A STUDY OF THE ANTIGENICITY OF T_3 AND T_4 COLI-DYSENTERY BACTERIOPHAGES DURING THE VEGETATIVE STAGE OF DEVELOPMENT

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One of the fundamental problems in the field of phage research concerns the events which take place within a phage-infected bacterial cell prior to its burst. Immediately following infection and for a period of some minutes thereafter it is not possible to detect infectious virus by any of the techniques presently employed (1). When cells are prematurely lysed, however, it can be demonstrated that intracellular infectious units do appear several minutes before the normal burst occurs. From their investigation on T_2 coli-dysentery bacteriophage and *Escherichia coli* (strain B) Hershey and Chase (2) have concluded that the infecting phage particle does not penetrate the bacterium; only the nucleic acid portion of the virus enters the cell and the viral membrane remains attached to the surface. Rountree has shown, furthermore, that two antigens are present in the tailed coli-dysentery bacteriophage T_5 and in the staphylococcal phage 3A (3). The work of others (4), however, has suggested that additional antigens are present in mature phage.

It is the purpose of this investigation to determine by immunological means whether the viral component which elicits the production of neutralizing antibodies (hereafter referred to as the neutralizing antigen) is synthesized during the vegetative stage of phage growth when no intracellular infectious virus is detectable. In the present report, T_3 and T_4 coli-dysentery bacteriophages and the host cell *Escherichia coli* (strain B) were chosen for study.

Materials and Methods

Bacterial Cultures.—Strain B of *Escherichia coli* used for viral assays was subcultured daily on fresh nutrient agar slants. The benzylpenicillin-resistant mutant strain of *Escherichia coli* was obtained by serial passage of the sensitive microorganism in nutrient broth containing 100 μg. per ml. of the drug. The resistant strain was subsequently maintained by daily subculture on fresh agar slants containing the same concentration of benzylpenicillin.

Bacteriophages.—Fresh stocks of T_3 and T_4 coli-dysentery bacteriophage were prepared by infecting *E. coli* B in the usual manner (5). Viral assays were performed by the poured-agar-layer method (6).

Antisera.—Antisera to T_3 and T_4 phages were obtained from groups of rabbits which had been injected intravenously with normal phage lysates of *E. coli* B, with prematurely induced slysates, or with phage-infected host cells as the case might be. The animals received a series

of three or more courses of immunization. For the first course a total of 4.5 ml. of material was administered during a 5-day interval, followed by a rest period of 7 days. The second course of immunization consisted of four daily injections of 1 ml. of antigen, again followed by a rest period. Succeeding courses consisted of three 1.0 ml. daily injections. Bleedings were taken at the end of each rest period and the antisera were tested for their virus-neutralizing properties.

Media.—*E. coli* B was cultivated in 1 per cent nutrient broth. In certain of the experiments an enriched medium was used which contained nutrient broth and 1 per cent casamino acids (Difco technical grade) at pH 7.0 (7). A synthetic medium M-9 (8), modified by the addition of 0.2 per cent glucose, was likewise employed.

Neutralization Tests.—The tests used to determine the potency of the various T_3 and T_4 phage antisera were conducted in the following manner. To a series of tubes, each containing 0.9 ml. of phage (1×10^6 P/ml.), was added at 1-minute intervals 0.1 ml. of a serial dilution of the antiserum to be tested. The tubes were incubated for 30 minutes at 37°C. 0.1 ml. aliquots were withdrawn at 1-minute intervals and diluted 2×10^{-1} in broth; a second dilution of 1.5×10^{-1} was then made. Unneutralized phage was determined by plating 0.1 ml. samples of the latter in duplicate, using *E. coli* B as host. After incubating the plates for 8 hours at 37°C. in the case of the T_3 phage, and overnight in the case of the T_4 virus, plaque counts were made and the results were recorded. The dilution of antiserum which neutralized 50 per cent of the phage in 30 minutes was taken as the end-point.[1]

EXPERIMENTAL

Experiments with T_4 Coli-Dysentery Bacteriophage

In order to ascertain whether a neutralizing antigen is elaborated by infected *E. coli* B prior to the appearance of mature phage, it is necessary to determine the time at which the latter first makes its appearance. This has been accomplished by Doermann (1), who studied the rate of formation of T_4 in *E. coli* B by lysing the cells with the heterologous phage T_6 r+. It has been found in this laboratory that infected cells can also be lysed by the addition of penicillin and sodium citrate; in this manner it is possible to release and to determine the number of newly formed virus particles prior to the burst.

The procedure used for determining the rate of intracellular virus formation by lysing cells infected with T_4 phage during the latent period with benzylpenicillin is outlined below.

[1] The potency of the antisera can also be expressed in terms of the rate at which they neutralize phage. The relationship between the 50 per cent neutralization point and the rate constant of phage neutralization is given by the equation

$$k = \frac{2.3D \log P_0/P}{t}$$

in which D = dilution of the antiserum,
t = time in minutes,
P_0 = initial phage concentration,
P = final phage concentration,
k = neutralization constant.

An actively growing culture of *E. coli* B was obtained by seeding 10 ml. of nutrient broth with 0.3 ml. of a suspension of this organism which had been washed from a slant with 3 ml. of the same medium. The tube was incubated at 37°C. with aeration for 2 hours. The bacterial concentration at this time was 1.9×10^8 B/ml. as determined by colony count.

To 1.8 ml. of the bacterial suspension at 37°C. was added 0.2 ml. of T_4 phage containing 8.8×10^7 P/ml. (adsorption tube A, *cf.* Fig. 1). After allowing 2 minutes to elapse for the adsorption of phage, two separate aliquots (0.2 ml.) were withdrawn. One was added to 0.6 ml. of T_4 antiserum (diluted 1:37.5) at 37°C. (tube B); the second was added to the same volume of antiserum at 0°C. (tube C). The dilution of the first aliquot in antiserum at 37°C. (tube B) served to neutralize all free virus, but permitted intracellular virus growth to continue. Dilution of the second aliquot at 0°C. (tube C) not only served to neutralize free virus but stopped the further development of intracellular virus. After permitting tube B to stand an additional 4 minutes two separate 0.1 ml. aliquots were removed and each was diluted 1.25×10^{-2} into broth, one at 37°C. (tube D), the other at 0°C. (tube E). Thus, in the latter tube the development of virus was arrested 6 minutes after the initiation of infection. From this tube was immediately withdrawn a 0.2 ml. sample which was added to 1.8 ml. of penicillin broth (1.6 mg. benzylpenicillin/ml. and 0.1 M potassium citrate) at 0°C. (tube 2).

At the time intervals shown in Fig. 2 (curve II), 0.2 ml. aliquots were withdrawn from the dilution tube maintained at 37°C. (tube D) and likewise added to 1.8 ml. of penicillin broth at 0°C. (tubes 3, 4, etc.). After standing for 16 minutes a 0.1 ml. aliquot was now withdrawn from the serum tube (C), maintained at 0°C., and diluted 1.25×10^{-2} in 0°C. broth (tube F). A 0.2 ml. aliquot was withdrawn from this dilution and added to 1.8 ml. of penicillin broth at 0°C. (tube 1). Each penicillin dilution tube was kept at 0°C. for 20 minutes to permit adsorption of the drug to the bacteria; the tubes were then incubated at 37°C. for 40 minutes. During the incubation period the bacteria lysed. Assays were then made of the viral content of the various dilution tubes containing the lysed bacteria. A penicillin-resistant mutant of *E. coli* B was used as the host organism. This bacterial mutant has a plating efficiency of 1; *i.e.*, the same plaque count is obtained by plating T_4 with the mutant *E. coli* B/Pen G as with *E. coli* B.

A normal one-step growth curve (9) was also determined from a sample removed from the dilution tube (D) 10 minutes following infection. Thus it was found that the average burst size per bacterium was 59; this value was ascertained by dividing the final yield of phage by the number of infected cells. The multiplicity of infection, determined by dividing the number of adsorbed phage particles by the total number of bacteria, was 0.46.

In Fig. 2 is shown a plot of the rate of release of phage particles from infected *E. coli* B cells during normal lysis (curve I) and from cells which had been prematurely lysed with penicillin (curve II). It can be seen that singly infected cells under conditions of normal lysis release new phage approximately 23 minutes after the initial infection. When lysis is induced with penicillin, however, new phage makes its first appearance within 12 to 13 minutes (curve II). When cells are lysed before this time, no infectious particles are present; not even the original infecting virus can be detected. The points plotted prior to 11 minutes (curve II) represent a background count of the phage particles which were unadsorbed and had survived neutralization by the serum, and were not newly synthesized phage. The rate of release of intracellular phage as determined by penicillin lysis is, within 1 minute, the same as that observed by Doermann (1, 10). The penicillin method has not proved satisfactory for

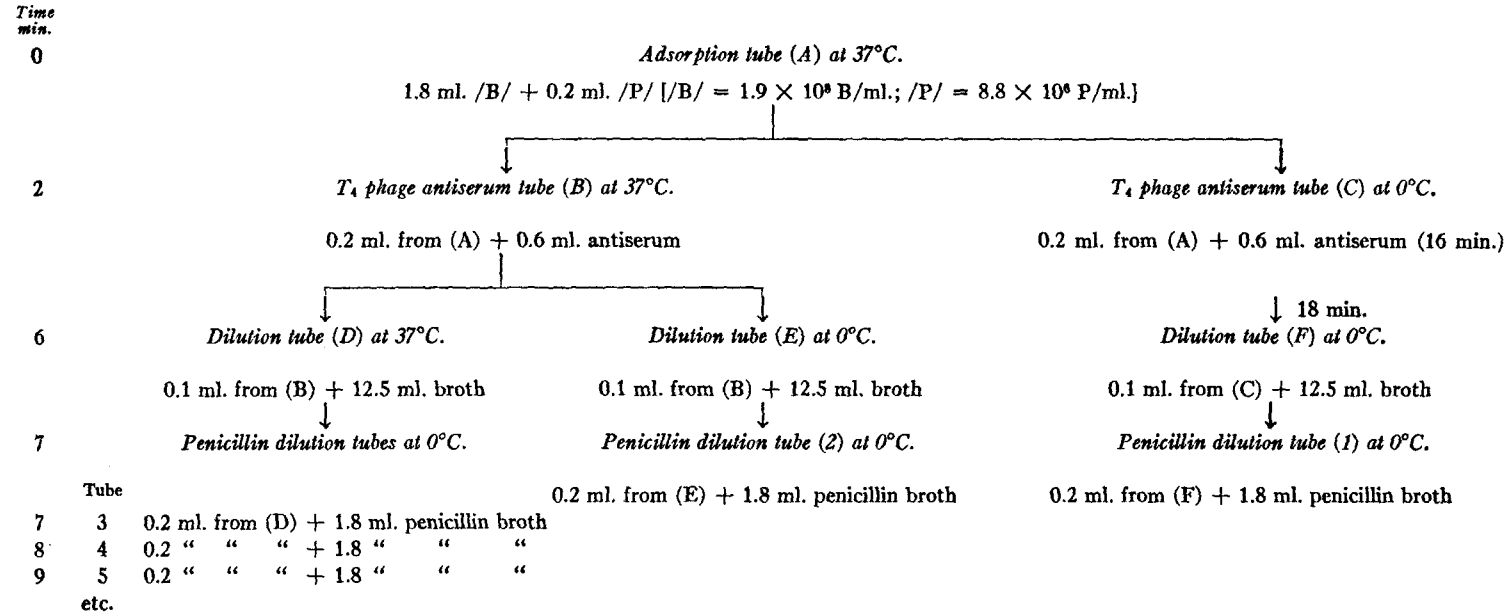

FIG. 1. Procedure outline for determining the number of intracellular phage particles by penicillin lysis.

lysing virus-infected bacteria at concentrations greater than 10^8 B/ml. because of the release of phage which presumably arises from appreciable numbers of resistant mutants present in the culture.

From the results of these experiments it is apparent that 11 minutes is the minimum time required for the maturation of a new phage particle within an infected host cell. In view of this, it was thought that the interval in which no viable virus could be detected might be utilized by the cell to accumulate

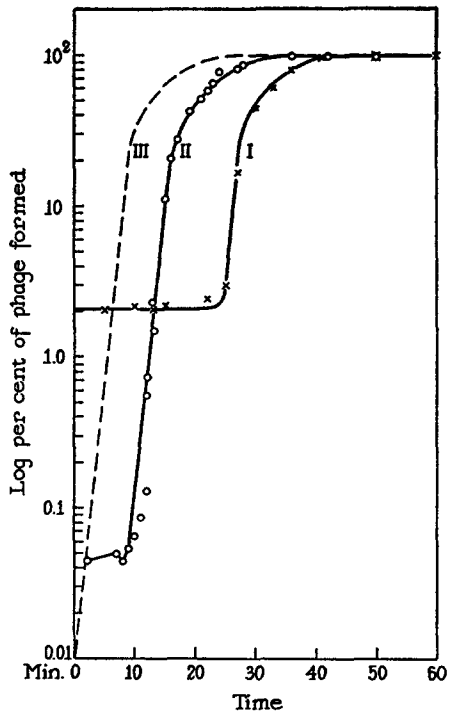

FIG. 2. Rate of release of phage from T_4-infected *E. coli* B during normal and penicillin lysis at 37°C. I, normal lysis. II, penicillin lysis. III, hypothetical rate of synthesis of the neutralizing antigen.

precursor materials needed for the synthesis of new phage. Since infectious T_4 phage can elicit the production of neutralizing antibodies in rabbits, it was considered likely that phage precursor material also might elicit an immune response in experimental animals. If it were possible to detect a specific antigen, a new stage in the developmental cycle of phage would be established.

Method of Assay for Neutralizing Antigen.—An immunological assay method for the estimation of the neutralizing antigen was developed by measuring the level of the immune response in the sera of rabbits which had been injected with known concentrations of virus.

In Table I are shown the phage-neutralizing titers of antisera obtained after inoculating rabbits with increasing concentrations of T_4 virus. It can be seen that injection with 2×10^6 P/ml. failed to stimulate a significant antibody response after three courses of immunization. Even after 30 courses of immunization with this concentration of phage the animals failed to yield sera with higher neutralizing titers. However, when these same animals were subsequently inoculated with a solution containing 2×10^8 P/ml., their antisera, after three courses of injections, showed a high concentration of viral neutralizing antibodies. This fact demonstrates clearly that the animals were not incapable of responding to adequate amounts of the virus antigen. In contrast to this, inoculation with 2×10^7 P/ml. gave, after three courses, antisera having titers of 1:2,560, whereas immunization with 2×10^8 P/ml. gave titers of 1:20,480. From the results of these experiments it is apparent that the extent of the immune response of a given group of animals which had received

TABLE I

Fifty Per Cent Neutralization End-Points of Antisera Obtained from Rabbits Immunized with T_4 Phage at Various Concentrations

Rabbits immunized with T_4 phage	Titers of antisera after:					
	1st course		2nd course		3rd course	
P/ml.						
2×10^6	1:10	1:10	1:10	1:10	1:10	1:10
2×10^7	1:10	1:10	1:80	1:80	1:2,560	1:2,560
2×10^8	1:40	1:40	1:2,560	1:2,560	1:20,480	1:20,480

a restricted course of immunization should serve as an index of the amount of neutralizing antigen present in an infected culture.

Fate of the Adsorbed Viral Antigen.—It was of interest to know whether the infecting virus retains its capacity to elicit immune bodies during the vegetative stage of phage growth. It is obvious that the adsorbed virus must be taken into account in any experiments designed to demonstrate the synthesis of new neutralizing antigen. In order to learn the fate of added virus, the latter was adsorbed to a concentrated suspension of bacteria under conditions in which absorption was rapid and in which no bacterial growth could occur. At a specified time after the infection, formol was added to kill the microorganisms and to inactivate the phage. The suspension was chilled to 0°C. and was used for the immunization of animals.

170 ml. of broth was seeded with 0.6 ml. of a 4 ml. slant washing of a 16-hour culture of *E. coli* B and the flask was incubated at 37°C. with aeration for 2⅓ hours. The bacteria were collected by centrifugation and then resuspended in 9 ml. of nutrient broth. The bacterial concentration was 7.4×10^9 B/ml. as determined by colony count. 3.9 ml. of a culture of

E. coli B was infected at 37°C. with 0.1 ml. of T_4 phage containing 9.6×10^{10} P/ml. After 6 minutes, 0.2 ml. of 4 per cent formol was added. The tube was cooled to 0°C. and the culture left overnight in the chill room. The following day it was diluted 1.2×10^{-1} in nutrient broth and then stored in the refrigerator. The concentration of input phage in the diluted sample was equivalent to 2.0×10^8 P/ml. The culture contained neither viable bacteria nor infectious phage.

A separate virus assay made 6 minutes after infection showed that the supernatant fluid contained 8.1×10^6 P/ml. of unadsorbed phage particles. Thus 99 per cent of the T_4 phage had been adsorbed to the bacterial cells. Since the formolized preparation had been diluted twelvefold, the amount of unadsorbed killed virus present in the material used for the immunization of animals was 6.7×10^5 P/ml., or an amount well below the antigenic threshold.

A second sample was prepared in a similar manner except that formol was added 9 minutes after the initial infection. In addition, formolized phage at a concentration of 2.0×10^8 P/ml. was likewise prepared. This material was used to inject a control series of animals. Rabbits were now immunized with these various preparations and the sera were assayed for their ability to neutralize virus.

TABLE II

Fifty Per Cent Neutralization End-Points of Antisera Obtained by Immunization of Rabbits with T_4 Phage Adsorbed to E. Coli B

Rabbits immunized with:	Titers of antisera after 3 courses of immunization		
(2×10^8 P/ml.) formolized phage (control)	1:10,240	1:10,240	—
Phage-infected *E. coli* B formolized after 6 min.	1:640	1:640	1:640
Phage-infected *E. coli* B formolized after 9 min.	1:80	1:160	—
E. coli B/4 (6×10^8 B/ml.) and T_4 phage (2.4×10^8 P/ml.) formolized after 15 min.	1:320	1:160	1:320

The 50 per cent neutralization end-points of the various antisera are recorded in Table II. It will be noted that the ability of adsorbed phage to stimulate antibodies diminishes shortly after infection is initiated.

The antisera of rabbits injected with the preparation which had been formolized 6 minutes after infection, gave neutralization values of 1:640 whereas the preparation inactivated after 9 minutes yielded antisera having neutralization values of 1:80 and 1:160 respectively. It should be noted that these values are considerably lower than those obtained with formolized phage alone. The results indicate clearly that following infection the bacteria exert some inhibitory action upon the antigenicity of the adsorbed phage, for its capacity to incite viral neutralizing antibodies is considerably impaired. That this effect is not due to mere physical adsorption of the phage to the host but to the presence of bacteria was demonstrated by immunizing animals with a mixture of the phage-resistant variant *E. coli* B/4 and T_4 virus. In this case the phage remains free, yet it can be seen from Table II that a similar interference phenomenon takes place.

Immunization with Phage-Infected Bacteria.—In order to determine whether newly synthesized intracellular virus is capable of eliciting specific antibodies, rabbits were injected with *E. coli* B infected with T_4 phage killed at a predetermined time prior to the burst. Although the previous experiments indicate that adsorbed phage or mixtures of phage and phage-resistant bacteria lose their ability to elicit a maximum antibody response, it seemed desirable to arrange the conditions of immunization for the detection of any newly synthesized phage antigen in such a manner that the original adsorbed virus would not of itself stimulate any measurable antiviral response. This was accomplished by injecting animals with infected formol-killed bacilli which had been diluted so that the concentration of adsorbed virus was not greater than 2×10^6 P/ml. (*cf.* Table I). Animals were immunized with T_4-infected *E. coli* B cells killed with formol 11 and 15 minutes after the initial infection.

100 ml. of broth was seeded with 0.6 ml. of a 3 ml. slant washing of a 16-hour culture of *E. coli* B; the flask was incubated with aeration at 37°C. for 1½ hours. The bacteria were collected by centrifugation and resuspended in 15 ml. of fresh broth containing 1 per cent casamino acids. The bacterial concentration was 1.1×10^9 B/ml. as determined by colony count. To 9 ml. of this culture, maintained at 37°C. with aeration, was added 1 ml. of T_4 phage containing 2.5×10^9 P/ml. 1 ml. aliquots were now withdrawn from the original adsorption tube at the 11- and 15-minute intervals and diluted 10^{-1} in ice cold broth containing 0.5 per cent formol. After standing overnight at 5°C. the samples were sterile; they were then further diluted 10^{-1} in nutrient broth, stored at 5°C., and used for the immunization of rabbits. The concentration of adsorbed virus in these samples was equivalent to 2.5×10^6 P/ml.

In order to ascertain the percentage of adsorbed virus and the number of infected bacteria, a sample (0.2 ml.) was withdrawn from the original adsorption tube 5 minutes after the addition of virus. 0.1 ml. of this aliquot was diluted 10^{-2} in broth at 0°C. and the remaining 0.1 ml. was diluted 10^{-1} at 37°C. in nutrient broth containing an appropriate dilution of T_4 antiserum. The first dilution was centrifuged at 0°C. and the free virus was determined in the supernatant fluid. The assay showed that 2×10^6 P/ml. of virus particles remained unadsorbed, which is less than 1 per cent of the input virus. An aliquot of the second sample, after 5 minutes exposure to the antiserum, was diluted 10^{-4} in broth and was also assayed. This assay indicated that 2.4×10^8 Bp/ml. of infected cells were present in the adsorption tube.

A normal one-step growth experiment was then performed by diluting an aliquot taken from the original adsorption tube in nutrient broth at 37°C. and plating for phage after 50 minutes. This assay revealed that the equivalent of 1.66×10^{10} P/ml. newly formed phage particles had been released in the original adsorption tube.

The average burst size, determined by dividing the final phage yield by the number of infected cells, was found to be 70. The multiplicity of infection, determined by dividing the number of adsorbed phage particles by the total number of bacteria, was 0.24. Using the Poisson formula

$$P(r) = \frac{n^r}{r!e^n}$$

in which n = multiplicity of infection
r = number of viruses per bacterium
$P(r)$ = probability of any number of particles adsorbed to the cells,

the value of $P(0) = \frac{1}{e^n} = 0.78$ in which $n = 0.24$

$$P(1) = \frac{0.24}{e^{0.24}} = 0.19$$

From this it is apparent that 78 per cent of the bacteria remained uninfected, whereas 22 per cent were infected with one or more virus particles. This value is in good agreement with the actual assay, which showed that 24 per cent of the cells had been infected. It should be emphasized that 87 per cent of the infected bacteria were singly infected.

From their studies on the incorporation of radioactive S^{32} in T_4 phage, Mäaløe and Symonds (11) have suggested that the average time required for the assimilation and incorporation of this material into phage particles is 6 to 7 minutes. The rate of S^{32} assimilation was also found to parallel roughly the rate of development of intracellular phage. If the assimilation of sulfur

TABLE III

Fifty Per Cent Neutralization End-Points of Antisera Obtained by Immunization of Rabbits with T_4-Infected Bacteria Inactivated at Various Intervals during the Latent Period

Rabbits immunized with:	Titers of antisera after 6 courses of immunization		
(2×10^6 P/ml.) formolized phage (control)	1:10	1:10	—
(1×10^7 P/ml.) formolized phage (control)	1:640	1:1,280	—
Phage-infected *E. coli* B formolized after 11 min.	1:10	1:10	1:10
Phage-infected *E. coli* B formolized after 15 min.	1:10	1:640	1:10

can be considered an index of the synthesis of the neutralizing antigen, then the rate at which the latter is produced must parallel the production of infectious units. On this basis a hypothetical curve has been constructed (Fig. 2, curve III) which represents the rate of synthesis of this antigen 7 minutes before the appearance of intracellular infectious units. This curve shows the earliest time, following infection, at which the antigen can appear; the latest time would of course be represented by the appearance of the infectious units themselves.

If the synthesis of the neutralizing antigen does indeed proceed at the rate shown by curve III, then considerable amounts of this material must be present both at 11 and at 15 minutes following infection of the cells. However, the experimental results presented in Table III indicate that the neutralizing antigen either does not accumulate in the infected cells, or if it does, its antigenic potentialities are masked by the host cell. It can be seen from the table that no immune response was obtained in animals immunized with material withdrawn at 11 minutes.

From the values shown in Fig. 2, curve II, it is apparent that about 8.5

per cent of the total phage (1.6×10^8 P/ml.) was formed within 15 minutes following infection. The concentration of intracellular phage which one might expect in this sample is 1.4×10^7 P/ml., an amount quite sufficient to stimulate antiviral immune bodies in rabbits. It must be emphasized, however, that this value was arrived at by assuming that normal phage development took place in the original adsorption tube during the latent period. This assumption must be made for it is not possible to lyse T_4-infected cells instantaneously. It will be seen from the table that the serum of only one animal inoculated with the preparation taken at 15 minutes showed a significant rise in titer,—a neutralization end-point equivalent to that of a control animal inoculated with 1×10^7 P/ml. The fact that but one of three animals showed a response suggests that this particular rabbit might have been oversensitive to the phage. It should be emphasized, furthermore, that six courses of inoculations were required to stimulate the antibody response. These results suggest that either there is no significant amount of preformed neutralizing antigen within the cells 15 minutes following infection, or the antigen is combined with some other cellular component which renders it incapable of eliciting a specific immune response.

Synthesis of the Neutralizing Antigen in T_4-Infected Cells Treated with Proflavine.—It has been demonstrated (8) that 3,6-diamino-acridine hydrochloride (proflavine) will inhibit the production of viable phage in infected bacteria without preventing normal lysis. It was of interest, therefore, to determine whether the neutralizing antigen is synthesized in the presence of proflavine. Experiments were carried out in which the dye was added to T_4-infected *E. coli* B cells, and assays were made of the phage yield after lysis. The rate of growth of the bacteria alone, and that of bacteria in the presence of proflavine, were also ascertained. A typical experiment is as follows:

10 ml. of modified M-9 medium was seeded with 0.05 ml. of a 16-hour culture of *E. coli* B and the tube was incubated with aeration for 3 hours at 37°C. The seeding culture had previously been adapted to the medium by some 30 successive serial passages. The bacteria were now collected by centrifugation and resuspended in 30 ml. of isotonic saline. The bacterial concentration was 2.7×10^7 B/ml. as determined by colony count. 27.2 ml. of modified M-9 medium and 3 ml. of the bacterial suspension were added to each of 4 tubes (A, B, C, D). To tubes A and C was added 0.2 ml. of proflavine dissolved in saline so that the final concentration was 1.25 μg./ml. To tubes A and B was now added 0.3 ml. T_4 phage containing 6.3×10^8 P/ml. All 4 tubes were incubated with aeration for 90 minutes at 37°C. The bacterial concentration in tubes C and D, containing no virus, was determined by colony count. The two tubes A and B containing phage and bacteria were assayed for their virus content. To the lysed culture B, lacking the dye, was now added an amount of proflavine equal to that in the other two tubes, A and C. Both lysed cultures (A and B) were stored at 5°C. after diluting 1:1 in M-9 medium. Periodic assays of the stored proflavine lysates showed no loss in phage titer.

In Table IV are recorded the results of a series of experiments in which proflavine at various concentrations was employed to inhibit the development

of the virus. It will be noted that the inhibition of phage production and bacterial growth occurred when the concentration of proflavine was 1 μg./ml. or greater. At lower levels no significant inhibition could be detected. It will also be observed that the inhibition of phage was always considerably greater than that of the bacterial growth.

In column 5 are recorded the ratios of newly formed phage to the input phage in the tubes in which no proflavine was added. Thus, in the tube con-

TABLE IV

Effect of Proflavine on Uninfected E. Coli B and on Cells Infected with T_4 Phage

Amount of proflavine	Viable T_4 count		Ratio B/A	Ratio B/IP*	Viable bacterial count		Ratio D/C
	A (proflavine)	B (no proflavine)			C (proflavine)	D (no proflavine)	
μg./ml.							
0.75	—	—	—	—	5.8×10^7	5.9×10^7	0.99
1.00	3.5×10^7	5.9×10^8	16.9	13	5.4×10^7	6.0×10^7	0.90
1.25	4.0×10^6	2.9×10^8	72.5	46	3.4×10^7	4.3×10^7	0.80
1.50	1.4×10^6	4.1×10^8	294	75	6.6×10^7	9.7×10^7	0.68

* IP represents input phage.

TABLE V

Fifty Per Cent Neutralization End-Points of Antisera of Rabbits Immunized with Proflavine-Inhibited T_4-Infected E. Coli B

Rabbits immunized with:	Concentration of phage in lysate	Titers of antisera after 3 courses of immunization		
	P/ml.			
Proflavine lysate of T_4-infected *E. coli* B	3.2×10^6	1:10,240	1:10,240	1:10,240
Normal lysate of T_4-infected *E. coli* B (control)	1.4×10^8	1:20,480	1:20,480	—

taining 1.25 μg./ml. of proflavine, there was present 1/72.5 of the amount of phage antigen (determined as viable phage) as compared to that present in the normally lysed culture. It will also be noted from columns 6 and 7 that under these same conditions the bacterial inhibition as determined by colony count was but 1/1.25 of that in the control tube. Thus the concentration of bacteria in both cultures was essentially the same.

Rabbits were now immunized with both lysates and the neutralization end-points of their respective antisera were determined. The results are recorded in Table V. Here it is seen that both antisera had essentially the same titer. It should be noted, however, that in the proflavine lysate the known total

phage antigen present, consisting of the sum of the input phage and of released phage, was only 3.2×10^6 P/ml. This amount of virus is insufficient to elicit an immune response in rabbits (*cf.* Table I). Thus it would appear that the mechanism concerned in the synthesis of the neutralizing antigen is not inhibited by proflavine.

Experiments with T_3 Coli-Dysentery Bacteriophage

At 37°C. coli-dysentery bacteriophage T_3 has a latent period of 13 minutes when grown in *E. coli* B. This interval is too short to permit a study of the rate of intracellular virus formation by induced lysis; at this temperature any pool of neutralizing antigen which might form during the early part of the latent period would be present for so short a time that its detection would be difficult. If the growth temperature is reduced to 27°C., however, the latent period is doubled and these difficulties are to some extent overcome.

Anderson and Doermann (12) have shown that T_3 phage is relatively resistant to sonic vibration and they have made use of this fact to study the development of T_3 in infected *E. coli* B cells. In the experiments reported here it was found that 5 minutes of sonic vibration of 10 kc./sec. destroyed only 10 to 15 per cent of the T_3 virus, whereas 95 per cent of the *E. coli* B cells were destroyed. This procedure could not be employed for the liberation of the neutralizing antigen from T_4-infected cells, however, for not only is the infectivity of the virus rapidly destroyed but its antigenicity is also lost.

One of the limitations resulting from the selection of T_3 for study was its slow rate of adsorption to *E. coli* B. This difficulty can be overcome by adsorbing the phage to the host cells under conditions in which no growth occurs, —in an unaerated buffer system containing no nutrients (13). After a suitable period the cells can be collected by centrifugation and the supernatant fluid, containing the unadsorbed free virus, discarded. The cells, now free of most of the unadsorbed phage, can be used for the study of phage growth after resuspension in fresh nutrient.

105 ml. of broth enriched with 1 per cent casamino acids was seeded with 0.3 ml. of a 3 ml. slant washing of *E. coli* B and the flask was incubated at 37°C. with aeration for 2⅓ hours. The bacteria were collected by centrifugation and resuspended in 6 ml. of 0.1 M phosphate buffer at pH 7.0. To 3.8 ml. of the resuspended bacterial culture contained in a centrifuge tube was added, at 27°C., 0.2 ml. of T_3 phage in buffer and containing 3.2×10^{10} P/ml. After 20 minutes the culture was centrifuged. The supernatant fluid was decanted and the bacteria were resuspended in 3.9 ml. of fresh buffer. The free virus content of the first supernatant fluid was determined and that in the supernate of the resuspended bacteria. The assays showed that 83 per cent of the input virus had been adsorbed; only 2.0×10^7 P/ml. remained in the supernate of the resuspended organisms.

30 minutes after the initial infection, 1.5 ml. of the bacterial suspension was added to 298.5 ml. of casamino acid–enriched broth, and the flask was incubated with aeration at 27°C. The time at which the dilution in the nutrient medium was made represents the beginning of phage synthesis, or 0 time. At 13.5, 15, 18, 20, 22, 24, 26, 28, 30, 35, 38, 42, and 45 minutes

1.0. ml aliquots were withdrawn from the flask and diluted to 20 ml. in broth a 0°C. Each sample was sonically vibrated at 10 kc./sec. in a Raytheon magnetostriction oscillator for 5 minutes at 9°C. and the lysate was assayed for intracellular phage. The per cent of the final total phage released at various time intervals is plotted in Fig. 3 (curve II). A one-step growth curve was also determined at 27°C. from a sample removed at 17.5 minutes (curve I). From the data presented in curve II it is apparent that at 16 minutes, when only 2 per cent of infectious phage is present, there should be considerable amounts of neutralizing antigen (curve III) if this substance is preformed. A sample of 100 ml. was, therefore, removed at 16 minutes, cooled rapidly to 0°C. by immersion of the container in dry ice–alcohol mixture, and the

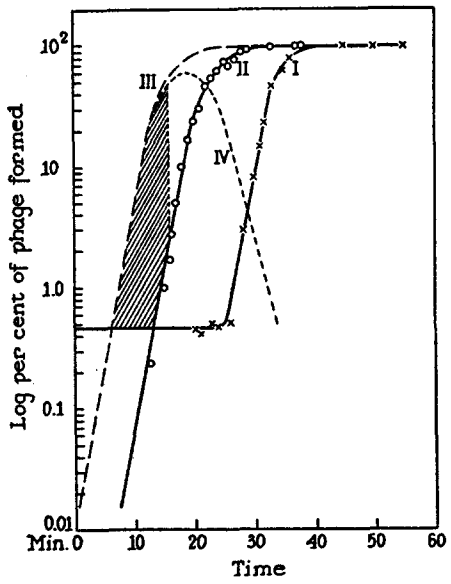

FIG. 3. Rate of release of phage from T_3-infected *E. coli* B during normal and sonic lysis at 27°C. I, normal lysis. II, sonic lysis. III, hypothetical rate of synthesis of the neutralizing antigen. IV, hypothetical pool of neutralizing antigen.

sample was then sonically vibrated. The lysate was assayed for phage, and stored at 0°C after the addition of 0.5 ml. of formol.

It will be noted that the multiplicity of infection was 0.17 and the average burst size was 187. By using the Poisson formula it was found that 88 per cent of the infected bacteria were singly infected.

It was pointed out earlier in this communication that in the case of T_4 some 7 minutes is required for the assimilation and incorporation of S^{32} into the phage particles (11). Since the latent period of T_3 is 25 to 26 minutes at 27°C., an interval only some 3 minutes longer than that of the latent period of T_4 at 37°C., it has been assumed that the synthesis of T_3 viral protein likewise commences approximately 7 minutes prior to the appearance of infectious

units. On the basis of this assumption curve III in Fig. 3 has been constructed to represent the earliest time interval at which viral neutralizing antigen synthesis could occur within infected cells if the synthesis begins 7 minutes prior to the appearance of infectious units. At the time when mature virus first makes its appearance part of the neutralizing antigen must of necessity be bound to infectious particles and part should be considered either free or bound to immature non-infectious virus. The rate of conversion of the neutralizing antigen from the free or non-infectious state to the condition in which it occurs in the mature virus particle is illustrated in curve IV. The values expressed in this curve were obtained by subtracting the amount of the neutralizing antigen bound to mature phage particles (determined by assay) from the total maximum amount of neutralizing antigen estimated to be present (curve III) for any particular time interval. Thus, of the total amount of antigen finally formed, some 50 per cent should be present in the unbound state 16 minutes after growth has commenced. At this same time interval 2.5 per cent is bound to the viable phage particles which have already formed. The concentration of unbound antigen is equivalent, therefore, to 6×10^7 P/ml. (*i.e.*, 50 per cent of the final phage yield of 1.2×10^8 P/ml.). By direct assay, the sample actually contained 2.4×10^6 viable phage particles or roughly 2 per cent of the final phage yield. Adsorbed to the bacteria, however, is the input phage antigen, equivalent to 6.6×10^6 P/ml. This value is calculated by dividing the adsorbed virus (1.35×10^9 P/ml.) by 200, the dilution factor of the infected cells in the growth tube. Thus the total amount of known phage antigen in the sonically lysed sample is 9.0×10^6 P/ml. Since the adsorbed viral antigen has been shown to lose its antigenic efficacy, any immune response elicited by animals infected with the sonic lysate should arise from preformed antigen, and from the small amount of infectious phage known to be present.

Three series of animals were now immunized, one with the lysate and the other two with control phage solutions containing 6×10^7 P/ml. and 9×10^6 P/ml. respectively. All samples were sterilized prior to their use by the addition of 0.5 ml. of formol per 100 ml.

The results of these experiments are recorded in Table VI where it can be seen that the neutralization titers of the antisera obtained from animals inoculated with the sonic lysate did not exceed 1:20. The titers of the antisera of the two control animals which received 9×10^6 P/ml. were 1:160 and 1:320; those which received 6×10^7 P/ml. either as free virus or as a mixture of sonically disrupted bacteria and phage showed titers of 1:1,280. It is clear from these results that the sonic lysate contained no pool of viral antigen of sufficient concentration to yield a specific immune response.

The immunization procedure is sufficiently sensitive to detect as few as 9×10^6 P/ml., an amount equivalent to 7 per cent of the final phage yield. Since there is no indication that this amount of antigen is present at the 16-

minute interval, curve III, which represents the rate of synthesis of the neutralizing antigen, can now be displaced to a parallel position 5 minutes to the right. Thus it is concluded that the neutralizing antigen must be synthesized within 2 minutes of the appearance of infectious units at 27°C. The procedure employed does not permit a more precise determination.

In order to determine whether sonic vibration destroys the antigenicity of intracellular virus, or whether antigenicity is masked by bacterial components the following experiment was performed.

TABLE VI

Fifty Per Cent Neutralization End-Points of Antisera of Rabbits Immunized with Sonically Lysed T_3-Infected E. Coli B Taken during the Latent Period

Rabbits immunized with:	Titers of antisera after 4 courses of immunization		
Sonic lysate made 16 min. after infection	1:20	1:20	1:20
(9×10^6 P/ml.) formolized phage (control)	1:160	1:320	—
(6×10^7 P/ml.= formolized phage (control)	1:1,280	1:1,280	—
Sonic lysate plus 6×10^7 P/ml. added and formolized (control)	1:1,280	1:640	—

TABLE VII

Fifty Per Cent Neutralization End-Points of Antisera Obtained by Immunization of Rabbits with Sonically Lysed and Formol-Killed T_3-Infected E. Coli B

Rabbits immunized with:	Titers of antisera after 4 courses of immunization		
Sonic lysate made 24 min. after infection	1:5,120	1:5,120	1:2,560
Formol-killed cells 24 min. after infection	1:320	1:640	1:320
(1.5×10^8 P/ml.) formolized phage (control)	1:5,120	1:5,120	—

The intracellular virus in a culture of T_3-infected *E. coli* B bacteria was permitted to develop for a period of 24 minutes at 27°C. Half the culture was then sonically disrupted and the remaining half killed with 1 per cent formol at 0°C. In the lysed sample the virus assayed 1.5×10^8 P/ml. or 62 per cent of the phage which would have formed had maturation proceeded to completion. By means of a one-step growth curve it was shown on a control aliquot taken from the adsorption tube that the bacteria, prior to the addition of formalin, contained 102 phage particles per infected cell. Animals were immunized with both preparations after adding 1 per cent formol to the sonically lysed culture.

The results of these experiments are recorded in Table VII. It can be seen that the titers of the sera of animals immunized either with the formolized, sonically lysed, infected culture, or with formolized virus itself were identical. On the other hand the titers of the sera obtained from animals infected with formolized, infected, whole bacteria were considerably less. Even after three

additional courses of injections with the formolized infected cells, the sera failed to show antiviral titers greater than 1:1,280.

These results demonstrate that the immunization of animals with bacterial cells containing intracellular virus elicits an antibody response which is considerably weaker than that obtained with comparable amounts of free virus. This feeble response can be attributed to the presence of large amounts of bacterial antigens, as was shown in the case of both T_4 and T_3. It is possible to eliminate this interference phenomenon in the case of T_3 infections by selectively destroying the bacterial components with sonic vibration (7).

DISCUSSION

It is evident from the data which have been presented that when *E. coli* strain B was infected with either T_3 or T_4 virus, the presence of a neutralizing antigen could not be detected during the vegetative stage of phage development by immunological means. In the case of both these phages it has been demonstrated that when animals are immunized with a mixture of large amounts of *E. coli* B and virus they show a marked diminution in their ability to develop viral antibodies. In those experiments, however, in which T_3-infected cells were disrupted by sonic vibration and the lysates used to inject rabbits, there was no untoward inhibition of their antiviral immune response. The reason for this probably resides in the fact that the antigenic efficacy of the bacterial components was impaired by the treatment to which the cells had been subjected, whereas that of the virus remained essentially unaffected.

Although the neutralizing antigen cannot be demonstrated during the early stages of phage development, this does not necessarily mean that synthesis of other phage protein might not take place. It has been shown by Levinthal and Fisher (14) that "doughnut"-shaped particles believed to be intimately related to phage, can be obtained from infected cells which have been prematurely lysed and that these same bizarre forms occur in lysates of infected cultures inhibited with proflavine. These particles contain sulfur and nitrogen but no nucleic acid and although they have the ability to fix complement, they do not react with the neutralizing antibody of phage immune serum (15). In contrast to this, it has been shown that tailed phage ghosts, which contain no nucleic acid, readily react with the neutralizing immune body of phage antisera (2). On the basis of these observations it has been suggested that the neutralizing antigen of the mature virus particle is confined mainly to the tail.

The experiments reported here with T_4-infected bacteria inhibited with proflavine indicate that the neutralizing antigen is elaborated to the same extent as it is in infected cultures containing no proflavine, although few new phage particles are released in the presence of the dye. Thus proflavine appears

to interfere specifically with the attachment of the neutralizing antigen to the immature phage particle. This concept is in accord with Foster's observations (8) that proflavine hinders some late stage in phage synthesis.

Failure to detect the neutralizing antigen in virus-infected cells during the vegetative stage of T_3 phage formation by the methods employed indicates strongly that this component does not accumulate in any appreciable amounts prior to the appearance of infectious virus. It should be borne in mind, however, that during this stage of development the antigen might be labile and hence be destroyed by sonic vibration. Nor is it out of the question that the neutralizing antigen might remain combined with bacterial debris following sonic treatment, thus rendering it incapable of eliciting a specific immune response in experimental animals. It should not be forgotten, however, that the mature virus is resistant to sonic vibration.

In conclusion it may be stated that in the case of T_3 coli-dysentery bacteriophage it has not been possible to demonstrate by immunological means the accumulation of a neutralizing antigen in infected cells during the vegetative stage of phage growth. If indeed such an antigen were present, it is believed that its detection could readily have been made by the experimental procedure employed.[2] The experiments recorded here indicate that the synthesis of new virus particles and the appearance of an antigen capable of eliciting viral neutralizing antibodies take place nearly simultaneously.

SUMMARY

The development of viral neutralizing antibodies in animals injected with T_3 or T_4 phage is considerably inhibited by the presence of bacterial antigens.

A new procedure has been described to liberate phage from infected *E. coli* B bacteria by inducing lysis with penicillin.

By immunological means it has been shown that T_4-infected cultures of *E. coli* B, in which phage development has been inhibited with proflavine, contain the viral neutralizing antigen after lysis.

In contrast, it has not been possible to demonstrate by immunological means the appearance of viral neutralizing antigen in *E. coli* B infected with T_3 prior to the appearance of intracellular phage.

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[2] Since this manuscript was completed, a statement has appeared in the literature (16) to the effect that the neutralizing antigen of T_4 phage is demonstrable within infected cells a few minutes prior to the appearance of infectious units. However, the details of these experiments have not been described.

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