ELECTRON MICROGRAPHIC COUNTS OF BACTERIOPHAGE PARTICLES[1]

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All measurements of virus activity give titers expressed in "infectious units," that is, in multiples of the smallest amount of virus needed for virus infection under the conditions of the measurements. Statistical arguments have been used (Feemster and Wells, 1933; Parker, 1938; Ellis and Delbrück, 1939; Luria, 1940; Lauffer and Price, 1945) to show that the number of infectious units is a linear function of the number of material particles supposedly bearing virus activity. This relation is not unequivocally established in all cases, however (Bryan and Beard, 1940; Kleczkowski, 1950), and the factor of proportionality between infectious units and particles is in some cases fairly low—between 10^{-4} and 10^{-8} for viruses such as tobacco mosaic virus or rabbit papilloma virus. Some doubts may, therefore, be justified as to the general validity of the identification of the minimal infectious unit with *one* material virus particle.

Bacteriophage particles, as identified by electron microscopy (Ruska, 1940; Luria and Anderson, 1942), come closer to representing an ideal case. Phage activity assays by plaque count give a perfectly linear relation with the amount of phage material assayed, which proves that each plaque stems from the action of one material particle; this does not necessarily mean that every phage particle present is counted as a plaque, but only that a constant fraction of the particles produces plaques.

Luria *et al.* (1943) compared the number of particles of phage T2 visible in electron micrographs on the edges of infected cells with the number of infectious units adsorbed per bacterium in the same suspension and found a reasonable agreement. This method, of course, merely compares the number of infectious units with the number of particles that are adsorbed by the host; if a phage suspension contained an appreciable proportion of inactive, *nonadsorbable* particles, they would not be detected on the edges of the bacteria. The presence of such inactive particles would affect the result of chemical analyses on phage preparations and the quantitative determination of the chemical composition of the phage particle.

The present paper presents a comparison of plaque count for different phages with the number of specific particles determined by the "spraying technique" (Williams and Backus, 1949; Backus and Williams, 1950). In this method, use

179

is made of indicator particles of polystyrene latex, whose number concentration in a standard aqueous suspension is known. The standard suspension is mixed in known proportions with the virus suspension, and the mixture is sprayed upon specimen screens for electron microscopy. The spray drops, upon drying, leave droplet patterns sufficiently small to allow the image of a whole pattern to lie fully within one micrograph. Counts are made of the numbers of the latex particles and of the virus particles, and from the ratio of these numbers the particle assay of the virus suspension is obtained. The assumption that both types of particles are distributed at random, without clumping or mutual repulsion, is confirmed in most cases by the statistics of the actual counts.

MATERIAL AND METHODS

Preparation of phage concentrates. Lysates of coliphages T1, T2, T4, and T6 (Delbrück, 1946) were prepared in M-9 synthetic medium (KH_2PO_4, 3.0 g; Na_2HPO_4, 6.0 g; $MgSO_4$, 0.12 g; NH_4Cl, 1.0 g; glucose, 4.0 g; and H_2O, 1,000 ml) or in Difco nutrient broth containing 0.5 per cent NaCl. After filtration through Mandler filters (tested under 8 lbs pressure) the lysates were kept at 5 C, and titrations were made every 2 or 3 days until the titer had risen, as it generally does, to a steady maximum. The phage was then concentrated and washed by differential centrifugation at 5 C in the multispeed attachment of a refrigerated centrifuge (International Equipment Company). High-speed centrifugation cycles lasted 30 to 60 minutes at 19,000 rpm, corresponding to about 16,000 $\times$ g. This treatment collects over 90 per cent of phages T2, T4, and T6, and 70 to 80 per cent of T1, in compact pellets. Low-speed cycles lasted 10 to 15 minutes at about 1,000 g in the same centrifuge. The phage was washed and resuspended in cold phosphate buffer M/15 plus $MgCl_2$ 10^{-3} M; for phage T1, $CaCl_2$ 10^{-4} M was also added. The final concentrates contained about 10^{12} infectious units per ml or more. For each sample the supernatants were pooled and used to rinse all pipettes and centrifuge tubes employed in the preparation of that sample.

Phage assays. The original lysates, the concentrated samples, and the supernatants were repeatedly assayed by plaque counts on *Escherichia coli* strain B by the agar layer method. The total activity of each original sample was compared with that of the corresponding concentrate plus supernatant, to assess possible losses during centrifugation; the losses varied between 0 and 30 per cent for different samples. The major source of loss is probably the incomplete recovery of the pellets, fragments of which have an annoying tendency to remain on the wall of the pipettes. The possibility of losses by inactivation or clumping during the treatment cannot be excluded.

The precision of the activity titers determined by plaque counts is difficult to assess. Plaque counts on a series of similar plates, with 100 to 400 plaques per plate on the average, seldom give results as reproducible as expected on the basis of sampling errors only. Moreover, every titration involves several dilutions, whose inaccuracies may contribute to the total variance. The coefficient of variation for each assay is probably lower than 20 per cent; repeated assays give aver-

ages whose coefficients of variation are probably of the order of 10 to 15 per cent.

Latex preparation. The standard suspension of polystyrene latex particles was assayed for the number of particles per ml according to a method previously described (Backus and Williams, 1950). It is estimated that the absolute assay is in error by less than 6 per cent.

Preparation of mixtures for microscopic examination. The phage concentrates were suitably diluted (1:50 to 1:400) in distilled water and added to the latex suspensions in a concentration such as to give 2.23×10^{10} latex particles per ml in the mixed suspensions sprayed. These dilutions are sufficiently great to reduce the content of salt and other impurities below the level where it would interfere
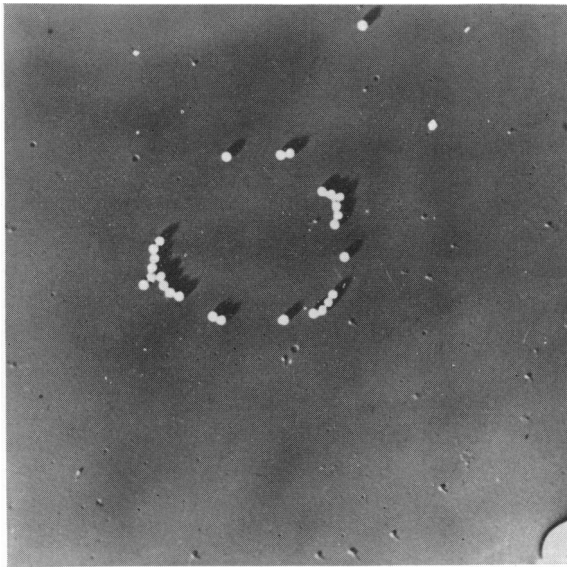

Figure 1. Drop pattern from a mixture of latex and phage T2. Magnification 5,000 $\times$.

with the micrographic observations, and are sufficiently small to provide 15 to 80 phage particles per droplet pattern.

Several of the phage preparations were impure enough to cause some of the particles in the dry droplet patterns to be masked by dried aggregates of suspending material adhering to the particles. This masking made counting uncertain, and an attempt was made to prevent the dried material from clinging to the phage particles. The addition of purified bovine albumin in a concentration of about 0.001 per cent before spraying caused the nonvolatile material to spread more evenly over the drop patterns, leaving the phage particles relatively unobscured. The reason for this behavior is probably that the albumin caused the droplets to dry more evenly, whereas, without the albumin, the late drying areas centered around the particles, thus causing materials to clump upon drying.

The phage-latex mixture was sprayed onto collodion-coated specimen screens, which were then shadow-cast with uranium and examined. Several drop patterns

were photographed for each sample (magnification 2,500 X), and all particles were counted in those patterns that lay fully within one field (figure 1). Unless otherwise specified, at least 500 phage particles were counted for each sample; this corresponded to 10 to 25 drop patterns, containing also 500 or more latex particles.

As a test of the assumption of random distribution of particles of both types, we calculated for each sample the correlation coefficient r between the counts of particles of the two types in individual drop patterns. A high correlation ($P <$ .05) was found in most cases; for several samples, however, the correlation was poor, suggesting nonrandom distribution of particles. The values for r and P for each sample are included in tables 1 and 2.

Calculation of particle titer. The titer P in "phage particles per ml" was calculated as follows:

$$P = \frac{\text{Phage particle count } (A)}{\text{Latex particle count } (B)} \times \text{Phage dilution} \times \text{Latex titer } (L).$$

The standard deviation of P was calculated in the following way: First, the standard deviation of the ratio A to B was calculated by the approximate formula (Dahlberg, 1948, p. 94): $\sigma_{A/B} = \pm \sqrt{\dfrac{\sigma_A{}^2}{B^2} + \dfrac{\sigma_B{}^2 A^2}{B^4}}$, where σ_A is the standard deviation of A and σ_B that of B. To estimate these, we considered the total counts of phage particles and those of latex particles for each preparation (sum of the counts in individual drop patterns) as single samples from two Poisson distributions ($\sigma_A = \pm \sqrt{A}$; $\sigma_B = \pm \sqrt{B}$). Next, the standard deviation of the phage particle titer P was estimated by the formula:

$$\sigma_P = \pm \sqrt{\sigma_{A/B} L^2 + \sigma_L{}^2 (A/B)^2} \times \text{phage dilution}$$

where L was taken as 2.23×10^{10} and σ_L as $6L/100$. The values of σ_P are underestimated because of neglecting possible errors in phage dilution and overestimated by assuming for the estimate of L a standard deviation of 6 per cent, a value probably too high. The accuracy of P is, of course, much lower for preparations with poor correlation between counts of particles of the two types, since then the assumption of Poisson distributions of particles is probably not valid.

Ultraviolet absorption measurements. A sample of each concentrated phage preparation was diluted in phosphate buffer to contain between 1 and 5×10^{10} activity units per ml; the absorption spectrum was determined in the Beckman spectrophotometer for wave lengths between 240 and 420 mμ, using fused silica cells 1 cm thick. Most samples contained phage in a pure enough state to give absorption spectra rather typical for these phages, with a sharp rise in absorbancy between 240 and 260 mμ, a rapid fall above 260 mμ, and a hardly noticeable singularity at 280 mμ; some samples, however, gave anomalous absorption spectra and were clearly too impure for the data to be significant.

The absorbancy index per particle at wave length 260 mμ was calculated in the following way: (a) the absorbancy was read; (b) a correction for scattering was obtained by plotting log absorbancy versus log wave length between 340

and 420 mμ (where absorption by phage is negligible), extrapolating the straight-line plot to the value corresponding to the 260 mμ abscissa, and subtracting this value from the absorbancy read at 260 mμ to give the corrected absorbancy; this correction was generally between 10 and 20 per cent of the total absorbancy; (c) the absorbancy index per particle, a_p (260 mμ), in cm^2 per particle, was obtained by dividing the corrected absorbancy by the concentration (particles per ml), the thickness of the samples in our measurements being 1 cm.
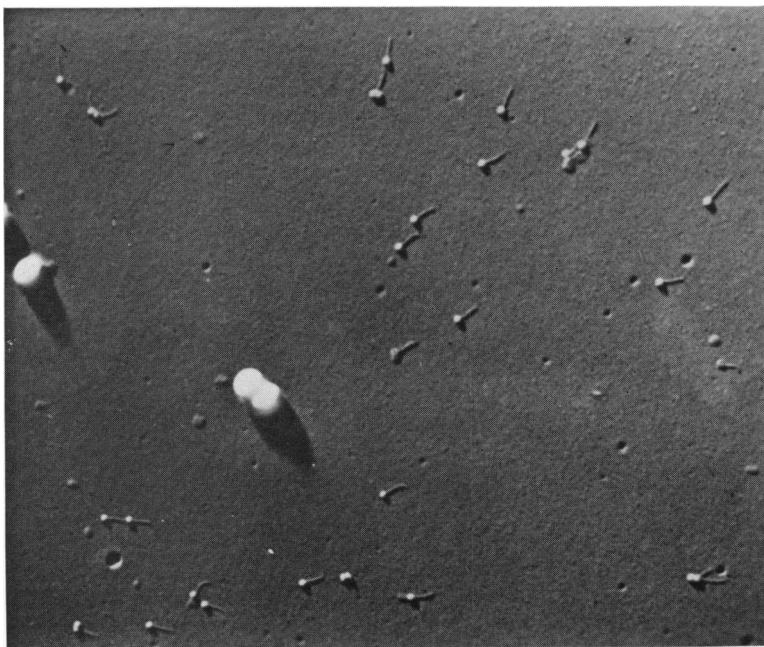

Figure 2. Part of a drop pattern from a mixture of latex and phage T1. Magnification 15,500 ×.

RESULTS

Droplet patterns of phage-latex mixtures. Figure 1 shows a pattern typical for bacteriophages T2 (or T4, or T6); figure 2 shows part of a drop pattern for phage T1. Notice that most of the sperm-shaped phage particles are oriented radially with their heads toward the center of the patterns. This is probably due to the fact that, as a droplet dries, the latex particles are pushed centerward by the surface tension, and the phage particles orient themselves with their thicker part toward the central area, where the liquid layer remains thicker as the droplet dries from the periphery inward.

Counts of phage particles. The results of the first group of tests are given in table 1. The ratio "infectious units/particles" ranges from 1.4 to 0.45. The value 1.7 for T1 is not significant, since it is based on very poor counts; it indicates, however, that particle count and infectious unit count are of the same order of magnitude for this phage as for the others. The particle counts for phages T2, T4,

TABLE 1

PHAGE	PREPARATION NO.	MODE OF PREPARATION*	PLAQUE COUNT, UNITS PER ML	ACTIVITY LOSS DURING PREPARATION, % OF TOTAL	ELECTRON MICROGRAPHIC COUNT, PARTICLES PER ML	CORRELATION BETWEEN PHAGE AND LATEX PARTICLES			RATIO (INFECTIOUS UNITS/PARTICLES)	a_p (260 mμ)
						Droplets examined	r	P		
T2	36	3HS, 3LS	2×10^{12}	29	$3.95 \times 10^{12} \pm 4.5 \times 10^{11}$	14‡	0.85	$<.01$	0.51	4.5×10^{-12}
T2	37	1HS†	3.3×10^{12}	0	$7.5 \times 10^{12} \pm 6.0 \times 10^{11}$	22	0.66	$<.01$	0.44	6.9×10^{-12}
T2h	11	2HS, 1LS	3.7×10^{12}	6	$6.55 \times 10^{12} \pm 5.9 \times 10^{11}$	20	0.67	$<.01$	0.56	7.5×10^{-12}
T4	27	2HS, 1LS	2.6×10^{12}	7	$1.8 \times 10^{12} \pm 1.5 \times 10^{11}$	24	0.79	$<.01$	1.4	—
T6	23	2HS, 2LS	3.25×10^{12}	11	$4.8 \times 10^{12} \pm 3.7 \times 10^{11}$	22	0.70	$<.01$	0.68	4.95×10^{-12}
T1	1	2HS, 1LS	6.1×10^{11}	19	(3.6×10^{11})	11§	<0.2	$>.5$	(1.7)	—

* Number of cycles of high-speed (HS) and low-speed (LS) centrifugation.
† Pellet redispersed in a small volume of supernatant; no purification.
‡ Only 230 phage particles and 262 latex particles counted.
§ Only 103 phage particles and 323 latex particles counted.

TABLE 2

PHAGE	PREPARATION NO.	MODE OF PREPARATION*	PLAQUE COUNT, UNITS PER ML	PLAQUE COUNT AFTER ZnCl₂ TREATMENT, UNITS PER ML	ACTIVITY LOSS DURING PREPARATION, PER CENT OF TOTAL	ELECTRON MICROGRAPHIC COUNT, PARTICLES PER ML	CORRELATION BETWEEN PHAGE AND LATEX PARTICLES			RATIO (INFECTIOUS UNITS/PARTICLES)	a_p (260 mμ)
							Droplets examined	r	P		
T2	α (broth lysate)	1HS	2.4×10^{12}	3.6×10^{11}	20	$6.7 \times 10^{12} \pm 6 \times 10^{11}$	21	0.85	$<.01$	0.54	4.0×10^{-12}
T2	β (broth lysate)	2HS	4.65×10^{11}	1×10^{12}	0	$1.54 \times 10^{12} \pm 1.5 \times 10^{11}$	17†	0.78	$<.01$	0.65	(6.0×10^{-12})‡
T4	γ	1HS	1.7×10^{12}	1.8×10^{12}	12	$4.6 \times 10^{12} \pm 4.4 \times 10^{11}$	16†	0.54	.03	0.40	2.9×10^{-12}
T2	2	2HS, 1LS	1.7×10^{12}	1.7×10^{12}	33	$1.84 \times 10^{12} \pm 1.75 \times 10^{11}$	19	0.36	$>.1$	0.91	(1.0×10^{-11})
T2	My	2HS, 1LS	3.2×10^{12}	(4.6×10^{12})‡	24	$3.2 \times 10^{12} \pm 3.0 \times 10^{11}$	16	0.06	.9	1.4	(1.1×10^{-11})
T4	Ma	2HS, 1LS	2.35×10^{12}	3.6×10^{12}	0	$3.54 \times 10^{12} \pm 3.0 \times 10^{11}$	18	0.31	.2	1.0	(5.6×10^{-12})‡

* See table 1.
† Only about 300 particles of each type were counted.
‡ Ultraviolet absorption spectrum was atypical.

and T6 were technically satisfactory, as shown by the good correlation between phage and latex counts.

The values for the absorbancy index per particle $a_p(260 \text{ m}\mu)$ are also given in table 1. Although the values for several preparations of phage T2 fall within a factor of 1.7 from one another, they are not constant enough for the absorbancy of a sample to be used as an accurate indication of the number of phage particles present. Our phage preparations were clearly not "clean" enough, and it is only because of the very high ultraviolet opacity of the phage that the values are at all close to one another. An estimation of $a_p(260 \text{ m}\mu)$ on a partially purified preparation of one of these phages could, however, be used as an approximate indication of the number of particles present (within a factor of 2), thus providing an easy check on the activity titers obtained by plaque counts. The values of a_p (260 mμ) for T4 are lower than those for T2 and T6 (see table 2); this may be involved in determining the lower ultraviolet sensitivity of this phage.

The problem arises of interpreting the ratios between infectious units and characteristic phage particles. The ratio higher than 1 for T4 is probably not significant and may be attributed mainly to inaccuracy of the plaque counts, which were determined only twice on this sample. The values for the T2 samples, however, indicate that only about one-half of the visible particles of this phage are counted as infectious units. Three elements may be involved in producing a ratio lower than unity: (a) low precision of the determinations of infectious titer, or of particle titer, or of both; (b) the presence of inactive phage particles; (c) low "efficiency of plating" (probability lower than unity that one active particle produces a plaque). This in turn could depend either on inefficiency of the plating method or on clumping of particles.

It has been observed occasionally that the plaque counts of phage T2 may be increased by a factor 2, if the phage is diluted and kept for some time in distilled water prior to plating. Recently, Bertani (1950) found that a rapid increase in titer was obtained for phage T2, and to a lesser extent for phage T4, by a 1- to 2-hour pretreatment with 0.8 per cent $ZnCl_2$ or $ZnSO_4$. Two groups of phage concentrates were prepared, assayed by plaque count both in the usual way and after $ZnCl_2$ or distilled water treatment, and counted electron micrographically. The results are given in table 2. It is clear that the refinement in activity titration did not improve the agreement between particle count and plaque count. The first group of samples in table 2, for which the micrographic counts were satisfactory (see the correlation columns), gave ratios "plaques after Zn/particles" between 0.7 and 0.4. For the second group of samples, the ratios are closer to unity, but the micrographic phage counts were poor (see correlation columns) and probably too low, as suggested by the values of $a_p(260 \text{ m}\mu)$, almost twice as high as for the previous samples. It should be noticed that the $ZnCl_2$ and distilled water effects on plaque count are rather elusive, being present mainly for freshly prepared stocks, for which they apparently accelerate the natural increase in titer by a factor 2 or 3, which would occur upon standing. Once a stock has naturally reached its steady maximum titer, zinc or distilled water treatment may or may not increase the plaque count any further (see table 2), de-

pending probably on the completeness of the natural rise. Our data indicate that these treatments do not raise the plaque counts to the full value of the particle count.

DISCUSSION

Our results as a whole show a good degree of correspondence between the activity titers and the numbers of characteristic virus particles for several phages of the T group, with ratios between 1.4 and 0.4 for different samples. This confirms the observations of Luria *et al.* (1943) and the impression prevalent among phage workers that they possess an almost ideal method of titration, more efficient than even the best method for the titration of an animal virus. This efficiency is made possible by the well-known modalities of phage bacterium interaction, with very high chances of successful infection of the bacterial cell, whose surface is studded with easily accessible phage receptors.

Because of the closeness of the agreement between titers and particle numbers, it seems legitimate to inquire into the possible reasons for the residual discrepancies. The plaque counts reveal between 100 per cent and 40 per cent of the characteristic particles present in a suspension. The errors in the micrographic particle counts are probably relatively small, so that we should consider what can make plaque counts lower than particle counts. The imprecision of the plaque counts might be responsible for ratios between 1 and 0.7, but is unlikely to be responsible for ratios 0.5 or lower. Low efficiency of plaque formation on agar by otherwise fully active single particles, which could effectively attack a bacterium in liquid, seems improbable in view of the well-established one-to-one relationship between plaque counts and number of units able to kill a bacterial cell (Delbrück and Luria, 1941). This relationship holds true also after $ZnCl_2$ or distilled water treatment; the increase in plaque count is paralleled by a similar increase in bacterial killing ability (Bertani, 1950).

This leaves two alternatives: (a) the presence of clumps of active particles, each clump killing one bacterium only and producing one plaque; (b) the presence of inactive particles, either intrinsically such or prevented from infecting bacteria by some extrinsic mechanism, for example, by combination with inhibitors, possibly of bacterial origin. Our results cannot distinguish between these possibilities. On the one hand, no obvious evidence for clumping was found on our photographic plates, but the presence of clumps of the size and frequency required to account for a ratio around 0.5 between plaques and particles cannot easily be excluded or confirmed by observation of our micrographs. Moreover, if clumping was involved, and if the increases in plaque count, both natural and after distilled water treatment, were due to breaking up of clumps, it is conceivable that the dilution in distilled water included in the spraying method might further disaggregate the clumps. On the other hand, removal of inhibitors could also be effected by the treatments that increase the plaque count titers.

It should be remembered that the phage samples used in the group of experiments reported in the present paper were ordinary lysates, filtered through Mand-

ler filters and concentrated or partially purified by mild treatments, unlikely to produce inactivation, as shown indeed by the small percentage of losses in activity titers during treatment. The total losses in activity during purification, even if all due to clumping or inactivation of particles were included in the final concentrated samples, could account only for a fraction of the excess of particles over plaque counts found for several samples. The possibility of inactivation during the initial Mandler filtration was not tested and cannot be excluded.

We were more interested for the time being in checking the relation between plaque count and particle count in ordinary lysates, such as are used routinely by phage workers, than in the preparation of "fully infectious" phage preparations. Particle counts on phage preparations carefully submitted to all sorts of treatments that may either disaggregate clumps or remove inhibitors of infectivity should be attempted before the conclusion is accepted that in some phage stocks—and not in others—a large proportion of irreversibly inactive particles are present. If the latter are indeed present, it is important to decide whether they have become inactivated after being liberated by the bacteria or if they have been liberated already in inactive form.

SUMMARY

Electron micrographic counts of bacteriophage particles are presented. The ratio between the titer in infectious units and the number of phage particles varied between 0.4 and 1.4 for several preparations of phages T2, T4, T6. The interpretation of ratios different from unity is discussed. The absorbancy per phage particle at wave-length 260 mμ was found to be between 2.9×10^{-12} and 7.5×10^{-12} cm^2.

REFERENCES

BACKUS, R. C., AND WILLIAMS, R. C. 1950 The use of spraying methods and of volatile suspending media in the preparation of specimens for electron microscopy. J. Applied Phys., 21, 11–15.

BERTANI, G. 1950 *Unpublished.*

BRYAN, W. R., AND BEARD, J. W. 1940 Host influence in the characterization of response to the papilloma protein and to vaccinia virus. J. Infectious Diseases, 67, 5–24.

DAHLBERG, G. 1948 Statistical methods for medical and biological students. George Allen and Unwin, London.

DELBRÜCK, M. 1946 Bacterial viruses or bacteriophages. Biol. Revs. Cambridge Phil. Soc., 21, 30–40.

DELBRÜCK, M., AND LURIA, S. E. 1941 Interference between bacterial viruses. Arch. Biochem., 1, 111–141.

ELLIS, E. L., AND DELBRÜCK, M. 1939 The growth of bacteriophage. J. Gen. Physiol., 22, 365–384.

FEEMSTER, R. F., AND WELLS, W. F. 1933 Experimental and statistical evidence of the particulate nature of the bacteriophage. J. Exptl. Med., 58, 385–391.

KLECZKOWSKI, A. 1950 Interpreting relationships between the concentrations of plant viruses and numbers of local lesions. J. Gen. Microbiol., 4, 53–69.

LAUFFER, M. A., AND PRICE, W. C. 1945 Infection by viruses. Arch. Biochem., 8, 449–468.

LURIA, S. 1940 Méthodes statistiques appliquées à l'étude du mode d'action des ultravirus. Ann. inst. Pasteur, **64**, 415–438.

LURIA, S. E., AND ANDERSON, T. F. 1942 The identification and characterization of bacteriophages with the electron microscope. Proc. Nat. Acad. Sci. U.S., **28**, 127–130.

LURIA, S. E., DELBRÜCK, M., AND ANDERSON, T. F. 1943 Electron microscope studies of bacterial viruses. J. Bact. **46**, 57–76.

PARKER, R. F. 1938 Statistical studies of the nature of the infectious unit of vaccine virus. J. Exptl. Med. **67**, 725–738.

RUSKA, H. 1940 Ueber ein neues bei der bakteriophagen Lyse auftretendes Formelement. Naturwissenschaften, **29**, 367–368.

WILLIAMS, R. C., AND BACKUS, R. C. 1949 Macromolecular weights determined by direct particle counting. I. The weight of the bushy stunt virus particle. J. Am. Chem. Soc., **71**, 4052–4057.