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A Comparative Study of Three Mycobacteriophages

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A Comparative Study of Three Mycobacteriophages

by

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Phages Bol, Bo2, and Bo2h were studied with respect to seven characteristics. After three days of incubation the plaques of phage Bol were circular, 4-5 mm in diameter, and made up of a series of up to four concentric rings. After ten days the plaques were circular, 8-9 mm in diameter, and composed of up to seven concentric rings. In three days phage Bo2 formed hazy, circular, 3-4 mm diameter plaques. After ten days the plaques were circular, 5-6 mm in diameter, and had a light halo around the central clear area. After three days phage Bo2h formed 4-5 mm circular, clear plaques. After ten days the plaques were circular, 8-9 mm in diameter, and also had a light halo around the central clear area. In addition there were many small "satellite" plaques around the outer edge of the halo.

Bol is a specific Mycobacterium smegmatis phage. Bo2 and Bo2h are polyvalent phages lysing Mycobacterium phlei, Mycobacterium smegmatis, and Mycobacterium avium.

The GC ratios of phages Bo2h and Bo2 and Mycobacterium phlei SN109 and F89 were 72% (identical to that of Micrococcus lysodeikticus). Phage Bol had a GC ratio of 71%, and Mycobacterium smegmatis SN2 had a GC ratio of 66%. They all were found to be composed of double-stranded DNA.

The heat inactivation of phage Bo2 was more rapid than that of phages Bol or Bo2h at 55 C, 56 C, and 60 C. Heat inactivation of phages Bol and Bo2h was similar, but phage Bol was slightly more sensitive than phage Bo2h. Thus, the order of heat sensitivity was phage Bo2 > phage Bol > phage Bo2h.

Under the electron microscope phage Bol had an elongated hexagonal head 99 nm in length and 41 nm in width, and a tail 10 nm in width and 308 nm
in length. Phage Bo2 had a hexagonal head 54 nm in diameter, and a tail 9 nm in width and 221 nm in length. Phage Bo2h had a hexagonal head 58 nm in diameter, and a tail 9 nm in width and 225 nm in length.

All three phages adsorbed very poorly to their host bacteria. When the single-step growth curves were performed, a lengthened adsorption period was used.

Phages Bo1, Bo2, and Bo2h all showed cross-neutralization, which was most pronounced between phages Bo2 and Bo2h. Phage Bo2h was very strongly neutralized by anti-Bo2 serum, whereas the neutralization of phage Bo2 by anti-Bo2h serum was much weaker.

In heart infusion broth without Tween 80, phage Bo1 had a latent period of 150 minutes, a rise period of 230 minutes, and a burst size of 61. In heart infusion broth plus 0.03% Tween 80, the latent period of phage Bo1 was 110 minutes, the rise period was 120 minutes, and the burst size was 4. Phage Bo2 had a latent period of 260 minutes, a rise period of 180 minutes, and a burst size of 11 in heart infusion broth without Tween 80. When 0.03% Tween 80 was added, phage Bo2 had a latent period of 320 minutes, a rise period of 240 minutes, and a burst size of 8. In heart infusion broth without Tween 80, phage Bo2h had a latent period of 320 minutes, a rise period of 110 minutes, and a burst size of 20. In heart infusion broth with 0.03% Tween 80, phage Bo2h had a latent period of 150 minutes, a rise period of 300 minutes, and a burst size of 4.
INTRODUCTION

In 1915 the British bacteriologist F.W. Twort described an acute "infectious disease" of staphylococci that produced marked changes in colonial morphology. The infective agent was filterable, and could be passed indefinitely from colony to colony (63). In 1917 Felix d'Herelle published his independent discovery of an agent which he called a "bacteriophage" (20).

It was not until approximately two decades after the discovery of the bacteriophage that the first report of what could have been a bacteriophage active on the genus Mycobacterium appeared in the literature. Steenken, in 1938, reported spontaneous lysis of old cultures of human tubercle bacilli (53). In 1947 Gardner and Weiser isolated a bacteriophage active against saprophytic mycobacteria (16). Their report was the first in which a mycobacterial lytic agent was actually proven to be a bacteriophage.

This was followed by the isolation of many mycobacteriophages that were active on saprophytic strains of mycobacteria (19, 21, 22, 39 & 66). It was not until 1953 that Froman and Bogen reported the first mycobacteriophage that was active against pathogenic mycobacteria (13). Since then a great number of mycobacteriophages active on both virulent and saprophytic mycobacterial strains have been described.

Following discovery and isolation, attempts were made to characterize and classify these mycobacteriophages. The early reports contained very little as far as characterization of the phage itself. They contained mainly details of the isolation procedure along with the host range of these phages. Most of the studies of the 1950's were motivated by the possibility that a phage-typing system could be developed for the identification and classifica-
tion of mycobacteria. Unfortunately, workers attempting to establish a phage-typing system for pathogenic mycobacteria did not attain complete success.

There are a number of reasons for this: 1) Very few mono-specific phages have been reported; mycobacteriophages are usually polyvalent. 2) Most of the mono-specific mycobacteriophages reported do not lyse all of the strains in a particular species, but only part of them.

Mycobacteriophages other than those which I have investigated have been studied to varying degrees. Some of the workers have used criteria similar to those which I have chosen in order to characterize the phages, such as: 1) plaque morphology (5, 6, 12, 14, 16, 21-23, 35, 37, 41, 42, 51 & 57); 2) host range (2, 3, 6, 12-14, 16, 19, 22-24, 34, 37, 41, 42, 50, 51, 54, 55, 57, 60 & 64); 3) DNA base composition (9, 33, 44, 50, 59, 61, 62 & 65); 4) heat inactivation (10, 12, 16, 21-23, 35, 41, 42, 48, 51, 64 & 66); 5) electron microscopic morphology (5, 6, 10, 12, 15, 17, 22, 29, 33, 35-37, 39, 40-42, 44, 47, 54, 56, 58, 61 & 66); 6) neutralization by homologous and/or heterologous anti-mycobacteriophage sera (4, 5, 7, 8, 10, 12, 22, 34, 35, 37, 41, 42, 48, 51, 57, 59 & 64); 7) single-step growth (5, 6, 34, 42 & 49).

The three mycobacteriophages with which I have been working have been described in several papers (18, 25-28, 45 & 52). The plaque morphology, heat sensitivity, host range and electron microscopic morphology of phages Bo2 and Bo2h (25-27) and the host range and electron microscopic morphology of phage Bol (25) have been reported. These studies were performed to a more limited extent, and under different circumstances.

It was important that more work be done on these phages, not only because relatively little is known about them, but also because phage Bo2 is being used extensively in transduction experiments in our laboratory.
**MATERIALS AND METHODS**

**Bacterial strains.** *Mycobacterium smegmatis* strain SN2 is the propagating host of phage Bo1*, *Mycobacterium phlei* strain SN109 that of phage Bo2*, and *Mycobacterium phlei* strain F89 the propagating host of phage Bo2h*. Strains SN2 and SN109, and all the SN strains listed later in the table on the host range studies, are from the Borstel Collection, Borstel, West Germany. *Mycobacterium phlei* strain F89 is from the Froman Collection, Olive View, California. All strains were maintained on Löwenstein-Jensen slants.

**Bacteriophage stocks.** Mycobacteriophages Bo1 and Bo2 were isolated in Borstel, West Germany, in 1963 (25). Phage Bo2h was obtained from a *Mycobacterium phlei* F89 strain lysogenic for phage Bo2 (26). All phages were tested for plaque forming ability upon their respective propagating host.

In order to purify the phage stocks, three single-plaque transfers of each phage were made. The phage suspension was serially diluted to single plaques, and a well isolated typical plaque was picked up with an inoculating loop and suspended in HIB. This again was diluted to single plaques, and the process was repeated twice.

In order to produce high titer phage stocks, 0.3 ml of each of the purified phage preparations was spread over the top of three plates, each of which contained a bacterial lawn of its own propagating host. The large confluent lytic zones obtained after three days of incubation were scraped off with a bacteriological loop and suspended in a tube of HIB, one plate per

Phages Bo1, Bo2, and Bo2h were originally designated B1, B2, and B2h. The label was re-designated in order to avoid confusion with phages B1 and 2 of the Japanese authors (18).

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2 ml. This was centrifuged to remove agar particles and debris. The supernatant was used to lyse 15 other plates. Phages were harvested and centrifuged as above. The supernatant was filtered through a 0.45 μm millipore filter into a sterile screw-capped tube. In this way more than 10 ml's of three phage stocks were prepared. These phage stocks were the only ones used throughout the entire set of experiments.

Phage stocks were titrated in HIB immediately, and also assayed at three week intervals to determine if there was any loss in titer. Storage at 4 C minimized this loss.

Media. Bacterial cultures were maintained on Löwenstein-Jensen slants and plates (Colab, Glenwood, Illinois). Phage stocks were stored in heart infusion broth, (HIB), (Difco, Detroit, Michigan), and kept at 4 C. Dilutions of the phages were made also in HIB, except for the heat inactivation experiments where physiological saline (0.85% NaCl) was used. For assaying plaque formation, a modification of the medium first described by Froman (13 & 14) was used. The ingredients are as follows:

**Basal plating medium (bottom agar):**

- 30 g Bordet-Gengou (Difco) agar base
- 10 g Protease peptone No. 3 (Difco)
- 10 ml Glycerol (Eastman; reagent grade) suspended in 1000 ml distilled H2O

**Semi-solid medium (top agar):**

- 7.5 g of agar (Difco) suspended in 1000 ml of nutrient broth (Difco)

Chemicals and Reagents. Saline-citrate buffer is composed of 0.15 M sodium chloride and 0.015 M sodium citrate (Difco). This buffer was routinely used for dialysis, as well as dissolving and storing the DNA preparations.
Deoxyribonuclease (DNase), Ribonuclease (RNase), and cesium chloride (CsCl)
optical grade, were purchased from Calbiochem, Los Angeles, California.
Phenol (89%), liquid reagent (Allied Chemical Company, Morristown, New
Jersey) was saturated with water at 4°C.

Equipment. Genie vortex mixer (Scientific Products); dialysis tubing,
½ inch in diameter (Union Carbide); glass tubes (Pyrex screw-capped tubes);
glass beads, 0.10 to 0.11 mm in diameter (Quigley-Rochester, Inc., Rochester,
New York); B. Braun cell disintegrator (Quigley-Rochester, Inc., Rochester,
New York); water bath (Precision Scientific); spectrophotometer (Beckman DB-G);
commercial film (Kodak, 2½ x 3½ inches); and densitometer (Beckman model
R-110). Centrifuges. A desk top Clay-Adams centrifuge was used in the prep-
aration of the phage stocks. A size 2 I.E.C. (International Equipment
Company) centrifuge was used for the adsorption studies and the DNA prepara-
tions. For the CsCl density gradient centrifugation studies, a Beckman
(Spinco) model E analytical ultra-centrifuge, with an AN-D rotor (maximum
speed 59,780 rpm) was used.

Plaque assay. The soft agar overlay method of counting plaques, describ-
ed in Adams (1) was employed. The bottom agar was the above-described
Bordet-Gengou agar basal medium. This was overlayed by 0.75% nutrient agar
which contained the suspended bacterial indicator strain. Upon solidifica-
tion of the semi-solid top agar, 0.1 ml of phage suspension was dropped on
top of the bacterial lawn. The bacterial indicator strain was grown on
Löwenstein-Jensen plates for one week. The bacteria were removed from the
surface of the plate with a sterile scalpel blade and suspended in HIB in a
glass screw-capped tube with glass beads in the bottom. This was placed on
a vortex mixer at top speed for 10 minutes to break up the larger bacterial
clumps. The tube containing the background bacteria was placed in a 37°C
incubator for 24 hours in order to obtain great amounts of cells in the logarithmic phase of growth. After sedimentation of the larger clumps, the suspension of finer bacterial particles was used as background. 0.5 ml of these finer particles was added to 3 ml of top agar. This was shaken for 10 seconds on the vortex mixer, poured over the Bordet-Gengou bottom agar, and allowed to solidify. 10-fold dilutions of phage in HIB were employed. 0.1 ml of each dilution was placed on the bacterial lawn until single plaques (about 200) could be counted. The total number of phage per ml in the undiluted suspension was calculated by multiplying the total dilution by the number of plaques formed.

Preparation of specimens for the electron microscopic morphological examination. A zone of confluent lysis was obtained by dropping a few drops of the concentrated phage stock upon a background of bacterial indicator. The Petri dish was inverted and 3 ml of a 2% solution of OsO₄ was put on a piece of filter paper which had been placed in the cover. For two hours the vapors arising from this cover were allowed to fix the phage in the zone of confluent lysis just above it. At the end of this time, the Petri dish was again placed right side up and a small volume of phage was washed from the surface of the plate with distilled water. A drop of this was placed on a 300 mesh copper grid, which had previously been coated with 0.5% formvar. After the drop dried, the phage on the grid was stained with 0.2% uranyl acetate for 10 seconds and allowed to dry. The specimens were then viewed on a Hitachi HU-11A Perkin-Elmer electron microscope.

Production of anti-phage antibodies in rabbits. 1 ml of a given mycobacteriophage suspended in physiological saline was injected into a rabbit which received injections twice a week, alternately intravenously and intraperitoneally, for fifteen weeks. The concentrations of phage used were
about $7 \times 10^{10}$ plaque forming units (pfu) per ml for phage Bol, $1 \times 10^{10}$ pfu/ml for phage Bo2h, and $7 \times 10^9$ pfu/ml for phage Bo2. Blood samples were obtained from the ear vein of each rabbit weekly and tested for neutralizing activity. The first blood sample (20 ml) was taken from each rabbit before injection began, and this serum was saved and used as a control serum. When the neutralizing activity appeared to reach a plateau, the rabbits were bled by cardiac puncture. The blood samples were collected in 20 ml screw-capped tubes and allowed to set at room temperature for 30 minutes. The clot was removed from the sides of the tube with a sterile glass rod and the tube was placed in the refrigerator for 24 hours to allow the serum and blood cells to separate. The tube was then removed and centrifuged, and the supernatant serum was carefully pipetted off of the sedimented blood cells, placed in screw-capped tubes (5 ml per tube), and stored in the refrigerator. These antisera were used in cross-neutralization studies and single-step growth studies of the phages.

**Isolation of bacteriophage DNA.** DNA was isolated from all three phages by the cold phenol method of Marmur (32). 5 ml phage suspensions containing $1 \times 10^{11}$ pfu/ml for phage Bo2h, $2 \times 10^{11}$ pfu/ml for phage Bol, and $6 \times 10^9$ pfu/ml for phage Bo2 were treated with equal volumes of water saturated phenol. After this, the tube was inverted and quickly turned right side up several times, and placed in an ice water bath for 10 minutes. This was repeated twice. Denatured protein was sedimented by centrifugation at 5,000 rpm for 10 minutes. The top aqueous layer was carefully removed with a wide-bore pipette and transferred to a sterile screw-capped tube. This was dialyzed against 2 liters of SSC for 24 hours in order to remove the extracting solvent. The content of the dialysis tubing was transferred to another sterile screw-capped tube, in which the DNA was precipitated with two volumes of cold
isopropanol. This was centrifuged and the supernatant was drawn off, leaving
the precipitated DNA which was re-suspended in SSC and refrigerated until
used.

Isolation of bacterial DNA. Mycobacterial cells taken from Löwenstein-
Jensen plates with a scalpel (as described above) were washed with distilled
water, centrifuged, and collected in .1 SSC to a final concentration of 1
gm/ml. An equal weight of glass beads was added to this suspension, which
was poured into a disintegrator bottle and cooled in an ice water bath. The
cells were then broken for 15 seconds in a cell disintegrator operating at
4,000 cycles per second. Unbroken cells, glass beads, and larger cell debris
were removed by centrifugation at 5,000 rpm for 10 minutes, and the super-
natant fraction was carefully pipetted off and placed in a sterile screw-
capped tube. Just as for the DNA extracted from the phage, equal volumes of
H2O saturated phenol were added to this supernatant fraction. The contents of
the tube were gently rocked back and forth ten times, and placed in an ice
water bath. This was allowed to stand for 10 minutes, and the procedure was
repeated twice. The aqueous layer was removed after centrifugation at 5,000
rpm for 10 minutes, and the DNA was precipitated by two volumes of cold iso-
propanol, and collected by centrifugation. This was then dissolved in SSC
buffer and treated with RNase which was boiled for 15 minutes to remove any
trace of contaminating DNase. The RNase treatment was done at a concentration
of 50 \( \mu \text{g/ml} \) at 37 C for one hour. The DNA was again precipitated with two
volumes of cold isopropanol, centrifuged, the supernatant removed, and the
DNA was re-suspended in SSC. Finally, this was dialyzed against two changes
of 2 liters of SSC for 48 hours. The dialysate was removed with a wide-bore
pipette, put into a sterile screw-capped tube, and stored in the refrigerator.

Determination of buoyant density by cesium chloride density gradient

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centrifugation. Buoyant densities of bacterial and phage DNA preparations were determined by centrifugation at 44,770 rpm at 25°C in CsCl as described by Schildkraut, Marmur and Doty (46) with an analytical ultra-centrifuge. The DNA of Micrococcus lysodeikticus (ε = 1.7310) was used as a marker. Also, DNA of Mycobacterium smegmatis strain SM46 was used as an additional marker. Pictures of the DNA bands formed were taken after 24 hours of centrifugation, using ultra-violet adsorption photography. Tracings were made of the film (containing the bands of DNA) with a densitometer. The difference between the distance that the unknown DNA bands moved and the distance that the marker DNA moved was computed, and the buoyant density was calculated using the formula (46):

\[ \epsilon = \epsilon_0 + 4.2 \omega^2 (r^2 - r_0^2) \times 10^{-10} \text{g cm}^{-3} \]

At 44,770 rev/min this formula converts to:

\[ \epsilon = \epsilon_0 + 0.0092 (r^2 - r_0^2) \text{ g cm}^{-3} \]

where \( \epsilon \) = the density of the unknown DNA

\( \epsilon_0 \) = the density of the standard DNA

\( r_0 \) = the distance of the standard DNA from the center of rotation

\( r \) = the distance of the unknown DNA from the center of rotation

To determine the buoyant density, the following formula was used:

\[ \epsilon = 0.098 (GC) + 1.660 \text{ g cm}^{-3} \]

or \( GC = \frac{\epsilon - 1.66 \text{ g cm}^{-3}}{0.098} \)

where \( \epsilon \) = buoyant density in g cm\(^{-3}\)

and \( GC \) = mole fraction guanine plus cytosine
Samples were treated with DNase and then analyzed on the model E to prove that what was being measured was indeed a band of DNA. This was done even though the banding and adsorption characteristics were quite typical of DNA. Also, hyperchromicity tests were performed to prove that the DNA is double-stranded. The DNA solution was read on a spectrophotometer at 260 nm; the solution was then boiled for 15 minutes and quick-cooled in an ice bath, and the OD260 was again read.

A 12 mm standard cell (which held 0.83 ml of the CsCl-DNA solution in the rotor) was used. Enough CsCl was added to bring the mean density of the DNA solution to 1.710 gm cm\(^{-3}\). Also, the concentration of DNA was adjusted to between 3 and 10 \(\mu\)g/ml, using the Molar Extinction Coefficient of DNA at 260 nm of 20 (i.e., 20 OD260 units = 1000 \(\mu\)g/ml DNA). This concentration of DNA was used in the cell because it resulted in a band that was sharp and not too wide.

**Procedure for heat inactivation studies.** Dilutions had to be made in physiological saline because of the heat shielding effects of HIB. The concentrated phage stock was diluted in physiological saline to make a suspension of about \(1 \times 10^4\) pfu/ml. This suspension was maintained in a covered water bath at the temperature at which I wished to measure the effect. At ten minute intervals for 2 hours, samples were withdrawn with a sterile pipette. All tests were done in duplicate and plated out undiluted, 1:10 diluted, and 1:100 diluted. The heat inactivation was scored as percent of survival of unheated controls.

**Procedure for adsorption experiments.** The method followed was that described in Adams (1). Host bacteria were grown on Löwenstein-Jensen plates and dense suspensions were prepared in screw-capped tubes containing 10 ml sterile HIB. This stock was placed in the refrigerator after titration.
(This same stock was used for both the adsorption and the single-step growth curve studies). At the time of testing, 0.1 ml of refrigerated stock was added to 0.9 ml of HIB and pre-incubated for 24 hours to bring the culture into early logarithmic phase (this was determined by growth curves done earlier in our laboratory). After pre-incubation the phage was added to the bacteria at a multiplicity of infection (moi) of .1 and .001. At ten minute intervals for one hour, 0.1 ml of the incubation mixture was withdrawn and placed into 9.9 ml HIB in order to dilute the mixture and stop adsorption. This dilution was centrifuged at 3000 rpm for ten minutes. 0.2 ml was carefully withdrawn from the top, and 0.1 ml of undiluted and 1:10 diluted suspension was placed upon the background indicator strain. One control was the sample withdrawn and centrifuged immediately, after adding the phage to the bacteria. Another control involved adding the phage to HIB without bacteria and centrifuging and titrating this. The percent of phage remaining unadsorbed was calculated as the number of phage/ml in the supernatant at \( t_n \) over the number of phage/ml at \( t_0 \) (control) \( \times 100 \). Percent adsorption was then computed by subtracting the percent remaining unadsorbed from 100 percent.

**Neutralization test procedures.** Neutralization tests were performed following the methods described in Adams (1). The stock phage was diluted in HIB to a concentration of about \( 1 \times 10^6 \) pfu/ml. The rabbit serum was first heated to 55 C for 30 minutes to destroy complement, allowed to cool, and then tested at undiluted strength and at dilutions of 1/10 and 1/100 in HIB. 0.1 ml of the diluted, pre-warmed (37 C) phage preparation was then added to 0.9 ml of pre-warmed (37 C) antibody solution and incubated at 37 C. At determined time intervals, the number of free phage was determined by diluting the reaction mixture 1:100 with HIB and plating 0.1 ml of this upon a lawn of bacterial indicator. The amount of neutralization was determined.
by comparing the number of plaques formed after exposure of the phage to the antiserum with the number of plaques formed in the controls. Two controls were used: one in which there was no antiserum, HIB alone was added to the phage suspension; the other in which normal rabbit serum replaced the antiphage serum. All tests were done in duplicate.

Procedure for the single-step growth experiments. The method followed was largely that described in Adams (1). Host bacterial suspensions were prepared as described above in the adsorption procedure. Adsorption was allowed to take place at a multiplicity of infection of 0.001, since adsorption of over 50% could only be obtained at this low multiplicity. After 24 hours preincubation of 9 ml of host cells (2-5 x 10⁹ colony forming units per ml) suspended in HIB, 1.0 ml of phage (1-4 x 10⁷ pfu/ml) was added and adsorption was begun. Adsorption was allowed to proceed for 60 minutes, and then 0.1 ml was withdrawn from the tube and added to 0.9 ml of the appropriately diluted homologous complement inactivated antiserum. Antiserum with over 95% neutralization capacity was used in all cases. After 30 minutes of exposure to the antiserum, a 1:100 dilution was made in HIB. 0.1 ml samples were withdrawn at 20 minute intervals for the first 2 hours, and at 30 minute intervals for the remaining 10½ hours, and placed on a background of host bacteria. All tests were done in duplicate.
RESULTS

Titers of phage stocks used. The titer of phage Bol in HIB was $2 \times 10^{11}$ pfu/ml. In physiological saline it was $8 \times 10^{10}$ pfu/ml. The titer of phage Bo2 in HIB was $6 \times 10^9$ pfu/ml, and in physiological saline the titer was $7 \times 10^8$ pfu/ml. The titer of phage Bo2h in HIB was $5 \times 10^{11}$ pfu/ml, while the titer in physiological saline was $1.3 \times 10^{10}$ pfu/ml. After six months of storage at 4°C, there was no significant loss in titer. The loss of titer of phage suspended in HIB was slightly less than that of phage suspended in physiological saline.

Plaque morphology. Phage stocks were diluted so that there were 5-30 single, well-separated, easily observable plaques per plate. After three days the plaques had the following appearance:

Phage Bol formed circular plaques 4-5 mm in diameter, composed of a series of concentric rings. The center of the plaque was very clear, almost devoid of bacteria. Proceeding centrifugally the next ring was dark, followed by a third thicker ring which was lighter than the second, but not as clear as the first. Many times a fourth (halo) ring was observable. This ring was quite dark in comparison to the other three, and the edge of this fourth ring was well demarcated against the surrounding bacterial lawn (Fig. 1). After ten days the plaques of phage Bol were still circular, but reached a diameter of up to 9 mm with up to 7 concentric rings.

Phage Bo2 formed circular plaques 3-4 mm in diameter. These plaques were quite hazy, due to the presence of many survivor bacteria within the plaque (Fig. 2). This was demonstrated by scraping across the center of the plaque with a sterile bacteriological needle and inoculating a Löwenstein-Jensen
plate with the scrapings. After ten days the central lytic area was lined with a light halo and the total diameter of the circular plaque was 6 mm.

Phage Bo2h formed circular plaques 4-5 mm in diameter. The plaque was almost entirely devoid of bacteria, resulting in a clear rather than a hazy plaque. The periphery of the plaque was demarcated (Fig. 3). After 10 days smaller satellite plaques appeared around the periphery of the 9 mm circular plaque in the light halo outside the central lytic area.

Fig. 1. Plaque morphology of phage Bo1 after three days of incubation at 37 C.
Fig. 2. Plaque morphology of phage Bo2 after three days of incubation at 37 °C.
Fig. 3. Plaque morphology of phage Bo2h after three days of incubation at 37°C.
**Electron microscopic morphology.** Under the electron microscope phage Bo1 had an elongated hexagonal head 99 nm in length and 41 nm in width, and a tail 10 nm in width and 308 nm in length (Fig. 4). Phage Bo2 had a hexagonal head 54 nm in diameter, and a tail 9 nm in width and 221 nm in length (Fig. 5). Phage Bo2h had a hexagonal head 58 nm in diameter, and a tail 9 nm in width and 225 nm in length (Fig. 6). The tails of phages Bo1, Bo2, and Bo2h appear to be striated. The tails of phages Bo2 and Bo2h terminate in a knob-like structure.

![Electron microscopic morphology of phage Bo1 (magnified 240,000 x)]
Fig. 5. Electron microscopic morphology of phage Bo2 (magnified 79,800 x)

Fig. 6. Electron microscopic morphology of phage Bo2h (magnified 111,000 x)
Host range. The host range studies are summarized in Table 1. Twenty-two different strains, including ten different species of *Mycobacterium* were tested. Phage Bol lysed only *Mycobacterium smegmatis* strains, and it lysed all of those tested. Phages Bo2 and Bo2h lysed four of the five *Mycobacterium smegmatis* strains tested, one of the two *Mycobacterium avium* strains, and both of the *Mycobacterium phlei* strains tested.

DNA base composition. The buoyant densities and GC ratios of phages Bol, Bo2, and Bo2h and their respective host strains *Mycobacterium smegmatis* SN2, *Mycobacterium phlei* SN109, and *Mycobacterium phlei* F89 are presented in Table 2. Phages Bo2, Bo2h, and their propagating hosts *Mycobacterium phlei* SN109 and F89, banded coincident with *Micrococcus lysodeikticus* on the CsCl density gradient, and showed GC ratios of 72%. *Mycobacterium smegmatis* SN2 had a GC ratio of 66%, and phage *smegmatis* Bol had a GC ratio of 71%. When phage Bol DNA was boiled for 15 minutes it showed a hyperchromic shift of 32%, phage Bo2 DNA a 28% shift, and phage Bo2h DNA a 30% shift (Table 3). This high hyperchromic shift is characteristic of the separation of two DNA strands, and lends evidence to double-strandedness (31).

Heat inactivation. Heat inactivation studies are presented in Figures 7-9. At 55°C, 56°C, and 60°C, phage Bo2 was the most sensitive; next was phage Bol. Phage Bo2h was the most heat resistant. The only exception to this occurred when, in the early samples at 10 and 20 minutes, at 55°C, phage Bo2h was slightly more heat sensitive than phage Bol. The curves all had an initial rapid inactivation portion, followed by a less rapid one that almost levelled off. At 60°C phage Bol was totally inactivated within 20 minutes, phage Bo2 within 12 minutes, and it took almost 30 minutes to totally inactivate phage Bo2h.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Phage Bol</th>
<th>Phage Bo2</th>
<th>Phage Bo2h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I Photochromogen SN531</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II Scotochromogen SN650</td>
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<tr>
<td>Group II Scotochromogen SN703</td>
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<td>0</td>
</tr>
<tr>
<td>Group II Scotochromogen SN721</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II Scotochromogen SN801</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III M. intracellulare SN403</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III M. triviale (27)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III M. avium SN304</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Group III M. avium SN327</td>
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<td>0</td>
<td>0</td>
</tr>
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<td>Group IV M. smegmatis SN2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>Group IV M. smegmatis SN5</td>
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<td>+</td>
<td>+</td>
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<td>Group IV M. smegmatis SN13</td>
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<td>Group IV M. smegmatis SN38</td>
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<td>+</td>
</tr>
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<td>Group IV M. smegmatis SN46</td>
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<td>+</td>
</tr>
<tr>
<td>Group IV M. phlei SN109</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Group IV M. phlei F89</td>
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<td>+</td>
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<td>Group IV M. fortuitum SN203</td>
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<td>0</td>
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<td>Group IV M. vaccae SN901</td>
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<td>Group IV M. vaccae SN920</td>
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<td>0</td>
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<td>Group IV M. vaccae SN961</td>
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<tr>
<td>M. bovis ECG</td>
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<tr>
<td>M. tuberculosis H37Ra</td>
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</table>

+ = Strain sensitive to lysis by the phage
0 = Strain not sensitive to lysis by the phage
TABLE 2

Buoyant Densities in CsCl and Computed GC Ratios of Phages Bol, Bo2, Bo2h, and of M. smegmatis Strain SN2, and M. phlei Strains SN109 and F89

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>AVERAGE BUOYANT DENSITY</th>
<th>AVERAGE GC RATIO*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micrococcus lysodeikticus</td>
<td>1.7310</td>
<td>72</td>
</tr>
<tr>
<td>(marker)</td>
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<td></td>
</tr>
<tr>
<td>Phage smegmatis Bol</td>
<td>1.7297</td>
<td>71</td>
</tr>
<tr>
<td>M. smegmatis SN2</td>
<td>1.7244</td>
<td>66</td>
</tr>
<tr>
<td>Phage phlei Bo2</td>
<td>1.7309</td>
<td>72</td>
</tr>
<tr>
<td>M. phlei SN109</td>
<td>1.7310</td>
<td>72</td>
</tr>
<tr>
<td>Phage phlei Bo2h</td>
<td>1.7310</td>
<td>72</td>
</tr>
<tr>
<td>M. phlei F89</td>
<td>1.7310</td>
<td>72</td>
</tr>
</tbody>
</table>

* Rounded to the nearest whole number

TABLE 3

Hyperchromic Shifting of the Phage Nucleic Acid

<table>
<thead>
<tr>
<th></th>
<th>WAVE LENGTHS</th>
<th>% HYPERCHROMICITY</th>
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<tbody>
<tr>
<td></td>
<td>CD at 220 nm</td>
<td>OD at 235 nm</td>
</tr>
<tr>
<td>before heating</td>
<td>0.400</td>
<td>0.300</td>
</tr>
<tr>
<td>after heating</td>
<td>0.410</td>
<td>0.340</td>
</tr>
<tr>
<td>before heating</td>
<td>0.300</td>
<td>0.370</td>
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</table>
Fig. 7. Heat inactivation of phage Bol at 55°C, 56°C & 60°C
Fig. 8. Heat inactivation of phage Bo2 at 55°C, 56°C & 60°C.
Fig. 9. Heat inactivation of phage Bo2h at 55 °C, 56 °C, and 60 °C
Adsorption rates. Adsorption experiments were attempted at an moi of 0.1 in HIB, HIB + 0.2M CaCl₂, and HIB + 0.2M CaCl₂ + 0.03% Tween 80. After thirty minutes none of the three phages adsorbed over 21% in any combination of media. Subsequently, a much lower moi was used. At an moi of 0.001 the degree of adsorption in HIB after one hour for phage Bol was 52%, for phage Bo2, 56%, and for phage Bo2h, 46%. When adsorption experiments were performed in HIB + CaCl₂ and HIB + CaCl₂ + Tween 80 at an moi of 0.001, the adsorption of phage Bo2h was inhibited. The addition of CaCl₂ aided in the adsorption of phages Bol and Bo2.

Neutralization studies with homologous and heterologous antisera. The neutralizing effect of homologous antisera has been established as a necessary step to performing the single-step growth experiments. At 30 minutes incubation and 5 times dilution, phage Bol showed 95% neutralization by homologous anti-Bol serum. At 30 minutes incubation and 10 times dilution, phages Bo2 and Bo2h were neutralized 99% by their homologous antisera. Results of neutralization by homologous antisera and control sera and cross-neutralization by heterologous antisera can be seen in Table 4. Cross-neutralization of phage Bol by anti-Bo2 serum was slightly stronger than by anti-Bo2h serum. Cross-neutralization of phage Bo2 by anti-Bo2h serum was stronger than by anti-Bol serum. Cross-neutralization of phage Bo2h by anti-Bo2 serum was much stronger than by anti-Bol serum. Neutralization of the three phages by the control serum was insignificant when compared to the anti-phage sera.

Single-step growth. In HIB without Tween 80, phage Bol had a latent period of 150 minutes, a rise period of 230 minutes, and a burst size of 61 (Fig. 10). When 0.03% Tween 80 was added phage Bol had a latent period of 110 minutes, a rise period of 120 minutes, and a burst size of 4 (Fig. 10).
<table>
<thead>
<tr>
<th>Time</th>
<th>Dilution</th>
<th>AntSi-Bol</th>
<th>Anti-Bo2</th>
<th>Anti-Bo2h</th>
<th>Control</th>
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<tbody>
<tr>
<td>Bol</td>
<td>10 min. undiluted</td>
<td>97</td>
<td>22</td>
<td>16</td>
<td>16</td>
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<tr>
<td></td>
<td>20 min. undiluted</td>
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<td>40</td>
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<td>100</td>
<td>49</td>
<td>20</td>
<td>23</td>
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<tr>
<td></td>
<td>10 min. 10 x dil.</td>
<td>62</td>
<td>14</td>
<td>13</td>
<td>16</td>
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<tr>
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<td>73</td>
<td>28</td>
<td>10</td>
<td>20</td>
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<tr>
<td></td>
<td>30 min. 10 x dil.</td>
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<td>31</td>
<td>15</td>
<td>25</td>
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<tr>
<td>Bo2</td>
<td>10 min. undiluted</td>
<td>29</td>
<td>99.6</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>20 min. undiluted</td>
<td>44</td>
<td>100</td>
<td>54</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>30 min. undiluted</td>
<td>63</td>
<td>100</td>
<td>61</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>10 min. 10 x dil.</td>
<td>15</td>
<td>95</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>20 min. 10 x dil.</td>
<td>16</td>
<td>98</td>
<td>27</td>
<td>16</td>
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<tr>
<td></td>
<td>30 min. 10 x dil.</td>
<td>30</td>
<td>99.6</td>
<td>51</td>
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<tr>
<td>Bo2h</td>
<td>10 min. undiluted</td>
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<td>99.4</td>
<td>100</td>
<td>14</td>
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<td></td>
<td>10 min. 10 x dil.</td>
<td>8</td>
<td>82</td>
<td>89</td>
<td>9</td>
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<tr>
<td></td>
<td>20 min. 10 x dil.</td>
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<td>90</td>
<td>93</td>
<td>11</td>
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<tr>
<td></td>
<td>30 min. 10 x dil.</td>
<td>20</td>
<td>98.2</td>
<td>99.3</td>
<td>11</td>
</tr>
</tbody>
</table>
Fig. 10. Single-step growth curve of phage Bol in HIB with, and without, Tween 80
In HIB without Tween 80 phage Bo2 had a latent period of 260 minutes, a rise period of 180 minutes, and a burst size of 11 (Fig. 11). When 0.03% Tween 80 was added, phage Bo2 had a latent period of 320 minutes, a rise period of 240 minutes, and a burst size of 8 (Fig. 11). In HIB without Tween 80 phage Bo2h had a latent period of 320 minutes, a rise period of 110 minutes, and a burst size of 20 (Fig. 12). When 0.03% Tween 80 was added Phage Bo2h had a latent period of 150 minutes, a rise period of 300 minutes, and a burst size of 4 (Fig. 12). It can be seen that Tween 80 drastically affected the growth cycle of all three phages. The latent period of phages Bol and Bo2h was shortened by the addition of Tween 80. The latent period of phage Bo2, as well as the rise periods of phages Bo2 and Bo2h, was lengthened. The rise period of phage Bol, on the other hand, was shortened. Finally, the addition of Tween 80 reduced the burst size of all three phages.
Fig. 11. Single-step growth curve of phage Bo2 in HIB with, and without, Tween 80
Fig. 12. Single-step growth curve of phage Bo2h in HIB with, and without, Tween 80
DISCUSSION

Phages Bol, Bo2, and Bo2h have been studied and compared according to seven characteristics including: plaque morphology, electron microscopic morphology, host range, DNA base composition, heat inactivation, neutralization and cross-neutralization, and single-step growth. It was shown that the plaque morphology of phages Bol, Bo2, and Bo2h are quite different. Phage Bol formed concentric rings, while phages Bo2 and Bo2h exhibited a light halo around the clear central area. Although there have been reports of concentric ring formation by Mycobacterium phlei phages, mine is the first report of a Mycobacterium smegmatis phage which forms concentric rings. Hnatko induced concentric ring formation in Mycobacterium phlei phages by incubating them at different temperatures. The rings were thought to be due to: 1) adsorption of the phage to cells under conditions unfavorable to lysis (cold), 2) viral interference, and 3) differing abilities of the phage to diffuse (21). No change in temperature was needed to induce ring formation by phage Bol. The phage was held at a constant 37°C temperature in the incubator. However, the size and appearance of the rings of phage Bol could be altered by exposure of the plates to 37°C, 4°C, and then 37°C. I would like to suggest that the formation of concentric rings is due to the intrinsic phage development cycle, coupled with the physiological condition of the bacterial host at the time of burst and re-adsorption.

Phage Bo2 forms hazy plaques, while phage Bo2h forms clear plaques. This is due to the number of bacteria that survive phage lysis. Phage Bo2h is more virulent than phage Bo2, i.e., phage Bo2h lyses the bacterial lawn more completely than phage Bo2.
It was possible to see plaque formation by phage Bol after 16 hours, and by phage Bo2h after 30 hours. No plaque formation by phage Bo2 was visible until after at least 48 hours.

Titers of over $2 \times 10^{11}$ pfu/ml could be obtained with both phages Bol and Bo2h on their respective host strains. However, I never obtained a titer of more than $6 \times 10^9$ pfu/ml for phage Bo2, even though I attempted to raise the titer by passing it many times on its host or the host strains of the other phages. Also, the size of the phage Bo2 plaque never reached that of either phages Bol or Bo2h. It seems that phage Bo2 is an excellent candidate for the mediation of genetic transfer, since it is not so destructive for its host.

The satellite effect described occurring around the edge of the phage Bo2h plaque after 10 days of incubation has also been described by Murohashi (37). No explanation for this phenomenon was given. I feel that this is merely a dilution effect. As the lytic zone proceeds from the center, it is diluted out so that eventually a ring of small, individual plaques forms. The satellite plaques were very small, probably because the agar was drying out. The plaques at the edge of the plates were the smallest, due most likely to the fact that this is the driest area. This observation was made many times.

The electron microscopic morphology of phages Bol, Bo2, and Bo2h was similar to that reported in the literature by Juhasz and Bonicke (25 & 27). The measurements which I obtained for phages Bo2 and Bo2h were slightly larger. This is probably due to the fact that the method of specimen preparation was different. Also, the average of only a small number of phages was taken. Takeya (58) reports that "All mycobacteriophages tested can be divided into two distinct morphological groups: 1) those with regular
hexagonal heads, and tails varying in length from 1300A-3300A; and 2) those with oval heads, and tails about 1700A long". If the oval heads described by Takeya are the same as those referred to in more up-to-date papers as elongated hexagons, then phage Bol belongs to group 2. Phages Bo2 and Bo2h belong to the first group of Takeya. Head ranges reported in the literature vary between 850A-1650A length and 450A-650A width for oval heads, and between 450A-1150A for phages with hexagonal heads. Tail widths vary from 90-200A. Two literature reports show tail lengths outside the ranges of Takeya's two groups. Guba and Vandra (17) report that Phagus butyricus has a tail length of 4000A. Cater and Redmond (12) report a spherical-headed DS1 phage with a tail length less than 1000A. Our phage Bol had a 3080A tail, which falls outside of Takeya's tail range for the oval-headed phages of group 2 (around 1700A).

In order to establish host range, both undiluted and diluted phage suspensions should be used on a variety of hosts. The most reliable method appears to be testing with ten-fold serial dilutions, beginning from the undiluted stock and going to single plaques (although this cannot always be achieved). Full dilution must be used because "non-specific" lysis can also occur when very large numbers of phage are used in testing. Upon further titration these zones of non-specific lysis do not form single plaques - which appears to indicate that it is rather a bacteriocin or defective phage which affected the strain, and not true phage lysis. The RTD (routine test dilution) i.e., the highest dilution of a phage suspension still producing confluent lysis, is now utilized by workers who are attempting to phage-type mycobacteria (42).

Phage Bol turned out to be a monospecific Mycobacterium smegmatis phage, confirming an earlier report (25). It had no lytic activity against any
other species, even at a high concentration. Phages Bo2 and Bo2h had identical host ranges. They are polyvalent phages, since they lysed strains of Mycobacterium phlei, Mycobacterium smegmatis, and one strain of Mycobacterium avium.

While phage Bo2h was polyvalent upon its isolation and remained polyvalent (26), the originally polyvalent phage Bo2 could be rendered specific upon serial passage on Mycobacterium phlei strain F89 (25). My fellow laboratory worker, Sheldon Gelbart, and I decided to perform experiments to uncover why the phage Bo2 had a different host range than that described in an earlier report by Juhasz and Bonicke (25). The only difference in the treatment of the phage was that it was propagated on a different host. We obtained the originally described phage Bo2 from Borstel and propagated part of the suspension on Mycobacterium phlei strain F89, and the other part on Mycobacterium phlei strain SN109. The portion of the original phage Bo2 propagated on Mycobacterium phlei strain F89 (designated Bo2•F89) did not lyse Mycobacterium smegmatis when it was tested. The portion of the original phage Bo2 suspension propagated on Mycobacterium phlei strain SN109 (designated Bo2•SN109) lysed many of the Mycobacterium smegmatis strains. We can offer no explanation as to why the host range of phage Bo2 is broadened by passage through Mycobacterium phlei strain SN109.

The literature is replete with evidence that mycobacteriophages are poor antigens for the production of antiserum. The K values which have appeared in the literature for anti-sera prepared against mycobacteriophage are far below those obtained in other phage systems. Although not elaborated in the Results Section, my K values for the homologous systems were lower than some of those reported for anti-mycobacteriophage sera (7, 34, 35, & 48). However, my K values fell within the range of others reported (8, 37, & 60).
The relative strength of the antisera is unimportant here, since it was sufficiently strong to perform the tasks for which it was needed, i.e., to study the serological relatedness of the phages and, more importantly, to neutralize unwanted free phage in the single-step growth curve experiments.

Phages Bo2 and Bo2h were closely related, which was entirely expected since phage Bo2h has been derived from phage Bo2. One unexpected result was the much stronger cross-neutralization of phage Bo2h by the anti-Bo2 serum than cross-neutralization of phage Bo2 by anti-Bo2h serum. This suggests that phages Bo2 and Bo2h share many common antigenic determinants. Moreover, phage Bo2 seems to possess most of the antigenic determinants of phage Bo2h as far as neutralization is concerned, plus some which in phage Bo2h no longer elicit antibody (either due to loss or blocking). Or, it may be that phage Bo2 possesses more active sites that must be tied up before neutralization can occur. This is derived from the fact that antibodies formed against the antigens on the surface of phage Bo2 will neutralize phage Bo2h rather efficiently at the times and dilutions tested (82-100%), whereas the antibodies produced against phage Bo2h neutralize phage Bo2 only at 50-60% efficiency at the same times and dilutions. Perhaps the difference in surface antigenic determinants and/or active sites has been instrumental in causing some of the differences between the two phages regarding plaque morphology, virulence or adsorption. The control sera obtained from un-injected rabbits had very little neutralizing effect upon phages Bo1, Bo2, and Bo2h. Thus, the neutralizing data listed above are due to the antibody produced, and not to other intrinsic factors in the sera.

The GC ratios that I have presented for Mycobacterium phlei and Mycobacterium smegmatis fall in between various values reported in the literature. I found that Mycobacterium phlei had a 72% GC ratio, whereas other
workers report from 63% to 73% GC ratios (9, 30, 31, 46, 59 & 65). Different strains of *Mycobacterium smegmatis* were reported to have GC contents ranging from 61.4% to 68% (9, 50, 59 & 65). I obtained a 66% GC content for *Mycobacterium smegmatis*. Phage Bol had a 71% GC ratio, and phages Bo2 and Bo2h had 72% GC ratios. The GC ratios of these particular phages have not appeared in the literature. The GC content reported for other mycobacteriophages ranges from 60% to 68% (33, 44, 50 & 62). Our values are slightly higher than those reported. The wide variability in the bacterial, as well as in the phage GC ratios, is probably due to the fact that different strains and different methods of DNA preparation were used.

Phage Bo2 was the most heat sensitive of the three phages tested, next was phage Bol, and then phage Bo2h. These phages are slightly more heat sensitive than most of those reported in the literature. A good comparison cannot be made with the work of others, because they used different suspending media than I did. However, the shapes of the inactivation curves drawn are quite comparable. These inactivation curves are made up of two portions: a rapid inactivation component, and a slower inactivation component. This is probably due to inherent population heterogeneity. There are probably two or more heat-mediated reactions involving different components of the phages. This is also the opinion expressed by Sellers (48). Also, it may be simply that the most heat resistant phages in the population were selected out after the rapid inactivation part of the curve, and a longer time was needed to inactivate the resistant ones. When phages Bo2 and Bo2h were tested for heat sensitivity by Juhasz and Bonicke (27), they were found to be slightly more heat sensitive than when I tested them. However, I could confirm their finding that phage Bo2 was more heat sensitive than phage Bo2h.

The adsorption rate of phages Bol, Bo2, and Bo2h was relatively poor.
Thus, various ingredients were added to the HIB in an attempt to improve adsorption. Under identical conditions, the adsorption rate of phage Bo2 was slower than that of phage Bol which was slower than that of phage Bo2h. At an moi of 0.001 phage Bo2h was inhibited by the addition of 0.2M CaCl₂, while adsorption of phages Bol and Bo2 was enhanced by the presence of CaCl₂ (both with and without 0.03% Tween 80). At 60 minutes the adsorption of phages Bol, Bo2, and Bo2h in HIB were quite comparable. This adsorption time was chosen subsequently for the single-step growth curves. Some workers have had no problem obtaining high adsorption rates, while others have encountered difficulties. Penso (40) states that, "The phage lysis of mycobacteria, unlike that of E. coli, is a matter of hours rather than minutes". Under our conditions, the Mycobacterium-phage host system behaved as described above by Penso. Bowman (5 & 6) and Mohelska (36) also found poor adsorption in their Mycobacterium-phage host system.

The three mycobacteriophages with which I have been experimenting exhibit long growth cycles as compared to coli phages. However, the single-step growth curves are comparable to those reported by others working with mycobacteriophages. Bowman (6) reports a minimum latent period of 240 minutes. In another paper (5) he reports that phage D29S has a latent period of 90 minutes, and a burst size of 18. We also obtained a comparable low burst size for phages Bo2 and Bo2h, as well as a step-wise curve very similar to that which he has obtained. Bowman explains the low burst size and the step-wise growth curve in the following manner: "Several preliminary one-step growth experiments (Adams, 1950) did not give the classical one-step growth results. A sharp leveling or plateau (defining the single bursts) did not occur; instead, a gradual two-step or three-step growth occurred. Cycling usually prevented by the high dilution from the adsorption tube to the
first growth tube. Apparently cycling occurred independently of (1) the low adsorption rate constant for D29S on *Mycobacterium ranae* and (2) the high dilution made from the adsorption tube to the first growth tube. A consideration of the clumped state of the bacteria in the first growth tube led to the hypothesis that adsorption of new phage progeny could occur independently of the two factors mentioned above, because phage progeny from a cell contained within a bacterial clump would only have to diffuse over relatively short distances to infect adjacent bacteria. Also, he reports that the addition of Tween 80 to the medium gave him a one-step cycle instead of two or three-step cycling. I have found this to be the case, even though Tween 80 also changed the length of the various phases of phage growth. This is probably due to the effect of Tween 80, or some factor extracted by it, on the host rather than on the phage.

My experimental results in regard to single-step growth experiments were quite similar to Bowman's. Sellers, Baxter and Runnals (49) also report a step-wise growth curve when done in the absence of Tween 80, and a single-step growth curve when Tween 80 was added. Their latent periods are shorter, burst sizes higher, and adsorptions faster than those which I, or Bowman, attained.

Castelnuovo states that, "The supernatant of the Tween 80 treated mycobacteria showed marked inhibitory activity against the phages, and the plaques were greatly reduced in number. This activity was probably not due solely to the presence of Tween 80 because our controls, which contained Tween 80 at higher concentration (1%) than that present in the supernatant, showed a much weaker inhibitory activity. These findings would seem to suggest that some substance has been removed from the surface of the bacteria and that, as a result of this, the phage is no longer able to attach itself to the host" (10).
As mentioned earlier, pinpoint plaques did not appear until after 3/4 day for phage Bol, more than one day for phage Bo2h, and about two days for phage Bo2. Thus, the relatively long growth cycles which I observed are quite sensible. These might also be due to the slow growth of the host bacterial strains. The relatively low burst sizes I have obtained are probably accounted for by the fact that not all of the infected bacteria burst. (Sheldon Gelbart, in this laboratory, did single burst experiments on phage Bo2, and found that more than 1/3 of the infective centers did not burst).

Mycobacteriophages act in several ways differently from the phages of other phage-host systems. There is much work to be done before one can arrive at an understanding of the many characteristics exhibited by mycobacteriophages. Genetic work using these as agents of transfer is in its infancy. Mycobacteriophages provide an excellent opportunity for studying age properties in detail because, compared to other phages, mycobacteriophages do everything in "slow motion".

In conclusion, it can be seen that phages Bol, Bo2, and Bo2h, although they share common antigens, are different in many respects. They exhibit differences in: 1) single plaque morphology, 2) electron microscopic morphology, 3) host range, 4) DNA base composition, 5) adsorption rate and the effect that CaCl₂ and Tween 80 have on them), 6) cross-neutralization, and 7) single-step growth cycle (including the effect of Tween 80 on ).
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The thesis submitted by James P. Kraiss has been read and approved by the members of the Advisory Committee listed below.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

Nov. 2, 1971
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