AN ANALYSIS OF THE ACTION OF PROFLAVINE ON BACTERIOPHAGE GROWTH[1]

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The bacterium-bacteriophage system offers many advantages for the study of the general principles of the host-virus relationship. The availability of precise quantitative methods for analyzing phage growth makes this system suitable for the study of the nature of the inhibition of virus growth by drugs.

A majority of the compounds with suppressive activity on phage multiplication have likewise shown suppressive effects on bacterial multiplication (Delbrück and Luria, 1943; Spizizen, 1943; Bailey, 1943; Jones and Schatz, 1946; Cohen and Anderson, 1946; Cohen and Fowler, 1947). In these cases the inhibition appeared to be indirect through inhibition of the metabolism of the host, and possibly aspecific. Recently a reverse phenomenon has been reported, namely, increased yields of phage occurring at bacteriostatic concentrations of penicillin (Price, 1947). Temperatures above optimum for the growth of the host inhibit phage growth with some degree of specificity (Luria, 1943). What appears to be a specific inhibitory effect of a number of acridines and an anti-acridine action of ribose nucleic acid has been reported by Fitzgerald and collaborators (1946). The present paper is concerned with the results of further studies on the action of the acridine compound proflavine and with their interpretation in relation to the problem of phage multiplication.

MATERIAL AND METHODS

The bacteriophages studied were members of the "T" series whose common host is *Escherichia coli*, strain B (Delbrück, 1946). Plaque counts by the agar layer method with nutrient agar were used to determine phage activity. The "one-step growth experiment," described in detail by Delbrück and Luria (1942), was the basic technique adopted. In this type of experiment, known numbers of phage particles are mixed with an excess of sensitive bacteria, so that most bacteria adsorb a single particle. After a few minutes the mixture is highly diluted to stop further adsorption. The plaque count at this time, before lysis begins, comprises two sources of "infective centers": the infected bacteria and the free, unadsorbed phage. By determining the number of plaques obtained by plating the supernatant of a centrifuged sample, the number of free particles can be determined and the number of infected bacteria estimated by difference.

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The final plaque count, after lysis of all infected cells, is a measure of the total yield of phage. From it the average phage yield per infected bacterium can be calculated.

To determine the actual yields of phage from individual cells, the "single burst experiment" (Burnet, 1929; Delbrück, 1945) was used. Before lysis begins, the infected bacteria are highly diluted and distributed to a large number of small tubes, so that each tube receives on the average less than one infected bacterium. By plating the entire contents of each tube after lysis has taken place, the phage yield from individual bursts is obtained.

To study macroscopic lysis, a "multiplicity of infection" of about four particles per bacterium was found suitable. The "r" mutants of the T-even phages were employed to avoid "lysis inhibition" (Doermann, 1948).

The medium used was M-9[3], in which the generation time of *E. coli* B is approximately 45 minutes, and the latent periods for lysis by T2, T4, T6, and T7 are 21, 28, 30, and 15 minutes, respectively. In experiments with T4 and T6, 20 μg per ml of L-tryptophan were added to permit phage adsorption (Anderson, 1945).

The proflavine (di-amino-acridinium sulfate, Abbott Laboratories) was dissolved in distilled water and adjusted to pH 7 with sodium hydroxide.

RESULTS

Action of proflavine on free phage and on bacterial growth. Exposure of free phage to proflavine in concentrations sufficient to inhibit phage growth (1.0 mg per 100 ml for T2, 0.35 mg per 100 ml for T6; see below) produced a negligible loss of free phage activity in 60 minutes at 37 C.

Plate counts taken at intervals after the addition of proflavine to growing cultures of *E. coli* B, similar to those used in one-step growth experiments, showed that in those concentrations in which growth inhibition was present the inhibition was evident 1 hour after the addition of the drug. Complete inhibition of reproduction was produced by a concentration of 0.5 mg per 100 ml (table 1).

Concentration of proflavine preventing production of active bacteriophage. The addition of proflavine to a bacterial culture 2 minutes before the addition of bacteriophage either reduces the average yield of phage or suppresses all phage production, depending on the concentration. The amount of proflavine that, in a one-step growth experiment, completely suppresses the ability of infected bacteria to produce active phage will be called the "completely inhibitory concentration" (C.I. concentration).

The data assembled in table 1 show the C.I. concentrations for T2, T4, T6, and T7 to be 0.35, 0.1, 0.05, and 0.75 mg per 100 ml, respectively. Thus, the least sensitive phage is T7, the most sensitive, T6; T4 and T2 have intermediate sensitivities. Growth of T7 is prevented only by concentrations of proflavine that are completely inhibitory for bacterial multiplication, whereas T6 and

[3] Formula for M-9 Medium: *Solution A*—KH_2PO_4 3 g, $MgSO_4$ 0.2 g, NaCl 0.5 g, NH_4Cl 1 g, anhydrous Na_2HPO_4 6 g, dissolved in 900 ml of distilled water in the order above. *Solution B*—4 g of glucose dissolved in 100 ml of distilled water. Solutions A and B are sterilized separately and mixed in a ratio of 9:1 as needed. The pH of the final solution is 7.0.

T4 are completely suppressed at concentrations that allow uninhibited multiplication of the host.

To estimate the sensitivity of other phage types we used a screening test consisting of plating on nutrient agar in which increasing concentrations of proflavine were incorporated. In this way gross differences in the effectiveness of proflavine can be demonstrated. T1 and T3 appear to be similar to T7; T5 appears intermediate between T4 and T2.

Each phage type is adsorbed by sensitive bacteria at the same rate in the presence as in the absence of proflavine. T2, T4, and T6, the related large phage

TABLE 1

Growth-limiting concentrations of proflavine

The data for various phages represent the average yield per bacterium in one-step growth experiments, measured at the time of maximum yield. The data for *E. coli* B are percentage ratios between viable counts in the presence of proflavine and viable counts in its absence, the conditions of the measurements being identical with those in the corresponding one-step growth experiments.

PROFLAVINE CONCENTRATION (MG/100 ML)	AVERAGE YIELD IN PERCENTAGE OF YIELD IN CONTROL				GROWTH OF E. COLI B IN 1 HOUR, PERCENTAGE OF CONTROL
	T2	T4	T6	T7	
0.005			91		
0.0125			40		
0.025			8		
0.05		3	*		100
0.1		*	*		100
0.125	55	*			
0.2					50
0.25	18				40
0.3	4				
0.35	*			39	
0.5	*			5	0
0.75				*	

* Decrease of infective centers below input.

types, are all more sensitive to proflavine than the small phages T1, T3, and T7 and differ in absolute sensitivity among themselves. The "r" mutants have the same sensitivities as the corresponding wild types.

It should be noted in table 1 that, as the proflavine concentration is reduced, the average phage yield per bacterium gradually increases. Analysis of individual cell yields of T2 in the presence of 0.25 mg per 100 ml proflavine—a not completely inhibitory concentration—showed that the loss in phage yield could be accounted for by a reduction of the individual yields.

Analysis of the action of completely inhibitory concentrations of proflavine. Phage growth, as studied in the one-step growth experiments, can be divided into

a "latent period" of intracellular reproduction, a "rise period" corresponding to phage liberation, and a "stationary period," after liberation is completed.

If the C.I. concentration of proflavine is added to the growth tube 2 minutes before the addition of phage and the infective centers are assayed at intervals, a progressive fall in the plaque count is found, indicating an actual loss of the adsorbed phage. A number of experiments of this kind were done. Table 2 shows the results of representative experiments in which the plaque counts at various times were determined. Data on the yield in the absence of proflavine are presented in the same table. It should be remembered that plating for plaque count results in diluting the proflavine well below inhibitory concentrations.

The data in table 2 show three characteristics of proflavine action: first, there is no loss of infective centers during the latent period; second, there is a progressive loss of infective centers during the rise period; and third, at the stationary period the plaque count reaches approximately the value of the unadsorbed phage, indicating loss of all adsorbed phage. Moreover, as seen clearly in figure 1, in which the results from several experiments are summarized, the loss of infective centers in the presence of proflavine closely parallels the rise in titer in the control without proflavine. This indicates a parallelism between liberation of phage by lysis in the absence of proflavine and loss of ability to produce active phage in the presence of the drug.

These experiments suggested that in the presence of the C.I. concentration of proflavine the cells may lyse at the same time as they would in the absence of the drug, but release no active phage. This would explain the loss of infective centers and the presence of the unadsorbed phage alone at the end of the rise period.[4]

According to this interpretation, the presence of proflavine should allow lysis to occur after a normal latent period. We tested this point by macroscopic observation of lysis. Parallel cultures with and without proflavine were inoculated with enough phage to give multiple infection (about 4 particles per bacterium). Clearing occurred at the same time in both cultures. Proflavine neither prevents lysis of the host nor causes delay in lysis.

Another type of experiment gave further support to the hypothesis. Bacteria were infected with phage in the presence of the C.I. concentration of proflavine, as in the experiments of table 2. Then, at various intervals, samples were highly diluted with medium containing no proflavine. The concentration of proflavine was thus reduced below the inhibitory level. All samples were incubated and plated at a time when the yield in the control tube had reached the stationary period. If the conclusion previously reached is correct, we may expect a full yield if proflavine is removed during the latent period. With dilution after the lysis of cells has begun, the yield should vary inversely with the time of dilution, since, as the time before dilution is extended, more and more cells will have lysed

[4] Phage T6 shows the peculiarity that the rate of loss of adsorbed phage in the presence of proflavine is more rapid than the rate of increase in phage titer without proflavine. The latter value, however, can only be considered as an estimate of the number of bacteria that have lysed by assuming that the average yield per bacterium is the same for bacteria lysing early or late, which is by no means certain.

TABLE 2

Action of completely inhibitory concentrations of proflavine

Proflavine in C.I. concentration was added to bacteria 2 minutes before adding phage. After 7 to 10 minutes phage adsorption was interrupted by a dilution of 1:2,000 in proflavine medium containing the C.I. concentration. Platings were made at intervals. The data in columns 2 and 4 were obtained in parallel experiments for T6 and T7. For T2, they were from different experiments. The control value represents the phage input in the absence of proflavine.

TIME AFTER INFECTION, MINUTES	NO PROFLAVINE		C.I. CONCENTRATION OF PROFLAVINE	
	Plaque count at time indicated	% rise completed	Plaque count at time indicated	% loss adsorbed phage
T2 (latent period 21 minutes, C.I. concentration 0.35 mg/100 ml)				
8	7.8×10^6	0	2.7×10^6	0
14	7.6×10^6	0		
21	9.9×10^6	0.55	2.9×10^8	0
24	1.3×10^7	1.3		
27	1.2×10^8	28		
30	2.8×10^8	68	1.1×10^6	71
40	3.8×10^8	93	8.1×10^5	85
50	4.0×10^8	100	3.4×10^5	100
Free			5.0×10^5	
Control			2.9×10^6	
T6 (latent period 30 minutes, C.I. concentration 0.05 mg/100 ml)				
12	6.04×10^6	0	6.74×10^6	0
25	5.52×10^6	0	7.16×10^6	0
30	6.4×10^6	0	6.36×10^6	0
33	8.0×10^6	0.2	5.82×10^6	15
35	1.6×10^7	1	4.44×10^6	39
37			3.5×10^6	55
39	6.2×10^7	6	2.36×10^6	75
41	2.1×10^8	22	1.92×10^6	82
43	3.1×10^8	33	1.66×10^6	86
45	3.8×10^8	40	1.6×10^6	87
47	4.9×10^8	52	1.22×10^6	94
49	5.6×10^8	60	1.08×10^6	96
55			9.6×10^6	98
60	7.7×10^8	83		
65	8.1×10^8	86	8.0×10^5	100
75	9.3×10^8	100	6.2×10^5	
90	9.2×10^8		5.0×10^5	
Free			8.6×10^5	
T7 (latent period 15 minutes, C.I. concentration 0.75 mg/100 ml)				
9	5.6×10^6	0	4.4×10^6	0
18	1.4×10^7	12	4.3×10^6	7
23	4.0×10^7	47	3.5×10^6	60
30	6.9×10^7	87	3.2×10^6	80
40	7.3×10^7	100	2.8×10^6	100
50	7.2×10^7		2.0×10^6	
Free			2.9×10^6	

without releasing any phage. Representative experiments presented in table 3 show that the anticipated results were obtained. With T2, the yield is reduced by 61 per cent for dilution at 30 minutes and by 96 per cent for dilution at 40 minutes. With T6, the reduction in yield is 34 per cent at 39 minutes and 79 per cent at 55 minutes. This emphasizes the correlation of the longer latent period for T6 with the longer time required before phage is lost in the presence of proflavine.

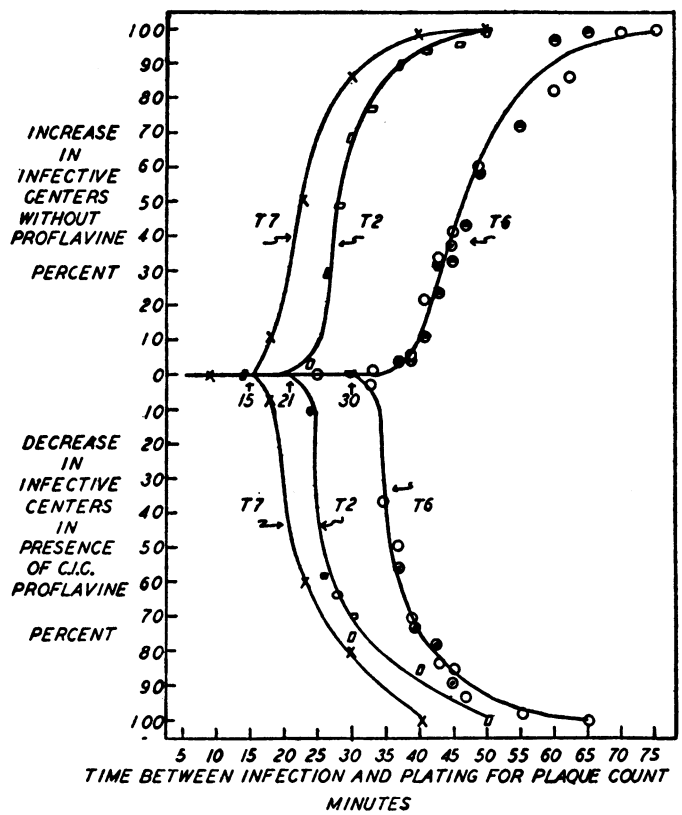

Figure 1. Correlation between loss of infective centers in the presence of the C.I. concentration of proflavine and liberation of phage in its absence. Results for phages T2, T6, and T7 are presented. Note that the first loss of infective centers occurs at the end of the latent period, and that complete loss is observed at the end of the normal rise period for each phage type.

We may then conclude that, in the presence of the C.I. concentration of proflavine, the phage inside the infected cells is not lost until a time shortly before—or corresponding to the time of—the lysis of the host. Until this time phage can be liberated if proflavine is removed. Lysis in the presence of proflavine does not produce active phage, and even the original infecting particle is lost.

It was possible that bacteria lysed in the presence of proflavine, although releasing no active phage, might liberate particles that could be adsorbed by host cells

but that failed to multiply. Since adsorption of phage is generally followed by death of the host cell, we attempted to detect host-killing activity in proflavine lysates. Parallel bacterial cultures—one with and the other without proflavine —were inoculated with phage, incubated for 2 hours, and filtered. They were tested, immediately and after storage, for plaque production and bactericidal

TABLE 3

Phage liberation after removal of completely inhibitory concentrations of proflavine by dilution

The technique is the same as described in table 2 with additional dilutions to remove proflavine. These dilutions were 1:20 for T2 and T7 and 1:50 for T6. The final titers were determined at the times indicated in the table. The control values represent the yield in the absence of proflavine in a parallel growth tube. The lower control value for T7 is characteristic.

TIME OF REMOVAL FROM PROFLAVINE (MINUTES AFTER INFECTION)	FINAL TITER FOLLOWING DILUTION AT TIME INDICATED	YIELD AFTER DILUTION IN PERCENTAGE OF MAXIMUM YIELD	TIME OF ASSAY (MINUTES AFTER INFECTION)
T2 (latent period 21 minutes)			
10	1.4×10^8	100	57
20	1.4×10^8	100	59
30	5.4×10^7	39	65
40	5.2×10^6	4	75
50	1.2×10^6	0.9	85
Free	4.0×10^5		
Control	1.7×10^8		55
T6 (latent period 30 minutes)			
10	3.2×10^8	100	67
20	3.3×10^8	100	69
30	3.0×10^8	94	71
39	2.1×10^8	66	75
55	6.6×10^7	21	85
65	2.6×10^7	8	95
Free	1.4×10^6		
Control	5.1×10^8		65
T7 (latent period 15 minutes)			
11	1.5×10^8	100	54
18	1.1×10^8	73	56
23	9.4×10^7	63	58
30	2.9×10^7	19	60
40	6.4×10^6	4	62
Free	4.5×10^6		
Control	7.2×10^7		50

activity. All bactericidal activity could be accounted for by the active phage present. This does not exclude the possibility that bacteria lysed in proflavine liberate some kind of inactive, nonbactericidal phage particle.

The time of occurrence of the proflavine-inhibited reaction. We have seen that, in the presence of proflavine, ability to liberate phage is not lost until approxi-

mately the time when the infected bacterium is lysed. This may indicate either that the infecting particle remains, as it were, in a dormant state, as found for 5-methyl tryptophan (Cohen and Anderson, 1946; Cohen and Fowler, 1947), or that the processes leading to phage multiplication go on inside the bacterium but that some late or even final reaction needed for production of active phage is inhibited. This point was tested by determining the length of the interval between dilution in M-9 medium to remove proflavine and the initiation of the rise in titer. The results shown in table 4 indicate that phage liberation begins almost immediately upon dilution and proceeds at a normal rate.

TABLE 4

Absence of latent period after removal of proflavine

The procedure was the same as described in table 2 with an additional dilution 1:20 from proflavine at the times indicated in column 1. The titer of this growth tube was then determined at the times specified in column 2.

TIME OF REMOVAL FROM PROFLAVINE (MINUTES)	TIME OF ASSAY (MINUTES AFTER REMOVAL FROM PROFLAVINE)	TITER
T2 (latent period 21 minutes)		
28	0	3.8×10^6
	3	1.1×10^7
	5	$>4.0 \times 10^7$
40	0	2.7×10^6
	2	3.2×10^6
	4	4.3×10^6
	6	1.1×10^7
	8	2.0×10^7
T6 (latent period 30 minutes)		
34	0	1.2×10^7
	4	1.9×10^7
	6	2.2×10^7
	8	4.1×10^7
	11	2.2×10^8
	16	7.9×10^8
	31	1.5×10^9

We may then conclude that some of the reactions leading to phage production proceed in the presence of proflavine, and that the inhibitory action is exerted on some late step in the process. If so, the addition of proflavine at various intervals after infection should only suppress phage growth to the extent that the reactions inhibited by proflavine have not yet taken place. Several experiments of this type were performed. Table 5 shows the results of two representative experiments, in which the C.I. concentration of proflavine was added at various intervals after infection and all tubes were assayed at a time when the rise in the control tube had been completed. It will be seen that, both with T2 and T6, by

17 minutes the process of phage formation has already reached a stage at which some active phage can be produced in the presence of proflavine. A progressive

TABLE 5

Addition of the C.I. concentration of proflavine at various intervals after infection

The procedure was that of the one-step growth experiment. The mixture of phage and bacteria was diluted after 8 minutes and divided into a series of tubes, one of which received the C.I. concentration of proflavine at one of the times indicated in column 1. The yield of phage in the presence of proflavine was measured between 52 and 60 minutes for T2, and between 60 and 70 minutes for T6. The value for the time "−2 minutes" was obtained by a parallel experiment similar to the one of table 2.

TIME BETWEEN INFECTION AND ADDITION OF PROFLAVINE (MINUTES)	FINAL TITER AFTER LYSIS	YIELD IN PERCENTAGE OF YIELD IN CONTROL
T2 (latent period 21 minutes). Input 1.6×10^7		
−2	2.0×10^8	0
10	1.9×10^8	0
16	1.2×10^8	12
20	3.5×10^8	35
24	7.7×10^8	77
28	8.1×10^8	81
32	8.9×10^8	89
36	9.3×10^8	93
40	9.2×10^8	92
44	9.7×10^8	97
No proflavine	1.0×10^9	100
T6 (latent period 30 minutes). Input 5.2×10^6		
−2	4.2×10^8	0
15	3.4×10^6	0.8
17	9.1×10^6	2.3
20	2.8×10^7	7
25	9.1×10^7	23
30	2.9×10^8	74
35	3.3×10^8	85
40	4.2×10^8	108
45	4.5×10^8	115
50	4.8×10^8	123
No proflavine	3.9×10^8	100*

*Since the yield in the control tube for this experiment was abnormally low, in plotting these results in figure 2 we used the values as percentages of the maximum titer reached with proflavine input at 50 minutes. The apparent increase in yield over the control is not significant.

increase in phage production is found as proflavine is added at later times. Extrapolation from these and other data not included in the table suggests that the latest input time for complete suppression is about 12 to 15 minutes after infec-

tion, this time being possibly earlier for T2 than for T6. The results of experiments of this type are plotted in figure 2, which shows the progressive increase in phage liberation as a function of the time of addition of proflavine.

The data in table 5 and figure 2 may be interpreted as suggesting that the particles inside the bacteria reach at various times a stage at which proflavine can no

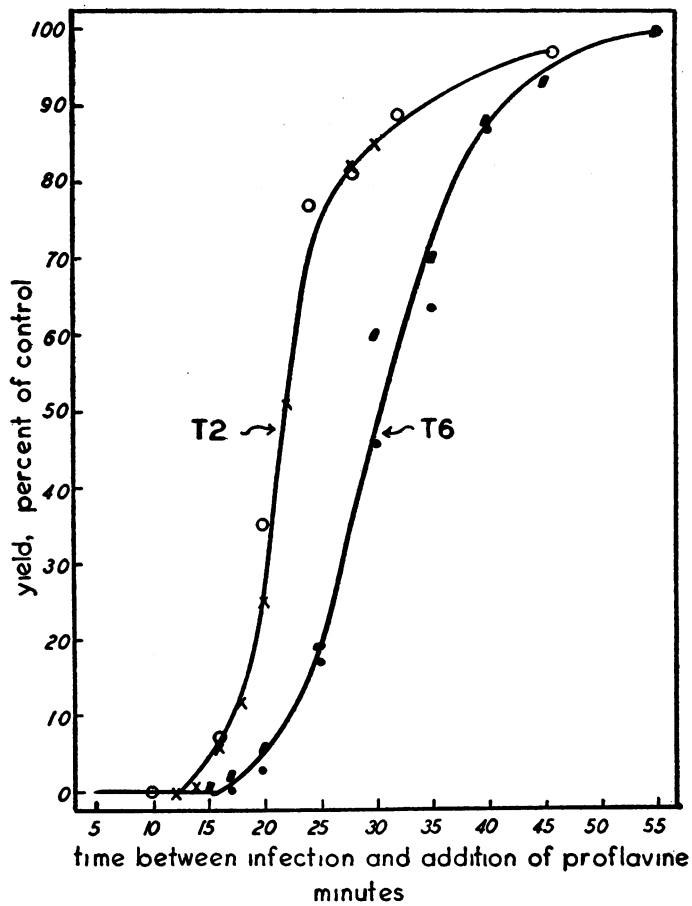

Figure 2. Yield of active phage (measured at the end of the normal rise period) when proflavine is added at different times after infection. No viable phage is produced if the drug is added during the first 12 to 15 minutes after infection. If proflavine is added later, the yield progressively increases with the interval between infection and the addition of the drug.

longer prevent the further steps necessary to produce viable phage. They may, therefore, give some information on the rate of phage growth. Luria and Latarjet (1947), using ultraviolet light to inactivate phage T2 during intracellular growth, found that a multiple-hit curve begins to appear between 7 and 12 minutes after infection, from which they concluded that, in the multiplication of this phage, the number of particles begins to increase at this period. Confirmation of this conclusion appears to be provided by the use of X-rays (Latarjet,

1948). Although these observations may support the hypothesis that by the addition of proflavine at intervals after infection we can measure the actual increase in phage within the culture during the latent period, our data are insufficient to justify a quantitative analysis of this type.

The fact that no phage liberation occurs if proflavine is added before a critical time in the latent period suggests the possibility that no fully active phage is present as such in the cell up to that time. This suggestion is in agreement with the recent findings of Luria (1947) and Doermann (private communication). The stage in phage production blocked by proflavine may be connected with the appearance of fully active phage particles.

The slow rise in phage yield as a function of the time of input of proflavine (figure 2) might be due to one or two reasons: (1) the sequel of reactions involved in phage production reaches, earlier in some cells than in others, a stage that can no longer be blocked by proflavine, just as some cells lyse before others; or (2) the processes leading to the production of individual phage particles inside each cell reach at different times a stage that can no longer be blocked by proflavine, there being in each cell a gradual accumulation of phage particles whose production has reached this stage. This hypothesis does not in itself imply any specific mechanism of phage reproduction.

In an attempt to distinguish between these two possibilities, single burst experiments were performed, adding proflavine at different times during the latent period before the samples were diluted in proflavine and distributed to individual tubes. All tubes were plated at the time at which the rise period in the absence of proflavine was completed. The number of cells yielding active phage in the presence of proflavine can be obtained directly from the number of samples without bursts. If the fraction of samples without bursts is e^{-x}, then the number of bursts per sample is given by x. By the first hypothesis—difference in critical stage among bacterial cells—a few tubes should show a normal burst, but the others should show no phage. By the second hypothesis—difference in critical stage among phage particles—many tubes should show a burst, but this burst should be small. Only one single-burst experiment was done for each time of proflavine addition. Table 6 shows the results of these experiments for T2 and T6. Although more experiments would be desirable, a tentative conclusion seems justified.

With both phages the fraction of infected cells capable of liberating phage in the presence of proflavine (table 6, column 8) increases rapidly, but the average yield per fertile burst (table 6, column 10) is much lower than the average yield per burst in the control, even when a majority of the infected cells already give active phage in the presence of proflavine. Both the number of bacteria that liberate phage and the amount of phage liberated per bacterium increase as the time of the addition of proflavine approaches the end of the latent period. Since we have already concluded that the inhibitory action of proflavine is exerted on some late step in the process of phage production, we incline to the belief that individual phage particles reach at various times the stage which is blocked by proflavine, and that a bacterium liberates active phage in amounts corresponding

to the number of particles that had completed this stage at the time of the addition of proflavine.

The effect of addition of proflavine to bacteria prior to infection with T2. In the experiments discussed above proflavine was added to bacteria 2 minutes before adding the bacteriophage. When the host, however, is exposed to the C.I. concentration of proflavine for longer periods of time before infection with T2 (45 minutes to 4 hours) and the phage is then added and liberation tested at intervals, some phage production occurs in the presence of the C.I. concentration, the more so the longer the time during which the bacteria have been in contact with the drug. The effect is not due to reduction in drug concentration by previous

TABLE 6

Single burst experiments: proflavine added at intervals during the latent period

Bacteria were mixed with phage in the absence of proflavine. Eight minutes later, the mixture was diluted in M-9 medium to stop adsorption. At the time indicated in column 2, the C.I. concentration of proflavine was added to the diluted mixture. The mixture was further diluted in medium with the C.I. concentration of proflavine in such a way that, when distributed in single-drop samples to a large number of tubes, it would give a suitable number of bursts per tube. The final plating of the entire contents of each tube was done at the time of the maximum titer in the control.

PHAGE TYPE	TIME BETWEEN INFECTION AND ADDITION OF PROFLAVINE (MINUTES)	NUMBER OF SAMPLES	INFECTED BACTERIA IN ALL SAMPLES	INFECTED BACTERIA PER SAMPLE	SAMPLES WITHOUT FERTILE BURSTS	PROPORTION OF SAMPLES WITHOUT FERTILE BURSTS, e^{-x}	AVERAGE NUMBER OF BURSTS PER SAMPLE, x	INFECTED BACTERIA YIELDING PHAGE (%)	TOTAL YIELD IN ALL SAMPLES MINUS FREE PHAGE	AVERAGE YIELD PER FERTILE BURST	RANGE OF YIELDS IN INDIVIDUAL FERTILE BURSTS
T2	16	36	236	6.56	6	0.167	1.79	27	387	6.0	3–35
	20	36	140	3.9	3	0.083	2.48	63.7	1,328	14.9	3–114
	No proflavine	36	59	1.64	7	0.194	1.64	100	2,066	35	5–367
T6	20	24	34	1.42	16	0.666	0.41	28.8	148	15.1	2–44
	27	36	23.5	0.65	19	0.5277	0.64	98	671	29.1	3–82
	No proflavine	24	20.9	0.87	10	0.417	0.87	100	2,045	98	48–322

contact with bacteria, since all samples are diluted in fresh medium containing the C.I. concentration 8 minutes after infection. The reduction in inhibitory effect of proflavine after prolonged exposure of the bacteria is even more pronounced when tested with dilution from proflavine 35 minutes after infection.

Although proflavine-resistant bacterial mutants can be obtained, the phenomenon discussed here was shown not to be due to a selection of mutants. We incline to the belief that during growth in the presence of proflavine an adaptation of the host occurs, which makes the cells more suitable for phage growth. This may be caused, either by the accumulation during this period of some material needed for phage growth, or by the activation of an alternate metabolic pathway removing the block to phage production (for adaptation to proflavine see Hinshelwood, 1946).

Bacteriophage mutants resistant to proflavine. By plating large amounts of the most sensitive phage types, T4 and T6, on agar containing inhibitory concentrations of proflavine a few plaques were obtained. Stocks prepared from these plaques in medium without proflavine showed higher proflavine tolerance than the respective wild type. The process could be repeated, with further increase in tolerance. The danger of contamination of the stocks with other phages used in the laboratory was eliminated by sensitivity tests with a number of indicator bacterial strains (Delbrück and Luria, 1942). The differences in proflavine tolerance between mutants and wild types, as measured by the C.I. concentrations, were significant. A threefold increase in resistance was observed for T6, and a twofold increase for T4.

These experiments, by showing that proflavine sensitivity of a phage can be brought about by a heritable change in the phage, suggest that some of the mechanisms of intracellular growth which are prevented by proflavine may be carried by the phage particle itself.

Antagonism of proflavine inhibition of T2 multiplication. In addition to ribose nucleic acid, polymerized and depolymerized desoxyribose nucleic acid (DNA)[5] were tested as antagonists of proflavine. Complete reversal of inhibition of phage growth was obtained with both preparations of DNA at 0.25 mg per 100 ml. The ribose nucleic acid was slightly less effective. Casein hydrolyzate or riboflavin produced very little reversal. Cohen (1948) measured the increase in DNA in bacteria following infection with bacteriophage T2, and found that increase in DNA began several minutes after infection. It seems possible that the rise in DNA may parallel the appearance of phage that has completed the proflavine-inhibited step, and that the chemical step subject to proflavine inhibition is related to the formation or utilization of DNA for phage synthesis (McIlwain, 1941).

DISCUSSION AND SUMMARY

The use of proflavine as an inhibitor of phage growth has demonstrated that the characteristics both of the host and of the phage affect the response of infected bacteria to the drug. On the one hand, adaptation of the host may permit phage growth in the presence of the drug. On the other hand, the growth sensitivity to proflavine is characteristic of each phage type and can be altered by mutation of the phage. Confirming the observation of Fitzgerald and collaborators (1946) we found that the growth of several phages, particularly those of the "large particle" type, is completely inhibited by drug concentrations that allow normal or subnormal multiplication of the host.

Proflavine appears to block a late reaction necessary for the production of active phage, whereas some of the earlier processes leading to phage production are completed in the presence of the drug, since the removal of proflavine allows phage liberation without any appreciable delay. Phage-infected bacteria in which the production of phage is inhibited by proflavine are lysed after a normal

[5] Kindly supplied by Dr. Seymour S. Cohen.

latent period, but fail to liberate any active phage particles. In these respects, the inhibition by proflavine differs from the inhibition of T2 growth by 5-methyl tryptophan (Cohen and Anderson, 1946; Cohen and Fowler, 1947), which appears to interrupt phage growth completely. It is not known whether or not infected bacteria inhibited by 5-methyl tryptophan undergo lysis, like those inhibited by proflavine. Particularly in view of the different modes of action of the two inhibitors, it is interesting that in both cases complete inhibition can be obtained only if the inhibitor is introduced within 12 to 14 minutes after infection. It may be that this represents the minimum time of appearance of active phage particles inside the bacterium. It is possible that the proflavine-blocked reactions, which we have shown to take place relatively late in the course of infection, involve the formation or utilization of desoxyribose nucleic acid, the synthesis of which has been found (Cohen, 1948) to begin several minutes after infection with bacteriophage.

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