

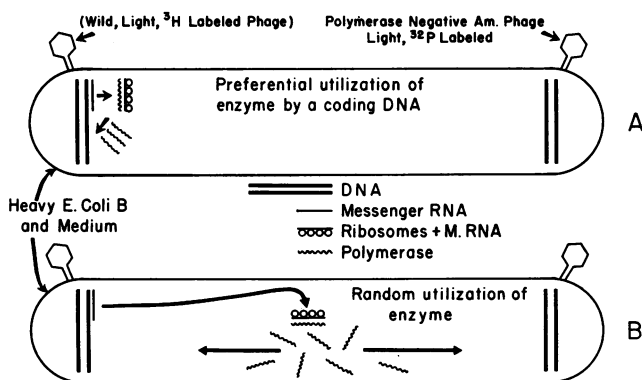
EARLY INTRACELLULAR EVENTS IN THE REPLICATION OF T4 PHAGE DNA, III. RANDOM UTILIZATION OF PHAGE-CODED ENZYMES BY SIMULTANEOUSLY INFECTING PHAGE*

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Enzymes involved in the synthetic processes in T4 infected bacteria are well characterized in the timing of their appearance. Many have been isolated and partially purified, and their mode of action has been simulated *in vitro*.¹ The topography in the distribution of phage-coded enzymes within the infected cell is unknown. In a previous paper² we proved that DNA injected soon after infection enters a proteinaceous complex. We postulated at this time that the complex could play a significant regulatory role during the process of replication and recombination. A tight association of DNA with protein might provide a milieu in which DNA replicates in an orderly manner, without the "hairpins," covalently attached new strands, or possibly other artifacts that appear during an *in vitro* synthesis,³⁻⁶ but not during T4 DNA replication within the host cell.^{7, 8} It is also justifiable to visualize that enzymes participating in the replication might be coded for, and produced, in the direct vicinity of the parental DNA. This could be an efficient arrangement in which a "factory," which is only a short distance from its substrate, would provide all of the precursors and polymerizing enzymes *in situ*. One could speculate that such enzymes could, like polymerase, be integrally built into a structure involved in replication of DNA. (This structure might involve fragments of cell membranes or a complex of replicative DNA with ribosomes.) Alternatively, one could visualize that injected DNA codes for the production of messenger RNA's which randomly diffuse throughout the cell, settles on available ribosomes, and produces specific proteins at a point in the cell which is random with respect to the location of the original parental DNA molecule. These two possibilities are represented in Scheme 1. According to model A (Scheme 1), new



proteins are formed in the direct neighborhood of the DNA and are incorporated into a proteinaceous complex *in statu nascendi*, thus being preferentially used for the needs of replication of the DNA molecule, which originally coded for its production.

Model *B* schematizes a system in which messenger RNA and the resulting enzymes randomly permeate the cell. In this case the enzymes are equally likely to become involved in the replication of the parental DNA from which the original message was transferred, as with any other phage DNA. In order to discriminate between these two hypotheses, we designed an experiment in which the relative rate of replication of wild phage and the polymerase negative amber mutant, Am. N122, were analyzed after simultaneous infection of the nonpermissive host *Escherichia coli* B.

Materials and Methods.—The bacterial strain used was *E. coli* B. The bacteriophage used were the T4-amber mutants: Am. N122, defective at the gene locus 42, and lacking hydroxymethylase and polymerase; and bacteriophage Am. 453, defective at gene 32 and deficient in thymidylate synthetase.⁹ *E. coli* B is a nonpermissive host for both of these amber mutants. The osmotic shock resistant bacteriophage T4BO,¹ was used as the wild phage in this system.

Light and heavy TCG media (see list of abbreviations) were described before.²

CsCl fractionations were performed by admixing 0.3 ml of a sample of DNA to 3.0 ml of 9.3 *M* CsCl. After conclusion of the centrifugation, fractions were collected directly on glass fiber filters which had been placed in scintillation vials. The distribution of the isotopes was measured in a liquid scintillation spectrometer.

Extraction leading to the isolation of replicative DNA was performed according to the SDS-pronase-phenol technique.²

Results.—*Comparison of the rate of replication of amber N122 in a nonpermissive host after simultaneous infection with T4BO_r¹:* The purpose of this experiment was to discriminate between the two possible modes of the distribution of phage-coded enzymes which were outlined in the introduction. Heavy bacteria *E. coli* B, a permissive host for T4BO,¹ but a host which does not support replication of amber N122,^{10a} were grown in heavy TCG to 3×10^8 bacteria/ml, and were infected simultaneously with an MOI of 3.0 of H³-labeled T4 wild phage, and an MOI of 0.5 of P³²-labeled amber N122. Samples of the infected suspension were iced chilled in EDTA, at different times after infection, centrifuged, and extracted by the SDS-pronase-phenol method, and replication of both parental DNA's was followed in a CsCl density gradient. At the conclusion of the run, fractions were collected in scintillation vials and, after drying, were counted in a scintillation spectrometer. (It should be mentioned that the relative proportion of both isotopes remained constant prior to and after extraction with phenol, thus eliminating the possibility of preferential losses of either phage DNA during the extraction procedure.) The results are shown in Figure 1.

The result of the experiment with phage Am. N122, which, due to the lack of polymerase, does not replicate, shows virtual identity in the kinetics of replication and recombination. The conclusion, therefore, is that polymerase coded only by the wild mutant becomes equally available for the polymerase negative mutant. However, the molecular recombination, which followed replication, could have been catalyzed by an enzyme coded by phage N122, as this phage does undergo extensive "nicking" and interparental recombination.¹⁰ Amber mutant 453 is an exception in this respect since, although it undergoes nicking, the resulting DNA fragments lack single-stranded regions and do not recombine in a biparental recombination experiment.¹⁰ Simultaneous infection with Am. 453 and the wild strain, in a heavy

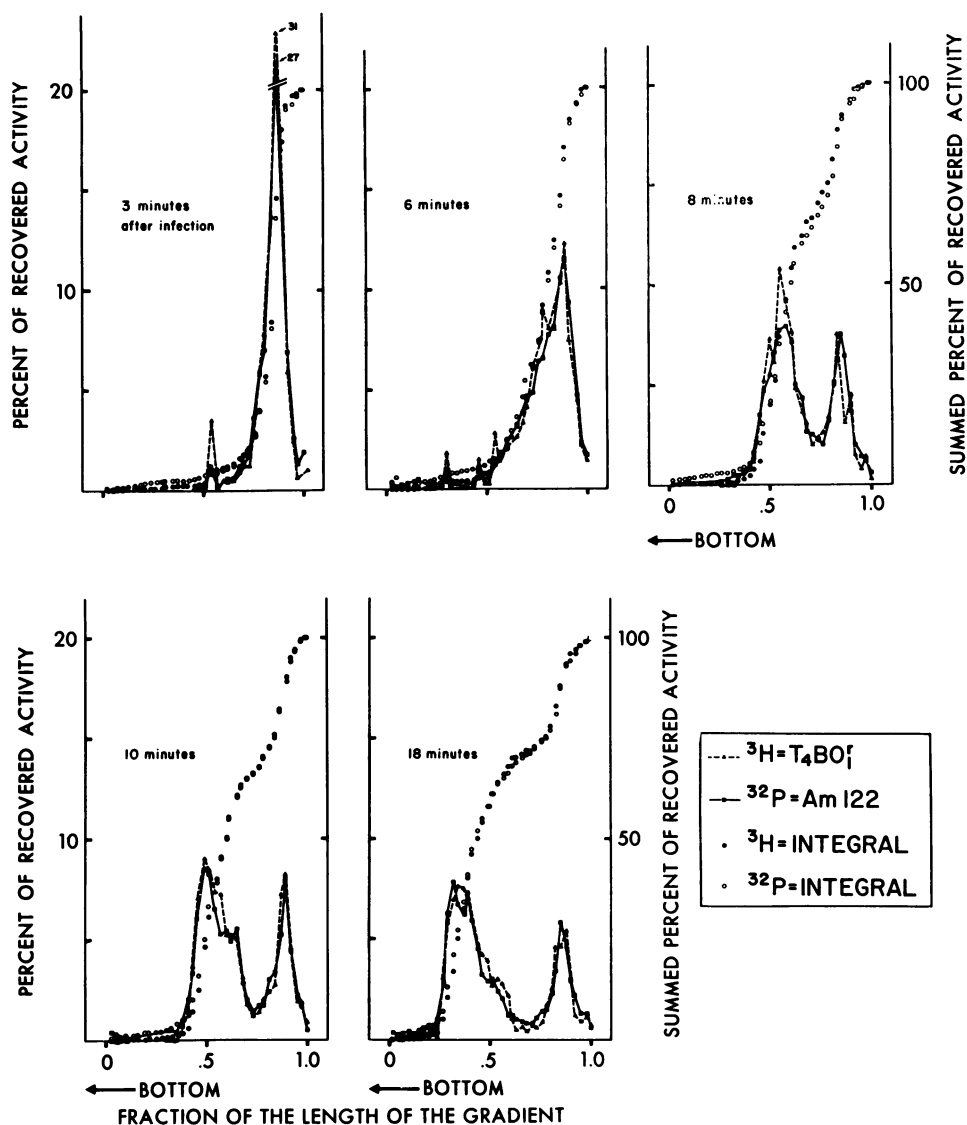


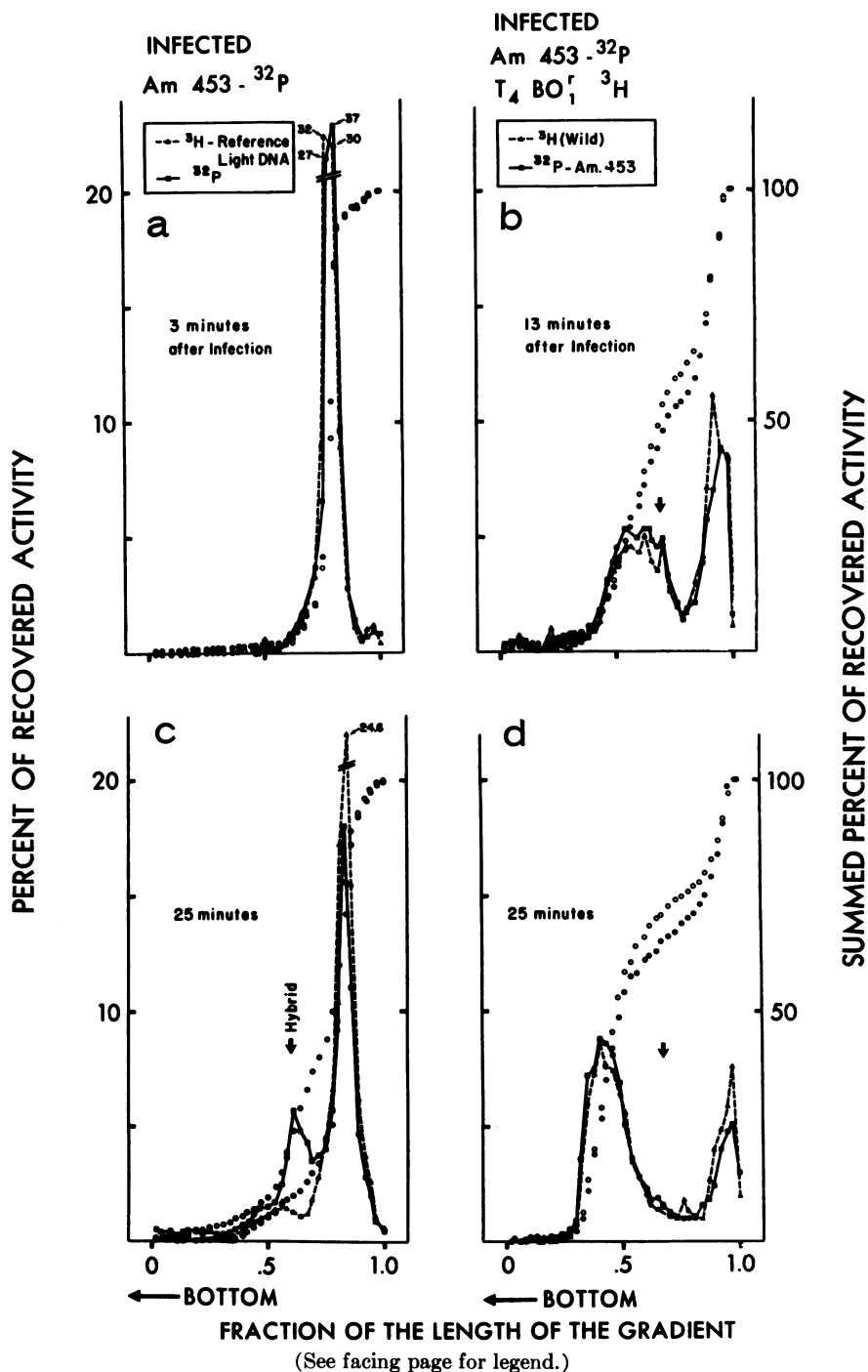
FIG. 1.—CsCl density equilibrium analysis of intracellular replicative DNA in heavy bacteria simultaneously infected with H^3 -labeled wild phage and P^{32} -labeled amber N 122. The sigmoid curve represents the summed recovery of either isotope in successive fractions. Note a perfect overlap in the summed recoveries of both isotopes.

host (which is nonpermissive for Am. 453) and with conditions otherwise identical to those used in the first experiment, should provide a model for following the relative kinetics of parent to progeny molecular recombination.

Heavy *E. coli* B was grown to 3×10^8 bacteria/ml and the suspension was divided into two groups. One part was infected with an MOI of 1.0 of P^{32} -labeled Am. 453 bacteriophage, and the other part was infected with a mixture of an MOI of 1.0 of P^{32} -labeled Am. 453 and an MOI of 3.0 of H^3 -labeled wild phage. Samples

were chilled at intervals, extracted by the SDS-pronase-phenol method, and analyzed in CsCl. The results are shown in Figure 2.

Consistent with our previous data,¹⁰ Am. 453 parental phage DNA, after in-



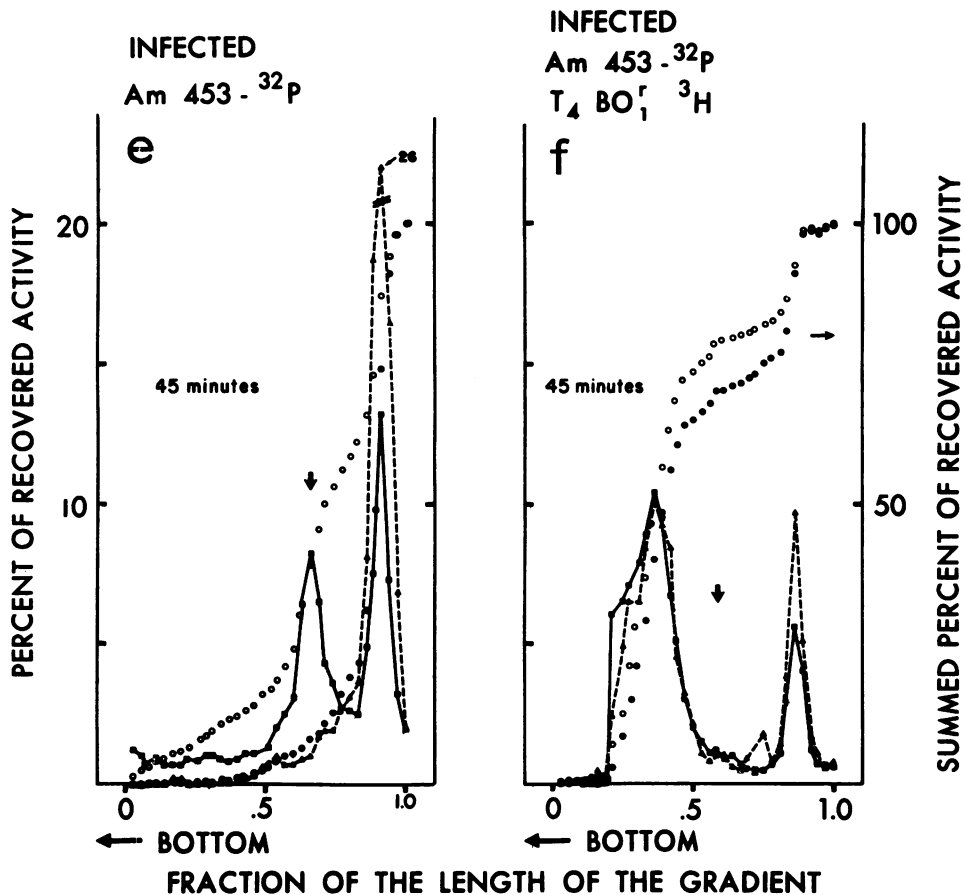


FIG. 2.—CsCl density equilibrium analysis of intracellular replicative DNA in bacteria simultaneously infected with H^3 -labeled wild phage and P^{32} -labeled amber 453. (a, c, e) The intracellular fate of the P^{32} -labeled, light phage Am. 453 DNA, in the nonpermissive host *E. coli* B. Note: The light H^3 is a reference added prior to the centrifugation. (b, d, f) The intracellular events in *E. coli* B infected simultaneously with P^{32} -labeled, light phage Am. N122 (MOI 1.0) and H^3 -labeled, light wild phage (MOI 3.0). Note: H^3 is not a reference. The thick arrow indicates the location of hybrid. The horizontal arrow in (f) points to the summed value representing the total amount of replicated parental DNA (80%).

fecting a nonpermissive host, replicates once and reaches only a hybrid density. If rescued with the wild phage, however, it undergoes molecular recombination to the same extent as the wild phage and with identical kinetics.

Discussion.—The work of DeWaard and co-workers¹¹ clearly proved the exclusion of host DNA polymerase in the replicational events of T4-phage DNA, but it left unanswered whether the phage-coded polymerase was randomly distributed within the cell or was synthesized and utilized in close proximity of the coding DNA molecule.

Our data clearly demonstrate that H^3 -labeled wild and the P^{32} -labeled polymerase negative Am. N122 phage DNA's replicate and at later times recombine with the same kinetics. Even at early times after infection, both parental DNA's engage in

replicational events, and proceed through partially replicated molecule (PRM)⁷ hybrid and the recombined stages with remarkable identity. In order to emphasize the identity of both the replicational and recombinational events of both phages, there is in each graph a sigmoid curve showing the summed per cent of both isotopes. With respect to the integral graph shown in Figure 1, we would like to emphasize that more than 80 per cent of the injected DNA was replicated within the host cell. (With a very good preparation of parental phage, the figure for replication can go above 90 per cent.) This obviously contradicts any hypothesis which would postulate replication of one strand of T4 phage DNA and breakdown of the other. The conclusion is obvious. DNA polymerase is equally available for either of the infecting phages, although only coded by the H³-labeled phage. The hypothesis of a close proximity of newly formed polymerase with the replicative DNA is therefore disproved in favor of the hypothesis represented in Scheme 1 *B*, in which polymerase is formed outside of the replicative focus of DNA. The same argument applies to the part of the experiment described for Am. 453, in which molecular recombination was tested between parental and progeny molecules. This experiment indicated that "recombinase,"¹² coded by the wild mutant, became equally available for both infecting phages.^{12a} If polymerase is found within the fragments of cell membranes or associated with replicative DNA, it must have found its way there by some random process.

There is another possible explanation for the identity of both parental labels—extensive material recombination between the injected parental molecules prior to replication. This, however, was disproved in an experiment in which the parental phage, labeled with different density markers, were used for simultaneous infection of bacteria, and the extent of biparental recombination was estimated in a CsCl density gradient analysis. At no time after infection was there any joining between simultaneously infecting phage DNA's.¹² Biparental recombination could only be detected if there was extensive inhibition of DNA synthesis by the addition of 5-fluorodeoxyuridine (FUDR), and under these conditions, not earlier than 15–20 minutes after infection.¹³

We should comment here on a rather important aspect of this experiment which is relevant for interpretation, and that is the length of the bacteria used in the experiment. After two generations in 5-BU medium, heavy bacteria cease to divide, and become filamentous. These filaments have an average length up to ten times that of normal bacteria. We have shown that biparental molecular recombination was significantly reduced in long bacteria simultaneously infected with phage of different densities.¹⁰ For that matter, even in short bacteria, interparental recombination only occurs when at least ten bacteriophage are used for infection and cannot be detected with an average MOI of 4, which was the highest multiplicity of infection used in these experiments. Therefore, polymerase diffuses through the infected cell much more readily than fragments of parental DNA.

Summary.—DNA of Am. N122 labeled with P³² and DNA of the wild mutant labeled with H³ replicate in perfect synchrony after simultaneous infection of *E. coli* B, a nonpermissive host for amber mutants. This contradicts the possibility of the production of polymerase in close proximity of the parental DNA, and preferential use of the polymerase by the phage which coded for it. Our results strengthen the hypothesis that polymerase is formed at random locations with re-

spect to the parental DNA which coded for it. Thus, after synthesis, polymerase is equally likely to initiate replication of any phage DNA within the infected host cell.

A similar argument was presented for the model of molecular recombination, using the nonrecombining mutant Am. 453.

Abbreviations: 5-BU, 5-bromodeoxyuridine; "light," not substituted with 5-BU; "heavy," substituted with 5-BU; EDTA, solution of 0.015 M EDTA, 0.15 M NaCl, pH 8.2; MOI, multiplicity of infection.

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^{10a} This was reconfirmed by the infection of a heavy host with the light, radioactive amber N122. The extracted intracellular DNA overlapped perfectly, in CsCl, with the H³ light reference DNA even after 2 hr after infection.

¹¹ DeWaard, A., A. V. Paul, and I. R. Lehman, these PROCEEDINGS, 54, 1241 (1965).

¹² Kozinski, A. W., P. B. Kozinski, and P. Shannon, these PROCEEDINGS, 50, 746 (1963).

^{12a} It is important to note that the progeny phage, resulting from the simultaneous infection of the nonpermissive host with the wild and amber mutants, contain particles of both genetic types.

¹³ Kozinski, A. W., and P. B. Kozinski, these PROCEEDINGS, 52, 211 (1964).