GENE LINEARITY AND NEGATIVE INTERFERENCE IN CROSSES OF ESCHERICHIA COLI

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THE discovery of sexual reproduction and genetic recombination in *Escherichia coli* strain K-12 was made possible by controlled crosses of mutant lines, differing in growth factor requirements (TATUM and LEDERBERG 1947; LEDERBERG 1947) or resistance to drugs and phages (LEDERBERG 1950). Where reversion rates are low, recovery of nutritionally independent (prototroph) or doubly resistant cells from mixedly infected selecting media is dependent upon genetic exchange between complementary mutant strains. Thus in crosses of threonine leucine dependent (diauxotrophic) W-677 (T^-L^-) and biotin methionine dependent 58-161 (B^-M^-) only prototroph recombinants ($B^+M^+T^+L^+$) are recovered as colonies from minimal medium. The segregation of additional unselected markers into the selected recombinant class may be studied.

Using these methods LEDERBERG in 1947 showed that *B, M, L, T,* and unselected loci controlling lactose fermentation (*Lac*) and bacteriophage T_1 resistance (V_1) are linked according to the map (*BM*) 22 *Lac* 38 V_1 20 (*LT*). The order of loci within brackets was not established. Since recombination of (*BM*) and (*LT*) is obligatory, map distances had to be based on the relative frequencies of single and triple crossovers. Interference cannot be estimated where only two loci are studied between selected loci (BAILEY 1951) and where a third one (V_6) was considered there were strong indications of interference.

Further linkage data on derivatives of LEDERBERG's lines were published by NEWCOMBE and NYHOLM (1950a, b, and c). A locus controlling streptomycin response recombined to 5 or 10 percent with (*BM*) and segregation independently of *Lac* and V_1 placed it to the left of (*BM*). Various fermentation loci (*Mal, Xyl, Gal, Ara*) apparently located between *S* and (*BM*) could not be mapped unequivocally. Assuming the existence of linearity, it appeared that multiple crossovers were in excess (negative interference). Similarly high coincidence in crossing over between fermentation loci was also observed by LEDERBERG, CAVALLI and ALLEN (LEDERBERG *et al.* 1951; CAVALLI 1950).

Since these anomalies left bacterial linearity unproven, it was decided to reinvestigate the region to the right of (*BM*) involving the loci *B, M, Lac,* V_1, *L,* and *T*. The order of *L* and *T* would be decided on the basis that monoauxotrophs for the factor mapped to the right should show the better agreement with prototroph segregation of *Lac* and V_1. Critical data would be

obtained by supplementing crosses with leucine and threonine one at a time and scoring for growth factor requirements as well as fermentation and resistance characteristics. Furthermore by supplementing both threonine and leucine and selecting for recombinants between S and (BM) one could recover all recombination classes of Lac, V_1, L, and T and test for linearity and interference in a progeny unbiased except for possible interference across (BM). The results and conclusions from unsupplemented, singly supplemented and doubly supplemented crosses are described in the following report.

MATERIAL AND METHODS

The parental lines were LEDERBERG's W-677 and a streptomycin resistant mutant (NEWCOMBE and NYHOLM) of LEDERBERG's 59-161 of *E. coli* K-12. These lines were reported to possess the following mutant characters:

W-677: Lac^-, Mal^-, Xyl^-, Gal^-, Ara^-, $V_1{}^r$, $B_1{}^-$, T^-, L^-
58-161 S^r: B^-, M^-, S^r

where the symbols represent, respectively, lactose, maltose, xylose, galactose, and arabinose non-fermentation, bacteriophage T_1 resistance, thiamin, threonine, leucine, biotin, and methionine requirements, and streptomycin resistance.

The characteristics of the parent lines were verified with the following exceptions:

1) The thiamin requirement of W-677 was not clearly indicated. Nonetheless, to safeguard against possible selective advantages of $B_1{}^+$ recombinants, a supplement of 2.5 μg/l was added to all cross and test media. Thus, henceforth, when minimal medium is spoken of, this means thiamin minimal medium.

2) The leucine requirement of W-677 could only be filled with L-leucine. This, for supplemented crosses, needs to be entirely free of methionine with which most commercial samples are contaminated. Full growth is obtained with a supplement of 18 μg/ml. D-leucine is inhibitory, and growth fails in all concentrations of the racemic mixture.

3) The biotin requirement of 58-161 could not be demonstrated. In the presence of methionine, the addition of biotin to the medium did not improve growth of 58-161 and the addition of raw egg white, containing avidin, did not inhibit growth. After considerable experimentation it was concluded that in our crosses, B is not operative as a selecting locus. Thus, in unsupplemented crosses, only the loci M, T, and L are relied upon for selection of recombinants. Fortunately the reversion rate to methionine independence is very low, and no prototrophs have been found in controls.

Unsupplemented crosses were made by spreading washed suspensions of the two parents (M^- and T^-L^-) on minimal agar (LEDERBERG 1947). After 48 hours incubation prototroph recombinants ($M^+T^+L^+$) were picked, diluted, and test-streaked directly on eosine-methylene blue (EMB) agar plates with appropriate sugars and cross-streaks of bacteriophage and streptomycin.

Threonine supplemented crosses were carried out in an analogous manner on minimal agar supplemented with 18 μg/ml of threonine. Recombinant

colonies ($M^+T^+L^+$ and $M^+T^-L^+$) were tested as in unsupplemented crosses, but in addition were scored for threonine requirement by streaking on minimal agar with and without threonine supplement.

Leucine supplemented crosses were made on minimal agar containing 18 μg/ml of leucine. Recombinants from this type of cross had to be picked against a heavy background growth which could not be eliminated. Categorizing of recombinant colonies ($M^+T^+L^+$ and $M^+T^+L^-$) was as in unsupplemented crosses. The leucine requirement was tested for by streaking on minimal agar with and without leucine.

Threonine-leucine supplemented crosses were screened for S^rM^+ recombinants. The washed parents were plated together on doubly supplemented minimal medium. Under these conditions W-677 grew very rapidly. Following eight hours preliminary incubation for initiation of zygote formation and recombination, cross plates were sprayed with 20 mg of streptomycin. After a further 36 hours incubation S^rM^+ colonies were picked from a perfectly clear background. These were subjected to single colony reisolation on the cross medium to eliminate W-677 parental contaminants. "Purified" colonies ($M^+T^+L^+$, $M^+T^-L^+$, $M^+T^+L^-$, $M^+T^-L^-$) were then tested for fermentation and resistance characteristics on the standard set of EMB plates, and for growth factor requirements on four minimal agar plates containing, respectively, no supplement, threonine alone, leucine alone, and both threonine and leucine.

RESULTS AND CONCLUSIONS

The unsupplemented cross

When W-677 and 58-161 are crossed on minimal agar, four types of progeny are recovered through recombination in the right end of the linkage map. These are due to single crossovers in regions a ($Lac^+V_1^s$), b ($Lac^-V_1^s$), c ($Lac^-V_1^r$), and triple crossing over in abc ($Lac^+V_1^r$) (see figure 1). Three

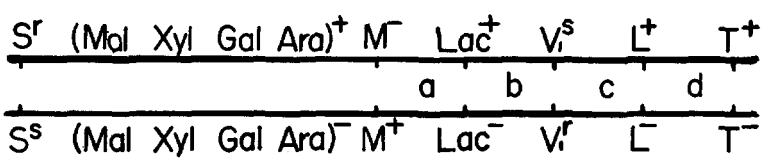

FIGURE 1.—Linear sequence of gene loci M, Lac, V_1, L, T in lines of *Escherichia coli* K-12. (Above, 58-161 S^r; below, W-677.)

replicate experiments were in close agreement and the totals only are shown in table 1.

Contamination with prototroph mutants is considered negligible since: (1) in regard to 58-161, the reversion rate to methionine independence is low and S^r(*Mal Xyl Gal Ara*)$^+$ colonies are infrequent and their proportion no higher among the $Lac^+V_1^s$ than among the other three classes combined; and (2) in regard to W-677 a simultaneous reversion to leucine and threonine independence is improbable and cannot be demonstrated when large populations of this parent alone are plated on minimal medium.

TABLE 1

Segregation of Lac, V_1, T, and L in unsupplemented, threonine supplemented, leucine supplemented and threonine leucine supplemented crosses of W-677 × 58-161 S^r.

Recombination classes	Supplement							
	None		T		L		TL	
	Number	Fraction	Number	Fraction	Number	Fraction	Number	Fraction
T^+L^+ $Lac^+V_1^s$	409	.337	286	.343	343	.500	40	.288
$Lac^+V_1^r$	39	.032	32	.038	20	.029	8	.058
$Lac^-V_1^s$	497	.410	310	.372	191	.278	57	.410
$Lac^-V_1^r$	267	.220	206	.247	132	.192	34	.245
Total	1212	.999	834	1.000	686	.999	139	1.001
T^-L^+ $Lac^+V_1^s$			60	.165			5	.227
$Lac^+V_1^r$			9	.025			0	.000
$Lac^-V_1^s$			138	.380			9	.409
$Lac^-V_1^r$			156	.430			8	.364
Total			363	1.000			22	1.000
T^+L^- $Lac^+V_1^s$					5	.013	7	.137
$Lac^+V_1^r$					8	.021	6	.118
$Lac^-V_1^s$					7	.018	6	.118
$Lac^-V_1^r$					366	.948	32	.627
Total					386	1.000	51	1.000
T^-L^- $Lac^+V_1^s$							14	.012
$Lac^+V_1^r$							20	.017
$Lac^-V_1^s$							39	.033
$Lac^-V_1^r$							1115	.939
Total							1188	1.001

Assuming absence of interference, recombination percentages a, b, and c were calculated from the four class totals from the equations:

1. $a - ab - ac + abc = 40900/1212\,x$
2. $b - ab - bc + abc = 49700/1212\,x$
3. $c - ac - bc + abc = 26700/1212\,x$
4. $abc = 3900/1212\,x$

where x is a correction factor for eliminated non- and double crossovers. These equations yield the values a = 25.1, b = 30.4, c = 19.0, (x = 2.22), in fair accord with LEDERBERG's mapping.

The T-supplemented cross

Since *L* and *T* are linked (LEDERBERG 1947) the percentage frequency of L^+T^- recombinants will equal the percentage recombination over *L-T*, provided there is no interference. On the same assumption, if *T* is to the right, the *Lac* V_1 segregations will be alike among the L^+T^+ and the L^+T^-. If *T* is to the left, a relative excess of $Lac^-V_1^r$, the only single crossover progeny, is expected among the L^+T^-. In five replicate experiments the proportion and genetic composition of the L^+T^- varied somewhat, presumably through

the inclusion of variable numbers of W-677 reverts to leucine independence. Since heterogeneity was not such as to affect conclusions on the sequence of *L* and *T*, the totals only are shown in table 1.

The data indicate approximately 30 percent recombination over *L*-*T*. The *Lac* V_1 segregations of the L^+T^+ agree very well with those from the unsupplemented cross but among the L^+T^- there is a significant excess of $Lac^-V_1{}^r$. This excess appears inadequate to justify mapping *T* to the left of *L* since this procedure would render one triple crossover type ($Lac^-V_1{}^s$) almost as common as the presumed single. On the other hand, mapping *T* to the right of *L*, the excess of $Lac^-V_1{}^r$ among the L^+T^- might be accounted for by (1) contamination of the cross with a considerable number of W-677 reverts to leucine independence and (2) the operation of negative interference, double recombinants over cd being more common than expected. Reversions were anticipated since controls inoculated only with W-677 yield colonies. That they did occur is indicated by the relative excess of $S^s(Mal\ Xyl\ Gal\ Ara)^-$ among the $Lac^-V_1{}^rL^+T^-$. Reality of the interference phenomenon is supported by the relative excess of $Lac^-V_1{}^s$ (bd) over $Lac^+V_1{}^s$ (ad) among the threonine dependents. Thus, since there is experimental support for the efficacy of both postulated mechanism, *T* is provisionally mapped to the right of *L*.

The effect of mutants on the estimate of map distance d may be eliminated by ignoring the $Lac^-V_1{}^r$ both among the L^+T^+ and the L^+T^-. This procedure yields 207 T^- colonies in a total progeny of 835 and the recombination percentage d = 25, ignoring interference.

The L-supplemented cross

If the sequence of *L* and *T* inferred above is correct the *L*-cross is expected to yield an abundance of $Lac^-V_1{}^r$ among the leucine dependents. Five replicate experiments differed somewhat in the proportion of L^+T^+ and L^+T^- and the *Lac* V_1 segregations within both groups. Variations were not such as to affect seriously mapping of *L* and *T* and totals only are shown in table 1.

The prototrophs may be considered first. Their *Lac* V_1 segregations should be independent of the crossing medium, the excess of $Lac^+V_1{}^s$ on leucine minimal medium (table 1) therefore requires an explanation. It is not due to excessive methionine reversion in the presence of leucine as there is no change in the proportion of $Lac^+V_1{}^s$ that are $(Mal\ Xyl\ Gal\ Ara)^+$. It would appear, therefore, that either certain L^+T^+ recombinants (largely $Lac^+V_1{}^s$) which form colonies in the presence of leucine do not do so in its absence or, more probably, that some L^+T^+ (largely other than $Lac^+V_1{}^s$) which grow on unsupplemented medium are inhibited by leucine. On the likeliest interpretation the prototrophs comprise two genetic types differing in a modifier allele (L_m) such that $L^+L_m{}^-T^+$ is inhibited by leucine while $L^+L_m{}^+T^+$ is not. The original K-12 (and the derived 58-161) grow both in the presence and absence of leucine ($L^+L_m{}^+$). Irradiation presumably produced an $L^-L_m{}^+$ stock which did not grow in the absence of leucine and which grew poorly in its

presence but sufficiently so to collect the L_m^- modifier mutation which enabled it to grow well. This is the condition of W-677 ($L^-L_m^-$). Interaction of L and L_m is such that the crossover product $L^+L_m^-$ grows only in the absence of leucine. The elimination in the L plates of approximately one half the $Lac^-V_1^s$ recombinants suggests that the locus L_m is halfway between *Lac* and V_1. This, however, is not conclusive evidence of its position. The basic interpretation in terms of a modifier locus is borne out by the results of the doubly supplemented cross where it is found that certain prototrophs grow well on minimal medium, grow moderately well on TL-supplemented medium, but do not grow at all on L-supplemented medium. Among these types $Lac^-V_1^s$ and $Lac^-V_1^r$ predominate.

Turning next to the L^-T^+, the expected vast excess of $Lac^-V_1^r$ is indeed observed; in fact, in one of five replicate experiments no other types were obtained. This finding immediately supports mapping L to the left of T. However, two phenomena must be taken into account which contribute $Lac^-V_1^r$ by means other than the linkage *M, Lac, V_1, L, T*. These are: (1) segregation of the modifier L_m favoring the $Lac^-V_1^r$ with the parental combination $L^-L_m^-$; and (2) reversion of W-677 to threonine independence. The latter effect is significant as judged by (a) the number of $Lac^-V_1^r$ that are S^s(*Mal Xyl Gal Ara*)$^-$ and (b) crude mutation rate studies which indicate approximately 10^{-8} reversions per bacterial division. That appreciable numbers of $Lac^-V_1^r$ are not mutants is clear since 5 percent carry one or more of the 58-161 markers in addition to T^+. Furthermore, with either sequence of L and T, the proportion of L^+T^- in the T-cross (30 percent) and of L^-T^+ in the L-cross (36 percent) should be of the same order of magnitude (ad + bd + cd + abcd against d + abd + acd + bcd). This relation cannot be maintained if almost all $Lac^-V_1^r$ are considered mutants.

It is concluded, therefore, that the L-cross yields a real excess of $Lac^-V_1^r$ consistent with the mapping of L to the left of T. This conclusion is borne out by the results of the TL-cross described in the next section.

The TL-supplemented cross

In the doubly supplemented cross S^rM^+ recombinants are selected and the confusing effects of reversions to threonine and leucine independence are eliminated. On the other hand W-677 S^r mutants are scored as non-crossovers and 58-161 methionine reverts as single recombinants in a. The crossover distribution in the freely segregating region to the right of M is recorded for five replicate experiments in table 2 on the provisional sequence of figure 1. Possible mutant types were scored as non-c.o./$S^s - S^r$ ($Sugar^-V_1^rL^-T^-$) and single c.o. a/$M^- - M^+$ ($Sugar^+V_1^sL^+T^+$) as opposed to *bona fide* non-c.o. and single c.o. a with mixed parental fermentation markers.

An attempt was made to determine the proportion of mutants and segregants in the two possible mutant classes. The number of recombinants among the 25 single c.o. a/$M^- - M^+$ was estimated using the following ratios of

TABLE 2

Segregation of Lac, V_L, L, and T in S^rM^+ recombinants from threonine leucine supplemented cross. (5 replicate experiments. For crossover regions a, b, c, d, see figure 1.)

Crossover (CO) type	Experiment no.					Total	Total minus mutants
	1	2	3	4	5		
0 CO/S^s-S^r	80	112	37	152	67	448	
0 CO	248	117	69	162	79	675	1115
0 CO (all)							1115
1 CO a/M^--M^+	4	0	14	2	5	25	
1 CO a	8	9	3	4	4	28	40
1 CO b	6	17	8	20	6	57	57
1 CO c	3	10	7	5	9	34	34
1 CO d	11	5	3	7	6	32	32
1 CO (all)							163
2 CO ab	5	2	1	7	5	20	20
2 CO ac	5	1	0	2	6	14	14
2 CO ad	2	0	0	2	1	5	5
2 CO bc	7	6	4	15	7	39	39
2 CO bd	4	1	1	3	0	9	9
2 CO cd	4	3	0	0	1	8	8
2 CO (all)							95
3 CO abc	0	2	3	3	0	8	8
3 CO abd	1	3	0	1	1	6	6
3 CO acd	1	3	0	2	1	7	7
3 CO bcd	0	2	0	2	2	6	6
3 CO (all)							27
4 CO (all)	0	0	0	0	0	0	0
Total	389	293	150	389	200	1421	1400

(*Mal Xyl Gal Ara*)$^+$/total in known recombination classes: single c.o. a (incl. mutants): 25/53, single c.o. b: 12/57, single c.o. c: 4/34, single c.o. d: 3/32, all doubles: 20/95, all triples 2/27. On the basis of the single c.o. b at least 12 of the "possible mutants" are regarded as recombinants (raising the total single c.o. a to 40) and the remaining 13 as mutants. Most of the latter occurred in the single run No. 3 in which, for unknown reasons, the ratio of mutants and recombinants appears shifted.

A direct attempt was made to estimate the relative frequencies of S^r mutants and recombinants. In experiment No. 4, 20 TL plates were inoculated with both parents, another six plates were inoculated with W-677 ($S^s M^+$) only. On spraying with streptomycin the cross plates yielded 500 colonies. The controls yielded no S^r mutants. Similarly, in experiment No. 5, 10 cross plates gave 200 colonies and eight control plates gave two independent S^r mutants.

Thus, whereas a total of 30 cross plates yielded 700 well separated colonies, the 14 control plates gave only two mutants and an experiment of the order of the total of table 2 may be expected to include only about eight S^r mutants.

An indirect approach also was made using the following ratios of (*Gal Ara Xyl Mal*)$^-$/total in known recombinant groups: non c.o. (incl. mutants): 448/1123 = .39, all singles (excl. a): 27/123 = .22, all doubles: 26/95 = .27, all triples: 8/27 = .30. The ratio does not differ significantly among the single, double, and triple recombinants and averages .25. If this is valid also for the noncrossover segregants, only 900 of the non-c.o./$S^s - S^r$ are recombinants and 223 must be eliminated as mutants. However, if there is negative interference and particularly an excess of non-crossovers through irregular and incomplete pairing, the number of mutants would be overestimated by this procedure. More weight is attached, therefore, to the direct evidence of the control experiment and in table 2 only eight of the 448 non-c.o./$S^s - S^r$ are eliminated as mutants. The implied very low mutation rate is in accord with the experience of NEWCOMBE and HAWIRKO (1949) who, with maximum population densities on complete media, had difficulty getting S^r mutants.

The results of the doubly supplemented cross as shown in the last column of table 2 support the linear sequence of *M Lac* V_1 *L T* inferred from the singly supplemented crosses, for: (1) of the 16 possible combinations of *Lac* V_1 *L* and *T*, the only one not found in 1400 segregants is $Lac^+V_1{}^rL^+T^-$, the presumed quadruple crossover, all others being found at least five times; and (2) here, where reversions to threonine and leucine independence are not selected for, there is a distinct excess of $Lac^-V_1{}^r$ among the L^-T^+ (table 1), whereas the *Lac* V_1 segregations of the L^+T^- are like those of the prototrophs. However, while the linear sequence *M Lac* V_1 *L T* is confirmed, the provisional assumption of absence of interferences can be shown to be inadequate and map distances must be revised. These points are considered in the following discussion.

DISCUSSION

In the TL-supplemented cross S^rM^+ recombinants alone survive and all recombination classes involving *Lac*, V_1, *L*, and *T* (non-, single, double, triple, quadruple crossovers) are recovered. Valid estimates of crossover frequencies are obtained only if there is no interference across *M*. The best prospect of minimizing and estimating the possible effects of limited interference lies in the study of recombination distal to selected loci.

Using this approach NEWCOMBE and NYHOLM (1950c) attempted to place the loci *S Mal Xyl Gal Ara* in a linear array to the left of *M*. However, depending upon the method (1, interaction with *Lac;* 2, recombination with *S;* 3, recombination with *M;* 4, comparison of factors three at a time, and choosing as middle factor that one undergoing recombination least often with respect to the other two), five different and conflicting sequences were obtained (two from method 4). The number of colonies in their "unselected" crosses was too small to base a sequence on the identification of the quintuple

crossover type, but the finding of all *Gal Ara Xyl Mal* combinations among the S^r is contrary to the suggested sequence *S* (*Mal Gal Ara Xyl*) *M* and the demonstrated interaction across *M* of all fermentation loci, and *Lac* is in itself anomalous. Since no sequence could be chosen as being the most probable, the authors proceeded to show that, granting linearity, their evidence for conflicting sequence implied an excess of multiple crossovers, no matter which sequence is, in fact, the correct one.

In the present study the sequence *M Lac* V_1 *L T* is strongly indicated, yet there is evidence of the same kind of anomaly which was observed by NEWCOMBE and NYHOLM. (1) Among the 1400 recombinants of table 2 recombination between *M* and the unselected loci *Lac*, V_1, *L*, and *T* occurred with the following frequencies: *M-Lac:* 100, *M-*V_1*:* 177, *M-L:* 161, *M-T:* 190. Thus frequency of recombination with *M* yields the sequence *M Lac L* V_1 *T*, contrary to the linked segregations of *Lac* and V_1 in unsupplemented crosses. (2) Determining the sequence by the "middle locus method" nine of the ten possible comparisons of loci three at a time are compatible with the sequence *M Lac* V_1 *L T*, but the tenth comparison places *L* between *M* and V_1. In both cases conflict with the accepted sequence is largely due to bc being more common than expected. Since excessive numbers of bc were introduced through the single run No. 4 no assurance is felt that the evidence for conflicting sequence is significant.

On the other hand, striking interference effects are revealed in the relative frequencies of non-, single, double and triple recombinants. In 1400 recombinants 100 crossovers were scored in a, 145 in b, 116 in c, and 73 in d. Thus, the TL-cross yields the recombination percentages a = 7.1, b = 10.4, c = 8.3, d = 5.2, as compared to a = 25.1, b = 30.4, c = 19.0, d = 25.0 from non- and T-supplemented crosses assuming absence of interference and differential death. In table 3 the expected and observed frequencies of all recombination classes are compared. It is clear that there is (1) a relative deficit of all single recombinants, and (2) a tendency for multiple crossovers to occur close together, that is in terms of survivors crossing over is rare, but where one crossover occurs it tends to be accompanied by a second and a third nearby.

NEWCOMBE and NYHOLM have attempted to decide whether excess survival or excess formation of multiple recombinants is responsible for this type of anomalous distribution. Among genetic mechanisms leading to selective survival they have discussed: ring chromosomes, position of loci between lethals or in inversions, and loss phenomena from zygotes carrying the *Het*-factor (LEDERBERG 1949, and 1950b). They have concluded that no simple structural or genic modification of chromosomes would alone account for the anomalous segregations to the left of *M*.

However, more unorthodox complexities may be involved. Thus LEDERBERG *et al.* (1951) propose that zygotes of W-677 and 58-161 are interchange hybrids, a cross-shaped quadrivalent pairing configuration being the physical counterpart of a branched four-armed linkage map. This suggestion gains

TABLE 3

Segregations compared with expectations: 1. in absence of interference 2. with excess of non-crossovers (1000/1400) due to non-pairing. (For crossover regions a, b, c, d, see figure 1.)

Crossover type	Observed	Expected			
		Non-interference		Excess non-CO	
	Number	Number	χ^2	Number	χ^2
O (all)	1115	1013.07	10.26	1111.00	.014
a	40	77.93	18.46	37.00	.243
b	57	117.05	30.81	63.12	.593
c	34	91.53	36.16	45.36	2.845
d	32	55.73	10.10	24.76	2.117
all single	163	342.24		170.24	
ab	20	9.00	13.44	21.04	.051
ac	14	7.04	6.88	15.12	.083
ad	5	4.29	.12	8.28	1.300
bc	39	10.57	76.47	25.76	6.805
bd	9	6.44	1.02	14.12	1.856
cd	8	5.03	1.75	10.12	.444
all double	95	42.37		94.44	
abc	8	.81	63.83	8.60	.042
abd	6	.50	60.50	4.68	.372
acd	7	.39	112.03	3.36	3.943
bcd	6	.58	50.66	5.76	.010
all triple	27	2.28		22.40	
abcd (all quadruple)	0	.04	.04	1.92	1.922
Total	1400	1400.00	492.53[1]	1400.00	22.640[1]

[1] 15 degrees of freedom.

substance from DELAMATER's evidence (1951) that *E. coli* K-12 has two chromosomes per nucleus. Interchange heterozygosity might explain conflicting indications of sequence and the nearly equal non-linear linkage of several loci with *M* (one breakage point near *M*). It does not account for the interaction between *Lac* and other fermentation loci nor for the significant interaction between all of the loci studied by NEWCOMBE and NYHOLM. In the present study it is possible to place the loci *M*, *Lac*, V_1, *L*, and *T* in an unambiguous sequence, and interaction among these loci is of a higher order than between them and other loci. Thus, these factors would be linearly arrayed on one arm of the linkage map, and interchange hybridity is not relevant to an analysis of their recombination.

A second complexity which may contribute to linkage anomalies is the as yet uninterpreted behavior of persistent heterozygotes. The relevant published facts are as follows: In EMS (synthetic medium) crosses of certain stocks

(W-466, etc.) carrying the hypothetical *Het*-factor considerable numbers of persistent diploids are discovered (10 percent). By segregation and reversion tests on EMB each diploid clone is seen to be heterozygous for certain factors, homozygous for others and hemizygous for yet others. Thus, genetic recombination and elimination precede diploidization. Since clonal segregants are of more than two or even four types, recombination must again occur in the formation of haplonts. Furthermore, segregations from the heterozygous state deviate widely from the 1 : 1 ratio. Diverse diploid clones isolated from the same cross, differ in the distribution of, at least, homo- and heterozygosity, and in the segregation of identical heterozygous markers. Segregants backcrossed to parental standard stocks show altered linkage relations. Special screening techniques have allowed the identification in standard crosses (W-677 and 58-161) of rare (.1 percent of prototrophs) diploids, qualitatively like those of the *Het*-series.

On the relevance of these aberrant heterozygotes one may take one of two stands. (1) It is possible that a similar duplex meiosis and gene loss precede the segregation of transient zygotes in standard crosses. This might be inferred from the abnormal linkage relations in backcrosses of 58-161 and L^-T^- recombinants (ostensibly non-*Hets*) extracted from TL-crosses (LEDERBERG, personal communication). Similarly it is suggestive that selection in appropriately supplemented crosses of complementary diauxotrophic recombinants yields complementary segregations for some loci (*Lac*, V_1) but not for others (*Mal*) which are generally eliminated in the persistent diploids (data of P. FRIED, cited in LEDERBERG 1951). If standard and *Het* segregants differ only in the duration of the diplophase and if there is in addition structural hybridity, attack on the linkage problem must be renewed with unrelated stocks, and the present discussion is premature. (2) It is possible, however, that the nuclear cycles of *Het* and standard zygotes are entirely different, the former being initiated only by special crossover configurations in structurally hybrid zygotes. Thus crossing over in relatively inverted segments might lead to bridge formation and delay of cytoplasmic division resulting, on bridge breakage, in two complementary hypodiploid cells. One or both of these might propagate mitotically in diplophase and eventually segregate haplonts meiotically. Such diploids would be homo-, hemi- or heterozygous for any given locus depending upon crossover and bridge breakage relations. Alternatively, if the standard zygote is an interchange hybrid, the *Het*-cycle might be started simply by a partial (3 : 1) nondisjunction. However, since elimination affects only certain loci (*Mal*, *S*) and since the *Het*-character originated in a single mutation-like step, non-recurrent in the parent strain, nondisjunction is likely to be directed by a second superimposed chromosomal change such as an inversion. It should be noted that with a system of double hybridity the *Het*-character might be perpetuated in inbred clones and haploid progeny could exhibit altered linkage relations whether they had persisted for a time as heterozygotes or not.

If this general interpretation is correct, then where persistent diploids are rare (W-677 × 59-161) linkage anomalies are quite independent of chromosomal aberrations. This is the view adopted by us at the present. This discussion will be continued, therefore, on the contention that the excess of multiple crossovers between all loci so far studied is not due to the elimination of certain crossover types nor to life cycle complexities of the *Het*-type but reflects largely interference at the level of crossing over.

Whether there is significant interference between two regions may be judged by the calculation of interaction, *i.e.*, the product of non- and double crossovers divided by the product of single crossovers. Interactions of unity indicate absence of interference, interactions significantly smaller than unity indicate positive interference, and interactions larger than unity indicate negative interference. The six possible comparisons, taking crossover a, b, c, d, two at a time, are shown in table 4. Interactions are very much larger than

TABLE 4

Interference between crossover regions a, b, c, d two at a time. (For crossover regions a, b, c, d see figure 1.)

Crossover type	Number of segregants in paired regions					
	ab	ac	ad	bc	bd	cd
0 CO	1189	1213	1245	1192	1203	1232
1 CO (region 1)	66	71	82	92	124	95
1 CO (region 2)	111	87	55	63	52	52
2 CO	34	29	18	53	21	21
Interaction[1]	5.518	5.695	4.969	10.900	3.918	5.237
Chi squared[2]	68.76	64.37	38.21	170.34	28.57	43.09

[1]Interaction equals the product of the non- and double crossovers divided by the product of the single crossovers. Where the interaction is significantly greater than 1 there is negative interference.

[2]From 2 × 2 tables. $\chi^2 = 6.6$ is significant at the 1 per cent level.

unity in every case. Thus, the observed crossover distributions in table 3 are formally correctly described as showing negative interference.

Two biological models are visualized which individually or jointly would give negative interference. (1) It may be assumed that crossing over in paired regions is free of interference but pairing itself is irregular and incomplete through random initiation and the operation of a time limit, or through structural hybridity following mutagenic treatment. Thus zygotes may undergo pairing both in *S-M* and *M-T*, or in *S-M* or *M-T* alone, or in neither. Unselected progeny, if recoverable, would show a degree of interference, negative for double crossovers in adjacent regions and positive for doubles far apart. Selection of $M^+T^+L^+$ (prototroph recovery technique) would yield an overestimate of map distances through exclusion of zygotes not paired in *M-T*. If pairing were all or none in *M-T*, there would be no internal evidence of interference but if it occurred in smaller variable blocks prototroph progeny would

show negative interference but no actual numerical excess of doubles over either single. On the other hand, in S^rM^+ selections, zygotes with pairing restricted to *S-M* will produce non-crossovers only and will largely be responsible for the apparent negative interference. Regardless of the frequency of extra non-crossovers from this source double crossovers will not exceed singles. Crossover frequencies throughout *M-T* may be underestimated and will certainly appear lower than when recombination is made obligatory over the region in which crossing over is scored (unsupplemented cross). (2) Alternatively it may be assumed that pairing is complete but that the occurrence of one crossover directly increases the probability of another occurring nearby through weakening of the chromosome strand, production of extra tension, or other means. Thus in relation to single crossovers and segmental crossover frequencies there will be too many non- and double crossovers. If crossing over in bacteria were four-strand, the phenomenon known as negative chromatid interference in higher organisms would provide a model. Depending upon the magnitude of the effect, double crossovers may or may not exceed one or both single types.

It is difficult to establish criteria which discriminate between these interpretations. Excess multiple crossing over can only be proved where there is an excess of double crossovers over at least one of the two single types. The corresponding tests are negative for the present data no matter which regions are paired (table 4).

Conversely in the TL-cross the adequacy of the incomplete pairing hypothesis may be tested by ignoring the non-crossovers and considering the interrelations of singles, doubles and triples. If there is no interference, and pairing in *M-T* is all or none, for each region the product of singles and triples divided by the product of the doubles should be unity. Tests were carried out for regions a, b, c, and d, three at a time and compounded in all possible ways. The interactions are generally close to unity and where they deviate they are sometimes larger and sometimes smaller, the mean being .94. In the last columns of table 3, the specific hypothesis is tested that pairing had occurred in 400/1400 zygotes both in *S-M* and *M-T*, and in the remaining 1000 zygotes in *S-M* only. The agreement between the observed and expected distribution is surprisingly good.

Main support for excess multiple crossovers comes at present from the data of NEWCOMBE and NYHOLM. It is possible that conflicts of sequence may be resolved by accommodation of loci in a branched interchange map (LEDERBERG *et al.* 1951). In such a system unselected loci in any two arms should be linearly linked, whatever the position of selected loci. Thus, mapping (from the free ends) *T L* V_1 *Lac M* on one arm, *Mtl* (Mannitol), *Xyl* on a second, and *S*, *Mal* on a third, the following three assemblies should meet tests of linearity: (1) *T L* V_1 *Lac M; Mal S.* (2) *T L* V_1 *Lac M; Xyl Mtl.* (3) *S Mal; Xyl Mtl.* With markers in all four arms, six linear and mutually reconcilable linkage assemblages are expected. If such mapping should prove to eliminate

ambiguities of sequence it would still leave evidence of residual interference quite similar to that obtained in the present work. To give just one example; where *S-Mal-Xyl* segregated in "unselected crosses" NEWCOMBE and NYHOLM found 2224 non-crossovers, 62 single crossovers of one type and 37 of the other, and 38 double crossovers. Thus, if allowance is made for the possibility that in the study of NEWCOMBE and NYHOLM markers were distributed in several arms of a branched map while in the present study, they were confined to one, anomalies in segregation might become comparable and explicable by irregular pairing alone. On the other hand excess multiple crossing over alone or in conjunction with irregular pairing is not excluded in either study. In view of these uncertainties map distances for unselected crosses cannot be inferred from the present data. If incomplete pairing is involved prototroph recovery yields overestimates and SM selection may yield underestimates. If excess multiple crossing over is responsible even SM selections may give overestimates according to the degree of negative interference across *M*. In either case uncorrected mapping from unsupplemented crosses leads to exaggerated map distances. Thus LEDERBERG's 1947 map is not valid for unselected progeny. On the other hand, the existence of linearity which had to be assumed by NEWCOMBE and NYHOLM appears consolidated for the loci *M-Lac-*V_1*-L-T* through the relative insignificance of anomalies in segregation.

SUMMARY

Segregation of the linked gene loci *M, Lac,* V_1, *L,* and *T* was studied in crosses of *E. coli* strain K-12. Evidence from all crosses unambiguously supports the above linear seriation. In a critical five point cross recombination over the region *S-M,* not overlapping *M-T,* was made obligatory and all recombination classes involving *M, Lac,* V_1, *L,* and *T* could be recovered. The distribution of recombinants was anomalous in that, relative to segmental crossover frequencies, there was an excess of multiple crossovers. This could not be accounted for by the familiar consequences of crossing over in a structurally changed chromosome segment. The view is favored that the apparent excess of multiple crossovers is due to an excess of non-crossovers through irregular and incomplete pairing, although negative interference in completely paired chromosome regions is not ruled out.

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