STEPWISE LIBERATION OF POORLY SORBED BACTERIOPHAGES

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It is rather commonly accepted that the process of phage regeneration begins with the fixation to the bacterial cell of a phage particle, which "multiplies" in or on the growing cell until lysis occurs, when the newly formed particles are liberated into the medium. If this is the only mechanism involved, no secretion of phage by intact, living bacteria is possible.

The process described above is in accord with two facts. Most phages are strongly sorbed by susceptible bacteria and, with suitable methods, the liberation of phage can be shown to occur in steps in such a way as to suggest that the lysis of a single bacterium liberates anywhere from a few to a hundred or more active particles.

The stepwise liberation of phage, first described by d'Herelle, has been confirmed by Burnet (1929) for a number of phages, including a staphylococcal phage (Burnet and Lush, 1936), and the same phenomenon has been studied in detail by Ellis and Delbruck (1939) and by Delbruck (1940b) for two coliphages.

Krueger and Northrup (1930) and Northrup (1939), on the other hand, have expressed the idea that the phage is secreted continuously by growing bacteria. Experiments on the sorption of a staphylococcal phage (Krueger, 1931) were in accord with the view that the phage is distributed between bacteria and the external medium by simple reversible partition. These conclusions are, of course, directly opposed to the concept of liberation by lysis. The interpretation of these authors was questioned by Delbruck (1940a), but was tentatively accepted by him later (1940b) with the conclusion that in the case of *Staphylococcus aureus* and *Bacillus megatherium* "lysis from within either does not exist and is replaced by continuous phage secretion; or it exists but leads only to a slow equalization of the refractive indices of the cell interior and milieu."

Delbruck (1940b) pointed out a correlation between the number of particles of phage liberated during the lysis of a single bacterium and the sorptive capacity of the bacterium. This relationship suggests that during propagation the newly formed phage is held to the bacterial cell solely by chemical forces of adsorption, and if the latter were sufficiently weak, it might be supposed that the phage would be liberated from the cell just about as fast as it is formed. Under these conditions one could expect the phage to be secreted by living bacteria, which would undergo lysis only after the external concentration of phage had become very high. This is precisely the course of events postulated by Krueger and Northrup for *Staphylococcus*.

The possibility exists that different phages may be liberated from the infected cell by two separate processes; in the one case by lysis, in the other by continuous secretion. The present paper describes some attempts to demonstrate continu-

ous secretion. As materials favorable to this purpose, we have chosen a coli-dysentery phage which is poorly sorbed by susceptible bacteria, and the staphylococcal phage of Krueger and Northrup.

EXPERIMENTAL

Three phages were used: the coli-dysentery phage P9H of our collection, a staphylococcal phage received from Krueger in 1933, and a second strain of the same origin received from Northrup in 1938. Phage P9H was cultivated on an intermediate coliform strain designated 3A, the staphylococcal phages on two cultures of *Staphylococcus aureus* received with the phages.

Phage P9H is of the greatest interest because no definite sorption of this phage by the bacterial cells can be demonstrated with the usual methods. Small amounts of phage P9H added to living 18-hour broth culture are recovered quantitatively (± 10 per cent) in the supernate by centrifugation 10 minutes after mixing. The same is true if the culture is concentrated 10 or 20 times by preliminary centrifugation, and is true also if killed bacteria are used and the time allowed for sorption is increased to 24 or 48 hours. Similarly, no sorption of this phage by growing (2 or 4 hour) cultures containing about 10^8 bacteria per ml can be detected in 5 minutes. As will be shown below, however, a slow sorption can be demonstrated by special methods. This behavior is peculiar to the phage itself. Thus among six additional strains of bacteria susceptible to this phage, only one, *Shigella flexneri*, takes up appreciable though small amounts of the phage in brief exposure.

Stepwise liberation of phage P9H

Approximately 100 lytic units of phage are added to 100 ml. of 2 per cent Bacto-tryptose broth containing 0.5 per cent NaCl. The broth is seeded from 24-hour broth cultures with about 5×10^7 bacteria per ml. and immediately distributed in amounts of one ml. in serological tubes, so that each tube receives approximately one phage particle. Within 15 minutes of the time of seeding, the tubes are placed in a water bath at 37°C. At stated intervals, five tubes are plated, the contents of each tube being mixed with 2.5 ml. of one-per-cent agar and poured over the surface of a plate containing solidified one-per-cent agar (Hershey, Kalmanson, and Bronfenbrenner, 1942). The condensed results of a typical experiment are shown in table 1, and indicate that phage P9H is liberated by lysis under these conditions, and that the processes of infection and lysis of one bacterium initiated by one particle of phage occupies about one hour and results in liberation of 100 or more lytic units.

We consider this finding to be significant because of the peculiarity of phage P9H. As we shall show in detail in a subsequent paper, the sorption of this phage is further reduced in the absence of salt. Phage produced in the absence of salt might be expected, therefore, to be more readily desorbed from the bacterial cells, and perhaps under these conditions to be secreted before lysis takes place.

However, in the absence of added salt only one per cent or less of the phage-

particles known to be present undergo regeneration in culture, so that it is not feasible to study the process directly. On the other hand, after the phage has received minimal exposure to specific antiserum, it is readily taken up by bacteria, and is capable of regenerating in the absence of salt and free antibody (Hershey and Bronfenbrenner, 1942). But since the phage particles newly formed under these conditions are not sensitized and possess the original salt-sensitivity, they should be readily liberated from the cells if bound only by adsorption-forces.

Sensitized phage was prepared by mixing equal volumes of phage (10^8 lytic units per ml.) and 1:100,000 antiserum. After 2 hours at 37°C. the reaction

TABLE 1

Stepwise growth of coliphage P9H in the presence of salt

MINUTES AFTER SEEDING	COUNTS OF INDIVIDUAL TUBES				
20	0	0	0	1	0
30	1	1	1	3	1
40	3	1	3	1	0
50	1	201	135	0	0
55	0	1	205	206	0
60	128	205	0	0	lost
65	600	283	615	273	0

TABLE 2

Stepwise growth of coliphage P9H without added electrolyte

MINUTES AFTER SEEDING	SENSITIZED PHAGE					MINUTES AFTER SEEDING	NATIVE PHAGE				
	Counts of individual tubes						Counts of individual tubes				
30	2	1	0	4	3	75	1	1	0	1	0
40	2	4	2	3	3	100	1	0	0	1	1
50	2	3	4	3	90	180	0	1	1	0	2
60	116	237	58	3	81	210	3	1	1	0	2
75	3	476	2	144	131	255	1	0	2	0	0
90	154	2	366	80	421	300	1	0	0	0	0

was stopped by a thousand-fold dilution in broth. The phage retained at least 90 per cent of its original activity; and, whereas unsensitized phage is counted with an efficiency of about 0.001 on agar without salt, about half the sensitized preparation could be counted.

Using the sensitized phage, the experiment of table 1 was repeated without change, except that broth without added salt was used. For comparison, a second series of tubes were prepared with unsensitized phage. The results obtained in both series are shown in table 2. It will be seen that the regenerating phage is retained by the cells until lysis even in the absence of salt. Native phage fails to regenerate under these conditions (or rather does so too infrequently to be observed in an experiment of these dimensions).

It may be concluded that liberation of phage P9H occurs exclusively by lysis, and further that, during regeneration, phage is held by cellular structures not readily available for the adsorption of external phage. Phage P9H may provide an exception, therefore, to the relation between sorptive capacity and burst size postulated by Delbruck (1940b). On the other hand, our results may indicate merely that this phage penetrates the cell very slowly in either direction, although this explanation is difficult to reconcile with the observed effects of salt and antibody.

Demonstration of sorption as a condition of regeneration

It remains to explain the fact that the poorly sorbed phage P9H nevertheless promptly undergoes regeneration in the growing bacterial culture. For a time it appeared that free phage, perhaps by momentary contact with the bacterial cell, was capable of initiating intracellular regeneration. Especially the observation (recently confirmed by Dr. Kalmanson) that sensitized, rapidly sorbed phage initiates regeneration no more promptly than unsensitized phage, suggested that sorption might be only an incidental phenomenon, not essential to the growth of phage. Because of the importance of this conclusion, if true, the following rather elaborate experiments were carried out.

It was necessary to design an experiment in which the measurement of sorption would be uncomplicated by the simultaneous increase of phage. This condition was realized by studying the fate of individual particles of phage in cultures containing initially very few particles. Growth of phage under these conditions can be recognized immediately because the lysis of a single bacterial cell liberates many times more phage than that initially present.

Broth (beef extract, 0.5 per cent NaCl) containing precisely one lytic unit per ml. (see below) was seeded at 37°C. with about 10^8 bacteria per ml., and distributed in amounts of 1 ml. in serological tubes. Two experiments were performed, each comprising 50 tubes, the cultures being carried through in batches of 10 at a time. The tubes were incubated for 30 minutes (experiment 1) or 25 minutes (experiment 2) and immediately sedimented in the angle centrifuge. The supernates were decanted within 10 minutes of the beginning of centrifugation, and either plated entire (experiment 1), or poured into 2 ml. of lightly seeded broth which was then separated into three portions for incubation (experiment 2). To each of the sediments, one ml. of broth was added and the tubes were incubated for the detection of phage. Results were read after 36 hours, when lysis was sharp. All unlysed tubes were tested for the presence of phage by spotting on seeded agar plates. All were negative.

In the first experiment, the supernates of only four cultures yielded more than three plaques, the counts of these four falling between 19 and 100. The counts of the remaining samples accorded with the expectations of random sampling (only one yielded three plaques) and probably no increase of phage had occurred in these tubes. In the second experiment, growth was assumed to have occurred if phage was detected in all three portions of the supernate. The procedure followed in the second experiment is preferable, since it is not always certain

whether the efficiency of plating and counting in broth is identical (however, see below).

To test the possibility of contamination with phage, ten cultures without added phage were run through the operations of centrifugation, decantation, etc., collected and diluted to 50 ml. with broth, and distributed among 50 tubes for incubation. No contamination with phage was detected.

Plate counts corresponding to 40 ml. of the culture fluid used in the two experiments yielded 1.02 and 1.00 lytic units per ml. Six broth counts of about 50 tubes each (including the two sedimentation experiments described above) containing one ml. of the original culture fluid lightly seeded gave 0.99, 0.88, 0.97, 0.96, 1.23, and 0.66 lytic units per sample. The total result was 124/316 tubes

TABLE 3

Distribution of phage between sediments and supernates

	EXPERIMENT 1	EXPERIMENT 2
Both negative	19	26
Both positive	8	6
Sediment only	14	12
Supernate only	9	6
Total	50	50
Growth	4*	2*

* These tubes certainly belong in the class "sediment positive," and therefore cannot be subtracted without prejudicing the data. Since about one-third of the tubes with positive sediments have also positive supernates, tubes showing growth have been divided in this ratio between the groups "both positive" and "sediment only."

negative or 0.94 lytic units per tube. These and other calculations were made by means of the Poisson distribution

$$P_r = \frac{e^{-m} m^r}{r!}.$$

In this

P_r = probability of obtaining r particles in a specified sample.

m = mean number of particles per sample.

Thus

$$m = 2.3 \log \frac{\text{total number of samples}}{\text{number of samples negative}}$$

$1 - P_0$ = expected fraction of samples positive

$1 - P_0 - P_1$ = expected fraction of samples containing more than one particle, etc.

The crude data of the two experiments are shown in table 3.

For further analysis, a gravimetric determination was carefully made of the amount of supernate retained by the sediment on decantation under the condi-

tions of the experiment. This proved to be 6.5 per cent. With this information, the calculations shown in table 4 were made. The recovery of the phage was

TABLE 4

Relation between sorption and infection

	SAMPLES POSITIVE		LYTIC UNITS PER SAMPLE	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Combined samples				
Expected	30	30	0.94	0.94
Found sediment or supernate	31	24	0.97	0.66
Found sediment + supernate			1.03	0.73
Supernates				
Expected,* less 6.5 per cent	30	23	0.91	0.62
Found	17	12	0.41	0.28
Difference (sorbed)			0.50	0.34
Sediments				
Expected,* 6.5%	3	2	0.06	0.04
Found	22	18	0.58	0.45
Difference (sorbed)			0.52	0.41
Both positive				
Expected†	7	4		
Found	8	6		

* Computed from numbers found in the combined samples assuming no sorption.

† Computed from the expected frequency of 2 or more particles per sample, assuming 50 per cent sorption, e.g., one-half of those tubes containing two particles should show both sediment and supernate positive.

TABLE 5

Stepwise growth of staphylococcal phages

MINUTES AFTER SEEDING	PHAGE FROM KRUEGER					MINUTES AFTER SEEDING	PHAGE FROM NORTHRUP				
	Counts of individual tubes						Counts of individual tubes				
30	2	2	0	1	2	35	1	1	1	1	2
45	1	4	0	1	4	60	2	0	2	0	1
60	1	2	1	0	0	80	0	3	0	1	1
80	0	0	1	35	0	95	0	0	1	3	1
90	54	29	61	0	24	115	108	2	12	1	0
100	0	2	41	43	1	140	3	180	287	1	89
110	71	1	51	23	7	155	1	0	218	0	1
120	88	42	83	35	18	180	39	351	0	1	71

satisfactory in both experiments, and the sum of the phage found in the sediments and supernates was not significantly greater than that expected from the total

number of positive samples. Considering the supernates alone, 52 per cent of the phage was adsorbed in each of the two experiments. Considering the sediments alone, the apparent adsorption was 54 and 62 per cent respectively. Probably no infection of the sediments without permanent adsorption of the phage occurred. Similarly, the number of cultures showing both infected sediments and supernates was only slightly greater than that expected on the hypothesis of infection by adsorption.

We conclude that infection with phage P9H takes place exclusively by fixation to bacteria, but the latter occurs too slowly to be detected easily by the ordinary methods in which growth of phage must be prevented.

It may be noted that these experiments confirm in detail the previous conclusion that phage P9H is liberated exclusively by lysis.

Stepwise growth of staphylococcal phage

Stepwise growth in tryptose broth containing 0.5 per cent NaCl was observed by the same methods with the two staphylococcal phages previously mentioned. The results are shown in table 5.

DISCUSSION

The experimental results are sufficiently clear-cut to require no comment. The question arises, however, whether the stepwise liberation of phage necessarily implies the lysis of a bacterial cell. It is of course conceivable that the phage is liberated discontinuously by living cells, corresponding to some natural rhythm of bacterial growth. However, Delbruck (1940b) found that the period of the phage-growth from adsorption to lysis was one and one-half times longer than the division-time of bacterial cells under comparable conditions. In unpublished experiments, we ourselves found some years ago that bacterial cultures diluted after infection with approximately one phage-particle per cell virtually stopped respiring at the end of 30 minutes of decelerating growth at 37°C., and under these conditions visible lysis began at about this time. According to Delbruck (1940b), 70 per cent of individual bacterial cells (probably all those initially infected) became invisible within 70 minutes at 25°C. under similar conditions. The possibility of stepwise liberation of phage without lysis seems rather remote.

CONCLUSIONS

A poorly sorbed coliphage has been found to exhibit stepwise increase in the manner of other phages. Regeneration occurs in the same manner even in the absence of salt, where adsorption normally does not occur. It may be concluded that the newly formed phage is held by structures not readily available for the sorption of external phage; presumably, it is formed intracellularly.

Liberation of phage by lysis was also demonstrated with the staphylococcal phage of Krueger and Northrup. This finding is incompatible with their interpretation of the growth process.

A method is described for correlating infection and sorption of phage in growing cultures, which also serves to demonstrate fixation of poorly sorbed phages. No evidence was obtained that lysis may be initiated without sorption.

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