The Properties of a Star Mutant of Phage T2

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SUMMARY: The properties of a plaque-type variant, or 'star' mutant, of coliphage T2 are investigated. This mutant forms sectoried plaques which are composed of approximately equal numbers of the parental star phage particles and of rapid-lysing (r) mutants. The star mutant is shown to be a one-step mutant of wild-type, possessing a mutation at a locus, s_1 , that is close to the r_1 locus of phage T2. The r mutants occurring in the star plaques are all double-mutants, containing as well as the s_1 mutation, a mutation at some further r locus. Rapid-lysing mutants isolated from different star plaques possess r mutations at different loci. The high proportion of r mutants occurring in the star plaques is due to selection of r mutants that are formed spontaneously during replication of the star phage. This selection occurs during phage multiplication in *Escherichia coli* that is no longer multiplying exponentially (old bacteria), but not in exponentially growing bacteria. The slow multiplication of star compared to r phage in old cultures of *E. coli* is not due to an inability of the old bacteria to synthesize star phage particles at the same rate as r phage particles, but is caused by two, probably related, factors. First, the star phage has a prolonged latent period in old bacteria, and secondly, the star phage particles that are eventually released when the old cells lyse re-adsorb very slowly to old bacteria in the culture. During multiplication in old bacteria the r phage therefore goes through many more cycles of growth than the s phage, and thus accumulates more rapidly.

Wild-type coliphages of the related strains T2, T4 and T6 form plaques which have a small dark centre surrounded by a pale halo. The most common type of mutation that affects plaque-type in these phages is the so-called r , or rapid-lysing mutation. These r mutations occur with a frequency of approximately one per thousand duplications, and give rise to a distinctive type of plaque that is much clearer than that of the wild-type phage (Hershey, 1946). Another plaque-type variant, which only occurs rarely, was discovered by Hershey (1946) in a stock of T6. The plaques of this variant have the dark centre surrounded by a pale halo that characterizes the wild-type plaques, but surrounding the halo are a number of areas of rapid lysis which give the plaques a characteristic sectoried appearance. When these variant plaques are picked and replated one always obtains the same sectoried plaques in a mixture with about an equal number of typical r plaques. On picking these secondary sectoried plaques one again obtains a mixture of sectoried and r plaques, but picking from the secondary r plaques yields pure r stocks. Hershey suggested that the high proportion of r mutants found in these sectoried plaques was due to a mutational instability in the parental phage, and he called this variant an 'unstable' mutant.

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Subsequently other 'unstable' mutants were found in coliphage T4 by Doermann & Dissoway (1949) and in phage T2 by Hershey (Visconti, Garen & Symonds, 1953). It was shown with these mutants that the high proportion of *r* mutants found in the sectorized plaques was not due to a mutational instability, but was caused by a selective mechanism which allowed the *r* mutants to multiply much faster than the 'unstable' phage under the physiological conditions existing on agar plates during plaque formation. As these mutants do not have a mutational instability the name 'unstable' is inappropriate. We shall call variants whose plaques contain a high proportion of mutant types, star or *s* mutants. More recently other star mutants, all of which form plaques with a somewhat similar morphology, have been reported in coliphage T2 (Baylor, Hurst, Allen & Bertani, 1957; McFall & Stent, 1958), in coliphage T1 (Bresch, 1953; Tessman, 1956), and in salmonella phage (Terada, Kondo & Ogawa, 1956).

In this paper we shall discuss the properties of the star mutant discovered by Hershey (Visconti *et al.* 1953). Two main problems will be investigated: (1) the genetic structure of this phage and of the mutants that are found in the star plaques; (2) the mechanism of selection that is responsible for the large proportion of mutants found in the star plaques. Some preliminary results of this work, carried out in collaboration with Visconti, have already been published (Visconti *et al.* 1953).

METHODS

Bacteria. *Escherichia coli* strain R2 (Hershey, Dixon & Chase, 1953) was used throughout the experiments.

Phage. All the phage strains were mutants of T2H. The viral mutations used as markers were:

(a) *Host-range* (*h*). Hershey & Rotman (1949). The genetic factor *h* was carried in all the phage stocks used in the experiments described in this paper, therefore its presence will not be specifically indicated.

(b) *Plaque-type*. The different mutants employed were:

(1) Wild-type (*w*).

(2) Rapid-lysing (*r*). The 3 mutants r_1 , r_7 , r_{13} , isolated and mapped by Hershey & Rotman (1949), were used as reference markers. The r_7 and r_{13} loci are linked with a frequency of recombination of about 14%; the r_1 locus is unlinked (i.e. the frequency of recombination is $\geq 30\%$) both to r_7 and r_{13} .

(3) Minute (*m*). This was also used as a reference marker. The *m* locus is unlinked to r_1 , r_7 and r_{13} .

(4) Star (*s*). This was isolated by Hershey (Visconti *et al.* 1953). Photographs of plaques of these mutants are presented in Pl. 1.

Preparation of phage stocks. Because of the large number of *r* mutants normally present in the star plaques, it was not possible to prepare stocks of *s* in the usual way. In order to obtain relatively pure high-titre stocks of *s*, a strain of *Escherichia coli*, N-1, that had been isolated by Nelson (Streisinger, 1956) was employed. On plates seeded with this coli, the *s* mutant formed very

small plaques containing about 10^7 phage particles, less than 1% of which were *r* mutants. When all the phage particles comprising one of these plaques was inoculated directly on to a single plate seeded with coli N-1, confluent lysis occurred. From such plates, stocks of the *s* mutant were obtained that contained less than 0.2% of *r* mutants and had titres greater than 10^{11} /ml. Stocks of the other mutants were prepared by standard procedures (Adams, 1950).

Growth medium (broth). Bacto-peptone 2% (w/v), Lemco meat extract 0.3% (w/v), glucose 0.1% (w/v), 0.1 M-NaCl, 10^{-3} M-MgSO₄, pH 7.0.

Phage crosses. Crosses were performed by infecting bacteria suspended in buffer with an equal multiplicity of the two parental types, according to the procedure described by Visconti & Delbrück (1953). In all crosses the average multiplicity of infection with each parent was 5. Burst sizes in different experiments ranged from 80 to 400. Equal yields of both parental types were obtained in the progeny of all the crosses.

Growth conditions. When investigating the growth of the star phage in broth cultures, experiments were performed when the bacteria in the cultures were in two distinct physiological conditions. For convenience we shall term the bacteria in these two physiological conditions either 'young' or 'old'. Young bacteria are those which have been grown, with aeration, to a concentration of 10^7 organisms/ml. from a 10^{-3} dilution of an unaerated overnight culture. Old bacteria are those from an unaerated overnight culture, at a concentration of about 10^9 organisms/ml., and are aerated from the time phage is added. All experiments were performed at 37°.

Phage adsorption. In all growth experiments, adsorption was carried out by adding phage particles to bacteria growing in broth, and after a short time (from $\frac{1}{2}$ to 2 min.) adding antiserum to neutralize the unadsorbed phage particles and so limit the adsorption time. Usually about 2% of the phage particles were adsorbed in 1 min. either to young bacteria at a concentration of 10^7 /ml., or to old bacteria at a concentration of 10^9 /ml. All growth experiments were performed under conditions of single infection.

Intracellular development of phage. The average number of phage particles/bacterium that was present in infected cultures at different times during the latent period was determined by adding cyanide to samples of the culture, and assaying the number of phage particles released after lysis (Doermann, 1952). In order to determine the rate of increase in the fraction of infected bacteria in the culture which contained at least one phage particle, a streptomycin technique was used. At various times after infection samples from the infected bacteria (which, like the *Escherichia coli* R2, were sensitive to streptomycin) were removed from the culture and immediately plated on agar plates containing 2000 μ g. equivalents streptomycin/ml. and seeded with a streptomycin resistant indicator strain of *E. coli* strain B. At this concentration the streptomycin requires 2-3 min. to stop phage maturation, and only those infected bacteria which contain at least one mature phage particle by the time streptomycin kills the bacteria are able to form plaques (Symonds, to be published).

Ultraviolet irradiation of infected bacteria. The method used for irradiating infected bacteria which had been withdrawn from an infected culture at

different times during the latent period was similar to that described by Benzer (1952). To stop phage development at the required times, samples from the infected culture were diluted 100-fold into buffer chilled in an ice-bath. A 3 ml. volume from these samples was then irradiated with ultraviolet light on a watch-glass kept chilled by resting in a Petri dish full of crushed ice and water. The fraction of infected bacteria which survived the irradiation was determined from the number of irradiated bacteria that were still capable of originating plaques.

RESULTS

Mutant types arising in s plaques

When an *s* plaque is resuspended in buffer, and after an appropriate dilution, plated on agar plates, one always finds approximately equal numbers both of *s* plaques and typical *r* plaques. In some *s* plaques one also finds a few ($<1\%$) particles that appear to be wild-type from their plaques, and also a few ($<1\%$) other star variants that can be distinguished from the parental *s* because their plaques contain a smaller amount of sectored growth. These star variants were called 'derived star' or *s_d* mutants. The plaques of these *s_d* mutants contain about 90% *s_d* phage and 10% of typical *r* mutants. Two such *s_d* mutants were isolated whose plaques (Pl. 1) could be distinguished both from each other and from *s*. Fig. 1 shows schematically the way these various mutants originate from a single *s* plaque. In the succeeding sections we shall investigate the genetic structure of these mutants.

Fig. 1. Schematic representation of the different types of mutants that dissociate from a star plaque. The numbers refer to the percentage of each phage in the parent plaque.

Genetic structure of the s mutant

The genetic structure of the *s* mutant was first examined by performing a cross between *s* and wild-type. No recombinants were found in the progeny of this cross, which suggested that *s* was a one-step mutant of wild-type.

Further evidence supporting this idea came from crosses between *s* and reference mutants. At least 15% wild-type recombinants were found in the progeny of the crosses $s_1 \times r_7$, $s_1 \times r_{13}$, $s_1 \times m$ and 2.5% in the cross $s_1 \times r_1$. These results showed that the mutation responsible for the *s* phenotype behaved like a single locus that was located at a distance of 5 recombination units from r_1 , and was thus unlinked to either r_7 , r_{13} or *m*. We shall designate this star locus as s_1 (the subscript suffix 1 will be used to distinguish this from other star mutants). In Fig. 2 the location of s_1 on the T2 linkage map is shown with respect to the reference loci.

In the last four crosses described above only wild-type recombinants were observed. As will be shown later this is because the double recombinants $s_1 r_1$, $s_1 r_7$, and $s_1 r_{13}$ are phenotypically indistinguishable from *r* phage and thus could not be detected amongst the parental *r* phage in the progeny, while the double mutant *sm* is difficult to distinguish phenotypically from *m*.

Genetic structure of the 'wild-type' phage occurring in the s plaques

In order to see whether the wild-type plaques that were observed in the *s* plaques originated from phage particles that were genetically identical with wild-type, stocks isolated from these plaques were crossed with the standard wild-type stock. No recombinants were found in these crosses; it appears therefore that these plaques did originate from genuine wild-type phage.

Genetic structure of the s_d mutants

Further information that supported the identification of *s* as a one-step mutant of wild-type came from crosses performed with the two s_d mutants that had been isolated from *s* plaques. These two mutants could be distinguished by their plaque-type from *s*, *r*, and wild-type phage. When a cross was made between either of these s_d mutants and wild-type, equal numbers of two recombinants could be distinguished in the progeny. One of these recombinants was *s*, while the other was an *r* mutant. This finding indicated that the s_d were double-mutants of wild-type that contained, as well as a mutation at the s_1 locus, a mutation at some specific *r* locus. That is, the cross $s_d \times$ wild-type could be represented:

Phenotype	Parentals		Recombinants
	$s_d \times w$		$s + r$
		→	
Genotype	$s_1 r \times s_1^+ r^+$		$s_1 r^+ + s_1^+ r$

A test was made of these genotypes by isolating the recombinant *r* mutant in the above cross, and then making the reverse cross $s \times r$. The progeny of this cross contained both wild-type and s_d phage particles with the same frequency of recombination as was found in the cross $s_d \times w$. This is the result that would be expected from the proposed genotypes. The details of these crosses for both of the s_d mutants, which have been designated $s_1 r_{31}$ and $s_1 r_{32}$, are presented in Table 1 (in order to avoid confusion with the *r* loci already described by Hershey & Rotman (1949), we shall start numbering from 31 the *r* loci isolated from the star mutant).

Table 1. Percentage of recombinants in crosses with two s_d mutants, $s_1 r_{31}$ and $s_1 r_{32}$

Type of cross	Parentals	Percentage recombinants		Plaques scored
		s	r	
$s_d \times w$	$s_1 r_{31} \times w$	2.6	2.9	1700
	$s_1 r_{32} \times w$	14.5	16.0	550
$s \times r$	$s_1 r^+ \times s_1^+ r_{31}$	s_d 2.5	w 3.0	2500
	$s_1 r^+ \times s_1^+ r_{32}$	14.5	17.0	700

The location of the r_{31} and r_{32} mutations was found in two-factor crosses of the reference mutants with the recombinant r mutants $s_1^+ r_{31}$ and $s_1^+ r_{32}$. In this way r_{31} was shown to be linked to the locus r_1 with a frequency of recombination of 1%, while r_{32} was unlinked to either r_1 , r_7 , r_{13} or m . The positions of r_{31} and r_{32} on the coliphage T2 linkage map are shown in Fig. 2.

Fig. 2. Linkage map of coliphage T2 showing the locations of s_1 and of the r mutations occurring in the $s_1 r$ mutants. The numbers refer to the number of recombination units (i.e. the recombinant frequency in two-factor crosses) between the various loci. Approximately one-third of the r loci occurring in the $s_1 r$ mutants are in the A region, and two-thirds in the B (or rII) region. The indicated single-linkage group for coliphage T2 is based on the results of Baylor *et al.* (1957) and Streisinger (personal communication). Present evidence is insufficient to determine on which side of r_1 the s_1 locus is situated. Not drawn to scale.

Various checks can be made of these locations, and also of the genotypes that have been ascribed to s , s_d and the recombinant r mutants. First, it can be seen from Fig. 2 that the loci s_1 and r_{31} are approximately 6 recombination units apart, while the s_1 and r_{32} loci are unlinked. A verification of these map distances comes from the data of Table 1, where the frequency of recombination for the two-factor cross $s_1 \times r_{31}$ is given as 5.5%, and that for the cross $s_1 \times r_{32}$ as 31.5%. Secondly, it follows from Fig. 2 that the r_{31} and r_{32} loci should be unlinked. Experimentally this can be tested in two-factor crosses either between the r mutants r_{31} and r_{32} , or between the s_d mutants $s_1 r_{31}$ and $s_1 r_{32}$. The first of these crosses resulted in 14% wild-type recombinants as expected. In the second cross 16% star recombinants were found as expected, and in addition 17% r recombinants, which showed that the triple mutants $s_1 r_{31} r_{32}$ had the r phenotype.

The results of these different crosses therefore all confirm the idea that the star mutant possesses a single mutation, s_1 , while the s_d are double mutants with the genotypes $s_1 r$.

Genetic structure of the r mutants occurring in s and s_d plaques

The genetic structure of the *r* mutants that were isolated from *s* plaques was next investigated. When any one of these *r* mutants was crossed against wild-type, *s* recombinants always appeared in the progeny. This finding suggested that these, like the *s_d* mutants, were double-mutants with the genotype *s₁r*. As the *s₁⁺r* recombinant in the above cross could not be distinguished phenotypically from the *s₁r* parent, it was not possible to test the proposed genotype of these *r* mutants, as was done in the case of the *s_d* mutants, by performing the reverse cross *s₁r⁺* × *s₁⁺r*. However, the proposed genetic structure of these *r* mutants was verified, and the *r* loci of different mutants located, in 3-factor crosses with the reference mutants of the type *s₁r* × *r₁*. From these crosses it was found that some of the *r* loci in the *s₁r* mutants were closely linked to *r₁*, while some were linked to *r₇*. Typical data for one such mutant, *s₁r₃₆*, in which the *r₃₆* locus is closely linked to *r₇*, is presented in Table 2.

Table 2. *Percentage of recombinants in crosses with an r mutant, s₁r₃₆, derived from an s₁ plaque*

Cross	Percentage recombinants		Plaques scored
	<i>s</i>	<i>w</i>	
<i>s₁r₃₆</i> × <i>s₁</i>	—	—	2000
<i>s₁r₃₆</i> × <i>w</i>	15.0	—	300
<i>s₁r₃₆</i> × <i>r₁</i>	11.5	1.5	1600
<i>s₁r₃₆</i> × <i>r₇</i>	0.2	0.2	7000
<i>s₁r₃₆</i> × <i>r₁₃</i>	6.0	5.0	1400

Table 3. *Percentage of recombinants in crosses with an r mutant, s₁r₃₁r₆₃, derived from a plaque of the s_d mutants s₁r₃₁*

Cross	Percentage recombinants			Plaques scored
	<i>s</i>	<i>s_d</i>	<i>w</i>	
<i>s₁r₃₁r₆₃</i> × <i>s₁r₃₁</i>	—	—	—	1400
<i>s₁r₃₁r₆₃</i> × <i>s₁</i>	—	12.0	—	700
<i>s₁r₃₁r₆₃</i> × <i>w</i>	1.0	10.0	—	1700
<i>s₁r₃₁r₆₃</i> × <i>r₇</i>	0.1	0.5	0.5	1250
<i>s₁r₃₁r₆₃</i> × <i>r₁₃</i>	0.2	3.3	2.7	1100

Except for the few wild-type phage particles, all the mutants that occur in the *s* plaques therefore appear to be double-mutants with the genotype *s₁r*. For most *r* mutations (including *r₁*, *r₇*, and *r₁₃*) these *s₁r* mutants have the *r* phenotype. However, there are some *r* mutations for which the *s₁r* mutants form plaques that still possess some star characteristics; these are the *s_d* mutants. Since the *r* mutants occurring in the *s* plaques still carry the parental *s₁* mutation, one would expect that the *r* mutants arising, for example, in plaques of the *s_d* mutant *s₁r₃₁*, would still carry both the *s₁* and the *r₃₁* loci. That is, they would be triple mutants with the genotype *s₁r₃₁r_d*. If this were so, in a cross between one of these mutants and wild-type both the *s_d* mutant *s₁r₃₁* and the star mutant *s₁r⁺* should be recognizable as recombinants. Table 3

presents the result of this and other crosses performed with one of these r mutants. These results are consistent with the idea that this is a triple mutant with the genotype $s_1 r_{31} r_{63}$ in which the r_{63} locus is closely linked to r_7 .

The genotypes of the various mutants that dissociate from an s plaque are shown schematically in Fig. 3.

Fig. 3. Genotypes of the various mutants dissociating from the star plaques. The phenotypes of these mutants are shown in brackets.

Distribution of the r mutations occurring in the s plaques

In order to obtain some idea of the distribution of r mutations among the $s_1 r$ mutants found in s plaques, use was made of the discovery of Benzer (1956), that coliphages having a mutation in the rII region of the T2 chromosome (which includes the locus r_7), are not able to form plaques on plates seeded with the strain K12 (λ) of *Escherichia coli*.

First, a number of r mutants, all isolated from a single s plaque, were tested for their ability to form plaques on coli K12 (λ). Of 12 s plaques tested in this way, all yielded some r mutants that were able to form plaques on coli K12 (λ), and some that were unable to do so. This showed that at least two independent r mutations were present among the r mutants found in any one star plaque.

Next, 100 r mutants, each isolated from different s plaques (and therefore originating from independent mutations), were tested on coli K12 (λ). Approximately two-thirds of these were found to possess r mutations in the rII region. Ten of these mutants from the rII region were then crossed together in pairs. In all of these crosses s progeny were found, indicating that all 10 of these $s_1 r$ mutants possessed different r mutations. Ten of the mutants that were able to grow in coli K12 (λ) were then investigated further. It was found that their r mutations were all located within one recombination unit of the r_1 locus, and while 5 of the mutants were genetically distinct, 5 were either very closely linked or were identical.

In a parallel experiment performed on 100 spontaneous r mutants isolated from different wild-type plaques, again approximately two-thirds of the mutations were found to be in the rII region (this is roughly the same distribution as Benzer (1956) found for spontaneous r mutations in the coliphage T4). Ten of the mutants that could grow on coli K12 (λ) were investigated in more detail, and found to be all genetically different, and to be all linked to r_1 with recombinant frequencies of between 0.5 and 4%.

On the basis of these results the distribution of the r mutations occurring in different s plaques is roughly similar to that found among the spontaneous r mutants. Since it has also been shown that the mutation rate from $s_1 r^+ \rightarrow s_1 r$ is approximately the same as the spontaneous rate $r^+ \rightarrow r$ (Visconti *et al.* 1953), it seems likely that the $s_1 r$ mutants found in the s plaques originate from spontaneous r mutations. Since an s plaque contains more than 10^8 phage particles, and since the mutation rate from $r^+ \rightarrow r$ is one in 10^3 duplications (Hershey, 1946), a number of independent r mutations will occur during the early growth of the plaque. If those $s_1 r$ mutants that are formed multiply at the same rate as s , then the s plaques would still contain only about 0.1% of these mutants. The high proportion of r mutants that do accumulate by the time the s plaques are fully grown must, therefore, be due to some efficient process of selection by which some at least of the $s_1 r$ mutants that have been formed can grow faster than the s phage under the physiological conditions holding during plaque formation. It is the purpose of the rest of this paper to investigate the mechanism which is responsible for this selection.

Growth of s phage in broth cultures

Since it was difficult to perform experiments during plaque formation that would give any information concerning the selective mechanism which enabled r phage to multiply faster than s phage in the star plaques, an attempt was made to determine growth conditions in broth cultures of bacteria in which there was a similar selective advantage for the multiplication of the r phage.

(a) *Growth of s and r phage in cultures of young bacteria.* First, the relative rates of multiplication of s and r phage were measured in a culture of young bacteria. A mixture of $s_1 r_{34}$ phage (which is a typical r mutant isolated from an s plaque, the r_{34} mutation being near the r_1 locus) and s phage was added to a culture of young bacteria. Antiserum was added 2 min. later, and after a further 6 min. (by which time the unadsorbed phage had been inactivated), a sample from this culture was diluted 500-fold into another culture of young bacteria. The subsequent rates of multiplication of the s and r phages in this culture was then followed by immediately assaying samples taken from the culture at various times after infection. The result of this experiment is presented in Fig. 4a. It can be seen that both the s and r phage multiplied in the young bacterial culture at roughly the same rate, and that when the culture lysed 80 min. after infection both types of phage had increased in titre 10^6 -fold. During phage multiplication in young bacterial cultures the r phage therefore has no selective advantage over the s phage.

(b) *Growth of s and r phage in cultures of old bacteria.* The relative rates of multiplication of s and r phage in a culture of old bacteria was next measured in an experiment similar to the previous one. The result of this experiment is shown in Fig. 4b from which it can be seen that in the old bacterial culture the s phage multiplied much slower than the r phage. By 80 min. after infection, (which was the latest time any s phage particles could be detected in the culture), there was only an increase of 12-fold in the s titre compared to an increase of 2000-fold in the r titre. Because of this slow multiplication of s there

is thus a strong selective advantage for r phage during phage growth in cultures of old bacteria.

These experiments show that it is possible to obtain growth conditions in broth cultures of bacteria in which r phage multiplies considerably faster than s phage. This selective advantage for the multiplication of r phage occurs during phage growth in cultures of old bacteria, but not in cultures of young bacteria. It occurs because the s phage multiply at a very slow rate in the old bacterial cultures.

Intracellular growth of s and r phage in old bacteria

One possible reason for the slow multiplication of s compared to r phage in old bacterial cultures was that there was a considerable delay in the time at which maturation of s phage particles began in old infected bacteria. Fig. 5

Fig. 4a

Fig. 4b

Fig. 5

Fig. 4. Growth of s ($\blacktriangle$) and r ($\bullet$) phage in the same bacterial cultures; (a) growth in young bacteria, (b) growth in old bacteria.

Fig. 5. Intracellular growth of s ($\blacktriangle$) and r ($\bullet$) phage in the same culture of old bacteria.

shows the result of an experiment to test this possibility, using the streptomycin technique. Although the s_1r_{34} infected bacteria formed their first plaques on the streptomycin plates about 2 min. earlier than the s_1 infected bacteria, there was no significant difference in the time at which phage development commenced in the greater proportion of the s_1 and s_1r_{34} infected bacteria. In a further experiment on a similar culture it was shown also that the number of intracellular s_1 and s_1r_{34} phage particles increased at approximately the same rate.

Intracellular s phage particles were thus produced at about the same time and rate in old bacteria as intracellular s_1r_{34} phage particles. The slow multiplication of s compared to that of r in a culture of old bacteria was therefore not due to any marked difference in the ability of the old bacterial cells to synthesize s and r phage particles.

Latent periods of s and r phage in old bacteria

Another possible reason why s phage multiplied slower than r phage in cultures of old bacteria was that in old bacteria s had a considerably longer average latent period than r . When a one-step growth experiment with s phage

was performed in old bacteria some lysis occurred as early as 25 min. after infection, but the number of infective centres in the culture increased slowly over a period of 2 hr. In order to determine the average latent period of s it was thus necessary to estimate the fraction of the infected bacteria which had not lysed at any particular time after infection. Two methods, as described below, were used to obtain this information.

Antiserum inactivation of free phage. When assays are made, at different times after infection, from an infected culture to which strong antiserum has been added, there is a considerable increase in plaque count near the time when lysis is occurring (as many of the infected bacteria are then unstable and lyse during the dilution and warming to 45° that occurs before they can be plated), followed by a continual decrease in plaque count as more and more of the infected bacteria lyse and the particles they liberate are neutralized by the antiserum. Table 4 presents the result of an experiment in which this method was used to compare the latent periods of s_1 and s_1r_{34} phage in the

Table 4. *Number of s_1 and s_1r_{34} infective centres present at different times after infection in a singly infected culture of old bacteria that lyses in the presence of strong antiserum*

Minutes after infection	Number of infective centres in a fixed dilution	
	s_1	s_1r_{34}
11	57	64
15	56	57
19	61	140
24	200	2000
30	400	400
45	3000	8
75	120	4

same culture of old bacteria. Lysis of the s_1r_{34} infected bacteria is seen to have started by 24 min. after infection, and at 45 min. after infection the number of r infective centres had dropped to 10% of its original value; hence at least 90% of the s_1r_{34} infected bacteria must have lysed by this time. There is a slight increase in the titre of s_1 infective centres as early as 24 min. after infection, but the increase is most marked after 45 min. and still noticeable as late as 75 min. after infection. This result clearly indicates that the average latent period of s_1 in old bacteria is much longer than that of s_1r_{34} .

Irradiation experiment

It is possible to modify the previous experiment so that a more accurate estimate can be made of the number of s_1 infected bacteria that have not lysed after a certain period of time. It has been shown (Symonds, 1957) that at times later than 10 min. after infection the resistance to ultraviolet light of old bacteria infected with s_1 phage (i.e. the ability of these bacteria to produce at least one phage particle after irradiation), is much greater than the resistance of free T2 coliphage. Therefore if a sample is taken later than 10 min. after infection from an old infected culture of s_1 to which antiserum has been added,

it can be irradiated with a dose of ultraviolet light that will inactivate any intra- or extracellular phage particles present in the sample, but have very little effect on the plaque-forming ability of the bacteria which have not lysed. The fraction of infected bacteria which still yield plaques after the irradiation then places a lower limit on the fraction of unlysed cells present in the culture at the time the sample was taken. Table 5 presents the result of an experiment of this kind in which the fraction of old bacteria infected with either s_1 or s_1r_{34} phage that survived an ultraviolet dose of 20 T2 lethal hits was compared at different times during the latent period. By 25 min. after infection only 3% of the s_1r_{34} infected bacteria were still viable after the irradiation, compared to 90% of the s_1 infected bacteria. Even as late as 50 min. after infection 30% of the s_1 infected bacteria were still viable. Since these figures set a lower limit to the number of infected cells in the culture which have not lysed, they confirm the result that the latent period of s_1 in old bacteria is considerably longer than that of s_1r_{34} .

Table 5. *Percentage of old bacteria, singly infected with s_1 or s_1r_{34} phage, that are still viable at different times after infection after being irradiated with an ultraviolet dose of 20 lethal T2 hits*

Minutes after infection	Percentage of infected cells that survive the irradiation	
	s_1	s_1r_{34}
12	70	50
18	80	10
25	90	3
35	80	< 1
50	30	< 1

These experiments thus point to one reason why s phage multiplies so much slower than r phage in cultures of old bacteria. Since the latent period of s in old bacteria is longer than that of r , the intracellular s phage particles which are formed are unable to re-infect other bacteria in the culture as quickly as are the r phage particles; the s phage therefore carries out fewer cycles of growth and so multiplies more slowly in the culture.

Adsorption of freshly liberated s and r phage

An additional factor which might contribute to the slow multiplication of s phage in cultures of old bacteria was that the s phage particles which were eventually released when the cells lysed were unable to re-adsorb at the normal rate to other bacteria in the culture. This possibility was tested by infecting a culture of old bacteria with s phage and allowing phage growth to continue for 120 min. The culture was then centrifuged, and the rate of adsorption to old bacteria of the phage particles in the supernatant fluid was immediately measured. This supernatant fluid, and also a 1/10 dilution of it in buffer, were then stored at 4°, and the adsorption rates of both these phage suspensions were measured the next day. The results of two experiments of this type are

presented in Table 6. In the 120 min. of phage growth the *s* titre increased about 100-fold. During this time the proportion of *r* phage in the cultures increased from 0.2% in the original inoculum to be almost equal to that of the *s* phage. In the experiments the adsorption rates of both the freshly liberated *s* and *r* phage particles were therefore measured at the same time. The results show: (a) the ordinary stocks of *s* and *r* adsorbed at approximately the same rate; (b) the freshly liberated *s* phage particles adsorbed 15–25 times more slowly than freshly liberated *r* phage particles; (c) in the broth supernatants that were stored for 24 hr. there was little change in the adsorption rates of either *s* or *r*. However, in the dilute buffer suspensions the adsorption rate of *s* increased to about one-half that of *r*.

Table 6. *Percentage of s and r phage particles that are freshly liberated from old bacteria, which are able to re-adsorb to old bacteria in 5 min. at 37°. The control was a mixture of the standard stocks of s₁ and s₁r₃₄ phage.*

Type of phage			Percentage of adsorbing phage particles	
			<i>s</i>	<i>r</i>
First day	—	Control	10	7.5
	Supernatant of	{ Culture 1	3.5	50
		{ Culture 2	1.5	36
	—	Control	10	6
Second day	Supernatant of	{ Culture 1	3	20
		{ Culture 2	2.5	17
	1/10 buffer dilution of the supernatant of	{ Culture 1	18	20
		{ Culture 2	10	21

One plausible explanation for these findings would be that old bacteria infected with *s* phage do not liberate their intracellular phage particles abruptly at the end of their long latent period. Consequently pieces of bacterial membrane adhere to the phage particles as they are liberated and so hinder them from re-adsorbing to bacteria. However, on being left in dilute suspension for 24 hr. most of the bacterial debris dissociates from the phage particles, which then adsorb much more quickly.

These results, in conjunction with those of the previous sections, show that two factors contribute to the selective advantage which allows *r* phage to multiply much faster than *s* phage during growth in old cultures of *Escherichia coli*. First, in old bacteria the latent period of *s* is much longer than that of *r*, and secondly, the *s* phage particles that are eventually released from the old bacteria adsorb about 20 times more slowly to old bacteria than do freshly liberated *r* phage particles.

DISCUSSION

The star mutant discovered by Hershey and discussed in this paper is a single-step mutant of coliphage T2, having a mutation at a locus, *s*₁, that is close to the *r*₁ locus. This *s*₁ mutation has no effect on phage multiplication in cultures

of young bacteria. However, it does have two probably related effects on phage growth in cultures of old bacteria. Under these conditions the star phage has a considerable extended latent period, and in addition the star phage particles which are eventually released from the old bacteria re-adsorb very slowly. These two effects cause the star phage to multiply at a very slow rate in cultures of old bacteria. Consequently any mutants that happen to be in the same old culture as the *s* phage, and can grow at the normal rate, have a marked selective advantage.

If it be assumed that these effects of the s_1 mutation also manifest themselves in the same way during plaque formation, then the characteristic appearance of the star plaques can be easily understood. While the bacteria on the plate are still young there is considerable multiplication of the star phage, and during this time a number of spontaneous mutations occur. As the bacteria on the plate become older, the *s* phage in the plaques multiplies more slowly, and any of the mutants that can multiply at the normal rate have a strong selective advantage. These mutants then grow out around the edge of the plaques forming clear sectors of growth which give the star plaques their characteristic appearance.

It seems probable that most other star mutants owe their distinctive plaques to some similar mechanism as this. That is, they have a mutation at one (or more) loci which exerts some inhibitory effect on phage multiplication under the conditions of plaque formation. Any spontaneous mutants that are formed in which this inhibition is released then have a strong selective advantage during the subsequent growth of the plaque. In star mutants in which the inhibitory effect is due to only one *s* locus there are two different kinds of mutation that can release the growth inhibition. The first kind is the back-mutation $s \rightarrow s^+$. This back-mutation automatically releases the growth inhibition and yields phage that no longer contains a mutation at the *s* locus. The other kind is a mutation at some further locus. This kind of mutation does not automatically release the inhibition, and always produces double mutants which still contain a mutation at the *s* locus. Whether or not these double mutants can multiply at the normal rate depends on both of the mutations involved. With the star mutant discussed in this paper, a further mutation at almost any of the numerous class of *r* loci yields $s_1 r$ phage that has the *r* phenotype and thus can grow normally. However, there are some *r* mutations which do not completely overcome the effect of the s_1 locus, and these produce 'derived star' plaques that still show some star characteristics. As the spontaneous mutations $s_1 r^+ \rightarrow s_1 r$ are more frequent than the back mutation $s^1 \rightarrow s_1^+$, most of the mutants found in these star plaques are *r* mutants which still contain the s_1 locus and have *r* mutations at different loci. However, it is possible with other star phage that mutants of the sr type are still growth inhibited. In these cases the phage selected for in the star plaques will either be due to back mutations, or to mutations at some other class of loci which are able to overcome the inhibitory effect of the *s* locus. An example of the first of these types is reported in the accompanying paper by McFall & Stent (1958). They have investigated a star mutant of coliphage T2 first isolated by

Litman, and find that the phage selected in these star plaques was all wild-type; in this case therefore the only frequent mutation that could overcome the inhibitory effect of the *s* locus was the back mutation $s \rightarrow s^+$. McFall & Stent have also investigated a star mutant of coliphage T2 discovered by Baylor (Baylor *et al.* 1957) which is of the second of the types mentioned above. In the plaques of this star phage the mutation which releases the inhibitory effect of the *s* locus is not an *r* mutation, but a mutation at a locus which lies very close to the *s* locus.

The mechanisms which cause growth inhibition in different star phages no doubt differ considerably. The one described here for s_1 is interesting in that it points clearly to two new factors which arise during the repeated cycles of growth that go to make up a lysate or a plaque, and which do not arise in one-step growth experiments. First, the physiological conditions of the host bacteria are continually changing; and secondly, the adsorbing phage particles do not come from a purified phage suspension but are always phage particles which have been freshly liberated from infected bacteria. It seems possible that these two factors might be important in understanding some processes of infection which occur in animal and plant virus systems.

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EXPLANATION OF PLATE

Photographs of plaques of various coliphage T2 mutants. The different phenotypic types (with their corresponding genotypes) are: (a) wild-type (r^+), (b) r mutant (r_1), (c) mixture of minute (m) and an r mutant (r_1), (d) star mutant ($s_1 r^+$), (e) derived-star mutant ($s_1 r_{31}$), (f) derived-star mutant ($s_1 r_{32}$).

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