REPLICATIVE FRAGMENTATION IN T4 BACTERIOPHAGE DNA, 
II. BIPARENTAL MOLECULAR RECOMBINATION*

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Although deoxyribonucleic acid (DNA) replication of T4 bacteriophage is basically of a semiconservative pattern, it is accompanied by extensive fragmentation and the dispersion of parental polynucleotide over a large fraction of the progeny molecules. The parental contribution to a progeny molecule represents a single subunit of a semiconservative nature averaging 5–7 per cent of the total mass of the molecule. The incorporated parental fragment is firmly associated with the rest of the polynucleotide backbone, since denaturation by heat or alkali does not separate the parental fragment in pure form. This phenomenon can be explained by the high efficiency of mating of these bacteriophage.

It has also been shown that, by adding chloramphenicol (CM) to the infected bacteria 5–7 min after infection, it is possible to inhibit the fragmentation of parental phage DNA without affecting its semiconservative replication. We postulated, therefore, that recombination between T4 DNA molecules requires a specific enzyme, "recombinase," which can be detected in the infected bacteria later than DNA polymerase. In the same paper we tried to determine whether there is recombination between simultaneously infecting phages which is efficient enough to be detected in an experiment which, in brief, was performed as follows: light bacteria in cold medium were simultaneously infected with a single, light, hot (P32-labeled) phage and several cold, heavy, substituted with 5-bromodeoxyuridine (5BU) bacteriophages. If there is any significant recombination between light and heavy bacteriophage DNA, a fraction of radioactivity, when analyzed in cesium chloride (CsCl) gradient, would assume the intermediate position between the light and the heavy location of the extracted DNA. In this experiment we only followed the fate of radioactive DNA for 15 min, since maturation and withdrawal of phage from the pool occur after that time.

In our current work described here, prolonged contact between parental DNA's and reduction of the size of the DNA pool by 5-fluorodeoxyuridine (FUDR) made it possible to demonstrate the existence of interparental molecular exchanges, though a semiconservative replication still needs to be a closely accompanying process.

Materials and Methods.—The bacterial strain used in these experiments was E. coli B3, a thymineless mutant. The phage was T4 BO3. Light, heavy, radioactive and starvation (the medium from which energy sources, i.e., dextrose, and casamino acids were omitted) synthetic TCG medium, along with the method for purification and plating of bacteriophage, CsCl density gradient techniques, DNA extraction from bacteriophage and from the infected hosts have been

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described before.\textsuperscript{1-3} In the present experiments, \( \text{H}^3 \)-labeled light bacteriophage or its DNA was added as reference and used in association with the centrifugation of DNA and phage resulting from the experiment in which the parental phage was labeled with \( \text{P}^{32} \). \( \text{H}^3 \) was incorporated as radioactive thymidine having a specific activity of 10 \( \mu \text{c}/\mu\text{g} \) of thymidine. \( \text{P}^{32} \) and \( \text{H}^3 \) were estimated in a Packard liquid scintillation counter adjusted for double channel counting. Samples from \( \text{CsCl} \) and sucrose gradient were collected, in this case directly on the Fiberglas filter, and after drying, were overlaid with 3 ml of toluene scintillation liquid (1000 ml toluene, 4 gm PPO, and 0.3 gm POPOP). No refractions were measured in collected fractions so that in the figures they were normalized to a fraction of the length of the gradient. The top of the gradient, therefore, represents 0 and the bottom (first fraction) 1.

\textbf{Results}.—(a) Recombination between infecting DNA’s—light, radioactive and heavy, cold parental phage: \( \text{E. coli B3} \) was grown in nutrient broth medium to \( 3 \times 10^8 \). Bacteria were then sedimented and resuspended in starvation medium of one half the original volume. Further manipulations are shown on the diagram.

The resulting intracellular DNA or progeny phage has been subjected to the cesium chloride density analysis.

The results of these experiments (Figs. 1–3) indicate that biparental recombination occurs if the pool is reduced by the decrease in the net synthesis of DNA inside the infected bacteria. That this contribution was not caused by the depolymerization and random dispersion of infecting DNA was indicated by the fact that shearing reduced its contribution and caused the \( \text{P}^{32} \) to move toward the light location. The results also indicate that large numbers of both \( \text{P}^{32} \) and heavy parental fragments are incorporated, with repair, into the same polynucleotide chain (PNC), since alkali treatment leading to the separation of strands does not cause separation of the heavy and light moieties of the progeny DNA molecule. This phenomenon is similar to that described for the progeny DNA in a double density-labeling system.

Sucrose gradient analysis of intracellular DNA indicates that, although the dis-
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Fig. 1.—Biparental DNA contribution to a single progeny phage—light host infected with light, radioactive phage in the minority, and heavy, cold phage in the majority. Light bacteria grown in light medium, prestarved for 45 min, were infected in the presence of FUDR (15 μg/ml) with a multiplicity of 1 of light, radioactive phage and with a multiplicity of 10 of heavy, cold T4 bacteriophage. After 6 min of incubation in starvation medium, the infected bacteria were supplemented with an energy source and some subdivided into a thymidine-containing medium (a), while the rest remained in a thymidineless medium (b).

The progeny obtained 60 min after infection in the medium containing thymidine was supplemented with H3-light reference phage and centrifuged in a CsCl gradient (a). The progeny obtained in the absence of thymidine is analyzed in (b).

The displacement of P32 versus reference H3 of light phage, as seen in (b), is nonexistent if one purifies the progeny obtained under the thymidineless conditions without carrier. In this case, plating and P32 overlap closely (c). The possibility that the presence of FUDR induces production of heavier phage (due to different proportions of protein to DNA) has been ruled out by (d).

Under these conditions (d), the control group of bacteria was infected in the presence of FUDR with light, radioactive phage at a multiplicity of 1, and light, cold bacteriophage at a multiplicity of 10 and purified with an excess of light, cold carrier phage. As can be seen, there is a perfect overlapping of plating of the reference bacteriophage and of P32.

Phage were isolated from the significantly displaced fraction [marked by thin arrows on (b)] after dialysis against a solution of 0.015 M sodium citrate in 0.15 M NaCl, pH 7.6 (CS), supplemented with light carrier phage, DNA extracted, and used in CsCl analysis represented in Fig. 2.

In all figures, the thick arrow represents the location of heavy reference.

tribution of DNA is significantly broader than that extracted from the purified phage preparation, the majority of the molecules are single and intact.

(b) Recombination between infecting phage DNA—heavy, radioactive and light, cold parental phage: The previous results could be interpreted as evidence either for a conservative exchange with the repair of the continuity of the PNC, or for semiconservative exchanges in which both the parental molecules recombine simultaneously with the semiconservative replication. Since neither the residual synthesis of DNA nor the significant host contribution could be excluded, an experiment was designed to discriminate between the “conservative” type of recombination, and recombination occurring pari passu with replication. The experimental procedure is essentially the same as that followed in those experiments described
Fig. 2.—Biparental contribution of phage DNA to a single progeny molecule (light, radioactive × heavy, cold). DNA was extracted from the fraction of phage indicated by arrows in Fig. 1 b. After extraction with H3-phage DNA as a reference, the DNA was analyzed in a CsCl gradient without any further treatment (a), after shearing in a Virtis homogenizer, the setting at which the major part of the molecules are sheared to 1/4 or 1/8 of their size (b), or shearing in a Raytheon sonicator 10,000 cycles/min for 15 min (c). DNA was also subjected to denaturation by adjusting the pH to 12.5 with KOH for 15 min at 37°C, followed by dialysis against CS supplemented with 1% HCHO (CSF) and centrifuged in a CsCl gradient supplemented with 1% formaldehyde (d). All concentrations of DNA used in this experiment were below 1 μg/tube.

before (see diagram) except that bacteria were simultaneously infected with 1 heavy, radioactive P32 bacteriophage and with 10 light, cold bacteriophages. Under these conditions, replication of DNA will lead to the formation of a hybrid, and any further fragmentation will lead to a dispersion, as found under normal conditions of T4 phage DNA replication. However, if fragments of nonreplicating DNA molecules are exchanged in the conservative type of recombination, shearing by sonication would reveal a moiety of double-stranded DNA, both strands of which would be heavy and radioactive and easily identifiable in a CsCl gradient. (Figs. 4–6).

Discussion.—In a previous publication, the experimental approach for the analysis of recombination between input parental phages was described: light bacteria were simultaneously infected with light, radioactive phage in a minority, and heavy, cold bacteriophage in a great majority. The only change toward the heavy density should be interpreted as a recombination between the two parental DNA's. The negative results obtained then, however, could have been caused by the relatively extensive replication of DNA, on the one hand, and by the short period of contact, on the other. The positive results reported in the present paper indicate that a class of biparental recombinants was discovered in the presence of DNA synthesis inhibitor—FUDR. If CM was added at 9 min after infection with light, radioactive and heavy, cold phage, approximately 10–15 per cent of the total input of P32 was displaced toward the heavy side of the gradient. This shows that under these conditions there was some recombination between parental DNA molecules.
The intracellular fate of parental phage DNA in bacteria infected simultaneously with light, radioactive and heavy, nonradioactive phage. This experiment system was the same as that used and demonstrated in Figs. 1 and 2. DNA was extracted at 45 min of the experiment (see diagram). (a) represents Pt in starved bacteria. (b) represents Pt in bacteria supplemented with energy sources to which CM was added at 0 min. (c) represents Pt in bacteria supplemented with energy sources to which CM was added at 9 min. (The addition of CM at 5 min is not shown in the figure, as the distribution of Pt is identical to that shown in (b).) (d) represents Pt in bacteria where CM and thymidine were added at 9 min.

within 45 min. If, however, CM was added between 0 and 5 min after infection, no recombination was detected. This latter fact is in agreement with our previous observation that CM added early after infection prevents molecular fragmentation of parental DNA. Also, in starving infected bacteria, no recombination between the parental DNA's could be detected.

Fig. 3—Biparental DNA contribution to a single progeny phage—light host infected with heavy, radioactive phage in the minority, and light, cold phage in the majority. The experimental approach was identical to that described in Fig. 1 and shown on diagram, except that the bacteriophage were labeled in reverse, i.e., the minority of the phage was heavy and radioactive, whereas the majority was light and cold. The resulting progeny obtained in the presence (a) or absence (b) of thymidine was analyzed in CsCl. The reference phage is H3 light phage. The fraction marked with thin arrows was dialyzed, supplemented with light and heavy phage for extraction of DNA, and analyzed in Fig. 5.
Fig. 5.—Biparental contribution of phage DNA to a single progeny molecule (heavy, radioactive × light, cold phage). Progeny resulting from the infection of light bacteria with heavy, radioactive phage and light, cold phage was fractionated in CsCl gradient, and the displaced fractions containing parental P32 (indicated by the thin arrows on Fig. 4 a and b) were used after dialysis for DNA extraction. After extraction and dilution to the concentration of 1 μg/ml, DNA was analyzed in CsCl gradient: (a) represents native progeny phage DNA obtained in the absence of thymidine; (b) represents sonicated progeny phage DNA obtained in the absence of thymidine; (c) represents sonicated progeny phage DNA obtained in the presence of thymidine; (d) represents sonicated progeny phage DNA obtained in the presence of thymidine.

Analysis of DNA extracted from the progeny produced in the absence of thymidine indicated displacement of the parental P32-containing moiety toward the banding side of heavy DNA. The contribution of DNA from both parents to the single molecule could either be in the form of a relatively large subunit, similar to that observed during the normal parent–progeny transfer, or DNA’s of both parental phages could be depolymerized and randomly dispersed within the progeny molecule. Sonication or shearing in the homogenizer caused the heavy and light radioactive moiety of the progeny molecule to be separated in CsCl gradient. This fact contradicts the notion of extensive fragmentation and random incorporation of parental material into the progeny molecule.

Heating of DNA or treatment with alkali leads to the separation of strands. After denaturation, recombinant DNA still possesses contributions from both parents—a large fraction of the parental contribution thus being incorporated into the same strand. Although biparental contribution to a single strand is beyond question, the broad distribution of the P32-containing fraction in CsCl gradient makes it necessary to consider the possibility that a certain percentage of single strands possess only one parental fragment.

On the basis of the results described above, there is no way to differentiate between conservative and semiconservative recombination, i.e., recombination between nonreplicating double-stranded parental molecules or recombination between the replicative hybrid form of both parental DNA’s. In order to dis-
The intracellular fate of parental phage DNA in bacteria infected simultaneously with a minority of heavy, radioactive and a majority of light, cold phage. (a) represents the fate of intracellular heavy, radioactive DNA derived from bacteria in the absence of thymidine but with CM added at 0 min. (b) represents the fate of heavy, radioactive DNA in bacteria to which CM was added at 9 min after infection. The smaller figure in the upper right corner of (b) represents an identical experiment in which thymidine was added along with CM. (Note the good coincidence of the replicative and H\(^3\) reference peaks.) The displaced fraction (indicated by thin arrows) was isolated and rerun without further treatment (c) and after shearing with sonication (d). Note the hybrid location of the P\(^{32}\).

criminate between these two possibilities, a reverse experiment was performed, i.e., light, cold bacteria were infected in the absence of thymidine with one heavy, radioactive bacteriophage and many light, cold bacteriophages. Under these conditions, there was a significant displacement from the heavy toward the light location. This displacement was less pronounced when compared with that of control progeny obtained in the presence of thymidine. Fractions containing paren-

Fig. 7.—Models of biparental recombination of DNA. (a) represents exchanges between two intact, conservative input phage DNA molecules without the repair of PNC continuity. (b) represents similar exchange accompanied by repair of the continuity of the PNC. These possibilities have been excluded by the group of experiments represented by Figs. 4 and 6, indicating that no significant fraction of conservative parental-like material could be detected within the recombinant class. Model (c) represents a semiconservative type of fragmentary recombination in which the parental fragment is incorporated as a hybrid and the continuity of the PNC repaired, as shown by the alkali denaturation experiment, Fig. 2 (d). —, parental light strand; ---, parental heavy strand; ————, progeny (newly synthesized) strand.
tal P\textsuperscript{32} in a highly dispersed form were isolated, and then DNA was subjected to shearing. If recombination in the absence of thymidine is of a conservative nature, one should expect to obtain smaller subunits in which both strands would be radioactive and heavy and, therefore, would be easily separated in CsCl gradient. The experiments showed that after sonication there was an undetectable or very small, parental-like peak (Figs. 5 and 6). The majority of the P\textsuperscript{32} banded at the hybrid density, proving that the parental fragments are not incorporated as conservative subunits.\textsuperscript{2}

In summary, our results lead to the conclusion that, in most cases, the recombination process in T4 bacteriophage infection has to be preceded, accompanied, or followed immediately by replication of the phage DNA. Recombination could occur on the level of the single strand, in which case the single strand contains both parental contributions of heavy and light fragments; more than likely those fragments are separated by an area of nonradioactive progeny DNA, since shearing efficiently separates these two parental fragments. The continuity of the PNC on either end of the integrated parental pieces, both heavy and light, must, of course, be repaired since the contribution remains associated after strand separation with alkali. Under these experimental conditions, the average size of a single parental contribution to the recombinant molecule or recombinant progeny phage is 10 per cent, as estimated by the displacement of the recombinant class. It is difficult to evaluate data obtained with the single-stranded DNA since there is a difference of opinion concerning the integrity of the PNC in T4 phage\textsuperscript{4} (Fig 7).

Summary.—When a light, thymine-dependent strain of E. coli B is infected simultaneously with light, P\textsuperscript{32} at a low multiplicity, and heavy 5 BU-labeled, cold phage at a high multiplicity in the absence of thymine and in the presence of FUDR, the resulting progeny phage and its DNA contain a biparental contribution. This is revealed in CsCl gradient as a displacement of the P\textsuperscript{32}-containing moiety of DNA toward the heavy side. Shearing of this DNA separates heavy and light fragments; denaturation by alkali does not. These results indicate that parental contributions are integrated into the recombinant molecule as discrete subunits and that after exchanges the continuity of the PNC is repaired and a large fraction of subunits derived from both parents is located within the same single strand of DNA.

If infected bacteria are starved, or CM is added between 0 to 5 min after infection and intracellular parental DNA analyzed, no recombinant molecules can be detected. However, the addition of CM at 9 min after infection permits a certain degree of recombination. If the labeling system is reversed, i.e., light bacteria are infected with heavy and radioactive phage in the minority, and cold, light phage in the majority, and the displaced moiety of DNA is separated in CsCl gradient and then subjected to shearing, no parental-like conservative subunits, composed of both heavy and radioactive strands, could be detected in the recombinant molecule. This indicates that the phenomenon of semiconservative replication must be closely associated with molecular recombination. Models of recombinant molecules are presented.

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