THE FREQUENCY DISTRIBUTION OF SPONTANEOUS BACTERIOPHAGE MUTANTS AS EVIDENCE FOR THE EXPONENTIAL RATE OF PHAGE REPRODUCTION[1]

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The phage geneticist is faced with the task of constructing a satisfactory model of phage reproduction, in the absence of direct morphological evidence similar to the one available to the macro-geneticist. Cell division, mitosis, meiosis, fertilization have a solid basis of morphological observation that the modern geneticist takes for granted. The virologist, on the other hand, begins where the cytogeneticist ends; in a sense, he deals directly with the units of genetic material whose existence the macro-geneticists (including the bacterial geneticists) must infer. Here lies his weakness, since little is known of the performance of such units—and also his strength, since he can manipulate this subcytological world. He is not limited to dealing with integrated units of reproduction at the cellular level, but can control to a certain extent what goes into his cells. Because of this, virology's methods may lead more directly to solving the problem of the mode of replication of genetic material.

Penetration of one phage particle into a susceptible bacterium leads to production of a large number of similar particles. The intervening steps are unknown. We conjecture a reorganization of the viral material, because of its nonrecoverability in infectious form early after penetration (Doermann, 1948). We conjecture an integration of the viral material into the cell machinery at the genetic level (Luria and Human, 1950), because cell syntheses are redirected toward the production of virus specific substances. The genetic complexities of bacteriophage tell us that the viral specificities to be replicated are multiple (Hershey, 1946b) and that the new virus may receive imprints from more than one viral ancestor within the same cell (Delbrück and Bailey, 1946; Hershey, 1946b). There is evidence suggesting a discrete and regularly assorted nature of the material determinants of these specificities (Hershey and Rotman, 1948). A theory proposed by this writer (Luria, 1947) assumed independent replication of these determinants (or groups of determinants) followed by their assembly into mature virus particles. This theory originally aimed at accounting for the reactivation of ultraviolet inactivated phage inside multiple-infected bacteria (Luria and Dulbecco, 1949) and was extended to account for some features of genetic recombination (Hershey and Rotman, 1949). The main ground for proposing the theory, namely, the belief that the reactivation resulted from genetic exchange, has been weakened by new evidence (Dulbecco, 1952), and the theory has as little left to support it as to disprove it.

Yet, all this concerns the organization of virus material during reproduction, not the elementary process of replication. The latter cannot yet be attacked at the chemical level by any tool except speculation. It can be attacked on a limited front, however, by strictly genetic means. The experiments described in this paper were done to investigate the rate of replication of individual genetic determinants of the virus. They indicate that reproduction is exponential, each replica acting as a source of new replicas.

Theory

Phage mutations occur only in the intracellular state, presumably during replication. If a phage mutation occurs in a bacterium, that bacterium will liberate one or more mutants (assuming that no loss occurs intracellularly). Delbrück pointed out several years ago that the actual numbers of mutants would depend on the mode of phage replication—more specifically, on the mode of replications of the determinant or "gene" involved (see Luria, 1945b). We shall analyze a few possible mechanisms; other mechanisms may be proposed, but do not seem to lead to any simple picture.

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I. *Exponential Reproduction*

One gene produces n genes, each of which in turn gives n genes, and so on. After r generations, the number will be n^r. For n = 2 (duplication mechanism), the situation is analogous to bacterial reproduction. Let us assume this to be the case. N gene copies will derive from one gene by N-1 acts of replication. Let the last generation have the order number 0, the second last the order number 1, the third last 2, and so on. Suppose a mutation occurs at generation k, either as an "error of replication" affecting one of the two products of duplication, or as a change in one gene during interphase. If the replication process is completely synchronized (the consequences of nonsynchronization can, if necessary, be analyzed), the resulting clone of mutants will at generation 0 consist of 2^k individuals. If the total number of individuals (at generation 0) in the population is N, at generation k there were $N/2^k$ individuals. Assuming a constant probability m of mutation per individual, the number of mutations occurring at generation k is $mN/2^k$. The relation between the number x of mutants in a clone and the frequency y_x of such clones will then be obtained as follows:

$$y_x = \frac{mN}{2^k};\ x = 2^k;\ y_x = \frac{mN}{x}. \qquad (1)$$

Synchronization is expressed by the requirement that for $x \neq 2^k$, $y_x = 0$.

The frequency distribution (1) is, of course, identical to that of bacterial mutants in a series of similar populations (Luria and Delbrück, 1943; Lea and Coulson, 1949). If we limit our observation to one intracellular cycle of phage production, the maximum clone size will be the maximum "burst size." Each phage burst with 2^K particles represents a population. For $m \gg 2^{-K}$, there will practically never be more than one mutation per bacterium; the frequency distribution (1) will then be that of the mutant clones among a large number of bacterial yields, with limitations imposed by the inequality of burst size from cell to cell.

II. *Independent Successive Replications*

Let us suppose that each new replica of a gene is produced independently of the preceding one, for example, by a series of successive acts of replication controlled by the initial gene brought in by the infecting phage particle.

(a) If one of the copies mutates (at the time of its formation or later) the probability of mutation in other copies produced in the same cell should not be affected. Assuming a uniform mutation probability m, the mutants will be distributed at random among phage bursts (Poisson distribution).

(b) If the initial gene, the pattern, mutates while turning out replicas, we may assume that afterwards it produces only mutants. If the mutation rate is constant in successive replications, there will occur as many cases of mutation just before the production of the last viable gene copy as before the production of the second last, the third last, and so on. The mutants will be in clones, and the frequency of clones of different sizes will be uniform, at least up to the value of the minimum burst size.

Experimental Work

Previous data on the number of host range mutants in individual phage cultures (Luria, 1945a; Hershey, 1946a) did not allow the desired type of analysis, because the quantitative detection of the mutants was uncertain and because their low frequency made it necessary to look for them in mass cultures, where more than one cycle of intrabacterial growth of some mutants could take place.

The experiments here reported consisted in making single infection of *Escherichia coli* B with phage T2L (Hershey, 1946a; for the experimental methods used, see Adams, 1950) and counting the mutants *r* or *w* produced in phage bursts from individual bacteria. These mutant phenotypes (Hershey, 1946b) were chosen because they occur with suitable frequency, can be recognized and scored efficiently, and can be tested for genetic allelism or nonallelism (Hershey and Rotman, 1948). Previous extensive tests by Dulbecco (1949) and other tests made in the course of this work established the following technical points.

1. Mutant plaques *r* and *w* can be detected and scored without difficulty after six to eight hours of incubation at 37° C on nutrient agar plates under standard conditions (plating in a 0.6% agar layer over 24 hour old 1% agar plates, with about 2×10^8 young bacteria per plate). Over 100 single plaque isolations and replatings confirmed the scorings made by plaque type.

2. A plaque of *r* type can be distinguished without difficulty from a "mottled plaque" stemming from a bacterium infected with a mixture of *r* and wild-type, even when *r* is in moderate ex-

cess. Thus, it is possible to distinguish a plaque originating from an *r* particle from one resulting from a mutation that occurs on the plate during the development of a wild type plaque, unless the *r* mutation occurred in the first bacterium infected after plating and produced within that bacterium a large majority of *r* mutants—an improbable occurrence, as the results of the present work will show.

3. As for the *w* mutants, mixed plaques of *w* and wild type closely resemble the plaques of wild type, so that the chances of mistaking a mixed plaque for a pure *w* one are rather small.

Several single burst experiments of T2L on B, with single infection in Difco nutrient broth, gave average yields per bacterium around 60–100; the burst size distributions are shown in Table 1. The reasons for the lower average yield of T2L in Difco nutrient broth, as compared with the yields obtained several years ago in the same system (Delbrück and Luria, 1942), are unknown; they may have to do with changes in medium composition, in the bacterial host, or in the virus itself.

Mass phage lysates often contain more phage particles than plaque-forming units; most of the particles can be caused to form plaques by treatment with distilled water or Zn^{++} before plating (Bertani, unpub.). Electron micrographic counts give, for carefully assayed lysates of T2L, ratios "particles/plaques" between 1.0 and 2.0 (Luria, Williams and Backus, 1951 and unpub.). The failure of some phage particles to form plaques is apparently a peculiarity of phage in mass lysates, probably due to a combination between phage and inhibitors of bacterial origin. Repeated attempts to reveal, by various treatments, any increase in the plaque count of dilute lysates similar to those used in the experiments here reported, constantly failed. It is likely that plaque counts reveal nearly 100 per cent of the phage particles plated.

In our experiments, we plated the full content of tubes in which one or several bacteria had lysed. The loss of phage remaining in each tube after plating is of the order of five per cent or less. Thus, we feel that we recovered and examined practically the totality of the active phage produced by the bacteria.

Preliminary experiments indicated that mutants were present in about one burst out of 200. This made it possible to examine on each plate the phage yields from several infected bacteria, and yet to have almost never more than one mutant clone per plate. As many as 20 bursts per plate were examined in some experiments, particularly when only the *r* mutants were scored, since these are more easily recognized than the *w* mutants.

Independently isolated *r* mutants are generally found to be nonallelic, yielding wild-type recombinants in mixed infection (Hershey and Rotman, 1948). To test for allelism among *r* mutants isolated in our experiments, stocks were prepared from individual mutant plaques and used, separately or in mixtures, to infect bacteria. The yields were examined for wild-type plaques.

TABLE 1. BURST SIZE DISTRIBUTION, T2L ON B, SINGLE INFECTION

Date	Number of plates	Average number of bursts per plate	Average yield per burst	Median	Range*
12/7/50	96	0.93	69	101	13–472
12/8/50	105	0.89	71	84	5–347
4/10/51	96	0.73	92	106	9–477
4/23/51	48	0.85	87	111	10–254

*The maximum values are almost certainly too high, since they probably represent plates with two or more bursts.

Similar tests with *w* mutants are technically more difficult and were therefore not attempted.

The Frequency Distribution of Mutants

A total of 16 experiments, done between April 1949 and April 1951, yielded the data to be reported. In five experiments, only the *r* mutants were scored. The results are presented in Tables 2 and 3. A total of 90 plates with mutants *r* and 103 plates with mutants *w* were observed. The expected number of plates with both *r* and *w* mutants (calculated from the assumption of independent incidence of *r* mutations and *w* mutations, either in the same bacterium or in different bacteria whose bursts were pooled on one plate) was seven; six were found.

Let us observe the frequency distributions of the numbers of mutants (Table 3). The total number of mutants observed in all experiments (Table 3; total clone frequency) was 766, distributed among 2874 plates containing about 1.8×10^6 normal phages. The proportion of mutants is about 4×10^{-4}, and their average number per plate is about 2.5×10^{-1}. If the mutants were distributed at random, there would be about 550 plates with one mutant, about 90 with two or three mutants, and only four with four mutants or more.

TABLE 2. SYNOPSIS OF EXPERIMENTS ON DISTRIBUTION OF *r* AND *w* MUTANTS

Date	Stock phage, number	Number of plates	Number of bursts	Average number of bursts per plate	Number of plates with *r* mutants	Number of plates with *w* mutants
4/26/49	33	144	660	4.6	3	10
5/6/49	"	144	660	4.6	7	9
3/9/50	44	142	1120	7.9	12	7
3/14/50	"	143	1180	8.25	4	15
3/31/50	"	144	2020	14	6	18
5/2/50	"	143	250	1.75	2	3
5/19/50	46	144	1060	7.5	9	12
5/26/50	"	144	1800	13	15	9
12/18/50	51	605	540	0.89	2	1
1/11/51	"	144	800	5.5	7	10
2/12/51	"	144	820	5.7	4	9
3/1/51	"	192	1500	7.9	1	..*
3/8/51	"	192	2400	17.5	1	..
3/20/51	"	180	3400	19	7	..
3/29/51	"	173	4400	25.5	9	..
4/10/51	"	96	70	0.73	1	..
		Totals	22620		90	103

*Mutants *w* not scored.

There is no doubt that the distributions are not random, but clonal.

As a test of the nature of the mutants appearing on the same plate, 11 pairs of *r* mutants were isolated from 11 plates, which contained between 2 and 59 mutants. In all cases, the mutants in each pair proved allelic (probably identical); no wild-type recombinant was observed among at least 1000 plaques of the yield from mixed infected bacteria. In 11 out of 12 crosses between mutants isolated from different plates we observed wild-type recombinants; the twelfth cross failed to show recombinants. It may have represented either a case of repeated occurrence of the same mutation or a case of two mutations with recombination frequency lower than 0.2 per cent, the lowest frequency detectable in our rather crude tests. These results, then confirm the clonal nature of the mutants produced within a given bacterium. We will now consider the clonal distributions.

Inspection of Table 3 shows that the mutant distribution, though clonal, fails to fit the uniform frequency predicted for small clones of various sizes by the hypothesis of independent gene replication, with mutations occurring in the pattern.

Let us turn next to the distribution predicted by the hypothesis of exponential duplication.

Inspection of the data shows: (a) there are clones with two mutants; therefore, if exponential reproduction occurs, the elementary process is probably one of duplication (from 1 to 2) rather than triplication or quadruplication; (b) there are clones with 3, 5, 6, 7...in addition to clones with 1, 2, 4, 8...mutants. Thus, exponential reproduction, if present, must be nonsynchronized, a conclusion also suggested by the well-known distribution of the total burst size.

For a quantitative test of the hypothesis of exponential reduplication we shall use, instead of Equation (1), the following expression (accumulated distribution) suggested by Dr. Howard Levene:

$$Y_x = \sum_{\geq x} y_x = \Sigma \frac{mN}{x} = \Sigma \frac{mN}{2^k} = mN \Sigma \frac{1}{2^k} \approx \frac{mN}{2^{k-1}} = \frac{2mN}{x} \quad (\text{for } N = 2^K \gg x). \qquad (2)$$

Y_x is the number of clones with x or more mutants. The product $Y_x \times x$ is constant and a plot of log Y versus log x gives a straight line with slope −1; the vertical intercept for x = 1 is the logarithm of the total number of mutant clones. This plot has the advantage that it is hardly affected by nonsynchronization. A clone with three mutants can be considered either as a clone that should have had two mutants and underwent one extra reduplication, or as a four-mutant clone that lagged one reduplication behind. In either case, the clone will contribute to the value Y_2 and will be deducted from the value Y_4.

A correction should be made before comparing the results with the theoretical expectation. The stocks of phage used to infect bacteria contained small, known numbers of *r* and *w* mutants. Since we plated more than one burst per plate, some of the mutant clones observed may have stemmed from bacteria infected solely with one mutant particle. These clones ought to be eliminated (since they derive from mutant particles originated in a mass lysate, where different conditions obtain) but they cannot be recognized. The expected number of such "full mutant" clones was 10. Assuming that a "full mutant" clone would b least as large as the minimum burst size, we eliminated from the experiments where such clones were expected to be present an appropriate fraction of the largest clones (see Table 3). This correction is indeed a small one.

Figure 1 compares the experimental distribution of clones with one or more mutants with the expected distribution from the hypothesis of exponential reduplication. The data corresponding to the "corrected" columns in Table 3 are plotted for *r* mutants, for *w* mutants and for the two together. They fit well the expected relationship (linear relation between log Y_x and log x with slope −1) for low clone size, up to mutant clone sizes of the order of 10–15. Above that point, the frequency of mutant clones falls below the theoretical values. This behavior is precisely what we should expect. The linear relation between log y and log x—see Equation (1)—should only obtain for clones so small that they have equal chances to be formed in all bacteria. For clone sizes of the order of the burst size, a limitation is placed on the frequency with which these clones can be observed. In the curve log Y_x versus log x this limitation will manifest itself as a downward concavity, which becomes appreciable around the value corresponding to the lowest class

TABLE 3. THE DISTRIBUTION OF MUTANTS IN INDIVIDUAL PLATES

Clone size	Clone frequency, *r* mutants		Clone frequency, *w* mutants		Clone frequency, total, corrected for full mutant clones
	All plates	Corrected for full mutant clones	All plates	Corrected for full mutant clones	
1	47	47	46	46	93
2	9	9	18	18	27
3	11	11	11	11	22
4	2	2	4	4	6
5	2	2	1	1	3
6			7	7	7
7	2	2	2	2	4
8	1	1	1	1	2
9					
10	2	2	1	1	3
11			1	1	1
12	1	1			1
13					
14	1	1	1	1	2
15	1	1	1	1	2
16			1	1	1
20			2	1	1
22	2	2			2
25			1		
26			1	1	1
30	1	1			1
34			1	1	1
37			1		
39	1				
40	1		1		
41	2	1			1
47	1	1	1		1
53	1				
59	1	1			1
100	1				
Total	90	85	103	98	183

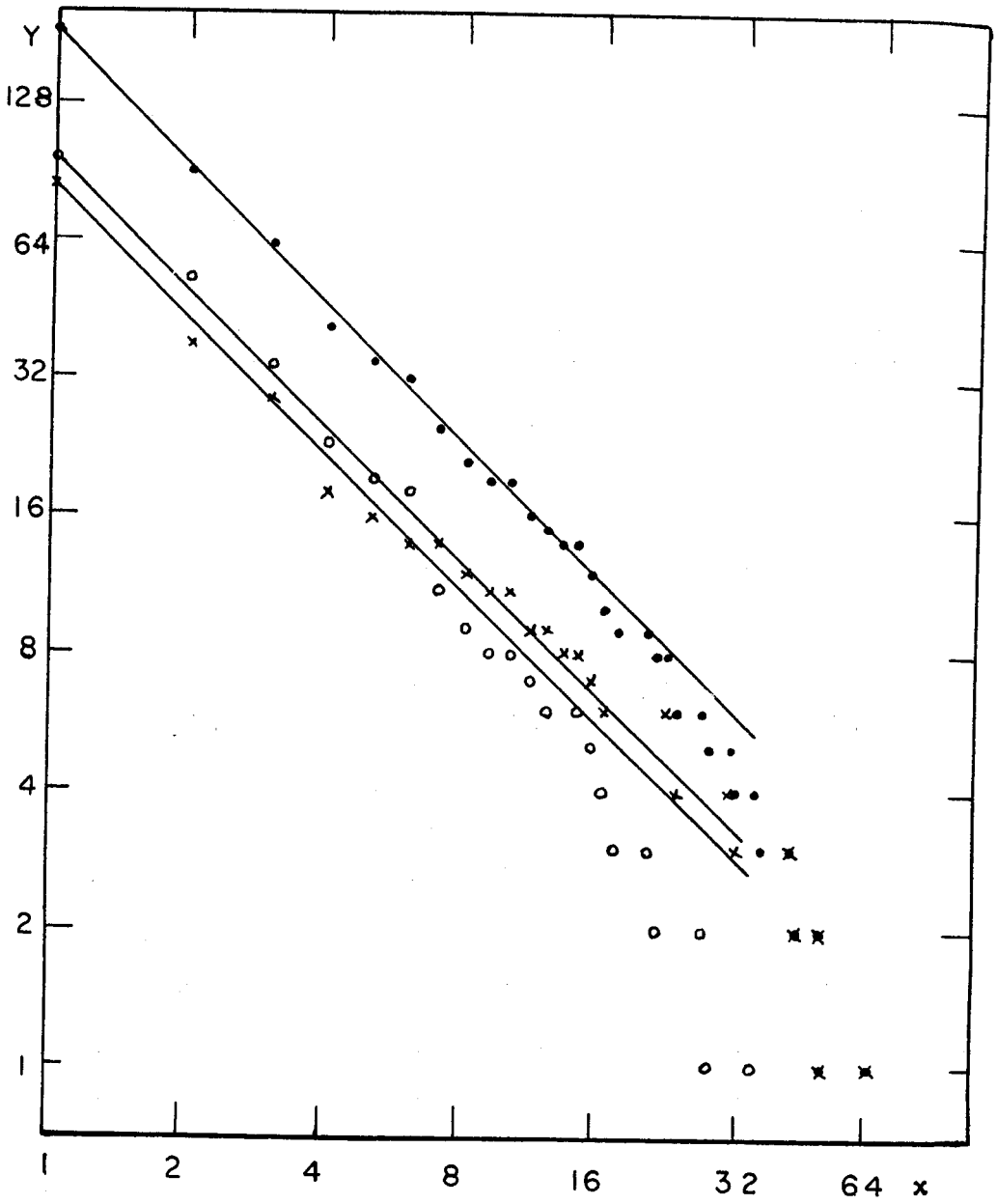

FIG. 1. The distribution of mutant clones. x = number of mutants in a clone. Y = number of clones with x or more mutants. X : *r* mutants. C : *w* mutants. ● : *r* and *w* mutants. The solid lines represent the theoretical distributions, with slope −1.

of frequent burst sizes (about 20 phages per bacterium in our experiments) and progressively more pronounced as the median burst size is approached. Of course, there cannot be any clone larger than the maximum burst size. An additional factor (suggested by Dr. S. Dancoff) that works in the same direction is nonsynchronization itself; in fact, this results in the existence, within each burst, of subclones that have originated at the same generation but have different sizes, thus producing effects similar to those of the burst size differences.

No closer analysis of the concave portion of the distribution frequency curve is feasible beyond these qualitative considerations, since the clones in this region are few and fluctuations affect the results strongly. Altogether, our results fit quite well the hypothesis that the genes

responsible for the investigated phenotypes reproduce exponentially by successive reduplications. Let us now analyze some of the factors that might affect the experimental results.

1. Failure to recognize mutants. This cause of error is difficult to assess; we believe the error to be very small. All plates were scored by the same observer after the optimum incubation period, and every plate that might have presented difficulties in scoring, because of crowding or of faulty layering, was discarded before examination. All the doubtful plaques were picked and replated for the phenotype test. Any residual error from this source would probably result in underestimation of the frequency of clones with one mutant, since the finding of the first mutant on a plate might sharpen the alertness of the observer, thereby increasing the chances of detecting other mutants on the same plate; the classes of clones with more than one mutant might thus have been favored in our observations.

2. A more definite and more easily evaluated source of error is the coincidence of more than one clone of a given mutant type (*r* or *w*) on the same plate because of coincidence of two mutations, either in the same bacterium or in the group of bacterial bursts examined on one plate. The expected coincidences ("doubles") were calculated to be 2.6 *r* and 4.3 *w*. It is not easy to correct for these "doubles," which cannot be recognized by inspection. A first approximation can be made by assuming that all "doubles" include a clone of one mutant, and that they all occur among the most frequent clone size classes observed (plates with 2, 3, 4 mutants). The resulting corrected distribution has a slight excess of ones, but does not deviate significantly from Equation (2), in spite of the fact that the correction is an extreme one, which concentrates all the distortion in the initial, most critical portion of the frequency distribution curve.

3. Failure of the plaque count method to reveal the full number of mutants is unlikely to result from technical reasons, but might be due to intrinsic properties of phage reproduction. For example, only a fraction of the gene copies produced might appear in active phage particles because of loss or inactivation of a certain proportion of phage within the bacteria before liberation. Such "sampling losses," superimposed on a logarithmic distribution, would make the initial slope of log Y versus log x steeper; a sampling loss of 50 per cent would give a quite appreciable deviation from the initial slope of −1. This is in conflict with our results. Sampling losses could be superimposed on any other distribution, but it is hard to visualize how the results could simulate those of an exponential distribution.

A variety of alternate hypotheses was considered in an attempt to find one, besides that of exponential reproduction, that could lead to the results found experimentally. No sensible hypothesis could be devised. Altogether, the hypothesis of mutations occurring at a constant rate in the course of exponential nonsynchronized gene reduplication appears adequate to account for our results.

Mutation Rates

Our results permit fairly accurate estimations of mutation rates. A total of 87.6 *r* mutations (85 mutant clones plus 2.6 coincidences) occurred in 23,000 bursts, producing approximately 1,850,000 active phage particles. The mutation frequency per reduplication is around 5×10^{-4}. 102.3 *w* mutations in 11,000 bursts, or 880,000 particles, correspond to a mutation frequency of 1.2×10^{-4}. These mutation rates, of course, are the sums of the mutation rates at all the individual loci that can mutate to give either the *r* or the *w* phenotype.

Incidental Observations

1. The mutants of T2L classified as *r* or *w* are generally clearly recognizable as such. The *r* phenotype, however, is not uniform; different *r* mutations give plaque types often distinguishable from one another. The *w* phenotype, though generally sharply distinct from *r* and from wild-type, is even more variable. Only one clone (consisting of one plaque), even after repeated replatings, could not be classified with certainty as either *w* or *r*; since at any rate it seemed to represent a novel phenotype, it was excluded from the analysis.

2. Several other types of mutants were observed in the course of our experiments, mainly "minute" or "sharp" plaque types. These were not included in the results.

3. Mottled plaques were often seen on our plates. They generally derive from mutations occurring during plaque formation. In several cases, mottled plaques were present on the same plate with *r* mutants. Three such mottled plaques were replated and the *r* component strain was isolated and crossed with a strain from a pure *r* plaque on the same plate. In two cases wild-type recombinants appeared, indicating that the mottled plaque probably stemmed from an independent *r* mutation that occurred on the plate; in the third case no recombinant was found. The latter type of mottled plaque could appear in the yield of a bacterium containing a clone of *r* mutants, owing to

some limitations to the complete segregation of virus particles or of their genetic components (Hershey and Chase, 1951).

Discussion

The exponential rate of gene reproduction in phage, suggested by our analysis of the clonal distribution of spontaneous phage mutants, simply means that the initial gene copy brought in by the infective virus does not possess the monopoly of replication. Its copies act in turn as sources for new replications and the elementary replication process is a reduplication. This conclusion, if correct, represents a step further in our analysis of phage reproduction.

Our results do not contribute any information as to whether the mutations occur only at reduplication or between reduplications, in the "interphase." They are compatible both with production of phage particles as such and of individual genetic components, although the apparent lack of "sampling losses" is more easily reconciled with the former.

There is some interest in comparing the clonal distribution of spontaneous mutants with the almost random distribution of recombinant phage from mixed-infected bacteria (Hershey and Rotman, 1949). The very first active phage particles that appear inside mixed-infected bacteria include recombinants (Doermann and Dissosway, 1949), whose distribution is also intermediate between a clonal, reduplicational one and a random one. The facts suggest the following conclusions:

1. Recombination occurs late in reproduction of the genetic material of phage. This may be due either to the coincidence of recombination with some terminal step in phage maturation or to an increased probability of recombination when large numbers of phage elements are present in a cell.

2. Recombinants detectable as mature particles around the middle of the intracellular growth period do not reduplicate as such; otherwise, by giving rise to clones, they would cause the later population of recombinants to be distributed more and more like spontaneous mutants, contrary to experimental finding (terminal distribution of recombinants almost random).

3. Reproduction of the genetic material of phage, therefore, takes place mainly by reduplication of elements that are not yet in the form of mature phage particles.

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DISCUSSION

ALTENBURG: I suggest that the conclusion to be drawn from Dr. Luria's experiments is that the reproduction cycle of viruses conforms with that of organisms in general, and that therefore viruses are to be considered organisms.