INFECTION BY BACTERIOPHAGE T5 AND ITS INTRACELLULAR GROWTH—A STUDY BY COMPLEMENT FIXATION[1]

YVONNE THÉRY LANNI[2]

Department of Bacteriology, University of Illinois, Urbana, Illinois

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Infection of a susceptible bacterium by a virulent phage particle is followed, after a latent period, by cell lysis and liberation of newly formed phage particles. The infecting particle loses its infectivity shortly after adsorption to the host, a fact attributed to dissociation of the particle into a cell penetrating fraction, consisting chiefly of nucleic acid, and an externally persisting fraction, consisting chiefly of protein and containing at least some of the principal phage antigens (Hershey and Chase, 1952).

The study of the intracellular development of virus antigens, initiated with influenza virus (Hoyle, 1948, 1952; Henle and Henle, 1949; Liu and Henle, 1951), has now been extended to phage (Rountree, 1951, 1952; DeMars *et al.*, 1953). At present, it is generally agreed that virus antigens appear intracellularly before infectious virus. Studies with phage have been aided considerably by the discovery of methods such as the cyanide-T6 technique of Doermann (1952) for lysing infected cells at given times during the latent period. In the only available quantitative studies of the role of phage antigen in the infective process, it has been reported (Rountree, 1951, 1952) that the complement-fixing antigen of coliphage T5 and of the staphylococcal phage 3A disappears from the system shortly after adsorption; with phage 3A, a second antigen is believed to persist externally.

The present paper describes a quantitative method for complement fixation with phage and its application to the study of infection and intracellular growth. Complement fixation was selected because of its sensitivity and precision. Phage T5 was selected because of its failure to cross-react with T6, an ingredient of the medium used to induce premature lysis.

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[2] Present address: Department of Bacteriology, University of Illinois, College of Medicine, Chicago, Illinois.

MATERIALS AND METHODS

Bacteria. Escherichia coli, strain B, the common host for the T-phages, was used throughout, except that in experiments on premature lysis with T6 present in some samples all infectivity assays were made on the mutant strain B/6 resistant to T6 but sensitive to T5.

Phage. A mass lysate prepared from a single plaque of phage T5 was plated by the agar-layer technique so as to give barely confluent lysis. The virus was eluted from the agar layer into broth and purified by two cycles of differential centrifugation. The final suspension, in M/15 phosphate buffer plus 2×10^{-3} M $MgSO_4$, had a plaque-count titer of 1.7×10^{13}, a value which did not change during storage for one year at 4 C. Unless otherwise noted, this stock preparation was used throughout, both as inoculum and as reference material in complement fixation.

Details of standard methods in phage work are given by Adams (1950).

P^{32}-labeled phage T5 was purified by three cycles of differential centrifugation from a mass lysate prepared on bacteria grown in tryptose medium (5 g Bacto-tryptose + 5 g NaCl per liter + 5×10^{-4} M $CaCl_2$) supplemented with 22 microcurie P^{32} per ml.

Adsorption. To promote adsorption of the poorly adsorbing phage T5, bacteria grown to about 1×10^8 in nutrient broth at 37 C were collected by centrifugation and resuspended in 1/60 the original volume of fresh broth. Adsorption by the concentrated bacteria was 95 to 98 per cent complete in 4 to 6 minutes at 37 C. For one step growth experiments, the adsorption mixture was then diluted 1:50 or

more in pre-warmed broth and incubation was continued at 37 C.

Premature lysis. Aliquots of the infected culture were mixed in an ice-bath with 0.05 vol of lysing mixture, so as to give a final concentration of 0.01 M KCN and about 500 T6 particles adsorbed per cell. The T6 stock was a purified preparation with a plaque-count titer of 1.1×10^{13}. After 10 minutes in the cold, the mixtures were incubated one hour at 37 C, then assayed by plaque count and complement fixation.

Complement fixation. Materials. The diluent, buffer-albumin, was a veronal-bicarbonate buffer containing 0.1 per cent bovine albumin (Mayer *et al.*, 1948).

Complement and hemolysin were commercial preparations (Sharp and Dohme). Sheep blood was collected aseptically into an equal volume of glucose-citrate-saline solution (DeGowin *et al.*, 1949) and stored at 4 C. When needed, a suitable quantity was centrifuged, and the red cells were washed three times with buffer-albumin and made up to 5 per cent by volume. The washed cells were sensitized by treatment for 10 minutes at 37 C with an equal volume of diluted hemolysin, sufficient to give optimal sensitization. Then they were stored at 4 C for not longer than two hours. Details are given by Kabat and Mayer (1948), whose general procedure we have followed. All glassware was cleaned in sulfuric acid-dichromate mixture and rinsed thoroughly.

A serum with high complement-fixing activity against T5 was obtained from a rabbit after repeated subcutaneous and intravenous injections of purified virus. The crude serum fixed complement also with disrupted host-cell preparations obtained by sonic vibration and with crude T2 lysates. To minimize heterologous reactions, the serum was diluted 1:5 in buffer-albumin, heated 30 minutes at 56 C to destroy the complement present, and absorbed three times, each time with 1.2×10^9 washed host cells per ml diluted serum. The absorbed serum, at a further dilution of 1:10 or greater, failed to react with host-cell preparations, crude T2 lysates, or purified T6, but showed undiminished activity against T5. The serum was sterilized then by filtration through sintered glass and stored at 4 C. In phage neutralization, this serum had a k value (Adams, 1950) of 87 min^{-1} (measured at a serum dilution 1:1,000).

Fixation and lysis. Mixtures containing 0.2 diluted antigen, 0.2 ml diluted serum, and 0.2 ml complement (diluted to give 1.5 50 per cent units) were prepared in photometrically standardized tubes (75 mm by 12 mm OD) in an ice-bath and refrigerated for about 18 hours. This procedure yielded a threefold increase in sensitivity over one involving incubation for one hour at 37 C. A volume of 0.4 ml chilled sensitized cell suspension was added then, and the mixtures were kept one hour at 37 C with frequent agitation. They were chilled, diluted to 2.5 ml with cold buffer without albumin, and centrifuged at low speed to deposit unlysed cells. The transmission of the supernatants at 520 mμ was measured in a Coleman Junior spectrophotometer. Where desirable, the degree of lysis was determined by photometric reference to graded standards, prepared by lysing sensitized cells in distilled water and diluting to give a linear series covering 0 to 100 per cent lysis in steps of 10 per cent.

In general, anticomplementary effects were limited to high concentrations of the test materials and could be avoided by suitable dilution. However, the high anticomplementary effect of early premature lysates, due to bacterial debris, could not be avoided by dilution since the antigen content was low. In these experiments, therefore, all the samples were diluted in a cyanide-T6 lysate of uninfected bacteria, so as to maintain a constant anticomplementary background.

RESULTS

Complement fixation in the T5:anti-T5 system. The dependence of complement fixation on the amounts of antigen and antiserum is shown as a family of constant serum curves in figure 1. It is convenient to plot the per cent transmission rather than the amount of complement fixed; these two quantities, although not proportional, increase together. The following observations can be made:

(1) Except at the highest serum concentration, the curves show a maximum with inhibition of fixation in excess antigen. As the serum concentration increases, the maximum increases toward a limit set by the available complement, and its position shifts toward higher antigen concentration. Other experiments show that in the region of low serum concentration the maximal fixation is roughly proportional to serum

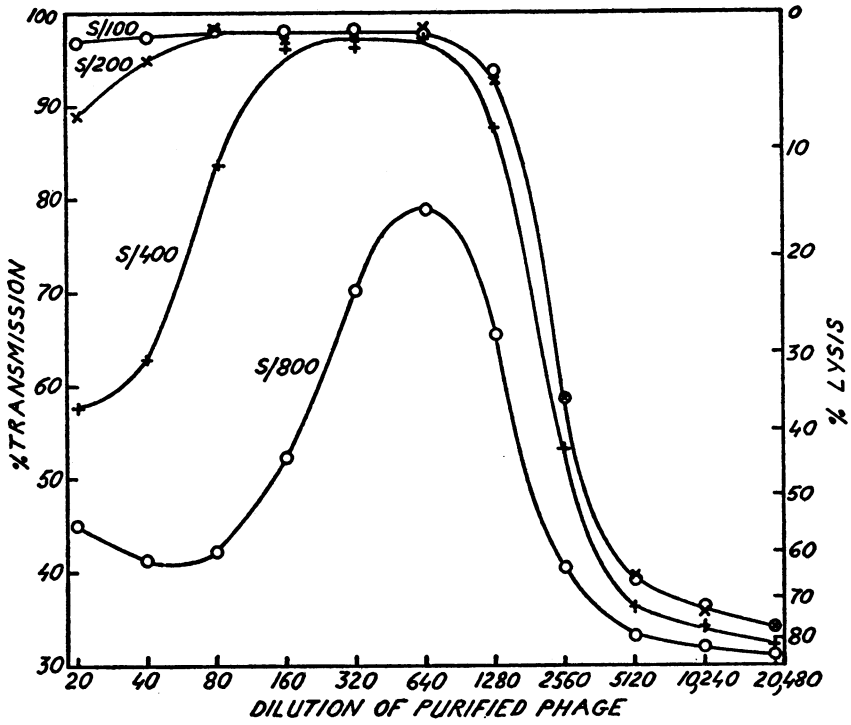

Figure 1. Complement fixation in the T5:anti-T5 system, showing the dependence on phage and serum concentrations at constant complement (1.5 50 per cent units). Complement fixation increases with per cent transmission; the corresponding degree of red-cell lysis is shown at the right. S/100, etc, refers to the dilution of serum added. The purified phage diluted 1:20 contains 1.7×10^{10} plaque forming units in the 0.2 ml volume employed in the test. The baseline, 30 per cent transmission, corresponds to the complement control (no complement fixed). The upper limit, 97 per cent transmission, is determined by colored matter liberated in the red-cell control (no complement present).

concentration. There is no evidence of inhibition of fixation in excess serum (at constant antigen). Similar results have been obtained with T2:anti-T2 (Lanni and Lanni, *unpublished data*), but Rountree (1951, 1952) has failed to observe antigen-excess inhibition with either phage T5 or phage 3A.

(2) With serum diluted 1:800 and less so at 1:400, the curve shows an aberration at high antigen concentration due to anticomplementary effect of the antigen. At the extreme right, all curves approach basal levels set by the anti-complementary effect of serum at various dilutions.

(3) At the maximum for 1:800 serum, approximately 50 per cent (0.75 out of 1.46 units) of the total complement has been fixed. The system is thus extremely sensitive to the fixation of small amounts of complement. On the other hand, levels of complement fixation ranging from 60 to 100 per cent are discriminated poorly since the corresponding transmissions range only from about 85 to 100 per cent.

(4) Significant fixation is observed with 1:5,120 antigen, corresponding to about 7×10^7 total plaque forming units. Variations in total complement, resistance of sensitized cells to lysis, and possibly other factors cause some variations in sensitivity from experiment to experiment.

(5) At constant antigen, the dependence of fixation on serum concentration is most pronounced at high antigen concentration. Hence, the relative activity of two sera is best determined with antigen diluted not more than about 1:640. The relative activity of two antigen preparations, the measurement that will concern us most, is best determined with serum diluted not more than about 1:200 in order to minimize the importance of volume errors in the distribution of the serum; we have used 1:200 serum. The

relative activity of two antigen preparations can be obtained directly from the displacement of the corresponding curves (transmission versus log antigen dilution or its equivalent) along the dilution axis.

More detailed information about antigens can be obtained by the use of serum diluted beyond the level recommended for routine quantitative analysis. This modification, employed profitably in work with phage T2 (Lanni and Lanni, 1953), limits the maximal possible fixation and permits observations in the region of antigen-excess inhibition. The complement fixation curves thus obtained have value both for the identification of antigens and for the detection of multiple antigens in a mixture. Similar considerations apply to the study of sera. A technical disadvantage of the modified system is its greater sensitivity to the dosage of complement, the fragility of red cells, and other factors.

Conditions for premature lysis. Doermann (1952) has shown that bacteria infected with T4 can be lysed throughout the latent period by treatment with 0.01 M cyanide and a high multiplicity of T6. Kay (1952*b*) has obtained similar results substituting glycine for T6. In the absence of T6 or glycine, cyanide promotes lysis only during the second half of the latent period when mature phage is already present intracellularly (Doermann, 1952; Weigle and Delbrück, 1951; Kay, 1952*b*; *see*, however, Rountree, 1951, 1952). We have confirmed Doermann's findings with T5. Cyanide alone, however, while not causing cell lysis early in the latent period, had a definite effect on the survival of infected bacteria as plaque forming units. When cyanide was added 8 minutes after mixing bacteria and phage, the recovery of plaque forming bacteria was less than 10 per cent. As the addition of cyanide was delayed, the recovery gradually increased, reaching about 35 per cent of the control value in 15 minutes. It would appear that the development of infection passes through a cyanide sensitive stage. This effect, whose mechanism is unknown, may be related to phenomena described below. The results confirm and extend certain observations of Doermann (1952) with T4.

Intracellular development of phage antigen and active phage. Aliquots of a culture of *E. coli*, strain B, infected with about 2.8 active T5 particles per cell, were lysed at intervals with cyanide + T6 and assayed by plaque count and complement fixation. Other aliquots of the culture were plated directly for plaque formers (normal lysis control). The results of these tests, illustrated by the experiment of figure 2, demonstrate the following:

(1) The normal lysis control (curve A) shows a latent period of about 45 minutes, a rise period of about 15 minutes, and a burst size of 95. Before the onset of lysis, the plaque count was constant at about 1×10^8 and was distributed between infected cells (88 per cent) and unadsorbed phage (12 per cent; arrow 1).

(2) With premature lysis (curve B), a shorter constant period of about 22 minutes is followed by a prolonged rise period of about 40 minutes. The final yield is experimentally identical with the control. The plaque count during the constant period, 1.25×10^7, corresponds to unadsorbed phage (arrow 1), which was about 5 per cent of total input (arrow 2).

(3) The complement-fixing activity of the premature lysates (curve C) shows a nearly constant period of about 18 minutes, during which the activity is almost identical with the total input activity (arrow 2). There is no evidence that the input antigen disappears during infection (*see* Rountree, 1951, 1952). The small apparent rise in complement-fixing activity between 6 and 18 minutes should not be emphasized at present because of the low concentration of antigen during this interval and the high anticomplementary effect of bacterial debris.

(4) After 18 minutes, the complement-fixing activity rises abruptly and eventually attains a maximal level 2.2 times greater than that which would be expected if all the activity were associated with plaque forming particles. This discrepancy, which will be considered further below, means that crude lysates contain more complement-fixing activity per plaque forming particle than does the purified phage stock. In the premature lysates, the complement-fixing activity per plaque forming particle, after both extimates have been corrected for initial levels, is maximal at about 20 minutes and decreases steadily thereafter, approaching the value noted above.

(5) A linear plot of complement-fixing activity and plaque count of the premature lysates (figure 2, insert), with each quantity expressed as

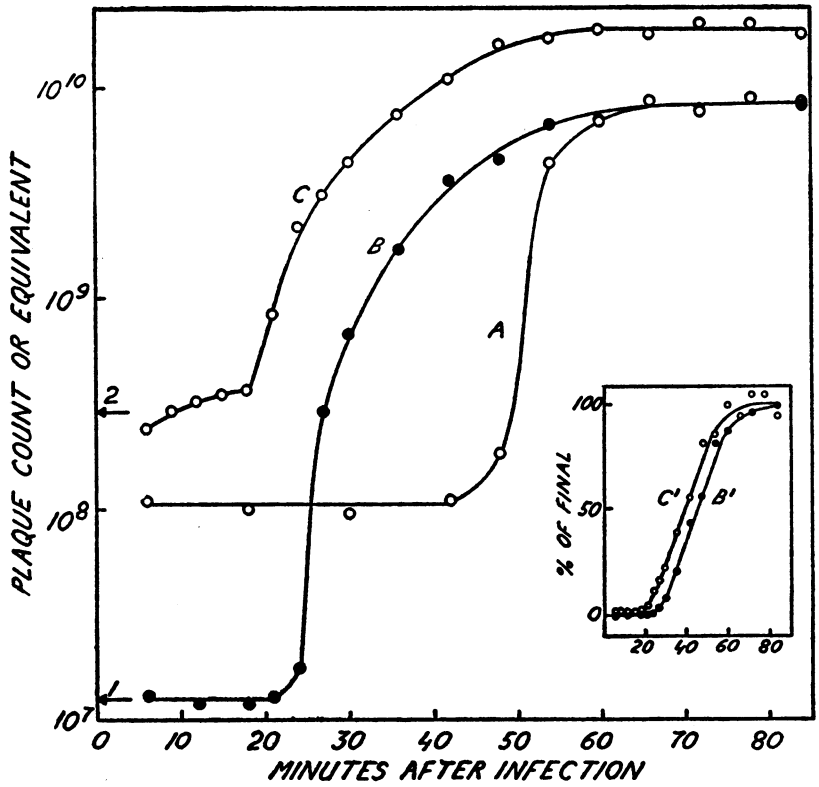

Figure 2. Intracellular growth of phage antigen and mature phage. Curve A: normal one step growth curve (no premature lysis). Curve B: mature phage in premature lysates. Curve C: total antigen in premature lysates, converted to plaque-count titer of purified phage at equivalent serological activity. Arrow 1: level of unadsorbed phage. Arrow 2: level of input phage (and antigen). Insert: linear plot of the data of curves B and C, with the ordinates expressed as per cent of final values.

per cent of final, shows that the two rise curves are essentially parallel, being displaced about 6 minutes, and approximately linear through most of their range. In the linear region, *total* antigen evidently increases slightly more than twice as fast as total mature phage. Further analysis shows that the excess of antigen over that present in mature phage also increases linearly during most of the growth period. There is, therefore, no evidence of an early completion of a pool of antigen later incorporated into phage (*see* Rountree, 1951).

The process of infection. Experiments of the type shown in figure 2 suggested that the infectivity but not the serological activity of the infecting phage is lost shortly after adsorption. Since, however, the infected cultures were lysed artificially, the experiments gave no evidence as to whether the antigen entered the host cell as suggested by Rountree (1951, 1952) or remained outside as suggested by findings of Hershey and Chase (1952) with T2.

Further experiments showed that when an infected culture is treated early in the latent period with cyanide alone, all the input antigen remains available for complement fixation, within the limits of experimental error (about 5 per cent); yet, the adsorbed fraction of the antigen, about 75 per cent of the input, is sedimentable at low speed. By contrast, the adsorbed antigen of infected cultures lysed with cyanide + T6 is found in the low speed supernatant. These experiments were supplemented by others employing a Waring blendor (Hershey and Chase, 1952) in an effort to separate the adsorbed antigen from the host cell. The results of numerous experiments can be summarized as follows:

(1) Under the usual conditions for adsorption, 95 to 98 per cent of the phage and about 75

per cent of the total antigen are adsorbed. The unadsorbed antigen fraction may represent phage particles inactivated and rendered unadsorbable during purification.

(2) The actual multiplicity of infection, usually two to three, is theoretically sufficient to cause infection of 85 to 95 per cent of the bacteria. Occasionally we recovered 85 to 100 per cent of the expected count of infected bacteria; more often the count was only about 50 per cent of the expected value (*see* Benzer, 1952).

(3) Treatment of infected cultures during the latent period in the semimicro-Waring blendor reduced the count of plaque forming bacteria and liberated antigen from the cells. These effects were nearly complete in one minute and complete in three minutes with no further change up to 10 minutes (running time at 10,000 rpm). The treatment had no effect on free phage. A standard period of three minutes was adopted.

(4) If the adsorption mixture was diluted 1:25 in cold broth (or diluted in warm broth and chilled immediately) and then blended, the count of plaque forming bacteria was only 1 to 3 per cent of that in unblended controls. This result contrasts markedly with the resistance of infected bacteria to blending observed by Hershey and Chase (1952) with T2.

(5) Those plaque forming bacteria that survived treatment in the Waring blendor gave a normal yield of phage. Those that did not were also inactive as colony formers. Hence, sterilization of the host appears to be distinct from the establishment of infection resistant to blending (*see* Herriott, 1951).

(6) If the adsorption mixure was diluted 1:25 in warm broth and samples were taken at intervals during the latent period, chilled, and blended, the fraction of plaque forming bacteria increased progressively (*see*, for example, figure 3) but was only about 65 per cent of the unblended control after twenty-seven minutes total pre-blending incubation at 37 C (including the adsorption period). At all times, the low speed supernatant of blended materials gave a plaque count equal to the initially unadsorbed phage and, within experimental error, contained all the initially adsorbed plus unadsorbed antigen. The above evidence indicates (a) that the adsorbed phage is not removed in infectious form by blending; (b) that the removability of the bulk of the adsorbed antigen is unrelated to the infective state of the bacteria; and (c) that the establishment of blendor-resistant infection with T5 is a slow process under our experimental conditions.

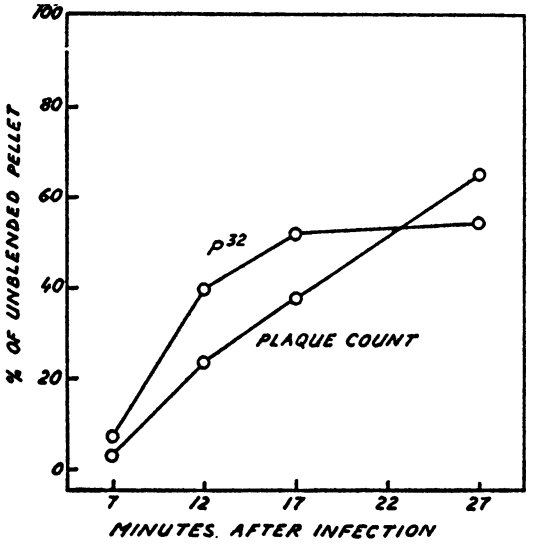

Figure 3. Incorporation of phage P^{32} into the host cell and establishment of infection resistant to blending.

Bacteria, grown in broth to 1.5×10^8 and concentrated 60-fold, were mixed in broth at time zero with 2.5 P^{32}-labeled phage per cell. The bacterial concentration was 7.2×10^9. After 6 minutes at 37 C with aeration, 98 per cent of the phage activity and 72 per cent of the antigen had been adsorbed. The mixture then was diluted 1:25 in broth and kept at 37 C. At intervals (7, 12, 17, 27 minutes) samples were chilled in an ice-bath and treated as follows. One portion was assayed for total infective centers and P^{32}, which were found to remain constant. A second portion was centrifuged at low speed, and the supernatant and pellet were assayed for infective centers and P^{32}, which again remained constant. A third portion was blended for 3 minutes in the cold. Part of this material was assayed as such. Another part was centrifuged at low speed, and assays were made on the pellet and supernatant fractions. The supernatants from blended materials were tested also in complement fixation and found to contain 102, 108, 97, and 98 per cent of the input antigen at the successive samplings. The present figure shows P^{32} content and the plaque-count titer of pellets from blended samples expressed as per cent of values in unblended control pellets.

(7) When the adsorption mixture was diluted 1:200 instead of 1:25, or when adsorption was

allowed to proceed at reduced bacterial concentration in nutrient broth (4×10^8 rather than 6×10^9), the establishment of blendor-resistant infection was only slightly accelerated.

(8) When the adsorption mixture was allowed to stand *undiluted* for 17 minutes at 37 C, then diluted in cold broth and blended, the recovery of plaque forming bacteria was only 2 to 4 per cent of the control (instead of about 40 per cent for a sample diluted after 6 minutes, chilled after 17 minutes, and blended). This effect is not due to a decay of plaque formers in the concentrated mixture since unblended samples gave constant plaque counts over a period of 40 minutes at 37 C. The establishment of infection resistant to blending is thus largely inhibited in the concentrated mixture.

Many of the foregoing observations can be explained by the assumption that the failure to recover the full number of infected bacteria after blending is related to the failure of attached phages to "inject" their desoxyribonucleic acid (Hershey and Chase, 1952). Experiments with P^{32}-labeled T5 phage indeed showed a close but not perfect correlation of phosphorus incorporation, measured by failure of removal of P^{32} by blending, with establishment of infection resistant to blending. An example is given in figure 3, where the blendor-resistant P^{32} content of phage treated bacteria is seen to increase slowly as the preblending incubation is prolonged, but somewhat more rapidly at first than the number of successfully infected bacteria. The rate of P^{32} incorporation was greatly reduced during incubation of undiluted adsorption mixture, just as was the establishment of infection resistant to blending (see above).

We conclude that the establishment of infection resistant to blending by phage T5 is a slow process under the most favorable of the investigated conditions and is correlated, though probably not perfectly, with a similarly slow incorporation of phage nucleic acid by the bacteria.

These results necessitate a reexamination of serological data in reference to the question whether any of the antigen removed by blending belongs to successfully infecting particles. It is necessary to estimate the fraction of antigen initially adsorbed to cells that are actually found infected at the time of blending, and to compare this estimate with the observed recovery of adsorbed antigen. For simplicity, we assume that antigen and phage are distributed together in random fashion among the cells of the culture. We assume further that the probability of irreversible infection of a given cell, with phage attached, during a given period of incubation is independent of the number of phage particles, or amount of antigen, attached to the cell; it will prove unnecessary at the moment to consider alternative assumptions. If *none* of the antigen initially adsorbed to irreversibly infected cells and *all* of the antigen adsorbed to the remainder are recovered by blending, we can calculate an expected deficit of antigen in the low speed supernatant of blended material.

Experiments in which all the necessary measurements were made are summarized in table 1. The second-last column shows the total antigen (eluted plus initially unadsorbed) found in the low speed supernatant. The last column shows the corresponding expectation if no antigen were recovered from irreversibly infected cells. The upper two or three entries are obviously not interesting since the irreversibly infected fraction of all cells receiving phage (B^*/B_p) was small at the time of blending. The other entries show systematically greater recovery than expected on the assumptions stated; the differences are, however, small. In experiment 2, table 1, with the prevailing limit of sensitivity being about 10 per cent of input, no antigen was detected in the low speed pellet from the blended material. It seems likely, therefore, that at least some of the antigen initially adsorbed to irreversibly infected cells was removed by blending. Since many of the cells adsorbed more than one phage and since our purified phage may contain some noninfectious, serologically active, adsorbable particles, it is less certain that any antigen of successfully infecting particles actually persists externally. On the other hand, the experiments give no evidence that any of the adsorbed antigen enters the host cell or is otherwise lost (*see* Rountree, 1951, 1952).

When the serological data of experiment 1, table 1, are compared with the radioactivity data of the same experiment (figure 3), much stronger evidence is obtained that antigen and nucleic acid dissociate during the infective process. Thus, at a time (17 to 27 minutes) when half the initially adsorbed phosphorus is irreversibly adsorbed, essentially all the initially

TABLE 1

Removal of adsorbed phage antigen in the Waring blendor in relation to the recovery of irreversibly infected bacteria

EXPERIMENT	B_0	P_b	A_b	m	$\frac{B_p}{B_0}$	$\frac{B^*_{max}}{B_p}$	BLENDED AT	$\frac{B^*}{B^*_{max}}$	$\frac{B^*}{B_p}$	A_s	A_s, CALC
							min				
1	7.2×10^9	0.98	0.72	2.5	0.92	0.29	7	0.028	0.008	1.02	0.99
							12	0.23	0.067	1.08	0.95
							17	0.38	0.11	0.97	0.92
							27	0.65	0.19	0.98	0.86
2	7.9×10^9	0.95	0.80	1.55	0.79	0.61	17	0.45	0.27	0.95	0.78
3	5.7×10^9	0.96	0.70	2.7	0.93	0.59	17	0.35	0.21	1.18	0.86

B_0 = bacteria/ml adsorption mixture; P_b = fraction of input phage adsorbed; A_b = fraction of input antigen adsorbed; m = adsorbed phage per bacterium; $B_p/B_0 = 1 - e^{-m}$ = fraction of input bacteria receiving phage; B^*_{max}/B_p = fraction of B_p yielding plaques in unblended control; B^*/B^*_{max} = fraction of B^*_{max} irreversibly infected at time shown; A_s = fraction of input antigen found in supernatant from blended sample; A_s, calc $= 1 - A_bB^*/B_p$ (see text). In experiments 1, 2, and 3 the adsorption mixture was diluted, respectively, 1:25, 1:40, and 1:25 in broth after 6, 4, and 7 minutes; and incubation was continued at about 37 C.

adsorbed antigen can be eluted. The evidence for dissociation is even stronger if we consider that the incorporation of phosphorus is nearly complete at 17 minutes and that the elutable P^{32} fraction may be altogether incapable of incorporation (*see* Hershey and Chase, 1952). The suggestion, needed for an alternative explanation, that about 90 per cent of the total antigen of our phage preparation is associated with noninfectious particles could probably be dealt with by comparing the count of total particles, obtained by electron microscopy, with the plaque-count assay.

Composition of terminal lysates. We have seen in figure 2 that the ratio antigen:phage, set at unity for purified phage, was 2.2 for crude lysates. Whatever the content of noninfectious, serologically active material in the purified preparation, the proportion of such material is evidently greater in the lysates. Additional information is given by the following experiments.

Two one step lysates (A and B), similar to the control of figure 2 at 80 minutes, were clarified by low speed centrifugation and centrifuged one hour at 12,000 × G. The pellets were eluted into nutrient broth with gentle agitation; and supernatant and pellet fractions were assayed for active phage and for antigen. As a control for stability of phage during centrifugation, purified phage was diluted in nutrient broth and centrifuged in a similar way. In addition, a portion of lysate B was incubated for 15 minutes at 37 C with host cells concentrated from a growing culture and added to give 1.7×10^9 cells per ml in the mixture and a phage:bacterium input ratio of 1.0. A sample of this mixture plated before lysis for total infective centers gave a count of 1.1×10^9. The remainder was centrifuged at low speed, and the supernatant was assayed for unadsorbed phage (1.6×10^7) and for antigen. The count of infected bacteria (65 per cent of input) was very close to the theoretical value ($1 - e^{-1} = 0.63$), an exceptional but useful result.

The results of the activity assays given in table 2 show that:

(1) The plaque forming activity of purified phage was not reduced appreciably by centrifugation; most of the activity (84 per cent) was recovered in the pellet.

(2) The total recovery of phage in the lysate fractions was only 44 and 58 per cent. It is suggested that the loss of activity occurs when phage and bacterial components are brought together in the high speed pellet; no loss occurs when lysates are allowed to stand uncentrifuged for comparable or longer periods.

(3) The antigen of the lysates was recovered quantitatively during centrifugation, more than half remaining in the supernatant. It seems

TABLE 2

Fractionation of crude lysates

MATERIAL*	ACTIVITY	ONE HR AT 12,000 × G			ADSORPTION TO HOST CELLS
		Per cent recovered			
		Pellet	Supernatant	Total	Supernatant
Lysate A (4.1×10^9)	Phage	51	7.7	58	
	Antigen	34	65	99	
Lysate B (2.0×10^9)	Phage	36	8.5	44	0.9
	Antigen	42	57	99	43
Purified phage (4.4×10^9)	Phage	84	11	95	

* Lysates A and B were used after initial low speed centrifugation to sediment debris; about 15 per cent each of phage and antigen were thus removed or lost. The purified phage was the stock preparation diluted in broth. The numbers in parentheses indicate the phage concentration during high speed centrifugation.

unlikely that the excess supernatant antigen was derived from the phage inactivated during centrifugation although this possibility is not quantitatively excluded.

(4) After adsorption of lysate B with host cells, 1 per cent of the phage and 43 per cent of the antigen remained in the supernatant. The missing phage is accounted for by infected bacteria (see above).

The results indicate that a large fraction of the total antigen of crude lysates does not sediment with phage and a large fraction is not host-adsorbable. The relation of these fractions to each other, or to the noninfectious serologically active materials previously described in phage lysates (for references, see Lanni and Lanni, 1953), has not been studied.

DISCUSSION

Infection of a sensitive bacterium by phage is generally considered to follow rapidly on adsorption if phage and host are mixed in a medium where both processes can occur. Under the conditions of our experiments, however, irreversible infection by phage T5 lags considerably behind adsorption. The rate of establishment of infection throughout a culture is related, though not perfectly, to the rate of incorporation of phage phosphorus (hence, nucleic acid) by the bacteria. Suggestive evidence, needing extensive confirmation, has been obtained (figure 3) that the simple uptake of phage phosphorus is not sufficient to establish infection. The manner in which the over-all effects, summed over the whole culture, reflect the properties of individual phage-bacterium interactions cannot be judged from the available data.

The further inhibition both of phosphorus incorporation and of establishment of infection that occurs in highly concentrated phage-bacterium mixtures is apparently a distinct phenomenon and seems to be related to availability of calcium (Luria and Steiner, 1954), an element known to be essential for the growth of T5 (Adams, 1949; Kay, 1952*a*,*b*).

The concept (Hershey and Chase, 1952) that the infecting particle dissociates into cell-penetrating and externally-persisting fractions is supported in our work by evidence that phage antigen, active in complement fixation, can be stripped almost quantitatively from the cells of an infected culture at a time when considerable phosphorus has been incorporated. When the recovery of antigen is compared, not with phosphorus uptake but with the recovery of actually infected cells, the evidence for dissociability is weaker, for reasons already noted. The latter comparison could be improved somewhat by changing some of the experimental conditions. For example, Luria and Steiner (1954) found that a better recovery of plaque forming bacteria is obtained if chilled samples are prewarmed at 37 C before plating for plaque count. Under any conditions, the experiments should include a critical study of the composition of the phage inoculum, for analysis of the data requires knowledge of the content of serologically active, noninfectious material. The absence of such a study in the experiments by Hershey and Chase (1952) with T2 somewhat weakens their arguments for dissociation of the infecting particles.

Our experiments on intracellular growth yield the type of evidence that one can reasonably expect from a straightforward serological analysis unsupplemented by other devices. Newly formed complement-fixing antigen appears intracellularly a few minutes before newly formed mature phage. For a long period, total

antigen and mature phage increase almost linearly, the former about twice as fast as the latter. Hence, the infected culture contains at all times an excess of antigen over that present in phage. The production of antigen does not appear to cease until phage liberation is complete. The precise meaning of the antigen estimations is obscured by the lack of information about the physical form in which the antigen is present and the serological value of antigen in different forms. Altogether, the results support, although not in a crucial way, the hypothesis that the production of mature phage proceeds through a stage of production of noninfectious, serologically active elements. The discovery and isolation of immature, serologically active particles of T2 and T4 (Levinthal and Fisher, 1952; DeMars *et al.*, 1953; Maaløe and Symonds, 1953) suggest that quantitative serology may be combined profitably with other techniques in future work. If T5 turns out, like staphylococcal phage 3A (Rountree, 1952) and phage T2 (Lanni and Lanni, 1953), to possess multiple antigens, the serological task, although multiplied, will be aided by the opportunity of discerning the development of linkage among the distinct antigens.

SUMMARY

Quantitative complement fixation has been applied to the study of the reaction of phage T5 with homologous rabbit antibody, of the infection of *Escherichia coli*, strain B, with this phage, and of the intracellular development of phage antigen. The infective process can be interrupted by treatment of infected bacteria in a Waring blendor early after phage adsorption. The proportion of bacteria in which infection can be arrested by blending decreases slowly with time. Incorporation of phage phosphorus into the bacteria, as measured by nonremovability of phosphorus by blending, is also slow. Phosphorus incorporation and the establishment of infection resistant to blending are both strongly inhibited at high bacterial concentration. In all cases, blending removes from infected bacteria practically all the complement-fixing antigen of the adsorbed phage in noninfectious form. New phage antigen appears within infected bacteria a few minutes before infectious phage and continues until lysis to increase at a faster rate than infectious phage. Phage lysates contain at least one-half of their phage antigen in noninfectious form.

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