Seymour Benzer’s work changed our notion of the concept of the gene, by demonstrating that the gene had a fine structure consisting of a linear array of subelements. At the time Benzer began his classic work, the concept of the gene was different from what it is today. Genes were thought to be indivisible and to be the smallest units of recombination, mutation, and function. Genes could have different allelic states, but these alleles represented the whole gene, not parts of it. In one sense, genes were thought of as beads on a necklace, the necklace being the chromosome. This picture of the gene proved to be at odds with the physical structure of DNA elucidated by Watson and Crick in 1953, which revealed the physical structure of the gene to consist of a sequence of nucleotides. Each nucleotide should be able to mutate and should also be the smallest unit of recombination. Benzer’s work bridged the gap between the classic view of the gene as an indivisible unit and the physical structure of DNA. By exploiting the rII system of phage T4 and refining high-resolution genetic selection, Benzer was able to show that the subelements in the gene could mutate and recombine with one another. The smallest unit of mutation and recombination was now shown to be on the order of only a few nucleotides or less, based mainly on genetic analysis. Benzer also refined the cis-trans test for use with phage T4, and he defined the cistron as a unit of gene function, a term and concept that were used for many years.

Benzer developed the use of deletions in genetic crosses, which opened up the entire field of fine structure analysis. It laid the groundwork for his subsequent studies, which defined the concept of mutational hot spots, and profoundly influenced work on mutagenesis for a generation.

Jeffrey H. Miller

THE ELEMENTARY UNITS OF HEREDITY*

SEYMOUR BENZER

Biophysical Laboratory, Purdue University, Lafayette, Indiana

INTRODUCTION

The techniques of genetic experiments have developed to a point where a highly detailed view of the hereditary material is attainable. By the use of selective procedures in recombination studies with certain organisms, notably fungi (14), bacteria (2), and viruses (1), it is now feasible to “resolve” detail on the molecular level. In fact, the amount of observable detail is so enormous as to make an exhaustive study a real challenge.

A remarkable feature of genetic fine structure studies has been the ability to construct (by recombination experiments) genetic maps which remain one-dimensional down to the smallest levels. The molecular substance (DNA) constituting the hereditary material in bacteria and bacterial viruses is also one-dimensional in character. It is therefore tempting to seek a relation between the linear genetic map and its molecular counterpart which would make it possible to convert “genetic length” (measured in terms of recombination frequencies) to molecular length (measured in terms of nucleotide units).

The classical “gene,” which served at once as the unit of genetic recombination, of mutation, and of function, is no longer adequate. These units require separate definition. A lucid discussion of this problem has been given by Pontecorvo (13).

The unit of recombination will be defined as the smallest element in the one-dimensional array that is interchangeable (but not divisible) by genetic recombination. One such element will be referred to as a

*This research has been supported by grants from the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council, and from the National Science Foundation.
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"recon." The unit of mutation, the "muton," will be defined as the smallest element that, when altered, can give rise to a mutant form of the organism. A unit of function is more difficult to define. It depends upon what level of function is meant. For example, in speaking of a single function, one may be referring to an ensemble of enzymatic steps leading to one particular physiological end-effect, or of the synthesis of one of the enzymes involved, or of the specification of one peptide chain in one of the enzymes, or even of the specification of one critical amino acid.

A functional unit can be defined genetically, independent of biochemical information, by means of the elegant cis-trans comparison devised by Lewis (12). This test is used to tell whether two mutants, having apparently similar defects, are indeed defective in the same way. For the trans test, both mutant genomes are inserted in the same cell (e.g., in heterocaryon form, or, in the case of a bacterial virus, the equivalent obtained by infecting a bacterium with virus particles of both mutant types). If the resultant phenotype is defective, the mutants are said to be non-complementary, i.e., defective in the same "function." As a control, the same genetic material is inserted in the cis configuration, i.e., as the genomes from one double mutant and one non-mutant. The cis configuration usually produces a non-defective phenotype (or a close approximation to it). It turns out that a group of non-complementary mutants falls within a limited segment of the genetic map. Such a map segment, corresponding to a function which is unitary as defined by the cis-trans test applied to the heterocaryon, will be referred to as a "cistron."

The experiments to be described in this paper represent an attempt to place limits on the sizes of these three genetic units in the case of a specific region of the hereditary material of the bacterial virus T4. A group of "rII" mutants of T4 has particularly favorable properties for this kind of analysis. Mutants are easily isolated. Recombinants can be detected, even in extremely low frequency, by a selective technique. The system is sufficiently sensitive to permit extension of genetic mapping down to the molecular (nucleotide) level, so that the recon and muton become accessible to measurement. The rII mutants are defective in the sense of being unable to multiply in cells of a certain host bacterium (although they do infect and kill the cell). The cis-trans test can therefore be readily applied.
**Method of Construction.**

The construction of a genetic map of an organism starts with the selection of a standard ("wild") type. From the progeny of the wild type, mutant forms can be isolated on the basis of some heritable difference. When two mutants are crossed, there is a possibility that a wild-type organism will be formed as a result of recombination of genetic material. The reciprocal recombinant, containing both mutational alterations, also occurs. The proportion of progeny constituting such recombinant types is characteristic of the particular mutants used. The results of crosses involving a group of mutants can be plotted on a one-dimensional diagram where each mutant is represented by a point. The interval between two points signifies the proportion of recombinants occurring in a cross between the two corresponding mutants. Usually, it is not possible to construct a single map for all the mutants of an organism; instead the mutants must be broken up into "linkage groups." A linear map may be constructed within each linkage group, but the mutant characters assigned to different linkage groups assort randomly among the progeny. The number of linkage groups, in some cases, has been shown to correspond to the number of visible chromosomes.

The procedure for constructing a genetic map for a bacterial virus is much the same (9). A genetically uniform population of a mutant can readily be grown from a single individual. Two mutants are crossed by infecting a susceptible bacterium with both types and examining the resulting virus progeny for recombinant types. Virus T4 has been mapped in some detail (3, 1), and behaves as a haploid organism with a single linkage group (18).

**Relativity of Genetic Maps.**

A genetic map is an image composed of individual points. Each point represents a mutation which has been localized with respect to other mutations by recombination experiments. The image thus obtained is a highly colored representation of the hereditary material. Alterations in the hereditary material will lead to noticeable mutations only if they affect some phenotypic characteristic to a visible degree. Innocuous changes may pass unnoticed, leaving their corresponding regions on the map blank. At the other extreme, alterations having a
Fig. 1. Photographs of plaques formed on E. coli B by T4 "wild-type" and nine independently arising r mutants.
Fig. 2. The same mutants as used in Fig. 1, plated on E. coli K.
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lethal effect will also be missed (in a haploid organism). The map represents, therefore, only cases which fall between these extremes under the conditions of observation. By varying these conditions, a given mutational event may be shifted from one of these categories (innocuous, noticeable, or lethal) to another, thereby appearing on, or disappearing from the map.

This effect may be illustrated by the "r" mutants of bacterial virus T4. Wild-type T4 produces small, fuzzy plaques on *Escherichia coli* B (Fig. 1). From plaques of wild-type T4, r-type mutants can be isolated which produce a different sort of plaque. Fig. 1 shows the plaques of nine r mutants, each isolated from a different plaque of the wild type in order to assure independent origin. The similarity of plaque type of these r's on B disappears when they are plated on another host strain, *E. coli* K (a lysogenic K12 strain (10) carrying phage lambda), as shown in Fig. 2. Here, they split into three groups: two mutants form r-type plaques, one forms wild-type plaques, and the remaining six do not register. Thus, with B as host, all three types of mutation lead to visible effects, while with K as host, the effects may be visible, innocuous, or lethal.

When the same set of mutants is plated on a third strain, *E. coli* S (K12S, a non-lysogenic derivative (10) of K12) or BB (a "Berkeley" derivative (17) of B) the pattern of plaque morphology is different from that on either B or K (Table 1).

Table 1: Plaque Morphology of T4 Strains (Isolated in B) Plated on Various Hosts

<table>
<thead>
<tr>
<th>PHAGE STRAIN</th>
<th>B</th>
<th>S</th>
<th>K</th>
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<tbody>
<tr>
<td>wild</td>
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<td>r I</td>
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<td>r II</td>
<td>r</td>
<td>wild</td>
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</tr>
<tr>
<td>r III</td>
<td>r</td>
<td>wild</td>
<td>wild</td>
</tr>
</tbody>
</table>

If a genetic map is constructed for these mutants, using B as host, the three groups fall into different map regions, as indicated in Fig. 3. On strain S, the rII and rIII types of mutation are innocuous. Thus, if S had been used as host in the isolation of r mutants, only the rI region would have appeared on the map. On K as host, the rIII mutation is innocuous, and the rII mutation is (usually) lethal, so that only the
THE CHEMICAL BASIS OF HEREDITY

Fig. 3. Dependence of the genetic map of T4 upon the choice of host. Three regions of the map are shown as they probably would appear if *E. coli* strains B, S, or K were used as the host.

*rI* region would appear. Actually, a few *rII* mutants are able to multiply somewhat on K, producing visible tiny plaques. If K were used as host in the isolation and testing of mutants from wild-type T4, these mutants could be noticed and would probably be designated by some other name, perhaps "minute." The map would then appear as in the bottom row of Fig. 3. The distribution of points on the map within this "minute" region would be very different from those for the *rII* region using B as the host.

The appearance of a genetic map also depends on the choice of the standard type, which is, after all, arbitrary. For example, suppose an *r* form were taken as the standard type and non-*r* mutants were isolated from it. Then a completely different map would result. An example of this is to be found in the work of Franklin and Streisinger (5) on the *h* → *h+* mutation in T2, as compared with that of Hershey and Davidson (7) on the *h+* → *h* mutation.

Another way in which the picture is weighted is by local variations in the stability of the genetic material. Certain types of structural alterations may occur more frequently than others. Thus, a perfectly stable genetic element (i.e., one which never errs during replication) would not be represented by any point on the map.

**Determination of the Sizes of the Hereditary Units by Mapping.**

Determination of the recom requires "running the map into the ground" (Delbrück's expression), that is, isolation and mapping of so large a linear density of mutants that their distances apart diminish to the point of being comparable to the indivisible unit. With a finite set of mutants, only an upper limit can be set upon the recom, which
must be smaller than (or equal to) the smallest non-zero interval observed between pairs of mutants.

To determine the length of map involved in a mutational alteration, a group of three closely linked mutants is needed. Since map distances are (approximately) additive, a calculation of the “length” of the central mutation can be attempted (15) from the discrepancy observed between the longest distance and the sum of the two shorter ones, as shown in Fig. 4. The upper limit to the size of the muton would be

![Diagram of three linked mutants](image)

Fig. 4. Method for determining the “length” of a mutation. The discrepancy between the long distance and the sum of the two short distances measures the length of the central mutation.

the smallest discrepancy observed by this method, which can be determined accurately only if the three mutants are very closely linked. It should be noted that since the degree to which the genetic structure can be sliced by recombination experiments is limited by the size of reon, the size of the muton will register as zero by this method if it is equal to or smaller than one reon. A second method for determining the muton size is by the maximum number of mutations, separable by recombination, that can be packed into a definite length of the map.

For the eistron size, only a lower limit can be set with a finite group of mutants. The eistron must be at least as large as the distance between the most distant pair within it. Its boundaries become more sharply defined the larger the number of points which are shown to lie inside them.

Thus, the determination of the sizes of all three units requires the isolation and crossing of large numbers of mutants. The magnitude of this undertaking increases with the square of the number of mutants, since to cross $n$ mutants in all possible pairs requires $n(n-1)/2$ crosses, or approximately $n^2/2$. Fortunately, however, the project can be shortened considerably by means of a trick.
The Method of Overlapping "Deletions."

Certain rII mutants are anomalous in the sense that they cannot be represented as points on the map. The anomalous mutants give no detectable wild recombinants with any of several other mutants which do give wild recombinants with each other. An anomalous mutant can be represented (Fig. 5) as covering a segment of the map. Reversion of such an rII mutant has never been observed; also, no mutant which does revert has been found to have this anomalous character. The properties of an anomalous mutant can be explained as owing to the deletion (i.e. loss) of a segment of hereditary material corresponding to the map span covered. However, anomalous behavior and stability against reversion are not sufficient to establish that a deletion has occurred. Similar properties could be expected of a double mutant when crossed with either of two different single mutants located at the same points. An inversion also would show the same behavior. However, the occurrence of a deletion seems to be the only reasonable explanation in the cases of several of the rII mutants, since they fail to give recombinants with any of three or more (in one case as many as 20) well-separated mutants.

Fig. 5. Illustration of the behavior of an "anomalous" mutant. Mutant no. 6 is anomalous with respect to the segment of the map indicated by the bar; it fails to give wild recombinants with mutants (nos. 2, 3, and 4) located within that segment. A + signifies production, and 0 lack of production, of wild recombinants in a cross.
Whether a given mutation belongs in the region covered by a deletion can be determined by the appropriate cross. If wild recombinants are produced, the mutant must have a map position outside the region of the deletion. This eliminates the need to cross that mutant with any of the mutants whose map positions lie within the region of the deletion. The problem of mapping a large number of mutants is greatly simplified by this system of “divide and conquer.” The mutants can first be classified into groups that fall into different regions on the basis of crosses with mutants of the deletion-type. Further crossing in all possible pairs is then necessary only within each group.

Suppose that three deletions occur in overlapping configuration, as shown in Fig. 6A. Fig. 6B represents the results that would be obtained in crosses of pairs of these three mutants. A diagonal element (representing a cross of a mutant with itself) is, of course, zero, since no wild recombinants can be produced. An overlap is reflected by the pattern of non-diagonal zeros. These results would establish a unique order of the deletions (without resort to the three-factor crosses that would ordinarily be necessary). With a sufficient number and appropriate distribution of deletions, one could hope to order a large length of map. The reader will note an analogy (not altogether without significance!) to the technique used by Sanger (16) to order the amino acids in a polypeptide chain by means of overlapping peptide segments.
TAXONOMY OF T4 MUTANTS

T4 Standard ("Wild") Type

m, tu, h, etc

KINGDOM

PHYLUM

CLASS

SUB-CLASS

SPECIES

VARIETY

Fig. 7. Classification scheme for r mutants of T4.
Mapping the rII Region of T4

Taxonomy of r Mutants.

In classifying the mutants of T4, classical terminology may be conveniently used for the taxonomic scheme shown in Fig. 7. Mutants of the r "kingdom," isolated on B, can be separated into three "phyla" by testing on K. For the present purposes, our attention will be limited to mutants of the rII phylum, which are inactive on K.

A pair of rII mutants may be subjected to the cis-trans test. The cis configuration (mixed infection of K with double mutant and wild-type particles) is active, since the presence of a wild particle in the cell enables both types to multiply. The trans configuration (mixed infection of K with the two single mutants) may be active or inactive. If inactive, the two mutants are placed in the same "class." Since the members of a class fail to complement each other, they can be considered as belonging to a single functional group. On the basis of this test, the rII mutants divide into two clear-cut classes. The map positions of the mutants in each class have been found to be restricted to separate map segments, the A and B "cistrons."

Arbitrary sub-classes can be chosen (from among the available deletions) for convenience in mapping; mutants falling within the map region encompassed by a particular deletion form a sub-class.

Reverting mutants are considered as of different "species" if crosses between them yield wild recombinants. Among a group of mutants which have not yielded to resolution by recombination tests, "varieties" can in some cases be distinguished by other criteria (reversion rate or degree of ability to grow on K).

Procedures in the Classification of r Mutants of T4.

(1) Isolation of mutants

Each mutant is isolated from a separate plaque of wild-type T4 (plated on B) and freed from contaminating wild-type particles by replating. Stocks of mutants are prepared by growth on S (to avoid the selective advantage which wild type revertants would have on B). Mutants are numbered in the order of isolation, starting with 101 to avoid confusion with mutants previously isolated by others.

(2) Spot test on K

In this first test of a new r mutant, 10^8 particles are plated on K and then the plate is spotted with one drop (10^6 particles) of r164 (a
mutant having a "deletion" in an A cistron and one drop of r196 (a mutant having a deletion located in the B cistron). Typical examples of the results of this test are shown in Fig. 8. If the new mutant belongs either to the rI or the rII phylum, the plating bacteria will be completely lysed (except for a background of colonies formed by mutants of K which are resistant to T4), as typified by mutant X in Fig. 8.

Mutant Y in Fig. 8 is typical of a stable rII mutant. The background shows no plaques, indicating that the proportion of revertants in the stock is less than $10^{-8}$. The spot of r196 is completely clear, in contrast to the r164 spot. This massive lysis is caused by the ability of mutant Y and r196 to complement each other for growth on K. From this result, it may be concluded that mutant Y belongs to the A class. Within the r164 spot, however, some plaques may be seen. These are due to wild recombinants arising from r164 and mutant Z (by virtue of very feeble growth of rII mutants on K). Therefore, mutant Y is not in the subclass defined by r164.

The third test plate is typical of a reverting rII mutant. The stock contains a fraction $10^{-6}$ of wild-type particles which produce the plaques seen in the background. It is evident from the spot tests that the mutant Z belongs in the B class, and appears to lie within the r196 subclass.

(3) Spot test on a mixture of K and B cells

Once the class of a new mutant is known, it can be tested on a single plate against several mutants of the same class. For this purpose, the sensitivity of the test may be increased enormously by the addition of some B cells (about one part in a hundred) to the K used for plating. The additional growth possible for the mutants on B cells enhances their opportunity to produce wild recombinants. This test gives a positive response down to the level of around 0.01 per cent recombination. A negative result does not, of course, eliminate the possibility that recombination occurs with a lower frequency.

(4) Preliminary crosses

A semiquantitative measure of recombination frequency may be obtained by mixedly infecting B with two mutants and plating the infected cells on K. B cells which liberate one or more wild-type particles can produce plaques. This method is convenient for preliminary testing for recombination in the range from 0.0001 to 0.1 per cent.
**Fig. 8.** Spot test used in classification of $r$ mutants. To test a mutant, $10^8$ mutant particles are plated on bacterial strain K and the plate is spotted with one drop of $r164$ (left) and one drop of $r196$ (right). Mutants X, Y, and Z illustrate typical results. Mutant X is of the $rI$ phylum. Mutant Y is a stable mutant of the $rII$ phylum and the A class, but is not in the $r164$ sub-class. Mutant Z is a reverting mutant (wild-type plaques in background) of the $rII$ phylum, B class, and $r196$ subclass.
With higher frequencies of recombination, approaching the point where a large fraction of the mixedly infected cells liberate recombinants, saturation sets in.

(5) Standard crosses

Standard measurements of recombination frequency are made in conventional crosses. B cells are infected with an average of three particles per cell of each phage. The infected cells are allowed to burst in a liquid medium, and the progeny are plated on K and on B to determine the proportion of wild-type particles. The reciprocal recombinant (double rII mutant) does not, in general, produce plaques on K, but since the two recombinant types are produced in statistically equal numbers (θ), the proportion of recombinants in the progeny can be taken as twice the ratio of plaques on K to plaques on B (corrected for the relative efficiency of plating of wild type on these two strains, which is close to unity).

(6) Reversion rates

The reversion rate of a mutant is reflected in the proportion of wild-type particles present in a stock. This value is an important characteristic of each mutant, varying over an enormous range for different mutants. It may be less than $10^{-8}$ for “stable” (i.e., non-reverting) mutants or as high as several per cent. (For one exceedingly unstable mutant the proportion of revertants averages 70 per cent, even in stocks derived from individual mutant particles.) The precision with which a mutant can be localized on the map is inversely related to its reversion rate; only relatively stable mutants are useful for mapping. In the experiments here reported, it has been assumed that the reversion rate of a mutant is not altered during a cross; the reversion contribution is subtracted from the observed percentage of wild particles in the progeny. In most cases, this correction is negligible.

(7) “Leakiness” of rII mutants

rII mutants differ greatly in their ability to grow on K cells. A sensitive measure of this ability can be obtained by infecting K cells and plating them on B. Any K cell that liberates one or more virus particles can give rise to a plaque. The fraction of infected cells yielding virus progeny, which is a characteristic property of each mutant (when measured under fixed conditions), may vary from almost 100 per cent down to less than one per cent for different mutants.
Leakiness has the effect of limiting the sensitivity of $K$ as a tool for selection of wild recombinants, thereby hampering the mapping of very leaky mutants.

### TABLE 2

**Classification of an Unselected Group of 241 $r$ Mutants of T4**

The number of mutants in each classification is given in parentheses. An asterisk indicates that reversion of the mutant to wild type has not been detected. A few mutants (indicated as "not determined") could not be further classified due to excessively high reversion rate or leakiness.

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<th>Subclass</th>
<th>Species</th>
<th>Variety</th>
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<td>8 sp. (1 ca.)</td>
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</table>

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Classification of a Set of 241 r Mutants.

A set of r mutants was isolated, using B as host, and given numbers from r101 to r338; the mutants r47, r48, and r51, isolated by Doerrmann (3), were added to this set, making a total of 241 r mutants. These were analyzed according to methods already described.

The results are shown in Table 2. Of these mutants, 134 fell into the rII phylum. Each of these (with the exception of one very leaky mutant) could be assigned unambiguously to either of two classes on the basis of the test for complementary action of pairs of mutants for growth on K. Mutants within each class were crossed with stable mutants of the same class; those giving no detectable wild recombinants with a particular stable mutant were assigned to the same subclass. Mutants of each subclass were crossed in all pairs. When two or more mutants were found to be of the same species (i.e., showed, in a "preliminary" type cross, recombination of less than about 0.001 per cent, or less than the uncertainty level set by the reversion rate, whichever was greater), one was used to represent the species in further crosses. Those mutants not falling into any of the subclasses defined by the available stable mutants were crossed with each other in pairs. By these procedures, the classification was carried to the species level for the entire set of mutants, except for six highly revertible or leaky mutants whose subclass was not established.

Several of the species showed evidence of splitting into varieties distinguishable by reversion rate or degree of leakiness. Some mutant varieties recurred frequently (e.g., 19, 11, 9 times). These recurrences were far outside the expectation for a Poisson distribution, and are indicative of local variations of mutability. The fact that many species were represented by only one occurrence suggests that many other species remain to be found.

The 33 species found in the A cistron and the 18 species found in the B cistron are sufficient to define reasonably well the limits of each cistron. The minimum size of a cistron in recombination units is determined by the maximum amount of recombination observed in standard crosses between pairs of mutants within it. On the basis of the standard crosses performed so far, this value is about 4 per cent recombination for the A cistron, and 2 per cent for the B cistron.

Study of 923 r Mutants.

While the study of the foregoing 241 mutants yielded a good idea of the sizes and complexity of the A and B cistrons, it fell short of
“saturating” the map sufficiently to provide the close clusters of mutants required for the determination of the sizes of the recon and muton. To this end, it was decided to isolate many more mutants. By confining attention to those falling into the r164 subclass, a more exhaustive study could be made of a selected portion of the map.

In a group of 923 r mutants (r101 through r1020, plus Doermann's three), 149 were found to belong to the r164 subclass. Four of those were stable. The remaining 145 mutants separated into the 11 species shown in Fig. 9. One of the species accounted for 123 of the mutants! As shown in Table 3, this species included three varieties as distinguished by their reversion rates; two were of roughly equal abundance, while the third occurred only once.

Results of standard crosses between the mutants of the r164 subclass are presented in Fig. 10. The smallest recombination distance, setting an upper limit to the size of the recon, is around 0.02 per cent (between

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Reversion Index (units of 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>variety α</td>
<td></td>
</tr>
<tr>
<td>r200</td>
<td>0.47</td>
</tr>
<tr>
<td>r120</td>
<td>0.55</td>
</tr>
<tr>
<td>r174</td>
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</tr>
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<td>r380</td>
<td>0.17</td>
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<td>r255</td>
<td>420</td>
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<tr>
<td>r151</td>
<td>540</td>
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<tr>
<td>r176</td>
<td>520</td>
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<tr>
<td>r119</td>
<td>2000</td>
</tr>
<tr>
<td>r888</td>
<td>610</td>
</tr>
<tr>
<td>variety γ</td>
<td></td>
</tr>
<tr>
<td>r91.3</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

*The “reversion index” is the proportion of wild-type particles in a lysate prepared from a few mutant particles (to avoid introduction of any revertants present in the original stock) using S as host. The measurement is subject to large fluctuations due to the clonal growth of the revertants formed. Therefore, four separate lysates are made for each mutant. Parentheses indicate extreme fluctuations. An asterisk indicates a background of tiny plaques (smaller than those produced by wild type) when the lysate is plated on K.

In addition to the examples listed in the table, 67 other mutants of this species are also of variety α, as judged by the proportion of revertants (from 0.2 \times 10^{-6} to 4.0 \times 10^{-6}) in single lysates; 45 additional mutants apparently are of variety β (having values from 300 \times 10^{-6} to 4.000 \times 10^{-6}).

The mutants of variety α give less than 0.001 per cent recombination with r274. For the more highly revertible mutants of variety β, this limit can only be set at less than 0.02 per cent recombination with r274. The mutant r97.3, of variety γ, gives less than 0.005 per cent (the limit set by background on K) recombination with r274.
Fig. 9. Grouping into species of mutants within the segment defined by the "deletion" r164. The arrow indicates 103 additional mutants of the r131 species. The different-sized boxes of the r131 species indicate two distinct varieties. The number assigned to each location is that of the first mutant to indicate it.
r240 and r359). Since only one interval has this value, the possibility of smaller values is not ruled out.

One procedure for measuring the size of the muton requires a group of three closely linked mutants in order to compare the long distance with the sum of the two shorter ones. If the central mutation has an appreciable size, there should be a discrepancy between these values. There are eight cases in Fig. 10 for which the distances have been measured for three adjacent mutants: 240-359-391, 359-391-279, 391-279-271, 279-271-539, 271-539-385, 385-155-131, 155-131-596, 131-596-106. The discrepancies for these groups are, reading from left to right, + 0.14, − 0.03, − 0.02, − 0.03, − 0.02, − 0.03, + 0.03, and + 0.05 per cent recombination. The average of these values is + 0.01, with an average deviation of ± 0.05. Since each measurement of recombination frequency is subject to experimental error of the order of 20 per cent of its magnitude, these determinations of mutation size (each derived from three measurements) are uncertain to plus or minus about 0.05 per cent recombination. Therefore, the latter is the smallest upper limit than can be set upon the size of the muton by these data.

Another measure of muton size can be attempted by finding the number of species that can exist within a given length of the map. As shown in Fig. 10, a map length of 0.8 per cent recombination includes 9 separable mutant species, or no more than 0.09 per cent per species. Both this determination and the previous one suffer uncertainty due to imperfect additivity of map distances (“negative interference”—see Discussion).

Stable Mutants.

Since the problem of mapping large numbers of mutants is greatly facilitated by the use of “deletions,” particular attention has been paid to non-reverting mutants, in the hope of obtaining a complete set of

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Fig. 10. Map of the mutants in the r164 segment. The numbers give the percentage of recombination observed in standard crosses between pairs of mutants. The arrangement on this map is that suggested by these recombination values; it has not yet been verified by three-point tests. Stable mutants are represented as bars above the axis; the span of the bar covers those mutants with which the stable mutant produces no detectable wild recombinants. The stable mutant r223 appears to be a double mutant having one mutation at the highly mutable r131 location and a second mutation at a point in the B cistron. Mutants r131 and r973 are separated on the map so that the data for each can be indicated. Some of the data here given differ from (and supersede) previously published data based upon unconventional crosses which turned out to be incorrect.
overlapping deletions. Among the series of 923 r mutants, 72 stable rII mutants were found, 47 in the A ciston and 24 in the B ciston. One mutant (r928) was exceptional: it failed to complement mutants of either the A or B cistrons and therefore belongs to both classes.

The stable mutants of the B ciston have been crossed (by spot tests on K plus B) in all possible pairs. The results are shown in Fig. 11.

![Recombination matrix](image)

**Fig. 11.** Recombination matrix for stable mutants of the B class. A + indicates production of wild recombinants (around 0.01 per cent recombination or more would be detected) in the cross between the indicated pair of mutants (by spot test on K plus B). All diagonal elements (self-crosses) are zero; non-diagonal zeros indicate overlaps. Two of the mutants are derived, not from the original wild type, but from a revertant of r199.
Fig. 12. Preliminary locations of stable $rII$ mutants. Mutants producing no wild recombinants with each other are drawn in overlapping configuration. Pairs which produce small amounts are placed near each other. Since there remain some gaps, the order shown depends upon that established by Doermann (4) for the mutants shown on the axis. The scale is somewhat distorted in order to show the overlap relationships clearly. Brackets indicate groups, the internal order of which is not established. Ten stable mutants of the A class and six of the B class were not sufficiently close to any others to permit them to be placed on the map. (A class equals A cistron; B class equals B cistron.)
Overlapping relationships are indicated by non-diagonal zeros. Fig. 12 shows the genetic map representation of these results together with the results derived from an analysis (as yet incomplete) of the stable mutants of the A cistron. Unfortunately, gaps still remain in the map.

The mutant r638 is of particular note. No B class mutant has been found that gives wild recombinants with it, so that it appears to be due to deletion of the entire B cistron. In spite of this gross defect, it is capable of normal reproduction on E. coli strains B and S.

In order to characterize a stable mutant of a “deletion” type, it is necessary to show that it gives no wild recombinants with at least three other mutants that do give recombination with each other (to exclude the possibilities that it is a double mutant or has an inversion). This criterion cannot be applied unless a suitable set of three mutants is available. Only some of the stable mutants (164, 184, 221, 196, 782, 638, 832, 895, 951) have as yet been shown to satisfy this criterion.

Stable mutations tend to occur “all over the map.” However, as in the case of reverting mutants, certain localities show a strikingly high recurrence tendency, as illustrated by r102, et al., and by r145, et al.

**DISCUSSION**

*Relation of Genetic Length to Molecular Length.*

We would like to relate the genetic map, an abstract construction representing the results of recombination experiments, to a material structure. The most promising candidate in a T4 particle is its DNA component, which appears to carry the hereditary information (6). DNA also has a linear geometry (20). The problem, then, is to derive a relation between genetic map distance (a probability measurement) and molecular distance. In order to have a unit of molecular distance which is invariant to changes in molecular configuration, the interval between two points along the (paired) DNA structure will be expressed in nucleotide (pair) units, which are more meaningful for our purposes than, say, Ångstrom units.

Unfortunately, present information is inadequate to permit a very accurate calculation to be made of map distance in terms of nucleotide units. First, it is not known whether the probability of recombination is constant (per unit of molecular length) along the entire genetic structure. Second, there is the question of what portion of the total DNA of a T4 particle constitutes hereditary material (for a discussion,
of this problem, see the paper by Delbrück and Stent in this volume). The result of Levinthal's elegant experiment (11) suggests a value of 40 per cent. Since the total DNA content of a T4 particle is $4 \times 10^5$ nucleotides (8), it would seem that the hereditary information of T4 is carried in $1.6 \times 10^5$ nucleotides. We do not know, however, whether the information exists in one or in many copies. If there is just one copy, and if it has the paired structure of the model of Watson and Crick (20), the total length of hereditary material should be $8 \times 10^4$ nucleotide pairs.

There are difficulties on the genetic side as well. The total length of the genetic map is not well established. The determination of this length requires a number of genetic markers sufficient to define the ends of the map. It also requires a favorable distribution of markers in order that the intervals between them can be summated; if the distance between two markers is sufficiently large, the frequency of recombination between them approaches that for unlinked markers and therefore loses its value as a measure of the linkage distance. Unfortunately, the linkage data presently available for T4 leave much to be desired. The experiments of Streisinger (18) indicate that the map of T4 consists of a single linkage group. Adding up the intervals between markers (corrected for successive rounds of mating according to the theory of Visconti and Delbrück, 19) leads to a total value of the order of 200 per cent recombination units. This estimate is very rough, since the number of available markers upon which it is based is small.

A further difficulty arises from the fact that the map distances measured in standard crosses are not quite additive: a large distance tends to be less than the sum of its component smaller distances. For distances of the order of 10 per cent recombination units and more, the deviations from additivity, referred to as "negative interference," can be accounted for by the Visconti-Delbrück considerations. However, a "negative interference" effect, not accountable for by their theory, persists, and apparently gets worse, at very small distances (4). This presents a serious obstacle for our purposes, since we are interested in knowing what fraction of the total map is represented by a small distance. According to preliminary data on this point, summation of the smallest available distances between rII mutants yields a total length for the rII region which is several fold greater than that found for crosses involving distant rII markers. If the total T4 map length could be obtained by a similar summation of small distances, the indi-
cations are that it might be of the order of 800 per cent recombination units in length.

Thus, there are plenty of uncertainties involved in relating the genetic map quantitatively to the DNA structure. The best we can do at present is to make a rough estimate based upon the following assumptions: (1) the genetic information of T4 is carried in one copy consisting of a DNA thread 80,000 nucleotide pairs long; (2) the genetic map has a total length of about 800 per cent recombination units; (3) the probability of recombination per unit molecular length is uniform. According to these assumptions, the ratio of recombination probability (at small distances) to molecular distance would be 800 per cent recombination divided by 80,000 nucleotide pairs, or 0.01 per cent recombination per nucleotide pair. That is to say, if two mutants, having mutations one nucleotide pair apart, are crossed, the proportion of recombinants in the progeny should be 0.01 per cent. This estimate is greater, by a factor of ten, than one made a year ago, in which it was assumed that all the DNA was genetic material and that the effect of negative interference was negligible. It should become possible to improve this calculation as more information becomes available.

The estimate indicates that the level of genetic fine structure which has been reached in these experiments is not far removed from that of the individual nucleotides. Furthermore, the estimate is useful in that it defines an "absolute zero" for recombination probabilities: if a cross between two (single) T4 mutants does not give at least 0.01 per cent recombination, the locations of the two mutations probably are not separated by even one nucleotide pair.

Molecular Sizes of the Genetic Units.

Recon: The smallest non-zero recombination value so far observed among the rII mutants of T4 is around 0.02 per cent recombination. If the estimate of 0.01 per cent recombination per nucleotide pair should prove to be correct, the size of the recon would be limited to no more than two nucleotide pairs.

Muton: Evidently, among the stable mutants, mutations may involve varied lengths of the map. The muton is defined as the smallest element, alteration of which can be effective in causing a mutation. In the case of reverting mutants, it has not been possible, so far, to demonstrate any appreciable mutation size greater than around 0.05 per cent.
recombination. This would indicate that alteration of very few nucleo-
tides (no more than five, according to the present estimate) is capable
of causing a visible mutation.

_Cistron:_ A cistron turns out to be a very sophisticated structure. The
function to which it corresponds can be impaired by mutation at many
different locations. In the study of 241 r mutants, 33 species were
found to be located in the A cistron, of which 6 were in the r164 subclass.
In extending the survey to 923 r mutants, the number of known species in
the r164 subclass was doubled. Consequently, it may be expected
that about 60 A cistron species will be found—when the analysis of the
923 mutants is completed. Since many species are represented by only
one occurrence, implying that many more are yet to be found, it seems
safe to conclude that in the A cistron alone there are over a hundred
“sensitive” points, i.e., locations at which a mutational event leads to
an observable phenotypic effect. Just as in the case of the entire genetic
map of an organism, the portrait of a cistron is weighted by considera-
tions of which alterations are effectual. It should be fascinating to try
to translate the “topography” within a cistron into that of a physi-
ologically active structure, such as a polypeptide chain folded to form
an enzyme.

REFERENCES

   41, 359 (1955).
4. Doermann, A. H., and collaborators, pers. commun.
17. Stent, G. S., pers. commun.
   18, 123 (1953).