GENETIC INTERFERENCE AND RECOMBINATION IN PHAGE T4: THE ROLE OF HETEROZYGOTES¹

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HIGH negative interference over short spans of the genetic structure of phage T4 was first observed by CHASE and DOERMANN (1958). To explain their observations these authors proposed the existence of switch areas located at random over a fractional length of the genetic structure and over which recombination has a higher probability of occurring than in equivalent regions with no switch area. These authors reviewed the data on other organisms such as *Aspergillus*, *Neurospora*, and *Drosophila* and generalized that switch areas are a widespread genetic phenomenon, providing that sufficiently minute genetic spans are studied.

In this communication we test the hypothesis that all recombination in phage proceeds solely via the formation of heterozygous structures (LEVINTHAL 1954) and, derivative from this, that the high negative interference (HNI) as usually calculated for short spans ignores the existence of heterozygotic structures and leads to overestimates of genetic interference.

THEORETICAL

It has been demonstrated for phage T2 by LEVINTHAL (1954) and for phage T1 by TRAUTNER (1958) that heterozygote frequencies are sufficient (although not necessary) to account for all genetic recombination. Consistent with LEVINTHAL's hypothesis regarding the role of heterozygotes as intermediates in recombinant production, and with modifications of HERSHEY's (1958) theory for phage crosses we write:

$$R = 2 ab f_1 f_2 (1 - e^{-mhp}) \quad (1)$$

where R is the observed recombination frequency, p is the probability of a switch per marked region, m is the average number of phage generations, h is the frequency of phage heterozygous at any given point, a and b are frequencies of input phage, f_1 is the finite input factor of LENNOX, LEVINTHAL and SMITH (1953), and f_2 is the measure of the extent to which the population is panmictic. Equation 1 is an approximation since it does not consider spread in maturation time, or other experimental variables. However, we regard this approximate

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form as sufficient for our needs and do not use the more exact equation, which can be derived from HERSHEY's paper (1958).

Since $2abf_1f_2$ is a constant, in part by our experimental design, if we measure m (via burst size) and assume that p remains constant for any given pair of markers, then for any two closely linked markers:

$$R \simeq mhp \quad (2)$$

Thus, if our hypothesis is true that heterozygotes are necessary for recombinant formation, we predict that observed recombination frequencies will be dependent upon heterozygote frequencies. Consider two extreme cases: (1) that heterozygotes formed between two distant points lead directly to recombinant formation; and (2) that heterozygotes which encompass two close points can or cannot lead to recombinant formation depending upon p . In the first case, distant markers of map-span large relative to overlap length, our approximations do not hold, p approaches unity, and recombinant frequency is dependent solely upon h when $2abf_1f_2$ and m are held constant. When map distance is small relative to heterozygote length we hypothesize that recombinants are not formed immediately, but only upon heterozygote segregation with probability p . It is this latter case to which we have restricted our observations, calculations and conclusions.

EXPERIMENTAL

We have performed under various experimental conditions two-factor crosses of *rII-B* cistron markers $1f \times 1g$ and $1g \times 1h$ which are 0.39 and 0.37 map units in span, and we have computed for each cross R , recombination frequency; m , average number of generations as determined by burst size; and h , heterozygote frequency. Properties of *rII* mutants $1f$, $1g$, and $1h$, and standard cross procedures were presented by FOLSOME (1965).

Heterozygote frequencies were measured by plating about 60 progeny phage per plate (for a minimum of 30 plates) on *E. coli* B. After 24 hr incubation B progeny plates were replica plated, using sterile velvet pads, to fresh plates seeded with *E. coli* K-12 (λ *str-r*, plus 0.5 mg streptomycin. The K replicas were matched to B master plates after 24 hr incubation. For each original progeny r plaque one of four responses was scored: true r^+ recombinant; negative; revertant r^+ (rare); or heterozygous r^+ . Negative r plaques are purely parental genotype. Revertant r^+ responses were rarely observed on control plates (each parental r plated separately and then replica plated) and when present, generally did not manifest large numbers of r^+ . Heterozygous r^+ responses are detected as r master plaques that transferred many r^+ phage (formed by recombination during plaque development on B) to K plates. The distinction between r master plaques containing r^+ revertants and those containing r^+ due to heterozygote segregation and subsequent recombination is definite and clear. For two-factor crosses $f \times g$ and $g \times h$, heterozygote frequency was measured by this replica-plating method. Averages from both crosses are presented in Table 1.

5-fluorodeoxyuridine (FUDR) treatment of multicomplexes followed basically the method of HERTTEL (1963) and employed the following special media and solutions: M9C, minimal medium M9 plus 0.5% Difco Casamino acids; solution I, 40 μ g FUDR plus 80 μ g uracil per ml distilled water; solution II, 10, 40, or 80 μ g FUDR per ml M9C; chloramphenicol, 2.5 mg per ml M9. Bacteria B, adapted to growth in M9C were grown from small inocula to 10^8 cells/ml. For each cross at time 0, 0.8 ml B containing 20 μ g/ml L-tryptophan was mixed with 0.2 ml solution I and aerated at 37°C to time 2 min. Input phages at a multiplicity of 5 each were then added and the adsorption mixture was incubated to time 13 min. Then 0.2 ml chloramphenicol stock was added to adsorption tubes and incubation continued to time 133 min. At this time multicomplexes were chilled, centrifuged, and washed with cold solution II, resuspended in 0.9 ml M9C plus 0.1

ml solution II, incubated a further 60 min, then treated with one drop of chloroform, and assayed. Control FUDR experiments were always performed serially according to the above protocol except that FUDR was omitted from solutions I and II. Average burst sizes ranged from 10 to 20 for most experiments (FUDR and controls). Variation in FUDR concentration from 1 to 8 $\mu\text{g/ml}$ during post-chloramphenicol incubation of complexes from time 133 to time 193 min did not affect average burst sizes in any consistent manner.

Multiplicities of input phages were varied in otherwise standard broth crosses by varying the input volumes of each parental phage stock. Thus the total multiplicity remained constant while the ratio of minority to majority input phages was varied. No corrections were made for bacteria receiving 0 phage of the minority genotype since it is the uncorrected data which we require to test our hypothesis concerning the relationships between h , R , and P . Variable minority to majority ratios provide, within limits, a means of reducing h and measuring the effect on R and p .

Heterozygote frequencies were varied by ultraviolet irradiation of multicomplexes (as by FOLSOME 1962).

Media, phage and bacterial strains, and general procedures are in FOLSOME (1962, 1965).

Results: Two-factor crosses. Table 1 presents the effect of these various treatments to multicomplexes upon R , h , and m . Also in Table 1 is the calculated value of p by equation 1 which should by our hypothesis be constant. Figure 1 illustrates R as a function of h for the two two-factor crosses $f \times g$ and $g \times h$, and is linear within experimental error. Thus, two lines of evidence support our predictions embodied in equation 2: first, p , the recombination probability is constant although h and R vary; second, a definite linear relationship exists between R and h .

Figure 2 presents p plotted against h . If p is a constant we would expect such a plot to generate a straight line of slope 0. Although some scatter of points does exist owing to compounding of experimental errors and to approximations, no

TABLE 1
Analysis of two-factor crosses: heterozygote frequency varied

Conditions	$R(h-g) \times 10^{-2}$	$R(f-g) \times 10^{-2}$	$h \times 10^{-2}$	m	$p(h-g)^*$	$p(f-g)^{\dagger}$
Standard cross	0.37	0.39	1.4	7.2	0.07	0.08
FUDR I Control	0.20	0.16	0.4	4.4	0.23	0.18
FUDR I (4 $\mu\text{g/ml}$)	8.60	7.68	7.0	3.9	0.59	0.52
FUDR II Control	0.27	0.37	1.1	4.4	0.11	0.15
FUDR II (1 $\mu\text{g/ml}$)	1.72	1.52	5.4	3.9	0.16	0.15
FUDR II (4 $\mu\text{g/ml}$)	3.08	2.34	9.1	3.9	0.17	0.13
FUDR II (8 $\mu\text{g/ml}$)	4.06	3.84	6.5	3.9	0.32	0.30
UV Control	0.28	0.29	1.3	7.2	0.06	0.06
UV (65 sec)	1.59	1.00	3.6	6.9	0.13	0.08
UV (90 sec)	2.24	1.37	3.0	7.0	0.21	0.13
UV (120 sec)	2.42	3.36	3.8	6.0	0.21	0.30
MOI (0.26 min./maj.)	0.22	0.15	0.2	7.7	0.29	0.20
MOI (0.20 min./maj.)	0.08	0.05	0.3	7.7	0.70	0.04

R : recombination frequency as $2 \times r^+$ phage/total phage. h : heterozygote frequency from cross $f \times g$ and $g \times h$ averaged (see text). m : from 2^m = average burst size. p : calculated from equation 1, assuming ($ab f_1 f_2 = 1$).

Standard and UV crosses: see FOLSOME (1962). FUDR crosses: see HERTEL (1963).

* $\bar{p}(h-g) \pm \text{se}_M = 0.20 \pm 0.04$.

$\dagger \bar{p}(f-g) \pm \text{se}_M = 0.18 \pm 0.04$.

FIGURE 1.—Recombination frequencies, R , graphed as a function of heterozygote frequency, h , for two-factor crosses $f \times g$ (●) and $g \times h$ (○).

marked deviation or trend from a line of 0 slope is evident. $\bar{p} \pm s_{EM}$ values of $0.20 \pm .04$ ($h \times g$) and $0.18 \pm .04$ ($f \times g$) obtained from all calculated p values of Table 1 indicate that p is constant for any pair of markers and is independent of h .

FIGURE 2.—Recombination probability per mating region, p , calculated as described in the text, graphed as a function of heterozygote frequency, h , for two-factor crosses $f \times g$ (●) and $g \times h$ (○).

Results: Three-factor crosses. Crosses of $fh \times g$ (FOLSOME 1965) in which two simultaneous events must occur to produce an r^+ had been performed simultaneously with the two-factor crosses $f \times g$ and $g \times h$, discussed above. Since our replica-plating method cannot measure heterozygote frequencies from three-factor crosses of this type, we make the assumption that two- and three-factor crosses performed under equivalent experimental conditions on the same day manifest the same heterozygote frequencies.

Customarily S , the measure of genetic interference, is calculated (after CHASE and DOERMANN 1958) as:

$$S = r_d / R_{xy} R_{yz} \quad (3)$$

where, r_d is the observed frequency of double recombinants, R_{xy} and R_{yz} frequencies of recombinants for regions x - y and y - z .

However, we hypothesized and presented above some evidence that R is a function of h and that for short spans h is *the only* fraction of the entire phage pool capable of generating recombinants. S appears, then, to be misleading since we can write:

$$S = \frac{r_d}{R_{xy} R_{yz}} = \frac{r_d}{m^2 h^2 P_{xy} P_{yz}} \quad (4)$$

since $R \simeq mhp$ (assuming m and h to be constant among any three cross sets). S is incorrect by the fraction $1/mh$ on our hypothesis, since the values P_{xy} and P_{yz} , and not R_{xy} and R_{yz} should be used to calculate S .

To determine the extent and direction of interference when solely the mating fraction (heterozygotes) from the entire phage pool is considered, we can perform two tests. First, we plot S (as customarily calculated from equation 3 against experimentally varied h). We predict a linear relationship will be realized in the form: $S \propto 1/mh$. Second, we calculate S_p where

$$S_p = \frac{r_d}{mh P_{xy} P_{yz}} \quad (5)$$

We predict for this equation that S_p will be constant value independent of variations in h and that S_p will be a minimal measure of the interference that exists among the mating fraction of the phage pool, assuming all heterozygotes can generate recombinants.

Figure 3, in which h and S are graphed, clearly demonstrates that S is a value inversely proportional to h . Table 2 presents the data used for Figure 3 and the calculated S_p interferences according to equation 5. Although S ranges from 3.8 to 79.1 and h from 0.2×10^{-2} to 9.1×10^{-2} , S_p values are quite consistent with an average S_p of 0.32 for all experiments. Hence we conclude: (1) S_p calculated from p and h values from a set of crosses provides a valid minimal and uniform estimate of interference for the mating (heterozygous) proportion of the vegetative phage pool; (2) high negative interference as usually calculated represents only the fact that a *small fraction* (h) of the vegetative phage pool are capable of generating recombinants; (3) genetic interference for short spans among the mating population may be slightly positive, slightly negative, or unity (see DISCUSSION).

FIGURE 3.—Genetic interference values, S , calculated according to text equation 3, graphed as a function of heterozygote frequency, h , for three-factor crosses $f + h \times + g +$.

TABLE 2

Biparental three-factor crosses of $f-h \times g$ performed simultaneously with equivalent two-factor crosses as listed in Table 1

Condition	r_d^*	$S_1^\dagger$	$h^\ddagger$	$S_p^\S$
Standard cross	1.91	13.2	1.4	0.34
FUDR I Control	1.72	21.8	0.4	0.24
FUDR I ($\mu\text{g/ml}$)	252.0	3.8	7.0	0.30
FUDR II Control	2.42	24.4	1.1	0.30
FUDR II (1 $\mu\text{g/ml}$)	19.8	7.6	5.4	0.39
FUDR II (4 $\mu\text{g/ml}$)	48.8	6.8	9.1	0.62
FUDR II (8 $\mu\text{g/ml}$)	98.2	6.3	6.5	0.40
UV Control	1.74	21.4	1.3	0.52
UV (65 sec)	10.7	6.8	3.6	0.41
UV (90 sec)	15.3	4.9	3.0	0.27
UV (120 sec)	30.8	3.8	3.8	0.21
MOI (0.26 min./maj.)	1.03	31.2	0.2	0.12
MOI (0.20 min./maj.)	0.29	79.1	0.3	0.05

* $f-h \times g$ recombination frequency $\times 10^{-4}$.
 † Interference value calculated according to equation 4.
 ‡ Heterozygote frequency $\times 10^{-2}$ from matching crosses of Table 1.
 § Calculated assuming heterozygotes only generate recombinants by $S_p = r_d / (m h p_{xy} p_{yz})$ using p , m , and h values from Table 1.

DISCUSSION

Our data support the concept that the vegetative phage pool is one which, for any generation, can be compartmented into mating and nonmating sectors for any given (short) genetic span. We are led to believe that the mating sector of the population is composed either of a constant fraction or solely of nonrecombinant or recombinant heterozygotes, depending upon the relationship of the overlap region to the genetic region studied. This concept of recombinant formation is not new, as LEVINTHAL (1954) has demonstrated heterozygotes to be sufficient to account for all recombination, while EDGAR (1961) has shown that nonrecombinant heterozygotes can act to generate recombinants with greater efficiency than a mixedly infected cell.

We do believe that our results point out that recombination frequencies for two- or three-factor crosses respond in a specific manner consistent with experimentally altered heterozygote frequencies. Thus, our results lend credence to the hypothesis that heterozygotes *alone* are necessary intermediates of recombination.

Assuming the truth of the statement that only heterozygotes can generate recombinants, we then calculated genetic interference for three-factor crosses in which heterozygote frequencies were varied and corrected for the nonmating fraction of the vegetative phage population. The result is that genetic interference is not negative but appears slightly positive. However, the conclusion regarding positive interference rests upon the assumption that all heterozygotes can form recombinants. Were this not the case, the value of h employed in our equations would be decreased and S_p would be correspondingly increased. Thus the S_p values we have calculated are minimal. If a constant fraction, F , of observed heterozygotes produced recombinants, then our S_p average value of 0.32 should in actuality be increased by the term $1/F$. Thus our data cannot show whether S_p is slightly positive, slightly negative, or unity. The data do indicate that a constant interference value near unity is obtainable when overall heterozygote frequency is assumed to be a determinant of recombination frequency. Of interest is the observation of NOMURA and BENZER (1961) that there appear to exist two types of heterozygote, one which can ($1/3$) and one which cannot ($2/3$) form an extended mismatch. If only one of these two types could generate recombinants, our S_p average would be increased from 0.32 to 0.48, or to 0.96 ($0.32 \times 3/2$; $0.32 \times 3/1$).

DOERMANN (1963) has demonstrated that recombination values derived from single bursts of single heterozygous phages of multi-factor T4D *rII* crosses are considerably elevated. When these data are analyzed for interference, little difference is found between expected and observed double recombinant frequencies. Thus, DOERMANN's examination of the recombinant output of heterozygous structures agree well with our conclusions as to the role of phage heterozygotes and with our independent determinations that one recombinant event does not influence an adjacent one.

Whether one considers *Drosophila*, *Neurospora*, *Aspergillus*, or other organisms, it is evident that two levels of mating opportunity exist. The first level in-

volves relatively large spans which embrace *at least one* effective pairing region (PRITCHARD 1960) between any two given distant markers. In this instance, any pair of homologous chromosomes has a high probability of being in a configuration in which recombination could occur. Thus, mating (effective-pairing-region or heterozygote formation) between homologous chromosomes is not limited to a fraction of these chromosomes.

The second level, involving closely linked markers, is one in which only a fraction of all homologous chromosomes have opportunity to mate by effective-pairing-region formation. Therefore, in this second case the entire population of chromosomes actually is two subpopulations, one which can and one which cannot offer opportunity for recombination. High negative interference would be apparent upon consideration of the entire population, but not of just the mating fraction.

Since the phage vegetative pool contains a limited small number of heterozygous structures per short genetic span, the high negative interference of CHASE and DOERMANN (1958) appears to result from calculations performed on the basis of the entire population rather than on the basis of the heterozygous structures within the population. Our treatment of the data yields constant interference values near unity.

SUMMARY

When phage T4 *rII* multicomplexes are treated during growth with 5-fluorodeoxyuridine, or are ultraviolet-irradiated, both heterozygote and recombination frequencies, and the extent of high negative interference, are altered. Recombination frequencies can be expressed as a variable dependent upon heterozygote frequencies. We have employed an approximate method for the calculation of switching frequency based upon the hypothesis that heterozygous structures are necessary for genetic recombination. Switching frequencies thus calculated were constant for any close pair of markers, although recombination and heterozygote frequencies had been both experimentally altered. High negative interference as customarily calculated also proved to be a variable dependent on heterozygote frequencies. A mode of calculation of interference based upon the presumed functional role of heterozygotes has been evolved and applied; the result is that genetic interference does not appear to be high, but slightly positive, or slightly negative, or unity.

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