THE INITIAL CHARACTERIZATION OF GW6210, A NEWLY ISOLATED BACTERIOPHAGE FOR GLUCONOBACTER

by

Pamela Jean Churn

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APPROVED:

G.W. Claus, Chairman	A.A. Youatten
R.C. Bates	S.A. Tolin

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INTRODUCTION

In October of 1976, a rotting apple sample was collected from Catawba Creek Valley of Botetourt County by G.W. Claus. He and his colleagues were interested in obtaining a phage for the genus Gluconobacter. C.A. Baker subjected the apple sample to an enrichment using Gluconobacter oxydans subspecies suboxydans ATCC 621. Lawns of the indicator strains for the detection of plaques were usually disposed of after 24 hours of incubation. The plaques of GW6210 did not become visibly distinct until 48 hours. The plaques were noticed on an older plate that had not been thrown away.

Preliminary observations by Baker and Claus demonstrated that this phage was extremely large. This investigator thought it would be interesting to more extensively characterize this unusually large phage, especially since the host was of known industrial importance. The only known publication on phages for the Acetic Acid bacteria is that by Bradley (1965a), however the strict identification of the host was not determined. Since there is no other known work on Gluconobacter phages, I decided to undertake the initial characterization of the phage GW6210.

The proposed primary objectives were to analyze the plaque morphology and the morphology of the phage particle. These analyses would be followed by the determination of the phage's host range, density, and nucleic acid type.

LITERATURE REVIEW

The Discovery of Bacteriophages

The concept of a virus was a controversial issue in the late 1890's (77). There were two schools of thought concerning this issue. Beijerinck described tobacco mosaic virus as a subcellular infectious agent (77). This idea challenged the concept of a virus as a tiny nutritionally fastidious organism (77). Stent points out that the controversy of virus versus bacterium motivated many bacteriologists to seek *in vitro* propagation of viruses, one such man being Frederick W. Twort. Twort inoculated the fluid of a smallpox vaccine onto nutrient agar, hoping to isolate a nonpathogenic, nutritionally fastidious, ancestral strain of the virus. However, the only growth that occurred was that of a micrococcal contaminant. Upon further incubation, these colonies became clear and liquescent. Twort also observed that this glassy material could be transferred to normal cells and induce the metamorphosis of heat-killed cells.

Felix d'Herelle, a French-Canadian microbiologist, also received credit for the analogous discovery of phages (3). Adams (3) described the series of events that led to d'Herelle's discovery. D'Herelle took stool filtrates of patients with dysentery and inoculated them into a culture of Shiga's bacillus. His intention was to inject the bacterium-virus composite into laboratory animals, but upon overnight incubation, the cultures exhibited no turbidity and were bacteria-free. Filtrates of the lysed cultures were inoculated into freshly prepared cultures of Shiga's bacillus, resulting in a second clearing of the culture.

According to Duckworth (28), most people give Twort and d'Herelle equal credit for having made independent discoveries. However, "some have made illusions which suggest that d'Herelle may not have been altogether honest in claiming to have no knowledge of Twort's 1915 discovery when he published his 1917 work."

The virus theory, as proposed by the experiments of Twort and d'Herelle, was very controversial and still unaccepted through 1922 (15). Bacteriophage work continued and brought together three men in the late 1930's, Alfred D. Hershey, Salvador E. Luria, and Max Delbrück. These men, in 1940, originated "the phage group" -- a small group of scientists dedicated to the physiology and genetics of phage reproduction (77). It must be said, that preceding the 1940's, phage research consisted primarily of abortive attempts to develop therapeutic uses for phages (15). During this time, the phage group grew very slowly until Delbrück's assembly of the annual summer phage course at Cold Spring Harbor in 1945. Interest in the group's phage work skyrocketed, compounded by the introduction of new technological developments such as the electron microscope, the ultracentrifuge, and radioactive tracers (77).

Since then, bacteriophages have been energetically investigated as to their morphological characteristics, infective process, chemical nature, and effects on the host organism (15). One of the first characteristics usually noticed about a phage concerns the effect of the phage on the appearance of the host bacterial lawn -- the plaque. Since the plaque type is easily analyzed macroscopically, it will

logically be the first topic in this review of the characterization of bacteriophages.

Plaque Morphology and Size

A plaque has been defined by Douglas (26), as a clear zone of lysis on a bacterial lawn. It results from the following events of a bacteriophage lytic cycle. A bacteriophage infects a host cell and replicates within the cell, causing the cell to lyse. The released progeny phages diffuse through the agar to infect neighboring cells. This process occurs repeatedly resulting in a plaque that can be easily observed macroscopically. The simplicity in observing plaques makes the analysis of plaque morphology a desirable starting point for a phage characterization study.

An investigator should be aware of factors that affect plaque morphology. Elford and Andrewes (1932) believed that one should standardize the conditions of plaque size measurements, so that comparative studies with other phages may be made.

The first and most basic factor affecting plaque size and morphology is the specific combination of phage and bacterial host (3). That is, the plaques resulting from the infection of a specific phage on one host may appear different from the plaques of the same phage on a different host. Likewise, the plaques on a lawn of bacteria may look different from those resulting from the infection of a different phage on the same host.

Adams (3) feels that the size of the plaque is greatly influenced by the metabolic state of activity of the host organism. Adams

supports this idea with the work of Elford and Andrewes (32) who found that the deeper the base layer of media, the larger the plaque formed. Their findings relates plaque size to the supply of nutrients. Exhaustion of nutrients slows the growth of bacteria, which in turn, reduces the burst size, until ultimately, the plaque ceases to increase in diameter. Douglas (26) feels that this explanation along with the accumulation of toxic metabolites is not satisfactory to explain all cases, however, he does not suggest any alternatives. He simply states that "no satisfactory explanation has been advanced."

Sagik (69) described another phenomenon that affected plaque morphology. He found that the phage T2 would produce small irregular plaques as well as normal plaques on lawns of E. coli B. He explained the presence of these plaques as having resulted from the slow adsorption of phage that have combined with specific host materials.

Elford and Andrewes (32) did a study that indicated an inverse relationship between plaque size and phage size. They filtered several different phage through colloidal membranes to determine approximate sizes of the particles. They correlated this data with plaque size measurements. They found that their smallest phage, S13, produced the largest plaques, whereas their largest phage, a Staphylococcus phage, produced the smallest plaque. The coli-phage C36 was intermediate in size and produced an intermediate-sized plaque. They suggested that this inverse relationship between plaque size and phage size may be related to the diffusion rate of the phage through the agar. They felt it logical that the smaller the phage, the faster it will diffuse throughout the agar, thus resulting in a larger plaque (32).

Adams (3) agreed with Elford and Andrewes, stating that, "Since the diffusion rate is often the limiting factor, anything that interferes with diffusion will reduce the plaque size. The concentration of agar in the plating medium is important for this reason, a more dilute agar permitting the development of larger plaques".

Sertic and Boulgakov (70) studied another factor that altered plaque morphology, the production of a hydrolytic enzyme. They were working with Fcz, a phage specific for strain Fb of E. coli, and found that turbid halos existed around the plaques produced by this phage-host combination. They were able to extract plates of barely confluent lysis and separate the enzyme from the phage by ultrafiltration through colloidal membranes or by high-speed centrifugation. They showed that heat-killed bacterial cells as well as chloroform-killed cells were altered by the enzyme. They showed that the enzyme was inactivated at 70°C and neutralized with anti-phage serum. Anti-bacterial serum did not neutralize the enzyme. This enzyme was not found in the extracts or cultures of the host.

Adams and Park (3) found a similar situation with phage strain Kp type 2 and its host Klebsiella pneumoniae. They were able to eliminate the halo around the plaques formed by this phage-host pair, by isolating the enzyme by high-speed centrifugation and spreading the enzyme preparation over half of a phage-infected plate. Following incubation, halos were present on the side that had been treated with the enzyme.

Another factor that may affect plaque morphology is the concentration of divalent cations in the media where adsorption of a phage to its

host takes place. Fildes, Kay and Joklik (37) performed experiments that showed how the burst size was affected by the concentration and the time of addition of Ca^{++}. As the concentration of Ca^{++} was reduced, so was the burst size. As the addition of Ca^{++} was delayed, the burst was also delayed and was less in magnitude. It would follow, then, that a reduction in burst size and a delay in the burst would reduce plaque size.

Adams (4) mentioned other factors affecting plaque morphology: 1) mutations of the phage genome, 2) the susceptibility of the bacterial host to the phage and 3) the ability of the phage to establish lysogeny with its bacterial host. Since all of the factors mentioned above may influence the morphology of a plaque, it is my opinion that plaque morphology alone is of limited value as a taxonomic criterion. However, I also feel that if growth conditions of the specific phage-host mixture are adequately controlled, plaque morphology may prove valuable, along with other phage characteristics, in providing an accurate description of the phage for classification purposes.

Phage Morphology

The basic morphological types of bacteriophages have been described in two very similar classification schemes. The most predominant scheme was devised by Bradley (15). He divided bacteriophages into six morphological groups. The first of these, group A, represents phages with polygonal heads and contractile tails. The head may or may not be elongated and the rigid tail, surrounded by a contractile sheath, may terminate in a variety of structural processes

(Fig. 1A). Group B contains phages with polygonal heads and long, flexible, noncontractile tails. Terminal appendages may or may not be present (Fig. 1B). Group C also is composed of phages with polygonal heads and noncontractile tails, but the tails are shorter than those found in group B (Fig. 1C). Group D represents tailess phages with polygonal heads displaying large capsomeres at the apices of the polygon (Fig. 1D). Group E is comprised of phages with polygonal heads, no tails, and no large capsomers at the apices of the head (Fig. 1E). The last class, group F, is made up of long filamentous phages (Fig. 1F). These phages are the simplest phages, lacking any attachment structures.

The second classification scheme, a modification of Bradley's, was described by Tikonenko (80). Her morphological groupings are as follows: type I = filamentous phages; type II = phages with tail analogues; type III = short-tailed phages; type IV = phages with non-contractile tails; and type V = phages with contractile tails. The main difference in the two classification schemes is that Tikonenko combined Bradley's groups D and E into one group, group II. She justified this change by stating that in Bradley's group D, some phages exist in which the large capsomeres at the apices of the head take on the same function as a tail. In this same reference, she also expressed the opinion that some phages in Bradley's group E had tail-like structures.

These two classification schemes arose out of the need to find some order in the chaotic area of phage taxonomy. The Linnaen form of classification could not be applied to the bacteriophages, because the

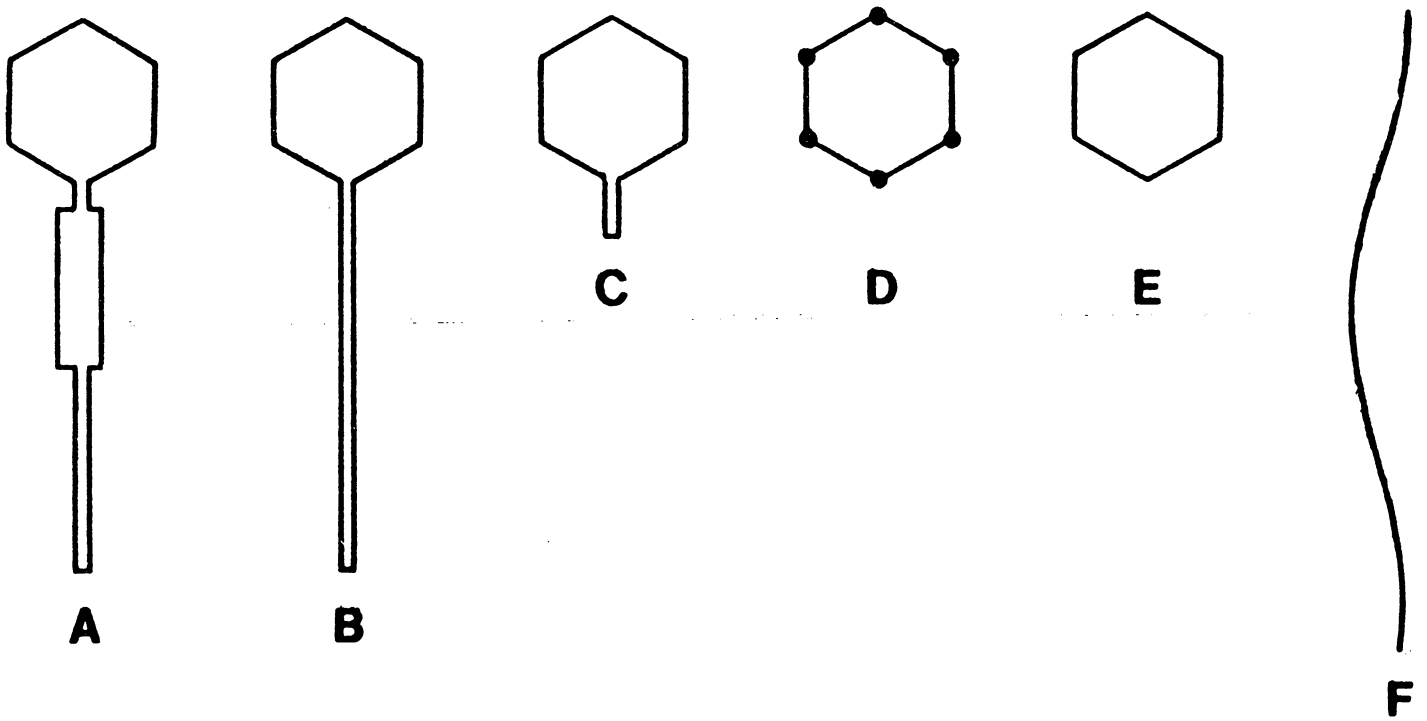


Fig. 1. Morphological groups of bacteriophages (15).

phylogenetic relationships between phages, between phages and viruses, and between phages and their bacterial hosts, have not been determined (80).

Although the classification of phages by morphology seems to be useful, there are dangers in utilizing morphology as the sole taxonomic criterion. For example, Anderson (5) described a Salmonella phage, P221, which can establish lysogeny with its host. He pointed out that the host lacks some of the genes for the production of infectious virions and that phage P22 can provide the host with these genes during superinfection. After superinfection, the resulting virions were morphologically identical to P22 (exhibiting a long tail), and a small portion were like P221 (exhibiting a short tail). The P221 particles have been shown to be different serologically from P22 and can be grown on hosts that are resistant to P22, but 46% of their DNA is homologous to P22 DNA (85). Based upon a morphological judgment alone, these two phages could have mistakenly been described as being unrelated (5).

In light of the present state of phage taxonomy, phage morphology, like plaque morphology, takes on an important role in the accurate description of bacteriophages. This role, albeit necessary, must be seen as an integral part of a series of investigations leading to an adequate characterization.

Unpublished observations by Baker and Claus prior to this study demonstrated that this phage belonged to Bradley's group A. This structural type is usually placed in the family Myoviridae (36). The name of this family was derived from the word mys or myos, meaning

"muscle", referring to the contractile tail characteristic of phages of this family (36). The cryptogram D/2:137/40:X/X:B/O is a code describing some of the general characteristics of this family (84). The first term, D/2, defines the viral genome to be double stranded DNA. The second term, 137/40, refers to the molecular weight of the genome (137 x 10^6 daltons) representing 40% of the total weight of the virus particle. The third term, X/X, is interpreted to mean that the structure of the particle and the nucleocapsid is complex. The fourth term, B/O, indicates that the host for the virus is a bacterium, and that there is no vector mediating infection of the host.

A summary of the dimensions of the phages in this group is given in Table 1. The basis for this table was found in Tikonenko's book (80). Dimensions of phages added to the values Tikonenko presented are indicated by an asterisk (*). This table illustrates a range of head sizes for phages in this morphological group. Head lengths and widths range from 35 nm to 160 nm. Tail lengths range from 60 nm to 455 nm. Tail diameters range from 15 to 25 nm.

Host Range

Adams (1959) defined host range as "the range of action of a phage measured in terms of the varieties of bacteria in which it can be grown." Host range appears frequently as a criterion for phage characterization. Before considering this role, it seems appropriate to discuss the factors that affect the host range.

Host-range mutations can occur by either phenotypic or genetic modification. These host-range mutants differ from the wild type

Table 1. Dimensions of Bradley's Group A Phages From Available Literature

Host	Phage	Head Dimensions (nm)[a]		Tail Dimensions (nm)		Ref.
		Length	Width	Length	Width	
Alcaligenes faecalis	A11/A79		65	130	19	(55)
Alcaligenes faecalis	A6		90	110	16	(55)
Bacillus species	G		160	455		(1)*
Bacillus subtilis	SP50		100	230		(64)
Bacillus subtilis	GA-2		35	210		(11)
Bacillus subtilis	GF-2		115	280		(11)
Bacillus subtilis	SP8		100	165	20	(24)
Bacillus subtilis	SP3	110	115	289	22	(31)
Bacillus subtilis	PBS1		120	200	22	(31)
Bacillus thuringiensis			90.5	232	16.5	(22)
Bacillus thuringiensis var. galleriae	GV-6	92	85		18[b]	(23)*
Caulobacter crescentus	Cr24		140	140		(47)*
Caulobacter crescentus	Cr26		160	160		(47)*
Caulobacter crescentus	Cr30		80	140		(47)*
Caulobacter crescentus	Cr35		80	140		(47)*
Caulobacter crescentus	Cr40		80	60		(47)*
Clostridium species	HM3		82	100		(1)*
E. coli	T4			90-95	16-17	(50)*
E. coli	T2	100	70	110	25	(53)
E. coli	T2	95	65	115	20	(49)
E. coli		54	54	140		(49)
E. coli	3T	90	65	95	15	(16)
E. coli	C4	100	75	100	20	(16)
E. coli	11F	90	65	92	15	(16)
E. coli	66F	90	70	95	15	(16)
E. coli	02	72	66	110	22	(16)
E. coli	Vi1	90	95	110	18	(16)
Myxococcus xanthus	MX-1		75	100		(18)
Pasteurella species	PST	125	95	115	25	(51)

Table 1 (continued)

Host	Phage	Head Dimensions (nm)[a]		Tail Dimensions (nm)[b]		Ref.
		Length	Width	Length	Width	
Proteus hauseri	13_{vir}		60	101.9	16.7	(65)
Proteus hauseri	57		61.9	88.2	17.8	(65)
Proteus hauseri	67b		58.9	98.1	17.4	(65)
Proteus hauseri	78		61.1	88.9	16.3	(65)
Proteus morganii	50	97	67	125.2	15.9	(65)
Proteus morganii	5845	102.2	76.3	125.2	15.6	(65)
Proteus rettgeri	8893	92.6	74.1	129.6	17.8	(65)
Providence species	9266	119.3	82.2	215.2	24.8	(65)
Pseudomonas aeroginosa	E79		66	150	17	(73)
Pseudomonas aeroginosa	F116	58	70	79	8.0	(73)
Pseudomonas aeroginosa	7v	65	55	90	20	(34)*
Pseudomonas aeroginosa	BP1		75	150		(34)*
Pseudomonas syringae	12S		80	100		(10)
Pseudomonas syringae	6C		65	125	14	(8)
Saprospira grandis	no name	110	120	105	22	(53)
Serratia marcescens	SMP		135	235	27.5	(12)
Serratia marcescens	SM2		110	190		(12)
Serratia marcescens	SMB3		57	125		(12)
Serratia marcescens	SMP5		57	125		(12)
Shingella sonnei	PK_{vir}		48-52	104-110	15-17	(46)
Vibrio cholerae	VA-1	50	58	87	26	(82)*

[a] If only one dimension is given, a spherical shape is assumed.

[b] Tail diameter measured in contracted state.

*References not obtained from Tikonenko's table (80).

phage in the structure of the adsorption appendages. This usually results in an expansion of the phage host range (Stent, 1963).

The phenotypic types of host range alterations can occur by host-induced adaptation or by phenotypic mixing (Stent, 1963). Anderson and Felix (1952) performed an experiment concerning Vi-phage typing of the typhoid bacillus which illustrated a host-induced phenotypic modification of a phage resulting in the modification of host range. They found that the Vi II phage strain, type A, was specific for bacteria exhibiting the A type pattern of phage specificity. When they tested a high titer inoculum of this phage on C type strains, a few plaques appeared on the plates. Phage isolated from these plaques were said to be "adapted", having an equal ability to infect typhoid strains of types A and C. However, when they plated this phage on an A-type strain of S. typhi, this adapted phage underwent a 100% reversion to the wild type phage, indicating that this host range mutation was a phenotypic one.

Novick and Szilard are given credit for the disclosure of phenotypic mixing as another means by which host-range is altered (Novick, 1966). This discovery came about through their investigation of an apparent paradox in a paper written by Delbrück and Bailey (25). Lawns of E. coli were doubly infected with phages T2 and T4. Lawns of B/4 (E. coli B resistant to T4) yielded T2 plaques. Lawns of B/2 (E. coli B resistant to T2) yielded T4 plaques. Lawns of B/4 + B/2 yielded cloudy plaques where bacterial cells released one type of phage, and clear plaques where both types of phage were released from the cells. Normally, one would expect there to be more plaques on

the B/4 plate than clear plaques on the B/2 + B/4 plate since the clear plaques on the latter type of plate represent only the portion of cells that released both phage types. However, more clear plaques appeared on the B/2 + B/4 plates than on the B/4 plates. Novick and Szilard (63) began to suspect that phenotypic mixing was occurring, resulting in, not only the release of T2 and T4, but also phages with the phenotype of T4 and the T2 genotype. Confirmation of their hypothesis followed in their experiments that showed that these T2 particles with T4 genomes were more sensitive to T4 antisera than were normal T2 particles (62). Therefore, they concluded that the T2 host range mutant had a protein in common with the T4 particle.

Another type of mutation that can affect host range is a genetic mutation of the vegetative phage (3). That is, an error may occur in the replicative process by which the parent viral information is copied to generate a mutated progeny virus. The phage may acquire by this mutation the ability to adsorb bacteria resistant to the nonmutated type (3).

The fact that the host range of a phage may vary by phenotypic modification of the phage or by genetic modification of the phage limits its value as a taxonomic criterion for phages. However, Adams (3) points out the value of host range determinations in differentiating related phages of similar characteristics, such as the coliphages, T2, T4, and T6.

The importance of utilizing host range in bacterial taxonomy is emphasized by Stocker (78) who points out that different bacterial species or strains of a species that have the capacity to lysogenize

with a particular phage, are closely related. He describes the use of phage typing, whereby a phage that is capable of modification to a number of specificities, is used to provide a pattern of infectivity that can be used to identify the bacterial species or genus of an unidentified organism. Host range was found useful by Smith and co-workers (74) in that it correlated with biochemical and morphological characteristics of the bacteria. However, Stocker (78) feels that this should not be used as a primary basis for species identification, because the phage's host range can be altered by phenotypic or genotypic modification of the phage.

Phage Concentration and Purification

The chemical analysis of bacteriophages usually requires higher quantities of viruses than are available in crude lysates (48). Several techniques are frequently used to achieve appropriate viral quantities. Each technique has advantages and possibly disadvantages that must be taken into account. A review of these methods follows.

Virus Concentration by PEG Precipitation. Polyethylene glycol (PEG, 6000) may be utilized in the precipitation of viruses. The PEG molecule is randomly coiled in solution and is capable of holding water within these molecular folds (79). Thach and Newberger (79) describe the mechanism of action of PEG as follows: When an aqueous solution of PEG is mixed with a suspension of virus and a salt, the salt ions are hydrated and the proteinaceous material is displaced from the solution. This mechanism is called "salting out". This phenomenon, however, is not the only mechanism working in this technique. The coiled PEG molecule in solution swells in response to an

osmotic gradient. This swelling reaches a point of equilibrium between the osmotic forces and the intermolecular attractive forces. When a virus suspension is added to the PEG, the virus particles will become entrapped within the coils of the PEG molecule. As the concentration of PEG is increased, the hydrated PEG molecules will contract to equalize the osmotic forces, excluding virus particles into intramolecular spaces, and the newly added PEG molecules will take up solvent, concentrating those particles in the intramolecular spaces. As the PEG concentration is further increased, a critical level is reached in which the protein particles are no longer soluble, resulting in their precipitation.

The advantage of utilizing this method is that high percentages of recovery of viruses are possible and that this method can be adapted to large-scale virus purification (86). Also, this method remains efficient regardless of the titer of the virus lysate used (86).

Concentration of Viruses by Ammonium Sulfate Precipitation. Although Sinsheimer (72) was not the first to use ammonium sulfate to concentrate viruses, he used it to concentrate X174 from E. coli C lysates. Thach and Newberger (79) explain this principle of "salting out" proteins in terms of the relationship between the protein molecules and the water molecules surrounding them. They reason that because of their polarity, water molecules may react with charged groups of proteins, electrostatically. Hydrogen bonds may be formed between water molecules and uncharged groups of proteins. The solubility of a protein is determined by these interactions. When an

inorganic salt is added to the solution, the salt, which is capable of establishing a charge-dipole interaction with water, competes with the proteins for these interactions. Proteins excluded from these interactions are no longer soluble and will precipitate (79).

Ammonium sulfate is commonly used to salt out viruses, as well as unstable enzymes, because of its mildness and its high solubility in water (79). This method, however, requires long incubation periods making it somewhat less attractive as a concentration method (72).

Concentration of Viruses by Ultrafiltration. The method of membrane ultrafiltration may be used for the concentration of proteinaceous solutions. Wang (81) describes the technique which involves the use of a sample reservoir that is pressurized with nitrogen for providing the flow of sample into a polycarbonate cylinder. The bottom of the cylinder consists of a supportive porous disc upon which is placed a polyelectrolyte membrane of the desired pore size to retain the protein of a given molecular weight. A magnetic stirrer just above the membrane reduces buildup of the concentrate on the membrane surface. This technique was applied by Wang to the concentration of bacteriophage SPO-1 from Bacillus subtilus lysates with a resulting concentration of 140%. According to Wang (81), this technique is useful in that moderate volumes of lysates may be filtered at high rates of filtration and the membranes may be used repeatedly. However, this technique does not appear often in the literature as a phage concentration technique because of some of its inherent problems. Some disadvantages are that clogging of the membrane may slow the

process of filtration, and that loss of phage may occur due to absorption of phage to the membrane or deactivation of the phage (33).

Concentration of Viruses by Differential Centrifugation. Differential centrifugation may be used to separate phage particles from cellular debris and soluble protein contaminants as described by Kay (1972): The larger cell debris is centrifuged out at a low speed of about 5,000 rpm for 15 minutes. The pellet of whole cells and cellular debris is discarded and the phages which remain in suspension are separated from soluble proteins by sedimenting the former at high speed (80,000 x g) for two hours. The pellet can be resuspended in a small volume of media or buffer to concentrate the phage.

When using this method, one must be wary of the possibility of damage of the phages due to high speed centrifugation (Kay, 1972). Kay (1972) suggests the examination of the fractions at each stage of purification by electron microscopy, however, infectivity studies would be helpful in determining damage that results in deactivation of the phage.

Purification of Phages by Density Gradient Centrifugation. When a high degree of purity is needed, virus preparations are frequently subjected to density gradient centrifugations (26). There are two major techniques used, rate-zonal centrifugation and equilibrium centrifugation (26).

Rate zonal centrifugation involves the use of a gradient forming device that delivers a solution, usually sucrose, into the centrifugation tube so that the solute concentration varies from 25% (w/v) at the bottom to 0% (w/v) at the top (48). A sample is layered

over the gradient and centrifuged at a speed that will separate the components of the sample according to density and shape, as they move down through the tube without sedimenting the virus to the bottom of the tube (26). The tube is then fractioned and assayed for the virus (48). It seems, therefore, that this method is appropriate for the purification of a virus, but not for the determination of its density.

The alternative method, equilibrium centrifugation, allows the density gradient to be formed as the sample is added to the gradient solution, usually cesium chloride (26). The virus will band at the point in the gradient that corresponds to its buoyant density making this method useful in both purification of a virus and in the determination of density (26).

Determination of Nucleic Acid Type

Fluorescent Staining with Acridine Orange. The method most often used by bacteriophage investigators for the determination of nucleic acid type, is Bradley's method of fluorescent staining with acridine orange (13). Some bacteriophage investigators, who have utilized this technique are Markel and Curtis (54), Driggers and Schmidt (27), Ackermann and co-workers (2).

According to Bradley's technique (13), crude phage lysates were purified by differential centrifugation. Small spots of the purified phage were placed on glass slides and fixed with Carnoy's fluid. The slides were stained with an acridine orange solution and rinsed with McIlvaine's buffer. The slides were observed under a U.V. lamp.

Spots of phages with double-stranded DNA or double-stranded RNA, will appear "apple- or yellow-green" whereas, spots of phages with single-stranded DNA or single-stranded RNA will appear "flame red" (13). For confirmation of this color reaction, the slides are subsequently stained with molybdic acid. No color changes are observed for the double-stranded nucleic acids. Spots of phages containing single-stranded DNA will change from flame red to a pale green. Spots of single stranded RNA phages will change in color from flame red to pale red. To distinguish between DNA and RNA species, freshly prepared phage spots were treated with DNAse or RNAse and the staining procedure repeated. The susceptibility to an enzyme was indicated by the development of no color after fluorescent staining.

This method was developed in attempt to simplify nucleic acid analysis by eliminating extensive purification procedures and nucleic acid extraction procedures (13). However, necessary control phages of known nucleic acid content must be tested along with the organism of interest, which requires the propagation of these phages to titers of approximately 10^{10} particles/ml (59).

Colorimetric Estimation of Nucleic Acids. The colorimetric determination of nucleic acids by the diphenylamine and/or orcinol tests requires the extraction of the nucleic acid from phage proteins (Shatkin, 1969). The phenol extraction used frequently for this purpose is described by Thach (1972) and is briefly described here. Hydrophobic groups are located in the center of protein molecules. When phenol is added to a viral suspension, the hydrophobic phenyl groups of the phenol move into the cores of the proteins where they

are more soluble. The proteins swell, uncoil, and denature. This uncoiled protein molecule exposes its hydrophobic groups and the phenyl groups. The nucleic acid molecules are more soluble in the phenol phase than in the aqueous phase. The denatured protein will, therefore, precipitate along the water-phenol interface, or diffuse into the phenol phase. The nucleic acid, having more polar groups, remains in the aqueous phase. After the nucleic acid has been extracted from the phage proteins, the colorimetric reactions may be employed. Both the diphenylamine and orcinol tests make use of standard curves for the determination of nucleic acid quantities (68).

Shatkin (68) explains that the diphenylamine reaction is based on the interaction of diphenylamine and 2-deoxyribose, which results in the formation of a blue color. In this test, the pyrimidine-sugar linkage is not hydrolyzed, leaving the purine nucleotides free to react with the diphenylamine. In this procedure, contaminating RNA does not interfere with the color reaction (68).

In the orcinol reaction, RNA is hydrolyzed to its bases, phosphate, and ribose (79) when heated with orcinol, HCl, and the catalyst, $FeCl_3$ (68). A green color develops when the ribose reacts with orcinol (79). Since DNA reacts with orcinol to give a green color (68) it is essential that measures be taken to rid the RNA sample of contaminating DNA.

These colorimetric determinations of nucleic acid type are useful when microgram quantities of the material can be extracted (68). When working with some viruses, however, the quantity of nucleic acid in each particle may be so small that this method would require

extensive concentration procedures. In cases such as these, the following procedure would be profitable:

Radioisotopic Labeling of Nucleic Acids. To bypass the problem of low virus titers in nucleic acid work, many investigators are now determining nucleic acid types by radioisotopic labeling. Labeled thymidine is used for the detection of DNA; labeled uridine is used for the detection of RNA (52). Usually, the label and phages are added to the host organism during an appropriate phase of growth (29). The virus sample may then be concentrated and centrifuged in a density gradient. Fractionation of the gradient follows centrifugation (7). The low-energy β-emissions of ^{3}H-labeled substances are easily detected with a liquid scintillation counter (41). Radioactive profiles relate peaks of radioactivity to density which are then correlated with infectivity assays. The peak of radioactivity should correspond closely to the peak of infectivity (7).

Although this system is very sensitive, there are problems that arise, such as adsorption of phages to cellular material, that are inherent in working with density gradients. This can cause bands of differing densities. Cesium ions bind to some viruses, causing an apparent increase in density (7). Also, thymidine is not easily incorporated into many organisms, such as Salmonella typhimurium (20). One solution to this problem would be the use of a thymidineless mutant in the assay (30). Cairns (20) ensured thymidine uptake in a similar fashion by growing E. coli B3, by selecting for a thymidineless mutant and by blocking the enzyme thymidylate synthetase that

was produced even in mutant strains. By these manipulations, the amount of thymidine incorporated was proportional to the amount of [^{3}H] thymidine added.

MATERIALS AND METHODS

Host Organism and the Preparation of Bacterial Stock Cultures

The host organism used during the course of this study was Gluconobacter oxydans subspecies suboxydans ATCC 621. The organism was obtained in March of 1974 from the American Type Culture Collection (Rockville, MD) and had been lyophilized since April 8, 1970. The lyophilized cells were cultured and placed into liquid nitrogen on March 6, 1974. The culture was transferred once a year and checked for exhibition of typical Gluconobacter characteristics. The culture was obtained by this investigator in the spring of 1977 for this study.

In the preparation of stock cultures, the organism was grown in 50 ml of 5% sorbitol medium contained in a 500 ml Bellco nephelometer flask. The culture was shaken at 28°C in a New Brunswick Psychotherm Model Incubator at a rate of 200 reciprocations per minute with a stroke amplitude of 1.5 inches. When the optical density ($O.D._{620}$) of the culture reached 1.0, one milliliter of the culture was added to a sorbitol stock vial (83). The vials were then vortexed until the contents were uniform and stored at -20°C.

Growth Media

Five Percent Sorbitol Medium.

For most experiments in this study, cells were cultured in 5% sorbitol medium which contained 5% (w/v) sorbitol, 1% (w/v) yeast extract, and 1% (w/v) peptone. The medium was also acidified to a pH of 6.0 with glacial acetic acid before autoclaving.

Base Layer Medium. For the enumeration of phage, the solid medium used as a base layer contained 1% (w/v) peptone, 1% (w/v) yeast extract, 5% (w/v) sorbitol, and 1.5% (w/v) Bacto agar. The pH was adjusted to 6.0 with glacial acetic acid. The medium was usually made up in three liter batches and autoclaved for one hour before cooling and dispensing into plastic petri dishes.

Overlay Agar. For uniform distribution of the phage and its host over the base layer medium, 15 ml screw capped tubes containing 2.5 ml of sterile overlay medium were prepared. This medium contained 1% (w/v) peptone, 1% (w/v) yeast extract, 5% (w/v) sorbitol, and 0.7% (w/v) Bacto agar. Glacial acetic acid was used to adjust the pH to 6.0 before autoclaving.

Agar Overlay Technique for the Enumeration of Phage Particles

A 0.05 ml sample of the sorbitol stock culture was inoculated into a roller drum tube containing 5 ml of sterile 5% sorbitol medium. The culture was incubated on a roller drum at 28°C until the culture reached 0.2 $O.D._{620}$. Appropriate dilutions of the phage to be titered were made into 15 ml screw capped tubes containing 5% sorbitol medium. Tubes containing 2.5 ml of sterile overlay agar were melted and tempered to 45-50°C in a waterbath. To a tube containing soft agar, 0.2 ml of the bacterial cells was added along with 0.1 ml of phage of a specific dilution. The mixture was briefly vortexed and poured over a plate of sorbitol base layer medium and allowed to harden. The plates were incubated at 28°C for 48 hours before the plaques were counted.

Preparation of High Titer Bacteriophage Stocks

The method of preparing high titer phage stocks ($\sim 10^{11}$pfu/ml) used throughout the course of this study, was patterned after the method outlined by Hershey, Kalmanson, and Bronfenbrenner (42). Utilizing the standard overlay technique described by Adams (3), various dilutions were made of the phage stock. A 0.1 ml aliquot of the diluted phage was plated with 0.2 ml of the bacterial host that reached an $O.D._{620}$ of 0.2. The dilution of phage that caused barely confluent lysis on host lawns was the dilution used to infect lawns for phage prepagation. Barely confluent lysis is characteristic of a plate that has been inoculated with enough phage to lyse most, but not all of the bacteria on the plate. On this type of lawn, bacterial growth can be seen only along the peripheral edges of the base layer agar. All other areas of the plate appear clear or free of growth, indicating bacterial lysis.

With the proper dilution used as the phage inoculum, 50 plates of barely confluent lysis were prepared by the agar overlay technique. After a 24 hour incubation, 5 ml of sterile 5% sorbitol medium was added to each plate. The entire agar overlay was then dislodged with a bent glass rod made from a Pasteur pipette. The liquid was decanted with a 10 ml pipette into sterile 15 ml screw capped tubes. The tubes were centrifuged for twenty minutes at 4000 rpm in a Damon IEC HN-S clinical centrifuge with a fixed-angle rotor. This centrifugation removed bacteria, agar, and cellular debris. The supernatant fluids from each of these centrifuge tubes were pooled, and saturated with a

few drops of chloroform to prevent bacterial growth. This will be referred to as a "phage stock."

Separation of Two Different Plaque Types

To separate the small turbid plaque (type B) from the clear, pinpoint plaque surrounded by a large turbid region (type A), a plaque of type B was picked with an inoculating needle. This was added to a tube containing melted and tempered soft agar and 0.2 ml of the bacterial host culture ($O.D._{620} = 0.2$). A plaque of type B from the resulting plate was again plated on a lawn of the host organism in the same manner. Plaque type B was serially picked and plated with the host organism. This method was recommended by Adams (3). This procedure was repeated 8 times.

Plaque Diameter Measurements

Diameter measurements of plaques were made both at 48 and at 72 hours of incubation. The measurements of the turbid region of plaque type A and of the turbid plaque type B were measured with dial calipers. A loupe, which had higher resolution than dial calipers, was used to measure the pinpoint, clear region of plaque type A. The turbid region of plaque type A was measured on 100 different plaques. The same number of measurements were made on the clear central regions of plaque type A. The turbid plaque type B diameter was measured on 429 plaques. Average values for both plaque diameters were calculated. The standard deviation was also calculated according to the following equation (75):

$$\sigma = \sqrt{\frac{\Sigma(x)^2}{N}}$$ where, σ = standard deviation
x = deviations from arithmetic mean $(x - \bar{x})$
N = total number of items

Determination of Phage Location Within the Plaque

An agar plug containing the clear central region from an A type plaque was removed with a micropipette constructed by drawing out a sterile Pasteur pipette over a flame. The approximate diameter of this pipette was 0.5 mm. This agar plug was vortexed in 2.0 ml of 5% sorbitol medium. Using the agar overlay technique, 0.2 ml of the resulting suspension was plated with 0.2 ml of the host organism that had reached an $O.D._{620}$ of 0.2. The plate was incubated at 28°C for 48 hours and observed for the presence of plaques. The same procedure was followed for an agar-plug extraction of the turbid region of plaque type A, and also for the central and peripheral regions of plaque type B.

Photography of Plaques

In preparation for the photography of plaques, G. oxydans subspecies suboxydans (ATCC 621) was inoculated into a 5 ml roller drum tube and grown to an $O.D._{620}$ of 0.9. Two-tenths milliliter of this culture plus 0.1 ml of an appropriate dilution of the phage was plated utilizing the agar overlay technique as previously described. The bacterial culture was grown to an $O.D._{620}$ of 0.9 instead of the standard 0.2, because the higher density allowed for greater contrast between the plaque and the bacterial lawn, and this provided better photographic definition of the plaques.

The plate to be photographed was mounted on a ring stand so that the ring was in a vertical position. A hole with the diameter of a petri plate was cut in the center of a piece of black construction paper. This piece of paper was taped onto the ring so that the petri dish fit snugly through the hole. Black electrician's tape was used to prevent light from leaking in between the edge of the petri plate and the construction paper. This apparatus was placed between a glow box and a Pentex 35 mm camera. Since contrast between the plaque and the bacterial lawn was so low, the room was darkened during photographing of plates, allowing light from the glow box to penetrate the plaques, thus achieving maximum photographic contrast. Plaques were photographed with Kodak SO-410 monochrome film, and the film was developed using HC110 developer (dilution D). The negatives were enlarged for printing using an Omega Pro-lab enlarger (model D-6) and a 50 mm lens. Printing was done on Kodak A light weight Ektamatic SC paper.

Electron Microscopy

Coating of Grids with Parlodian Support Films. Rhodium-backed copper mesh grids (400 mesh, Ernest F. Fullam, Inc.) were cleaned by placing about 50 grids in a 250 ml beaker containing 25 ml of concentrated HCl, and swirling once. Fifty milliliters of double distilled water (ddH_2O) was then immediately added, swirled, and the entire acidic solution decanted. The grids were rinsed 4-5 times with an identical volume of ddH_2O, rinsed once with acetone, and air dried on filter paper.

Parlodian was dissolved in amylacetate overnight (10% w/v). A Buchner funnel was fitted with a clamped piece of rubber tubing. The funnel was filled with distilled water and the clamp released and re-closed so as to remove all air from the tubing. Three test tube caps were placed on the bottom of the funnel and covered by a circle of aluminum screening. The acid-cleaned grids were placed below the water surface, copper-side-up, onto an aluminum screen. Additional ddH_2O was added until the funnel was as full as possible without running over. A drop of 10% Parlodian was added to the water surface, and, after it had spread, the film was pulled off the surface with a wooden applicator stick. After removing surface dust in this manner, another drop of 10% Parlodian was added. As soon as the Parlodian crinkled around the edges, the water was rapidly released into a beaker. The Parlodian-coated grids were allowed to dry overnight before being carbon coated in a Kinney (Model KDG-N) vacuum evaporator. The evaporation of carbon was executed under a pressure of 0.2 μ Hg with an alternating current of approximately 30 amp at 15 v passing through the electrodes, as recommended by Hyat (45).

Procedure for Sampling and Staining Grids. A lawn exhibiting barely confluent lysis was prepared as previously described. One drop of TMV (0.1 mg/ml) in 0.01 M phosphate buffer was diluted in 9 drops of doubly distilled H_2O and added to a parlodian- and carbon-coated grid. A drop of ddH_2O was added to the plate on which the bacterial lawn showed barely confluent lysis. The agar overlay beneath this drop of H_2O was probed gently with a sterile toothpick. The toothpick was then touched to the buffer-TMV suspension on the grid and moved in

a circular fashion for 30 seconds. The drop was allowed to stand for 10 minutes, and the excess fluid was removed with cut pieces of filter paper. Another drop of ddH_2O was added to the grid and immediately drawn off with filter paper. One drop of 1% uranyl acetate was applied to the grid and allowed to stand for 7 minutes. The stain was removed with filter paper, and the grids were allowed to dry.

Observations. All electron microscopic observations were made with a Jelco 100C electron microscope at an accelerating voltage of 80 KV. Kodak Electron Image Film 4463 was used for photographing the images, and the film was developed with Kodak D-19 developer (diluted 1:2). The negatives were enlarged for printing with an Omega Pro-lab enlarger, model D-6, using a 50 mm lens. Printing was done on Kodak A-surface, light weight, Ektamatic, SC paper.

Use of TMV for the Determination of Phage Size. TMV was placed on coated grids along with the phage in preparation for electron microscopy, as previously described. The diameters of fifty TMV particles were measured with a loupe on electron micrographs taken at a magnification of 20,000 X. A histogram of these measurements was constructed, and the diameter of highest frequency was chosen to represent 15 nm (38), a value characteristic for the width of a TMV particle (43). It was then possible to calculate the number of nanometers represented per millimeter measured from a micrograph print. From this conversion factor, it was also possible to calculate the dimensions of the phage particles in nanometers from the measurements made from the micrograph prints.

Host Range Studies

The procedure for growing bacteria for host range studies was the same for strains of Gluconobacter, Acetobacter, and Pseudomonas. The only exception was: Gluconobacter and Acetobacter species were grown in the 5% sorbitol medium previously described, and Pseudomonas species were grown in nutrient broth (Difco).

All strains were provided by C.A. Baker from the Acetic Acid culture collection maintained in our laboratory.

Some bacterial strains were obtained from liquid nitrogen stocks. One milliliter of these stocks was inoculated into roller-drum tubes, containing 5 ml of sterile growth medium, and incubated on a roller drum at 28°C for approximately 8-10 hours, in order to allow the cells adequate time to begin actively growing. The cultures were then diluted back to about 0.1 $O.D._{620}$ and allowed to incubate at 28°C on a roller drum until 0.2 $O.D._{620}$ was reached.

Strains that were obtained from slants were inoculated with a loop into roller-drum tubes containing 5 ml of the growth medium. The cells were incubated at 28°C on a roller drum until 0.2 $O.D._{620}$ was reached.

Other strains were obtained from sorbitol stocks. A 0.05 ml volume of a stock was inoculated into a roller drum tube containing 5% sorbitol medium. This tube was incubated at 28°C on a roller drum until 0.2 $O.D._{620}$ was reached.

After the bacteria had been cultured by one of the procedures above, the cells were then ready to be inoculated onto lawns. A 0.2 ml

volume of cells (0.2 $O.D._{620}$) was the standard inoculum for host range lawns.

Lawns of the test organisms were spotted with one drop of a filtered stock of GW6210 ($\sim 10^8$ PFU/ml). If a clear zone of lysis developed, lawns of the test organism were again prepared and spotted with each dilution of the phage stock ranging from 10^{-1} to 10^{-9}. These lawns were then observed for plaques.

Concentration of Phage by Polyethylene Glycol Precipitation

A phage stock of approximately 100-130 ml was prepared by the extraction of plates exhibiting barely confluent lysis as previously described. The polyethylene glycol (PEG) precipitation procedure used was as described by Yamamoto (86). One hundred milliliters of the phage stock was incubated with 0.03 mg/ml each of Bovine Spleen DNAse (Sigma, St. Louis, MO) and Bovine Pancreas RNAse (Sigma, St. Louis, MO), for one half hour at 28°C. Sodium chloride was added to a final molarity of 0.5 M. The stock was equally divided into three 33 ml volumes. Each volume was mixed with 10% (w/v) PEG 6000 (Baker, Phillipsburg, NJ) in a 50 ml Sorvall centrifuge tube. The tubes were allowed to stand at 4°C overnight. These mixtures were spun at 8000 xg for 10 minutes in a Sorvall RC2B centrifuge (SS-38 rotor) at 4°C. The supernatant fluid was recovered by aspiration. All pellets were resuspended and pooled in a total of 1 ml of 5% sorbitol medium. This preparation will be referred to as the "PEG concentrate".

Determination of Phage Density

A phage stock was prepared and concentrated with PEG as previously described. A step gradient was prepared as follows: Five milliliters of cesium chloride (CsCl) with a density of approximately 1.5 g/ml, was pipetted into the bottom of a 17 mm nitrocellulose ultracentrifugation tube. Two milliliters of 1.0 M sucrose was layered on top of the CsCl. One-tenth milliliter of phage sample ($\sim$ 10^{11} PFU/ml) was layered over the sucrose. The gradient was centrifuged using an SW27 swinging bucket rotor for 24 hours at 26,000 rpm in a Beckman L5-50 ultracentrifuge. The position of the resulting visible band in the gradient was approximately 8 mm from the bottom of the tube, or 1/3 the distance from the bottom of the tube to the sucrose-CsCl interface. The band was removed with a pipette and mixed with 5 ml of CsCl to give a final density of 1.5 g/ml. This was put into a 5 ml nitrocellulose tube and centrifuged for 24 hours utilizing an SW50.1 swinging bucket rotor. The resulting gradient was fractionated with an ISCO fractionator (Model 184). The density of the fractions was determined with a Bausch and Lomb refractometer (Model ABBE-3L). The absorbance of the fractions was read with a Gilford spectrometer (Model 240) at 280 nm.

Colorimetric Estimation of Phage Nucleic Acid

The procedure for the colorimetric estimation of phage nucleic acid involved a phage-adapted modification (R.C. Bates, et al, personal commun.) of Marmur's technique (56) for the extraction of DNA from microorganisms and Shatkin's procedure (68) for the diphenylamine

colorimetric reaction. These procedures are briefly described below.

Phage Sample Preparation. Fifty plates of phage-infected G. oxydans subspecies suboxydans exhibiting barely confluent lysis were prepared and extracted as previously described. Several 5 ml nitrocellulose tubes containing this phage stock were spun at 30,000 rpm for 2 hours to sediment the phage. Each resulting pellet was resuspended in 2.0 ml of 0.01 M TRIS, pH 8.0. Each 1.0 ml of these suspensions was called "phage sample".

Phenol Extraction of Phage Nucleic Acid. Since Marmur was interested in protecting cellular DNA from nucleases, following lysis of the cells by sodium lauryl sulfate (SLS), he added EDTA as a chelator to remove divalent cations essential for the activity of nucleases. No chelator was added in the phage-adapted nucleic acid extraction performed in this study, for cellular DNA was to be removed from the phage sample. For this same reason, the cofactors for deoxyribonuclease (DNAse), $CaCl_2$ and $MgCl_2$, were added at a concentration of 0.5 mM to 2.0 ml of the phage sample, and 100 µg/ml of Bovine Spleen DNAse II (Sigma, St. Louis, MO) was added. This mixture was incubated for 1 hour at 37°C. One percent SLS was added and incubated for 30 minutes at 37°C to dissociate the phage DNA from phage protein. An equal volume of phenol, a strong deproteinization agent, was added per unit volume of sample and was mixed by repeated inversion for 3 minutes at room temperature. This mixture was centrifuged at 3000 rpm for 5 minutes in a Damon IEC HN-S centrifuge, to break up the emulsion. The upper aqueous phase was withdrawn by aspiration

with a Pasteur pipette whose tip was bent back 180°. This phenol extraction procedure was repeated twice.

A control experiment was performed to test the activity of the DNAse on cellular DNA. To artificially break up uninfected cells, the culture was exposed to ultrasonic sound waves of 90 watts for six 15-second bursts at 30-second intervals, using a Virsonic Cell Disrupter, model 150. This brie was then centrifuged at 4000 rpm with a Damon IEC HN-S centrifuge for 20 minutes to remove unbroken cells. The supernatant fluid was then subjected to the same phenol extraction procedure as was the phage stock.

Ethanol Precipitation of Phage Nucleic Acid. Sodium acetate was added to phenol extracts to a final concentration of 0.3 M. Cold 95% ethanol was then added to a volume equal to 2.5 times the volume of the extract. This mixture was allowed to stand overnight at -20°C. The resulting alcohol-precipitated nucleic acid was centrifuged for 10 minutes at 10,000 rpm in a Sorvall RC2B centrifuge (SS-B4 rotor) at -2°C using a rotor. The supernatant fluid was decanted, and the pelleted nucleic acid was resuspended in 2.0 ml of 0.01 M TRIS, pH 8.0.

Diphenylamine Reaction. A standard curve was constructed using calf thymus DNA (Calbiochem, San Diego, CA) for the purpose of estimating nucleic acid quantities (Fig. 2). A series of dilutions of the DNA were made. Final reaction mixtures contained 2 ml of diphenylamine reagent (Shatkin, 1969), 0.01 ml of aqueous acetaldehyde (16 mg/ml) and 1 ml of a diluted DNA sample. These mixtures were allowed to stand for 20 hours at room temperature. Absorbance$_{600}$ was read with

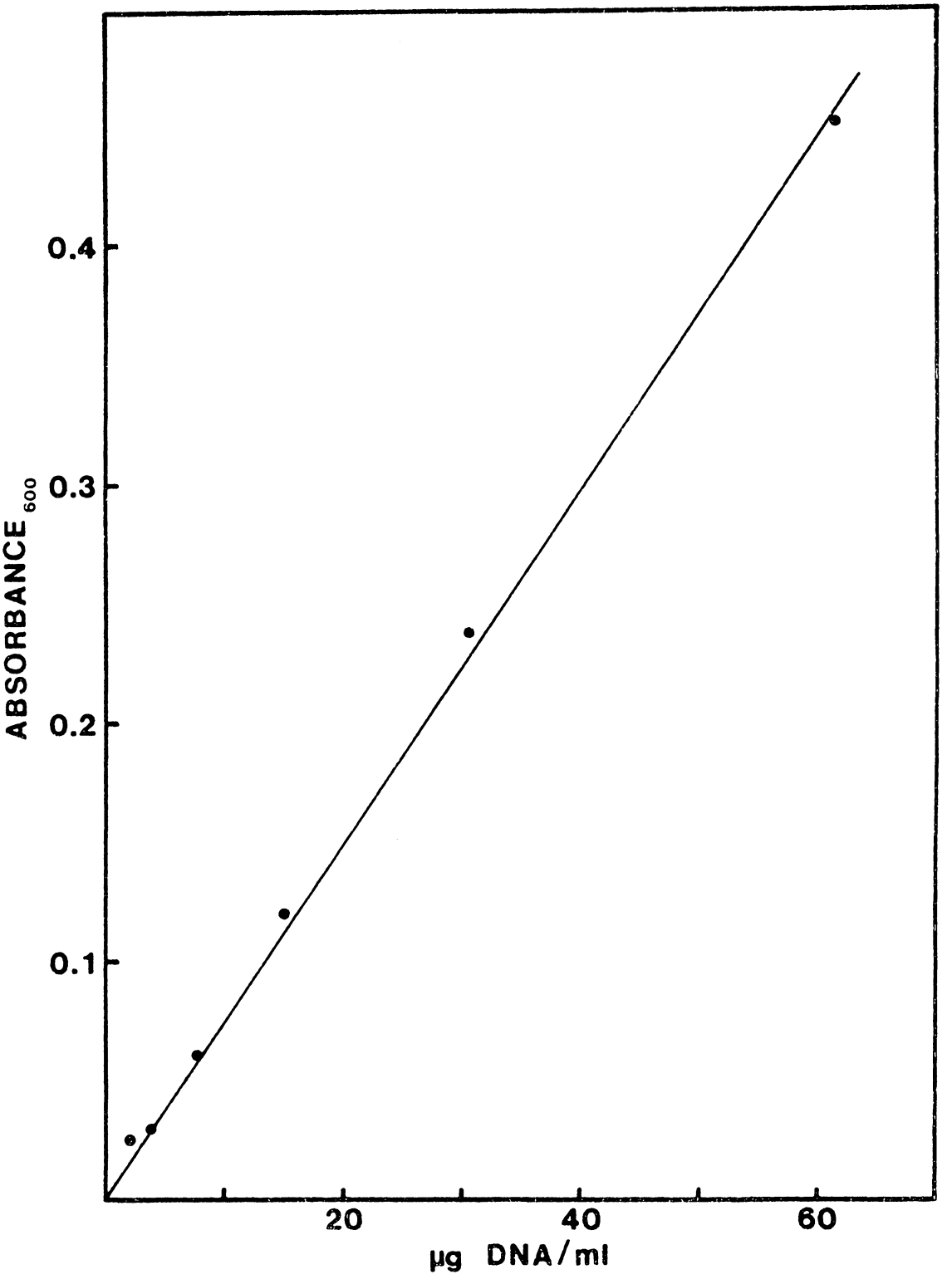


Fig. 2. Standard curve of calf thymus DNA.
This curve was constructed using Calf Thymus DNA for the purpose of estimating phage nucleic acid quantities. Each point represents a single determination.

a Bausch and Lomb Spectrophotometer 20 (standard model 33-31-71). Voltage was externally regulated with a Bausch and Lomb 33-29-10 voltage regulator. Reaction mixtures containing phage of cellular DNA were prepared as described for the standard curve DNA samples.

Radioactive Labeling of Phage Nucleic Acid

The first attempt to incorporate label into the phage nucleic acid, required the propagation of this phage in liquid media. The host organism was grown in two roller drum tubes, each containing 5 ml of 5% sorbitol medium until 0.2 $O.D._{620}$ was reached in both tubes. Four milliliters of the cell culture was infected with 1.14 ml of a phage stock (3.5×10^9 PFU/ml) to give a multiplicity of infection of 5.0. Simultaneous to the addition of phages to the cells, was the introduction of label. To one tube, 5 µci of ^{3}H-thymidine (ICN Pharmaceuticals, Irvine, CAL) was added. The other tube received 5 µci of ^{14}C-uridine (Amecsham Corp., Arlington Heights, ILL). After 10 hours of incubation on a roller drum at 28°C, the cells were centrifuged at 4000 rpm for 20 minutes in an IEC HN-S clinical centrifuge. A 0.10 ml fraction of the supernatant fluid was layered over 5 ml of CsCl and centrifuged for 24 hours at 35,000 rpm. The gradient was fractionated with an ISCO gradient fractionator. Each fraction was tested for radioactivity utilizing a Beckman LS-230 Liquid Scintillation Counter.

The second attempt to incorporate label into phages replicating in liquid media, required growing the cells as described above, only to an $O.D._{620}$ of 0.1. Then 25 µci of ^{3}H-thymidine was added to one tube of cells, and 10 µci of ^{14}C-ruidine was added to the other tube

of cells. The cells were allowed to grow (approximately 1 hour) until they reached 0.2 $O.D._{620}$. At this time, 1.14 ml of a phage stock (3.5×10^9 PFU/ml) was added to the cells to give a multiplicity of infection of 5.0. Also at this time, the tube containing labeled thymidine received an additional 25 µci of ^3H-thymidine. The tube containing labeled uridine received an additional 10 µci of ^{14}C-uridine. Following 10 hours of incubation, the cells were recovered by centrifugation as previously described. The same procedure as used in the first attempt for making the gradient and assaying for radioactivity was followed.

The third attempt to incorporate label into the phages required propagation of the phages on solid media. The host organism was grown in 2 tubes to 0.1 $O.D._{620}$ as previously described. At this point, one tube received 16 µci of ^3H-thymidine. The other tube received 10 µci of ^{14}C-uridine. The cells were allowed to grow to 0.2 $O.D._{620}$. Sixty microcuries of additional ^3H-thymidine was added to 1 ml of the cell suspension containing ^3H-thymidine. Thirty microcuries of additional ^{14}C-uridine was added to 1 ml of the cell suspension containing ^{14}C-uridine. Each of these two 1-ml suspensions fortified with label was used in a separate agar overlay techniques to produce 5 plates of barely confluent lysis. Each plate consisted of a radioactive host inoculum of 0.25 ml infected with 3.5×10^8 PFU's. Following 24 hours of incubation, the plates were extracted as previously described. Cellular debris was removed by centrifugation at 4000 rpm for 20 minutes on an IEC HN-S clinical centrifuge. The lysate was dialyzed against 0.01 M TRIS to remove free radioactive nucleotides.

Ten microliters of the tritium-containing concentrate was counted with a liquid scintillation counter and contained only 79 counts/minute. Only 251 counts/minute were detected in 50 µl of the concentrate containing ^{14}C. Because of these low counts, fractions of gradients of these concentrates were not assayed for radioactivity.

RESULTS

Plaque Morphology

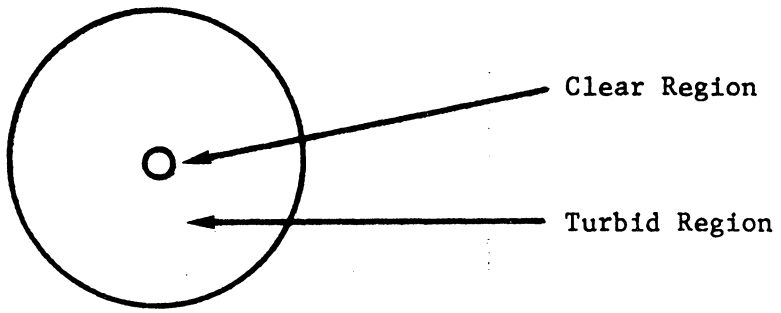
Utilizing the standard overlay method for the preparation of bacterial lawns (3), the phage-infected plates showed two different plaque types (Fig. 3). The clear, pinpoint plaque surrounded by a large turbid region is referred to as plaque type A, and the small turbid plaque is called plaque type B.

The morphological regions of the two plaque types are diagrammed in Fig. 4. The pinpoint zone of lysis in the center of plaque type A is designated as the clear region. The zone surrounding the clear region is called the turbid region. The two areas of plaque type B are referred to as the central region and the peripheral region.

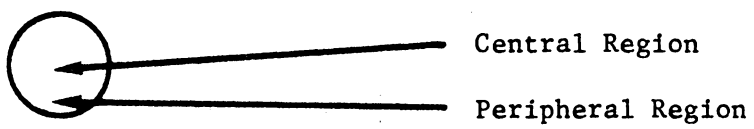
In characterizing the two plaque types, diameter measurements were made after 72 hours of incubation (Table 2). The clear region of plaque type A had a mean diameter of 0.3 mm and was surrounded by a turbid region 3.5 mm in diameter. The small turbid plaque, type B, had a mean diameter of 1.4 mm. The clear region of plaque type A deviated little from the mean diameter, whereas the turbid regions of both plaque types exhibited large standard deviations.

Since the initial observations indicated that there were two plaque types, attempts were made to separate two phage by serially picking and plating from the B type plaques, as shown in Fig. 5. The relative percentages of plaque types A and B on the resulting plates fluctuated between 40 and 60%. No significant separation trend was observed. Failure to separate plaque types by this method therefore,

Fig. 3. Lawn of Gluconobacter oxydans subspecies suboxydans infected with GW6210. Two plaque types may be seen: a clear pinpoint plaque surrounded by a large turbid region (Type A), and a small turbid plaque (Type B). Magnification = 3.2 X.



Plaque Type A



Plaque Type B

Fig. 4. Morphological regions of plaque types A and B.

Table 2. Plaque Diameter Measurements of GW6210[a]

Plaque Type	Area Measured	No. Plaques Measured	Mean Diameter(mm)	Standard[b] Deviation(mm)	Range[c] (mm)
A	Clear center	100	0.3	0.1	0.2-0.5
A	Turbid zone	100	3.5	0.7	1.9-5.0
B	Turbid zone	429	1.4	0.8	0.3-4.0

[a]All diameter measurements were made after 72 hours of incubation. Plaques were measured with dial calipers.

[b]Standard deviations were calculated with use of the following formula (75):

$$\sigma = \sqrt{\frac{\Sigma(x)^2}{N}}$$

where, σ = standard deviation
x = deviations from arithmetic mean $(x - \bar{x})$
N = total number of items $\Sigma(f)$

[c]The range represents the smallest diameter measurement to the largest diameter measurement.

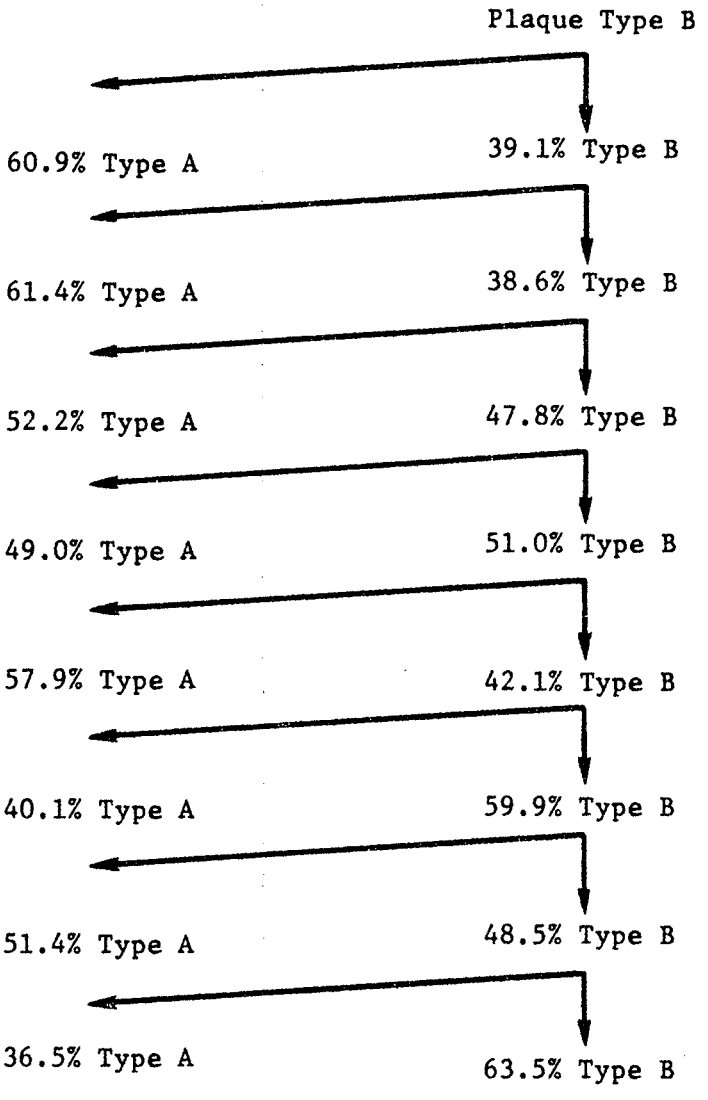


Fig. 5. Attempts to separate plaque types A and B by serially picking and plating the B type plaque. Relative numbers of plaque types A and B are reported as percentages.

indicated that there was another explanation for the presence of two plaque types, possibly size variation of a single type of plaque.

Several experiments were performed to test this hypothesis. If the plaques differed only in size, they would have several characteristics in common. One of these characteristics would be the presence of phage in the same morphological regions of both plaques. Infectivity assays performed on extracts of specific regions of each plaque type indicated that phage were found only in the clear region of plaque type A (Table 3). Plaque type B also showed phage to be present only in the central region (Table 3). It is possible, then, that a central zone of lysis was visible for the larger plaque type A, but was below the threshold of visibility for the smaller plaque type B. Also supporting this possibility was the data shown in Table 4. that indicates that the clear region of plaque type A did not increase in diameter from 48 to 72 hours of incubation. If the larger plaque type A had reached its maximum size, it follows that the smaller plaque type B, could have possessed a zone of lysis below the threshold of visibility. Correlating well with this finding, the corresponding rates of increase in diameters of the turbid zones of plaque types A and B, indicated another similarity between the two plaque types. The turbid region of plaque type A increased from 3.0 mm at 48 hours, to 3.8 mm at 72 hours. This is a 27% increase in diameter. There was a very similar 29% increase in diameter of the entire plaque type B. Type B increased from 1.5 mm at 48 hours, to 1.94 mm at 72 hours. This similarity could have been the diffusion

Table 3. Location of GW6210 in Plaque Types A and B

Plaque Type	Area of Extraction[a]	Replicate	No. Plaques		
			Trial 1	Trial 2	Trial 3
A	Clear center	1	TNTC[b]	TNTC	TNTC
		2	TNTC	TNTC	TNTC
		3	TNTC	TNTC	TNTC
		4	TNTC	TNTC	TNTC
		5	TNTC	TNTC	TNTC
A	Turbid zone	1	0	0	0
		2	0	0	0
		3	0	0	0
		4	0	0	0
		5	0	0	0
B	Central area	1	108	3	TNTC
		2	108	3	TNTC
		3	120	3	TNTC
		4	167	2	TNTC
		5	104	7	TNTC
B	Periphery	1	0	0	0
		2	0	0	0
		3	0	0	0
		4	0	0	0
		5	0	0	0

[a] Refer to Fig. 4.

[b] TNTC = plaques in numbers too numerous to be counted. A 10^{-1} dilution of phage from a single plaque was plated with the host organism utilizing the standard overlay technique (3).

Table 4. Plaque Diameter Measurements at 48 hrs. and at 72 hrs.

Plaque Type	Area of Plaque Measured[a]	Diameter at 48 hrs. (mm)	Diameter at 72 hrs. (mm)
A	Clear region	0.4 ± 0.1[b]	0.4 ± 0.1
A	Turbid region	3.0 ± 0.3	3.8 ± 0.4
B	Turbid region	1.5 ± 0.4	1.94 ± 0.5

[a] Turbid regions of plaque types A and B were measured with dial calipers. The clear region of plaque type A was measured with a loupe.

[b] Standard deviations were calculated with use of the following formula (3):

$$\sigma = \sqrt{\frac{\Sigma(x)^2}{N}}$$

where, σ = standard deviation
x = deviations from arithmetic mean $(x - \bar{x})$
N = total number of items $\Sigma(f)$

rate of a common hydrolytic enzyme, whose spread throughout the bacterial lawn, resulted in a zone of partial lysis.

Additional support of these findings became apparent with electron microscopic examination of the phage stocks. The phage particles appeared morphologically identical. It should be recalled from Fig. 5 that the relative percentages of A type plaques to B type plaques ranged from 40-60%. If the 2 plaque types were caused by morphologically different phages, one would expect to see approximately the same percentage of morphological heterogeneity in the phage stock as observed by the electron microscope. However, this was not observed. Observation of crude samples showed also a population of phage that were homogeneous morphologically.

All of the experiments mentioned above, those dealing with plaque morphology, the failure to separate the two plaque types and the electron microscopic observation of the phage samples, all indicate the presence of only one type of phage, regardless of the presence of two plaque types. With this understanding of the plaque morphology, the investigator felt that the next appropriate step in the characterization of this phage, was the analysis of phage morphology.

Phage Morphology

Initial electron microscopic observations of GW6210 by C.A. Baker indicated that this phage should be placed morphologically into Bradley's group A (Fig. 1). This group contains phage with polygonal heads and contractile tails (15).

In determining phage dimensions, Tobacco Mosaic Virus (TMV) was utilized as an internal standard as suggested by Bos (1975), and by Ford (38). Electron micrographs were taken of mixtures of TMV and GW6210. Width measurements of TMV particles were measured with a loupe directly on the micrograph prints at 60,000X. All loupe measurements were accurate within 1.7 nm. Histograms were constructed and the diameter measurement of highest frequency was used to represent 15 nm, the standard width of a TMV particle (43). Measurements of the length and width of the phage head and tail length were made with a metric ruler on photographs printed at 60,000X; these measurements were accurate within 9.0 nm. Tail width was measured with a loupe on prints of the same magnification. Fifty measurements of each dimension were made. These values were then converted to nanometers by utilizing the internal standard. The length of the phage was 306 nm, in other words it was found to be over 1/5 the length of its 1600 nm host (Fig. 6). The phage head was 170 nm in both length and width. The tail was 136 nm long and 34 nm in diameter.

A typical head conformation seen in electron micrographs is shown in Fig. 7. This micrograph also illustrates a collar located at the base of the head. Other structures observed but not illustrated included tail pins extending off the base plate and a tail structure composed of a central core surrounded by a contractile sheath. The tail sheath is shown in a contracted state in Fig. 8, which also illustrates the central core being exposed.

In an attempt to purify phage GW6210, a polyethylene glycol concentrate of the phage was layered over a sucrose-CsCl step gradient.

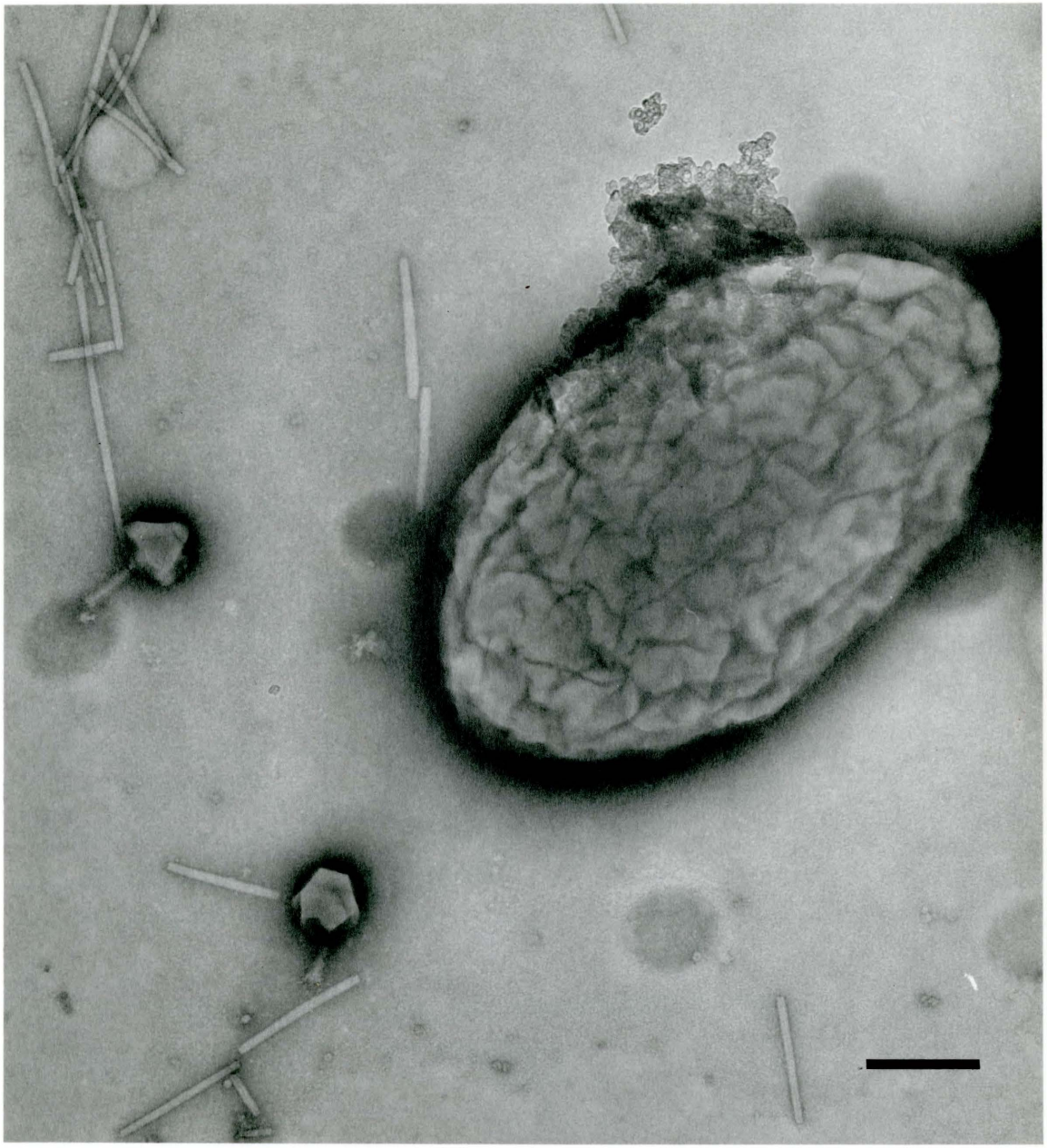


Fig. 6. Bacteriophage GW6210 and its host, Gluconobacter oxydans subspecies suboxydans. The rods present in the micrograph are tobacco mosaic virus (TMV) particles that were used as internal standards. The bar represents 200 nm.

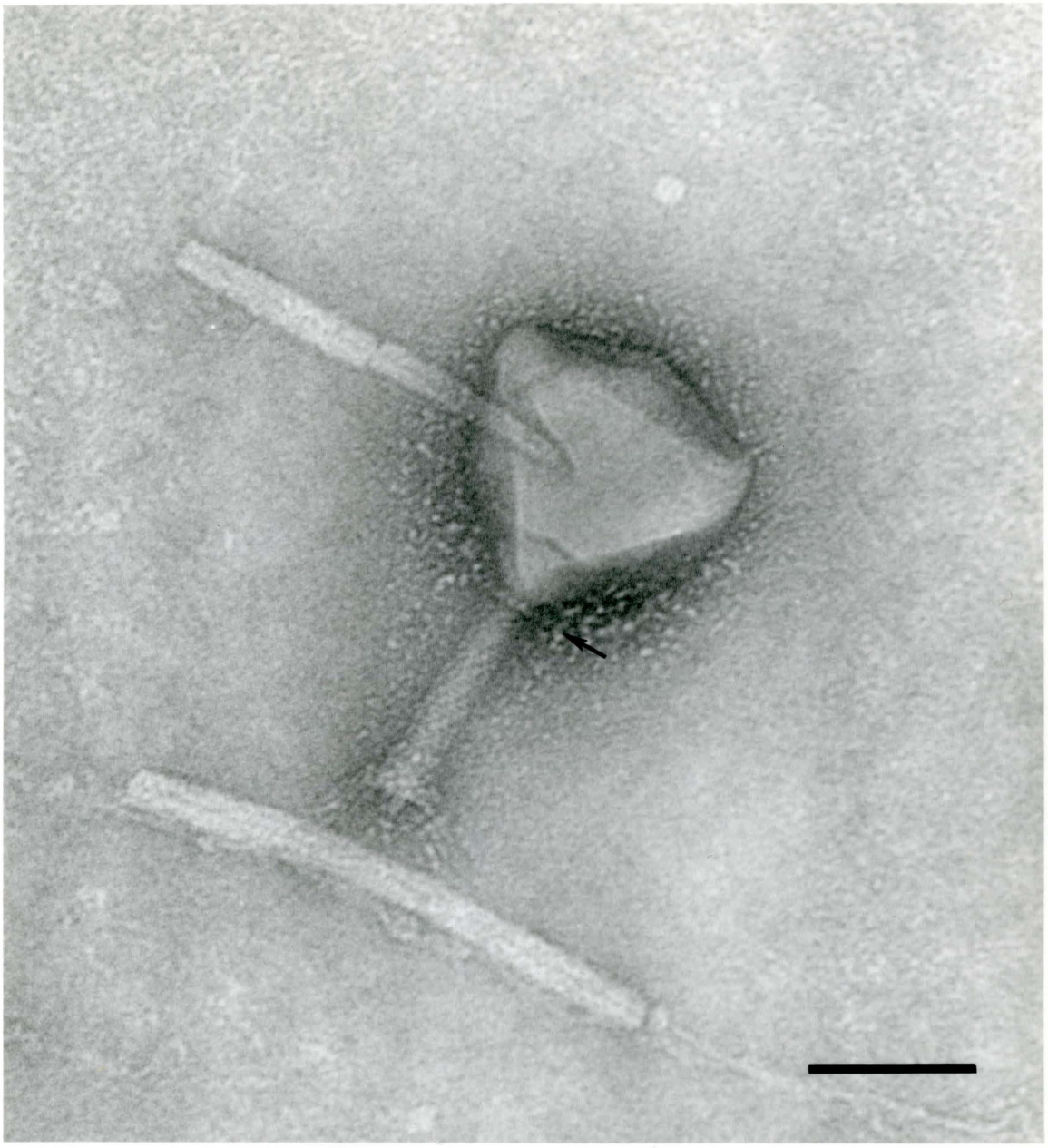


Fig. 7. Bacteriophage GW6210 exhibiting typical head conformation. The collar at the base of the head is indicated by the arrow. The rods are TMV particles used as internal standards. The bar represents 100 nm.

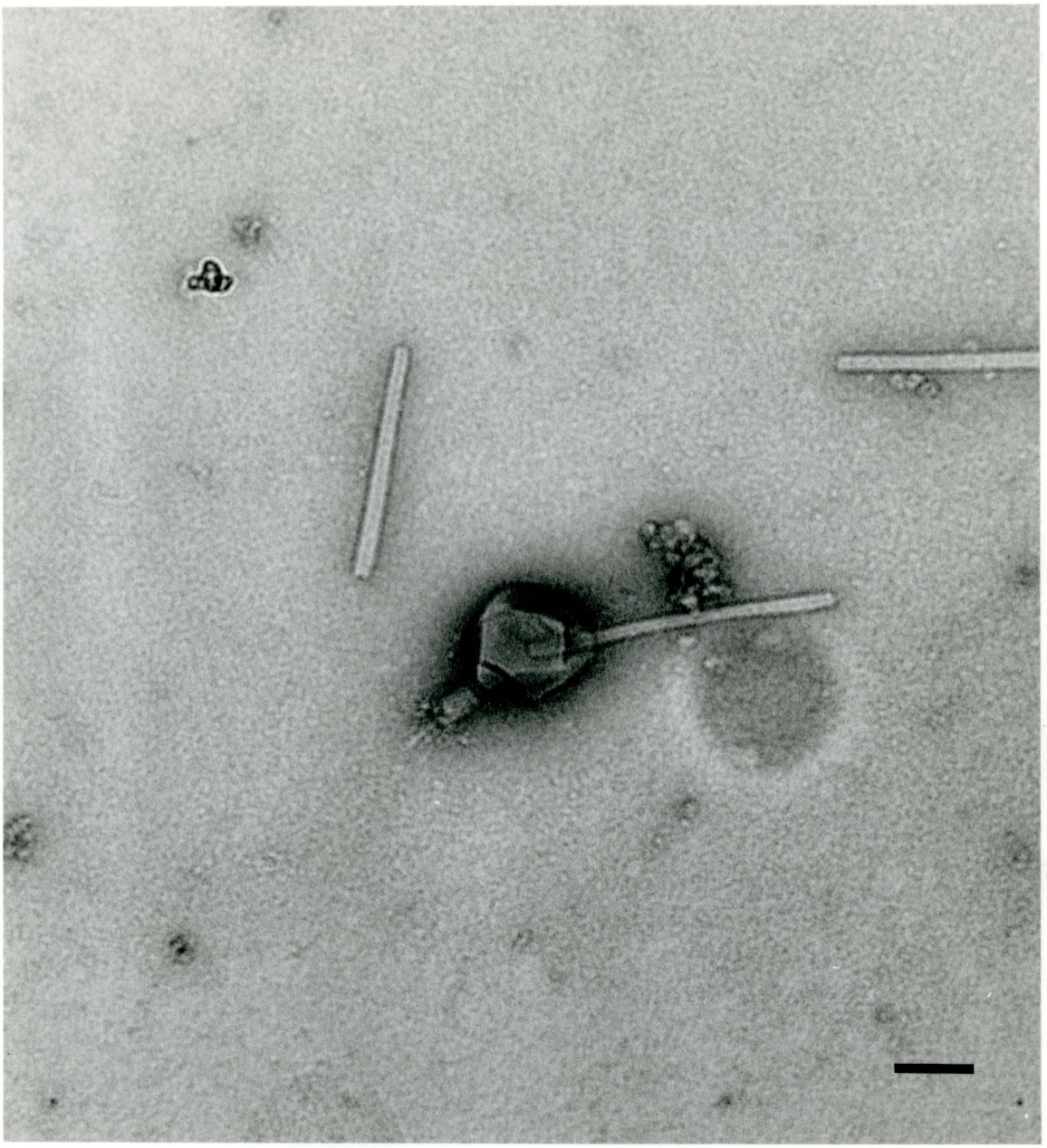


Fig. 8. Bacteriophage GW6210 exhibiting a contracted tail sheath, exposing the central core. The rods present are TMV particles used as internal standards. The bar represents 100 nm.

The resulting band that was formed in the gradient was extracted and examined electron microscopically. The material in these bands contained GW6210 particles, as well as structures that resemble the purified tail sheaths (Fig. 9) described by Horne (43).

Host Range Study

Fifty-three strains of Gluconobacter, 52 strains of Acetobacter, and three strains of Pseudomonas were tested for susceptibility to infection by GW6210. These three genera of organisms are similar in that they are Gram negative and can grow at low pH levels. The criteria for choosing these strains were: (A) the availability of the strains in our bacterial collections, (B) the listing of these strains in Bergey's Manual of Deteminative Bacteriology, and (C) in the case of the acetic acid bacteria, the degree to which each strain conformed to reactions of biochemical tests typical of the bacterial genera.

The fifty-three strains of Gluconobacter and their reactions with the phage are listed in Table 5. The 52 strains of Acetobacter tested are listed with their reactions in Table 6. Table 7 summarizes the positive data listed in Tables 5 and 6. The first organism listed in Table 7 was the host organism used to propagate the phage. The next three organisms listed in Table 7, G. suboxydans var. α, A. melanogenus, and G. rubiginosis, were the only organisms found to form distinguishable plaque when infected by GW6210. Of the remaining 50 Gluconobacter tested (Table 8), 45 strains were unaffected by the phage. This type of reaction was called "negative". Lawns of five strains developed areas of lysis when spotted with the phage stock,

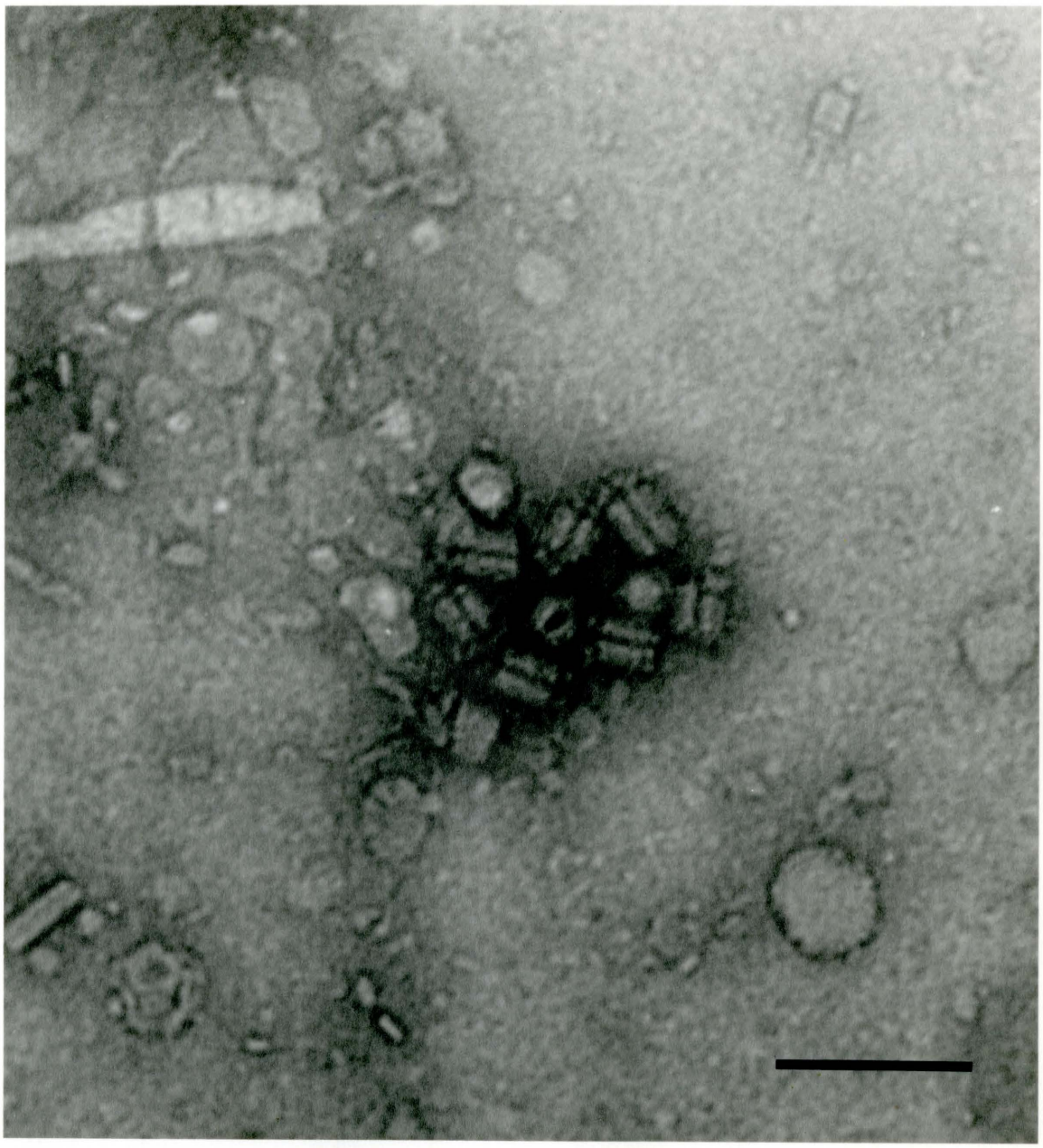


Fig. 9. Electron micrograph of partially purified GW6210 following equilibrium centrifugation, showing tail sheath-like structures. This material was found in the visible band came from the sucrose-CsCl gradient. The bar represents 200 nm.

Table 5. Host Range of Phage GW6210 on *Gluconobacter* species

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
oxydans	*suboxydans*	*G. oxydans* subsp. *suboxydans*	Wellcomb	1221	-	-
		G. oxydans subsp. *suboxydans*	NCIB	9498	-	-
		G. oxydans subsp. *suboxydans*	ATCC	12303	-	-
		G. oxydans subsp. *suboxydans*	ATCC	14960	-	-
		G. oxydans subsp. *suboxydans*	ATCC	19441	-	-
		G. oxydans subsp. *suboxydans*	ATCC	23771	+	-
		G. oxydans subsp. *suboxydans*	ATCC	23773	+	-
		G. oxydans subsp. *suboxydans*	ATCC	23777	-	-
		G. suboxydans var. α	IFO	3254	-	-
		G. suboxydans var. α	IFO	3255	-	-
		G. suboxydans var. α	IFO	3256	-	-
		G. suboxydans var. α	IFO	3257	-	-
		G. suboxydans var. α	IFO	3258	-	-
		G. suboxydans var. α	IFO	3289	-	-
		G. suboxydans var. α	IFO	3290	-	-
		G. suboxydans var. α	IFO	12528	+	+
		G. suboxydans var. α	IFO	3291	-	-
		A. suboxydans	ICPB	3313	-	-
		A. suboxydans subsp. *hoyerianum*	Wellcomb	1150	-	-
		G. dioxyacetonius	IFO	3271	-	-
		G. dioxyacetonius	IFO	3272	-	-
		G. dioxyacetonius	IFO	3273	-	-
		G. dioxyacetonius	IFO	3274	-	-
		G. nonoxygluconicus	IFO	3275	-	-
		G. nonoxygluconicus	IFO	3276	-	-

Table 5 (Continued)

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
oxydans	suboxydans	G. cerinius	IFO	3263	-	-
		G. cerinius	IFO	3264	-	-
		G. cerinius	IFO	3265	-	-
		G. cerinius	IFO	3266	-	-
		G. cerinius	IFO	3268	-	-
		G. cerinius	IFO	3269	-	-
		G. cerinius	IFO	3270	-	-
		A. albidus	IFO	3250	-	-
		A. albidus	IFO	3251	-	-
		A. albidus	IFO	3253	-	-
oxydans	oxydans	G. oxydans	IFO	3287	+	-
		G. oxydans subsp. oxydans	ATCC	15179	-	-
		G. oxydans subsp. oxydans	ATCC	19357	-	-
		G. oxydans subsp. oxydans	ATCC	23651	-	-
oxydans	industrius	G. industrius	IFO	3261	-	-
		G. oxydans subsp. industrius	ATCC	11894	-	-
		G. oxydans subsp. industrius	ATCC	11895	-	-
		G. oxydans subsp. industrius	ATCC	12302	-	-
		G. oxydans subsp. industrius	ATCC	23772	-	-
		G. oxydans subsp. industrius	ATCC	23775	-	-
		G. oxydans subsp. industrius	ATCC	23776	-	-
		A. capsularus	NRRL	B-1225	-	-
oxydans	melanogenes	A. melanogenus	ICPB	2096	+	+
		G. melanogenum	IFO	3294	+	-

Table 5 (Continued)

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
oxydans	melanogenes	G. oxydans subsp. melanogenes	ATCC	8147	-	-
		G. oxydans subsp. melanogenes	ATCC	9937	-	-
		G. oxydans subsp. melanogenes	NRRL	B-58	+	-
		G. rubiginosis	IFO	3244	+	+

[a]Strain of bacterium being tested for susceptibility to GW6210.

[b]One drop of a millipore filtered stock of GW6210 (10^8 PFU/ml) was applied to a lawn containing the test organism in exponential growth. Following 48 hr incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[c]The phage stock was serially diluted to a point where individual plaques should develop. One drop of each dilution (10^{-1} to 10^{-9}) was applied to a lawn of the test organism. Following incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

Table 6. Host Range of Phage GW6210 on *Acetobacter* Species

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
aceti	*aceti*	*A. aceti*	Guinness	c.100	-	-
		A. aceti	ICPB	2574	-	-
		A. aceti subsp. *aceti*	ATCC	15973	-	-
		A. aceti subsp. *aceti*	ATCC	23747	-	-
		A. aceti subsp. *aceti*	ATCC	23748	-	-
aceti	*orleanensis*	*A. aceti* subsp. *orleanensis*	NCIB	8746	-	-
		A. aceti subsp. *orleanensis*	ATCC	23755	-	-
		A. mesoxydans	Guinness	c.108	-	-
aceti	*xylinus*	*A. xylinum*	NRC	17010	-	-
		A. xylinum	NCIB	6658	-	-
		A. xylinum	NCIB	8756	-	-
		A. aceti subsp. *xylinus*	ATCC	10245	-	-
		A. aceti subsp. *xylinus*	ATCC	23768	-	-
		A. aceti subsp. *xylinus*	NCIB	6659	-	-
		A. aceti subsp. *xylinus*	NCIB	6661	-	-
aceti	*liquifaciens*	*A. aceti* subsp. *liquifaciens*	ATCC	14835	-	-
		A. aceti subsp. *liquifaciens*	ATCC	23749	-	-
		A. aceti subsp. *liquifaciens*	ATCC	23750	-	-
		A. aceti subsp. *liquifaciens*	ATCC	23751	-	-
pasteur-ianus	*pasteurianus*	*A. pasteurianus*	NCIB	6423	-	-
		A. pasteurianus	NCIB	6430	-	-
		A. pasteurianus	NCIB	8133	-	-
		A. pasteurianus	NCIB	8163	-	-
		A. pasteurianus	NCIB	8555	-	-

Table 6 (Continued)

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
pasteurianus	pasteurianus	A. pasteurianus	NCIB	8758	-	-
		A. pasteurianus	NCIB	8759	-	-
		A. pasteurianus	NCIB	8957	-	-
		A. pasteurianus	NCIB	9896	-	-
		A. pasteurianus	NRRL	B570	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	9432	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	12877	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	12879	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23650	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23758	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23759	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23760	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23761	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23764	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23765	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	23757	-	-
		A. rancens	Guinness	c.103	-	-
		A. rancens	ICPB	2573	-	-

Table 6 (Continued)

Species	Subspecies	Host[a]	Collection	Strain	Spot[b]	Plaques[c]
pasteurianus	pasteurianus	A. turbidans	Lock	86	-	-
		A. pasteurianus ssp. pasteurianus	ATCC	9322	-	-
		A. pasteurianus ssp. lovaniensis	ATCC	12875	-	-
		A. pasteurianus ssp. estunensis	ATCC	23753	-	-
		A. ascendens	NRRL	B-56	-	-
		A. ascendens	IFO	3299	-	-
		A. ascendens	ATCC	9323	-	-
		A. pasteurianus ssp. ascendens	ATCC	23754	-	-
		A. paradoxum	ATCC	23756	-	-
		A. peroxydans	ATCC	12874		

[a]Strain of bacteria being tested for susceptibility to GW6210.

[b]Using a Pasteur pipette, one drop of a millipore filtered stock of GW6210 (10^8 PFU/ml) was applied to a prepared lawn of the test organism. Following incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[c]The phage stock was serially diluted. One drop of each dilution (10^{-1} to 10^{-9}) was applied to a lawn of the test organism. Following 48 hour of incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

Table 7. Host Range of Phage GW6210 on Susceptible *Gluconobacter* Species[a]

Species	Subspecies	Host	Collection	Strain	Spot[c]	Plaques[d]
oxydans	*suboxydans*	*G. oxydans* subsp. *suboxydans*[b]	ATCC	621	+	+
		G. suboxydans var. α	IFO	12528	+	+
oxydans	*melanogenes*	*A. melanogenus*	ICPB	2096	+	+
		G. rubiginosis	IFO	3244	+	+

[a]Summarized from Table 5. Organisms are named as received from the culture collections.

[b]Host used for the propagation of GW6210 in the preparation of phage stock.

[c]Using a Pasteur pipette, one drop of a millipore-filtered stock of GW6210 (10^8 PFU/ml) was applied to a prepared lawn of the test organism. Following incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[d]The phage stock was serially diluted. One drop of each dilution (10^{-1} to 10^{-9}) was applied to a prepared lawn of the test organism. Following 48 hour incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

Table 8. Host Range of Phage GW6210 on *Gluconobacter* Species[a] Giving False Positive or Negative Tests

Species	Subspecies	Host[b]	Collection	Strain	Spot[c]	Plaques[d]
oxydans	*suboxydans*	32 strains			-	-
		G. oxydans subsp. *suboxydans*	ATCC	23771	+	-
		G. oxydans subsp. *suboxydans*	ATCC	23773	+	-
oxydans	*oxydans*	3 strains			-	-
		G. oxydans	IFO	3287	+	-
oxydans	*industrius*	8 strains			-	-
oxydans	*melanogenes*	2 strains			-	-
		G. melanogenus	IFO	3294	+	-
		G. oxydans subsp. *melanogenes*	NRRL	B-58	+	-

[a]Summarized results from Table 5. Organisms are named as received from the culture collections.

[b]Strain of bacterium being tested for susceptibility to GW6210.

[c]One drop of a millipore filtered stock of GW6210 (10^8 PFU/ml) was applied to a prepared lawn of the test organism. Following incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[d]The phage stock was serially diluted. Using a Pasteur pipette, one drop of each dilution (10^{-1} to 10^{-9}) was applied to a lawn of the test organism. Following 48 hour incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

but did not produce individual plaques upon being spotted with serial dilutions of the phage stock ranging from 10^{-1} to 10^{-9}. This type of reaction was called a "false positive" reaction.

The host range reactions of the Acetobacter are condensed in Table 9. This table indicates that all 52 strains of Acetobacter gave negative reactions when infected with the phage.

The only organisms tested that were not acetic acid bacteria, were three strains of Pseudomonas. These organisms are close taxonomically to the acetic acid bacteria in that they tolerate low pH levels and are gram negative rods. The three strains of Pseudomonas tested, as shown in Table 10, were P. vesiculare, P. diminuta ATCC 11568, and P. diminuta ATCC 13184. These strains were unaffected by GW6210.

The host range of GW6210 seems, therefore, to be limited to four strains of the genus Gluconobacter.

Determination of Phage Density

Ten attempts were made to determine the density of the phage by density gradient centrifugation. Several variations of techniques were tried. The technique normally used involved two density gradient centrifugation steps. The first density gradient consisted of two milliliters of one molar sucrose layered over five milliliters of CsCl of density 1.5 g/ml. The gradient was formed over 24 hours at 26,000 rpm using an SW 27 rotor. The visible band of material was then extracted from the gradient and mixed with 5 ml of CsCl with a density of 1.5 g/ml. After a 24 hour centrifugation at 35,000 rpm using an SW 50.1 rotor, the gradient was fractionated. The $absorbance_{260}$ of each

Table 9. Host Range of Phage GW6210 on *Acetobacter* Species[a] Giving Negative Tests

Species	Subspecies	No. Strains	Spot[b]	Plaques[c]
aceti	*aceti*	5	-	-
	orleanensis	3	-	-
	xylinus	7	-	-
	liquifaciens	4	-	-
pasteurianus	*pasteurianus*	25	-	-
	lovaniensis	1	-	-
	estunensis	1	-	-
	ascendens	4	-	-
	paradoxum	1	-	-
peroxydans	none given	1	-	-

[a]Summarized results for Table 6. Organisms are named as received from the culture collections.

[b]One drop of a millipore filtered stock of GW6210 (10^8 PFU/ml) was applied to a prepared lawn of the test organism. Following incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[c]The phage stock was serially diluted. Using a Pasteur pipette one drop of each dilution (10^{-1} to 10^{-9}) was applied to a prepared lawn of the test organism. Following 48 hour incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

Table 10. Host Range of Phage GW6210 on Selected *Pseudomonas* Species

Host[a]	Collection	Strain	Spot[b]	Plaques[c]
P. vesiculare	ATCC	11426	-	-
P. diminuta	ATCC	11568	-	-
P. diminuta	ATCC	13184	-	-

[a]Strain of bacterium being tested for susceptibility to GW6210.

[b]One drop of a millipore filtered stock of GW6210 (10^8 PFU/ml) was applied to a prepared lawn of the test organism. Following incubation, the lawn was examined for the presence of an area of bacterial lysis where the drop was applied.

[c]The phage stock was serially diluted. Using a Pasteur pipette, one drop of each dilution (10^{-1} to 10^{-9}) was applied to a lawn of the test organism. Following 48 hour incubation, the lawn was examined for the presence of individual plaques within each area where the drop was applied.

fraction was read with a Gilford spectrophotometer and plotted against fraction number as shown in Fig. 10. Refractive indices were also measured for each fraction to determine fraction densities (Fig. 10). Figure 10 also indicates that partial phage counts were made for the fractions containing the most UV absorbing material, as well as for fractions at three other points along the gradient that showed little UV absorbance. Since the fractions were so small (10 drops), two adjacent fractions were combined in order to determine a phage count. From Fig. 10, it would appear that the most of the infective particles represented by a phage count of 1.15×10^9 PFU/ml present in fractions 9 and 10, indicated the density of the phage to be 1.47 g/ml. However, the phage counts for fractions 12 and 13 were incomplete. It is therefore possible that the majority of infective particles were in fractions 12 and 13. Also, it should be noted that UV absorbing material was found to collect at different densities of CsCl during subsequent density gradient centrifugation. Also, when the step gradient was fractionated and the fractions were assayed for infectivity by spotting the fractions on lawns of G. oxydans subspecies suboxydans, most of the infective phage particles were detected in the sucrose layer. Therefore, it may have been possible that the phage was adsorbing to cellular material of differing densities.

Colorimetric Estimation of Phage Nucleic Acid

A phage sample (5×10^{11} PFU) was treated with DNAse prior to phenol extraction. In this preparation, only 13-15 µg/ml of DNA were detected (Table 11). DNA quantities were determined with the use of a

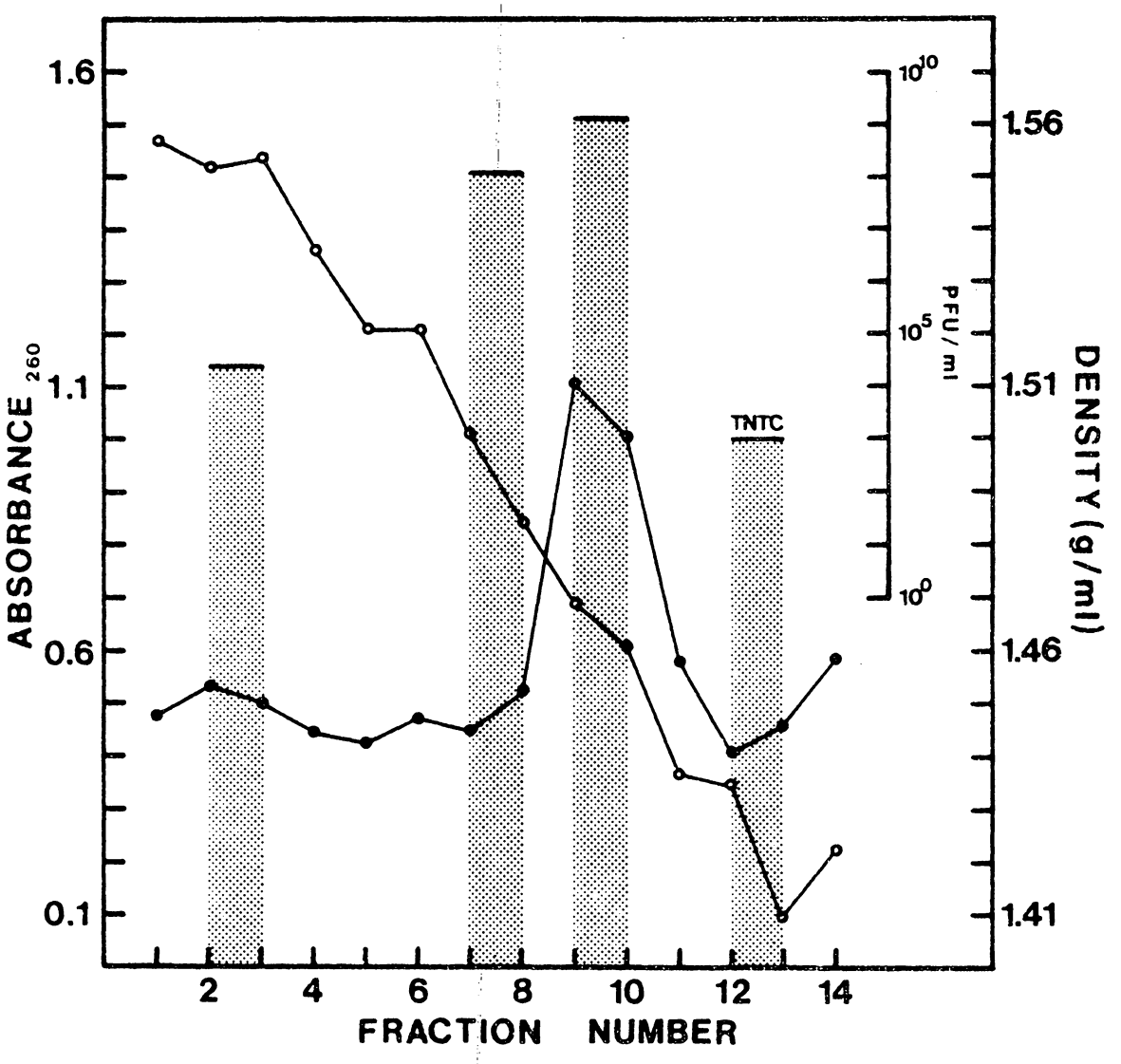


Fig. 10. Density, absorbance$_{260}$, and partial phage counts for fractions obtained from density gradient of phage GW6210. The closed circles (●) illustrate the position of nucleoprotein in the gradient (Absorbance$_{260}$) in relation to the densities of the fractions ($CsCl_2$, g/ml) represented by the open circles (o). The number of plaque forming units (PFU/ml) of combined fractions, represented by the stippled area (⁘), is also shown. TNTC = plaques in numbers too numerous to be counted.

Table 11. Colorimetric Estimation of Phage GW6210 Nucleic Acid

Source of Nucleic Acid	DNAse Treatment[a] in Phenol Extraction		Diphenylamine Reactions - DNA (μg/ml)[b]		
	Present	Absent	Replicate 1	Replicate 2	Replicate 3
Phage lysate[c]	X		13	15	15
Phage lysate		X	16	4	3.5
Sonically-treated[d] uninfected cells	X		2	2	2
Sonically-treated uninfected cells		X	3	2.5	3

[a]Phenol extraction procedure included the addition of $CaCl_2$, and $MgCl_2$, DNAse, Protinase K, and sodium lauryl sulfate before phenol treatment as described in the Materials and Methods.

[b]DNA quantities obtained from a standard curve (Fig. 10).

[c]A single phage stock obtained as previously described for the preparation of high titer phage stocks, served for all 3 replicates.

[d]One culture of uninfected cells were divided into 3 parts and separately treated with sonic oscillation using six 90 watt, 15-second bursts at 30 second intervals. Each sonically treated culture was divided into 2 parts; one part was treated with DNAse during phenol extraction and the other part was not.

standard curve. In a similar phage sample that had not been treated with DNAse, DNA quantities were surprisingly lower in phenol extracts. It should be noted that this test was accurate for DNA quantities of 50 µg/ml or more. Also, control reactions of artificially broken uninfected cells showed no cellular DNA released without DNAse treatment, indicating that the cell breakage was not extensive enough to release cellular DNA.

Radiosotopic Labeling of the Phage Nucleic Acid

Attempts to label the nucleic acid of GW6210 in liquid culture and on solid media were unsuccessful. Phage propagated in liquid media and on solid media were subjected to density gradient centrifugation. Following fractionation of the gradient, liquid scintillation counting showed no peaks of radioactivity to be detected in any of the fractions.

Following propagation of the phage in liquid media, a phage sample containing ^{3}H-thymidine and phage, and another sample containing ^{14}C-uridine and phage were subjected to density gradient centrifugation. The gradient was fractionated and assayed for radioactivity. In the gradient containing phage and ^{3}H-thymidine, fraction counts ranged from 20 to 92 counts/minute against a background of 22 counts/minute. In the gradient containing phage and ^{14}C-uridine, counts ranged from 15 to 63 counts/minute against a background of 10 counts/min.

Following the propagation of the phage on solid media, the two phage samples containing different labels were concentrated 5-fold as

previously described. The concentrates were counted with the liquid scintillation counter. Ten microliters of the concentrate containing phage and ^{3}H-thymidine contained only 79 counts/minute. Only 251 counts/minute were detected in 50 µl of the concentrate containing phage and ^{14}C-uridine. Because of these low counts, fractions collected from density gradients containing these concentrates were not assayed for radioactivity.

DISCUSSION

Plaque Morphology

At first, the observation of two plaque types appearing on lawns of the host organism infected with GW6210 led this investigator to question the purity of the phage stock. Plaques displaying different characteristics could have indicated the presence of different phage (26). The appearance may vary according to the specific combination of phage and host (3). For example, a plaque formed by a phage infection of one host may appear different from a plaque formed by the same phage on a different host.

If two plaque types differ morphologically because they are caused by different phages, it is possible to separate the phage by serially picking and plating one plaque type. The idea of one phage giving rise to one plaque is the basis for this enrichment procedure. In this study, however, failure to separate plaque types by this method indicated that there was another explanation for the plaque variation. According to Adams (3), plaque variation may be caused by varying rates of adsorption of individual phage on the bacterial lawn. In the case of lytic phages, after a lawn is seeded and infected, phages that adsorb soon thereafter will result in larger plaques and phages that adsorb later will form smaller plaques. The size of a plaque is dictated by the number of lytic cycles that can occur while the bacterial host is still actively growing within the agar overlay. However, this relationship of plaque size and phage adsorption time may not apply to temperate phages whose lytic cycles

may not be initiated after infection until spontaneous induction occurs (44).

Several experiments were performed to test the hypothesis that plaque appearance was being affected by size variation. In the first experiment, agar plugs were removed from the central regions of both plaque types, and from the peripheral regions of both plaque types (Table 3). Extracts of these plugs were plated on lawns of the host organism. Phage were present only in the central regions of both plaque types. No phage were found in the peripheral regions of either type. These results indicate that the location of phage in both plaque types was the same, regardless of the different appearances of the plaques. It is possible, then, that a clear region of lysis was visible for the larger plaque type A which may have originated from a phage that had adsorbed early in the growth of the bacterial lawn. The clear region of lysis could have existed in the smaller plaque type B, yet have been below the threshold of visibility, due to late adsorption of the phage.

The second experiment also indicated the two plaque types to be size variants. The clear region of plaque type A (Table 4) did not increase in diameter from 48 to 72 hours of incubation. This indicated that the clear region of plaque type A had reached its maximum size. It follows, then, that the smaller plaque type B could have possessed a clear region of lysis below maximal size and thus, below the threshold of visibility. If the growth of the bacterial cells had reached the point where the cells were no longer actively growing, the clear region of plaque type A would stop increasing, as observed.

The clear region of plaque type B would stop increasing and therefore, never reach the threshold of visibility. In accordance with these findings, the turbid regions of plaque types A and B increased at the same rate (Table 4), indicating another similarity between the two plaques. This similarity may possibly have been the diffusion rate of a common hydrolytic enzyme, whose spread throughout the bacterial lawn resulted in a zone of partial lysis.

Various microscopic examinations supported these data. All phages observed in both crude samples and samples banded on CsCl gradients were morphologically identical. Although this type of observation would not distinguish between genetically different phages of identical morphology, it is useful, with the support of other methods, to check the purity of a phage sample.

Phage Morphology

The phage, GW6210, displays the binal symmetry typical of the T-even phages and of Bradley's morphological group A (14). The unusual characteristic possessed by this phage is its size. Comparisons with other phages in Group A (Table 1), show this phage to be possibly the largest phage to be reported in this group. The Caulobacter phage, Cr26, is the phage most similar in size to GW6210, exhibiting a 160 nm head and a tail 160 nm long (47). The Bacillus phage GA-2 is the smallest of the group A phages having a head 35 nm in diameter and a tail 210 nm in length (11). GW6210 exhibits a 170 nm head, and a tail 136 nm in length. The head is almost twice the size of the head of a T2 phage particle (16).

The significance of the head size could be amplified if this phage was shown to be temperate and capable of transduction, for transduction has proven to be very useful in the mapping of bacterial genes (39). Transducing phages are equal in density to nontransducing phages of the same type indicating that the same amount of DNA is included in each phage head (58). Since up to 2% of the genome of E. coli can be transduced in a single P1 particle, which corresponds to 2/3 of the phage genome (39), it is possible that some transducing phages contain only bacterial DNA from the host donor (58). This has been found to be true with a mutant of phage P1 (60). Mise states that "the production of active transducing phages is probably limited by the size of the DNA molecule which can be packaged into a mature phage particle." If this is indeed the case, and GW6210 could be shown to be a transducing phage, then the size of this phage's head would take on a significant role in the transducing ability of the phage, and in the genetic analysis of the host organism. The speculation is interesting; however, much work would have to be done to determine if the phage was capable of transduction. As the first of several steps, the phage would have to be proven capable of establishing lysogeny, as lysogeny and transduction are interrelated (39). This could be done by infecting various strains of the host organism, followed by the induction of the phage from the bacterial culture with mitomycin C (61, 71) or UV irradiation (61). The phage released would then be tested against an indicator strain susceptible to lysis by the phage (71). The next step would be to select a genetic marker to be transduced, such as the trp gene and to obtain trp^- mutants of

the host organism. The phage would then be used as a trp^+ donor during infection (61). Transduction would be detected on the plates by the presence of prototrophic colonies (61).

Host Range Study

The host range study of GW6210 involved testing bacterial strains in 3 genera of bacteria, the Gluconobacter, Acetobacter and Pseudomonas. The members of these genera are gram negative, rod-shaped organisms, that are capable of growth at a pH of 6.0 or below (17).

The strains of acetic acid bacteria chosen for the host range study of GW6210 were chosen on the basis of 3 criteria. First, the strain had to be available in our culture collection. Second, the name of the organism or a synonym for that name had to be listed in Bergey's Manual of Determinative Bacteriology. Third, the organism had to display biochemical reactions characteristic of its genus. The 3 Pseudomonas strains were chosen on the basis of the first 2 criteria. The biochemical reactions performed by Baker (unpublished observations) that typified the acetic acid bacteria will be briefly described. The organisms had been checked for growth at pH 4.5. Gluconobacter strains and Acetobacter strains grew well at the low pH (17).

The organisms had been tested for oxidation of ethanol on $CaCO_3$ media. Production of acid was typical of both genera and was indicated by the clearing of $CaCO_3$ from the plate (21). The Gluconobacter species take the reaction no further because they lack a

complete Tricarboxcylic Acid Cycle (21). The Acetobacter species have a complete TCA cycle and therefore metabolize the acid and redeposit the $CaCO_3$ (21). This process is called "irisation" (Frateur, (40).

A test to confirm the above results utilizes a calcium lactate medium. Acetobacter species will utilize the lactate and precipitate the $CaCO_3$ whereas Gluconobacter will not precipitate the $CaCO_3$ (21).

Two other tests used in the identification of the acetic acid bacteria are the production of dihydroxyacetone from glycerol and the production of acid on glucose (Baker, personal communication). However, it should be recognized that these reactions do not differentiate between the Gluconobacter and the Acetobacter species. They do, however, help in conjunction with the reactions previously described, to confirm that an organism is an acetic acid bacterium.

The Gluconobacter species usually produce calcium ketoglutarate crystals from glucose media containing $CaCO_3$ while the Acetobacter may or may not exhibit this ketogenesis (17). The Pseudomonas species do not undergo ketogenesis (17). Production of acid from glucose is pronounced for the Gluconobacter and variable for the Acetobacter and Pseudomonas (17). Production of dihydroxyacetone from glycerol is produced strongly by the Gluconobacter species and to a lesser degree by the Acetobacter species and not at all by the Pseudomonas species.

With this background on how the test organisms were chosen, it is important to note that the 4 strains susceptible to GW6210 (Table 7) were identical in their responses to the biochemical tests

described above. All 4 strains gave the following biochemical reactions (Baker, personal communication):

1. The organisms grew at pH 4.5
2. The oxidized ethanol with resulting precipitation of $CaCO_3$; no irisation occurred.
3. Organisms grew on calcium lactate-medium, but did not precipitate the $CaCO_3$.
4. The organisms produced calcium ketoglutarate crystals on glucose-$CaCO_3$ medium.
5. The organisms produced dihydroxyacetone from glycerol medium.

The above responses to the biochemical tests described are typical for organisms of the genus Gluconobacter. The organisms shown to be susceptible to GW6210 in this host range study, Gluconobacter oxydans subspecies suboxydans, G. suboxydans var. α, A. melanogenus, and G. rubiginosis, are all species of Gluconobacter (17). The only characteristic offsetting any of the species from the others is the production of a soluble brown pigment of A. melanogenus and G. rubiginosis when grown on glucose medium. Since the organisms susceptible to GW6210 and the similar biochemical characteristics, the experimental results from this host range study support Smith's (74) statement that the host range of a phage should correlate well with the biochemical characteristics of the host organisms.

Determination of Phage Density

Buoyant density is a property that is found to be frequently determined by investigators involved in phage characterization. Some examples of typical values for phage buoyant densities are as follows. The Caulobacter phage Cd1 has a density of 1.51 g/cm^3 (60). The Myxococcus phage, MX8 has a density of 1.43 g/cm^3 (57). A T4 particle has a density of 1.45 g/cm^3 (48).

At first, this investigator was utilizing a step gradient as previously described to partially purify the phage, followed by a second continuous gradient to determine the phage's density. This procedure was performed 4 times, resulting in bands collecting at densities varying from 1.47 g/cm^3 to 1.55 g/cm^3. A partial titer was determined for fractions of one continuous gradient as shown in Fig. 10. A peak of absorbance appeared at a density of 1.47 g/cm^3, in fractions 9 and 10. It appears that most of the infective phages were detected in these two fractions. These results were not repeatable and are therefore inconclusive. Three subsequent attempts to detect phage in the continuous gradient by spotting the fractions on lawn of the host organism, showed only very low concentrations of phages throughout the gradient. The presence of phage throughout the gradient was an indication of possible adsorption of the phage to material of different densities, and loss of most of the phage population during some part of the purification process. In attempt to locate the rest of the phage population, this investigator fractionated the step gradient and found a peak of absorbance in the sucrose layer. The fractions were spot-assayed for infectivity, and most of

the infective phage were detected in the sucrose layer. This may have been an indication that phage had adsorbed to cellular material of low density.

The failure to collect a homogeneous band of phages at a repeatable density of $CsCl_2$ may, therefore, be attributed to the impurity of the phage sample. If this explanation is correct, the problem could possibly be overcome by the purification of the phage particles before placing them on a density gradient. One purification procedure that has been applied to viruses is the technique of chromatography on calcium phosphate as described by Burness (19). Calcium phosphate will adsorb at low concentrations to viruses, proteins, and nucleic acids. After adsorption of this kind on a Sephadex column, these materials may be eluted from the column with higher concentrations of $CaPO_4$ buffer. A linear column gradient should be constructed to determine at which $CaPO_4$ concentration the virus is eluted. Fractionation with $CaPO_4$ is followed by taking absorbance$_{260}$ readings of each fraction. Each fraction is also assayed for infectivity. Once the fraction that contains the virus is located, as opposed to the fractions that contain other UV absorbing material, another column may be constructed so as to allow for maximum separation of the two entities.

Another possible purification procedure that could be utilized prior to density gradient centrifugation, is gel filtration using agarose gel as described by Reiland (66). This liquid column chromatographic technique separates molecules in solution by molecular size. The agarose gel possesses a porous structure whose dimensions

are a function of gel-particle concentration in an appropriate solvent. The application of the phage stock to the column allows for molecules smaller than the phage to percolate faster through the column, and larger molecules to be retained for a longer period of time. Fractionation of the column effluent, followed by measurement of absorbance and infectivity of the fractions, will allow for the detection of the purified phages (66).

Colorimetric Estimation of Phage Nucleic Acid

The diphenylamine reactions of the phage nucleic acid were inconclusive (Table 11). The source of phage nucleic acid was a high titer phage stock prepared as previously described. Three samples of this stock were subjected to the phenol extraction previously described with DNAse treatment, and 3 additional samples of this stock were subjected to the phenol extraction without DNAse treatment. As shown in Table 11, the diphenylamine reactions indicated that phenol extracts of phage lysates treated with DNAse during the extraction procedure gave higher diphenylamine readings than did phenol extracts of phage lysates that were treated with DNAse. This sheds doubt on whether the DNAse was acting on cellular DNA. To test the activity of the DNAse on cellular DNA, control reaction mixtures were prepared whereby one attempt was made to artificially break up uninfected cells by sonic oscillation, thus releasing cellular DNA, and mimicking the conditions of phage-induced lysis. A strong diphenylamine reaction in the phenol extracts of artificially broken cells that had not been exposed to DNAse treatment, would have

indicated that the sonic oscillation was releasing cellular DNA. A negative diphenylamine reaction in the phenol extracts of artificially broken cells that had been exposed to DNAse treatment, would have indicated that the DNAse treatment was destroying the cellular DNA, thus validifying the phage diphenylamine reactions as having resulted from phage DNA only. Unfortunately, an equally negligible amount of DNA was detected in both DNAse-treated and DNAse-untreated phenol extracts of artificially broken cells. The DNA indicated by the diphenylamine reaction of the phage lysates can not be conclusively labeled as phage DNA.

Radioisotopic Labeling of the Phage Nucleic Acid

Experimental results indicated that radioactive isotopes were not taken up by the phage, GW6210. There are several possibilities to explain why this uptake did not occur.

One problem with radioactive isotope uptake may occur due to the presence of intracellular nucleotide pools, as well as exogenous supplies of nucleotides (67). During growth, the bacterial cells may utilize endogenous and exogenous sources of nucleotides, inhibiting labeled nucleotide uptake by competition (67). If the bacterium does not take up the label, the viral progeny will not incorporate it into their genome. I feel that this may have been the problem with uptake by the phage GW6210. The endogenous nucleotide competition was probably reduced by the introduction of the label in an early phase of bacterial growth as previously described. This should have allowed for the incorporation of labeled nucleotides into

intracellular pools, however, no radioactivity was detected. Therefore, it appears as if the bacterial cells had not incorporated the label and had thus, blocked the incorporation of the label by the phage. Possible solutions to this problem would include the use of a defined thymidine-deficient mutant as described by Cairns (20). Starving the cells for thymidine would therefore force cellular uptake of the label.

Another suggestion for further study is to obtain a one-step growth curve for the phage-host combination. Knowing the exact time when the maximum number of phages are released from the culture, would allow for maximal detection of labeled phage. The third suggestion would be applicable if one does not find the uptake problem to be host-related. The nucleic acid of the phage could be isolated and then analyzed for base content. The replacement of a common base with an unusual one may indicate the presence of phage-induced enzymes that alter the nucleotides of the parent phages (52). In this case, the appropriate radioactive precursor to the unusual base might not be taken up by the host organism, and an alternate method of nucleic acid determination should be utilized.

CONCLUSIONS

From this characterization study of the Gluconobacter phage, GW6210, it may be concluded that:

1. The phage, GW6210, produces a small pinpoint plaque surrounded by a large turbid region. The plaque and the turbid region surrounding it are highly variable in size. The turbid region surrounding the clear region is caused by some mechanism other than phage-induced lysis from within.

2. Phage GW6210 possesses a polygonal head, contractile tail, collar, tail sheath, base plate and tail fibers. The head of this phage is 170 $\pm$ 9.0 nm in diameter. The tail is 136 $\pm$ 9.0 nm long and 34 $\pm$ 9.0 nm in diameter.

3. The host range of this phage includes the normal host, G. oxydans subspecies suboxydans ATCC 621, and three other Gluconobacter species: G. suboxydans var. α, IFO 12528; A. melanogenus, ICPB 2096; G. rubiginosis, IFO 3244. Fifty strains of Gluconobacter and 52 strains of Acetobacter were as well as 3 strains of Pseudomonas.

4. Further research is necessary to determine the density and nucleic acid type of this phage.

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THE INITIAL CHARACTERIZATION OF GW6210, A NEWLY ISOLATED BACTERIOPHAGE FOR GLUCONOBACTER

by

Pamela Jean Churn

(ABSTRACT)

The Gluconobacter phage GW6210, infects the ATCC strain 621 of G. oxydans. This phage possesses a polygonal head and contractile tail; therefore, it can be placed morphologically into Bradley's group A and tentatively placed in the virus family Myoviridae. This intensive electron microscopic study shows phage GW6210 to have a polygonal head, collar, tail sheath, baseplate, and tail pins. This phage is primarily unusual because of its large size. Using Tobacco Mosaic Virus as an internal standard, the head was found to be 170 nm in diameter and the tail was 136 nm long and 34 nm wide. The phage preparation seemed to consist of two plaque types: a small clear plaque having a 0.3 $\pm$ 0.1 mm diameter surrounded by a large turbid region that measured 3.5 $\pm$ 0.7 mm wide, and a small turbid plaque that varied greatly in size (1.4 $\pm$ 0.8 mm). However, subsequent data indicated that these types represented size variation of a single plaque type. In addition to the normal host of GW6210, G. oxydans subspecies suboxydans ATCC 621, the host range of this phage includes: G. suboxydans var. α, IFO 12528; A. melanogenus, ICPB 2096; G. rubiginosis, IFO 3244. Fifty strains of Gluconobacter and 52 strains of Acetobacter were unaffected by the phage as weel as 3 strains of Pseudomonas tested.