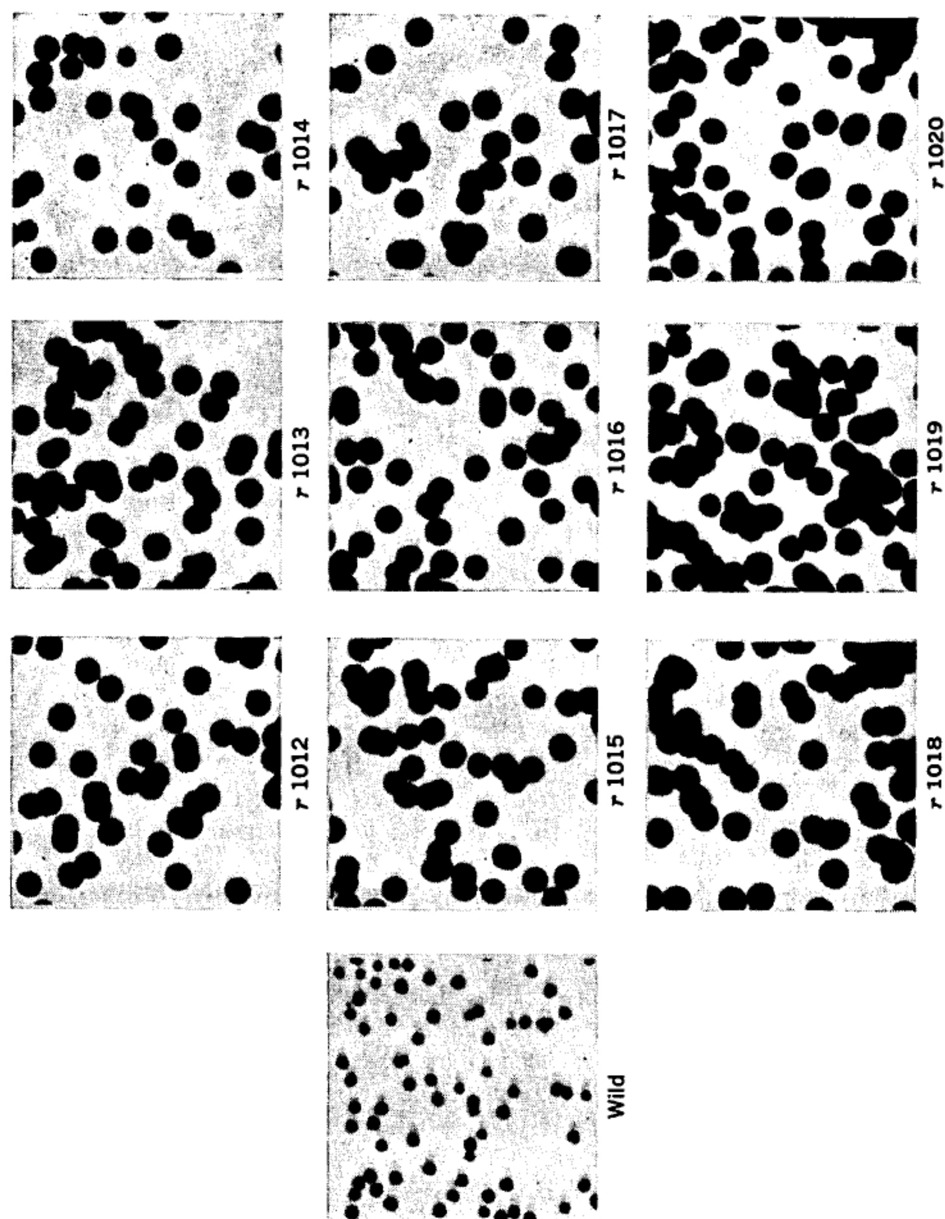
chapter 4

THE SUBSTRUCTURE OF GENES

As we pointed out in Chapter 1, most evolutionists feel that all levels of evolution can be interpreted in terms of a single basic process, the natural selection of organisms whose phenotypes have been modified in some favorable way by gene mutation. During the earliest stages of evolutionary development, the sudden appearance of new genes must have been a frequent and important occurrence. However, it is, perhaps, not too heretic to suggest that the major proportion of evolution has been the result of a continual process of modification, and integration into new systems of organization, of genetic potentialities already present in our extremely distant ancestors. (We shall consider the evidence for the antiquity of some of our own genes in a later chapter.) Although evolution of the sort undergone by the *Gryphaea* (page 7) is generally referred to as microevolution, such a process is many orders of magnitude more complex than the process that can be demonstrated in favorable test objects like bacteriophages. With bacteriophage we may detect "ultramicroevolutionary" changes caused by even extremely infrequent single-gene mutations. The morphology and general physiology of these most elementary "organisms" are discussed in the following pages, together with a consideration of the progress of "fine-

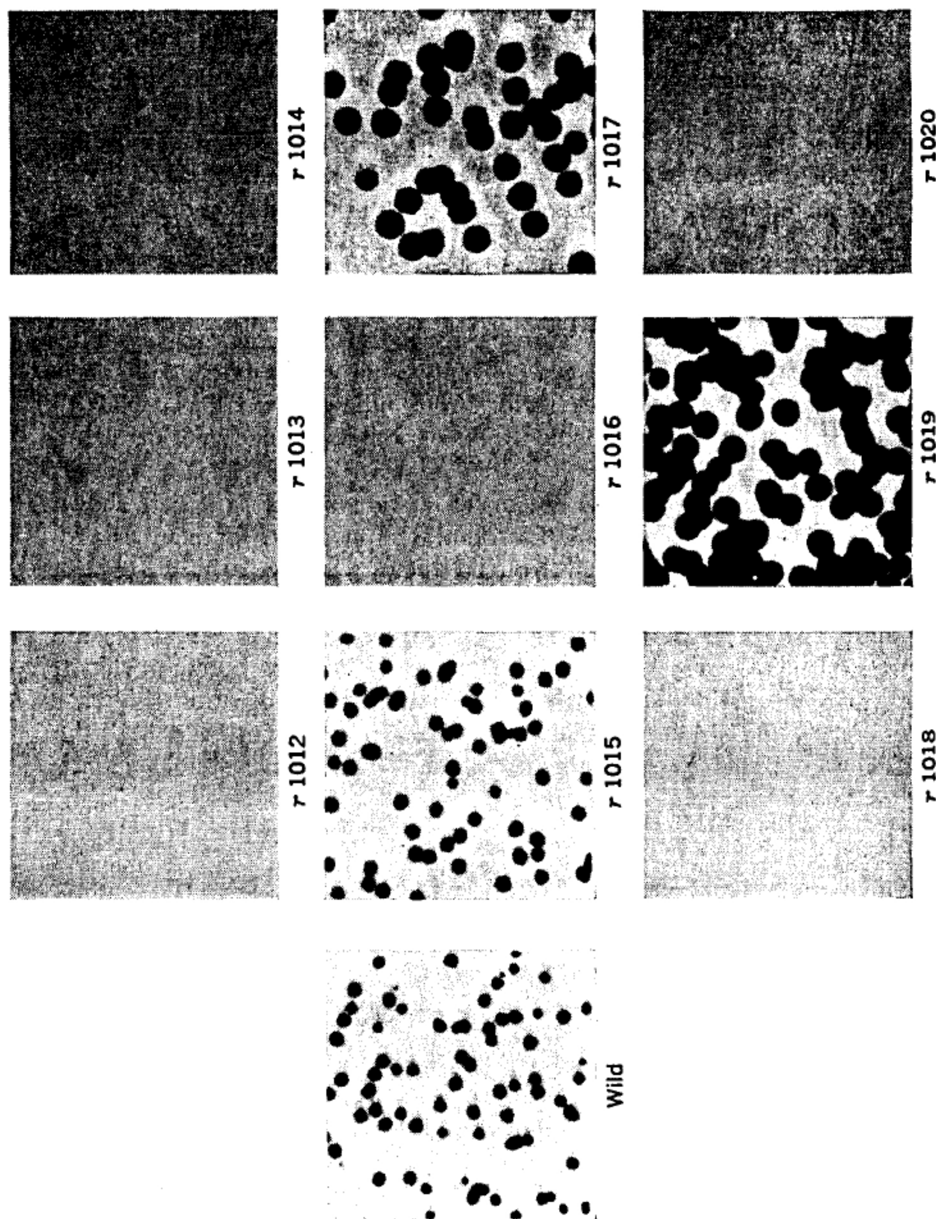


structure genetics" which has been so stimulated by work on bacteriophages and on their bacterial cell hosts.

Bacteriophages were discovered by D'Herelle in 1917. He demonstrated that these were extremely small particles, invisible by ordinary microscopy, which multiply parasitically within their host and escape in a burst when the bacterial cell wall ruptures. Later studies by a number of investigators, among whom Delbrück, Demerec, Luria, and Burnet deserve particular mention, showed that the bacteriophage bacterial cell microcosm exhibited many elegant examples of evolutionary adaptation.

A particularly cogent example is presented by the case of the *r* mutants of bacteriophage T4. Each wild-type T4 particle produces a small, fuzzy plaque (Figure 34), when grown on agar containing *E. coli* of strain B. This plaque type is one of the phenotypic characters of this particular phage. From time to time these wild T4 undergo a mutation which introduces a new phenotypic character. Thus we may isolate, from individual plaques of wild T4, mutants that produce quite a different sort of plaque, nine of which are shown in Figure 34. In spite of the identical appearance of plaques produced by all these *r* mutants on *E. coli* B, we may easily distinguish several different sorts of *r* mutants by employing a new bacterial host. Thus, as shown in Figure 35, when the nine *r* mutants are plated on *E. coli* K rather than B, three subgroups become evident; two of the mutants still form *r*-type plaques, one forms wild-type plaques, and six do not multiply at all. Whereas on strain B all the mutants produce a visible effect, on K some are visible (the *r* plaques), some are "innocuous" (wild type), and some are lethal. *Escherichia coli* B, therefore, supplies something which is not supplied by K, at least for the latter *r* mutants. In a sense, K may be thought of as an ecological niche which is not attainable by these "species" of T4 bacteriophages until a mutation has occurred which makes the "something" in B no longer of importance.

Figure 34. Plaques formed on *E. coli* B by wild-type bacteriophage T4 (left) and by nine independently arising *r* mutants. As described in the text, wild-Type T4 particles infect and multiply within *E. coli* cells of strain B to produce the small plaques shown. The progeny infect and lyse neighboring bacterial cells to produce plaques, which are actually areas in the *E. coli* B–agar mixture that have become translucent owing to lysis of the bacterial suspension; *r* mutants produce the large plaques shown in the nine photographs at the right of the figure. The plaques may easily be distinguished from those produced by the wild-type bacteriophage particles by their characteristic plaque morphology. From S. Benzer, *The Chemical Basis of Heredity*, Johns Hopkins Press, 1957.

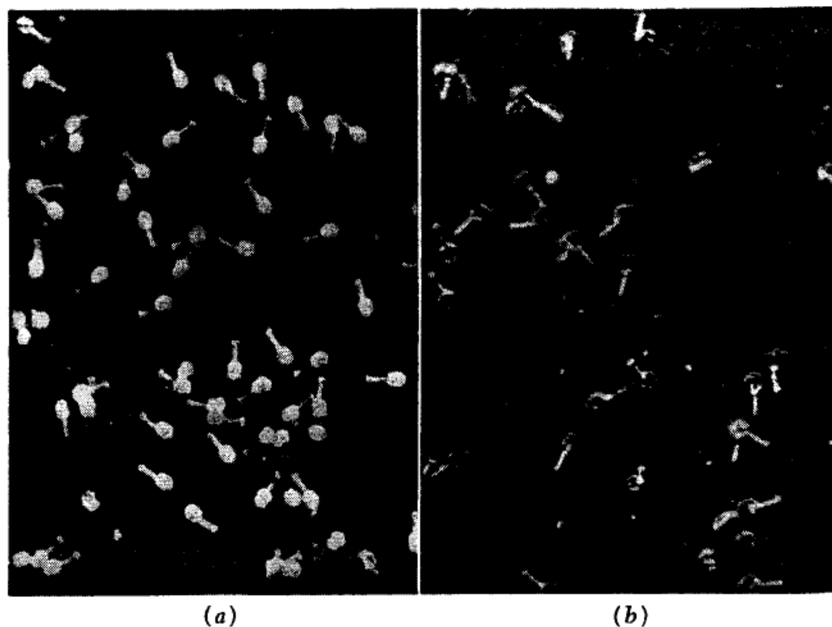


Another point of evolutionary interest can be illustrated by these observations. The *r* mutants which *behave* like wild-type phage on *E. coli* K are *not* wild type as is shown by their behavior on B. In terms of evolutionary adaptation, these particles are very much analogous to the reptiles which we discussed in Chapter 1. The reader will recall that changes occurred in the structure of the jaw of reptiles which were not of apparent adaptive value until millions of years later when certain of the bones of the reptilian jaw became important units in the assembly of the characteristic mammalian ear. These changes (call them "aristogenic" if you like) could not be taken advantage of until the proper ecological situations made their presence of great value to the budding mammal. The "false" wild-type phage mutants, in a similar manner, are the possessors of mutations which are, at least superficially, neither "detrimental" nor "advantageous" in a world of *E. coli* K, but which facilitate a marked change in phenotype in a world of *E. coli* B. These *r* mutations might be preserved for a long time, to become of critical importance when environmental (host cell) conditions accidentally changed from K to B. More likely, the abnormal gene would eventually back-mutate to the normal allelic form and be forgotten as an evolutionary ripple.

The Chemistry and Enzymology of Bacteriophages

Although a number of different sorts of bacteriophages have been studied with respect to their chemical composition, morphology, and genetic constitution, we shall restrict our discussion to the so-called T-even (and particularly T2) phages since these have been most commonly chosen for study by virologists and are by far the best understood. These viruses, which infect and kill cells of *E. coli* B, may be divided into major groups on the basis of their ability or inability to invade *E. coli* B cells of certain types. Thus *E. coli* B6 will support growth of T2 and T4 viruses, B4 will support T2 and T6 but not T4, and so on. They may be further distinguished on the basis of their plaque morphology, the rate at which such plaques

Figure 35. When the nine *r* mutants, shown in Figure 34, are plated on *E. coli* K rather than on strain B, three subgroups become distinguishable. Two mutants continue to form plaques similar to those formed on *E. coli* B, one forms wild-type plaques, and six do not multiply at all. From S. Benzer, *The Chemical Basis of Heredity*, Johns Hopkins Press, 1957.



(*a*) (*b*)

Figure 36. Electron photomicrographs of (*a*) bacteriophage T2 and of (*b*) "ghosts" prepared by osmotic shock. These photographs were obtained through the kindness of Dr. Roger Herriott. From R. M. Herriott and J. L. Barlow. *J. Gen. Physiol.*, **40**, 809 (1957).

become visible under standard conditions of agar plate culture, and their specific serological characteristics.

The virus particles can be visualized in the electron microscope, and the morphology of T2 coliphage, which is more or less typical for all the T phages, is shown in Figure 36. The particles are made up of a rounded hexagonal head and a tail. The tip of the tail is slightly expanded into a knob-like structure. This can be deduced from the outlines of some of the shadows in the electron micrograph.

Intact phage particles consist of approximately 60 per cent protein and 40 per cent DNA, together with traces of lipid material. The nucleic acid component is entirely of the DNA variety and, as discussed earlier, contains the unusual pyrimidine 5-hydroxymethylcytosine partly in glucosidic combination with glucose. It was believed for some time that the DNA and protein were tightly conjugated in nucleoprotein form. It can be shown, however, that the DNA is actually physically enclosed within a protein shell, the so-called bacteriophage "ghost," and can be released from the interior of the particle by suitable treatment, whereupon it becomes digestible

by deoxyribonuclease. In Figure 36 is shown a collection of such ghosts which have been prepared by submitting virus particles to "osmotic shock," that is, rapid dispersion into distilled water after prior equilibration with strong salt solution. The ghosts may be prepared in a form essentially devoid of polynucleotide by proper washing. The separability of protein and DNA is elegantly demonstrated by the fact that, when phages are doubly labeled with P^{32} and S^{35}, the P^{32} is found entirely in the DNA released by "shock" and the S^{35} in the material of the ghosts.

Doubly labeled phage have also been used by Hershey and Chase to study the fate of the various components of phage during infection and rupture of bacterial cells. These investigators allowed the labeled particles to infect *E. coli* cells and, after a brief period, exposed the mixture to rapid agitation in a Waring Blendor. The P^{32}-labeled DNA was found entirely within the infected bacterial cells, whereas a large part of the protein was sheared off. The small amount of S^{35}-labeled protein which adhered to the bacterial cells after the agitation procedure did not contribute to the structure of the progeny phage produced when the infected cells were allowed to incubate and rupture spontaneously. Since the infected cells which were exposed to the blender produced a normal yield of progeny, it may be concluded that DNA alone is capable of directing the synthesis of new particles, genotypically identical with the parent phage, and that the protein shell around the DNA makes no contribution to the process of genetic information transfer. Although lacking any genetic role, the protein ghost is responsible for many features of bacteriophage infection in addition to its function as an enclosure for DNA. Ghosts can attach to compatible host cells or cell walls, that is, they bear the host specificity of the intact virus (see Chapter 8). Cells which have been "infected" with ghosts are killed and subsequently lyse, although no progeny phage are produced. Ghosts also have the mysterious ability to cause inhibition of RNA synthesis and protein synthesis within the host cell.

It has been known for some time that small molecular fragments of the bacterial cell wall are released shortly after infection by an enzyme present in both intact viruses and ghosts. This material has been identified as a mucopeptide derivative having the interesting structure shown in Figure 37. Now, although the nature of the natural substrate for the enzyme, lysozyme, is not known with precision, a number of observations suggest that the function of lysozyme is to attack mucopolysaccharide or mucoprotein compounds. It will degrade chitin, for example, which is a long-chained poly-N-acetyl glucosamine, although it will not attack this substance after de-

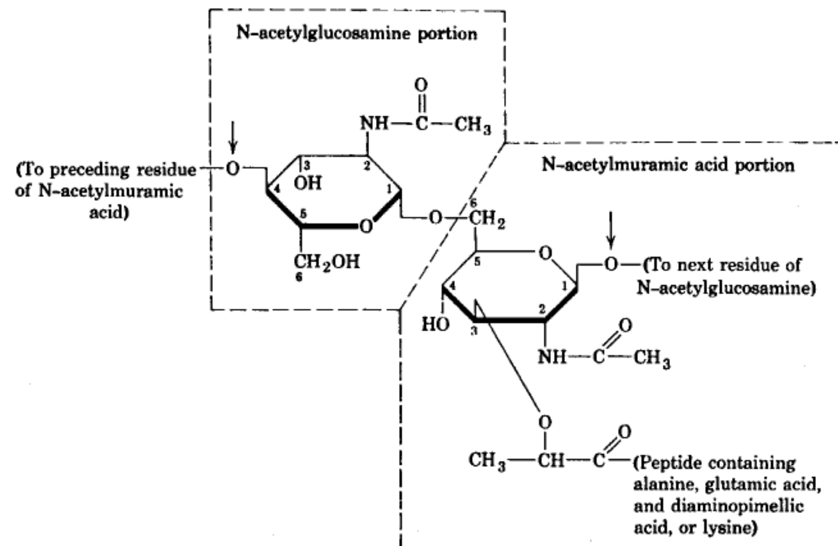


Figure 37. A proposed structure for the fragment which is produced from the cell walls of certain Gram-positive bacteria by digestion with lysozyme. The arrows indicate the probable site of attack by the enzyme. Redrawn from W. Brumfitt, A. C. Wardlaw, and J. T. Park, *Nature*, **181**, 1783 (1958).

acetylation. The chemical nature of the fragment split off from the cell wall is sufficiently reminiscent of chitin to suggest that the viral enzyme responsible for bacterial penetration is, indeed, of the lysozyme variety. Dreyer and Koch[1] have recently compared the action of crystalline egg white lysozyme and virus ghosts on bacterial cell walls and have found that a very similar cleavage product is released by both catalytic agents. Further, the enzymatic activity in the ghosts distributes in the same way as the egg white enzyme during purification with the specific adsorbant, Bentonite, and can be chromatographed on the ion exchange resin, XE-64, to give a similar elution pattern. Chemical comparison of the two lysozymes has not yet been made, so we cannot state categorically that the virus enzyme has structural features in common with the egg white enzyme. The chromatographic properties and similar molecular size of the phage enzyme (slowly dialyzable through cellophane membranes) makes this similarity rather likely, however, and sequential analysis should give very interesting results. It will be of obvious importance, in connection with the "age" of genes and with the evolutionary process in general, to determine whether or not an enzyme designed

to digest cell wall material is an essential component even in "semi-living" biological systems like the coliphages.

Morphology of T Phages

Some idea of the number of morphological components making up the bacteriophage particle may be obtained by electron microscopic

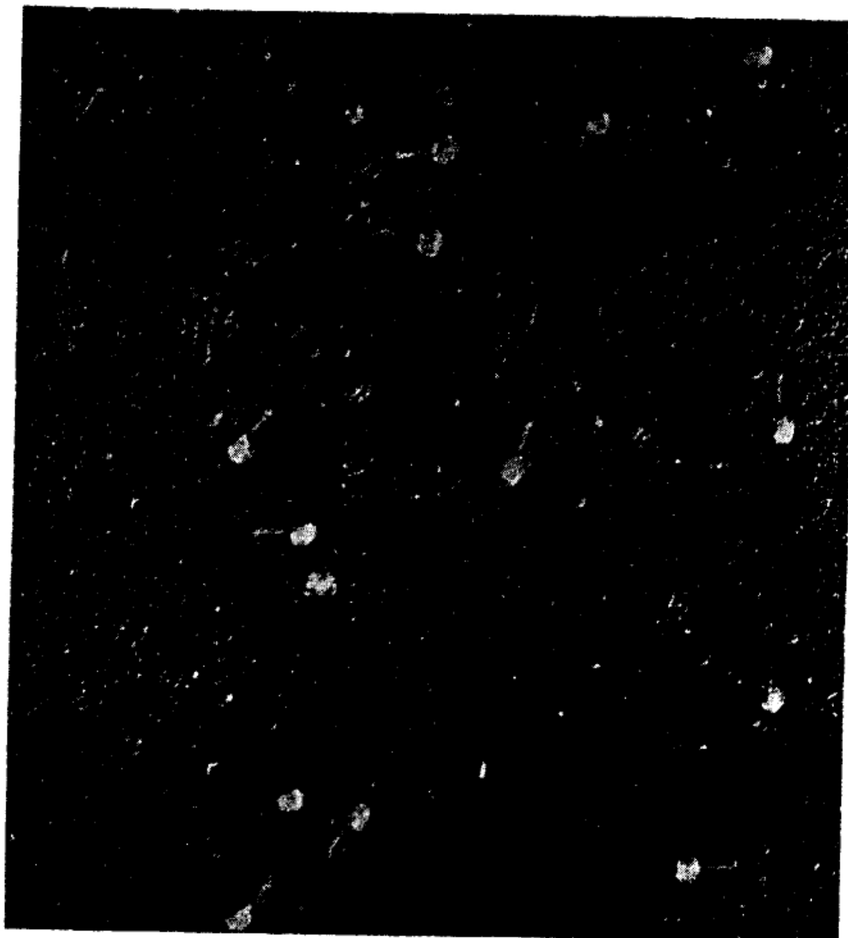


Figure 38. An electron photomicrograph of a crude lysate of bacteriophage T2 prepared by treatment of infected *E. coli* cells with chloroform. The photograph shows intact bacteriophage particles and a collection of "heads," rods, and filaments. This photograph was obtained through the kindness of Dr. E. Kellenberger of the University of Geneva.

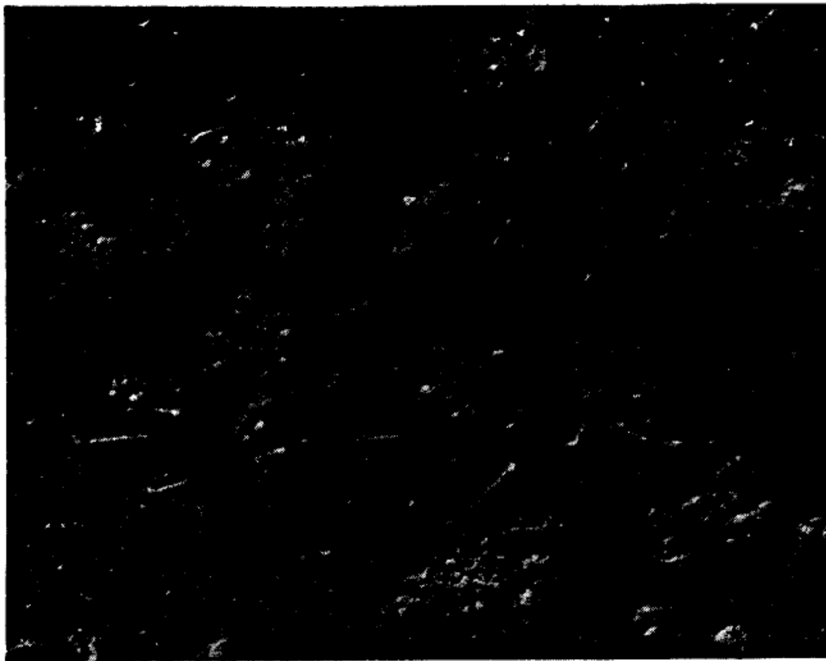


Figure 39. An electron photomicrograph of partially purified rods from the lysate shown in Figure 38. Many of these rods may be seen to terminate in a brush-like arrangement. This photograph was obtained through the kindness of Dr. E. Kellenberger of the University of Geneva.

examination of a phage which has been subjected to various degradative treatments (peroxide, cadmium cyanide, N-ethylmaleimide) which split off from the body of the phage certain parts of the tail structure. We may also examine the contents of artificially lysed bacterial cells which are still in the process of synthesizing phage. The electron micrograph shown in Figure 38, for example, was made by Kellenberger and Sechaud on the crude lysate of T2 prepared by treatment of infected *E. coli* cells with chloroform. The photograph shows, in addition to intact phages, a collection of empty heads, rods, and filaments, the latter very likely being strands of DNA which have not yet been wrapped in their protein envelope. These phage components may be concentrated as shown in Figure 39. The rods, which must constitute a large part of the tail structure, are seen in many cases to terminate in a wire-brush-like arrangement. In some electron micrographs we can distinguish rod-like structures which are somewhat smaller in diameter than the intact viral tail

and which have been interpreted as a sort of plug in the tail which confines the DNA to the head of the phage. Two other phage components, not visualized by the electron microscope, are the penetration enzyme (lysozyme?) and the protein material responsible for host range specificity. The latter may be associated with one of the morphologically distinguishable tail components.

The drawing in Figure 40 summarizes, in a schematic way, the subunits of structure which have been detected microscopically. A slightly different reconstruction has been proposed by Kozloff, Lute, and Henderson.[2] This diagram helps to point out one of the major puzzles of bacteriophage biosynthesis, namely, the problem of how the DNA strand or strands are gotten into the preformed heads, if these are indeed synthesized before DNA is. We might visualize that either an enzyme system, rich in genetic information, is able to

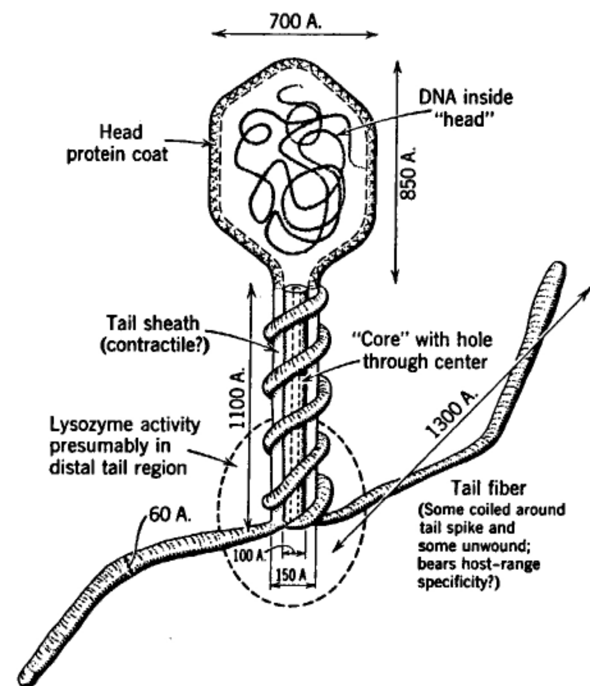


Figure 40. A schematic reconstruction of the morphology of a bacteriophage particle. The dimensions shown are approximate and are averaged from several sources (see, for example, the excellent work of R. C. Williams and D. Fraser, *Virology*, **2**, 289 (1956).

carry out DNA synthesis within the head (perhaps in cooperation with the protein of the head itself) or that DNA can thread itself into the head after synthesis on the outside. The possibility must not be overlooked, however, that phage fragments really represent abortive attempts at synthesis and that the protein shell and tail of completed phage particles are assembled around a preformed mass of DNA. The latter possibility is supported by a certain amount of experimental evidence and certainly makes the best sense, metabolically and mechanically. For example, Hershey has shown with isotopic tracer methods that the DNA of phage is synthesized before the protein precursors and that the small amount of protein synthesis which occurs before DNA synthesis may form some essential enzymes (perhaps for the synthesis of the unique pyrimidine, or for the formation of a ribonucleic acid "template") which are not otherwise present in the bacterial host.

The Molecular Heterogeneity of Coliphage DNA

The total phosphorus content of a single T2-phage particle is about 2×10^{-17} grams. If all this phosphorus resides in a single DNA molecule, we may calculate the total molecular weight of this DNA to be approximately 110 million. Should the Watson-Crick type of structure apply in phage, we are faced with the problem of how each enormously long polynucleotide strand becomes replicated during phage multiplication and how the two intertwined strands in the double helix unwind during, or in preparation for, this event.

This problem has been somewhat simplified by the observations of Levinthal and Thomas.[3] Their experiments indicate that the DNA of phage may not be in a single large unit but may be made up of a number of smaller parts, the largest one of which makes up about 40 per cent of the DNA. The technique employed by them is a particularly elegant example of simplicity combined with sensitivity and precision. Levinthal and Thomas embedded bacteriophage particles, previously labeled with P^{32}, in an electron-sensitive emulsion in which the passage of fast electrons, such as are emitted by P^{32} during radioactive decay, causes the formation of a track of silver grains. A single phage particle, acting as a "point source," can thus cause the formation of a "star" in the emulsion, each arm of which is produced by the decay of a single atom of phosphorus within the DNA of the particle (Figure 41). The sensitivity of the detecting method is such that the radioactivity of a phage particle, emitting

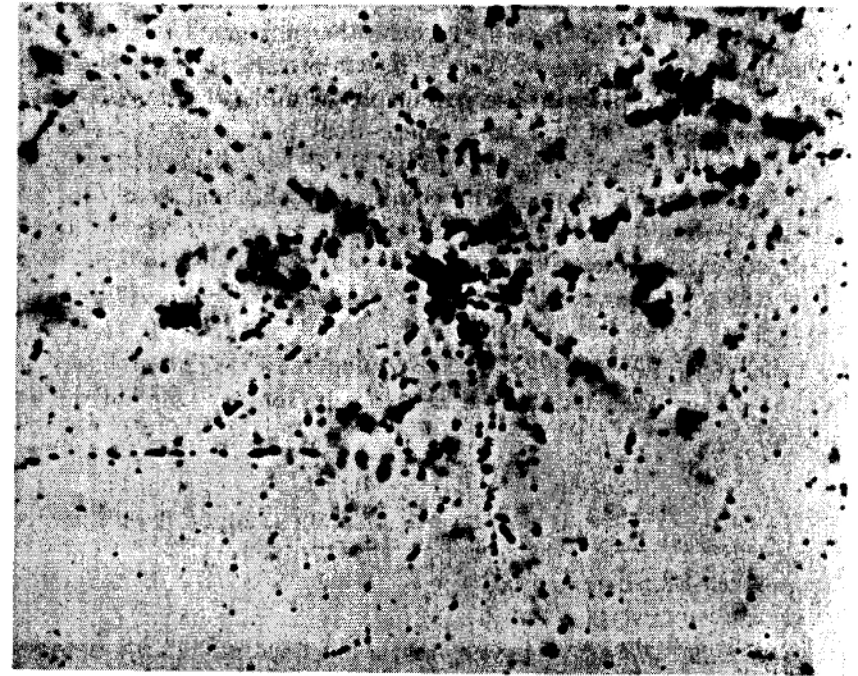


Figure 41. Photomicrograph of a "star" produced by radioactive decay of labeled phosphorus within the DNA of a phage particle embedded in a photographic emulsion. This photograph was obtained through the kindness of Professor Cyrus Levinthal, Massachusetts Institute of Technology.

only about fifteen disintegrations per month, can be estimated. In uniformly labeled electron sources (e.g. the DNA of a single phage particle or fragments thereof) the number of tracks per star gives a measure of the molecular size of the emitting source, relative to the intact phage particle.

The number of tracks per star was determined for intact phage and for first- and second-generation progeny before and after osmotic shock. The results for the various fractions are shown in Table 2, expressed in terms of the number of P^{32} atoms in the star-forming particle, normalized to 100 for the original uniformly labeled phage. (Put in another way, if each star formed by intact parent phage particles has 100 tracks, each star produced by particles in suspensions of "shocked" phage, having half the content of DNA, will show 50 tracks.) The results indicate that (*a*) there is a single large piece of DNA comprising about 40 per cent of the total DNA (i.e.,

about 45 million in molecular weight), (*b*) after one generation in nonradioactive bacteria there are, among the progeny, a few particles in which this large piece of DNA has approximately half the P^{32} atoms of the large piece in the parent phage, and (*c*) after a *second* generation the size of this large piece does not change (the same number of tracks per star is observed). Since some 100 to 200 progeny are formed from each parental phage, the *number* of labeled particles in second generation shockates was too few to permit accurate detection and track counting.

TABLE 2

The Number of Arms per "Star" for Various Suspensions of Intact or Ruptured Phage Particles, Normalized to 100 for the Original, Intact Particles[3]

	Intact Phage	After Osmotic Shock
Uniformly labeled parental phage	100	40 ± 4
First-generation progeny	24 ± 3	23 ± 3
Second-generation progeny	26 ± 3	Too dilute for study

The simplest and most appealing interpretation of these data is that the large piece represents the "chromosome" of the phage and that the two halves of its double helical structure serve as templates for the synthesis of new DNA in the progeny. This wishful picture is completely analogous to the scheme growing out of the Meselson and Stahl experiments on *E. coli* DNA. There is, however, no compelling evidence to indicate that the DNA of the T phages is in the form of a double helix, although this is generally assumed in most discussions of the subject for the obvious reason that it makes discussion possible.

The Levinthal-Thomas experiments suggest a "conservative" mechanism of replication during which the structure of the large piece of DNA is not degraded or involved in exchange with external sources of nucleotides.

It would have been pleasant, in connection with this discussion of these experiments, to have been able to present some evidence for the chromosome-like nature of the "big piece." Attempts have been made to show that, during the production of progeny phage, genetic markers, chosen at random along the genetic strand, always accompany the large star-forming fragment. The early experiments along

this line looked quite encouraging but, of late, the picture has become rather murky and, after consultation with some of the investigators in the field, it appears that the subject is best avoided at the present time. There are, for example, several lines of evidence which suggest rather strongly that the "40 per cent piece" is *not* a single molecule but that various mild treatments can cause this aggregate to dissociate into still smaller units having molecular weights in the neighborhood of 12 to 15 million. The results of the original "star" experiments seem to be unequivocal and, indeed, chromatographic separation on special columns of ion exchange resin have also shown the presence in T2 DNA of an entity containing about 40 per cent of the total phosphorus. Nevertheless, any attempt to relate the chemical structure of phage DNA to genetic behavior must be deferred until much more data have been obtained.

The Life Cycle and Biosynthesis of T Phages

It may be useful at this point to consider the events that occur following infection of *E. coli* with T phages. Most of the metabolic consequences of infection are summarized in Figure 42, which is a slightly modified form of that given by R. Hotchkiss in his excellent review of 1954, and in Figure 43, taken from an article by L. Kozloff in the *Cold Spring Harbor Symposia* for 1953. Figure 42 gives a condensed view of the fates of various morphological components of the infecting phage and the host cell and also indicates, in a rough way, the manner in which the metabolism of the combined DNA, protein, and ribonucleic acids of the infected cell is altered. It is assumed that the RNA of the host is, in some way, involved in the biosynthesis of phage protein. This assumption is based on certain general observations on protein synthesis which we shall discuss in more detail in Chapter 10.

Kozloff's pictorial summary of the origins of phage DNA and protein (Figure 43) emphasizes that the major share of phage protein is derived from the nutrient medium. Of the newly synthesized phage DNA, it would appear that most of the phosphorus is also exogenous in origin, but that a considerable amount of host DNA is reutilized as a source of purines and pyrimidines.

The kinetics of the various metabolic processes that commence after phage infection are of particular importance in connection with the elucidation of the way in which the genetic information in phage DNA is translated into the chemical structure of progeny phage.

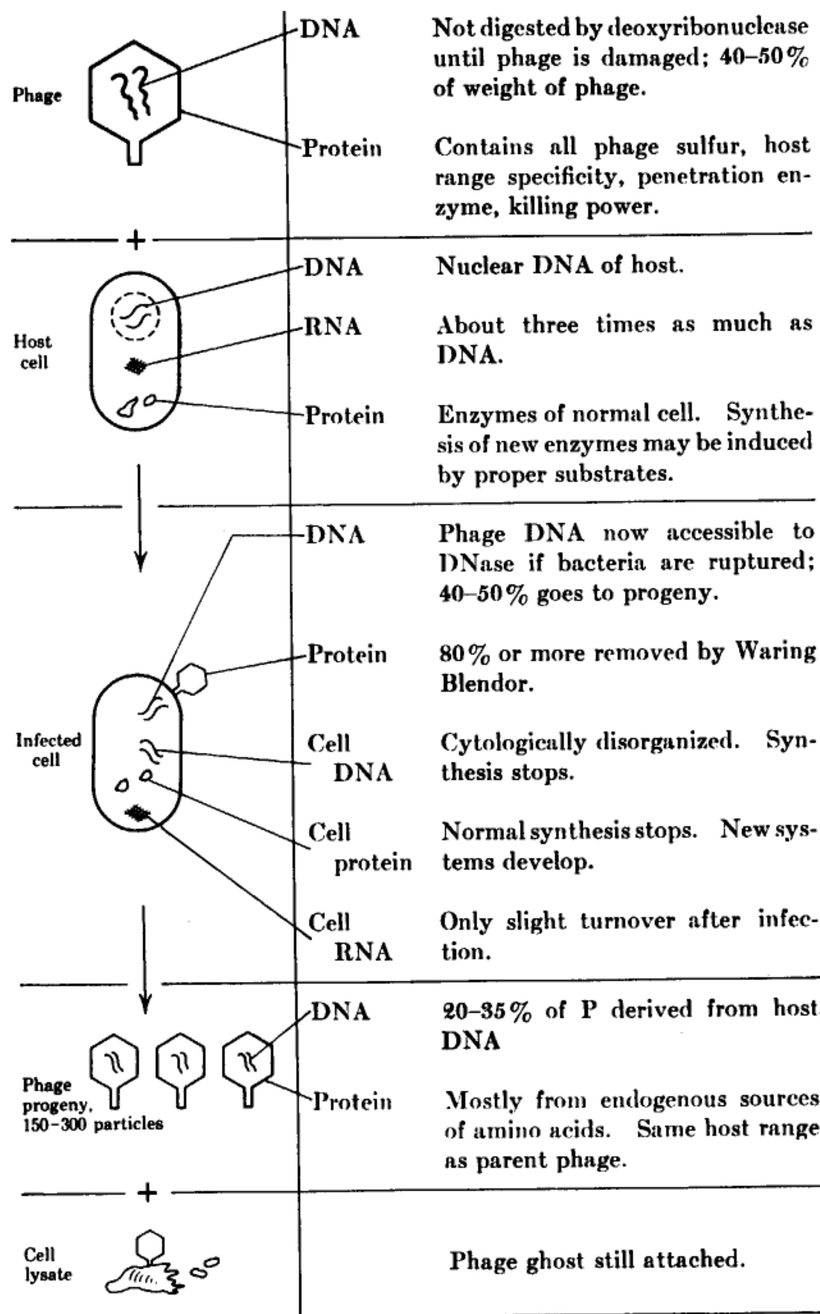


Figure 42. The course of infection of *E. coli* with bacteriophages of the T series. Redrawn after R. D. Hotchkiss, *The Nucleic Acids*, volume 2, Academic Press, 1954.

When the host cell becomes infected, its metabolic machinery appears to become completely devoted to phage synthesis. Protein synthesis goes on but produces a new set of proteins. After a short delay, DNA synthesis proceeds actively. However, if the initial protein synthesis is prevented by the addition of a suitable inhibitor such as chloramphenicol, the synthesis of DNA does not take place. On the other hand, if the inhibitor is added *after* protein synthesis has gotten under way, DNA synthesis *can* proceed. These observations suggest that an essential set of enzymes must first be formed, perhaps for the synthesis of the unique 5-hydroxymethylcytosine or its glucose conjugate, before DNA assembly is possible.

Stent and his colleagues have carried out interesting experiments[4] which have a direct bearing on the problem of the order of events during information transfer. They infected P^{32}-labeled cells with P^{32}-labeled phage, both having a sufficiently high level of isotope to cause chemical modification of a significant fraction of the DNA when the phosphorus atoms decayed to form sulfur atoms. The infected cells were incubated in a P^{32} medium so that the progeny phage were also heavily labeled. Samples of infected cells were frozen at —196°C. at various times after infection and were stored in this state of suspended animation. During this time P^{32} decay took place. The surprising result was obtained that, in those samples frozen approximately 10 minutes after infection, there was no detectable change in the ability of the infected cells to produce infective phage progeny in spite of the destruction of a considerable portion of the DNA by radioactive "suicide." The results have been interpreted in several ways, the most provocative being the hypothesis that, early in the synthesis of progeny, information in the DNA of the infecting phage particle is transferred to some substance, perhaps protein in nature, which is not susceptible to radioactive decay. Another speculation which could explain the results would require that the structure of DNA be such that, in spite of occasional breaks in the sugar-phosphate backbone of the molecule caused by P^{32} decay, hydrogen bonds hold the macromolecule intact and in a configuration adequate for purposes of replication (Figure 44).

The possible conflicts in logic that might arise from a comparison of the P^{32}-decay data with the experiments of Levinthal and Thomas or with the mass of evidence supporting the central role of a specific

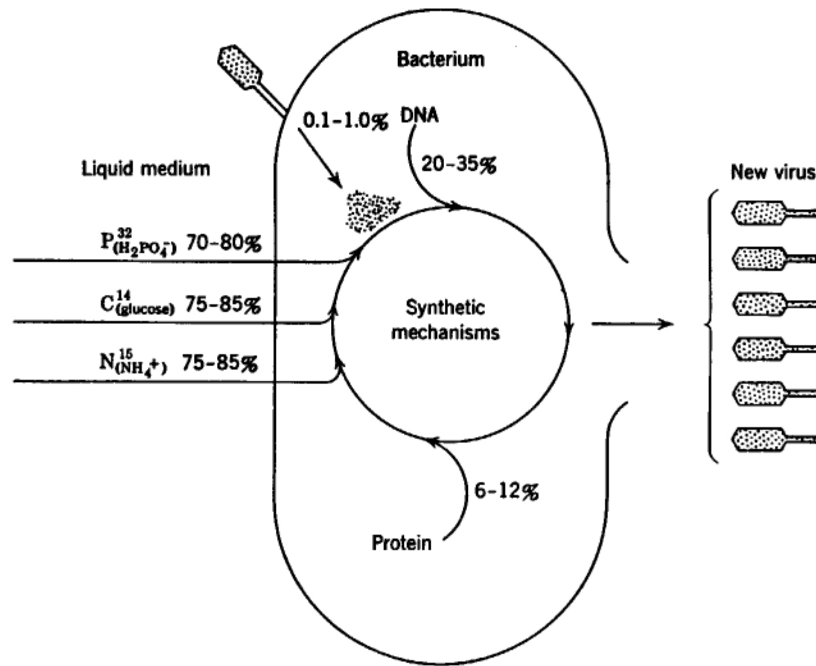


Figure 43. Schematic representation of the origin of the phosphorus, nitrogen, and carbon of bacteriophages T2, T4, and T6. Percentages show origin of material found in viral offspring. Redrawn from L. N. Kozloff, *Cold Spring Harbor Symposia Quan. Biol.*, **18**, 209 (1953).

DNA structure in the determination of heredity are not, to my knowledge near resolution. Fortunately for the following discussion of genetics in phage, we do not need an answer to the fascinating problem of phage replication. Although the behavior of phage during "mating" may only superficially resemble the process as it occurs in more respectable organisms, the phenomena of recombination and segregation, and of linkage, are exhibited in qualitatively the same way.

Genetic Mapping in Bacteriophage

In most organisms it is not possible to construct a single gene map, but instead we find that the various genes fall into several linkage groups (see Chapter 2). However, in bacteriophage T4 and T2, for which quite a few "genes" have been mapped, only a single

linkage group seems to be present, and these phages behave genetically as an organism with a single haploid chromosome. The sort of gene map obtained depends, naturally, on the strain of bacteria chosen as host. As we have already shown, the plaque characteristics of mutants that are *r* type on *E. coli* B may be quite different when the mutants are plated in *E. coli* K. The map obtained with a particular host cell, therefore, includes only the loci that cause a detectable change in phenotype in this chosen environment.

The construction of a genetic map for bacteriophage involves more or less the same manipulations that have been used for such organisms as *Drosophila* or peas. A wild-type strain is chosen arbi-

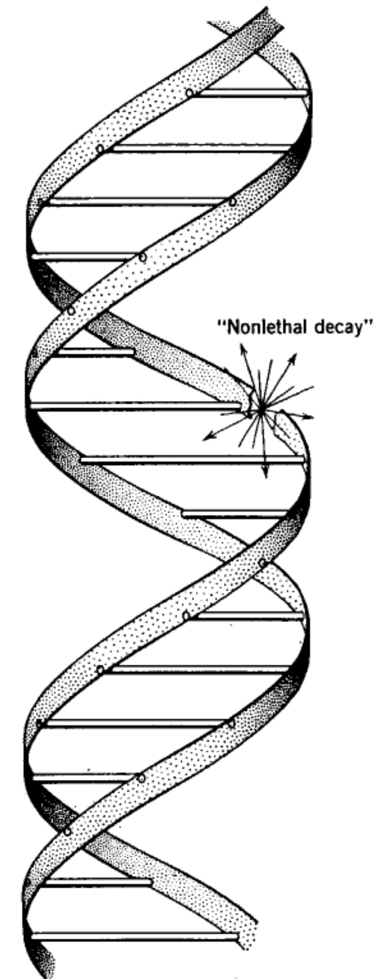


Figure 44. A schematic representation of how breaks in the chains of the DNA dougle helix, caused by radioactive decay of P^{32}, might occur and still permit the maintenance of a relatively intact structure through the forces of hydrogen bonding between complementary pairs of purine and pyrimidine bases.

trarily. Mutants are then chosen on the basis of some easily recognized phenotypic character such as plaque morphology on a particular strain of *E. coli*. The relative position of two mutant loci on the "chromosome" of the phage may then be established by "mating" the mutants and observing the proportion of the progeny that differ from the two parental mutants. True mating does not, of course, occur with phage. Instead, bacterial cells are mixedly infected with the two phages in question, and recombination is allowed to take place within the cell by some obscure process that has the superficial characteristics of crossing over in higher organisms. As we discussed earlier, conventional crossing over results in the production of the two possible recombinants, the wild type and the double recombinant containing both mutant loci. It has been shown that, in phage, such reciprocal crossover does not take place, but that in any single recombination event only one or the other recombinant is formed [perhaps because of a "copy-choice" replication (Figure 45)]. However, since a single phage particle gives rise to several hundred progeny, and since there appears to be about the same probability for the formation of the two kinds of recombinants, the usual statistical treatment may be applied.

Seymour Benzer[4] has, over the past few years, been in the process of mapping a very large number of *r* mutants of bacteriophage T4. These studies represent the first real attempt to determine the ultimate limits of recombination and involve "running the map into the ground," to use an expression attributed by Benzer to Max Delbrück. The detection and mapping of mutations in bacteriophages are facilitated by several factors, including the sensitivity of the technique for detecting mutants and the occurrence of certain mutations which involve the "deletion" of a considerable portion of the map. The latter factor, as we shall see, has permitted Benzer to group many point mutations into "families," thus greatly decreasing the number of crosses required.

In any population of T4 there occurs from time to time a spontaneous mutation which leads to the production of an *r*-type plaque on *E. coli* B agar plates. Since an astronomical number of phage particles may be plated on a single Petri dish surface, the presence of a single mutant in as many as 10^9 particles is easily observed by its *r* phenotype. When a suitable number of such mutants has been accumulated, they may be further subdivided into three subgroups as mentioned earlier, by plating on *E. coli* K. By this trick (see page 69) we may distinguish those that still show *r* morphology on K, those that show the wild-type phenotype, and those that fail to grow

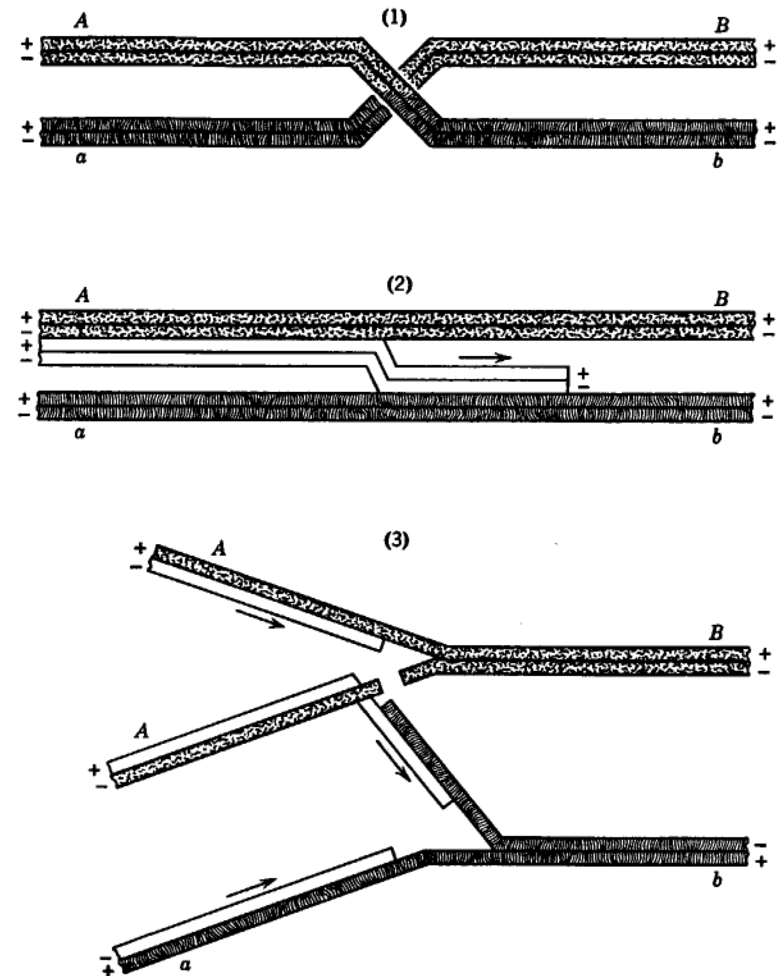


Figure 45. Three possible mechanisms of genetic recombination: *A* and *B* represent two genetic loci and *a* and *b* their alleles. (1) Fragmenting crossing over; the two parental duplexes synapse and break between the loci in question, and heterologous pieces rejoin. (2) Nonfragmenting copy choice. (3) Fragmenting copy choice; parent duplexes unwind and semiconservative replication occurs along the single strands. A switchover occurs between loci *A* and *B* as the parent chains break, and the daughter chains continue to grow complementary to a single chain of the second duplex. Redrawn from M. Delbrück and G. Stent *The Chemical Basis of Heredity*, Johns Hopkins Press, 1957.

Figure 46. The order of the three subunits of the *r* region in the genetic material of bacteriophage T4. Since *r*II and *r*III are farther apart than *r*II and *r*I, the frequency of recombination between *r*II and *r*III mutants should be greater than that between *r*II and *r*I mutants.

at all. These three groups, termed the *r*I, *r*II, and *r*III categories, respectively, may be arranged in the proper linear order by performing mixed-infection crosses. As may be deduced from the scheme in Figure 46, the frequency of recombination between members of the *r*II and *r*III groups would be greater than between mutants of the *r*II and *r*I varieties, making the usual assumption of a linear arrangement of "genes."

Benzer has chosen to concentrate mainly on the *r*II region for his genetic analysis. The *r*II mutants have one clear advantage from the point of view of detection, in that they do not grow on K at all and therefore give an unequivocal phenotypic test. Some thousand mutants of the *r*II type could thus easily be selected for the mapping project.

The *r*II mutants were further subdivided on the basis of the *cis-trans* test. It will be recalled that this genetic trick enables us to determine whether or not two mutants which show a similar phenotypic abnormality are concerned with the same functional unit of heredity or with two separate functions which interact, cooperatively, in producing the change. As we discussed in connection with the "lozenge" genes of *Drosophila* (Chapter 2), three closely linked genetic loci all appeared to be part of the same functional unit since crosses between double heterozygotes bearing both mutant loci on the *same* strand of the chromosome duplex (i.e., in the *cis* arrangement) produced wild-type recombinants, whereas crosses between double heterozygotes in the *trans* arrangement did not. The adequacy of the *cis* arrangement was explained on the assumption that the one, unmarred, strand of the chromosome supplied the required unit of physiological function in sufficient quantity to satisfy the needs of the organism even in the presence of one "nonfunctional" chromatid.

In phage, although the genetic material behaves as though the organism were haploid, the *cis-trans* test can still be applied since mixed infection with two mutants appears to simulate the more conventional diploid situation in higher organisms. Thus, when a mutant

phage containing an *r* mutation is introduced into an *E. coli* K host cell along with a wild-type phage particle, the cells are lysed and both kinds of parent phage are liberated. The presence of the wild-type presumably supplies the function missing in the *r* mutant (the *r*II mutation is "recessive"). Although the test has not been made, it is also assumed that mixed infection with a wild type, and with a double *r* mutant bearing both mutations on the same strand, would permit the formation of both sorts of progeny, and for the same reason. When two *r* mutants are used, however, lysis of host cells and progeny production will take place only if the two mutants are deficient in *different functional units.* The various situations are schematized in Figure 47. When two *r* mutants fail to lyse *E. coli* K following mixed infection, the mutations are said to belong to the same "cistron" (the name derived from the test employed). When they supplement one another and cause lysis, they are said to belong to different cistrons. By this sort of technique, Benzer divided the *r*II mutants into two functionally different groups which he names the A and B cistrons (Figure 48). (We might visualize that each of the various phage-synthesizing enzymes, whose formation is induced in bacteria by phage infection, is represented by a different cistron.)

Now it is clear that to map a large number of mutant loci, even after such further subdivision, would require an impossible number of crosses, and Benzer has taken advantage of the fact that some of the mutations appear to involve a change in much more than a point locus. These unusual mutations are detected by the fact that, when

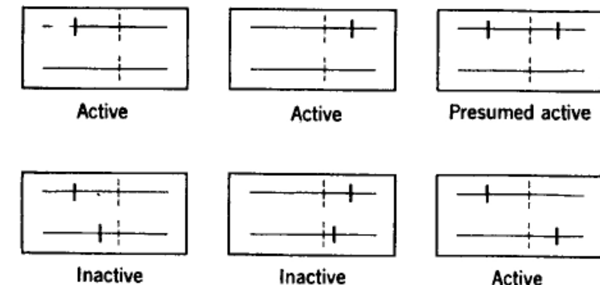


Figure 47. Schematic diagram showing the results of *cis* and *trans* configurations of double "heterozygotes" bearing two *r*II mutations. *Active* means extensive lysis of the doubly infected cells. *Inactive* means very little lysis. The presence of one "unmarred" strand is pictured as being sufficient to supply the required unit of physiological function.

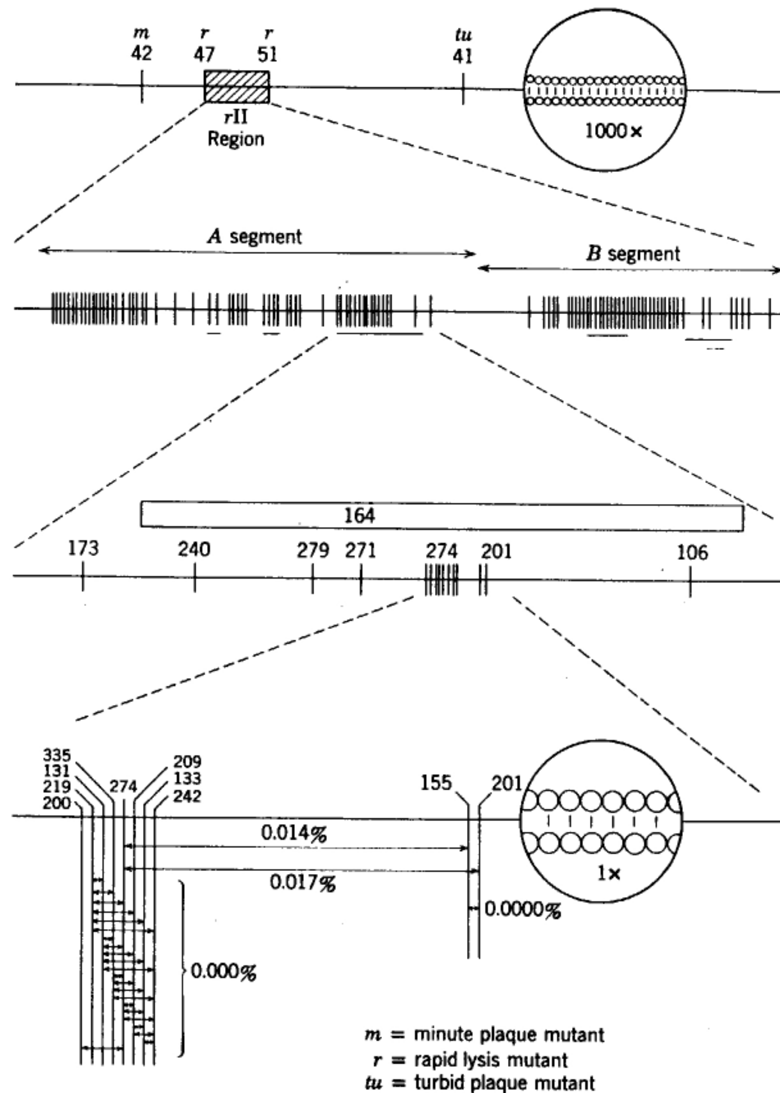


Figure 48. A partial linkage map of T4 bacteriophage. The successive drawings indicate increasing orders of magnification of the *r*II region as revealed by the fine-structure analysis of this region. This figure is highly schematic, and for details of its construction the reader should consult the article by S. Benzer in "Mutation," *Brookhaven Symposia in Biol.*, No. 8 (1956). Redrawn by permission of the author.

they are crossed with several other mutants which give wild-type recombinants when crossed with one another, no detectable wild-type recombinants are observed. The situation is explainable on the basis of the chart shown in Figure 49. If mutant 6 represents a "deletion" type of mutation, and 1 through 5 represent various other ordinary point mutations, crosses between 6 and either 2, 3, or 4 will not yield wild-type recombinants since 6 is deficient in the same properties that are missing in the other three. Wild-type recombinants *could*, however, be obtained from crosses between 6 and either of 1 and 5 since there is no overlap. Using "deletion" mutants for further screening within each of the two cistrons, Benzer was able to simplify the task of mapping since it was then necessary to perform crosses in all combinations only with the mutants that fell within any given deletion region, rather than with *all* the mutants in each cistron. We shall not describe, in detail, this part of the mapping project. In spite of the shortcuts devised, the job was obviously extremely laborious. The schematic map shown in Figure 50 was constructed from crossover data obtained on some of the 923 *r* mutants and shows, in addition to the location of "point" mutations, the location of various of the deletion mutants (the horizontal bars or lines) which were of such value in mapping. The map also

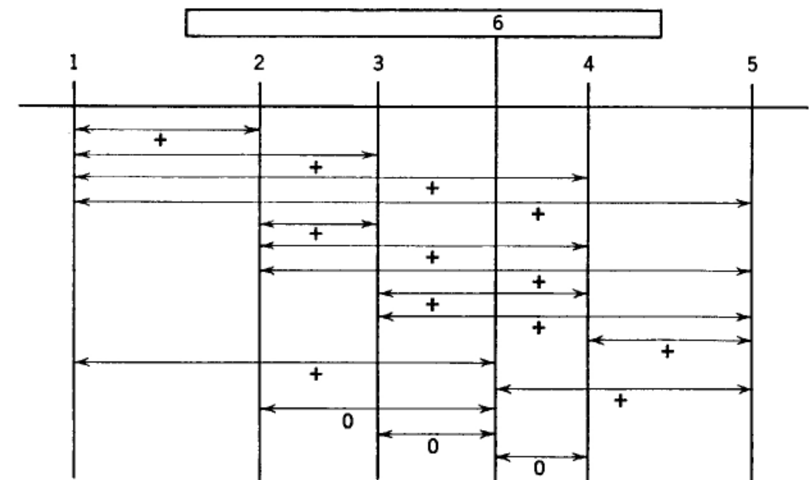


Figure 49. Schematic diagram showing the behavior of an "anomalous" mutant. Mutant 6 fails to give wild-type recombinants with mutants 2, 3 and 4 located within the same segment of the genetic strand. Wild-type recombinants are given, however, with 1 and 5.

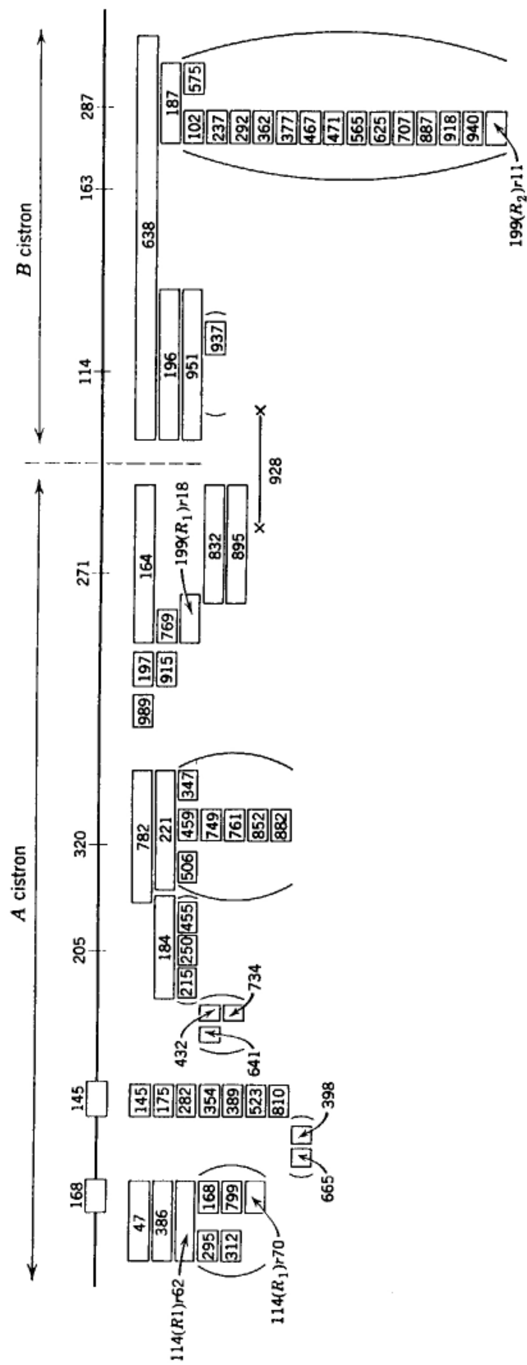


Figure 50. A genetic map showing the locations of a number of *r*II mutants. Some regions of the genetic material appear to be "hot spots," and at such localities a large series of mutants may be isolated, all of which are probably at the same locus (e.g. the series which begins with 102). Parentheses indicate groups in which internal order was not established. To appreciate this map properly the reader should consult the article by S. Benzer in *The Chemical Basis of Heredity*, Johns Hopkins Press, 1957.

incorporates the observation, made by Benzer, that a great many separate mutations turn out to be in *the same* location. In other words, some regions of the genetic material of the *r*II zone appeared to possess a much higher mutability than others. The word "appeared" is used advisedly since it must be remembered that the detectability of a mutant depends on the phenotypic test. We have seen, for example, that some *r* mutants behave on *E. coli* K as though the mutation were lethal to the phage in this environment. Others behave like wild-type phage. These classes are, of course, not associated with the *r*II region as was just discussed. However, we may postulate (and the postulation is supported in part by certain observations which we shall discuss in Chapter 6) that the substances whose synthesis is controlled by one or the other of the two cistrons in the *r*II region contain some structural features of greater importance than others. A mutation leading to modifications in the functionally less critical parts of structure in a phenotypic protein might be much less frequently detected since its influence on the "*r*-ness" of an *r* mutant growing on K might be slight in comparison with the effects of the mutations that cause a complete inability to grow on K; that is, those that have caused modifications in the chemistry of a phenotypic protein of a magnitude which completely abolishes function.

The *cistron*, conceived of by Benzer as the genetic unit of function, is clearly a very sophisticated structure. It contains many distinguishable genetic subunits as is indicated by the number of individual loci, within its length, that can be detected by recombination. Benzer, who like Levinthal is a converted physicist, has coined names for the operational subunits within the cistron that have a delightfully physical ring. The *recon* is defined as the smallest element in the one-dimensional array that is interchangeable, but not divisible, by recombination. The *muton* is defined as the smallest element of the cistron that, when altered, gives rise to a mutant form of the organism.

The size of the recon can be estimated by isolating and mapping so large a number of mutants within any given region that the distance between individual points on the map begin to approach the indivisible unit. The recon is thus an empirical unit smaller than or is equal to the smallest nonzero interval between pairs of mutants. The length of the muton is determined by estimating the discrepancy in map distances between closely linked sets of mutants. As shown in Figure 51, the "length" of mutation 2 is equal to the discrepancy between the long distance and the sum of the two short dis-

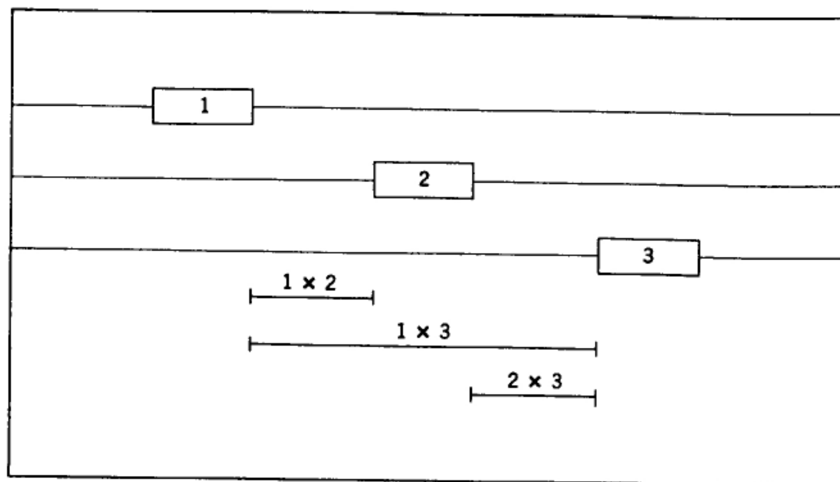


Figure 51. Determining the "length" of a mutation. The discrepancy between the long distance and the sum of the two short distances measures the "length" of the central mutation.

tances. The muton would also be measurable by determining the maximum number of mutations, separable by recombination, that can be packed into a portion of the map.

It must be emphasized that the genetic units proposed by Benzer are completely operational in meaning and origin. They serve the important function of pointing up the complexity of the word "gene" by making a clear distinction between the several operational components of this word, namely, recombination, mutation, and function. Although it is far too early to suggest that the observations made on bacteriophages may be directly applicable to higher organisms, the results at least give a rational basis for the consideration of such phenomena as pseudoallelism. More important, the high density of mapping attained and the indications therefrom of the size of the recon now make it possible to discuss chemical structure and genetics in the same breath. By making assumptions about the chemical length of the genetic strand of DNA in phage (perhaps on the order of 80,000 nucleotide pairs, in a double helix) and the total "genetic" length (perhaps on the order of 800 recombination units), Benzer is able to suggest that a single nucleotide pair might correspond to about 0.01 recombination units. Since this distance is of the same magnitude as that observed between some of the most closely linked loci on the map (about 0.02 units), it is possible that the techniques

employed have, indeed, been able to distinguish the genetic effect of a modification in as few as two pairs of nucleotides in a DNA molecule. If, as we may hope, the details of protein structure in an organism are the reflections of the chemical structure of its genetic material, these calculations would suggest that a mutation may be detectable in the phenotype when as little as a single amino acid replacement has occurred. The reader must view these speculations with absolute candor. The hypothesis is under active experimental test, and we must now rely on the protein chemist to help close the gap between genetic speculation and chemical fact.

Before leaving the subject of genetic fine structure, brief mention must be made of another technique for mapping the substructure of genes. This technique, discovered by Zinder and Lederberg[5] in 1952, relies on a phenomenon known as transduction, in which genetic information in bacterial chromosomes is transmitted from cell to cell by bacteriophage particles. Certain bacteria may be inhabited by so-called temperate bacteriophages which exist in a symbiotic arrangement with their host, wherein they only rarely lyse the bacterial cell. When such lysis *does* occur, the liberated phage particles can infect new host cells and, in the process, carry with them some of the genetic peculiarities of the original host. Only a single phenotypic character is generally transduced at a time, as though the phage were carrying with it a single gene. However, there is occasional transfer of more than one genetic marker in a bundle, and such events enable the investigator to determine the closeness of linkage between two or more functional units of heredity.

The explanation might be offered for transduction in general that parts of the "chromosome" of the phage have exchanged with parts of the bacterial chromosome and that, following the establishment of a new symbiosis, this newly acquired information is unloaded on the new host. Many characteristics of *transduction* are highly reminiscent of *transformation*. In both, transfer of genetic information appears to depend on the physical transfer of DNA, either packaged as in phage or free. Both phenomena, although generally involving only a single genetic marker, may occasionally involve several. Transduction has been employed by a number of investigators for the mapping of genetic material in several bacterial species and permits a degree of discrimination comparable to that achieved by Benzer through bacteriophage crossing. However, since the concept of "fine-structure genetics" is quite elegantly illustrated by the bacteriophage approach, we shall defer to the excellent reviews listed below and proceed to the next item of business, the protein molecule.

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4. Summarized in *The Chemical Basis of Heredity*. See Suggestions for Further Reading.
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