TABLE I.
Age of Culture, Duration of Guinea Pig Passage and Resistance to Streptomycin of Tubercle Bacilli Isolated from Patients by Direct Culture and by Guinea Pig Inoculation.

	Direct culture		Culture from guinea pig*		
Case	Age of culture, days	Resistance to streptomycin†	Days in guinea pig	Age of culture, days	Resistance to streptomycin†
1‡	102	0.15	42	60	0.15
2‡	161	0.15	67	94	0.15
3	31	100.0	64	42	100.0
4	24	>2000.0	22	55	500.0
5	114	25.0	71	43	25.0
6	181	25.0	67	114	25.0
7	34	>2000.0	68	30	>2000.0
8	114	10.0	63	50	10.0
9	24	>2000.0	61	37	>2000.0
10	74	0.08	93	32	0.31
11	70	>2000.0	58	33	>2000.0
12	98	1500.0	65	33	>2000.0
13	95	25.0	76	135	25.0
14	112	0.15	60	52	0.15

* Inoculated the same days as direct cultures were made with a portion of the same material.
† Greatest concentration of streptomycin allowing growth in micrograms per milliliter of medium.
‡ Untreated patients.

ment has been carried out for several months.

It is evident from the data presented in Table I that cultures of tubercle bacilli from patients receiving streptomycin retain their resistance to the drug even after residence in guinea pigs for 10 weeks or more and subsequent maintenance on glycerinated egg yolk agar for many weeks.

In another experiment each of 14 guinea pigs was inoculated subcutaneously with 0.1 mg of tubercle bacilli found to be resistant to at least 2,000 μg of streptomycin per milliliter of medium. Cultures isolated from 9 of these that died after periods of 61 to 164 days of infection were found still to be resistant to at least 2,000 μg. In all 14 animals there was gross and microscopic evidence of progressive tuberculosis indistinguishable from that seen in a similar group of guinea pigs inoculated with a sensitive strain from the same patient.

15682

Uniformity of Size of Bacteriophage Particles.

A. D. HERSHEY, FRANCES KIMURA, AND J. BRONFENBRENNER.
From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Mo.

As measured by the sintered glass boundary method of Northrop and Anson, Kalmanson and Bronfenbrenner[1] found the apparent diffusion coefficient of coliphage PC (hereafter called T2K) to be about 1.7×10^{-7} cm^2/sec, corresponding to an equivalent spherical diameter of about 16 mμ. By fractional ultrafiltration, a very small fraction of the phage was obtained which behaved in the same measurement like particles having a diameter of about 4 mμ.

Immunological analysis[2] revealed a re-

[1] Kalmanson, G. M., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 203.

[2] Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 281.

markably high combining power of the phage for neutralizing antibody, about 4600 molecules of antibody per lytic unit of phage. This may be taken as evidence that the preparations of Kalmanson and Bronfenbrenner,[1] weighing about 10^{-13} mg per lytic unit, consisted of remarkably pure antigen derived from the phage. The weight 10^{-13} mg is equivalent to about 30 particles of 16 mμ diameter, and since the measurement of effective antigenic surface seemed to give independent support for this assumption, it was concluded that the total number of phage particles, each measuring 16 mμ in diameter, was about 30 times higher than that indicated by the lytic titre.[2]

Northrop,[3] both by diffusion and centrifugal analysis, estimated a diameter of about 10 mμ for a staphylococcal phage in dilute solution (in concentrated solution it behaved like particles 100 mμ in diameter). A few phages, namely S13 and C13, have a similar small size (8 to 20 mμ) by either filtration or centrifugal analysis.[4] Otherwise, and particularly for phages belonging to the serological group (group 11 of Burnet[5]) homologous with T2K, much larger sizes have been found by all workers. For example, phage WLL has a diameter of 50 to 75 mμ by filtration, and 60 mμ by sedimentation.[4] T2K itself has a diameter of about 50 mμ estimated from the ratio of weight to lytic titre of the best preparations,[1] and T2 (a variant of T2K now known as T2L[6]) has a diameter of about 50 mμ from the X-ray sensitive volume,[7] and measures about 65 × 80 mμ[8] or 80 × 100 mμ[9] in electron-micrographs. In addition, approximate one to one correspondence between lytic titre and particle count revealed by means of the electron-microscope.[8] contradicts our conclusion[2] regarding the infectivity of the particles.

In view of this unsatisfactory state of affairs,[4] we began several years ago some additional measurements of the size and uniformity of phage T2K. We have only now been able to correlate our results in a fairly satisfactory way.

We have used 3 methods: diffusion through agar, centrifugation, and diffusion across the sintered glass boundary. In all 3 methods, another coliphage, T1, has been used as reference, the 2 phages being counted separately in mixtures by plating on suitable indicator strains of bacteria each sensitive to only one of the phages.

Diffusion Through Agar. Diffusion through agar was studied as follows. Suitable quantities of melted 3% washed agar in distilled water, and the phages in broth, were mixed at 45°C to give a 0.6% agar solution containing about 10^7 particles each of T1 and T2K per ml. The phages consisted of filtered broth lysates diluted about 1:1000. At the same time a similar solution was prepared containing broth without added phage. The boundary was set up by allowing 10 ml of the phage-free solution to solidify in the lower end of a length of glass tubing (16 mm internal diameter) fitted with rubber stoppers, and then adding an equal volume of the phage-containing solution. After this had solidified, a layer of melted paraffin about 2 cm long was pipetted directly onto the agar surface. This formed a plug serving to minimize mechanical movements of the gel during diffusion, which was allowed to proceed at 5°C in the horizontal direction.

Ultrafiltrates were prepared by passing broth lysates through a collodion membrane of sufficient density to reduce the lytic titre by the factor 10,000.[1] The diffusion measurements were conducted with these fractions in the way already described, except that the ultrafiltrates were incorporated into the agar gel without further dilution, yielding a titer of about 10^5 per ml, and the corresponding phage-free solution contained the same ultrafiltrate inactivated by heating, instead of broth. In these experiments also, unfractionated T1 admixed in equal titer

[3] Northrop, J. H., *J. Gen. Physiol.*, 1938, **21**, 335.

[4] Elford, W. J., in *Handbuch d. Virusforschung*, Doerr and Hallauer, Editors, Vienna, 1939, p. 126.

[5] Burnet, F. M., *J. Path. and Bact.*, 1933, **36**, 307.

[6] Hershey, A. D., *Genetics*, 1946, **31**, 620.

[7] Luria, S. E., and Exner, F. M., *Proc. Nat. Acad. Sci.*, 1941, **27**, 370.

[8] Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.

[9] Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

TABLE I.
Diffusion Coefficients of T1 and T2K in Agar.

Material	Time (days)	Phage per ml	$D_1 \times 10^8$	$D_2 \times 10^8$	D_2/D_1
Whole T1 + Whole T2K	7	*ca.* 10^7	1.6	0.98	0.61
	14		0.82	0.53	0.65
	28		0.61	0.44	0.72
	43		0.69	0.50	0.72
Whole T1 + Ultrafiltered T2K	14	*ca.* 10^5	1.9	1.4	0.74

D_1 = diffusion coefficient of T1.
D_2 = diffusion coefficient of T2K.

served as reference.

For analysis of the contents of the diffusion cell, the agar was cut into segments with a fine stretched wire, beginning from the phage-free end of the column, the latter being expelled from the tube without backward movements by means of the plunger formed by anchoring a wire handle in the paraffin plug. The segments, collected in numbered weighing bottles, were weighed, and their length and position with reference to the boundary fixed by summing the weights. The phage content of the segments was determined by suspending the agar in broth by aspiration with a pipette, and plating after suitable dilution. The recovery of both phages under the conditions of the experiments was quantitative.

Owing to the nature of the analytic method, and to the slow rate of diffusion, only the data for the region of low phage concentration proved useful for the calculation of diffusion coefficients. This disadvantage, as compared with optical methods of analysis in the diffusion of purified proteins for instance, was more than offset by the advantages of being able to use very dilute solutions, and to make analysis from the region of 10^{-1} down to about 10^{-5} times the initial concentration of phage.

For this region, the distribution of phage about the boundary, after correction for the lengths of the individual segments (by graphical integration of the ideal diffusion curve), conformed nearly but not quite to that expected for ideal diffusion. The deviations were systematic, and might be attributed to heterogeneity of the phage, to the curvature of the boundary, or to seepage of fluid along the walls of the tube.

The pertinent results of 2 experiments are summarized in Table I. It will be seen that the method clearly distinguishes T1 and T2K in mixture, the latter diffusing more slowly. The diffusion coefficients, calculated for the largest distance from the boundary at which dependable analysis could be made, vary from 0.61 to 1.9 $\times$ 10^{-8} cm^2/sec for T1, and from 0.44 to 1.4 $\times$ 10^{-8} for T2K. The variation must arise either from errors in locating the boundary, or to differences in the quality of the 2 preparations of agar gel, since the relative coefficients of the 2 phages vary only from 0.61 to 0.74. Since this ratio is not altered by ultrafiltration of T2K, we evidently failed to obtain any fractionation with respect to size of particles of this phage by this method.

The actual size of the particles cannot be estimated from these data, owing to the impeding effect of the gel on diffusion. It is of some interest to note the magnitude of this effect. For T2K, the diffusion coefficient of 0.5 $\times$ 10^{-8} corresponds to an equivalent spherical diameter of about 500 mμ, calculated for diffusion in water. This is about 6 times the probable true diameter,[8] as if the particles were diffusing freely in the agar gel only about one-sixth of the time allowed. On the other hand, the relative sizes estimated from these data, namely T1 about two-thirds the diameter of T2K, is in good agreement with the corresponding X-ray sensitive volumes[7] and electron-micrographs.[8]

Other experiments using this same method, in which a second ultrafiltrate of T2K was compared with whole T1, and an ultrafiltrate

TABLE II.
Sedimentation of Fractions of T2K in Mixture with T1.

Source of material	No. of Exp.	% sedimented			
		T1		T2K	
		Mean	Extremes	Mean	Extremes
Whole broth lysates	7	14	4-28	84	61-99
Ultrafiltered T2K + whole T1	6	13	5-34	91	76-99
Supernates, T2K	3			87	69-97

of T1 with whole T2K, yielded less presentable data, but nevertheless confirmed our principal conclusion, that ultrafiltration does not affect the speed of diffusion of these 2 phages relative to each other. The 3 ultrafiltrates examined in this way were prepared with 3 different collodion filters.

Centrifugation. The sedimentation experiments were also essentially qualitative, but yielded unequivocal results owing again to the use of a second phage as standard of reference. The method follows.

Three ml volumes of the mixed phages in broth (about 10^5 lytic units per ml) were spun for one hour at 18,000 r.p.m. in 6 ml tubes in an international centrifuge equipped with "multispeed" angle head. The mean distance from the axis of rotation was about 6.5 cm, corresponding to a centrifugal field of 23,500 g. When spun in a cool room, the temperature in the tubes did not exceed 45°C, and the phage could be recovered quantitatively by mixing the contents of the tube at the end of the run. Supernates were sampled by withdrawing 0.5 ml with a pipet from the upper portion of the fluid. Assays of these samples were compared with the same materials uncentrifuged, and in many of the experiments an assay was made also of the mixed residue in the bottom of the tube, as a parallel check against inactivation. Table II shows the results of several experiments with T1 and T2K from lysates prepared in broth, with ultrafiltrates of T2K prepared with 2 different collodion filters, and with T2K from supernates of previous centrifugations. We have omitted from these data a few discrepant experiments, in which both phages failed to sediment appreciably, since this happened just as frequently with unfractionated as with fractionated phage, and can safely be attributed to accidents of convection.[10] Excluding these, the results show clearly that:

(1) T2K sediments rather completely under the conditions of these experiments, whereas admixed T1 is left largely in the supernate.

(2) The few particles of T2K passed by 3% collodion membranes sediment just as rapidly in the ultrafiltrate as whole phage diluted to the same titer in broth. This conclusion must be qualified somewhat, owing to inaccuracies of the method, but it is clear that we have failed to obtain a fraction of T2K containing particles anywhere near as slowly sedimenting as those of T1.

(3) T2K in supernates from which the bulk of the phage has been removed by previous centrifugation, is not less easily sedimentable than the unfractionated material. In another experiment not shown in the table, 3 successive centrifugations of an undiluted lysate threw down respectively 98.5, 96, and 97% of the residual unsedimented phage.

We conclude that the particles of T2K in dilute solution are essentially homogeneous in size, or at any rate all are appreciably larger or denser than those of T1.

Passage through the sintered glass boundary. In a single experiment, carried out as described by Kalmanson and Bronfenbrenner,[1] it was found that whole T2K in broth, and ultrafiltered T2K from a broth lysate, passed through the sintered glass boundary into broth, or into heated ultrafiltrate, respectively, at an identical rate corresponding to a spherical diameter of about 4 mμ. In both cells, the admixed T1 and T2K passed in precisely the same proportion.

[10] Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 267.

Since particles of this size would not be sedimentable under the conditions of the centrifugations described above, it is evident that we are not here measuring diffusion, and this conclusion is confirmed by the failure to distinguish T1 and T2K in the same mixture. Probably what we are measuring is actual circulation of fluid through the boundary.

A repetition of the experiments with phage propagated in synthetic medium nevertheless confirmed the earlier results, namely, that whole lysate diluted in synthetic medium passed the boundary at an appreciably slower rate than undiluted ultrafiltered phage, which passed at essentially the same rate as phage in broth. This result is evidently inconsistent with the conclusions reached above.

It was noticed, however, that the synthetic medium used in these experiments[1] was supersaturated with respect to magnesium ammonium phosphate, and eventually deposited crystals on standing in the refrigerator. Since no crystals formed in ultrafiltrates of lysed cultures prepared in the same medium, it was suspected that the difference in the rate of passage through the glass boundary might be due to the clogging of the porous plate by crystal formation, which might be expected to occur preferentially within the pores of the glass. The following experiment seems to show that this is what happens.

Parallel measurements of the rate of passage through the glass boundary were made of the same stock of phage T2K diluted respectively in a "saturated" and a "supersaturated" medium. The supersaturated medium was prepared by mixing the 2 stock solutions[1] composing the medium, and placing the mixture at 5°C for about 12 hours, at which time crystallization had not yet begun. The saturated medium was prepared from the same materials, but was stored in the refrigerator for several days, or as long as necessary, until crystallization was complete, and filtered. Two experiments were done, switching the cells used for the measurement of saturated and supersaturated solutions, and cleaning the cells between runs with chromic-sulfuric acid mixture. The results, expressed as equivalent spherical diameters calculated from the apparent diffusion coefficients, were as follows: in saturated medium, 6.8 and 9.8 mμ, and in supersaturated medium, 22.2 and 24.4 mμ. This difference is clearly an artifact, and very likely has the explanation we have suggested.

Discussion. As a result of the experiments described in this paper, it must be concluded that the unit particles of the phages T1 and T2K in diluted lysates are of very large size. They are probably identical with those identified in undiluted lysates by electron-micrography,[8,9] analytical centrifugation,[9] and other means.[4,7] There is no evidence that these particles are heterogeneous in size, or that they disaggregate appreciably on dilution. The previous conclusion,[2] based partly on immunological evidence, that lysates are composed of antigenically similar particles about 30 times more numerous than indicated by the lytic titer, has also to be withdrawn. The infectivity of the phage particles must be very nearly perfect, as has been found also by other means.[8]

The very large antibody absorbing capacity[2] of bacteriophage T2K, which is equally great for several members of the same serological group,[6] may now be accounted for in terms of the analytical data for T2 reported by Hook and co-workers.[9] These data, which refer to morphologically and centrifugally homogeneous preparations, indicate a considerably larger size than any of the previous estimates. The electron-micrographs show particles 80 $\times$ 100 mμ or 86 $\times$ 113 mμ, exclusive of tail, depending on the source of material. From the sedimentation constant (which, however, unaccountably decreases at low concentrations) one estimates an equivalent spherical diameter of 60 to 130 mμ for the densities 1.5 to 1.1 respectively. From the weight per lytic unit of 10^{-15} gm, and assuming perfect infectivity, one estimates a spherical diameter between 100 and 150 mμ, depending on the density and hydration assumed. A sphere of diameter of 100 mμ, plus a tail of 20 $\times$ 120 mμ,[8] provide a primary physical surface of about 4 $\times$ 10^{-10} cm^2, sufficient to accommodate 4000 molecules of antibody each requiring an area of 10^{-13} cm^2. This

size is therefore compatible with the observed combining ratio of 4600 molecules per lytic unit,[2] a correlation which also holds with fair exactness for antigens as diverse in size and shape as ovalbumin, thyroglobulin, hemocyanin,[11] and tobacco mosaic virus.[12]

These experiments have some bearing on the question of the utility of the porous plate boundary method of Northrop and Anson,[13] for the measurement of coefficients of diffusion. In this method, the estimated coefficient is directly proportional to the fraction of the solute diffusing in unit time. Under our conditions, the rate of flow of fluid through the boundary, measured in terms of the amount of bacteriophage passed, is about 0.01 ml per hour, equivalent to a diffusion coefficient of about 4×10^{-7} cm^2/sec. This is of the same order as the diffusion coefficient of hemoglobin.[13] It is evident that this rate of flow would not seriously affect the measurement of diffusion of substances diffusing 5 to 10 times faster than hemoglobin, *e.g.*, of the HCl or other electrolyte used in calibration. For proteins of the size of hemoglobin, the error would be about 100% for solutions having a viscosity near that of water. Actually, protein solutions of sufficient concentration for analysis of the diffusate have a considerably higher viscosity, and the data of Northrop and Anson[13] suggest that under their conditions, the flow of fluid is negligible compared with the diffusion of hemoglobin, which means that the flow is at least several times slower than in our experiments. Bacteriophage T2, taken to be a sphere of about 90 $m\mu$ in diameter, would have a diffusion coefficient of about 2.7×10^{-8} cm^2/sec in water, and would pass through glass discs such as we have used at a rate equivalent to about 6×10^{-4} ml of solution per hour by diffusion alone. Observed amounts in excess of this would provide a very accurate measure of the speed of flow of liquid. A small amount of bacteriophage included in the solution whose diffusion is to be measured would thus permit a very simple correction for what is probably the principal source of error in the use of this method.

Summary. Neither fractional ultrafiltration nor fractional centrifugation have revealed any heterogeneity of size among the particles of the bacteriophage T2K (formerly called PC). The methods used for the measurement of size, namely, diffusion through agar and sedimentation in an angle centrifuge, were essentially qualitative, but the experimental arrangements were such as to yield unequivocal results.

It must be concluded therefore that this bacteriophage has a minimal particle diameter of 60 $m\mu$ or more, as has been found previously for unfractionated phages of the same group by a variety of methods.

It is necessary also to withdraw the conclusion previously reached that this bacteriophage has a low infectivity for susceptible bacteria.

An explanation is suggested for some anomalous results previously obtained in attempts to measure the diffusion of bacteriophage through the porous glass boundary. The use of bacteriophage to measure the rate of flow of fluid as a means of control in the application of this method to other materials is described.

[11] Hershey, A. D., *J. Immunol.*, 1941, **42**, 485.

[12] Schramm, G., and Friederich-Freksa, H., *Z. physiol. Chem.*, 1940, **270**, 233.

[13] Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1929, **12**, 543.