THE B. MYCOIDES N HOST-VIRUS SYSTEM

II. Interrelation of Phage Growth, Bacterial Multiplication, and Lysis in Infections of the Indicator Strain of B. mycoides N with Phage N in Nutrient Broth*

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As part of a survey of a new bacterium-bacteriophage system, the following studies deal with changes in bacterial and virus numbers when lag phase suspensions of *B. mycoides* N containing 10^0 to 10^7 cells per ml. are infected with *mycoides* phage N in nutrient broth.

A. Phage Growth, Bacterial Multiplication, and Lysis in Concentrated Virus-Infected Suspensions (1–5 × 10^7 Cells per Ml.)

In Experiments A1 to A4 (Figs. 1–4) the phage was present in excess at the onset of infection. Experiment A2 is a typical one for multiple phage infections of this system. Readings on the Klett colorimeter remained constant until 1.2 hours. Evidently no appreciable multiplication of infected cells occurred. Incomplete lysis started at 1.2 hours and continued until 2.0 hours. The drop in bacterial numbers coincides with the release of the main amount of phage.[1] From 2.0 hours until 2.8 hours, the number of cells (about 5 per cent of the original number) did not change a great deal.

The increase in bacterial numbers in Experiment A1 (Fig. 1) during the first 0.5 hour is not characteristic of multiple infections for this system. Experiment A1 probably does not represent a true case of multiple infection. The low initial phage:bacteria ratio (only 2:1) and a temperature of 28°C. for the first 0.25 hour (instead of 31°C. as in Experiment A2) presumably resulted in a failure of many cells to be infected until 0.5 hour.

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[1] In discussing the phage titers in section A, 0.1 hour should be added to each time recorded in the graphs to allow for the time elapsing between withdrawal of the sample and completion of plating for plaque count.

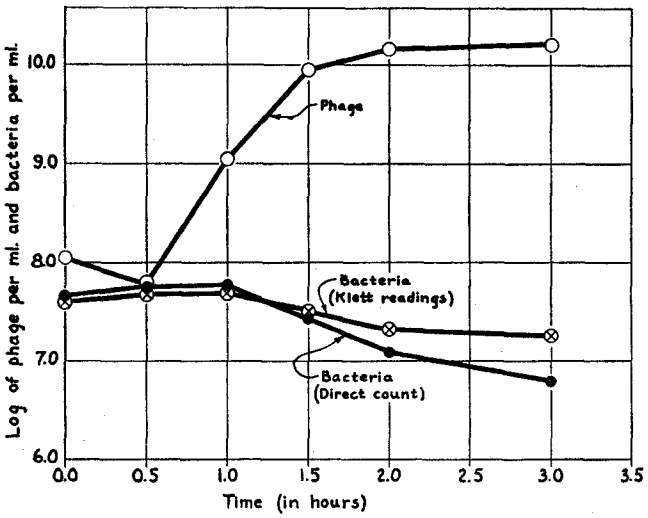

FIG. 1. Experiment A1. Phage multiplication in "concentrated" cell suspensions; initial virus to cell ratio, 2:1.

At the end of 2.8 hours the phage-infected suspension was incubated at 32°C. for 6.2 hours longer (without shaking). At the end of this time it was observed that there was a very heavy growth in the tube, consisting of clumps of cells in otherwise clear broth.

The time given represents the time at which the sample was taken. Actual completion of plating for plaque count occurred 0.1 to 0.2 hour later.

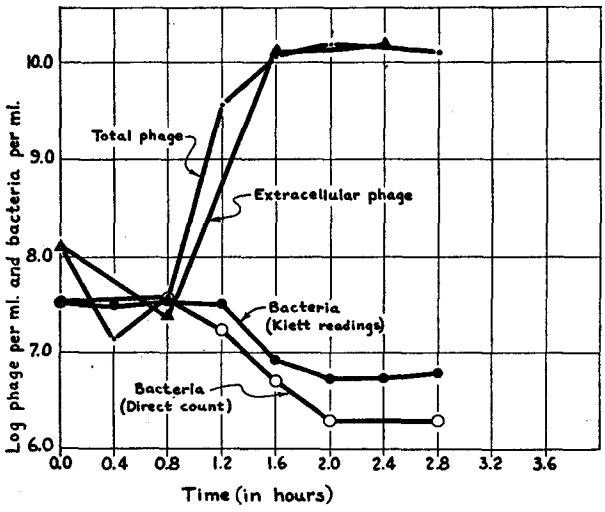

FIG. 2. Experiment A2. Phage multiplication in cell suspensions giving visible turbidity; initial virus to cell ratio, 4:1.

In Experiments A1 to A3, there was a pronounced drop in the plaque count for the first 0.6 to 0.8 hour followed by a sudden rise in the free phage titer. The release of phage continued until 2.0 hours in Experiment A1 (Fig. 1), 1.7 hours in Experiment A2 (Fig. 2). The titer then remained constant until the end of both experiments (at 2.8 hours).

With multiple infection then, phage production in concentrated suspensions with lag phase *B. mycoides* N in nutrient broth is stepwise. The exaggerated initial dip is a consequence of (1) continuous adsorption of free phage to chains of cells and (2) absorption of more than 1 phage particle to a bacillus. A flat

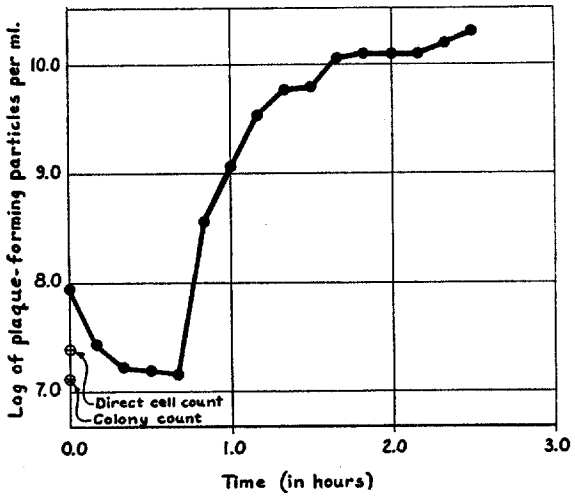

FIG. 3. Experiment A3. Phage multiplication in suspensions of cells giving visible turbidity; initial virus to cell ratio, 4:1. Phage-bacterium mixture at onset of infection contained 9.3×10^7 virus particles per ml., 2.4×10^7 cells per ml.

initial phase was obtained in Experiment A4 (Fig. 4) when adsorption was stopped after 0.25 hour by 1:100 dilution in nutrient broth.

The average phage yields per bacterium in Experiments A1, A2, A3, and A4 were 395, 296, 875, and 275 particles respectively, as calculated from the formula of Delbrück and Luria (1942). The yields in Experiments A1 to A4 (10^6 to 10^7 cells per ml., 31–32°C.) are significantly higher than those in section B (10^8 to 10^4 cells per ml., 30–31°C.) and Experiment C1 in section C (4.6 cells per ml., 30°C.). The cell numbers reported in section A may be too low because of clumps originally present in the suspensions, which were not gauze-filtered, a procedure producing clump-free suspensions and used as routine in preparing experimental cultures in sections B, C, and D.

In Experiments A5 to A8 (Figs. 5 to 8), the bacteria were initially present in excess. In all four experiments, the phage titer remained fairly constant for at least 0.6 hour. In those experiments in which extracellular phage was deter-

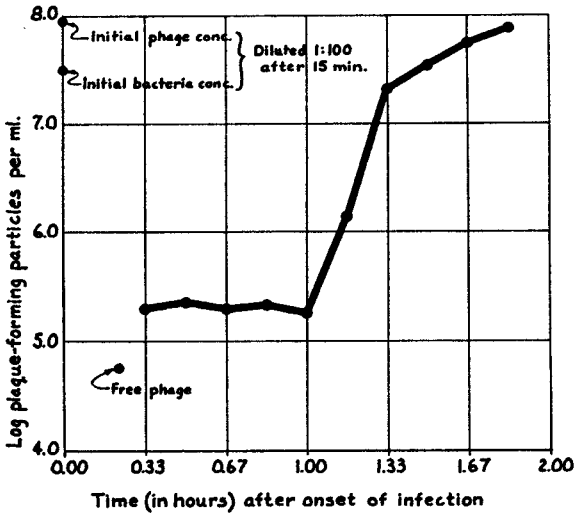

FIG. 4. Experiment A4. Phage multiplication in a suspension of *B. mycoides* N containing 10^8 cells per ml.; initial phage to cell ratio, 3:1.

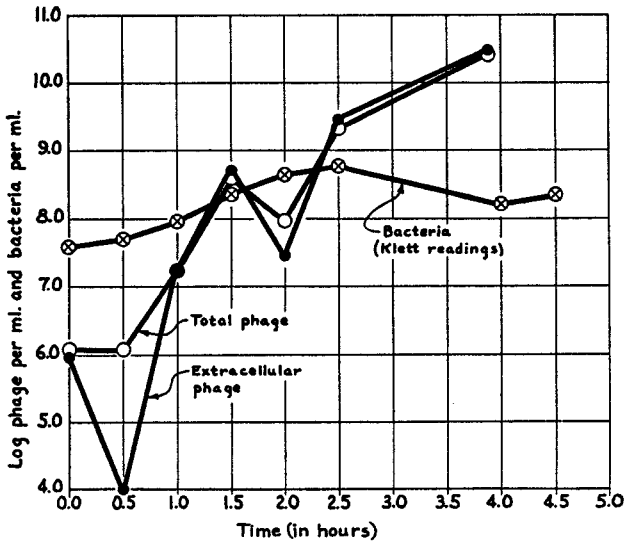

FIG. 5. Experiment A5. Phage multiplication in a suspension of *B. mycoides* N containing 10^7 cells per ml. when the original virus to cell ratio is 1:30.

The time given represents the time at which the sample was withdrawn. Actual completion of plating for plaque count occurred 0.1 to 0.2 hour later.

mined (A5 to A7), 98 per cent of the total phage was intracellular. As in experiments in which virus was initially present in excess, phage was released between 0.6 and 1.1 hours.

Fluctuations in phage and bacterial numbers after 1.1 hours will be described for Experiment A7 (Fig. 7). At 1.1 hours, the total phage count was 10 times greater than at 0.6 hour but still largely represented infected bacteria. Probably most of the phage released between 0.6 and 1.1 hours was adsorbed to uninfected cells. By 1.6 hours, however, the virus: cell ratio was 6:1, insuring infection of all cells. (The dip in total and free virus at 2.1 hours may be due to adsorption of liberated phage.) Incomplete lysis occurred from 2.0 to 3.5 hours. From 3.5 to 4.5 hours, the bacterial numbers stayed fairly constant—10^7 cells per ml.—although the titer had reached over 10^{10} plaque-forming particles

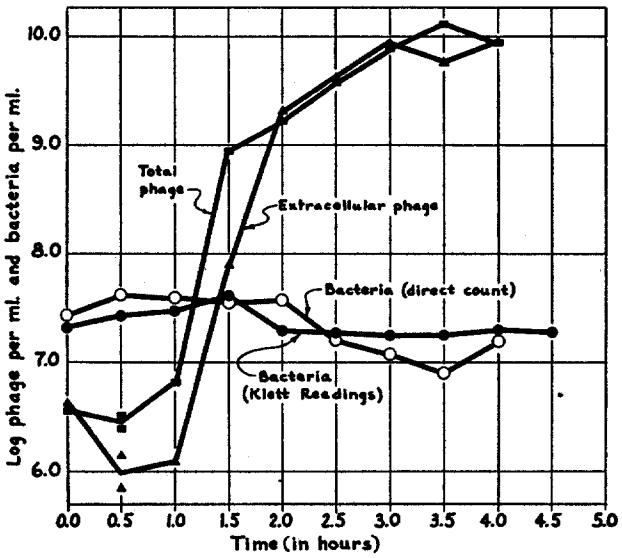

FIG. 6. Experiment A6. Phage multiplication in suspensions of *B. mycoides* N, containing 10^7 cells at the onset of infection, when the original virus to cell ratio is 1:6.

Plaque counts in this experiment merely indicate orders of magnitude. Old agar plates were used, and the plaques were indistinct and difficult to count.

before 3.1 hours. (From 1.6 to 4.5 hours the free and total phage titers are practically identical.)

Further visual observation of the culture in this experiment and in many others showed that no further clearing followed the incomplete lysis occurring at 2 to 4 hours after infection. The cell numbers always increased again. Additional readings were not made after 4.5 hours in Experiment A7 nor in other experiments because the clumping of the cells—which always began prior to lysis and release of phage—was so marked by this time that further cell counts or turbidity readings would have had no semblance of accuracy.

Experiment has proven that those cells which survive initial phage infection and are able to multiply in the presence of 10^9 to 10^{10} free phage particles per

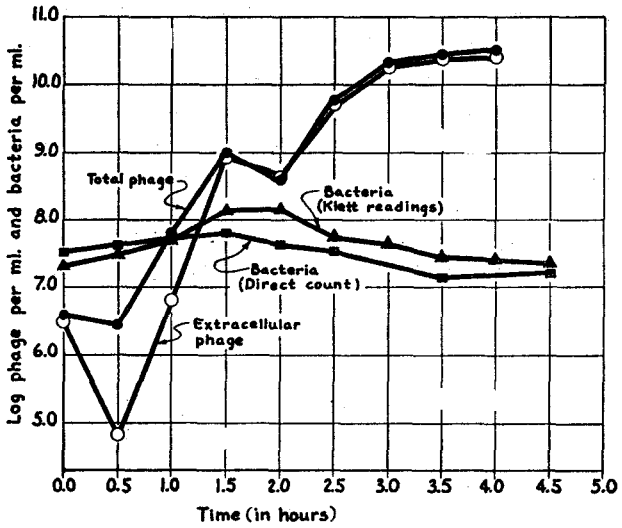

FIG. 7. Experiment A7. Phage multiplication in suspensions of *B. mycoides* N (containing 10^7 cells per ml. at onset of infection) when original virus to cell ratio is 1:8. Turbidometric readings and direct counts were made on a suspension in which clumping became progressively worse and therefore merely indicate trends in the increase or decrease of bacterial numbers.

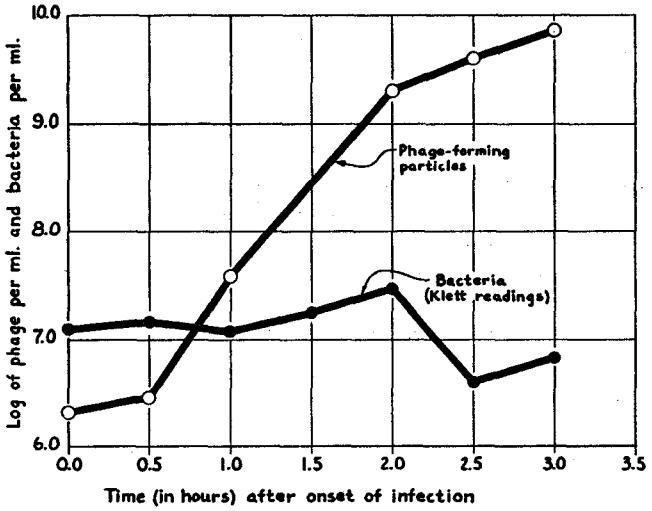

FIG. 8. Experiment A8. Phage multiplication in "concentrated" cell suspensions when initial virus to cell ratio is 1:7. All samples were iced after withdrawal. They were plated for plaque count at the end of the experiment. Samples on which turbidity readings were to be made were formolized before being placed in ice water.

ml. are themselves internally infected (Baer and Krueger, 1952); that is to say, they are lysogenic.

In Experiment A5 (Fig. 5) the initial phage:bacteria ratio was 1:16 rather than 1:7 as in Experiments A6 to A8. This is reflected in a more impressive increase in the total number of cells and in the number of unlysed cells remaining.

B. Phage Multiplication in Dilute Cell Suspensions (10^2 to 10^4 Cells per Ml.); One-Step Growth Experiments

One-step growth curves confirm what has been learned using cell-virus mixtures containing 10^7 cells per ml. The average constant period of intracellular

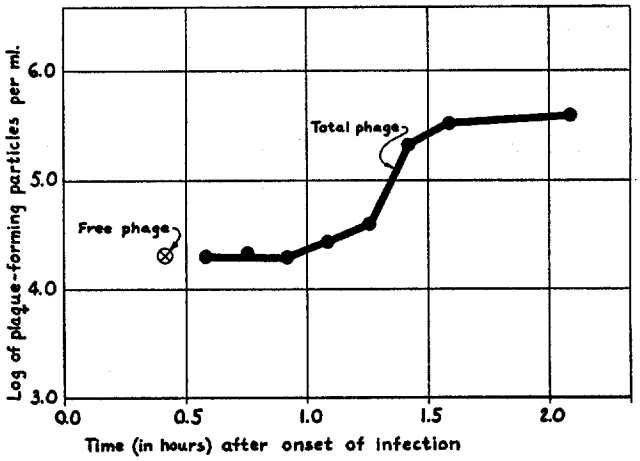

FIG. 9. Experiment B1. One-step growth curve for log phase, singly infected cells of *B. mycoides* N.

phage growth in Experiments B1 to B5 (Figs. 9 to 13) was 1.2 hours. It was essentially the same:

1. For lag phase (Figs. 10 to 12) and log phase (Figs. 9 and 13) cells.
2. For multiple (Figs. 12 and 13) and single (Figs. 9 and 11) infections.
3. When adsorption was carried out at 0°C. for 1.6 hours (Fig. 11) rather than at 30°C. for 0.4 hour (Figs. 9 and 10).
4. When dilution after 0.4 hour adsorption at 30°C. was further delayed by a 0.2 hour period of centrifugation, followed by resuspension of the cells in fresh medium (Figs. 12 and 13).

The latent period in Experiments B1 to B5 (Figs. 9 to 13) is succeeded by a "steep-rise period" during which rapid phage liberation occurs. This is in turn followed by a slower, continuous increase in the virus titer or a plateau.

Experiments A1 to A8, as well as Experiments B1 to B5, were conducted

several months prior to Experiments B6 to B9 (Figs. 14 and 15) in which the cell concentrations ranged from 10^2 to 10^7 cells per ml. These later experiments

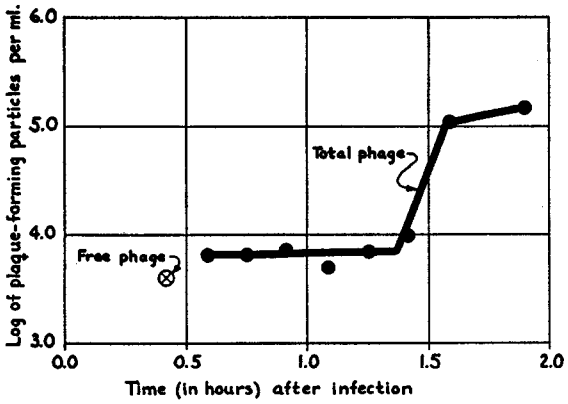

FIG. 10. Experiment B2. One-step growth curve for lag phase, singly infected cells of *B. mycoides* N.

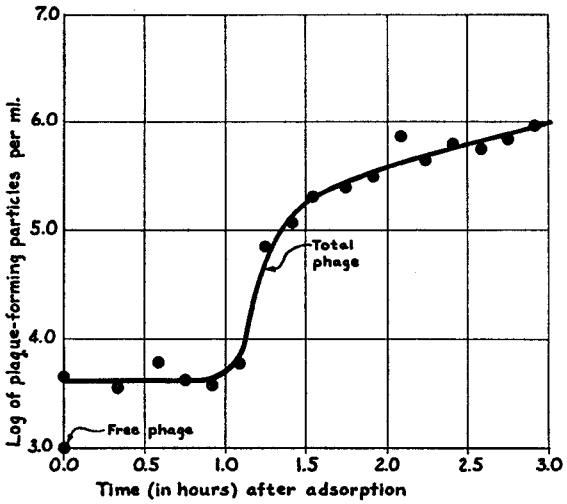

FIG. 11. Experiment B3. One-step growth curve for lag phase, singly infected cells of *B. mycoides* N; adsorption at 0°C. for 1.5 hours. Free phage titer at zero time was 1×10^3 per ml.

show the same general sequence of events with respect to changes in phage titer, but there may be an increase in the length of the latent period (Experiments B6, B8, B9; Figs. 14 and 15) and in the character of adsorption.

The average yields per bacillus in Experiments B1 to B4 and B6 to B8 were

calculated from the formula of Delbrück and Luria (1942). They form the following series: 25, 43, 52, 67, 86, 100+, 127. This probably does not represent

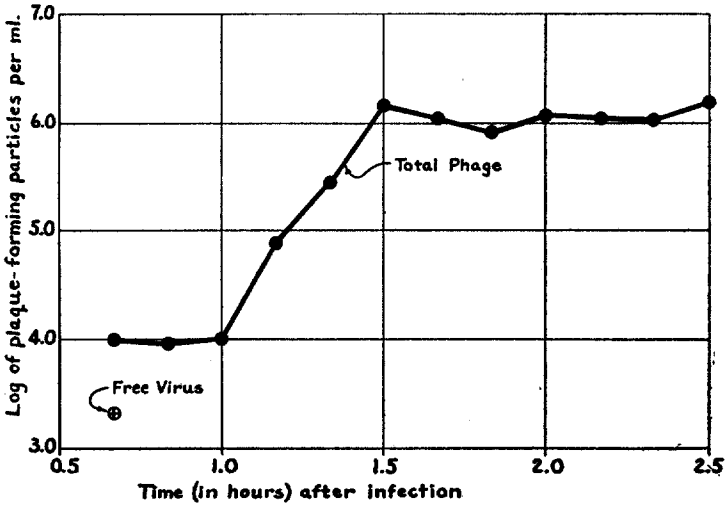

FIG. 12. Experiment B4. One-step growth curve for multiply infected lag phase cells of *B. mycoides* N.

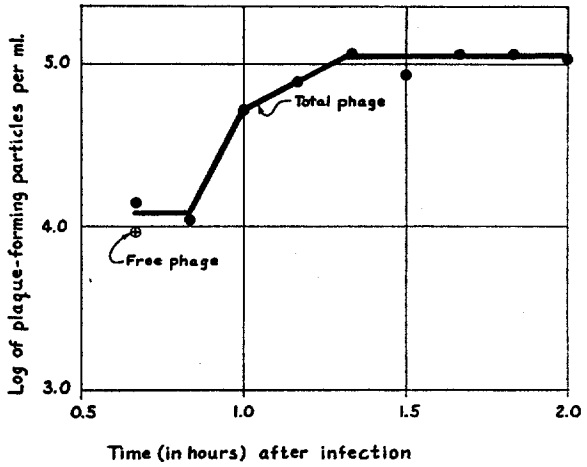

FIG. 13. Experiment B5. One-step growth experiment with log phase cells of *B. mycoides* N; multiple infection.

a real variation in the average yield in different experiments so much as it reflects a quantitative weakness of the data, resulting from the following facts:

1. The host used is a chain former.
2. These yields do not take into consideration the number of intact lysogenic

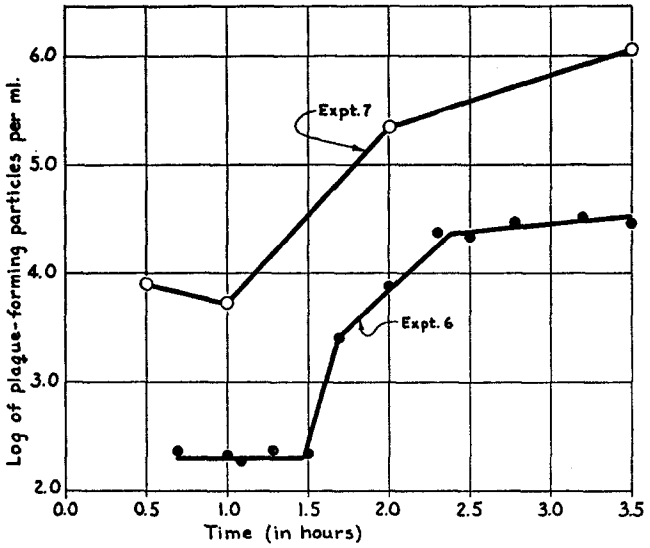

FIG. 14. Experiment B6 and B7. One-step growth experiments with lag phase suspensions of *B. mycoides* N.

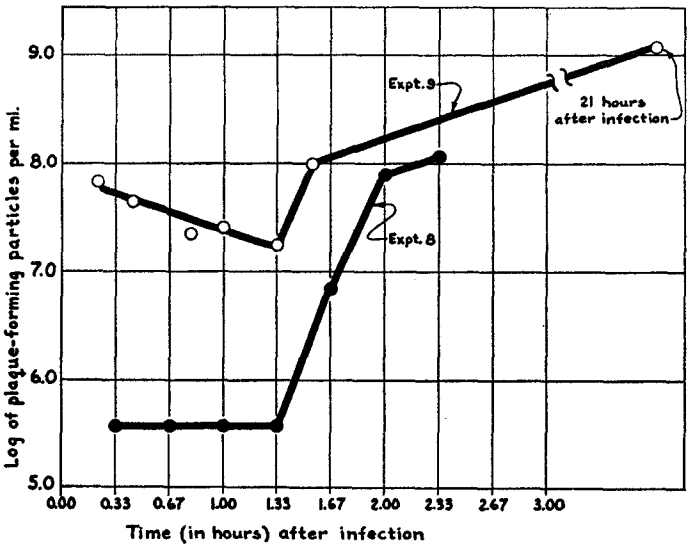

FIG. 15. Experiments B8 and B9. Phage multiplication in suspensions of cells containing 10^6 and 10^7 cells per ml. respectively.

cells left because of the inability to follow cell numbers in dilute bacterial suspensions.

3. There is a considerable amount of free phage present at the end of the adsorption period in Experiments B1 and B2.

4. During the latent period, in single infections (even when the string of cells is quite short and adsorption essentially complete), the number of infected filaments plus the extracellular phage fell quite short of the input of free phage. In Experiment B6, the initial concentration of phage was 6.1×10^7 particles per ml.; of filaments, 1.1×10^8 per ml.; of cells, 1.5×10^8 ml. Over 99 per cent of the free phage introduced was adsorbed, yet the plaque count during intracellular virus growth was only 3.1×10^7 particles per ml. The same type of results was obtained in Experiment B7.

C. Absolute Yield of Virus per Indicator Bacillus

The virus yield from individual bacteria is obtained by distributing "single drops" of a greatly diluted suspension of phage-infected cells to a large number of small tubes and then plating the entire contents of each tube for plaque count at the end of a suitable incubation period (Burnet, 1929).

Although the Burnet technique was not applied on a scale sufficiently large to indicate accurately the distribution of the absolute yields per cell, the series of 29 samples reported in two separate experiments (Tables I and II) seems to show that in this system the majority of bacilli release about the same amount of phage.

In the 1×10^{-7} dilution in Experiment C1 (Table I) in which there was an average of 2.3 infected filaments per sample (or about 4.6 infected cells altogether), the phage yields only varied from 59 to 523. In the 3×10^{-7} dilution, in which there was about 1 bacterium per sample, five out of the six absolute yields are in close agreement: 62, 69, 72, 76, 87. This series is consistent with the average of 76 particles per chain observed in the 1×10^{-7} dilution and with yields of 52 particles per cell in Experiment B6 (10^2 cells per ml.) and of 67 particles per cell (10^4 cells per ml.) in Experiment B7.

In Experiment C2 (Table II), there was a 3° fall in temperature for about 0.5 hour during phage multiplication; this may explain the lowered yields. Here, also, there is a limited range of distribution of absolute yields, 8–191.

D. "Simultaneous Titers"

An experiment, D1, was conducted to determine whether titers obtained in multiply infected suspensions giving visible turbidity were really disproportionately higher than those obtained in more dilute suspensions when run simultaneously and with the same adsorption mixture. After adsorption of phage to bacteria (initial virus:cell ratio, 1.5) for 1.0 hour at 0°C. and then at 0.3 hour at 30°C., the cells were sedimented down and resuspended in fresh broth. Six successive tenfold serial dilutions were made. All dilutions and the original

resuspension were shaken for 2.8 hours at 30°C. Each dilution was then plated for plaque count.

As demonstrated in Table III, there is no consistent trend toward an increase or decrease in titer upon diluting the mixture. The computed average yields per cell, 67–146, are more in keeping with those obtained in sections B and C than in A.

TABLE I

Experiment C1. Yield of Virus per Infected Cell of the Indicator Strain (30°C.)

1×10^{-7} dilution		3×10^{-7} dilution	
0.0 hr.	2.8 hrs.	0.0 hr.	2.8 hrs.
13 tubes*	12 tubes*	13 tubes*	13 tubes*
Initial plaque count of individual tubes	Final plaque count of individual tubes	Initial plaque count of individual tubes	Final plaque count of individual tubes
1	189	1	0
1	0	0	0
2	0	0	76
3	149	1	87
3	523	3	212
1	115	1	62
3	96	1	69
1	59	1	72
3	142	0	0
0	471	0	0
5	321	0	0
2	193	1	0
5		3	0

Average yield of phage per infected filament = 76 phage particles.

Microscopic examination of culture: single cells, diplobacilli, few chains of 3 or 4, no clumps.

* Each tube contained a 0.5 ml. sample. Entire contents of each tube was plated.

Experiments A4 and D1 indicate that manipulation of the culture merely by diluting will not in itself lower the yield. Further handling of the culture—sedimentation and fresh broth—as in Experiments B4, C1, and D1 did not produce significantly lower yields than in those merely diluted (Experiments B1 to B3, B6 to B8). The multiplicity of infection in Experiment D1 was low, yet the yield of the undiluted suspension was not increased. Most likely, the presence of clumps in the suspensions used in Experiments A1 to A4 was responsible for the higher yields in section A. When the host in a phage-bacterium system is a chain-forming, clump-forming organism, the system can only be investigated "somewhat quantitatively" (Van Iterson *et al.*, 1940).

TABLE II

Experiment C2. Absolute Yield of Virus per Cell

Plaque count			
1×10^{-7} dilution		1×10^{-8} dilution	
0.5 ml. in each tube: entire contents of each tube plated			
13 tubes	12 tubes	7 tubes	8 tubes
0.0 hr.	4.0 hrs.	0.0 hr.	4.0 hrs.
3	42	0	0
1	175	0	0
0	8	0	57
1	24	0	0
5	89	0	0
0	11	0	0
1	3	1	0
2	48		0
2	31		
4	0		
1	191		
2	21		
1			

Average yield per infected filament = 27 phage particles.

TABLE III

Effect of Dilution of Final Titer Obtained

Dilution of resuspended bacteria	Time after dilution	Relative titer dilution of resuspended cells × titer at end of 3.0 hrs.	Average yield of phage per bacillus
	hrs.		
Undiluted	0.0	4.4×10^{7}	
Undiluted	3.0	3.2×10^{9}	67
10^{-1}	3.0	5.6×10^{9}	117
10^{-2}	3.0	7.0×10^{9}	146
10^{-3}	3.0	6.5×10^{9}	135
10^{-4}	3.0	5.6×10^{9}	117
10^{-5}	3.0	5.6×10^{9}	117
10^{-7}	0.0	4.6×10^{7}	
10^{-7}	3.0	3.5×10^{9}	73

Direct cell count of resuspended cells = 4.8×10^{7} filaments per ml.

Microscopically: suspension consisted predominantly of single cells and diplobacilli.

Undiluted phage-bacterium resuspension cleared (incompletely) at end of 2.3 hours at 30°C.

Experimental Procedure

For Section A.—Suitable amounts of bacteria and phage were mixed so that, in the first four experiments, the phage was present in excess; in the last four, the bacteria in excess (initial virus to cell ratio from 4:1 to 1:16). The initial bacterial concentrations in the experiments varied from 1 to 5 $\times$ 10^7 cells per ml., the phage titers from 1 $\times$ 10^6 to 1 $\times$ 10^8 particles per ml. Phage-bacterium mixtures were rocked in a water bath usually at 31°C. Samples were taken at intervals. Total and free phage titrations were conducted according to the plaque count method of Gratia (1936) immediately upon withdrawal of the sample. Extracellular phage was determined by titrating filtrates of samples passed through a filter mat of supercel (Krueger *et al.*, 1946). Because of the time involved in making four to ten serial dilutions and in layering two or three dilutions onto the surface of nutrient agar in plating for plaque count, it should be noted that, while the times given in Figs. 1 to 8 for the release of phage are the times at which the samples were withdrawn, the actual completion of plating for plaque count occurred 0.1 to 0.2 hour later.

Bacterial numbers were determined by turbidity readings or direct counts or both. Turbidity readings were made immediately upon withdrawal of the sample; suspensions to be counted were formolized, refrigerated, and direct counts made the following day.

In these particular experiments, determinations of the bacterial numbers are somewhat inaccurate.

The most important source of error in judging bacterial numbers lay in the tendency of *B. mycoides* N cultures to clump. Phage-infected cultures always showed agglutination to some degree prior to and during lysis. Whereas this aggregation probably had only a minor effect on the turbidity readings, it played havoc with cell counts. Vigorous shaking, even with glass beads, did not disperse the clumps.

Moreover, the original cell suspension before phage addition was not entirely clump-free. Cultures were prepared in Experiments 1 to 8 by growing cells on the surface of nutrient agar in Roux flasks at 30°C., scraping the growth off with a large platinum loop and glass beads, and resuspending the cells in nutrient broth. Although the suspension was mixed by pulling it in and out of a pipette several times, there were many clumps still present. These were allowed to settle and the less aggregated supernatant material was pipetted off and used in Experiments A1 to A8.

In the work reported in sections B and C, liquid cultures, filtered through gauze and usually homogenized in a Waring blendor, were employed. When cultures of the indicator strain of *B. mycoides* N were treated in this way, the resulting suspension was clump-free and usually consisted predominantly of diplobacilli.

Lag phase cells were used in these experiments because log phase cells, grown in nutrient broth, usually showed marked agglutination. Since these experiments were performed before the knack of homogenizing and gauze-filtering cultures was hit upon, it was not possible to use log phase cells and hope for any degree of accuracy.

For Section B.—The procedure in one-step growth experiments is to mix virus and bacteria at high concentrations so that adsorption of the virus by the bacteria occurs rapidly (Delbrück and Luria, 1942). Some minutes after preparing the mixture a sample is diluted sufficiently in broth to prevent further infection of bacteria by virus. Samples from the diluted culture are assayed for virus content at intervals.

For Section C.—Two separate experiments were carried out. In Experiment C1 (Table I), a liquid culture of the indicator strain of *B. mycoides* N was mixed with phage. The adsorption mixture (initial phage:bacteria ratio, 3:1) was shaken for 1.0 hour at 0°C., for 0.3 hour more at 30°C., and then centrifuged for 0.3 hour at 3400 R.P.M. to throw down the cells. The supernatants were discarded and the cells resuspended in an equal quantity of fresh nutrient broth. The long adsorption period at 0 and 30°C. insured infection of almost all cells; discarding the supernatant eliminated the free phage remaining.

The resuspended culture contained 4.8×10^7 filaments per ml. (direct count). The suspension was then diluted to 1×10^{-7} and also to 3×10^{-7}. 0.5 ml. aliquots of each dilution were pipetted into 26 small test tubes. Thirteen tubes of each dilution were assayed immediately. The full contents of each tube were plated for plaque count by adding 0.5 ml. of a plating suspension and 0.2 ml. of hot agar to the 0.5 ml. of the phage-infected suspension already in each tube. The entire 1.2 ml. volume in each tube was then layered onto the surface of a nutrient agar plate.

The remaining 13 tubes in each dilution were shaken for 2.8 hours at 30°C., in a water bath and then plated for plaque count as just described.

In Experiment C2 (Table II) a similar procedure was followed. However, the temperature during the period of incubation (4 hours) of the small tubes fell from 30°C. to 27°C. for about 0.5 hour.

In both experiments, it took 0.6 hour to dilute the adsorption mixture and distribute it to the 52 small tubes. The suspension and tubes were kept at 0°C. during this period.

SUMMARY

In nutrient broth at 30 to 32°C., the cycle of virus growth (following adsorption) in lag phase cells of *B. mycoides* N included a period of intracellular multiplication, ranging from 0.8 to 1.3 hours, succeeded by a sharp rise in the free phage titer and then by a slower rise or a plateau in the extracellular phage content. The yield of virus per infected cell at 30°C., as determined by a modified Burnet dilution technique, was about 76 plaque-forming particles.

During the latent period, multiply infected cells showed no change in numbers. Coinciding with phage release, incomplete clearing occurred. The unlysed, remaining cells multiplied and the turbidity rose again. These survivors and their progeny were lysogenic.

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