A RAPID SLIDE PLAQUE TECHNIC FOR BACTERIOPHAGE ASSAY

By D. JONES and A. P. KRUEGER

(*From the Department of Bacteriology and Office of Naval Research, Task XXI, University of California, Berkeley*)

(Received for publication, May 27, 1950)

The most commonly used technic for enumerating bacteriophage particles has been the plaque count of Gratia (1), in which 1 ml. portions of phage-infected cells suspended in a low concentration (0.5 per cent) agar at 55°C. are pipetted over the surface of a solid agar medium in a Petri dish. This method was modified and analyzed statistically by Hershey *et al.* (2), who added 0.5 ml. of phage-infected cells to a volume of 3.5 ml. of 0.7 per cent agar at 45°C. and poured the entire amount over the surface of the supporting 1 per cent agar base. It has been experimentally demonstrated by several investigators that the development of plaques in different phage-host systems is affected by such variables as the composition of the medium, including co-factors for adsorption, concentration of electrolytes, number and metabolic condition of bacterial cells, concentration and depth of suspending agar layer, oxygen supply, and temperature of incubation. Thus the time required for appearance of plaques, and their ultimate size, are dependent upon the regulation of these conditions and are characteristic for each individual phage and host cell indicator.

With the technic of Gratia, the *Staphylococcus aureus* phage K has required over 8 hours' incubation at 37°C. for the development of readable plaques. In addition, it has been observed on many occasions that despite attempts to maintain uniform conditions, large discrepancies often occurred in the counts on replicate plates. It was thought that perhaps one of the causes for this variation might be the fact that plaque development is extremely sensitive to the depth of the agar layer spread over the supporting base. It is possible to run the 1 ml. sample over the entire surface of the Petri dish, but in this case the plaques developing around the edges are very difficult to read.

In order to overcome these difficulties and yet maintain a simple and rapid technic for assay of our strain, a method was developed which (1) eliminates the supporting agar base, (2) and the need for large numbers of Petri plates; and (3) makes use of a known volume of phage-infected cells suspended in a 0.3 to 0.4 per cent agar medium spread on ordinary microscope slides, which are then stored during incubation in moist chambers. A somewhat similar

method has been described by Dreyer and Campbell-Renton (3) for staphylococcus and *coli* phage systems, whereby plate glass slides of a larger size (photographic quarter plates) were used to support the agar base on which a liquid suspension of cells was spread and infected with phage by means of platinum loops. The authors reported appearance of some plaques within a few hours and were able to record the process of plaque development by direct photography.

Using the present technic, it has been found possible to observe plaques within $2\frac{1}{2}$ hours and to make counts either macro- or microscopically. Statistical analysis indicates low variation among replicates prepared from individual samples, indicating that the procedure of plating in a thin agar layer over a constant area is reliable for enumerating the number of particles in a phage-cell suspension of the staph. K system.

Assay Procedure

1. Host Cells.—Young (12 to 24 hour) cultures of *Staph. aureus* on tryptose phosphate agar slants are harvested by suspending in tryptose phosphate broth, pH 7.2–7.4, and the suspension is adjusted to contain 6×10^7 cells/ml. by Klett turbidimetric reading. In the course of the assay the cells are diluted to a final concentration of 3×10^7/ml., since this concentration lies within the optimal range (2.5 to 6×10^7) for plaque production under the conditions of the test. See Fig. 1.

2. Agar Medium.—Sterile tryptose phosphate broth containing 0.75 per cent agar is melted and kept in a boiling water bath. Five ml. pipettes, graduated in 0.1 ml., are used to pipette the agar, which is diluted to a final concentration of 0.3 per cent upon mixture with the cells and phage. Experiments testing the effect of agar concentration indicated that low amounts, from 0.3 to 0.6 per cent, allowed optimal plaque size and number. Concentrations of 1 per cent reduced the size and those of 2 per cent prevented production of readable plaques (Fig. 2).

3. Phage Dilutions.—Dilutions are prepared in tryptose phosphate broth at pH 7.2–7.4 and are kept at room temperature.

4. Slides.—Microscope slides are cleaned with bichromate-sulfuric acid solution, followed by a thorough tap water rinse, acetone rinse, distilled water rinse, and are dried at 55°C. between paper hand towels. No precautions to achieve sterility are essential; however, the slides may be flamed prior to use if desired. When samples are to be plaqued, the slides are placed in rows on the surface of a flat glass through which may be seen a set of ruled areas of constant size (2×3.5 cm.) as shown in Fig. 3.

5. Plating Suspensions.—2.5 ml. of cell suspension containing 6×10^7 cells/ml. is pipetted into 20×150 mm. test tubes with a 10 ml. pipette graduated in 0.1. To this is added 0.5 ml. of phage dilution delivered from a pipette graduated in 0.01. Each dilution is mixed 10 times with the sampling pipette prior to adding it to the host cells. The mixture is then shaken rapidly by hand and stored at room temperature until plated (about 5 to 10 minutes). With a 5 ml. pipette 2.0 ml. melted boiling agar is delivered to the infected cell suspension by touching the pipette to the side of the glass about midway and allowing the hot agar to run down the side. The final tem-

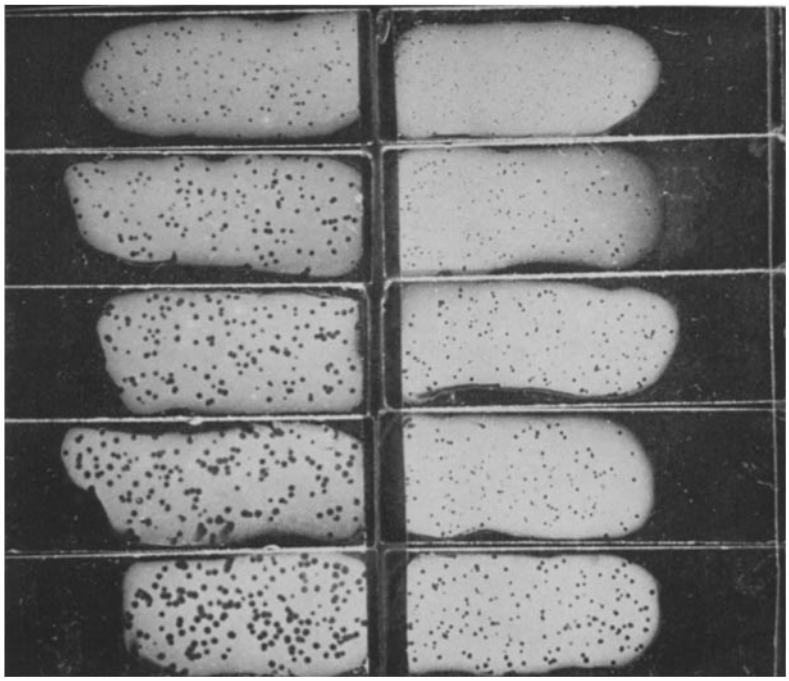


FIG. 1. Effect of cell concentration on plaque size at 28°C. and 37°C. Left side: slides incubated at 28°C., cell concentrations range from 20 × 10^7 (top) to 2 × 10^7 (bottom). Right side: incubated at 37°C., same cell concentrations.

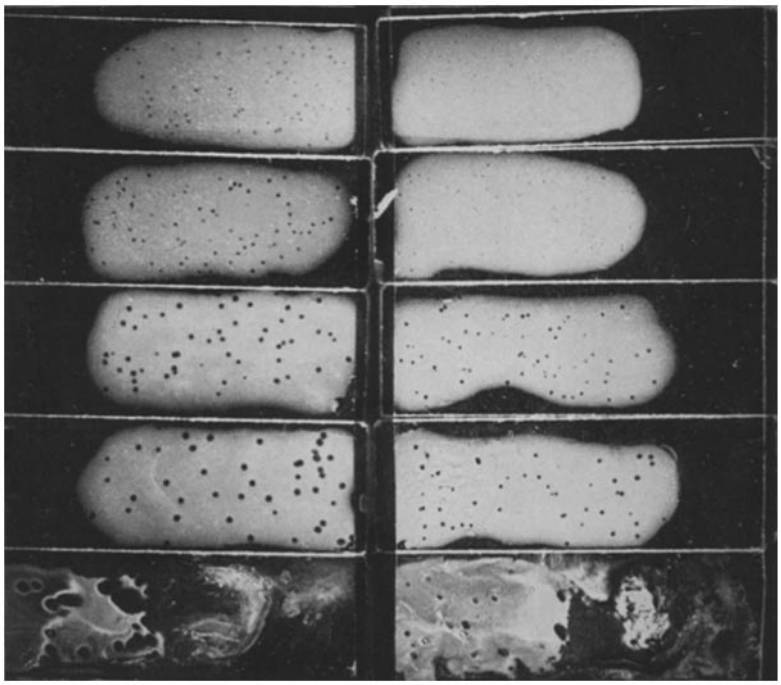


FIG. 2. Effect of agar concentration on plaque size at 28°C. and 37°C. Left side: slides incubated at 28°C., agar concentration ranging from 2 per cent (top) to 0.2 per cent (bottom). Right side. 37°C., using the same agar concentrations.

perature of the assay mixture is never over 45°C. Immediately, the tube is shaken, a 1 ml. pipette graduated in 0.01 is warmed slightly and used to mix the contents 7 to 10 times before removing the aliquot of 0.5 ml. and spreading it over the appropriate area of the slide. It has been found most convenient to measure the 0.5 ml. from the 0.48 to the 0.98 mark on the pipette.

In this way, if desired, eight slides can be prepared from one assay mixture (cells + phage + agar) within a minute or so. The replicates are allowed to solidify for approximately 5 minutes and are then placed face up on glass rods in a 150 mm. Petri dish, the bottom of which contains moistened blotting paper. Thus, instead of eight

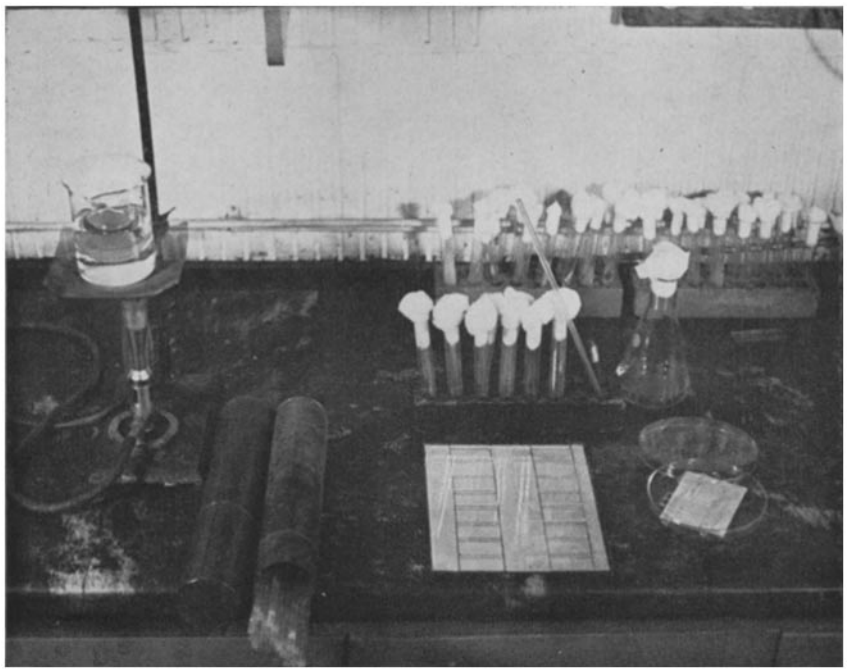


FIG. 3. Working materials arranged for plating by slide technic.

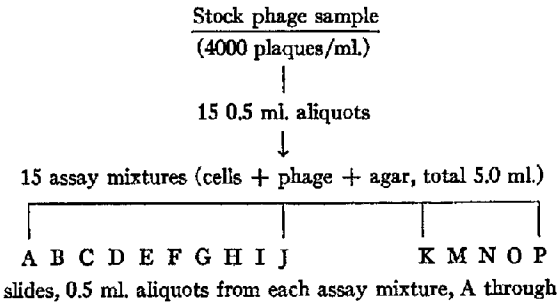
dishes, eight slides are stored in two dishes. The samples are then incubated at the desired temperature: 37°C. for rapid development, 28°C. for optimal plaque numbers, as discussed in the section on statistical analysis.

6. Reading of Plaques.—An ordinary bacterial colony counter was found convenient to view the plaques. Since the final concentration of agar is only 0.3 per cent, the thin layer of agar tends to slide if tilted too steeply; therefore, the counter should be set in a more horizontal position. Light coming through may be regulated by placing translucent strips of paper on either side of a black strip used as a background in such a way as to eliminate unnecessary glare and yet show up the plaques to best advantage. Each slide may be placed over a second slide ruled in areas to facilitate accurate counting.

With this technic up to 400 plaques per 0.5 ml. can be read on any slide. It is an excellent procedure for observing the characteristics of individual plaques, their edges, resistant growth, halo formation, etc., being at once apparent through the thin glass slide.

Statistical Analyses

Precision of the Method.—In order to determine the reproducibility of the method and the effects of time and temperature of incubation on plaque development, fifteen aliquots of 0.5 ml. each were taken from an original sample containing approximately 4000 particles per ml. and were mixed with appropriate amounts of cells and agar to make fifteen individual assay mixtures, each representing a 10^{-1} dilution of the stock phage sample. Eight replicates of 0.5 ml. were removed from each assay mixture and spread on slides in the manner described above. Of these, the sets of slides from ten assay mixtures were incubated at 37°C. and read after 4 and 24 hours; and the remaining five sets of eight each were incubated at 28°C. and counted after 24 hours. The procedure is represented in the following diagram:



All slides from mixtures A–J inclusive 37°C. All slides from mixtures K–P inclusive 28°C.

Since bacterial growth is slower at 28°C., no count was attempted after 4 hours on these sets. However, it was found that this temperature increased the plaque size, and, as will be considered in the analysis, caused a significant increase in the mean number of plaques per ml. In Tables I, II, and III are recorded the counts, means, and standard deviations for each assay mixture tested.

The results of the plaque counts made on the same phage suspensions incubated at different temperatures indicated that incubation at 28°C. resulted in higher mean counts, with less variation from one assay mixture to another. Thus a mean of 202/0.5 ml. was obtained with the set at 28°C. in contrast to one of only 175/0.5 ml. at 37°C. (for the identical dilutions incubated for 24 hours). The smaller standard deviation of 7.59 to be attached to each assay mixture mean for those sets incubated at 28°C. reflects the closer agreement between individual means at the lower temperature.

TABLE I

Plaque Count on Slides Incubated for 4 Hours at 37°C.

Slide No.	Plaques/slide, 0.5 ml. assay mixture									
	A	B	C	D	E	F	G	H	I	J
1	162	150	158	140	175	156	171	146	158	148
2	160	144	151	139	186	148	164	140	159	141
3	171	153	170	157	162	147	142	155	152	155
4	—*	—*	164	148	178	149	168	162	155	150
Mean/0.5 ml.	164.3	160.7	160.7	146.0	175.2	150.0	161.2	150.7	156.0	148.5
S.d. slide	5.87	4.58	8.14	8.37	9.98	4.09	13.15	9.71	3.16	5.81
Grand mean	156.2									
S.d. of the mean of each assay mixture	9.17									

* Slide lost

Calculated from: S.d. slide = $\sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}}$, where S.d. = standard deviation; n = No. slides; x_i = slide count; $\bar{x}$ = mean count

Calculated from: Grand mean $\bar{\bar{x}} = \frac{\Sigma x_i}{n}$

Calculated from: S.d. mean of each assay mixture = $\sqrt{\frac{\Sigma(\bar{x} - \bar{\bar{x}})^2}{n-1}}$,

where n = number of means; $\bar{\bar{x}}$ = grand mean

TABLE II

Plaque Count on Slides Incubated for 24 Hours at 37°C.

Slide No.	Plaques/slide, 0.5 ml. assay mixture									
	A	B	C	D	E	F	G	H	I	J
1	197	184	181	163	196	156	180	156	172	173
2	167	175	204	174	172	154	160	161	162	169
3	185	173	164	172	188	165	180	177	161	162
4	158	193	178	171	175	166	188	179	179	168
5	172	170	192	175	201	167	171	165	171	167
6	182	202	169	186	206	180	167	175	193	162
7	189	192	177	175	190	160	178	154	163	178
8	—*	—*	165	179	191	183	175	165	158	149
Mean/0.5 ml.	178.5	185.4	178.7	174.3	189.9	166.4	174.8	166.5	169.8	166.0
S.d. slide	13.55	10.65	13.75	6.59	11.75	9.75	8.72	9.56	11.67	8.60
Grand mean	174.9									
S.d. mean of each assay mixture	8.24									

Samples incubated for only 4 hours at 37°C. resulted in incomplete counts, as evidenced by the lower mean (156). That this was not due to chance difference between assay mixtures in each temperature set was proven by statistical analysis.

Two separate analyses of variance were performed by Mr. Eugene Harris of the statistical department of the School of Public Health. The first determined whether any significant differences in plaque count occurred in readings made at 4 hours and 24 hours. Observation of plaque development had already indicated that appearance of plaques was incomplete at 4 hours and it was desirable to test the significance of the difference in order to gain some basis for early comparison of test samples in experiments in which a 4 hour reading could be used to obtain a rapid estimate of the final (24 hour) results.

TABLE III

Plaque Count on Slides Incubated at 28°C. for 24 Hours

Slide No.	Plaques/slide, 0.5 ml. assay mixture				
	K	L	N	M	O
1	189	209	203	202	194
2	194	224	202	216	202
3	195	223	198	210	205
4	185	—*	215	224	208
5	191	204	201	204	186
6	204	206	191	—*	194
7	207	203	191	211	190
8	184	195	191	209	199
Mean count.....	193.6	209.1	199.0	210.8	197.2
S.d. slide.....	8.31	10.70	8.26	7.40	7.57
Grand mean.....	201.6				
S.d mean of each assay mixture.....	7.59				

The second analysis tested the significance of the effect of temperature of incubation, 37°C. and 28°C., on the final plaque count at 24 hours. In addition, differences between samples (assay mixtures) independent of time and temperature were analyzed. Data for the analyses are recorded in Tables IV and V.

From the results of the F tests, the following may be concluded: (1) Counts made at 4 hours are significantly lower than those at 24 hours. (2) Incubation at 28°C. produced a significantly higher count than at 37°C. for 24 hours. (3) There are small but significant differences between means of slide counts from individual assay mixtures.

On the basis of the above data a more cautious estimate of the standard deviations to be attached to means of assay mixtures can be made, which takes into account the fact that a significant variation existed between means of

TABLE IV

*Effect of Time on Plaque Development**

Slides incubated at 37°C.

Source of variation	Sums of squares	Degrees of freedom	Mean square	*F*
Time	8,960.737	1	8,960.737	89.63‡
Samples	6,648.233	9	738.6926	7.39‡
Interaction (time × samples)	1,034.804	9	114.9782	1.15
Residual	9,597.976	96	99.9789	
Total	26,241.750	115		
Corrected term for means	3,303,281.250	1		

* Analysis of variance performed by Mr. Eugene Harris, School of Public Health, University of California, Berkeley.

‡ Significant at 1 per cent level of probability.

TABLE V

*Effect of Temperature on Plaque Development**

Slides incubated 24 hours at 28°C. and 37°C.

Source of variation	Sums of squares	Degrees of freedom	Mean square	*F*
Temperature	18,171.955	1	18,171.955	180.356‡
Samples	6,478.957	13	498.381	4.946‡
Residual	10,176.398	101	100.756	
Total	34,827.310	115		
Correction term for mean	3,911,120.690	1		

* Analysis of variance performed by Mr. Eugene Harris.

‡ Significant at 1 per cent level of probability.

assays made on individual mixtures of cells + phage + agar, prepared as described.

These deviations are as follows:—

1. Standard deviation of means, of four slides, incubation 4 hours, 37°C. = ±13.59.
2. Standard deviation of means, of eight slides, incubation 24 hours, 37°C. = ±9.61.
3. Standard deviation of means, of eight slides, incubation 24 hours, 28°C. = ±7.89.

The above values are calculated by using the residual mean square as an unbiased estimate of the variance of each single observation, a procedure

validated by the non-significance of the interaction terms. Then the standard deviation of a mean equals

$$\pm \sqrt{\frac{\text{Mean square samples}}{\text{Mean No. slides/assay mixture}}}$$

From these estimates, standard deviations of the grand mean, *i.e.* the mean of individual assay mixture means, plated on any given number of slides can be calculated and used for confidence limits for means of counts in future experiments. It is to be emphasized, however, that these estimates are valid only for tests in which experimental conditions approach those used for this analysis.

For tests run at 37°C., the standard deviation to be applied to a mean of an assay mixture of four slides per mixture is estimated:

$$\pm \sqrt{\frac{738.6926}{4}} = \pm 13.59$$

Similarly, the standard deviation of a mean of an assay mixture of eight slides per mixture would be:

$$\pm \sqrt{\frac{738.6926}{8}} = \pm 9.61$$

For tests run at 28°C., the standard deviation of a mean of an assay mixture of eight slides per mixture is estimated:

$$\pm \sqrt{\frac{498.381}{8}} = \pm 7.89$$

DISCUSSION

With the Gratia technic as modified by Hershey *et al.* (2), a precision of ± 41 (standard deviation of means), or 12.5 per cent of the grand mean was reported for sample means resulting from duplicate plates of eight separate experiments. In comparison, calculation of the standard deviation for eight separate samples plated in duplicate by the modified slide technic results in a figure of ± 12.03 or 6.9 per cent of the grand mean, with variations from 6.3 to 7.5 per cent of the mean on two similar determinations. The former study was carried out with an *E. coli* phage and its host; therefore any comparison of the two technics must not neglect the possibility that biological differences between the two systems might affect the apparent precision of the tests. Moreover, Hershey's samples were prepared on different days (from the same phage stock), whereas those used in our studies were made from a stock phage on the same day. Thus the authors are aware that no critical comparison is justified, and can only report that the apparent precision of the

slide method, using the staph. K system, is comparable to and perhaps greater than that obtained by Hershey *et al.* with an *E. coli* system by the modified Gratia method.

The analyses performed by Hershey indicated that there was no significant variation from one sample mean to another. This observation is interesting in view of the fact that by the slide technic small but significant differences between samples were detected among mean counts that were much less spread about their grand mean than were those obtained by Hershey. This fact is reflected by the lower standard deviation of sample means (calculated

TABLE VI

Precision of Slide and Plate Phage Assay Technics

Sample	Plate method (Hershey *et al.*)						Slide method					
	Count per plate			Grand mean	Standard deviation*	Per cent S‡	Count per slide			Grand mean	Standard deviation	Per cent S
	1	2	Mean				1	2	Mean			
1	276	288	282	327	±41	12.5	197	167	182	174	±12	6.9
2	307	375	341				184	175	180			
3	221	302	262				181	204	193			
4	340	370	355				163	174	169			
5	330	370	350				196	172	184			
6	308	364	336				154	156	155			
7	309	426	368				180	160	170			
8	329	319	324				156	161	159			

* Calculated from formula. S means $= \sqrt{\frac{d^2}{n}}$, where d = differences from mean and n = number of means.

‡ Per cent $S = \frac{\text{Standard deviation}}{\text{Grand mean}} \times 100.$

for means of duplicate slides obtained by the slide technic). A comparison of basic data is shown in Table VI.

A possible explanation for this observation may lie in the fact that the test for homogeneity of individual means depends upon the spread of values (on plates or slides) within each sample compared with the spread of the means—or differences between samples. Thus, a wide variation between readings on individual plates might obscure the fact that there are actually significant differences existing between sample means. That this might be so is suggested by an inspection of the range of readings obtained by Hershey on individual plates. With the slide technic the range was on the whole narrower, standard deviations for slides of each set being in many cases smaller than the standard deviation of sample means. Consequently the precision of counts within a

sample was great enough so that one could detect differences between the values of the means.

SUMMARY

1. A modified rapid plaque-counting procedure is described, whereby staphylococcal phage host-cell suspensions in agar are spread over constant areas on glass slides and are incubated in moist chambers.

2. Statistical analysis indicated a precision of ±8.2 for means of sets of eight slides incubated at 37°C. and ±7.6 for means of sets of eight incubated at 28°C.

3. With the staphylococcus system, a temperature of 28°C. yielded significantly higher counts than at 37°C.

4. Counts made after 4 hours may be used as an estimate of the final plaque count bearing in mind the fact that the yield at this time is significantly less than at 24 hours.

5. The data presented compared favorably with the results obtained by the plate method used by Hershey, and suggest that a greater precision may be obtained by this method—at least for the staph. K phage system.

The authors wish to express their sincere appreciation to Mr. Eugene Harris of the School of Public Health for his statistical analyses of the experimental data, and to Professor John H. Northrop, Member of The Rockefeller Institute for Medical Research and Visiting Professor of Bacteriology, University of California, for his helpful criticism.

BIBLIOGRAPHY

1. Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.
2. Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 267.
3. Dreyer, G., and Campbell-Renton, M., *J. Path. and Bact.*, 1933, **36**, 399.