MUTUAL EXCLUSION BETWEEN AN INFECTING PHAGE AND A CARRIED PHAGE

J. J. WEIGLE AND M. DELBRÜCK

California Institute of Technology, Pasadena, California

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When bacteria are simultaneously or consecutively infected by two dissimilar phages, any one bacterium yields, upon lysis, phage particles of one or the other of the parental types, never of both types (Delbrück and Luria, 1942; Delbrück, 1945). This phenomenon has been called mutual exclusion; the mechanism responsible for it is at present unknown.

Recent work by Lwoff and his collaborators has opened for quantitative studies a new field of phage research: lysogenesis. Lwoff and Gutmann (1950) have shown that in lysogenic strains of bacteria the ability to yield phage is carried by the bacteria through many generations. The phage is presumed to be carried intracellularly in a noninfective form called prophage. The conversion from prophage into infective phage can be initiated simultaneously in all the bacteria of a culture by irradiation with a small dose of ultraviolet light (Lwoff, Siminovitch, and Kjelgaard, 1950), or by exposure to certain compounds containing sulfhydryl groups (Lwoff and Siminovitch, 1951). About 60 minutes after applying the inducing treatment, each bacterium lyses, liberating many phage particles.

We were interested in testing whether mutual exclusion would occur between carried phage, on its way to maturity by virtue of the inducing treatment, and a different phage introduced from the outside at various times during the maturation period. If the external phage can exclude the carried phage, then any mechanism of exclusion operating by erecting a block at the invasion stage of the bacterium by the phage would be ruled out.

A system which is suitable for such studies consists of the *Escherichia coli*, strain K12, its carried phage λ, and one of the phages of the T series. Strain K12 is sensitive to the seven phages of the T series, but T5 was employed in this study because of a combination of two advantageous features. First, it registers on strain K12 with the same efficiency of plating as on strain B, its normal host strain. Second, its long latent period gives greater freedom for a number of manipulations. A disadvantage of phage T5 is its very low rate of adsorption on strain K12, even lower than on strain B. This complicates the techniques in various ways and necessitates additional controls.

MATERIALS AND METHODS

Bacteria. Escherichia coli, strain K12, well-known from the important experiments on genetic recombination, was used in the wild-type form. Its lysogenicity was discovered only recently by E. Lederberg (personal communication). The generation time of strain K12 in our nutrient medium is 20 minutes at 37 C.

As an indicator strain for λ (the phage carried by K12), we used a bacterial strain we call S, kindly supplied us by Mrs. Lederberg. Strain S is a derivative of strain K12 which arose in certain experiments of Lederberg after treatment with ultraviolet light.

Although all the phages of the T series will produce plaques when plated with strain K12, their efficiencies of plating are widely different (Benzer *et al.*, 1950). Phages T1 and T5 plate with an efficiency of 100 per cent, T2, T4, T6 with intermediate efficiencies, T3 and T7 with very low efficiency. In the cases of low efficiency of plating, strain K12 may be resistant to the wild type phage and sensitive to host range mutants. Strain S is resistant to T1 and T5; its sensitivity to the other phages of the T series is similar to that of K12.

Phage λ. High titer stocks of this phage were obtained from K12 by induction of phage production with UV (the Lwoff effect). These stocks were purified by differential centrifugation. Electron micrographs made from the purified preparations show phage λ to be a large one (head: 1,000 A diameter; tail: 3,000 A long, 200 A thick) about the same size and shape as T5. Notwithstanding the similarity in size between λ and T5, the two phages differ enormously in their resistance to ultraviolet light (UV), λ being the more resistant by a factor of 13 (see figure 1). Chemical studies on purified λ have not yet been made but are certainly desirable in view of this radiological abnormality of λ. A specific antiserum with high neutralizing activity against λ was obtained by injection of rabbits with the purified λ preparations. The serum showed no neutralizing activity against any of the phages of the T series, nor did antisera against any of the phages of the T series show any activity against λ.

Phage T5. This phage has been studied in detail by Adams (1950). The specific antiserum against T5 used in the present experiments gave a neutralization of 99.98 per cent in 10 minutes at a dilution of 1:100.

Plating of λ *on strain S.* Plating is done by the agar-layer technique (Adams, 1950). The plaques are fully developed in about 10 hours at 37 C. The plaque diameter averages around 1 mm and varies greatly. Adsorption of λ on S is fast (about 80 per cent in 8 minutes at a bacterial concentration of 5×10^7 per ml). The relation between strain S and λ exhibits a curious feature. In any culture of S a certain proportion of the bacteria is resistant to λ in the sense that these bacteria can form colonies in the presence of excess λ. This resistance appears to be in the nature of a physiological state. Its inheritance is, at best, very fleeting. The resistant forms adsorb λ without being killed by it and without reproduction of λ taking place. To obtain good plaques of λ it is essential that the proportion of these resistant forms in the plating mixture be small. Saturated cultures of strain S contain approximately 10 per cent of the resistant forms while young cultures are almost 100 per cent resistant (Lieb, unpublished).

Media. (a) Culture medium: Difco tryptone broth, 8 g per liter, plus 0.5 per cent NaCl. For solid media 1.1 per cent agar in the bottom layer, 0.7 per cent in the top layer.

(b) Buffer: 1/15 M phosphate buffer, plus 10^{-3} M $MgSO_4$, plus 0.5 per cent NaCl.

Irradiations with UV. A 15 watt G. E. "germicidal" lamp, emitting 80 per cent of its energy in the wave length 2537 A, supplied by a "sola" voltage stabilizer, was used as a UV source. Samples were irradiated in a layer 1 to 2 mm thick at a distance of 80 cm from the center of the lamp. The intensity at the sample was of the order of 3×10^2 ergs per sec per cm^2. A more convenient reference for the intensity may be the fact that the irradiation gave 99 per cent inactivation of phage T2 in 40 seconds.

Plating with mixed indicators. The indicator strains B and S were grown overnight in aerated test tubes and were used in the amounts of 2 drops of B plus 1 drop of S per plating tube. The plaques of λ came out a little better if the S bacteria were added to the plating tubes about half an hour before plating and were kept at 45 C during this half hour. Since the plaques produced by T5 and by λ are both small, there is very little danger of confusion between true mixed yielders and accidental overlaps. The truly concentric plaques stand out at a glance on a plate with several hundred plaques.

EXPERIMENTAL RESULTS

Lysogenicity of K12. In a normal culture of K12 very few bacteria lyse and liberate phage. In contrast to the relation between lysogenic *Bacillus megaterium* and its carried phage, we find that phage λ is not adsorbed at a measurable rate on K12. The liberated phage thus remains free in the culture. In a growing culture there is an approximately constant proportion of one free phage to one thousand bacteria. One can account for this constant proportion by assuming that there is a constant probability per time unit for any one of the bacteria to be induced to λ production, thus liberating, after a maturation period, a number of phages. If we assume a burst size value of 150 phages per induced bacterium and a maturation period of 60 minutes (as observed when λ production is induced by irradiation with UV), this probability is 5×10^{-5} per division time.

K12 bacteria plated on S should form plaques with a central colony, one such plaque for each bacterium plated. Because of the very low rate of phage liberation from normal K12, only about 1 per cent of these centered plaques reach a size visible to the naked eye.

The Lwoff effect: Induction of phage production by ultraviolet light. Lwoff, Siminovitch, and Kjelgaard (1950) have found that a small dose of UV given to *B. megaterium* initiates the production of mature phage and lysis of the bacteria. K12 exhibits the same phenomenon. Only the facts relevant to the technique of our experiments will be described here. Bacteria from a growing culture are diluted in buffer (or centrifuged and resuspended in buffer) before irradiation. After irradiation the bacteria are returned to broth and incubated at 37 C.

Under our conditions of irradiation a dose of 80 seconds induces more than 95 per cent of the bacteria to phage production. This is indicated by the increase in the number of plaques and by the decrease in the number of colonies produced by a sample of the irradiated bacteria, as compared to the corresponding num-

bers obtained from parallel samples of noninduced bacteria. The plaque count rises from its background value of 1 per cent to a number equal to the colony count before induction. The plaques formed by the induced bacteria are large and very regular in size compared to those formed by single λ particles. The colony count drops to about 0.2 per cent of its value before induction. With a dose of only 10 seconds, about 30 per cent of the bacteria show up as plaque formers. With doses larger than 80 seconds, the number of induced bacteria showing up as plaque formers decreases exponentially. In figure 1 the fraction of induced bacteria is given as a function of the UV dose. Once induction is established, the decrease in the number of induced bacteria follows a multiple target curve with a multiplicity between 3 and 4 and an ultimate slope equal to that of the inactivation curve of free λ. A detailed discussion of this important

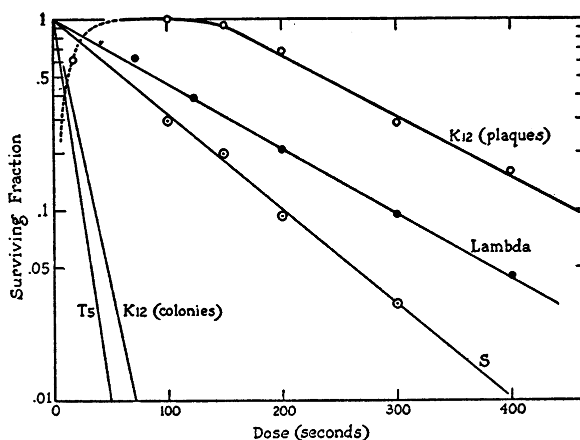

Figure 1. Semilogarithmic plot of the UV surviving fractions of free T5, of free λ, of S, of K12 as a colony former, and of K12 as a plaque former on S. The doses are given in seconds of irradiation as described in the text.

curve is deferred to a later paper concerned with quantitative aspects of the radiobiology of the Lwoff effect.

Precise measurements of the number of induced and noninduced bacteria are complicated by the fact that the handling of the bacteria involved in plating may produce a reversal of the induction in a small fraction of the bacteria. The amount of this reversal depends on the time of incubation in broth after irradiation. If the bacteria are incubated for 50 minutes in broth at 37 C, there is practically no reversal. Therefore measurements of induced bacteria and of surviving bacteria are best made at this time.

The induction effect is reversible by irradiation with visible light. In the experiments reported here the amount of visible light received by the bacteria was kept below the limit causing appreciable reversal.

It is pertinent to compare the dose of UV causing induction (and thereby death of the bacteria) with the dose that would cause death of the bacteria in

the absence of the carried phage. The only appropriate strain available for this comparison is the indicator strain S. The dose of 80 seconds which kills more than 99 per cent of K12 by induction kills only 50 per cent of the phage-free indicator strain S (see figure 1). This makes it seem unlikely that induction is an effect of processes that would lead to the death of the cell even in the absence of the carried phage.

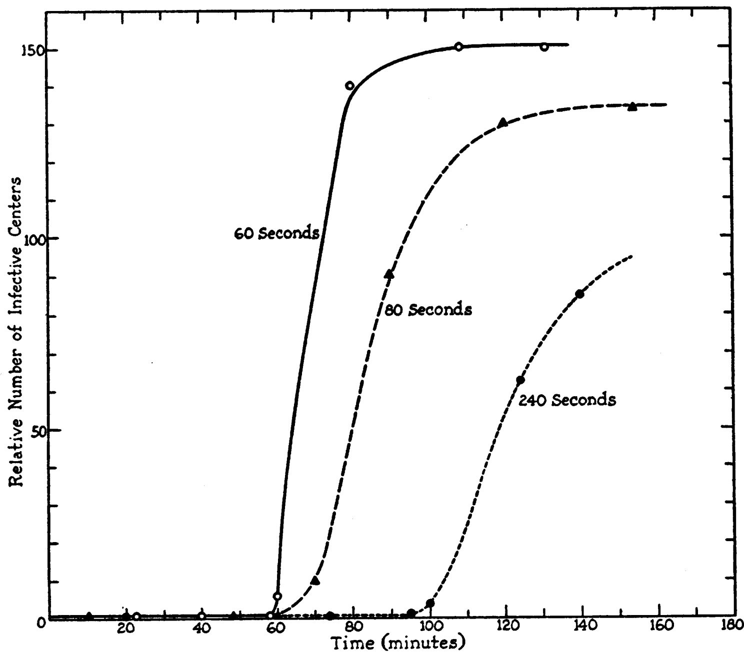

Figure 2. One step growth curves of λ liberated by K12 induced with different doses of UV. A growing culture of K12 containing 1.3 × 10^8 bacteria per ml was diluted 1:100 in buffer and irradiated for 60, 80, and 240 seconds. After irradiation dilutions of 1:500 into broth at 37 C for the first growth tubes and further dilutions of 1:150 for the second growth tubes were made. From these tubes aliquots were plated on S at the times after irradiation indicated on the abscissae. The ordinates give the ratio between the number of infective centers at the different times and the average number of infective centers before the onset of lysis.

Microscopic observations show that the induced bacteria double in length and thickness during the latent period between irradiation and lysis. They do not divide. At the end of the latent period they suddenly assume a spherical shape and burst a few seconds later, leaving a mass of debris.

Figure 2 gives growth curves of λ liberated from K12 after induction with different doses of UV. For a dose of 80 seconds or less, the latent period is 60 minutes, the burst size about 150, and the rise period 20 to 40 minutes. For larger doses, at which an appreciable fraction of the induced bacteria are killed as infective centers, the latent period and the rise period increase and the burst size decreases.

If the induced bacteria are incubated in broth at 45 C, the latent period is reduced to about 38 minutes.

Premature lysis of induced bacteria. Noninduced bacteria infected with T5 lyse, after 35 minutes, yielding only T5 and no λ. After induction there must be some stage in the maturation process of λ at which infection with T5 cannot any longer prevent the production of mature λ by the bacterium. It is important

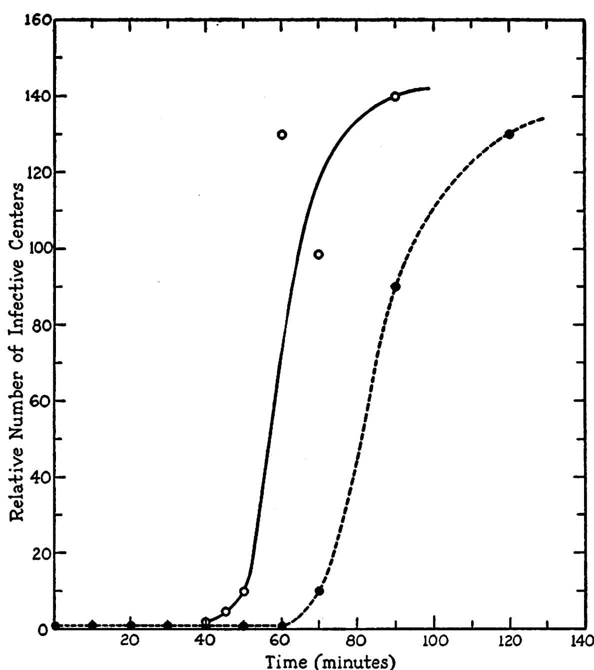

Figure 3. Premature lysis of induced K12. A growing culture of K12 was centrifuged, resuspended in buffer, and irradiated for 80 seconds. The suspension was recentrifuged and resuspended in broth at 37 C. Dilutions in broth at 37 C were made for the first and second growth tubes. At the times after induction indicated on the abscissae, aliquots were plated on S for the control growth curve of λ. At the same times, other dilutions were made into 0.01 M KCN diluted in broth. These tubes were kept at 37 C for 30 minutes. After a further dilution of 1:100, aliquots were plated on S. The ordinates represent the ratio of the number of infective centers at various times to the average number of infective centers before this number begins to rise. The solid line represents the growth of λ in the control culture and the broken line its growth in the bacteria treated with KCN.

to relate this stage to the stage at which mature λ first makes its appearance within the cell. Since the prophage is not infective, this stage can be ascertained by Doermann's technique of stopping phage growth and causing the cells to lyse prematurely by exposure to 0.01 M KCN (Doermann, 1948, 1949). We have performed such experiments with induced K12 bacteria, applying the KCN at various times after irradiation, and leaving the samples for 30 minutes at 37 C in KCN before plating. The results are shown in figure 3, together with a control growth curve of λ in the absence of KCN treatment.

The control growth curve shows a latent period of 60 minutes and a burst size of 130. The experimental curve shows a constant number of infective centers, equal to the number of treated bacteria, during the first 40 minutes. This is analogous to Doermann's finding with strain B infected with a phage of the T series. According to Doermann it is interpreted not as a content of one infective particle per bacterium, but as a failure of the KCN treatment to break open bacteria prior to the appearance of mature phage. At these earlier stages the KCN treatment merely inhibits the bacteria from further development, and the

TABLE 1

Premature single bursts of induced K12 bacteria

Bacteria (1.3×10^8 per ml) induced by a dose of 80 seconds of UV were suspended in broth at 37 C and aerated. After 40 minutes of incubation and a dilution in broth by 10^7, one drop of suspension (4.5×10^{-9} of the original suspension) was put into each of 98 dilution tubes and kept at 37 C. At 50 minutes these tubes were chilled and one drop of a 0.02 M solution of KCN in broth was added to each tube. The tubes were put again at 37 C and one hour later the content of each tube was plated on S.

NO. OF PLAQUES	NO. OF PLATES	NO. OF PLAQUES	NO. OF PLATES
0	62	14	1
1	7	16	1
2	4	18	1
3	5	20	1
5	3	21	1
6	1	23	1
7	1	25	1
9	1	26	1
11	2	27	1
12	1	38	1
13	1		
		Total 342 plaques	98 samples 36 samples with λ yields

Average number of bacteria per sample
= 0.58 (from colony count of noninduced bacteria)
= 0.57 (from plaque count of induced bacteria)
= 0.47 (from fraction of samples without λ yield).
Average burst size = 7.5.

inhibition is removed when the KCN is diluted at the time of plating. When the bacteria at these stages are actually lysed by other means, no infective particle is found.

Between 40 and 45 minutes there is a sharp increase in the number of infective centers. We conclude that infective phage particles appear in some of the induced bacteria between 40 and 45 minutes after irradiation.

The time of appearance of the first infective particle might vary greatly within the bacterial population. The growth curve just given does not tell us what fraction of the bacteria contained infective particles. The question can be answered by a single burst experiment combined with KCN treatment. Table 1

gives the result of such an experiment in which the induced bacteria were exposed to KCN 50 minutes after irradiation. The results show that at the time of treatment 28 out of 36 bacteria, or approximately 80 per cent, yielded more than one phage particle. The remaining 7 bacteria gave one infective center. From the burst size distribution, it appears likely that most of these 7 were not bacteria containing just one infective λ particle but bacteria that had not been lysed by the KCN treatment. Presumably, then, they represent the fraction of bacteria which does not yet contain a mature phage particle at 50 minutes.

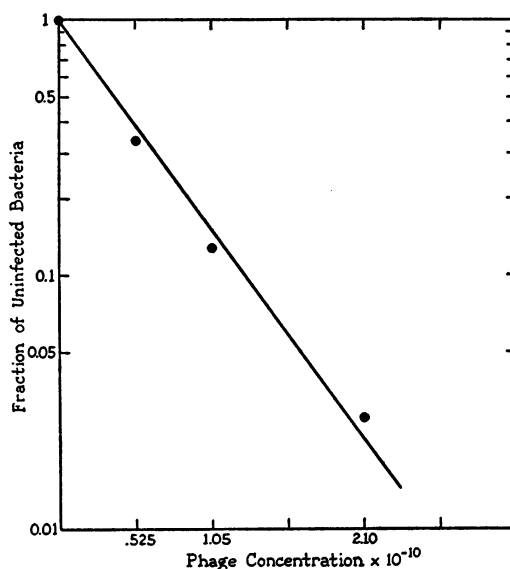

Figure 4. Measurement of adsorption of T5 on K12 by counting noninfected bacteria, at various concentrations of T5. Mixtures were made of a growing culture of K12, at a concentration of 1.06×10^8 per ml, and T5 at various concentrations (indicated on the abscissae). After five minutes at 37 C, dilutions were made into anti T5 serum (diluted 1:100) and after 10 minutes of serum action aliquots were plated for colony count of bacteria not infected with T5. The figure is a semilogarithmic plot of the fraction of the bacteria not infected versus the concentration of phage in the adsorption tubes. No corrections were made for growth of the uninfected bacteria before plating.

We conclude from the two experiments on premature lysis that: (1) mature phage particles begin to appear in induced bacteria between 40 and 45 minutes after irradiation, and (2) at 50 minutes after irradiation, about 80 per cent of the bacteria contain more than one mature phage particle.

Adsorption of T5 and K12. For the experiments on mutual exclusion between T5 and λ it is of crucial importance to know with a fair degree of accuracy how many bacteria have actually been infected with T5. As mentioned in the introduction, T5 is very slowly adsorbed on K12, of the order of 2 to 4 per cent in five minutes at bacterial concentrations around 10^8 per ml. Obviously the adsorption cannot be determined by measuring the decrease of free phage. The com-

plementary method of measuring the number of infected bacteria after eliminating the free phage with specific antiserum can be used for noninduced bacteria; but it is open to doubt in the case of induced bacteria since in some of the infected bacteria T5 may not be produced, by virtue of the very phenomenon we want to study, mutual exclusion, in this case λ excluding T5. In noninduced bacteria this is not likely to occur, for several reasons. First, T5 plates on K12 with the same efficiency of plating as on strain B. Second, infection of K12 with T5 does not lead to an increased plaque count of λ on S. Third, the application of this method gives results which are in agreement with an independent estimate of the rate of adsorption of T5 on K12, obtained by making a multiple infection with T5 and measuring the fraction of surviving bacteria. According to Poisson's distribution, the fraction of uninfected cells is e^{-n}, where n is the average multiplicity of infection. Figure 4 gives the result of an experiment in which bacteria at a concentration of 1.06×10^8 per ml were exposed for 5 minutes to various concentrations of T5, as indicated on the axis of abscissae. Figure 4 shows that the surviving bacteria decrease approximately exponentially as required by Poisson's law. A survival of $e^{-1} = 0.37$, corresponding to a multiplicity of 1, is obtained at a ratio of phage to bacteria of about 50 to 1. This corresponds to an adsorption of 2 per cent. This estimate of the adsorption is probably a little too low since the estimates of surviving bacteria were not corrected for growth before plating.

With this approximate information on hand, we now turn to the method of measuring infective centers after elimination of free phage with specific antiserum. Table 2 gives an experiment in which bacteria at a concentration of 8.3×10^7 per ml were exposed for 5 minutes to a low concentration of T5. The adsorption period was terminated by a dilution of 1:10 into specific antiserum. This experiment was run in parallel with noninduced bacteria and with induced bacteria incubated in broth after irradiation for various lengths of time. The count of infective centers represents bacteria infected with T5 and liberating T5. For infections with T5 taking place up to 24 minutes after induction, the count of T5 infective centers is sensibly the same as for noninduced bacteria. It amounts to slightly less than 4 per cent of the phage input. This is in reasonable agreement with the estimate obtained by the first method. The fact that the count does not drop appreciably up to 24 minutes after induction indicates that at least up to this time λ does not exclude T5. It does not say whether any of the T5 yielders are or are not also λ yielders. The counts of infective centers obtained from adsorptions occurring at 40 and 51 minutes after induction are very much lower than those from noninduced bacteria. The experiment does not tell whether this is caused by a reduced ability of these bacteria to adsorb T5 or by a failure of a proportion of the bacteria to yield T5 when infected at such a late stage in the maturation process of λ.

The point of greatest interest with respect to mutual exclusion is the proof that the residual T5 yielders obtained from infections at 40 and 51 minutes actually represent bacteria which in the absence of infection with T5 would have proceeded to liberate λ. This is shown convincingly by the drop in the count

of T5 yielders obtained from still later infections. As seen in table 2, this count drops down during the rise period of λ to the background level of serum survivors.

TABLE 2

T5 yielders among induced bacteria singly infected with T5 at various times after induction

A growing culture of K12 was centrifuged and resuspended in buffer. Half of the suspension was irradiated for 80 seconds and both the irradiated and nonirradiated suspensions were centrifuged and resuspended in broth at 37 C and aerated. The times given in the first column are the incubation times in broth between induction and infection with T5. The nonirradiated suspension was assayed by colony count for the number of bacteria. The irradiated suspension was assayed at 46 minutes by plating on S to measure the number of induced bacteria. At the times indicated in the table (at time zero for the nonirradiated suspension) 0.9 ml of the suspension in broth was mixed with 0.1 ml of phage suspension. Five minutes at 37 C were allowed for adsorption, then 0.1 ml of the mixture was placed in 0.9 ml of anti T5 serum diluted 1:100 in broth. After ten minutes in the serum a convenient dilution was plated on B; the number of plaques on these plates thus gives the number of T5 infective centers.

Exp. 108 In adsorption tube of noninduced bacteria 8.3×10^7 bacteria per ml
6.3×10^7 T5 per ml
In adsorption tube of induced bacteria 6.9×10^7 bacteria per ml
6.3×10^7 T5 per ml.

INFECTION WITH T5 AT	T5 PLAQUES PER ML $\times 10^{-6}$	PER CENT OF T5 INPUT	PLAQUES FROM IND. BACT. / PLAQUES FROM NONIND. BACT.
4 minutes	2.5	4.0	1.05
24 minutes	2.1	3.4	0.92
40 minutes	0.6	1.0	0.26
51 minutes	0.4	0.6	0.08
noninduced bact.	2.4	3.8	1.00

Exp. 118 In adsorption tube of noninduced bacteria 5.4×10^7 bacteria per ml
7.9×10^8 T5 per ml
In adsorption tube of induced bacteria 5.8×10^7 bacteria per ml
7.9×10^8 T5 per ml.

INFECTION WITH T5 AT	T5 PLAQUES PER ML $\times 10^{-6}$	PER CENT OF T5 INPUT	PLAQUES FROM IND. BACT. / PLAQUES FROM NONIND. BACT.
1 minute	16	2.0	1.00
23 minutes	11	1.4	0.69
40 minutes	5.4	0.69	0.34
51 minutes	2.0	0.25	0.12
73 minutes	0.51	0.07	0.03
85 minutes	0.26	0.03	0.016
noninduced bact.	16	2.0	1.00

Multiple infection of noninduced or induced K12 with T5 causes an appreciable amount of lysis from without (Benzer *et al.*, 1950) at an average multiplicity of infection as low as five. This may be seen from the experiment reported in table 3, in which noninduced and induced bacteria (at various times after

induction) were exposed to an excess of T5 and the infective centers were counted after reducing the free phage to a low level with specific antiserum. Parallel samples were plated on strain B, the indicator for T5, and on strain S, the indicator for λ. In each case the sum of the counts of infective centers amounts to only about 40 per cent of the total number of treated bacteria. The fraction of uninfected bacteria is negligible in these cases. Presumably, the remaining 60 per cent perish as a result of lysis from without. The counts of λ plaques are very small (around 1 per cent) compared to the counts of T5 yielders in the case of noninduced bacteria and for induced bacteria infected with T5 at 2.5 and 20 minutes. In the case of infection with T5 at 37 minutes, the T5 yielders are still four times more numerous than the λ plaque count. It is likely that these λ plaques are not caused by true λ yielders but by bacteria in which

TABLE 3

T5 and λ infective centers from induced bacteria multiply infected with T5 at various times after induction

Procedure was similar to that described in table 2, except that additional aliquots were plated on S to measure the number of λ infective centers. Spontaneous lysis always produces a certain number of free λ particles in a culture of K12. In this experiment the number of free λ particles in the nonirradiated suspension before infection with T5 was 1.7×10^5 per ml. Free T5 surviving the serum action amounted to about 0.4×10^7 per ml (compared with from 6.6×10^7 to 5×10^7 infective centers).

INFECTION WITH T5 AT	INPUT		INFECTIVE CENTERS	
	bact./ml	T5/ml	T5/ml	λ/ml
2.5 minutes	1.5×10^8	2.8×10^{10}	6.6×10^7	4.5×10^5
20 minutes	1.5×10^8	2.8×10^{10}	6.1×10^7	21.0×10^5
37 minutes	1.5×10^8	2.8×10^{10}	5.0×10^7	130.0×10^5
noninduced bact.	1.4×10^8	2.8×10^{10}	6.5×10^7	3.0×10^5

some mature λ particles were present at the time of infection with T5 and that these mature λ particles were liberated by lysis from without by T5. This is shown by the data presented in table 4, in which the λ counts are given for samples from the tube infected at 37 minutes, plated after various times of further incubation. Table 4 shows that the λ count stays constant up to 73 minutes and then increases only slightly by an amount that can be accounted for by liberation of λ from bacteria that had escaped infection with T5.

We conclude that infection with T5 causes either multiplication of T5, or lysis from without with liberation of mature λ particles present at the time of infection. Completion of the maturation of λ occurs in at most one per cent of bacteria multiply infected with T5 at 37 minutes after induction.

Mutual exclusion between T5 and λ. The experiments reported so far have shown that infection with T5 leads to the production of T5 in every noninduced bacterium, and also in the majority of the induced bacteria when the infection is made prior to the time when mature λ particles begin to appear in some of the bacteria. We now turn to the question whether or not any of the T5 yielders

also yield λ. Manifestly, this does not occur if the T5 infection is made during the early part of the maturation period of λ; the experiments on multiple infection with T5 show that the count of λ yielders is quite negligible for early infections. For later infections the number of λ yielders among the bacteria infected with T5 cannot be assessed, for several reasons. If a multiple infection with T5 is made, a considerable amount of lysis from without ensues, liberating mature λ particles present in the cells. These liberated particles will simulate cells in which T5 was excluded and λ production went on to completion. If the infection with T5 is lowered to an average of one adsorbed T5 per bacterium, so as to minimize lysis from without, 30 to 40 per cent of the cells will not get infected with T5, and will certainly produce λ. It is then impossible to assess whether

TABLE 4

Plaque counts of λ from bacteria multiply infected with T5 at 37 minutes after induction

This experiment followed the growth of λ in the suspension of the experiment described in table 3 in which infection with T5 occurred at 37 minutes after induction. The times noted below measure the time of incubation in broth at 37 C after induction. The normal latent period of λ is 60 minutes. The number of bacteria induced by the dose of 80 seconds of UV was 1.5×10^8 per ml as given in table 3. The number of bacteria not induced by this dose is approximately 5×10^5 per ml.

TIME BETWEEN INDUCTION AND PLATING (MINUTES)	T5 INFECTIVE CENTERS PER ML	λ INFECTIVE CENTERS PER ML
52	5.0×10^7	1.3×10^7
65		1.3×10^7
73		1.4×10^7
78		3.4×10^7
90		13.4×10^7

there is an additional small number of λ yielders among the cells that did get infected with T5.

We therefore confine our attention to the possible existence of mixed yielders, i.e., bacteria yielding both T5 and λ. The most sensitive technique for the detection of such bacteria consists in plating the infected bacteria before lysis on a mixture of the indicator strains for the two phages. On such a mixture, only bacteria that liberate at least one phage particle of each kind give a clear plaque. The infections with T5 have to be done as late as possible during the maturation period of λ to get any λ yielders at all. Here we are limited by technical reasons, however. If the infection with T5 is made with a multiplicity of approximately one, plating must be completed before 60 minutes, at which time the bacteria not infected with T5 begin to burst, swamping the plates with λ plaques. With an adsorption period of 5 minutes, and a serum action period of 10 minutes, and about 7 minutes for plating operations, this condition fixes 37 minutes as the latest point at which a low multiplicity infection with T5 can safely be made. If the infection with T5 is made so highly multiple that there is only a negligible number of bacteria not infected with T5, then we are free of the

limitation just mentioned. However, multiple infection causes lysis from without and liberation of mature λ present at the time of infection. Thus, also in this case, the infection with T5 must not come too late or else the plates will again be swamped by plaques due to free λ particles.

Table 5 summarizes the results of three experiments designed according to the arguments just given. In two of the experiments the multiplicity of infection with T5 was high, causing a certain amount of lysis from without. The infections were made at 45 and 50 minutes, respectively. In the first experiment, the ratio of T5 plaques to λ plaques was about 1:2, in the second experiment about 1:8. In the third experiment the average multiplicity of infection with T5 was close to one, and little, if any, lysis from without occurred. This infection was made at 37 minutes after induction. In this experiment the T5 plaques were slightly in excess of the λ plaques. In each of the three experiments only about 1 per cent of the T5 plaques contained a concentric λ plaque.

TABLE 5

Mixed indicators experiments

The same procedure was followed as described in table 2, except that in exp. 86 the anti T5 serum was allowed to act during 8 minutes only. The bacteria of exp. 98 were tested in a parallel experiment at the time of infection with T5 for mature phage particles. The results of this test with KCN have already been given in table 1.

	Exp. 86	Exp. 98	Exp. 119	
T5 per ml	3.2×10^{10}	4×10^{10}	2.7×10^{9}	
Induced bacteria per ml	2.3×10^{8}	1.2×10^{8}	1.1×10^{8}	
Approximate multiplicity	6	13	1	
Infection with T5 at	45	50	37	
T5 yielders per ml	5×10^{7}	1×10^{7}	4.2×10^{7}	
λ yielders per ml	1×10^{8}	7.9×10^{8}	3.1×10^{7}	
Number of plates	24	10	16	16
Av. no. of T5 plaques per plate	50	9	84	42
Av. no. of λ plaques per plate	100	790	77	39
Total no. of T5 plaques	1200	88	1280	640
Total no. of concentric plaques	10	2	11	7

We conclude that λ is excluded from at least 99 per cent of the T5 yielders even when infection is made at a time when the majority of the bacteria already contain mature λ.

Our next experiments are aimed at a closer characterization of the influences of λ on T5, specifically, on the latent periods and burst sizes of the T5 yielders. In the case of mixed infections with dissimilar phages it is known (Delbrück, 1945) that the excluded virus may exert a strong depressor effect on the yield of the infecting virus.

One step growth curves of T5 on noninduced and on induced K12. Infections were made at low multiplicity of T5 (about 0.02), and complete growth curves were obtained by plating samples at various times after infection. Some of the growth curves are shown in figure 5. The latent periods could be determined with fair accuracy, and they are collected in table 6. The latent period of T5 is

TABLE 6

Latent periods of T5 in noninduced and in induced bacteria

These latent periods measure the time interval between infection and the onset of T5 liberation. They were obtained from one step growth curves of the type given in figure 5.

INFECTION WITH T5 AT	LATENT PERIOD
6 minutes	32–35 minutes
25 minutes	34–35 minutes
40 minutes	28–30 minutes
50 minutes	20–25 minutes
noninduced	34–36 minutes

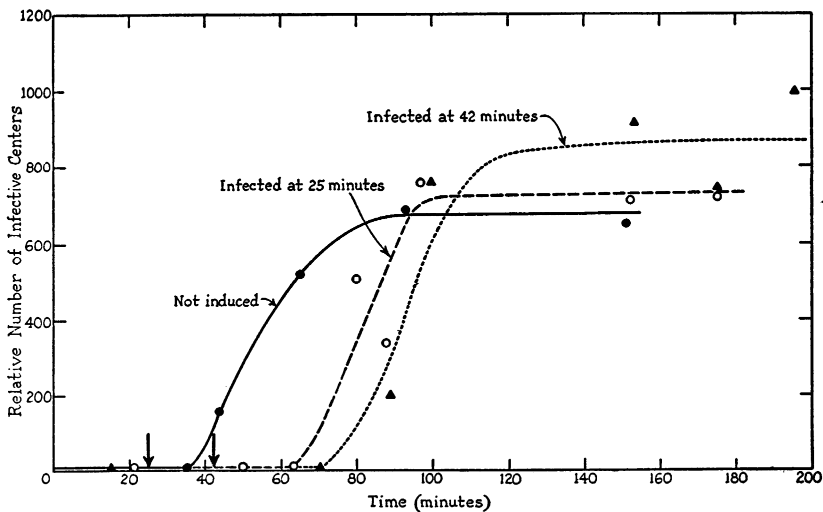

Figure 5. One step growth curves of T5 infecting K12 at various times after induction. The ordinates indicate the number of infective centers divided by the average number of infective centers before the onset of lysis.

35 minutes for noninduced bacteria. For induced bacteria, the latent period remains at the same value for infections made up to 25 minutes after induction, i.e., up to the time at which the onset of liberation of T5 coincides with the time (60 minutes after induction) at which liberation of λ begins in the bacteria that are not infected by T5. When infection with T5 is made still later, the length of the latent period is slightly reduced.

The average burst size of T5 on noninduced and induced K12 varies considerably from experiment to experiment (between 500 and 2,000). However, no

trend towards smaller burst sizes with later infections has been noted, even when the infections take place as late as 50 minutes after induction.

We conclude that if T5 is not excluded, it multiplies as though no λ were present, except that the latent period is slightly reduced when infection takes place during the last stage of the maturation period of λ.

In previous experiments on interference it had been observed that the latent period of the successful phage retains the value it has in unmixed infection. In our experiments the latent period of T5 becomes shorter when the infection with T5 takes place in bacteria containing almost mature λ, although the yield of T5 does not seem to be affected. One might ask whether the presence of almost mature λ particles in a bacterium infected with T5 and liberating T5 with a normal yield, facilitates the multiplication of T5. Only a detailed analysis of growth curves of T5 obtained from infections made during the rise period could answer this question. We have mentioned that on this phase our experiments were not reproducible from day to day.

Chacterization of the T5 particles multiplied in induced bacteria. The T5 particles multiplied on induced bacteria infected with T5 at 45 minutes after induction were tested for their similarity with the parental T5 particles, multiplied on strain B, by measuring their resistance to UV and their sensitivity to specific anti T5 serum. The UV sensitivity was followed down to a survival of 10^{-3}. No difference between the sensitivity of T5 particles multiplied on induced K12 bacteria and those multiplied on strain B was detected. Similarly, the serum inactivation tests revealed no difference between the two types of T5 particles.

DISCUSSION

When mutual exclusion was first discovered, for the pair of phages T1 and T2 (then called *alpha* and *gamma*, Delbrück and Luria, 1942), the phenomenon was interpreted in terms of a key enzyme, present in a single copy in each cell, necessary for the production of phage, and completely monopolized by the first phage particle that engages it. This hypothesis was dropped (Delbrück, 1945) when it was found in experiments with the pair T1 and T7 (then called *alpha* and *delta*) that mutual exclusion operates with an efficiency better than 99 per cent. It was argued that a key enzyme would have to duplicate some time before each cell division, and that it was not reasonable to assume the presence of only one key enzyme in more than 99 per cent of the cells of a growing culture. In the paper just quoted the hypothesis of the key enzyme was replaced by the penetration hypothesis. It was assumed that the first phage which penetrates into the cell causes the entire cell membrane to become impermeable to other phages. Very recently this hypothesis has received a striking support for the large phages T2, T4, T6, and the unrelated phage T5 (Lesley, French, Graham, and van Rooyen, 1951 *a* and *b*). When strain B is infected with any one of these large phages, and this primary infection is followed a few minutes later by a secondary infection with T2 labeled with P^{32}, the labeled T2 is rapidly degraded into low molecular weight fragments. The first infection stimulates the bacteria to break down the late comers.

To understand the relation of this observation to mutual exclusion, we must

discuss in more detail observations concerning mixed infections with pairs of *related* phages. Mutual exclusion was first studied for pairs of very dissimilar phages (T1 and T2, T1 and T7). It was assumed that it would also occur between similar phages and, in fact, between several phages of the same strain. The reason for making this assumption was the observation that the latent periods are exactly the same in the case of single infection as in the case of multiple infection with several particles of the same kind. If, because of mutual exclusion, the multiple infections were in fact single infections, the identity of the two latent periods would be explained. This notion was shattered, however, when Hershey (1946) discovered that T2 and T2r will multiply together in the same bacterium. This finding was soon vindicated by others and generalized to other cases of pairs of mutants and to pairs of serologically related phages. In fact, the entire field of study of recombination of genetic markers in phages is predicated on the nonoccurrence of mutual exclusion in these mixed infections. There is, however, a residue of mutual exclusion also in this case. It becomes apparent when infections with T2 and T2r are spaced with a time interval. If T2r arrives on the bacterium two minutes later than T2, only a small fraction of the bacteria will carry the r marker in any of its phage progeny (Dulbecco, 1951). It is this exclusion we see the reflection of in the experiments of Lesley *et al.* (1951 *a* and *b*) on the breakdown of labeled phage.

We find it hard to believe, however, that this stimulation of the bacterium by an infecting phage particle, causing it to break down later coming phage particles, contains the whole of the explanation of the mutual exclusion effect. In fact, the small phages, T1, T3, and T7, do not stimulate the bacterium to the point of causing the breakdown. It might be argued that the stimulus given by these phages is a weaker one, and that the corresponding response of the bacterium is weaker too, carrying the bacterium only to the point where it becomes impenetrable for the other phages without actually breaking them down. In support of this supposed weakness of the stimulus one might cite further the fact that the small phages are certainly slower than the large phages in establishing exclusion. This is shown by the fact that the large phage, even if it arrives several minutes after the small one, can still exclude it. To explain the miraculous efficiency of mutual exclusion solely by the effect of the first phage rendering the cell membrane impermeable to other phages, one is forced to assume further that a small phage, even though it excludes with some delay, does so at the precise moment at which it itself enters the bacterium. Otherwise, the absence of cases in which, say, both T1 and T7 multiply in the cell, would be unexplained.

The penetration hypothesis runs into even greater difficulties when the specificity of the mutual exclusion effect is considered. The experiments require, in fact, that exclusion is specific for *dis*similar phages. Thus, if the two phages arrive simultaneously, T2 will never exclude a phage that differs from it by only one or two mutational steps. It will exclude the serologically related phage T4 in a certain fraction of the bacteria, and it will completely exclude the totally dissimilar T1.

Let us now turn to the discussion of the results of the present investigation. We find in the first place that the carried phage in the prophage condition, i.e., in noninduced bacteria, does not exclude T5. This in itself is not a new result since it has been shown many times that a lysogenic strain of bacteria may be sensitive to infection with phages that differ from the carried one. It is also not a contradiction to the penetration hypothesis. One merely has to assume that the prophage does not stimulate the reaction in question. Of much greater interest is the finding that even induced bacteria, for which we know that the carried phage is in the process of maturation, cannot be made to give mixed yields of the carried and the infecting phage. A bacterium infected in this stage with T5 seems to be capable of three alternatives: to yield T5 after a proper latent period, or to be lysed from without, liberating any mature λ particles present at the time of infection with T5, or, possibly, to yield λ after completion of the maturation period. Our experiments do not permit any conclusions as to the frequency of occurrence of this last alternative, except that at high multiplicity it must be very small (see table 4). In any case, the alternative which, a priori, would seem to be the most likely, i.e., mixed growth, occurs with a frequency below 1 per cent. For a discussion of this finding, let us focus our attention on bacteria infected with T5 rather late in the maturation period of λ, say, at 40 minutes. We know that at about this time the first mature λ particles begin to appear in some of the induced bacteria. It is possible that the bacteria already containing mature λ are the ones that will respond preferentially with exclusion of T5, either by lysis from without or, very rarely, by completion of the maturation of the λ particles. Let us consider, then, those other bacteria (at 40 minutes still the majority) which do not yet contain mature λ particles. In most of these, T5 multiplies and the yield does not contain any mature λ particles. Here, obviously, λ is not excluded by a barrier to penetration since λ has been inside the bacterium long before the excluding phage arrived and is, in fact, near completion. Our experiments thus furnish conclusive proof that mutual exclusion must involve some mechanism other than the establishment of a barrier to penetration. They do not furnish any clue as to the nature of this other mechanism. It would seem likely, however, that it does not act by creating a block at the beginning of the production of new phage particles, but at a terminal stage, where perhaps only one more step is needed to complete the phage.

SUMMARY

Escherichia coli, strain K12, carries a phage, λ, which is large, morphologically complex, and unusually resistant to ultraviolet rays. It is serologically unrelated to any of the phages of the T series. Strain K12 does not adsorb λ at a measurable rate.

In a normal culture of strain K12, λ is in the prophage condition in all but a very small fraction of the bacteria. Maturation from prophage into phage can be induced in every bacterium of a culture of K12 by irradiation with small doses of ultraviolet rays (Lwoff effect). The latent period is 60 minutes, and the

yield of λ per bacterium about 150. During the maturation period the bacteria do not divide, but increase considerably in volume.

Premature lysis experiments show that 40 minutes after irradiation some bacteria contain mature λ particles. Fifty minutes after irradiation about 80 per cent of the bacteria contain more than one mature phage particle, and the average number of phage particles per bacterium at this time is about 8.

Strain K12 is sensitive to phage T5 with a high efficiency of plating. The rate of adsorption of phage T5 on strain K12 is very small. The latent period is 35 minutes; the burst size is high (ranging between 500 and 2,000 per bacterium). Multiple infection of strain K12 with phage T5 causes appreciable lysis from without at multiplicities around five.

Infection of induced K12 with phage T5 leads either to multiplication of T5 or to lysis from without (or, very rarely, to the maturation of λ particles). Under all conditions the fraction of bacteria liberating both T5 and λ is below 1 per cent.

The findings are discussed in relation to previous experiments and theories on mutual exclusion. It is concluded that the penetration hypothesis, according to which a phage may stimulate the bacterium to a reaction which blocks the entry of other phages (or even causes their breakdown), is probably correct for some cases but cannot account for the principal finding reported in this paper, the exclusion of phage λ by T5 in the case of infections with T5 occurring at an advanced stage of the maturation of λ.

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