A CRITICAL TEST OF A CURRENT THEORY OF GENETIC RECOMBINATION IN BACTERIOPHAGE[1,2]

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Received September 22, 1961

HIGH *Negative Interference:* In an analysis of closely linked markers of bacteriophage T4, CHASE and DOERMANN (1958) found a phenomenon which they called "high negative interference" (HNI): the frequency of genetic recombinants whose formation requires multiple exchanges is much greater than would be predicted if exchanges in neighboring regions were statistically independent. This is not a marginal effect. For three-factor biparental crosses in which the outermost markers are less than two map units apart, the fraction of double recombinants exceeds the random expectation by a factor of 14–31. For four-factor crosses the factor is still greater.

EDGAR and STEINBERG (1958) concluded that excess double recombinants due to HNI were produced in a single mating event. This conclusion was based principally upon the results of a series of biparental, three-factor crosses in which the relative frequency of the two parents was systematically varied. Using the type of reasoning common in chemical kinetics, it was demonstrated that the excess recombinants derived their three selected markers from only two parents. It is also possible to design triparental, three-factor crosses so that the selected double recombinant must derive its three markers from three parents. In this case, there is little, if any, negative interference (STEINBERG and EDGAR 1961).

Possible explanations for HNI: Since HNI is such a striking phenomenon, we will center our discussion of recombination over short distances around the question: how can we account for HNI? We can conceive of three general directions in which an answer can be sought.

The first possibility might be called The Trivial Hypothesis. By this we mean an explanation which has little to do with the mechanism of recombination *per se.* As examples, we might mention technical artifacts—that the experiments do not really measure what we think they measure—and various types of statistical inhomogeneities. Inhomogeneity of "mating experience" can satisfactorily account for the small degree of negative interference found for crosses involving loosely linked markers. It is easy to imagine more insidious types of inhomogeneities which would be important only when relatively rare events are being con-

[1] Taken from a Ph.D. Thesis submitted to the California Institute of Technology by C. M. Steinberg.

[2] This investigation was aided by a grant from the National Foundation and by a grant (RG-6965) from the U.S. Public Health Service.

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sidered. A particularly simple example would involve a small fraction of infected bacteria which liberate large bursts of recombinants. This can be ruled out by the results of single burst experiments (STEINBERG and STAHL 1961). The surprising result here is that there are so few large bursts. In the triparental crosses cited above, recombinants are very rare indeed; thus mere rarity itself is not a sufficient condition for HNI.

The second possibility may be entitled The Hypothesis of the *Deus ex Machina*. In this type of hypothesis, HNI is assumed to be a more or less direct reflection of the mechanism of recombination among closely linked markers, but this mechanism bears little or no relation to the mechanism of recombination among loosely linked markers. CHASE and DOERMANN (1958) were able to rule out one model of this type, which they termed "muton conversion": the wild-type "allele" of a marker converts the mutant allele to the wild-type state at a rate which is independent of the other markers in the cross. [This definition seems to be the content of what is usually termed "gene conversion." Some authors, however, insist that the term is simply a name for all of the aberrant phenomena associated with intragenic recombination. Hence our preference for the newer term, muton conversion.] Another explanation, is the suggestion of HERSHEY (1958) that exchanges are stimulated by the markers themselves; hence, an excess of multiple exchanges would occur in a multifactor cross because there are more markers in a multifactor cross. This explanation appears unlikely (EDGAR, FEYNMAN, KLEIN, LIELAUSIS and STEINBERG 1962).

The third possibility we will call The Hopeful Hypothesis. Such a hypothesis assumes that HNI is a more or less direct reflection of the mechanism of recombination among closely linked markers, and that, furthermore, this mechanism is intimately related to the mechanism of recombination among loosely linked markers. We can imagine that what is counted as a "single exchange" in a cross involving only distant markers is in reality a complex event which is distributed over a finite region of the genome. We would not expect the results of a cross to be strongly dependent on the detailed structure of the exchange process unless the distances between the markers were of the same order as or smaller than the distance over which the exchange process is distributed. While the hypotheses previously considered cannot be rigorously excluded, the present hypothesis is, in fact, the only one for which there is reasonably cogent experimental support. It includes, but is somewhat broader than, the hypothesis of the "switch region" as conceived by several authors. Indeed, we will presently arrive at a prediction which is diametrically opposed to that of the "switch theory" of FREESE (1957).

Heterozygosis: Further specification of The Hopeful Hypothesis requires some knowledge of the process of recombination among loosely linked markers. In a formal sense, our information about this process comes from the study of phage heterozygotes. If bacteria are mixedly infected with mutant and wild-type phages, most of the progeny phage are homozygous in the sense that upon further growth, each gives rise to a single type—either mutant or wild type, but not both. A few particles, however, give rise to both mutant and wild-type offspring and are therefore called heterozygotes. For phage T2 the heterozygous fraction is about

two percent; for the closely related phage T4, this fraction is somewhat smaller (1.4%), a result which is probably due to technical difficulties of scoring heterozygotes with this phage. With *r*-type plaque morphology markers, heterozygous particles yield "mottled" plaques which are distinguishable from both *r* and r^+ plaques. It was the observation of such mottled plaques that led to the discovery of the phenomenon of heterozygosis in phage T2 by HERSHEY and CHASE (1951). These authors described several fundamental features of heterozygosis:

(a) Heterozygosis occurs for all markers with about the same frequency.

(b) Heterozygotes are unstable. A mottled plaque does not contain more than two percent heterozygotes.

(c) When two unlinked markers are used, phage which are heterozygous for one marker are rarely (6%) heterozygous for the other.

(d) When two closely linked markers are used, phage heterozygous for one are often heterozygous for the other. The actual frequency of double heterozygosis varies inversely with the distance between the markers and presumably approaches 100 percent for exceedingly closely linked markers.

(e) When linked markers are involved, there are always two predominant genotypes in a mottled plaque. In plaques originating from double heterozygotes, the two minority genotypes are presumed to arise secondarily from recombination during growth in the plaque. The structure of the original heterozygote is thus inferred from the predominant genotypes in the plaque.

(f) One of the predominant genotypes in a mottled plaque is always parental; the other may or may not be recombinant.

These results are easily understood by assuming that a phage heterozygote is heterozygous for a short segment of the genome.

The relationship between heterozygosis and recombination was demonstrated by LEVINTHAL (1954). Using phage T2 genetically marked with three linked factors, LEVINTHAL showed that phage heterozygous for the middle marker were predominantly recombinant in genotype for the two outside markers. That they are not *all* recombinant is easily accounted for on the basis of multiple matings. This finding resulted in the overlap model for the phage heterozygote shown in Figure 1. It is reasonable, of course, to assume that overlaps occur whether or not appropriate genetic markers are present to reveal them as heterozygotes. Overlaps are recombinant for outside markers and, *ipso facto*, form one mechanism of recombination. Are there other mechanisms? LEVINTHAL calculated that the frequency of overlaps should be sufficient to account for all observed recombination among loosely linked markers. Hence, it can be assumed that recombination among loosely linked markers occurs by means of the overlap mechanism. Similar conclusions were reached by TRAUTNER (1958) on the basis of experiments with phage T1.

It should be emphasized that the overlap is a purely formal conception. Of course, a heterozygous region must be diploid in some sense, but it is entirely possible that the entire genome is diploid in the same sense. According to this view, an overlap represents an overlap of "information sources" and would not be physically distinguishable unless it were heterozygous. The most popular

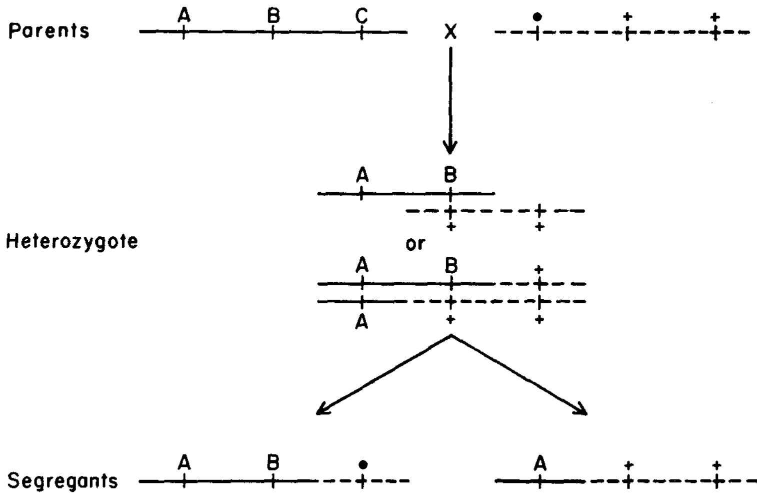

FIGURE 1.—The LEVINTHAL (1954) model for heterozygosis and recombination for distant markers (after EDGAR 1961).

physical model for the phage heterozygote has been of this type. Following LEVINTHAL (1954), it has usually been assumed that the diploidy of the phage genome reflects the duplex nature of the DNA molecule, and that the heterozygote is structurally a "heteroduplex." It is equally possible, however, that an overlap represents a structural singularity. DOERMANN and BOEHNER (1961), on the basis of recent experiments, assert that this latter view is correct and that the overlap represents an overlap of material structures. We will not pursue further this question.

Relationship of heterozygosis and recombination: To return now to The Hopeful Hypothesis, it is obvious that an overlap represents an exchange which is distributed over a finite region of the genome. Since the distance between what we have termed very closely linked markers is considerably less than the overlap length, about two percent of the particles in the vegetative pool will be heterozygotes of the type which segregate principally the two parental genotypes; i.e., nonrecombinant heterozygotes (NRH's). It should be apparent that in order to have a sufficient explanation for HNI, it is only necessary to postulate that an NRH segregates recombinants with an appreciable frequency. EDGAR (1961) has provided cogent evidence that this is indeed the case. EDGAR begins with a lysate of phage T4 containing the progeny from a biparental cross involving two exceedingly closely linked markers. He demonstrates that this lysate contains a small fraction of particles which are not recombinants but which give rise to recombinants upon singly infecting a sensitive bacterial host. The only type of particle in the lysate which could have this property is the NRH. An NRH would be expected to segregate the two parental types which, in turn, would be expected to produce recombinants just as in a mixedly infected cell. The crucial observation is a quantitative one. The probability that an NRH will give rise to a recombinant is considerably greater than the corresponding probability for a mixedly

infected cell. Thus it is concluded that while the principal segregants of an NRH are parental in genotype, there is an appreciable probability that an NRH will segregate recombinants. As EDGAR points out, however, mixedly infecting a cell is not quite comparable to infecting it with an NRH. The question of topography arises. The genomes of the phages infecting a mixedly infected cell are far apart compared to the strands of the heterozygote. One can imagine that the heterozygote segregates into the two parental types which are also physically adjacent. One can further imagine that this physical proximity will lead to "incestuous matings" among the segregants, resulting in an excess of recombinants being produced. If our objective is to account for HNI, this question is not necessarily relevant. The NRH's which are formed during the course of a mixed infection would also be expected to segregate parental types which would be physically adjacent and which would undergo incestuous matings. The only precaution is that the phrase, "segregate recombinants," must be interpreted in a sense sufficiently broad to include the result of incestuous matings.

On the basis of the observations reviewed above, EDGAR (1961) proposes the following unified mechanism of recombination for all distances. At all distances, a heterozygote (or overlap) is assumed to be an intermediate in the process of recombinant formation. Depending upon the relative position of the overlap region and the markers, there are three distinct ways in which recombinants can be formed in a two-factor cross; these ways are diagrammed in Figure 2. In the first or "direct" route, the overlap lies completely in the region between the markers and no heterozygous intermediate is detectable. In the second of "recombinant heterozygote" (RH) route, one end of the overlap lies between the markers, forming an RH; the RH has a recombinant type as one of its principal segregants. In the third or "NRH" route, both markers lie in the overlap region; the NRH, with some frequency, is able to segregate recombinants—perhaps directly, more likely through an RH intermediate. The relative contribution of each of these routes to the production of recombinants in any given cross will

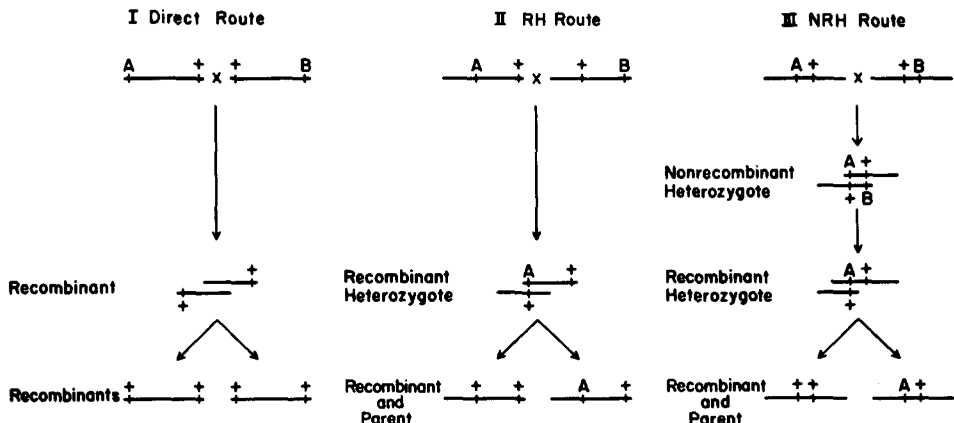

FIGURE 2.—EDGAR's generalized scheme for the production of recombinants for distant (Route I), intermediate (Route II) and close (Route III) markers (after EDGAR 1961).

depend upon the distance between the markers. A complete enumeration of the possible routes for the formation of double recombinants in three-factor crosses would be too lengthy to present here; these routes, moreover, would consist of obvious combinations of the three basic routes.

A critical test: The EDGAR scheme leads to an unusual prediction. Consider a biparental cross involving a group of very closely linked markers and, in addition, outside markers loosely linked on either side. We select a class of progeny from the cross which are recombinant for the closely linked markers and ask: what is the genotypic composition of this selected class with respect to the outside markers? We reason as follows. The selected recombinants arise as segregation products of heterozygotes. Furthermore, heterozygotes are predominantly recombinant for outside markers. Therefore, we would predict that *the selected recombinants will be predominantly recombinant for the outside markers, irrespective of how many exchanges are required to produce the selected recombinant class.* In the cross schematically shown in Figure 3, three closely linked *r*II markers are flanked by the markers A and B in coupling. According to the EDGAR scheme, r^+ recombinants are formed from an intermediate NRH which is either A+ or +B in genotype. Secondary recombinational events would produce AB and ++ as minority genotypes. Translated into the language of classical genetics, this means that triple exchanges would be more frequent than double (or quadruple) exchanges—a most unusual prediction indeed. The classical rules lead, of course, to the prediction that AB (double exchange) will be more frequent than A+ or

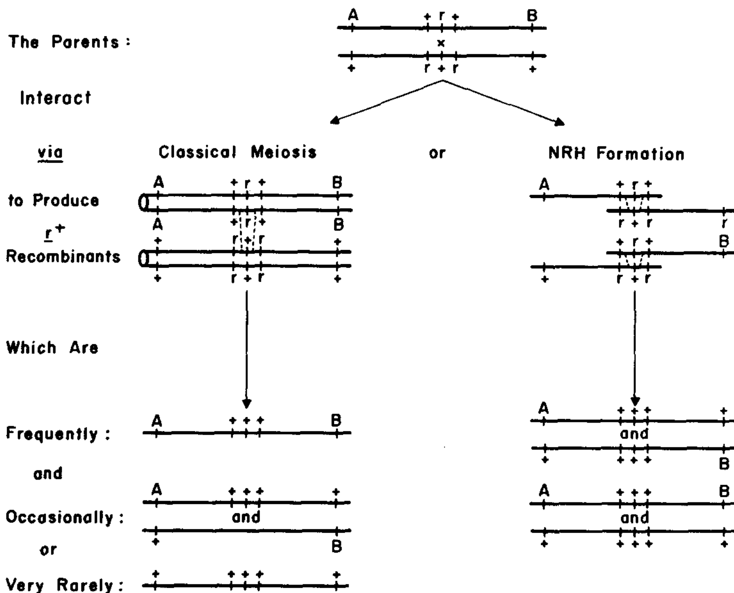

FIGURE 3.—A cross involving three closely linked *r*II markers and the two loosely linked outside markers. The segregation patterns for the outside markers predicted by the rules of classical genetics and by the EDGAR scheme are shown.

+B (triple exchange) which in turn will be more frequent than ++ (quadruple exchange). Thus the prediction of the EDGAR scheme is far from trivial. It differs so strongly from the classical picture that we thought that crosses of the type shown in Figure 3 would provide a critical test of our current ideas about the relationship of heterozygosis and recombination. Experiments along these lines will be presented in the sequel. Before proceeding further, however, let us state the result. The principal result of this paper will be that the segregation pattern of outside markers is always in accordance with the simple rules of classical genetics. Hence we will conclude that something is wrong with our current ideas.

MATERIALS AND METHODS

Bacterial strains: Several strains of *Escherichia coli* were used. Strain B was used as a host for crosses. Strain S/6 (DOERMANN and HILL 1953) was used as the standard nonselective indicator strain. Strain K is the K-12 derivative 112–12 (WOLLMAN 1953) lysogenized with phage lambda by J. J. WEIGLE. Strain F, a hybrid between K-12 and B, was given to us by P. FREDERICQ (1956) and lysogenized with lambda. Both F and K support the growth of wild-type T4 but do not support the growth of *r*II mutants (BENZER 1955) and were used as selective indicator strains.

Phage strains: The wild-type strain T4D and various mutant derivatives were employed. T4D *r*II mutants were isolated and mapped in our laboratory (EDGAR, FEYNMAN, KLEIN, LIELAUSIS and STEINBERG 1962): *r*EDa41, *r*EDb45, *r*EDb46, *r*EDb48. (In the sequel, only the numerical part of the designation will be used.) T4D *ac*41 was isolated by plating a high titer stock of T4D on acriflavine plates (see below). T4D *tu*41 is described by DOERMANN and HILL (1953).

T4D *ti* originated in the following manner. A "partial revertant" of *r*41 was selected by plating a high titer stock on strain F. This revertant, designated *r*41*su*4, gives *r*-type plaques on S/6, small plaques with sharp margins on F (with good efficiency), and "pinpoint" plaques on K. Similar partial revertants had been shown by R. P. FEYNMAN (personal communication) to be due to closely linked suppressor mutations; he further showed that the suppressor was itself a standard *r*II mutant. Our isolate, *r*41*su*4, was backcrossed to wild-type T4D; *r* plaques were picked and tested for ability to grow on K. Any recombinants would not be expected to grow on K. Out of 800 plaques tested, all grew on K. Thus, at a confidence level of 99 percent, the suppressor must be within 0.4 map units of *r*41. For our purpose this justifies treating the suppressed *r* as if it were a single mutant, designated "tiny" (*ti*) since it gives tiny plaques on strain F.

Media. H-broth: Bacto nutrient broth, 8 g; Bacto peptone, 5 g; sodium chloride, 5 g; glucose, 1 g; tap distilled water, 1 liter. H-broth was used as a liquid nutrient medium in all experiments.

EHA-bottom layer: Bacto agar, 10 g; Bacto tryptone, 13 g; sodium chloride, 8 g; sodium citrate (dihydrate), 2 g; glucose, 1.3 g; tap distilled water, 1 liter. Approximately 40 ml per plate of EHA-bottom layer is used.

EHA-top layer: Bacto agar, 6.5 g; Bacto tryptone, 13 g; sodium chloride, 8 g;

sodium citrate (dihydrate), 2 g; glucose, 3 g; tap distilled water, 1 liter. Approximately 2 ml of EHA-top layer is used in the agar overlay technique for plating.

Acriflavine supplemented plates contained 0.25 mg/liter "acriflavine neutral, NF IX" (Nutritional Biochemicals Corporation) in both bottom and top layers.

Preparation of phage stocks. All phage stocks were clonally derived from single plaques. Phage of the desired genotype are plated on S/6, and the plates are incubated at 30°C for four hours. A plug of agar containing a single well isolated plaque is removed with a capillary tube and transferred to about 25 ml of a growing culture of bacteria in H-broth. The growing culture is obtained by diluting a saturated culture of S/6 1:500 or 1:1000 into H-broth and incubating for 2½ hours at 30°C with aeration. After addition of phage, incubation is continued until lysis. The lysate is shaken with chloroform, centrifuged in the cold (ca. 4,000 × *g* for 20 min), and then filtered through a Mandler candle.

Plating conditions: Cultures of the indicator strains in the late exponential phase of growth were used for plating. A saturated culture is diluted 1:100 into H-broth, incubated for 2½ hours at 30°C with aeration, centrifuged in the cold, and resuspended in 1/10 volume of fresh broth. Such cultures may be used for several days if kept in the cold. Two or three drops were used for plating.

S/6 plates were incubated at 30°C and K plates at 37°C for 18 to 24 hours. For routine assays, F plates were incubated at 37°C for 18–24 hours. When cross progeny were plated for scoring of genotypes, another regime was used in addition: incubation at 37°C for one hour followed by incubation at 25°C for two days. At the lower temperature, the *ti* plaques are much smaller and more easily distinguishable; the preliminary incubation is necessary, however, to improve the efficiency of plating and uniformity of size. The distinguishability of the *tu* character is also better at the lower temperature, at least in a ti^+ background. Unfortunately, plaque size is not reproducible from day to day, and *ti* plaques were often too small to permit scoring of the *tu* character as well, particularly on acriflavine plates. Accordingly, both incubation regimes were usually used. Appropriate control mixtures were used to test the reliability of scoring each day. The efficiency of plating of each genotype was also determined each day by plating appropriate mixtures. When the frequency of r^+ in the progeny of a cross is low, the possibility exists that markers from parental *r* phage are "rescued" into r^+ phage from accidental mixed infections occurring on the plate. Hence, control platings in which the parental mixture was also added were performed each day. It was always possible to plate a sufficiently small amount of the cross progeny for this sort of contamination to be indetectable.

Standard cross procedure: Our procedure follows, in general, that of CHASE and DOERMANN (1958). A saturated culture of B bacteria is diluted 1:1000 into H-broth, incubated for 2½ hours at 30°C with aeration, centrifuged in the cold, and resuspended in a small volume of fresh broth. The cells are counted in a Petroff-Hausser counting chamber under the phase contrast microscope and diluted to a concentration of 4×10^8 bacteria/ml. A few minutes before the addition of phage, sufficient potassium cyanide is added to make the bacterial suspension 0.004 M.

Equal volumes of the parental phage mixture (usually containing about 6×10^9 total phage/ml) and bacterial suspension are mixed. Ten minutes is allowed for adsorption to take place, and during this interval the mixture is agitated by gentle aeration at 30°C. After the adsorption period, the infected culture is diluted at least 10^4-fold into a growth tube (GT) containing H-broth prewarmed to 30°C. An aliquot of the GT is plated immediately for total infective centers. To another aliquot, chloroform is added to kill infected cells. Unadsorbed phage are enumerated by plating the chloroform treated aliquot, and the number of infected cells is obtained by difference. Usually more than 99 percent of the parental phage are adsorbed. The GT is aerated at 30°C for 90 minutes, at which time chloroform is added to sterilize the tube. Progeny phage are counted and scored for genotype by diluting and plating under appropriate conditions.

Premature lysis procedure: When it is desired to examine the progeny produced before the normal lysis time, the standard cross procedure cannot always be used since insufficient numbers of progeny phage may be present to yield a statistically reliable sample of recombinants. The simple expedient of diluting the infected cells less after adsorption is not satisfactory due to residual cyanide. When the adsorption mixture is diluted only 10^3-fold, there is an appreciable delay in the appearance of the first intracellular phage. This is somewhat puzzling in view of the finding of BENZER and JACOB (1953) that fivefold higher concentrations of cyanide do not appreciably delay the earliest steps of intracellular development as measured by sensitivity to ultraviolet light. One could speculate that maturation is a particularly cyanide-sensitive process, but we have not pursued the matter further.

To avoid the residual cyanide effect, the adsorption mixture is diluted fortyfold into ice-cold H-broth and centrifuged at 0–4°C. The supernatant is decanted, and the upper part of the tube is wiped with sterile cotton swabs. During this procedure, particular care is taken to avoid warming the infected cells in the pellet. The pellet is resuspended in about 2 ml of cold H-broth. Development begins when the resuspended cells are transferred to a growth tube (GT) containing at least 100 ml of H-broth prewarmed to 30°C. This concentrated GT is continuously aerated, and infected cells and unadsorbed phage are measured in the usual way. It should be noted that only about ⅓ of the infected cells are recovered. Most of the loss presumably arises from centrifuging a dilute suspension in a fairly large tube. It should also be noted that despite the fact that no more than one percent of the original adsorption medium could be carried over into the concentrated GT, the ratio of apparently unadsorbed phage to infected cells changes very little. This presumably means that desorbed phage make an appreciable contribution to the unadsorbed phage. Hence, correction of the progeny yields for unadsorbed phage is likely to be rather inaccurate, and we have disregarded all samples for which this correction is more than ten percent.

Premature lysates are obtained by adding chloroform to aliquots of the concentrated growth tube at various times. When it is desired to store undiluted lysates, they are filtered.

EXPERIMENTAL RESULTS

The linkage relations of the markers used in the present experiments are shown in Figure 4. With the exception of *tu*41, these markers were all isolated

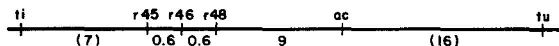

FIGURE 4.—Map showing approximate linkage relationships of the markers used in the crosses described in the text. Map distances not in parentheses are frequencies of recombination in two-factor crosses at normal lysis. Map distances in parentheses are similar measures but are based upon crosses to adjacent markers.

by us; a detailed description was given above. The map distances given in Figure 4 are values obtained after spontaneous lysis.

In order to minimize the effects of multiple matings, all of the crosses to be reported below were executed according to the premature lysis procedure described above. Genotypic frequencies were determined in lysates in which the average phage yield was 7.5 to 15 phage per cell. The lysate was plated on strain F. One set of plates was supplemented with acriflavine, and another set of plates was not. On both sets of plates, plaques were scored for the *ti* and *tu* characters by inspection of plaque morphology. The frequencies of genotypes bearing the *ac* allele are thus determined directly, while the frequency of genotypes bearing the ac^+ allele are determined by difference. As explained in Materials and Methods, small efficiency of plating (e.o.p.) corrections were usually necessary. When the selected r^+ recombinants are predominantly *ac*, small volumetric errors and errors in the determination of e.o.p. are amplified by the subtraction of one large number from another, and a high order of accuracy is not to be expected. Little difficulty was experienced in scoring plaque types on standard plates. On acriflavine plates, the scoring is somewhat difficult, particularly when both the *ti* and the *tu* characters must be scored simultaneously. Accordingly, the *tu* marker was not included in all crosses. The total number of r^+ recombinant plaques scored ranged from 4,000 to 10,000 in various crosses (except for cross no. 42 in which about 2,700 plaques were scored). Hence, statistical sampling is not the limiting source of error in any frequency exceeding a few percent.

Lysates were also plated on the nonselective indicator strain S/6 on standard plates and on acriflavine plates. The ratio of plaques on F to plaques on S/6 gives the fraction of r^+ recombinants. The *ti* marker cannot be scored in a *r* background on S/6. Hence the only additional information obtained in the crosses involving closely linked *r*II markers is the frequency of exchanges in the *ac–tu* interval for crosses in which the *tu* marker is present. One cross involved only a single *r*II marker; in this case more information was obtained from the S/6 plating.

Crosses involving three closely linked rII markers: We have performed four crosses, each involving three closely linked *r*II markers arranged in such a fashion that two exchanges are required to produce the selected r^+ recombinant type. Each cross involves a different one of the four possible arrangements of mutant and wild-type alleles at the *ti* and *ac* loci. The *tu* marker is included in only two of the crosses. Genotypic frequencies in the progeny from each cross are given

TABLE 1

Crosses involving three closely linked rII markers

1	+ r +	1	1
	×		
2	r + r	2	2

Cross no.	Genotype of +r+ parent (111)	Genotype of r+r parent (222)	Frequency, among selected r^+ recombinant class, of genotype: (111)	(112)	(211)	(212)	(122)	(121)	(222)	(221)
32	*ti* + +	+ *ac* *tu*	0.477	0.020	0.266	0.013	0.134	0.031	0.052	0.0072*
			0.497		0.279		0.165		0.059	
33	+ *ac* +	*ti* + +	0.528		0.129		0.276		0.068	
40	+ + *tu*	*ti* *ac* +	0.550	0.074	0.137	0.021	0.167	0.013*	0.034	0.0040*
			0.624		0.158		0.180		0.038	
42	*ti* *ac* +	+ + +	0.518		0.189		0.193		0.100	

* Frequency based upon a plaque count of less than 100.

in Table 1. Inspection of Table 1 reveals that qualitatively normal linkage relations are maintained in all crosses. It will be noticed that there is considerable variation from cross to cross and that this variation is not random. The variability and other quantitative considerations will be discussed below.

Crosses involving two closely linked rII markers: We have performed two crosses, each involving two closely linked *r*II markers (*r*46 and *r*48). In these crosses, a single exchange is sufficient to produce the selected r^+ recombinant type. The *ti* and *ac* markers are included in both crosses; the *tu* marker is included in only one. Genotypic frequencies in the progeny from each cross are given in Table 2. Inspection of Table 2 reveals that qualitatively normal linkage relations are again maintained.

A cross involving only one rII marker: We have performed one cross involving only one *r*II marker (*r*46). This cross differs from the crosses previously considered in that by plating on strain F, we select the parental r^+ allele, rather than an r^+ recombinant type. The results are given in Table 3. It is apparent that a few of the genotypes can be determined independently by plating on strain S/6, and we shall have occasion to refer to such results later. The frequencies given in Table 3, however, are based solely upon the platings on F; thus, the data of Table 3 are directly comparable to those obtained from the selective crosses and given in Tables 1 and 2.

Heterozygosis: Let us return to the crosses involving three closely linked *r*II markers. In this section, we will ignore the *tu* marker and concern ourselves solely with the markers adjacent to the *r* region. For each of these crosses, we calculate from the data in Table 1 the frequency of exchanges in the *ti*–*r* interval (interval I), the *r*–*ac* interval (interval II), and the frequency of simultaneous exchanges in I and II. These exchange frequencies are given in Table 4. The average frequencies of exchange are quite similar for the two intervals. As noted previously, however, there is considerable variation from cross to cross. When the data from Table 4 are rearranged as in Table 5, it is seen that a considerable part of the variation is not random. When an exchange in interval I (or II) results in a recombinant type which bears the mutant allele at the *ti* (or *ac*) locus, the apparent frequency of exchange is considerably smaller than when the exchange results in the recombinant being wild type. The effect is still more drastic

TABLE 2

Crosses involving two closely linked rII markers

1	$r+$	1	1
	×		
2	$+r$	2	2

Cross no.	Genotype of $r+$ parent (111)	Genotype of $+r$ parent (222)	Frequency, among selected r^+ recombinant class, of genotype: (211)	(212)	(111)	(112)	(222)	(221)	(122)	(121)
47	+ + *tu*	*ti ac* +	0.469	0.043	0.230	0.045	0.140	0.022	0.041	0.0100*
			0.512		0.275		0.162		0.051	
49	*ti ac* +	+ + +	0.571		0.214		0.191		0.024	

* Frequency based upon a plaque count of less than 100.

TABLE 3

A cross involving only one rII marker

1	+	1	1
	×		
2	r	2	2

Cross no.	Genotype of r^+ parent (111)	Genotype of r parent (222)	Frequency (percent), in selected class bearing r^+ allele, of genotype: (111)	(112)	(211)	(212)	(122)	(121)	(222)	(221)
50	*ti* + *tu*	+ *ac* +	86.407	7.896	2.659	0.461*	1.800	0.416	0.318	0.054*
			94.30		3.12		2.22		0.36	

* Frequency based upon a plaque count of less than 100.

TABLE 4

Exchange frequencies for intervals I and II

Cross no.	Frequency of exchanges in* Interval I (*ti-r*)	Interval II (*r-ac*)	Intervals I and II
32	0.338 (B)	0.224 (A)	0.059 (D)
33	0.197 (A)	0.344 (B)	0.068 (D)
40	0.196 (A)	0.218 (A)	0.038 (C)
42	0.289 (B)	0.293 (B)	0.100 (E)
Average	0.255	0.270	0.066

* Letter in parentheses beside each frequency is used for reference in text and in Table 5.
Data in this table are based upon Table 1. Reading from left to right, the three columns of exchange frequencies are the frequencies of genotypes 21· + 22·, 12· + 22·, and 22·, respectively. The dot (·) in the genotype formulae indicates that the *tu* allele is disregarded (*e.g.*, 21· = 221 + 222).

TABLE 5

Asymmetry in exchange frequencies for intervals I and II

Apparent frequency of exchanges in* Interval I or interval II when recombinant type is: Mutant (A)	Wild type (B)	Interval I and interval II when recombinant type is: Double mutant (C)	Single mutant (D)	Wild type (E)
0.224	0.338	0.038	0.059	0.100
0.197	0.344		0.068	
0.196	0.289			
0.218	0.293			
0.209 (Average)	0.316 (Average)	0.038	0.064	0.100

* Data in this table are taken from Table 4. Listed under column A, for instance, are all of the values marked "(A)" in Table 4.

when double exchanges are considered. The double recombinant may bear zero, one, or two mutant alleles, and the apparent exchange frequency decreases in the same order.

An asymmetry between mutant and wild-type alleles is shown by one of the crosses (cross 47) involving two closely linked *r*II markers (Table 2), but not by the other cross. That is, one of these crosses is not only inconsistent with the obervations made in the preceding paragraph but also with the other cross of the same type. In view of this, we will not further consider the crosses shown in Table 2.

The asymmetry between mutant and wild-type alleles is not due to selection or to different efficiencies of plating (e.o.p.'s), at least not in the ordinary sense. When *ac* and *ti* are backcrossed to wild type, no selective effects are demonstrable. Furthermore, the data have already been corrected for e.o.p. differences as determined by plating control mixtures of different genotypes. Special selective or e.o.p. effects which are only demonstrable when recombinants for closely linked markers are selected cannot be excluded.

A more reasonable explanation in terms of known phenomena could be given, however, if a significant fraction of the selected r^+ recombinants were heterozygous for *ti* or *ac* (or both). It is apparent that an adequate explanation of the data in terms of heterozygosis requires that particles *heterozygous* for *ti* or *ac* be

phenotypically wild type. A direct determination of the phenotype of *ac* or *ti* heterozygotes is not feasible. However, if cells are mixedly infected with high equal multiplicities of *ti* and ti^+ phage and plated on strain F, more than 99 percent of the plaques are ti^+ in morphology. Similarly, of cells mixedly infected with high equal multiplicities of *ac* and ac^+, less than one percent give rise to plaques on acriflavine plates. Hence, *ti* and *ac* are both recessive in the sense defined above, and it is reasonable to assume that heterozygotes for either marker will be phenotypically wild type. The recessiveness of *ti* is readily explicable in terms of selective forces which are operative during the course of plaque formation on strain F. It is, in fact, such selection that makes the direct determination of the phenotype of a *ti* heterozygote impossible. It is more difficult to understand why *ac*, an allele for resistance, should be recessive to ac^+, an allele for sensitivity, but the effect is quite real and reproducible (EDGAR and EPSTEIN 1961).

In order to test the "heterozygote hypothesis," as well as for its intrinsic interest, let us attempt to calculate the frequencies of the various classes of heterozygotes which must be postulated in order to account for our results. At either locus (*ac* or *ti*), a given particle may be homozygous for allele 1 (see Table 1), homozygous for allele 2, or heterozygous. Thus, there are three states at each locus or nine classes in all. We shall assume strict symmetry, which reduces the number of different frequencies to be found to six. Since the frequencies of all classes must sum to unity, only five of the frequencies are independent. The five quantities, A, B, C, D, and E, in Table 5 are thus sufficient to calculate the frequencies of all nine classes. For example, C, the frequency of the double recombinant when that type is a double mutant (*ti ac*), must be the frequency of the "pure," nonheterozygous double recombinant. We shall not go through the algebra involved in calculating the other quantities here. The results and numerical calculations are given in Table 6.

TABLE 6

Calculation of the frequencies of heterozygous and pure types

	Allele(s) present at locus* *ti*	*ac*	Frequency: Formula†	Calculated value
Doubly heterozygous class	1,2	1,2	C −2D +E	0.010
Singly heterozygous classes	1,2	1	−A +B +D −E	0.071
	1	1,2	−A +B +D −E	0.071
	1,2	2	−C +D	0.026
	2	1,2	−C +D	0.026
Nonheterozygous classes	1	1	1 −2B +E	0.468
	1	2	A −D	0.145
	2	1	A −D	0.145
	2	2	C	0.038
			1	1.000

* Numerical designations for alleles present at *ti* and *ac* loci refer to Table 1.
† Letters in formulae for calculation of frequencies refer to Table 5. Average values from Table 5 were used for numerical calculations.

Turning now to Table 6, we note that the "heterozygote hypothesis" is reasonable in the sense that the calculated frequencies of all classes of particles are positive. The total frequency of heterozygotes is calculated to be 20.4%, a quite significant fraction. Aside from this, no great reliance should be placed on the numerical values. With this warning, however, we proceed to note a few points. Most of the heterozygotes are heterozygous for only one marker. Indeed, the frequency of double heterozygosis (1.0%) is close to the square of the frequency of heterozygosis for an individual marker (10.7%), and in this sense, heterozygosis occurs independently for each locus. The frequency of heterozygosis among our selected recombinants is considerably greater than the frequency (2%) observed in the whole population. In view of the observations of EDGAR (1958) that a considerable fraction of the phenotypically r^+ are actually heterozygotes, it seems reasonable to assume that the particles heterozygous for an outside marker are also heterozygous for at least one of the *r*II markers. That is, the heterozygotes observed here are actually quite long. The only relevant data on the distribution of lengths of overlap regions are those of DOERMANN and BOEHNER (1961). From these data we estimate that, of particles heterozygous for one of the *r*II markers, 15–20 percent would also be heterozygous for one of the outside markers. If we assume that the distribution of overlap regions in our case is similar, then at least 50 percent of the selected r^+ recombinants are actually heterozygotes. Alternatively, we could assume that less than 50 percent of the selected r^+ recombinants are heterozygotes, but these heterozygotes would then be longer, on the average, than in the entire population.

It is also possible to calculate genotypic frequencies for the separate "strands" of the heterozygotes. Such calculations show that qualitatively normal linkage relations are maintained for the heterozygous classes as well as for the pure types. The exchange frequencies calculated for the heterozygous strands are somewhat greater than for the nonheterozygous classes. In the Discussion, we shall seriously consider a hypothesis which in the present context amounts to saying that the pure types arise from heterozygotes. The numerical calculations, for what they are worth, do not contradict this hypothesis.

An effect of heterozygosis is also evident in the cross in which the selected r^+ class is not necessarily recombinant (cross 50, Table 3). The apparent frequencies of exchange are given in Table 7. The exchange frequencies in this cross are very much lower than in the crosses previously considered. Hence, we would expect heterozygotes occurring with the normal frequency to influence the results. For

TABLE 7

*Exchange frequencies for intervals I and II**

Exchanges in interval	Frequency	Number of mutant alleles in recombinant type
I	0.0348	0
II	0.0258	1
I and II	0.0036	1

* Data in this table are taken from Table 3; they are all based upon platings on indicator strain F.

this cross, it is also possible to determine the frequency of exchange in interval I by plating on indicator strain S/6. On strain S/6, selection against the *ti* allele is not strong, and *ti* heterozygotes (usually) give rise to mottled plaques. The frequency of exchange in interval I determined by plating on S/6 is 0.0274; this value is considerably smaller than the value of 0.0348 obtained by plating on F and is quite close to the frequency of exchange in interval II determined by plating on F, 0.0258.

Interference: In this section, we will consider several types of interference relationships. Before proceeding further, however, a few cautionary notes should be introduced. In view of the conclusion that a significant fraction of the r^+ recombinant progeny are heterozygous for the outside markers, the meaningfulness of any interference calculation is open to question. We shall, wherever possible, use average values to eliminate some of the biases due to heterozygotes. All of our data concern cross progeny obtained by premature lysis of the infected cells. With our procedure, it is impossible each day to lyse the cells at exactly the same point in the phage life cycle. Thus the data are nonhomogeneous to this extent. Furthermore, as noted previously, some of the procedures used in order to obtain frequency data are not intrinsically very accurate. Therefore, we should not expect to be able to make fine comparisons and should restrict ourselves to rather gross effects.

First, let us consider interference in the production of r^+ recombinants, without reference to the outside markers. In Table 8, we give the frequency of r^+ recombinants in each of our crosses. The data in Table 8 allow us to estimate the frequency of double exchanges and the frequency of exchanges in one of the two intervals. No comparable data are available for the other interval. Since the frequencies of recombination are essentially the same in the two intervals at the time of normal lysis (see Figure 4), it is reasonable to assume that they are also

TABLE 8

Negative interference in the production of r$^+$ *recombinants*

Exchanges selected in interval(s)	Cross no.	Frequency of exchanges*	Random expectation†	Interference index‡
	47	1.98×10^{-3}		
r46–r48	49	1.84×10^{-3}		
	Average	1.91×10^{-3}		
r45–r46		Assumed to be equal to interval *r46–r48* (see text)		
	32	2.15×10^{-4}		
r46–r48	33	1.87×10^{-4}		
and	40	1.85×10^{-4}		
r45–r46	42	1.93×10^{-4}		
	Average	1.95×10^{-4}	3.65×10^{-6}	53.4

* In each cross, the frequency of exchanges is twice the observed frequency of r^+ recombinants in order to take into account the reciprocal recombinant class.

† The random expectation for the frequency of double exchanges is the product of the exchange frequencies in the single intervals.

‡ The interference index is the ratio of the observed frequency of double exchanges to the random expectation.

the same at the lysis times used here. Thus, we calculate in Table 8 that the frequency of double exchanges exceeds random expectation by a factor of slightly over 50. This factor is three to four times the interference index obtained by CHASE and DOERMANN (1958) for markers at comparable distances. The discrepancy is, of course, explicable in terms of different lysis times in the two cases. It is perhaps more instructive to look at the absolute values rather than the interference index. Given an exchange in one interval, there is a probability of about ten percent that there will be an exchange in the other interval.

Let us now consider interference in the intervals adjacent to the *r*II markers. This can be looked at in two ways. First, we can compare the frequency of exchange in interval I (or II) among the selected r^+ recombinant class with the corresponding frequency of exchange among all particles. The latter must be determined in a separate cross (cross 50). Furthermore, we can compare the frequency of double exchanges in interval I and interval II with a random expectation based upon the frequencies of exchanges observed in the separate intervals among the r^+ recombinants. The first comparison gives us a measure of interference due to selection of r^+ recombinants, while the second gives a measure of additional interference within this class. The relevant data and calculations are shown in Table 9. The data in Table 9 are based upon the crosses involving three closely linked *r*II markers together with data from the cross with selection for an r^+ parental allele for comparison. We have assumed that the markers are symmetrically placed and have averaged all individual frequencies. An examination of Table 9 reveals that the frequency of exchange for a single interval (I or II) is over eightfold greater among the r^+ recombinant class than among the entire progeny. On the other hand, there is essentially no additional interference for double exchanges within the r^+ recombinant class.

Thus far we have neglected the *tu* marker. We will refer to the *ac–tu* interval as interval III. The frequency of exchanges in interval III (unlike in intervals I and II) may be determined over the entire progeny by plating on S/6 on acriflavine plates and scoring the *tu* character. In Table 10, we present the frequency of exchanges in interval III in both the selected r^+ class and in the entire popula-

TABLE 9

Negative interference for exchanges in intervals I and II

	Frequency of exchanges when selected r^+ class is: Parental allele	Doubly recombinant	Interference index‡
	(a)§	(b)§	(I_2)
Single interval (I *or* II)	0.0303	0.262	8.6
Both intervals (I *and* II)	0.0036	0.066	
Random expectation*	0.00092	0.069	
Interference index, I_1†	3.9	0.96	

* "Random expectation" for each column is the square of the frequency for the corresponding single interval.
† Interference index, I_1, is the ratio of the observed frequency of exchanges in both intervals to the corresponding random expectation.
‡ Interference index, I_2, is the ratio of the frequency in column (b) to that in column (a).
§ Data in column (a) are based on Table 7. Data in column (b) are based on Table 4. Average values are used in both cases.

TABLE 10

Exchange frequencies for interval III

Cross no.	No. of selected exchanges	Frequency of exchanges in interval III: Selected class*	All classes†
32	2	0.071	0.097
40	2	0.076	0.080
47	1	0.117	0.081
Average of above		0.088	0.086
50	0	0.087	0.084

* The "selected class" data are based on Tables 1, 2, and 3; they are derived from platings on strain F.
† The "all classes" data are derived from platings on strain S/6.

tion; all crosses involving the *tu* marker are included in this table. We first note that for the cross in which the selected r^+ class is not necessarily recombinant (cross 50), the two methods of determination agree quite well. If we consider the average of the other crosses (in which the selected r^+ progeny are recombinants), the two determinations are not only similar to each other, but to the former determinations as well.[4] That is, there is no interference. This is a rather surprising and intriguing result. It has invariably been true that when *any* recombinant class is selected, the frequency of any other recombinant class is greater than for the population as a whole. This is explained in terms of heterogeneity in "mating experience." In view of this, our finding that there is no interference, if true, would really mean that there is *positive* interference in a single "mating." We will not present further interference calculations in detail. In the cross in which the selected r^+ type is not necessarily recombinant, the expected negative interference for exchanges in interval III is demonstrable when considering any recombinant class. In the selective crosses, there is a comparable degree of negative interference for exchanges in interval III among particles recombinant in the adjacent interval II. Among particles recombinant in interval I, there is, if anything, positive interference. The significance of these findings is by no means clear.

DISCUSSION

The new experimental results reported here concern the segregation pattern of loosely linked outside markers within a selected class of particles which are recombinant for closely linked internal markers. We can summarize our observations as follows:

(a) The outside markers maintain qualitatively normal linkage relations with the internal markers and with each other. This is true whether one or two exchanges are required to produce the selected recombinant class.

(b) The region of HNI extends into the intervals adjacent to those in which the exchanges are selected but does not extend into a nonadjacent interval.

(c) Multiple exchanges within the selected class occur with little, if any,

[4] Taken at face value, there would appear to be a difference between the results when one exchange and when two exchanges are selected. Whether this is significant or not cannot be said.

additional interference. That is, given the frequencies of exchanges for the separate intervals within the selected class, the frequency of multiple exchanges is essentially equal to the random expectation.

(d) A significant fraction of the selected recombinant class is heterozygous for at least the adjacent outside markers. The evidence upon which this conclusion is based is indirect and is, to this extent, weaker than the preceding observations.

The fact that, aside from the complications of negative interference and heterozygosis, the segregation pattern of outside markers is normal would, of itself, require little comment. In view of the previously discussed current notions concerning the mechanism of recombination in phage, the "complications" are to be expected, while the "normal" result leads us to a paradox. We are now faced with three apparently well founded assertions:

(a) According to EDGAR (1961), recombinants for very closely linked markers arise as segregation products of heterozygotes—either directly or as a result of incestuous matings among the primary segregation products of heterozygotes.

(b) According to LEVINTHAL (1954) and to TRAUTNER (1958), heterozygotes are recombinant for outside markers.

(c) According to our observations, recombinants for closely linked markers are not necessarily recombinant for outside markers.

We do not think that all of these assertions can be strictly correct.

We do not pretend to be able to resolve the paradox at the present time. We can only examine it a little more closely and speculate about the direction from which an eventual resolution might come. It is possible, of course, that the paradox is the result of an error of fact, but we consider this to be unlikely. If there is no error of fact, there must be an error of interpretation.

First of all, it should be noted that we have tacitly assumed that there is only one type of heterozygote. Next, we consider the assertions individually. In reality, the three paradoxical assertions are not highly derivative. They follow very closely from experimental observations, and were we not faced with an apparent paradox, we would be tempted to refer to them as "facts" rather than as "assertions." The factual basis for EDGAR's assertion is that an NRH, upon singly infecting a host cell, segregates recombinants with a frequency great enough to account for the production of recombinants for closely linked markers in a mixedly infected cell. It is assumed that an NRH which arises in a mixedly infected cell behaves in an identical manner. At first sight, the other two assertions might appear to be direct statements of the experimental observations, but in fact they involve a similar type of assumption. The factual basis for the LEVINTHAL-TRAUTNER assertion is that the *progeny* of heterozygotes (in mottled plaques) are predominantly recombinant for outside markers. It is assumed that the heterozygous particles (which give rise to mottled plaques) are themselves recombinant. A similar *caveat* is applicable to our own assertion. We do not directly examine recombinants for closely linked markers; we examine the progeny of such recombinants at some remove. These would seem to be the important assumptions. There may be others.

If any of the above mentioned assumptions were not strictly correct, it would

be easy to resolve the paradox. If there were two types of heterozygotes, for instance, then LEVINTHAL and TRAUTNER could be studying one type and EDGAR the other type, and there is no paradox. If an NRH which arises during the course of a mixed infection behaves somewhat differently from a singly infecting NRH, there are several possibilities. One of the more likely ones involves the previously discussed notions of topography and incestuous matings. We might imagine that an NRH which arose during the course of a mixed infection produces recombinants as a result of an incestuous mating with one (or both) of the parental types which gave rise to the NRH. This could not happen, of course, with a singly infecting NRH.

We could speculate, however, that the fallacy lies in reasoning that a heterozygote is recombinant for outside markers because its offspring are so recombinant. If the primary heterozygote is not recombinant for the outside markers, what is its genotype? We would not wish to argue that it is parental in genotype; hence, it must be heterozygous for one or both of the outside markers as well. We have already pointed out that it is possible that the entire phage genome is diploid in the same sense that a heterozygous region must be diploid. We now note that it is not even necessary to assume that the major portion of the genome is homozygous. We might postulate that phage T4 is a diploid organism but that it replicates vegetatively in such a fashion that all of its progeny usually bear the same allele at any given genetic site. Over a short region of the genome, both alleles may appear in the progeny, thus enabling us to recognize that the progenitor was heterozygous. In this light, our finding that a significant fraction of the selected recombinants are heterozygous for the outside markers assumes a new importance. Compatible with the diploid postulate is a recent result of DOERMANN and BOEHNER (1961). They have analysed the phage present in mottled plaques arising from particles heterozygous for several fairly closely linked markers. They find that, in a given plaque, the frequency of the mutant allele in the plaque is different for different markers. In particular, a given allele may be present in very low frequency. This suggests that the original particle was also heterozygous for other markers which, however, did not appear at all in the progeny. We should note that DOERMANN and BOEHNER interpret their results in quite a different fashion. In any event, it is likely that a resolution of our paradox will emerge from further studies on heterozygotes.

SUMMARY

Results are reported concerning the segregation pattern of loosely linked outside markers within a selected class of particles which are recombinant for closely linked internal markers:

(a) The outside markers maintain qualitatively normal linkage relations with the internal markers and with each other. This is true whether one or two exchanges are required to produce the selected recombinant class.

(b) The region of HNI extends into the intervals adjacent to those in which the exchanges are selected but does not extend into a nonadjacent interval.

(c) Multiple exchanges within the selected class occur with little, if any, additional interference.

(d) A significant fraction of the selected recombinant class is heterozygous for at least the adjacent outside markers.

In summary, aside from the "complications" of negative interference and heterozygosis, the segregation pattern of outside markers is "normal."

In view of current notions concerning the mechanism of recombination in phage, the "complications" are to be expected, while the "normal" result leads to a paradox. According to observations of other workers, recombinants for very closely linked markers arise as segregation products of phage heterozygotes, and furthermore, such heterozygotes are recombinant for outside markers. On the other hand, result (a) above means that recombinants for closely linked markers are not necessarily recombinant for outside markers. Some possible resolutions of this paradox are discussed.

ACKNOWLEDGMENTS

The authors are indebted to PROFESSOR MAX DELBRÜCK for his encouragement of this work. They also wish to thank DR. R. H. EPSTEIN and MR. MILLARD SUSMAN for criticism and discussion and MRS. ILGA LIELAUSIS for technical assistance.

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