Purification of B. megatherium Phage G and Evidence for a Muralytic Enzyme as an Integral Part of the Phage

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ABSTRACT The purification of B. megatherium G phage is described and it is shown that DEAE cellulose chromatography combined with conventional methods gave a phage preparation which was at least 95 per cent pure, and contained 2.16 μg nitrogen/10^{11} infective particles. The phage particle weight in molecular weight units was 91 $\times$ 10^6. The small amount of contaminating material appeared to represent phage "ghosts." An essentially 1:1 ratio of particles to infective units was found when data from electron microscopic counts or data from chemical analysis were related to phage infectivity. Comparison, by several methods, of the G phage and coliphage T2 shows that T2 is 2.6 times larger than G phage. The specific activity of the muralytic component obtained by disintegration of phage preparations with urea was unchanged by the purification indicating that the phage-"bound" muralytic activity is an integral part of the phage structure.

INTRODUCTION

Lytic activity against the bacterial cell has been found in phage lysates of several bacteria (1–5). The muralytic activity has been shown to be of enzymatic character and not associated with the phage particle itself but associated with the phage infection of the host cell (3, 4). In a previous communication a muralytic component from B. megatherium phage G was described, which appeared to be closely attached to the phage particle (6). This component showed enzymatic properties and could be clearly distinguished from the "soluble" lytic substance formed in the same system. To obtain conclusive evidence that this enzyme is an integral part of the virus structure, considerable purification of the B. megatherium G phage is necessary. The present investigation presents methods for extensive purification of B. megatherium phage G and the physicochemical characteristics of the purified product. It also compares the enzymatic activity obtained with purified virus to the activity obtained with less pure material.

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MATERIALS AND METHODS

Bacteriophage The bacteriophage G used in the present study was isolated from *B. megatherium* KM as described by Murphy (3).

Plate Preparations Plates were made with 7 per cent peptone and 2 per cent agar. Each plate contained 30 ml. Plates for counting plaques were prepared by the double agar layer method (7).

Bacterium All experiments were done with *B. megatherium* KM maintained by daily transfers on slants composed of 2 per cent peptone and 2 per cent agar.

Heavy suspensions of bacteria were prepared by inoculating about 10^8 bacteria suspended in 2.5 ml of 5 per cent peptone on a Yeast Dextrose Calcium Medium (Y.D.C.)[1] plate which was incubated overnight at 33°C. A suspension resulting from washing off the plate with 5 ml of sterile tap water, when used in phage assay, provided a plating efficiency up to 2.5 times higher than the usual methods (7) and a standard phage suspension plated daily showed variations no greater than predicted by the Poisson distribution.

Broth Cultures All broth cultures were grown in 5 per cent bacto-peptone at pH 7.4 in 175 × 22 mm test tubes shaken in a water bath at 30°C at 400 cycles/minute through a distance of 20 mm. The bacterial generation time was approximately 40 minutes with a culture volume of 15 ml per tube. All turbidimetric measurements were made in a Klett-Summerson colorimeter which was modified to accept these tubes.

Cell Walls Cell walls were prepared by the method of Salton and Horne (8), then treated with 0.1 mg/ml of trypsin for 5 to 15 hours and submitted to differential centrifugation.

Chromatographic Technique The anion exchange DEAE (*N*,*N*-diethylaminoethyl)-cellulose was prepared as described by Peterson and Sober (9). The flow rate of the column was about 50 ml/hour. The buffer system was 0.02 M phosphate buffer,

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[1] Yeast Dextrose Calcium Medium

Distilled water	1000 ml
Difco bacto-yeast extract	5 gm
$MgSO_4$	1 gm
K_2HPO_4	0.5 gm
Difco bacto-agar	20 gm
Dextrose	20 gm
$CaCO_3$ precipitated (Merck)	20 gm
1.0 M NaOH	1.75 ml

Dextrose is mixed with 150 ml distilled water. The other ingredients are mixed with 850 ml of distilled water in a separate flask which is heated to dissolve the agar. The flasks are autoclaved separately at 15 pounds for 15 min. After autoclaving the two mixtures are combined and the pH adjusted to 7.5 using sterile technique. The medium is then dispensed aseptically into smaller flasks making sure that the $CaCO_3$ is uniformly suspended before pouring. The medium is then resterilized by heating in flowing steam for 35 minutes.

pH 7.4. Except when stated otherwise, elution was carried out with a salt gradient in NaCl from 0.12 to 0.33 M. The samples were adsorbed to the column in 0.12 M NaCl buffered with 0.02 M phosphate at pH 7.4, after preliminary equilibration of the column in the same ionic environment. The column was regenerated with 0.5 M NaOH before it was used again. Optical density in arbitrary units was automatically recorded in the eluates at 260 mμ on a Canalco ultraviolet (UV) absorption meter.

Enzyme Assay The bound enzyme was assayed in a reaction mixture containing 0.03 ml of a cell wall suspension (5 mg of dried material/ml), 0.1 ml of 0.1 M glycine buffer pH 9.5, 0.1 to 0.2 ml of the enzyme preparation, and demineralized water to a final volume of 1 ml. One unit of enzyme is defined as the amount that causes 50 per cent lysis of the cell walls in 45 minutes at 20°C. The lysis of the cell walls was followed turbidimetrically and it was found that the rate of lysis was dependent on enzyme concentration within a range of 0.5 to 4 units of enzyme in the reaction mixture. The assay was also carried out in 5 per cent peptone at pH 7.4. The reaction mixture then contained 0.1 to 0.2 ml enzyme preparation, 0.03 ml of a cell wall suspension, and peptone to a final volume of 1 ml. Enzymatic activity was also tested in peptone after the enzyme was made non-sedimentable by treatment with 10 per cent $(NH_4)_2SO_4$ in peptone. The enzyme preparation was also tested for remaining activity of the soluble enzyme previously described (3) at the 4.5 pH maximum of this activity.

Electron Microscopy Purified phage suspensions containing 5 to 8 × 10^{12} phage/ml were placed on carbon-coated grids. The phages were "stained" with uranylacetate and/or phosphotungstic acid at pH 5.0 and examined in a Phillips or a Siemens electron microscope according to the procedure of Brenner and Horne (10).[2]

Chemical Tests Nitrogen was measured by the Kjeldahl procedure and phosphorus was determined by a modification of the procedure of Fiske and SubbaRow (11). Optical density was generally measured in a Beckman DU spectrophotometer and spectra were recorded in a Carey automatic spectrophotometer.[3]

Ultracentrifugation Density gradient centrifugation was carried out in a cesium chloride gradient according to the technique of Meselson *et al.* (12). The phage was mixed with cesium chloride to obtain a measured density of 1.47 and centrifuged at 97,238 g (average) for 18 to 20 hours in a swinging bucket rotor SW 39L in a Spinco L ultracentrifuge. Drops were collected from the bottom of the tube after pinhole perforation and the density was measured by weighing fixed amounts in a micropipette. Optical density and phage titer were also determined on the fractions so obtained.

Sedimentation coefficients were calculated after centrifugation in an analytical ultracentrifuge model E with the AN-D rotor at a speed of 20,410 RPM.

Diffusion Coefficient[4] The diffusion coefficient of the phage was measured by two

methods. (A) The spreading, by diffusion, of a boundary between a 0.1 per cent phage solution and the phosphate buffer against which it had been dialyzed, was followed at 25°C, with the aid of Rayleigh interferometry (13) in a sheared boundary cell having channels of 3 × 25 mm cross-section. Although this cell has not yet been described it is similar to the one of Antweiler (14) except for the channel dimensions. The refractive index difference between phage solution and buffer was such as to give a total of 9.7 fringes. The concentration distribution in the boundary was not markedly non-Gaussian and fringes 2 to 8, inclusive, were used in the evaluation of a diffusion coefficient for each of six patterns recorded at intervals over a 48 hour period. (B) The diffusion coefficient was also determined at low speed centrifugation (6,000 RPM) in the analytical E centrifuge with Rayleigh interferometry.

TABLE I

PURIFICATION STEPS OF *B. MEGATHERIUM* PHAGE G

	Procedure
Step 1	Crude lysate
Step 2	Sedimented at 44,330 g(average) for 150 min. Pellet allowed to resuspend without agitation
Step 3	Supercel filtered and centrifuged at 105,400 g(average) for 60 min. Pellet resuspended without agitation
Step 4	DEAE chromatography

RESULTS

The Purification of B. megatherium Phage G   B. megatherium KM was infected in broth cultures with a multiplicity of 10 phage G particles/ bacterium and the lysates harvested 60 to 180 minutes after infection. The steps used in the purification procedure are given in Table I. The method of the supercel filtration is that of Northrop (15). After two cycles of centrifugation and filtration through supercel the pellets of phage were still brownish and not completely translucent. Chromatography on DEAE cellulose was therefore tried as a further purification step. Fig. 1 shows a typical elution diagram based on the optical density and the phage titer when a gradient from 0.12 to 0.33 M NaCl was used with material from step 3 (*cf.* Table I). The recovery of infectious phage in the eluate was from 74.5 to 78.5 per cent of the input when a gradient was used, but only 30 to 50 per cent when the DEAE cellulose chromatography was carried out in a batch procedure at 0.12 or 0.14 M NaCl. The purification factor of the chromatography step was 2 with regard to nitrogen content of the material, and 1.8 with regard to optical density at 260 mμ. Since only the purified phage elutes from the column, it seems that most impurities remain adsorbed to the column. The similarity of the purification factors for nitrogen content and for optical density at 260 mμ suggests that a large part of the impurities removed is nucleic acid.

When the eluates of phage were centrifuged, the pellets were colorless and translucent. To obtain further purification of the eluates they were subjected to density gradient centrifugation in cesium chloride. As seen in Fig. 2 the phage showed a single sharp peak in the cesium chloride gradient. Total recovery of optical density was obtained in the fractions from the gradient, but only 65 per cent of the infective phage input was recovered. This may have been due to inactivation of phage by the high concentration of cesium chloride. Most of the phage was found in the fraction with a density of 1.50.

An ultracentrifugal analysis of the eluates from the column showed one
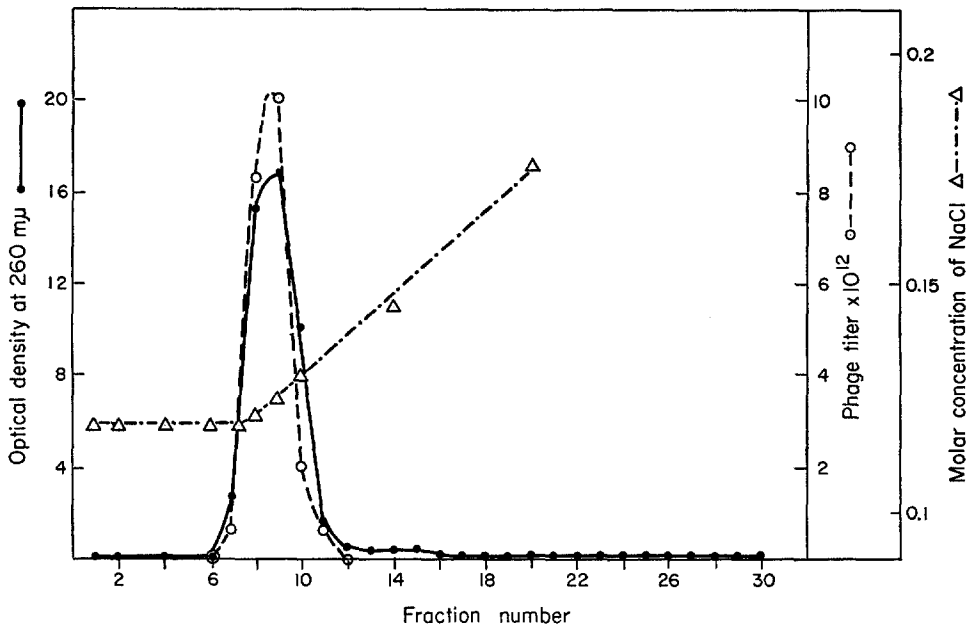

FIGURE 1.   Elution diagram of *B. megatherium* phage G from DEAE cellulose.

major peak in all preparations and a second smaller peak in all but one preparation, constituting between 3 to 9 per cent of the major component. The major peak was sharp and symmetrical by the schlieren pattern and showed extensive absorption in ultraviolet (UV) light. This peak was probably due to phage particles. The second component appeared as a symmetrical peak in the schlieren pattern but showed no significant ultraviolet absorption and was therefore presumably protein in nature. It is likely that the second component represented phage ghosts. Fig. 3 records the concentration dependence of the $s_{20, w}$ values for the two peaks. The S values at infinite dilution are 321 for the major component and 118 for the minor one. The diffusion coefficients ($d_{20, w}$) for the phage suspension (method A) and the major peak (method B) were also determined, and they were found to be

$2.5 \pm 0.06 \times 10^{-8}$ and $2.8 \pm 0.2 \times 10^{-8}$ cm^2/sec., respectively. The value of 2.6 was used in the calculation of the molecular weight of the phage which was found to be 91×10^6.

Since the whole phage suspension and the major peak have similar diffusion coefficients it is most likely that the minor peak represents phage ghosts. The
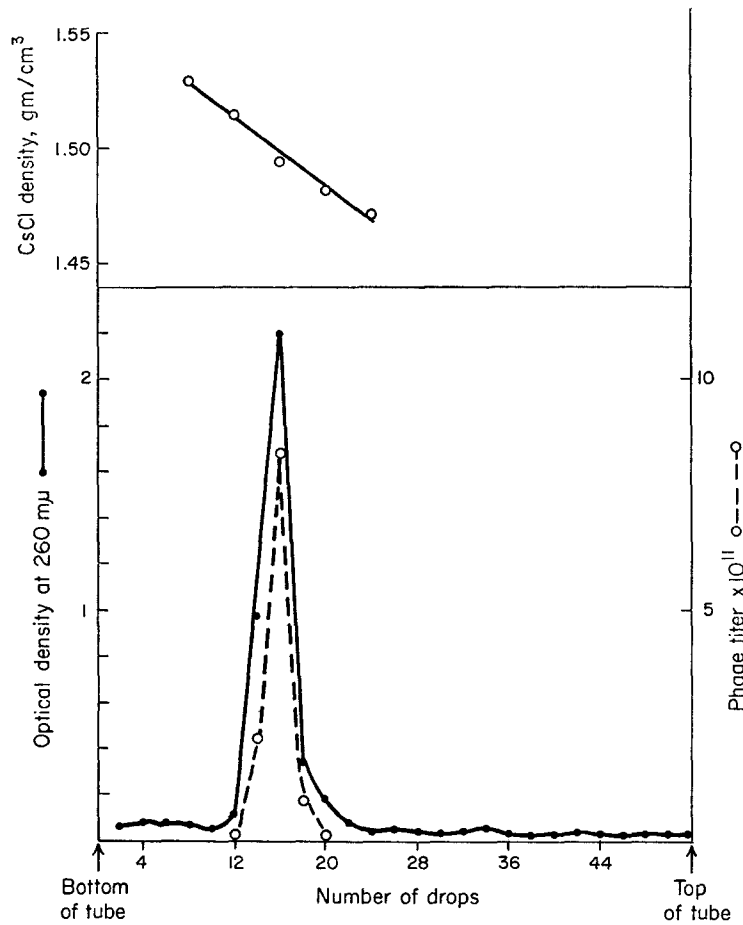

FIGURE 2. Cesium chloride density gradient centrifugation of B. megatherium phage G

ghost fraction increased by storing the purified preparation at 4°C. In the best preparations, the minor fraction was 3 per cent of the major fraction, but it increased to 20 per cent on storing for 2 weeks. A DNA fraction could also be resolved in the ultracentrifuge after the preparations had been stored.

When the absorption spectrum for the eluates of the column was recorded, a spectrum characteristic of a nucleoprotein with a maximum at 260 mμ and minimum of 238 mμ was obtained as seen in Fig. 4. The OD 260/OD 280 ratio was 1.57 and the OD 230/OD 260 ratio was 0.85.

The nitrogen and phosphorus analyses gave 2.16 μg nitrogen and 0.69 μg phosphorus per 10^{11} infective phage particles. Table II summarizes the chemical characteristics found for the purified material after DEAE cellulose chromatography.

Electron Microscopy of the B. megatherium Phage G The appearance of the G phage in uranylacetate-stained preparations is shown in Fig. 5. The shape of the head is very close to spherical, but at the higher magnification it appears to be a hexahedron. The tails are extremely long and thin; they are
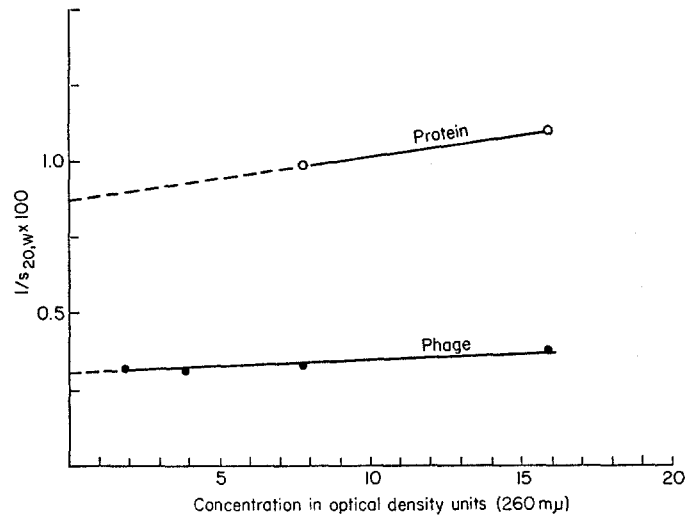

Figure 3. Concentration dependence of sedimentation coefficients for the phage particles of *B. megatherium* phage G and a protein component (phage ghosts).

approximately 4 to 5 times longer than the diameter of the head, but only 7.6 mμ wide. A central hole in the phage tail could be resolved and its diameter was found to be only 23 A. Table III summarizes the measurements made on the structural units of the G phage and the calculated volumes of these units.

Correlation between Number of Particles and Infective Phages To obtain conclusive evidence that the phage preparation obtained after DEAE cellulose chromatography is essentially pure, an estimate of the number of particles per infective phage was made. This formed a basis for comparison of chemical and physical data with the infectivity titers.

The *B. megatherium* phage G was sedimented onto electron microscopic grids from a suspension containing known concentrations of polystyrene spheres of an average diameter of 256 mμ. The technique used was essentially the same as described by Overman and Tamm (16). The disadvantage of this technique with this virus is that since the polystyrene and the phage sediment

with different speeds the ratio of polystyrene to phage varies too widely. Furthermore, the adsorption to the sides of the cell of the two particles might be different and give erroneous results. The method, however, was expected

$$\frac{O.D. 230}{O.D. 260} = 0.85$$
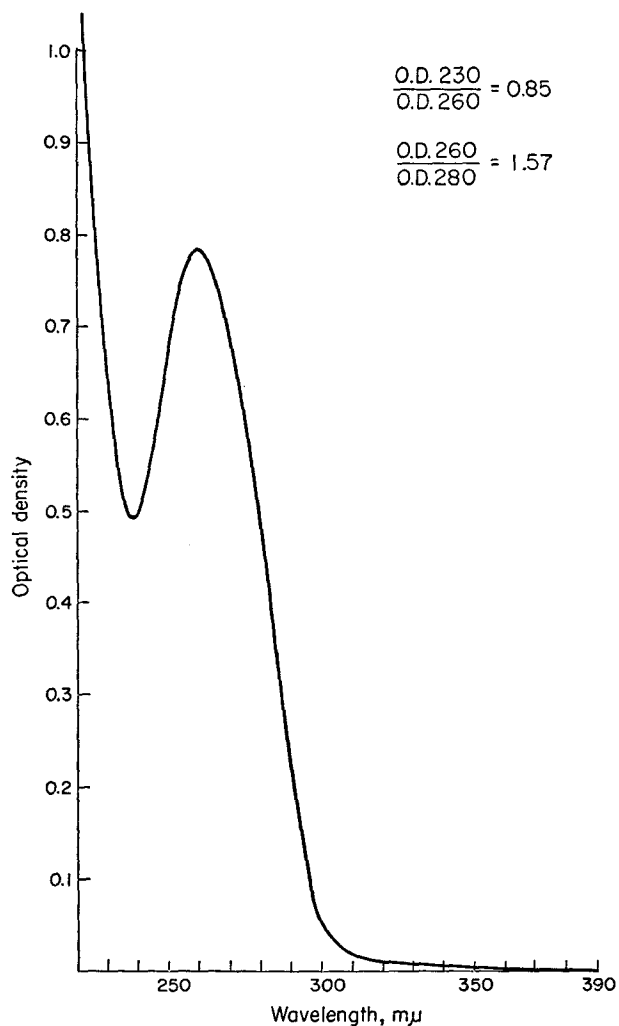
$$\frac{O.D. 260}{O.D. 280} = 1.57$$

FIGURE 4. Ultraviolet absorption spectrum for a purified preparation of *B. megatherium* phage G (step 4, Table I).

to give a crude estimate of the ratio of particles to infective phage. As seen in Table IV this ratio varied from 0.79 to 1.53 depending on how the particles were counted. Since these data were obtained with only partially purified material, the ratio 0.99, based on a comparison of the actual numbers of polystyrene and phage particles, appears most reliable. Thus, the ratio

between the numbers of particles and infective phages is close to 1 in the present system.

This ratio makes meaningful a direct comparison of data obtained by chemical and physical techniques with values computed on the basis that one infective unit of phage corresponds to one physical particle (*cf.* Table V). The partial specific volume obtained by cesium chloride gradient sedimentation is slightly lower than that obtained by postulating that all the phosphorus is in the DNA of the phage. There is agreement as to the mass of the infective particle among all three methods used. The values for the DNA fraction obtained by phosphorus analysis agree with calculations based on the extinction coefficient but the value obtained from the partial specific volume is higher. The phage thus contains between 45 to 55 per cent DNA. The higher value is more likely because the phosphorus determination is probably

TABLE II

CHEMICAL CHARACTERISTICS OF PURIFIED
B. MEGATHERIUM PHAGE G

OD $260/10^{12}$ phage $= 1.53$	$s^{\circ}_{20, w} = 321 \times 10^{-13}$
Nitrogen/10^{11} phage, $\mu g = 2.16$	"Molecular" weight $= 91 \times 10^{6}$
Phosphorus/10^{11} phage, $\mu g = 0.69$	Density $= 1.50$
OD $260/$OD $280 = 1.57$	N/P ratio $= 0.31$
OD $230/$OD $260 = 0.85$	$d_{20, w} = 2.6 \times 10^{-8}$

somewhat low, which is not unexpected since dialysis of the phage suspension was necessary and may have resulted in losses due to adsorption. The extinction coefficient should give a slightly low value since the extinction of DNA in a phage envelope presumably is less than that of free DNA.

A similar comparison of experimental and theoretical data for *E. coli* phage, T2, and the experimental data for phage G are given in Table VI. The best experimental data have been calculated or taken directly when available. The G phage values *observed* give a ratio between 2.4 and 2.9 with the *theoretical* T2 values, with an average of 2.6. The data suggest that the phosphorus values obtained for phage G are probably slightly low.

In summary, phage G, purified according to the procedure described showed essentially a 1:1 ratio between physical and infective particles, and was homogeneous by the chemical and physical criteria generally used to characterize phage preparations. In mass phage G is 2.6 times smaller than phage T2.

The Recovery of Muralytic Activity from the Purified B. megatherium Phage G It has been shown earlier that intact phage particles in a concentration of 3 × 10¹³/ml (3) have no enzymatic activity against the cell walls. However, after urea treatment the DNA is released and the "bound enzyme" becomes active
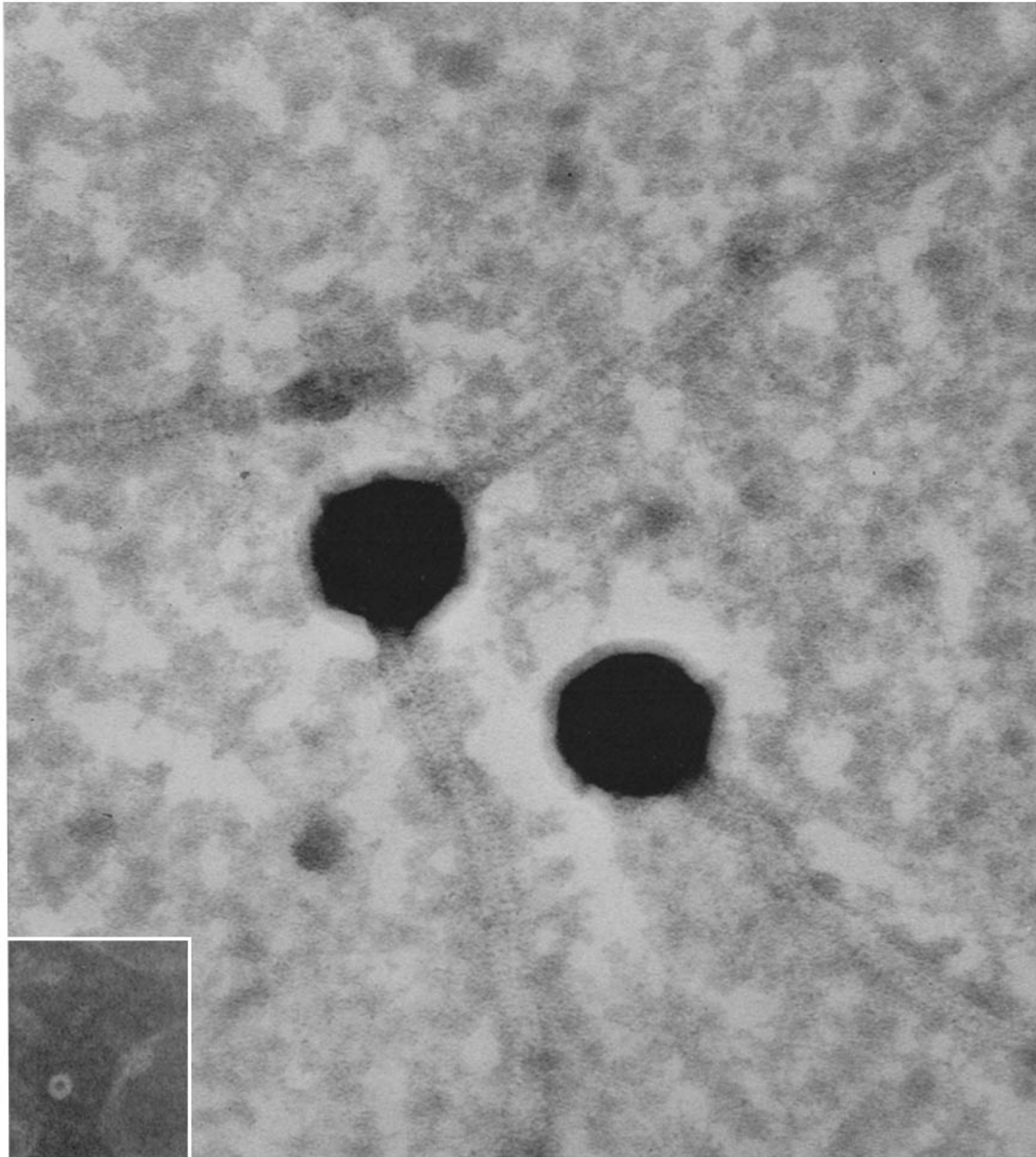
FIGURE 5.   Electron micrograph of *B. megatherium* phage G stained by uranylacetate. × 473,000. Insert, phage tail end on. × 473,000.

The phage preparations of step 3 or step 4 in Table I were centrifuged at 105,400 *g* (average) for 60 minutes and, after resuspension in 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate (saline), were diluted with an

TABLE III

THE SIZE AND VOLUME OF *B. MEGATHERIUM* PHAGE G
AS DETERMINED BY ELECTRON MICROSCOPY

Structure	Size	Volume
	$m\mu$	$m\mu^3$
Head, diameter	55.1 ± 1.1	88.1×10^3
Tail, length	226 ± 6.2	10.2×10^3
width	7.6 ± 0.2	
	Total	98.3×10^3

TABLE IV

THE RELATIONSHIP BETWEEN NUMBER OF
PARTICLES AND INFECTIVE PHAGES AS DETERMINED
BY ELECTRON MICROSCOPY

Calculation method	Ratio particles/infective phage	
	Counting resolved phages	Counting all phage-like particles
Theoretical polystyrene concentration	0.79	1.00
Counted polystyrene concentration	0.99	1.53

TABLE V

PROPERTIES OF *B. MEGATHERIUM* PHAGE G
FROM RELATED EXPERIMENTAL DATA

Property	Experimental		Estimation	
	Observation	Method	Value	Method
Partial specific volume, $\bar{V}$	0.67	CsCl gradient	0.69	Phosphorus analysis
Mass, gm	1.41×10^{-16} 1.47×10^{-16}	Nitrogen analysis Electron microscopy and density	1.4×10^{-16}	Molecular weight determination
DNA fraction	0.45	Phosphorus analysis	0.55 0.46	$\bar{V} = 0.67$ Extinction coefficient at 260 $m\mu$

equal volume of 10 M urea. This preparation was subsequently dialyzed against 0.15 M saline. The enzymatic activity of the preparation against cell walls was tested at pH 9.5 (glycine buffer) and in peptone. The activity was still sedimentable at 114,560 g (average) after 60 minutes. However, solubilization was achieved by resuspending the sediment in, or dialyzing against 10 per cent $(NH_4)_2SO_4$ in peptone.

TABLE VI

COMPARISON OF CHEMICAL CHARACTERISTICS OF
B. MEGATHERIUM PHAGE G AND COLIPHAGE T2

Characteristics	T2 experimental	T2 theoretical	G phage experimental	Ratio $\dfrac{\text{T2 theoretical}}{\text{G phage experimental}}$
Mass, gm	—	3.6×10^{-16}*	1.4×10^{-16}	2.6
N/10^{11} phage, μg	8.3‡	5.75*	2.16	2.6
P/10^{11} phage, μg	2.0§	2.0*‡	0.69	2.9
OD 260/10^{12}	6.0§	3.6§	1.53	2.4
Molecular weight	—	215×10^{6}*	91×10^{6}	2.4

* Cummings, D. J., and Kozloff, L. M., 1960, *Biochim. et Biophysica Acta*, **44**, 445.
‡ Herriott, R. M., and Barlow, J. L., 1952, *J. Gen. Physiol.*, **36**, 17.
§ Hershey, A. D., Dixon, J., and Chase, M., 1953, *J. Gen. Physiol.*, **36**, 777.

The number of phage particle equivalents representing one unit of enzymatic activity was compared for phage preparations before and after the chromatographic step in the purification and, as seen in Table VII, no decrease in enzymatic activity after chromatography was observed when the activity was measured at pH 9.5. However, it appears that with the lower concentrations of phage obtained after purification, and, with the much lower concentration of contaminating material, the enzyme was rendered less stable and more difficult to solubilize. This may have caused the major losses of specific enzyme activity which were observed in all subsequent steps of the enzyme isolation. However, a sufficient quantity of enzyme survived the procedure to give measurable activity in the assay against cell walls.

The findings that after the chromatographic step in phage purification, the preparation had retained its enzymatic activity in full, coupled with the previous demonstration of the non-identity of the soluble and bound enzymes (6), indicate that the bound enzyme is, in all probability, a functional component of the virus particle.

TABLE VII

ENZYMATIC ACTIVITY OF PURIFIED PHAGE
COMPARED TO LESS PURE MATERIAL

Preparation of G phage*	Phage equivalents $\times 10^{12}$ per enzyme unit			
	Conditions for assay			
	pH 9.5 glycine	pH 7.4 peptone	Solubilized‡ enzyme	Non-solubilized enzyme
Step 3	2.5	1.5	0.6	9.2
Step 4, preparation 1	—	4.1	9.4	4.8
Step 4, preparation 2	2.9	3.8	52.5	15.7

* *Cf.* Table I.
‡ Refers to enzyme solubilized by $(NH_4)_2SO_4$-peptone.

DISCUSSION

The purification of *B. megatherium* phage G is described and it is shown that chromatography on DEAE cellulose increases the purity twofold compared to methods used previously. The elution characteristics of G phage from the cellulose column are similar to those reported for T2 phage and ECTEOLA cellulose (17). The purity of the phage G eluates after chromatography seems to be high but the phage appears to be 3 to 9 per cent contaminated with a protein component which probably represents phage ghosts. When the combined chemical data or electron microscopic phage counts are related to the number of infective units of phage, a 1:1 ratio between particles and infective units is obtained. The value for the ratio of nitrogen to infective units agrees with previously published data on purified *B. megatherium* T phage (15).

The purity of the preparations described made it possible to obtain evidence of whether or not the muralytic activity associated with the phage structure is an integral part of the phage. It has previously been shown in this and the T2 phage systems that purified intact phage has no muralytic activity (3, 18), but that after chemical alteration phage particles are capable of causing the release of nitrogen and carbon from cell walls in the T2 phage system (18) or of lysing cell walls in the *B. megatherium* system (6). The present investigation has provided evidence in favor of the contention that the muralytic enzyme of the *B. megatherium* G phage is an integral part of the phage, since after purification of the phage, the enzymatic activity is essentially unaltered as determined by phage equivalents per enzyme unit at pH 9.5. Therefore, it appears that the *B. megatherium* phage G system has two distinct enzymes involved in the multiplication cycle of the phage; the bound enzyme of the phage being involved in the penetration of the nucleic acid, and the soluble enzyme, produced in the bacterial cell during phage infection, functioning in the release of the phage.

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