further within the membrane will, of course, be higher. From the numerical value of ∂c one can estimate the *p*H at the inner edge of the thin layer ∂x, taking into consideration the acidic dissociation constant of RCOOH ($pK_A = 3.38$) (*5*). The value obtained, $[H^+] \simeq 5 \times 10^{-5}$, is several *p*H units lower than the *p*H of the outer part. Even if we consider ∂x to be 0.1 percent of the thickness of a papain layer, $[H^+] \simeq 5 \times 10^{-6}$. The hydrogen ion concentrations in the inner layers of the membrane will obviously be greater. The foregoing calculation thus leads to internal *p*H values differing from those of the external *p*H to the extent of several *p*H units, as indicated by the *p*H-dependence of esterase activity and the experiments with neutral red. In this simple estimate the reaction of H^+ with OH^- entering the membrane from the outside has been neglected, since their concentrations are low in comparison to that of RCOOH.

It has been pointed out (*6, 7*) that the many native enzyme systems which are embedded in particles or membranes (such as the mitochondrial enzymes) may well be situated in a microenvironment considerably different from the aqueous solutions in which isolated enzymes are usually investigated. Doscher and Richards (*8*), studying the enzymic activity of crystals of ribonuclease S, have indicated that, although the enzyme is active in the crystalline phase, its activity is limited by the rate of diffusion of substrate into the crystal. Goldstein *et al.* (*7, 9*), studying the action of trypsin embedded in a polyanionic gel, showed that drastic changes in internal *p*H and substrate concentration result from the electrostatic potential of the gel phase also (*6, 10*), and pointed out the relevance of these findings in the investigation of native particulate enzymes. Our data demonstrate that an enzyme embedded in a membrane can, by its action, change its environment markedly, and thereby its own activity. Such effects may play a part in feedback and control systems at the cellular and intracellular level. Moreover, it is conceivable that a wave of reaction may be propagated in the plane of a membrane, as the result of the acid released by one enzyme molecule serving to trigger the action of an adjacent enzyme molecule which might, even in the presence of substrate, be initially inactive. It should be realized that such propagation would be a much faster process than simple diffusion, because each enzyme molecule activated would serve as a fresh source of the diffusing species. Such a wave could take the form of a pulse if the local substrate concentration were sufficiently small.

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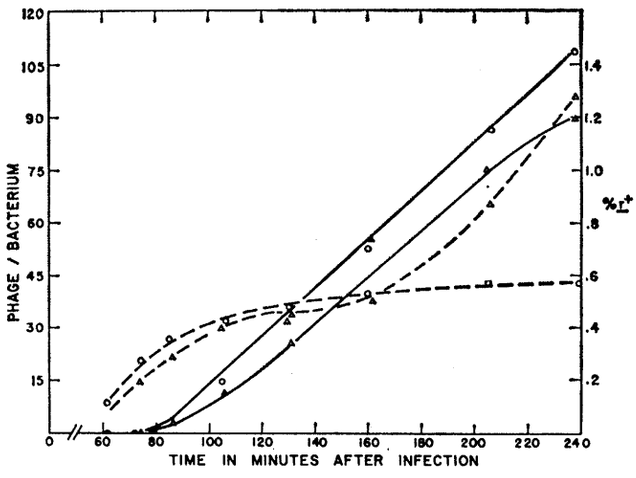
Recombination in Bacteriophage T4: a Mechanism

Abstract. *Genetic recombination between* r*II mutants of T4 bacteriophage grown in* Escherichia coli *can occur under conditions where DNA synthesis is strongly inhibited by 5-fluorodeoxyuridine. The small amount of DNA synthesized under these conditions cannot account for the observed frequency of recombinants. The major mechanism of recombination in this system is a process of breakage and rejoining.*

Early workers on the mechanism of recombination in bacteriophages noted that reciprocal recombinants are found in equal frequencies in a mass lysate; this was not the case when the yield from single cells was observed (*1*). This observation, along with other considerations reviewed by Luria (*2*), led to the conclusion that recombination occurred by a copy-choice process in which a new DNA molecule is copied first from one parent and then from another. This view generally prevailed until 1961, when Meselson and Weigle (*3*) demonstrated that recombination in the temperate bacteriophage λ at least occasionally involved a breakage mechanism. Later work showed that the process involved both breakage and rejoining (*4*). At about the same time, Kozinski (*5*), using physical techniques, demonstrated that the parental T4 molecule was dispersed among the progeny particles in pieces with molecular weights of 10^6 to 10^7. Tomizawa and Anraku (*6*) correlated physical and genetic evidence for recombination by a break-rejoin process. Genetic evidence that recombination in bacteriophage T4 occurs principally by a process of breakage and rejoining is presented in this report.

Since recombination by a copy-choice mechanism would require extensive synthesis of DNA, while recombination by a breakage and rejoining process would require little or none, the problem can be reduced to the question whether recombination would occur in the absence of DNA synthesis. The experiment required four steps. (i) Inhibit maturation and permit accumulation of a pool of phage precursor DNA. (ii) Stop further DNA synthesis. (iii) Allow the pool to mature into viable phage particles. (iv) Examine the frequency of recombinants as a function of time after maturation has begun. Maturation was prevented (step i) by the addition of chloramphenicol shortly after infection (*7*). DNA synthesis was inhibited (step ii) by addition of 5-fluorodeoxyuridine (FUDR). Step iii was accomplished by removing the chloramphenicol by centrifugation, and step iv by means of suitable genetic markers (*8*).

All experiments were carried out with *Escherichia coli* strain B Berkeley (designated BB) grown in tris-buffered glucose medium (*9*). The *r*II mutants were supplied by S. Benzer. In most experiments, *r*227 and *r*147 were used; both are spontaneously revertible A-cistron mutants located about 1 map unit apart (*10*). The *td*8 and *td*9 mutants were isolated by Simon and Tessman (*9*) and are also 1 unit apart. The *r*II and *td* loci are separated by about one-fourth of the T4 genome (*9*). All stocks were grown in BB and prepared from cultures lysed 4 hours



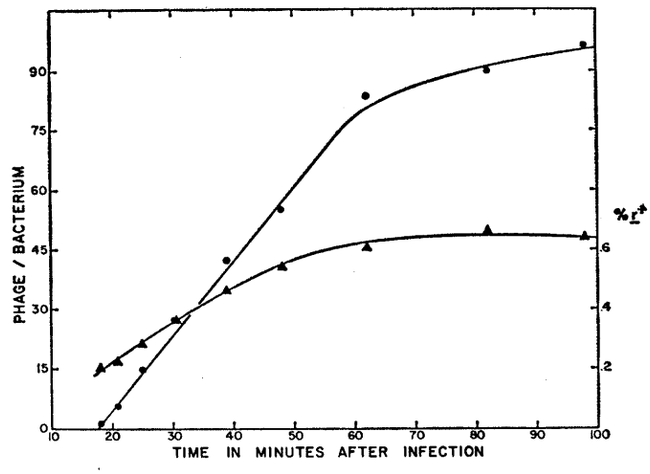


Fig. 1 (left). Cells were infected with three to five each of *r*147 and *r*227 bacteriophage and treated as described in the text. Solid lines represent the yield in phage per bacterium and dotted lines the fraction of r^+ among the progeny. At indicated times after infection, 0.1 ml of the culture was sampled into broth, and the cells were lysed with chloroform. (○). Virus from chloramphenicol-treated cells; (△) virus from chloramphenicol-FUDR-treated cells. Fig. 2 (right). Cells were infected with three to five each of *r*147 and *r*227 bacteriophage. At indicated times after infection, 0.1 ml of the culture was sampled in broth, and the cells were lysed with chloroform. (●) Phage/bacterium; (▲) fraction of r^+.

after infection. The total virus was determined by assay on *E. coli* strain B. The r^+ recombinants were scored on *E. coli* strain K 12 (λ) and td^+ recombinants on the thymine-requiring *E. coli* B3. The *td* mutants were detected by spot tests (*9*), and DNA was assayed by the method of Hershey and Melechen (*7*). Bacteria labeled with P^{32} were precipitated with 0.3*M* trichloroacetic acid, and the RNA was hydrolyzed with KOH. At least 80 percent of the radioactivity remaining after reprecipitation of the hydrolyzate with 0.3*M* trichloroacetic acid was contained in DNA (*7*).

At time zero, the bacteria were infected with 3 to 5 plaque-forming units (PFU) of each of two viruses. Chloramphenicol (40 μg/ml) was added 9.5 minutes later; 60 minutes after infection the chloramphenicol was removed by centrifugation (*11*). During this interval, from 100 to 160 phage-precursor DNA molecules were synthesized. The cells were then divided into two portions, one suspended in warm tris-buffered glucose and the other in the warm buffer containing FUDR (7 μg/ml). Samples were taken periodically, lysed with chloroform to release intracellular virus, and plated to determine total phage and the fraction of recombinants (Fig. 1). For comparison, Fig. 2 shows the result of a standard cross. Maturation started about 15 minutes after the chloramphenicol was removed and continued for at least 3 hours. The rate of maturation for virus from chloramphenicol and chloramphenicol-FUDR cells is approximately equal. The frequency of r^+ recombinants among the first phage matured after removal of chloramphenicol is about 0.2 percent from both kinds of cells. The same frequency of recombinants was seen among the first phage arising from a standard cross involving neither drug (*12*). DNA synthesis in FUDR was no more than 10 percent of the control (Fig. 3). At face value, the experiments shown in Figs. 1 and 3 would prove that recombination can occur by a break-rejoin mechanism. However, it was possible that the residual P^{32} uptake represented synthesis of molecules of phage-precursor DNA, and if so, these could account for all of the observed recombinants. To determine if DNA synthesis in fact occurred in the presence of FUDR, cells were infected with HB122—an *r*II mutant highly revertible by 2-aminopurine (*10*)—and treated as described above except that 2-aminopurine was added along with the FUDR. A significant number of revertants appeared in the progeny. If the purine acts as a mutagen by its incorporation into DNA, then some DNA synthesis must have taken place (*13*). Hence the possibility that all or most of the observed recombinants resulted from the residual DNA synthesis had to be eliminated.

Although FUDR-treated cells synthesized less than 10 percent as much DNA as the control, the viruses synthesized in them had a frequency of recombination greater than, or equal to, the control virus (Figs. 1 and 2). Therefore, if newly synthesized molecules contained all of the recombinants, each newly synthesized molecule must have had a far great probability of being recombinant than the average molecule. In other words, if one out of a hundred phage were recombinant, the overall fraction of recombinants was 1 percent; but if only ten of the hundred phage were capable of being

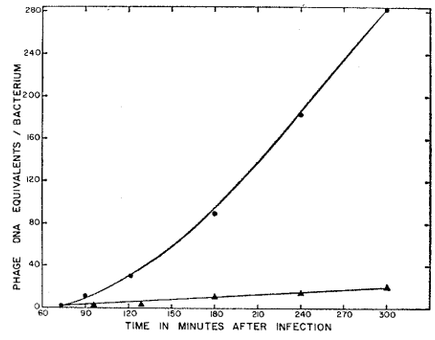


Fig. 3. Cells were infected as described in the legend to Fig. 1. After the chloramphenicol was removed by centrifugation the cells were resuspended in tris-glucose medium, and P^{32} was added. The culture was immediately divided and FUDR (5 μg/ml) added to one portion. At indicated times, 1 ml of the culture was added to 6 ml of ice-cold 0.35*M* trichloroacetic acid. Incorporation of P^{32} into DNA was determined as described, (text and 7). The unit is 2×10^{-11} μg of phosphorus assimilated during the labeling period. (●) Chloramphenicol-treated cells; (▲) chloramphenicol-FUDR-treated cells.

recombinant, the fraction of recombinants among those capable of recombination was 10 percent. If one assumes that all the observed recombinants arose from molecules which were synthesized in the presence of FUDR, the problem could be solved by isolating phage containing this DNA and examining them for recombinants. If the assumption is correct, and the recombinants arose by a copy-choice process, then phage containing DNA molecules synthesized in the presence of FUDR would have a frequency of recombinants 10 to 20 times greater than control virus.

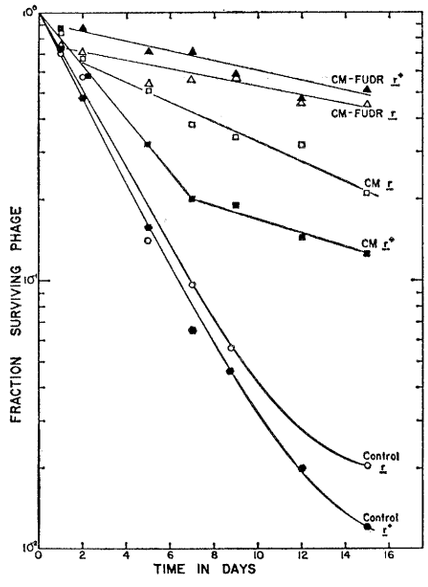


Fig. 4. All cells were infected with three to five each of *r*147 and *r*227 bacteriophage. *Control experiment.* After centrifugation, some cells were resuspended in phosphate-free tris-buffered glucose and the rest in normal tris-buffered glucose medium. Immediately after infection, P^{32} (150 μc/ml) was added to the phosphate-free medium. Sixty minutes after infection, portions of both cultures were diluted 100-fold into broth, and the cells were lysed with chloroform. The average yield of each infected center was about 100 in normal medium and 25 in phosphate-free medium. *Chloramphenicol–FUDR experiment.* The cells were treated as described in the legend for Fig. 1 except that, after the chloramphenicol was removed, the cells were suspended in medium with and without phosphate. P^{32} (150 μc/ml) was added to the phosphate-free medium, and FUDR (10 μg/ml) was added to the indicated cultures; 240 minutes after infection, cultures were sampled into broth, and the cells were lysed with chloroform. All cultures yielded about 100 phage for each infective center. Samples were stored at 4°C and assayed periodically for total and recombinant viruses. Unlabeled virus was stable under these conditions.

Table 1. Analysis of four-factor cross. Cells were infected as described in the legend to Fig. 3. The numbers in parentheses represent the number of plaques on which the percentage is based. The fourth column represents the number of phage DNA equivalents synthesized in the period 0 to 60 or 60 to 240 minutes after infection. The seventh column shows the fraction of r^+ plaques which are also td^+. CM, chloramphenicol.

Treatment	Time after infection (min)	Phage/infected center (No.)	P^{32}/infected center (units)	r^+ (%)	td^+ (%)	td^+/r^+ (%)	Summary of all experiments (% td^+ of r^+)
None	60	60	160	1.0	0.5(14)	1.2(9)	1.1 (19)
CM	240	65	62	0.95	0.6(5)	1.9(13)	1.8 (30)
CM-F	240	56	3.3	1.5	1.5(23)	2.3(12)	1.45(22)

Phage containing new molecules can be studied selectively in at least two ways. (i) Add a large amount of P^{32} with the FUDR after the chloramphenicol treatment. Phage containing newly synthesized molecules of DNA would then be killed when the P^{32} disintegrated (*14*), but unlabeled molecules would remain undamaged. If all recombinants were newly synthesized, the r^+ phage would be inactivated preferentially. In fact, however, the r and r^+ phage were inactivated at about the same rate (*15*) (Fig. 4). (ii) Perform a four-factor cross of the type $a1b1 \times a2b2$ where $a1$ and $a2$ and $b1$ and $b2$ are closely linked, but the a and b loci themselves are unlinked; then select for a^+ recombinants and look for b^+ recombinants among the a^+ fraction. This method permits the problem to be unequivocally solved; for if the hypothesis is correct, all or most of the a^+ recombinants are in phage containing newly synthesized DNA, and the fraction of b^+ among the a^+ recombinants should be at least ten times greater than in the controls. However, if the DNA molecules made in the presence of FUDR have a normal recombination frequency, then the fraction of b^+ recombinants among the a^+ recombinants should be about equal to the control.

Accordingly, *r*147*td*8 and *r*227*td*9 double mutants were prepared, and a standard cross (Fig. 1) was made. The amount of DNA synthesized before and after chloramphenicol removal was determined by addition of P^{32} to a specific activity of 0.01, 30 minutes after infection, and by increasing the level of P^{32} to a specific activity of 0.2 immediately after the chloramphenicol was removed. The cultures were sampled and lysed 240 minutes after infection (180 minutes after removing the chloramphenicol); they were then plated to determine total virus and the frequency of r^+ and td^+ recombinants. The r^+ recombinant plaques were picked and spotted on *E. coli* B3 to determine if they were *td* or td^+. The complete results of one experiment of this type are shown in the first seven columns of Table 1. The last column of the table shows a summary of all experiments of this type, including some where there was less complete inhibition of DNA synthesis. As expected, the frequency of recombination of td^+ and r^+ phage from cells treated with chloramphenicol-FUDR is about twice that from control cells, and the final frequency of recombinants in chloramphenicol-treated cells is nearly equal to the control (see Figs. 1 and 2). The yield of phage per infected cell in the chloramphenicol-treated cultures is greater than from cells treated with both drugs. In more recent experiments, the yield from cells treated with the combination of drugs has usually exceeded those treated with chloramphenicol alone. The reason for this is unknown, but the difference in frequency of recombination has been maintained. The key data are given in the seventh column and show that the frequency of td^+ phage among the r^+ population (selected td^+) is about the same from cells treated either way. This proves that most of the recombinants in cells with both agents did not arise from newly synthesized DNA. The frequency of td^+ recombinants among the r^+ recombinants should be somewhat greater than the frequency of td^+ recombinants in the whole population, since they have had more mating experience (*16*).

Thus recombination occurring in the presence of FUDR cannot result from the appearance of a special class of highly recombinant molecules. This work, together with that of Kozinski and of Tomizawa (*5, 6*) shows conclusively that recombination can occur

by a process of breakage and rejoining. Furthermore, since normal recombination frequencies can be attained in the absence of DNA synthesis (Fig. 1), breakage and rejoining is probably the major mechanism for recombination in this virus. The possibility that a small amount of DNA synthesis may be involved in the recombination process is not excluded. However, the basic process is clearly one of physical exchange between molecules rather than a copying of genetic information from two molecules in the process of making a third (*17*).

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23 July 1965

Cestode in North Dakota: Echinococcus in Field Mice

Abstract. *Field mice* (Microtus pennsylvanicus *and* Peromyscus maniculatus) *are naturally infected with* Echinococcus multilocularis. *Thus, the sylvatic cycle (fox to field mice to fox) has been established in North Dakota. This cestode is expected to extend its range to other agricultural regions of the continental United States where similar conditions favorable for the completion of its life cycle exist.*

Echinococcus multilocularis (Leuckart, 1863) was reported first from the continental United States by Leiby and Olsen (*1*) who found the adult cestodes in red foxes (*Vulpes vulpes*) from Ward County, North Dakota. Biologic and morphologic studies (*2*) on the larval stages which developed in experimentally infected cotton rats (*Sigmodon hispidus*) have confirmed that the North Dakota cestode is *E. multilocularis* and is indistinguishable from the species found in Alaska (*3*).

Preliminary investigations in North Dakota, during the late spring of 1965, have revealed a high prevalence of sylvatic multilocular echinococcosis. Of 47 field mice examined for natural infections, 3 of 32 *Microtus pennsylvanicus* and 3 of 15 *Peromyscus maniculatus* harbored *Echinococcus multilocularis*. With the exception of a single *Peromyscus maniculatus*, in which the cystic stages occurred in both the liver and spleen, the infections were confined to the liver. In all cases, the larvae seemed normally developed with large numbers of scolices. To the best of my knowledge, this constitutes the first report of *E. multilocularis* occurring naturally in the above rodents; however, experimental infections with the Alaskan strain have been established in both *Microtus pennsylvanicus* (*4*) and *Peromyscus maniculatus* (*5*).

The work by Leiby and Olsen (*1*) and my study confirm that the sylvatic cycle of *Echinococcus multilocularis* (fox to field mice to fox) is well established in North Dakota. In view of present knowledge, it should be expected that its range will eventually extend throughout the agricultural regions of the United States where conditions for completion of its life cycle are favorable.

Vogel (*6*) ascertained that dogs, cats, and foxes served equally well as definitive hosts for the Eurasian strain of *E. multilocularis*. Therefore, it is reasonable to assume that dogs and cats in rural areas will become infected when they feed upon field mice harboring the larval cestode, and occasional infection of man in the United States could occur as a result of his association with these domestic animals. Also, as indicated by Rausch (*3*), there is some risk in the handling of foxes by trappers, hunters, and fur handlers.

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14 July 1965

Periodic Respiratory Pattern Occurring in Conjunction with Eye Movements during Sleep

Abstract. *With each flurry of rapid eye movements during the sleep of human subjects there is a decreased amplitude of respiration and a slight increase in rate. Occasionally the rhythmic breathing pattern may even resemble Cheyne-Stokes respiration. The consistency of this breathing pattern suggests that respiration in this stage of sleep is not a direct function of dream content.*

The initial report (*1*) regarding the occurrence of a rapid eye movement (REM) stage of sleep indicated that the cardiac and respiratory rates were slightly elevated during that stage as compared with either the preceding or following stages. In the decade that ensued, these results were essentially confirmed, although great stress (*2, 3*) was placed on the purported irregularity of the respiratory pattern in the REM stage of sleep. Inasmuch as respiration (sighing, hyperventilation, compulsive breath-holding, and so forth) reflects