ADSORPTION OF BACTERIOPHAGE UNDER VARIOUS PHYSIOLOGICAL CONDITIONS OF THE HOST

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(Received for publication, January 29, 1940)

INTRODUCTION

The first step in the growth of bacteriophage is the combination of phage with the susceptible bacterial host. The rate of this combination is, under simple conditions, proportional to both the bacterial concentration and to the phage concentration. Various aspects of this process have been studied quantitatively by previous workers (1, 2). Their results will be analyzed and discussed in the sections entitled "Residual free phage," and "Theory of adsorption rates." The main purpose of this paper was the study of a detail of the adsorption process that had not previously received attention, namely the dependence of the rate constant of adsorption on the physiological state of the bacterial host. Such a dependence must be anticipated for two reasons. First, it is known that the size of a bacterium changes very considerably depending on its phase of growth in a given culture medium, and an increased cell surface should lead to an increase of the adsorption rate on to a given number of bacteria. Second, for motile bacteria, like *B. coli*, the adsorption will be faster when the bacteria move about rapidly than when their motility is reduced by adverse physiological conditions.

Our experiments show that the rate constant under optimal conditions is more than sixty times greater than under poor conditions.

Adsorption Rates

The main difficulty in the measurement of adsorption rates is of course the fact that the adsorption process starts off the growth of the phage. Later the phage will be liberated from the bacterial host and will then interfere with the determination of the unadsorbed fraction. For this reason most measurements of adsorption rates have been carried out either

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with dead bacteria or at a temperature sufficiently low to prevent growth. Another method of evading this difficulty consists in making use of the short latent period which elapses between the infection of a bacterium and the release of newly grown phage from it. This method has been used by Schlesinger (reference 2, page 145) and by Ellis and Delbrück (3) and was also used in the present experiments. The limitation in time set by this condition enforces the use of comparatively high bacterial concentrations in order that the decrease of free phage will be measurable. It also requires accurate definition of the time interval to which the measured adsorption is to be referred. This can be achieved by reducing the adsorption rate at the desired moment to one hundredth of its former value by a 100-fold dilution of a test sample.

TABLE I

Adsorption Rate Constants in Cm.^{3}/Min.

Type of bacteria	Physiological state	k cm.^{3}/min.	Observer
B. coli	Live, resting	15×10^{-10}	Schlesinger (2)
"	Heat-killed	6×10^{-10}	"
"	Live, small	15×10^{-10}	Ellis and Delbrück (3)
Staphylococcus aureus	Live, resting	2.2×10^{-10}	Krueger* (1)
"	Heat-killed	2.2×10^{-10}	"

* The numbers in Table IX of Krueger's paper are by a misprint too low by a factor of 100.

We give first a table of adsorption constants as determined by previous observers (Table I).

It will be seen from Table I that the adsorption constants given do not differ very greatly. This is plausible since all the phages used have been selected for their great "activity," so that the adsorption rates are likely to be near the maximum rates attainable, and this maximum rate is determined by the diffusion constant and the size of the adsorbing organism, as will be shown later. Krueger reports no difference between the rates of adsorption on live and on heat-killed bacteria, whereas Schlesinger finds a 2.5-fold greater rate for live than for heat-killed bacteria. This difference may be due to the fact that Krueger's measurements with live bacteria were done at 10°, when they were truly resting, whereas Schlesinger measured at 37°, where the bacteria, though in their lag period and not dividing, were probably actively growing in size.

In Fig. 1 we give some results obtained with our new strains, B_2 and P_2,

of *B. coli* and homologous phage.[1] They show that the free phage falls off exponentially in all cases and that the rate constants derived from the slope

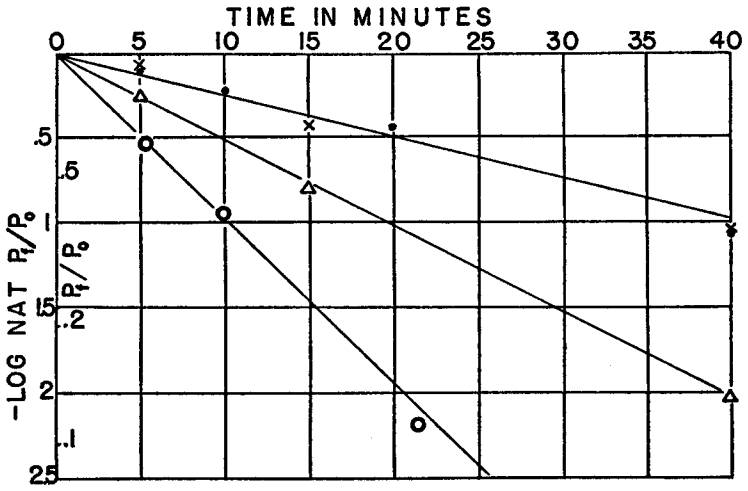

FIG. 1. Adsorption of phage by live bacteria in or near the stationary growth phase, at 25°C.

The bacteria were grown in broth. At time zero they were mixed with the phage. At the intervals given by the experimental points a sample was taken and at once diluted 1:100 in broth to prevent further adsorption. The diluted sample was then in some cases at once, in others at the end of the experiment centrifuged for 4 minutes to throw down the bacteria with those phage that had been adsorbed. A sample from the supernatant was then plated with bacteria. The latent period of phage growth at 25°C. is considerably longer than 40 minutes.

The bacterial concentrations, their cultural conditions, and the adsorption rate constant derived from the experiment were as follows:

	$[B]$ cm.$^{-3}$	k cm.^{3}/min.	Cultural conditions
●	5×10^7	5.4×10^{-10}	Grown with aeration up to a density of 10^9/cc. Diluted 1:20 in broth
×	1.1×10^7	23×10^{-10}	Grown for 30 hrs. without aeration, diluted 1:10 in broth 30 min. before experiment
○	10×10^7	10×10^{-10}	Grown with aeration up to a density of 10^8/cc.
△	10×10^7	5.2×10^{-10}	Grown in broth without aeration for 24 hrs. Diluted 1:2 in broth 10 min. before experiment

of the straight lines of the plot on a logarithmic scale are again similar to those obtained previously on other organisms although there are undoubt-

[1] For a description of these strains see the following paper (*J. Gen. Physiol.*, 1940, **23**, 643).

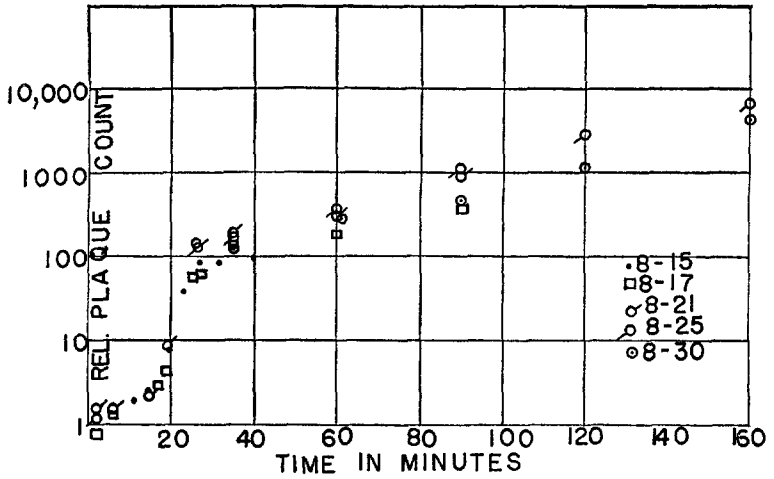

FIG. 2. One step growth in broth at 37°C.

At time zero phage are added to an actively growing aerated broth culture of bacteria, which had been inoculated 3 hours earlier. At time zero it contains about 10^8 *B*/cc. After 5 minutes about 90 per cent of the phage are adsorbed. The mixture is then diluted $1:10^4$ or $1:10^5$ in order to reduce the rate of infection of bacteria by phage set free in the first rise. It is seen that even at this very high dilution the plaque count increases slowly. That this represents true growth is proven by the next two experiments.

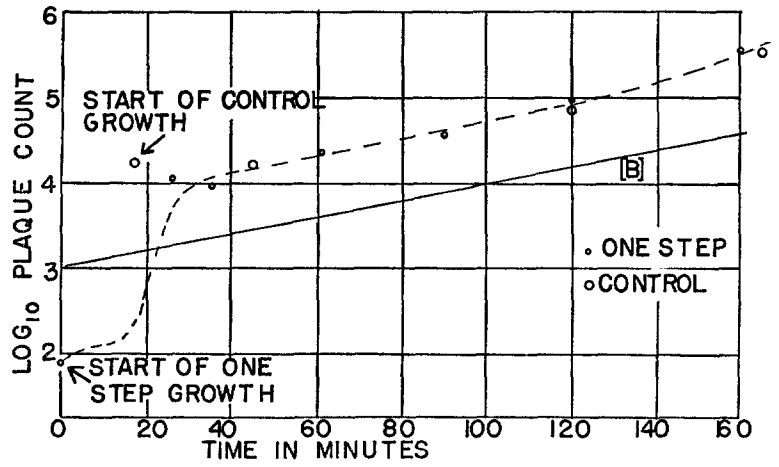

FIG. 3. Proof that true growth of phage occurs at the low bacterial concentrations of the one step experiments.

A one step experiment was carried out with a control, which contained the same concentration of bacteria, cultured under identical conditions and diluted simultaneously with the main culture. But whereas the main culture was mixed with phage 5 minutes *before* the dilution ($1:10^5$), the control was mixed with phage *after* the dilution with such an amount as to make the phage concentration nearly equal to the concentration in the main culture at the end of the step. It is seen that the phage grow equally in both cultures. That this growth proceeds by the normal mechanism, involving, however, an abnormally high adsorption rate is proven by the next experiment.

edly differences between the various batches of bacteria depending on their previous history. Such variations in rate arising from differences in the physiological state will lead to deviations from the simple exponential decrease of the free phage in cases where the physiological state is changing during the course of the adsorption experiment. The failure of some of the earlier observers to establish the simple adsorption mechanism which finds its expression in the straight line dependence is probably due to this complication.

We first obtained an indication that the adsorption rates under optimum physiological conditions might be considerably larger than those measured under the usual conditions in experiments designed to obtain one step growth curves. In these experiments a growth mixture was highly diluted before the occurrence of the first rise in order to prevent reinfection of new bacteria by the phage set free in the first rise. It was found that a 10^4 or 10^5-fold dilution does not suppress further phage increase in the expected degree (Fig. 2). That this increase represents real growth caused by the phage set free in the first rise, and was not due to delayed liberation of phage from bacteria infected before the dilution, was shown by a control in which the phage was added *after* the dilution and to an amount approximating the concentration attained previously by the first step. This control showed the same increase (Fig. 3). We then proceeded to make direct tests of the adsorption rate of the bacteria under these optimal growth conditions, taking points between 1 and 10 minutes. The results are given in Fig. 4 and summarized in the legend. It will be seen that the rate constants are indeed very much higher than in any previous experiments and are of the right magnitude to explain the secondary rise obtained in the one step growth curves after longer intervals.

This result may have some bearing on the activity method of assay invented by Krueger and applied by him and by Northrop in most of their work. In this method the time required to lyse a standard batch of bacteria is taken as a measure of phage concentration. The time interval in question is in part spent by the phage on diffusion preceding the adsorption. It is clear that any slight change in the physiological state of the test sample of the bacteria themselves may greatly influence the adsorption rate and thereby the whole scale of the assay. This is taken care of in Krueger's method by always running a known sample parallel with the unknown in order to establish the scale of the day, but possibly the great day to day fluctuations in this scale are partly caused by this factor.

It should also be noted that by this method adsorbed phage will be assayed higher than free phage, because adsorption of phage is the first stage in the process of lysis. Also the bacterium to which the phage is adsorbed

in the experimental tube will differ physiologically from those used in the assay, and this will alter its growth characteristics and thus its assay value by the activity method.[2] Adsorbed phage and free phage as measured by

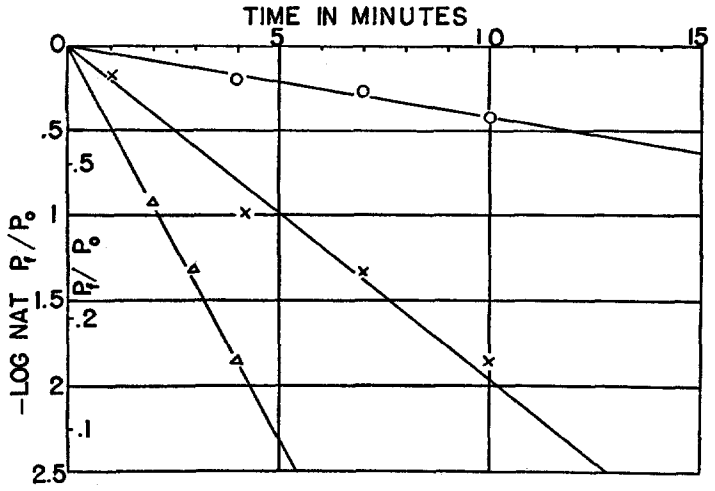

FIG. 4. Adsorption of phage by live bacteria under optimum conditions at 37°C.

The bacteria were grown in broth with aeration at 37°C. They were well in the logarithmic growth phase. Aeration was continued during the adsorption experiment. Technique of determination of free phage as in previous experiment. The bacterial concentrations and the adsorption rate constants derived from the experiments were as follows:

	$[B]$ cm.$^{-3}$	k cm.^{3}/min.
○	1.3×10^8	310×10^{-10}
×	7.7×10^8	210×10^{-10}
△	4.2×10^7	100×10^{-10}

this method are not comparable. Adsorbed phage may simulate increase in phage where there is only a change in the scale of assay.

[2] This is a point which also disturbs the reliability of the plaque count method. Sometimes differing assay values are obtained for free and for adsorbed phage, although the number of infective units has not changed, particularly when the adsorbing bacteria are in a different physiological state from the plating bacteria. Compare the discussion of the "initial rise" in (3). Also the transfer of the infected bacterium from the experimental tube to the agar plate or to the activity assay tube will be accompanied by a change of *milieu*, sometimes favorable, sometimes injurious or even fatal to the progress of phage production in the host. Refinement of technique in this respect will depend upon a closer study of the conditions of phage growth.

Residual Free Phage

If one follows the adsorption of phage over longer times one finds that the rate soon slows down and a few per cent or a fraction of a per cent will always be found unattached. Quantitative studies of this free fraction at equilibrium have been published by Krueger in 1931 (1) and by Schlesinger in 1932 (2), the first working with staphylococcus using the activity assay, the second working with *B. coli* using the plaque count assay. Their results, viewed together allow a more complete interpretation than is given by the authors.

The free fraction becomes of course very large when the phage are more than a hundred times in excess of the bacteria. This is due to saturation of the bacteria with phage and will not concern us here.

But also when the rate P/B is less than one, not all the phage will be adsorbed. We discuss first the experiments with *heat-killed bacteria*.

(*a*) *Krueger's Results.*—In his experiments the ratio P/B varies between 0.4 and 400. They are all done with the same concentration of bacteria. Beginning with small concentrations of phage it is found that the free phage is very nearly a constant fraction of the total phage (about 0.3 per cent). It increases very slowly to about 0.8 per cent when $P/B = 40$, and then very rapidly, on account of the saturation described before. Krueger interpreted these results in terms of a true equilibrium between adsorption and desorption. If this were the case the free fraction should be inversely proportional to the bacterial concentration. No experiments are given to test this point, but one experiment is given as a test whether or not desorption occurred. An adsorption mixture was diluted 1:10 after "equilibrium" had been reached. No desorption was found. Krueger believes that this is due to the extreme slowness of the desorption. It is indeed true that, proceeding on the assumption of a true adsorption equilibrium, the desorption rate would have to be more than a hundred times slower than the adsorption rate, but since also the amount of bound phage is more than a hundred times higher than the free phage a few per cent of desorbed phage would increase the free phage by a few hundred per cent. Indeed a simple calculation, using Krueger's data, shows that in his experiment the free phage should have increased about eightfold. His experiment therefore disproves the assumption of an adsorption-desorption equilibrium.

(*b*) *An Alternative Interpretation Has Been Proposed and Proven by Schlesinger.*—He shows that the free fraction arises from an inhomogeneity among the phage particles themselves. He distinguishes and actually isolates three groups of phage from a fresh lysate. The first and largest group exhibits a uniform and fast adsorption rate and is irreversibly bound. The second fraction is slowly and reversibly bound, the third fraction is not bound at all by heat-killed bacteria.

The last fraction (and part of the second fraction) is that which is found at equilibrium. It is independent of the bacterial concentration when the bacteria are in excess (reference 2, page 140) and it can be isolated and shown to have little affinity to heat-killed bacteria (reference 2, page 144).

The experiments of Krueger and of Schlesinger on free phage in equilibrium with heat-killed bacteria are therefore compatible and can be interpreted with Schlesinger's assumptions of an inhomogeneity among the phage particles.

On the other hand the situation is not so clear with respect to the equilibrium between phage and *live bacteria*.

TABLE II

Test of Affinity of Residual Phage Fractions

	1st Tube 0.4 cc. *B*		2nd Tube 0.4 cc. *B*		3rd Tube 0.4 cc. *B*		4th Tube 0.4 cc. *B*	
Time, *min*.....	0	10	16	26	32	42	48	58
	Add 0.1 cc. stock phage, assay stock phage	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant →	Add 0.1 cc. of previous supernatant	Centrifuge, assay supernatant
$P_f = \frac{P \text{ in supernatant}}{P \text{ added}}$		0.16		0.35		0.36		0.38
Fraction of stock phage-free		0.16		0.05		0.018		0.007

24 hrs. aerated bacteria were used for adsorbing the phage. The free phage in the supernatant of one tube were tested for their affinity to the bacteria in the next tube. 25°C. The experiment shows that the residual phage is qualitatively different from the main bulk.

The difficulty of such measurements is of course the fact that the phage can grow in the presence of living bacteria and that this must either be prevented or taken into account in an unambiguous way.

Schlesinger makes use of the latent period of growth. He leaves the phage in contact with a batch of bacteria for only 10 minutes, then removes these bacteria by centrifugation, and tests the remaining free phage for their affinity to a new batch of bacteria. He finds that the first batch leaves 2 per cent free phage, the second batch leaves 10 per cent free, finally the sixth batch leaves 70 per cent free (reference 2, page 145). This proves that there is a small fraction of phage with little affinity even to live bacteria. We have repeated these experiments with similar results. (See Table II.)

In Krueger's experiments with live bacteria growth of phage was prevented by working at 10°C. He finds that 2 hours after mixing phage and bacteria equilibrium is attained. The free phage is then proportional to the total initial phage (as he and Schlesinger found for heat-killed bacteria), but he also finds that the free phage is inversely proportional to the bacterial concentrations (in contrast to Schlesinger's result with heat-killed bacteria when the free fraction was independent of the bacterial concentration). This would suggest a simple adsorption-desorption equilibrium, a view which is further supported by one experiment, which indicates that desorption occurs at

TABLE III

Desorption Test

0.1 cc. stock phage — 0.9 cc. 24^h aerated *B*
(2.1×10^7 plaques/cc.)
wait 14 min.

Centrifuge assay supernatant	Dilute 1:100 in supernatant of 24^h aerated *B*
1.7×10^8 plaques/cc.	(2.1×10^6 plaques/cc.)
P_f = 8 per cent	wait 14 min.
	Centrifuge assay supernatant
	1.8×10^4 plaques/cc.
	P_f = 8.5 per cent

Phage are left in contact with a thick culture of 24^h aerated bacteria (2.5×10^9/cc.) until adsorption has become slow (14 minutes). A sample is then used for free phage assay, another is diluted 1:100 in the same medium, namely 24^h aerated culture, from which the bacteria have been removed by centrifugation. Free phage is again determined after 14 minutes. No desorption is found.

the required rate. We have found no trace of desorption. (See experiment, Table III.)

Although Krueger's experiments are quite consistent in themselves and could not easily be explained otherwise, we hesitate to accept the interpretation of their author because it requires us to accept that phage can be desorbed from live resting but not measurably from heat-killed bacteria, although the *ad*sorption rates are found to be equal in both cases.

Theory of Adsorption Rates

Schlesinger (reference 2, page 155) has given an interesting theoretical discussion of the adsorption rate by adapting the theory of coagulation of

von Smoluchowski (4) to this case. We will give a simplified presentation of the theory and compare it with some of the experimental results.

Let us calculate the average density distribution around a resting adsorbing sphere of radius a (bacterium) suspended in a medium that contains initially a uniform density of particles which may stick to the sphere when they encounter it. The average density near the sphere will decrease because the particles are constantly withdrawn by adsorption and soon a stationary density gradient will be set up. The density c as a function of r, the distance from the center of the sphere, is then

$$c(r) = c_\infty(1 - a'/r)$$

where c_∞ is the average density at a great distance from the sphere, and a' is a constant which depends on the probability of adsorption once a particle comes close enough to the surface of the sphere for direct interaction. If every approach to the surface leads to adsorption the density near the surface must drop to zero and we must therefore have $a' = a$. If not every approach is successful, the density near the surface will be positive and $a' < a$. This stationary density distribution is quite independent of the diffusion constant. It causes, however, a constant flow F towards the surface which is proportional to the density gradient *and* to the diffusion constant D.

$$F = D \cdot 4\pi r^2 \cdot \frac{\partial c}{\partial r} = c_\infty \cdot 4\pi D a'$$

This flow towards the spheres (bacteria) represents the loss of free particles by adsorption. The adsorption rate constant according to this model is therefore

$$k = 4\pi D a'$$

where a' is smaller or equal to the radius a of the adsorbing particle. Since the theory gives only an upper limit for a', it gives also only an upper limit of the adsorption rate, which will be attained when every collision with any part of the bacterial surface leads to adsorption

$$k_{\max.} = 4\pi D a$$

Let us evaluate this upper limit for the staphylococcus phage studied by Northrop and Krueger. The diffusion constant was determined by Northrop (5). He found values between 15×10^{-7} and 0.75×10^{-7} cm.^{2}/ min. The higher value was found at small concentrations of phage, corresponding to those in adsorption experiments. The radius of the coccus we can take to be 5×10^{-5} cm. With these values we obtain as the upper limit of the rate constant $k_{\max.} = 18 \times 10^{-10}$.

This is about eight times greater than the value determined by Krueger (see Table I). The lower value of the diffusion constant would make the observed k greater than the $k_{\max.}$ allowed by the theory and can therefore be ruled out on the basis of this theory.

Our own much higher rate constants for *B. coli* under optimum conditions can be ascribed to two contributing factors. First the considerably larger

surface of the bacteria (perhaps ten times the minimum value). Second, the model underlying the theory is not quite adequate. The *B. coli* will not be stationary under the conditions mentioned but will be actively moving and moreover their range of action is extended through their flagellae. Both the mobility as such and the movement of the flagellae as such will cause the solution near the bacterial surface to circulate rapidly. The average density near the surface will be raised by this circulation to nearly the average density in more distant parts, and the adsorption rate will thus be increased.

Krueger (1) has recorded experiments, in which he varied the viscosity of the suspension by the addition of glycerol. This should, according to Einstein's formula, alter the diffusion constant and according to the above theory cause a proportionate change in the adsorption rate. This conclusion is quite independent of the type of reaction that takes place at the bacterial surface itself. Krueger's experiments do not show this expected dependence of the adsorption rate on the viscosity. This seems quite inexplicable.

DISCUSSION

The study of the adsorption of phage onto its sensitive host is a study of the diffusion of phage through the medium and of its specific attachment to a host. This attachment has many analogies to the antigen-antibody reaction (Burnet). The whole process of adsorption is a necessary forerunner of the multiplication of phage under ordinary conditions. In itself it has nothing to do with the growth process, but in non-lysogenic strains it is intercalated between the true growth reactions and necessitates a careful separate study of the adsorption before one can draw relevant inferences from growth experiments. The adsorption interferes in two ways. First in the experimental vessel. Here the fact, that the adsorption proceeds continuously, has the effect of smoothing out the steps that arise from the sudden liberation of new phage from infected bacteria. A judicious adjustment of the bacterial concentrations, so as to make adsorption either "infinitely fast" or "infinitely slow" in comparison to the latent growth in the bacteria, is therefore necessary to bring out the steps described by d'Herelle, by Ellis and Delbrück (3), and in the following paper by Delbrück (6).

Second, it interferes in the assay of phage, whether the plaque count or the activity method is used. The effect on the plaque count is small, and this can probably be understood by the following argument. In order that a phage particle may form a plaque it must infect a living bacterium *before* the bacterial layer has grown so thick, that growth conditions for phage

become unfavorable. In general there will be ample time for the majority of phage to fulfill this condition even if the bacteria are of the poorly adsorbing variety. Adsorption rate is therefore not a limiting factor for the efficiency of plating. This is borne out by the observation, that the efficiency of plating is only slightly changed if rapidly growing bacteria are used for plating instead of the standard 24 hour aerated cultures.

The converse is true for the activity method. Here the time spent by the phage particles on adsorption is an integral part of the quantity measured, namely the time between the addition of phage and lysis, and any factor that changes the adsorption rate (or eliminates it, as in the assay of adsorbed phage) will shift the scale of the assay. As we have shown, the adsorption rate does in fact change with the physiological state of the bacteria, and it is different for different fractions of the phage. In fact, if Schlesinger's discovery holds true also for staphylococcus phage, it would appear that the activity method was ill-suited for the quantitative determination of the residual free phage, since the experiment selects those particles whose assay value is least comparable with those of the main bulk.

SUMMARY

1. The adsorption rate constant of phage to bacterium is found to change between wide limits, depending on the physiological state of the bacterium.

2. The experiments of Krueger and of Schlesinger on the residual free phage in contact with an excess of bacteria are discussed and the view of Schlesinger, that they represent phage particles with reduced affinity to the bacterial host is supported by experiments.

3. The theory of von Smoluchowski and Schlesinger is compared with the experiments.

4. The implications of these findings for the assay methods currently used are discussed.

The author wishes to express his appreciation for the hospitality extended to him by the Biology Department of the California Institute of Technology during the tenure of a Fellowship of The Rockefeller Foundation. In particular he wishes to record his indebtedness to Dr. E. L. Ellis for constant help and advice and to Mr. F. Gardner for technical assistance.

REFERENCES

1. Krueger, A. P., *J. Gen. Physiol.*, 1931, **14,** 493.
2. Schlesinger, M., *Z. Hyg. u. Infectionskrankh.*, 1932, **114,** 136, 149.
3. Ellis, E. L., and Delbrück, M., *J. Gen. Physiol.*, 1939, **22,** 365.
4. von Smoluchowski, M., *Z. phys. Chem.*, 1917, **92,** 129.
5. Northrop, J. H., *J. Gen. Physiol.*, 1938, **21,** 335.
6. Delbrück, M., *J. Gen. Physiol.*, 1940, **23,** 643.