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Molecular Mechanism of Genetic Recombination in Bacteriophage T7

by

Yoshihide Tsujimoto

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Part A

INTERMEDIATES IN GENETIC RECOMBINATION OF BACTERIOPHAGE T7 DNA

Summary

DNA molecules formed by interaction of two molecules of phage T7 DNA were isolated from infected bacteria incubated under the condition which did not permit phage DNA replication. After 594 su^- bacteria were infected with ^{32}P - and 5-bromodeoxyuridine (BrdU)-labelled T7 phage carrying 5 amber mutations in genes, 1.3, 2, 3, 4, and 5, intermediate density molecules were isolated by CsCl density gradient centrifugation. The intermediate density fraction contained dimeric T7 DNA which was purified by CsCl band centrifugation. The dimeric DNA molecules had "H" or "X" configuration. Two branches at each branch point were equal in length, and the sum of the lengths of one of the branches at each branch point and the length between two branch points was equal to the unit length of T7 phage DNA.

When doubly branched molecules obtained by co-infection of [^{32}P]T7 phage with an EcoRI-sensitive mutation (Tsujiimoto & Ogawa, 1977) and BrdU-T7 phage without the mutation were digested by EcoRI restriction endonuclease, only one among four branches of the molecule was cleaved by EcoRI endonuclease. This result indicates that the doubly branched molecules consisted of two parental DNA molecules. By using the EcoRI-cleaved site as a marker, the regions between two branch points were mapped. The regions did not seem to distribute randomly on the molecules. The size of the region varied with the mean length of about 1000 nucleotide pairs.

The product of gene 6, 5'-exonuclease, is required for the formation and/or maintenance of doubly branched molecules.

Models of genetic recombination that involve formation of a branched molecule as an intermediate are discussed.

Introduction

One of the approaches for understanding the molecular mechanism of genetic recombination of bacteriophage is to isolate and characterize intermediate DNA molecules in the recombination process. For the phage to be used in such an approach, it is desirable that: the genome is small enough to be isolated as an intact DNA molecule; exists in the infected cells in simple forms; and the efficiency of recombination of the genome is high. Using phage T4 (Tomizawa & Anraku, 1964; Broker & Lehman, 1971) and recently phage λ (Tsujimoto & Ogawa, manuscript in preparation), molecules which were presumed to be formed as intermediates in genetic recombination were isolated. However, the large size of T4 phage genome makes isolation of a whole structure of interacting molecules difficult and the variety in conformations of the phage λ genome in infected cells introduces complication in the analysis of the structures. In contrast, the system with phage T7 will be shown to satisfy the above mentioned conditions and provides a more suitable system to study the mechanism of genetic recombination. The genome of phage T7 is a linear duplex of molecular weight 2.5×10^7 (Freifelder, 1970), about a fifth of the T4 phage genome, which in the absence of DNA replication or even in the early stage of replication presents mostly in monomeric linear structures (Wolfson *et al.*, 1972). The T7 system shows high recombination efficiency: The genetic map comprises about 200 map units (Studier, 1969) and the recombination efficiency in the standard cross is about that of T even phages. Moreover, considerable informations have accumulated concerning the physiology of the phage (Studier, 1972). Many mutants were isolated (Studier, 1969; Hausman & Gomez, 1967) and several genes involved in DNA replication and genetic recombination were identified (Studier, 1969; Powling & Knippers, 1974; Kerr & Sadowski, 1975).

In this paper, isolation and characterization of molecules formed by interaction of two T7 DNA molecules are described and the role of the molecules as intermediate in genetic recombination of phage T7 is discussed.

Materials and Methods

(a) Bacteria

The following Esheria coli K12 strains were used: Q1 suII (Signer & Weil, 1968) used as the host for preparation of phage stocks and for phage crosses; C600 suII thy⁻ (Ogawa & Tomizawa, 1967) was used to prepare BrdU- or ³H-labelled phage : 594 su⁻ (Campbell, 1965) used as a host for the isolation of the branched DNA molecules of T7 phage. A745 (Col E1) (Sakakibara & Tomizawa, 1974) for preparation of colicine E1 plasmid (Col E1) DNA.

(b) Bacteriophage

T7 amber mutants used were am64 in gene 2 (DNA synthesis), am29 in gene 3 (Endonuclease), am208 in gene 4 (DNA synthesis), am28 in gene 5 (DNA polymerase), am233 in gene 6 (5'→3' exonuclease) and amHA13 in gene 1.3 (ligase). T7 amHA13 (Masamune et al., 1971) was obtained from Dr. Y. Masamune, and the rest of the mutants (Studier, 1969) were obtained from Dr. W. Studier. The mutation, sRI, which renders T7 DNA sensitive to restriction by EcoRI endonuclease is described elsewhere (Tsujimoto & Ogawa, 1977).

(c) Buffers and Media

Tryptone broth (T-broth) contains 1% Bactotryptone (Difco) and 0.25% NaCl. It was used for the preparation of phage stocks and the growth of the host cells for recombination. Cas-λbroth contains 1% Casamino acids (Difco), 0.5% NaCl and 1 μg/ml thiamine. TCG-medium contains 50 ml of 20% Casamino acids (containing 20 μg phosphorus/ml), 2 g glucose, 5.4 g NaCl, 3.2 ml 0.1 M-Na₂SO₄, 3 g KCl, 1.1 g NH₄Cl, 2.58 ml 0.1 M-KH₂PO₄, 1 ml 1 M-MgSO₄ and 100 ml 1 M-Tris- HCl, pH 7.4, per liter. The phosphorus content of the medium is 9 μg/ml.

SSC contains 0.15 M-NaCl and 0.015 M-sodium citrate, pH 7.2. T7 buffer contains 10 mM-Tris-HCl, pH 7.4, 5% NaCl, 0.01% gelatine and 1 mM-MgSO₄.

(d) Preparation of labelled phages

[³²P]phage; An over-night culture of Q1 in TCG medium was diluted 40 times into the same medium supplemented with ³²PO₄ (final specific radioactivity, 0.2 to 0.3 Ci/mMole) and grown at 37°C to 3 × 10⁸ cells/ml. The cells were infected with phage T7 at a multiplicity of 0.1 and incubated until lysis of most of the cells (about 90 min). NaCl was added to the lysate to a final concentration of 5% with a few drops of chloroform. After removal of cell-debris by centrifugation, the supernatant was further centrifuged for 60 min at 30,000 rev./min in a Spinco #30 rotor. The pellet was suspended in 0.1 ml of T7 buffer and layered onto a sucrose gradient (5 to 20%) in 4.6 ml of T7 buffer. After centrifugation at 20,000 rev./min for 45 min at 15°C in a Spinco SW50.1 rotor, the tube was punctured and phage was collected by drop collection. Phage was dialysed against T7 buffer over-night.

[³H]phage; C600 thy⁻ cells were grown to 3 × 10⁸ cells/ml in T-broth supplemented with 2 µg/ml thymidine and 8 µCi/ml of [³H]thymidine and infected with phage T7 at a multiplicity of 0.1. The lysate was prepared and phage was purified as described above for [³²P]phage.

BrdU-phage; C600thy⁻ cells were grown to 1 to 2 × 10⁸ cells/ml in Cas-λ broth supplemented with 5 µg/ml thymine, harvested by centrifugation and resuspended in Cas-λ broth containing 9 µg/ml 5-bromodeoxyuridine (BrdU, Sigma) and 1 µg/ml thymine. After cultivation for 90 min, cells were infected with phage T7 at a multiplicity of 0.1. The infected cells were incubated for about 90 min, when complete lysis occurred, and NaCl and chloroform were added. After removal of cell debris by centrifugation, the lysate was centrifuged

further at 30,000 rev./min for 60 min in a Spinco #30 rotor. The phage pellet was suspended in T7 buffer.

(e) Mixed infection with labelled phages and extraction of DNA

Cells of 594 su⁻ were grown to a density of 2 to 3 × 10⁸ cells/ml in T-broth at 32°C and infected with an average of 20 each of ³²P- and BrdU-labelled T7 phages per cell. After 5 min of adsorption at 32°C, 50 to 60% of the phage particles were adsorbed. The cells were incubated further for 15 to 20 min and then one-fifth volume of 0.1 M KCN was added. The cells were harvested by centrifugation in cold and resuspended in SSC containing 50 mM EDTA and 500 µg/ml of lysozyme to 1.5 × 10⁹ cells/ml. The suspension was immediately frozen in a dry-ice acetone bath. A frozen sample was thawed in a water bath at the room temperature. Freezing and thawing were repeated 3 times. Then one-tenth of the volume of 10% sodium N-lauroyl sarcosinate and of 1 mg/ml pronase (self-digested for 4 hr at 37°C and then heated at 80°C for 3 min) were added. After incubation at 37°C for 60 min, CsCl was added and the density of the solution was adjusted to 1.72 g/cm³. The sample was centrifuged at 36,000 rev./min for 40 hr at 15°C in a Spinco #40 rotor. One centrifuge-tube contained the material from 2 to 3 × 10⁹ infected cells in 5 ml of the solution. Re-centrifugation of an intermediate density fraction in a CsCl gradient was carried out in the presence of 0.01% of sodium N-lauroyl sarcosinate.

DNA molecules in intermediate density fraction were fractionated by band sedimentation in a CsCl density gradient. The 4 ml of CsCl solution containing 0.1 M NaCl and 0.01 M sodium citrate with a density of 1.48 g/cm³ was centrifuged in a Spinco SW 50.1 rotor at 30,000 rev./min for 2 hr at 15°C to form a density gradient. After application of 0.1 to 0.25 ml of a sample (previously dialyzed against 0.1 M NaCl and 0.01 M sodium citrate) on the top

of the gradient, the sample was further centrifuged for 3 hours and fractionated by drop collection.

(f) EcoRI endonuclease reaction

A mixture of T7 DNA and Col E1 DNA, approximately 1.7 ng/ml and 2 ng/ml respectively, was treated with EcoRI endonuclease at 37°C in 0.1 M-Tris-HCl, pH 7.5, 10 mM-MgSO₄ for 60 min to 80 min. During the incubation, more than 90% of Col E1 DNA were cleaved. The reaction was terminated by adding EDTA to 20 mM. The endonuclease was kindly supplied by Drs. Y. Takagi and K. Matsubara. Col E1 DNA was prepared from A745 (Col E1) as described in Clewell and Helinski (1970).

(g) Electron microscopy

The DNA was mounted for electron microscopy by the aqueous technique described by Davis et al. (1971). Fractions from a CsCl density gradient were dialyzed against SSC or 0.25 M ammonium acetate. Col E1 DNA was usually added for calibration of the length of the molecule. The DNA sample treated with EcoRI was directly spread on 0.25 M-ammonium acetate after addition of cytochrome C.

Results

(a) Isolation of branched DNA molecules formed in the absence of DNA synthesis

With T4 phage, Tomizawa and Anraku (1964) found that the early process of recombination is separable from replication. Their methods allowed analyses of DNA molecules formed by recombination without complication caused by DNA replication. In this study we followed their principle.

It is known that six genes of T7, namely genes, 1.3 (ligase), 2 (DNA synthesis), 3 (endonuclease), 4 (DNA synthesis), 5 (DNA polymerase) and 6 (5'-^{Masamune et al}, 1971). exonuclease), are involved in T7 DNA replication (Studier, 1969; [^]To observe the events which might occur in the infected cells in the absence of DNA replication it seems to be most desirable to use T7 phage carrying mutations in all of the above six genes. However, as it will be presented in section (c), the function of gene 6 was found to be necessary for the formation of a stable interaction between T7 DNA molecules. Therefore, a mutant phage simultaneously defective (amber mutations) in genes, 1.3, 2, 3, 4 and 5 was used as the phage in the cross under the standard DNA negative condition. Cells of non-permissive bacteria, 594, were infected with ³²P- and BrdU-labelled T7 phages carrying above multiple amber mutations and incubated for 20 min at 32°C. DNA molecules were extracted and centrifuged in a CsCl gradient (Fig. 1(a)). Fractions 19-24 in Fig. 1 (a) which were heavier in density than the light DNA, were pooled and recentrifuged in a CsCl gradient. As shown in Fig. 1 (b), two peaks were obtained: one was at the fully-light position and another at the half-heavy position. Fractions 23-25 in Fig. 1 (b) were pooled and, after dialysis, the DNA was layered on a preformed CsCl density gradient and centrifuged. Two distinct peaks were observed; one peak sedimenting with or slightly faster, and the other sedimenting 1.3 to 1.4 times faster, than T7

phage DNA of the unit length (Fig. 2). DNA molecules in the faster-sedimenting peak, fractions 17-18 in Fig. 2, were examined in the electron microscope. Out of 53 molecules scored, 38 molecules (72%) were doubly branched molecules, and 12 (23%) were singly branched molecules, and the remaining 3 (5%) were linear molecules without a branch. Almost all doubly branched molecules (35 among 38) had "H" like structure (Plate I (a)(b)) and 3 had "X" like structure (Plate II (a)). The measurement of length of doubly branched molecules revealed that, with most of the molecules (82%), the lengths of two branches from the same branch point were equal, and that the sum of length of one of branches at each branch point and the length of the region between two branch points made the unit length of T7 phage DNA (data not shown; see the following section). These results suggest that the branched molecules are composed of two parental DNA molecules. The lengths of two branches in a singly branched "Y" like molecule were equal but varied in length from molecule ^{to molecules} _A (data not shown). The sum of the length of one of the branches and that of the stem part of the "Y" like molecule was equal to the unit length of T7 DNA. They probably arose by the loss of one of the branches of the "H" like molecules.

With some of the "H" like molecules (2 among 28) a very short DNA strand projected from a branch point was observed (see Plate I(a)). It probably formed by branch migration (Lee *et al.*, 1970; Broker & Lehman 1971) which occurred in bacteria or during the preparation of the molecule.

The molecules in the slow sedimenting peak in Fig. 2 (fraction 21-23) sedimented together with the reference T7 phage DNA in a sucrose gradient (5 to 20% in SSC). These were probably linear monomeric molecules which might ^{have} _A arisen by the loss of one each of the paired branches of the "H" like molecules. Further investigation on these molecules are in progress.

(b) Doubly branched molecules are biparental

To show that the doubly branched molecules are biparental, we used a mutant phage which has a sensitive site to EcoRI endonuclease (Tsujiimoto & Ogawa, 1977), as one of the parents. The mutation was located between gene 5 ^{am28 in} and gene 6, _{am233 in} and at the position of 0.46 fractional length from the left end of T7 DNA. The DNA is cleaved by the endonuclease at that position.

The crosses were performed between ³²P-labelled T7 phage carrying this EcoRI sensitive site, 1.3, 2, 3, 4, 5, sRI, and BrdU-labelled T7 phage not carrying the site, 1.3, 2, 3, 4, 5, sRI^o. If doubly branched molecules were replicating molecules, they would not be cleaved by EcoRI endonuclease since BrdU-DNA of the sRI^o phage alone would have entered into the hybrid density fraction in the light medium. On the other hand, if they were biparental, one among the four branches would be cleaved by EcoRI endonuclease. Following the same procedures described in the section (a), the fraction containing branched molecules was obtained. DNA molecules in the fraction, with or without treatment with EcoRI endonuclease, were examined by electron microscope. A line diagram of the doubly branched molecules without the EcoRI endonuclease treatment, is shown in Fig. 3 (a). The molecules are arranged arbitrarily so that the branch point is in the left half of the diagram. For the EcoRI endonuclease treated molecules, 20 out of 21 molecules which were randomly picked had a branch with an end at or very near the position of 0.46 ^{or 0.54} fractional length from the expected end of the branch of the "H" like molecules which would have not been cleaved by the endonuclease. The photographs of representatives of these molecules are presented in Plate II (a)(b). A line diagram of 21 molecules observed is presented in Fig. 3 (b) where molecules are arranged so as to locate the cleaved end at or near the position of 0.46 fractional length from the left end. These results clearly indicate that the

doubly branched molecules are biparental.

Specific cleavage of doubly branched molecules by the endonuclease allowed us to locate the region between the branched points. As shown in Fig. 4, the region distributed from the extreme left to the extreme right but the location did not seem to distribute randomly on the molecules. The length of the regions between the branched points varied from apparent zero to 0.08 fractional length with the mean of 0.03 fractional length (about 1000 nucleotide pairs). The distribution of the length of the regions was shown in Fig. 5.

(c) Necessity of gene 6 product for formation of the doubly branched molecules.

T7 phage performs recombination normally on any rec^- hosts examined (Powling & Knippers, 1974; Kerr & Sadowski, 1975), suggesting that they have a recombination system of their own. The partial loss or the suppression of the activity of any one of the products of genes, 3, 4, 5 and 6 in T7, reduces the frequency of recombination greatly (Powling & Knippers, 1974; Kerr & Sadowski, 1975). As presented above, branched molecules were formed even under the simultaneous absence of gene 3, gene 4 and gene 5 products. However, we found that additional loss of gene 6 product reduced the formation of molecules banded at the intermediate density fractions significantly. DNA extracted from the infected cells in the cross with gene 6^+ or gene 6^- phages was centrifuged in a CsCl density gradient and fractions encompassing the half heavy density region were pooled. The pooled fractions contained 2.1% and 1.2% of the total amount of [32 P]DNA recovered on the first centrifugation in the cross with gene 6^+ and gene 6^- phage, respectively. These fractions were recentrifuged in CsCl density gradients and the results are presented in Fig. 6. About 60% of the [32 P]DNA recovered in (a), and about 20% of that in (b), were found in

the intermediate density fractions. If one assumes random loss of molecules during the handling, approximately 5 times more $[^{32}\text{P}]\text{DNA}$ would have been in the intermediate density fraction in the presence, than in the absence, of gene 6 product. The DNAs in these intermediate density fractions (fractions 20-22 in Fig. 6(a) or (b)) were analyzed further by band centrifugations. As seen in Fig. 7(a), in the presence of gene 6 products, about 60% of the intermediate density molecules sedimented 1.3 to 1.4 times faster than the reference $[^3\text{H}]$ T7 DNA. When they were examined by electron microscope most of them were found to be doubly branched molecules (data not shown) similar to the preparation described in section (a). In contrast, most of the molecules obtained under the absence of gene 6 product, sedimented with the same velocity as the reference T7 phage DNA, and only 13% of the molecules sedimented faster than the reference DNA (Fig. 7(b)). Therefore, in the absence of gene 6 product, the amount of the half-heavy density molecules sedimenting 1.3 to 1.4 times faster than T7 phage DNA was less about one twentieth than that obtained in the presence of gene 6 product. These results indicate that the product of gene 6, 5' to 3' exonuclease, is needed for the formation and/or maintenance of doubly branched molecules at least in the absence of the products of genes, 1.3, 3, 4, and 5.

Discussion

DNA molecules with intermediate density were isolated from nonpermissive bacteria infected with ^{32}P - and BrdU-labelled T7 phage, both carrying amber mutations in genes, 1.3, 2, 3, 4 and 5. A majority of the molecules sedimented 1.3 to 1.4 times faster than T7 DNA of the unit length. When examined by the electron microscope, about 70% of the faster sedimenting molecules were doubly branched molecules having an "H" or "X" configuration. Two branches at each branch point had an equal length.. The result of the measurement of the length of these molecules suggested that they consisted of two DNA molecules of the unit length paired at the region between two branch points. When one of the parental phage has an EcoRI-sensitive mutation, only one among four branches of the molecule was cleaved by the endonuclease, indicating that the doubly branched molecule consists of two parental DNA molecules. The region between two branch points seems to be double stranded since the width of the DNA strand at this region in the electron microscopic photograph is the same as that of the other parts of the molecule. Therefore we concluded that "H" or "X" shaped molecules consisted of two homologous DNA molecules paired with each other at exposed complementary strands. The length of the paired regions varied from apparent zero to 0.08 fractional length. The mean length was 0.03 fractional length that corresponds to about 1000 base pairs.

Those "H" and "X" shaped molecules are most likely the intermediates in recombination: the amount of these molecules in infected bacteria is either increased or reduced when a certain function which is known to affect the frequency of genetic recombination of phage T7 is inactivated by a mutation (sections, (a) and (c)); they are biparental (section (b)). This conclusion is supported by the successful transfection to the bacteria with doubly

branched molecules which were prepared by the infection of ^{32}P - and BrdU-labelled phages. Approximately a half of the progeny clones formed by the transfection of the branched molecules consisted mostly of recombinants and the rest of the clones consisted mostly of progeny particles of one of the parental types with a small fraction of recombinants (described in Part C).

(a) Synapsis

An amber mutation in gene 6 greatly reduced the amount of doubly branched molecules in DNA isolated after T7 phage infection. Since gene 6 codes 5' to 3' exonuclease which can initiate hydrolysis from a nick or gap (Kerr & Sadowski, 1972), a single-stranded region expanded by this enzyme would allow pairing of homologous DNA at the region (Pathway A in Fig. 8). For this to occur, strand breaks have to be introduced on the opposite strand of two DNA molecules to be paired. Labile pairing might first occur without formation of a single-stranded gap (Pathway B in Fig. 8) or without even a nick (Pathway C in Fig. 8). The exonuclease, together with other appropriate nucleases, might expand the pairing region and thus enhancing or stabilizing the pair formation (Pathway B & C). Mapping of the paired regions showed that they were not distributed at random. Pairing regions might be distributed at random along the genome at first, and only relatively stable pairs alone might be remained, or, by branch migration, pairing regions might be fixed at relatively stable regions. Alternatively, this non-randomness might directly reflect the primary non-random events. For instance, a putative endonuclease might introduce non-random nicks.

(b) Maturation

Requirement of the products of genes, 3, 4 and 5 for recombination is suggested by genetic results (Powling & Knippers, 1974; Kerr & Sakowski, 1975). Our results show that these gene products are not required for the formation of the doubly branched intermediates. Therefore, the products of genes, 3, 4 and 5 are probably involved in maturation of the intermediates formed by participation of the gene 6 product. Gene 3 endonuclease which digests single-stranded DNA more efficiently than double-stranded DNA (Center & Richardson, 1970) may be a candidate for the nuclease to trim branches of a doubly branched molecule by endonucleolytic cleavage of a single stranded portion at the branch point. In consequence, four kinds of mature recombinant molecules would be formed: both side of the exchange region which is heterozygous, would be either one of the recombinant types or one of the parental types (Fig. 8). DNA polymerase, specified by gene 5 (together with the product of gene 4) may fill gaps remained after trimming of branches of "H" or "X" shaped molecules, and final nicks may be sealed by viral or bacterial DNA ligase.

In phage T4, using a similar method, DNA molecules formed by interaction of two parental DNAs have been isolated although almost all of isolated molecules were not in an intact form because of their large size (Tomizawa & Anraku, 1964). Some of them closely resembled branched molecules mentioned above (Anraku & Tomizawa, 1971; Broker & Lehman, 1971). DNA binding protein (T4 gene 32 product) is necessary for the interaction to occur (Tomizawa *et al.*, 1966; Alberts & Frey, 1970).

Although a variety of models of genetic recombination have been proposed with various systems (see: Signer, 1971; Hotchkiss, 1974), an intact form of the intermediate DNA molecules in recombination have not been isolated. The isolation of an intact form of the intermediates with biological activity will allow us to elucidate the mechanism of each step in genetic recombination of

the DNA molecules.

REFERENCES

- Alberts, B. M. & Frey, L. (1970). Nature, 227, 1313-1318.
- Anraku, N. & Tomizawa, J. (1971). In Methods in Enzymology (Grossman, L. & Moldave, K., eds.), vol. 21, pp. 289-296, Academic Press, New York.
- Broker, T. R. & Lehman, T. R. (1971). J. Mol. Biol. 60, 131-149.
- Campbell, A. (1965). Virology, 27, 329-339.
- Center, M. S. & Richardson, C. C. (1970). J. Biol. Chem. 245, 6285-6291.
- Clewell, D. B. & Helinski, D. R. (1970). Proc. Nat. Acad. Sci., U.S.A. 62, 1159-1166.
- Davis, R. W., Simon, M. & Davidson, N. (1971). In Methods in Enzymology (Grossman, L. & Moldave, K., eds.), vol. 21, pp. 413-428, Academic Press, New York.
- Freifelder, D. (1970). J. Mol. Biol. 54, 567-577.
- Hausman, R. & Gomez, B. (1967). J. Virol. 7, 779-792.
- Hotchkiss, R. D. (1974). Annu. Rev. Microbiol. 28, 445-468.
- Kerr, C. & Sadowski, P. D. (1972). J. Biol. Chem. 247, 311-318.
- Kerr, C. & Sadowski, P. D. (1975). Virology, 65, 281-285.
- Lee, C. S., Davis, R. W. & Davidson, N. (1970). J. Mol. Biol. 48, 1-22.
- Masamune, Y., Frenkel, G. D. & Richardson, C. C. (1971). J. Biol. Chem. 246, 6874-6879.
- Ogawa, T. & Tomizawa, J. (1967). J. Mol. Biol. 23, 225-245.
- Powling, A. & Knippers, R. (1974). Mol. Gen. Genet. 134, 173-180.
- Sakakibara, Y. & Tomizawa, J. (1974). Proc. Nat. Acad. Sci., U.S.A. 71, 802-806.
- Signer, E. R. & Weil, J. (1968). J. Mol. Biol. 34, 261-271.
- Signer, E. R. (1971). In The Bacteriophage Lambda (Hershey, A. D., ed.), pp. 139-174, Cold Spring Harbor Laboratory, New York.

Studier, F. W. (1969). Virology, 39, 562-574.

Studier, F. W. (1972). Science, 176, 367-376.

Tomizawa, J. & Anraku, N. (1964). J. Mol. Biol. 8, 516-540.

Tomizawa, J., Anraku, N. & Iwama, Y. (1966). J. Mol. Biol. 21, 247-253.

Tsujimoto, Y. & Ogawa, H. (1977). Mol. Gen. Genet. in press.

Wolfson, J., Dressler, D. & Magazin, M. (1972). Proc. Nat. Acad. Sci., U.S.A.

69, 499-504.

Legend to Plate

Plate I (a) and (b) Doubly branched molecules which have "H" configuration.

Magnification 14,000 \times .

Plate II (a) and (b) EcoRI endonuclease cleaved product of doubly branched molecules, obtained from the cross between T7 sRI and T7 sRI^o. Arrows indicate the EcoRI endonuclease cleaved ends. Short fragments were ColE1 DNA added before incubation in the reaction mixture to estimate the extent of digestion.

(a) "X" configuration

(b) "H" configuration

Magnification 14,000 \times .

Legned to Figures

Fig. 1 (a)

CsCl density gradient centrifugation of T7 DNA from cells infected with 20 each of ^{32}P - and BrdU-labelled T7 1.3, 2, 3, 4, 5, phage particles per cell and incubated for 20 min at 32°C . Centrifugation was carried out in a Spinco #40 rotor at 36,000 rev./min for 40 hr at 15°C .

Fig. 1 (b)

Recentrifugation in a CsCl density gradient of the pooled fraction, 19 to 24 in Fig. 1 (a). The conditions of centrifugation were as described for (a). The position of the peak of the light DNA was determined with [^3H]T7 phage DNA added before centrifugation, and the positions of the heavy and half-heavy DNA were deduced from the density of BrdU-T7 DNA.

—○—○— : ^{32}P -radioactivity, _____ : Density

Fig. 2

Band centrifugation in CsCl density gradient of fractions 23 to 25 in Fig. 1 (b) with [^3H] T7 phage DNA. Centrifugation procedures were described in Materials and Methods (e).

—○—○— : ^{32}P -radioactivity, ---○---○--- : ^3H -radioactivity

Fig. 3

Line diagrams of doubly branched molecules of "H" and "X" configurations before (a) and after cleavage ^{with} EcoRI endonuclease (b).

Molecules of "H" or "X" configuration were obtained after crossing [^{32}P] T7 1.3, 2, 3, 4, 5, sRI and BrdU-T7 1.3, 2, 4, 5, sRI^o, followed by purification

by equilibrium and band centrifugation in CsCl solutions as described in Materials and Methods, and the legends to Fig. 1 and 2. The DNA molecules were cleaved by EcoRI endonuclease and examined by the electron microscope.

In (a), the molecules are arranged arbitrarily so that the branch points distributed at the left half, and in (b) the molecules are arranged as described in the text. A vertical line represents the position of 0.46 fractional length from the left end of the molecule.

Fig. 4

A line diagram showing distributions of paired regions of doubly branched molecules. The location of paired region was determined by cleaving the branch at the mutation site sensitive to EcoRI endonuclease.

Fig. 5

Histogram showing length distribution of the regions between two branched points of the doubly branched molecules. Thirty nine molecules shown in Fig. 3 were analyzed.

Fig. 6

Recentrifugation profiles in CsCl density gradient of the intermediate density molecules obtained by the cross of ^{32}P - and BrdU-labelled T7 phage in the presence (a) and absence of the gene 6 product. The phages used were defective in the 5 functions related to DNA replication. The experimental methods were described in Materials and Methods and the legend for Fig. 1. DNA extracted from the infected cells were centrifuged in CsCl density gradients and fractions at or near the half heavy density were pooled as described in Fig. 1 (a). The pooled fractions were mixed with [^3H] T7 phage DNA,

centrifuged in CsCl density gradients.

--- o --- o --- : ^3H -radioactivity
 —●—●— : ^{32}P -radioactivity

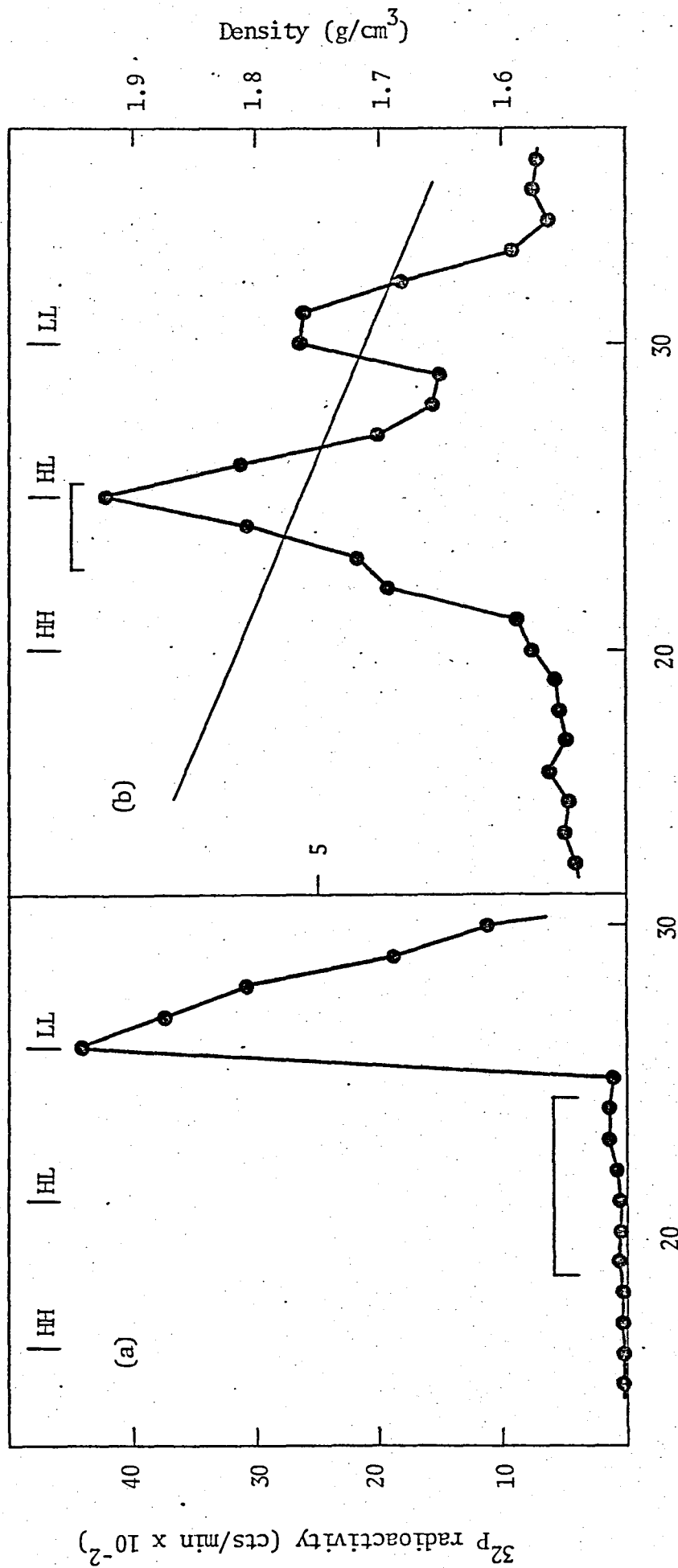
Fig. 7

Band centrifugation in CsCl density gradient of fractions, 20 to 22, in Fig. 6 (a) and (b). The total amount of the input ^{32}P -radioactivity was 1210 cts/min and 221 cts/min in (a) and (b), respectively. The recovery of the radioactivity after centrifugation was 97% and 83% in (a) and (b), respectively. [^3H] phage DNA were added before centrifugation.

—●—●— : ^{32}P -radioactivity, --- o --- o --- : ^3H -radioactivity.

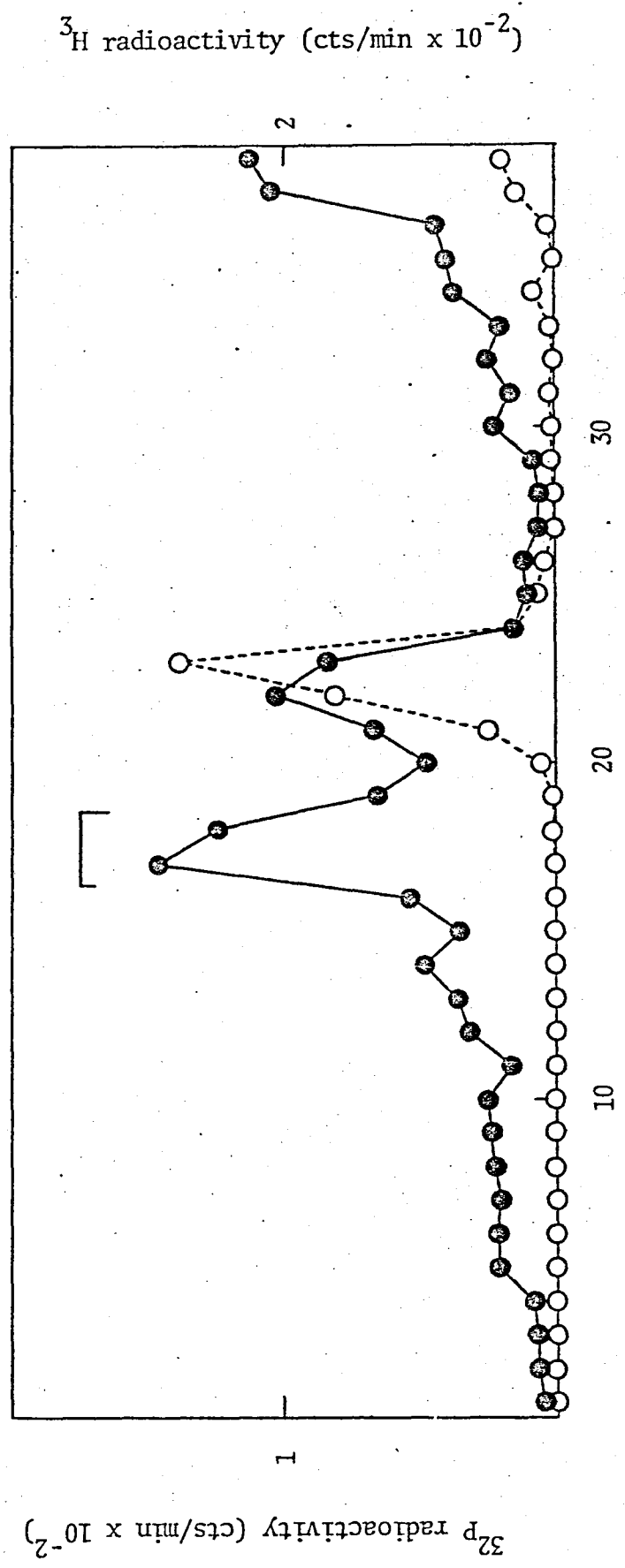
Fig. 8

Schematic representation of DNA recombination involving doubly branched molecules as intermediates. From modes of interaction between two parental DNA, three possible pathways are proposed: pathway A requires gap; pathway B requires only nick; pathway C requires neither gap nor nick. Doubly branched intermediates are processed to mature recombinant DNA by trimming of branches, followed by repair of remaining nicks and gaps.

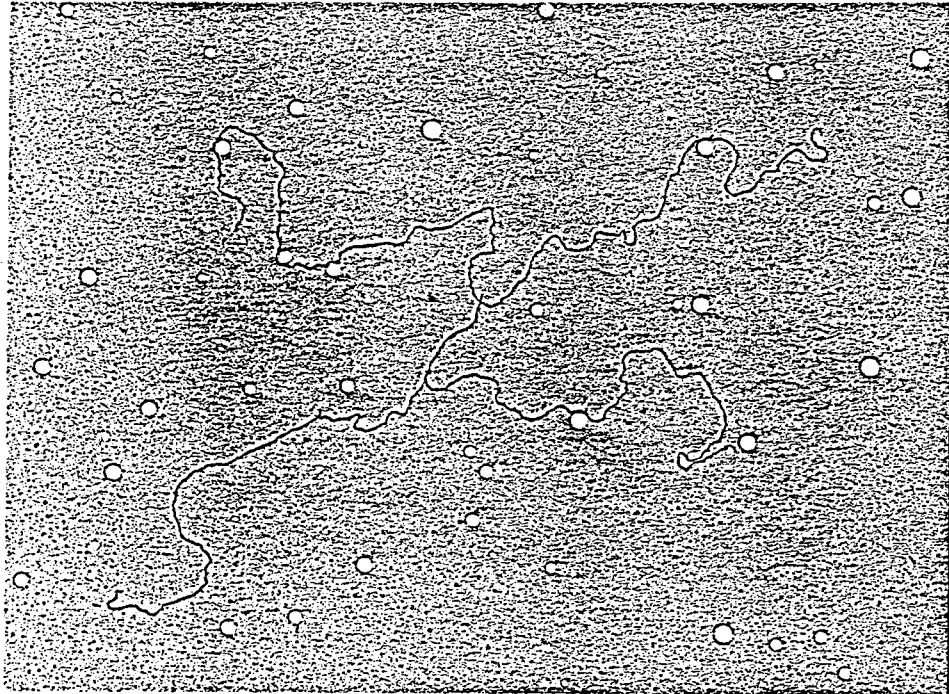


Fraction no.

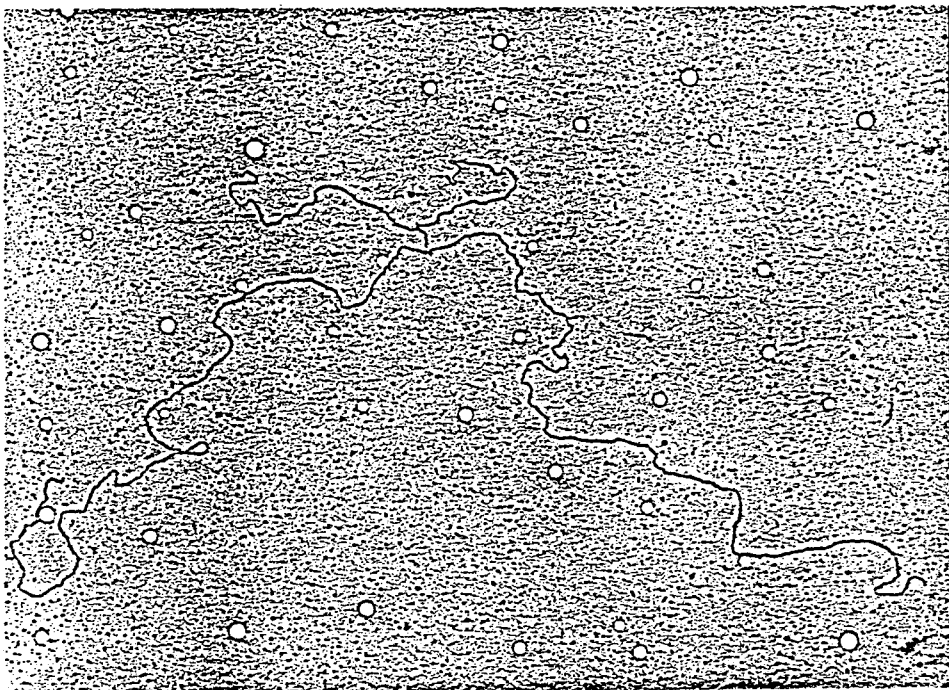
Figure 1.



Fraction no.
Figure 2.

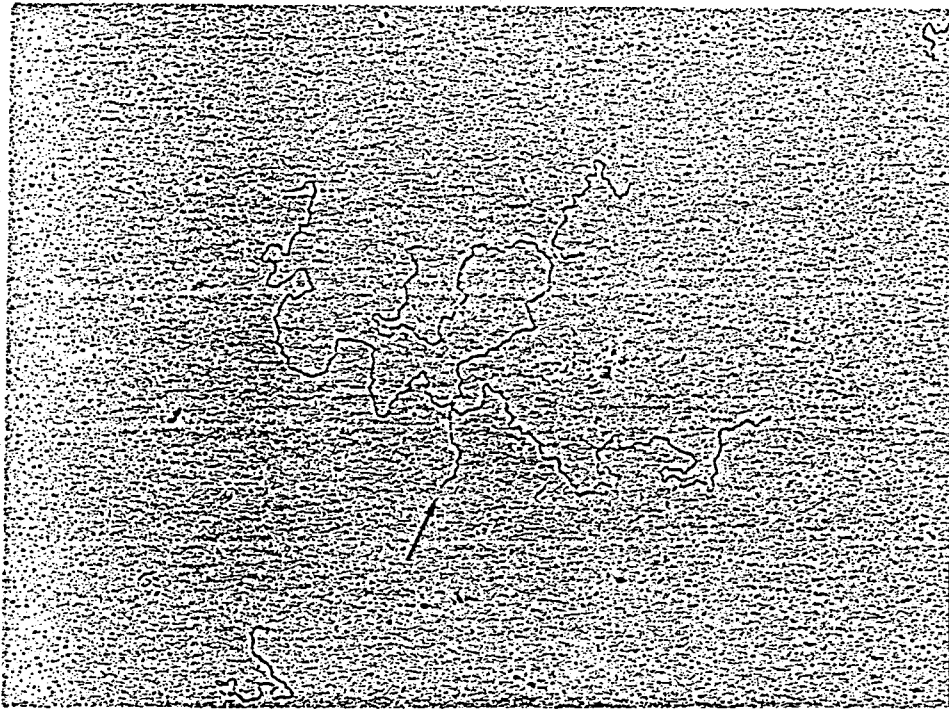


(a)

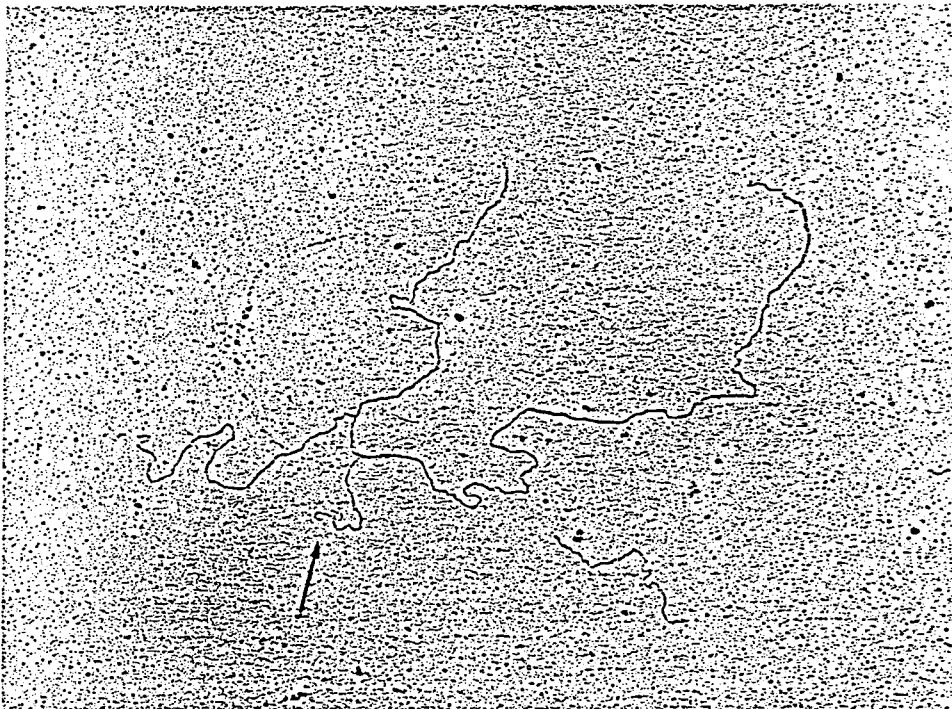


(b)

Plate I

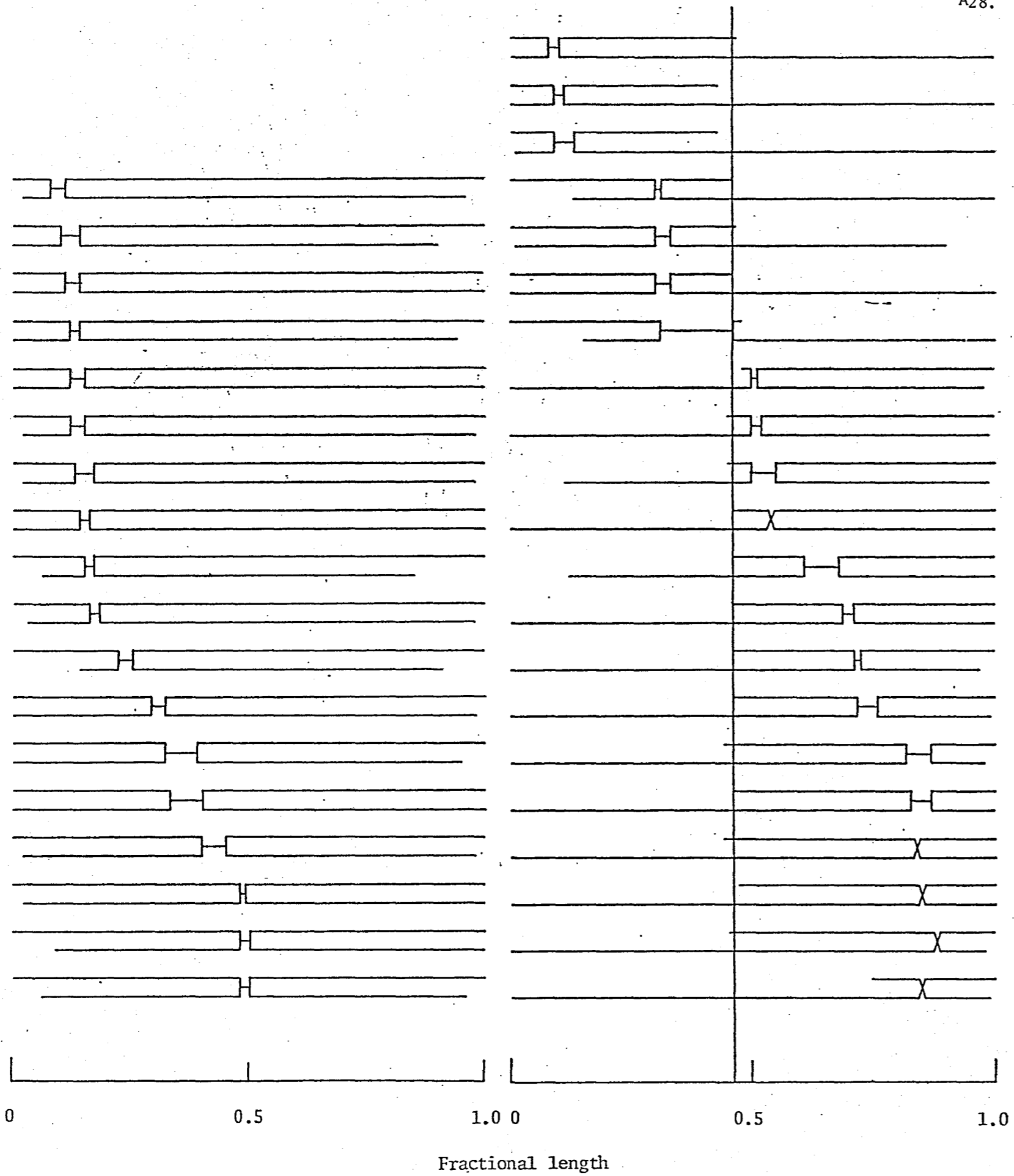


(a)

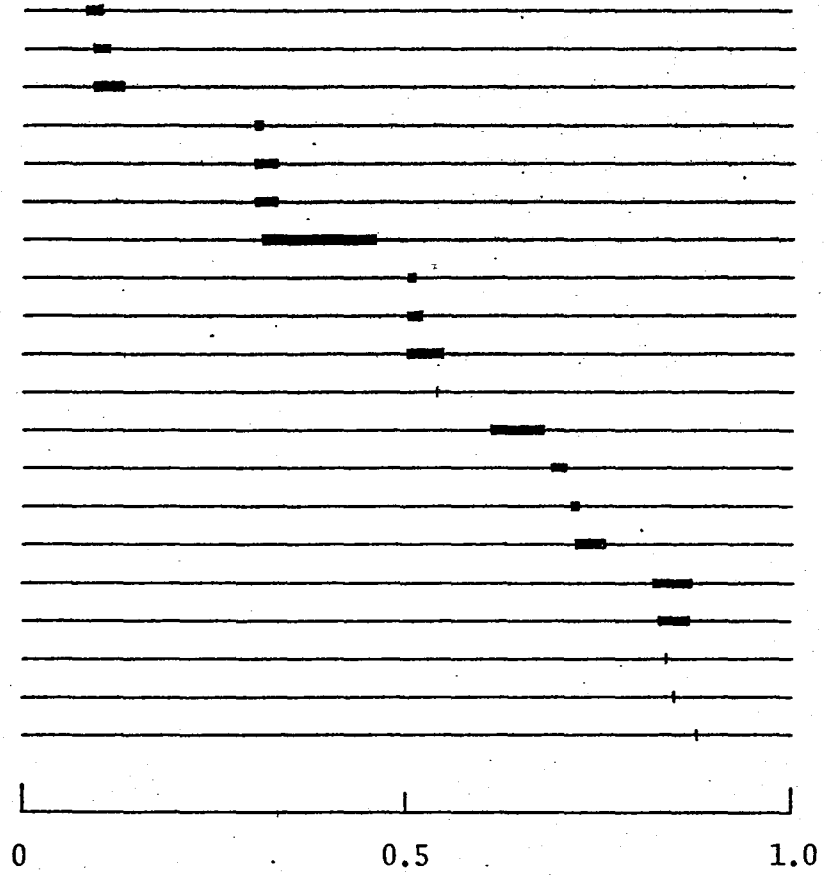


(b)

Plate II.



Fractional length



Fractional length

Figure 4.

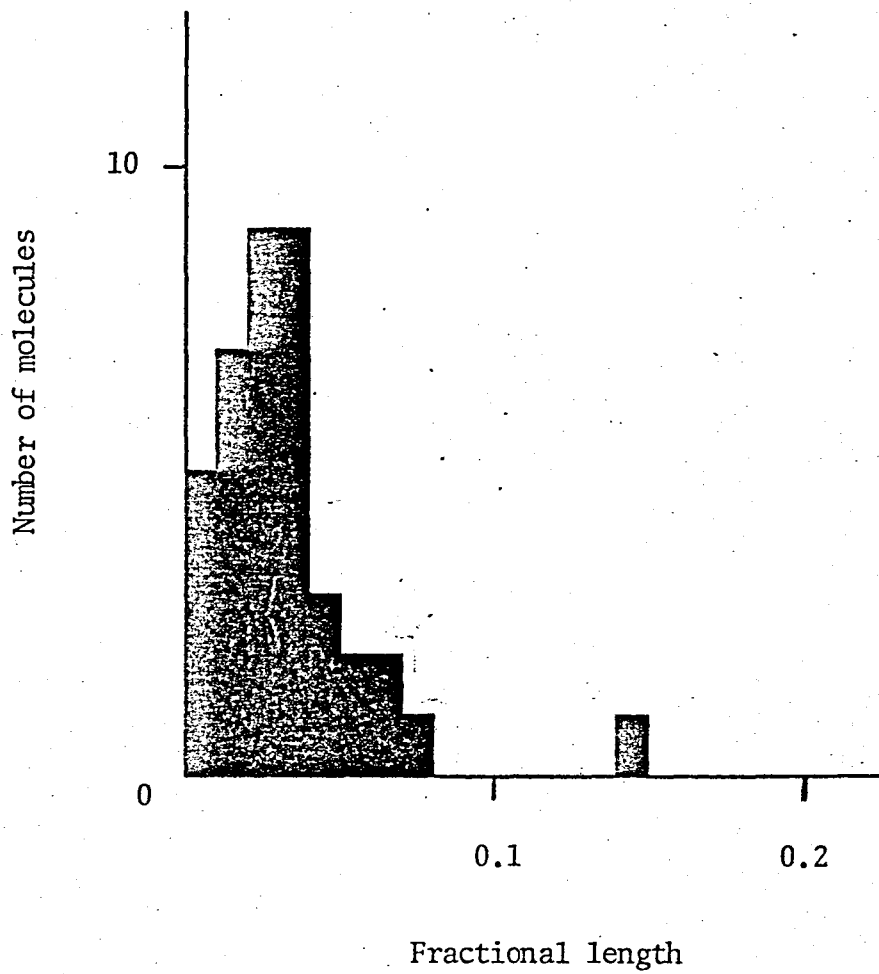


Figure 5.

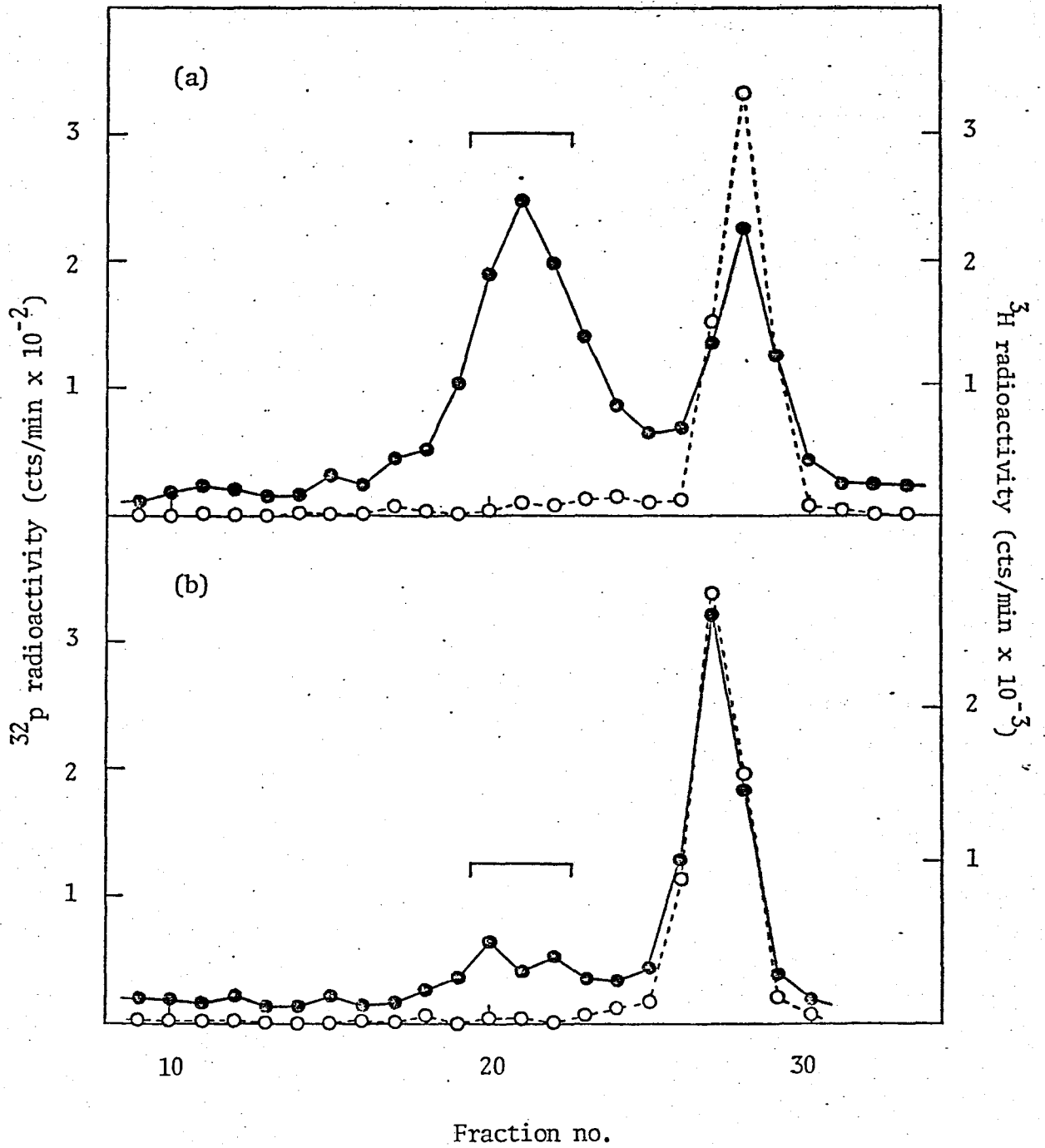


Figure 6.

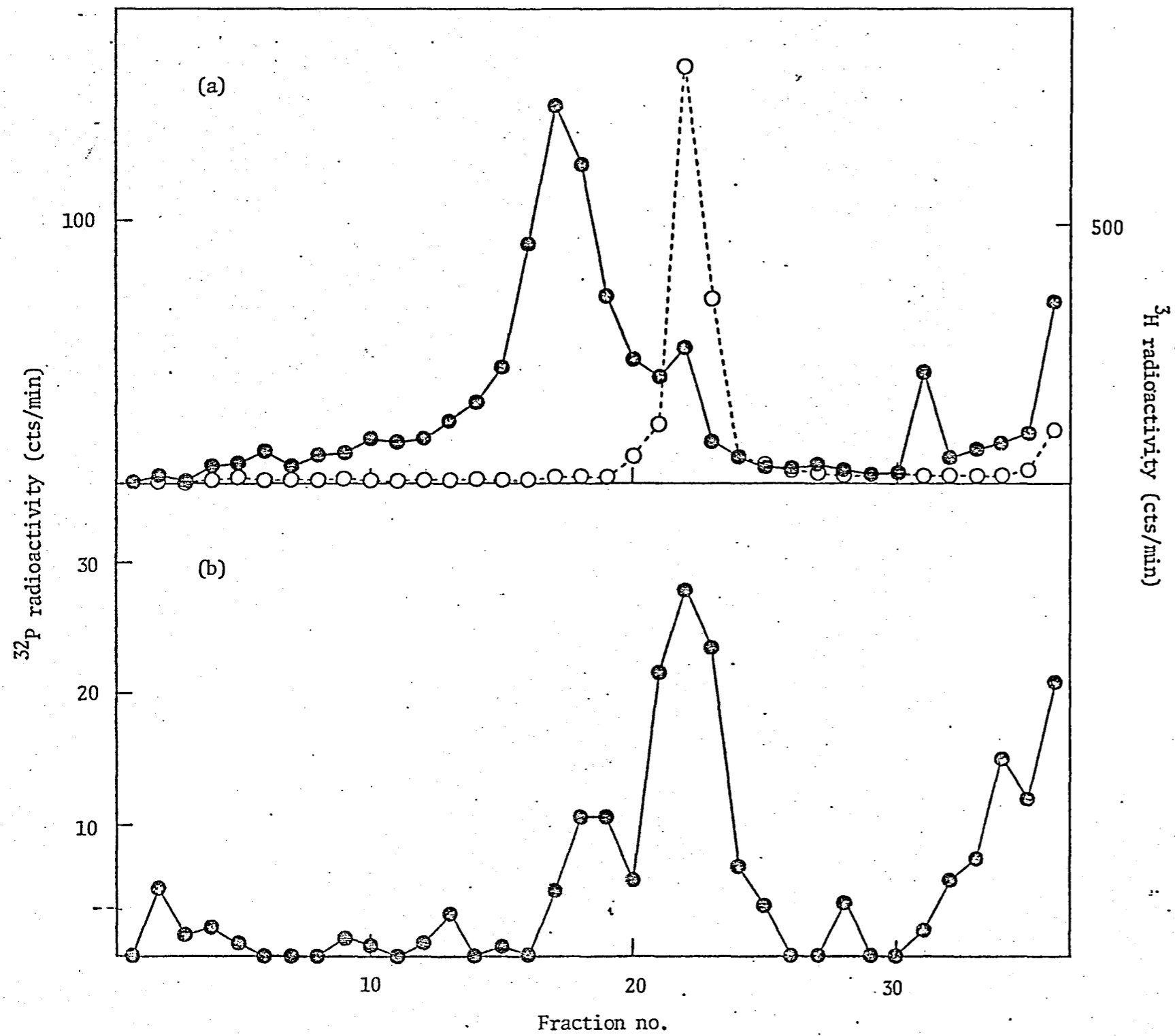


Figure 7.

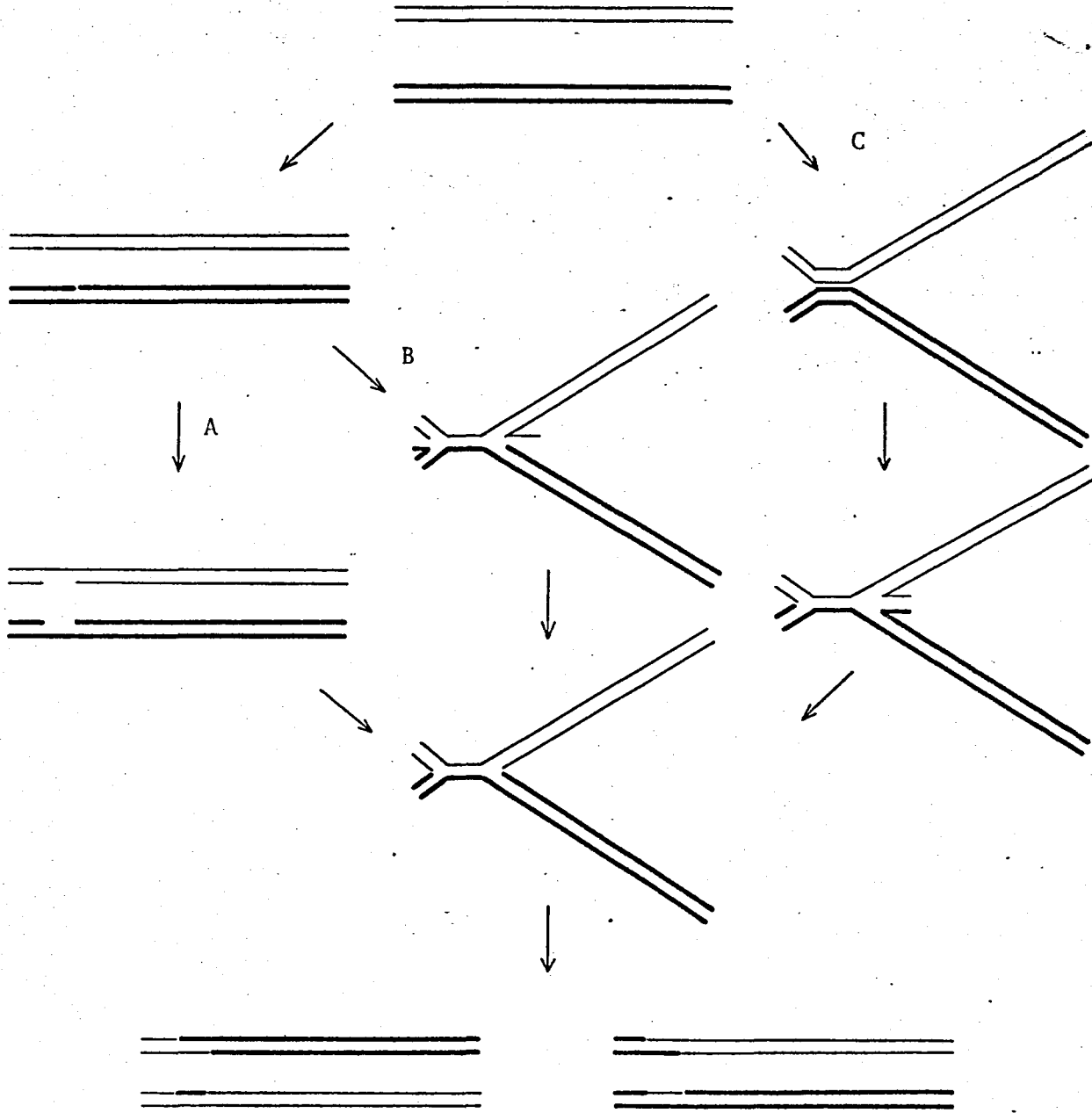


Figure 8.

Part B

EcoRI-SENSITIVE MUTATION OF T7 PHAGE

Summary

It is known that DNA from phage T7 is not cleaved by restriction endonuclease EcoRI. However, DNA from phage T7am28 (gene 5) mutant (Studier, 1969) was found to be cleaved by the endonuclease at one site. The site is located at 0.46 fractional length from the left end of the molecule. The mutation which makes T7 DNA sensitive to the endonuclease is separable from the amber mutation and located between am28 and am233 (gene 6).

Restriction endonuclease EcoRI acts on a double strand DNA at the unique base sequence, GAATTC, producing a limited number of fragments which are characteristic of any particular DNA molecules (Mertz & Davis, 1972). Although T7 DNA has a molecular weight of 2.5×10^7 daltons (Freifelder, 1970), it has been shown to resist cleavage by the endonuclease (Salser, 1974). However, DNA from a mutant phage, T7am28 carrying an amber mutation in gene 5 (DNA polymerase), one of the collections of Dr. W. Studier (1969), was found to be cleaved by the endonuclease.

DNA of phage T7am28 was treated with EcoRI endonuclease and mounted for electron microscopy by the basic protein film technique (Kleinschmidt, 1968; Tsujimoto & Ogawa, 1976). The histogram of the length distribution of fragments obtained from the cleaved molecules is presented in Fig. 1. The mean length of the two classes of fragments found were $5.83 \pm 0.18 \mu\text{m}$ and $6.87 \pm 0.31 \mu\text{m}$. The sum of these mean lengths is equal to the mean unit length of T7 DNA, $12.75 \pm 0.28 \mu\text{m}$, which was measured with the intact DNA molecules. These results showed that the cleavage site is located at 0.46 fractional length from one of the ends.

To map the EcoRI restriction site physically, two deletion mutants, T7LG37 and T7LG30 (Simon & Studier, 1973) which have a deletion of 5.8% and 5.3% of the genome, respectively, at the regions covering gene 1.3 (ligase), were crossed with T7am28 mutant. A recombinant, LG37am28 or LG30am28 was selected from the progeny as the mutant which was unable to complement either am28 phage on 594su⁻ bacteria, or amHA-13 (gene 1.3) phage (Masamune *et al.*, 1971) on 594ligts7 (Pauling & Hamm, 1969; Gottesman *et al.*, 1973) at 32°C. T7amHA-13 can not grow on the bacteria having a temperature-sensitive ligase even at 32°C. Cleavage by EcoRI endonuclease of T7am28LG37 DNA or T7am28LG30 DNA produced two classes of fragments of 0.43 and 0.57 or 0.44 and 0.56,

respectively, of the mean fractional length of the respective deleted T7 DNA (Fig. 2). These lengths correspond 0.41 and 0.54 or 0.41 and 0.53, respectively, of the fractional length of the undeleted T7 DNA. Thus the deletions reduced the length of the shorter EcoRI-fragment of T7 DNA. Since the deletions located at the region between 0.152 and 0.227 of the fractional length from the left end of the DNA (Simon & Studier, 1973), the restriction site of T7 DNA was located at 0.46 fractional length from the left end.

The mutation in T7am28 phage which rendered T7 DNA sensitive to EcoRI endonuclease (called sRI and resistant allele called sRI^o) was genetically mapped by two or three factor crosses involving am28, am233 (gene 6) and sRI and their respective wild type alleles. The use of the bacteria carrying a drug resistant factor RI-1 plasmid allowed us to distinguish between phage having the mutation sRI and that having the wild type allele. The comparison of the results of two factor crosses show that the sRI site is located between am28 and am233 (Table 1). This conclusion is confirmed by the low frequency of sRIam⁺ progeny in the three factor cross that reflects requirement of double crossover for the formation of the am⁺sRI recombinants.

In conclusion, DNA molecule from phage T7am28 (gene 5) was cleaved by the restriction endonuclease EcoRI at a site located at 0.46 fractional length from the left end of the molecule. The mutation site is separable from the amber mutation and mapped between am28 and am233 (gene 6) (Fig. 3). EcoRI sensitivity of DNA can be lost by a mutation (N. Murray & K. Murray, 1974; Rambach & Tiollais, 1974; Thomas et al., 1974). It can also be created by a mutation.

References

- Campbell, A. (1965). Virology, 27, 329-339
- Freifelder, D. (1970). J. Mol. Biol., 54, 567-577
- Gottesman, M. M., Hicks, M. L. & Gellert, M. (1973). J. Mol. Biol., 77, 531-547
- Kleinschmidt, A. K. (1968). In Methods in Enzymology (Grossman, L. & Moldave, K., eds.), vol. 12 B, pp. 361-377, Academic Press, New York, London.
- Masamune, Y., Frenkel, G. D. & Richardson, C. C. (1971). J. Biol. Chem., 246, 6874-6879
- Mertz, J. E. & Davis, R. W. (1972). Proc. Nat. Acad. Sci., U.S.A., 69, 3370-3374
- Murray, N. E. & Murray, K. (1974). Nature, 251, 476-481
- Pauling, C. & Hamm, L. (1969). Proc. Nat. Acad. Sci., U.S.A., 64, 1195-1202
- Rambach, A. & Tiollais, P. (1974). Proc. Nat. Acad. Sci., U.S.A., 71, 3927-3930
- Salser, W.A. (1974). Annu. Rev. Biochem., 43, 923-965
- Signer, E. R. & Weil, J. (1968). J. Mol. Biol., 34, 261-271
- Simon, M. N. & Studier, F. W. (1973). J. Mol. Biol., 79, 249-265
- Studier, F. W. (1969). Virology, 39, 562-574
- Thomas, M., Cameron, J. R. & Davis, R. W. (1974). Proc. Nat. Acad. Sci., U.S.A., 71, 4579-4583
- Tsujimoto, Y. & Ogawa, H. (1976). J. Mol. Biol.
- Yoshimori, R. N. (1971). Ph.D. Thesis, University of California, San Francisco Medical Center.

Table 1

Two- and three-factor crosses of T7 phage involving sRI mutation

cross	Ratio of the number of plaques formed on 594(RI-1) to that on Q1su ⁺	Ratio of the number of plaques formed on 594 to that on Q1su ⁺	Fraction of <u>sRI</u> ^o phage among progeny grown on 594(RI-1)	Fraction of am <u>sRI</u> ^o in the total progeny particles
LG37am28 <u>sRI</u> ^o × <u>sRI</u>	7.3×10^{-2}	-	0.57 (20/35)*	4.2×10^{-2}
am233 <u>sRI</u> ^o × <u>sRI</u>	4.5×10^{-2}	-	0.50 (20/40)	2.3×10^{-2}
am28 am233 <u>sRI</u> ^o × <u>sRI</u>	3.5×10^{-2}	-	0.11 (10/90)	3.9×10^{-3}
am28 <u>sRI</u> × am233 <u>sRI</u> ^o	-	6.2×10^{-2}	-	-

* The number of the sRI^o progeny over the total number of tested progeny

Legend to Table 1

Bacteriophages used were T7am233sRI° (Studier, 1969); LG37am28sRI°, which was obtained by the cross between LG37sRI° (Simon & Studier, 1973) and am28sRI (Studier, 1969); am⁺sRI, an am⁺ revertant of phage am28sRI; and am28am233sRI°, which was obtained by the cross between am28sRI and am233sRI°. Bacteria used were 594su⁻ gal⁻ strA (Campbell, 1965); 594(RI-1): 594 strain carrying a drug resistant factor, RI-1, which was derived from RY13 (Yoshimori, 1971); and Q1 suII (Signer & Weile, 1968).

All crosses were performed at 37°C. Bacteria Q1 su⁺ grown to 2×10^8 cells/ml in Trypton broth were infected with parental phages at a multiplicity of infection of 10 each. After 5 min, they were treated with anti-T7 phage serum (final value of K was 2 min^{-1}) for 5 min. The mixture was diluted 10^4 -fold into Trypton broth and shaken for 60 min followed by addition of a few drops of chloroform. The total number of the progeny particles was measured on Q1 su⁺. The number of am⁺sRI° recombinants was measured as follows. First the progeny particles were plated with 594su⁻ (RI-1). It has been shown that the plating efficiency of T7sRI on the bacteria is approximately 6×10^{-2} . The plaque formers on 594(RI-1) consisted of am⁺sRI° recombinants and am⁺sRI modified by the EcoRI modification methylase. Therefore, the plaques formed on 594 su⁻ (RI-1) were transferred to plates seeded with 594 by stabbing to eliminate the host modification, and the character, sRI or sRI°, of each phage on 594 was determined by plating with 594 and 594(RI-1).

Legend to Figures

Fig. 1 Histogram of the length distribution of the fragments formed by EcoRI endonuclease cleavage of T7am28 DNA. The reaction mixture (0.1 ml) contained 0.1 M-Tris-HCl buffer (pH 7.5), 10 mM-MgSO₄, 0.18 µg/ml of DNA and 10 µl of EcoRI endonuclease (gift from Drs. Y. Takagi and K. Matsubara). The condition of the enzyme treatment which produces the limited digest of the DNA was previously determined. After incubation at 37°C for 80 min, the reaction was stopped by the addition of EDTA to 20 mM. The DNA was mounted for electron microscopy by the basic protein film technique (Kleinschmidt, 1968). The number of molecules examined was 42.

Fig. 2 Histograms of the length distribution of the fragments formed by cleavage of T7LG37 am28sRI DNA (a) and T7LG30am28sRI DNA (b) by EcoRI endonuclease. The conditions of treatment with EcoRI endonuclease were as described in the legend to Fig. 1 except that the reaction mixture contained 0.29 µg/ml (a) or 0.45 µg/ml (b) of DNA. The number of molecules examined: 49 (a) and 40 (b).

Fig. 3 The location of the sRI mutation.

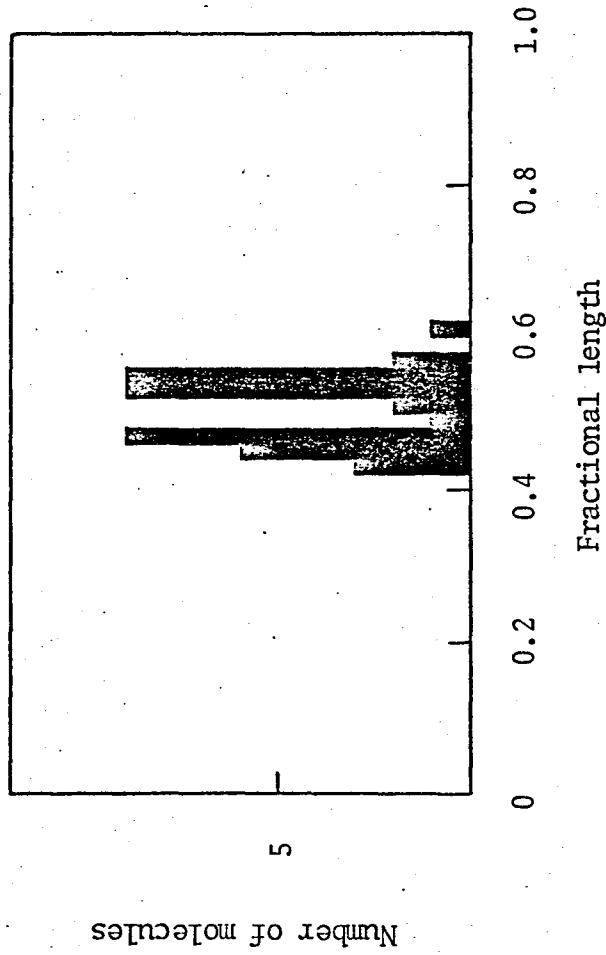


Figure 1.

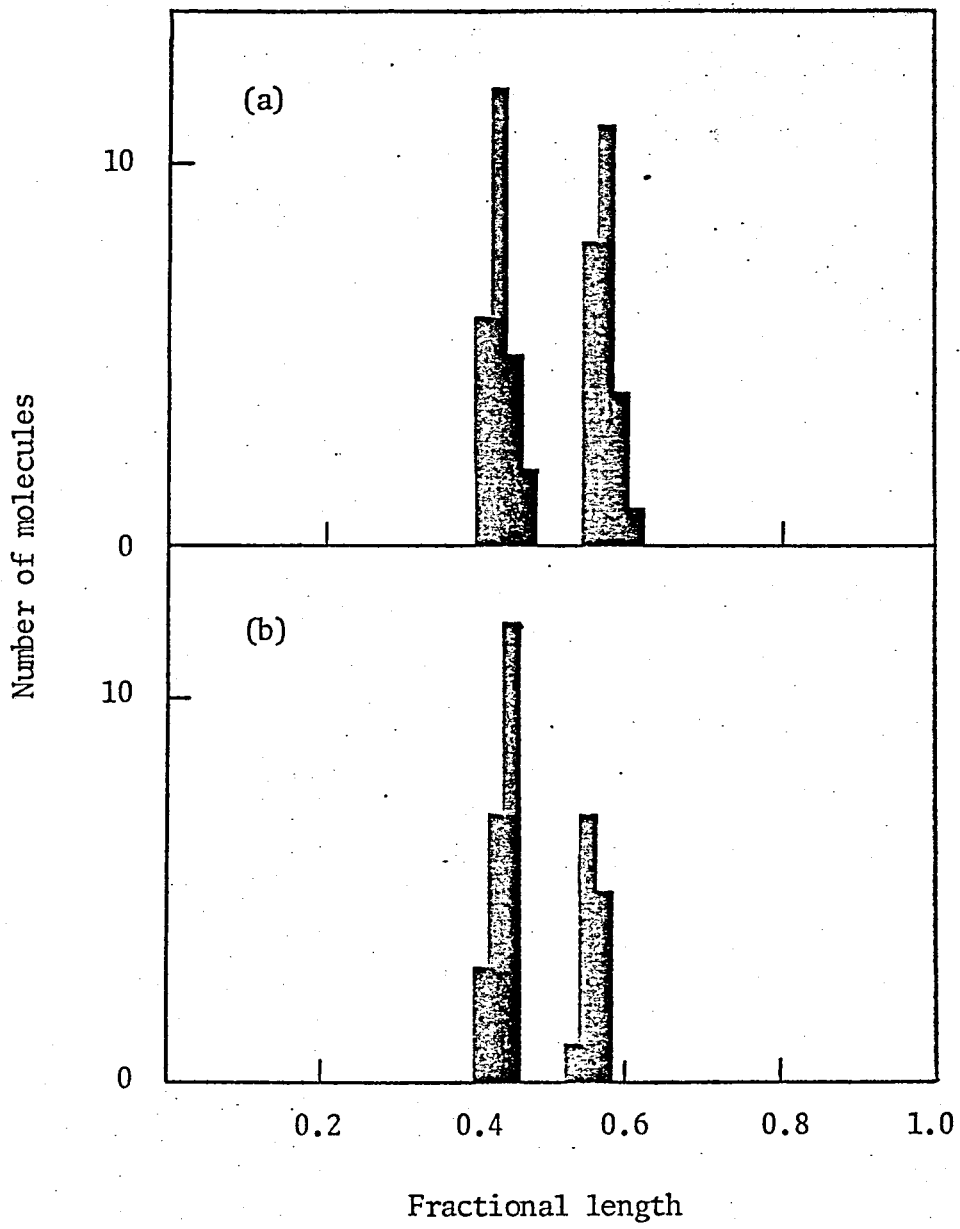


Figure 2.

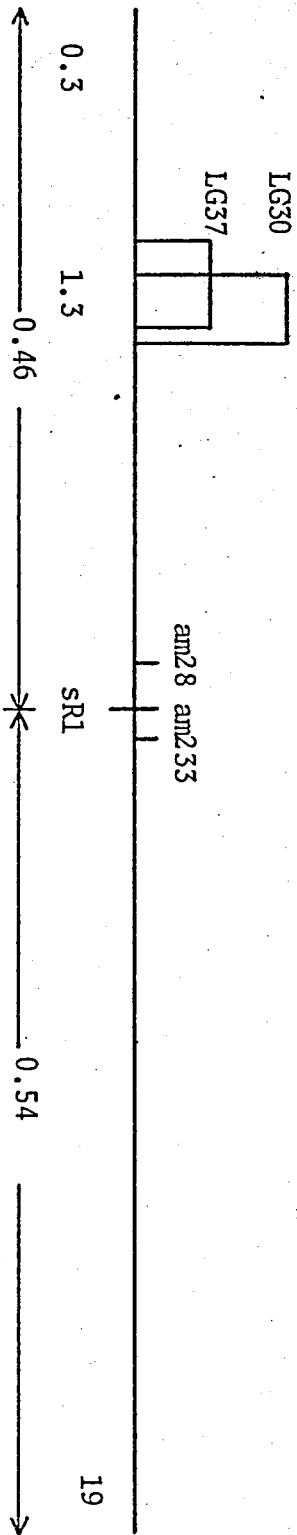


Figure 3.

Part C

MOLECULAR MECHANISM OF GENETIC RECOMBINATION IN BACTERIOPHAGE T7:

SYNOPSIS AND MATURATION

Summary

Dimeric T7 DNA molecules with H or X configuration were accumulated after infection of E. coli cells with T7 1.3, 2, 3, 4, 5 phage and they were composed of two parental DNA (Tsujiimoto & Ogawa, 1976). These dimeric DNA molecules formed in the cross of two phages marked with different mutations were purified by two cycles of CsCl equilibrium density gradient centrifugation followed by CsCl zone sedimentation and the transfection assay of them was carried out. Genetic analysis of progeny in infective centers obtained by transfection showed that these dimeric DNA molecules yielded recombinant phages at a high frequency.

To elucidate the roles of the products of gene 3 (endonuclease I) and gene 5 (T7 DNA polymerase) in the recombination process, the intermediate DNA molecules were isolated in the presence or absence of these gene products and comparative analyses of their structures were carried out.

T7 DNA polymerase was found to stimulate the interaction of parental DNA in the absence of the products of genes 2, 3 and 4. At an early stage of infection in the absence of T7 DNA polymerase, few dimeric T7 DNA were formed, while in the presence of T7 DNA polymerase, dimeric DNA molecules were already formed and at a late stage of infection more multiply branched molecules were accumulated.

Endonuclease I showed the remarkable effect on the intermediate DNA molecules in the presence of T7 DNA polymerase and seemed to participate in the processing of the branched intermediates to linear recombinant DNA by trimming branches. In the absence of endonuclease I, multiply branched molecules accumulated while in the presence of endonuclease I, almost of all the intermediate DNA molecules were linear monomer T7 DNA. These linear monomer DNA molecules were arisen by recombination as judged from (i) the fragmentation of these molecules shifted almost their density toward to fully light and 50% of ³²P-DNA was found to be

covalently linked to BrdU-DNA, (ii) all these linear monomer DNA molecules marked with different mutations yielded recombinant phages.

Introduction

One of the direct approaches for understanding the molecular mechanism of genetic recombination of bacteriophage is to isolate and characterize intermediate DNA molecules in the recombination process. For such an approach, T7 phage is very desirable since the genome is small enough to be isolated as an intact molecule, the structure in infected cells is simple linear form and the recombination efficiency is high as T even phages (Hausman, 1973).

It was previously shown (Tsujimoto & Ogawa, 1976) that doubly branched molecules with H or X configuration were accumulated after infection of E. coli with a high multiplicity of phage T7 carrying amber mutations in genes 1.3, 2, 3, 4 and 5 which are all involved in DNA replication and these doubly branched molecules were composed of two parental DNA, strongly suggesting that they are intermediates in recombination.

Analysis of the structures of the intermediate DNA molecules using a series of mutational blocks in recombination and transfection experiment of ^{the} intermediate DNA molecules are useful ways to elucidate the molecular mechanism of recombination. The product of gene 6, 5' -exonuclease has been previously shown to be necessary for the formation and/or maintenance of doubly branched molecules (Tsujimoto & Ogawa, 1976). The products of genes 3, 4 and 5 are involved in DNA replication (Studier, 1969) and also suggested to be involved in recombination (Powling & Knippers, 1974; Kerr & Sadowski, 1975).

In this paper, the roles of gene 3-coded endonuclease I and gene 5-coded DNA polymerase in the recombination process were investigated and transfection experiments with two DNA species, doubly branched molecules and linear monomer DNA molecules with intermediate density, were carried out.

Materials and Methods

The materials and methods used in this paper were generally the same as those described in preceding paper (Tsujimoto & Ogawa, 1976).

(a) Bacteria and phage strains

The following Escherichia coli strains were used.

(i) E. coli K12: Q1 suII (Signer & Weil, 1968); 594 su⁻ (Campbell, 1965); C600 (Appleyard, 1954); C600 suII recA recB; 594 ligts7 (Tsujimoto & Ogawa, 1977); C600 rK⁻ mK⁻, obtained, via Dr. R. W. Davis, from Dr. M. Meselson

(ii) E. coli C (Bertani & Weigle, 1953)

The phage strains used here were T7 phage strains described in preceding paper (Tsujimoto & Ogawa, 1976), T7am10 (gene 19, maturation), T7 wild type and $\lambda_{c_{60}}$ (Ptashne & Hopkins, 1968). T7 phage strains (Studier, 1969) were obtained from Dr. W. Studier.

(b) Media and buffers

Tryptone broth contains 1% Bactotryptone (Difco) and 0.25% NaCl. It was used for the growth of a host for recombination. P medium (Radding & Kaiser, 1963), modified slightly, contains 0.02M-potassium phosphate (pH 7.0), 0.015M-(NH₄)₂SO₄, 1mM-MgSO₄, 1.8 x 10⁻⁶M-FeCl₂ and 0.2% glucose. It was used for the growth of the host for transfection. L broth (Ikeda & Tomizawa, 1965) contains 1% Polypeptone (Takeda), 0.5% Yeast extract (Difco) and 0.5% NaCl and used for the growth of indicator cells for infective centers obtained by transfection.

(c) Isolation of DNA

(i) From T7 phage

T7 phage was purified by differential centrifugation followed by CsCl equilibrium centrifugation. DNA was extracted twice with phenol saturated with SSC by gentle shaking for 60 min at room temperature. The preparation was then dialyzed for 3 days against 0.1M-Tris-HCl (pH 7.4). DNA concentration was determined by absorbance at 260nm. Absorbance 0.02 corresponds to one μg DNA/ml.

(ii) From T7 infected cells

The procedure of DNA extraction from T7 infected cells was described previously (Tsujimoto & Ogawa, 1976).

(d) Electron microscopy

The sample for electron microscopy was made by aqueous or formamide technique described by Davis et al. (1971).

(e) Transfection

The method of Mandel and Higa (1970) modified by Cameron et al. (1975) was generally followed. C600 suII recA recB was grown to 5×10^8 to 6×10^8 cells/ml at 37°C in P medium supplemented with 0.05% Casamino acids (Difco), 20 μg L-threonine/ml, 20 μg L-leucine/ml and 1 μg thiamine/ml. The bacteria were chilled, harvested by centrifugation in cold at $3,000 \times g$ for 10 min and suspended in 0.5 volume of cold 50 mM-CaCl₂, kept on ice for 5 min, then centrifuged, resuspended in one-fifteenth volume of cold 50 mM-CaCl₂ and kept on ice for 5 min. One volume of chilled DNA solution in 0.1 M-Tris-HCl (pH 7.4) was added to two volumes of the competent cell suspension. The mixture was further kept on ice for 5 min, heat-pulsed at 50°C for 2 min, chilled and plated with Q1 grown in L broth.

progeny in
 (f) Analysis of genetic markers of λ infective centers obtained by transfection
 Genetic markers, amHA13 in gene 1.3 and am10 in gene 19 were analyzed for
 infective centers obtained by transfection with T7 DNA molecules.

The plaque obtained by transfection was picked up with Pasteur pipette
 and suspended in 1 ml of T7 buffer. The concentration of phage was about 10^7 to
 10^8 /ml. The genetic markers were tested by complementation with T7am10 phage
 on 594 and with T7amHA13 phage on 594ligts7 at 32°C . Genetic markers of isolated
 plaques from individual transfectant were tested by replica stabbing with tooth-
 picks on plate seeded with 594 and T7am10 and ^{on} plate seeded with 594ligts7 and
 T7amHA13.

(g) Fragmentation of T7 DNA

About 70 μl of DNA sample (2.8 ng/ml) was mixed with 1.5 ml of SSC containing
 0.05 M-EDTA and 0.14 μg cold T7 phage DNA/ml. The mixture was then put into a
 bottle with a diameter of 2.1 cm and sheared by revolving a plastic disc with
 a diameter of 1.2 cm at 10,000 revs/min for 30 min in ice-water bath. Under this
 condition, T7 phage DNA was fragmented to about one-third to one-fourth of unit
 size (determined by neutral sucrose density gradient centrifugation, data not
 shown).

equilibrium
 (h) Alkaline CsCl λ density gradient centrifugation

About 50 μl of DNA sample was denatured in the solution composed of 0.4 ml
 $\times 10$ SSC, 0.04 ml 0.5 M-EDTA, 0.02 ml 10% sodium N-lauroyl sarcosinate, 0.4 ml
 1 N NaOH and 3 ml distilled water. CsCl was added and the density was adjusted
 to 1.75 g/cm^3 . This preparation was centrifuged in polyallomer tube in a Spinco
 40 rotor at 36,000 revs/min for 40 h at 15°C .

Results

(a) Biological activity of H or X-shaped molecules obtained under 2, 3, 4, 5 condition

(i) Single infection of T7 phage DNA

The transfection assay used in this work is ^{the} one developed by Mandel and Higa (1970) modified by Cameron et al. (1975). We have used a strain C600 suII recA recB. The competent cells were prepared as described in Materials and Methods, section (e). One volume of the T7 wild type phage DNA with various concentrations, was mixed with two volumes of competent cells and infective centers were titrated. As shown in Figure 1, the number of infective centers obtained was linearly increased until a concentration of 1 $\mu\text{g/ml}$. The DNA solution alone or competent cells alone yielded no plaque. In this assay, transfection efficiency was about 15 infective centers per ng T7 DNA, corresponding to 6.3×10^{-7} infective centers per genome equivalent.

Using the procedure of Mandel and Higa (1970) with some modifications, Ehrlich et al. (1976) have showed that transfection efficiency of T7 DNA was influenced by host restriction system (rK^+) and that transfection efficiency was about 10^{-6} or 10^{-9} infective centers per genome equivalent on restriction-deficient or on restriction-proficient host, respectively. However, in our assay using restriction-proficient host (rK^+), a high transfection efficiency, 6.3×10^{-7} infective centers per genome equivalent, was usually obtained. In Table 1, are shown the results of confirmation of our strain with restriction-proficient character.

Linear relationship between the number of infective centers obtained and the input DNA concentrations suggests that the infective centers were produced by a single infection of T7 DNA. To confirm this, competent cells were mixed with T7 DNA solution containing T7am10 and T7amHA13 phage DNA on equal amount. Each

100 infective centers obtained with DNA concentration of 0.125, 0.25 and 0.5 $\mu\text{g/ml}$ were tested for complementations of am10 and amHA13 as described in Materials and Methods, section (f). Each 100 infective centers complemented only one of two phages, T7am10 (143 out of 300 infective centers) or T7amHA13 (157 out of 300). Thus at least up to DNA concentration of 0.5 $\mu\text{g/ml}$, the chance of mixed infection of T7 DNA was less than 1% in our system.

(ii) Transfection with doubly branched molecules

As reported previously (Tsujiimoto & Ogawa, 1976), doubly branched molecules with H or X configuration were accumulated under the condition of the absence of DNA replication, 1,3, 2, 3, 4, 5 and shown to be composed of two parental DNA.

To test whether or not these H or X-like DNA molecules possess infectivity and produce recombinant phages, a large number of doubly branched molecules were purified from nonpermissive cells (total 6×10^{10} cells) infected with ^{32}P -labelled T7 1,3, 2, 3, 4, 5 and BrdU-labelled T7 2, 3, 4, 5, 19 phage. DNA extracted from the infected cells was centrifuged in CsCl equilibrium density gradient and intermediate density fractions were pooled and recentrifuged in CsCl equilibrium density gradient (Figure 2 (a)). The fractions 18 to 20 in Figure 2 (a) were pooled and after dialysis, sedimented by CsCl zone centrifugation (Figure 2 (b)). The pooled sample of fractions 20 and 21 in Figure 2 (b) was used for transfection after dialysis against 0.1M-Tris-HCl (pH 7.4). The same sample was examined in the electron microscope. Fourteen (54%) out of randomly picked up 28 molecules were H or X-shaped, 4 (14%) molecules were Y-shaped, 6 (21%) molecules were simple linear and 3 (11%) were molecules with many free ends.

The DNA concentration of the sample was estimated as follows.. From the ^{32}P -radioactivity and the specific radioactivity, the concentration of DNA was calculated as 23 ng/ml and since DNA molecules in this sample had half-heavy density, the DNA concentration of the sample was estimated as about 46 ng/ml. It is unlikely

that heavy DNA was brought into this sample during preparation since, as described below, heavy parent type of progeny was not preferentially produced by transfection.

One volume of the DNA solution with the concentration of 46 ng/ml or 23 ng/ml and two volumes of competent cells were mixed and infective centers were titrated. DNA solution with 46 ng/ml yielded 6 ± 3 infective centers per 0.1 ml and that with 23 ng/ml, 4 ± 1 infective centers per 0.1 ml. The DNA solution or competent cells alone yielded no plaque. The transfection efficiency was about 4 infective centers per ng DNA. Although this efficiency per ng DNA was 3 to 4 times less than that of phage DNA, transfection efficiency per DNA molecule seemed to be comparable to that of phage DNA, since the majority of molecules in this sample were dimeric T7 DNA. Considering the enough low DNA concentration and the reasonable transfection efficiency per DNA molecule, mixed infection of cell with T7 DNA molecules seemed to be negligible.

Fifty-five infective centers obtained by transfection were picked up, suspended in T7 buffer and analyzed for the presence or absence of gene 1.3 and 19 as described in Materials and Methods, section (f). Twenty out of 55 infective centers could complement both T7_{am10} and T7_{amHA13} phage on restrictive host and 8 infective centers could complement neither T7_{am10} nor T7_{amHA13} phage. Seventeen and 10 infective centers complemented only T7_{am10} and T7_{amHA13}, respectively. Thus at least 28 (51%) out of 55 infective centers had the markers derived from both parents simultaneously.

To examine whether these individual infective centers consisted of one type of phage or of the mixture of two or more types of phage, each suspension of infective center was plated with permissive host and 30 separate plaques were tested for complementations of am10 and amHA13. The results are summarized in Table 2. Twenty (36%) out of 55 infective centers were heterogeneous, consisting of two or more types of progeny and the rest of 35 infective centers were

homogeneous as far as 30 plaques were tested. Eighteen (90%) of 20 heterogeneous infective centers consisted of one parent and one recombinant type of progeny and the rest of 2 had all four markers derived from two parents. Furthermore in almost heterogeneous infective centers, one type of phage was predominantly found over the other(s). Heterogeneous infective centers (17) consisting mostly of $1.3^- 19^+$ or $1.3^+ 19^+$ phage were more frequently found than the other two, $1.3^+ 19^-$ or $1.3^- 19^-$ phage (3 infective centers). On the other hand, the number of infective centers consisting of one type of phage was 11 for $1.3^- 19^+$, 8 for $1.3^+ 19^-$, 8 for $1.3^+ 19^+$ and 8 for $1.3^- 19^-$ phage and no inclination among them was observed.

The fact that two or more types of phage were found in an infective center suggests that at least heterogeneous infective centers were formed by transfection of doubly, singly or multiply branched molecules which were composed of two parental DNA.

To know whether asymmetry of phage type and asymmetry on relative amount of phage observed in heterogeneous infective centers were characteristic of transfectants of doubly, singly or multiply branched molecules, a transfection experiment was carried out where competent cells were coinfecting with free two parental DNA. Competent cells (9×10^9 cells/ml) were mixed with DNA solution (about 2 μ g/ml) containing T7 1.3, 2, 3, 4, 5 DNA and BrdU-labelled T7 2, 3, 4, 5, 19 DNA in equal amount so that frequency of mixed infection was 6.7%. Assuming Poisson's distribution, about 83% of mixed infection would be arisen by two DNA molecules each of which was from different parent. The relative amount of progeny phage in infective centers obtained by mixed infection with two parental DNA was analyzed and the results were summarized in Table 3. Two types of asymmetry stated above were observed also in this case. Thirteen (72%) out of 18 infective centers were heterogeneous. Ten (77%) out of 13 heterogeneous infective centers consisted of one parent and one recombinant type of phage. In almost heterogeneous infective centers, one type

of phage was predominantly found. Heterogeneous infective centers (12) consisting mostly of $1.3^- 10^+$ or $1.3^+ 19^+$ phage were more frequently found than the other two, $1.3^+ 19^-$ or $1.3^- 19^-$ phage (1 infective center).

Previously H or X-shaped molecules have been shown to be physically biparental (Tsujimoto & Ogawa, 1976). Here it was further shown that they possessed infectivity and were composed of two parental DNA.

(b) The role of gene 3 and gene 5 in recombination process

Genetic results suggest that the products of genes 3, 4, 5 and 6 are involved in recombination (Powling & Knippers, 1974; Kerr & Sadowski, 1975) but the roles of these gene products in the recombination process have not been known. Previously we have shown that the product of gene 6, 5'-exonuclease was required for the formation and/or maintenance of H or X-shaped intermediates in the absence of the products of genes 1, 3, 2, 3, 4 and 5 (Tsujimoto & Ogawa, 1976). We attempted to elucidate the roles of the products of gene 3 and gene 5 in our system, whose products have been purified and enzymatic activities were well known.

(i) Role of gene 5-coded DNA polymerase

It is very likely that from its enzymatic characteristics, T7 DNA polymerase repairs polynucleotide gaps on intermediate DNA molecules in recombination. However, as described below, the remarkable difference between the structures of intermediate density molecules obtained under 2, 3, 4, 5 condition and those under 2, 3, 4 condition was observed, suggesting that T7 DNA polymerase plays an additional role besides gap filling.

Nonpermissive host 594 su⁻ were infected with ³²P- and BrdU-labelled T7 2, 3, 4 phage and incubated for 20 min at 32°C. DNA was extracted and intermediate density molecules were purified by two cycles of CsCl equilibrium density gradient centrifugation. About 0.5 to 0.9% of total input of ³²P-radioactivity was recovered in intermediate density fractions which was about 2 to 3 times more than that (about 0.3%) of 2, 3, 4, 5 cross. Purified intermediate density molecules were sedimented by a CsCl zone centrifugation (Figure 3 (a)). As described above, under 2, 3, 4, 5 condition, two peaks were observed, a minor one sedimenting with or slightly faster and a major one sedimenting 1.4 times faster than T7 unit length DNA. The latter consisted mostly of H or X-like dimer molecules and the former

consisted of monomeric T7 DNA. On the other hand, under 2, 3, 4 condition, about 64% of ^{32}P -radioactivity sedimented faster than dimeric T7 DNA molecules and few dimeric molecules were found. This sedimentation profile suggests that in this cross, molecules bigger than dimeric T7 DNA were accumulated. The same DNA molecules as used in Figure 3 (a) were examined in the electron microscope and the result is presented in Table 4. DNA molecules which had five or more free ends were classified as multiply branched molecules. As expected from the profile of CsCl zone sedimentation (Figure 3 (a)), frequently multiply branched molecules were observed. The representative photographs were presented in Figure 4 (a) and (b). Eighteen out of 23 multiply branched molecules were accompanied by one or more cores. It should be also noted that on many molecules projected single-stranded segments were found. On some multiply branched molecules, the similar structure to D-loop was found (Figure 4 (b)) which had been described by Wolfson & Dressler (1973) who observed it after infection with T7 phage defective in gene 4. Thus in the presence of T7 DNA polymerase, interaction of parental DNA and of DNA strands on paired DNA molecules seemed to be stimulated.

Shortning incubation time after infection reduced the extent of interaction of DNA. Nonpermissive cells were infected with ^{32}P -labelled T7 1.3, 2, 3, 4, 5 and BrdU-labelled T7 2, 3, 4 phage at a multiplicity of 20 each and incubated for 10 min at 32°C . DNA was extracted and intermediate density molecules were purified as stated above. DNA molecules in half-heavy density ^{fraction} were sedimented by a CsCl zone centrifugation. As shown in Figure 3 (b), in contrast to the case of a late stage of infection mentioned above, molecules sedimenting faster than dimeric T7 DNA were greatly reduced and a peak was formed in the fractions where dimeric T7 DNA sedimented, while 10 min after infection with T7 1.3, 2, 3, 4, 5 phage, only about one-tenth amount of dimeric T7 DNA molecules were found. These results also strongly support that in the presence of T7 DNA polymerase, interaction of parental

DNA molecules are stimulated. DNA molecules in fractions 18 and 19 in Figure 3 (b), were examined in the electron microscope. Eight (58%) out of randomly picked up 12 molecules were dimeric DNA and this frequency was almost equal to that in dimeric DNA fractions obtained 20 min after infection with T7 1.3, 2, 3, 4, 5 phage. The compositions of various dimeric DNA molecules in both crosses are presented in Table 5. In both cases, were observed H, X-like molecule and molecule consisting of two T7 DNA crosslinked each other with a short DNA segment (called crosslinked molecule). On crosslinked molecules, the sum of length of two branches at each branch point equalled the length of monomeric T7 DNA molecules and the length of one of two branches at a branch point was equal to one of two branches at the other branch point. The length of cross-bar linking two DNA was generally very short and less than about 0.01 fractional length. A representative photograph was presented in Figure 5. Although the amount of crosslinked molecules varied from experiment to experiment, in the presence of T7 DNA polymerase crosslinked molecules and X-shaped molecules seemed to be more frequently formed than in the absence of T7 DNA polymerase. However an alternative possibility is not excluded that these molecules might be formed preferentially in an early stage of infection (10 min).

The multiply branched molecules observed late after infection in the presence of T7 DNA polymerase would be arisen by accumulation of these three types of interaction between parental DNA and between DNA strands on already interacting DNA molecules.

(ii) Role of endonuclease I coded by gene 3

T7 gene 3 codes endonuclease I which digests single-stranded DNA preferentially than double-stranded DNA (Center & Richardson, 1970). This endonuclease I has been suggested to be involved in recombination (Powling & Knippers, 1974; Kerr & Sadowski, 1975) but since in the absence of the endonuclease, branched intermediate DNA molecules were accumulated, this endonuclease, if required for recombination also in our system, would play a role on maturation step of branched intermediates to complete recombinant DNA molecules. The enzymatic activity of preferential digestion of single-stranded DNA over double stranded DNA allows us to hypothesize that endonuclease I trims branches of branched intermediates by endonucleolytic cleavage of single-stranded DNA at branch points. To test this hypothesis intermediate density molecules obtained under 2, 4 condition were compared with those under 2, 3, 4 condition.

Nonpermissive cells were infected with ^{32}P - and BrdU-labelled T7 2, 4 phage at a multiplicity of 20 each and incubated for 20 min at 32°C . DNA was extracted and intermediate density molecules were purified. About 0.6 to 1.0% of total input of ^{32}P -radioactivity was recovered at the intermediate density fractions. The purified intermediate density molecules, after dialysis, sedimented by a CsCl zone centrifugation (Figure 6). In contrast to the case of 2, 3, 4 cross, only one sharp peak was formed, sedimenting slightly faster than ^3H -labelled T7 phage DNA and few molecules sedimenting more faster were found. Sedimentation of ^3H -labelled T7 phage DNA was carried out in ^{an} _{another} ^{tube} _^. DNA molecules in fractions 24 and 25 in Figure 6 were examined in the electron microscope. Out of 110 molecules picked up randomly, 108 (98%) molecules were simple linear and the rest 2 (2%) were Y-shaped molecules. Contour length of twenty five linear molecules were

measured using colicin E1 plasmid DNA as length calibrator. Sixteen (64%) out of 25 molecules were in a unit length and the rest 9 were shorter, probably arising by breakdown of linear monomer DNA. Since these linear monomer DNA were recombinant DNA as described below, they would be accompanied by single-stranded gaps and might be easily broken down. Thus in this cross the majority of molecules with intermediate density were linear monomer T7 DNA and accumulation of branched molecules as seen in the case of 2, 3, 4 or 2, 3, 4, 5 cross did not occur, suggesting that endonuclease I was required for the trimming of branches of branched molecules. The slightly faster sedimentation of these linear monomer DNA than ³H-labelled T7 phage DNA in CsCl zone sedimentation was probably due to the heavier density of the molecules.

To determine that these linear monomer DNA molecules were not formed by replication of parental DNA, DNA molecules in fractions 24 and 25 in Figure 6 were fragmented by shearing force to about one-third to one-fourth of a unit length (determined by neutral sucrose density gradient centrifugation, data not shown) and centrifuged in CsCl equilibrium density gradient (Figure 7). If formed by replication, even after fragmentation, ³²P-radioactivity should be banded at the original half-heavy density. As shown in Figure 7 (a), before fragmentation about 83% of ³²P-radioactivity was banded at the original density and the rest banded at fully light density, while after fragmentation, a few of ³²P-radioactivity was found at the original density and at heavier density, and almost all of the ³²P-radioactivity was shifted toward fully light density (Figure 7 (b)). To test covalent joining of parental DNA in these linear monomer DNA molecules, DNA molecules in fractions 24 and 25 in Figure 6 were centrifuged in an alkaline CsCl equilibrium density gradient. The profile is shown in Figure 7 (c). About 53% of ³²P-radioactivity was banded at intermediate density and the rest banded at fully light density. Thus covalent joining of at least 53% of parental DNA strands occurred in linear monomer DNA molecules.

From these results together with the results of transfection experiment described in section (c), it is conclusive that linear monomer DNA molecules with intermediate density obtained under 2, 4 condition arose by recombination and at least a half of them were complete recombinant DNA.

As mentioned above, comparison of intermediate density molecules obtained under 2, 3, 4 condition and those under 2, 4 condition suggested that endonuclease I was required for trimming of branches of branched intermediate DNA molecules. At DNA level, covalent joining of two parental DNA would be the final structure through the sequential recombination processes. Therefore if endonuclease I plays any role at maturation step of intermediate DNA, defect of gene 3 would result in failure in covalent joining of parental DNA. Based on this criterion, we tested further the involvement of endonuclease I in recombination.

Nonpermissive cells were infected with ^{32}P - and BrdU-labelled T7 2, 4 or T7 2, 3, 4 phage and incubated for 20 min at 32°C . DNA was extracted and the intermediate density molecules were purified. About 1% and 0.8% of total input of ^{32}P -radioactivity was recovered in intermediate density fractions under 2, 4 and 2, 3, 4 condition, respectively. To test the occurrence of covalent joining of ^{32}P -labelled DNA and BrdU-labelled DNA, the purified intermediate density molecules were recentrifuged in alkaline and neutral CsCl equilibrium density gradients with ^3H -labelled T7 phage DNA. The profiles of neutral CsCl density gradient centrifugation are presented in Figure 8 (a) and (c). In both cases all ^{32}P -radioactivity was banded at the original density. The profiles of alkaline CsCl density gradient centrifugation were presented in Figure 8 (b) and (d). In the presence of endonuclease I, 51% of total recovered ^{32}P -radioactivity was found at the intermediate density fractions and the rest was banded with light ^3H -labelled T7 DNA (Figure 8 (b)). On the other hand, in the absence of endonuclease I, all ^{32}P -radioactivity was found at light T7 DNA and no radioactivity was found

at the intermediate density fractions (Figure 8 (d)).

Therefore the endonuclease I coded by gene 3 is necessary for the covalent linkage of parental DNA at least in the absence of the products of genes 2 and 4 and was concluded to be involved in recombination, especially in the maturation step of intermediate DNA molecules.

(c) Biological activity of linear monomer DNA molecules with intermediate density obtained under 2, 4 condition

As described above, the intermediate density molecules obtained under 2, 4 condition consisted mostly of linear monomer T7 DNA and at least a half of them were complete recombinant DNA.

To test whether or not these linear monomer DNA possesses infectivity and produces recombinant phages, transfection of these DNA molecules was performed. About 260 ml of 594 su⁻ bacteria at 2×10^8 cells/ml were infected with 20 ³²P-labelled T7 1.3, 2, 4 and 20 BrdU-labelled T7 2, 4, 5, 19 phage particles per cell and incubated for 20 min at 32°C. After DNA extraction, linear monomer DNA molecules with intermediate density were purified by two cycles of CsCl equilibrium density gradient centrifugation followed by CsCl zone sedimentation as described in section (b) (ii) and assayed for biological activity by transfection. The DNA concentration of the obtained sample, estimated from ³²P-radioactivity, specific activity of ³²P and their half-heavy density, was 139 ng/ml and low enough to avoid mixed infection of competent cell. In fact, as shown in Table 6, the number of infective centers was linearly dependent on DNA concentration. The efficiency of transfection was 15 infective centers per ng T7 DNA and equal to that of T7 phage DNA. The markers of progeny phage in the obtained infective centers were examined and the results were summarized in Table 6. As shown in Table 6, at a high frequency of about 50%, the infective centers carrying recombinant 1.3⁺ 19⁺ or 1.3⁻ 19⁻ phage were produced.

The infective centers carrying recombinant $1.3^+ 19^+$ or $1.3^- 19^-$ phage were produced at a constant frequency with a serially diluted DNA solution. This fact further confirmed that the obtained infective centers were arisen by a single infection with DNA molecule. The $1.3^+ 19^+$ infective centers were further analyzed for the included progeny phage whether they were heterogeneous or not with genes, 1.3 and 19. Out of 32 individual infective centers analyzed, 2 consisted of a mixture of 19^+ and 19^- phage and one infective center a mixture of 1.3^+ and 1.3^- phage. The remainder 29 (91%) infective centers consisted only ^{of} $1.3^+ 19^+$ phage as far as 20 purified plaques from individual infective centers were tested. As seen in Table 6, at the frequency of about 14%, were also found the infective centers which seemed to be formed by recombinant produced by double crossover. All parent type infective centers had markers from heavy parental DNA but not from light parental DNA. These would be due to heavy parental DNA brought into the DNA sample during preparation. If it is the case, all infective centers obtained by transfection of linear monomer DNA with intermediate density would be recombinant type. Since the possible content of heavy parental DNA was about 38% of total DNA, DNA concentration of the sample for transfection calculated above might be underestimated by that extent. Among recombinant infective centers, asymmetry was observed, that is, $1.3^+ 19^+$ infective centers were dominant over the reciprocal recombinant type $1.3^- 19^-$ ones. This might be due to advantage in propagation of $1.3^+ 19^+$ over $1.3^- 19^-$ phage under our transfection condition.

Thus linear monomer DNA with intermediate density obtained under 2, 4 condition produces recombinant phages at very high frequency by transfection and seems to be complete recombinant DNA.

Discussions

(a) Transfection with dimeric T7 DNA molecules

Dimeric T7 DNA molecules were accumulated after infection with T7 2, 3, 4, 5 phage. These dimeric T7 DNA molecules marked with different mutations were purified by two cycles of CsCl equilibrium density gradient centrifugation followed by CsCl zone sedimentation and transfection experiment of them was carried out. When the DNA molecules in the same sample were examined in the electron microscope, about a half of them were doubly branched dimeric DNA molecules and the rest consisted of multiply branched, singly branched and linear molecules. Linear and singly branched molecules were probably arisen from dimeric DNA molecules by loss of branch(es) during manipulation. At least 51% of the infective centers obtained had markers derived from both parents. About 36% of total infective centers were heterogeneous, that is, consisted of two or more types of phage. This heterogeneity suggests that heterogeneous infective centers were formed by transfection of doubly, singly or multiply branched molecules and these DNA molecules were composed of two parental DNA. Almost heterogeneous infective centers consisted of one parent and one recombinant type of phage and one of them was predominantly found. These two types of asymmetry were not characteristic of transfectants of doubly, singly or multiply branched molecules and also observed in infective centers obtained by mixed infection with two parental DNA. Why these types of asymmetry occurred is not known. It might be due to many cycles of propagation of progeny derived from transfectants or reflect the initial burst of transfectants.

Infective centers consisting of one type of phage were equally yielded with four possible phage types. They might be arisen from in vivo processing of doubly, singly or multiply branched molecules or from artificial loss of branches of branched molecules and removal of branches from dimeric DNA molecules seemed to occur randomly.

(b) The role of T7 DNA polymerase in recombination

T7 DNA polymerase seemed to stimulate and/or stabilize the interaction of parental DNA at least in the absence of the products of genes 2, 3, 4. At an early stage of infection in the absence of T7 DNA polymerase few dimeric T7 DNA molecules were found, while in the presence of T7 DNA polymerase, dimeric T7 DNA molecules consisting of H, X-shaped and crosslinked molecules were formed and at a late stage, more multiply branched molecules were accumulated. The stimulation and/or stabilization of interaction of DNA molecules was consistent of increase of intermediate density molecules.

Many multiply branched molecules were accompanied by core(s) containing single-stranded DNA, suggesting that T7 DNA polymerase stimulates and/or stabilizes the interaction of DNA strand on paired DNA molecules.

One of the ways to stimulate the DNA interaction would be to increase single-stranded DNA. T7 DNA polymerase has 5' to 3' polymerizing activity and 3' to 5' exonucleolytic activity but not 5' to 3' exonucleolytic activity (Grippo & Richardson, 1971). Therefore, if T7 DNA polymerase acts on nick or gap and performs repair synthesis, single-stranded DNA would be displaced as described in Figure 9, A. This type of reaction was observed in T5 induced DNA polymerase (Fujimura & et al., 1973) Roop, 1976) and a mutant DNA polymerase I in *E. coli*. (Heijnecker [^]) which has the same enzymatic characteristics as T7 DNA polymerase. Whether or not T7 DNA polymerase has a capacity to perform this displacement DNA synthesis has not been rigorously tested (Oey et al., 1971; Grippo & Richardson, 1971). After infection with T7 phage defective in gene 4, displacement loop (Figure 9, B) was frequently observed (Wolfson & [^] Dressler, 1973) and also on intermediate density molecules obtained under 2, 3, 4 condition, D-loops were observed. It might be ^{also} possible that single-stranded gaps are formed by 3' exonucleolytic activity of T7 DNA polymerase. These single-stranded structures arisen by T7 DNA polymerase might stimulate interaction of T7

DNA by making it facile for parental DNA to be trapped.

Synapsis could be formed using these single-stranded structures together with single stranded gaps formed by 5' -exonuclease coded by gene 6. It might be also possible that before T7 DNA polymerase and/or 5' -exonuclease act on DNA, interaction of parental DNA was formed and then these enzymes act on interacting molecule to make its interaction more stable.

(c) The role of endonuclease I in maturation step of recombination

Covalent joining of polynucleotide chain of parental DNA would be the final step of the sequential recombination process. This assumption makes it possible to test whether a certain enzyme is necessary for recombination, especially for maturation steps of intermediate DNA to complete recombinant DNA.

Alkaline CsCl equilibrium density gradient centrifugation analysis of intermediate density molecules obtained under 2, 3, 4 and those under 2, 4 condition showed that endonuclease I was necessary for covalent joining of parental DNA, indicating that endonuclease I is involved in at least in maturation step of recombination.

Suggestion for role of the endonuclease I in recombination process was obtained by comparing the structure of intermediate density molecules formed in the presence and absence of the endonuclease I. Under 2, 3, 4 condition, multiply branched molecules were accumulated while under 2, 4 condition, almost of all molecules were linear monomer T7 DNA. These linear monomer T7 DNA molecules were recombinant DNA and at least a half of them were complete recombinant DNA as judged from (1) fragmentation of these molecules shifted almost their density from original to lighter one and about 53% of ³²P-DNA was covalently linked to BrdU-DNA, indicating that these linear monomer DNA molecules were not formed by semiconservative replication, (2) by transfection, all these molecules marked with ^{different} mutations yielded

recombinant phage. These results suggest that endonuclease I participates in the processing of branched intermediates to linear recombinant DNA by trimming of branches. Since the endonuclease I acts more preferentially on single-stranded DNA than on double stranded DNA (Center & Richardson, 1970), it is likely that this endonuclease performs such a processing of trimming branches by cleaving single-stranded regions at branch points. This is supported by the fact that less dimeric T7 DNA molecules were found under 1.3, 2, 4, 5 than under 1.3, 2, 3, 4, 5 condition and more linear monomer DNA was found in the presence than in the absence of endonuclease I (unpublished result).

As shown in Results, section (a), even in the absence of endonuclease I some linear monomer T7 DNA molecules were found. These molecules would be arisen from branched molecules by loss of branches by nonspecific endonucleas and/or mechanical force during DNA extraction and/or manipulation.

References

- Appleyard, R. K. (1954). Genetics, 39, 440-452
- Bertani, G. & Weigle, J. J. (1953). J. Bact. 65, 113-121
- Campbell, A. (1965). Virology, 27, 329-339
- Cameron, J. R., Panasencko, S. M., Lehman, I. R. & Davis, R. W. (1975). Proc. Nat. Acad. Sci., U.S.A. 72, 3416-3420
- Center, M. S. & Richardson, C. C. (1970). J. Biol. Chem. 245, 6285-6291
- Davis, R. W., Simon, M & Davidson, N. (1971). In Methods in Enzymology (Grossman, L. & Moldave, K., eds), vol. 21, pp. 413-428
- Ehrlich, S. D., Sgaramella, V. & Lederberg, J. (1976). J. Mol. Biol. 105, 603-609
- Fujimura, R. K. & Roop, B. C. (1976). J. Biol. Chem. 251, 2168-2175
- Grippe, P. & Richardson, C. C. (1971). J. Biol. Chem. 246, 6867-6873
- Hausman, R. (1973). Annu. Rev. Microbiol. 27, 51-67
- Heilinecker, H., Ellens, D., Tjeerde, H., Glickman, B., von Drop, B. & Powels, P. (1973). Mol. Gen. Genet. 124, 83-96
- Ikeda, H. & Tomizawa, J. (1965). J. Mol. Biol. 14, 85-109
- Kerr, C. & Sadowski, P. D. (1975). Virology, 65, 281-285
- Mandel, M. & Higa, A. (1970). J. Mol. Biol. 54, 159-162
- Oey, J. L., Stratling, W. & Knippers, R. (1971). Eur. J. Biochem. 23, 497-504
- Powling, A. & Knippers, R. (1974). Mol. Gen. Genet. 134, 173-180
- Ptashne, M. & Hopkins, N. (1968). Proc. Nat. Acad. Sci., U.S.A. 60, 1282-1287
- Radding, C. M. & Kaiser, A. D. (1963). J. Mol. Biol. 7, 225-233
- Singer, E. R. & Weil, J. (1968). J. Mol. Biol. 34, 261-271
- Studier, F. W. (1969). Virology, 39, 562-574
- Tsujimoto, Y. & Ogawa, H. (1976). J. Mol. Biol. in press
- Tsujimoto, Y. & Ogawa, H. (1977). Mol. Gen. Genet. in press
- Wolfson, J. & Dressler, D. (1973). In DNA Synthesis in Vitro (Wells, R. D. & Inman, R. B., eds). pp. 448, University Park Press, Baltimore

Table 1

Efficiency of plating of phage λ_{c60} variants
on different host strains

Host	Efficiency of plating	
	λ grown on K12 C600	λ grown on C
E. coli K12		
C600	6.3×10^{-1}	1.3×10^{-4}
C600 recA recB	4.0×10^{-1}	1.4×10^{-4}
C600 rK ⁻ mK ⁻	7.0×10^{-1}	3.4×10^{-1}
E. coli C	1	1

Table 2

Analysis of phage in infective centers obtained by
transfection with doubly branched molecules

Infective center	Number of phage			
	Parent type		Recombinant type	
	1.3 ⁻ 19 ⁺	1.3 ⁺ 19 ⁻	1.3 ⁺ 19 ⁺	1.3 ⁻ 19 ⁻
1	27		3	
2	25		5	
3	17		13	
4	25			5
5	20			10
6	29			1
7	27			3
8	23			7
9 - 19	30			
20		24		6
21		24		6
22 - 29		30		
30	2	1	27	
31	1		29	
32	1		29	
33	6		24	
34	10		18	2
35	14		16	
36	7		23	
37		7	23	
38		3	27	
39 - 46			30	
47	1			29
48 - 55				30

Table 3

Analysis of phage in infective centers obtained by
mixed infection with parental DNA

Infective center	Number of phage			
	Parent type		Recombinant type	
	1.3 ⁻ 19 ⁺	1.3 ⁺ 19 ⁻	1.3 ⁺ 19 ⁺	1.3 ⁻ 19 ⁻
1	29		1	
2	28		2	
3	23		7	
4	15		15	
5	5	25		
6	13		17	
7	7		23	
8	5		25	
9	1		29	
10	10		20	
11	8		22	
12		11	16	3
13	10	1	16	3
14			30	
15			30	
16			30	
17			30	
18			30	

Table 4

Composition of DNA molecules in half-heavy density
fractions obtained under 2, 3, 4 condition

	No. of molecules
Linear	16
Y-shaped	3
H or X-shaped	9
Multiply branched	23
Total no. of molecules scored	51

Table 5

Composition of various doubly branched molecules obtained
in the presence or in the absence of T7 DNA polymerase

Condition of cross	Number of molecules	
	<u>2</u> , <u>3</u> , <u>4</u>	<u>2</u> , <u>3</u> , <u>4</u> , <u>5</u>
Doubly branched molecules		
H-shaped	3	13
X-shaped	5	4
Crosslinked	3	3
Total number of molecules scored	11	20

Table 6

Analysis of phage in infective centers obtained by transfection
with linear monomer T7 DNA isolated under 2, 4 condition

	Number of infective centers		
	DNA concentration (ng/ml)		
	139	70	35
No. of total infective centers obtained per 0.1 ml	71	39	16
Genotype			
1.3 ⁺ 5 ⁻ 19 ⁺ a)	17	18	15
1.3 ⁺ 5 ⁺ 19 ⁺	3	5	2
1.3 ⁻ 5 ⁺ 19 ⁻ a)	1	3	1
1.3 ⁻ 5 ⁻ 19 ⁻	2	2	2
1.3 ⁻ 5 ⁻ 19 ⁺ b)	0	1	0
1.3 ⁺ 5 ⁺ 19 ⁻	7	10	3
1.3 ⁻ 5 ⁺ 19 ⁺ c)	0	0	0
1.3 ⁺ 5 ⁻ 19 ⁻	17	31	12
Total number of infective centers analyzed	47	70	35

a) recombinant type formed by odd number of crossover

b) recombinant type formed by even number of crossover

c) parent type

Legend to Figures

Fig. 1

Linearity of transfection assay of T7 phage DNA in CaCl_2 -treated E. coli. Competent cells of C600 suII recA recB were prepared as described in Materials and Methods, section (f). Phenol-extracted T7 phage DNA was diluted to the indicated concentrations in 0.1M-Tris-HCl (pH 7.4). An 0.1 ml of the T7 DNA was mixed with 0.2 ml of competent cells and infective centers were titrated. One μg T7 DNA is equal to 2.4×10^{10} T7 genomes.

Fig. 2 (a)

Recentrifugation profile in CsCl equilibrium density gradient of the intermediate density molecules obtained under 2, 3, 4, 5 condition. DNA extracted from nonpermissive cells infected with 20 each of ^{32}P -labelled T7 1.3, 2, 3, 4, 5 and BrdU-labelled T7 2, 3, 4, 5, 19 phage particles per cell and incubated for 20 min at 32°C , was centrifuged in CsCl equilibrium density gradient. The fractions at or near the half-heavy density were recentrifuged in CsCl gradient. Centrifugation in CsCl density gradient was carried out in a Spinco 40 rotor at 36,000 revs/min for 40 h at 15°C . The position of the half-heavy (HL) DNA was deduced from the density of the light (LL) and BrdU-labelled T7 DNA (HH).

—●—●—, ^{32}P -radioactivity;

(b)

Zone sedimentation in a CsCl density gradient of fractions 18 to 20 in Fig. 2 (a). The pooled fractions were dialyzed against 0.1M-NaCl, 0.01M-sodium citrate and layered on a CsCl density gradient and then centrifuged in a Spinco SW 50.1 rotor at 30,000 revs/min for 3 h at 15°C . Arrow indicates the position of T7

linear monomer DNA, sedimented in another tube simultaneously.

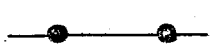
 , ^{32}P -radioactivity.

Fig. 3

Zone sedimentation in CsCl density gradient of intermediate density molecules obtained in the presence of T7 DNA polymerase. (a) Analysis of intermediate density molecules obtained 20 min after infection with ^{32}P - and BrdU-labelled T7 2, 3, 4 phage. (b) Analysis of intermediate density molecules obtained 10 min after infection with ^{32}P -labelled T7 1.3, 2, 3, 4, 5 and BrdU-labelled T7 2, 3, 4 phage. Before centrifugation, ^3H -labelled T7 phage DNA was added. The experimental methods are the same as those described in the legend to Fig. 2.

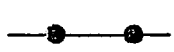
 , ^{32}P -radioactivity; ---O---O--, ^3H -radioactivity.

Fig. 4

Multiply branched molecules. The purified intermediate density molecules obtained under 2, 3, 4 condition were spread by aqueous (a) and formamide technique (b). Small circular DNA is colicin E1 plasmid DNA added before spreading. Arrows indicate D-loop structure. Magnification (a) 12,000 X ; (b) 16,000 X

Fig. 5

Crosslinked molecule. Magnification 14,000 X

Fig. 6

Zone sedimentation in a CsCl density gradient of the intermediate density molecules obtained by the cross of ^{32}P - and BrdU-labelled T7 2, 4 phage. The experimental procedures are the same as those described in the legend to Fig. 2. DNA was extracted from the infected cells and intermediate density molecules were

purified by two cycles of CsCl equilibrium density gradient centrifugation. The purified DNA molecules were sedimented in a CsCl density gradient. Arrow indicates the position of T7 linear monomer DNA.


 , ^{32}P -radioactivity.

Fig. 7

Analyses of linear monomer T7 DNA molecules with intermediate density obtained under 2, 4 condition in CsCl equilibrium density gradient centrifugation. DNA molecules in fractions 24 and 25 in Fig. 6 were used. (a) Centrifugation in a neutral CsCl gradient; (b) centrifugation in a neutral CsCl gradient after fragmentation; (c) centrifugation in an alkaline CsCl gradient. Before centrifugation, ^3H -labelled T7 phage DNA was added. The procedure of fragmentation by shearing force is described in Materials and Methods, section (f). Centrifugation procedures are the same as those described in the legend to Fig. 2 (a) and in Materials and Methods, section (f).

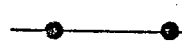

 , ^{32}P -radioactivity;  , ^3H -radioactivity.

Fig. 8

Analyses of intermediate density molecules obtained in the presence (a, b) and absence (c, d) of gene3 endonuclease I in CsCl equilibrium density gradient centrifugation. The phages used were defective in gene 2 and 4. DNA was extracted from the infected cells and intermediate density molecules were purified by two cycles of CsCl equilibrium density gradient centrifugation. (a) and (c): recentrifugation in neutral CsCl gradient of the purified intermediate density molecules. (b) and (d): alkaline CsCl density gradient centrifugation of the same sample as in (a) and (c). Before centrifugation, ^3H -labelled T7 phage DNA was added. Centrifugation procedures are the same as those described in the legend to Fig. 2 (a)

and in Materials and Methods, section (f).

—●—●—, ^{32}P -radioactivity; ---○---○---, ^3H -radioactivity.

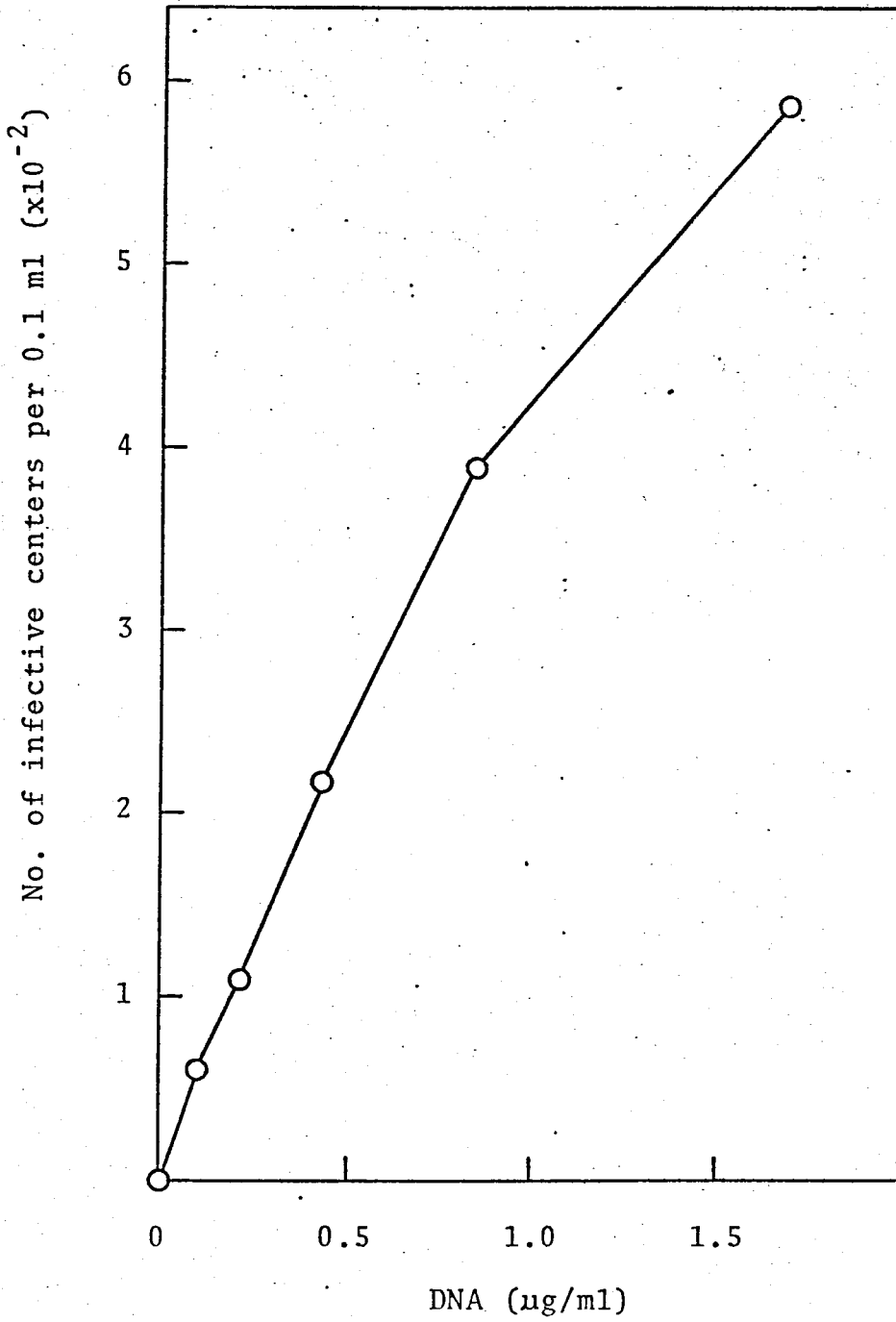
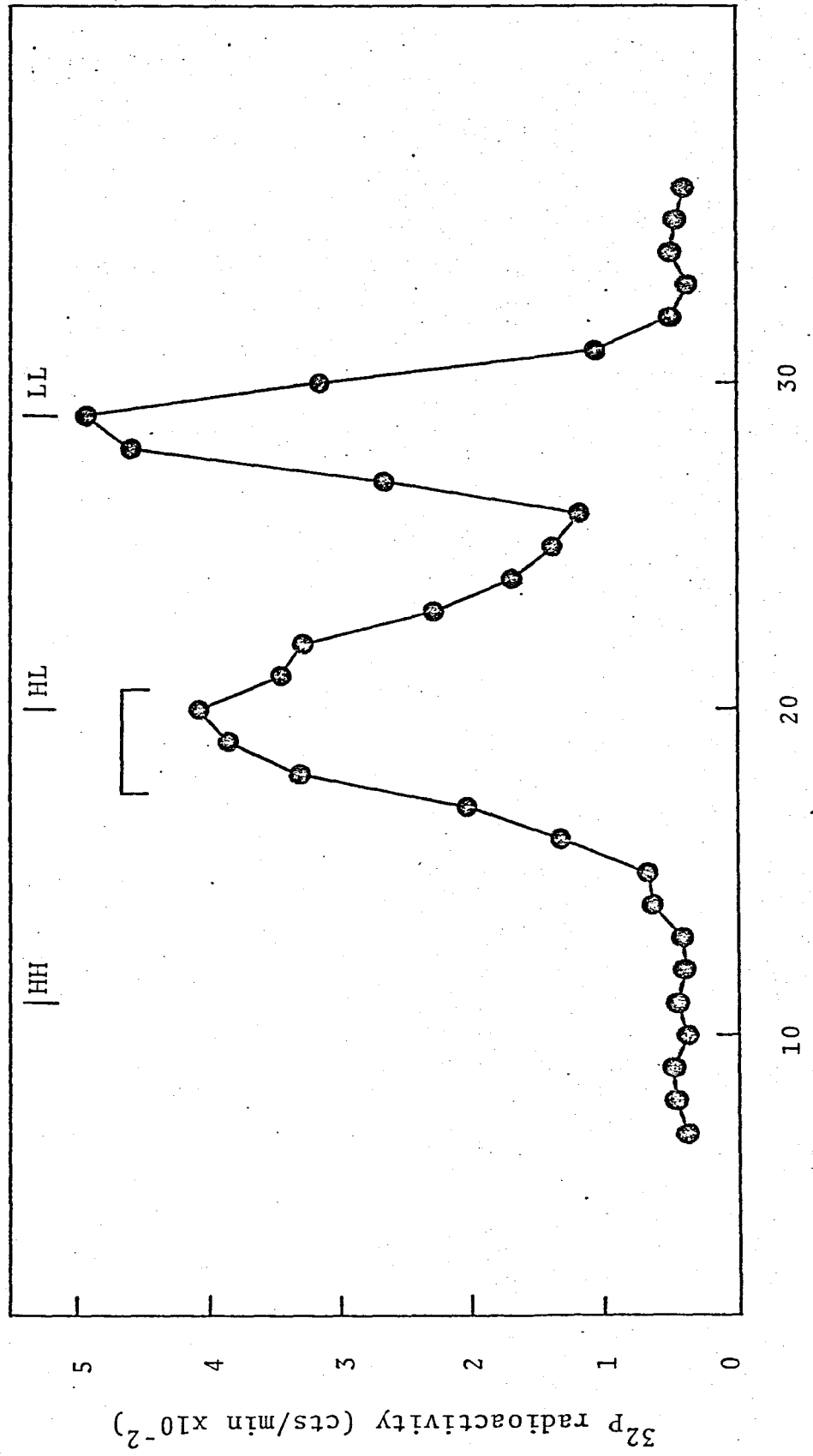


Figure 1.



Fraction number
Figure 2 (a)

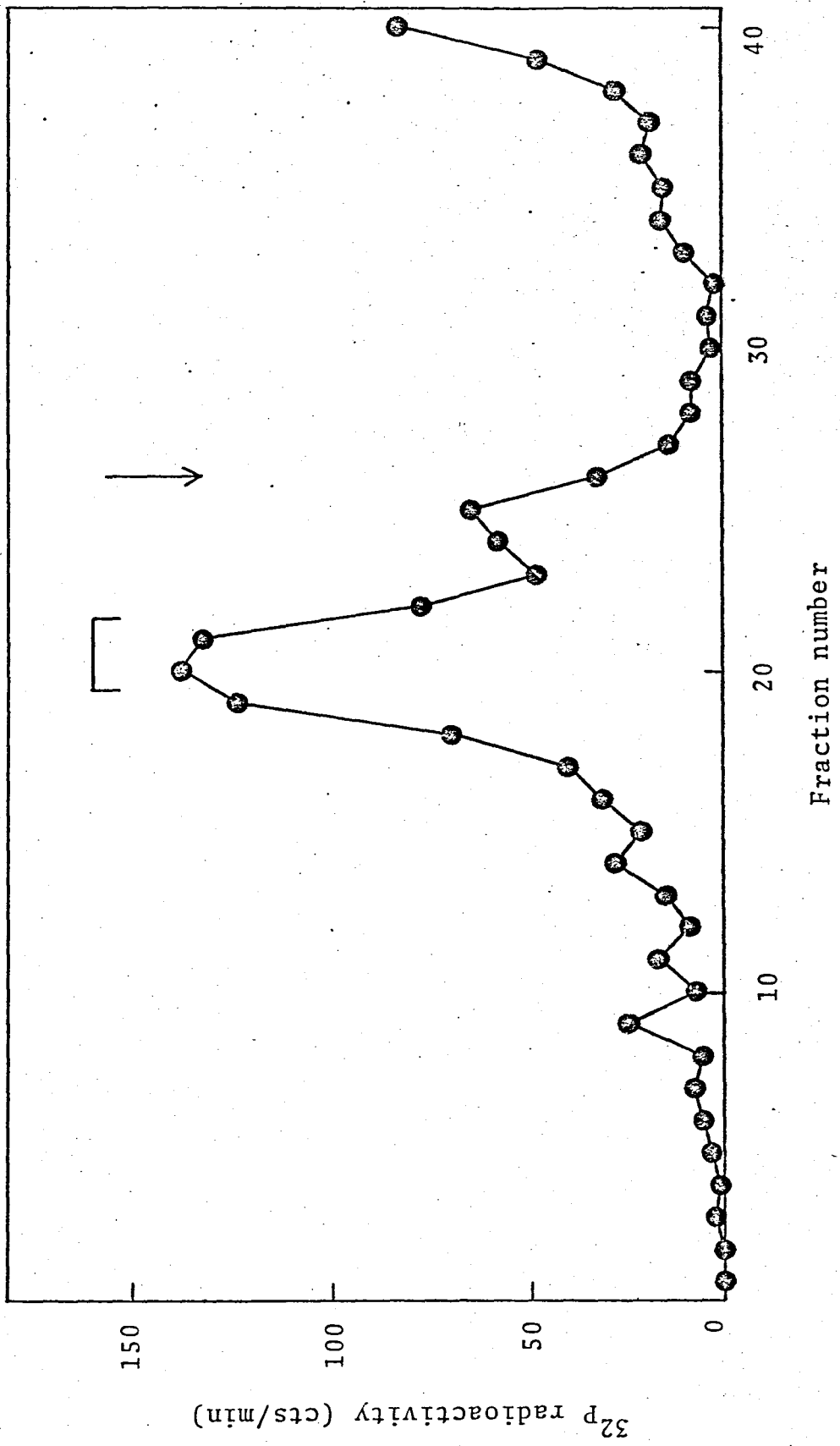
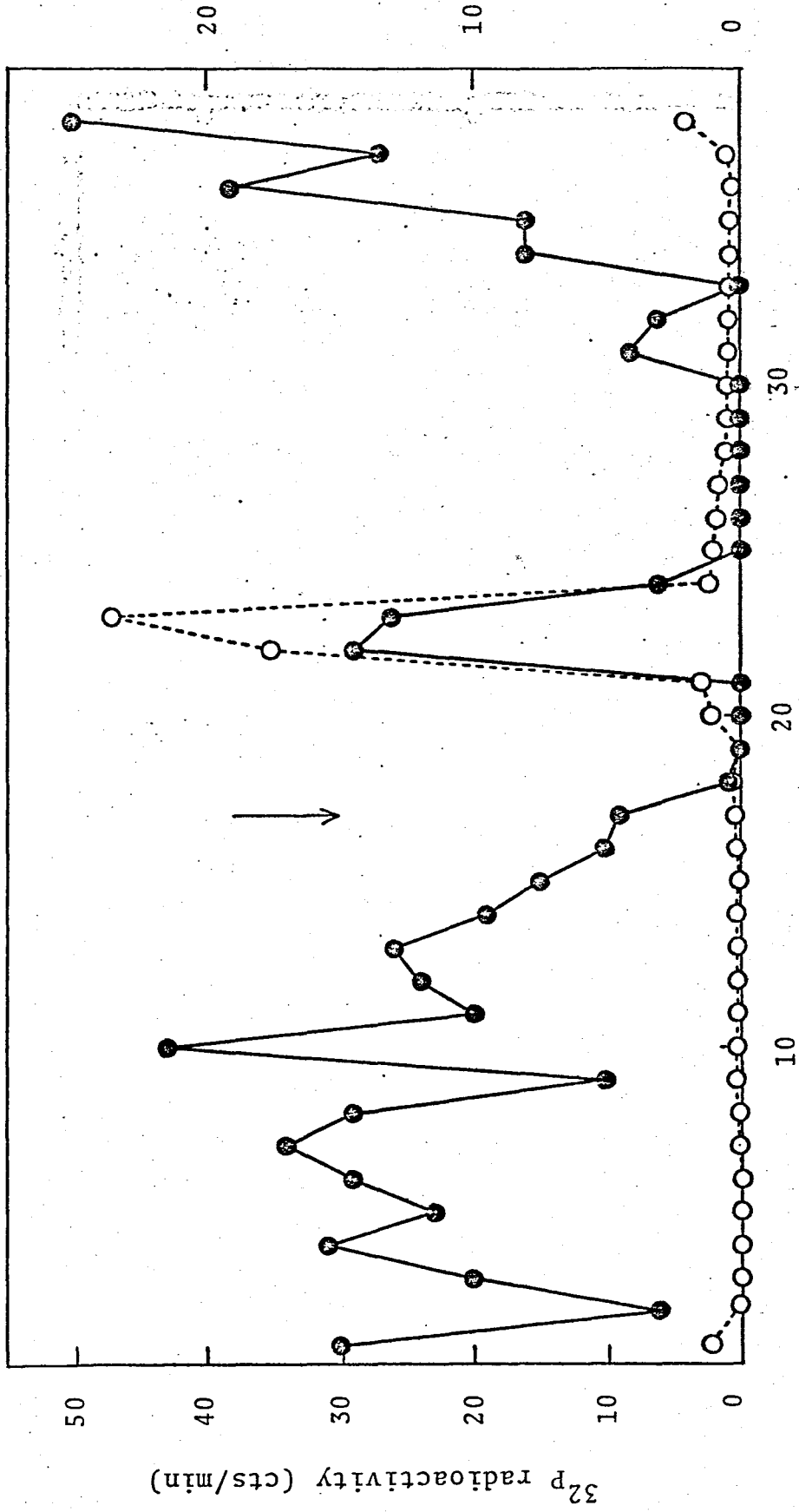


Figure 2 (b)

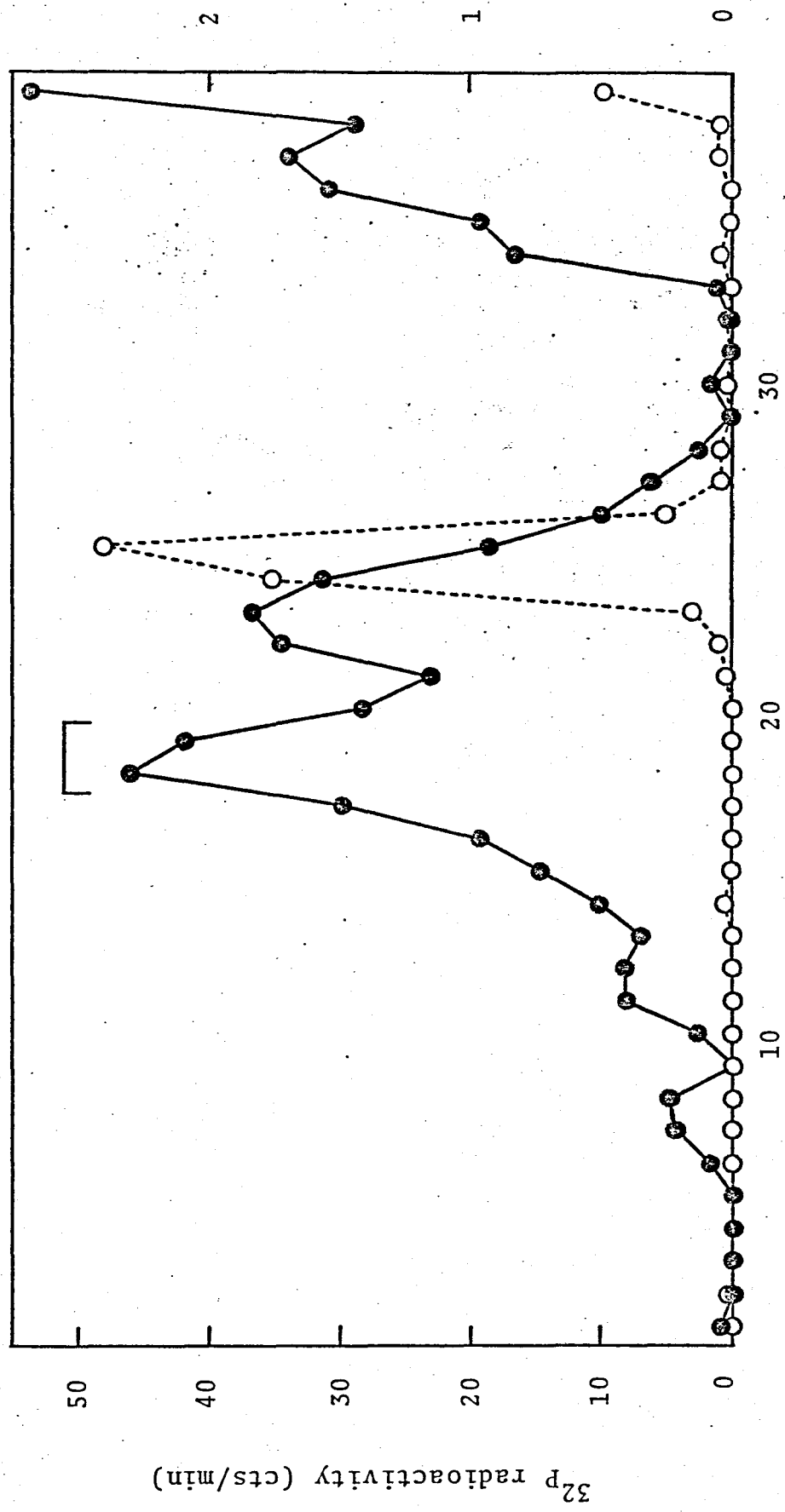
^3H radioactivity (cts/min $\times 10^{-2}$)

C39.



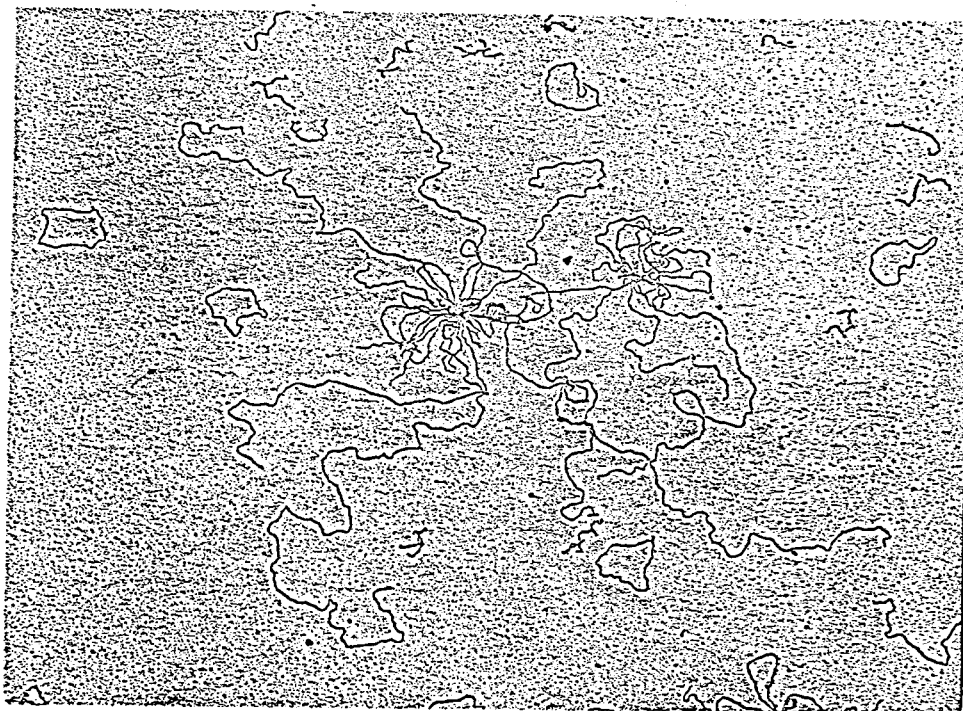
Fraction number

Figure 3 (a)

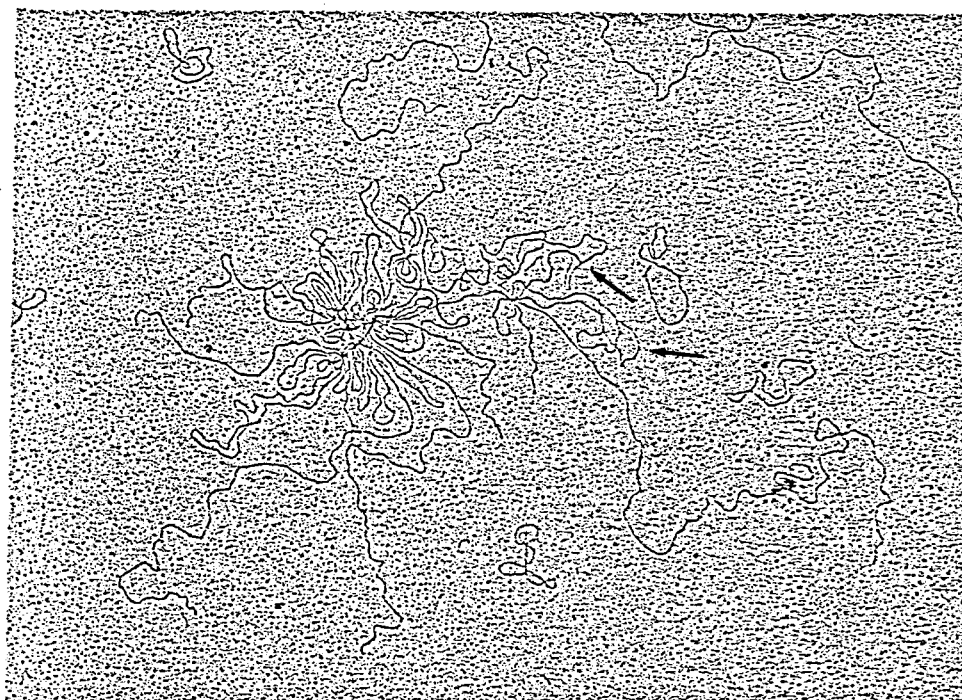


Fraction number

Figure 3 (b)



(a)



(b)

Figure 4

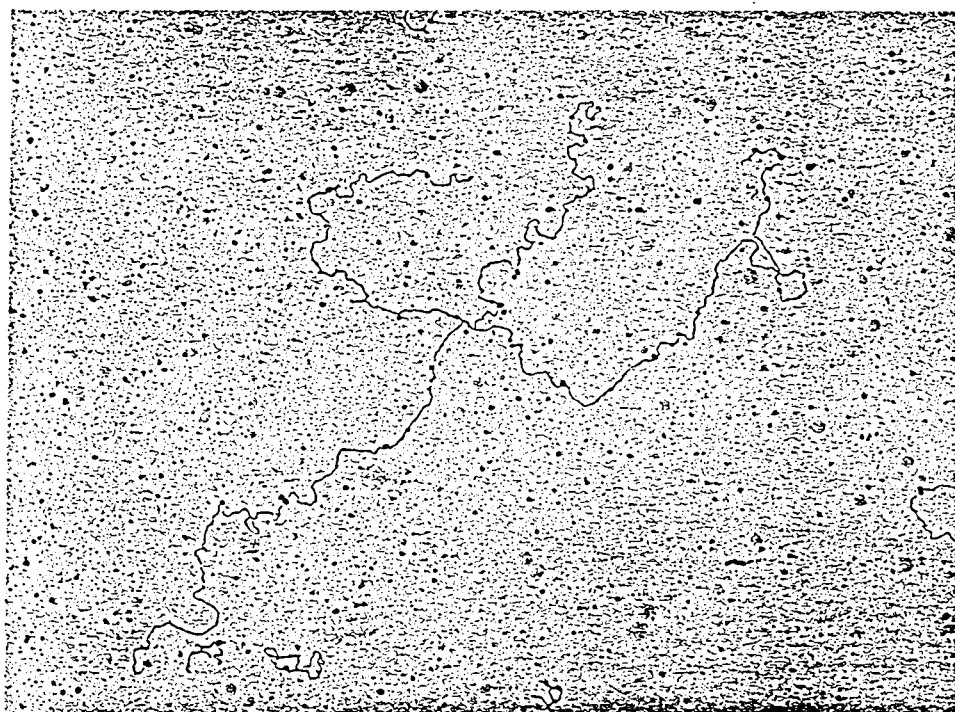


Figure 5

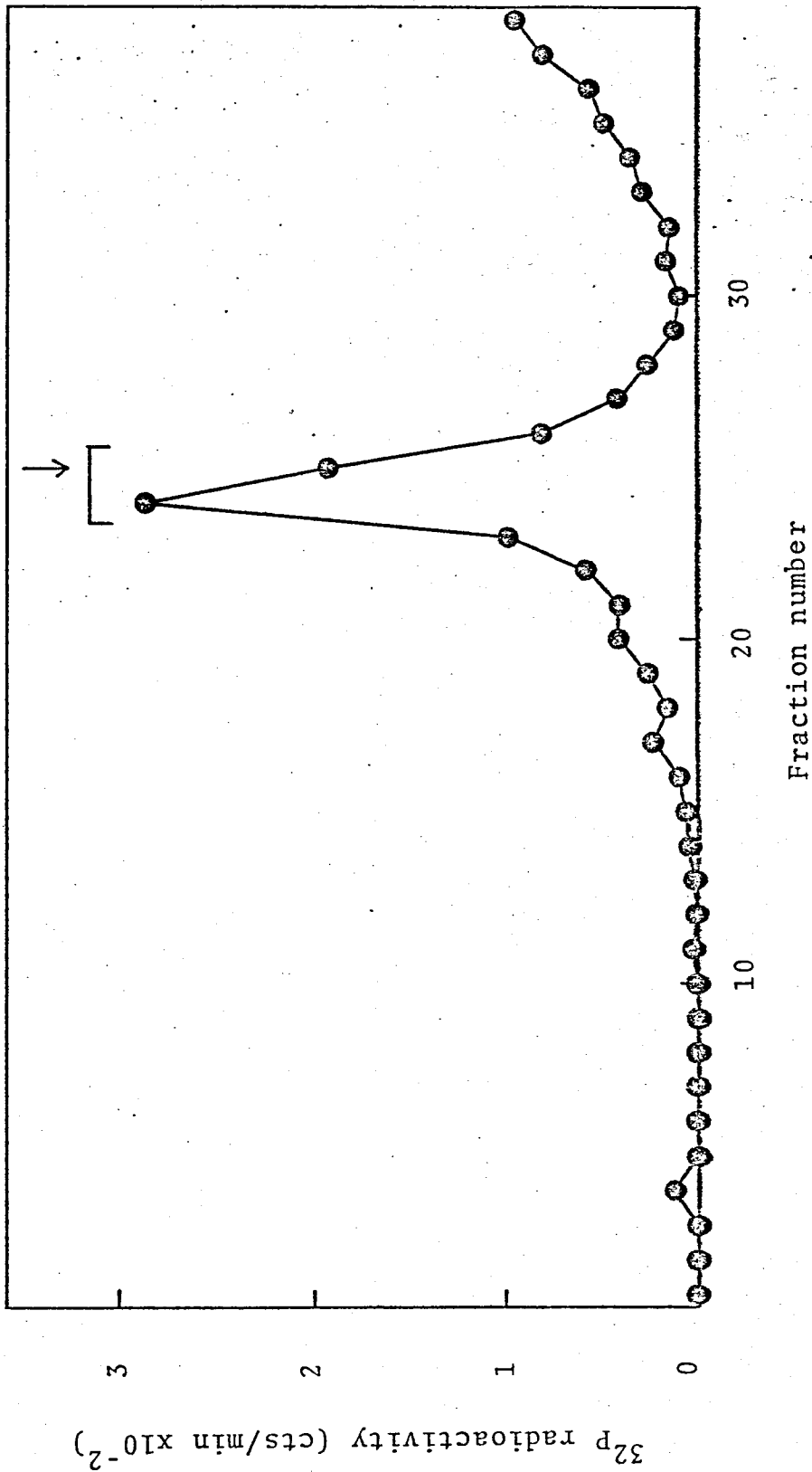


Figure 6

^3H radioactivity (cts/min $\times 10^{-3}$)

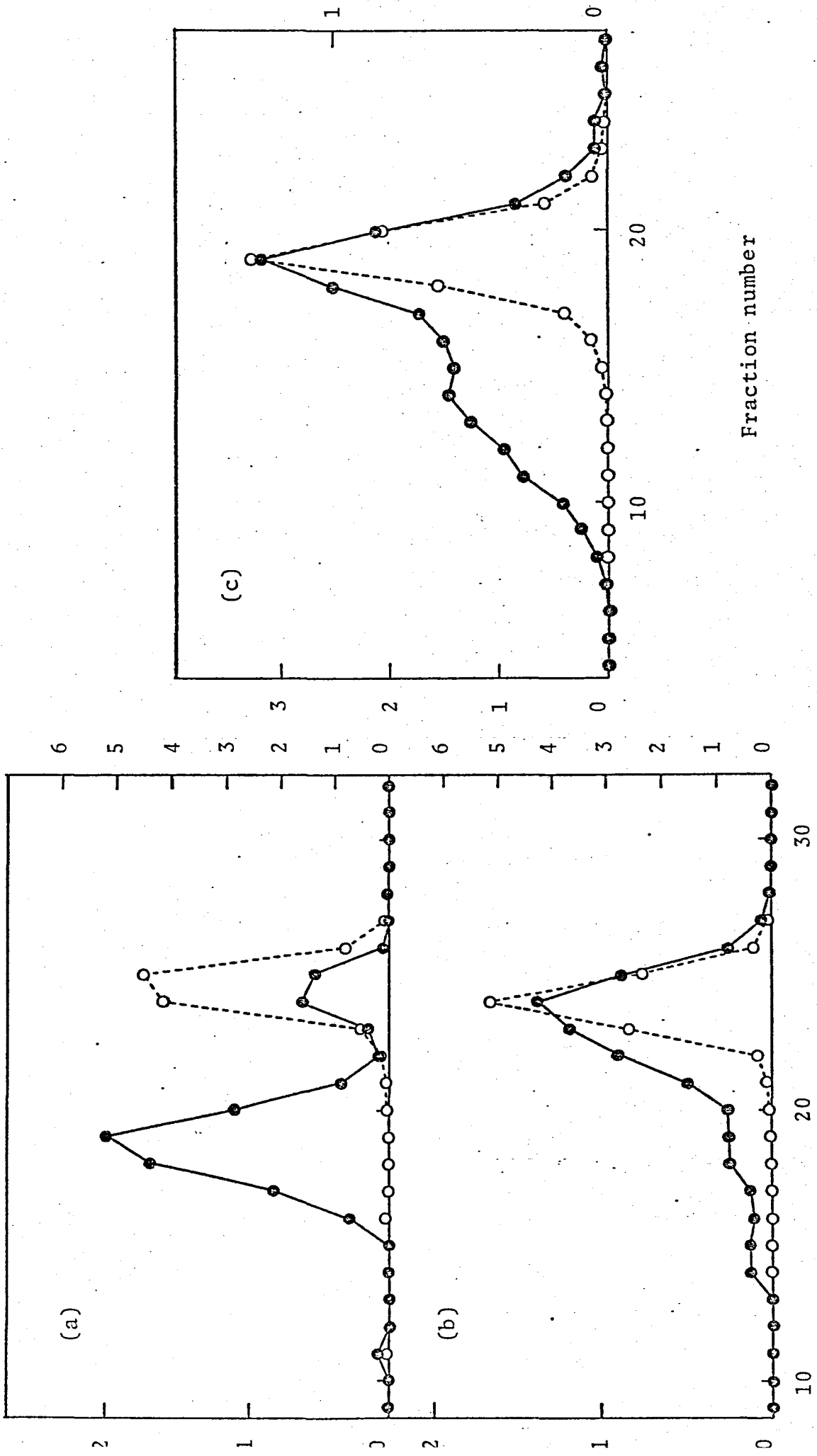


Figure 7

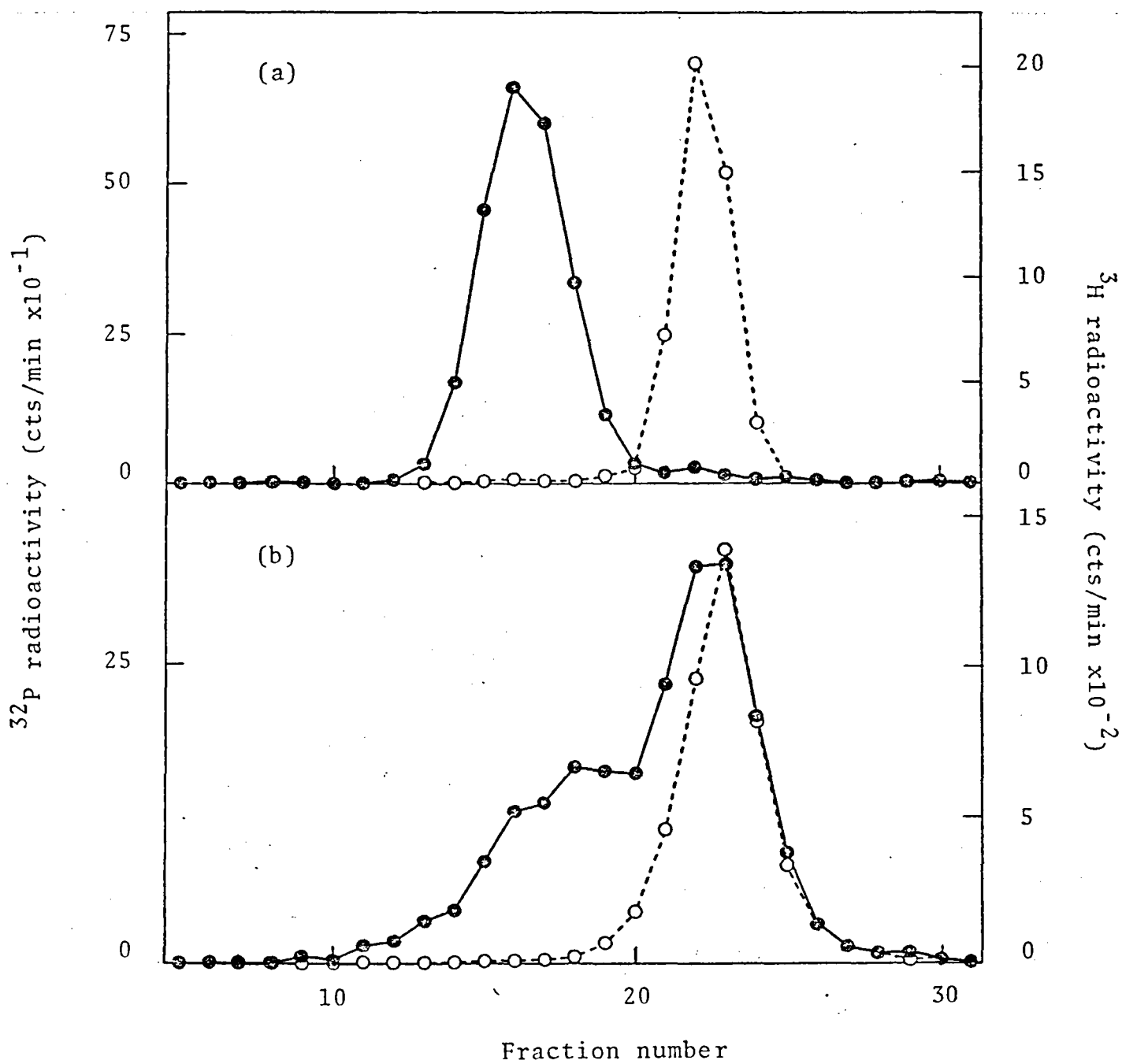


Figure 8 (a) (b)

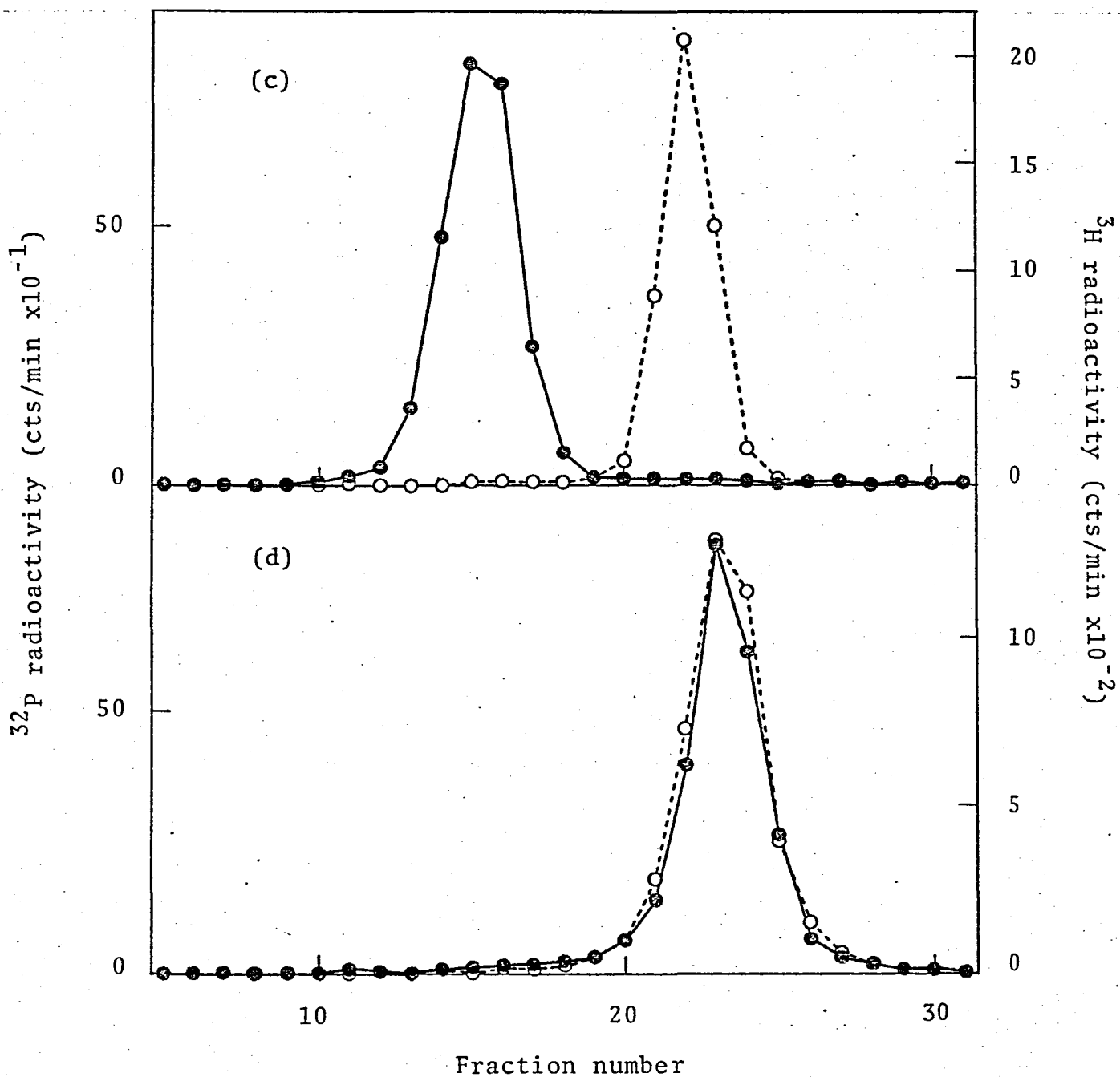
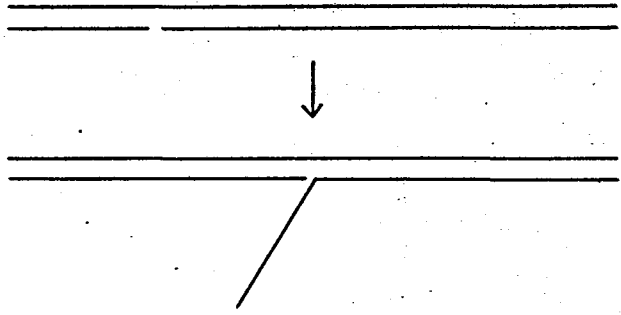


Figure 8 (c) (d)

A



B

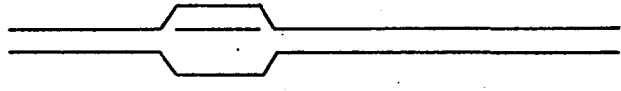


Figure 9

Concluding remarks

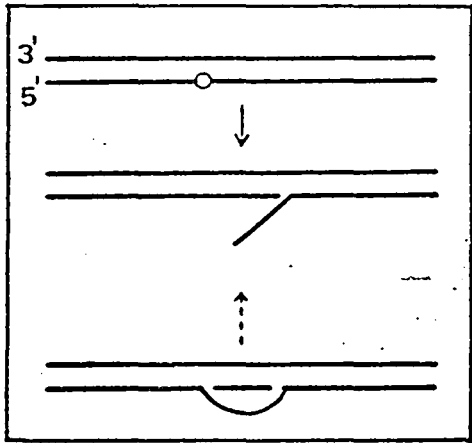
From the results mentioned in part A and part C, the process of genetic recombination in bacteriophage T7 would be summarized as follows (Figure CR-1). The organized summary is based on the results from the comparative analyses of the structures of the intermediate density molecules obtained in the presence or absence of genes suggested to be involved in recombination.

Synapsis step of formation of dimeric intermediate DNA molecules from parental DNA requires 5'-exonuclease (gene 6). Single-stranded gaps (Figure CR-1 (a)) formed by the exonuclease would allow parental DNA to interact each other. This synapsis step is stimulated by T7 DNA polymerase (gene 5) and in result, multiply branched molecules are accumulated. Presynaptic event for the stimulation of DNA interaction would be accumulation of single-stranded structures as described in Figure CR-1 (b). These two types of single-stranded structures are formed through displacement DNA synthesis by T7 DNA polymerase with nick or without nick.

Then, endonuclease I (gene 3) which digests single-stranded DNA preferentially acts on branched intermediates and processes them to linear recombinant DNA by cleaving single-stranded region at branch points.

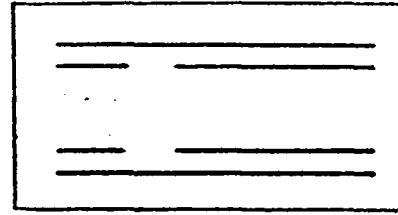
These linear recombinant DNA are then matured to complete recombinant DNA through repair by bacterial or phage DNA polymerase and ligase.

(b)



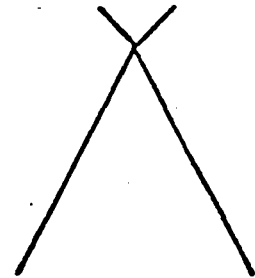
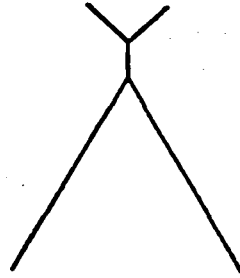
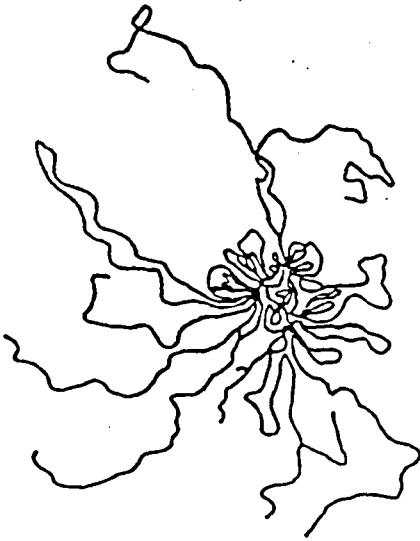
PARENT DNA

(a)



DNA POLYMERASE
GENE 5

5' EXONUCLEASE
GENE 6



ENDONUCLEASE 1
GENE 3

BACTERIAL LIGASE

BACTERIAL DNA POLYMERASE 1

T7 LIGASE
GENE 1.3

T7 DNA POLYMERASE
GENE 5

RECOMBINANT DNA

Figure CR-1

Acknowledgement

My appreciation and deep thanks are extended to Dr. Hideyuki Ogawa, Dr. Tomoko Ogawa and the late Professor Ichiro Haruna for their supports, encouragements and helpful discussions during this work. I am also indebted to Dr. Jun-ichi Tomizawa for his critical discussions and suggestions, to Dr. Joseph Inselburg for his critical reading of the manuscripts, during the preparation of part A and part B of this dissertation.