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GENE RECOMBINATION AND LINKED SEGREGATIONS IN ESCHERICHIA COLI[1]

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THE occurrence of factor recombination in the bacterium, *Escherichia coli*, has been described in previous reports (LEDERBERG and TATUM, 1946 b, c, TATUM and LEDERBERG, 1947). In an attempt to elucidate further the genetic structure of this organism, these studies have been extended to crosses involving several characters, and to the quantitative enumeration of various recombination classes. The results described in this paper provide evidence supporting the sexual basis of factor recombination and of the existence of an organized array of genes comparable to that of higher forms.

MATERIALS AND METHODS

The parent "wild-type" strain, K-12, of *E. coli* used in these experiments and the production and behavior of biochemical mutants have been described (GRAY and TATUM, 1944, LEDERBERG and TATUM, 1946a, ROEPKE, LIBBY, and SMALL, 1944, TATUM, 1945). Specific requirements, notation, and other data pertinent to the biochemical mutants are summarized in tables 1 and 2. In general, a biochemical deficiency resulting from mutation is designated by the initial of the substance required (e.g. B^- for biotinless), while the wild type alternative is written with a "+" sign (e.g. B^+ to emphasize the alternative to B^-). The term "prototroph" (RYAN and LEDERBERG, 1946) has been devised for strains exhibiting the nutritional behavior of the wild type, which for *E. coli* implies independence of any specific growth factors. Prototroph is, however, not synonymous with "wild type" since it refers (a) only to the phenotypic appearance of a culture and (b) only to nutritional and not to other possible mutant characteristics.

K-12 as a coliform is capable of fermenting, or producing acid, from a variety of sugars, including glucose, galactose, maltose, lactose and mannitol; however, it ferments glycerol only weakly, and sucrose even less so. Because of the ease of scoring and their biochemical specificity, mutants unable to ferment various sugars have been looked for. Particular attention was paid to the isolation of "lactose-negative" or "*Lac*$^-$" mutants, because of the taxonomic significance which has been attached to this character.

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The detection of fermentation mutants is readily accomplished by the use of indicator media. The medium "EMB-lactose" used in routine bacteriological work was found to be highly useful. It consists of the following (in g/l): peptone (or "N-Z-Case") 10, yeast extract 1, lactose 10, agar 15, eosin Y 0.4, methylene blue 0.06, sodium chloride 5, dipotassium phosphate 2. On this medium, colonies of bacteria which can ferment lactose (or any other sugar added in its place) rapidly turn a deep purple color, while colonies of non-fermenting organisms remain white or pink but may slowly turn light blue.

Lac$^-$ mutations have been recovered in two instances. Among 15,000 colonies

TABLE 1

Symbols used for various loci.

1. Nutritional requirements. Allele for requirement of a given substance is designated by the superscript "$-$"; independence by "+". E.G., B^- is biotinless; B^+ is biotin-independent.

B	biotin	*L*	leucine	*Pa*	phenylalanine (ϕ was used previously, but has been modified for typographical reasons)
B_1	thiamin	*M*	methionine		
C	cystine	*P*	proline		
		T	threonine		

2. "Sugar" fermentations. The ability to ferment is designated "+"; the inability "$-$".

Lac	lactose	*Gly*	glycerol

3. Bacteriophage resistance. Resistance is designated by the superscript "r"; sensitivity by "s". E.G. V_1^r.

V_1	resistant to *T1*, *T5*
V_{1a}	resistant to *T1*; sensitive to *T5*
V_{1b}	resistant to *T1*; mucoid colonies
V_6	resistant to *T6*.

4. Resistance to chemical agents. Resistance and sensitivity "*r*" and "*s*" respectively, as *Cla*^{r}.

Cla	sodium chloroacetate
A	sodium azide

of strain Y-10 ($T^-L^-B_1^-$) obtained by spreading a culture previously treated with ultraviolet light on EMB-lactose agar, a single pink colony was noted. It proved to be the same, nutritionally, as Y-10 and was therefore regarded as a *Lac*$^-$ mutant; this stock is labelled Y-53. Among 30,000 colonies of Y-40 ($B^-M^-V_1^r$) a single *Lac*$^-$ was recovered following treatment with nitrogen-mustard (TATUM, 1946), and was designated as Y-87. Tests showing that these independent mutations are probably allelic will be described in a later section (see table 5). Strains Y-53 and Y-87 differ in the rate at which the *Lac*$^-$ character reverts to the *Lac*$^+$ condition, but whether this is due to different allelic states or to differences at other loci, cannot be definitively asserted.

Attempts to obtain maltose, mannitol, and galactose-negative mutants were not successful, presumably because the populations tested were too small. A glycerol-negative strain has been obtained, but the wild type ferments this polyalcohol so poorly to begin with that accurate scoring is difficult; studies on this character will not be further reported here.

been reported by ANDERSON (1946) for the corresponding mutants of *E. coli B*. In this paper, the designation V_1^r will be used for the more frequent T_1-resistant mutant, which is also resistant to T_5. The symbol V_{1a}^r is reserved for the T_5-sensitive, T_1-resistant mutant, but the evidence that distinct loci are involved will be presented *in extenso* in another place.

In addition to V_1^r and V_{1a}^r, just mentioned, a third type of "secondary colony" has been found among populations treated with the virus T_1. This type, V_{1b}^r is characterized by an exceedingly slimy or mucoid colony confor-

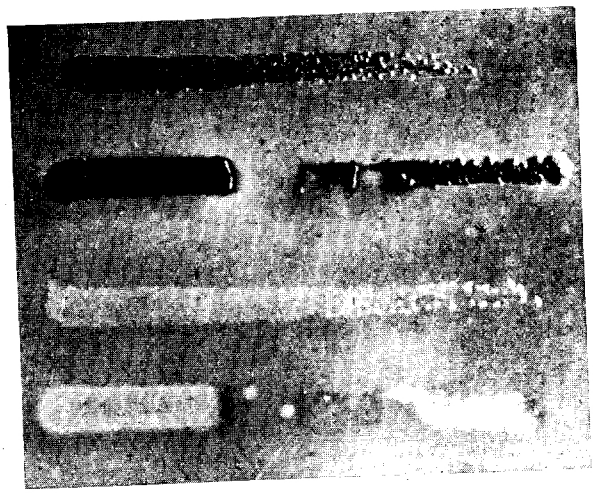


FIGURE 1.—The phenotypes of the four combinations of *Lac* and *V* are illustrated. In order they are: $Lac^+V_1^r$; $Lac^+V_1^s$; $Lac^-V_1^r$; $Lac^-V_1^s$. An EMB-lactose agar plate was first streaked vertically with the virus T_1. Subsequently, each of the bacterial types was streaked, from left to right, perpendicularly across the virus streak. After 16 hours incubation, both the *Lac* and V_1 phenotypes are well developed. Developing in the zone where $Lac^-V_1^s$ has been lysed can be seen two colonies of resistant mutants: $Lac^-V_1^r$.

mation. Recombination studies on this mutant are complicated by its genetic instability; V_{1b}^r rapidly reverts to the wild type, and in addition may also be strongly selected against in competition with V_{1b}^s. However, the locus of V_{1b} can be distinguished from the locus of the other V_1 mutants by the demonstration of a different recombination frequency with *Lac*. These data are summarized in order to emphasize the importance of genetic tests to insure the allelic identity of phenotypically similar mutants.

It is particularly fortunate that resistance tests can be conducted on EMB agar, since this allows the characterization of a strain with respect to virus-resistance and to lactose fermentation with a single streaking (see fig. 1).

Mutants resistant to sodium chloroacetate (Cla^r) were obtained by streaking a large number (about 10^7) of bacteria on nutrient agar to which filter-sterilized chloroacetate has been added to make a final concentration of 2 mg/ml. At this concentration, the wild type is substantially inhibited, while resistant mutants grow luxuriously. This mutation is accompanied by deficiencies in

the metabolism of pyruvic and acetic acids, which will be described in more detail elsewhere. Independent mutations to other inhibitors, including iodoacetate, azide, streptomycin, streptothricin, mercuric chloride, and Brilliant Green, can be secured in a similar fashion, but genetic analysis of these mutations has not been completed.

Morphological variation has occasionally been noted (exceedingly rough or very mucoid colonial form) but is relatively unsuitable for genetic work because the presumably random choice of prototroph recombinants may be influenced.

In addition to the EMB agar already described, a number of other natural or "complete" media have been used. The Difco product "Penassay Broth" has been used most extensively, and is satisfactory for the preparation of inocula, except that it must be supplemented with cystine for the growth of cystineless organisms, such as strain Y-24. Other satisfactory media include a broth consisting of: peptone 5, glucose 5, yeast extract 3, g/l, as well as Difco Nutrient Broth, and diverse concoctions containing peptone or casein hydrolysates and meat or yeast extract.

The synthetic or minimal medium contains, in g/l: NH_4Cl 5, NH_4NO_3 1, Na_2SO_4 2, K_2HPO_4 3, KH_2PO_4 1, glucose 5, asparagine 1.5, $MgSO_4$ 0.1, trace elements (GRAY and TATUM 1944), and $CaCl_2$, a trace. The medium is made solid by the addition of agar in a concentration of 1.5 percent.

To avoid flocculation when used with agar, the glucose and agar in solution should be autoclaved separately, and mixed with the other components just before using. Unwashed agar (Difco) is sufficiently free of the growth factors under consideration to be satisfactory for many experiments; the use of washed agar, however, is recommended for the cleanest results.

The detection of recombinants is based upon the inability of biochemical mutant bacteria to proliferate in the absence of their specific growth substances. Plating in minimal agar, therefore, has the effect of a sieve for prototroph cells. To insure against contamination with prototrophs derived by reverse mutation, which has been noticed at certain loci, it has been desirable to use multiple biochemical mutants as the parental stocks in recombination studies. Coincidental reversion at two or more loci is theoretically improbable, and experimentally undemonstrable (RYAN, 1946, TATUM and LEDERBERG, 1947). For example, plating either $B^-M^-T^+L^+B_1^+$ or $B^+M^+T^-L^-B_1^-$ separately into minimal agar did not lead to the appearance of prototrophs, $B^+M^+T^+L^+B_1^+$. When, however, a mixture of these cell types was so "sieved," one prototroph was found for about each 10^7 cells inoculated. These have been assumed to arise from the recombination of "+" alleles to form the prototroph.

In previous experiments, the two multiple mutants were inoculated together into a complete medium and allowed to grow in mixed culture before plating into minimal agar. This method is not satisfactory for present purposes because it allows possible selective differentials to alter the relative frequencies of different recombination classes. A modified procedure has been developed, which will now be described in detail.

The mutant stocks are maintained on "complete" agar slants, transferred

at intervals of 6–8 weeks. They are inoculated separately into test-tubes containing about ten ml of liquid complete medium and incubated overnight at 30°C with gentle shaking. The following morning, an additional ten ml of the same medium is added to each culture, and the tubes are incubated in the same manner for an additional three to five hours. These cultures contain from 1–4× 10^9 cells per ml. They are then washed in the following manner: the cotton plugs are replaced with sterile corks which have been kept in 95 percent alcohol and the alcohol flamed off just before using. The cultures are then centrifuged at about 2500 r.p.m. for 20 minutes, which suffices to pack the cells in the bottom of the test tubes. The supernatant medium is carefully poured off, and the tube is rinsed with about 10 ml sterile distilled water, care being taken not to disturb the pellet. The cells are then resuspended in an additional 15–20 ml sterile water, and recentrifuged. The supernatant wash water is decanted and replaced with an equal volume of fresh sterile water, in which the cells are suspended. In the meantime, minimal agar plates are prepared. A bottom layer of about 15 ml minimal agar is poured into each Petri plate and allowed to solidify. Cell suspensions of different mutant stocks are mixed at this time and measured quantities (usually about 10^8–10^9 cells) are pipetted onto the agar surface. At this time also, one may add such growth factor supplements as are desired to permit the growth of recombination types other than prototrophs. The cell suspensions are then mixed into a layer of about ten ml molten minimal agar (at 45–50°C) which is poured onto the plates. After the agar hardens, the plates are incubated at 30°C for a period of 48 hours. At this time prototroph colonies will be found distributed throughout the plate, many of them at or near the surface and accessible to picking for further characterization.

The procedure may be varied in several ways. It is important however that the inoculum consist of "young" cells, since cultures of 24 hours or older have given quite inconsistent results. It is possible to store the inoculum in distilled water for at least twenty-four hours without appreciably affecting the yield, which suggests that the aggregation of genetic types leading to the recombination process occurs in the molten or the solidified agar. This occurrence must, however, take place within a few hours, since the recombinant prototrophs are not appreciably slower to appear than wild type cells in a similar physiological state which may be streaked on the surface of the plates. Presumably, therefore, one could increase the yield of prototrophs by making conditions more favorable for the free contact of the cells, as by packing them together in a centrifuge tube in minimal liquid medium. However the complication of proliferation of prototrophs already formed would interfere with the interpretation of such an experiment. Many physiological factors may interfere with the recombination process, and, for example, the yield may be reduced markedly by inoculating too heavily, or by omitting an under-layer of agar into which, presumably, deleterious metabolic products may diffuse. Instead of mixing the cells in semisolid agar, it is possible to streak the mixture on the surface of slightly dried minimal agar plates. Under these conditions, however, the prototroph colonies are likely to be more heavily contaminated with the residual parental mutant types.

For most purposes, however, this contamination may be ignored, as will be shown in a later section. Prototroph colonies are then fished and streaked directly on EMB plates, or otherwise tested, to classify them with respect to other factors that may be segregating.

RESULTS AND CONCLUSIONS

In most organisms inheritance is studied by the examination of zygotes carrying the gene alternatives determining a character. The segregants are chosen at random, and factor linkage is recognized by deviations in the frequency of parental and new couplings of a series of characters. In the absence of a random method of separating zygotes in *E. coli*, one is limited here to the members of specific recombination classes, namely the prototrophs. It is however, possible to introduce other factor differences into the biochemical mutants from which prototrophs are obtained, and to determine how such factors segregate into this recombination class. It was hoped in this way to obtain information concerning the haploid or diploid condition of the bacterial cell, and to determine whether factors segregated at random, or according to specific, perhaps linear chromosomal laws.

The first factor pair to which this approach was applied was V_1^r/V_1^s (LEDERBERG and TATUM, 1946b). In the cross $B^-M^-P^+T^+V_1^r \times B^+M^+P^-T^-V_1^s$, ten $B^+M^+P^+T^+$ were isolated. Eight proved to be V_1^r while two were V_1^s. This at once suggested that the vegetative cell of *E. coli* is haploid, since segregation could be observed in the first filial generation clone. It was noted also at that time that the "reversed" cross: $B^-M^-P^+T^+V_1^s \times B^+M^+P^-T^-V_1^r$ gave quite a different ratio of r/s in the prototrophs, namely 3:7. Results on so small a sample are of doubtful significance, but they suggested the technique by which the basis of this character "segregation" could be elucidated. For this reason, the study of "reversed" crosses was extended to include numerically more data, using various combinations of mutants, and involving in addition to V_1^r/V_1^s, Lac^+/Lac^-. The information which was obtained is summarized in tables 3 and 5. The data show clearly that neither of the factor alternatives V_1^r/V_1^s or Lac^+/Lac^- segregates at random into the prototroph recombination class. However, the occurrence of all factor combinations, albeit with different frequencies, is evident, at least with respect to *Lac* and V_1. It seemed clear that there are only two alternative explanations for the unequal frequencies with which alternative alleles are manifested in the prototrophs: (a) that the alleles were characterized by some differential physiological property, such as dominance, or preferential segregation, or (b) that the nonrandom segregation was due purely to the mechanics of factor recombination, which is to say a linkage system.

The results of "reversed crosses" have a distinct bearing on this problem. If nonrandom segregation into prototrophs were due to some physiological property of the allele concerned, its particular coupling in the parent in which it is introduced should have no great effect on the segregation frequency; if on the other hand, the effect were purely mechanical, the segregation would reflect entirely the couplings of the parents, and the substitution of one allele for

another in the parents (as in reversed crosses) should lead to a corresponding inversion in the ratios with which that allele is found in the prototrophs. The tables cited show that in every case there is no agreement between the ratios found in reversed crosses, unless the comparison is made with one of the ratios inverted, in which case there is reasonably good agreement. This result is in accord with the hypothesis that the genes in *E. coli* are arranged in one or more linkage groups, and is in disagreement with the postulation of a diploid con-

TABLE 3

*Comparisons of V_1^r segregations when introduced with alternative parents.**

PARENTS			PROTOTROPHS $[B^+M^+Pa^+C^+T^+L^+B_1^+P^+]$		
			V_1^r	V_1^s	% V_1^r
$B^-Pa^-C^-T^+P^+$		$B^+Pa^+C^+T^-P^-$			
$\cdots V_1^r$	×	$\cdots V_1^s$	76	6	92
$\cdots V_1^s$	×	$\cdots V_1^r$	30	107	22
$B^-Pa^-C^-T^+L^+B_1^+$		$B^+Pa^+C^+T^-L^-B_1^-$			
$\cdots V_1^r$	×	$\cdots V_1^s$	80	23	77
$\cdots V_1^s$	×	$\cdots V_1^r$	53	133	28
$B^-M^-T^+P^+$		$B^+M^+T^-P^-$			
$\cdots V_1^r$	×	$\cdots V_1^s$	49	8	86
$\cdots V_1^s$	×	$\cdots V_1^r$	5	19	21

* See LEDERBERG (1947) for a statistical analysis of tables 3, 5, and 6.

dition, or with a state of indefinite "ploidy" which would be characteristic of a system of cytoplasmic inheritance.

The results of these experiments seemed sufficiently secure that one could adopt the existence of a linkage system as a working hypothesis and on this foundation, an attempt has been initiated to "map" a number of markers in *E. coli*. It was hoped at first that there might be found linkage groups which would be independent of one another, so that recombination between biochemical markers in one group could be used to detect recombinants, yet not interfere with the segregations in the other group(s). There was, however, no immediate prospect that these relationships could be found initially, so it was decided to study linkage relationships in a single pair of mutant stocks, and their derivatives. The stocks which were selected for this study were 58–161 (B^-M^-) and Y-53 ($T^-L^-B_1^-Lac^-$) and their V_1^r mutants. Since *Lac* and V_1 could be so readily scored, using only a single streak from each prototroph colony which appeared, it was hoped that the collection of an adequate volume of data could be accomplished with greater facility than if biochemical markers only were used.

It was, however, necessary to determine the relationships of the biochemical mutant loci of which at least four must be used to obtain recombinants. Mixtures were, therefore, plated into minimal medium supplemented with a single

nutritional requirement, i.e., either biotin, methionine, threonine, leucine, or thiamin, allowing the proliferation of the corresponding single mutant as well as the prototrophic type. Colonies were then picked at random and scored according to their nutritional requirements. The results are summarized in table 4. Unfortunately, it was found that the addition of methionine to the minimal medium allowed excessive growth of B^-M^-, presumably because of a degree of contamination of the methionine with biotin. This datum is, however, not essential for the argument. In general, it will be seen that the + classes are markedly and significantly more frequent than the single mutant types, with

TABLE 4

Relative frequency of various biochemical recombination classes in the cross.

$B^-M^-T^+L^+B_1{}^+ \times B^+M^+T^-L^-B_1{}^-$*

FROM PLATES SUPPLEMENTED WITH	NUMBER OF COLONIES TESTED	RECOMBINATION CLASSES FOUND				RATIO	χ^2
		TYPE	NUMBER	TYPE	NUMBER		
Biotin	70	B^-	10	B^+	60	0.17	36
Threonine	46	T^-	9	T^+	37	0.24	17
Leucine	56	L^-	5	L^+	51	0.096	38
Thiamin	87	$B_1{}^-$	79	$B_1{}^+$	8	9.88	56

* Cells of the parental types were mixed and plated into agar supplemented with the growth factor indicated. On this medium, the two recombination classes indicated on each line of the table could form colonies. Contrasting alleles only are specified; other loci, unless otherwise specified, have the "+" configuration. The χ^2 for the ratio of single biochemically deficient types to prototrophs is calculated for a comparison with the 1:1 expectation of a random segregation. As can be seen from the χ^2 values, the probability that the deviations are due solely to chance is, in each case, less than .001.

the exception of $B_1{}^-$ which is nearly ten times as frequent as $B_1{}^+$. Writing the cross as $B^-M^-T^+L^+B_1{}^+ \times B^+M^+T^-L^-B_1{}^-$, these results may be interpreted as follows:

1. B^+M^+ $T^+L^+B_1{}^+$ more frequent than B^-M^+. Therefore B and M are linked.
2. T^+L^+ $B^+M^+B_1{}^+$ more frequent than either T^-L^+ or T^+L^-. Therefore T and L are linked.
3. $B_1{}^-B^+M^+$ T^+L^+ more frequent than $B_1{}^+B^+M^+$. Therefore B_1 is linked to B and M, but probably not between them.

One may therefore map these five loci onto not more than two linkage groups, according to the scheme in fig. 2a. In all that follows, the [B-M] and [T-L] combinations will be regarded as single units, since conclusive information as to their relative order has not been obtained. These data so far do not allow any conclusion to be drawn as to whether the regions $B_1{}^-$[BM] and [TL] are linked or are independent of each other, since a recombination between them is a necessary requirement for a detectable type.

TABLE 5

*Segregation of Lac and V_1 into prototrophs issuing from various parental combinations.**

PARENTS			RECOMBINATIONS			
$B^-M^-T^+L^+B_1^+$	$B^+M^+T^-L^-B_1^-$	$B^+M^+T^+L^+$	$Lac^-V_1^r$	$Lac^-V_1^s$	$Lac^+V_1^r$	$Lac^+V_1^s$
$Lac^+V_1^r$	$Lac^-V_1^s$	$\cdots B_1^+$	602 [45.8]	203 [23.1]	387 [29.4]	22 [1.7]
		$\cdots B_1^{-***}$	13 [45]	8 [28]	8 [28]	0 [0]
		$\cdots B_1^{-**}$	244 [42.8] (D)	157 [27.5] (E)	159 [27.9] (C)	10 [1.9] (triple)
$Lac^+V_1^s$	$Lac^-V_1^r$	$\cdots B_1^+$	107 [33.2]	145 [45.0]	9 [2.8]	61 [19.0]
		$\cdots B_1^{-**}$	134 [35.8] (E)	151 [40.4] (D)	9 [2.4] (triple)	80 [21.4] (C)
$Lac^-V_1^r$	$Lac^+V_1^s$	$\cdots B_1^+$	28 [23.9]	6 [5.1]	46 [39.3]	37 [31.6]
		$\cdots B_1^{-**}$	102 [25.4] (C)	7 [1.7] (triple)	201 [50.1] (D)	91 [22.7] (E)
†$Lac^+V_1^r$	$Lac^-V_1^r$	$\cdots B_1^{-**}$	128	0	33	0
†$Lac^-V_1^r$ (Y-87)	$Lac^-V_1^s$ (Y-53)	$\cdots B_1^{-**}$	134 Lac^-; not scored for V_1			

* Cell mixtures of the indicated composition were plated into minimal agar plates or into plates supplemented with thiamin. B_1^+ types refer to scores of prototrophs picked at random from minimal plates.

** B_1^- refers to colonies picked at random from thiamin supplemented plates. Although predominantly B_1^- they contain B_1^+ colonies in the proportion 1:10 as may be seen from table 4.

*** B_1^-. In this series, colonies were scored as to B_1, and only the B_1^- are recorded.

The letters (C), (D), (E), refer to crossover types corresponding to the regions [*B M*]–*Lac*; *Lac*–V_1; and V_1–[*T L*] respectively, according to the map of Fig. 2d.

† Test for allelism.

On the basis of table 5, the factors V_1 and *Lac* may be brought into the argument. In addition to the joint segregations of these factors, the effect of the B_1 segregation was studied in the following way. It would be uneconomical, in view of the relative paucity of B_1^+ types, to separate these from the B_1^- by nutritional testing of colonies which appear on thiamin supplemented agar. Instead, the entire sample was regarded as B_1^- with the proviso that it might be contaminated to the extent of ten percent with B_1^+. However, it has been found that the distribution of *Lac* and *V* on colonies picked from thiamin

supplemented agar is homogeneous with the distribution in prototrophs, so that the segregation of these factors is not influenced by the B_1 segregation.

The data in table 5 show that *Lac* is inclined not to separate from *BM*, and is therefore regarded as linked to it, while there is a similar linkage of V_1 to *TL*. Since the recombination of *Lac* with *BM* is not influenced by the interchange between B_1 and *BM*, they are on opposite sides of *BM* as suggested by map 2b. Finally, a scrutiny of the interaction between the *Lac* and *V* segregations shows that these are not independent of each other, particularly because of the rarity of the least frequent class. This suggests, then, that the two linkage groups of fig. 2b be combined to give the map of fig. 2c. (The locus of V_6 on this map is obtained from additional data.) According to this interpretation, the rarity of the least frequent *Lac-V* combination stems from the fact that a triple-crossover is necessary for its production. In fig. 2d, the cross Y-40×Y-53 is interpreted according to the map, with a table citing the regions in which interchange must take place to yield the given types.

That the first seven factors to be investigated should fall in the same linkage group leads to the inference that there is only a single chromosome in *E. coli*. This inference is supported by incomplete analyses of the segregations of 8 other markers referred to in table 1. None of these factors has been found to segregate independently of the factors which have already been described as belonging to a single linkage group. The possibility that segregation interactions may, in some cases, be based upon an inter-chromosomal type of interference (compare STEINBERG and FRASER, 1944), has not been ruled out, however.

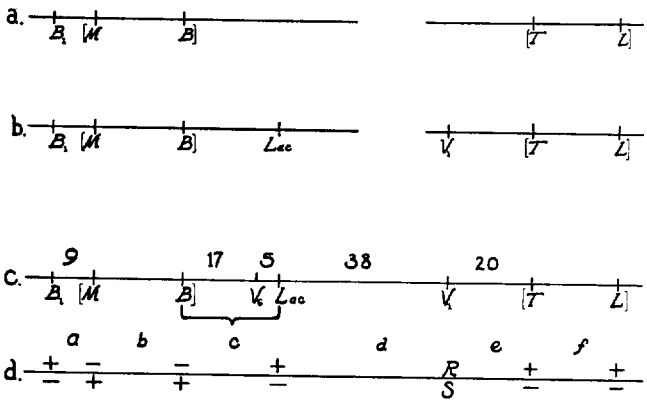
The distances recorded in fig. 2c are derived from the recombination totals in tables 5 and 6. However, the distance between [*BM*] and [*TL*] cannot be estimated directly, but only the partition of that distance among the regions *BM-Lac*, *Lac-*V_1, and V_1-*TL*. The relative frequency of the "triple-interchange" type can be used to estimate the absolute map distances, if it is assumed that there is no interference. This frequency, about 2.1 percent, is readily calculated to be consistent with a map length of between 75 and 80 units altogether either in a two-strand or a four-strand system (LEDERBERG, 1947). These values must be regarded as rough approximations, because they are extremely sensitive to error in the estimation of the proportion of the "triple" types.

Linearity

In constructing a map, and calculating distances, it has been taken for granted that there is in *E. coli* a system of linear linkage, such as has been demonstrated quite conclusively in Drosophila, and inferred in all higher organisms. What direct evidence may one bring to bear on this question?

The method which one is forced to employ in hybridizing this bacterium introduces certain complications. The classical proof of linearity is based on the additive character of distances, expressed in morgans, between loci occurring within the same linkage group. The determination of map distances is based upon a comparison between parental and new combinations of linked

genes, as determined in the progeny of zygotes selected at random. In *E. coli*, on the other hand, one is limited to the recovery of that recombination class in which there has necessarily been an interchange between certain biochemical loci, in the cases here discussed, betwen [*BM*] and [*TL*]. For this reason, it is not possible to obtain a direct measure of the absolute distance between factors which are located within this critical region, and any argument in favor of linearity which is based on the segregations of such factors may have the



Crossovers	*Recoverable type*
(*c*, *d*, or *e*)	$B_1-M+B+T+L+$
(*a*) (*c*, *d*, or *e*)	$B_1+M+B+T+L+$
(*a*) (*b*)	$B_1+M+B-T+L+$
(*a*) (*f*)	$B_1+M+B+T-L+$
(*a*) (*f*) (*c*, *d*, or *e*)	$B_1+M+B+T+L-$
(*a*) (*c*)	$B_1+M+B+T+L+ \cdots Lac^+V_1^r$
(*a*) (*d*)	$\cdots Lac^-V_1^r$
	$\cdots Lac^-V_1^s$
(*a*) (*c*) (*d*) (*e*)	$\cdots Lac^+V_1^s$

FIGURE 2.—*a*, *b*, and *c*. Mapping of genetic factors. *d*. The cross $B_1^+M^-B^-Lac^+V_1^rT^+L^+ \times B_1^-M^+B^+Lac^-V_1^sT^-L^-$ and some of the recoverable crossover classes. (See table 5.)

flavor of circular reasoning. It would be preferable to study the segregations of factors which are assigned to loci distal to the biochemical factors whose recombination is the basis of the detection of sexual offspring. The stocks with which this might be accomplished are not yet available, but it is hoped that they will be for future work.

That there does exist some sort of linkage system is made highly credible by the results of the "reverse crosses" tabulated in tables 3 and 5. The chief difficulty in proving that this system is linear has been to formulate the feasible alternatives, so that critical experiments, the results of which could discriminate between linearity and a given alternative, might be set up. Certain types of "linkage" can be disqualified by the data already at hand. For example, one might postulate that genes of bacteria are embedded in a two-dimensional

matrix, and there occasionally occurs a gene-for-gene interchange. This is equivalent to the "Konversion" theory once proposed by WINKLER (1932), to account for interchanges in Drosophila. While this type of arrangement would account for a tendency to preserve the parental configuration, it fails to explain either quantitative linkage intensities, or the interaction of segregations which is revealed by the data on *Lac* and *V* in table 5. Naturally, one could further modify the "Konversion" theory to take these exigencies into account, but in so doing one would be elaborating an exceedingly complicated theory which would, in fact, be a re-expression of a mechanical theory of linkage.

The interaction of the *Lac* and *V* segregations is perhaps the most critical datum with which a genetic system for *E. coli* can be formulated. The interaction may be expressed as follows: the frequency of interchanges between [*BM*] and *Lac* is dependent upon the interchanges between [*BM*] and *V*. Specifically, in the cross $B^+M^+T^-L^-B_1^-$ $Lac^-V_1^s \times B^-M^-T^+L^+B_1^+$ $Lac^+V_1^r$, one finds in the $B^+M^+T^+L^+B_1^+$ the following distribution of classes: $Lac^-V_1^s$ 23 percent, $Lac^+V_1^r$ 29 percent (for the parental combinations) and $Lac^-V_1^r$ 46 percent, $Lac^+V_1^s$ 2 percent (for the new combinations). With reference to $[B^+M^+]$, Lac^- is the parental, Lac^+ the interchange type. The proportion of V_1^r (representing an interchange between V_1 and [*BM*]) is different in the Lac^- and Lac^+ segregations: namely 46:23 = 2:1 and 29:2 = 14.5:1 respectively. This interaction between interchanges is most simply explained by the assumption that factors are located on a linear segment, so that interchanges between proximal factors also lead to the crossing over of more distal factors, barring the occurrence of additional interchanges.

Additional support for the theory of linear arrangement has been found in the segregation of V_6, summarized in table 6. It will be noted that the segregations of *Lac*, V_1, and V_6 are quite congruous in the B_1^- and B_1^+ classes. In the totals, one finds the ratios, for each factor separately, of Lac^- 78 percent; V_6^r 82 percent; V_1^s 36 percent; indicating that the first two are both linked to [*BM*] while the latter is linked to [*TL*]. V_6 cannot, however, be to the left of [*BM*] because it does not interact with B_1. If, therefore, there is a linear order of genes, V_6 must be to the right of [*BM*], and because of its greater linkage intensity, nearer [*BM*] than is *Lac*. This arrangement is indicated in the map in table 6, and in fig. 2c. The agreement of the data with the hypothesis can be examined at several points. In the first place, the single exchange types, as indicated in the table, should be the most frequent. Secondly, barring multiple exchanges, an interchange between V_6 and *Lac* should lead also to an interchange between V_6 and V_1. That is to say, the $Lac^+V_6^r$ class should be more often V_1^r than V_1^s. Finally, in view of the similarity in linkage intensities to [*BM*], *Lac* and V_6 must be closely linked. Although the "triple-interchange" types would seem to be rather frequent, reference to the table may suggest that these conditions are fulfilled. In particular, it will be noted that among the Lac^-, the ratio of $V_6^r : V_6^s$ is 94:3, or 31:1, while among the Lac^+, this same ratio is 10:29, or 1:3. This difference is interpreted to mean that *Lac* and V_6 are linked to each other, as demanded by the theory of linearity.

It is not, of course, proven that the gene order is not branched at some other

point. The most economical hypothesis at this time, however, is that there is a single unbranched chromosome as the physical basis of inheritance in *E. coli*.

Attempts to Induce Aberrations

Using a chromosomal theory as a working hypothesis, it was hoped that some verification could be found by the study of types in which the normal order of genes was disturbed. Since there is only one chromosome (from the

TABLE 6

Segregation of Lac, V_1 and V_6.

$B^-M^-T^+L^+B_1^+Lac^+V_1^rV_6^s \times B^+M^+T^-L^-B_1^-Lac^-V_1^sV_6^r$

$B^+M^+T^+L^+$	*Lac:*	−	−	−	−	+	+	+	+	
	V_1 :	*r*	*s*	*r*	*s*	*r*	*s*	*r*	*s*	TOTAL
	V_6 :	*r*	*r*	*s*	*s*	*r*	*r*	*s*	*s*	
$\cdots B_1^+$		24	16	1	0	2	1	10	2	56
$\cdots B_1^-$**		52	42	2	0	6	1	16	1	120
Total		76	58	3	0	8	2	26	3	176
%		43	33	1.7	0	4.6	1.1	15	1.7	
Crossover region		*e*	*f*	*cde*	*cdf*	*d*	*def*	*c*	*ced*	

B_1	$\cdots M$	$\cdots B$	$\cdots V_6$	$\cdots Lac$	$\cdots V_1$	$\cdots T$	$\cdots L$
	a	*b*	*c*	*d*	*e*	*f*	
+	−	−	*s*	+	*r*	+	+
−	+	+	*r*	−	*s*	−	−

** See footnote to table 4.

genetic evidence), the only types of rearrangements would be changes leading to a series of inversion-transposition types. It was thought that such types might be detected by genetical procedures by virtue of their effect on crossing over. In particular, the occurrence of an inversion in the region $B_1 \cdots [MB]$ would be expected to have the effect of eliminating the recombination classes involving interchanges in this region. In the cross $B^-M^-T^+L^+B_1^+ \times B^+M^+T^-L^-B_1^-$ this would be equivalent to the suppression of prototroph recombinants; B_1^- types, however, would be recoverable, and allow the investigation of the extent of the changes.

Preliminary attempts to find such aberration types have, to date, been unsuccessful. The procedure was as follows:

Following treatment with nitrogen mustard (TATUM, 1946) or 20,000 r of X-rays, cells of Y-40 and of Y-53 were incubated separately for 24 hours, to allow the separation of cells or nuclei that might have been associated at the time of treatment. The cultures were then streaked out on nutrient agar

plates. Single colonies of Y-40 were picked and streaked across a nutrient agar plate. Streaks of similarly treated Y-53 colonies were made from the opposite direction, so that in the center of the plate, cells of the two types were mixed, treated colony by treated colony. The plates were incubated for 24 hours, the mixed growth scraped from the plates, suspended in sterile water and plated into minimal agar. The occurrence of colonies which would not interact to produce prototrophs, as detected by plating into minimal medium, would be an indicator that the combination was heterogeneous for an aberration. Since in these experiments, both "parents" were exposed to treatment, each plating was equivalent to the testing of two chromosomes for the occurrence of an aberration. No marked variation in the yield of prototrophs was noted in tests involving 121 mustard- and 28 x-ray-treated chromosomes. This can scarcely be regarded as an adequate sample in view of the stringent selection imposed by the technique, which might be expected to eliminate any aberration types which are even slightly less vigorous than the normal. This consideration is especially relevant in view of the "hemizygous" condition of any aberrations in the probably haploid vegetative cells. These studies will be continued.

How Many Segregants per Zygote?

In the experiments detailed in this paper, recombinants were obtained from different cell types which were exposed to each other in an agar medium. Therefore each prototroph recombinant colony seen by the experimenter marks the site of formation of a zygote. The question may immediately be raised whether there are at that site other recombination classes which, by virtue of their biochemical deficiencies, remain dormant within the prototroph colony on the minimal selective medium. This is equivalent to inquiring whether there is but a single viable product of meiosis (as in megasporogenesis in many higher plants) or more than one, as in the ascomycetes. The solution to this problem would be of special interest in relation to the possible occurrence of four-strand crossing over. In addition, if an appreciable proportion of prototroph colonies consisted of two distinct segregation types, it would be necessary to isolate these types for the collection of segregation data.

There are at least three ways in which a zygote might yield more than one haploid recombinant. Firstly, the zygote might be capable of proliferation in the diplophase (or sporophyte), leading to the concurrence of several diploid cells, each of which might undergo meiosis independently, and by chance yield several segregation types. Secondly, a single zygote might produce, after meiosis, in addition to the prototroph, the *complementary* multiple mutant class. Thirdly, in a system of four-strand crossing-over, there might be two *supplementary* prototroph recombinants differing in the segregation of factors such as *Lac* and V_1 for which the diploid was heterozygous.

Obviously, the proper investigation of these possibilities requires that one stringently avoid contamination of one colony with another. For this reason, the cell suspensions used were diluted so as to yield only about five to ten recombination colonies per plate.

Crosses were made between Y-40 and Y-53 ($B^-M^-T^+L^+B_1^+Lac^+V_1^r \times B^+M^+T^-L^-B_1^-Lac^-V_1^s$) on B_1-containing minimal agar medium. As already noted, about 90 percent of the colonies from such a cross are $B^+M^+T^+L^+B_1^-$. The theoretical complementary class would be $B^-M^-T^-L^-B_1^+$. Because of its nutritional deficiencies, it could not be expected to proliferate on the minimal medium even had it been produced after meiosis. The possibility remains, however, that a few cells of this constitution might still be present among the 10^8 or so B_1^- cells of the predominant type in a colony. By plating such colonies into medium lacking B_1 but containing biotin, methionine, threonine and leucine, the B_1^- cells would be suppressed, while the postulated multiple mutant type could form colonies and be recovered.

The experiment just described was carried out, testing 52 colonies for their content of other cell types. In general, a thiaminless colony could be shown to contain from 10–100 cells capable of forming colonies on the *B*, *M*, *T*, *L* medium. However, in each case investigated these have been shown to be indistinguishable from the Y-40 parental B^-M^- type, and must be presumed to arise from a surprisingly low degree of contamination of the colony with these cells from the heavily seeded plate. A few colonies were found which could be characterized as reversions from B_1^- to B_1^+. These experiments are then, inconclusive with respect to the occurrence of complementary genotypes in the same colony. With appropriate stocks, not as yet available, it should eventually be possible to manipulate the situation so that the complementary type could be recovered selectively, excluding both parents and the predominant recombination class.

A search for supplementary types was conducted with the same crosses, except that colonies appearing on B_1 agar were streaked out directly on EMB-lactose agar to determine whether any of them were heterogeneous for *Lac*. In some cases, a number of isolated colonies from each EMB-test plate were then also tested for homogeneity with respect to *T1*-resistance. About 90 colonies were so tested; only one colony was found containing both Lac^+ and Lac^- cells. It is impossible to be certain that, with this low frequency, the single colony which was picked was not actually derived from two distinct zygotes. These experiments cannot be considered as bearing critically on the question of the occurrence of two- or four-strand crossing over because of the absence of information concerning (a) the viability of more than one meiotic product and (b) chiasma interference. The results do, however, justify the technique of picking the prototroph colonies directly, and testing them without further purification for the collection of segregation data.

A Comparison of Sexual Recombination and Transformation

The occurrence of recombination types has been interpreted by us (LEDERBERG and TATUM 1946c, TATUM and LEDERBERG 1947) as a consequence of cell fusion, "karyogamy" and meiosis with crossing over. This is, however, not the only allowable interpretation of the general phenomenon of the occurrence of new character combinations. By analogy with the systems which have been described in pneumococci (AVERY, MACLEOD and MCCARTY 1944) and other

strains of *E. coli* (BOIVIN and VENDRELEY 1946) one might postulate that genotypically distinct cells interact not through cell fusion, but through the release of "transforming substances" diffusing through the medium. Such transforming substances would have the property of inducing or directing mutational changes in the cell receiving them so as to lead to what appear to be recombination types. Our inability to separate such postulated transforming substances from the cells themselves is not proof of their absence but could be due to their lability in our hands.

In previous publications, certain reasons were given for the rejection of the transformation hypothesis in favor of a picture of cell fusion, and so forth. It was not our intention thereby to state, with clairvoyant insight, that no investigator will be able to duplicate the results which we have reported, using instead of living cells extracts specially prepared. It is, rather, our view that since we have been able to demonstrate no appreciable point of difference between the features of gene exchange in this strain of *E. coli* and in the classical materials of Mendelian experimentation, the most economical conclusion is that the mechanisms involved are also similar. In the absence of more detailed information on the behavior of transforming systems, a critic would be free to impute to such systems all of the properties which have been found to characterize the genetic system of *E. coli*, K-12. While this would be tailoring the cloth to suit the customer, it cannot be disputed that the only conclusive method by which it could be shown that cell fusion underlies gene recombination would be a direct cytological demonstration. The rarity with which the presumed zygote occurs, however (as indicated by the low frequency of effective recombination types) is very discouraging to attempts to find and characterize the "fusion-cell," at least in the present material.

Certain genetic experiments were performed in an attempt to characterize further the behavior of this system. On the transformation hypothesis, one must attribute the rarity of the imputed transformations primarily to restricted conditions for susceptibility to the transforming factors released into the milieu. Otherwise, one would expect to find "transformations" for single factors much more frequent than those involving more than two factors. A glance at tables 4 and 6 illustrates that certain "multiple transformed" types are much more frequent than singly transformed classes. Under these conditions, one might also anticipate that genetic materials from two different kinds of cells could mix in the medium and together transform a third. In a mixture of three cell types then, one should find cases where genes from all three have combined. Using *Lac* and V_1 as markers, this type of experiment was set up in several different ways, as summarized in table 7. Pairwise, prototrophs can be formed only from biochemically distinct and nonoverlapping parents. Combinations of B^-M^- and of $T^-L^-B_1^-$ were arranged so that taken two at a time they were heterozygous either for *Lac* or for V_1 but not both. For example, a mixture of $B^-M^-Lac^-V_1^r$, $T^-L^-B_1^-Lac^-V_1^s$ and $T^-L^-B_1^-Lac^+V_1^r$ was plated. Prototrophs could be formed by recombination between either of the two latter and the former types. In one case, only V_1 would be heterozygous, and the expected types would be $Lac^-V_1^r$ and $Lac^-V_1^s$. In the other, *Lac*

would be heterozygous, and prototrophs carrying the markers $Lac^+V_1^r$ and $Lac^-V_1^r$ could be produced. The type $Lac^+V_1^s$ would not be expected unless, indeed, genetic material from all three types could combine in a sort of ménage à trois. As recorded in table 7, no instance of such a three-way combination was found in 628 tests, a different class being vacant, as anticipated, in each of the four parts of the experiment. It may be concluded that genetic factors from different cells are not freely miscible, as would be demanded by the most economical version of the interpretation of transformations.

From all the experiments so far cited, it must be concluded that if trans-

TABLE 7

Pairwise occurrence of recombination in mixtures of three components.

PARENTAL TYPES		RECOMBINANT PROTOTROPHS* $B^+M^+T^+L^+B_1^+$ or B_1^-				
$B^-M^-T^+L^+B_1^+$	$B^+M^+T^-L^-B_1^-$	$Lac^-V_1^r$	$Lac^-V_1^s$	$Lac^+V_1^r$	$Lac^+V_1^s$	TOTAL
$Lac^-V_1^r$	$Lac^+V_1^r$ $Lac^-V_1^s$	173	49	4	0	226
$Lac^+V_1^r$	$Lac^-V_1^r$ $Lac^+V_1^s$	16	0	7	28	51
$Lac^+V_1^s$	$Lac^+V_1^r$ $Lac^-V_1^s$	0	136	37	40	213
$Lac^+V_1^s$ $Lac^-V_1^r$	$Lac^-V_1^s$	65	48	0	25	138
					Total	628

* Mixtures of the three types indicated in each experiment were plated into thiamin-containing agar. The prototrophs are therefore a mixture of B_1^- and B_1^+ types, as indicated in table 4, footnote.

forming factors are operating in this system, the diverse factors (or genes) are not independent of one another, but are grouped in separate and immiscible parcels. Such parcels would also be potentially capable of transmitting all of the genetic factors of a cell, so that there seems to be no compelling reason why such a parcel, speaking purely genetically, could not be regarded as a gamete. MULLER (1947) has interpreted the pneumococcus transformation in terms of "still viable bacterial chromosomes or parts of chromosomes floating free in the medium . . . these have penetrated the capsuleless bacteria and in part at least, taken root there, perhaps after having undergone a kind of crossing over with the chromosomes of the host." It remains to be seen whether this interpretation will be upheld by further studies on factor interaction in *bona fide* transforming systems.

Several attempts were made to determine whether "transforming activity" could be separated from the living cell under conditions comparable to the

platings in minimal agar medium, or after extraction of cells by BOIVIN's method (BOIVIN *et. al.*, 1946). No activity was found in the supernatant of a suspension of Y-40 and Y-53 together or separately in the same minimal liquid medium to which agar is added for plating experiments. The only manipulation involved here consists of the removal of most of the bacteria by ordinary centrifugation. It could thus be shown that the "activity" was associated with the cells. Equally negative results characterized attempts to reveal transforming activity on culture filtrates and cell autolysates prepared as crude fractions according to BOIVIN's procedure. Finally, the addition of desoxyribonuclease in a final concentration of .05 mg/ml to the mixing and plating medium had no effect on the number of prototrophs which appeared in the cross of Y-40 and Y-53. Tests for the destruction of enzymatic activity under these conditions were, however, not done.

The conclusions which we draw from these experiments are (a) that the existence of transforming factors is exceedingly unlikely and (b) it would be not worthwhile to go to extreme trouble to attempt to isolate such factors from this system until the study of *bona fide* transforming systems has progressed sufficiently that the genetical criteria already discussed might be applied.

DISCUSSION

Regardless of the stand that one takes on the issue of invisible zygotes versus non-extractable transforming factors, it can be asserted that *E. coli* K-12 provides a useful tool for genetic analysis. The use of biochemical mutants as parents allows crosses which are nearly as well controlled as in Neurospora. The segregational behavior of mutant factors seems to be closely analogous to that of higher forms, and seems to compel their admission into the same arena as the genes of Drosophila. However, it would be premature to transfer these conclusions to other genetic characters of other microorganisms, each of which must be examined on its own merits.

It may be wondered that the apparent recombination rate is so low. However, this is possibly not to be attributed to any sexual imperfections of *E. coli*, but to the method of enumeration. It seems likely that an analogous comparison of the number of somatic and generative cells in an organism like the oak-tree, or man (especially the female of the species) would give ratios similar to those prevailing in *E. coli*. It is also possible that the optimal conditions for zygote formation or germination have not yet been achieved and that by special procedures the rate of zygote-formation may be accelerated to the level where there might be some hope of finding it in the field of the microscope.

Attempts to detect recombination in two other strains of *E. coli*, B (DEMEREC and FANO, 1945) and L-15 (ROEPKE, LIBBY, and SMALL, 1944) by analogous methods have been unsuccessful (LURIA, 1947, TATUM and LEDERBERG, 1947). At least two strains then must be classified with the "*Fungi Imperfecti.*" This dismal conclusion is, however, illuminated by the fact that many heterothallic species have been eliminated from the *Fungi Imperfecti* with the discovery of the appropriate opposite mating-types. At the present time, one scarcely knows where to begin to look for the bacterial analogy. The

application of genetic techniques to the elucidation of unusual life-cycles in diverse bacteria (BRAUN and ELROD, 1946, DIENES, 1946) cannot fail, however, to be most fruitful.

The evolutionary significance of gene recombination has been made so widely familiar by DOBZHANSKY's book (1941), and adequately discussed, more recently, by MULLER (1947), that it would be impertinent to do more than simply refer to these papers.

SUMMARY

The recombination of genetic factors and their segregation into prototroph recombinants of *Escherichia coli* have been studied. It was found that genetic markers behaved as if they were part of a system of linked genes. Some evidence for linear order of genes was obtained. Each of 15 factors studied fell into the same linkage group. Data are given in detail for the segregation of factors involved in the biosyntheses of biotin, methionine, threonine, leucine, or thiamin; in the fermentation of lactose, and in resistance to bacterial viruses *T1* and *T6*. On the basis of these data a tentative 8-point genetic map of the chromosome of *E. coli* is presented.

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