from 10^{-7} and 10^{-8} dilution tubes. Hence, it stands to reason that the actual titer of a phage preparation estimated to show a tenfold drop in titer; that is, 1×10^{9} pfu/ml; be

determined by plating 0.1 ml samples from the 10^{-6} and 10^{-7} dilution tubes. However, the 1×10^{9} pfu/ml titer was obtained only if no inactivation occurred and no phage was lost in performing the mechanics of the procedure. Therefore, to screen for results ranging from zero to greater than 99% inactivation, another tenfold decrease in the dilution tube number was made. This meant 0.1 samples from the 10^{-5} and 10^{-6} dilution tube were plated on the 4 hour control specimen and all other specimens incubated up to 2 hours in the proposed inactivant. Furthermore, because the amount of inactivation was expected to increase with increase of incubation time and because this could manifest itself by failure to obtain plaques from samples of the 10^{-5} and 10^{-6} dilutions, another tenfold decrease in the dilution tube number was made on all specimens incubated 2 hours or longer in the inactivant which meant 0.1 ml samples from the 10^{-4} and 10^{-5} dilution tubes were plated from these specimens.

If the occasional situation arose where, instead of having an adverse effect, the incubation agent either had no effect or a stimulatory effect upon the phage such that the number of pfu/ml of the treated phage was greater than the number of pfu/ml of the control specimen, then these dilutions would be too low and the plaques too numerous to count. Hence, when results from first run experiments exposed these occasionally occurring situations, modification of the usually chosen dilutions were made and used in the duplication runs. For example,

only one incubation agent, 0.2M Tris(hydroxymethyl)aminomethane at pH 9.5, tested by this procedure exposed the activation effect. Therefore, before duplication runs were made with this agent, the dilutions previously used were modified such that every treated specimen was titered using 0.1 ml samples from the 10^{-5} and 10^{-6} dilution.

6) To see if the inactivant affected only the phage or if spore germination was also affected a control spore count was done as described for spore stocks in the Plate Counts procedure subsection.

7) The spore titer of Step (6) was repeated, but this time an aliquot of the most concentrated dilution of the particular chemical agent that was titered with the treated phage, as indicated in Step (5) of this procedure, was added to each plate. In cases where the agent had an inactivation effect upon the phage, a 0.1 ml aliquot of a 10^{-4} dilution of the inactivant was added; but in cases where the agent had an activation effect upon the phage, a 0.1 ml sample of a 10^{-5} dilution was used.

Modified General Inactivation Procedure

Since many of the inactivants were made and used at specific pH values, an experiment was devised to see if pH was a factor contributing to the results obtained with the inactivants. The following pH's were chosen: 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 8.75, 9.00, 9.50, 10.00, 10.50, 11.00,

11.50, and 12.00. The experiment was divided into these 3 parts: pH 3.00 through 7.00; pH 7.00 through 10.00; and pH 10.50 through 12.00. The neutral pH of 7.00 was selected for the control.

1) Step (1) of the General Inactivation Procedure was followed.

2) If necessary an aliquot of phage stock was diluted with peptone broth such that the quantity of input stock figured for 1×10^{10} pfu/ml was sufficient for the particular pH run. This input quantity was allowed to stand in a 30° C water bath for 15 minutes.

3) Step (3) of the General Inactivation Procedure was followed.

4) One ml of input phage was added to 9 mls of peptone broth and adjusted to the appropriate pH. The control consisted of 1 ml of the input phage added to 9 mls of peptone broth. All tubes were incubated in a 30° C water bath for 4 hours. So that all samples were not coming off at the same time, each specific inactivation tube was inoculated with the phage input at 15 minute intervals.

5) At the end of the incubation time 1 ml was removed from the tube and titered according to the following table.

pH Range Used for Incubated Specimen	Dilutions Sampled (0.1 ml aliquots titered)
4.50 through 8.50	10^{-5} and 10^{-6}
3.00 through 4.00	10^{-4} and 10^{-5}
8.75 through 12.00	10^{-4} and 10^{-5}

Triplicate 0. 1 ml samples from each dilution were plated as described in the Plate Counts procedure subsection for stock phage preps.

Selection of 10^{-5} and 10^{-6} dilutions to be titered was based on the same argument discussed in Step (5) of the General Inactivation Procedure. However, a different reason caused selection of 10^{-4} and 10^{-5} dilutions, for instead of expecting increase of inactivation with increase in incubation time as encountered in the General Inactivation Procedure, increase of inactivation was expected as the extremes of the pH scale were approached. Thus 0. 1 ml samples of 10^{-4} and 10^{-5} dilutions were titered on all specimens at or below pH 4. 00 and at or above pH 8. 75.

Unfortunately, some of these dilutions were inadequate, for data from the first run experiment at the alkaline pH levels exposed another example of the occasional situation, similar to the Tris buffer effect discussed in Step (5) of the General Inactivation Procedure, where the incubation agent did not produce the expected inactivation effects. As a matter of fact, instead of causing phage inactivation, a pH ranging from 8. 75 through 10. 50 had no effect on the phage and a pH of 11. 00 had an activation effect on the phage. Therefore, before duplication runs were made, the dilutions previously used were corrected by sampling from a tenfold increase of the original dilutions according to this new table.

pH Range Used for Incubated Specimen	Dilutions Sampled (0.1 ml aliquots titered)
3.00 through 4.00	10^{-4} and 10^{-5}
11.50 through 12.00	10^{-4} and 10^{-5}
4.50 through 11.00	10^{-5} and 10^{-6}

6) Step (6) of the General Inactivation Procedure was followed.

7) The spore titer of Step (6) was repeated, but depending upon the pH used, either 0.1 ml of a 10^{-4} or of a 10^{-5} dilution of the appropriate pH broth was added to each plate. To possibly conserve the time and energy of the researcher only the extreme pH values of each run group were checked first. If this preliminary screening revealed the extreme pH values had an effect on spore germination, then the entire range of pH values were tested, but if there was no effect at the extremes the chances of an effect were considered nil. In this latter case the entire range was not checked.

8) If necessary Step (8) of the General Inactivation Procedure was followed.

Freeze Thaw Inactivation Procedure

1) Step (1) of the General Inactivation Procedure was followed.

2) If necessary an aliquot of phage stock was diluted with peptone broth such that 10 mls of input stock gave 1×10^{10} pfu/ml. This was placed in a 500 ml round bottom flask which then was allowed

to stand in a 30° C water bath for 15 minutes. The purpose of using this large flask was to supply more surface area for more rapid freezing and thawing.

3) Step (3) of the General Inactivation Procedure was followed.

4) The freeze part of the cycle was performed by rotating the flask in a bath of acetone containing dry ice. The thaw part of the cycle was initiated immediately after freezing occurred by removing the flask from the dry ice bath and rotating it in a 30° C water bath. Each part of the cycle was accomplished within one minute after its commencement.

5) After 1, 2, 5, and 10 freeze thaw cycles were completed, 1 ml samples were removed and titered in the following manner.

Number of Completed Cycles Undergone by Phage	Dilution Sampled (0.1 ml aliquots titered)
1	10^{-6} and 10^{-7}
2	10^{-6} and 10^{-7}
5	10^{-6} and 10^{-7}
10	10^{-5} and 10^{-6}

Triplicate 0.1 ml samples from each dilution were plated as described for stock phage preparations in the Plate Counts procedure subsection.

Since there was no dilution factor associated with any of the previous steps in this procedure, a titer no greater than the initial one of 1 x 10^{10} pfu/ml was expected. Because this titer was tenfold greater than the one discussed in Step (5) of General Inactivation Procedure, the dilutions selected were tenfold greater than those in that procedure. Therefore, according to the same reasons discussed in that procedure, 10^{-6} and 10^{-7} dilutions, no 10^{-5} and 10^{-6} dilutions, were titered after 1, 2, and 5 freeze thaw cycles.

6) No spore controls were tested because nothing was added to the phage samples subjected to the freeze thaw procedure.

Osmotic Shock Inactivation Procedure

1) Step (1) of the General Inactivation Procedure was followed.

2) If necessary an aliquot of this stock was diluted with peptone broth such that 8 mls of input stock figured for 2 x 10^{10} pfu/ml was obtained. This was allowed to stand in a 30^{o} C water bath for 15 minutes.

3) Step (3) of the General Inactivation Procedure was followed.

4) Step (4) of the General Inactivation Procedure was followed.

5) At the end of the incubation time the contents of the tube was added to 170 mls of ice bath chilled, sterile distilled water in a 500 ml Ehrlenmeyer flask and rapidly agitated 100 times. The tube was rinsed with 10 mls of cold sterile distilled water and the contents

added to the flask, which was then agitated 100 times more. This rinse was repeated a second time.

6) One ml from this flask was removed and titered according to the following table.

Incubation Time of Phage Specimen	Dilutions Sampled (0.1 ml aliquots titered)
4 hr control (untreated)	10^{-4} and 10^{-5}
1/2 hr treated	10^{-4} and 10^{-5}
1 hr treated	10^{-4} and 10^{-5}
2 hr treated	10^{-3} and 10^{-4}
3 hr treated	10^{-3} and 10^{-4}
4 hr treated	10^{-3} and 10^{-4}

Triplicate 0.1 ml samples from each dilution were plated as described in the Plate Counts procedure subsection for stock phage preparations.

At this point an estimated titer of 1×10^{8} pfu/ml was expected. This resulted from adjustment of the estimated input titer of 2×10^{10} pfu/ml by the final dilution factor of $(2 \times 10^{2})^{-1}$ which was obtained by combining the $(1 \times 10)^{-1}$ dilution factor created in Step (4) and the $(2 \times 10)^{-1}$ dilution factor created in Step (5) of this procedure. Since this new titer was tenfold lower than the one discussed previously in the General Inactivation Procedure, 0.1 ml samples from the 10^{-4}

and 10^{-5} dilutions were titered on the control specimen and all specimens incubated up to 2 hours and 0. 1 ml samples from the 10^{-3} and 10^{-4} dilutions were titered on all specimens incubated 2 hours or longer.

Again, these dilutions, like the ones selected for the other procedures, were designed to indicate cases of inactivation only. Thus, if the occasional case arose where first run experiments revealed results other than inactivation, as actually occurred with concentrated sucrose solutions, 3M Na $C_2H_3O_2$, or 3M $MgSO_4$, these dilutions would be too low to permit the counting of specific phage plaques. Now because these problems resembled the one discussed in Step (5) of the General Inactivation Procedure, a parallel solution was employed. Therefore, before duplication runs were made, the original dilutions were modified such that every treated specimen was titered using 0. 1 ml samples from the 10^{-4} and 10^{-5} dilutions.

7) Step (6) of the General Inactivation Procedure was followed.

8) The spore titer in the above step was repeated, but an aliquot of the most concentrated dilution of the particular chemical agent that was titered with the treated phage in Step (6) of this procedure was added to each plate. Because the dilution factor of a 10^{-1} order created by Step (6) of this procedure also diluted the incubating agent, the value of the most concentrated dilution of this agent titered with the treated phage was more dilute by a factor of

a 10^{-1} order than the apparent 0. 1 ml samples from the 10^{-3} dilution represented. Therefore, in cases where the agent had an inactivation effect upon the phage, a 0. 1 ml aliquot of a 10^{-4} dilution of the inactivant was added; but in cases where the agent had either an activation or no effect upon the phage, a 0. 1 ml aliquot of a 10^{-5} dilution was used.

9) If necessary Step (8) of the General Inactivation Procedure was followed.

Grid Preparation for Electron Microscopy

Punched 200 mesh stainless steel grids from the Ernest Fullman Company were coated with a parlodion film. The concentration of parlodion dissolved in amyl acetate was sufficient to produce a film thin enough to give off a gray resolution color by reflected light when the film was cast onto a water surface. Since amyl acetate is very volatile, it was replenished to the stock parlodion solution each time a new set of films was cast. Otherwise the films became too thick for use. The film was produced by first dipping a glass slide wiped clean of dried Bon Ami into the parlodion solution and then air drying for at least 30 minutes.

During this drying period the grids to be coated were washed clean of any oxidized material by placing them in a Petri dish containing 20% formic acid. After 20 minutes the formic acid was poured off

these grids which were then maintained in acetone until they were to be coated. The acetone also served to remove any grease from the grid's surface.

Next the air dried film initially was loosened by scraping the edges of the slide with forceps and then holding the slide with a snap clothespin over steam from a beaker of boiling water containing glass beads. This step was continued until a condensed vapor covered and remained for a few seconds on the entire film area on the slide.

While the slide was cooling a glass bowl was filled with distilled water until it was brimming over. The water's surface was stripped to remove the lint and other debris that was present by drawing the cut edge of heavy bond paper across the surface as well as partly below the surface. To get the entire surface with one sweep, the length of the paper must be several inches wider than the diameter of the bowl.

Final loosening of the film from the slide was accomplished by exhaling through the mouth across the film until condensed vapor remained over the film's surface. Before the condensation evaporated the film was floated off the slide onto the surface of the stripped water by laying the flat side of the slide on the water's surface and passing the distal end of it downward into the water and rotating it through an angle of 180^{o}. After this slide air dried the same casting process with the film layer on the reverse side of the slide was

repeated.

After a usable film was cast about a dozen grids were removed by forceps from the acetone. To avoid any degradatory reaction between the parlodion film and the organic solvent, acetone, the excess unevaporated acetone was drained off these grids by placing them on filter paper. Then each of these grids was dropped such that the smooth side was against a gray resolution area of the film's surface that was free of lint and wrinkles. When the usable areas of the film were covered with grids the film was picked up by placing the flat side of a clean glass slide parallel and slightly above the film, touching the distal end of this slide to the distal end of the film, and then passing this end downward into the water and rotating it through an angle of 180^{o}. To prevent the film layer from folding back on itself the slide was drained of excess water and placed in a dessicating jar containing $CaCl_2$. After 24 hours these slides were removed and stored in covered Petri dishes. Only the grids where the film layer was not folded back on itself were used. Since the parlodion films weakened with time, only enough grids to last about 2 weeks were coated. Then another set was made as previously described. Films on inadequately coated grids were removed by dissolving them in acetone and then after following the washing steps the grids could be recoated.

Specimen Preparation for Electron Microscopy

The following procedures constituted the methods best suited for the preparation and observation of the coarse and fine structure of S. griseus phage with a RCA EMU 2D electron microscope.

Incidentally, as already cited in the Inactivant Preparation subsection, the concentration of all the solutions used for negative staining refer to their initial concentration.

Negative Stain Procedure for Centrifuged Phage

1) Two 10 ml aliquots of stock phage titering in the 5×10^9 to 5×10^{10} pfu/ml range were centrifuged in sterile heavy walled Servall tubes at 10,000 x g for 2 hours. Refrigeration was unnecessary.

This centrifugation step was included because it was thought that stocks of this phage were not concentrated enough for electron microscope work.

2) The soft pellets were removed to separate tubes with a sterile 1 ml pipette.

3) Each pellet was resuspended by gently mixing it for 15 minutes with 0.5 ml of a 0.4% sucrose solution of pH 7.00.

Apparently other investigators using the classical negative stain procedure of Brenner and Horne (1959) did not encounter the problem of getting even distribution of the phage particles over the entire grid's

surface. This problem continued to be a source of aggravation until it was found that resuspending the pellet in 0.4% sucrose decreased both the hydrophobic nature of the parlodion film on the grid's surface and the tendency of the resuspended phage to remain in small aggregated groups.

4) To one resuspended preparation 0.5 ml of 2% PTA; that is, phosphotungstic acid; pH 7.40 solution was added and to the other 0.5 ml of 2% PTA, 2% UA; that is, uranyl acetate; 3% Na_2CO_3 pH 8.40 solution was added. After the appropriate addition was made, the resulting solutions were again gently mixed for 15 minutes.

Unlike Brenner and Horne (1959), very little negative staining success was obtained by using equal volumes of a 1% $NH_4C_2H_3O_2$ pH 7.20 solution and a 2% PTA pH 7.40 solution. Since, as will be shown in the Experimental Results section, the ammonium ion and its derivatives had a deleterious effect on the cultural features of this phage, the 1% $NH_4C_2H_3O_2$ pH 7.20 solution was eliminated from these procedures. Therefore, only a volume of the 2% PTA pH 7.40 solution that was equal to the volume of the 0.4% sucrose resuspending solution used in Step (3) was added to the preparation.

Even though all these modifications supplied better negative contrast, inadequate contrast of the phage head still resulted. Therefore, another staining solution was devised. It contained 2% PTA to provide a good negative stain of the phage tail; 2% UA to provide a

heavier ion for better negative contrast to the phage head; and 3% Na_2CO_3 to remove the uranyl phosphate precipitate and to give an alkaline pH of 8.40 to the solution. As a matter of fact, pH was the prime reason this more complex solution was chosen, for a solution containing only 2% UA cannot be alkalinized above pH 3.50 without precipitating most of the uranyl ions.

5) To determine whether any visible alteration or any better negative staining parallelled the slight activation results obtained at pH 11.00, the pH of another set of specimens similarly treated through the previous steps of the procedure was raised to the 10.50 to 11.00 range by adding 0.01 ml of 0.1N NaOH to each ml of specimen immediately after the staining solution was added. After gentle mixing for 15 minutes, the remainder of the appropriate procedure was followed.

6) Using a 27 gauge needle and a 1 ml syringe a drop of one preparation was added to each of 4 parlodion coated grids on a piece of filter paper in an open Petri dish. After a 10 minute dessication the droplets were broken to allow the excess stain to drain off. If this was not done, the stain and the phage particles were too dense to permit sufficient electron beam penetration for viewing or for photography. Overnight dessication was then continued. Using another syringe and needle this same step was repeated on the other preparation.

7) The next morning the grids were removed from the dessicator and examined. Although immediate examination was the most desirable, circumstances such as no free time was available that day on the microscope or the microscope was shut down for repairs sometimes made that impossible. In that case, the grids were removed from the dessicator, covered, and stored in a desk drawer for no longer than one week. If the grids were kept beyond the week limit, the film became too unstable and thus broke very easily when the electron beam was turned on.

Negative Stain Procedure for Noncentrifuged Phage

1) A phage stock possessing a titer of at least 1×10^{10} pfu/ml was held at room temperature for 15 minutes before two 1 ml samples were removed and placed in separate tubes. Then 1 ml of a 2% PTA pH 7.40 solution was added to one specimen and 1 ml of a 2% PTA, 2% UA, 3% Na_2CO_3 pH 8.40 solution was added to the second prep. Each resulting solution was mixed gently for 15 minutes.

Since it was found that the concentration process involved in the centrifugation and resuspension steps of the previous Negative Stain Procedure represented only a ninefold increase of phage titer, but gave an overabundance of particles/field of vision and since it was suspected that centrifugation even at 10,000 x g caused some sort of alteration in the phage head protein, direct staining of

noncentrifuged stock phage was attempted. Thus because the phage did not need to be resuspended, the sucrose solution was not used.

2) If necessary Step (5), the Optional Step, of the previous Negative Stain Procedure was followed.

3) Steps (6) and (7) of the previous Negative Stain Procedure were followed.

Electron Micrography

Even though the following methodology used for the processing of electron micrographs only represents the standard photography operating procedure, the details of time and the kind of agents and equipment included in the procedures provide a basis for either duplication by later investigators or comparisons by other researchers who use modifications of the standard procedure.

Negative Processing Procedure

1) The electron micrograph negatives were developed for 3 minutes in Kodak Dektol.

2) This developing process was halted by dipping the film strip 3 times in a 3% acetic acid stop bath.

3) Each film strip negative was fixed for at least 10 minutes in Kodak hypo solution.

4) Finally the negative was washed with flowing water for a period of time ranging from a minimum of 4 hours to a maximum of 24 hours. Then about 1 more hour was required to permit air drying of the washed negative.

Actually the exact amount of time that the hegative was allowed to wash largely depended upon how anxious the researcher was to examine the prints for possible answers to the kind of substructure possessed by this phage. This in turn depended upon such factors as the contrast quality of the stained prep, the stability of the parlodion film to the electron beam, the behavior of the electron microscope, and the quality of the negative. In other words, if the prep was stained sufficiently to give a good contrast, the microscope was functioning properly, and the negatives were properly exposed and clearly focused, then the washing time was held to the minimum, for the researcher was very anxious to enlarge, make, and examine the prints of these negatives.

Print Processing Procedure

1) The negatives were magnified using a Beseler enlarger and exposed to double weight Kodak Kodabromide F4 contrast paper. The resulting prints were developed for 1 to 3 minutes in Kodak Dektol.

2) This developing process was halted by immersing the print in a 3% acetic acid stop bath for 15 seconds.

3) Step (3) of the Negative Processing Procedure was followed.

4) These prints were washed overnight in a flowing water bath.

5) Finally, either to obtain a glossy finish, these prints were placed face down on nickel plated slabs and air dried for 24 hours or to obtain a semi glossy finish, these prints were placed face up on a nickel plated drum and electronically dried for 45 minutes.

EXPERIMENTAL RESULTS

Expression of Data

Before analyses and interpretations of this section's data can be made, a few of the notations used should be explained. First, the encircled plaque or spore counts were not figured in the average counts because, as discussed earlier in the Plate Counts subsection, they were not considered to be in close numerical agreement with the other members of the triplicate set. In one instance, the evaluation of the plate counts of the untreated and treated host shown in Table IX, this rule appears to be violated. Actually, in this case, discarding the count not in close agreement would be wasted effort, for it would accomplish nothing. This is because plate counts for both the untreated and the treated host resulted in two low counts and one high count of similar numerical value, which meant that a similar inactivation value for the treated host would result regardless of whether the high counts were figured in the average count or whether they were discarded before the average count was computed.

Secondly, the methodology involved in Step (4) of both the General Inactivation Procedure and the Modified General Procedure or in Steps (4) and (5) of the Osmotic Shock Inactivation Procedure created certain dilution factors. Therefore, input titer (A) denotes the actual input titer and titer (B) represents the newly adjusted estimated titer

obtained by multiplying the actual input titer by the dilution factor encountered in the specific procedure. Hence, the actual input titer (A) of the General Inactivation Procedure represented in Tables I and II and the Modified General Inactivation Procedure results represented in Tables III, IV, and V was adjusted by the dilution factor of $(1 \times 10)^{-1}$ and the actual input titer (A) of the Osmotic Shock Inactivation Procedure results represented in Tables VIII, IX, X, and XI was corrected by the total dilution factor of $(2 \times 10^{2})^{-1}$.

Thirdly, the percent inactivation values are the same as saying percent loss of phage or host. Therefore, the percent inactivation value of the control phage specimen, which was incubated 4 hours in peptone broth, was obtained by comparing the control titer with the adjusted input titer by substituting in this general equation:

$$\frac{\text{adjusted input titer - control titer}}{\text{adjusted input titer}} \times 100 = \text{inactivation percent.}$$

More specifically, in Table I, $\frac{1.19 - 1.04}{1.19} \times 100 = 12.6\%$.
These values established the amount of phage loss due mainly to the mechanics involved in the procedure.

Next, the phage loss due to the inactivating agent alone was determined by comparing the treated phage titers with the 4 hour control titer by substituting in this similar general equation:

$$\frac{\text{control titer - treated specimen titer}}{\text{control titer}} \times 100 = \text{inactivation percent.}$$

Table I. Effects of general inactivation attempts using 6M urea upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs	111, 120, 127	119	(A) 1.19×10^{10} (B) 1.19×10^{9}	
Control 4 hrs	102, 105, 107	104	1.04×10^{9}	12.6
Treated Phage				
1/2 hr	No plaques	< 1.00	$< 1.00 \times 10^{6}$	>99.9
1 hr	No plaques	< 1.00	$< 1.00 \times 10^{6}$	>99.9
2 hrs	No plaques	< 1.00	$< 1.00 \times 10^{5}$	>99.9
3 hrs	No plaques	< 1.00	$< 1.00 \times 10^{5}$	>99.9
4 hrs	No plaques	< 1.00	$< 1.00 \times 10^{5}$	>99.9
Untreated Host* (Spore control)	144, 150, 163	152	1.52×10^{8}	
Treated Host* (Spores with 0.1 ml of a 10^{-4} dilution of 6M urea)	136, 148, 155	146	1.46×10^{8}	4.0

* All host data based on number of germinating spores/ml.

For example, the inactivation value of the spores treated with the proper dilution of 6M urea was obtained as follows:

$$\frac{1.52 - 1.46}{1.52} \times 100 = 4.0\%.$$

Fifthly, as can be seen from these equations, there is a positive inactivation value only as long as the treated titer is less than the control titer. On the other hand, when the treated titer is greater than the control titer, the inactivation value acquires a negative sign. Some examples of this occurred as shown in Table II when phage was subjected to 0.2M Tris buffer, pH 9.5. For instance, the specimen subjected for 1/2 hour titered 1.88×10^9, whereas the control titer was only 1.02×10^9. Substituting in the preceding formula gives $\frac{1.02 - 1.88}{1.02} \times 100 = \frac{-0.86}{1.02} \times 100 = -84.4\%$. Therefore, a negative inactivation value results when the treated titer is greater than the 4 hour control titer. This implies the test agent activates the specimen rather than inactivates or has no effect on it. Thus another way of interpreting negative inactivation data is that a -50% is equivalent to a 1 1/2 fold increase in phage titer; a -100% is equivalent to a 2 fold increase; and a -150% is equivalent to a 2 1/2 fold increase.

Finally, according to a previous statistical survey on dilution techniques, inactivation values ranging between 30% and -30% were not considered significant with regard to the effectiveness of the recovery procedure or the test agent because these values were most

likely due to errors involved in the dilution mechanics.

Effects of Various Chemical Agents

Cursory perusal of the results listed in this section's 11 tables revealed two general observations. First, in most cases, as indicated by the 4 hour control inactivation values, the phage loss due to the mechanics inherently present in these inactivation procedures was kept within the insignificant percent range. Secondly, in no case did the chemical test agents used affect the host, for the inactivation values of the treated spores also remained within the insignificant range. Therefore, only the phage must be affected by these selected physical or chemical treatments.

Closer investigation of the individual tables revealed that any one of three effects occurred; the selected agent definitely inactivated the phage; the selected agent had no effect on the phage; or the selected agent activated phage stocks. Results of high inactivation were obtained, as exemplified in Table I, with concentrated 6M urea solutions. Further results indicated this compound was so effective that even after only 5 minutes incubation greater than 99% of the phage had lost its power to form plaques. The high concentration of this compound may be responsible for this effect. However, identical results occurred with even a lesser concentrated solution of the immediate precursor of urea in the urea cycle, namely 0.2M arginine at

pH 9.5. Although a 15 minute incubation was required, a still lesser concentration of another agent, 0.05M hydroxylamine at pH 6.7, was also found to render greater than 99% of a phage stock inactive.

The process an agent uses to cause any one of the three possible effects is difficult to determine because when considered individually each agent possesses several chemical properties any one of which may be the factor responsible for the particular effect. However, by considering the agents which produce the same kind of effect on the phage as a group, the number of factors possibly responsible usually can be reduced to those mutually possessed by every member of the group. Of course, just because a chemical property is common to each member of the group does not necessarily mean it is always the factor responsible for causing the effect. One property common to urea, arginine, and hydroxylamine is the possession of nitrogen in the form of at least one amide group and/or one of its derivatives. Now since the nitrogen component in these groups bears a negative oxidation value, it appears that S. griseus phage is highly inactivated upon short contact with very small amounts of certain reducing agents.

Only 10 minute incubations with 3% hydrogen peroxide in 10% ethanol also caused greater than 99% inactivation of phage stocks. Although usually an oxidizing agent hydrogen peroxide solutions under certain conditions, such as an acid environment, may also act as a

reducing agent. In this ethanol-hydrogen peroxide solution an acid environment probably is evolved, for its components are mixed together before the phage is added. Thus part of the hydrogen peroxide is given a chance to oxidize part of the ethanol and release H ions in the process. Then by the time the phage is added, the acidity is at the level which permits the remainder of the hydrogen peroxide to behave as a reducing agent.

Another radical capable of breaking certain chemical bonds by reduction is cyanide. A 0.03M KCN and 0.01M $ZnSO_4$ solution yielding soluble Zn CN complexes as pH 11.25 was capable of giving a high degree of inactivation as exemplified by the data in Table I. The question of whether the high alkalinity, which was obtained by the addition of NH_4OH, contributed to the inactivation will be postponed until the results of the pH experiments are discussed.

Unlike the chemical agents previously mentioned, 0.2M Tris buffer at pH 9.5 failed to inactivate phage stocks. On the contrary, phage activation, as indicated in Table II by the 1 1/2 to 2 fold increase of the treated phage titers over the control titer, was caused by exposure to this chemical compound. In view of the fact that this agent possesses an amide group, these results are unexpected. However, it may be that the spatial configuration of this molecule prohibits close contact of the amide group with the phage particle and/or the three hydroxymethyl groups present may form

Table II. Effects of general inactivation attempts using 0.2M Tris (hydroxymethyl)aminomethane buffer upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs	136, 142, 150	143	(A) 1.43×10^{10} (B) 1.43×10^{9}	
Control 4 hrs	92, 111, ---	102	1.02×10^{9}	28.6
Treated Phage				
1/2 hr	181, 186, 197	188	1.88×10^{9}	-84.4
1 hr	145, 178, 195	173	1.73×10^{9}	-69.7
2 hrs	180, 191, 192	188	1.88×10^{9}	-84.4
3 hrs	135, 167, 167	167	1.67×10^{9}	-63.8
4 hrs	132, 159, 162	161	1.61×10^{9}	-59.0
Untreated Host* (Spore control)	80, 86, 91	85.6	8.56×10^{7}	
Treated Host* (Spores with 0.1 ml of a 10^{-5} dilution of 0.2M Tris, pH 9.5)	60, 77, 81	79.0	7.90×10^{7}	7.7

* All host data based on number of germinating spores/ml.

van der Waals interactions with the free hydroxymethyl groups present in the polysaccharide components of membranes.

Hydrogen Ion Effects

A review of most of these treatments indicated another condition, hydrogen ion concentration, was involved with the treatments. To find whether the pH alone would affect the phage, a series of experiments were run. The results, listed in Tables III, IV, V, and VI, showed pH's ranging from 6.00 through 10.50 had no significant affect on phage titers. However, increase in acidity from pH 6.00 to 3.00 caused steady increase in phage inactivation such that greater than 90% of phage activity was lost at pH 4.50 or lower. In a similar manner phage infectivity was decreased greater than 99% at alkaline pH's of 11.50 or higher.

The most unusual result obtained from these pH experiments was the small, but constant activation of phage stocks incubated at pH 11.00 for 4 hours. Whether pH 11.00 is the optimum pH for this activation or whether it occurs at some other pH greater than 10.50 but less than 11.50 is unknown at this time. Another question raised by this study is why is this phage much more stable to increasing alkaline pH's than to the corresponding increasing acid pH's. Also what enables this phage to be unaffected at alkaline pH's through a pH of 10.50, to be slightly activated at pH 11.00, and then immediately

Table III. Effects of increasing acidity upon S. griseus phage.

Specimen Type, Incubation Time, and pH of Peptone Broth	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs pH 7.00	36, 40, 41	39.0	(A) 3.90×10^9 (B) 3.90×10^8	
Control 4 hrs pH 7.00	33, 34, 38	35.0	3.50×10^8	10.2
Treated Phage (4 hrs)				
pH 6.50	268, 275, 283	275	2.75×10^8	21.5
pH 6.00	284, 286, 295	288	2.88×10^8	17.7
pH 5.50	183, 191, 195	190	1.90×10^8	45.7
pH 5.00	88, 97, 111	98.0	9.80×10^7	72.2
pH 4.50	8, 8, 13	9.66	9.66×10^6	97.2
pH 4.00	No plaques	< 1.00	$< 1.00 \times 10^5$	>99.9
pH 3.50	No plaques	< 1.00	$< 1.00 \times 10^5$	>99.9
pH 3.00	No plaques	< 1.00	$< 1.00 \times 10^5$	>99.9

Table IV. Effects of increasing alkalinity upon S. griseus phage.

Specimen Type, Incubation Time, and pH of Peptone Broth	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% In-activation
Untreated Phage				
Input 0 hrs pH 7.00	122, 130, 182	126	(A) 1.26×10^{10} (B) 1.26×10^{9}	
Control 4 hrs pH 7.00	72, 91, 97	86.6	8.66×10^{8}	31.2
Treated Phage (4 hrs)				
pH 7.50	86, 92, 96	91.3	9.13×10^{8}	-5.4
pH 8.00	69, 83, 86	84.5	8.45×10^{8}	2.4
pH 8.50	87, 93, 122	90.0	9.00×10^{8}	-3.9
pH 8.75	832, 888, ---	860	8.60×10^{8}	0.7
pH 9.00	694, 750, 1012	722	7.22×10^{8}	16.6
pH 9.50	698, 748, 792	746	7.46×10^{8}	13.9
pH 10.00	596, 748, 750	749	7.49×10^{8}	13.6

Table V. Effect of further increases in alkalinity upon S. griseus phage.

Specimen Type, Incubation Time, and pH of Peptone Broth	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs pH 7.00	67, 83, 85	84.0	(A) 8.40×10^9 (B) 8.40×10^8	
Control 4 hrs pH 7.00	93, 104, 115	104	1.04×10^9	-23.8
Treated Phage (4 hrs)				
pH 10.50	100, 105, 126	102	1.02×10^9	1.9
pH 11.00	135, 137, 148	136	1.36×10^9	-30.7
pH 11.50	4, 2, 2	2.66	2.66×10^6	99.7
pH 12.00	No plaques	< 1.00	$< 1.00 \times 10^6$	> 99.9

Table VI. Effects of various hydrogen ion concentrations upon S. griseus spore germination.

Specimen Type	Spore Count	Average # of spores/ml	Titer (spores/ml)	% Inactivation
Untreated Host (Spore control in pH 7.00 peptone broth)	29, 36, 40	35.0	3.50×10^8	
Treated Hosts				
Spores with 0.1 ml of a 10^{-4} dilution pH 3.00 peptone broth	33, 34, 36	34.3	3.43×10^8	2.0
Spores with 0.1 ml of a 10^{-5} dilution pH 10.00 peptone broth	32, 34, 40	35.3	3.53×10^8	-0.9
Spores with 0.1 ml of a 10^{-4} dilution pH 12.00 peptone broth	34, 35, 35	34.6	3.46×10^8	1.1

be totally inactivated at pH 11.50.

From these results it looks as if the high alkalinity necessary to keep the ZnCN complexes in solution was either substantially or entirely responsible for the phage inactivation obtained by treatment with a pH 11.25 solution containing 0.03 moles of KCN and 0.01 moles of $ZnSO_4$. Although inactivation data for values between pH 11.00 and 11.50 were not obtained experimentally, extrapolation from the graph plotted from the pH data in Table V indicated that a maximum of 35% of a phage stock would be inactivated by a pH of 11.25. In fact even a pH of 11.30 would be expected to cause no more than 50% inactivation. On the basis of this information at least 50% and probably more of the phage inactivation was due to the ZnCN complexes present in the highly alkaline solution.

Unfortunately it was impossible to obtain direct supporting evidence for further verification of this conclusion because any pH value within the range of 6.00 to 10.50 was not sufficient to keep the ZnCN complexes soluble. As a matter of fact, solubility of as little as a 10^{-2} dilution of the original concentration of complexes required a minimum pH of 10.85. However, this fact did not present an insurmountable problem, for by subjecting phage to this diluted solution it was possible to test the validity of the conclusion in an indirect manner. This indirect proof consisted of first calculating the theoretical inactivation effect and then comparing its value with the

experimental inactivation effect caused on phage by the diluted solution. Now if the conclusion were true, then at least 50% inactivation was expected to be contributed by the effect of the ZnCN complexes. Also by again extrapolating from the graph of the pH data it was observed that a pH of 10.85 was expected to cause at least a -20% inactivation. Thus, by combining these values, a theoretical minimum inactivation effect of 30% was expected. That a slightly smaller experimental inactivation effect of 23.7% occurred was probably due to the fact that the chances of the zinc complexes encountering and attacking a phage particle were reduced by using the 10^{-2} dilution of the original concentration of the agent.

Another piece of indirect evidence lending its support to the conclusion that ZnCN complexes indeed were able to inactivate this phage was supplied by the facts that 0.05M hydroxylamine destroys greater than 99% of the infective power of S. griseus phage and that hydroxylamine attacks the same kind of chemical bond as the ZnCN complexes.

Freeze Thaw Effects

The purely physical stresses involved with rapid, alternate cycles of freezing and thawing also inactivated this phage. In fact, even though more than one freeze thaw cycle was necessary to cause a significant inactivation value, the inactivation value increased with every increase in the number of cycles. Within certain limits the

volume of the phage solution had no influence on the amount of inactivation, for whether 10 mls of phage was subjected to 10 cycles before an aliquot was tested or whether 1 ml aliquots were extracted after 1, 2, and 5 cycles from the initial 10 mls, the inactivation value after the 10th cycle was between 84 and 90%.

Osmotic Shock Effects

Phage susceptibility to the combined effect of chemical agents and physical stress was tested by the osmotic shock trials. Results covered the entire range from complete inactivation through activation and depended upon the kind of chemical employed. For example, it was observed in Table VIII that even more activating than the Tris buffer was the osmotic shock treatment using 3M $MgSO_4$, for these treated phage stocks showed a 2 to 2 1/2 fold increase in titer over the control stocks. Duplication of this experiment indicated no specific time within the 4 hour test range diminished or accelerated the activation effect.

Completely opposite results were obtained using high concentrations of other divalent cations. For instance, almost complete inactivation was obtained after 1 hour incubation in any one of 3M $CaCl_2$, 3M $CoCl_2$, or 3M $MnSO_4$ (Table I). However, since the pH of aqueous solutions of $CoCl_2$ or $MnSO_4$ cannot be raised above 3.50 without precipitation of the solute, the effect may be due to a

Table VII. Effects of inactivation attempts by an increasing number of freeze thaw cycles upon S. griseus phage.

Cycle #	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
0 (Control)	75, 87, 101	87.6	8.76×10^8	
1	41, 63, 65	64.0	6.40×10^8	27.0
2	46, 48, 53	49.0	4.90×10^8	44.2
5	261, 279, 309	283	2.83×10^8	67.8
10	91, 96, 116	93.5	9.35×10^7	89.3

Table VIII. Effects of osmotic shock inactivation attempts using 3M magnesium sulfate upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs	176, 207, 212	210	(A) 2.10×10^{10} (B) 1.05×10^{8}	
Control 4 hrs	57, 62, 68	62.3	6.23×10^{7}	37.1
Treated Phage				
1/2 hr	109, 129, 139	126	1.26×10^{8}	-102.2
1 hr	129, 149, 162	147	1.47×10^{8}	-135.9
2 hrs	126, 132, 135	131	1.31×10^{8}	-110.2
3 hrs	149, 151, 185	150	1.50×10^{8}	-140.7
4 hrs	122, 133, 135	130	1.30×10^{8}	-108.8
Untreated Host* (Spore control)	67, 80, 92	79.6	7.96×10^{7}	
Treated Host* (Spores with 0.1 ml of a 10^{-4} dilution of 3M $MgSO_4$)	75, 81, 95	83.6	8.36×10^{7}	-5.1

* All host data based on number of germinating spores/ml.

combined effect of acid pH and osmotic shock or to the effect of the acid pH alone. In the case of $CaCl_2$ of pH 8.20, the effect was due to osmotic shock, for the pH was in the range ineffective to phage infectivity.

Somewhat less drastic inactivation data was obtained using monovalent cations. Repeated experiments employing either 3M Na_2SO_4 or 4M NaCl exemplified in Table IX showed that whether the incubation time was as short as 1/2 hour or as long as 4 hours, the amount of phage infectivity lost by the treated stock ranged between 70 and 80%. On the other hand, the inactivation data in Table X indicated that osmotic shock with 3M $(NH_4)_2SO_4$ was time dependent. In other words, over the 4 hour time period inactivation increased as the incubation time increased. In addition osmotic shock using the monovalent cation, NH_4, constantly produced higher inactivation levels than the Na ion. The facts that NH_4OH and either an NH_4 ion or an NH_3 molecule form an equilibrium reaction which strongly favors the NH_4OH component and that the inactivation effect is time dependent, suggest that the 10 to 20% increase may be attributed to an effect caused by the interaction of a phage component with an NH_4 ion or one of its derivatives.

Finally, as exemplified by the data in Table XI, no cultural effect was obtained with attempts at osmotic shock using either a 3M concentration of the salt of the organic acid, acetic acid, or 20, 40,

Table IX. Effects of osmotic shock inactivation attempts using 3M sodium sulfate upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% In-activation
Untreated Phage				
Input 0 hrs	247, 267, 284	266	(A) 2.66×10^{10} (B) 1.33×10^{8}	
Control 4 hrs	109, 120, 130	120	1.20×10^{8}	10.5
Treated Phage				
1/2 hr	34, 34, 35	34.3	3.43×10^{7}	71.8
1 hr	33, 38, 39	36.6	3.66×10^{7}	69.9
2 hrs	226, 243, 255	241	2.41×10^{7}	80.2
3 hrs	286, 351, 376	337	3.37×10^{7}	71.5
4 hrs	227, 239, 239	235	2.35×10^{7}	80.8
Untreated Host* (Spore control)	119, 119, 130	122	1.22×10^{8}	
Treated Host* (Spores with 0.1 ml of a 10^{-4} dilution of 3M Na_2SO_4)	111, 112, 134	119	1.19×10^{8}	2.5

* All host data based on number of germinating spores/ml.

Table X. Effects of osmotic shock inactivation attempts using 3M ammonium sulfate upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% In-activation
Untreated Phage				
Input 0 hrs	203, 218, 224	215	(A) 2.15×10^{10} (B) 1.08×10^{8}	
Control 4 hrs	101, 102, 113	105	1.05×10^{8}	2.8
Treated Phage				
1/2 hr	30, 30, 34	31.3	3.13×10^{6}	97.0
1 hr	17, 18, 19	18.0	1.80×10^{6}	98.2
2 hrs	100, 134, 146	138	1.38×10^{6}	98.7
3 hrs	115, 127, 137	127	1.27×10^{6}	98.9
4 hrs	66, 74, 118	70.0	7.00×10^{5}	99.4
Untreated Host* (Spore control)	40, 44, 57	47.0	4.70×10^{7}	
Treated Host* (Spores with 0.1 ml of a 10^{-4} dilution of 3M $(NH_4)_2SO_4$)	43, 45, 53	47.0	4.70×10^{7}	00.0

* All host data based on number of germinating spores/ml.

Table XI. Effects of osmotic shock inactivation attempts using 3M sodium acetate upon the S. griseus host-phage system.

Specimen Type and/or Incubation Time	Plaque Count	Average # of pfu/ml	Titer (pfu/ml)	% Inactivation
Untreated Phage				
Input 0 hrs	33, 41, 47	40.3	(A) 4.03×10^{10} (B) 2.02×10^{8}	
Control 4 hrs	118, 126, 128	124	1.24×10^{8}	37.5
Treated Phage				
1/2 hr	116, 127, 148	130	1.30×10^{8}	-4.9
1 hr	122, 122, 140	122	1.22×10^{8}	1.6
2 hrs	808, 884, 980	891	8.91×10^{7}	28.7
3 hrs	896, 964, 984	974	9.74×10^{7}	21.7
4 hrs	986, 1248, 1340	1294	1.29×10^{8}	-4.0
Untreated Host* (Spore control)	117, 142, 158	150	1.50×10^{8}	
Treated Host* (Spores with 0.1 ml of a 10^{-4} dilution of 3M $NaC_2H_3O_2$)	155, 159, 168	161	1.61×10^{8}	-7.3

* All host data based on number germinating spores/ml.

and 60% concentrations of the nonreducing sugar, sucrose. Once again the percent loss of the 4 hour control is higher than should be expected, but correction of all the results by reducing them 10% still keeps them in the no effect range. Therefore, these results were considered valid. Why agents like $NaC_2H_3O_2$, Tris buffer, and sucrose do not harm this phage is not known. However, certain relationships have been noted to exist between these agents. First, both sucrose and Tris buffer have hydroxymethyl groups in their chemical structure. Secondly, these agents are related to $NaC_2H_3O_2$ in that the hydroxymethyl group is the reduced form of the carboxyl group and the carboxyl group, its substituted derivative, and its ion are present in the $NaC_2H_3O_2$ solution.

Morphological Observations

Several other interesting features were denoted by examining the electron micrographs of negatively stained phage particles. For instance, the regular hexagonal configuration of the head first observed in shadowed preparations (Figure 1A) also was obtained by the noncentrifuged negatively stained phage particles (Figures 1B, 1C and 1D). Although head membranes frequently were distorted in the centrifuged negatively stained preparations, the most commonly occurring head shape was spherical (Figures 2D, 3B and 3D). These facts suggest the icosahedron most probably represents the third

dimensional shape of this phage's head.

Not only is head shape distorted with centrifuged specimens, but also substructure is obscured. This substructure, revealed by a nubby or bumpy appearing head, is best seen in noncentrifuged negatively stained specimens (Figures 1B, 1C, 1D and 4B). Actually closer examination of phage heads in centrifuged negatively stained preparations also reveals that some sort of electron transparent bodies appear to be associated with the head membrane (Figures 2B and 2C) or with the tail adjacent to the junction of head attachment (Figures 2A, 2B and 2D).

Another interesting feature was that phage aggregation by tails into the rozette patterns obtained for T even coliphages was never observed in any of the negatively stained specimens of S. griseus phage. On the other hand, aggregation of S. griseus phage particles by their heads was continually found, but only in the centrifuged preparations (Figures 3A, 3B and 3D). Furthermore, regardless of the kind of negative stain employed, electron transparency of similar intensity for both the S. griseus phage head and tail was not obtained with any centrifuged specimen (Figures 3A, 3B, 3C and 3D). In fact, only noncentrifuged PTA-UA stained preparations allowed similar electron transparency with sufficient contrast (Figures 1B, 1D and 4B). This again illustrates the existence of head membrane differences between the two phage types, because unlike S. griseus

phage, the head and tail of T even coliphages possess properties that permit sufficient contrast and equal electron transparency to be obtained when negatively stained with only PTA. Also it appears that S. griseus phage possesses a head membrane fragile enough to be disrupted by mild centrifugation such that the negative stain is able to diffuse into it to cause a decrease in its electron transparency.

Nevertheless, centrifuged specimens were not considered totally useless, for certain structural differentiation was still observed. First, some of the phage in Figures 3A, 3C and 3D indicate a body of slightly higher electron transparency is contained within the head membrane. Secondly, the tails of some of the particles in Figures 3C, 3D, 4A and 4B appear to narrow just before their insertion into the head membrane. Next, in agreement with the general tail morphology originally observed in the shadowed particles (Figure 1A), Figures 2C and 2D demonstrate this phage possesses a long, thin, wavy tail. Because of its tail being flexuous, the rigid tail core, which is present in T even coliphages, is not part of the substructure of S. griseus phage. Furthermore, neither tail striations, tail contraction, nor tail appendages have been observed to occur in this phage. However, the presence of a tail hole is suggested in the intact phage tail of Figure 4A and the free tail segment of Figure 4B.

Figure 1. Shape and substructure of the head component of S. griseus phage.

(A) S. griseus phage particles shadowed with palladium-platinum alloy. Magnification 30, 000 X. Scale marker denotes 0. 1μ.

(B-D) Noncentrifuged S. griseus phage negatively stained with 1% PTA-UA solution (final concentration), pH 10. 8. Magnification 192, 000 X. Scale marker represents 0. 1μ.

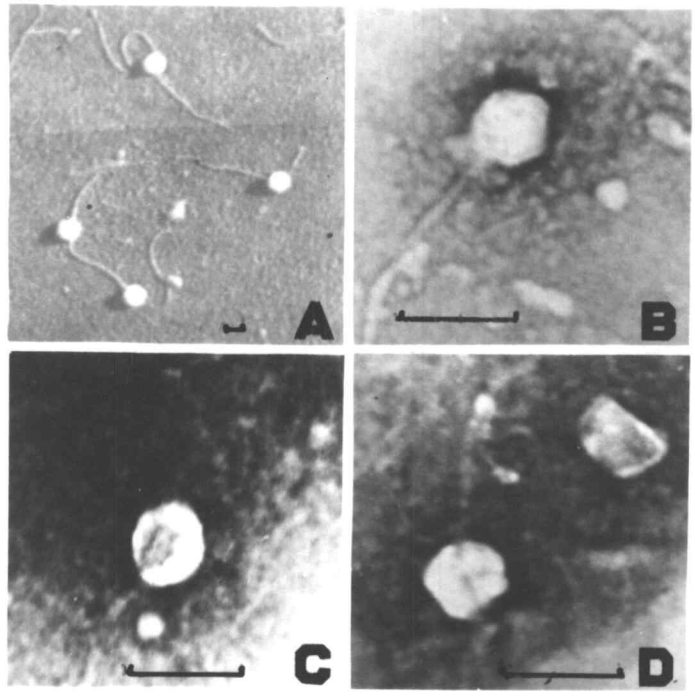

A
B
C
D

Figure 2. Presence and location of electron transparent bodies associated with S. griseus phage morphology.

(A, B and D) Centrifuged S. griseus phage particles negatively stained with 1% PTA (final concentration), pH 7. 4. Magnification 96, 000 X. Scale marker denotes 0. 1μ.

(C) Noncentrifuged S. griseus phage negatively stained with 1% PTA-UA solution (final concentration), pH 10. 8. Magnification 96, 000 X. Scale marker denotes 0. 1μ.

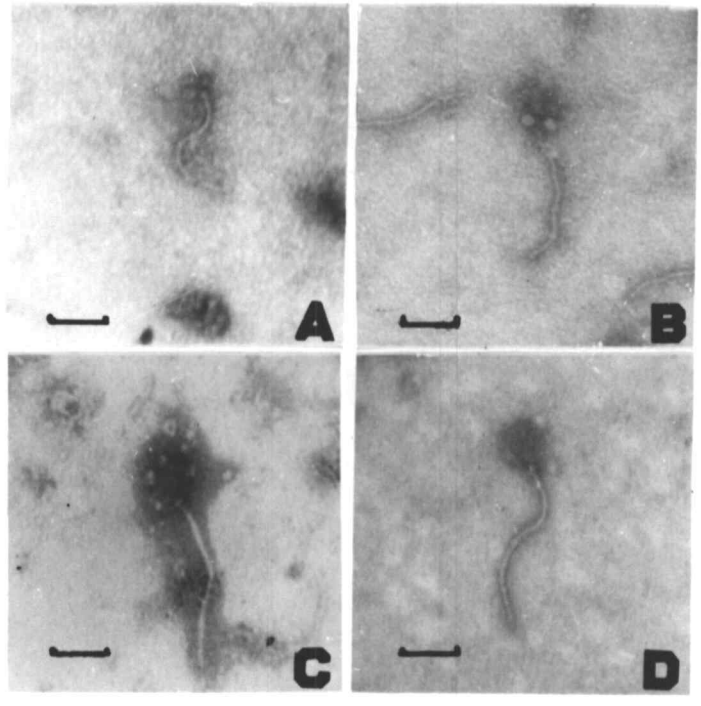

A
B
C
D

Figure 3. Aggregation and decreased electron transparency of the head component of centrifuged S. griseus phage.

(A, C and D) Centrifuged S. griseus phage particles negatively stained with 1% PTA-UA solution (final concentration), pH 10. 8. Magnification of A and C 192, 000 X and of D 96, 000 X. Scale marker represents 0. 1μ.

(B) Centrifuged S. griseus phage particles negatively stained with 1% PTA solution (final concentration), pH 7. 4. Magnification 192, 000 X. Scale marker denotes 0. 1μ.

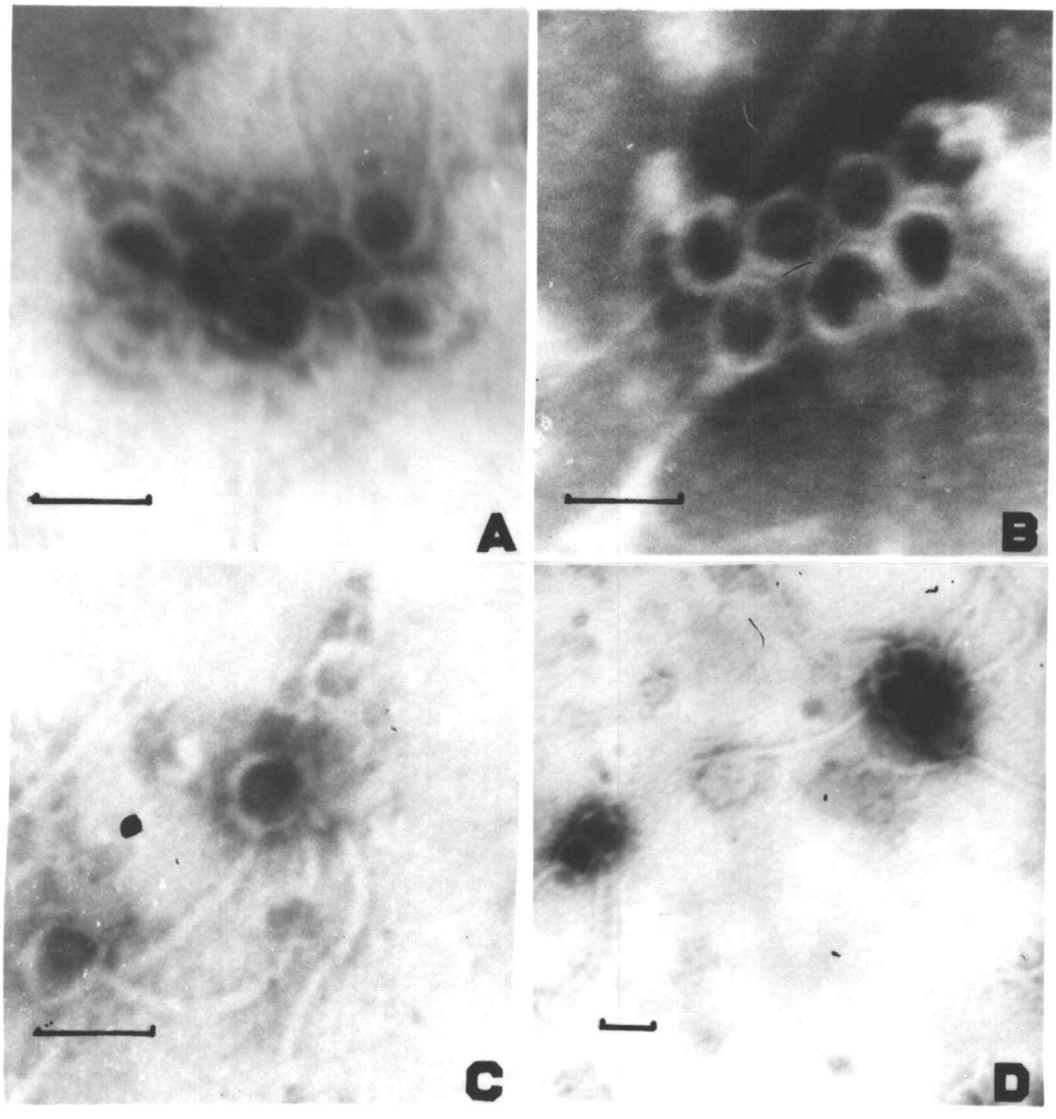
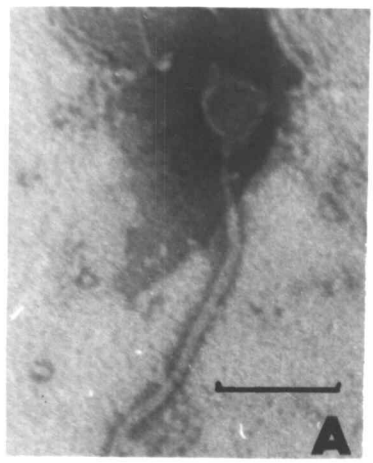
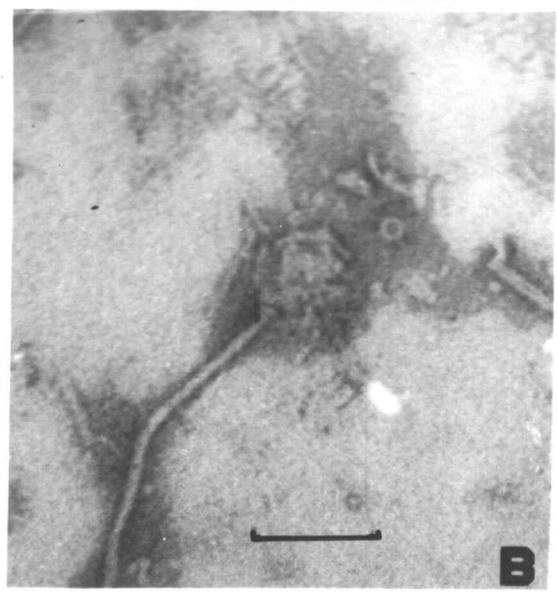

A
B
C
D

Figure 4. Substructure of intact S. griseus phage.

(A and B) Noncentrifuged S. griseus phage negatively stained with 1% PTA-UA solution (final concentration), pH 10.8. Magnification 192,000 X. Scale marker denotes 0.1μ.


A


B

DISCUSSION

Since phages are incapable of reproduction unless associated with their homologous host, the effect of subjecting them to specific chemical or physical agents is caused by intermolecular interactions with these agents on already existing components of the phages' structure. If these interactions are of sufficient strength as with chemical redox reactions or with physical osmotic pressures, then certain chemical bonds will be attacked and broken. This results in a certain amount of alteration or even total degradation of the phage structure which in turn renders at least one phase of the infective cycle nonfunctionable. On the other hand, if these interactions are of lesser strength as those involving van der Waals forces, then the chemical bonds will be reinforced instead of broken. This strengthening may be sufficient enough to aide phage in carrying out their infective processes or it may be of negligible significance upon the operation of the phage infective processes.

The effects of certain reagents upon S. griseus phage and the similarities and the differences between this phage and the previously studied T even coliphages are summarized in this section by two tables. The information in Table XII indicates that in all but one case a similar type of effect results from incubating either phage in any one of the first six reagents. This strongly implies that S. griseus

Table XII. Comparison of reactions to various chemical and physical agents by S. griseus phage and T even coliphages.

Treatment	T Even Coliphages		S. griseus phage
	Cultural & Morphological Effect	Ref. #	Cultural Effect
0.05M NH_2OH, pH 6.7	Inactivation (>99%, 2 hrs) Tail fibers unwound or removed; tail sheath contracted.	24	Inactivation (>99%, 15 min)
3% H_2O_2 in 10% C_2H_5OH	Inactivation (no value) Same alteration as with 0.05M NH_2OH	19, 10	Inactivation (>99%, 10 min)
Freeze thaw cycles	Inactivation (value increases as # of cycles increase) Same alteration as with 0.05M NH_2OH	28	Inactivation (value increases as # of cycles increase)
0.03M NaCN and 0.01M $ZnSO_4$, pH 8.0	Inactivation (>99%, 1 hr) Same alteration as with 0.05M NH_2OH	24	Inactivation (>50%, 1 hr, pH 11.25)
6M urea	Inactivation (no value) Head protein disrupted; DNA released	11	Inactivation (>99%, 5 min)
0.2M arginine, pH 8.75	Inactivation (>99%) Same alteration as with 6M urea	24	Inactivation (>99%, 1/2 hr)
0.2M Tris buffer, pH 9.5	Inactivation (>50%, 20 min) No information on alteration	24	Activation (78%, 1/2 hr)
pH 11.0 broth	No information		Activation (31%, 4 hrs)

phage possesses the same or similar kinds of chemical bonds as the T even coliphages. However, the degree of variation of inactivation indicates that differences in the number and/or in the position of these linkages exist between these two phages. More specifically, based upon this similarity of effect by the first four reagents listed in this table and upon Kozloff's argument that the thiol ester bonds present in T even coliphages are the only sites attacked by these reagents, it must be concluded that thiol ester linkages also are present in S. griseus phage structure.

Although a similar effect is caused upon both phage by urea and L-arginine, the exact site of chemical attack has not been determined. Actually several different bonds may be attacked. In both urea and L-arginine the specific reacting site involved is the nitrogen of the amide groups. This is because the electrons involved with the covalent bonding of an amide group are not equally shared. Thus the more strongly electronegative nitrogen atom in the group possesses a fractional negative charge, that is, a nucleophilic site. Since this site is characterized by an excess of electrons, it is attracted to and reacts with any chemical group that possesses a fractional positive charge or electrophilic site as does the thiol ester linkage or any positively charged atom such as the phosphorus atoms in ATP, acyl phosphates, guanidium phosphates, enolic phosphates, or in nucleic acids. Chemically this results in a form of oxidation-reduction

intermolecular interaction where the amide group acts as the initial reducing agent which loses electrons and is thus oxidized and the phage component which finally gains electrons and is thus reduced. Structurally this reduced phage component, which now possesses the amide group in place of the group it lost, is inadequate to maintain the normal functions of the phage. Therefore, both phage possess components containing electrophilic sites that can be attacked, reduced, and broken by chemical agents possessing these oxidizable nucleophilic sites. Incidentally, these reasons may begin to explain the specific chemical attack upon the phage structure by solutions of either cyanide complexes or hydrogen peroxide in ethanol. For instance, the cyanide ion is polarly linked, that is, the electrons involved in the union of the carbon atom with the nitrogen atom are not equally shared between the two atoms. This results in a partial positive charge or electrophilic site on the carbon and a partial negative charge or nucleophilic site on the nitrogen. Hydrogen peroxide also is capable of being a reducing agent when it is in an acid environment or a potentially acid environment such as ethanol.

If it is assumed that the inactivation effect only depended upon the presence of at least two amide groups or of one amide and one imide group per inactivant molecule, then no effect; that is, neither an inactivating nor an activating effect; should be expected from any molecule possessing only one amide group. However, the data shows

other factors must be involved, for while hydroxylamine will inactivate both phage, Tris buffer will inactivate only T even coliphage. These facts indicate the effect depends not only upon the number of amide groups present per molecule of inactivant, but also the stereochemistry of both the inactivation molecule and the components of the phage protein integument. Thus these results with Tris buffer is one piece of evidence that differences in the protein integument of these two phage exist. Whether these differences are a result of sequence variation of the same components or of variation in the components themselves is not presently known. Perhaps the components in the protein shell of S. griseus phage possess many hydroxymethyl groups. These groups could then form van der Waals intermolecular forces with any two of the three hydroxymethyl groups present in one Tris molecule. This would serve to hold the Tris molecule in a position with the amide group always directed away from the phage protein. Thus the occurrence of the redox reaction involving the amide group would be prevented as manifested by Tris having no effect upon S. griseus phage. However, that activation actually results suggests that Tris must also "mend" a certain amount of phage preexisting in the preparation which are "injured" enough to be unable to adequately perform some step in the infectious part of their life cycle. Possible explanations for this accomplishment may be by providing actual molecular level support or rigidity to a partially

weakened or collapsed protein membrane or by providing a more prominently protruding chemical group which happens to be involved in host receptor site attachment.

Due to a lack of information the results of subjecting T even coliphages to various pH levels is not known. However, by adjusting the pH of the incubation medium any one of the three possible effects--inactivation, no effect, or activation--may be inflicted upon S. griseus phage. That this phage can readily be inactivated by increasing the hydrogen ion concentration such that the pH falls below 6.00 is probably due to disintegration of the protein membrane by acid hydrolysis of its constituents. In a like manner, membrane disintegration by alkaline hydrolysis is probably the reason for almost complete inactivation of this phage by pH's of 11.50 or higher. However, explanation of the other pH effects upon this phage is not as simple. In the first place that pH's ranging from 6.00 to 10.50 have no effect on this membrane imply the phage constituents contain a strong excess of basic components. Interestingly enough contractile proteins also have been discovered to contain a majority of basic components. Furthermore, the slight but constant activation occurring at pH 11.00 may parallel the fact that relaxation of contracted tail sheath can be obtained by subjecting T2 coliphage to pH 10.00 buffer. Since this evidence strongly suggests S. griseus phage does possess contractile protein, then the slight activation which optimally occurs at some

point between pH 10. 50 and 11. 50 may be due to "mending" a small amount of pre-existing phage in the preparation by relaxing their contracted protein enough so as to unblock the step in the infectious process that had been preventing them from performing this process. That this activation is so slight indicates either that only a small amount of phage carry the "injury" or that the protein contraction has not proceeded far enough to initiate succeeding steps of the infectious process, that is, the point of no return has not been passed.

Since the susceptibility of phages to osmotic shock depends largely upon the toughness of the phages' head membrane, comparative analysis of osmotic shock results of T even coliphages with S. griseus phage may also reflect some similarities and differences in composition of this membrane. Before this can be done, the concepts involved with the mechanics of osmotic shock must be enumerated. In the first place successful osmotic shock manifests itself macroscopically through inactivation of phages which then characteristically appear under the electron microscope as "ghosts", that is, phage particles devoid of nucleic acid. This result has been obtained with T even coliphages by first subjecting them to incubation in highly concentrated solutions containing either ionic or nonionic solutes and then rapidly diluting and agitating them with a large quantity of cold water. Now these facts imply phage head membrane permeability is involved such that the membrane is permeable to both water and solute, but

much more permeable to the former than to the latter. Thus when the particle is placed in the concentrated solution, the solute molecules diffuse into the head membrane slower than the water molecules diffuse out of it. This continues until isotonic conditions are reached. With the rapid addition of a large volume of water this condition is again upset. Only now the water molecules diffuse into the head membrane more rapidly than the solute molecules can escape out of the membrane. Whereas the first unequal diffusion rate causes a net external force of a magnitude that has little if any effect on the phage particle, the second one creates a net internal force within the membrane that is physically great enough to cause small ruptures in the membrane which then allows nucleic acid to escape into the medium and ultimately results in "ghosts". Thus the toughness of the phage head membrane to osmotic shock is actually a resultant of two membrane components: 1) relative permeability to water and solute molecules and 2) relative rigidity or inelasticity to internal pressures. Therefore, assuming two membranes have equal permeability to an agent, the membrane that is structurally more rigid or less elastic is more easily subject to inactivation by osmotic shock. Likewise, assuming two membranes have equal rigidity, when the magnitude of difference between the initial diffusion rate of the water molecules and of the solute molecules is greater in a first membrane than in a second membrane, the first membrane is more easily inactivated

by osmotic shock than is the second one.

Keeping these details of osmotic shock in mind and returning to the comparative analysis of osmotic shock data in Table XIII, the evaluation regarding the similarities and differences between the head membranes of T even coliphages and S. griseus phage can be resumed. That S. griseus phage possess certain other compositional properties similar to T even coliphages additional to the ones previously mentioned is indicated by noting that inactivation can be inflicted upon both phage by osmotic shock with 4M NaCl, 3M Na_2SO_4, or 3M $(NH_4)_2SO_4$. However, the similarities soon cease. The first difference occurs in the higher degree of inactivation caused by osmotic shock with 3M $(NH_4)_2SO_4$ than with either 4M NaCl or with 3M Na_2SO_4 on S. griseus phage. Very possibly this inactivation is the additive result of an osmotic shock effect and the effect of one of the ammonium ion's derivatives upon the electrophilic sites possessed by this phage.

A second difference is that while osmotic shock with 3M $NaC_2H_3O_2$ inactivates T even coliphages, it has no effect upon S. griseus phage. However, unlike the previous instances with NaCl and Na_2SO_4, the solute particles responsible are not the ions resulting from the strong dissociation of these ionically bound salts. Instead the nonionic particles resulting from the covalent link between the Na and the $C_2H_3O_2$ components are responsible. Of course a small amount of Na and

Table XIII. Comparison of reactions to osmotic shock by S. griseus phage and T even coliphages.

Chemical Agent	T Even Coliphages		S. griseus phage
	Cultural & Morphological Effect	Ref. #	Cultural Effect
4M NaCl	Inactivation (no value) Alteration in all cases of inactivation by osmotic shock is ghost phage particles	3	Inactivation (>75%, 4 hrs)
3M Na_2SO_4	Inactivation (>99%,2 min)	17	Inactivation (80%, 4 hrs)
3M $(NH_4)_2SO_4$	Inactivation (>99%, 2 min)	17	Inactivation (>99%, 4 hrs)
3M $NaC_2H_3O_2$	Inactivation (>99%, 2 min)	17	No effect
Sucrose	Inactivation (no value)	3	No effect (even in 60% sucrose, 4 hrs)
3 M $MgSO_4$	Does not cause osmotic shock	17	Activation (135%, 1 hr)
3M $CaCl_2$	No information		Inactivation (>99%, 1 hr)
3M $CoCl_2$	No information		Inactivation (>99%, 1 hr)
3M $MnSO_3$	No information		Inactivation (>99%, 1 hr)

$C_2H_3O_2$ ions are released to the solution by dissociation. However, the $C_2H_3O_2$ ion pulls H ions from the solvent to immediately form an $HC_2H_3O_2$ association, which in itself is highly nondissociative. Thus since the sum total of ions represented by a small amount of Na ions, a lesser amount of OH ions, and a negligible amount of $C_2H_3O_2$ ions is too small to cause the osmotic shock effect reported in T even coliphage, then the large quantity of nondissociated $NaC_2H_3O_2$ and $HC_2H_3O_2$ particles must be the cause. This means these undissociated particles are able to permeate the T even coliphage head membrane at a rate that is sufficiently different than the rate of water molecule penetration. Therefore, to obtain no osmotic shock effect with this solution means the unionized particles either cannot permeate the S. griseus phage head membrane or permeate it at a rate similar to the diffusion rate of water molecules. Whichever the case, the fact remains that it is the membrane's chemical composition which dictates its permeability properties and is the ultimate factor responsible for the comparative osmotic shock results obtained.

That osmotic shock with concentrated sucrose solutions inactivate T even coliphages, but have almost no effect upon S. griseus phage again supplies evidence that differences in the head membrane permeability exist between the two phages. However, the explanation that S. griseus phage head membrane's permeability rate to water and

sucrose is not sufficiently different to cause formation of an internal pressure on the membrane walls has been discarded on the basis that centrifuged phage preparations resuspended and incubated overnight in 5% or lower sucrose solutions can indeed by inactivated (South, 1964). Instead, these facts plus the fact that the amount of effect remains fairly constant with increase in time, suggest the main factor involved is that this phage head membrane possesses properties that make it relatively impermeable to sucrose. Thus if sucrose passes very slowly into the membrane, the time necessary for an equal concentration of sucrose molecules on both sides of the membrane will be greater than four hours. Hence, if the concentration of the sucrose on the outside of the phage membrane after four hours incubation is still at least ten times greater than on the inside, then a rapid 1:20 dilution with cold water would not sufficiently lower the external solute concentration to cause internal pressure to build up and effect its forces upon the phage membrane.

Even though this provides the general explanation, other factors also may contribute. For example, perhaps the size of the sucrose molecule is barely small enough to squeeze through the membrane pores which in turn would increase the time necessary for passage into the phage. Finally perhaps the high number of sucrose molecules present in these concentrated solutions permit formation of "polymers" of varying lengths composed of sucrose molecules linked by

van der Waals intermolecular interactions from the hydroxymethyl group present on the glucose portion of one sucrose molecule to the hydroxymethyl group present on the fructose portion of another sucrose molecule. Since many of these "polymers" would be too large to pass through the membrane pores, the number of solute particles outside of the membrane is bound to be greater than on the inside. The addition of a 1:20 dilution of water would serve to break the van der Waals forces between the elements of the "polymers" and thus would not be able to decrease the external solute concentration sufficiently to cause internal pressure on the phage membrane.

In addition these data supply supporting evidence for the previously discussed data which indicates that this phage's integument chemically possesses reducible or electrophilic sites. If so, then a nonreducing sugar like sucrose would not be capable of disrupting these bonds. Thus inactivation would not be expected to occur.

Probably the most unique feature thus far exhibited by S. griseus phage is its reaction to osmotic shock attempts with 3M $MgSO_4$, for rather than being inactivated or unaffected, this phage is activated such that its titer is increased 2 to 2 1/2 times the control titer. Whether this feature is solely relegated to this phage cannot be maintained with complete assurance because reports do not indicate whether 3M $MgSO_4$ activates or has no effect on T even coliphages, but only that it does not cause osmotic shock. However, it seems

likely that if 3M $MgSO_4$ also activates coliphage, this fact would have been reported.

An abundance of experimental data about the 3M $MgSO_4$'s activation effect upon S. griseus phage permits an explanation regarding its mode of action upon the structure of this phage. This explanation can best be executed by describing what is not involved with the activation. In the first place since osmotic shock with 3M $MgSO_4$ activates this phage while osmotic shock with 3M Na_2SO_4 inactivates it, the Mg ion, not the common SO_4 ion must be the responsible factor. However, the mode of action bestowed upon the phage structure by the Mg ion is not due to a simple matter of its divalency, for osmotic shock with 3M $CaCl_2$ completely inactivates this phage. It should be noted that while complete inactivation was also obtained by osmotic shock with either 3M $CoCl_2$ or 3M $MnSO_4$, the effect was probably due to the high acidity necessary to maintain these divalent cations in solution, rather than to osmotic shock. Thirdly the Mg ion is not responsible for this activation by participating in exogenous enzymatic reactions or by providing a required adsorption factor, because adding Mg ion in concentrations varying by a factor 10 from 1.5×10^{-2}M to 1.5×10^{-7}M directly to the semisolid plating medium did not increase phage titers. Now all these negative factors imply permeability of the phage head membrane is involved. However, if the membrane were either relatively impermeable to Mg ion or permeable to nearly

the same degree to both water and Mg ion, then a neutral effect, not an activation one would result. Hence the Mg ion must not only combine with the phage membrane components to alter their reaction to osmotic shock, but also "mend" a certain amount of "injured" phage which prior to this treatment were incapable of infecting the homologous host. This first function may be accomplished by imparting a less rigid character to the membrane and/or changing the membrane's permeability to water molecules. Thus if after the Mg treatment the membrane became either less permeable or completely impermeable to water molecules, then osmotic shock could not occur because sufficient internal pressure could not build up. Before it can be shown that rigidity is indeed part of the answer, the concepts of freeze thawing must be explained. First of all these cycles occurred in water containing 0.25% peptone. Therefore, since water expands when it freezes, freezing of phage in this medium creates physical forces upon the chemical bonds of the membrane. Now if these bonds are relatively rigid, the membrane will rupture which in turn makes the protein component incapable of proper functioning and allows the internal phage contents to seep out upon thawing. Therefore, Mg ions must also impart greater elasticity to the membrane's chemical bonds because the amount of inactivation obtained by five freeze thaw cycles in the presence of peptone broth containing a final concentration of 1.5M $MgSO_4$ was 30 to 35% lower than that obtained by five

freeze thaw cycles in peptone broth alone. The other function of the concentrated Mg ion, the "mending" function, also may be accomplished by restoring a condition resembling normalcy to the phage head protein membrane and/or by furnishing cofactor necessary for performing endogenous enzymatic reactions, as kinase reactions.

Further membrane differences appear when electron micrographs of PTA stained preps are examined. For example, head membranes of centrifuged S. griseus phage preparations lack the electron transparency found in similarly treated T even coliphage head membranes, which, according to the interpretations of the negative contrast technique, indicates some alteration of the membrane has occurred to allow diffusion of nucleic acid out and this nondifferential stain, PTA, into it. Furthermore, an increase in electron transparency can be obtained either when centrifuged S. griseus phage preparations are stained with the PTA-UA mixture or when noncentrifuged specimens are stained with PTA and a still greater increase in transparency can be obtained when noncentrifuged samples of this phage are stained with the PTA-UA mixture. Hence, from these facts it is concluded that S. griseus phage head membrane possesses a certain fragility or delicacy such that even the forces of mild centrifugation rearrange some of its components to open up "pores" large enough to allow nucleic acid diffusion out and PTA diffusion into the membrane. Also the fact that only the PTA-UA mixture is able to

provide a certain amount of negative contrast on centrifuged preparations suggests the uranyl ions and complexes are too large to pass through these "pores" and/or these ions and complexes are able to combine with some membrane constituent to form a positive stain on the phage head membrane.

Information from these electron micrographs also can be used to illustrate the differences in tail morphology between phage types. In fact, except for tail length, the morphological differences between T even coliphages and S. griseus phage parallel those between T even coliphages and T1 or T5 coliphage. However, the structural similarities in T1 and T5 coliphage and S. griseus phage pose questions not easily reconciled with the classic T even coliphage concept of the infectious process. For example, how can the tail of T1 or T5 coliphage that does not appear to possess tail fibers be capable of tail attachment to its homologous host; that does not appear to possess rigidity in its distal portion be capable of performing a microsyringe injection of nucleic acid; and that measures 50 mμ longer and 10 mμ thinner than that of the T even coliphages be capable of tail contraction to the dimensions compensatory to the viscous drag on the nucleic acid molecule passing through a long slender tube? Furthermore, the T even coliphage tail can be easily disrupted with chemicals so that rozette patterns consisting of phage in wheel arrangements occur. In this pattern the distal tails form the hub and the heads the rim.

Frequently a wheel pattern is formed with resuspended, centrifuged, nontreated S. griseus phage, but here the heads form the hub and the distal tails the rim. Finally in contrast to the delicate structure of its head membrane, the tail of S. griseus phage neither loses its electron transparency when centrifuged and negatively stained nor is visibly altered in structure when subjected to centrifugation or purification procedures. In fact, so far no tail contraction, no tail striations, or no tail appendages have been observed.

On the bases of these arguments, it is hard to imagine that the tail of this kind of phage functions in the adsorption and infectious mechanism. In fact, despite numerous attempts, electron micrographs of tail attachment by this phage have never been obtained. However, in view of the fact that Mach (1958) published electron micrographs showing actinophages of both the T even coliphage morphology and the S. griseus phage type gathered around various streptomyces species in a tailward orientation, the argument for tail adsorption becomes considerably strengthened. Although this presents rather convincing evidence for tail adsorption, it cannot be considered conclusive until supporting micrographs of tail adsorbed phage with empty heads also have been provided.

Therefore, an impasse is reached. On one hand if tail adsorption does occur, how does infection occur, for even if tail contraction were observed, the extremely long, thin dimensions of the tail

make tail openings equivalent to those observed in T even coliphages highly unlikely. On the other hand, if tail adsorption does not occur why was Mach able to obtain those electron micrographs?

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