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The Process of Infection with Bacteriophage φX174

XIII. Evidence for an Essential Bacterial "Site"[1]

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The burst of a starved bacterium infected with several φX174 bacteriophage was usually found to contain genetic traits of only one of the possible parents; less often, two phage multiplied in the same host cell. Unstarved cells, in contrast, supported the growth of at least four parental phage types. The unproductive phage seemed to be able to undergo the intracellular transition from parental single-stranded deoxyribonucleic acid to the double-stranded "replicative form" (RF). These results are taken to mean that some bacterial factor required for a step between RF synthesis and maturation of progeny is limited in starved cells.

Experiments by Denhardt and Sinsheimer (2) on the inactivation of φX174-infected bacterial complexes by the decay of ^{32}P atoms, restricted to the parental viral deoxyribonucleic acid (DNA) strands, led to the conclusion that the "replicative form" (RF) of DNA, which includes the parental DNA strand, plays a special and essential role in the infection. For this role, it could not be replaced by daughter RF molecules. These experiments were performed in previously starved cells, in order to synchronize the bacteriophage growth in the manner first devised by Benzer (1).

One interpretation of the Denhardt and Sinsheimer experiments is that there may be a restriction upon the number of parental φX particles that can reproduce in a previously starved cell. Experiments have been performed to ascertain whether there are such restrictions upon φX reproduction under various physiological conditions. These experiments involve ultraviolet (UV) inactivation curves of φX-infected bacteria and, independently, assays of the genotype distribution of the progeny of single infected complexes which have been multiply infected with several phage genotypes.

MATERIALS AND METHOD

Bacterial strains. Escherichia coli C_N (ATCC 122) possesses the ability to perform host cell reactivation (*hcr*$^+$), and was used as host bacterium unless otherwise indicated.

E. coli C_s (*hcr*$^-$) is a derivative of *E. coli* C_N and was isolated by R. P. Boyce.

E. coli C_1 is resistant to φX *wt*, but susceptible to φX mutants *γh* and H_AH_B. It was selected from a strain of *E. coli* C by C. A. Hutchison III.

Bacteriophage. φX wild type (*wt*) is the virus described by Sinsheimer (16).

φX H_AH_B has an extended host range: it will form plaques at 30 or 40 C on *E. coli* C_1, a host insensitive to φX *wt*. Its isolation and properties were described by Dietrich Pfeiffer (14), who supplied our sample of this phage.

φX *tsγ* and *ts*41 are two temperature-sensitive mutants which multiply in *E. coli* C_N at 30 C, but not at 40 C; *γ* and 41 were isolated and assigned to the same cistron by C. A. Hutchison, who kindly supplied samples of these phages.

φX *tsγh* is a double mutant; it contains *γ* and also *h*, a mutation conferring the ability to form plaques on *E. coli* C_1; *γh* was also constructed and supplied by C. A. Hutchison.

A mixture of *wt*, H_AH_B, *γ*, or 41, and *γh*, were individually scored on a single agar plate by use of a 5:1 mixture of *E. coli* C_N and *E. coli* C_1 as indicator bacteria. The phage were spread in soft agar with the mixed indicator, incubated for 2 to 3 hr at 30 C, and then shifted to 40 C for 2 to 3 hr before scoring. Under these conditions, *wt* forms plaques which are large and turbid with fuzzy edge; H_AH_B gives a large clear, fuzzy-edge plaque; *γ* or 41, a small, turbid plaque with sharp edge; and *γh*, a small, sharp-edged, clear plaque.

Stocks of H_AH_B, 41, *γ*, and *γh* were prepared by use of *E. coli* C_N growing logarithmically at an appropriate temperature: an aerated culture in tryptone-KCl broth (TKB) plus 10^{-3} M $CaCl_2$ (10^8 cells per milliliter) was inoculated with the contents of a plaque. After clearing of the culture, the debris was pelleted by centrifugation (5,000 × *g*) and 2 to 5 ml of 0.05 M sodium tetraborate was added to the pellet to release the adsorbed particles. After storage with occasional

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shaking for several hours at 2 to 4 C, the suspension of debris was centrifuged; the supernatant fluid was the "stock." The clear supernatant fluid resulting from a 50-ml culture usually contained 2×10^{10} to 2×10^{11} plaque-forming units of virus per ml and was stable to storage at 4 C for a period of months.

^{32}P-labeled *wt* was made as described by Denhardt and Sinsheimer (3); $D^{15}N^{32}P$ *wt*, as by Sinsheimer et al. (17).

Media. Top and bottom agar for the phage assay were the tryptone media described by Sinsheimer (16), except that no NaOH was added to bottom agar.

Starvation buffer (SB) and TKB were described by Denhardt and Sinsheimer (2). Cells to be used as indicator or as hosts in a growth experiment were grown in TKB containing 10^{-3} M $CaCl_2$. However, *E. coli* C_1 was usually grown in TKB containing 5×10^{-4} M $CaCl_2$, to avoid the aggregation of these cells which occurs at higher Ca^{++} concentrations.

Samples containing phage alone were diluted in 0.021 M sodium tetraborate solution.

Medium 3XD (6) was the preferred growth medium for cells which were to be converted to spheroplasts. It contained, per liter of deionized distilled water: NH_4Cl, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; 1 M $CaCl_2$, 0.3 ml; glycerol, 24 ml; Casamino Acids (Difco), 15 g; gelatin, 0.03 g; KH_2PO_4, 0.9 g; Na_2HPO_4; 2.1 g. The last two were added after the former components were dissolved in the water.

PA and PAM media, used for growth of infected spheroplasts, were described by Guthrie and Sinsheimer (11). Bovine serum albumin (BSA), when required for stabilization of spheroplasts, was a dilution of Armour Bovine albumin, supplied by them as a sterile 30% solution.

Growth experiments. The starvation synchronization system was described by Denhardt and Sinsheimer (2). Briefly, cells grown to 10^8 per milliliter in TKB containing 10^{-3} M Ca^{++} were washed and suspended in SB, and aerated vigorously for 90 min at 37 C. This procedure yielded cultures which had stopped DNA, ribonucleic acid, and protein synthesis and cell division. Phage was then added, and, after a suitable time for both adsorption and eclipse (usually 5 min at 37 C), an equal volume of warm TKB was added to initiate development. When temperature mutants γ, 41, or γh were among the viruses used, growth was at 30 C and a longer time was allowed for adsorption. Time was reckoned from the addition of TKB. Time before this was given a negative sign; -7 min means 7 min before TKB was added. With this system, at 37 C, the first intracellular progeny appeared at 9 min, the eclipse period was 11 to 13 min, and the minimal latent period was 15 to 16 min. At 30 C, these times were 15, 21, and 28 min, respectively. Mean burst sizes were somewhat variable, but were seldom outside the range of 50 to 300. The rate and extent of phage adsorption to starved *E. coli* C were indistinguishable from those of the same cells before starvation.

Starved cells prepared in this way and infected with a small ratio of plaque-forming particles to bacteria yielded just as many infected cells as did log-phase bacteria. It seems, therefore, that every bacterium to which a potentially active phage adsorbs becomes infected; the restricted growth described below is not the result of an exceptional requirement that several ϕX particles infect a cell in order that one among them may yield progeny.

Alternatively, synchronization of infection was achieved with KCN. KCN was added to a final concentration of 0.009 M; 10 min later (at 30 C), phage were added. After another 10 min for adsorption, a $1:10^5$ dilution was made, to start development. The timing of ϕX growth under these conditions was similar to that seen with starved cells.

Infected cells to be converted to spheroplasts were chilled, washed at 0 C, and suspended in 0.5 M sucrose. They were then subjected to the lysozyme-ethylendiaminetetraacetic acid (EDTA) procedure described by Guthrie and Sinsheimer (11), except that the concentrations of lysozyme and EDTA were doubled and all operations were performed in ice-cooled tubes.

When high multiplicites of phage were used, the growth medium contained anti-ϕX serum ($K = 2$ to 3 min^{-1}) to inactivate unadsorbed phage. Sometimes, in addition, starved infected cells were filtered onto an HA membrane filter (Millipore Filter Corp., Bedford, Mass.) and washed with SB. The filter was then immersed in a volume of SB equal to that of the original culture and was swirled with a vortex orbital mixer to resuspend the infected cells. Recovery of cells from the filter was good (>50%), but not complete.

Use of very high phage-cell ratios resulted in the disappearance of potential infective centers. This effect became appreciable at a ratio of 15 to 20 plaque formers per cell. The affected cells did not reappear as colony-formers. To avoid selection against cells infected with many phage, the total phage multiplicities were kept well below these values. The observed recovery of most of the colony-forming cells as infective centers after exposure to several ϕX/cell assured that starved cells receiving several ϕX did survive and yield progeny.

Multiplicities of infection (MOI) were obtained by dividing the initial number of colony-formers into the titer of phage known to have entered the eclipse phase just prior to the time when TKB plus anti-ϕX serum was added. Eclipse was followed by taking small samples of the combined phage and bacteria, chilling, and lysing the cells by treatment with lysozyme and EDTA. This procedure has been described elsewhere (2), and yields smaller estimates of the effective multiplicity than are found by measurement of adsorbed virus.

Single-burst experiments. Infective centers were chilled during development by dilution into a large volume of cold TKB. They were held on ice until convenient and, after further dilution if required, 0.25- to 0.5-ml volumes were distributed into a number of small tubes by use of a semiautomatic pipette (no. 1250; Becton, Dickinson and Co., Rutherford, N.J.). These tubes were then incubated at 30 or 40 C, for at least 3 hr, and plated. Assay was performed by combining 200 ml of melted soft agar and 30 ml of the indicator mixture at 42 to 44 C and delivering 2.3-ml portions of this into the tubes by means of a semiautomatic pipette mounted on a stand. The contents

of each tube were then poured on a plate. In this way, 100 tubes could be assayed in 20 min or less; the seed bacteria survived this incubation at 42 to 44 C without harm.

In these experiments, no selection of a particular phage type was observed. The fractions of total phage yields which were of a given phage type corresponded approximately to the proportion of that phage among the input; that is, all types of phage used seem to be selected in accordance with their abundance. All the expected burst combinations appear; the restriction of the number of genotypes released from single cells (see below) is not a reflection of the exceptional vigor of one of the mutants chosen.

Irradiation of infected cells. Dilute suspensions of infected cells in SB were exposed to monochromatic UV radiation from a quartz prism monochromator and assayed under conditions selected to minimize photoreactivation. The techniques of irradiation and dosimetry may be found elsewhere (Yarus and Sinsheimer, *in preparation*).

Calculations. To construct a predicted UV inactivation curve for a cell containing several vegetative centers, data on the inactivation of singly infected cells at a similar stage in the phage replication cycle are used. The proportion of survivors after a UV dose to singly infected cells (SF) is the probability of a phage yield after exposure of a vegetative center to that amount of UV. Therefore, the probability of *inactivation* of a single vegetative center is: 1 − SF. If a cell contains X independent vegetative centers, the probability that they will *all* be inactivated (by the given dose) is: $(1 - SF)^X$. The probability that at least *one* vegetative center will have survived is then: $1 - (1 - SF)^X$. Inactivation data gathered from experiments at low multiplicity are used in this way and compared in Fig. 1 with the results of infections at high multiplicity.

In the limited-participation experiments, cells were infected with phage of four different genotypes. From the Poisson distribution, one can calculate the fraction of cells that have received one, two, three, or four types of phage. Assuming no selection and no limitation on phage reproduction, the probability of having been infected with phage a is taken to be $P_a = [1 - exp(-m_a)]$ in which m_a is the eclipsed multiplicity of phage a. The product of all the P values is taken to be the probability of a burst containing all four genotypes. The probability of each of the combinations of phages containing three types is taken to be the product of the P values for each of the included types times $(1 - P)$ for the missing type. For instance, bursts lacking only type d phage should occur in $[P_a \times P_b \times P_c \times (1 - P_d)]$ of the cells. The probability for each conceivable type of triple yield is then summed to give the total fraction of cells which would release three genotypes.

To compare these calculations to observed distributions when the total multiplicity of infection is small, it is sometimes necessary to normalize all calculations to the fraction of cells expected to yield phage of any kind. A similar calculation is performed to obtain the fraction of cells expected to yield two or one genotypes.

Some experiments, as in Fig. 3, are concerned with the fraction of pure bursts of wild-type phage observed when cells are infected with an average μ_w of w-type phage and μh of h-type phage. The theoretical curves in Fig. 3 are graphs of the function

$$\sum \left[\binom{w}{k} \Big/ \binom{h+w}{k} \right] \left[\frac{e^{-\mu_h}(\mu_h)^h}{h!} \right] \left[\frac{e^{-\mu_w}(\mu_w)^w}{w!} \right]$$

$w > 0; h > 0; \binom{w}{k}$ is $w!/[(w - k)!k!]$; which is the number of ways of selecting a group of things of number k from among a total of w things. Take h and w to be the number of h-type and w-type phages adsorbed to a bacterium; $\left[\binom{w}{k} \Big/ \binom{h+w}{k} \right]$ is then the probability that a bacterium which can allow k phages to multiply will select at random from among h h-type and w w-type phage, a group of size k which does not include an h-type.

This expression is not defined when $(h + w) < k$, but, of course, in this situation all phages will multiply.

The other two terms are the probabilities that a culture infected with an average multiplicity of μ_h h-type and μ_w w-type phage will yield cells infected by exactly h h-type and w w-type. Their product is the probability that a cell receives exactly h and w of the two phages. The sum above, then, gives the probability that cells infected with an average of μ_h and μ_w phage will yield bursts containing only w-type, if each bacterium will support the growth of a number of phage, k.

The above reasoning follows Dulbecco (5). At low total multiplicities, this expression must be normalized to the fraction of cells which yields bursts of any kind, $[1 - e^{-(\mu_w + \mu_h)}]$, in order to compare it with the observed fraction of pure bursts.

RESULTS

UV inactivation experiments. Other studies of the sensitivity of the ϕX-*E. coli* C complex have led to the conclusion that inactivation of the complex results from inactivation of the intracellular phage DNA (4). In Fig. 1 are results of the irradiation of starved *E. coli* C_N which had been infected with several ϕX per cell. At 0 min, that is, after phage adsorption and eclipse, but before the beginning of intracellular development, the inactivation of multiply infected cells followed, qualitatively, the course expected for several independent infections of the same cell (a multitarget survival curve). The infective center formed after infection at low multiplicity was inactivated exponentially according to the steep dashed line in Fig. 1. Infection with high multiplicity yielded infective centers with the same ultimate sensitivity (limiting slope), but the inactivation curve has a small shoulder near the origin. This shoulder, however, reaches its maximal size at a low ratio of phage to cells. The extrapolation of the exponential part of these latter curves to zero dose intercepts the ordinate

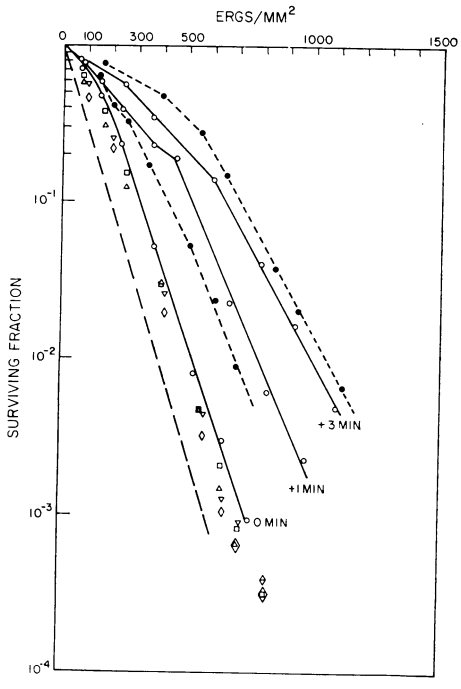

FIG. 1. *Ultraviolet (260 mμ) inactivation of starved Escherichia coli C_N infected with high multiplicities of φX, and inactivation curves predicted for limited participation. 0-min complexes infected with a low multiplicity of φX are indicated by the dashed line at left. (○) C_N complexes (MOI = 7.2) at 0, 1, and 3 min of development. The curve drawn through the 0-min data is a "three-hit" curve, calculated from the 0-min inactivation curve, obtained at low multiplicity. (●) Predicted inactivation curves at 1 and 3 min for three supposed vegetative phage per bacterium, calculated as described in Materials and Methods. The calculations were based upon data obtained from an inactivation performed on complexes of similar age infected at low multiplicity. (△) Observed inactivation of 0-min C_N complexes, MOI = 6.4 (□) Observed Inactivation of 0-min C_N complexes, MOI = 9.3. (▽) Observed inactivation of 0-min C_N complexes, MOI = 11.4. (◇) Observed inactivation of 0-min C_N complexes, MOI = 21.*

at 2.5 to 3, even at an input multiplicity of 21 (Fig. 1).

A similar situation exists when *E. coli* C_s cells are used. The young, singly infected complexes are also inactivated exponentially (4), although the survival curve has two distinguishable slopes (Fig. 2). After infection with several phage per cell, inactivation followed the course expected of cells containing an average of 1.5 to 2 independent vegetative phage.

Also shown in Fig. 1 is the inactivation of complexes after various times of early development. The dotted lines with solid circles were calculated assuming that the multiply infected complex contains just three copies of the sensitive structure observed in singly infected cells at a similar time in development. The observed and calculated curves agree satisfactorily.

In these Luria-Latarjet type of experiments, the survivors are those cells which release any progeny. Therefore, these experiments imply that only a limited number of the phages infecting a bacterium are capable of some function required for the production of progeny. Since the final slopes are independent of multiplicity, the capable vegetative viruses appear to be inactivated independently; they do not cooperate in such a way as to change the overall sensitivity of the infective center (e.g., as in multiplicity reactivation).

Limited-participation experiments. The limited extrapolation number of the UV curves suggests that, upon multiple infection, it should be possible to observe a restriction on the production of progeny types. That is, it could be that, upon

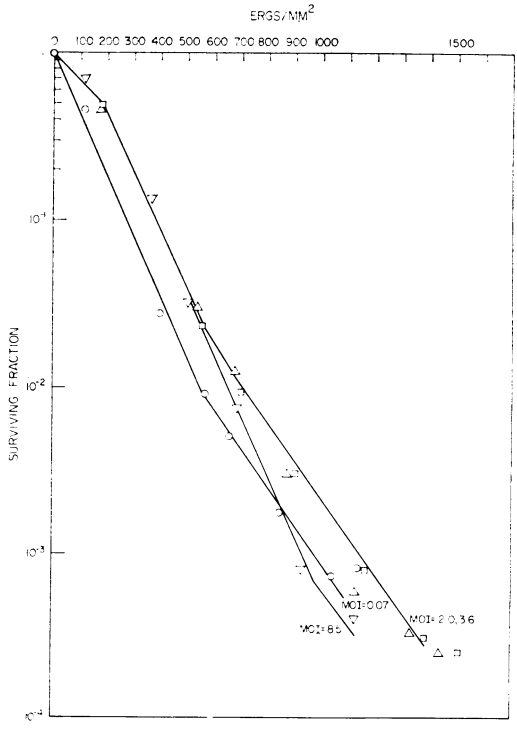

FIG. 2. *Ultraviolet (254 mμ) inactivation of starved E. coli C_s infected with various multiplicities of φX. No calculated curves are presented because of the simple form of the curves.(○) MOI = 0.07. (□) MOI = 2. 0 (△) MOI = 3.6. (▽) MOI = 8. 5. Complexes were harvested for irradiation after 3 min in the presence of nutrient, but curves indicate that development was slower than usual.*

multiple infection, only a few among the phages potentially able to grow in a cell are selected to reproduce themselves.

If this interpretation is correct, then the probability of release of progeny of one type of phage will be a function of the number of phages with which it has to compete. The result of an experiment designed to test this idea is shown in Fig. 3. A fixed number (1.5/cell) of H_AH_B, a host range mutant of θX, was used to infect starved *E. coli* C_s in the presence of various amounts of the wild-type phage. The fraction of cells which released only *wt* phage was measured. The result is compared to theoretical curves calculated by the method of Dulbecco (5) in which it is assumed that the phage which are to produce progeny are selected at random from among those which infect a bacterium, and that the distribution of infecting phage among bacteria is described by the Poisson distribution. The infected cells in this experiment were plated on a mixture of indicator bacteria, one of which was susceptible to both H_AH_B and *wt* and the other only to H_AH_B. Plaques corresponding to infective centers which released any H_AH_B could therefore be distinguished from those which yielded only *wt*. In addition, the phage released from single infected bacteria from several of these infections were plated on the mixed indicator after lysis, and the types of phage released were scored. Both types of data are presented in Fig. 3, and the

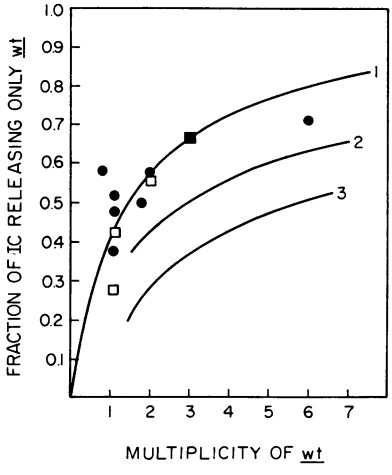

FIG. 3. *Occurrence of pure yields of wt from mixedly infected cells as a function of wt multiplicity. Starved C_s were infected with a fixed multiplicity of 1.5 φX H_AH_B per cell and a varying multiplicity of φX wt. Progeny were scored by plating on mixed indicator (●) and by observation of single bursts (□). The solid lines were calculated assuming that each bacterium will allow the propagation of 1, 2, or 3 parental phage.*

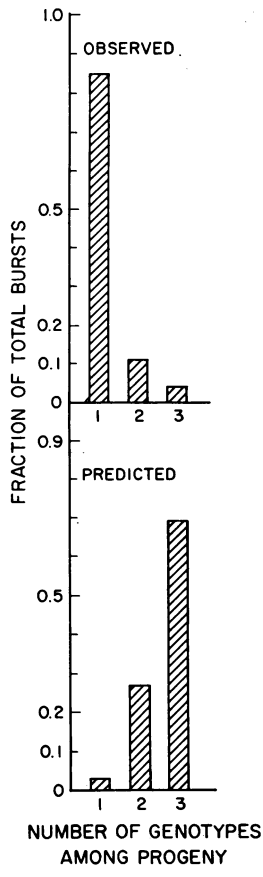

FIG. 4. *Number of different types of progeny released by single starved Escherichia coli C_N cells infected with φX γh (MOI = 1.7), 41 (MOI = 2.0), and wt (MOI = 3.6). The observed distribution is based on 53 bursts, 51 of which were scored among 130 tubes and 2 of which were found among 100 tubes at a higher dilution. A Luria-Latarjet curve of the 0-min complexes had an extrapolate of approximately 3. Mean burst size in culture was 75; in tubes, 50. Of the colony-forming bacteria, 83% were recovered as infective centers.*

results are consistent with the interpretation proposed for the irradiation experiment: only a small number (one or two, according to the theoretical curves) of the phages infecting a starved cell are selected to propagate themselves.

Another demonstration of this limitation on phage growth is presented in Fig. 4 and 5. In these experiments, each cell was infected with several phages of each of three or four genotypes. It can be shown by the Poisson distribution that most cells are infected with all four genotypes. However, the conclusion need not depend on a detailed quantitative argument, because the limitation on growth was so effective. When the infected cells were diluted and distributed into

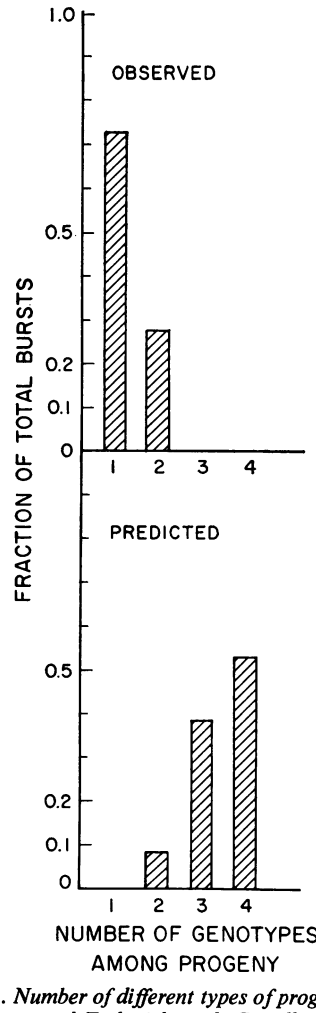

FIG. 5. *Number of different types of progeny released by single starved Eschericha coli C_s cells infected with ϕX γh (MOI = 1.6) and $H_A H_B$ (MOI = 1.3) and γ (MOI = 5.7) and wt (MOI = 2.5). The observed distribution is based on 52 bursts observed among a total of 400 tubes. A Luria-Latarjet curve of the 1-min complexes had an extrapolate of about 1.5. Mean burst size in culture was 63; in single bursts, 92. The infected cells were washed by filtration after phage adsorption; the number of infected cells recovered from the filter was >60% of the original number of starved cells.*

tubes so that after lysis most tubes contained phage derived from only one infected cell, the predominant class of cells did not release all genotypes. Most, instead, released only one genotype. A fraction of cells appeared to support the growth of two types of phage. The small number of bursts, observed in some experiments, which contained three parental types was no larger than the number expected as a result of chance dispensation of two or more infected cells into the same tube. The issue of a starved cell, then, are usually the descendants of only one of the viruses which infect it.

This restriction upon mixed infections is not a general property of ϕX growth in *E. coli* C. Depicted in Fig. 6 is the result of similar single-burst experiments in log-phase *E. coli* C_s infected with KCN synchronization or without synchronization of any kind. As a control, a portion of the same culture was washed, starved, and infected with the same phage mixture. As usual, the majority of starved cells yielded pure bursts. However, cells grown in broth and infected without synchronization, or cells infected in the presence of KCN, gave yields little different from the prediction made by assuming unlimited phage participation. The observation of a distribution which contains a number of quadruple and triple bursts in reasonable agreement with that predicted supports the calculations used to make the predictions and lends credence to the difference between the calculated distribution and that observed in starved cells.

E. coli C cells appear to be doublets in the phase microscope; the predominant type of cell is a rod with a constriction in the middle. It is unlikely that this doublet structure is related to the restricted maturation, however, since conversion of infected cells to spheroplasts, after which almost all bacteria appear to be undifferentiated spheres, has no effect on the genotype distribution (Fig. 7).

Formation of RF. None of this evidence indicates the stage of phage growth at which the limit to maturation is imposed. Experiments intended to determine whether the DNA of the debarred phage is converted to the RF are summarized in Table 1. RF is distinguishable from the parental single-strand DNA (SS) in a CsCl equilibrium density gradient by virtue of the density change accompanying its formation (17). Therefore, the appearance of isotopically labeled parental DNA at the density characteristic of RF in a neutral CsCl equilibrium density gradient was taken as the criterion of RF formation. The amount of RF formed by the labeled phage was measured in the presence and absence of a high multiplicity of unlabeled virus.

It appears that the conversion of the labeled phage DNA to RF is unimpeded by co-infection with a large excess of unlabeled phage, i.e., under conditions previously shown to restrict the ability of the labeled phage to form progeny (Fig. 3). This observation, of course, implies all that must come before: normal adsorption, eclipse, and access to intracellular enzymes. Normal adsorption and eclipse can also be ob-

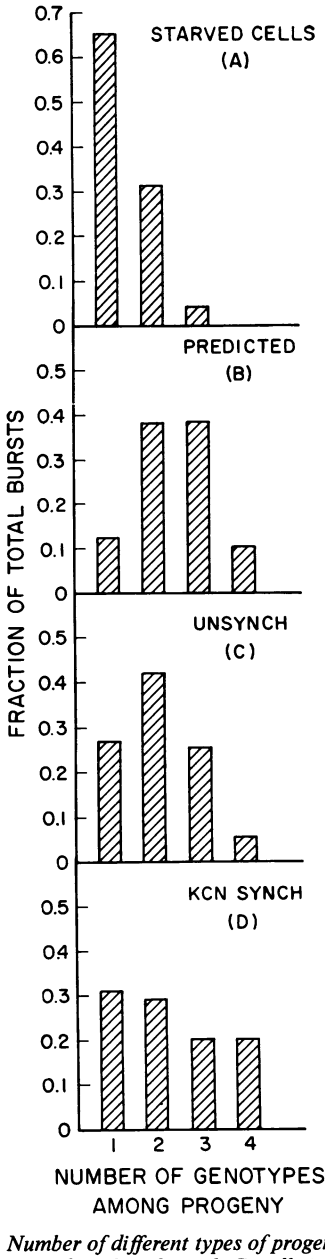

FIG. 6. *Number of different types of progeny released by single infected Escherichia coli C_s cells infected with φX γh (MOI = 0.4), and γ (MOI = 0.8) and wt (MOI = 2.4) and H_AH_B (MOI = 1.2). (A) A portion of the cells were starved and infected as in the experiment of Fig. 5. Among 100 tubes in this portion of the experiment, 26 bursts appeared; 86% of the starved cells appeared as infective centers. (B) Distribution of genotypes predicted from Poisson distribution, supposing no limitation on the number of phages which can propagate in a cell. (C) The phage mixture was added to cells growing logarithmically; no attempt was made to synchronize infection. Among 194 tubes, 55 bursts were scored; 98% of the colony-forming cells were recovered as infective centers. (D) KCN was added to logarithmically growing cells to a final concentration of 0.009 M; 20 min later, the phage mixture was added, and, after 10 min for absorption, cells were diluted 10^5 to initiate growth. Among 183 tubes, 35 bursts were scored; 45% of the colony formers were recovered as infective centers. Mean burst sizes were: starved cells in culture, 150 (in single bursts, 120); in unsynchronized infection in culture, 240 (in single bursts, 200); in KCN-synchronized infection in culture, 90 (in single bursts, 87).*

served directly when the minority phage is distinguishable genetically, as in experiment II (Table 1). The amount of parental ^{32}P found with washed complexes is unaltered by the presence of an excess of unlabeled phage.

DISCUSSION

It is clear from these results that, in the majority of previously starved cells, only one or, less often, two of a number of infecting φX174 phages are selected to give rise to progeny. The others are able at least to reach the stage of RF. The absence of such a strict limitation in unstarved cells suggests that the starvation procedure has limited the number of parental phages that can contribute to progeny. In this view, such a restriction may be present in unstarved cells also, but the number of φX allowed to reproduce is sufficiently large that it is not detected in these experiments.

Conceivably, the starved *E. coli* cell contains a single "site" with which an RF must become associated for the production of progeny. RF is formed from every parental phage; however, of those so formed, only the one in possession of the site will be able to donate genetic markers to the phage released on lysis.

Two interesting possibilities may be distinguished for the quality conferred on the possessor of a site. The distinction might be an "active" one; that is, a site might have a specific role in some obligatory reaction in maturation, and its occupant would be the only vegetative phage able to perform this reaction. Alternatively, the site could also be "passive" and shield its possessor from inhibition.

The existence of this restriction upon the RF formed as a result of several independent, simultaneous infections raises the likely possibility that only one among the replicas of RF may be capable of contribution to progeny in singly infected, starved cells. This restriction provides an explanation for the previously mentioned observations of Denhardt and Sinsheimer (3) concerning the sensitivity of complexes of starved *E. coli* C and heavily ^{32}P-labeled φX to the ^{32}P disintegration in

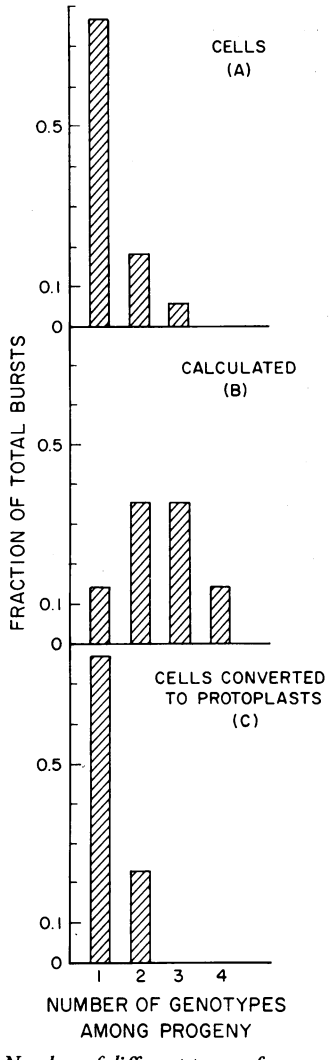

FIG. 7. *Number of different types of progeny released by single infected Escherichia coli C_s cells after conversion to spheroplasts. Infection with ϕX H_AH_B (MOI = 1.4) and γh (MOI = 0.7) and γ (MOI = 0.8) and wt (MOI = 1.1). (A) Starved, infected cells harvested after 10 min of development, diluted into PAM medium plus 0.5% BSA, and distributed into tubes without conversion to spheroplasts; 17 bursts found among 200 tubes were scored in this part of the experiment. At the end of phage adsorption, the cells were washed by filtration, and 70% of the original number of starved cells were recovered from the filter as infective centers. (B) Calculated distribution of genotypes, assuming no limit on bacterial capacity for phage growth. (C) The cells used in A were treated with lysozyme and EDTA at 0 C, diluted in PAM medium plus 0.5% BSA, and distributed into tubes; 22 bursts appeared among the 200 tubes scored in this part of the experiment. A 5-ml quantity of infected cells containing 10^8 infective centers per milliliter was added to 1 ml of 0.5 M sucrose before treatment with lysozyme. After fivefold dilution with PAM medium, which is a part of the spheroplast procedure (11), one microscopically visible cell body was recovered for each cell in the original suspension. More than 90% of these had the spherical shape of spheroplasts. The number of fertile tubes observed after dilution and distribution into tubes suggested a titer of about 6×10^7 infected spheroplasts per milliliter in this diluted suspension. Therefore, 60% of the original infected cells gave rise to a spheroplast which yielded phage. The diluted cells gave an average burst size of 67; the spheroplasts, 84. A concurrent single-step growth curve yielded a burst of 68. A Luria-Latarjet experiment performed on these complexes at 0 min had an extrapolate of about 1.5.*

the parental strand of an RF, even after unlabeled RF have been generated by RF replication in unlabeled medium. It appeared from this result also that at least one necessary function is reserved to a single RF, in this case the RF containing the parental SS, the first RF formed in the infected cell. Indeed, it might be suggested that association of the parental RF with the "site" is a prerequisite to replication of the RF; thus, the parental RF automatically becomes the essential RF in such experiments.

The evidently limited number of sites required for virus growth suggests that a site might be a bacterial DNA replication point or something closely associated with it. Oishi, Yoshikawa, and Sueoka (13) observed that, at the high growth rate prevailing in enriched medium, *Bacillus subtilis* appears to have more than one growing point per chromosome, whereas during slower growth a single point of replication was suggested (19). Similarly, Schaechter, Maaløe, and Kjeldgaard (15) found that the DNA content and number of nuclear bodies in *Salmonella typhimurium* increase with growth rate; Lark and Lark (12) reported that *E. coli* growing in glucose minimal medium replicates its two nuclear bodies simultaneously; in succinate minimal medium, which supports a slower growth rate, the cells retain two nuclear bodies, but replicate them one at a time, in sequence. These results recall our finding (Fig. 6) that cells growing in an enriched medium support the growth of several ϕX, but starved cells only one; the number of bacterial replication points might, then, determine the number of ϕX able to grow simultaneously.

The extrapolation (to zero dose) of UV inactivation curves of early complexes of C_s is usually 1.5 (Fig. 2), in good numerical agreement with the other measurements of its ability to support several infections. Similar irradiation of C_N complexes (Fig. 1), on the other hand, reproducibly yields extrapolates of 3; yet, the single-burst experiment (Fig. 4) indicates that only one type of phage progeny is matured in most cells. Our in-

TABLE 1. *Formation of RF by a minority parent in starved cells*

Expt	Host	Infection procedure	Minority parent	Majority parent	Time DNA harvested[a]	Counts/min in RF	Per cent of control
Ia	Starved *E. coli* C_N	Minority parent alone, −5 min, 37 C	φX ^{32}P *wt* eclipsed MOI = 0.027	—	12 min (37 C)	2,192	100
Ib		Minority parent and majority parent simultaneously, −5 min		φX *wt* eclipsed MOI = 6.4		1,738	79
IC		Majority parent, −10 min; minority parent, −5 min		φX *wt* eclipsed MOI = 6.4		1,719	79
IIa[b]	Starved *E. coli* C_s	Minority parent alone, −15 min, 30 C	φX $D^{15}N^{32}P$ *wt* eclipsed MOI = 0.45	—	19 min (30 C)	165	100
IIb		Minority parent, −15 min; majority parent, −8 min		φX *ts*γ eclipsed MOI = 3.3		148	90
IIc		Minority parent alone, −15 min, 30 C		—		120	100
IId		Minority parent, −15 min; majority parent, −8 min		φX tsγ eclipsed MOI = 3.3		129	107

[a] Method of DNA preparation: in experiments Ia, Ib, Ic, IIa, and IIb, the complexes were lysed with lysozyme-EDTA and centrifuged without further treatment; in experiments IIc and IId, the lysed complexes were treated with phenol before centrifugation.

[b] In experiment II, the reduced appearance of the minority parent among the progeny because of the presence of the *ts*γ could be directly measured: five independent dilutions and platings of *wt* infective centers with and without *ts*γ showed that only 57% of the *wt* infective centers observed when *wt* was used alone appeared when *ts*γ was also present. After addition of *ts*γ, 97% of the colony-formers appeared as infective centers. Therefore, the *wt* infective centers which did not appear in the presence of *ts*γ were not killed, but were recovered as pure γ bursts. Though entirely satisfactory in this respect, the fraction of *wt* infective centers which became γ infective centers was lower than expected from other experiments.

clination is to accept the lower estimate; the single-burst experiment is the more direct of the two. It may be that UV irradiation itself (specifically, in the *hcr*$^+$ strain) induces formation of a second site or allows a second RF to assume a previously occupied site after inactivation of the parental molecule, whereas, in the course of normal growth, the original RF would have remained unique. It is relevant that Hewitt and Billen (8) found evidence of new points of replication in the *E. coli* chromosome after UV irradiation. Denhardt and Sinsheimer (4) were able to reinfect φX-*E. coli* C (*hcr*$^+$) complexes after having inactivated the first infection with UV.

Generally similar conclusions relative to the limited number of phage T4 genomes which can participate in heterocatalytic function in T4-infected cells were drawn recently by Snustad (18) on the basis of the fractional success of mixed infections with various input multiplicities of wild-type and defective phage. Apparently, only four to six T4 phage genomes can be transcribed in such cells. This number is similar to the number of pre-existent, essential "cellular centers of virus reproduction" for T2 phage described by Epstein (6) from studies of the inactivation by radiation of the capacity of cells to support T2 reproduction.

Formation of RF by the minority phages in starved cells distinguishes limited participation

from temporal exclusion occurring after ϕX infection (10). If ϕX-infected cells are allowed a period of development before superinfection, genetic markers carried by the superinfecting phage do not appear among the progeny, and the superinfecting ϕX are unable to complement a genetic defect in the primary infection. However, in this case (temporal exclusion) the superinfecting SS DNA is recovered as SS on phenol treatment and does not appear in RF (10). The smaller fraction of quadruple bursts among the progeny of an unsynchronized infection than among those of cells infected in KCN (Fig. 6) may reflect a temporal effect of this kind.

Presumably, starved cells allowed enough time in broth would become indistinguishable from unstarved cells. The minimal latent period at 30 C in this system is about 28 min, the mean latent period, ca. 42 min. Since the excluded RF are unable, *ipso facto*, to produce progeny virus in this time, the formation of new sites must occur too slowly to permit their use by new RF during the latent period. Phage infection itself may block the transition to the unstarved state. Those starved cells which appear to contain two sites may be that fraction of the population which is able to form a second site before the end of the latent period.

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