STUDIES ON THE PURIFICATION OF BACTERIOPHAGE

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Many of the earlier studies on the purification of bacteriophage were undertaken with the object of demonstrating that phage is an autonomous, organized antigen distinct from the bacterium at whose expense it regenerates. Other investigators were inclined to consider the active agent as a chemical substance rather than a living organism and attempted to obtain the active agent in a pure state in order to study its chemical nature. The findings in these early studies failed to supply enough data to establish the chemical composition of bacteriophage, since no convincing criteria of purity were applied, nor could the activity of the purified preparation relative to the weight of the agent be ascertained (1–7). On the basis of the negative outcome of the usual protein color tests, most of these workers have come to the conclusion that phage is not protein in nature. However, the failure of a preparation to give protein color tests cannot alone be considered as a sufficient evidence to exclude the presence of a small amount of protein, especially when one is dealing with substances exhibiting activity in extremely low concentration.

A few workers did supply enough data to at least permit estimation of the activity corresponding to the nitrogen content of the preparation. Thus Kligler and Olitzki (8) purified a coliphage by adsorption on kaolin in acid solution with subsequent elution by rendering the reaction alkaline, and obtained a preparation with 0.014 mg. nitrogen and 10^9 active lytic units per cubic centimeter of concentrate (1.4×10^{-11} mg. of nitrogen per unit of lytic activity). Because the protein color tests were negative, they concluded that phage was not protein. Colwell (9), by extraction of "lysogenic" cultures with water, obtained a preparation containing 0.0027 mg. nitrogen and 10^7 units per cc. (2.7×10^{-10} mg. N per unit of lytic activity).

In 1927 one of us (10) attempted the purification of phage by fractional ultrafiltration and obtained as an end product a preparation which had the following composition:[1] C = 37.8 per cent; H = 5.74 per cent; N =

[1] Analyzed through the kindness of Dr. A. Elek of The Rockefeller Institute.

4.18 per cent; ash = 18.9 per cent. 360 liters of ultrafiltered phage of titre 10^6 active units per cc. yielded 7.7 mg. of dry residue, which corresponds to 1×10^{-12} mg. of nitrogen per active lytic unit. When concentrated to a syrupy mass, just before reducing to complete dryness, this concentrate retained practically all its original activity, and 1 cc. of this gave negative protein color tests, but positive copper reduction. When reduced to dryness the material was totally inactive, and the organic analysis given above was made on such inactive material. The analysis suggested that the material consisted either of a nitrosugar or of a polysaccharide contaminated with small amounts of nitrogenous material. However, there was still another possibility that could not be excluded, namely that this nitrogen might represent a small amount of protein contaminated with a relatively large amount of some non-nitrogenous material, and that the amount of protein present was insufficient to give protein color tests. This possibility seems the more likely in the light of recent observations of Northrop (11) that in his purified preparations of phage the active protein was contaminated with mucin-like carbohydrate which was difficult to remove completely.

Recent studies have indicated that phage might be protein in nature. Schlesinger (12–14) collected bacteriophage on a collodion membrane, purified it by repeated high speed centrifugation, and obtained a preparation which he described as having a density of 1.3 and consisting of spherical particles about 85 mμ in diameter. Calculated on the basis of the lytic activity of the original material, the dry residue represented 4.3×10^{-13} mg. of substance per unit of activity. Since the analysis of the residue showed that it contained 13 per cent of nitrogen, it appears that this preparation had 5.6×10^{-14} mg. of nitrogen per unit of lytic activity. Despite the fact that the Molisch test became very weak during purification, on the basis of the high phosphorus content (3.7 per cent), the positive Feulgen reaction, and insolubility in dilute alkali, Schlesinger concluded that it was a nucleoprotein. More recently Northrop (11), following the chemical procedure he has previously employed for purification of enzymes, isolated from a staphylococcus phage a nucleoprotein bearing the phage activity, and since hydrolysis of this nucleoprotein resulted in loss of activity, he considered that it represented the phage itself. He observed that this purified phage was quite labile and was quickly inactivated upon drying. About 5×10^{-13} mg. of this protein sufficed to produce lysis of a young culture of the homologous organism. On the assumption that this amount represented one molecule, he calculated the molecular weight to be about 300,000,000 and the diameter of the particles to be about 100 mμ. He

points out that "The active protein is of much higher molecular weight than most normal proteins and this is true of viruses in general."

On the other hand, by subjecting *B. coli* bacteriophage to fractional ultrafiltration, one of us (15) has shown earlier that particles endowed with lytic activity varied in size within wide limits but in general were of the order of magnitude of simple proteins (2 to 20 mμ in diameter). In attempting to explain this discrepancy, Northrop (11) investigated the diffusion rate of high diluted suspensions of his purified staphylococcus bacteriophage and found that it also contained smaller particles of about 5.6 mμ in radius corresponding to a calculated molecular weight of about 450,000. Since the small particles were apparently in equilibrium with the large ones (they were obtainable by simple dilution), he concluded that ". . . this protein exists in various sized molecules of from 500,000–300,000,000 molecular weight, the proportion of small molecules increasing as the concentration decreases."

In view of the evidence for the protein nature and relatively large molecular size of bacteriophage supplied by the experiments of Schlesinger (12–14), and particularly of Northrop (11), it was of interest to repeat the earlier (1927) experiments of Bronfenbrenner.

Methods

In our earlier experiments (1927) the Berkefeld filtrate of the lysed culture was forced through a relatively dense collodion membrane in order to remove the bulk of bacterial debris, and the ultrafiltrate was then concentrated on a double 7 per cent membrane. This procedure had two disadvantages: in the first place, the first ultrafiltration resulted in a considerable reduction in phage content so that the ultrafiltrate seldom contained more than 10^6 lytic units per cc. In the second place, collection of the ultrafiltered phage on a double 7 per cent membrane might have been responsible for the failure to remove some of the contaminating carbohydrate by subsequent washing. The more recent finding that bacterial protein is largely hydrolyzed during lysis (16–17), and the evidence that the particles of bacteriophage may reach the size of heavy protein molecules, suggested the possibility of collecting the phage directly on a collodion membrane of such a density that would just fail to let it pass without the preliminary fractionation.

Since the phage was to be collected directly on a collodion membrane, the medium of propagation obviously had to be completely diffusible through that membrane. To meet this requirement, the synthetic medium described by Hetler and Bronfenbrenner

(16) was used (modified to the extent that dextrose was substituted for saccharose). To avoid formation of a precipitate this medium was sterilized in two separate parts.

Part 1.—1 gm. Na_2HPO_4; 6 gm. ammonium succinate; 500 cc. distilled water. Titrate to pH 7.6. Autoclave.

Part 2.—20 gm. dextrose; 1 gm. $MgSO_4$; 1 gm. K_2SO_4; 500 cc. distilled water. Autoclave. When cool mix parts 1 and 2 and readjust to pH 7.2 with sterile NaOH.

The culture used throughout the work was a strain of colon bacillus (*B. coli* P.C.) which was known to be free of spontaneous lytic activity over a period of 15 years. This organism grew readily on the synthetic medium. When homologous phage (P.C.) was added to a young culture, regeneration of the phage was completed in 16–18 hours, though the culture was not sterilized so that in about 20–24 hours overgrowth of resistants usually became apparent.

All titrations of phage were carried out by a procedure which represents an adaptation of a method of counting bacteria described by McCrady (18), except when another procedure is indicated. This titration consists essentially in preparing serial tenfold dilutions of the active filtrate and testing five separate 1 cc. samples from each dilution for the presence of phage by addition of susceptible culture. As the dilution factor increases, the proportion of 1 cc. samples showing lysis will decrease. From data representing distribution of samples showing lysis (containing phage) the probable number of active units in the original suspension was calculated by means of probability tables (18).

The reinforced collodion membranes were prepared by depositing collodion[2] on the inner surface of an alundum thimble under pressure. In the preliminary experiments membranes of various densities were prepared by varying the concentration of the collodion and the time and pressure under which the collodion was deposited. The procedure which was chosen consisted of depositing the collodion under a vacuum of 15 inches of mercury for 1 minute, then emptying the thimble, and permitting it to drain for 2 minutes, after which it was solidified by admitting water and washed to remove the acetic acid for at least 24 hours before use.[3] These membranes were fairly reproducible as indicated by preliminary test of each membrane as to the rate of flow of water through it under pressure of 20 inches of mercury. All membranes which permitted the passage of a liter of water in about 1 hour and 15 minutes were found to be satisfactory.

EXPERIMENTAL

Approximately 16 hour *B. coli* P.C. phage lysate in synthetic medium was filtered through a Berkefeld filter in order to remove the bacteria and debris remaining after lysis. The phage was then concentrated by ultrafiltration on a 5 per cent collodion membrane, washed with distilled water until the filtrate was negative for ammonia

[2] The 5 per cent collodion solution was prepared according to the method of Bechhold and Gutlohn (19) and consisted of 50 gm. of nitrocellulose and 12.5 gm. of K_2CO_3 dissolved in 1000 cc. of glacial acetic acid. Incidentally, it should be noted that the membranes were prepared from a new batch of nitrocellulose, and were considerably less permeable than those prepared from a different lot of nitrocellulose in 1927.

[3] The essential details and precautions to be followed in this procedure have been described by Bronfenbrenner (20).

(Nessler's reagent), and finally concentrated on the membrane under vacuum to a few cubic centimeters. The concentrated phage was removed with a pipette and any phage adhering to the membrane was washed down by a stream of a few cubic centimeters of distilled water, the walls of the membrane were swabbed with a sterile cotton swab moistened with distilled water, and all phage thus collected was similarly removed with a pipette. This procedure was repeated two or three times with a fresh supply of distilled water and the phage so recovered was added to the original concentrate. As will be shown later, this procedure resulted in the recovery of practically all the phage as shown by the determination of the lytic activity, and of the nitrogen content (Tables I and III).

Since the total nitrogen content (0.6 per cent ammonium succinate) of the medium was about 1 mg. per cc., and the phage content at the end of incubation approximately 5×10^9 lytic units per cc., the original lysate (before ultrafiltration) represented $(1 \div 5 \times 10^9)\ 2 \times 10^{-10}$ mg. of nitrogen per each active lytic unit. However, since this nitrogen represents primarily unutilized ammonia as well as synthesized bacterial protein, and only a fraction of it might represent phage itself, it is evident that purification consisting of copious washing out of more diffusible extraneous constituents must result in a diminution of the nitrogen content per unit of lytic activity in the final product. This was actually found to be the case when the nitrogen content of crude and purified phage and of the phage-free ultrafiltrate, respectively, were determined (Table I). In these determinations the Parnas and Wagner (21) micro-Kjeldahl method was employed, all determinations being carried out in duplicate and an average figure recorded.

A typical experiment is herewith given in detail:

11,500 cc. of 16 hour lysate was filtered in three equal portions (for the purpose of efficiency in filtration) through three separate Berkefeld filters. The combined filtrates were concentrated on a 5 per cent membrane and then washed with distilled water until the ultrafiltrate gave a negative test for ammonia. The concentrate remaining in the thimble (70 cc.) was collected and the nitrogen content and phage activity of each fraction were measured (Table I).

TABLE I

Details of Purification and Concentration of B. coli Phage

	Volume	Phage titre per cc.	N/cc.	N per unit of activity
	cc.		*mg.*	*mg.*
Crude phage	11,500	5×10^9	1.274	2.55×10^{-10}
Berkefeld filtrate	11,500	5×10^9	1.267	2.53×10^{-10}
Ultrafiltrate	11,600	No activity	1.256	No activity
Purified concentrate	70	10^{12}	0.018	1.8×10^{-14}

Comparison of the nitrogen content per unit of activity in the original lysate (about 2×10^{-10} mg. per unit) with that of the washed concentrate (about 1×10^{-14} mg. per unit) shows that by this simple procedure a considerable degree of purification has been attained inasmuch as the concentrate possesses all the original activity of the crude phage while the nitrogen content per unit of activity was reduced approximately 20,000 times.

Since, as stated earlier, most of the nitrogen in the crude phage probably consists of unassimilated ammonium succinate, while some indubitably represents bacterial debris, it was of interest to determine the ammonia and non-ammonia fractions of a Berkefeld filtrate in order to estimate how much of synthesized bacterial protein is actually removed during the process of purification. To determine this, the ammonia in a sample of crude phage was measured by aeration into standard acid and the total nitrogen by micro-Kjeldahl; the difference between the two values was taken as representing synthesized nitrogen. As a control on the efficiency of recovery of ammonia, an equal volume (1 liter) of sterile medium from the same batch was similarly aerated. The results of three such experiments are given in Table II.

TABLE II

Proportions of Ammonia and Non-Ammonia Nitrogen in Crude Phage in Synthetic Medium

Exp. No.	Bacteriophage			Sterile medium (control)		
	Total N	Ammonia N	Difference* (synthesized nitrogen)	Total N	Ammonia N	Difference (experimental error)
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	1329	1288	41	1341	1336	5
II	1273	1264	9	1280	1279	1
III	1269	1252	17	1294	1292	2
	Average.....		22	Average.....		3

* The wide variations in synthesized nitrogen are probably due to the fact that growth of bacteria prior to onset of lysis might have proceeded further in some of the experiments, thus permitting the assimilation of different amounts of ammonia.

It is seen that on an average, approximately 22 mg. of non-ammonia (synthesized) nitrogen were usually present in a liter of crude phage. Since the control determinations on the sterile synthetic medium showed that all but about 3 mg. of ammonia was recovered, this amount represents the probable experimental error. Assuming that the amount of synthesized nitrogen in the crude phage approximates on an average 20 mg. per liter, and knowing that it contains approximately 5×10^{12} active lytic units per

liter, it is evident that the crude lytic filtrate contains ($20 \div 5 \times 10^{12}$) 4×10^{-12} mg. of synthesized nitrogen per active unit. Since the purified phage has been found to have but 1×10^{-14} mg. N per unit, therefore, the phage represents about 1/400 of the total synthesized non-ammonia nitrogen of crude phage. The remainder must represent bacterial debris.

In order to ascertain the validity of these calculations it was of interest to determine how much of the nitrogen present in the original material was accounted for in the process of purification.

10,000 cc. of 16 hour lysate was filtered through three Berkefeld filters, collected on a 5 per cent membrane, washed until free of ammonia, and concentrated to 33 cc. The résumé of the distribution of nitrogen is given in Table III.

TABLE III

Completeness of the Recovery of Nitrogen during Purification

Crude phage	13,020 mg. N
Berkefeld filtrate	12,670 "
Removed by Berkefeld filter	350 "
Concentrate	0.825 mg. N
Ultrafiltrate	12,640.000 "
Wash water	14.000 "
Accounted for	12,654.825 "
Berkefeld filtrate	12,670.000 mg. N
Accounted for	12,654.825 "
Unaccounted for	15.175 " N

While the procedure consisting of ultrafiltration is quite simple, it is apparently quite effective: practically all the activity (Table I), and practically all the nitrogen (Table III) of the original lysate can be recovered and it yields fairly reproducible results as can be seen from Table IV, which summarizes the results of a number of purification experiments carried out at different times over a period of about a year. From Table IV it can be seen that the average purified concentrate had a phage titre of the order of magnitude of 5×10^{12} lytic units per cc. and a nitrogen content of about 10^{-14} mg. per each lytic unit.

In order to secure a sufficient amount of material for chemical tests, and to calculate the weight of material per lytic unit, a number of purified preparations were reduced to dryness (the product being totally devoid of lytic activity). The results of seven such experiments are given in Table V.

Examination of Table V shows considerable differences in the dry weight per unit of activity and in the per cent nitrogen of different preparations. These differences are probably not as significant as they appear on first examination and probably represent the experimental errors involved in

TABLE IV

Summary of Purification Experiments with B. coli Phage

Original volume	Concentrate volume	Phage titre per cc.	N/unit of lytic activity
cc.	*cc.*		*mg.*
8,000	33.5	10^{12}	8×10^{-15}
11,500	70.0	10^{12}	1.8×10^{-14}
9,000	87.0	10^{12}	1.2×10^{-14}
10,000	33.0	3.5×10^{12}	7×10^{-15}
6,000	47.2	5×10^{11}	1.6×10^{-14}
6,000	45.2	6×10^{11}	1.5×10^{-14}
22,000	21.4	8.5×10^{12}	1.4×10^{-14}
20,000	26.3	5.0×10^{12}	1.5×10^{-14}
20,000	32.4	5.0×10^{12}	1.7×10^{-14}
18,000	17.3	8.0×10^{12}	1.4×10^{-14}
18,000	24.0	6.0×10^{12}	9.0×10^{-15}
18,000	25.0	5.0×10^{12}	1.2×10^{-14}
18,000	26.0	8.0×10^{12}	8.0×10^{-15}
14,000	39.0	2.0×10^{12}	1.2×10^{-14}

TABLE V

Nitrogen Content of Dried Purified Coliphage

Exp. No.	Original volume	Concentrate volume	Phage titre per cc. (conc.)	N/unit	Weight of dry residue	Dry weight/unit	Nitrogen of dry residue
	cc.	*cc.*		*mg.*	*mg.*	*mg.*	*per cent*
I	16,000	37.7	3.5×10^{12}	1.14×10^{-14}	9.58	7.26×10^{-14}	15.7
II	14,500	25.8	2.5×10^{12}	3.0×10^{-14}	8.88	1.4×10^{-13}	21.4
III	12,000	34.0	1.7×10^{12}	1.6×10^{-14}	5.27	9.1×10^{-14}	17.5
IV	8,000	43.0	7.0×10^{11}	2.0×10^{-14}	4.03	1.3×10^{-13}	15.4
V	8,000	33.0	9.0×10^{11}	2.0×10^{-14}	4.26	1.44×10^{-13}	13.9
VI	18,000	12.5	1.1×10^{13}	Average VI	6.98	5.07×10^{-14}	Average VI
VII	18,000	11.0	9.5×10^{12}	and VII = 7.8×10^{-15}	6.70	6.41×10^{-14}	and VII = 14.00

weighing of small samples, and inadequate washing of some of the residues. In round numbers these results indicate that each active lytic unit corresponds to approximately 6×10^{-14} mg. of dry residue containing 14–16 per cent of nitrogen.

This nitrogen content suggests the likelihood that the substance is a pro-

tein. In order to confirm this, protein color tests were carried out upon the dried material suspended in physiological saline. With suspensions containing about 2 mg. of dry residue per cc. it was possible to obtain positive biuret, Hopkins-Cole, and xanthoproteic reactions. The material contained sulfur and coagulated upon contact with strong acid. It is to be noted especially that this material contained no reducing sugars, even after hydrolysis with normal HCl at 100°C. for from one to several hours, in contrast to our own findings in 1927 and to the results of Northrop (11) who found about 1 per cent of glucose in his purified phage preparations.

In order to see whether the protein that has been isolated represents a specific substance synthesized only in the presence of bacteriophage, a similar procedure of purification and concentration was carried out upon filtrates of cultures grown on synthetic medium in the absence of bacteriophage.

B. coli P.C. was inoculated into 10 liters of synthetic medium and grown at 37° C. for 1 week in order to permit some autolysis to take place. This culture was filtered in equal portions through four Berkefeld filters, the filtrates were combined, concentrated, and washed upon a 5 per cent membrane exactly as were phage lysates. The last few cubic centimeters remaining on the membrane after copious washing and swabbing represented a water-clear solution which was divided into two portions and the total nitrogen of each determined. The results of three such experiments are given in Table VI.

TABLE VI

Nitrogen Content of Washed Concentrated Berkefeld Filtrates of B. coli P.C. Grown on Synthetic Medium in the Absence of Phage

Experiment No.	Original volume	Concentrate volume*	Average nitrogen content of ½ of sample†
	cc.	*cc.*	*mg.*
I	10,000	34.0	−0.0005
II	10,000	18.3	0.02
III	10,000	56.0	−0.001

* Since nitrogen content of the entire sample was determined, no attempt was made to keep the volume of concentrate constant.

† The apparently anomalous negative nitrogen value is an expression of the experimental error in nitrogen measurement.

These results show that the membrane which was used to collect bacteriophage in the purification procedure failed to retain any nitrogenous material from the filtrates of cultures of *B. coli* grown in the absence of bacteriophage, thus suggesting that the protein previously isolated is different from the protein present in autolyzed cultures of *B. coli*.

Properties of the Purified Phage
Activity and Stability

While the various preparations of purified bacteriophage differed somewhat in their activity, the greater number of concentrates gave a titre of about 5×10^{12} active units per cc., with a nitrogen content of about 10^{-14} mg. per unit of lytic activity.

In contrast to the marked stability of crude bacteriophage, which retains its activity for months even when reduced to dryness, the purified phage is very labile, losing its activity very rapidly even at ice box temperature and becoming completely inactive in 3 or 4 days. Addition of an equal volume of broth to a concentrate preserved the activity somewhat, but usually none was demonstrable beyond a week. If the preparation was dried or mixed with broth and then dried, the dry substance was always completely inactive.

The purified concentrated phage is antigenic, giving rise to antilytic antibodies upon parenteral injection into rabbits and guinea pigs. Formolized phage retains its antigenicity. However, if the phage is inactivated by drying, even in the presence of broth, as much as a total of 2.50 mg. of the phage protein injected in divided doses every other day for a total of four injections into each of six guinea pigs failed to stimulate the production of antilytic antibodies, while 1.80 mg. of a similar preparation, but not subjected to drying, injected into each of six guinea pigs was antigenic in every instance.

Chemical Properties

Two separate batches of 18 liters of phage each (representing a total of 2.32×10^{14} active lytic units) were purified and then dried in a vacuum desiccator. The dry weight of the combined residues was 13.1 mg. Some of this material (12.1 mg.) was analyzed through the kindness of Doctor A. Elek of The Rockefeller Institute, whose report was as follows:

The sample was dried *in vacuo* at 80°C. to constant weight. A sample of 3.092 mg. yielded 5.591 mg. CO_2, 2.182 mg. H_2O, and 0.030 mg. of ash, which corresponds to C = 49.31 per cent; H = 7.89 per cent; and ash = 0.97 per cent. A second sample of 3.602 mg. gave 0.442 cc. of N_2 at 25°C. and 757 mm., which corresponds to N = 14.00 per cent. A third sample of 3.580 mg. gave 0.195 mg. ammonium phosphomolybdate, which corresponds to P = 0.07 per cent.[4]

[4] It is of interest to note that the phosphorus content of our purified preparations is quite low (0.07 per cent) while Schlesinger (13), who also worked with a coliphage,

The material present in the bacteriophage concentrate is, therefore, apparently a protein. It contains 14–16 per cent nitrogen, gives positive protein color tests, and contains some sulfur. The extremely low phosphorus content of the sample analyzed by Doctor Elek (0.07 per cent) indicates that the substance as a whole is not a nucleoprotein, the phosphorus probably represents a trace of the phosphate from the medium which was not removed by washing.

Calculation of Molecular Weight

If a unit of lytic activity is assumed to be represented by one molecule of substance, the average molecular weight may be calculated from the weight of the particle by Avogadro's number. In the average preparation one unit of lytic activity was found to correspond to 10^{-14} mg. nitrogen (Table IV) or 6×10^{-17} gm. of dry residue (Table V) and, therefore, the gram molecular weight is of the order of magnitude of 36,000,000. The best preparation (7×10^{-15} mg. nitrogen per unit of activity), calculated as protein, would correspond to a molecular weight of 24,000,000.

Calculation of Particle Radius

With the average weight of substance corresponding to a unit of activity known (Table V), and assuming that each active unit represents: (1) one particle, (2) of spherical shape, and (3) of density 1.3,[5] the radius of the particle may be calculated:

Weight of particle (grams) $= \frac{4}{3}$ pi $R^3 \times 1.3$

Then:

$$R^3 = \frac{\text{Weight of particle in grams}}{\frac{4}{3} \cdot \text{pi} \cdot 1.3}$$

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analyzing a sample of about 4 mg. (approximately the same size as ours) found 3.7 per cent phosphorus, and concluded that he had isolated a nucleoprotein. Northrop (11), working with a staphylococcus phage, likewise found a high phosphorus content (4.8 per cent) and similarly considered it a nucleoprotein. However, the fact that our purified preparation contained only 1 per cent of ash is in itself a confirmation of the low phosphorus content, since in our earlier preparations (1928–1931), which were less pure, Doctor Elek found 5–8 per cent phosphorus and a correspondingly high ash content (12–30 per cent) in the different preparations.

[5] The density of the bacteriophage particle has been estimated by various workers to be from 1.14 to 1.3 (11, 22–24). Since both Northrop (11) and Schlesinger (13) assumed a density of 1.3 in calculating particle radius, we have likewise assumed this value to facilitate the comparison.

Substituting in this formula the weight of a particle of an average preparation containing 6×10^{-17} gm. of dry residue per lytic unit (Table V), the calculated radius would approximate 22 millimicra, and on the basis of data for the best preparation (calculated as protein, 4×10^{-17} gm. per lytic unit) the particles would have a radius of 19 millimicra.

Attempts at Purification of Bacteriophage from a Yeast Medium

As has been mentioned earlier, Northrop (11) has been able to purify a staphylococcus bacteriophage propagated on yeast medium by a procedure which was essentially the same as that used by him for the purification of enzymes. Because the procedure is rather complicated, it is not always successful. However, during the past year Mr. Gordon Moore in this laboratory was successful in six out of ten attempts in recovering on an average 22.5 per cent of the total activity of the original bacteriophage (the highest activity being 42 per cent in one instance) by the procedure of Northrop (11).

The fact that we have been able to recover practically 100 per cent of lytic activity by a simple ultrafiltration may be to a large extent due to the fact that the bacteriophage we used was propagated on a simple completely diffusible synthetic medium. It was of interest, therefore, to see how effective our procedure might be in purifying a bacteriophage propagated on Northrop's yeast medium.

Since this medium probably contained some diffusible matter, and since the process of purification consisted in collection of the active agent on a collodion membrane, it was essential to remove this non-diffusible material which otherwise would be retained on the membrane together with the phage. For this reason all the yeast extract medium used in these experiments was ultrafiltered through a 5 per cent collodion membrane and autoclaved before being seeded with the bacteria to propagate the phage. The same coliphage was propagated on the *B. coli* P.C. grown on the ultrafiltered yeast medium and purified by collection and washing on a 5 per cent collodion membrane.

The technique in these experiments was exactly the same as has been previously described except that all ultrafiltrations were carried out in the ice chest to minimize contamination of the relatively rich yeast medium. In each case, as a control, a liter of sterile ultrafiltered medium from the same batch was similarly concentrated and washed and the amount of non-diffusible nitrogenous material determined (Table VII). Since there was no means of judging when the washing was completed, the concentrates collected on the membrane were arbitrarily washed with 2 liters of sterile distilled water. The results of one of three such experiments with *B. coli* are given in Table VII.

TABLE VII

Concentration and Partial Purification of Coliphage from Yeast Medium

	Sterile medium (control)	Bacteriophage	Corrected values
Original volume, *cc*	1,000	1,000	—
Concentrate volume, *cc*	15.2	21.9	—
Phage titre/cc	—	10^9	—
Dry weight/cc., *mg*	0.5	1.0	—
Total nitrogen, *mg*	0.26	1.05	0.79
Total dry weight, *mg*	7.6	21.9	14.3
Nitrogen/unit activity, *mg*	—	4.8×10^{-11}	3.6×10^{-11}
Dry weight/unit activity, *mg*	—	1.0×10^{-9}	3.5×10^{-10}
Nitrogen of dry residue, *per cent*	3.4	4.8	5.5

These results show that both the nitrogen content and dry weight per unit of activity of phage propagated on the yeast medium (4.8×10^{-11} and 1.0×10^{-9} mg., respectively) are significantly greater than the values obtained when bacteriophage is purified from synthetic medium (1×10^{-14} mg. nitrogen and 6×10^{-14} mg. dry residue per unit of lytic activity) (Tables I, IV, and V).

Although the yeast medium was subjected to ultrafiltration prior to seeding with the bacteria, the possibility that some of the nitrogenous constituents might undergo aggregation or coagulation during the subsequent autoclaving and thus be retained on the membrane may explain why some material was recovered when the liter of sterile ultrafiltered medium was concentrated as a control. However, even when the findings for phage (Table VII, column 2) are corrected by subtracting the values obtained from the sterile medium (Table VII, column 1), the nitrogen per unit of activity (3.6×10^{-11} mg.), the dry weight per unit of activity (3.5×10^{-10} mg.), and the total yield (14.3 mg.) are still far greater (Table VII, column 3) than those obtained when the same phage is purified from synthetic medium (Tables IV and V). Furthermore, the phage recovered from the yeast medium is apparently contaminated with a large amount of some non-nitrogenous material, since the per cent nitrogen of the dry residue is quite low (5.5 per cent). These results indicate that when yeast medium is used for the propagation of coliphage, ultrafiltration is not an efficient means for the purification of such preparations.

Determination of Particle Size of Bacteriophage

Earlier in this study, on the basis of the experimentally determined weight of protein per unit of lytic activity, the radius of the active particles has been calculated to be from 19 to 22 millimicra. This figure certainly repre-

sents a maximum average size of particle rather than a true average, since the preparation, while considerably purified, most likely still contained some extraneous material. Other investigators have made attempts to determine directly the particle size of bacteriophage by such methods as ultrafiltration through collodion membranes of known porosity (25–27), optical methods (28–29), centrifugation (11, 22–23), and rate of adsorption of phage by susceptible bacteria (30). These authors have all either assumed or concluded that a bacteriophage suspension is homogeneous; that is, that all the particles in a given strain are of the same size. However, other workers, on the basis of the difference in diffusion rates of various fractions of a given phage (5), and on the basis of an observed difference in the rate of diffusion of phage in a concentrated as contrasted with a dilute suspension (11), have concluded that any one phage must exist as a mixture of particles of widely different sizes.

Since the latter method is apparently more efficient in that it permits the detection of definite differences in particle size, it was adopted in these experiments. The technique of Hetler and Bronfenbrenner (15), who first applied to bacteriophage the method originally described by Northrop and Anson (31) for the determination of the particle size of carbon monoxide hemoglobin, has been employed in this study.

The procedure consists in measuring the rate of diffusion of bacteriophage through a porous membrane whose porosity has been standardized against a substance with known diffusion rate such as HCl.

The diffusion coefficient "D" is defined as the quantity of substance that will diffuse across a permeable plane of unit dimensions, in unit time, under unit concentration gradient. Northrop and Anson (31) expressed it mathematically, thus:

$$D = \frac{KQ_{\infty}\ \text{cm.}^2}{t\ \text{day}}, \tag{1}$$

in which

D = diffusion coefficient.
K = diffusion constant of cell.
Q = cc. of substance diffused, expressed as a fraction of the original concentration.
t = time in days.

The relation between the diffusion coefficient, and the radius of the molecule, has likewise been derived as follows:

If water is used as a solvent, at 5°C.

$$r = \frac{1.148 \times 10^{-8}\ \text{cm.}}{D} \tag{2}$$

Three diffusion cells were standardized by measuring the rate of diffusion through them of HCl. The standardizations, as well as all further diffusion experiments, were carried out in the ice chest at 5° C., and the three cells were found to have the following constants (K): Cell I, 0.1736; Cell II, 0.1580; and Cell III, 0.1367.

The bacteriophage preparations used for measuring the diffusion rate were highly diluted so that the final titre was of the same order of magnitude (10^4 to 10^5 active lytic units per cc.) as that of the ultrafiltrates tested later (Table XI). The bacteriophage titre of the test solutions was measured by the McCrady (18) method previously described. In the experimental determination of the diffusion rate of phage, the cells were filled with a diluted phage and dipped into 30 cc. of the medium of dilution. Sixteen consecutive samples of the diffusates from each cell were secured at 30 minute intervals. The first four samples were discarded, and the remaining twelve were titrated for their phage content. The average of the twelve titrations gave the average number of units of lytic activity diffusing in 30 minutes. Since the number of lytic units diffusing through the porous disc in 30 minutes was rather small, a special method of titrating the activity (15) was used instead of the usual method of tenfold serial dilution. The 30 cc. sample of diffusate was thoroughly mixed and then added in amounts varying from 0.1 to 1.0 cc. to tubes of broth. Each tube was seeded with a drop of 1:10 dilution of an 18–24 hour broth culture of *B. coli*, incubated at 37° C., and examined for evidence of lysis the next day. From each tube which showed no evidence of lysis, one drop was deposited on a slant freshly seeded with *B. coli* P.C., incubated at 37° C., and examined for phage action 24 hours later. If no lysis was visible, it was considered that no phage was present. The concentration of phage in the test solution within the cell was so adjusted (10^4–10^5 units per cc.) that the number of units diffusing in 30 minutes could successfully be titrated by this method; *i.e.*, the end point of the titration (the volume representing one unit of phage) was between 1.0 and 0.1 cc. of diffusate.

Each diffusion experiment with a given phage preparation was carried out twice, using three cells simultaneously for each determination, thus resulting in six determinations on each test suspension. Below is described a typical diffusion experiment, employing as test solution a Berkefeld filtrate of *B. coli* phage in synthetic medium, diluted in synthetic medium. The titrations of the test suspension in duplicate are shown in Table VIII.

TABLE VIII

Titration in Duplicate of a Test Suspension of Coliphage in Synthetic Medium

Tube No.	1	2	3	4	5	6	7	Control
Amount of original diffusate, *cc.*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	0
Titration A.	x*	x	5†	3	4	1	0	0
Titration B.	x	x	5	4	2	1	0	0

* Dilution not tested.

† The number refers to the number of tubes (of the total of 5) showing lysis.

From the significant numbers (341 for titration A; and 421 for titration B), according to the probability tables (18), 10^{-4} cc. of the lysate contained

an average of 2.5 active lytic units, and its titre was therefore 25,000 active units per cc.

The details of the titration of the diffusates of one of the cells (cell I) are given in Table IX.

TABLE IX

Details of the Titration of Diffusates of Cell I

No. of tube..	1	2	3	4	5	6	7	8	9	10	Result
Amount of diffusate, *cc.*.....	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	Volume of diffusate containing 1 unit phage
Time of sampling *hrs.*											*cc.*
2.5	+*	+	+	+	+	−	−	−	−	−	0.6
3.0	+	+	+	+	+	−	−	−	−	−	0.6
3.5	+	+	+	+	−	−	−	−	−	−	0.7
4.0	+	+	+	+	+	−	−	−	−	−	0.6
4.5	+	+	+	−	−	−	−	−	−	−	0.8
5.0	+	+	+	−	−	−	−	−	−	−	0.8
5.5	+	+	+	+	−	−	−	−	−	−	0.7
6.0	+	+	+	−	−	−	−	−	−	−	0.8
6.5	+	+	+	+	−	−	−	−	−	−	0.7
7.0	+	+	+	+	−	−	−	−	−	−	0.7
7.5	+	+	+	+	+	−	−	−	−	−	0.6
8.0	+	+	+	−	−	−	−	−	−	−	0.8
Average.....											0.70

* + = lysis.
− = no lysis.

Since the total volume of diffusate in the beaker was 30 cc., and on an average 0.70 cc. contained 1 unit of phage (Table IX), it is clear that 30 ÷ 0.70, or 43 active lytic units, diffused through the disc in 30 minutes. Upon the assumption that each lytic unit represented one phage particle, the diffusion rate and the particle radius may be calculated. The fraction diffused (Q) was 43 ÷ 25,000 (original concentration of the test solution), or $Q = 0.00172$. From these data, knowing the cell constant (K), the diffusion rate may be calculated by means of equation 1.

$$D = \frac{KQ_{cc.}\ \text{cm.}^2}{t\ \text{day}}$$

$$= \frac{0.1736 \times 0.00172\ \text{cm.}^2}{0.0208}$$

$$= 0.01436\ \text{cm.}^2\ \text{per day.}$$

From the above diffusion rate (D), the average radius (r) of the particles is calculated by means of equation 2 to be equal to 8 millimicra as follows:

$$r = \frac{1.148 \times 10^{-8} \text{ cm.}}{D}$$

$$= \frac{1.148 \times 10^{-6} \text{ cm.}}{0.01436}$$

$$= 79.9 \times 10^{-8} \text{ cm.}$$

$$= 8.0 \text{ m}\mu.$$

By this procedure the rate of diffusion of both crude and purified phage was then measured. The test solutions were prepared in the following manner:

(A) *Crude Coliphage.—B. coli* P.C. phage was propagated in synthetic medium and filtered through a Berkefeld filter. The filtrate was diluted in sterile synthetic medium to give a phage titre of 10^4 to 10^5 active units per cc. This suspension was used as the test solution. (The results are given in Table X-A.)

(B) *Purified Coliphage.—B. coli* P.C. phage propagated in synthetic medium was concentrated on a collodion membrane, and purified by washing as previously described. This concentrate was diluted in synthetic medium to give a titre of 10^4 to 10^5 lytic units per cc. and this diluted suspension was used as the test solution (Table X-B).

It was of interest to compare the diffusion rate of coliphage with that of the staphylococcus phage (K) used by Northrop (11), but since the medium of suspension was different (staphylococcus will not grow on the synthetic medium) it was first necessary to measure the rate of diffusion of the coliphage when suspended in the yeast medium to see if the nature of the suspending medium had any significant effect on the rate of diffusion. These test suspensions were prepared as follows:

(C) *Crude Coliphage in Yeast Medium.—B. coli* P.C. phage was propagated in yeast medium, filtered through a Berkefeld filter, and then diluted with sterile yeast medium to give a test suspension with the same phage concentration as that used when phage in synthetic medium was employed (10^4 to 10^5 lytic units per cc.) (Table X-C).

(D) *Crude Staphylococcus K Phage in Yeast Medium.*—This test suspension was prepared exactly as was the crude coliphage in yeast medium (Table X-D).

The results of these experiments show that the average radius of the more rapidly diffusing phage particles in crude coliphage in synthetic me-

TABLE X

Diffusion Rates of Crude Bacteriophage Suspensions and Purified Bacteriophage

Test solution	Cell No.	Cell constant (K)	Total units phage in 30 cc. diffusate	Units phage in 1 cc. test solution	Fraction of phage diffused $\frac{\text{col. 4}}{\text{col. 5}}$ (Q)	Time in days (t)	Diffusion rate eq. (1) (D)	Average diffusion rate (Avg. D)	Average radius of particle in millimicra eq. (2). (r)
									mμ
A	I	0.1736	111	62,500	0.00177	0.0208	0.01477		
	II	0.1580	120	62,500	0.00192	0.0208	0.01458		
	III	0.1367	150	62,500	0.00240	0.0208	0.01577	0.01461	7.9
	I	0.1736	43	25,000	0.00172	0.0208	0.01436		
	II	0.1580	46	25,000	0.00184	0.0208	0.01398		
	III	0.1367	54	25,000	0.00216	0.0208	0.01419		
B	I	0.1736	91	60,000	0.00152	0.0208	0.01269		
	II	0.1580	100	60,000	0.00167	0.0208	0.01268		
	III	0.1367	107	60,000	0.00178	0.0208	0.01170	0.01242	9.2
	I	0.1736	63	42,500	0.00148	0.0208	0.01235		
	II	0.1580	70	42,500	0.00165	0.0208	0.01253		
	III	0.1367	81	42,500	0.00191	0.0208	0.01255		
C	I	0.1736	63	35,000	0.00180	0.0208	0.01477		
	II	0.1580	65	35,000	0.00186	0.0208	0.01413		
	III	0.1367	73	35,000	0.00208	0.0208	0.01367	0.01443	7.9
	I	0.1736	86	50,000	0.00172	0.0208	0.01436		
	II	0.1580	94	50,000	0.00188	0.0208	0.01428		
	III	0.1367	115	50,000	0.00230	0.0208	0.01512		
D	I	0.1736	81	42,500	0.00191	0.0208	0.01594		
	II	0.1580	91	42,500	0.00214	0.0208	0.01626		
	III	0.1367	111	42,500	0.00261	0.0208	0.01715	0.01614	7.1
	I	0.1736	88	47,500	0.00185	0.0208	0.01544		
	II	0.1580	100	47,500	0.00211	0.0208	0.01603		
	III	0.1367	115	47,500	0.00244	0.0208	0.01604		

dium was about 8 mμ. By similar calculations, the radius of the particle of the purified phage was found to be about 9 mμ. This very slight difference in calculated radius is probably not significant; in view of the lability of the purified phage it is likely that some of the pure phage particles diffusing through are inactivated, thus leaving a smaller number of active particles in the diffusate, which results in a correspondingly higher calculated radius.

The character of the suspension medium apparently has no significant effect, since the coliphage propagated and diluted in yeast medium had exactly the same rate of diffusion and presumably the same particle size as that propagated and suspended in synthetic medium.

The average radius of the more diffusible particles of the staphylococcus phage appears to be somewhat smaller (7 mμ) but of the same order of magnitude as that of the coliphage.

However, as has been previously pointed out, Hetler and Bronfenbrenner (15) have demonstrated that, at least in the case of a coliphage propagated in broth, any one preparation may consist of particles of widely different sizes. They have suggested the possibility that the bacteriophage may represent a relatively simple chemical substance adsorbed on inert colloidal particles of different sizes present in the lysate. Thus, according to them, the size of particles calculated on the basis of diffusion rate is referred to the size of the vehicle particles rather than of the bacteriophage proper. More recently Northrop (11) has found that bacteriophage purified by him and presumably devoid of extraneous material also consisted of particles of different size depending on the degree of dilution. It, therefore, seemed of interest to ascertain by means of fractional ultrafiltration whether particles of different size are present at all times, independent of the dilution.

For this purpose, crude and purified coliphage and crude staphylococcus phage were fractionated by ultrafiltration through a thin collodion membrane, and the diffusion rate of the particles present in the ultrafiltrates (presumably the smallest particles) was measured. The details of the preparation of the test solutions are as follows:

(*a*) Crude *B. coli* P.C. phage in synthetic medium was forced through a 2 per cent collodion membrane and the ultrafiltrate, with a titre of about 10^4 active lytic units per cc. was used for the determination of diffusion rate (Table XI-*A*).

(*b*) Staphylococcus phage in yeast medium was similarly forced through a 2 per cent membrane and the ultrafiltrate which also had a titre of about 10^4 lytic units per cc. was likewise used as the test solution (Table XI-*B*).

(*c*) Enough concentrated purified *B. coli* P.C. phage, from synthetic medium, was added to dialyzed yeast medium[6] to give a titre of 10^9 to 10^{10} lytic units per cc. and this suspension was forced through a similar 2 per cent membrane, and the ultrafiltrate with a titre of about 10^4 units per cc. was used as the test solution (Table XI-*C*).

The average radius of the particles coming through the 2 per cent membranes was, as was to be expected, quite small, those from crude coliphage being about 1.3 mμ; those from purified coliphage about 1.9 mμ; and those from staphylococcus phage 2.4 mμ. Here again the differences between the results on crude and purified phage are probably not significant and might again be explained on the greater lability of the purified phage.

[6] The purified phage was suspended in yeast medium in order to preserve its activity as much as possible.

TABLE XI

Diffusion Rates of Ultrafiltered Crude and Purified Bacteriophage

1	2	3	4	5	6	7	8	9	10
Test solution	Cell No.	Cell constant (K)	Total units phage in 30 cc. diffusate	Units phage in 1 cc. test solution	Fraction of phage diffused $\frac{\text{col. 4}}{\text{col. 5}}$ (Q)	Time in days (t)	Diffusion rate eq. (1) (D)	Average diffusion rate (Avg. D)	Average radius of particle in millimicra eq (2). (r)
									mμ
A	I	0.1736	111	12,000	0.00925	0.0208	0.07720		
	II	0.1580	115	12,000	0.00958	0.0208	0.07277		
	III	0.1367	136	12,000	0.01133	0.0208	0.07441	0.08539	1.3
	I	0.1736	107	9,000	0.01189	0.0208	0.09924		
	II	0.1580	111	9,000	0.01233	0.0208	0.09366		
	III	0.1367	130	9,000	0.01444	0.0208	0.09490		
B	I	0.1736	42	8,750	0.00491	0.0208	0.04098		
	II	0.1580	52	8,750	0.00594	0.0208	0.04512		
	III	0.1367	75	8,750	0.00857	0.0208	0.05584	0.04708	2.4
	I	0.1736	42	8,000	0.00475	0.0208	0.03964		
	II	0.1580	50	8,000	0.00625	0.0208	0.04745		
	III	0.1367	65	8,000	0.00813	0.0208	0.05343		
C	I	0.1736	100	13,000	0.00769	0.0208	0.06418		
	II	0.1580	107	13,000	0.00823	0.0208	0.06252		
	III	0.1367	120	13,000	0.00923	0.0208	0.06066	0.06126	1.9
	I	0.1736	59	8,000	0.00738	0.0208	0.06159		
	II	0.1580	60	8,000	0.00750	0.0208	0.05697		
	III	0.1367	75	8,000	0.00938	0.0208	0.06165		

The results indicate that the dimensions of these particles (about 2 mμ in radius) are significantly different from those of the whole phage (about 8 mμ) and, therefore, demonstrate that the active particles in a given phage suspension vary widely in size.

Calculation of Molecular Weights

If the radius of the particle is known (calculated from the diffusion rate) and the density again assumed to be 1.3, the molecular weight may be calculated by the formula:

$$\text{Molecular weight} = \frac{4}{3}\,\text{pi}\,R^3N \times 1.3$$

where R is the radius of the particle in centimeters and N is Avogadro's number (6.06×10^{23} molecules in a gram molecular weight). The molecular weights of the crude and purified phages whose particle radii are given in Table X were calculated by this formula. For the crude coliphage with

radius of about 8 mμ, the calculated molecular weight would approximate 1,500,000; for the purified coliphage with radius of about 9 mμ, the calculated molecular weight is slightly higher, namely 2,250,000; and for the staphylococcus phage with radius of about 7 mμ, the molecular weight would be about 1,000,000. Similarly, the calculated molecular weights of the smaller particles which passed through the 2 per cent collodion membrane (Table XI) would be: crude coliphage—about 7,000; purified coliphage—about 22,000; and crude staphylococcus phage—about 45,000. The values for sizes and molecular weights of different preparations, calculated on the basis of diffusion rates are summed up in Table XII.

TABLE XII

Diffusion Rates, Calculated Radii, and Calculated Molecular Weights of Various Bacteriophage Preparations

Test solution	Average diffusion rate cm.²/day	Calculated average radius	Calculated average molecular weight
		mμ	
Crude coliphage	0.01461	7.9	1,500,000
Purified coliphage	0.01253	9.2	2,250,000
Crude staphylococcus phage	0.01715	7.1	1,000,000
Ultrafiltered (2 per cent collodion) crude coliphage	0.08539	1.3	7,000
Ultrafiltered (2 per cent collodion) crude staphylococcus phage	0.04708	2.4	45,000
Ultrafiltered (2 per cent collodion) purified coliphage	0.06126	1.9	22,000

These figures for radius and molecular weight (Table XII) are at best only approximations, since determination of a radius to a fraction of a millimicron is subject to a large experimental error. Since the molecular weights vary as the cube of the radii, a small difference in the size of the radius would give rise to a relatively large difference in calculated molecular weight. However, it can be seen from Table XI that the particles passing through the 2 per cent membrane, with radius of 1.2 to 2.4 mμ, and a calculated molecular weight of less than 50,000 are of an entirely different order of magnitude from those in the whole original phage suspension with radius of 7 to 9 mμ (Table X), and calculated molecular weight of some one to two million. These results, as stated earlier, indicate that active particles in any one phage differ in size so much as to indicate the simultaneous presence of particles of different orders of magnitude.

DISCUSSION

While earlier studies (1–10) have indicated that bacteriophage might be obtained free of protein, or at least in a form which failed to give protein

color tests, recent more exact studies (11–14) concerning the chemical nature of bacteriophage have indicated that this active agent represents a new type of substance, the so called "heavy protein" with a molecular weight of many millions, similar to those isolated from tissues of animals (infected with equine encephalomyelitis (32) and with virus-induced rabbit papilloma (33)), and plants (infected with tobacco mosaic virus (32) and with cucumber viruses 3 and 4 (34), with potato virus "X" (35), and with tomato bushy stunt virus (36)).

The present studies on the concentration and purification of a coliphage by means of ultrafiltration have resulted in the isolation of a substance containing approximately 15 per cent of nitrogen, giving positive color tests, and therefore presumably a protein, and retaining all the activity of the original lysate. In this product there was five to ten times less nitrogen per unit of lytic activity (10^{-14} mg.) than was found by Schlesinger (13) for a coliphage and by Northrop (11) for a staphylococcus phage. If each unit of lytic activity is assumed to represent one molecule of protein, the molecular weight calculated from the weight of dry residue per unit (6×10^{-17} gm.) would be approximately 36,000,000, and the calculated radius of the molecule about 20 millimicra. These dimensions correspond quite well with the heavy protein previously mentioned.

However, earlier studies (Hetler and Bronfenbrenner (15)) have demonstrated on the basis of the measurement of diffusion rate that bacteriophage may exist as particles with a radius of the order of magnitude of 1 millimicron and a calculated molecular weight below 100,000, which possess all the properties of the original active agent. These findings were confirmed in the present study. Measurement of the diffusion rate of particles of purified phage in dilute solution demonstrated the existence of particles with an average radius of about 9 millimicra corresponding to a molecular weight of about 2,250,000. Since the concentrated, purified phage may not be completely free of inert material, the dimensions calculated from the total weight of material per unit of activity probably represent the maximum limits of average size rather than a true average. On the other hand, calculation from the measurement of diffusion rate probably gives dimensions that are somewhat low, since any environmental influences that increase the motion of the particles would tend to give a higher rate of diffusion than actually exists, and consequently a lower calculated value for particle size. Furthermore, when diffusion rates are measured, employing a suspension containing particles of various sizes, the measured dimensions do not represent a true average, but the average of the particles which diffuse faster; *i.e.*, the smaller particles. Thus, calculations of the radius of

particle from weight give a value that is too high (20 millimicra), while those from measurement of the diffusion rate tend to give values that are too low (9 millimicra), and the true average size probably lies somewhere between these two values.

However, our studies indicate, as Hetler and Bronfenbrenner (15) have demonstrated earlier, that in addition to these larger particles there are present also active particles of an entirely different order of magnitude. If the purified phage is fractionated by ultrafiltration through a collodion membrane which permits the passage of only the smaller particles, and the average size of the particles in this ultrafiltrate is measured on the basis of their diffusion rate, the average radius of the particles diffusing in this case is found to be about 2 millimicra, corresponding to a molecular weight of about 25,000. This figure is significantly different from the one obtained using purified but unfractionated phage, and may be taken as indicating that the active agent is distributed as particles of widely different size. Since the smaller particles possess the characteristic activity of bacteriophage, it seems that in calculating molecular weight it is more appropriate to take as a basis the smallest particle exhibiting the lytic property of the agent. The larger particles must then be either aggregations of the smaller particles which dissociate on dilution, as suggested by Northrop (11), or they represent inert protein to which a small molecule of the active agent is adsorbed, as suggested by Bronfenbrenner (20). In either case, the molecular weight of the active agent should not be calculated on the basis of the size of the largest particles, but on the smallest ones. Our results suggest that the small particles exist at all times and do not necessarily appear as a result of dilution, since both sets of experiments (those with the whole phage and those with the ultrafiltered fraction) were carried out with relatively dilute suspensions, the purified phage having about 20,000–60,000 active units per cc., while the ultrafiltrate of the purified phage had about 9,000–13,000 active lytic units per cc. If, as Northrop suggests, the presence of smaller particles is due to depolymerization taking place on dilution, it would seem probable that this depolymerization would have occurred by the time the phage was diluted 100,000,000 times from its original concentration of about 5×10^{12} to about 5×10^{4} units per cc. present in the material used in determining the diffusion rates of the whole phage, and that no more depolymerization would be expected to take place on a relatively slight (fourfold) further dilution from 40,000 to 9,000 active units per cc. Therefore, it is unlikely that the large particles represent aggregates which may be dissociated by dilution (since the size obtained by simple dilution is not the same as that obtained by fractionation by

ultrafiltration), but rather that they may consist of inert colloidal protein carrier particles to which the smaller active molecules of phage have been adsorbed. The calculated molecular weight of these smaller particles of phage (about 25,000) is well within the order of magnitude of that of proteins but, of course, of much smaller size than the heavy proteins previously mentioned. Whether these smaller particles actually represent free molecules of the active agent cannot be decided at present, although from a consideration of their small size and low calculated molecular weight it is possible that they do represent such free molecules.

It is interesting to note in this connection that Topley and Wilson (37) (1937) consider the relative uniformity of size among active particles of a given strain of bacteriophage as evidence in favor of the microorganismal nature of the agent. While a discussion of the living nature of bacteriophage is outside of the scope of this study, it should be pointed out that our findings contradict the assumption as to uniformity of size of particles, although not necessarily the living nature of phage. It must be admitted, however, that while the results of this investigation contribute no direct evidence to a solution of the problem of whether phage is or is not a living organism, it is rather difficult to imagine a relatively simple substance of molecular weight of only about 25,000, which probably represents but one protein molecule, to be a living organism.

SUMMARY

A simple method of concentrating and purifying bacteriophage has been described. The procedure consisted essentially in collecting the active agent on a reinforced collodion membrane of a porosity that would just retain all the active agent and permit extraneous material to pass through. Advantage was taken of the fact that *B. coli* will proliferate and regenerate bacteriophage in a completely diffusible synthetic medium with ammonia as the only source of nitrogen, which permitted the purification of the bacteriophage by copious washing.

The material thus obtained was concentrated by suction and after thorough washing possessed all the activity of the original filtrate. It was labile, losing its activity in a few days on standing, and was quickly and completely inactivated upon drying. This material contained approximately 15 per cent of nitrogen and with 2 or 3 mg. samples of inactive dry residue it was possible to obtain positive protein color tests.

The concentrated and purified bacteriophage has about 10^{-14} mg. of nitrogen, or 6×10^{-17} gm. of protein per unit of lytic activity. Assuming that each unit of activity represents a molecule, the calculated maximum

average molecular weight would be approximately 36,000,000, and on the assumption of a spherical shape of particles and a density of 1.3, the calculated radius would be about 22 millimicra.

By measurement of the diffusion rate, the average radius of particle of the fraction of the purified bacteriophage which diffuses most readily through a porous plate was found to be of the order of magnitude of 9 millimicra, or of a calculated molecular weight of 2,250,000. Furthermore, when this purified bacteriophage was fractionated by forcing it through a thin collodion membrane, which permits the passage of only the smaller particles, it was possible to demonstrate in the ultrafiltrate active particles of about 2 millimicra in radius, and of a calculated molecular weight of 25,000.

It was of interest to apply this method of purification to a staphylococcus bacteriophage. Since this organism does not readily grow in synthetic medium, a diffusate of yeast extract medium was employed. The better of two preparations contained about 10^{-12} mg. of nitrogen per unit of lytic activity. Although this is about one hundred times the amount of nitrogen found in an active unit of *B. coli* bacteriophage, nevertheless, the diffusion rate experiments gave results which paralleled those obtained with the coliphage. The diffusible particles of the crude staphylococcus bacteriophage had a radius of about 7 millimicra, and a calculated molecular weight of about 1,000,000, while the particles of the same phage which appeared in the ultrafiltrate through a thin collodion membrane had a radius of about 2.4 millimicra and a calculated molecular weight of about 45,000.

It appears, therefore, that the active principle is distributed as particles of widely different sizes. However, since the smaller particles have all the properties of bacteriophage, the larger particles probably do not represent free molecules, but either are aggregates, or more likely, inactive colloids to which the active agent is adsorbed.

The protein isolated, which bears the phage activity, is capable of stimulating the production of antilytic antibodies on parenteral injection into rabbits or guinea pigs. It retains its specific antigenicity when inactivated by formalin, but not when inactivated by drying.

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