FORMATION OF PARENTAL REPLICATIVE FORMS OF ϕX174: SYNTHESIS OF THE FIRST COMPLEMENTARY STRAND

Thesis by

Anthony Joseph Zuccarelli

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Abstract

Part I.

Mutants of the bacteriophage ϕX174 have been isolated that are less dense than wild-type ϕX phage particles in CsCl. When viral strands from the mutants are hybridized with wild-type complementary strands, the resulting duplex molecules have single-stranded loops characteristic of wild type-deletion heteroduplexes. The mutant phages fail to complement ϕX amber mutants in cistron E, but they do complement mutants in six other cistrons. Based upon contour measurements of phage DNA and duplexes, and the buoyant density of the particles, it is estimated that the mutant viruses have deleted approximately 7% of the ϕX genome in the region of cistron E.

Part II.

The formation of circular, double-stranded RF (replicative form) DNA in cells has been observed in the period from 15 seconds to 20 minutes after infection with the SS (single-stranded) DNA bacteriophage ϕX174. The kinetics of appearance of RF during the first few minutes lead to the conclusion that the new, complementary DNA strand is polymerized in less than 10 seconds (viz. about 600 nucleotides per second).

The structure of RFII (a circular duplex with at least one SS break), RF made after infection with UV damaged phage, and nascent RF (extracted 1 minute after infection) were determined by sedimentation analysis and observations made with the electron microscope. They led

to the following generalizations: (a) Normal RFII molecules usually have intact circular viral strands and unit-length linear complementary strands. (b) RF made on UV damaged templates also have circular viral strands, but the complementary strand is shorter than unit-length and regions of SS template are evident. (c) The new complementary strand contains many discontinuities immediately after its synthesis, but these are eventually sealed. (d) The viral strand in nascent RF also appears to be broken. These conclusions are incorporated into a proposed mechanism for the synthesis of the first complementary strand.

Part III.

Parental RF molecules were pulse-labeled with [^{3}H]thymidine under conditions expected to label the parts of the new complementary strand which are synthesized last. The RF were analyzed by digestion with a restriction enzyme isolated from Haemophilus influenzae. The pattern of ^{3}H label in the resulting fragments led to the following conclusions: (a) Synthesis of the complementary strand is ordered and begins at one or two specific initiation sites. (b) One initiation is located in or near cistron A. A second initiation near the junction of cistrons G and H may also exist. (c) Synthesis of the new strand is counter-clockwise on the genetic map, in the 5' → 3' direction catalyzed by the known DNA polymerases.

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GENERAL INTRODUCTION

Bacteriophage ϕX174 is a minute icosahedral virus which possesses a small, circular, single-strand of DNA as its only genetic inheritance. How this virus propagates in a biological universe dominated by double-stranded DNA has been a subject of research since 1959, when its physical properties were first reported (Sinsheimer, 1959a, 1959b). This introduction to a study of the initial stage of ϕX174 DNA synthesis will primarily review the early steps in the ϕX174 infection. Reviews of the entire infectious cycle should be consulted for details of other stages of virus multiplication (Sinsheimer, 1968, 1969).

The synthesis of ϕX DNA which occurs after the appearance of the viral genome in a host cell has been divided into three stages, briefly summarized below (Sinsheimer et al., 1962; Yarus & Sinsheimer, 1967; Lindqvist & Sinsheimer, 1968; Knippers et al., 1968; Komano et al., 1968; Sinsheimer et al., 1968; Knippers & Sinsheimer, 1968; Dressler & Denhardt, 1968).

(a) Synthesis of the complementary strand

Immediately after its entrance into the cell the viral single-strand ring is converted into a double-stranded ring. It is this step, in which the virus adjusts to the reality of a biological environment geared to double-stranded DNA, which is the subject of this thesis. The conversion is accomplished by host enzymes which synthesize a new strand of DNA, the "complementary" strand. The resulting double-stranded DNA ring is called the parental replicative form, or "parental RF," because it contains the original (parent) viral DNA strand.

(b) RF replication

After its completion, the parental RF molecule becomes attached to a special site within the host and there serves as a template for the manufacture of 10 to 20 new double-stranded rings called "progeny RF." The immediate products released from the replication site are RFII molecules: circular duplex DNA molecules with one or more single-strand breaks. Each is rapidly converted into RFI: a DNA duplex with both strands covalently closed. The replication process requires, in addition to host enzymes, a protein specified by one viral cistron.

(c) Single-strand synthesis

About 12 to 15 minutes after infection at 37°C, most of the progeny RFI molecules which have accumulated in the cell are converted to RFII. These begin an asymmetric DNA synthesis which produces single-stranded DNA of the kind which emerged from the original infecting virus. This stage requires, in addition to host enzymes, the presence of five viral proteins, three of which are found in the mature virus coat. The new single-stranded circles first appear in progeny virus particles within the host. 15 to 25 minutes after the infection started, they are released into the growth medium by the disintegration of the cell.

The synthesis of the first complementary strand, in making a parental RF, is preceded by interactions of the phage particle with the cell surface. These have been divided into three stages: adsorption, eclipse and injection.

The first stage involves a direct association of the phage with the cell wall of a susceptible host cell (Stouthamer et al., 1963). This attachment requires the presence of divalent cations (Sinsheimer, 1959a; Fujimura & Kaesberg, 1962) and occurs at a rate of 8×10^{-9} ml per minute per bacterium at 37°C in nutrient medium (Newbold & Sinsheimer, 1970b) and 6×10^{-9} ml per minute per bacterium in 0.1 M $CaCl_2$ (Fujimura & Kaesberg, 1962). The attachment is reversible in that all of the adsorbed phage eluted from the cells by repeated washing with borate-EDTA are fully infective (Newbold & Sinsheimer, 1970a). Attachment has been shown to be the result of an interaction between the phage and lipopolysaccharide components of the bacterial cell wall (Incardona & Selvidge, 1973). Three ϕX capsid proteins, found at the 12 vertices of the phage coat (Edgell et al., 1969) are considered to be the viral organelles of attachment (Edgell et al., 1969; Brown et al., 1971). Adsorption will occur at temperatures below 15°C (Newbold & Sinsheimer, 1970a, 1970b) and it may be limited to zones of the cell surface where the membrane is firmly attached to the wall (Bayer, 1971).

The next step, eclipse, occurs at temperatures above 17°C (Newbold & Sinsheimer, 1970b) and is considered to be a direct intermediate in the penetration of the cell wall by viral DNA. The kinetics of eclipse at 37°C are biphasic (Rueckert & Zillig, 1962). About 85% of the adsorbed particles eclipse at one rate ($K_e = 0.86\ min^{-1}$), and the remainder at a lesser rate ($K_e = 0.21\ min^{-1}$) (Newbold & Sinsheimer, 1970b). An irreversible conformational change occurs at eclipse since eclipsed phage, detached from the cell either spontaneously or chemically, are no longer infective and have viral DNA protruding from their

protein coats (Newbold & Sinsheimer, 1970a). Though phage are more difficult to remove from the cell after eclipse (Newbold & Sinsheimer, 1970a), they still appear to be associated with some outer layer of the cell wall (Knippers et al., 1969a).

The "cold-sensitive" mutants isolated by Dowell (1967) fail to eclipse at 26°C, but eclipse normally at 37°C. These mutants map in the ϕX cistron known to code for the main structural component of the phage coat (Hutchison, 1969). Unpublished data cited by Sinsheimer (1968) indicate that the formation of parental RF could be observed at temperatures as low as 2°C when a cold-sensitive mutant was used to synchronize infection.

In the final step, the viral DNA penetrates the cell wall. Knippers et al. (1969a) found that the single-stranded DNA entered the cells of a starved, thymine auxotroph when growth medium, without thymine, was provided. The detection of parental single strands within the cell in this experiment eliminates the possibility that the DNA is "pulled" into the cell by the synthesis of the complementary strand. This result could be interpreted to mean that active cell metabolism is necessary to uncoat the DNA or allow its penetration. However, double-stranded parental RF molecules are formed in cells even after exposure to lethal concentrations of potassium cyanide (Benbow et al., 1974) or sodium azide (unpublished results, see Knippers et al., 1969a). Therefore, it seems that starvation itself causes a condition which prevents ϕX DNA injection. The condition is reversed by a short exposure to growth medium.

The fate of all the capsid proteins of the infecting phage particle remains an active topic of study. Newbold & Sinsheimer (1970a) reported that the phage coat remains outside the cell since all but 2% of the protein label could be removed from the cell by seven washes with borate-EDTA. Brown et al. (1971) have reported that 15% of the parental coat proteins remained associated with the infected complexes after the cells were converted to spheroplasts by lysozyme-EDTA. The sedimentation properties of this residual protein indicated that it was in units much smaller than an intact phage coat. Electrophoresis of the protein remaining with the spheroplasts revealed that all of the capsid proteins were represented. They also reported that 7% of the proteins from the capsid of the parental phage with density-labeled DNA appeared in progeny phage particles containing unlabeled DNA. When T4 phage was used to superinfect ϕX infected cells, no ϕX parental protein label appeared in the T4 progeny. Brown et al. (1971) concluded that the parental coat proteins were not degraded to individual amino acids, but were preserved as intact polypeptides in the host cytoplasm and then incorporated into new phage particles.

Jazwinski et al. (1973) cite unpublished data which indicate that one or a few molecules of a unique phage coat protein remain associated with the injected viral DNA even after its conversion to RF. They speculate that, in analogy to the case of the filamentous phage M13, the coat protein may be essential for the synthesis of parental RF.

Formation of parental RF of ϕX174 does not require synthesis of any new proteins. Neither high levels of chloramphenicol (Tessman, 1966; Stone, 1967) nor starvation of an auxotrophic host for an

essential amino acid (Greenlee & Sinsheimer, 1968; Knippers & Müller-Wecker, 1970) prevents it appearance. These observations have led to the conclusion that the complementary strand is synthesized by pre-existing host enzymes, although the role of capsid proteins which may enter with the viral DNA has not been elucidated (see Jazwinski et al., 1973).

Starvation for energy sources or for thymine (Benbow et al., 1974), high doses of ultraviolet light (Newbold & Sinsheimer, 1970a), high caffeine concentrations (Hess et al., 1973), and the thymidine antagonist fluorodeoxythymidine (Sinsheimer et al., 1962) are treatments known to prevent parental RF formation.

Though the rate of conversion of infecting single strands to parental RF has not been measured directly, Knippers et al. (1969a) inferred that the synthesis of one complementary strand may take only 12 seconds on the basis of the number of parental RF found in cells early in the infection.

Direct observations of the conversion of viral DNA into RF have been hampered by the lack of effective means of synchronization (see Sinsheimer et al., 1962) and by the rapidity of synthesis. The starvation and cyanide synchronization procedures (Denhardt & Sinsheimer, 1965a) permitted the first studies of parental RF appearance. Denhardt & Sinsheimer (1965b) observed RF in cells 4 minutes after an infection synchronized by starvation. The incorporation of radioactive label into the complementary strand indicated that the new DNA was polymerized de novo from materials found in the medium at infection, rather than from

E. coli DNA made earlier. No free parental single-stranded DNA was observed in the cells.

Sinsheimer et al. (1965) reported that parental RFI molecules were present in cells 2 minutes after the removal of a cyanide block. However, since synthesis of the first complementary strand is not prevented by cyanide (Cairns & Denhardt, 1968; Denhardt & Sinsheimer, 1965b) their time estimate is invalid.

The double-stranded character of ϕX RF has been characterized by the hybrid density of molecules which are formed when cells in "light" medium are infected with "heavy," isotopically labeled phage (Sinsheimer et al., 1962) or when cells in "heavy" medium are infected with "light" phage (Stone, 1967). The infectivity of RF is ten times more resistant to inactivation by ultraviolet light than are single strands. In host cells which lack a UV repair enzyme (hcr$^-$) the UV resistance of RF is only twice as great as that of single strands (Jansz et al., 1963; Yarus & Sinsheimer, 1964). The base composition of RF also suggests that it is double-stranded (Siegel & Hayashi, 1967).

Evidence that the parental RF becomes associated with a specialized site within the cell was first presented by Denhardt & Sinsheimer (1965c). Highly radioactive ^{32}P phage were used to infect cells synchronized by starvation. During the first 15 minutes of the infection, portions of the culture were rapidly frozen in liquid nitrogen and stored at -196^{o}C to allow ^{32}P decay. The survival of the infected complexes was compared with the survival of purified ^{32}P phage preparations. As expected, conversion of the infecting single-stranded DNA to a double-stranded form during the first few minutes caused a 5-fold

decrease in the inactivation of the complexes by ^{32}P decays. However, it was observed that from 4 minutes to the end of eclipse (about 10 minutes) the inactivation rate did not decrease any further, in spite of the proliferation of progeny RF molecules in the cells during this period. A special functional role for the parental RF molecules, which could not be performed by the progeny RF, was proposed. It was suggested that the parental RF occupied a unique site which existed in previously starved cells. This hypothesis was supported by the work of Salivar & Sinsheimer (1969) which showed that even at high multiplicities of infection, only one or two of the infecting genomes participated in the formation of progeny phage in previously starved cells.

Yarus & Sinsheimer (1967) infected cells with four phenotypically different mutants simultaneously and assayed single-cell bursts to determine the number of mutants of each type which were released. In starved cells, over 70% of the bursts released only one mutant type and the remaining bursts released two. On the basis of the multiplicity of infection with each mutant, nearly all of the bursts were expected to produce phage of at least three different phenotypes. The limited number of functional sites in starved cells was considered responsible for the discrepancy.

Indirect evidence has shown that association with the site is necessary for an RF to be replicated or transcribed. After starvation, only a small fraction of the cells in a culture are capable of producing two types of progeny phage; most produce one type. However, complementation between phage mutants was observed only in those cells which generated two phage phenotypes, even at multiplicities which assured

that most cells received both mutant parents (Hutchison, 1969).

Knippers & Sinsheimer (1968) found that parental RF preferentially sedimented with a cellular component in gently lysed cells, whereas the numerous progeny RF in the cells sedimented as free molecules. The authors speculated that some parental RF were bound to a membranous cellular site which contained the replicating enzymes or which aided in unwinding ϕX RF during replication.

The rep⁻ mutation of E. coli (Denhardt et al., 1967) allows the synthesis of ϕX parental RF but does not permit RF replication. The nature of the defect in this mutant has not been identified, but it has been suggested that the essential membrane site needed for ϕX replication has been altered (Sinsheimer, 1968).

Several workers have studied the parental RF which accumulate when its replication is inhibited. Levine & Sinsheimer (1969) were able to isolate parental RF when cells were infected in high concentrations of chloramphenicol or when non-permissive host cells were infected with ϕX amber mutants in cistron A. Under both conditions, the parental RF appeared to be associated with a membranous cell component and they were largely RFII.

Francke & Ray (1971) observed that more than 90% of the parental RF were RFI, independent of the multiplicity of infection, when the cistron A protein was absent. The small proportion of molecules which were RFII appeared to be non-specifically nicked in either strand. However, when parental RF were made in the presence of the cistron A protein (by am3 infection of rep3 host cells) a constant number of molecules per cell were RFII. At high multiplicities of infection, the excess phage

DNA were converted to RFI. Furthermore, the structure of the parental RFII isolated under these conditions was highly specific. The complementary strand was invariably closed, and the viral strand was open. When complete synthesis of the first complementary strand was prevented by UV damage in the infecting strand, an open viral strand was not observed, even in the presence of cistron A protein. They concluded that after the synthesis of the complementary strand is completed the A gene product causes or maintains a break in the viral strand.

At least four conditions are essential for ϕX RF replication, the stage which follows parental RF formation:

(a) The DNA replication machinery of the host cell must be intact. UV irradiation of the host prior to infection, for example, inhibits RF replication (Sinsheimer et al., 1968). Various genetic alterations in the host DNA synthesizing capacity also effect ϕX RF replication (Dumas & Miller, 1973; Loos & Salivar, 1971; Greenlee, 1973; Taketo, 1973).

(b) The parental RF must be associated with a functional replication site on the membrane.

(c) Functional protein from the phage cistron A must be present in a cis configuration (Francke & Ray, 1972; Tessman, 1966).

(d) One of the strands must be open to allow strand separation. The fulfillment of condition (c) may satisfy this requirement, since it has been suggested that the effect of cistron A protein is to cause the appearance of a nick in the viral strand of some parental RF (Francke & Ray, 1971, 1972).

A controversy currently exists in the literature as to which of the two strands in the parental RF remains at the site of replication and which is displaced by each new round of synthesis. Some workers report that the viral strand remains circular while the complementary strand is nicked, elongated and eventually displaced into a progeny RF molecules (Knippers et al., 1969b; Knippers & Müller-Wecker, 1970). Others have described the complementary strand as being circular (Dressler & Denhardt, 1968; Dressler & Wolfson, 1970). All the reports agree that one of the two strands remains at the site of replication and is used as the template for successive rounds of synthesis. The displaced strand becomes double-stranded and eventually appears in the cytoplasm as an RFI molecule.

In this thesis, direct observations of parental RF synthesis are described. The structures of the molecules which accumulate when RF replication is inhibited by chloramphenicol and those which are made on UV damaged viral templates are investigated. A mechanism for the synthesis of the first complementary strand is proposed consistent with the structure of nascent parental RF molecules. Data which support the hypothesis that the new complementary strand has specific initiation site(s) are also presented. The isolation of a specific class of ϕX deletion mutants is included.

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Part I: Isolation of ϕX174 Mutants for Use as Markers in Electron Microscopy

Portions of this section have been published under the title "Deletion mutants of bacteriophage ϕX174" (Zuccarelli, Benbow & Sinsheimer (1972). Proc. Nat. Acad. Sci. U.S.A. 60, 1905-1910). The Introduction and Materials and Methods have been expanded to accommodate the discussion of ϕX174 insertion mutants.

Part I: Isolation of ϕX174 Mutants for Use as Markers in Electron Microscopy

I. 1 Introduction

Genetically characterized deletions have been used as physical markers in the heteroduplex mapping of the genomes of bacteriophage λ, T4, and φ80 by electron microscopy (Davis & Davidson, 1968; Westmoreland et al., 1969; Kim & Davidson, 1971; Fiandt et al., 1971). The loss of a region of DNA 100 nucleotides in length is detected as a single-strand loop with this technique (Davis & Parkinson, 1971). Genetically defined deletions and insertions of 100 or more nucleotides of bacteriophage φX174 DNA would be valuable tools for determining the locations of such structural features of the φX174 DNA molecule as the specific nick in the in vivo replicative form (Knippers et al., 1969), the initiation sites of complementary and viral DNA synthesis, the non-homologous regions in heteroduplexes with φX-like phages (S13, ST-1, α-3) and the binding sites of Escherichia coli RNA polymerase. The contour lengths of the DNA molecules from deletion or insertion mutants as measured by electron microscopy would distinguish them from normal-length molecules in genetic experiments involving mixed infections (Benbow et al., 1972a). In addition, deletion and insertion mutants may provide a means for generating specific fragments of φX DNA for nucleotide sequencing.

Since the genome of φX174 has been extensively mapped (Benbow et al., 1971), and recombination frequencies are proportional to

physical distances over much of the genetic map (Benbow et al., 1972b), a correlation could be made between the physical location of each structural feature and the known ϕX cistrons.

A deletion is a nonsuppressible genetic defect; such mutants are viable only if the lesion is limited to an untranslated region or to dispensible viral functions. Of the nine known ϕX174 cistrons (Benbow et al., 1971) only E is not essential to the production of infective viral particles (Hutchison & Sinsheimer, 1969; Hutchison, 1969). Mutants in cistron E are impaired in the lysis function. They produce large numbers of apparently normal virions which remain trapped with the walls of an intact nonsuppressing bacterial cell (Hutchison, 1969). This property has been exploited in this work to enrich phage stocks for deletions in the cistron E region of the genome. Further selection, based upon the buoyant density of mature virus particles, was done to obtain mutants that had lost an appreciable amount of DNA. Some of the physical and genetic characteristics of a virus stock obtained by these means are described in this report.

The insertion of several hundred nucleotides of extraneous DNA into a cistron would usually be expected to destroy the function of the cistron. For this reason, most insertions outside of cistron E, like deletions, would be incapable of generating infective phage particles. However, a small subclass of these mutants in which DNA has been inserted into untranslated regions (possibly between cistrons) would preserve all of the genetic information intact. In principle, insertions at several locations on the genome would be genetically complete. Transcriptional continuity and the structural limitations

of the phage capsid would determine their ability to produce infective phage particles.

Assuming that some insertions can have all of the normal phage functions, their isolation would depend entirely upon the change in buoyant density expected for a particle with an enlarged genome. One section of this report describes an attempt to isolate such insertion mutants.

I. 2 Materials and Methods

(a) Bacterial strains

(i) Escherichia coli C, BTCC No. 122 (Sinsheimer, 1959a) is the standard wild type, (hcr$^+$) nonpermissive host for ϕX174.

(ii) E. coli H502 is a thy$^-$, uvrA (hcr$^-$), endo I$^-$, su$^-$ host strain constructed by Dr. Hoffmann-Berling.

(iii) E. coli HF4714 is a C-K12 hybrid (Godson, 1971) with the multiple auxotrophic requirements arg$^-$, his$^-$, leu$^-$, thr$^-$, pro$^-$. It is su$^+_{UAG}$ and suppresses most ¢X amber mutants.

(iv) E. coli Su2$_{och}$ is a double suppressor su$^+_{UAG, UAA}$ (Person & Osborn, 1968) with the genotype thy$^-$, cyt$^-$, met$^-$, pro$^-$, trp$^-$ from its parent strain WWU (Person & Bochrath, 1964).

(v) E. coli CIT103 suppresses ϕX174 opal mutants and has the genotype su$^+_{UGA}$, lac$^-$, str^r (Hutchison, 1969).

(vi) E. coli C-110 is a thy$^-$ mutant of E. coli C (hcr$^+$) (Lindqvist & Sinsheimer, 1967a).

(b) Bacteriophage strains

(i) ϕX174 wt is the wild-type virus characterized by Sinsheimer (1959a).

(ii) ϕX174 am3 is a lysis deficient mutant which maps in cistron E (Hutchison, 1969).

(c) Other materials

(i) L-[^{14}C]leucine (316 c/mole), L-[4,5-^{3}H]leucine (2 c/mmole), [2-^{14}C]thymidine (50 c/mole), [methyl-^{3}H]thymidine (10 c/mmole), [methyl-^{3}H]thymine (16 c/mmole), sucrose (density gradient grade, ribonuclease free) and urea (ultrapure grade) were purchased from Schwarz/Mann, Orangeburg, N.Y.

(ii) Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (Temed), ammonium persulfate (all electrophoresis grade), Dowex 50W-X2 cation exchange resin (100 to 200 mesh, hydrogen form), and Bio-Gel A-1.5m agarose beads (100 to 200 mesh) were purchased from Bio-Rad Laboratories, Richmond, Calif.

(iii) Lysozyme (egg white, 3x crystallized), porous glass beads (pore diameter 240 Å ± 10%, mesh 120 to 200), pyruvic acid (type II, sodium salt), tris(hydroxymethyl)aminomethane and tris(hydroxymethyl)aminomethane hydrochloride (Tris-OH and Tris-HCl, respectively) were purchased from the Sigma Chemical Co., St. Louis, Mo.

(iv) Propidium iodide (A grade) and cytochrome C (equine heart, salt free, A grade) were purchased from Calbiochem, San Diego, Calif.

(v) Platinum-paladium wire (80:20) and copper grids (300 mesh, 3 mm diameter) were purchased from the Ted Pella Company, Tustin, Calif.

(vi) Cesium chloride (optical grade) was obtained from the Harshaw Chemical Company, Solon, Ohio.

(vii) Mitomycin C (with NaCl carrier) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

(viii) Disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA) was purchased from the G. Fredrick Smith Chemical Company, Columbus, Ohio.

(ix) Sodium lauryl sulfate (SDS, specially pure) was purchased from BDH Chemicals Ltd., Poole, England.

(x) NCS tissue solubilizer was obtained from the Amersham/Searle Corporation, Arlington Heights, Illinois.

(xi) Liquifluor and Aquasol were purchased from the New England Nuclear Corporation, Boston, Mass.

(xii) Formamide (99%) was purchased from Matheson Coleman and Bell, Norwood, Ohio.

(xiii) 2-mercaptoethanol was obtained from the J. T. Baker Chemical Company, Phillipsburg, N.J.

(d) Media

KC broth (Sinsheimer, 1959a), plating agar (Dowell & Sinsheimer, 1966) and TPG medium (Lindqvist & Sinsheimer, 1967a) have been described.

(i) TPA medium was made by the addition of 0.3 g of each of 20 individual L-amino acids to 1 liter of TPG.

(ii) TPA-Leu is TPA medium from which the amino acid L-leucine was omitted.

(iii) Borate dilution buffer contains 0.05 M sodium tetraborate.

(iv) Borate-EDTA contains 0.05 M sodium tetraborate and 5 mM EDTA.

(e) Virus stocks

The preparation of genetically pure stocks of φX174 amber mutants was described by Benbow et al. (1971). [^{3}H]leucine [^{3}H]thymidine labeled phage particles were prepared in H502 host cells growing in TPA-Leu medium with 2 μg/ml of thymine. DNA was extracted from purified viruses by the procedure of Sinsheimer (1959b). Rabbit serum against φX174 was prepared by Robert G. Rohwer by the procedure of Rolfe & Sinsheimer (1965).

(f) Isolation of φX wt complementary strands

Double-stranded φX replicative form (RF) DNA was prepared according to Komano & Sinsheimer (1968). RFI was isolated by exclusion chromatography on a Bio-Gel A-1.5 m column, followed by equilibrium buoyant density centrifugation in CsCl (final density, 1.530 g/cm^3) with 100 μg/ml of propidium iodide. Propidium was removed from the RFI pool by running it through a 5 ml Dowex-50 column. The RFI molecules were nicked by exposing them to x-rays generated by a General Electric industrial x-ray unit. Complementary strand DNA was enriched from the resulting RFII molecules by equilibrium buoyant density centrifugation in a solution of CsCl with an average density of 1.750 g/cm^3 and 0.1 M KOH (Siegel & Hayashi, 1967). Centrifugation was at 30,000 revs/min for 60 h in a Beckman Type 50, fixed-angle rotor.

(g) Isolation of deletion mutants

Deletion mutants were generated by infecting a culture of E. coli C in the presence of 1 μg/ml of mitomycin C with φX174 wt at a multiplicity of 10 phage/cell. Under these conditions any defective viruses produced by the mutagenic action of mitomycin C (Iyer & Szybalski, 1963; Lindqvist & Sinsheimer, 1967b) would be released into the medium by the action of the numerous wt virions also present in the cell. Phage concentrated from this lysate were used to infect a culture of HF4714 at a multiplicity of less than 0.2 wt pfu/cell. Under these conditions, lysis defective viruses which are not suppressed by the su^{+}_{UAG} gene in this strain would remain within intact host cells.

EDTA was added 15 min after infection to remove divalent cations from the medium and thereby reduce adsorption of newly released wt phage (Newbold & Sinsheimer, 1969). After 90 min of incubation, the cells were collected by centrifugation and washed five times (by resuspension and centrifugation) with borate-EDTA to remove phage which had been released into the medium (Newbold & Sinsheimer, 1969). The cells were disrupted by incubation with 300 μg/ml lysozyme for 30 min at 37°C. The lysate was treated for 30 sec with a Branson sonifier and the released phage were used to infect a second culture of HF4714 at a multiplicity of less than 0.2 wt pfu/cell. This culture was similarly incubated, washed and then lysed. The phage from it were used to infect yet another culture at low MOI. The cycle of infection, washing, lysis, and infection of a new culture was repeated sequentially 15 times.

The lysates from each step were monitored for pfu on E. coli C, HF4714 and $Su2_{och}$. The titers indicated that there was no significant accumulation of suppressible nonsense mutations during the procedure.

Lysates subsequent to the fifteenth infection cycle were centrifuged to equilibrium in CsCl (at a density of 1.410 g/cm^3) for 60 h at 25,000 revs/min in a Beckman SW41 rotor. Gradients were collected in fractions through a puncture near the bottom of the tube. The A_{260nm} of selected fractions was determined in a Zeiss spectrophotometer. Fractions from the "light" edge of the phage peak were pooled and used to infect a new culture of HF4714 which was then washed and lysed. The phages released from it were banded in CsCl and the "light" fractions used to infect yet another culture. This sequential infection procedure was repeated eight times. The final lysate was, thereby, the result of 23 sequential infections from which unsuppressed lysis defectives and phage with lower tuoyant density were repeatedly selected.

Virus particles from the final lysate were inoculated at a multiplicity of 8 phage/cell into a 200 ml culture of H502 at 4 x 10^8 cells/ml in TPA-Leu with 2 µg/ml thymine. After 5 min, 100 µc of [^{14}C]leucine or [^{14}C]thymidine was added to the medium. After 2 h of incubation, the cells were collected by centrifugation, washed twice with 200 ml volumes of borate-EDTA, and lysed with lysozyme. The phage were purified by equilibrium centrifugation in CsCl (at a density of 1.410 g/cm^3), followed by sedimentation in a linear 5 to 20% sucrose gradient in 0.05 M Tris (pH 7.4), 5 mM EDTA. Alternatively, the

isopycnic centrifugation in CsCl was replaced by exclusion chromatography on a 1 x 95 cm column of porous glass beads (Gschwender et al., 1969). These purified phage suspensions will be called the "deletion stock."

(h) Complementation-plaque assay of deletion stock

"Helper-phage" (specific amber mutants in any φX cistron) at a multiplicity of about 5 pfu/cell were adsorbed at 37° for 5 min in 2 ml aliquots of E. coli C at 1 x 10^8 cells/ml in KC broth containing 3 mM KCN. The suspension was divided in half, and a portion of the deletion stock was added to one of the two tubes. After both suspensions had been incubated at 37°C with aeration for 15 min, 5 ml of an E. coli culture at 4°C containing about 5 x 10^8 cells/ml in KC broth with 3 mM KCN was added as carrier, and the tubes were centrifuged at 4°C at 6,000 revs/min for 15 min. The pellets were washed twice with 5 ml volumes of KC broth with cyanide and were resuspended in 1.0 ml of KC broth with cyanide and anti-φX rabbit serum (K = 25 min^{-1}). After incubation for 10 min at 37°C, the suspensions were diluted 1:10^4 in KC broth without cyanide, and the tubes were transferred to an ice bath. Aliquots were plated by the agar layer technique with HF4714 plating bacteria.

(i) Electron microscopy of DNA heteroduplexes

Heteroduplex DNA molecules (Davis & Davidson, 1968; Westmoreland et al., 1969) were constructed by mixing 0.1 μg each of deletion stock single-stranded viral DNA and wt complementary strand DNA in 100 μl of 0.1 M NaOH, 0.02 M EDTA. After 10 min at room temperature, the sample

was neutralized with 10 µl of 1.8 M Tris-HCl, 0.2 M Tris-OH (pH 8.5), and 100 µl of formamide. The sample was permitted to renature at room temperature for 1 to 2 h.

The basic protein film technique (Davis et al., 1971) was used to prepare samples for viewing in the electron microscope. Spreading solutions contained about 50 ng of DNA in 50 µl of 0.1 M Tris (pH 8.5), 0.01 M EDTA, 50% formamide and 50 to 100 µg/ml cytochrome C. Approximately 50 µl was spread on 100 ml of hypophase containing 0.01 M Tris (pH 8.5), 1 mM EDTA and 10 or 17% formamide. The film was picked up with parlodion-coated grids. Preparations were stained with 5×10^{-5} M uranyl acetate in 90% ethanol and rotary shadowed with platinum-paladium. Grids were viewed and photographed at 17,500X in a Philips EM300 electron microscope using a 50 µm objective aperature and 60 kV accelerating voltage. The 35 mm negatives were enlarged 20X on a Nikon Shadowgraph, traced, and measured with a Keuffel and Esser map measurer.

(j) Polyacrylamide gel electrophoresis

15% Polyacrylamide gels containing 0.375 M Tris (pH 8.0), 0.1% SDS were used (Benbow et al., 1972b). Gels (9.0 cm long) were polymerized for 45 min in Pyrex glass tubes with an inner diameter of 7.0 mm. They were pre-run for 45 min at 4 mA/gel in a buffer containing 0.375 M Tris (pH 8.9), 0.1% SDS. The buffer was replaced with a solution containing 1.0 g SDS, 2.88 g glycine and 0.6 g Tris (pH 8.3) per liter.

Mixtures of [^{14}C] and [^{3}H]leucine phage particles were prepared for electrophoresis by mixing 25 µl of each phage suspension with 58.5 mg of urea, 5 µl of 10% 2-mercaptoethanol, and 2 µl of 1% SDS. The mixture was immersed in boiling water for 10 min immediately before electrophoresis.

Samples, mixed with 4 µg of bromphenol blue marker dye, were layered onto the tops of the gels. Electrophoresis was at 2 mA/gel for 10 min followed by 4 mA/gel for about 3 h. The gels were cut in 1 mm slices with a Mickle Gel-Slicer (Brinkman Instruments) and counted after incubation for 8 h in 5 ml of scintillation fluid which was 85.8% toluene, 9% NCS tissue solubilizer, 4.2% Liquifluor and 1% distilled water by volume.

(k) Selection for insertion mutants

A 100 ml culture of E. coli C-110 at 3 x 10^8 cells/ml was infected with am3 phage (MOI = 15) in TPA medium with 10 µg/ml thymine to which 5 µg/ml mitomycin C was added 5 min before the phage. After 10 min of infection, [^{3}H]thymidine (5 µc/ml, final concentration) was added and the culture was incubated for 3 h. The cells were harvested by centrifugation and lysed with 300 µg/ml of lysozyme in borate-EDTA. The phage suspension obtained from the lysate by exclusion chromatography on a 1 x 95 cm column of porous glass beads (Gschwender et al., 1969) was adjusted to a density of 1.410 g/cm^3 by the addition of 0.61 g of desiccated CsCl to each ml of phage. The solution was centrifuged at 25,000 revs/min for 60 h in a Beckman Type 50, fixed-angle rotor. The gradient was fractionated through a needle which was used to

puncture the bottom of the tube. Ten µl portions of each fraction were counted in 5 ml of Aquasol and assayed for pfu on E. coli HF4714.

Fractions from the gradient, at least 0.004 g/cm^3 more dense (as determined by refractometry) than the peak phage fraction, were pooled, dialyzed and used to infect a second culture of C-110 (without mitomycin C) at a multiplicity of 1 pfu/cell. After incubation for 3 h with [^{3}H]thymidine, the cells were disrupted and the lysate was chromatographed on a porous glass bead column as described above. The resulting phage suspension was centrifuged to equilibrium in CsCl and fractions at least 0.004 g/cm^3 more dense than the phage peak were pooled and dialyzed. These phages were used to infect still another culture of C-110 at a multiplicity of 1.0.

The process of infecting a culture and selecting particles which band at least 0.004 g/cm^3 more dense than the phage peak to infect the next culture was repeated five times. Mitomycin C was present only in the first infection cycle. Phage from the fifth lysate were banded twice, successively, in CsCl with selection for the particles in dense fractions. The pfu selected from the final centrifugation in this series were called "high-density enriched phage."

I. 3 Deletion Mutants of ϕX174

(a) Equilibrium centrifugation in CsCl

The "deletion stock" virus particles band at a lower buoyant density in CsCl than does wt ϕX174 (Fig. 1). Based upon refractometry of several fractions from the gradients, the mutant particles have a

density of 1.4035 g/cm^3 compared to 1.4079 g/cm^3 obtained for wt phage, an average density difference of 0.0044 ± 0.0003 g/cm^3 in four determinations. This characteristic difference was retained after phage from the "deletion stock" were propagated and purified (without selection on the basis of buoyant density) by chromatography on a porous glass bead column or when φX cistron E mutant am3 phage particles were substituted for wt φX phage particles.

The result suggests that the DNA-protein ratio of the deletion stock virus is different from that of wt or am3 viruses. Such an alteration may be explained either by a reduction in the molecular weight of the DNA in these virions or by an increase in the quantity of protein in the purified mutant particle.

(b) Sedimentation velocity of deletion particles in sucrose gradient

For investigation of the latter possibility, ^{14}C-labeled deletion stock phage were mixed with ^{3}H-labeled wt particles and centrifuged in neutral sucrose gradients. Figure 2 shows that the two viruses sediment together under these conditions, indicating that the hydrodynamic properties of the mutant virus are not detectably different from those of the wt virus.

(c) Polyacrylamide gel electrophoresis of bacteriophage particles

We have examined the coat proteins of the deletion stock viruses by disc electrophoresis in polyacrylamide gels. [^{3}H]Leucine-labeled wt particles and [^{14}C]leucine-labeled deletion particles were

mixed, disrupted with urea, and applied to 15% gels. The ^{14}C- and ^{3}H-specific counts in each slice of one such gel after electrophoresis are shown in Fig. 3. The deletion phage particles contain six electrophoretic components identical to those of the wt virus. Four of the proteins correspond to the products of cistrons F, H, G, and J identified by Benbow et al. (1972b), in spite of the fact that their relative mobilities (R_F) are somewhat greater than those previously reported (Benbow et al., 1972b; Mayol & Sinsheimer, 1970). The small peak (R_F = 0.56) appearing as a shoulder on the leading edge of the H protein (R_F = 0.54) is presumed to be the product of cistron B, which has been identified as a minor phage component (Benbow et al., 1972b; Godson, 1971). The peak at R_F = 0.75 has not been previously observed in ϕX virions, and may be a contaminant. However, its appearance as a constant fraction of the counts in both preparations, while there is a considerable variation in the small amount of radioactivity found in regions of the gel presumed to be lacking phage proteins, suggests that it is not a chance impurity.

The percentages of the total radioactivity found in each peak are shown in Table 1. With the exception of the cistron H product, the relative amounts of the remaining five proteins in the deletion stock particles do not differ significantly from those of the wt virus. The pattern of deletion phage proteins including the H product is nearly identical to that seen in the reported (Benbow et al., 1972b; Burgess, 1969) particles of cistron E mutants when the R_F = 0.75 component is added to the G peak (Table 1).

(d) Heteroduplex mapping in the electron microscope

In order to determine if the mutant and wt viruses contain the same nucleic acid moiety, we have constructed heteroduplex molecules containing one strand of DNA from each source. Examination of these preparations by electron microscopy revealed many double-stranded molecules with a small, single-stranded loop (Fig. 4) characteristic of a deletion-wt heteroduplex. 74 Such molecules on a single grid were photographed and measured. All the single-stranded circular molecules present in these photographs were also traced and measured. Figure 5 shows that the contour lengths of the single-stranded circles are distributed in two modes, representing the wt and mutant DNA molecules in the hybridization mixture. The peak of the shorter mode differs from the larger by 6.8%. The average length of the single-strand loops in the duplex molecules is 0.082 ± 0.018 of the wt φX genome (the larger mode in the distribution of single-strand circles in Fig. 5).

(e) The genetic defect of the deletion virus

The purified deletion phage stock contains 1.18 A_{260nm} units/ml, or a calculated 1.52×10^{14} phage particles/ml (Sinsheimer, 1959a). The deletion stock phage, however, do not form plaques efficiently on amber, ochre, or opal suppressor host strains under the conditions used to plate φX mutants (Table 2). Phage stocks not carrying deletions that have been subjected to the same purification procedure generally have specific infectivities of 0.05 to 0.5 pfu/particle (Sinsheimer, 1959a; Newbold & Sinsheimer, 1969), more than 100 times higher than that observed with the deletion stock.

In order to identify the ϕX cistrons that have been affected by the genetic lesion in the deletion phage DNA, we have modified the method used to detect complementation between mutants of ϕX174 (Hutchison, 1969). In addition, this technique serves as a quantitative plaque assay for the deletion phages. Under the conditions we use, ϕX deletion mutants will form plaques if the "helper phage" has a functional cistron at the position corresponding to the genetic defect of the deletion. A plaque is formed when a multiply-infected nonsuppressing host cell lyses and releases "helper phage" particles that are able to attack the amber suppressor host cells in the lawn.

Table 3 shows the plaque-forming ability of the deletion stock with "helper-phage" having mutations in each of seven ϕX cistrons. "Helper phage" with amber mutations in all of the cistrons except E increased the plaque-forming ability of the deletion stock from 4- to 200-fold. The deletion phage titers obtained with am3 and am27, mutants in cistron E, were identical to those observed when the deletion phage were plated on E. coli C without any "helper."

(f) Discussion

The data presented above suggest that we have isolated a collection of deletion mutants of bacteriophage ϕX174 that lack a portion of cistron E, the gene that controls the host lysis function.

The bimodal distribution of contour lengths (Fig. 5) obtained when wt and mutant stock single-strand DNA are examined in the same field in an electron microscope is strong evidence that the mutant virus genome is shorter than that of wt. The appearance and the lengths of

the single-stranded loops in heteroduplexes of wt and mutant DNA (Figs. 4 and 5) confirm this conclusion and indicate that the lesion in each DNA molecule is localized and not widely distributed over the genome. Indeed, the deletions are likely to be confined to cistron E, since the mutant viruses fail to complement amber mutants in that gene but are able to supply the defective functions of amber mutants in six other cistrons (Table 3).

We have attributed the change in buoyant density of the mutant virus particles (Fig. 1) to their loss of DNA. The sedimentation velocity of these viruses (Fig. 2) and the electrophoretic pattern of the viral proteins (Fig. 3 and Table 1) indicate that the protein structure of the mutant viruses is not sufficiently different from wt or am3 to account for the observed 0.0044 g/cm^3 decrease in particle density. Accordingly, we applied the relationship between increment in density and change in DNA content formulated by Weigle et al. (1959) for bacteriophage λ

$$\Delta\rho = \rho_o \alpha \frac{F_m - F_v}{1 + F_v} *$$

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*The values for wt φX174 used in this calculation are 1.408 g/cm^3 for the phage buoyant density (ρ_o), 0.269 for the mass fraction of DNA in the phage (F_m) (Sinsheimer, 1959a), and 0.220 for the volume fraction [F_v, the mass fraction, multiplied by the ratio of the phage buoyant density, 1.408 g/cm^3 to the DNA buoyant density, 1.725 g/cm^3 (Sinsheimer et al., 1962)].

to obtain a value of 6.3% for the percent of ϕX wt DNA deleted. This figure agrees very well with the estimate of deletion length obtained from contour measurements of single-stranded DNA (6.8%). The size of the deleted region calculated from the single-strand loops seen in heteroduplex molecules (8.2%) may be biased by the tendency to select molecules with large, obvious deletion loops, Nevertheless, it agrees within experimental error with the first two estimates.

Considering the manner in which the deletion viruses were selected and the fact that unsuppressed mutants in cistrons other than E are not expected to produce infectious phage under these conditions, all of the observed deletions in the mutant stock are probably confined to gene E. Benbow et al. (1972b) report that the presumed gene E product has a molecular weight of 17,500, representing 7.7% of the total protein coding capacity of a ϕX genome with 5,500 nucleotides. This figure might be considered the upper limit for the length of the deleted region in members of the mutant stock.

In our laboratory, am3, the cistron E mutant of ϕX174, is routinely used as a substitute for wt ϕX because its lysis defect facilitates the preparation of phage stocks of high titer (Hutchison & Sinsheimer, 1966). In some cases, this mutant has been grown for many generations under non-permissive conditions without cloning. After each propagation the cells were lysed artificially to release the phage particles. Such conditions permit the transmission of deletions in cistron E that may arise spontaneously. Indeed, the elimination of the cistron may confer an advantage upon the mutant by permitting it to replicate more quickly than its am3 ancestor.

In a companion paper, Kim, Sharp, & Davidson (1972) report their discovery of partially deleted ϕX DNA molecules that appeared spontaneously in samples of am3 RF grown under such conditions in our laboratory. We have denatured a portion of their "sample 1" and renatured it in the presence of viral DNA from the deletion stock described in this paper. Nearly all the molecules were perfect duplexes or had a single deletion loop. A few molecules were observed with one small single-strand loop, indicating some heterogeneity in the extent of the deletions in one or both of the component DNA stocks. We believe that the length of the deleted region varies in members of our mutant virus population. This is reflected, to some degree, in the asymmetric shape of the deletion loop histogram in Fig. 5. Apparently, many members of the stock have deleted the entire cistron, but others have lost smaller segments.

Benbow, Eisenberg, & Sinsheimer (1972a) measured the contours of single- and multiple-length DNA molecules in the RF preparation that Kim et al. (1972) have called "sample 2." The contours of the multiple-length molecules are exact multiples of either wt ϕX length or of the deletion DNA length. This observation suggests that the deletion DNA replicates in vivo, and that deletion viral strands are not generated by premature termination of replication from a wt RF molecule.

In summary, our results indicate that we have isolated a population of deletion mutations of bacteriophage ϕX174 that are (i) genetically defined within the cistron E region, (ii) physically defined as individual deletions with an average size of 7% of the ϕX genome (about 390 nucleotides), (iii) able to propagate as efficiently

as φX174 wt or am3, and (iv) possess an essentially normal viral coat structure and all viral functions except host cell lysis.

These mutants, which we shall call φX174 delE, represent the first reported genetically and physically characterized deletion mutants of a small DNA bacteriophage.

I. 4 A Search for φX174 Insertion Mutants

(a) Testing clones of the high-density enriched phage stock

The "high-density enriched phage" stock was plated on HF4704 and, after 5 hours of incubation at 37°C, isolated plaques were picked from the plates with sterile capillary tubes. Each plaque was individually inoculated into a 10 ml culture of HF4714 at 1 x 10^8 cells/ml in TPA. After 30 minutes incubation, 25 μc/ml of [^{3}H]thymidine was added, and the culture was incubated for another 2 hours. Collected by centrifugation, the cells were lysed in 2 ml of borate-EDTA with 300 μg/ml of lysozyme, and phage were purified from each lysate by exclusion chromatography on a 1 x 95 cm column of porous glass beads. The phage from each clone were further purified by banding in CsCl in a Type 50 rotor at 25,000 revs/min for 60 hours. A portion of the peak fraction from each gradient was mixed with ^{14}C am3 marker phage and again centrifuged to equilibrium in CsCl in a Type 50 rotor. The positions of the ^{14}C marker phage and the ^{3}H labeled clones were compared. Fifty phage clones were tested by this procedure.

The profiles of ten equilibrium buoyant density gradients, representative of the entire group, are shown in Fig. 6. None of the

clones had buoyant densities significantly greater than the am 3 marker. 47 Clones banded at positions which differed from the marker by no more than 0.0007 g/cm^3 (e.g., Figs. 6b, e, f, g, i, j). Two clones were 0.0007 to 0.0015 g/cm^3 less dense than the marker (Figs. 6c, h). One clone was lighter by approximately 0.0022 g/cm^3 (Fig. 6d).

(b) Bacteriophage propagated in the presence of mitomycin C

Figure 7a shows the equilibrium buoyant density profile of ϕX174 am3 phage grown in the continuous presence of 5 μg/ml of mitomycin C in E. coli C-110 (the first step in the preparation of the "high-density enriched phage" as described in the Methods). The yield of plaque-forming units per infected cell was quite high (about 600), in contrast to the results of Lindqvist & Sinsheimer (1967b). A peak of ^{3}H cpm and pfu is seen at the phage density position (1.408 g/cm^3). A second ^{3}H component banding at a lower density (about 1.398 g/cm^3) also contains pfu. Since it was found at a lower density, this second component was not expected to affect the selection procedure for insertion mutants. The fractions selected from this gradient for propagation in the subsequent step are indicated by the brackets. Phage propagated in the presence of 5 μg/ml of mitomycin C in E. coli C (hcr$^+$), C-110 (hcr$^+$), HF4704 (hcr$^-$) and H502 (hcr$^-$) all contain particles which band at both positions. The lighter component contained a large, but variable, fraction (30 to 80%) of the total pfu seen in the gradients.

Phage stocks produced in the absence of mitomycin occasionally contain a small amount of material (less than 20% of the total pfu) which bands near 1.398 g/cm^3. As an example, Fig. 7b shows the buoyant

density profile of phage after the fifth infection cycle in the preparation of the "high-density enriched phage stock." No mitomycin C was present during the infection. In this case, less than 15% of the ^{3}H cpm and pfu form a shoulder at the lighter density. The main phage peak is seen at 1.409 g/cm^{3}. In the absence of an internal, normal phage marker, that density cannot be considered significantly different from the buoyant density of normal phage. (The fractions indicated by the bracket were pooled and banded again as described in the Methods.)

When portions of the "dense" and "light" components of phage grown in mitomycin C (fractions 11 and 21 from Fig. 7a) were transferred directly from the collection vials to fresh CsCl solutions and individually centrifuged to equilibrium, each component reappeared at its original position, 1.408 or 1.398 g/cm^{3}, in the gradient. However, when the "dense" and "light" components were first dialyzed exhaustively against borate-EDTA at 4^{o}C, and then banded in CsCl, approximately 60% of the "light" component appeared as a peak of pfu at 1.408 g/cm^{3}. Dialysis had no effect upon the buoyant density of the "dense" component.

The stability of the "light" phage component was studied only briefly. It retained its original properties for 2 months at 4^{o}C, if the CsCl was not removed. Overnight dialysis against borate-EDTA converted more than half of it to the normal phage density. After this initial loss, the remaining "light" component was fairly stable at 4^{o}C in borate-EDTA. Two months after dialysis, 30% still banded near 1.398 g/cm^{3}.

(c) Discussion

(i) Insertion mutants

The failure to find insertion mutants by this procedure may be ascribed to any of the following possibilities:

(A) Insertion mutants were present in the high-density enriched phage stock but represented too small a fraction of the total phage population to be detected.

(B) Only a limited number of viable insertion mutants can exist. The population of phage propagated in the presence of mitomycin C was not large enough to allow an insertion mutant a reasonable probability of appearance.

(C) Insertion mutants of ϕX174 cannot exist.

The selection for insertion mutants was intended to be more intensive than the procedure used to isolate deletion mutants. Fractions at least 0.004 g/cm^3 more dense than the phage peak were chosen in each step. Such pools contained about 5% of the total pfu in the phage stock. The enrichment of dense phage was expected to be about 20-fold in each step compared to the 3- to 5-fold enrichment in "light phage" expected in each step of the deletion isolation. Nevertheless, if viable insertion mutants had been present in the high-density enriched phage stock at less than 2% of the total phage population, they would not have been detected among the tested clones. If, for example, a single insertion mutant had appeared during mitomycin treatment of the initial phage stock, it would have been enriched about 3×10^6-fold during the selection procedure, assuming that it grew as

well as its parent. The titer of final stock was nearly 3 x 10^9 pfu/ml.

The insertion of an extraneous piece of DNA into a cistron would be expected to destroy its function. Since the ability of ϕX DNA to generate infective phage particles is destroyed by the loss of any of the known cistrons, except E, only insertions between cistrons or into non-coding regions may be expected to survive if they do not have large polar effects. As a result, the number of viable insertion sites, including those in cistron E, may be as low as 500. (Cistron E is estimated to be about 420 nucleotides long [Benbow et al., 1972b], and the space between cistrons may be only a few codons.) Since the efficiency with which mitomycin C causes insertions is unknown, 10^{13} phage, grown in its presence, may not have been a sufficiently large population to allow a viable insertion to appear.

There is a substantial possibility that the insertions of the desired size (5 to 10% additional DNA) cannot be accommodated by the structure of the phage capsid. A more intensive search for ϕX insertion mutants must be made before this conclusion may be asserted with any certainty.

(ii) Buoyant density of phage particles prepared in the presence of mitomycin C

The reproducible appearance of pfu, from phage stocks grown in the presence of mitomycin C, with a buoyant density considerably less than that expected for normal phage, may be a significant observation. Since phage stocks propagated without mitomycin C sometimes contain particles which band at the light position, there is a possibility that

the mitomycin-particles represent naturally occurring intermediate structures which have been stabilized or accumulated by the action of mitomycin.

As one possibility, Wachman & Levitzki (1972) propose a mechanism for the assembly of spherical viruses in which the capsid protein initially organize into a cubeoctrahedral array. Subsequently, the structure collapses into a more compact icosahedron. The transformation from a less dense to a more dense structure agrees, at least qualitatively, with the observations reported here. Intra-strand cross-linking of the packaged ϕX single-stranded DNA by mitomycin C (Iyer & Szybalski, 1964) may conceivably inhibit the transition of the capsid superstructure.

The mitomycin-particles are about 0.010 g/cm^3 lighter than normal ϕX particles, a change in density which is more than twice as great as that exhibited by the ϕX deletion. A change in density of that magnitude would require a substantial alteration in the structure or composition of the particle. The nature of that alteration and the means by which it is reversed to generate phage of normal density may offer insights into the maturation of ϕX174 particles.

Footnotes

RF (replicative form) is the double-stranded circular form of ϕX174 DNA; RFI is the supercoiled double circle in which both polynucleotide strands are covalently closed; RFI is the circular duplex in which one strand (or both strands at different positions) is nicked; the viral strand is the polynucleotide having the same base sequence as the DNA found in the virus; the complementary strand has a base sequence complementary to the DNA in the virus; MOI (multiplicity of infection); pfu (plaque-forming unit).

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TABLE 1. Percent of total radioactivity in electrophoretic components of φX174 phage particles*

	Cistron	F	H	B?	C		-	J?
	Observed Mobility (R_F)	0.43	0.54	0.56	0.67		0.75	0.90
		(0.37)†	(0.50)†	(0.59)†	(0.63)†		-	(0.82)†
wt		64.5±0.5	4.0±0.3	3.9±0.3	21.0±0.3	(24.8)‡	3.8±0.2	2.8±0.1
deletion		65.9±0.3	6.7±0.1	3.4±0.1	17.9±0.1	(21.2)‡	3.3±0.2	2.8±0.1
am27† (E)		65.4	6.6	1.4	22.9		-	3.7
am3§ (E)		66.7±0.4	7.6±1.2	-	20.2±1.2		-	5.5±0.4

*Numbers are averages of four gels, with standard deviation from the mean.

†Benbow et al. (1972b); labeled with [^{3}H]leucine.

‡Sum of R_F = 0.67 and R_F = 0.75 components.

§Burgess (1969); labeled with 14 [^{3}H]aminoacid mixture.

TABLE 2. Plaque-forming ability of deletion and wt phages on suppressor host strains

Host strain	Suppressor	Deletion stock		wt φX174*	
		pfu/ml x10^{-10}	pfu/particle†	pfu/ml x10^{-10}	pfu/particle†
C	su^-	2.3	1.5×10^{-4}	9.3	0.13
HF4714	UAG	7.4	4.9×10^{-4}	8.4	0.12
$Su2_{och}$	UAG, UAA	7.8	5.1×10^{-4}	6.6	0.092
CIT103	UGA	1.3	8.6×10^{-5}	1.2	0.17

*Purified by the same method as the deletion stock.

†1.20×10^{13} phage particles/A_{260nm} unit in purified wt preparations (Sinsheimer, 1959a) corrected to $1.28 \times 10^{13}/A_{260nm}$ for particles with a 7% deletion of DNA.

TABLE 3. Plaque-forming ability of deletion stock with ϕX174 amber mutants as helper phage

Helper phage	Mutant cistron	Deletion stock pfu/ml	Relative number of pfu
none	-	2.3×10^{10}	1.0
am10*	D	4.5×10^{12}	200
am3*	E	$< 2 \times 10^{10}$	< 1
am27*	E	2.8×10^{10}	1.2
am88†	F	5.2×10^{11}	23
am87†	F	5.3×10^{11}	23
am9*	G	1.3×10^{12}	57
amN1‡	H	2.0×10^{12}	87
am86†	A	9.0×10^{10}§	3.9§
am14*	B	1.6×10^{12}	70

*Isolated by Dr. C. A. Hutchison (Hutchison, 1969).

†Isolated by Dr. F. Funk (Funk & Sinsheimer, 1970).

‡Obtained from Dr. M. Hayashi.

§This low value is probably a consequence of the asymmetric complementation observed for mutants of cistron A (Tessman, 1965).

Figure Legends

FIG. 1. Equilibrium buoyant density centrifugation of purified deletion and wt ϕX174 phage particles. [^{14}C]thymidine labeled deletion phage (O——O) and [^{3}H]thymidine labeled wt phage (●——●) were mixed in 8.0 ml of solution containing CsCl (ρ = 1.400 g/cm^3), 0.05 M borate, and 5 mM EDTA. The material was centrifuged for 60 h at 25,000 revs/min in a Beckman Type 50, fixed-angle rotor at 5°C. Fractions were collected directly into scintillation vials containing 10 ml of Aquasol. Several fractions throughout the gradient were collected directly into a small quantity of oil, and their density was determined by refractometry (▲——▲).

FIG. 2. Sedimentation of purified deletion and wt ϕX174 phage particles in a neutral sucrose gradient. [^{14}C]leucine labeled deletion phage (O——O) and [^{3}H]leucine wt phage (●——●) were mixed and layered onto a 13.0 ml, linear 5 to 20% sucrose gradient containing 0.05 M Tris (pH 8.1) and 5 mM EDTA. The preparation was centrifuged at 24,000 revs/min for 6.5 h in a Beckman SW40 rotor at 5°C. Fractions were collected into scintillation vials, which contained 10 ml of Aquasol.

FIG. 3. Electrophoresis of purified deletion and wt ϕX174 phage particles in polyacrylamide gels. [^{14}C]leucine labeled deletion phage (O——O) and [^{3}H]leucine wt phage particles (●——●) were mixed, disrupted with urea, and applied to 15% polyacrylamide gels. The gels

were cut into 1.0 mm slices, and each slice was digested and counted in NCS scintillation fluid. The arrow indicates the position of the bromphenol blue tracking dye.

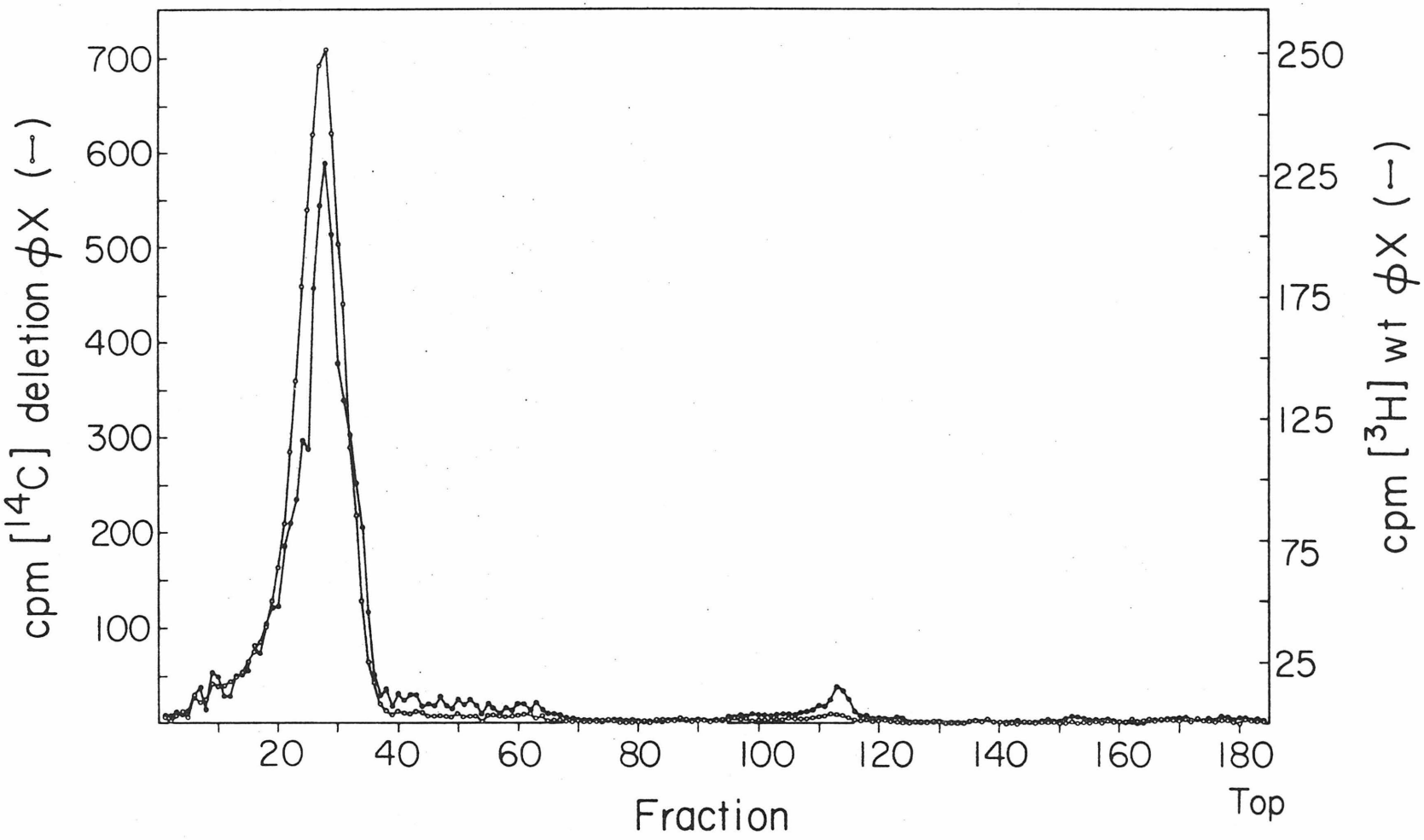
FIG. 4. Electron micrographs of heteroduplex DNA molecules produced by hybridization of deletion viral (+) strands with wt ϕX174 complementary (-) strands. Image magnification is 153,000X. Arrows indicate the single-stranded loops, which locate the deleted regions.

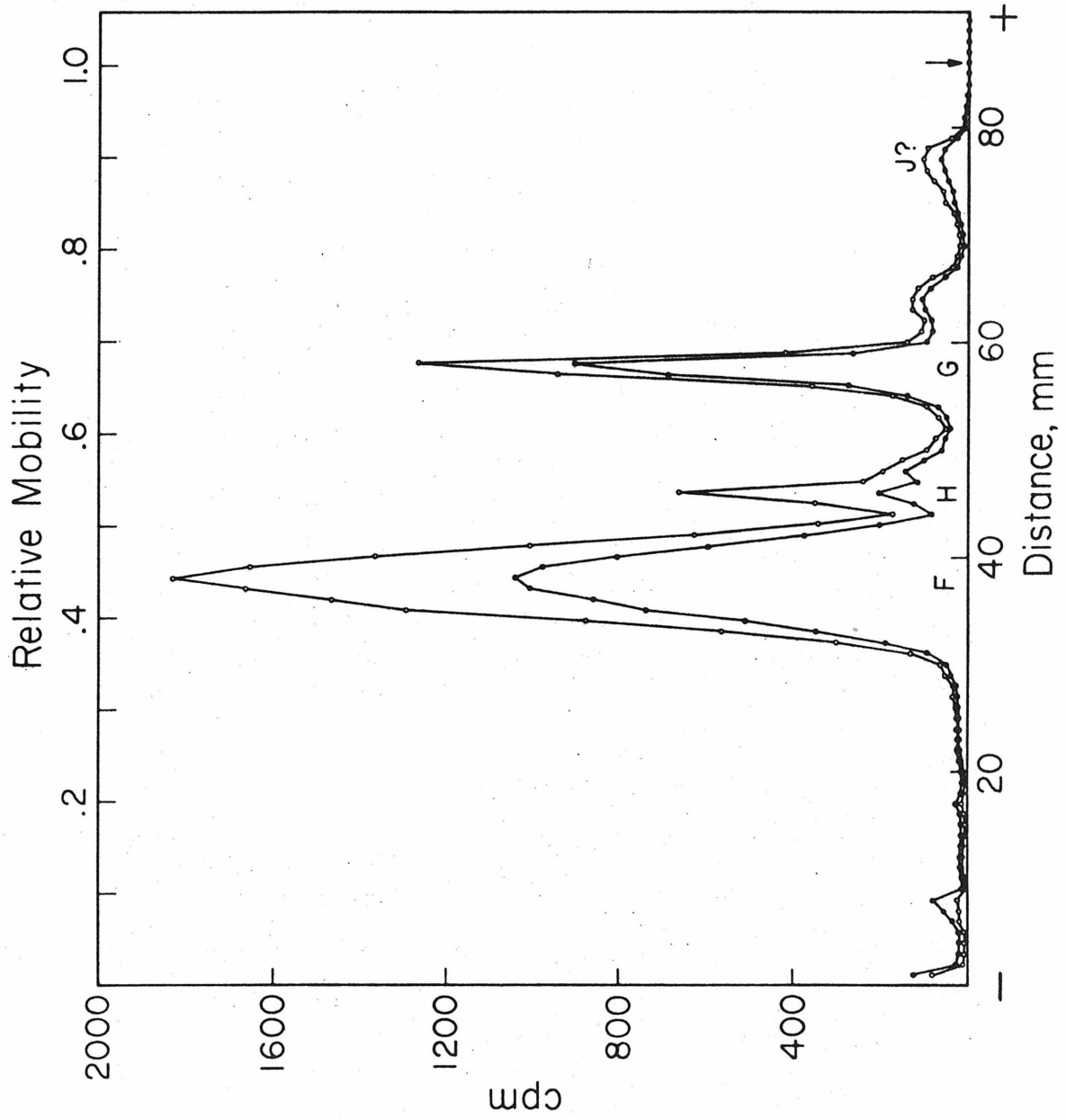
FIG. 5. Length distributions of single-stranded loops in heteroduplex molecules and of circular single-stranded DNA molecules in a partially renatured mixture of deletion viral (+) strands and wt ϕX174 complementary (-) strands. All measurements were made from photomicrographs of a single grid.

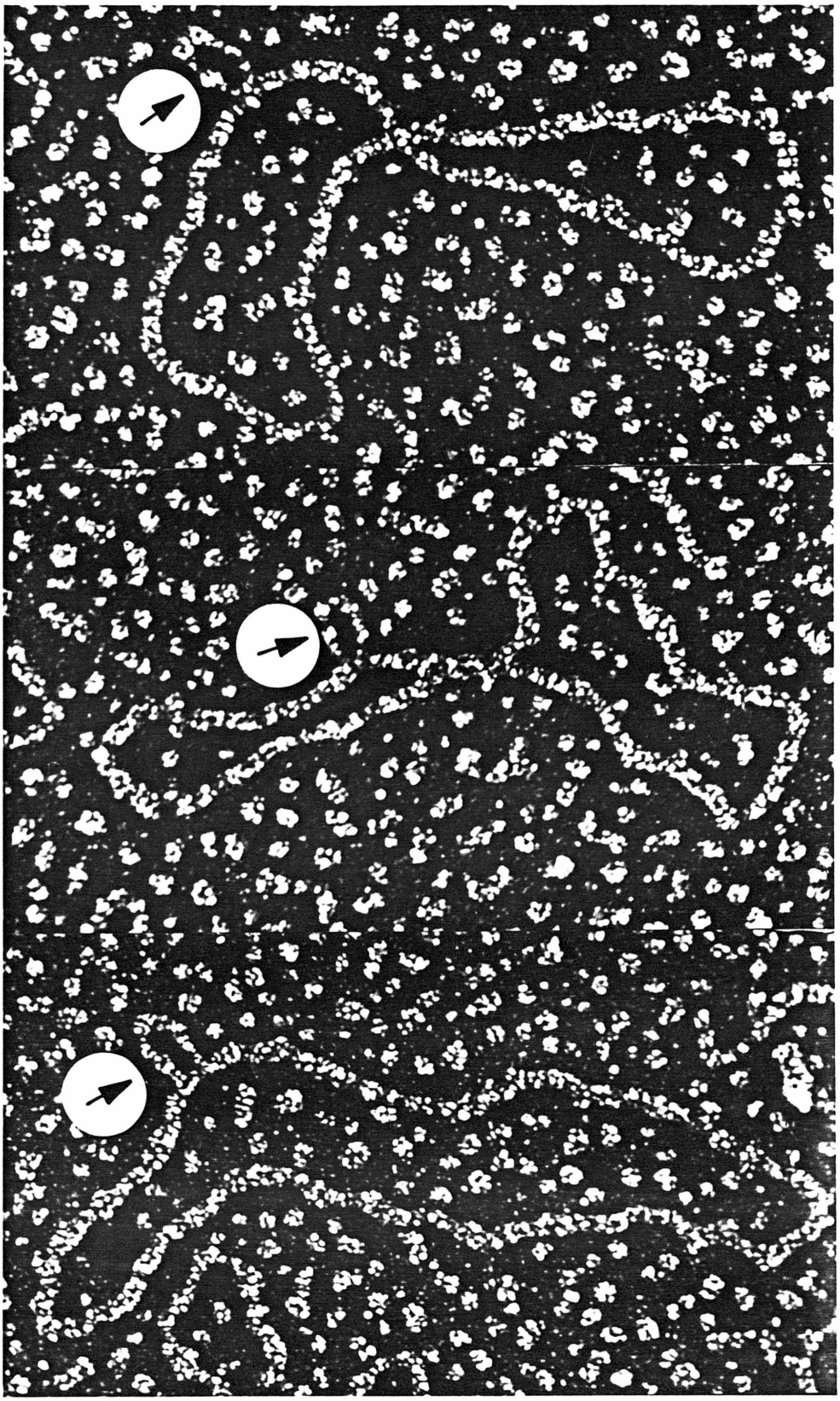
FIG. 6. Equilibrium buoyant density patterns of ten clones, isolated from the high-density enriched phage pool. Purified [^{3}H]thymidine phage (O——O), grown from single plaques, were mixed with ^{14}C am3 marker phage (-----) in CsCl (ρ = 1.410 g/cm^{3}), 0.05 M borate, 5 mM EDTA, and were centrifuged for 60 h at 25,000 revs/min in a Beckman Type 50, fixed-angle rotor. The gradients were collected into scintillation vials and counted in 5 ml of Aquasol. The position of the ^{14}C marker phage peak is indicated by the arrow in most of the profiles.

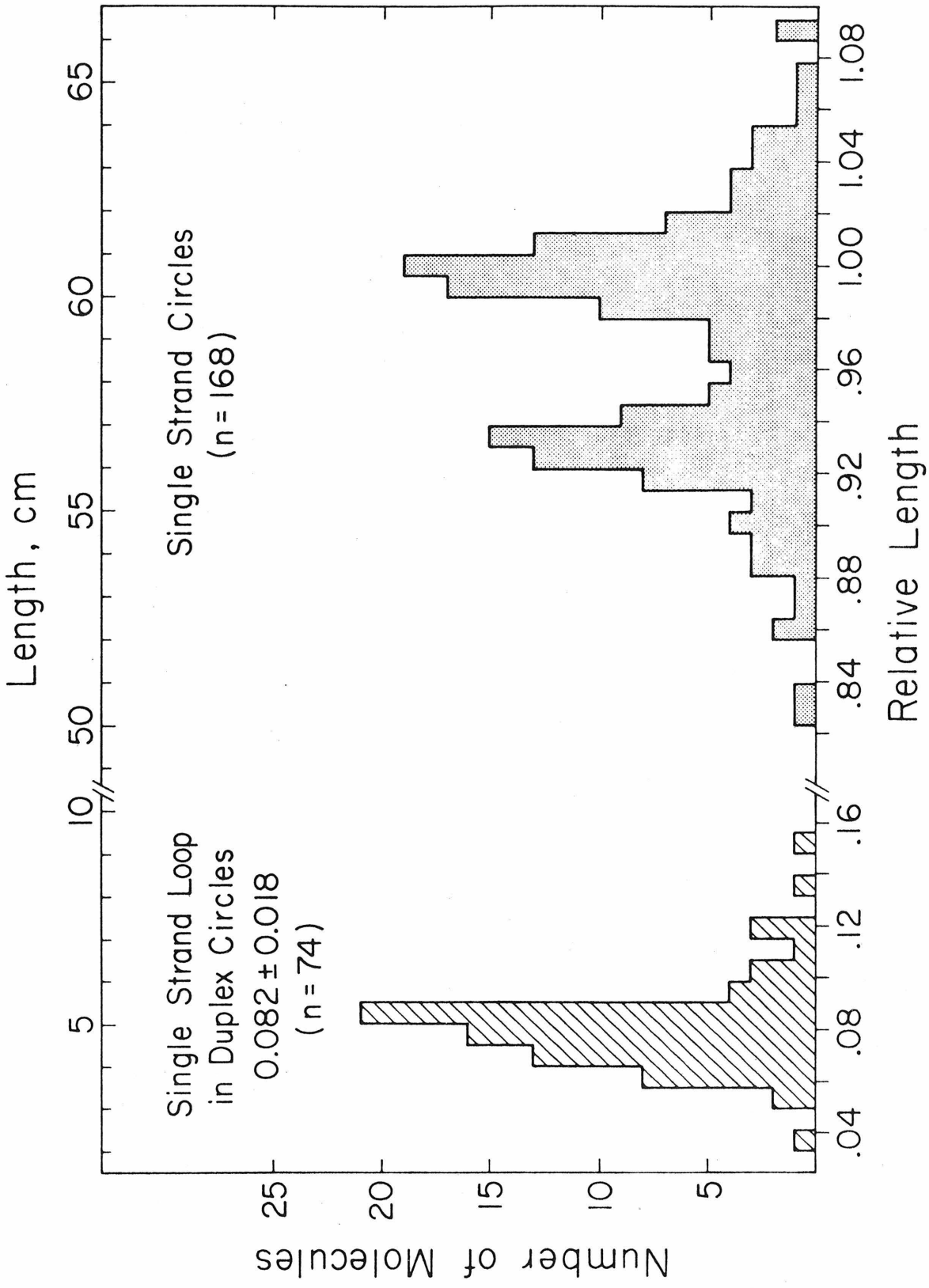
FIG. 7. Equilibrium buoyant density patterns of phage stocks grown in cultures of E. coli C-110, (a) in the presence of 5 μg/ml mitomycin C and, (b) in the absence of mitomycin C. [^{3}H]thymidine (5 μc/ml) was added 10 min after infection. After 3 h the cells were collected by centrifugation, lysed and phage purified from the lysate by exclusion chromatography on a porous glass bead column. The phage suspensions were adjusted to a density of 1.410 g/cm^3 with CsCl and centrifuged for 60 h at 25,000 revs/min in a Type 50 rotor. Portions of each fraction were counted in Aquasol to determine ^{3}H cpm (———) or plated on HF4714 to determine pfu (-----). The density of some fractions (▲——▲) was determined by refractometry.

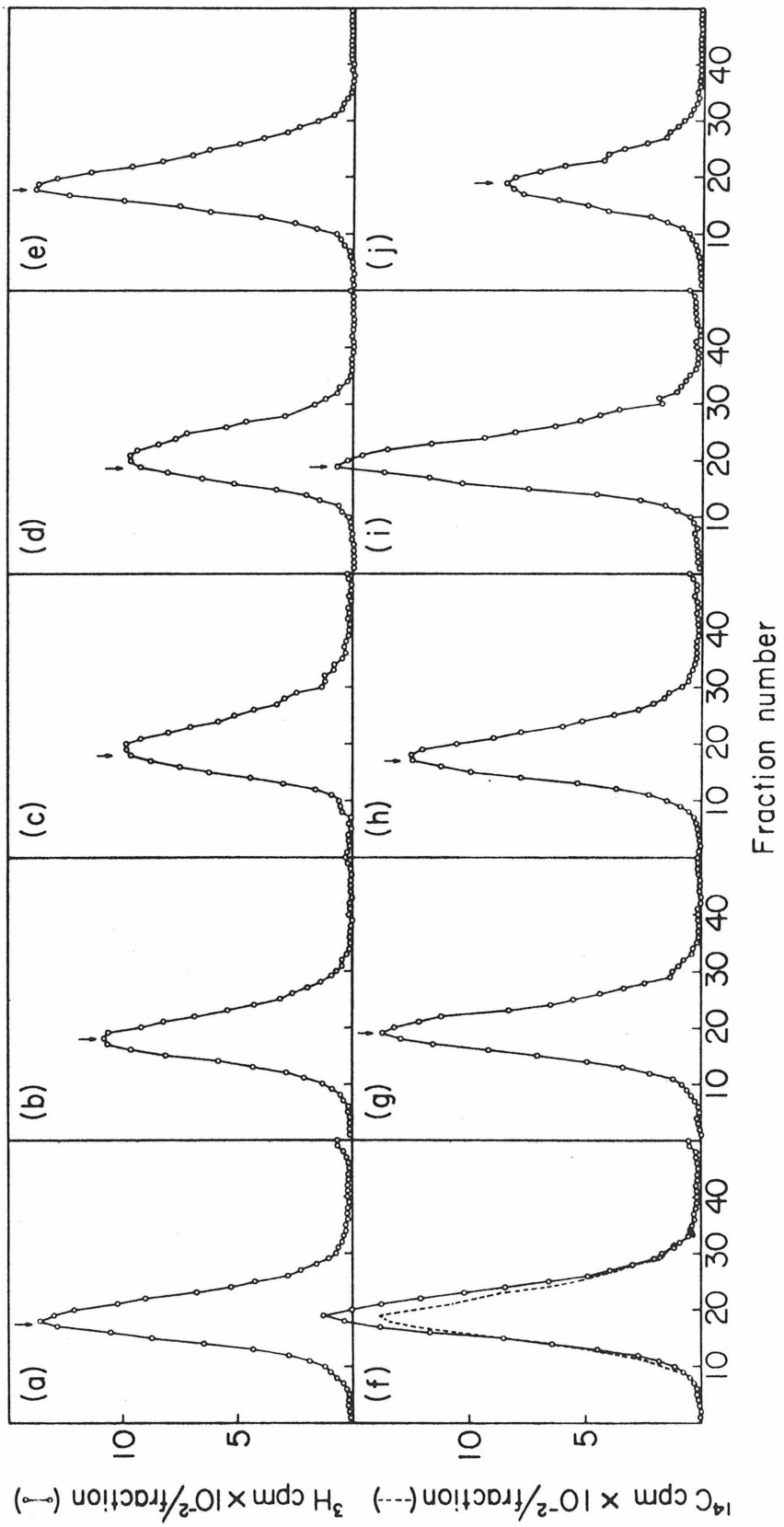
cpm [14C] deletion φX (-)
2500
2000
1500
1000
500
cpm [3H] wt φX (-)
4000
3500
3000
2500
2000
1500
1000
500
density, g/cm3 (-)
1.44
1.43
1.42
1.41
1.40
1.39
1.38
50
100
150
200
250
Top
Fraction

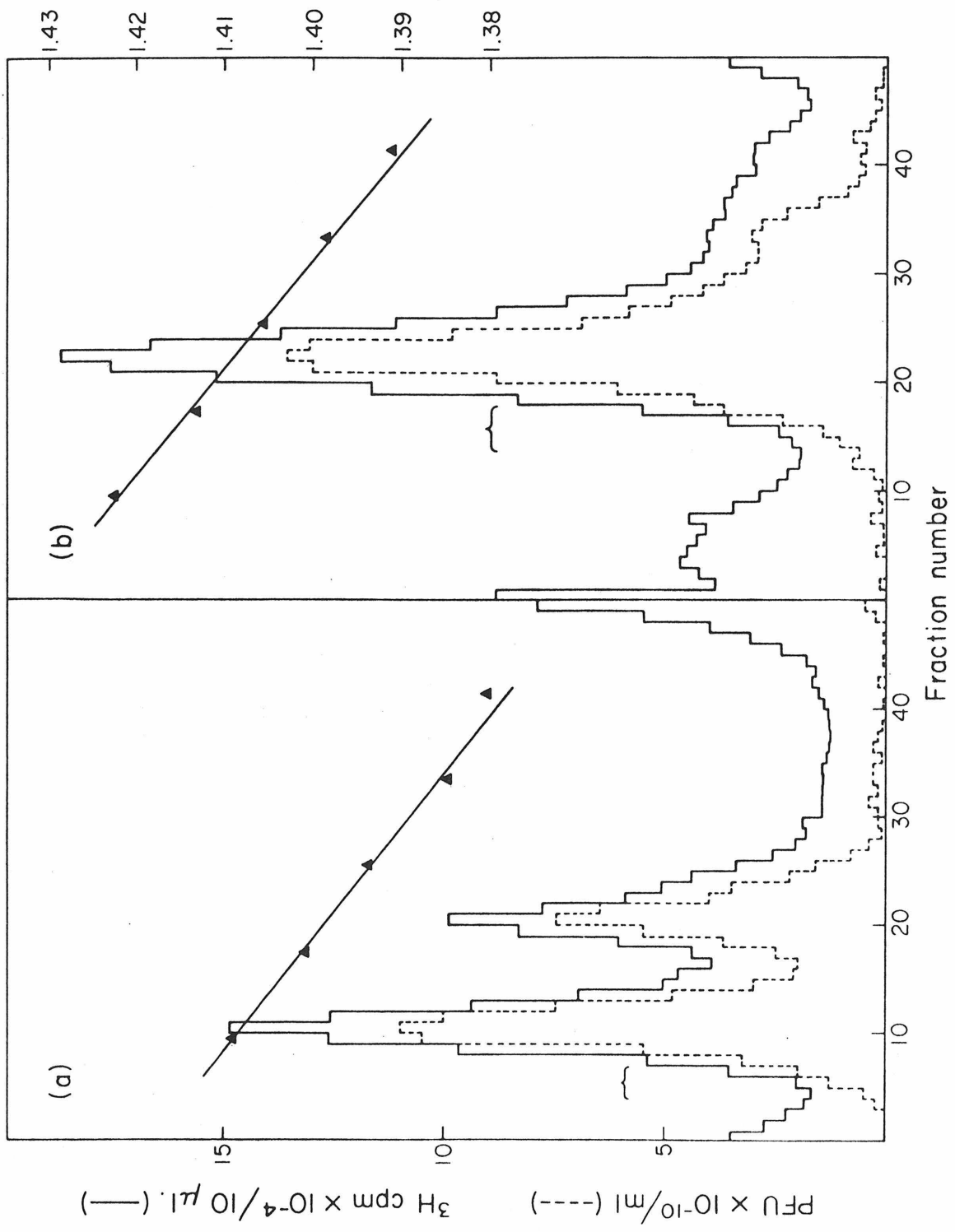

cpm [14C] deletion φX (-)
cpm [3H] wt φX (-)
700
600
500
400
300
200
100
250
225
175
125
75
25
20
40
60
80
100
120
140
160
180
Fraction
Top


Relative Mobility
.2
.4
.6
.8
1.0
cpm
2000
1600
1200
800
400
—
+
20
40
60
80
Distance, mm
F
H
G
J?




Length, cm
5
10
50
55
60
65
Single Strand Circles
(n = 168)
Single Strand Loop
in Duplex Circles
0.082 ± 0.018
(n = 74)
Number of Molecules
25
20
15
10
5
.04
.08
.12
.16
.84
.88
.92
.96
1.00
1.04
1.08
Relative Length


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
10
5
10
5
10 20 30 40
Fraction number
^{14}C cpm × 10^{-2}/fraction (---) ^{3}H cpm × 10^{-2}/fraction (—)


PFU x 10⁻¹⁰/ml (----)
³H cpm x 10⁻⁴/10 μl. (——)
Density, g/cm³ (▲——▲)
Fraction number
(a)
(b)
5
10
15
1.38
1.39
1.40
1.41
1.42
1.43
10
20
30
40

Part II: The Appearance and Structure of ϕX174 Parental Replicative Forms

II. 1 Introduction

The conversion of a circular, single-stranded DNA species into a duplex, superhelical DNA molecule is not a common biological event. It occurs in an early stage of the infectious cycles of a few animal viruses (Crawford, 1966) and two classes of bacterial viruses: the spherical and the filamentous single-stranded DNA phages. The first class of bacteriophages is represented by ϕX174, S13, ϕR and ST-1 (Sinsheimer, 1968). The filamentous, male-specific group includes M13, fd and f1 (Marvin & Hohn, 1969). The two classes are not serologically related and, indeed, their differences extend to their morphologies and their life cycles.

The formation of the duplex replicative form (RF) containing the infecting (parental) DNA strand has not been studied extensively *in vivo* (see the *General Introduction*). Considerably more information has come from examining the reaction in soluble extracts of *E. coli*.

The finding that parental M13 RF molecules do not appear in cells in the presence of rifampicin (Brutlag *et al.*, 1971), a specific inhibitor of *E. coli* RNA polymerase (Sippel & Hartman, 1968), suggests that RNA synthesis is involved in making the M13 complementary strand. The requirement for RNA polymerase function in this conversion was found to be unrelated to protein synthesis, since M13 parental RF is made in the presence of chloramphenicol. However, the conversion of ϕX single strands to RF *in vivo* is insensitive to rifampicin (Silverstein & Billen, 1971).

Using soluble extracts of E. coli, distinctly different mechanisms were found to operate in the two cases. In the M13 conversion, an initial period of RNA synthesis was followed by DNA synthesis. The first stage required the addition of the four nucleoside triphosphates and it was inhibited by rifampicin. The second stage resulted in the formation of the M13 complementary DNA strand, even in the presence of rifampicin (Wickner et al., 1972c). A primer function for the RNA was proposed.

The formation of ϕX RF in soluble extracts was unaffected by rifampicin (Wickner et al., 1972c). The produce contained a full-length linear complementary strand and an intact, circular viral strand (Schekman et al., 1972). However, all four ribonucleoside triphosphates had to be added to the enzyme preparation in order to obtain the maximum amount of RF. The product was found to contain ribonucleotides covalently linked to the new strand. Furthermore, the presence of actinomycin D, an antibiotic which binds to DNA templates (Sobell et al., 1971) and inhibits RNA synthesis by virtually all RNA polymerases, reduces ϕX RF formation when employed at a concentration not expected to affect DNA synthesis directly (Schekman et al., 1972). From this evidence, these workers concluded that ϕX parental RF formation in vitro requires RNA synthesis to generate a primer for DNA polymerization. They suggested that the RNA is made by an enzyme with properties different from those of the known E. coli RNA polymerase.

The data of Hurwitz et al. (1973) confirmed these differences between M13 and ϕX174. However, these workers did not detect any requirement for added ribonucleoside triphosphates to promote the conversion

of ϕX in their cell extracts (Wickner et al., 1972b).

The differences in the conversions of ϕX and M13 were found to extend beyond the type of RNA polymerase they use. Complementary strand synthesis on the ϕX template requires the products of five E. coli genes (dna B, C, D, E, and G), also needed by the host for replication of its own chromosome (Wickner et al., 1972a; Hurwitz et al., 1973; Taketo, 1973). The products of dna A and dna F are not essential. M13 is converted to RF when only dna E product, DNA polymerase III (Gefter et al., 1971), is available. All the other proteins are unnecessary (Wickner et al., 1972a). In this respect, the ϕX reaction is much more complex and resembles the synthesis of E. coli DNA more closely than does the M13 reaction (Schekman et al., 1972).

In this section various aspects of the synthesis of ϕX parental RF in vivo are investigated. The appearance of duplex ϕX DNA soon after infection is reported, and an estimate of the time it takes to polymerize the complementary strand is made. The structures of normal parental RFII, RF made on UV damaged templates and nascent parental RF are deduced from sedimentation data and their appearance in the electron microscope. These observations are used to construct a model for the synthesis of the first complementary strand. This investigation represents the first comprehensive study of the first stage of ϕX DNA synthesis in vivo.

II. 2 Materials and Methods

(a) Bacterial strains

Most of the bacterial strains have been described in Part I with the following exceptions:

(i) E. coli HF4704 is an hcr$^-$, thy$^-$, su$^-$, T_1^s, ϕX sensitive strain which requires 4 µg/ml of thymine for optimal growth (Lindqvist & Sinsheimer, 1967).

(ii) E. coli C1704 is a rep$^-$, thy$^-$, hcr$^-$, ilv$^-$ strain which contains the rep3 mutation of Denhardt et al. (1967) transduced into HF4704 by Calendar et al. (1970).

(iii) E. coli H502 is an endo I$^-$ derivative of HF4704 (hcr$^-$, thy$^-$, su$^-$) constructed by Dr. Hoffmann-Berling. This strain will grow satisfactorily in medium containing 1 µg/ml of thymine.

(b) Radiochemicals

(i) Thymidine [methyl-^{3}H] 24 c/mmole, 0.5 mC/ml in sterile aqueous solution with 2% ethanol was purchased from Schwarz/Mann, Orangeburg, N.Y.

(ii) Thymidine [2-^{14}C], 59 mc/mmole, 0.1 mc/ml in sterile aqueous solution was purchased from Schwarz/Mann, Orangeburg, N.Y.

(iii) $H_3{}^{32}PO_4$, carrier free, 30 to 60 mc/ml in 0.02 N HCl was purchased from the International Chemical and Nuclear Corporation, Irvine, California.

(c) Other materials

Most of the materials used in this section have been specified in Part I, with the following exceptions:

(i) Bovine pancreatic ribonuclease A (Type 1-A, 5X crystallized, protease free), and bovine pancreatic deoxyribonuclease I (chromatography prepared, lyophilized) were purchased from the Sigma Chemical Co., St. Louis, Mo.

(ii) Chloramphenicol (B grade) and pronase (B grade, nuclease free) were purchased from Calbiochem, San Diego, California.

(iii) The special natural L-amino acid mixture was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

(iv) Conical collection beakers (2 ml) were obtained from Scientific Products, Los Angeles, California.

(d) Media

(i) TPGAA medium was prepared by dissolving 0.5 g NaCl, 8.0 g KCl, 1.1 g NH_4Cl, 3.32 g Tris-OH, 11.44 g Tris-HCl, 0.23 g KH_2PO_4, 1.0 ml of 0.16 M Na_2SO_4 in 500 ml of deionized, distilled water. The pH was adjusted to 7.4 with HCl. In a second container 0.2 g $MgCl_2 \cdot 6H_2O$, 5 ml of 1 M $CaCl_2$, 2.0 g glucose, 0.8 g sodium pyruvate and 2.7 g of a special 20 amino acid mixture were dissolved in 500 ml deionized distilled water. The two solutions were autoclaved and then mixed.

(ii) TPGAA-low phosphate medium is TPGAA medium with the phosphate level reduced to 0.023 g KH_2PO_4 per liter.

(iii) Tris-EDTA contains 0.05 M Tris and 5 mM EDTA. This buffer has a high temperature coefficient and is pH 7.4 at 25°C, approximately pH 8.0 at 5°C and pH 7.1 at 37°C. Tris buffers at other pH levels will

be specifically mentioned.

(iv) Borate-EDTA contains 0.05 M sodium tetraborate and 5 mM EDTA.

(e) Preparation of ^{14}C, ^{3}H or ^{32}P DNA labeled am3 phage and single-stranded viral DNA

E. coli H502 was grown to 5 x 10^8 cells/ml with aeration at 37°C in TPGAA (or TPGAA-low phosphate for ^{32}P labeling) containing 2 μg/ml thymine. am3 Phage were added at a multiplicity of 5 to 10 per cell. ^{32}P (100 μc/ml, final concentration) was added 15 minutes before infection; [^{3}H]thymidine (100 μc/ml, final concentration) and [^{14}C]thymidine (10 μc/ml, final concentration) were added to the culture in approximately ten equal portions at intervals beginning at 10 min after infections and continuing for 2 h. (Assuming equilibration with the phosphorylated DNA precursors, 100 μc/ml of ^{32}P in TPGAA-low phosphate medium provides 0.3 ^{32}P atoms per single strand of φX DNA.)

After two hours of incubation the culture was adjusted to 5 mM EDTA and cooled in an ice bath. The cells were collected by centrifuging for 10 min at 5,000 revs/min in a Sorvall GSA or GS3 rotor at 0°C. The pellet was resuspended in 1/50 of the original volume in borate-EDTA and incubated for 15 min at 37°C with 300 μg/ml lysozyme. $MgSO_4$ was added to a final concentration of 10 mM. Bovine pancreatic RNase A and DNase I were added to final concentrations of 10 μg/ml each. The lysate was incubated for 30 min at 37°C and then EDTA was added to 20 mM.

Phage were purified from the lysate by exclusion chromatography on a 1 x 95 cm column of porous glass beads (pore diameter 240Å)

equilibrated with borate-EDTA as described by Gschwender et al. (1969). The purity of the resulting phage suspensions was sufficiently high for most purposes. At the highest multiplicities used (MOI = 200) the addition of phage introduced 5×10^{-6} M EDTA and 5×10^{-5} M borate into the culture, which is unlikely to affect the infection process.

Single-stranded DNA was obtained from phage stocks further purified by buoyant density centrifugation. Phage suspensions were adjusted to a density of 1.410 g/cm^3 by the addition of 0.61 g of dry CsCl per ml. These were centrifuged for 60 h at 30,000 revs/min in Beckman Type 50 or Type 30 fixed-angle rotors. The phage pools were dialyzed exhaustively against borate-EDTA.

The purified phage suspension was extracted three times with single volumes of phenol equilibrated with borate-EDTA at room temperature. The phenol phases were subsequently extracted once with one-half volume of borate-EDTA. The combined aqueous phase was extracted twice with single volumes of ethyl ether. Three volumes of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.5) were added to the aqueous phase and the mixture was cooled to -20°C for 15 h. The single-stranded DNA precipitate was collected by centrifugation for 1 h at 20,000 revs/min at -5°C. After the supernatant was aspirated and the remaining isopropanol removed by vacuum desiccation, the DNA was dissolved in a small volume of Tris-EDTA and stored at -20°C.

(f) Preparation of ^{3}H, ^{14}C, and ^{32}P DNA labeled RF

E. coli H502 was grown to 5×10^8 cells/ml with aeration at 37°C in 100 ml of TPGAA (or TPGAA-low phosphate for ^{32}P labeling)

containing 2 μg/ml thymine. am3 Phage were added at a multiplicity of 5 to 10 phage per cell. 10 mc of ^{32}P was added 10 min before infection; 2 mc of [^{3}H]thymidine or 100 μc of [^{14}C]thymidine was added 10 min after infection with 35 μg/ml of chloramphenicol (100 μc/ml of carrier-free ^{32}P in the low phosphate medium provides 0.3 ^{32}P atoms per single strand). Incubation and aeration were continued for another 60 to 90 min, at which time the culture was brought to 0°C by swirling the flask in a bath of methanol and dry ice.

The cells were harvested by centrifuging for 10 min at 5,000 revs/min in a Sorvall GSA rotor at 0°C. The pellet was washed twice with 20 ml volumes of borate-EDTA and then was resuspended in 2.0 ml of Tris-EDTA at 0°C. Lysozyme (3 mg/ml, freshly prepared in Tris-EDTA) was added to 300 μg/ml and the culture was incubated at 37°C for 15 min. ^{32}P labeled lysates were incubated with 200 μg/ml bovine pancreatic RNase A (heat-treated for 15 min at 80°C in 0.1 M Tris pH 8.0, 1 mM EDTA) at 37°C for 15 min. Sodium dodecyl sulfate (SDS, 10%) and pronase (10 mg/ml, self-digested 45 min at 37°C in Tris-EDTA) were added to produce final concentrations of 1% and 200 μg/ml, respectively. The lysate was mixed by rolling the tube gently and then was incubated for 2½ h at 37°C.

The lysate was poured directly onto a single 58 ml, linear, preformed, CsCl gradient (described below) and centrifuged at 25,000 revs/min for 15 h in an SW25.2 rotor. The RF peaks were pooled (about 15, one ml fractions) and dialyzed at least 5 h against one liter of Tris-EDTA. One-tenth volume of 3 M sodium acetate (pH 5.5) and two volumes of isopropanol were added and the mixture was cooled to -20°C

for 15 h. The DNA was pelleted by centrifugation at 20,000 revs/min for one h, dissolved in 0.2 ml of Tris-EDTA and then applied to a 13.5 ml neutral isokinetic sucrose gradient. The sample was centrifuged at 40,000 revs/min for 14 h in an SW40 rotor. Fractions containing RF were pooled, dialyzed overnight against 100 volumes of 0.01 M Tris pH 8.0 ($5^{o}C$), 1 mM NaCl and stored at $-20^{o}C$.

(g) Preparation of parental RF

E. coli HF4704 was grown to 5 x 10^{8} cells/ml with aeration at $37^{o}C$ in TPGAA (or TPGAA-low phosphate for ^{32}P labeling) containing 10 µg/ml thymine. A 3 mg/ml solution of chloramphenicol, freshly prepared in TPGAA medium, was added to the culture to bring the final concentration of chloramphenicol to 150 µg/ml (Tessman, 1966; Sinsheimer et al., 1967). The culture was incubated for 20 min with vigorous aeration. When specified in the experiment, [^{3}H]thymidine (1 min before infection) or $H_3{}^{32}PO_4$ (5 min before infection) was added to the culture at final concentrations of 20 µc/ml, unless otherwise stated. Infection was with labeled am3 phage at multiplicities ranging from 1.5 to 200. Incubation at $37^{o}C$ and aeration were continued. At the times indicated in each experiment, equal aliquots of the infected culture were removed and poured into ten volumes of borate-EDTA containing 0.05 M NaN_3, 5 mM KCN at $0^{o}C$ mixing vigorously on a magnetic stirrer.

Experiments with short infection periods could be performed with an accuracy of ±2 seconds. It is estimated that small volumes (5 to 50 ml) of culture were cooled to $5^{o}C$ in less than one second.

The cells were collected by centrifugation for 20 min at 5,000 revs/min in a Sorvall GS3 rotor at 0°C. The pellets were resuspended in 20 ml of borate-EDTA, 0.05 M NaN_3, 5 mM KCN at 0°C and centrifuged at 0°C in a Sorvall SS34 rotor for 5 min at 10,000 revs/min. Resuspension in 20 ml volumes of borate-EDTA, NaN_3-KCN and centrifugation was repeated four times to remove eclipsed phage particles (Newbold & Sinsheimer, 1969b) and to reduce the amount of unincorporated [^{3}H]thymidine in the cells.

Pellets from 10 ml and 50 ml of culture were resuspended in 1.0 and 2.0 ml of Tris-EDTA, respectively. When indicated, labeled RF marker was mixed with the washed cells. Lysozyme (3 mg/ml, freshly prepared in Tris-EDTA) was added to 300 µg/ml and lysis was allowed to proceed for 15 min at 37°C. The suspensions became very viscous within a few minutes. When specified, the lysates were digested with 200 µg/ml RNase (20 mg/ml, heat-treated at 80°C for 15 min in 0.1 M Tris pH 8.0, 1 mM EDTA) for 15 min, at 37°C, immediately after lysis. Sodium dodecyl sulfate (SDS, 10%) and pronase (10 mg/ml, self-digested 45 min at 37° in 0.05 M Tris pH 7.1, 5 mM EDTA) were added to produce final concentrations of 1% and 200 µg/ml, respectively. To prevent shearing the host DNA, these ingredients were mixed with the lysates by rolling the tube, gently, several times during the subsequent 2½ h incubation at 37°C. Shaking and pipetting the lysates were avoided. After this incubation the lysates were clear and colorless, but highly viscous. Each lysate was poured directly onto a preformed, linear, cesium chloride gradient at room temperature.

This preparation technique is a modification of that described by Francke and Ray (1971b) and it removes essentially all of the E. coli DNA in a single sedimentation. A low-speed centrifugation of pronase-SDS digested lysates, after the addition of 1 M NaCl (Hirt, 1967) results in the loss of a variable amount of the parental label (up to 40%) into the pellet (also observed by Godson & Vapnek, 1973), and therefore, was not included in the procedure.

(h) Centrifugation techniques

(i) Preformed, neutral, CsCl gradients contain 0.05 M Tris pH 8.0 (5°C), 0.015 M EDTA and have a linear density gradient from 1.20 to 1.35 g/cm^3. Gradients prepared in 1¼ x 3½ inch (SW25.2) and 1 x 3½ inch (SW27) cellulose nitrate tubes have volumes of 58 and 38 ml, and the samples applied to each had maximum volumes of 3.0 and 1.5 ml, respectively. Centrifugation was at 25,000 revs/min for 15 (SW25.2) or 13 (SW27) h at 5°C in a Beckman L2-65B centrifuge.

Centrifugation of pronase-SDS treated lysates in these gradients caused the host DNA to sediment into a large white pellet. The gradients were collected through a 20 gauge hypodermic needle which was used to puncture the tube near the bottom, beyond the edge of the pellet. Forty (SW27) or fifty (SW25.2) drop fractions (about 65) were collected into plastic 2 ml, conical, beakers or directly into scintillation vials. The entire fractions or measured samples from each fraction were counted in Aquasol.

It was not possible to estimate the efficiency of recovery of ϕX DNA species labeled during the experiment. Most of the new label

was incorporated into host DNA which was lost from the gradient in a pellet at the bottom of the tube. However, after the infected cells had been washed with borate-EDTA (see above), over 75% of the remaining parental counts from ^{3}H, ^{14}C or ^{32}P labeled phage was recovered from the gradients. More than 90% of the RF markers added to the cells just before lysis was also found in the gradients.

Preformed CsCl gradients were found to give better resolution of RFI, RFII and single-stranded DNA than high salt, neutral sucrose gradients when used for the removal of host DNA from lysates of ϕX infected cells. The high viscosity of lysates from cultures up to 200 ml in volume (about 2×10^{11} cells) apparently causes the ϕX DNA species to sediment further into the gradients, under the described conditions, than is observed for lysates prepared from fewer cells. This anomaly, which is minimized by treating the lysate with RNase, does not noticeably affect the recovery of the ϕX DNA species, nor does it greatly reduce the resolution.

(ii) Isokinetic sucrose gradients were generated by mixing 5% and 37.8% (w/v) sucrose solutions as described by Noll (1967) using a modification of the constant volume device proposed by Henderson (1969). Neutral isokinetic gradients are 5% sucrose at the top and were designed to sediment DNA at a constant velocity at 5°C. They contain 1 M NaCl, 0.05 M Tris pH 8.0 (5°C) and 5 mM EDTA. The gradients were prepared in 9/16 x 3-3/4 inch cellulose nitrate tubes and have a volume of 13.5 ml. Neutral gradients were centrifuged at 40,000 revs/min for 14 h at 5°C in a Beckman SW40 rotor and L2-65B centrifuge.

They were collected in approximately 60, ten drop, fractions through a 20 gauge hypodermic needle which was used to puncture the bottom of the tube.

(iii) Alkaline, isokinetic, sucrose gradients (pH 12.5 at 25^{o}C) were similarly prepared by mixing 5% and 37.8% (w/v) sucrose solutions which were approximately 0.1 M and 0.5 M KOH, respectively. The gradients, made in 9/16 x 3-3/4 inch cellulose nitrate tubes, also contain 5 mM EDTA and are 5% sucrose at the top. One-hundreth volume of 10 M KOH was added to the samples before layering on the gradients. Centrifugation was at 40,000 revs/min for 18 h at 5^{o}C and fractionation was accomplished as described above for neutral, isokinetic gradients.

(iv) Samples were prepared for equilibrium density centrifugation with propidium by adding 0.05 M Tris pH 8.0 (5^{o}C), 0.01 M EDTA to bring the sample weight to 5.700 g. Desiccated cesium chloride (5.190 g) and 300 µl of a 5 mg/ml propidium iodide solution were added to bring the volume to 7.3 ml, the propidium concentration to 200 µg/ml, and the final density to 1.530 g/cm^{3}. A 5/8 x 3 inch cellulose nitrate tube, containing the mixture, was filled with bayol oil and centrifuged for 50 h at 40,000 revs/min in a Beckman Type 65, fixed-angle rotor at 5^{o}C. The gradients were collected in approximately 50, ten drop, fractions through a 20 gauge needle which was used to puncture the bottom of the tube. When necessary, the propidium could be removed from fractions by running the sample through a 5 ml Dowex-50 column prepared in the shell of a disposable plastic syringe.

(v) Equilibrium buoyant density centrifugation in alkaline CsCl was used to separate ϕX viral and complementary DNA strands (Siegel & Hayashi, 1967). 0.05 M Tris pH 8.0 (5°C), 0.01 M EDTA was added to bring the sample weight to 5.000 g. The final density of the solution was adjusted to 1.755 g/cm^3 by adding 7.026 g desiccated CsCl and 50 µl of 10 M KOH. The sample, in a 5/8 x 2-1/2 inch cellulose nitrate tube filled with bayol oil, was centrifuged at 30,000 revs/min for 65 h at 5°C in a Beckman Type 50, fixed-angle rotor. After centrifugation, a capillary was gently lowered into the tube and the gradient was pumped from the bottom in ten drop fractions. The fractions were neutralized with 50 µl of 20% acetic acid before the scintillation fluid was added.

(i) Liquid scintillation spectroscopy

Unless otherwise stated, aqueous samples and fractions from CsCl and sucrose gradients were counted in 5 ml Aquasol scintillation fluid to which 0.5 ml of distilled water was added. One ml fractions from large, preformed, CsCl gradients required 10 ml of scintillation fluid and 0.5 ml of water for satisfactory counting efficiency. Samples from alkaline gradients were neutralized with 50 µl of 20% acetic acid before the addition of counting fluid.

Counting was done in Beckman LS233 and LS200B counters equipped with paper tape-punch mechanisms for computer interfacing. Counting times were adjusted to achieve a 10% standard deviation. To correct the channel overlaps, standards of the relevant isotopes were prepared for each gradient to match precisely the counting conditions of the samples.

Data from gradients containing two or three radioisotopes concurrently were processed by a computer program designed by Robert G. Rohwer. The program utilizes exact solutions to the discriminator ratio equations to separate the isotopes and it plots the corrected values in graph form. The data are presented as counts per min per fraction or measured volume. All figures are drawn with the bottom of the gradients at the left and the direction of sedimentation, therefore, from right to left.

(j) Ultraviolet irradiation of phage

Phage suspensions were diluted ten-fold in 0.05 M borate to contain less than 1.0 A_{260nm} per ml. Five ml was placed in a 10 cm plastic petri dish on a non-reflecting platform and exposed at 70 cm from two General Electric, germicidal, G8T5 lamps enclosed in a reflector housing. The phage suspension was constantly agitated during the exposure.

After a short (30 min) warm-up period, the phage killing rate of the lamps was highly reproducible. A one "hit" (1/e or 37% survival) dose was delivered in 45 seconds. The same apparatus was previously used by Benbow (1972) and Benbow, Zuccarelli & Sinsheimer (1974) to irradiate phage at 35 cm. Under their conditions (calibrated as 19 ergs/mm^2/sec), a "one hit" dose was delivered in 7 sec.

(k) Electron microscopy

ϕX174 RF DNA was prepared for visualization in the electron microscope by the aqueous, basic protein film technique (Davis, Simon & Davidson, 1971). Approximately 50 ng of DNA in 50 µl of 0.25 M

ammonium acetate and 100 µg/ml cytochrome C was spread on parlodion-coated grids, stained with 5 x 10^{-5} M uranyl acetate in 90% ethanol and rotary shadowed with platinum-paladium. Grids were viewed and photographed at 17,500X under a Philips EM300 electron microscope using a 50 µm objective aperature and 50 kV accelerating voltage.

II. 3 Results

(a) The appearance of parental RF after infection

The appearance of parental RF molecules in infected cells incubated in medium containing 10 µc/ml of [^{3}H]thymidine was detected by rapidly cooling 50 ml aliquots of the culture at various times after the addition of ^{14}C am3 phage. The lysate from each aliquot was pronase-treated in SDS and sedimented in a preformed CsCl gradient. Figure 1 shows the sedimentation profiles of infections terminated at 3, 5, 7, 10, 15, and 20 minutes. Peaks of ^{3}H counts at the positions of ϕX RFI and RFII are seen after 5 minutes of infection. An RFII peak is evident in the 3 minute lysate. In addition, all the lysates show a minor 22.5S component (near fraction 8) which sediments slightly faster than RFI (found at 20S in these gradients) and is labeled with both ^{14}C and ^{3}H. This component was designated "fast RF." ϕX single-strands have a velocity of 26S and would be expected to sediment very close to the bottom of the tube.

A peak of post-infection (^{3}H) label appeared near the top of the gradient in most experiments. It is presumed to be unincorporated label that was not removed from the cell before lysis, but it was not investigated.

The increasing amount of label sedimenting at the RF position is clearly represented in Fig. 2a. The ^{3}H cpm in the fractions composing the peaks in the gradients (see Fig. 1) have been added and plotted as a function of the infection period. It can be seen that the RFII molecules appear earliest, increase in number for 5 minutes and decrease slightly thereafter. The RFI species, first observed as a distinct peak in the gradients at 5 minutes, becomes the predominant form at 10 minutes and continues to be made at a gradually decreasing rate, for 20 minutes. Fast RF are clearly present at the earliest time point (3 minutes) and they remain roughly constant in number throughout the experiment.

The parallel increase of parental and post-infection labels in all three RF species can be seen in Fig. 2b. The amount of ^{3}H label found in the RFI and RFII regions in these experiments was on the order of one phage DNA equivalent per parental ^{14}C DNA molecule recovered. The isotopes in the fast RF component deviated somewhat from this ratio, having a 20 to 30% excess of parental label.

At 20 minutes the recovery of parental label from the gradient was 12% of the total phage cpm added to the culture. Most of the remaining parental label (84%) was found in TCA precipitates of the medium and borate-EDTA washes. Other workers have reported that 80% or more of the phage DNA label remained in the medium or borate-EDTA washes (Newbold & Sinsheimer, 1969b; Francke & Ray, 1971a).

The major features of these curves (Fig. 2a) were remarkably constant in several experiments. Figure 3 shows the post-infection label seen in RFI, RFII and fast RF in an infection performed with

ϕX174 wt phage at a MOI of 80. The results are very similar to those seen in Fig. 2a. One consistent variation was observed in experiments performed at multiplicities of from 1 to 5 phage per cell. At low multiplicities, RFI comprised as much as 90% of all the ϕX components at 20 minutes rather than the 60 to 70% seen in Fig. 2a and Fig. 3. In an infection performed at a multiplicity of 4 ^{32}P phage/cell, observed for 60 minutes, no significant changes in the ^{32}P labeled RF population occurred after 20 minutes.

Since RFII molecules were already present in appreciable amounts at 3 minutes after infection in these experiments, shorter infections with highly radioactive am3 phage were performed to determine the earliest appearance of parental RF in the cells. ^{32}P phage at a multiplicity of 1.5 were added to a culture mixing vigorously on a magnetic stirrer. At short intervals after infection 10 ml portions of the culture were removed and rapidly cooled in borate-EDTA.

The patterns of the parental ^{32}P label from six pronase-SDS treated lysates, sedimented in preformed CsCl gradients are shown in Fig. 4a. It can be seen that a small fraction (about 0.1%) of the total label in the infecting viruses sedimented as a peak at the position of RFII (indicated by the arrows) 15 seconds after the addition of phage. When the total radioactivity in the fractions of each peak is plotted against the infection period (Fig. 4b), an extrapolation of the first three data points to the abscissa, suggests that the first RFII molecules appear approximately 10 seconds after infection. Ten seconds is a maximum estimate of the time required for the synthesis of the first

complementary strand since the measured period also includes the time expended in adsorption, eclipse and injection, as well as synthesis.

These conclusions assume that cooling an infected culture in the manner described stops the incorporation of [^{3}H]thymidine into DNA immediately. To test this assumption, a culture at 5 x 10^8 cells/ml was infected with am3 phage at a multiplicity of 170. Chloramphenicol (150 µg/ml) and ^{32}P (1 µc/ml) were added 20 minutes and 5 minutes before infection, respectively. Seven minutes after infection, 20 ml aliquots were poured into 2, 5 or 10 volumes of borate-EDTA, 0.05 M NaN_3, 5 mM KCN at 0°C, containing 20 µc/ml [^{3}H]thymidine. At 8 minutes a 20 ml aliquot was poured into 2 volumes of TPA medium at 0°C, also containing [^{3}H]thymidine. Each of the mixtures was rapidly cooled to 0°C by swirling the vessel in a bath of methanol and solid CO_2. The amount of ^{3}H incorporation into ϕX RF would provide an estimate of the extent of DNA synthesis which occurred after the transfer.

The cultures were lysed, treated with pronase-SDS, and then sedimented in preformed CsCl gradients. The entire RF region of each gradient was pooled, dialyzed, precipitated with two volumes of isopropanol and then sedimented in neutral, isokinetic, sucrose gradients. No ^{3}H radioactivity was found in the RF from the cultures cooled in 10 or 5 volumes of borate-EDTA, NaN_3-KCN. The sedimentation pattern of the cultures cooled in two volumes borate-EDTA or TPA medium are shown in Fig. 5. About 550 cpm of ^{3}H is seen at the RF positions in Fig. 5a. A rough comparison with the rates of ^{3}H incorporation at 7 minutes seen in Figs. 2 and 3 (after making adjustments for different culture sizes and phage multiplicities) indicates that the equivalent of ½ second of

DNA synthesis at 37°C had occurred after dilution into two volumes of borate-EDTA, NaN_3-KCN at 0°C. The equivalent of about 5 seconds of synthesis occurred after a similar dilution into TPA medium at 0°C.

(b) The effect of ultraviolet irradiation of phage on the formation of parental RF

The presence of parental RF species in cells infected with ^{14}C am3 phage, irradiated with ultraviolet light, was determined by infecting 40 ml aliquots of cells, at a MOI of 50, with phage given 0, 20, 40, 60, 80 and 120 seconds of UV exposure corresponding to 0, 0.44, 0.89, 1.4, 1.8 and 2.7 lethal hits per phage (see Materials and Methods). The infections were performed in medium to which 10 μc/ml of [^3H]thymidine and 150 μg/ml of chloramphenicol had been added at 1 minute and 20 minutes before infection, respectively. After 20 minutes of infection the cultures were cooled to 0°C, lysed and digested with pronase and SDS. Figure 6 shows the sedimentation profiles of parental and post-infection labels in the six lysates sedimented through 38 ml, preformed, CsCl gradients.

The most dramatic effect of phage UV exposure is the rapid decrease of RFI accompanied by an accumulation of parental label close to the bottom of the gradient, near the position expected for single-stranded DNA (26S). The RFII and fast RF peaks decrease slowly with increasing UV dose.

Ultraviolet lesions in the viral template strand may prevent the complete synthesis of the complementary strand. The resulting, partially double-stranded molecules are expected to have sedimentation

properties intermediate between those of RFII and single-stranded rings (Benbow et al., 1974). Indeed, the change in the relative amounts of ^{3}H and ^{14}C cpm at the presumed RFI position indicates that not all of the molecules sedimenting at that velocity have complete complementary strands. The ^{3}H cpm at that position, however, may be used as an approximate measure of the number of RFI molecules present, overestimating the actual number at higher UV doses.

Figure 7a shows the ^{3}H cpm at each of the RF positions as a function of phage UV dose. All three components are reduced by increasing the exposure time with RFI decreasing most rapidly. Phage survival (pfu), also shown in Fig. 7a, appears to parallel the number of RFI molecules, while the total parental label (^{14}C) in the gradients shows no change at all.

The relative changes in the RF components are clearly seen in Fig. 7b. When plotted as a fraction of all the post-infection label (^{3}H) incorporated, RFII shows an increase with increasing UV dose. The changes in the fast RF seen in Figs. 7a and 7b may not be significant owing to the difficulty in estimating the amount of ^{3}H label in this minor component in the presence of large amounts of RFI at low UV doses and partially single-stranded molecules at high doses.

(c) The structure of normal parental RF and parental RF made from UV irradiated phage

Normal parental ϕX RF DNA was compared with parental RF made after infection with phage which had been exposed to ultraviolet light. Irradiation was for 60 seconds in borate-EDTA as described in the

Methods. Approximately 26% of the pfu survived the exposure calculated to give 1.3 lethal hits per phage (see Fig. 7a).

Two 60 ml cultures of HF4704 incubated with 150 μg/ml of chloramphenicol for 20 minutes were infected with either irradiated or unirradiated ^{14}C am3 phage for 20 minutes. RF was isolated from the lysates, after pronase-SDS treatment, by centrifugation in preformed CsCl gradients (Fig. 8). The sedimentation profiles of RF from unirradiated (Fig. 8a) and irradiated (Fig. 8b) phage are, predictably, similar to those seen in Figs. 6a and 6d using ^{14}C phage of much lower specific activity.

Regions of these gradients containing the RFI and RFII molecules were pooled separately, as indicated in the figure. The RFII pools were purposely extended to include part of the region between 18 and 21S (see below). Inevitably, some RFI molecules were included. Each pool was dialyzed, the DNA precipitated with isopropanol and then dissolved in a small volume of Tris-EDTA.

When the RFI pools from these gradients were centrifuged in alkaline isokinetic sucrose gradients, 94% (from Fig. 8a) and 83% (from Fig. 8b) of the parental label sedimented at 52S, the velocity of denatured RFI. The RFI pool obtained from the infection with UV irradiated phage contained parental label (15%) which sedimented between 16 and 14S (the positions of circular and unit-length, linear, single-stranded DNA). This contamination of the RFI pool may be explained by the sedimentation behavior expected of RF molecules with incomplete complementary strands.

Some of the RFII obtained from cells infected with normal phage and with UV irradiated phage (Fig. 8) were centrifuged to equilibrium in CsCl containing 200 μg/ml propidium iodide.

The buoyant density of circular double-stranded DNA molecules is quantitatively reduced by the binding of dyes which intercalate between the bases (Bauer & Vinograd, 1968) and unwind the duplex (Vinograd et al., 1968; Denhardt & Kato, 1973; Pigram et al., 1973). The uptake of dye by RFI molecules, however, is opposed by the formation of positive superhelical twists which result from the unwinding (Vinograd et al., 1968). The net effect is a lesser binding of dye by RFI than by RFII at the same free dye concentration (Bauer & Vinograd, 1968). This results in a lower buoyant density for RFII. At appropriate concentrations of dye, all three ϕX DNA species (RFI, RFII and single strands) band at densities sufficiently different to allow their mutual separation (Radloff et al., 1967; Fukuda & Sinsheimer, unpublished data).

Figure 4a shows that the parental RF made from unirradiated phage separates clearly into RFI and RFII components at positions which coincide with the added ^{32}P RF marker. (A small amount of contaminating ^{3}H host DNA at the RFII position may account for the difference in $^{3}H/^{14}C$ ratios in the RFI and RFII peaks.)

In contrast, about 1/3 of the ^{14}C label in parental RF made after infection with UV irradiated ^{14}C phage (Fig. 9b) is spread between the positions of RFII and single-stranded DNA. The post-infection label (^{3}H) is seen primarily at the locations of completely double

stranded species, RFI and RFII, with some skewing of the RFII peak toward the single-strand position.

Other portions of the two RFII pools indicated in Fig. 8 were sedimented in alkaline, isokinetic, sucrose gradients (Fig. 10). The viral strands (^{14}C label) in RFII from unirradiated phage sediment predominantly as closed circles at 16S (Fig. 10a). A smaller amount of parental label (19%) appears in a sharp peak at 14S, the sedimentation coefficient of unit-length, linear, single strands. About 24% sediments more slowly. (The small number of RFI molecules in these samples sediment to the bottom of the tube.)

The post-infection label (^{3}H) in RFII from unirradiated phage sediments largely at the position of unit-length linear DNA. About 35% of the post-infection label appears to be in shorter pieces, while 14% sediments as complete circles. The unique distribution of breaks in these terminal RFII molecules from unirradiated phage strongly indicates that they were not generated by random nicking of RFI during or after extraction.

The viral strands of RFII from phage exposed to UV are mostly intact. About 40% of the viral strands sediment as unit-length linears and smaller pieces as compared to the 43% seen in normal RFII. In contrast, nearly all (88%) of the complementary strand label in these UV molecules sediments heterogeneously at less than 14S.

When comparing the structures of RFII from irradiated and unirradiated phage, it can be seen that the viral strands of both molecules are largely closed; relatively few linear viral strands are found in the cell even after UV irradiation of the phage (1.3 lethal hits per

phage). The complementary strands of both these molecules are almost always open. However, normal RFII have predominantly unit-length complementary strands while RFII from phage exposed to UV are always found in shorter lengths.

In addition, three observations support the conclusion that the complementary strand in RFII, made from UV irradiated phage, is substantially incomplete as opposed to merely containing nicks:

(i) Some of the RFII made from UV irradiated phage have buoyant densities approaching that of ϕX single-stranded DNA when centrifuged to equilibrium in CsCl with propidium iodide (see Fig. 9b). The RF near the single-strand position are deficient in post-infection label (^{3}H).

(ii) The ratio of ^{3}H (post-infection) to ^{14}C (parental) labels is always less in RFII from UV irradiated phage than in normal RFII when both were prepared in parallel cultures with the same amount of [^{3}H]thymidine. (Compare the RFII in Figs. 6a and 6d). The ratio of post-infection to parental label in RFII was found to decrease progressively with increasing UV dose by Benbow, Zuccarelli & Sinsheimer (1974, see their Table 2) while the ratios in the RFI peaks remained constant.

(iii) When such molecules were observed in the electron microscope after spreading in a basic protein film (Plate I) it was found that a large fraction of the molecules contained single-stranded regions which collapsed into dense "bushes" when prepared for viewing. None of the RFII from unirradiated phage were found to have such "bushes" (Plate If).

To confirm our implicit assignment of the parental (^{14}C) and post-infection (^{3}H) labels to the viral and complementary strands, respectively, RFII from the CsCl-propidium gradients (see brackets in Fig. 9) were centrifuged to equilibrium in alkaline CsCl (Vinograd et al., 1963) after the propidium was removed. Since the profiles from both gradients were identical, only the analysis of RFII from unirradiated phage is shown in Fig. 11. The viral strand (^{14}C), having a buoyant density of 1.765 g/cm^3 in alkaline CsCl, bands below the peak of post-infection label (^{3}H) which is expected to have the density of ϕX complementary strands, 1.756 g/cm^3 (Rüst & Sinsheimer, 1967; Siegel & Hayashi, 1967). A slight skewing of the ^{3}H label toward the position of viral strand DNA was almost always observed. Approximately 5% of the ^{3}H label may be judged to band at the viral strand density. Three explanations for the appearance of a small amount of post-infection label at the viral strand position are possible:

(i) High concentrations of chloramphenicol are not absolutely efficient in preventing the subsequent steps in ϕX infection, i.e., RF replication. Some de novo synthesis of viral strands occurs in the presence of [^{3}H]thymidine.

(ii) Limited repair or nick-translation of the viral strands during the infection has caused some ^{3}H nucleotides to be incorporated into the DNA strands which came from the infecting viruses.

(iii) A small amount of contaminating host DNA, labeled with ^{3}H, has banded at the viral strand position. The buoyant density of E. coli DNA in alkaline CsCl (1.766 g/cm^3) is nearly identical to that of ϕX viral strands (Siegel & Hayashi, 1967).

RF purified from a lysate by a single sedimentation in a preformed CsCl gradient may contain some host DNA. The RFII peak from the CsCl-propidium banding (Fig. 8a), which was the source of the material used in the strand analysis, had a slightly higher $^{3}H/^{14}C$ ratio than the corresponding RFI peak: possible evidence of host contamination. This observation, however, does not deny the possibility of viral strand repair. A need for such repair, in spite of the apparent integrity of the viral strand 20 minutes after infection (see Fig. 10a), is revealed later in this section.

(d) The structure of nascent RFII molecules

Parental RFII molecules were isolated from 50 ml portions of a chloramphenicol-treated culture of HF4704 infected with ^{32}P am3 phage (MOI = 2.0) for 1, 5, or 20 minutes in the presence of 20 μc/ml of [^{3}H]thymidine. Each infection was stopped by pouring the culture into ten volumes of cold borate-EDTA, containing 0.05 M NaN_3 and 5 mM KCN at 0°C. The cells were washed, lysed, treated with RNase, and then with pronase and SDS. (The procedure is described fully in the Methods.) The lysates were applied to 58 ml preformed CsCl gradients which were centrifuged at 25,000 revs/min for 15 hours in a SW25.2 rotor. The fractions, collected in 2 ml conical, plastic beakers, were placed directly into scintillation vials and counted without the addition of scintillation fluid. (The counting of Cerenkov radiation from ^{32}P disintegrations in aqueous solutions has an efficiency of about 60% when compared with the counting rate after the addition of scintillation fluid.)

Figure 12 shows the sedimentation patterns obtained from lysates of cells infected for 20, 5 and 1 minute. A peak of single-stranded DNA at 26S (near fraction 8) is seen in these gradients due to the reduction in viscosity of the lysates caused by RNase treatment. (Compare with Fig. 1 which shows the sedimentation of lysates not digested with RNase.)

The RF and single-strand regions of each gradient were pooled separately, as indicated by the brackets. The pools were dialyzed, precipitated with isopropanol and dissolved in small volumes of Tris-EDTA.

A portion of each RF pool was mixed with ^{14}C RF marker and centrifuged to equilibrium in CsCl containing 200 μg/ml propidium iodide. As seen in Fig. 13, the RF isolated after 20 minutes of infection bands exclusively at the positions of the RFI and RFII markers (compare Fig. 9a). The RF molecules isolated after 5 minutes of infection produce a similar pattern, with a larger fraction of the parental label appearing at the RFII position. Cells infected for only one minute contain mostly RFII, no RFI and some of the ^{32}P label bands at densities intermediate between the position expected for single-stranded DNA and the RFII marker. (A similar result was shown for RFII made from phage exposed to UV in Fig. 9b.)

The remaining RF from the original sedimentation in CsCl (Fig. 12) was further purified by sedimentation in neutral isokinetic sucrose gradients. Figure 14 shows that the RF made in cells infected for 20 or 5 minutes sediments as distinct RFI and RFII peaks in sucrose. The molecules appearing in the cells after one minute of infection, however,

form a broad band which spans the positions of both RFI and RFII. At the RFII position the ratio of post-infection to parental labels is similar to that of RFI in the accompanying gradients, but the ratio decreases further down in the gradient.

When the RFII regions of the neutral sucrose gradients were pooled (as indicated by the brackets), precipitated with isopropanol, mixed with ^{14}C single-stranded marker and portions applied to alkaline isokinetic sucrose gradients, the profiles shown in Fig. 15 were obtained.

As seen in an earlier experiment (see Fig. 10), the RFII found in cells after 20 minutes of infection have predominantly closed viral strands and unit-length, linear, complementary strands (Fig. 15a). When the infection period was shortened to 5 minutes, much more of the viral strand material sediments as unit-length linears than was observed in the longer infection. About 70% of the post-infection label is seen at the position of unit-length linears; a small amount sediments more slowly.

After a brief infection period of 1 minute, the structure of both the viral and complementary strands in RFII differ, dramatically, from those seen in the RFII isolated at later times. None of the complementary (^{3}H) DNA is unit-length. Rather, it sediments in a broad spectrum which extends from 14S nearly to the top of the gradient. Viral strand label (^{32}P), in addition to circular (16S) and unit-length linear (14S) components, is also found in a peak which sediments at 11S. This last viral strand peak, containing about 60% of all the ^{32}P

label, is not as sharp as either the 16S or 14S components, but it is much more homogeneous than the ^{3}H labeled material in the same gradient.

To confirm these observations, several 1 minute infections were performed with the following individual variations in the method:

(i) To eliminate possible isotope effects, the radioactive labels were reversed: ^{3}H am3 phage were used to infect cells in medium containing $H_3{}^{32}PO_4$ (20 μc/ml, added 5 minutes before infection).

(ii) KCN (5 mM, added 10 minutes before infection) was used to inhibit RF replication in the place of chloramphenicol.

(iii) Since RF replication is not expected to occur during the first few minutes of infection, no chemical inhibitors of replication were added to a culture.

(iv) The four borate-EDTA, NaN_3-KCN washes of the cells before lysis were omitted.

(v) Digestion of the lysate with RNase was omitted (with [^{3}H]thymidine post-infection label).

(vi) The infection period with ^{3}H phage was reduced to 30 seconds in ^{32}P medium without chloramphenicol.

(vii) NaN_3 and KCN were eliminated from the procedure. The infection was terminated by pouring the culture into a vessel, precooled to -70°C, in a bath of methanol and solid CO_2. The cells were washed with borate-EDTA.

With the modifications listed, each culture was treated as described in the Methods, and the RFII were purified by sedimentations in preformed CsCl and neutral, isokinetic, sucrose gradients.

All of these nascent RFII preparations gave, qualitatively, the same results when analyzed by sedimentation in alkaline sucrose: most of the post-infection label sedimented slowly and heterogeneously between 5 and 14S while a large fraction of the parental label sedimented sharply at 11S in addition to the 16S (circular) and 14S (unit-linear) components. The actual proportion of each label, which appeared to be in fragments less than unit-length, varied considerably. Figure 16 shows the alkaline sedimentations of RFII made with the modifications described in paragraphs i, iii and vi above. It is not known if the variations observed in these gradients are significant, but shorter infection periods and the presence of chloramphenicol seemed to be correlated with the sedimentation of greater amounts of both labels at less than 14S.

In order to determine if phage infecting the cell after 1 minute also generate nascent RF containing viral strands which sediment at 11S, a super-infection experiment was performed. ^{3}H am3 phage were allowed to infect cells at a multiplicity of 6, in medium with chloramphenicol. After 4 minutes of incubation, ^{32}P am3 phage were added to the culture at a multiplicity of 2. One minute later the infection was terminated and RFII molecules were isolated from the cells by the procedure described in the Methods. As shown in Fig. 16d, the viral strands which come from the first phage (^{3}H) sediment at 16 and 14S in an alkaline sucrose gradient. The viral label from the phage which infected the cells for only 1 minute (^{32}P) forms a large peak at 11S and smaller peaks at the positions of ϕX circles and unit-length linears.

Clearly, the appearance of viral strand fragments is correlated with the age of the RFII molecules in which they appear and not with any specific time after the beginning of infection.

(e) Single-stranded DNA found in the lysates

Even when infected cells were diluted in ten volumes of borate-EDTA and then washed four times with the buffer, a substantial amount of parental label appeared in a peak at 26S when the lysates are sedimented in preformed CsCl gradients. The peak is seen as early as 1 minute after infection (Fig. 12c) and the amount of label it contains increases slowly for 20 minutes (Fig. 12a and Fig. 12b). The relationship of these molecules to the cell is not known. Seven washes of borate-EDTA are expected to remove all attached and eclipsed phage particles (Newbold & Sinsheimer, 1969b).

The single-strand peaks seen in Fig. 12 were pooled as indicated by the brackets, precipitated with isopropanol and applied to alkaline isokinetic sucrose gradients. Figure 17 shows that from 1 minute to 20 minutes the fraction of circular molecules decreases from 65 to 35%. Some exonucleolytic degradation of the linears is evident at later times in the trailing of the 14S peak toward the top of the gradient. By comparison, the DNA obtained directly from the ^{32}P phage stock by extraction with phenol is over 90% circles (Fig. 17d).

II. 4 Discussion

(a) The appearance of parental RF after infection

The appearance of parental RF molecules shortly after infection has been studied by several workers (Denhardt & Sinsheimer, 1965b). However, since synthesis of the complementary strand appears to be a very rapid process, attempts to study the kinetics of their formation or to isolate *in vivo* intermediates have been hampered by the technical problem of synchronizing the events of interest. The merits of the methods commonly used to synchronize bacterial and bacteriophage systems are presented below.

(i) Cyanide, frequently used to synchronize phage infections (Denhardt & Sinsheimer, 1965a), blocks ϕX174 infections *after* the formation of parental RF (Cairns & Denhardt, 1968). Cells incubated with 5 mM KCN for up to one hour in growth medium are capable of converting ϕX single strands to RFI and RFII fully as well as cells without KCN, in 150 μg/ml of chloramphenicol (data not shown; also Benbow *et al.*, 1974; unpublished data quoted by Knippers *et al.*, 1969b).

(ii) Similarly, amino acid starvation of an amino acid requiring host cell stops the ϕX infection process after the synthesis of the complementary strand (Knippers & Müller-Wecker, 1970; Stone, 1967; Greenlee & Sinsheimer, 1968).

(iii) Nalidixic acid, a naphthyridine derivative (Lesher *et al.*, 1962) is a specific inhibitor of DNA synthesis in some bacteria and bacteriophage systems, though its mechanism is not understood (see

review by Goulian, 1971). It prevents the replication of bacteriophage M13 RF but not the formation of M13 parental RF (Fidanian & Ray, 1972).

(iv) Starvation for energy source, a technique used to investigate adsorption (Newbold & Sinsheimer, 1969b) and other steps of ϕX infection (Denhardt & Sinsheimer, 1965a, 1965b; Newbold & Sinsheimer, 1969a) has been shown by Francke & Ray (1971a) to cause artifacts which may obscure the natural pathway of events. Their data indicate that the addition of growth medium to starved, infected cells results in the degradation of a substantial fraction of the eclipsed viral DNA. Though 30% of the parental label escaped damage, only a small fraction of these intact viral strands were converted into normal RF even after 30 minutes incubation in complete medium. The degradation does not seem to be merely the result of nicking caused by thymine starvation (Freifelder, 1969) since cells starved and infected in the presence of adequate concentrations of thymine uniformly exhibited an impaired capacity for the synthesis of parental RF over a range of multiplicities which extended to less than one phage per cell (Francke & Ray, 1971a).

(v) Low temperature was used by Newbold & Sinsheimer (1969a, 1969b) to study attachment, eclipse and abortive infection of ϕX174. The phage forms a stable attachment to cells at 15°C but eclipse is prevented below 17°C (Newbold & Sinsheimer, 1970). An experiment in which phage were adsorbed to cells in medium at 15°C, and then shifted to 37°C to study the synthesis of the first complementary strand, yielded results which indicated little synchronization (data not shown). Such a result is not unexpected in view of the kinetics of eclipse of phage adsorbed to cells (Newbold & Sinsheimer, 1970). With a rate

constant of 0.86 min^{-1}, approximately 50% of the previously adsorbed phage would eclipse during the first minute at 37°C. The data reported here, however, show that the first complementary strand is substantially complete in ten seconds. Clearly, cold synchronization is insufficient to observe such a rapid reaction in detail.

(vi) Caffeine has a high binding affinity to single-stranded DNA (Ts'o & Lu, 1964). At low concentrations it inhibits the repair of ultraviolet lesions (Sauerbier, 1964; Harm, 1970). At high concentrations (15 mg/ml) it has been used to prevent the formation of ϕX174 parental RF in nucleotide-permeable cells (Hess et al., 1973). Phage adsorb normally and appear to progress beyond eclipse in 15 mg/ml caffeine. After incubating phage with cells for 7 minutes at 35°C in the presence of caffeine, 38% of the viral DNA could not be removed from the cells by seven washes with borate-EDTA; this washing procedure is expected to elute attached phage and eclipsed phage DNA from cells completely (Newbold & Sinsheimer, 1969b). The DNA inaccessible to borate-EDTA had a buoyant density of single-stranded, viral DNA after the cells had been lysed and extracted with phenol (Hess et al., 1973).

Since caffeine completely inhibits all macromolecular synthesis at high concentrations (Hess et al., 1973), it is not known whether cells can regain their normal functions sufficiently fast to obtain synchronous parental RF synthesis when the caffeine is removed. Indeed, these workers reported a one minute lag in ϕX DNA synthesis when nucleotide triphosphates were added to caffeine-inhibited cells after ether treatment; ether-treated cells which were not exposed to caffeine began DNA synthesis without a detectible lag (Geider et al., 1972).

It is clear that the available techniques are unlikely to effectively synchronize the synthesis of the first complementary strand in vivo. Furthermore, the use of techniques or chemicals to enforce a limited synchronization may well cause aberrations in the formation of parental RF. The extent of such aberrations could be determined only by subsequently studying untreated, and therefore, unsynchronized infections. For these two reasons, no synchronizing agents were used in this study. Indeed, mitomycin C pre-treatment of the cells and phenol extraction of lysates were also avoided to simplify interpretation of the results.

The experiments in the Results sections (a) and (b) were performed to discover the basic outlines of parental RF synthesis in unsynchronized infections and to test a simple method for the detection and isolation of RF species (Francke & Ray, 1971b). Specifically, answers to the following questions were sought:

(i) How long does it take a culture to convert the infecting viral DNA into a stable, parental RF population in unsynchronized infections?

(ii) What are the kinetics of appearance of RF species?

(iii) What is the earliest time that any RF species can be detected in the cell?

(iv) How does exposure of phage to measured doses of ultraviolet light affect the terminal parental RF population?

(v) Can intermediates in the formation of a terminal RF population be detected?

The data presented provide some answers to each of these questions.

(i) Under the conditions used in this work, a stable (terminal) population of parental RF molecules was attained in, approximately, 20 minutes of infection. At low multiplicities of infection few changes occurred after 20 minutes. At multiplicities above 10 phages per cell some new RFI or RFII molecules may be made after that time.

The structural data provide some evidence that the terminal RF population is static rather than at equilibrium. An equilibrium implies that some RFI molecules are being opened while some RFII are ligated into covalently closed RFI. Unless nicking of RFI is presumed to occur only in the complementary strand, the high proportion of closed circular viral strands found in terminal RFII is not explained.

There is not sufficient information to decide if the RFII seen at 20 minutes persist from the earliest times or if they were made from phage DNA injected late in the experiment. Some molecules may maintain openings in the complementary strand due to a particular cellular attachment. However, from the data in this section, it does not appear that a constant number of RFII molecules are maintained per cell at varying multiplicities as is the case when the cistron A product is active (Francke & Ray, 1971b).

(ii) In unsynchronized infections some of the parameters of parental RF synthesis cannot be determined. The general pattern of appearance of RF was shown in Fig. 2a and Fig. 3. RFII were found in large numbers at 3 minutes (see Fig. 1a) while an RFI peak was not seen until 5 minutes. Extrapolation indicates that the first RFI was

made between 1 and 2 minutes after infection. In their investigation of RF replication in a host strain temperature-sensitive in DNA synthesis, Knippers & Müller-Wecker (1970) similarly found that the first progeny RFI molecules appeared less than two minutes after shift-down to a permissive temperature.

The structure of fast RF, also observed 3 minutes after infection, is not known. These molecules occupy a region of the preformed CsCl gradients containing excess parental label. Several other investigators report a species with similar properties. Benbow et al. (1974) observed that about 20% of the parental label sedimented in a peak faster than RFI in rec$^+$ cells but that the peak was not seen in recA hosts. An excess of parental label in this species, was also noted by Benbow (personal communication). The high-salt sucrose gradients of Francke & Ray (1971b, see their Fig. 1) show a species sedimenting either as a peak or shoulder on the leading edge of the RFI peak, precisely the position found to be occupied by "fast RF" in this work. They attribute the shoulder to the high viscosity of the samples applied to the gradients, but their subsequent centrifugal analysis of this region of the gradients reveals that, in addition to the expected RFI, it contains a 10 to 20% excess of parental label in the form of circles (50%), unit-length linears (20%), and shorter linears (30%). No post-infection label, other than that seen in RFI, was found in this region of the gradients. By comparison, in the present report, the RFI pool (Fig. 8a) from which the "fast RF" peak was specifically excluded, contained no material which sedimented slower than denatured RFI in alkaline sucrose gradients.

Greenlee (1970) described an intracellular ϕX DNA species sedimenting slightly faster than RFI which he observed when cellular protein synthesis was inhibited during infection (high chloramphenicol or amino acid starvation). He found that this species contained single-stranded regions and was more dense than RFI in CsCl. The molecules also contained excess post-infection label (probably complementary strand DNA). His centrifugal analysis led him to conclude that the viral strands in these molecules were in pieces less than unit-length, while post-infection label was found in DNA longer than unit-length.

Whatever their structure may be, distinct peaks at the fast RF position were found to contain 13% (Fig. 2a) and 21% (Fig. 3) of the post-infection label only 3 minutes after infection. The amount of fast RF is especially difficult to judge in the presence of a large RFI peak, but it appears to account for 10 to 20% of all ϕX forms in longer infection periods (Fig. 1 and Fig. 2). Its formation is much more resistant to UV irradiation of the infecting phages than is RFI (Fig. 7).

(iii) It is clear from Fig. 4 that RFII molecules have already been formed 15 seconds after the phage and cells are mixed. By extrapolation, the first RFII appears less than ten seconds after infection. Since the initiation of complementary strand synthesis is preceded by adsorption, eclipse, and possibly, injection, ten seconds is an overestimate for the duration of polynucleotide synthesis itself. Nevertheless, it indicates a rate of chain growth in excess of 600 nucleotides per second, or about one-half the <u>in vivo</u> elongation rate

of E. coli duplex DNA at 37°C (Bonhoeffer & Gierer, 1963; Cairns, 1963; Cooper & Helmstetter, 1968).

Unfortunately, the appearance of fast RF could not be seen in these gradients.

(iv) Exposure of phage to ultraviolet light greatly affects the parental RF population they are able to generate. The loss of plaque-forming units is highly correlated with the disappearance of RFI molecules from the terminal population. It suggests that a single lethal hit is sufficient to prevent the formation of an RFI molecule from the viral DNA template. Conversely, most (∿ 90%) of the lethality of UV irradiation appears to be due to the inability to generate RFI. Only 5% of the lethal hits are expected to cause DNA-DNA crosslinking, and perhaps, DNA-protein crosslinks which would prevent injection into the host (Francke & Ray, 1972). Lytle & Ginoza (1969) reported that 79% of the phage killed by UV were still able to generate RF (see their Table 1). However, they included the RFII peak in the category of functional RF.

The relative resistance of RFII to UV may be explained by assuming that molecules with appreciable single-stranded regions sediment near the position of completely duplex RFII (see Lytle & Ginoza, 1969). The survival of fast RF is not understood.

At higher doses parental label shifts from the RFI position (20S) to a position near the bottom of the tube (25S) where largely single-stranded ϕX DNA is expected to sediment. The amount of parental label found in the lysates after four borate-EDTA washes was not effected by the UV dose. Only a few percent of the lethal hits are

expected to cause DNA-DNA or DNA-protein crosslinking, and therefore, prevent injection into the host (Francke & Ray, 1972). Within 10%, the parental label seen in the gradients remained constant over the range from 0 to 2.7 hits per phage. This result is in substantial agreement with those of Lytle and Ginoza (1969). The data of Benbow et al. (1974) indicate that only 80% of the phage given 2.7 hits are still able to penetrate the cell.

(v) Since the actual assembly of nucleotides on the viral template to form the first complementary strand takes no more than 10 seconds, and may be as short as 4 seconds (at 15,000 nucleotides per second, the in vivo rate of E. coli polynucleotide chain growth) it is unlikely that intermediates in the synthesis will be observed in any but the shortest infection periods. However, in an unsynchronized infection, only 0.1% of the phage cpm added to the culture can be found in the cells after 15 seconds of infection, and only a fraction of the injected DNA would be expected to be in RF intermediates at that time. In the absence of synchronization, the yield of in vivo intermediates is very low. This dilemma was familiar to other workers studying parental RF formation (see Francke & Ray, 1971a).

For this reason, it was considered more feasible to study the structure of nascent parental RF molecules, in hopes of learning about the synthetic process, than to isolate and study the short-lived intermediates.

(b) The structure of parental RFII: The effect of an UV-damaged template

The structure of terminal parental RFII and parental RFII made from UV irradiated phage were investigated, primarily, to provide standards with which the data obtained from nascent RF could be compared. In addition, their structures may offer basic information about the synthesis of the first complementary strand.

(i) Normal RFII have primarily closed, circular viral strands and unit-length linear complementary strands. Most of the parental label sediments at 16S, the velocity of ϕX circular, single-stranded DNA in alkali. Similarly, most of the post-infection label sediments at 14S as unit-length, linear DNA. Some of the remaining parental and post-infection label is found in pieces which are smaller than unit-length.

(ii) RFII obtained from cells infected with UV irradiated phage have primarily closed, circular viral strands. The complementary strand is incomplete, i.e., substantial regions of the template remain single-stranded. As in normal RFII, most of the parental label in these molecules sediments with the velocity of ϕX circles. However, the post-infection label always sediments at less than 14S. The buoyant density, the ratio of parental to post-infection labels and the appearance of these molecules in the electron microscope indicate that they have single-stranded regions.

The assignment of parental-labeled DNA, exclusively to the viral strand, and the post-infection label to the complementary strand was supported by the banding pattern of the labels in alkaline CsCl.

The pattern of breaks in the normal, parental RFII reported here, is similar, but considerably more strand-specific than was observed by Francke & Ray (1971b). The structure deduced for molecules synthesized from UV irradiated phage agrees with the conclusions of Francke & Ray (1971b) and Benbow et al. (1974).

(c) The structure of nascent parental RF

Nascent, parental RF molecules were obtained from cells infected for short times. Molecules which sedimented as RFII and those which appeared in the general RFI-RFII region of the preparative gradients, were both studied. The following conclusions can be drawn:

(i) Intermediates in the synthesis of the first complementary strand can be observed in cells, transiently, during the first few minutes of an unsynchronized infection. Molecules which have properties substantially different from RFII, comprise about 10% of the total parental label in the cells at 1 minute after infection. In CsCl-propidium iodide, these molecules have buoyant densities intermediate between ϕX single strands and RFII. They sediment in a broad band in neutral sucrose gradients, covering the range from 18 to 24S. The molecules which band close to the single strand position or which sediment near 24S have lower post-infection to parental label ratios than do the molecules which behave like RFII. From these observations, it may be inferred that this population includes forms with varying amounts of complementary DNA and, consequently, regions of single-stranded template. The small number of these molecules which could be obtained free of host DNA contamination, prevented further analysis.

(ii) The first complementary strand is discontinuous shortly after its synthesis and the interruptions are sealed later. In unsynchronized infections, the post-infection label in molecules which behave like RFII appears to be in small pieces immediately after infection and in unit-length segments at later times. ϕX DNA species which sediment at 18S (like RFII) in neutral gradients, and which have parental to post-infection labels at ratios which indicate a largely double-stranded structure, can be isolated 1 minute after infection. In alkali, most of the post-infection label in these molecules sediments broadly in a peak from 5 to 12S. In RFII molecules isolated 5 minutes after infection, about 40% of the post-infection label is found in pieces which are less than unit-length, while only 20% of the terminal (20 minutes) RFII molecules contain complementary strand material which sediments at less than 14S.

The structure of nascent parental ϕX RF molecules from living cells has not previously been studied. In nucleotide-permeable cells the first complementary strand was found to be discontinuous shortly after its synthesis by Hess et al. (1973). The complementary strand DNA, appearing first as 5 to 6S fragments, gradually increased in size until it reached unit length. Some of the complementary strand pieces in purified nascent RF could be joined to form larger segments in vitro with T4 polynucleotide ligase, indicating that each molecule contained several adjacent complementary strand fragments (Hess et al., 1973).

RF replication, the process which follows parental RF synthesis under normal conditions, is also discontinuous in vivo (Knippers et al., 1969a; Eisenberg & Denhardt, 1974). The gaps in nascent RFII, obtained

from infected cells during RF replication, were filled with labeled nucleoside triphosphates by the action of T4 DNA polymerase. The incorporated label was found predominantly in small DNA fragments which sedimented broadly from 5 to 14S (Eisenberg & Denhardt, 1974). Similarly, Dürwald & Hoffmann-Berling (1971) found that nascent RF, synthesized in ϕX infected cells, treated with ether during the period of RF replication, contained 5S pieces which were precursors for full-length complementary strands.

Yokoyama et al. (1971) have concluded that the synthesis of viral single strands, late in the infection, is also discontinuous. They report the appearance of 5 to 12S DNA fragments in cells pulse-labeled 50 minutes after infection. When chased, the label in these fragments appeared in unit-length molecules.

(iii) The viral strands in RFII extracted during the first minute of infection are found in pieces which are about half-length. In cells infected for 1 minute, most of the parental label in nascent RFII sediments at 11S (± 0.2S in eight gradients). If the relationship between S value and molecular weight of single-stranded DNA formulated by Studier (1965) applies to DNA of this size, 11S is the velocity expected for a linear molecule one-half the length of ϕX DNA in alkali (see Hirose et al., 1973).

At 30 seconds after infection all of the parental label sediments at 11S. Five minutes after infection only unit-length linears and circles are found. The proportion of viral strand circles increases from 5 to 20 minutes. The progressive change in viral strands from 11S to 14S to 16S configurations is evident, however, since new phage

continue to adsorb and inject for most of the 20 minute period studied, it is not possible to conclude that the same parental DNA which appears in 11S fragments at 1 minute, is later found in 14 or 16S species.

Parental label sedimenting at 11S was seen when $H_3{}^{32}PO_4$ or [^{3}H]thymidine labeling, chloramphenicol, NaN_3-KCN termination, borate-EDTA washing and RNase digestion were individually eliminated from the procedure used to prepare nascent RFII. Alternatives to pronase-SDS digestion of the lysates were not tested. (It may be emphasized that the standard isolation procedure does not cause breaks in the viral strands of RFII isolated at 5 or 20 minutes after infection.)

The limited distribution of lengths of viral DNA sedimenting in the 11S peak is apparent from the width of the peaks. For example, very few counts are seen at 9S, and none at 8S, the sedimentation values predicted for linear molecules 1/3 and 1/4 the length of ϕX single strands. Furthermore, the peaks are completely symmetrical.

In their study of the effects of starving host cells for energy sources and thymine on parental RF formation, Francke & Ray (1971a) found that 60% of the viral DNA recovered from infected cells 20 minutes after the addition of nutrients to the medium was no longer intact. The alkaline sedimentation pattern of these viral strands (see their Fig. 3b) is identical in every important respect to that shown in Fig. 16a. A large fraction of the viral label appears in a sharp, symmetrical peak near 11S. It may be surmised that the absence of thymine during infection may have caused the breaks (Freifelder, 1969). However, they report that the same results were obtained when adequate amounts of thymine were present throughout the entire

experimental procedure. In light of the data presented here, it seems unlikely that the viral strand fragments observed by these workers are random degradation products or that they are unrelated to the normal process of infection.

The interpretation of these observations is not certain. At least three classes of explanations are possible:

(A) Breakage of the infecting viral strand is a normal step early in the infection process. Injection of the viral DNA into the cell or the initiation and elongation of the new complementary strand may require that the infecting DNA circle be opened. The data do not reveal whether the scissions are made before or after the DNA complement is synthesized. The finding that a large fraction of the single strands in the lysate are unit-length linears even at early times and the data from starved cells (Francke & Ray, 1971a) may support either of the two alternatives.

Several groups (Sinsheimer et al., 1968; Knippers et al., 1969a; Schaller et al., 1969; Forsheit & Ray, 1970; Iwaya et al., 1973) have proposed specific mechanisms for circularizing single-strand linears by means of intramolecular base pairing (Fiers & Sinsheimer, 1962). However, the viral strand fragments observed here appear to be approximately half-length. The efficient reconstitution of a unit-length circle from two single-stranded halves is difficult to envision without invoking extensive internal homology as well as specific nicking.

It is more likely that at least one of the breaks is made during the formation of the complementary strand. If the break is introduced into a duplex region, the circle remains intact. The nicks

may simplify complementary strand synthesis. Alternatively, since the mechanism by which the complementary strand is initiated in vivo has not yet been elucidated, one of the breaks in the template may be involved with starting the new strand.

All of the proposals in this category imply that the viral strand breaks are eventually repaired (very rapidly, according to the data). Such repair may account for the small amount of post-infection label which bands at viral strand density in alkaline CsCl. If viral strand breakage occurs at specific locations on the genome, the distribution of post-infection label would identify the sites.

(B) The relationship of the nascent parental RFII molecule to the infected complex is such that the viral strand is frequently broken during isolation. ϕX parental RF molecules are known to be attached to specialized (membraneous) cellular sites (Yarus & Sinsheimer, 1967; Knippers & Sinsheimer, 1968). This association occurs before RF replication. The mechanics of establishing this link between the cell and the nascent RF may make the molecule transiently susceptible to breaks in either strand. After the attachment is completed, the RF may be removed without breaking the viral strand.

Several other mechanisms, based upon qualities of the nascent parental RF which would make them exceptionally fragile during extraction and purification, may be proposed in this category.

(C) The breaks in the viral strands of nascent RFII are random nicks introduced during or after the isolation of the molecules. Many observations make this conclusion unacceptable. Breaks are only found in RFII isolated during the first minute of infection. This observation

indicates that the phenomenon is a function of the molecules rather than the method of isolation. Furthermore, the resulting fragments have a narrow, symmetrical distribution of sedimentation values in alkaline gradients. Such a distribution is not easily generated by random breakage. Finally, breaks appear in the viral strands from super-infecting phage when no breaks are seen in the viral strands from phage which had infected the cells 5 minutes earlier.

(iv) Initiation of the first complementary strand occurs at one or, at most, two specific positions on the viral strand template. The data from RF made from UV damaged phage described here, and their configuration in preparations studied by electron microscopy (Benbow et al., 1974) are best interpreted by proposing that the complementary strand is initiated at a single point on each viral template.

It was shown in the Results that the number of RFI and RFII molecules which could be generated decreased with increasing exposure of the infecting phage to UV light (see Figs. 6 and 7). In a further analysis of these gradients, the total amount of complementary strand material synthesized at each UV dose was determined by finding the total post-infection label observed in the regions of the gradients occupied by ϕX DNA species. On the basis of a single initiation hypothesis, the expected amount of complementary strand synthesis at each dose was calculated, relative to the unirradiated case, by making a few simple assumptions (see legend to Fig. 18). It can be seen in Fig. 18 that the curve expected from a single initiation closely approximates the observed values. For comparison, a second curve calculated on the basis of two equidistant initiations is also plotted.

Benbow et al. (1974) made an electron microscopic examination of the incomplete RF obtained from cells, infected with UV-damaged phage. All of the molecules observed were unit-length circles which contained one double-stranded region of variable length and, consequently, one single-stranded region. The average length of the duplex region was an inverse function of the phage UV dose. Even at low doses some molecules had a large single-stranded region. Since the new strand was frequently absent distal to a single UV damage site, it was inferred that synthesis of the complementary strand was initiated at a single position on each template and that the new chain stopped when it encountered a UV damage site.

In Part III of this thesis, parental RF synthesized in cells during a switch from cold to tritiated medium have a distribution of radioactivity which may be best interpreted by proposing that one or, at most, two specific initiation sites are used when generating an entire population of parental RF.

By comparison, initiation of the first complementary strand of the filamentous bacteriophage M13 appears to be specific. RFII made from the single-stranded DNA of M13 by a soluble enzyme extract from E. coli has been studied by Tabak et al. (1974). When the molecules were cleaved by a restriction enzyme from Haemophilus parainfluenzae, one of the nine expected fragments was absent. If the RFII were repaired with DNA polymerase I before digestion with the restriction enzyme, the fragment was restored and it was found to contain nearly all of the repair label. The pattern of pyrimidine tracts in the repaired region was much simpler than the pattern obtained from RF uniformly labeled in

the complementary strand. The linear complementary strand in the RFII was cleaved into one large and one small fragment by a restriction enzyme from H. influenzae. Digestion of the in vitro RFII with the single-strand specific endonuclease S_1 from Aspergillus oryzae yielded linear duplex molecules which remained linear after melting and annealing. These results led to the conclusion that the complementary strand of M13 is initiated by the extension of an RNA primer formed at a specific region on the circular M13 viral strand. The gap may be the result of partial degradation of the RNA primer. However, when ϕX174 RFII was synthesized in vitro and similarly examined, a specific gap in the complementary strand was not revealed (Tabak et al., 1974).

A comparison with the later stages of ϕX DNA synthesis is instructive. Two lines of evidence indicate that the replication of ϕX RF molecules and the synthesis of progeny ϕX viral strands, later in infection, both have specific and, possibly, identical initiation sites. Baas & Jansz (1972) observed that the efficiency with which mismatched regions in heteroduplex DNA molecules were repaired was related to the location of the mismatch on the ϕX genetic map. From their data they inferred that the replication of parental RF molecules begins at a specific location near cistron A and proceeds clockwise with respect to the genetic map (Benbow et al., 1971).

Johnson & Sinsheimer (1974) isolated RFII during the period of single strand synthesis, late in the ϕX infection in vivo. Their examination of these molecules revealed a structure similar to that observed for RFII made from M13 viral strands in vitro (see above). When ϕX RFII isolated late in the infection were cleaved with a restriction

enzyme from Haemophilus influenzae, one of the fragments had an altered electrophoretic mobility in polyacrylamide gels. If the RFII was repaired with DNA polymerase I before digestion with the restriction enzyme, the fragment was restored to its normal mobility and it contained nearly all of the repair label. Since many of the fragments produced by this restriction enzyme have been located with respect to the ϕX genetic map, Johnson & Sinsheimer (1974) were able to determine that the nicks and gaps in the original molecules were located specifically at a site in cistron A. They proposed that the initiation of viral single strand synthesis begins at this position. In addition, the fact that several other groups (Schröder & Kaerner, 1971; Schekman & Ray, 1971; Iwaya et al., 1973) have found that the linear viral strands in late RFII or phage particles produced in ligase-defective host cells appear to have specific ends by other criteria (infectivity or ability to circularize) seems also to support the idea that the last two stages of ϕX DNA replication have specific and possibly identical initiation sites.

With respect to the synthesis of the first complementary strand in parental RF, these results indicate that there is a possibility that all three stages of ϕX DNA synthesis have specific initiations (see Johnson & Sinsheimer, 1974).

(d) Proposal for the specificity of initiation of the ϕX complementary strand

A conflict exists between the conclusions of paragraphs ii and iv above. Discontinuous synthesis of the first complementary strand

implies that new polynucleotides are initiated at many sites on the template, yet the evidence for a unique initiation, summarized here, denies that conclusion. The same situation exists during RF replication and single strand synthesis, the last two stages of the ϕX infection. DNA synthesis during both periods is believed to be discontinuous (Knippers et al., 1969a; Eisenberg & Denhardt, 1974; Dürwald & Hoffmann-Berling, 1971; Yokoyama et al., 1971), yet single, specific initiation sites have been proposed (Baas & Jansz, 1972; Johnson & Sinsheimer, 1974; Iwaya et al., 1973).

This conflict emphasizes our inadequate understanding of the mechanisms of DNA initiation and chain elongation in E. coli. At present, one may speculate that a unique initiation event precedes or causes a sequence of secondary initiations which differ from the primary event in some way. Replication of the E. coli chromosome itself seems to fit this description. A new round of replication, beginning at a specific chromosomal origin, can be prevented by treatments which stop protein or RNA synthesis, e.g., chloramphenicol and rifampicin (Lark & Renger, 1969; Ward & Glaser, 1969; Lark, 1972). Once a new round of synthesis has begun, the secondary initiations, which generate Okazaki fragments with covalently bound RNA primers (Sugino et al., 1972; Sugino & Okazaki, 1973; Hirose et al., 1973), are not inhibited by these agents (Lancini & Sartori, 1968; Lancini et al., 1969; Silverstein & Billen, 1971).

An analogous mechanism for the synthesis of the first complementary strand of ϕX would require a protein to provide the specificity for the site of primary initiation. Such a protein could come from

the phage coat itself, entering the cell with the phage DNA at injection. Brown et al. (1971) have presented evidence that ϕX coat proteins do indeed enter the cytoplasm. Jazwinski et al. (1973) have observed that the filamentous phage M13 injects a minor coat protein (gene 3, molecular weight about 70,000 daltons) into the cell with its single-stranded genome. The protein is associated with the viral DNA after adsorption and remains with the DNA even after its conversion to RFI. Furthermore, these workers report that one or a few molecules of a unique phage protein are associated with the parental RF isolated after ϕX174 infection (unpublished results quoted in Jazwinski et al., 1973).

In contrast to M13, ϕX virions contain at least five different proteins (Benbow et al., 1972; Godson, 1971). Small amounts of several other virus-specific proteins have also been identified in highly purified phage preparations (R. G. Rohwer, personal communication). The ϕX cistron A protein, which is the only phage protein required for ϕX RF replication (Tessman, 1966; Lindqvist & Sinsheimer, 1967; Iwaya & Denhardt, 1971), has a molecular weight of about 62,000 daltons (Linney & Hayashi, 1973) and it has been detected in phage particles (R. G. Rohwer, personal communication). It seems an ideal candidate for the initiator protein.

This proposal agrees in many respects with the general model of DNA replication described by Denhardt (1972). The phage initiator protein corresponds to his π protein and it interacts with the specific initiation site (π sequence). Denhardt's model also predicts that a specific gap will remain in the new strand at the initiation site. The inability of Tabak et al. (1974) to detect such a gap may indicate that

the in vitro system does not perform the conversion exactly like a living cell. Clearly, in view of the growing number of specific protein cofactors and enzymes which are required for in vivo DNA replication in E. coli (Taketo, 1973; Wickner et al., 1973), it will be necessary to study the in vivo product more carefully.

(e) The involvement of RNA in the initiation of the first ϕX complementary strand

The mechanism of the primary initiation on the circular single-stranded DNA template in vivo is not yet completely understood. None of the known DNA polymerases is able to begin a new polynucleotide without a primer (Goulian, 1968; Kornberg & Gefter, 1972; Wickner et al., 1972d). The synthesis of RNA by E. coli RNA polymerase, however, is not governed by such a limitation (Maitra & Hurwitz, 1965). In the conversion of single-stranded DNA from the filamentous phage M13 into RFII, the action of E. coli RNA polymerase is required (Wickner et al., 1972c; Brutlag et al., 1971; Westergaard et al., 1973). Rifampicin, a specific inhibitor of RNA polymerase (Sippel & Hartmann, 1968), prevents the M13 conversion both in vivo (Brutlag et al., 1971) and in vitro (Wickner et al., 1972c). Furthermore, the M13 RF produced by cell extracts contains a ribonucleotide at the 5' end of the new strand (Wickner et al., 1972c). When these RFII are sealed by the action of T4 DNA polymerase and T4 DNA ligase, the resulting RFI are alkali-sensitive (Westergaard et al., 1973).

The discontinuous synthesis of DNA fragments during replication of the E. coli chromosome in living cells also involves short RNA priming chains which are covalently linked to the DNA product (Sugino et al.,

1972; Hirose et al., 1973). In E. coli however, discontinuous DNA synthesis by means of RNA primers does not appear to be inhibited by rifampicin except at the beginning of a new round of replication (Silverstein & Billen, 1971; Lark, 1972).

Rifampicin does not prevent the conversion of ϕX single strands to RF in vivo or in vitro (Silverstein & Billen, 1971; Wickner et al., 1972c), in contrast to the M13 case. This observation may be interpreted in two ways. Either RNA synthesis is not required for the conversion or, as appears to be the case with the E. coli chromosome itself, an RNA primer is synthesized by a yet unknown, rifampicin-insensitive mechanism. Schekman et al. (1972) report that the second alternative seems to be the case in the conversion of ϕX single strands to RF by a supernatant enzyme fraction from E. coli. They found that all four ribonucleoside triphosphates were required for the reaction and that the final product had a phosphodiester bond between a deoxyribonucleotide and a ribonucleotide. However, no report of an RNA primer covalently linked to DNA in ϕX parental RF from living cells has appeared. Though the involvement of an RNA primer in vivo is likely, until similar results are obtained from living cells, there is the possibility that ϕX complementary strand synthesis may be initiated by a mechanism which does not include RNA.

Footnotes

RF (replicative form) is the double-stranded circular form of ϕX174 DNA; RFI is the supercoiled double circle in which both polynucleotide strands are covalently closed; RFI is the circular duplex in which one strand (or both strands at different positions) is nicked; the viral strand is the polynucleotide having the same base sequence as the DNA found in the virus; the complementary strand has a base sequence complementary to the DNA in the virus; MOI (multiplicity of infection); pfu (plaque-forming unit).

II. 5 References

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Figure Legends

FIG. 1. The sedimentation in preformed CsCl gradients of pronase-SDS treated lysates from cells infected with ^{14}C am3 (MOI = 110) in medium containing [^{3}H]thymidine (10 μc/ml). Equal volumes of the culture were stopped at (a) 3, (b) 5, (c) 7, (d) 10, (e) 15 and (f) 20 min after infection by rapid cooling in borate-EDTA, NaN_3-KCN. The positions of ^{32}P RF markers, added to two of the cultures just before lysis, are indicated by arrows. A description of the gradients, centrifugation conditions, fractionation and counting procedures for this and the following figures may be found in the Methods.

●—● ^{14}C (parental) cpm fraction

O—O ^{3}H (post-infection) cpm fraction

FIG. 2. The label in RF components in the gradients of Fig. 1 plotted against the length of the infection. Panel (a) shows the post-infection label in RFI, RFII and fast RF, individually. Panel (b) compares the total parental label with the total post-infection label in all the RF species.

●—● ^{14}C (parental) cpm

O—O ^{3}H (post-infection) cpm

FIG. 3. The post-infection label in three RF species from pronase-SDS treated lysates from cells infected for 3, 5, 10 and 20 min with ϕX174 wt phage (MOI = 80) in medium containing [^{3}H]thymidine (10 μc/ml). The lysates were sedimented in preformed CsCl gradients and the ^{3}H cpm in

each of the RF peaks were summed and plotted against the length of the infection.

O—O ^{3}H (post-infection) cpm in RFI

Δ—Δ ^{3}H (post-infection) cpm in RFII

□—□ ^{3}H (post-infection) cpm in fast RF

FIG. 4. (a) ^{32}P parental label sedimenting as RFII after short periods of infection. Lysates of cells infected for short periods with ^{32}P am3 phage (MOI = 1.5) were treated with pronase-SDS and sedimented in pre-formed CsCl gradients. The position of ^{3}H RFII marker, added to two of the cell suspensions just before lysis, are indicated by the arrows.

O—O ^{32}P (parental) cpm/fraction after 15 sec of infection

Δ—Δ ^{32}P (parental) cpm/fraction after 30 sec of infection

□—□ ^{32}P (parental) cpm/fraction after 45 sec of infection

●—● ^{32}P (parental) cpm/fraction after 60 sec of infection

▲—▲ ^{32}P (parental) cpm/fraction after 120 sec of infection

■—■ ^{32}P (parental) cpm/fraction after 180 sec of infection

(b) The sum of the ^{32}P label in the RFII peaks shown in (a) plotted against the length of the infection period.

O—O ^{32}P (parental) cpm

FIG. 5. The isokinetic sucrose sedimentation profiles of parental RF from cells cooled in the presence of [^{3}H]thymidine. A culture was infected with am3 phage (MOI = 70) in medium containing ^{32}P (1 μc/ml). Twenty ml volumes were poured into (a) 40 ml of borate-EDTA, NaN_3-KCN at $0^{o}C$, 7 min after infection and (b) 40 ml of TPA medium at $0^{o}C$, 8 min after infection. Both the borate-EDTA and the TPA medium contained

[^{3}H]thymidine (20 μc/ml). The mixtures were rapidly cooled to 0°C by swirling in a bath of methanol and solid CO_2. The lysates were treated with pronase-SDS and then sedimented in preformed CsCl gradients. The entire RF region of each gradient was pooled and the DNA, concentrated by isopropanol precipitation, was applied to neutral, isokinetic, sucrose gradients in small volumes of Tris-EDTA buffer. The preparation, centrifugation and collection of isokinetic sucrose gradients are described in the Methods.

O—O ^{32}P cpm/100 μl

●—● ^{3}H cpm/100 μl

FIG. 6. The sedimentation profiles of pronase-SDS treated lysates from cells infected for 20 min in medium containing [^{3}H]thymidine (10 μc/ml) with ^{14}C am3 phage (MOI = 50) UV irradiated for (a) 0, (b) 20 sec, (c) 40 sec, (d) 60 sec, (e) 80 sec and (f) 120 sec. Sedimentation was in preformed CsCl gradients. ^{32}P RF markers, added to two of the cell suspensions just before lysis, are indicated by the arrows.

●—● ^{14}C (parental) cpm/fraction

O—O ^{3}H (post-infection) cpm/fraction

FIG. 7. The label in the RF components in the gradients of Fig. 6, plotted against the length of the phage UV exposure. Panel (a) shows the post-infection cpm in RFI, RFII, and fast RF, the total parental cpm in the gradients and the percent of pfu surviving each UV dose. Panel (b) shows the percent of the total post-infection label in RFI, RFII and

fast RF as a function of phage UV dose.

●—● ^{14}C (parental) cpm

O—O ^{3}H (post-infection) cpm

▲—▲ percent of pfu surviving

FIG. 8. Sedimentation patterns of pronase-SDS treated lysates from cells infected (MOI = 110) with (a) unirradiated ^{14}C am3 phage and (b) ^{14}C am3 phage irradiated with UV for 60 sec (26% survival). The infections were for 20 min in medium containing [^{3}H]thymidine (10 μc/ml). The pronase-SDS treated lysates were sedimented in 58 ml preformed, CsCl gradients. The brackets indicate fractions which were pooled, dialyzed, precipitated with 2 volumes of isopropanol and then dissolved in small volumes of Tris-EDTA.

●—● ^{14}C (parental) cpm/100 μl

O—O ^{3}H (post-infection) cpm/100 μl

FIG. 9. Equilibrium buoyant density centrifugation in CsCl with 200 μg/ml propidium of RF from (a) cells infected with unirradiated ^{14}C am3 phage and (b) cells infected with ^{14}C phage irradiated with UV for 60 sec. The DNA was prepared and purified as described under Fig. 8 and it represents a portion of the RF in the pool indicated by the brackets (II) in each gradient of Fig. 8. The positions of the ^{32}P RF or single-strand (SS) DNA markers, added before centrifugation, are indicated by the arrows. The fractions in each gradient indicated by the brackets were pooled, run through a 5 ml Dowex-50 column to remove the propidium, precipitated with two volumes of isopropanol and then

dissolved in a small volume of Tris-EDTA. The centrifugation conditions for these gradients can be found in the Methods.

●—● ^{14}C (parental) cpm/30 µl

O—O ^{3}H (post-infection) cpm/30 µl

FIG. 10. Sedimentation in alkaline, isokinetic, sucrose gradients of RFII from (a) cells infected with unirradiated phage and (b) cells infected with phage irradiated with UV for 60 sec. The DNA was prepared and purified as described under Fig. 8 and the material in these gradients represents a portion of the pools indicated by the brackets (II) in Fig. 8. The centrifugation conditions for these gradients may be found in the Methods.

●—● ^{14}C (parental) cpm/100 µl

O—O ^{3}H (post-infection) cpm/100 µl

FIG. 11. Equilibrium buoyant density centrifugation in alkaline CsCl of RFII from cells infected for 20 min with ^{14}C am3 phage in medium containing [^{3}H]thymidine. The RFII was obtained from the fractions indicated by the brackets in Fig. 9a. The DNA was prepared and purified as described under Figs. 8 and 9. The conditions of centrifugation for this gradient are described in the Methods.

●—● ^{14}C (parental) cpm/fraction

O—O ^{3}H (post-infection) cpm/fraction

FIG. 12. Sedimentation profiles of RNase and pronase-SDS treated lysates of 50 ml cultures infected for (a) 20, (b) 5 and (c) 1 min with ^{32}P am3 phage (MOI = 2.0) in medium containing [^{3}H]thymidine (20 μc/ml). The lysates were sedimented in preformed CsCl gradients and the Cerenkov radiation from ^{32}P disintegrations was counted for the whole fractions. Of the total 63 or 64 fractions in each gradient, only 1 through 50 are shown. The portions of each gradient indicated by the brackets were pooled, dialyzed, the DNA precipitated with 2 volumes of isopropanol and then dissolved in small volumes of Tris-EDTA.

●—● ^{32}P (parental) Cerenkov cpm/fraction

FIG. 13. Equilibrium buoyant density centrifugation of RF in CsCl with 200 μg/ml propidium iodide. The DNA was prepared as described under Fig. 12 and portions of the RF pools indicated by the brackets (RF) in Fig. 12 were used in this banding. The DNA represents the ϕX RF found (a) 20, (b) 5 and (c) 1 min after infection with ^{32}P am3 phage. ^{14}C RF marker was added before centrifugation.

●—● ^{32}P (parental) cpm/fraction

O—O ^{3}H (post-infection) cpm/fraction

----- ^{14}C (RF marker) cpm/fraction

FIG. 14. Sedimentation profiles of RF in neutral isokinetic sucrose gradients. RF found in cells infected with ^{32}P am3 phage for (a) 20, (b) 5, and (c) 1 min was prepared as described under Fig. 12. A portion of the material in the RF pools (indicated by brackets in Fig. 12) was applied to these neutral sucrose gradients. The RFII region of

each gradient (indicated by brackets) was dialyzed and the DNA concentrated by isopropanol precipitation. The first 50 of the total 65 fractions are shown. The remaining fractions do not contain any radioactivity.

●—● ^{32}P (parental) cpm/50 µl

O—O ^{3}H (post-infection) cpm/50 µl

FIG. 15. Alkaline isokinetic sucrose sedimentation patterns of RFII isolated from cells infected with ^{32}P am3 phage for (a) 20, (b) 5, and (c) 1 min. The RF were prepared as described under Figs. 12 and 14. The material indicated by the brackets in Fig. 14 was applied to these alkaline gradients. ^{14}C single-stranded viral DNA marker was added to each sample before centrifugation.

●—● ^{32}P (parental) cpm/fraction

O—O ^{3}H (post-infection) cpm/fraction

----- ^{14}C (single-strand marker) cpm/fraction

FIG. 16. Alkaline isokinetic sucrose sedimentation patterns of RFII isolated from cells infected for short times. These molecules were purified from pronase-SDS treated lysates as described in the Methods and text, with the following variations:

(a) Infection with ^{3}H am3 phage (MOI = 6) for 1 min in medium containing ^{32}P and 150 µg/ml chloramphenicol.

(b) Infection with ^{32}P am3 phage (MOI = 2) for 1 min in medium containing [^{3}H]thymidine and no chloramphenicol.

(c) Infection as in (b) for 30 sec.

(d) Infection with ^{3}H am3 phage (MOI = 6) followed, after 4 min, by ^{32}P am3 phage (MOI = 2) and an additional 1 min incubation.

●—● Parental cpm/fraction [^{3}H in (a) & (d); ^{32}P in (b) & (c)].

O—O Post-infection cpm/fraction [^{3}H in (b) & (c); ^{32}P in (a)].

Super-infection parental cpm/fraction [^{32}P in (d)].

----- ^{14}C (single-strand marker) cpm/fraction

FIG. 17. Alkaline isokinetic sucrose sedimentation of parental labeled single-stranded DNA isolated from lysates of cells infected with ^{32}P am3 phage for (a) 1 min, (b) 5 min, and (c) 20 min. The DNA represents a portion of the material contained in the SS pools indicated in Fig. 12. Panel (d) shows the sedimentation of DNA obtained from the ^{32}P am3 phage stock by phenol extraction. ^{14}C single-stranded DNA was added as a marker.

●—● ^{32}P (parental) cpm/fraction

----- ^{14}C (single-strand marker) cpm/fraction

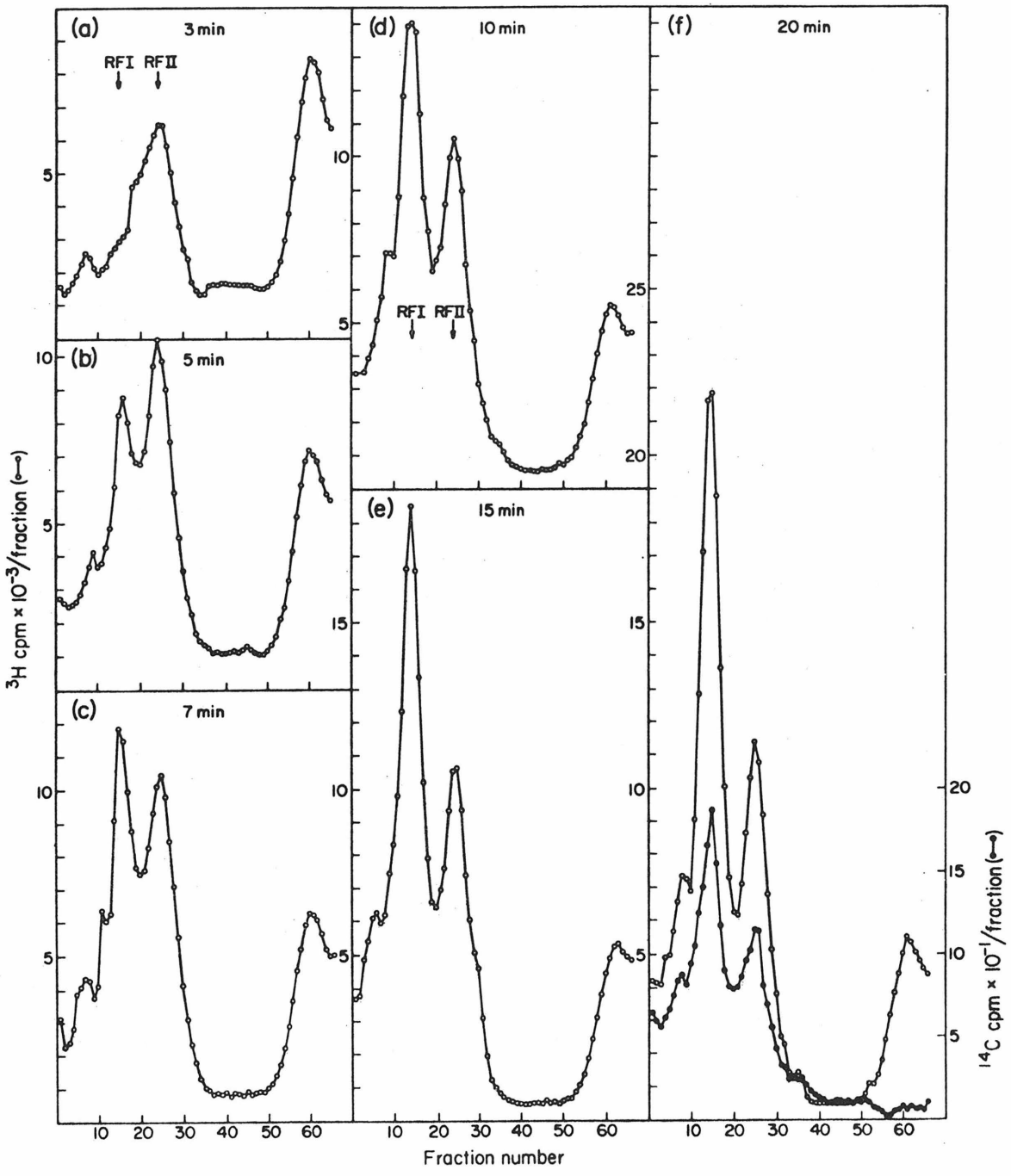
FIG. 18. The total post-infection label in all RF species in the gradients of Fig. 6 as a function of the UV exposure of the infecting phage particles. The values are plotted as a proportion of the unirradiated case. The two curves show the calculated amount of post-infection label expected at each dose when the new complementary strand is initiated at a single point on each viral template and when initiation occurs at two points, equally spaced, on the template. For simplicity, in making these calculations, all the lethal UV damage is assumed to cause termination of the growing polynucleotide. (Lesions which prevent

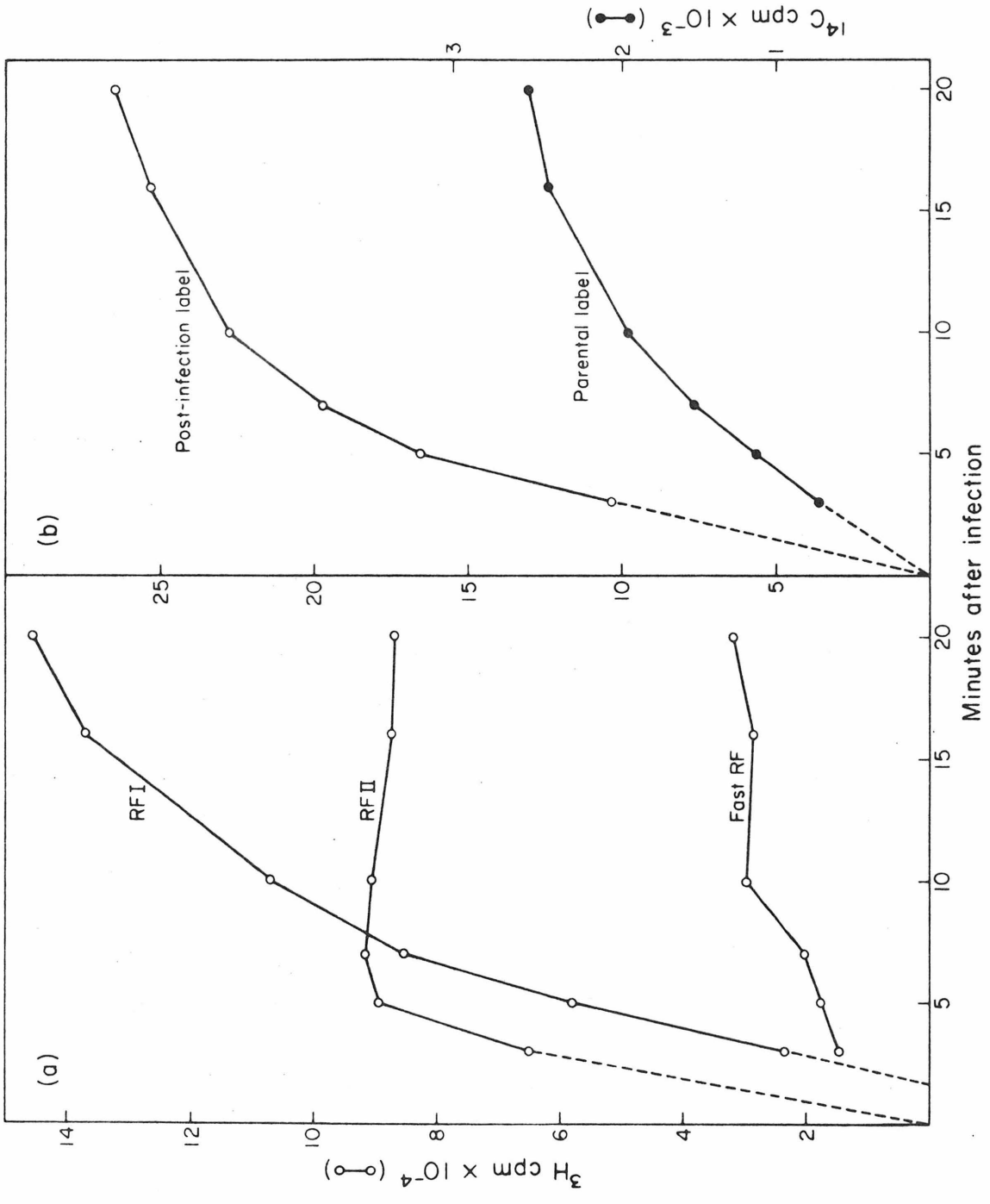
injection of the phage DNA and those which destroy gene function without affecting complementary strand synthesis, for example, have been neglected). UV lesions are assumed to be randomly distributed with respect to the initiation(s). If P_i is the fraction of viruses which have received $\underline{i}$ UV lesions according to the Poisson distribution, then the relative amount of complementary strand synthesized from a single initiation is $P_o + 1/2P_1 + 1/3P_2 + 1/4P_3 + 1/5P_4 \ldots$, and the amount synthesized from two equidistant initiations is $P_o + 3/4P_1 + 1/2P_2 + 1/3P_3 + 1/4P_4 + 1/5P_5 \ldots$

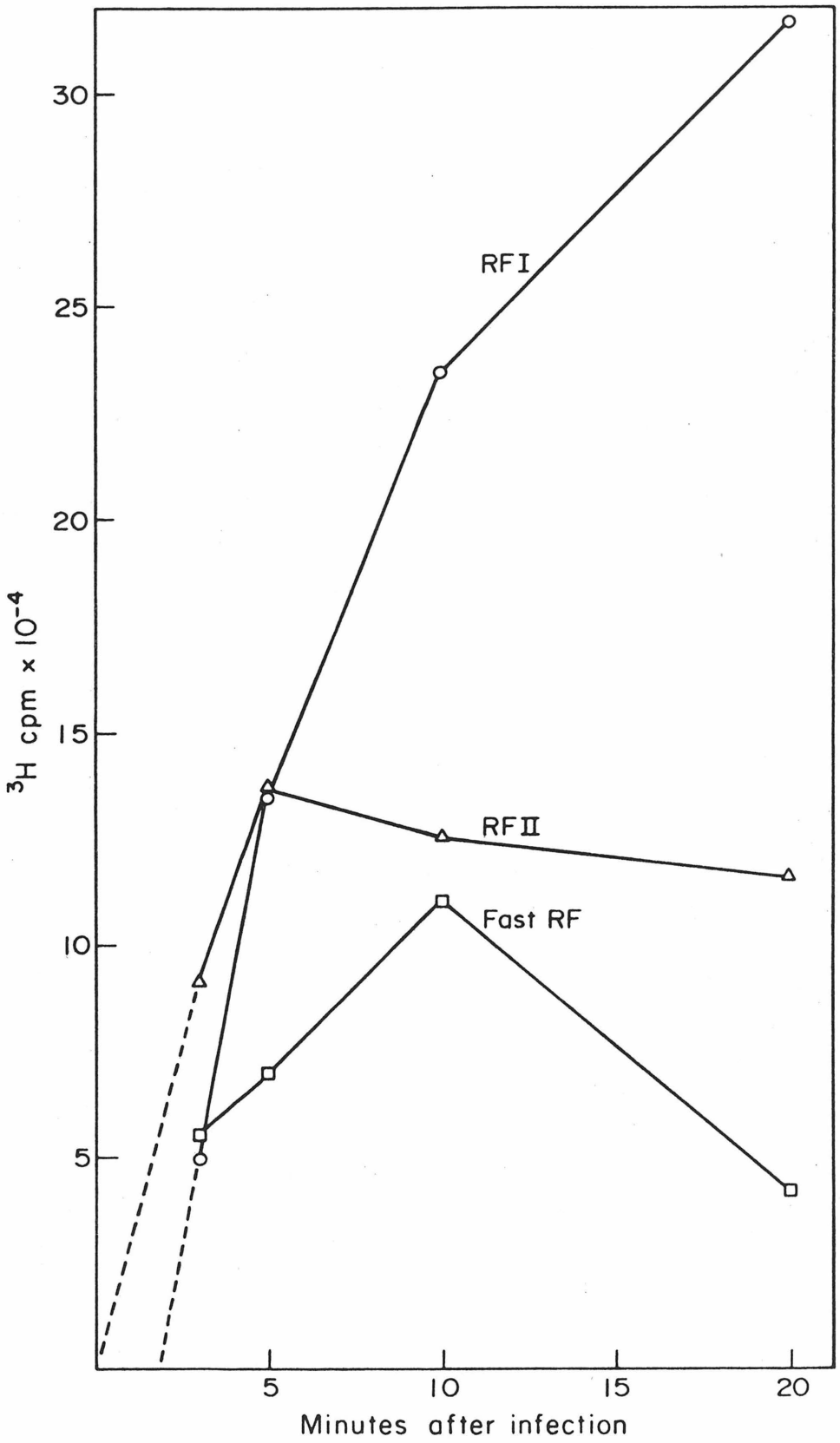
O O ^{3}H (post-infection) cpm

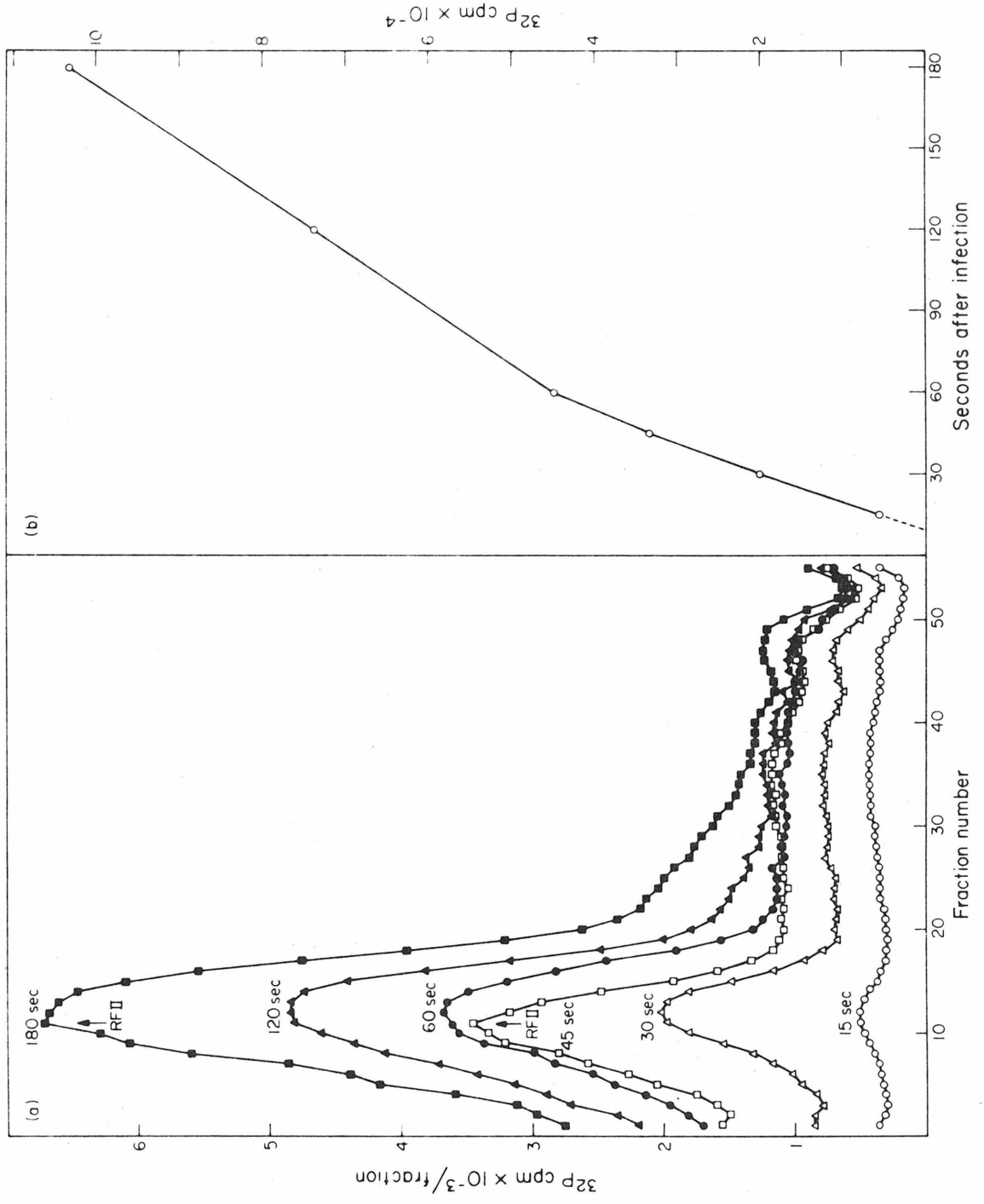
----- expected from single initiation

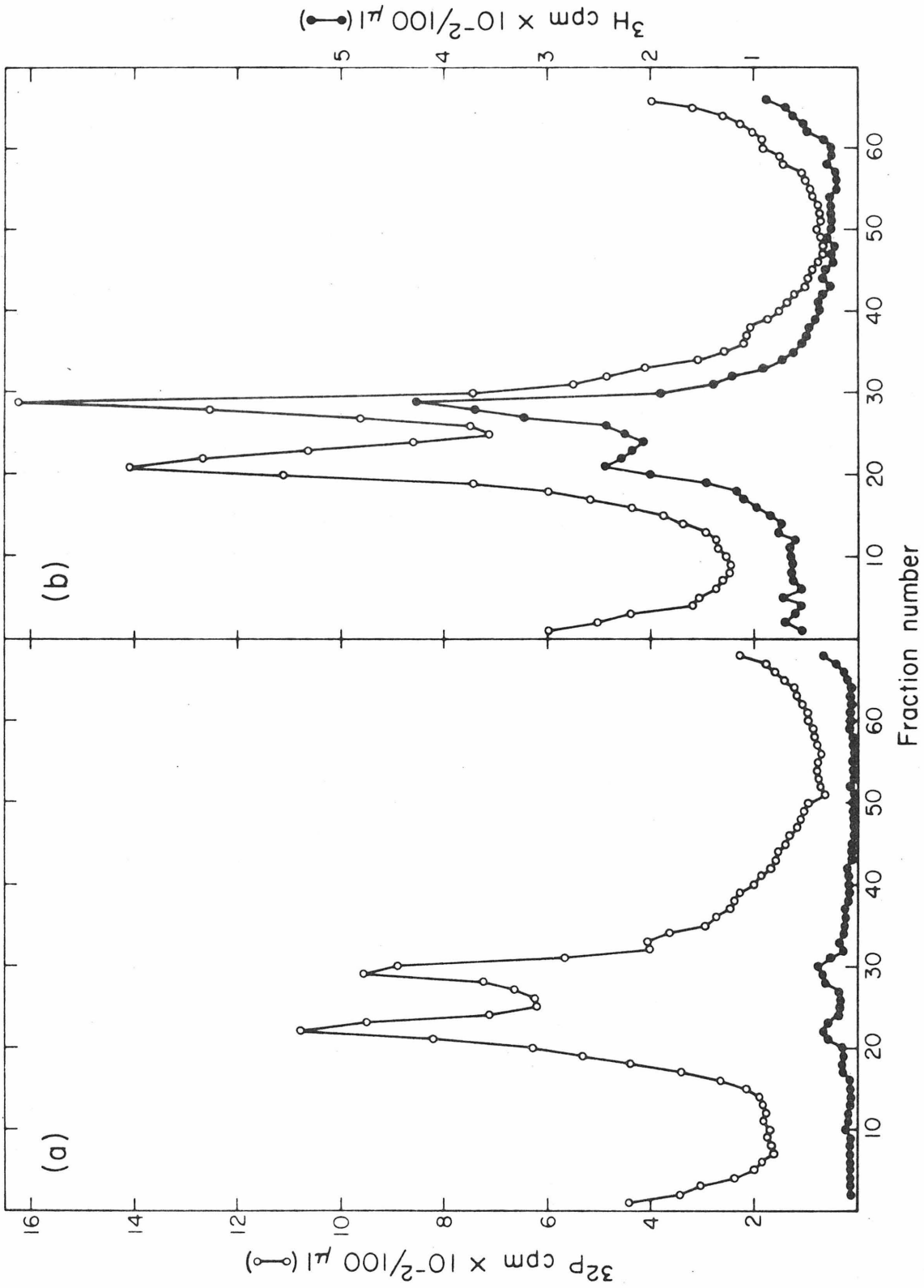
••••• expected from double initiations

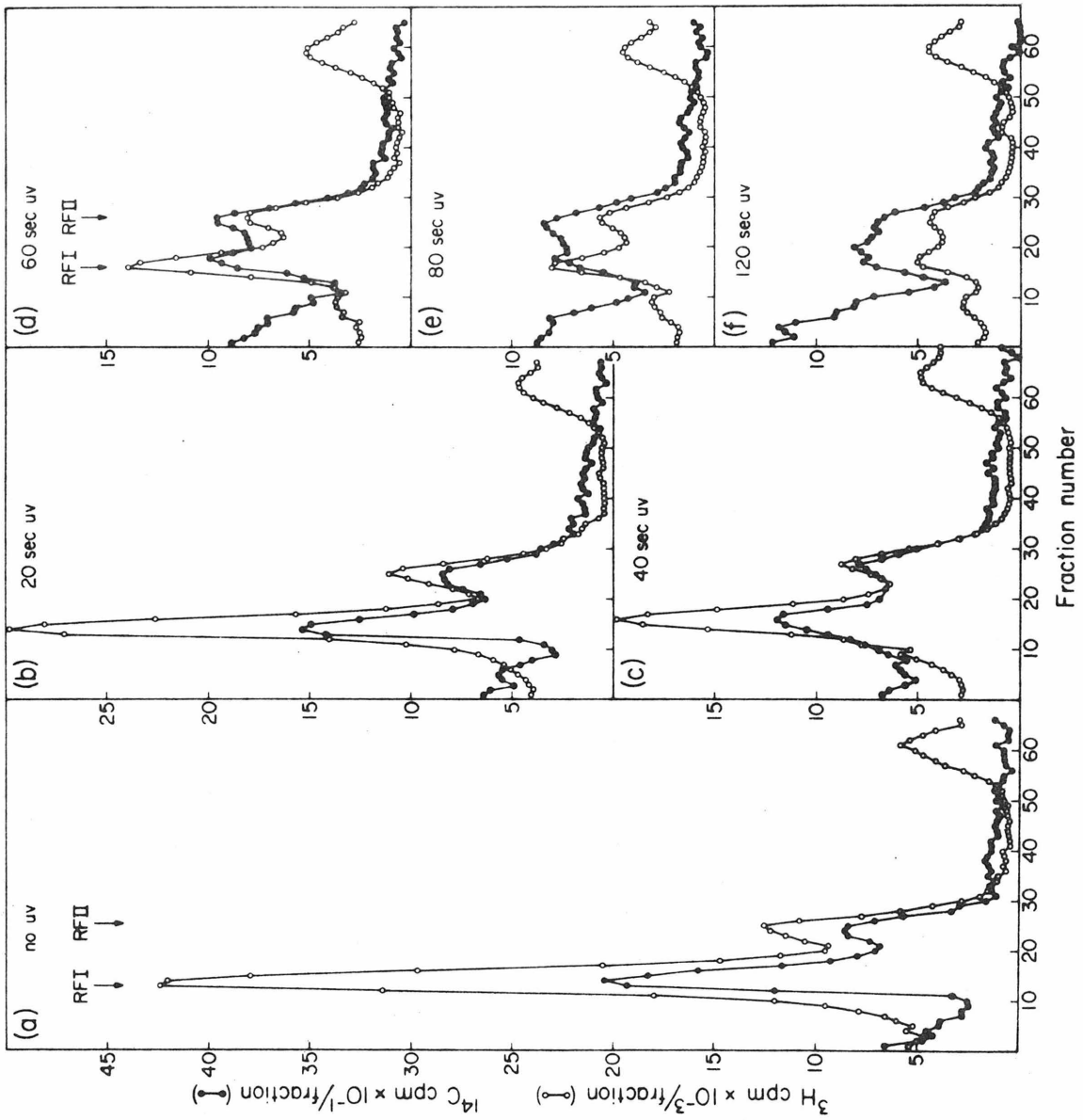

(a) 3 min
RFI RFII
(b) 5 min
(c) 7 min
(d) 10 min
RFI RFII
(e) 15 min
(f) 20 min
^{3}H cpm × 10^{-3}/fraction (•—)
^{14}C cpm × 10^{-1}/fraction (•—•)
Fraction number

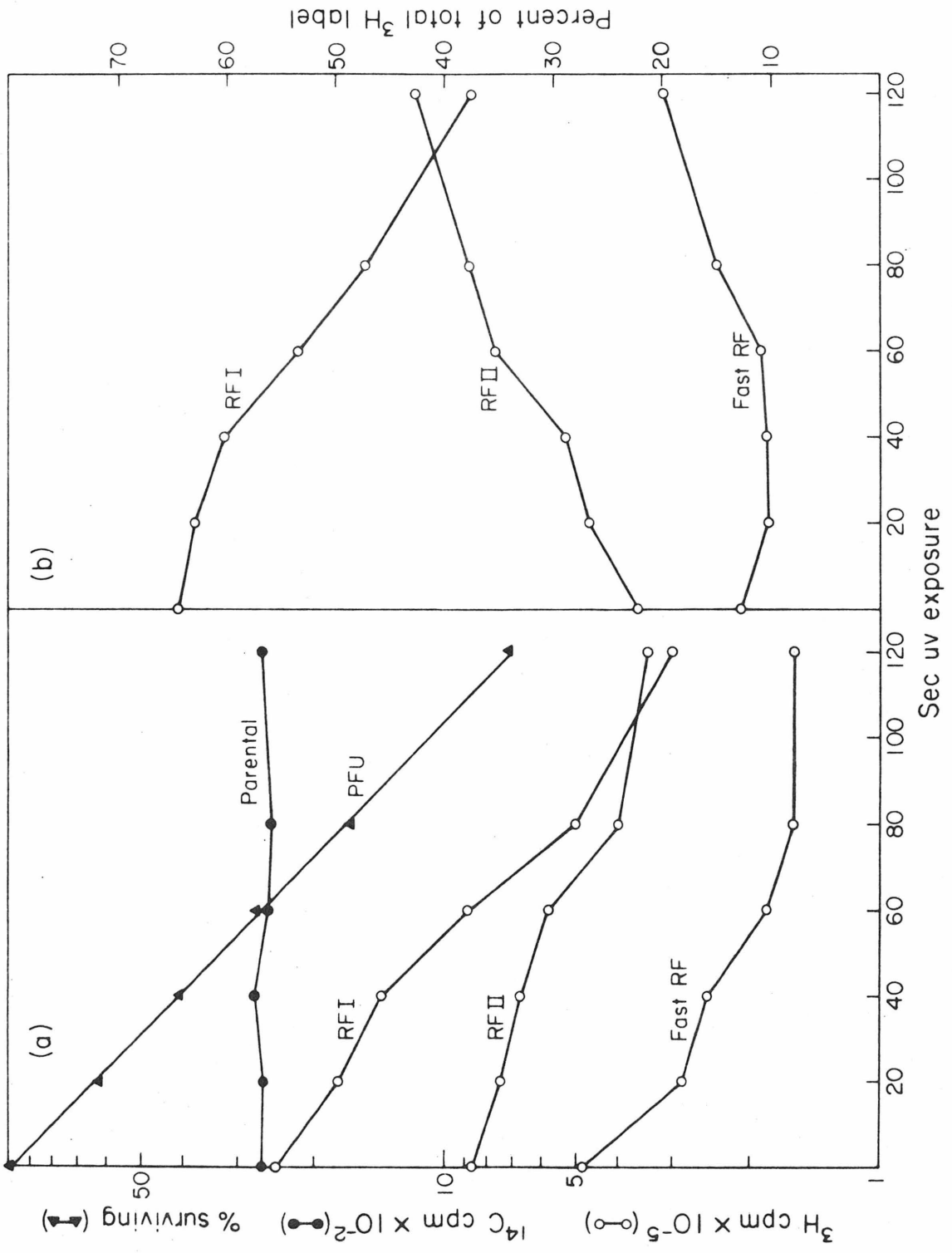

(a)
(b)
RF I
RF II
Fast RF
Post-infection label
Parental label
^{3}H cpm x 10^{-4} (●—●)
^{14}C cpm x 10^{-3} (●—●)
Minutes after infection


30
25
20
15
10
5
^{3}H cpm x 10^{-4}
RFI
RFII
Fast RF
5
10
15
20
Minutes after infection

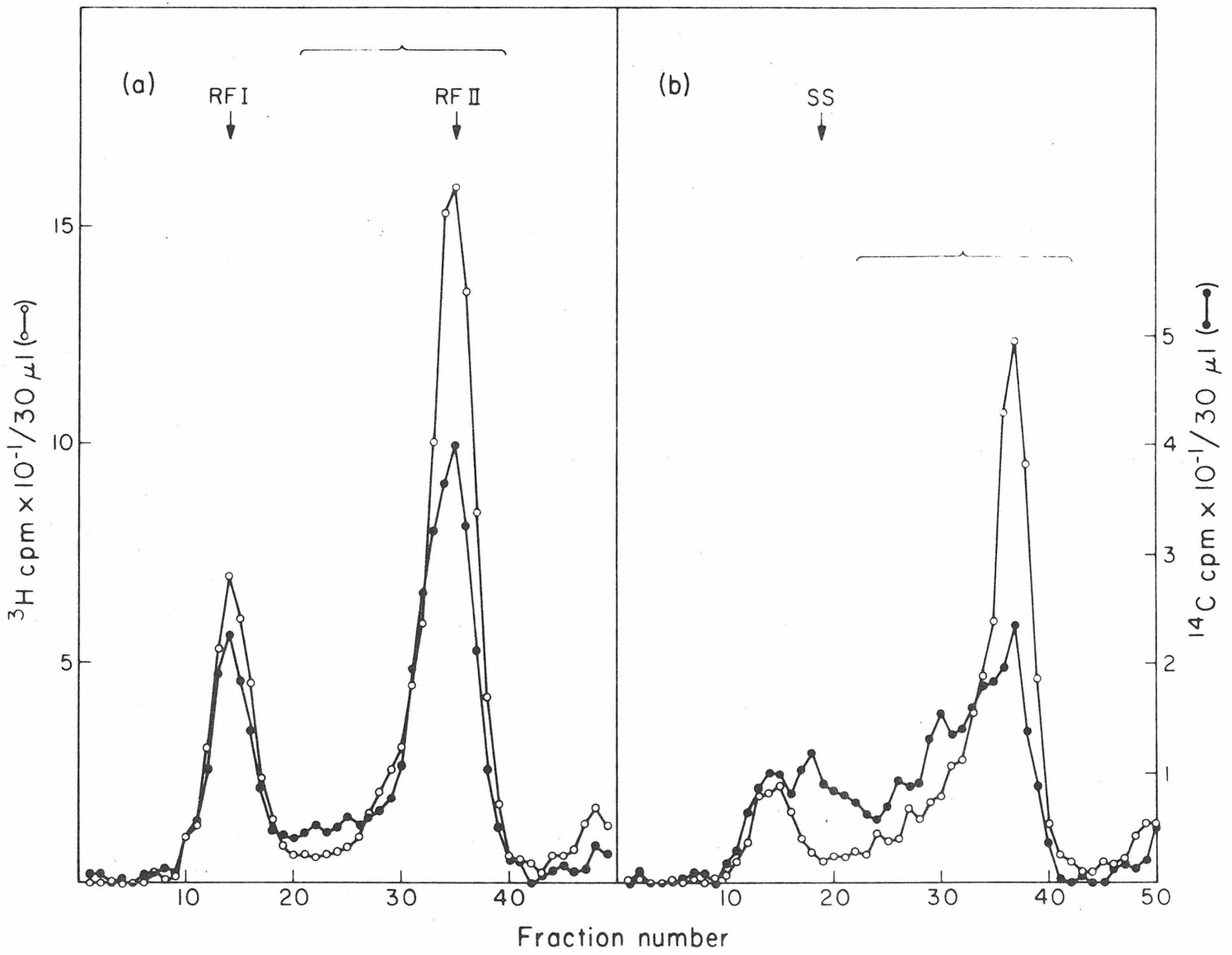

(a)
180 sec
RF II
120 sec
60 sec
RF II
45 sec
30 sec
15 sec
32P cpm x 10-3/fraction
Fraction number
(b)
32P cpm x 10-4
Seconds after infection

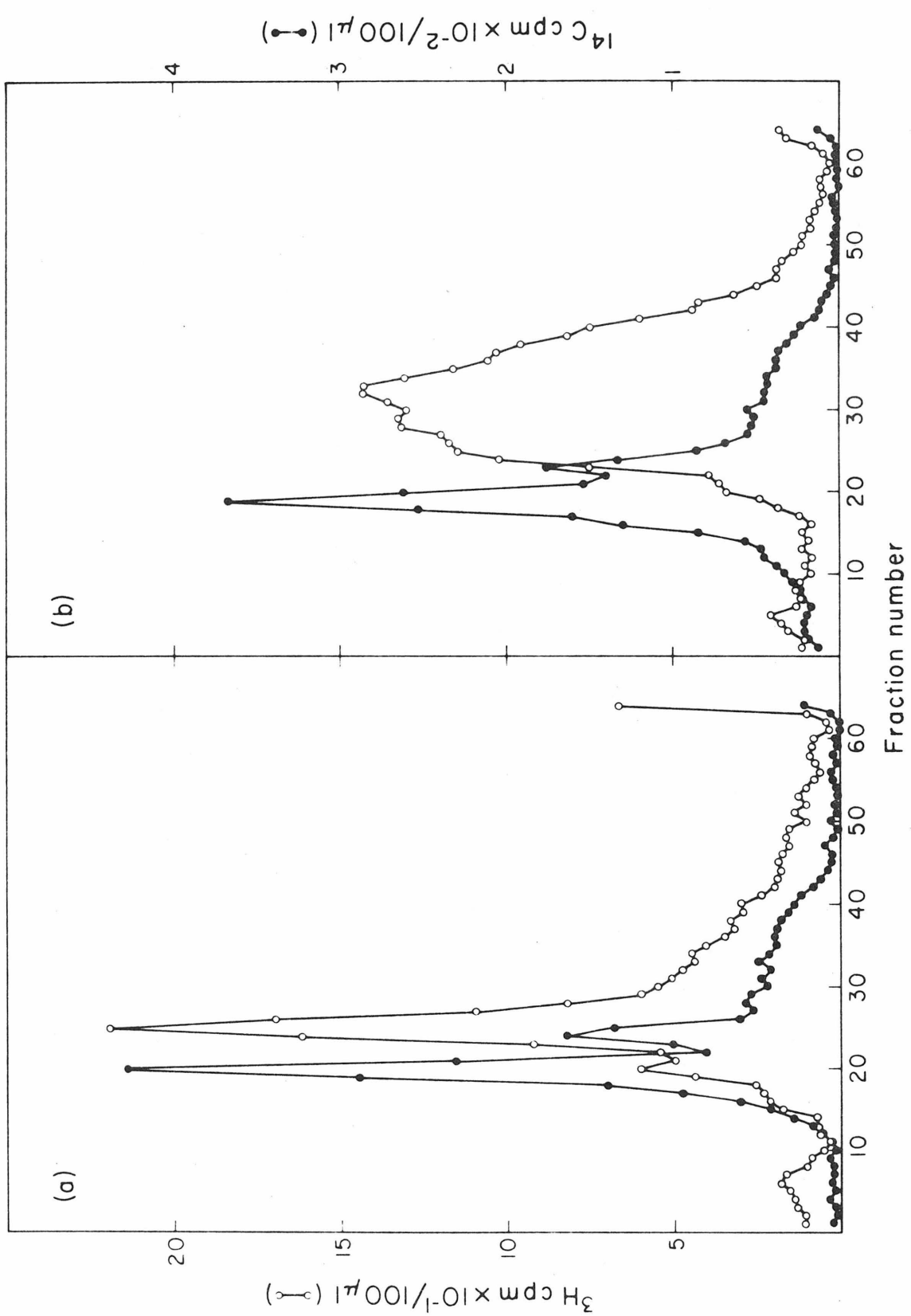

(a)
(b)
^{32}P cpm x 10^{-2}/100 μl (o—o)
^{3}H cpm x 10^{-2}/100 μl (•—•)
Fraction number

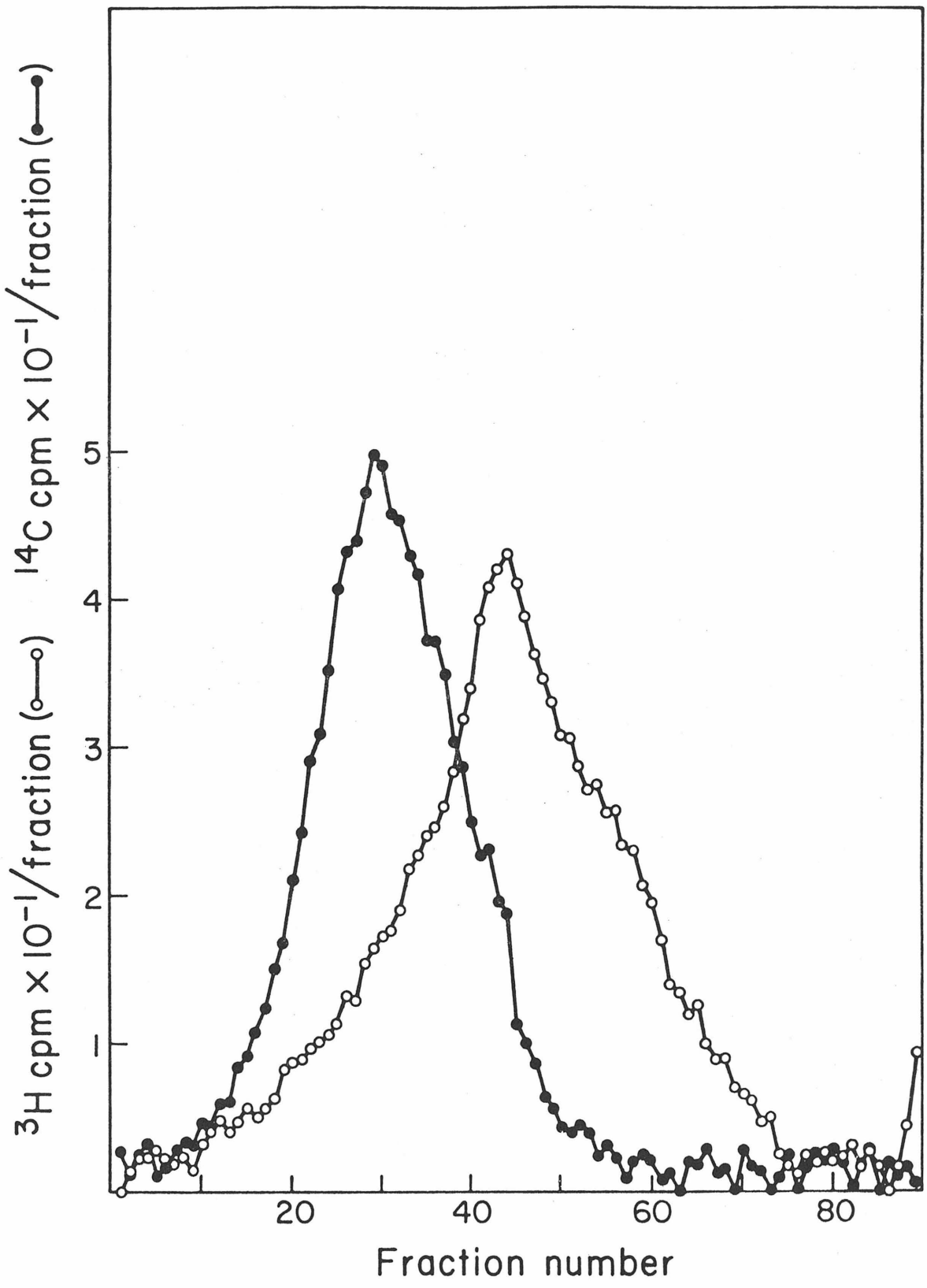

(a) no uv
RFI
RFII
(b) 20 sec uv
(c) 40 sec uv
(d) 60 sec uv
RFI RFII
(e) 80 sec uv
(f) 120 sec uv
^{3}H cpm × 10^{-3}/fraction
^{14}C cpm × 10^{-1}/fraction
Fraction number

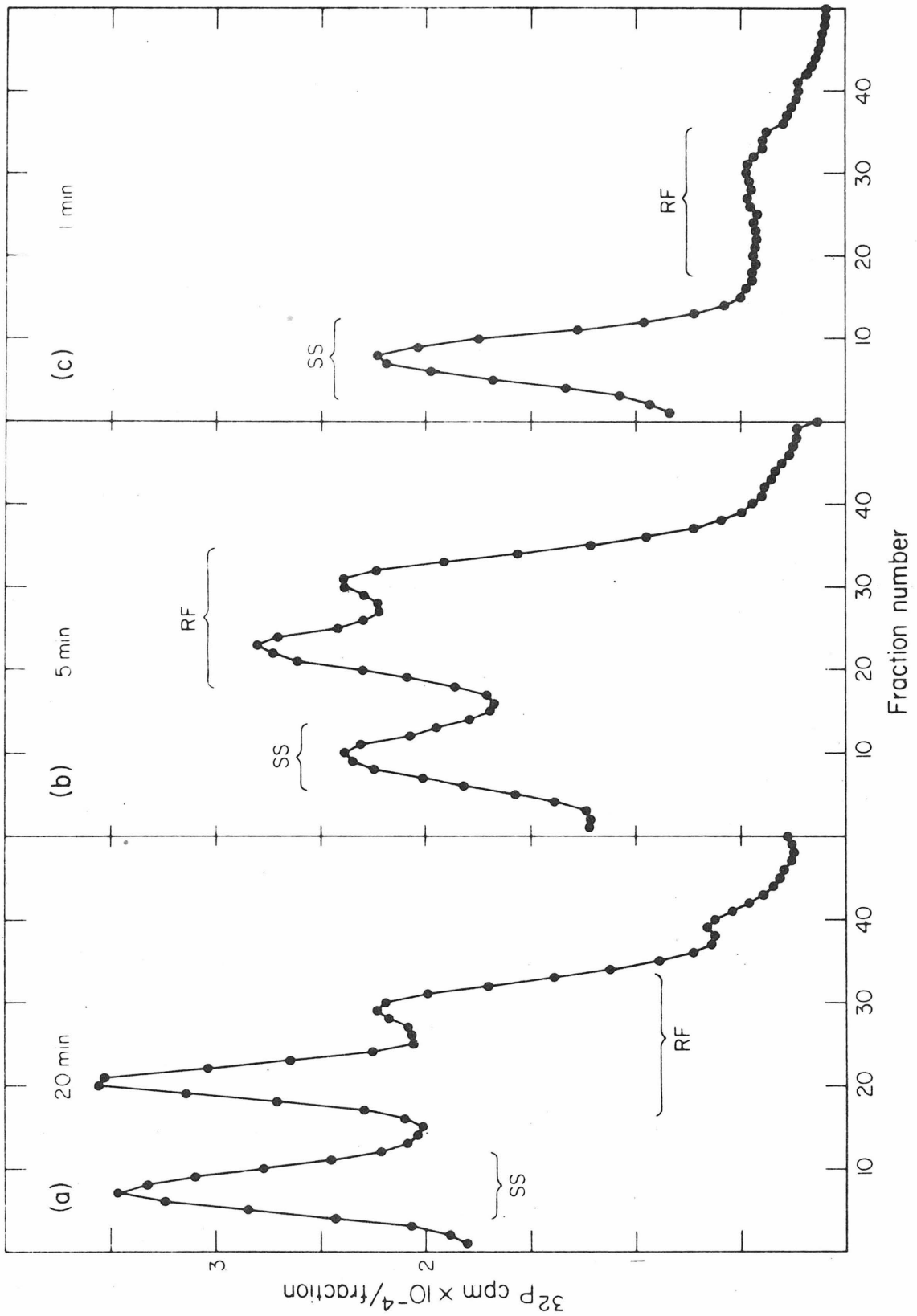

(a)
(b)
Parental
PFU
RF I
RF II
Fast RF
RF I
RF II
Fast RF
% surviving
14C cpm x 10-2
3H cpm x 10-5
Percent of total 3H label
Sec uv exposure

(a)
(b)
I
II
^{3}H cpm x 10^{-2}/100 µl (o—o)
^{14}C cpm x 10^{-2}/100 µl (●—●)
Fraction number

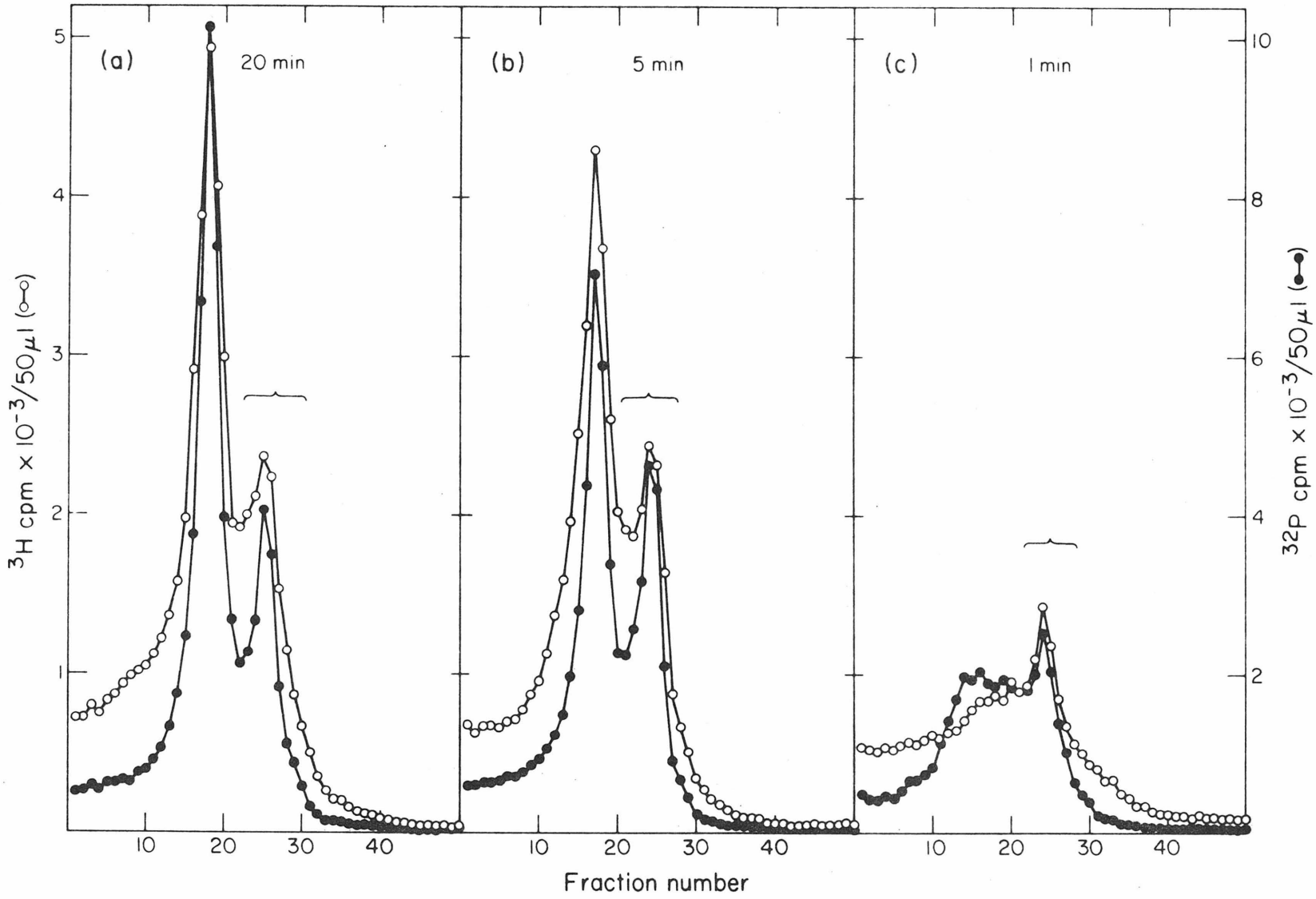

(a)
RF I
RF II
(b)
SS
15
10
5
5
4
3
2
1
10
20
30
40
50
Fraction number
^{3}H cpm x 10^{-1}/30 µl (o—o)
^{14}C cpm x 10^{-1}/30 µl (●—●)

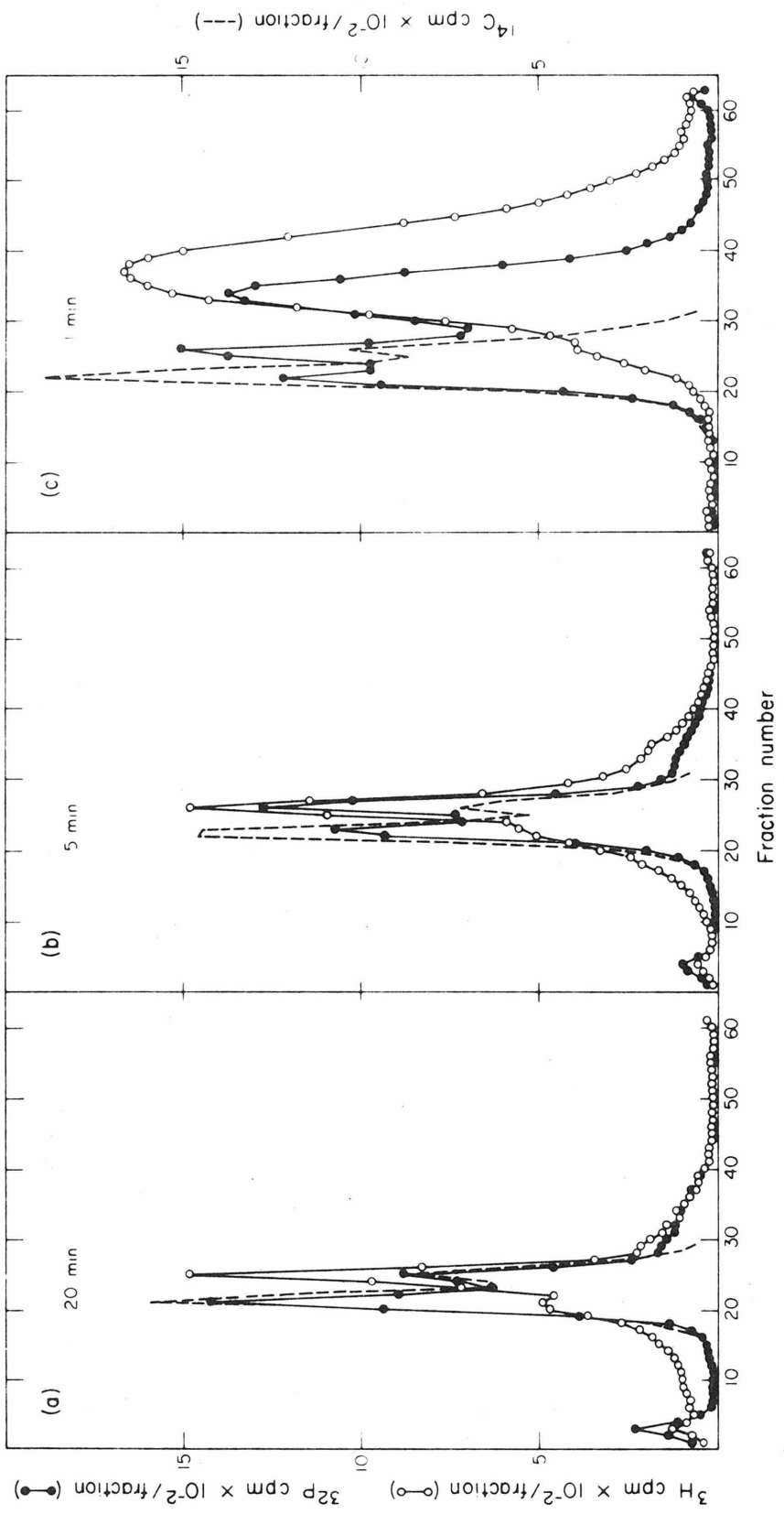

(a)
(b)
^{3}H cpm $\times 10^{-1}/100\mu l$ (○—○)
^{14}C cpm $\times 10^{-2}/100\mu l$ (●—●)
Fraction number

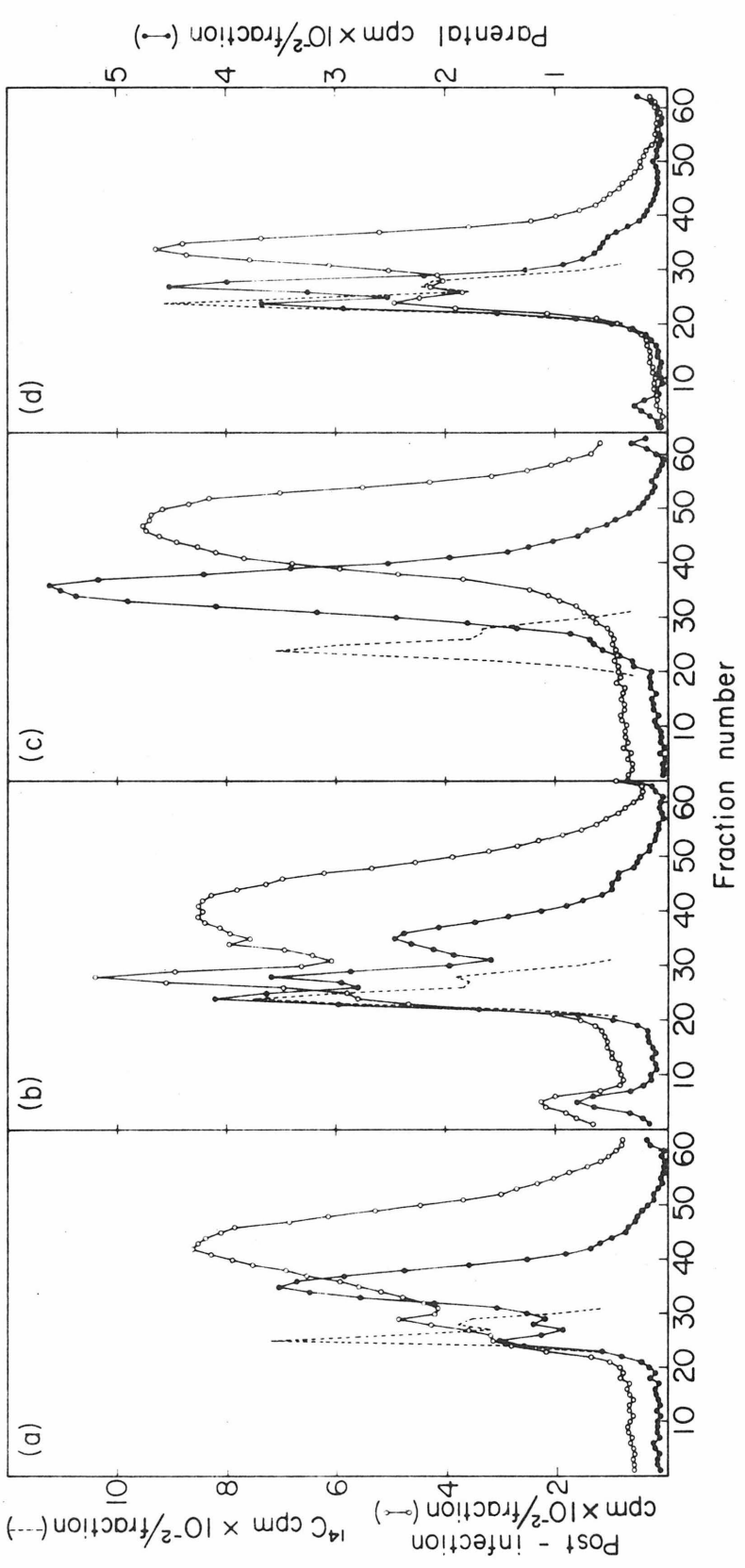

^{3}H cpm × 10^{-1}/fraction (o—o) ^{14}C cpm × 10^{-1}/fraction (•—•)
5
4
3
2
1
20
40
60
80
Fraction number

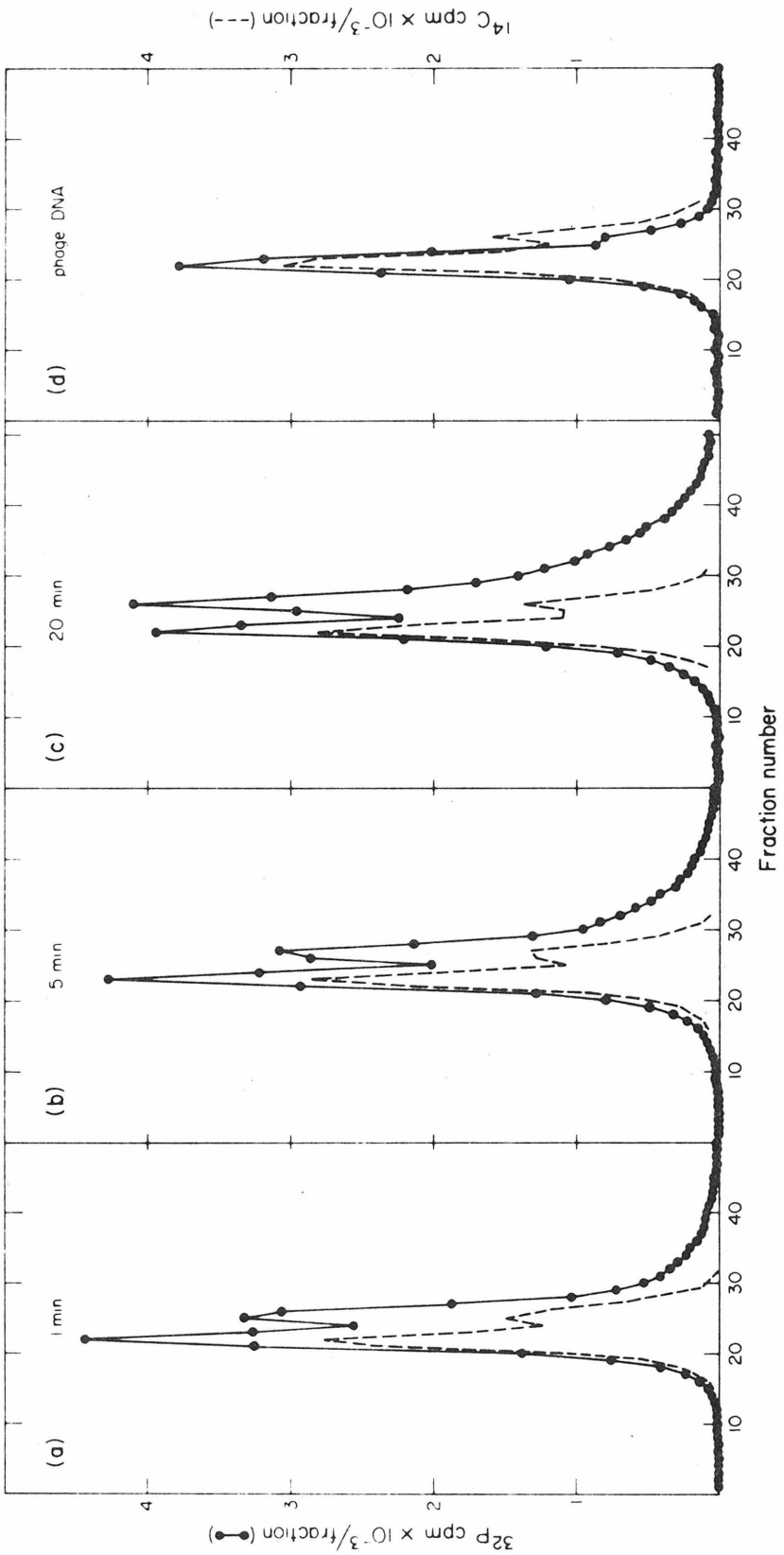

(a)
20 min
(b)
5 min
(c)
1 min
SS
RF
SS
RF
SS
RF
^{32}P cpm x 10^{-4}/fraction
Fraction number

^{3}H cpm × 10^{-2}/fraction (○—○)
^{32}P cpm × 10^{-2}/fraction (●—●)
^{14}C cpm × 10^{-2}/fraction (---)
(a) 20 min
(b) 5 min
(c) 1 min
Fraction number


(a) 20 min
(b) 5 min
(c) 1 min
^{3}H cpm $\times 10^{-3}/50\mu l$ (o—o)
^{32}P cpm $\times 10^{-3}/50\mu l$ (•—•)
Fraction number
1
2
3
4
5
6
8
10
20
30
40


(a)
20 min
(b)
5 min
(c)
1 min
^{3}H cpm × 10^{-2}/fraction (o—o)
^{32}P cpm × 10^{-2}/fraction (●—●)
^{14}C cpm × 10^{-2}/fraction (---)
Fraction number


(a)
(b)
(c)
(d)
Post - infection cpm × 10⁻²/fraction (⟡)
¹⁴C cpm × 10⁻²/fraction (---)
Parental cpm × 10⁻²/fraction (⟡)
Fraction number


(a)
1 min
(b)
5 min
(c)
20 min
(d)
phage DNA
^{32}P cpm × 10^{-3}/fraction (●—●)
^{14}C cpm × 10^{-3}/fraction (---)
Fraction number

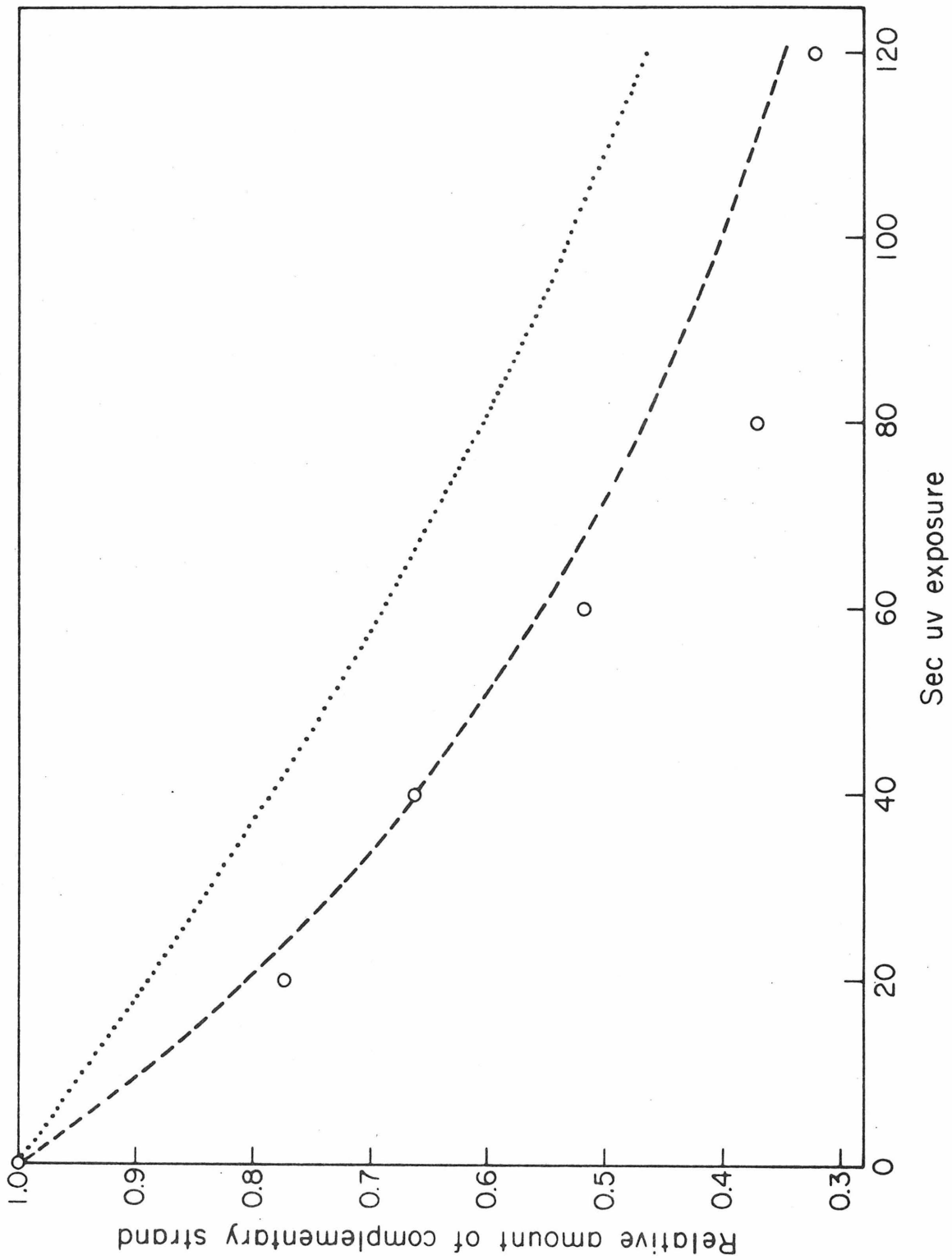
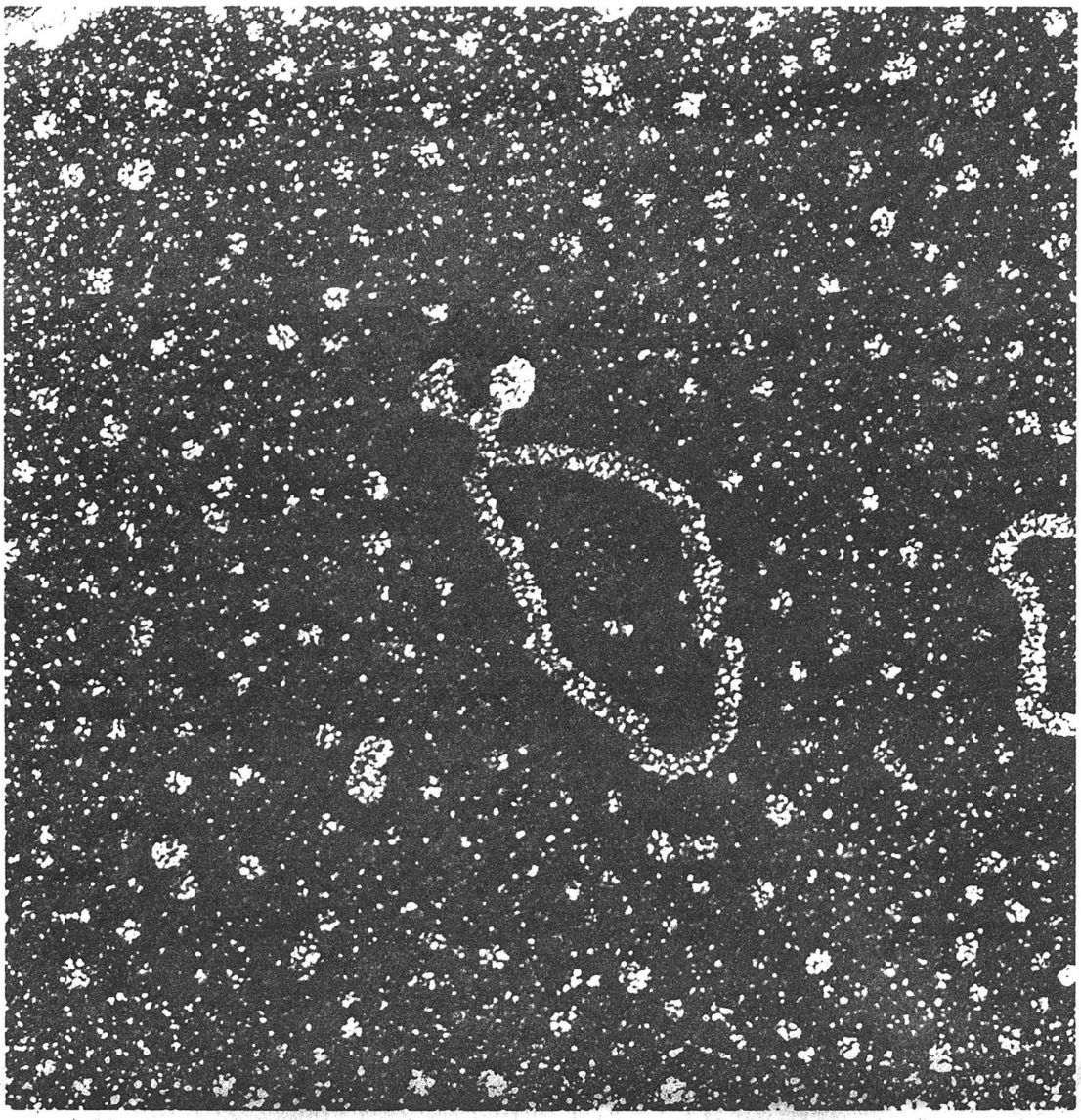
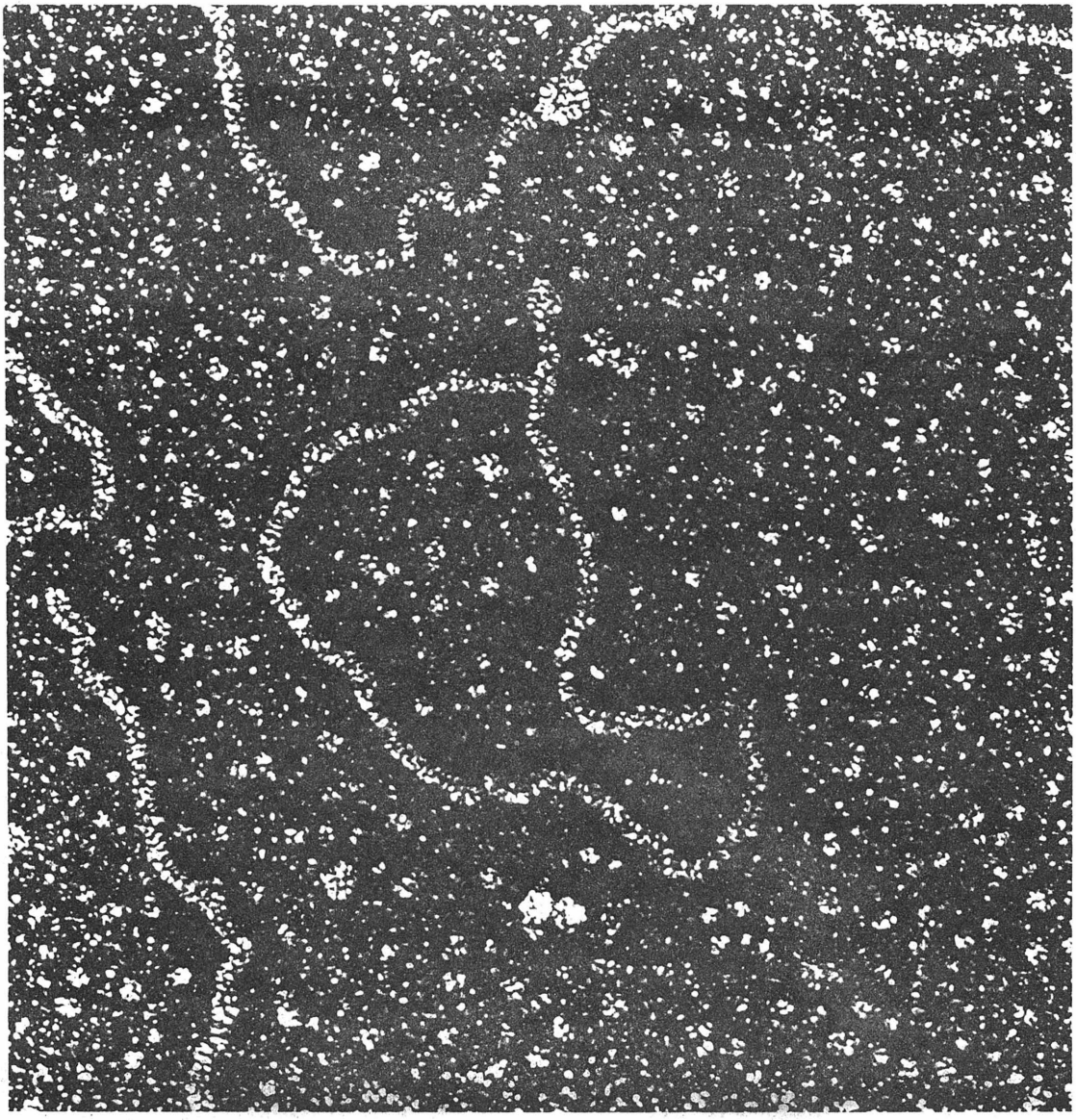
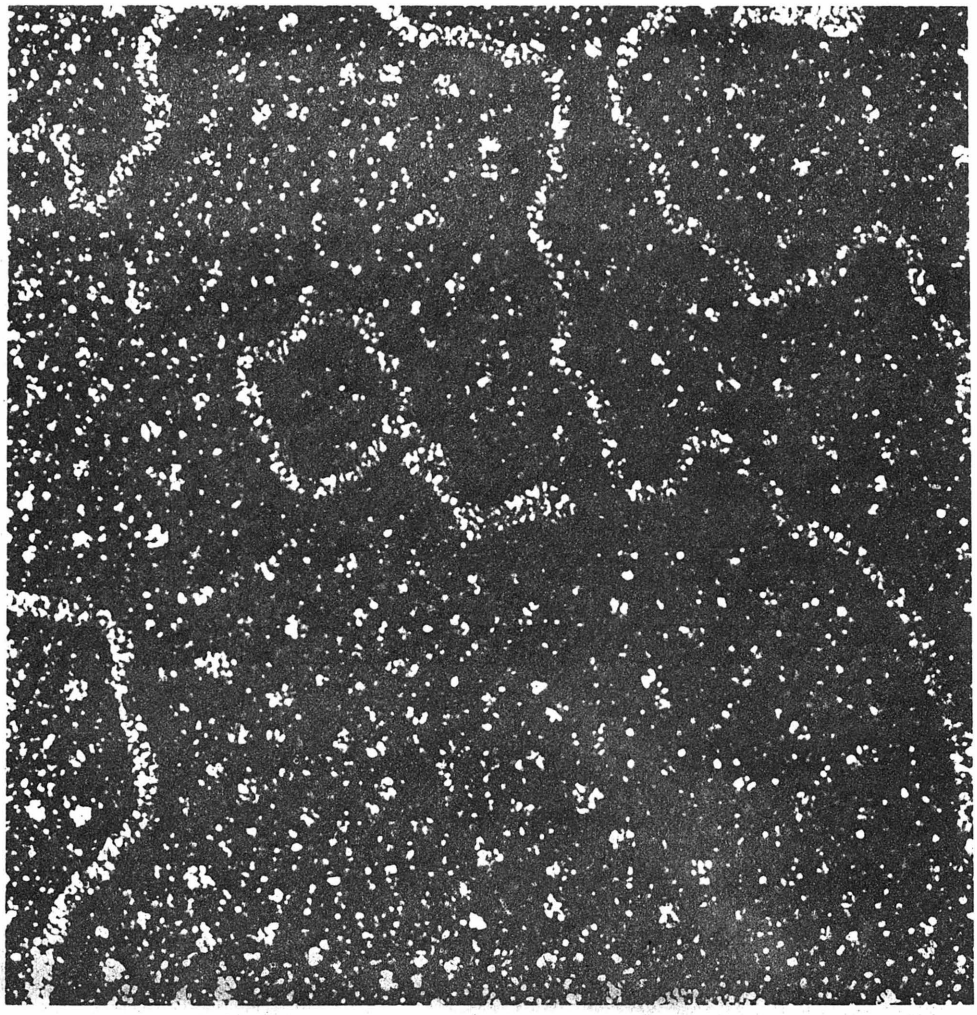

Relative amount of complementary strand
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
20
40
60
80
100
120
Sec uv exposure

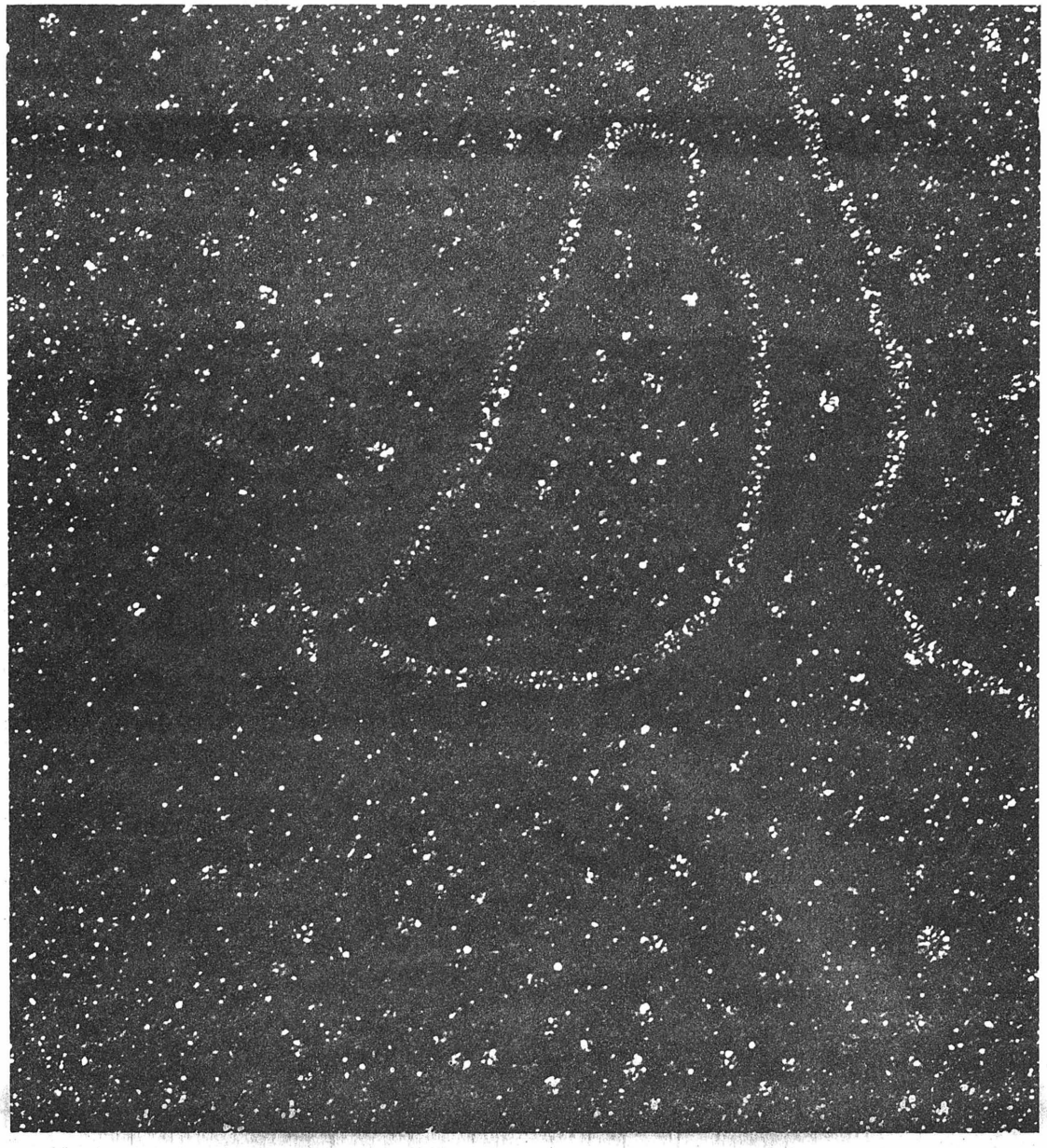
Plate I

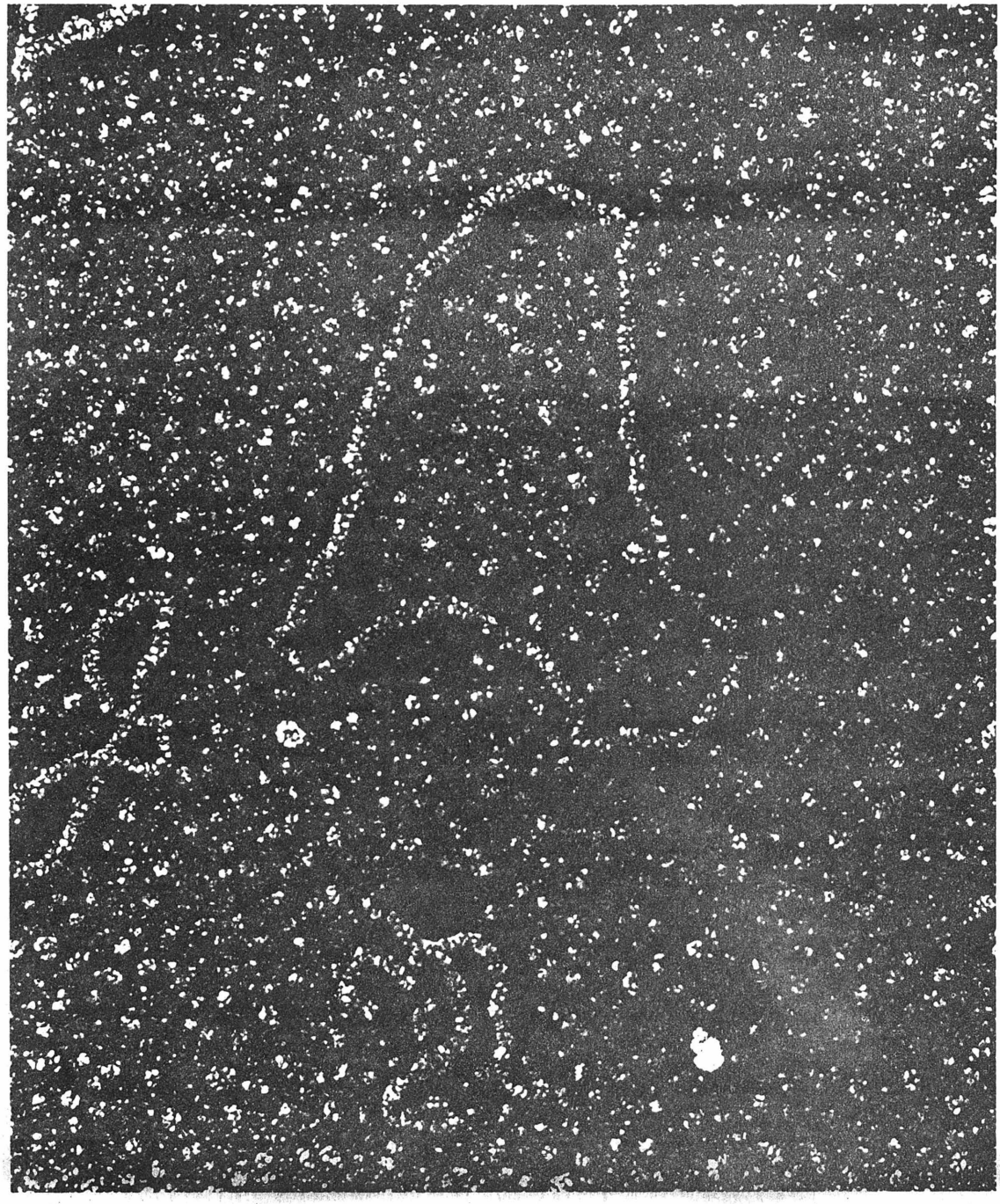
RF molecules isolated from cells infected with UV irradiated viruses. ^{14}C am3 phage were irradiated for 90 sec (2.0 hits/phage), were used to infect a culture (MOI = 7.0) containing 150 μg/ml of chloramphenicol for 20 min. The cells were lysed, treated with pronase-SDS and then sedimented in a preformed CsCl gradient. DNA from various positions in the gradient was prepared for viewing in the electron microscope as described in the Methods. The structures in (a), (b), (c), (d) and (e) were observed in the 18 to 22S region of the gradient. Photograph (f) is a RF made from unirradiated phage. Final magnification is 175,000X.











Part III: Origin of Synthesis of the First Complementary Strand

III. 1 Introduction

At present, all the evidence for specific initiations in any of the three stages of ϕX DNA synthesis is indirect. Baas & Jansz (1972) observed that the efficiency of repair of mismatched bases in artificially constructed heteroduplex RF molecules had a linear relationship to the position of the inhomology on the genetic map. They inferred that the cell could repair mismatched bases until the two strands in the duplex were separated by the process of RF replication. If the correction was not made before the replication fork reached the mutant site, it was likely that progeny phage with the genotypes of both the parent strands would be produced by the cell. Repair of the mismatch would result in the release of phage with only one of the two genotypes. This interpretation of their results allowed them to conclude that ϕX RF replication begins in or near cistron A and is unidirectional and clockwise with respect to the genetic map of ϕX174 (Benbow et al., 1971).

Johnson & Sinsheimer (1974) identified specific discontinuities in RFII isolated during the period of ϕX single-strand synthesis, late in an infection. Using a restriction enzyme isolated from Haemophilus influenzae, they determined that many of the RFII molecules had a nick or gap in the viral strand at a specific location. They characterized the site of the discontinuity as being in or near cistron A. Since the discontinuous viral strand in late RF has been shown to be the precursor of progeny single-stranded DNA (Knippers et al., 1969), they interpreted the specific break at cistron A as the initiation site for the synthesis

of progeny phage DNA.

A specific initiation for the first complementary strand has not been previously identified. Benbow et al. (1974b) predicted that the synthesis had a specific start on the basis of their examination of incomplete parental RF molecules made in cells infected with UV damaged phage particles. In the electron microscope, these molecules appeared to have only one region of duplex DNA and one region of single-stranded template over a range of phage UV doses. They interpreted the observation as indicating that the complementary strand begins at only one site on each template.

A study of M13 RF made from viral single strands in a cell-free extract of E. coli provides indirect evidence that synthesis of the first complementary strand on this template has a specific initiation in vitro (Tabak et al., 1974). RFII molecules isolated from the soluble enzyme mixture were analyzed by restriction enzymes from Haemophilus parainfluenzae and H. influenzae. A specific gap was detected in the new strand, but the genetic location of the discontinuity was not reported. Using the same cell extracts, ϕX174 viral DNA was also converted to RFII. No specific discontinuities were discovered in its new strand.

The experiments described in this section were performed to characterize the initiation site(s) of the first complementary strand of ϕX174 in vivo. The results support the hypothesis that initiation occurs at a small number of specific locations on the viral DNA template.

III. 2. Materials and Methods

The bacterial and bacteriophage strains and all of the materials used in this section have been described in Parts I and II.

(a) Preparation of parental RF pulse-labeled with [^{3}H]thymidine

E. coli HF4704 was grown to 5 x 10^8 cells/ml at 37°C in TPGAA (or TPGAA-low phosphate) medium containing 10 µg/ml thymine. Chloramphenicol was added to a final concentration of 150 µg/ml and, after 20 min of incubation, am3 phage were inoculated at a multiplicity of 200. In some experiments $H_3{}^{32}PO_4$ (5 µc/ml, final concentration) was added 5 min before the phage. At various times after infection, portions of the culture were poured into 10 volumes of fresh medium at 37°C containing 20 µc/ml of [^{3}H]thymidine and sufficient EDTA to chelate all the divalent cations in the medium (10 mM). After 30 sec of incubation, the infection was terminated by pouring the diluted culture into 5 volumes of borate-EDTA, 0.05 M NaN_3, 5 mM KCN at 0°C and swirling in a bath of methanol and solid CO_2.

Parental RF were obtained from the cells as described in the Methods of Part II. The RF were purified by sedimentation through a preformed CsCl gradient and a neutral, isokinetic, sucrose gradient.

(b) Digestion with Haemophilus influenzae restriction enzyme

The restriction enzyme from H. influenzae, endonuclease R (endo R), was prepared by Dr. P. H. Johnson and Lloyd H. Smith according to the method of Smith & Wilcox (1970). Digestions were performed in

a reaction mixture which contained up to 0.5 µg of ϕX RF DNA, 7 mM Tris (pH 7.4), 7 mM 2-mercaptoethanol, 7 mM $MgCl_2$, 50 mM NaCl and 0.01 unit of endo R in a total volume of 100 µl. After 3 to 4 hours at 37°C, the reaction was stopped by adding 5 µl of 1 M EDTA. The terminated reaction mixture was occasionally stored for several days at -20° without further treatment. Before electrophoresis, 5 µl of 20% (w/v) SDS, 10 µl of glycerol and 10 µg of bromphenol blue were added.

(c) Acrylamide gel electrophoresis

5% acrylamide gels were prepared according to the directions of Loening (1967) with the addition of SDS, by mixing 18 ml of a solution containing 15% (w/v) acrylamide, 0.75% (w/v) N,N'-methylene-bis-acrylamide with 10.8 ml of 5XA gel buffer (0.2 M Tris-OH, 0.1 M sodium acetate, 10 mM EDTA, 1% [w/v] SDS, adjusted to pH 7.8 with acetic acid), 25.2 ml deionized, distilled water and 0.44 ml Temed. The solutions were mixed and then 0.89 ml of freshly prepared 1% (w/v) ammonium persulfate was added. The final mixture was swirled in a beaker and 14.5 ml was immediately pipetted into 1 x 22 cm Plexiglas tubes, sealed at the bottom with a stopper. A flat-bottom well was formed at the top of the gel by inserting a Plexiglas cylinder (7 mm diameter) about 1 cm into the mixture and allowing it to remain in position during polymerization. After 45 min, the template for the well was removed and the stopper at the bottom of the gel was replaced with a piece of punctured dialysis tubing secured with a rubber band. Up to 8, 1 x 20 cm gels were placed in an electrophoresis apparatus with 200 ml of 1XA gel buffer (40 mM Tris-OH, 20 mM sodium acetate, 2 mM EDTA, 0.2% [w/v]

SDS, adjusted to pH 7.8 with acetic acid) in each buffer compartment. A 60V potential was applied to the gels for about 2 hours, at room temperature, to remove the persulfate.

After changing the buffer in both compartments, samples of digested RF (up to 125 µl), containing 0.05 M EDTA, 1% (w/v) SDS, 10% (v/v) glycerol and 10 µg bromphenol blue, were layered onto the bottom of the well. Electrophoresis was performed at 60V for 18 to 20 hours (until the marker dye began to leave the bottom of the gel).

The gels were removed from the tubes with gentle air pressure and were immediately frozen on a block of solid CO_2. A Mickle Gel-Slicer (Brinkman Instruments) was used to cut the gels, frozen to the platform with solid CO_2. Each 1 mm slice was placed in a scintillation vial and incubated at least 8 hours, in the dark, with 5 ml of NCS scintillation fluid which was 85.3% toluene, 10% NCS tissue solubilizer, 4.2% Liquifluor and 0.5% distilled water by volume. Radioactivity was counted in a Beckman LS233 scintillation counter equipped with a paper tape-punch mechanism. The data were processed as described in the Methods of Part II.

III. 3 Results

The data from Part II indicate that RFII molecules appear in cells less than 10 seconds after phage are added to the culture. The period of polynucleotide synthesis itself may be as short as 4 seconds if the new strand is formed at the rate of E. coli DNA elongation in vivo (Bonhoeffer & Gierer, 1963; Cairns, 1963; Cooper & Helmstetter,

1968). Some time may be expended in adsorption, eclipse and injection.

The data of Sugino and Okazaki (1972) and Denhardt (1969) indicate that [^{3}H]thymidine enters cells without any detectable lag. Indeed, the fact that pulses of [^{3}H]thymidine as short as 3 seconds have been successfully used to study E. coli DNA synthesis at low temperatuers (Okazaki & Okazaki, 1969; Sugino & Okazaki, 1972) indicates that [^{3}H] thymidine can enter cells and be incorporated into DNA in less time than it takes to make a ϕX complementary strand at 37°C.

If, during an unsynchronized infection, means can be found to prevent the adsorption and injection of ϕX particles after the introduction of [^{3}H]thymidine into the culture, it would be possible to selectively label the portions of the complementary strand which are synthesized last. The simplest techniques for retarding ϕX adsorption are dilution and removal of the divalent cations which facilitate phage attachment (Fujimura & Kaesberg, 1962). Since complementary strand synthesis is very rapid, while adsorption and eclipse are relatively slow (Newbold & Sinsheimer, 1970), a short labeling period would also selectively minimize the appearance of new templates in the cells.

A mixture of cells and am3 phage is active in parental RF formation during the first 10 minutes of infection at high multiplicities (see Figs. 2 and 3 in Part II). Portions of a culture were diluted ten-fold into 37° medium containing [^{3}H]thymidine and EDTA during the first 10 minutes of infection. Thirty seconds later, the diluted culture was rapidly cooled to 0°C. It was hoped that the synthesis of complementary DNA on some viral templates would begin in the unlabeled medium and be completed in the tritiated medium.

The isokinetic sucrose gradients of Figure 19 show the ^{3}H pulse label and the long-term ^{32}P label in RF from cultures diluted into ^{3}H medium 4, 6, and 8 minutes after infection. The ^{3}H appears predominantly in RFII (near fraction 28). In each case, about 25% of the ^{3}H label is also found at the position of RFI. In contrast, the relative amount of long-term ^{32}P label in RFI progressively increases, according to the pattern recorded in Part II (see Fig. 1).

The RFI and RFII peaks from similar preparations were pooled separately and dialyzed. The DNA was precipitated with isopropanol and subsequently resuspended in a small volume of 10 mM Tris (pH 7.4 at 25°C), 1 mM EDTA. Small quantities (less than 0.5 μg) of this DNA were digested with an Haemophilus influenzae restriction enzyme, endo R, and the products were applied to 5% polyacrylamide gels for electrophoresis.

Figure 20 shows the electrophoretic profiles obtained when the RFI and RFII preparations were separately digested with endo R. Nine discrete peaks, representing 12 fragments, appear in a pattern consistent with the observations of Edgell et al. (1972). Occasionally, an additional component was observed migrating about 1.4 times as fast as peak 9. In all the gels, peak 7 was sufficiently divided into two components (designated 7.1 and 7.2) to allow their separate quantitation. Each of the peaks contained ^{3}H as well as ^{32}P labels.

When the logarithm of the integrated ^{32}P counts in each peak was plotted against the distance it had migrated, the points fell close to a straight line (Figs. 21a and b). Since ^{32}P is expected to uniformly label the DNA and therefore, be proportional to the molecular

weight of the fragments, this observation confirms the inverse relationship between electrophoretic mobility and log[molecular weight] of DNA previously reported (Bishop et al., 1967; Peacock & Dingman, 1968; Edgell et al., 1972). The ^{32}P counts in peak 6 fell on the line only when it was divided by 3, consistent with the argument that it contains three different fragments of similar molecular weight (Edgell et al., 1972). The deviation of peak 9 from the linear relationship is not understood, but it may be explained by the loss of this small fragment (155 base-pairs) through diffusion into the thin aqueous layer between the acrylamide gel and the Plexiglas tube.

The pulse label in the fragments did not show the same relationship to mobility. It may be observed from the data shown in Figs. 21(c) and (d), that the amount of ^{3}H pulse label in each fragment is not a simple function of its molecular weight. By comparison, when the electrophoretic pattern of endo R fragments from parental RF synthesized in the presence of [^{3}H]thymidine for several minutes was similarly analyzed, the relationship between mobility and ^{3}H radioactivity was identical to that observed for the long-term ^{32}P label in Figs. 21(a) and (b). Thus, it appears that as a long-term label [^{3}H]thymidine is distributed uniformly. When used in a pulse under the conditions described, [^{3}H]thymidine incorporation is not proportional to molecular weight. These observations suggest that, during the pulse, some regions of the template were more frequently available for complementary strand synthesis than others.

The degree of synthetic activity during the pulse at various positions on the template may be estimated by the ratio of pulse label

to long-term label ($^{3}H/^{32}P$). Table 1 shows the $^{3}H/^{32}P$ ratios of endo R fragments of RFI and RFII from two different experiments. (Experiment #1 corresponds to the preparation seen in Figs. 20 and 21.) The fragments have been listed in order of decreasing ratio.

Variations in the experimental design have produced similar sequences. When parental RFII, labeled only with the ^{3}H pulse (no long-term label), were mixed with purified ^{32}P labeled RFII before endo R digestion, the ratios shown in Experiment #2 (Table 1) were obtained. With the exception of fragment 4 and, in one instance, fragment 1, the order is nearly invariable in the four cases.

The range of the ratios, especially in RFII, indicates that the effect is large. Some sections of the new complementary strand contain up to twice as much pulse label as others.

III. 4 Discussion

(a) Synthesis of the first complementary strand is ordered with respect to the genetic map. The order suggests a small number of specific initiation sites

The data show that some regions of the new complementary strand are synthesized later than others, after the appearance of the template in the cell. This observation has several possible interpretations. The conclusion proposed above is submitted as being most consistent with the studies of parental RF formation *in vivo* (see Part II). This discussion will attempt to show that the data in this section support this model. However, it is not possible, at this point, to rigorously exclude all competing interpretations.

If complementary strand synthesis is ordered, the ratios of ^{3}H to mass label may identify the order of synthesis, if the sequence is simple. Since the endo R fragments have been ordered with respect to one another on the RF molecule, this hypothesis may be tested.

The sequence ...3, 8, 5, 7.2, 6.3, 7.1, 1, 9, 2, 6.2, 6.1, 4... is suggested by Amy Shiu Lee and Dr. P. H. Johnson (personal communication) as the order of the endo R fragments in ϕX RF. This sequence has been deduced from (i) the "salvage" of genetic markers from the isolated fragments (Edgell et al., 1972; Hutchison et al., 1972; Chen et al., 1973), (ii) the sequence in which repair label appears in the fragments during in vitro displacement synthesis by polymerase I in late RFII (Johnson & Sinsheimer, 1974), (iii) the sequence in which labeled fragments appear when in vitro synthesis on viral template is primed with the isolated fragments (Amy Shiu Lee, unpublished data), (iv) the relationships (Amy Shiu Lee, unpublished data) between the fragments produced by endo R, endo HP from Haemophilus parainfluenzae (Johnson et al., 1973) and endo Z from H. aegyptius (Middleton et al., 1972), and (v) the appearance of specific fragments from larger fragments isolated from partial digests with each of the endonucleases (Dr. P. H. Johnson, personal communication). The relative positions of most of the endo R fragments, especially the larger ones, are supported by data from two or more of these different methods of analysis. For simplicity the order is shown as a linear sequence, but ϕX RF, and therefore the fragment sequence, are circular.

Table 2 compares the fragment order with the ratio order observed in RFII (from Table 1, Expt. #2). In Table 2(a), the two are listed in adjacent columns. The sequences are different, but a considerable homology may be detected. With a few exceptions, the fragments which have high ratios also appear near the top of the opposing column. Since there are a few uncertainties in the fragment order as well as insufficiencies and ambiguities in the ratio order, some of the discrepancies may be resolved with additional data.

An alternative analysis of the data, based upon a double-initiation hypothesis, is presented in section (b) of Table 2. Here the fragment order has been divided into two parts, representing complementary strand synthesis initiated at two points separated by about 1/3 of the genome. The first eight fragments form sequence 1, while the last four form sequence 2. The two are superimposed to produce the ratio order. With the exception of fragment 8, the observed ratio order agrees substantially with this arrangement. In the fragment sequence, the positions of 8, 7.1 and 6.3 have been judged to be the least secure (Amy Shiu Lee, personal communication). Small refinements in the deduced order may eradicate the remaining discrepancies.

(b) The complementary strand is polymerized counter-clockwise on the ϕX genetic map, in harmony with the capabilities of the known DNA polymerases

The ratios also show the direction in which synthesis occurs. Fragments with higher $^{3}H/^{32}P$ ratios were made last, according to this proposal and, therefore, the direction of synthesis would be from the

lowest to the highest ratios (from bottom to top in the columns of Table 2). According to the endo R fragment series which most nearly matches this order (Table 2b), the data indicate that the complementary strand is polymerized in a counter-clockwise direction on the ϕX174 map (Benbow et al., 1971).

ϕX messenger-RNA has been found to be copied exclusively from the complementary strand in vivo (Hayashi et al., 1963; Hayashi & Hayashi, 1970; Sedat et al., 1969) and therefore, it has the same polarity as the ϕX viral strand. Both transcription and translation occur in the same 5' → 3' direction, which Benbow et al. (1972) have found to be clockwise on the map. Therefore, the 5' → 3' polarity of the viral strand is clockwise. Inversely, the 5' → 3' direction of the complementary strand must be counter-clockwise. As a result, the ratio orders observed in this work indicate that complementary strand elongation proceeds in the 5' → 3' direction, the direction in which all the known DNA polymerases have been found to operate.

(c) All three stages of ϕX DNA replication may be initiated at the same site on the bacteriophage genome

[The genetic locations of the endo R fragments discussed below are based upon the fragment order deduced by Amy Shiu Lee and Dr. P. H. Johnson (see above), the "salvage" of genetic markers, and the ϕX genetic map of Benbow et al. (1974a)].

One of the initiation sites of the new strand appears to be near the junction of endo R fragments 3 and 4. The change in the position of R4 in the ratio order when the RFII is closed into RFI (see

Table 1), may indicate that some portion of the complementary strand in fragment 4 is synthesized just before ring closure.

About half of the endo R fragments have been reliably located on the ϕX genetic map by the marker "salvage" technique of Hutchison & Edgell (1971). When the results of these experiments (Edgell et al., 1972; Hutchison et al., 1972; Chen et al., 1973) are positioned on a refined genetic map of ϕX174 (see Fig. 1 of Benbow et al., 1974a), fragment 4 is found to be largely within cistron A. R3, the next fragment in the clockwise direction, contains the am33 marker, a mutation also in cistron A (Hutchison, 1969). Therefore, an initiation site near the junction of these two fragments is likely to be in or near the ϕX cistron A.

This same cistron has been suggested as the initiation site for RF replication (Baas & Jansz, 1972) and as the initiation site for asymmetric viral strand synthesis later in infection (Johnson & Sinsheimer, 1974). As suggested by the data, it seems likely that the first complementary strand is also initiated in this region. It is possible that all three stages of ϕX DNA synthesis have the same or closely associated initiation sites on the ϕX genome.

A second initiation site for complementary strand synthesis, near the junction of fragments 2 and 9, may also be suggested by the data. R9, a fragment only 155 nucleotides long, contains the am9 marker in cistron G (Hutchison, 1969). R2, the adjoining fragment in the clockwise direction, is about 735 nucleotides long. It is likely that fragment 2 begins in cistron G and extends some distance into cistron H. The high ratio order of R2 would indicate that most of it

is synthesized late in the polymerization. Therefore, this second initiation must lie in the region surrounding the junction between cistrons G and H.

The existence of two initiation sites for the synthesis of the first complementary strand disagrees with some of the observations reported in Part II. In particular, the amount of post-infection label seen in RF made after infection with UV irradiated phage (Fig. 18) and the structure of these molecules when observed in the electron microscope (Benbow et al., 1974b) indicate that only one initiation occurs. This conflict may be resolved if it is assumed that only one of the two possible initiation sites is used on a single template, but that in different members of the population both initiations are active. The means by which one of the initiations on each viral strand is inhibited is not understood.

III. 5 References

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TABLE 1. $^{3}H/^{32}P$ ratios of endo R fragments from ϕX174 RF

Experiment 1				Experiment 2			
RFI		RFII		RFI		RFII	
#	Ratio	#	Ratio	#	Ratio	#	Ratio
3	1.800	3	2.539	3	.831	3	1.102
2	1.728	2	2.323	2	.713	2	1.037
5	1.690	5	2.271	5	.694	5	.982
-	-	1	2.063	-	-	-	-
7.1	1.409	7.1	1.992	7.1	.592	7.1	.860
4	1.272	-	-	6	.535	6	.780
6	1.269	6	1.810	4	.518	-	-
7.2	1.244	7.2	1.726	7.2	.501	7.2	.712
1	1.226	-	-	1	.470	1	.634
-	-	4	1.456	-	-	4	.568
9	1.220	9	1.290	8	.462	9	.531
8	1.213	8	1.240	9	.459	8	.523

TABLE 2. Comparison of ratio order with fragment order

a		b		
Ratio Order	Fragment Order	Ratio Order	Fragment Order	
			Part 1	Part 2
3	3	3	3	
2	8	2		2
5	5		8	
7.1	7.2	5	5	
6	6.3	7.1	7.2	
7.2	7.1	6	6.3	6.2 6.1
1	1	7.2	7.1	
4	9	1	1	
9	2	4		4
8	6.2	9	9	
	6.1	8		
	4			

FIG. 19. Isokinetic sucrose gradient sedimentation of parental RF, pulse-labeled with [^{3}H]thymidine at various times after infection. Portions of a culture, infected with am3 (MOI = 200) were diluted ten-fold into fresh medium at 37^{o}C, containing [^{3}H]thymidine (20 μc/ml) and 10 mM EDTA. Thirty seconds later, the cultures were rapidly cooled to 0^{o}C. Chloramphenicol (150 μg/ml) and ^{32}P (5 μc/ml), added 20 minutes and 5 minutes before infection, respectively, were present throughout the experiment. The pronase-SDS treated lysates of each culture were sedimented through preformed CsCl gradients. The entire RF region of each gradient was pooled, dialyzed, and the DNA precipitated with isopropanol. The three DNA pools were applied to separate neutral isokinetic sucrose gradients. The procedures for preparation and centrifugation were described in the Methods of Parts II and III.

O——O ^{32}P (long-term) cpm/25 μl

●——● ^{3}H (pulse) cpm/25 μl

FIG. 20. Polyacrylamide gel electrophoresis of the ϕX RF DNA after digestion with the restriction enzyme, endo R. (a) RFI and (b) RFII molecules, prepared as described under Fig. 19, were digested with endo R. Each reaction mixture was applied to a 1 x 20 cm 5% acrylamide gel containing 0.2% SDS. After electrophoresis at 60V for 18 hours, the gels were frozen and cut into 1 mm slices. The radioactivity in each slice was counted in NCS scintillation fluid. The first 150 slices of each gel are shown. No radioactivity was detected in the remainder of the gels.

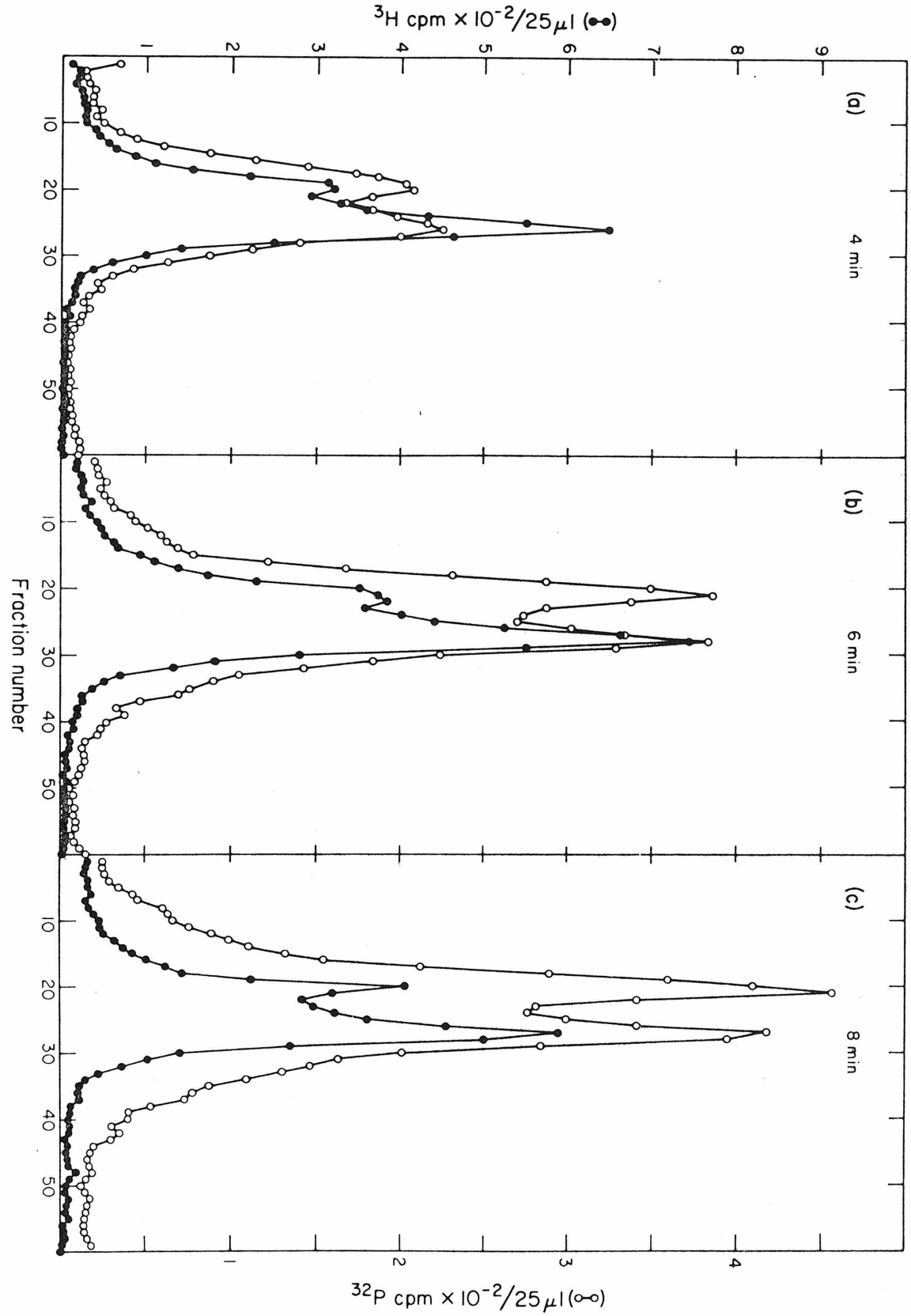
O—O ^{32}P (long-term) cpm/slice

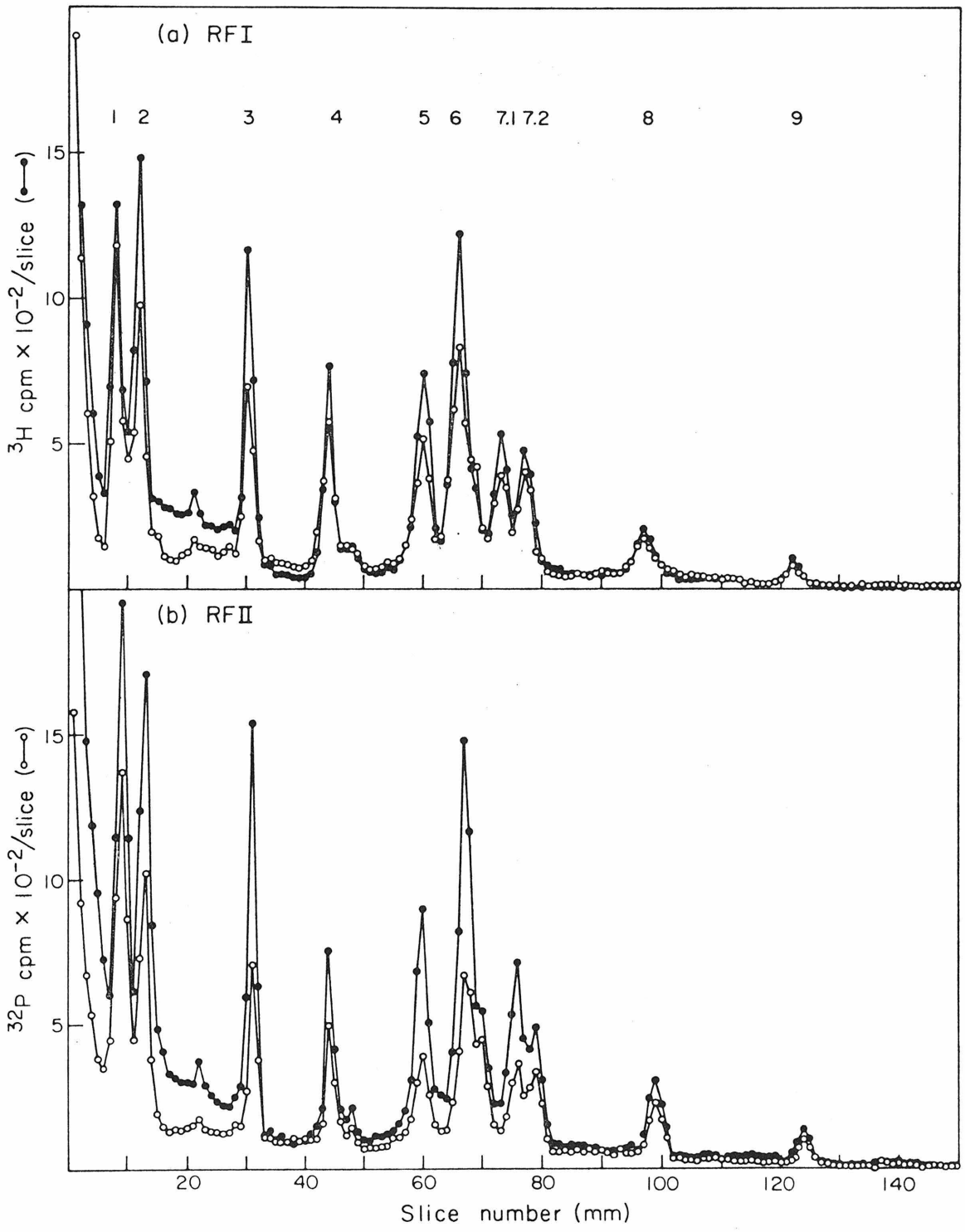
●—● ^{3}H (pulse) cpm/slice

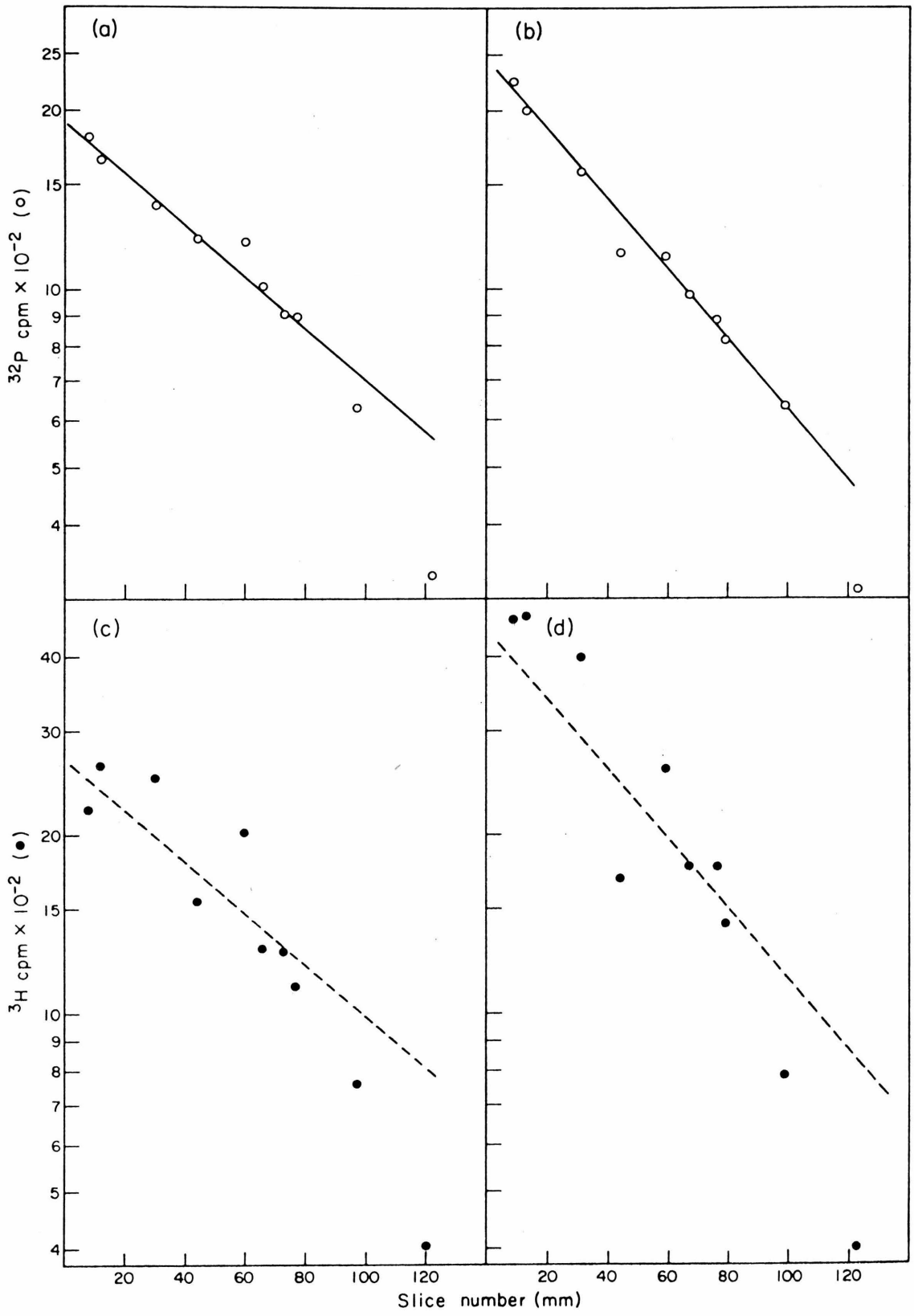
FIG. 21. Logarithm of the integrated radioactivity in endo R fragments as a function of electrophoretic mobility. The long-term (^{32}P) label in the peaks obtained from (a) RFI, (b) RFII and the pulse (^{3}H) label from (c) RFI and (d) RFII are plotted against the distance the peak has migrated from the cathode. The two components of peak 7 have been evaluated separately. The radioactivity in peak 6 has been divided by three, as described in the text. In (a) and (b) the data points have been interpreted as defining straight lines. In (c) and (d), lines with the same slopes as those seen in the corresponding ^{32}P label, have been arbitrarily drawn through the data.

O—O ^{32}P (long-term) cpm

●—● ^{3}H (pulse) cpm


^{3}H cpm × 10^{-2}/25μl (●–●)
(a)
4 min
(b)
6 min
(c)
8 min
Fraction number
^{32}P cpm × 10^{-2}/25μl (○–○)


(a) RFI
1
2
3
4
5
6
7.1
7.2
8
9
^{3}H cpm × 10^{-2}/slice (•—•)
15
10
5
(b) RFII
^{32}P cpm × 10^{-2}/slice (o—o)
15
10
5
20
40
60
80
100
120
140
Slice number (mm)


(a)
(b)
(c)
(d)
^{32}P cpm x 10^{-2} (o)
^{3}H cpm x 10^{-2} (•)
Slice number (mm)
25
20
15
10
9
8
7
6
5
4
40
30
20
15
10
9
8
7
6
5
4
20
40
60
80
100
120