8-1981

Electrophoretic Mobilities of Topoisomers of ΦX174 DNA

Virginia McKee Hanson

Abstract

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The analysis of nucleic acid structure by zone electrophoresis in agarose gels has become an increasingly important method in studies requiring the separation and identification of species differing only slightly in molecular weight or conformation. We have demonstrated the existence of several circular DNA forms that appear in cells infected with the bacteriophage ϕX174.

Wt/am3 heteroduplex molecules containing a single base mismatch (relaxed RFI molecules) were constructed in vitro, purified by sedimentation, and analyzed by zone electrophoresis on 1.4% agarose gels. In order to verify that the band of denaturation-resistant heteroduplex was indeed relaxed RFI, we compared it with relaxed RFI prepared from supercoiled RFI using the DNA relaxing enzyme (DNA topoisomerase) from Agrobacterium tumefaciens. To distinguish the product of the reaction from RF II molecules, it was analyzed by electrophoresis in 1.4% agarose with 0.5 μg/ml ethidium bromide. Under these conditions supercoiled RFI, relaxed RFI, RFII, RFIII and single-stranded DNA all migrate at different rates and can be readily identified with appropriate markers. Multiple length rings were also observed.

Much of the analyses of the mobilities confirmed our understanding of DNA migration in electrophoresis. The relaxed RFI migrates more

rapidly than natural supercoiled RFI in gels containing ethidium. In the absence of ethidium the supercoiled molecules migrate faster than relaxed RFI. Contrary to expectations, in the presence of ethidium bromide, the relaxed RFI and supercoiled RFI exchange positions.

LOMA LINDA UNIVERSITY

Graduate School

ELECTROPHORETIC MOBILITIES OF TOPOISOMERS of ϕX174 DNA

by

Virginia McKee Hanson

A Thesis in Partial Fulfillment of the Requirements

for the Degree Master of Arts in the Field of Biology

August 1981

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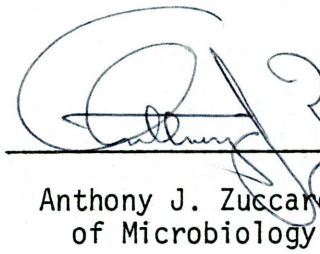

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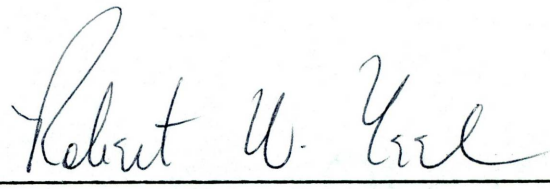

Robert W. Teel, Associate Professor of Physiology

ACKNOWLEDGEMENTS

I would like to gratefully thank:

- The faculty and staff of the Biology and Microbiology Departments of Loma Linda University for their friendship and warm suppoert during my educational endeavours.
- Dr. Robert Teel and Dr. Robert Nutter for their welcomed criticism and helpful suggestions for the preparation of this manuscript.
- Dr. Anthony Zuccarelli, my beloved teacher, for giving me so much of his knowledge, time and fascination with science. He has taught me to look beyond the obvious in the search for knowledge.
- my fellow comrades in research, for without them learning would not be exciting: Brenda Walden and Roberta Wright, for their friendship; to Mike Brown, whose expertise in photography and computers, made all my figures possible, and to the new fledglings who have just joined the group.
- Skip, my devoted husband, for giving support and encouragement and enduring love.
- And finally, our son, Joey, who was always content, even with a very part-time mother.

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INTRODUCTION

The DNA molecule has never been under more rigorous scrutiny by molecular biologists than in recent years when new tools have been devised to examine its physical and biochemical properties. Among these tools are enzymes which act on the DNA molecule only at sites with distinctive properties. In conjunction with the traditional chemical mutagens and the powerful capabilities of gel electrophoresis, these new methods have stimulated a resurgence of interest in the organization and function of the genetic material.

Spontaneous changes occur in genes, on the average, about once in every 10^6 duplications (or one error in every 10^9 to 10^{10} nucleotides). Since mutations are random and occur relatively infrequently, chemicals which stimulate their appearance have played a key role in genetics, and later in the study of DNA. External agents, such as ionizing radiation, ultraviolet light and certain chemicals can cause a variety of different DNA alterations. The shortcoming of all these mutagens is that they act indiscriminately. Until recently there were no physical or chemical agents that would act at the desired site and no others. The new methods of molecular biology have stimulated the development of methods for the production of site-specific mutations.

Several techniques for the production of highly specific genetic changes have found considerable usefulness in the study of DNA function. The best methods are those which are fairly simple and which can be applied to a wide variety of experimental systems. The most precise

technique (Hutchison et al., 1978; Gillam et al., 1979) allows one to convert one or more individual nucleotides in the molecule to any other nucleotides. The price for such capability is substantial. First, the sequence of bases in the DNA molecule must be known. Second, one of the two strands in the molecule must be isolated from its complement. Third, a specific oligodeoxyribonucleotide at least twelve nucleotides long must be synthesized in vitro.

The technique was first demonstrated using DNA from the bacterial virus ϕX174 (Hutchison et al., 1978; Gillam et al., 1979). The ϕX174 genome had been sequenced earlier in another laboratory (Sanger et al., 1977, 1978), and the DNA molecule in the mature virus capsid is single-stranded, thereby satisfying the first two requirements. The crucial step was the preparation of an oligonucleotide which was complementary except for one base, to the DNA sequence to be mutated. When the synthetic DNA fragment was annealed to the complete virus molecule, it created a short double-stranded segment within the largely single-stranded molecule. The duplex region, however, was not perfect since it contained a nucleotide which was not complementary to the corresponding nucleotide on the other strand. Using a DNA polymerase, the duplex segment was extended so that the entire molecule was double-stranded. E. coli cells, the normal host of ϕX174, were transfected with the molecules. Viruses produced by the transfection were either wild type (like the original virus) or mutant. The sequence of the synthetic oligonucleotide completely determined the location and character of the mutation (Hutchison et al., 1978; Gillam et al., 1979). Less demanding and more generally applicable techniques for producing site-specific mutations

have been devised utilizing restriction endonucleases (Muller et al., 1978; Shortle et al., 1978, 1980; Humayun et al., 1979; Green et al., 1980). In each case the original DNA molecule was cleaved into specific fragments using an appropriate restriction endonuclease. One of the fragments was isolated and exposed to a mutagenic agent. Shortle et al. (1978, 1980) treated an SV40 fragment having single-stranded ends with sodium bisulfite which causes the deamination of cytosine to uracil. The restriction fragment containing the altered ends was annealed to a single-stranded viral template and used to transfect an E. coli spheroplast. The resulting phage contained genetic differences within the restriction fragment selected for the mutagenesis. Borrias (1976) applied the same technique to the ϕX174 genome but used the mutagenic agent methoxyamine. Again the phage produced contained a mutational difference within the specific restriction fragment selected for treatment.

Mechanisms for in vivo repair of mismatched bases have been postulated. Naturally occuring repair systems are presumed to exist in many organisms, but those found in the Ascomycete fungi are of particular interest. In some of these (eg. Neurospora) products of each meiotic event preserve, in their order the sequence of events in meiosis: Analysis of the genetic markers found in each of the spores in the ascus (tetrad analysis) has added significant information to our understanding of meiosis.

In genetic experiments which are specifically designed to eliminate other causes, tetrad analysis of Neurospora and related fungi has shown that occasionally a copy of a gene is "lost" and replaced by a copy of

the alternative allele present in the zygote before meiosis. The result is an unequal number of the two alleles appearing in the four ascospores, (eg. 3 spores carrying one allele and one carrying the other). This phenomena is referred to as gene conversion. Conversion is regarded as a conservative process in that it preserves and does not generate new genetic information; only alleles which were present in the zygote can appear in the spores. Since gene conversion occurs during meiosis it is believed to be a consequence of genetic recombination. Genetic recombination involves a sequence of molecular events that includes breakage of DNA strands, formation of regions of hybrid DNA, limited degradation and repair of DNA and rejoining of ends. Recombination requires participation of nucleases, polymerases and ligases; cells lacking these enzymatic activities are often recombination-deficient.

The most persuasive models for gene conversion link the process to the repair of heteroduplex DNA regions produced during genetic recombination. A heteroduplex molecule contains a region in which one or more base pairs are noncomplementary. There is good evidence that every recombination event produces a limited region in which one strand is derived from one parent DNA molecule while the other strand is derived from the second parent molecule. If the two parent DNA molecules differed in sequence at that location the recombination process would give rise to a region of heteroduplex where the two strands do not match perfectly. (Similar molecules can be created <u>in vitro</u> by denaturing and renaturing a mixture of double-stranded DNA molecules which differ from each other at one or more points.) Gene conversion is likely to be the repair of heteroduplex regions by the enzymatic removal of one of the

mismatched bases, chosen at random, and its replacement by the base complementary to the one in the opposite, intact strand. The existence of heteroduplex repair mechanisms has been suggested by several experiments. For example, Doerfler and Hogness (1968) prepared two types of heteroduplex molecules and infected a susceptible host. Unexpectedly, the two types were equally infectious. They suggested that each of the heteroduplex molecules were converted to equal amounts of the parental homoduplexes by the host cells system involved in DNA repair. But the question of the frequency of repair remained unanswered. Russo et al. (1970) found that heteroduplexes formed in vivo disappeared upon DNA replication. However, they could not determine if the disappearance of the heteroduplex was entirely the result of DNA replication or if a repair process was also operating.

Baas and Janz(1970) deduced that a repair mechanism was working on ϕX174 heteroduplexes. They, too, constructed a heteroduplex and infected a susceptible host. The progeny phage were analyzed with respect to the genetic markers they inherited from the heteroduplex. They found that the majority (85 percent) of the spheroplasts infected with heteroduplex DNA produced phage with the genetic markers of one strand or the other, but not of both. This result was difficult to reconcile with any plausible replication mechanism. Rather, they postulated that the heteroduplex DNA was usually converted to homoduplex DNA prior to replication.

Howard (1973) performed an ingenious experiment whereby the frequency of conversion of heteroduplexes to homoduplexes by repair in vivo was determined. Heteroduplexes which differed at several known sites were

constructed *in vitro*. The results indicated that the repair mechanism operates independently at each site to produce progeny with genotypes different from either of the original parental homoduplexes. In this system heteroduplex repair occurred infrequently. A large proportion of the progeny had all the genetic markers of one of the two strands in the heteroduplex.

Wagner *et al*. (1976) created heteroduplexes of bacteriophage λ DNA that contained mismatches at four sites. These were used to transfect *E. coli* under recombinationless conditions. The output phage were analyzed to determine the extent of repair. According to the data, a wide range of repair frequencies was found. Repair was more apt to involve two or more close sites in the same strand than sites on opposite strands. The pattern of repair suggested that repair events initiate at mismatches and propagate preferentially in the 5'→3' direction, and extend about 3000 nucleotides.

The elimination of UV lesions from DNA has been well documented (Boyce and Howard-Flanders, 1964; Setlow and Carriet, 1964; Pettijohn and Hanawalt, 1964). If, as in the case of UV repair, heteroduplex repair involves excision of a single-strand segment with subsequent DNA repair synthesis, it is clear how a DNA heteroduplex could yield a homoduplex containing a mixture of markers originating in either of the strands of the heteroduplex. All of these results clearly indicate that cells contain enzymes which can recognize mismatched bases and initiate the repair porcess. At present, the enzymes which accomplish this recognition have not been identified. Since single strand nucleases purified from *Neurospora* and *Aspergillus* have been used in *in vitro*

repair manipulations, it is conceivable that these nucleases may also be responsible for *in vito* heteroduplex repair. It is also pertinent that these nucleases were extracted from the fungi in which the gene conversion studies were done.

Enzymes which cut the phosphodiester bonds of polynucleotide chains are called nucleases. Those nucleases which specifically cut internal bonds are called endonucleases, whereas those which remove nucleotides from the ends of chains are called exonucleases. Most nucleases are found to be relatively non-specific; their activity is not greatly affected by the nucleotide sequence of the substrate. Therefore, the discovery of type II restriction endonucleases (Smith and Wilcox, 1970; Kelly and Smith, 1970) was a major breakthrough, since these enzymes cleave DNA specifically. A given restriction enzyme recognizes a specific nucleotide sequence in the DNA and cuts the DNA wherever the sequence appears. Each enzyme produces a characteristic pattern of fragments from each DNA. Therefore, any change in a particular DNA molecule may result in the gain or loss of a site recognized by that restriction enzyme or it may alter the pattern of fragments produced by the digestion of the DNA by the enzyme. Thus, conceivable, any defect in the DNA molecule is detectable by restriction mapping. In the years since their discovery, restriction enzymes have greatly improved our ability to analyze changes in genes.

Endonucleases that specifically attack single-stranded DNA include the *Neurospora* endonuclease (Linn and Lehman, 1966), mung bean nuclease I (Kedzierski *et al.*, 1973), PI nuclease (Fugimoto *et al.*, 1974) and S1 nuclease from *Aspergillus oryzae* (Ando, 1966; Vogt, 1973). Many

experiments in molecular biology and molecular genetics include steps which require the removal of single-stranded DNA from a mixture of single- and double-stranded forms or, alternatively the removal of single-stranded regions from molecules which are only partially double-stranded. Several of the single-stranded endonucleases are commonly employed in such experiments but S1 from A. oryzae is the most widely used (Germond et al., 1974; Vogt, 1973).

In vitro studies of these various endocleases have shown that S1 has the advantage of being more easily purified from contaminating nucleases. By contrast, the enzyme from Neurospora has both endonuclease and exonuclease activities in a single molecule and therefore the two cannot be separated (Kato et al., 1973; Bartok et al., 1975).

S1 has a high affinity for single-stranded DNA as compared to double-stranded DNA. During the time it takes to completely digest ϕX174 single-stranded (ss)DNA to individual nucleotides (i.e. approximately 5400 bond cleavages) only two or three breaks are made in the double-stranded (ds) DNA molecule in the same reaction mixture (i.e. four to six phosphodiester bond cleavages) (Godson, 1973).

The important characteristic of S1 nuclease that has made it so useful in molecular studies is its ability to digest large single-stranded regions in DNA molecules containing limited duplex sections (Ando, 1966). The enzyme has been used to eliminate single-stranded regions in DNA-DNA or RNA-DNA hybrids and to obtain nuclease-resistant DNA fractions (Vogt, 1974). Harada and Dahlberg (1975) used the enzyme to cleave the anticodon loop in tRNA. Shenk et al. (1975) used it to map small deletions in SV40 viruses. Its discrimination between single- and double-

stranded DNA is demonstrated by its ability to detect regions of partial double-strandedness in supercoiled closed circular DNA molecules in SV40, T5 and ϕX174 (Germone et al., 1974; Shishido et al., 1975; Dodgson and Wells, 1977; Shenk et al., 1975). These supercoiled forms were converted to unit length linear molecules by the enzyme. Presumably, this occurs because unpaired, or weakly hydrogen-bonded regions which are susceptible to S1 nuclease occur in the strained superhelical molecule. Since nicked circular molecules appear to be intermediates in this process, the enzyme must also be capable of cleaving an intact strand opposite a break in the complementary strand. This property suggests that S1 nuclease could be used to map the location of small deletions, insertions, or in fact, any difference in base sequence between otherwise homologous DNA molecules.

Shenk et al. (1975) reported that S1 nuclease can be used to locate a deletion as short as 32 base pairs. Moreover, S1 was able to cleave heteroduplex molecules formed from the DNAs of SV40 temperature-sensitive mutants and either their revertants or wild type parents. It is not known if the base sequence of the temperature-sensitive mutant DNA differs from the wild type or revertant DNA by more than one, base, however, they postulated that S1 might be able to recognize a single base mismatch. If so, S1 could be used to accurately map point mutations. More recently, Shenk's procedures for the detection of deletion mutants have been modified to allow the detection of deletions or insertions as short as five bases (Gannon et al., 1980),

Experimental Protocol

We propose to synthesize and characterize a heteroduplex of ϕX174 DNA

that will be used to test the capabilities of S1 nuclease. The analysis of S1 specificity will show whether or not it is an efficient tool for detecting single base mismatches.

The DNA of bacteriophage ϕX174 is a circular, single-stranded molecule (Sinsheimer, 1959) containing 5386 nucleotides (Sanger et al., 1977; Sanger et al., 1978). Its infectious cycle and mode of replication have been adequately reviewed elsewhere (Denhardt, 1975).

The properties of circular DNA molecules are such that a single species of fixed molecular weight can assume any of several different topological conformations with distinctive physical properties. Four different states of the double-stranded replicative form (RF) of ϕX174 are mentioned in this work.

When both strands of the DNA are covalently closed polynucleotide circles with no interruptions, the molecule is called an RFI. Covalently closed DNA circles isolated from living systems are almost always "supercoiled"; the axis of the double helix is itself twisted in space. Wang (1968) measured 19 "negative" superhelical twists in natural ϕX174 RFI. Supercoiling can be artificially removed by breaking one of the strands, allowing the molecule to relax and then enzymatically resealing the broken bond so that both strands are again covalently closed circles. The result is a relaxed RFI. It can lie as a circle, unconstrained, on a plane. The intermediate in the process, having at least one interruption in either of its strands, but still maintaining its overall circularity is called an RFII. If both strands are broken near the same site a linear double-stranded form, called an RFIII, results.

RFII and relaxed RFI molecules, by virtue of their circular form,

have a smaller radius of gyration than the linear RFIII molecules; they are more "compact". This difference allows them to be separated from the latter in centrifugation and gel electrophoresis. As a result of supercoiling, natural RFI molecules are still more compact and move even more rapidly in centrifugation. Furthermore, since the individual strands are locked together topologically in both supercoiled and relaxed RFI, breaking the hydrogen bonds between the strands does not permit them to separate. The same treatment would cause RFII or RFIII to disassociate into their component single strands. General separation techniques rely upon these distinctions.

The strategy of the current study is to construct a heteroduplex DNA molecule that contains within its sequence a single base mismatch. ϕX174 ^{3}H wt RFI molecules were digested with the restriction endonuclease Xho I to produce RFIII molecules. (Xho I cleaves the ϕX DNA at only one point.) The linear RFIII molecules were denatured and renatured in the presence of a five-fold excess of am3 single-stranded, circular DNA. The resulting wt/am3 heteroduplex RFII molecules have a single nick in one strand at the position of the Xho I cleavage site. The nicks were closed with T4 DNA ligase, resulting in relaxed RFI molecules. A relaxed homoduplex RFI molecule was also produced employing a commercial enzyme and was used as a marker to compare electrophoretic mobilities of the topographical isomers in agarose gels containing ethidium bromide.

MATERIAL AND METHODS

A. Bacterial Strains

(i) *Escherichia coli* C, ATCC 25312, (Sinsheimer, 1959), is the standard wild type (hcr^+), non-permissive (*su-*) host for ϕX174.

(ii) *E. coli* H502 is a *thy-*, *uvr* A (hcr^-), *endo* I^-, su^-, host strain constructed by Dr. Hoffman-Berling (Zuccarelli *et al.*, 1972).

(iii) *E. coli* HF4714 is a hybrid of strains C and K12 (Godson, 1971) with the multiple auxotrophic requirements: arg^-, his^-, leu^-, thr^-, pro^-. It is su^+_{AUG}, and suppresses most "amber"mutations in ϕX174.

B. Bacteriophage Strains

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

(ii) ϕX174 *am3* is a lysis-deficient mutant in cistron E (Hutchison, 1969) which differs from wild type by having an A nucleotide instead of a G at position 587 in the revised nucleotide sequence of the viral DNA strand of ϕX174 (Sanger et al., 1978).

C. Chemicals

(i) Agarose (Type I),thymine, bovine serum albumin (BSA, fraction 5) diethyl pyrocarbonate, pyruvic acid (Type II, sodium salt), ATP (sodium salt), tris hydroxymethyl aminomethane (Tris-OH) and tris hydroxymethyl aminomethane hydrochloride (Tris-HCl.) were purchased from the Sigma Chemical Co., St. Louis, Mo.

(ii) Chloramphenicol (grade B), ethidium bromide (grade B), propidium

diiodide (grade A), and dithithreitol (Cleland's reagent) were purchased from Calbiochem, San Diego. CA.

(iii) Cesium chloride (optical grade) and sucrose (density gradient grade, ribonuclease free) were purchased from Schwartz/Mann, Orangeburg, N.Y.

(iv) Dowex 50W-X2 (200-400 mesh), disodium dihydrogen ethylenediamine-tetraacetate dihydrate (EDTA), toluene, 1-propanol, methanol, trichloro-acetic acid (TCA), 2,5-diphynyloxazol (PPO),1,4-bis-2-4-methyl-5phenyl-oxazole benzene (dimeth-POPOP, scintallation grade), formamide (99%), polyethylene glycol 6000 (PEG 6000), and other reagent grade chemicals were purchased from J.T.Baker, Phillipsburg, N.J..

(v) Dialysis tubing (size #8) was purchased from Union Carbide. Cellulose dialysis tubing was boiled three times in 10 mM $NaHCO_3$ and three times in 10 mM EDTA, rinsed with deionized water and stored in 50% ethanol, 5 mM EDTA at $5^{o}C$.

D. Radioactive Materials

(i) [methyl-^{3}H] thymidine in 70% ethanol, 20 Ci/mmole, was purchased from New England Nuclear Corp., Bostom, MA.

(ii) [2-^{14}C] thymine, 50 mCi/mmole, was purchased from Moravek Biochemicals, City of Industry, CA.

E. Enzymes

(i) Lysozyme (eggwhite, 3x crystallized, grade I) was purchased from the Sigma Chemical., St. Louis, MO.

(ii) Pst I, Xho I, E. coli DNA ligase, and T4 DNA ligase were purchased from New England Biolabs, Beverly, MA.

(iii) Nuclease S1 from Aspergillus oryzae was purchased from Miles laboratories.

(iv) Ribonuclease A (bovine pancreatic, RAF 55 P 548) and deoxyribonuclease I (bovine pancreatic DPFF 56 D 332) were purchased from Worthington Biochemicals, Treehold, N.J.

F. Media and Solutions

(i) TPG-high phosphate medium (Lindqvist and Sinsheimer, 1967) contains 8.0 g KCl, 0.05 g NaCl, 1.1 g NH_4Cl, 0.2032 $MgCl_2 \cdot 6H_2O$, 0.23 g KH_2PO_4, 0.8 g sodium pyruvate, 12.1 g Tris-OH, 0.147 g $CaCl_2 \cdot 6H_2O$, 0.0273 g Na_2SO_4 (anhydrous), and 2.0 g dextrose (anhydrous), dissolved in sufficient deionized water to make one liter. The pH is adjusted to 7.4 with HCl before autoclaving.

(ii) TPA-high phosphate medium contains all the ingredients of TPG-high phosphate medium with the addition of 15 ml of 20% (w/v) Casamino acids (Difco) to each liter before sterilization. The casamino acid solution was first shaken with powdered, activated charcoal and filtered to remove nucleotides.

(iii) KC Broth (Sinsheimer, 1959) contains 10 g Bacto-Tryptone, 5 g KCl, 0.147 g $CaCl_2 \cdot 6H_2O$ dissolved in one liter of deionized water.

(iv) Tris-EDTA is 0.05 M Tris-HCl- 5 mM EDTA. The pH of this buffer was 7.4 (25^oC) unless otherwise stated.

(v) Tris-$CaCl_2$ is 0.05 M Tris-HCl (pH 7.4) - 1 mM $CaCl_2$.

(vi) Borate-EDTA is 0.05 M $NaHBO_4$ - 5 mM EDTA.

(vii) Bottom agar contains 10.0 g Bacto-tryptone, 2.5 g NaCl, 2.5 g KCl and 10.0 g agar in one liter of deionized water. After autoclaving, add

1.0 ml sterile 1 M $CaCl_2$, mix and dispense 20 ml volumes into sterile petri plates.

(viii) Top agar contains 1.0 g Bacto-Tryptone, 5.0 g NaCl and 8.0 g agar in one liter of water. Heat in boiling water until the agar is dissolved, dispense into bottle and autoclave.

(ix) Tris-acetate gel buffer (50X) is 2 M Tris-OH, 250 mM sodium acetate, 50 mM EDTA, adjusted with acetic acid so that its pH is 8.2 when diluted 50-fold with deionized water.

(x) Tris-acetate gel buffer (1X) is 40 mM Tris-OH, 5 mM sodium acetate, 1 mM EDTA, Ph 8.2 ($25^{o}C$).

G. Centrifugation Techniques

(i) Isokinetic sucrose gradients were produced by mixing 5% and 33.78% (w/v) sucrose solutions in a device described by Noll (1967). Neutral isokinetic gradients had 5% sucrose at the top, and contained 1 M NaCl, 50 mM Tris (pH 7.4) and 5 mM EDTA. They were designed for the separation of ϕX174 DNA molecules at $5^{o}C$. Gradients with a total volume of 4.0 ml, 13.0 ml and 38.0 ml were prepared in polyallomer centrifuge tubes for the Beckman SW 56Ti, SW 27.1, and SW 27 rotors, respectively. Centrifugation was at 30,000 rev/min for 15 hours, 27,000 rev/min for 40-45 hours, and 27,000 rev/min for 23 hours in the SW 56Ti, SW 27.1, and SW 27 rotors, respectively. The tubes were punctured at the bottom with an Isco gradient fractionator and the gradients collected in 45 to 55 equal fractions through and LKB Multiperpex pump.

(ii) Samples were prepared for equilibrium density centrifugation with propidium diiodide by adding Tris-EDTA to bring the total sample weight

to 6.95 g. Dessicated cesium chloride was added to bring the solution to a density of 1.54 g/cm^3 for the purification of *in vivo* ϕX174 replicative forms or to 1.58 g/cm^3 for the isolation of relaxed heteroduplex replicative form I prepared *in vitro*. To this was added 0.35 ml of 5 mg/ml propidium diiodide. The final concentration of propidium was 200 µg/ml (Grossman *et al.*, 1974) and a total volume of the solution was about 8.5 ml. A 5/8" X 3" polyallomer tube containing the mixture, was filled with paraffin oil. Centrifugation was for 40-50 hours at 35,000 rev/min in a Beckman Type 65, fixed-angle rotor at 5^{o}C. The gradients were collected in 50 to 60 equal fractions by puncturing the tube at the bottom with an Isco gradient fractionator and LKB Multiperpex pump. Propidium diiodide was removed from selected fractions by running them through 2.5 ml of Dowex-50 (equilibrated with 1.5 M NaCl - 50 mM Tris (pH 7.4) - 5 mM EDTA) in a siliconized pasteur pipette.

(iii) Preformed CsCl step gradients for the purification of ϕX174 phage particles combine the benefits of both isopycnic and velocity centrifugation yet take only a few hours to run. In these gradients, the viable ϕX174 phage particles collect at the interface of two CsCl densities. The gradients were formed by layering the following series of CsCl solutions (in borate-EDTA) into an SW 27 cellulose nitrate tube: 10 ml of 1.45 g/cm^3, 10 ml of 1.35 g/cm^3, 5 ml of 1.20 g/cm^3, and 4 ml of 1.10 g/cm^3. Up to 10 ml of the phage suspension can be applied to the top. Centrifugation was at 25,000 rev/min at 5^{o}C for 4.5 to 5 hours. Since the density of the phage is close to 1.4 g/cm^3, it will sediment to the interface between the 1.45 g/cm^3 and 1.35 g/cm^3 layers. The gradients were collected by puncturing the tube at the bottom with an Isco fractionator and dispensing

40 to 50 equal fractions into plastic collection vials through an LKB Multiperpex peristaltic pump.

H. Liquid Scintillation Spectroscopy

Unless otherwise stated, the amount of ^{3}H-DNA in each fraction was determined by placing a 10 µl sample on 2.5 cm GF/A filters (Whatman). The filters were washed twice in 5% (w/v) TCA to precipitate the DNA, then twice with absolute methanol to remove the TCA and water. Filters were oven dried, then placed in vials containing 5 ml of scintillation fluid (4.0 g PPO and 0.2 g dimeth-POPOP per liter of toluene). Counting was done in a Packard-Tri Carb Scintillation Counter Model 2002 or Beckman LS 7500. To correct for channel overlap and backgrounds, standards of the relevant isotope were prepared for each gradient to match precisely the counting conditions of the samples.

The data are presented as counts per minute per fraction or 10 µl sample. All figures are drawn with the bottom of the gradient at the left and the direction of sedimentation from right to left.

I. Preparation of ^{3}H<u>am3</u> ϕX174 and Isolation of Single-Stranded Viral DNA

H5O2 was grown to 5 X 10^{8} cells/ml with aeration at 37°C in 5 liters TPA-high phosphate medium supplemented with 5 µg/ml thymine. After five minutes, purified <u>am3</u> ϕX174 was added to a multiplicity of infection (MOI) of 4 to 5. Ten minutes after infection 1 mCi [methyl-^{3}H] thymidine (20 Ci/mmole) was added to a final concentration of 0.2-0.5 µCi/ml After 3½ hours of incubation, the cells were placed on ice and harvested in an IEC centrifuge at 2600 rev/min for 15 min. at 23°C. The pellet was trans-

ferred to an SS34 centrifuge tube with Tris-EDTA. The cells were lysed according to Godson and Boyer (1976) with the following modifications. Each pellet ($2X10^{11}$ to $3X10^{11}$ cells) was resuspended in 20 ml cold 10% (w/v) sucrose, 0.05 M Tris (pH 8.0) and the cells were converted to spheroplasts by adding 2 ml of 10 mg/ml lysozyme (freshly prepared in Tris-EDTA) and 8 ml of 0.25 M EDTA. The mixture was kept on ice for ten minutes. Lysis was achieved by gently blowing 2 ml of 20% sodium dodecyl sulfate (freshly prepared) into the suspension with a pipette. One milliliter of $CHCl_3$ was added immediately to prevent viral adsorption to the debris. The solution became clear and extremely viscous within a few minutes. The lysate was kept packed in ice in the refrigerator overnight. The next morning the precipitate was removed by centrifugation at 12,100 g in a Sorvall RC5 centrifuge for 25 minutes at $0^{o}C$. Dry NaCl was added to the supernatant to bring the final concentration to 1 M. After three hours on ice, the second precipitate was removed by centrifugation at 9150 g at $0^{o}C$. The supernatant containing the free phage was dialyzed against Borate-EDTA overnight.

Dry PEG 6000 and NaCl were added to the dialysate to provide final concentrations of 0.5 M and 11% (w/v), respectively. After 1-2 hours in an ice bath, the mixture was centrifuged for 30 minutes at 9150 g in a Sorvall centrifuge. The pellet was washed three times with small volumes of Borate-EDTA to elute the phage. The pooled washes were applied to a 115 X 0.9 cm column of porous glass beads (pore size 229 A $\pm$ 4.2%, 200-400 mesh, Sigma) and eluted with Borate-EDTA. After voiding the first 25 ml, the eluate was collected into 40 - 2 ml fractions and 10 µl aliquots from each was counted as described in section H of the Methods. The

fractions containing the phage peak were pooled and glycerol was added to a final concentration of 10% (v/v). For extended storage, small samples were quickly frozen in a methanol-dry ice bath and placed in a -80°C freezer.

For the preparation of single-stranded viral DNA, the phage suspension was first treated with bovine pancreatic DNAse (10 mg/ml in 10 mM Tris, pH 7.4, 1 mg/ml heat-treated BSA, 50% glycerol) at a final concentration of 10 µg/ml, and then the mixture was extracted three times with single volumes of freshly distilled phenol, saturated with Tris-EDTA. The phenol layers were reextracted with ½ volume of Tris-EDTA to enhance the recovery of DNA (Sinsheimer, 1959). The pooled aqueous layers were shaken three times with ether to remove the phenol. The last traces of ether were evaporated under a stream of nitrogen. The DNA was precipitated by adding a tenth volume 3 M sodium acetate (pH 5.6), three volumes of 2-propanol and cooling the mixture to -20°C overnight. The precipitate was collected by centrifugation for 90 min at 12,100 g in a Sorvall GSA rotor, dried in a vacuum dessicator and resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA. The concentration of single-stranded DNA in the preparation was determined by its absorption at 260 nm. One absorption unit is equivalent to 39 µg/ml (Sinsheimer, 1953). The purity of the preparation was assessed by electrophoresis in 1.4% agarose gels. If significant contamination with small DNA fragments was detected, the single-stranded DNA was further purified by sedimentation in neutral isokinetic sucrose gradients (see Centrifugation Techniques, section G.) Fractions from the gradient containing intact single-stranded DNA were pooled and diluted by adding 3 volumes of Tris-EDTA. The DNA was precipi-

tated from this solution with 2.5 volumes of 2-propanol at -20°C as described above. The DNA precipitate was dissolved in 10 mM Tris (pH7.4), 0.1 mM EDTA and stored at -80°C.

J. Preparation and Linearization of ϕX174 WT RFI Molecules

E. coli H502 was grown to 5 X 10^8 cells/ml with aeration at 37°C in 4 liters of TPA-high phosphate medium containing 2μg/ml thymine (Godson and Vapnek, 1973; Godson and Boyer,1974). Purified wt ϕX174 phage were added at a MOI of 5 to 10. Ten minutes after infection, chloramphenicol (3 mg/ml, freshly prepared in TPA medium) was added to a final concentration of 30 μg/ml. Five to ten mCi of methyl-^{3}H thymidine was added to the culture in ten equal portions at five minute intervals beginning 10 minutes after infection. At 70 minutes, non-radioactive thymine was added to bring its final concentration in the medium to 7 μg/ml. After 2½ hours of incubation at 37°C, the culture was placed on ice and the cells harvested. The pellet (2 X 10^{11} cells to 3 X 10^{11} cells) was lysed according to the procedure of Godson and Boyer (1974). The cells were resuspended in 80 ml of 10% (w/v) sucrose, 50 mM Tris (pH 7.4) at 5°C. To this was added 8 ml of lysozyme (10 mg/ml, freshly prepared in Tris EDTA) and 30 ml of 0.25 M EDTA. The mixture was kept on ice for 15 minutes and then the cells were lysed by adding 8 ml of a freshly prepared 20% SDS (w/v) solution. Lysis was effected by blowing the SDS into the spheroplast suspension and gently stirring with the pipette. The suspension cleared almost immediately and became extremely viscous. The suspension was packed in ice overnight and centrifuged at 9150 g for 90 min in the Sorvall GSA rotor. Solid NaCl was

added to the supernatant to make it 1 M. After 3 hours at 0°C the precipitate was removed by centrifugation at 9150 g for 30 minutes. The supernatant was dialyzed against 2 liters of Tris-EDTA overnight.

Particulate material in the dialysate was removed by centrifugation at 9150 g for 30 min in the GSA rotor. The supernatant was extracted three times with freshly distilled phenol, precipitated with alcohol and dried exactly as described for the preparation of viral DNA from purified phage particles (section I). The DNA precipitate was dissolved in 6.95 ml of Tris-EDTA. CsCl and propidium diiodide were added and the mixture was centrifuged as described under Centrifugation Techniques (section G). Fractions containing RFI DNA were pooled and the propidium removed by running the sample through a 2.5 ml Dowex-50 column. DNA was precipitated from the solution at -20°C overnight after adding 2.5 volumes of Tris-EDTA and 3 volumes of isopropanol. The precipitate was collected by centrifugation for 2 hours at 12,100 g at -15°C in a Sorvall GSA rotor. After aspirating the supernatant, the RFI DNA pellet was dried in a vacuum dessicator and then dissolved in 10 mM Tris (pH7.4), 0.5 mM EDTA, 10% (w/v) glycerol and stored at -20°C. The RFI DNA was cleaved into linear RFIII molecules with Xho I restriction endonuclease (see Enzyme Incubation Conditions, section L) and visualized on 1.4% agarose gels.

K. Wt/am3 Heteroduplex Formation

The experimental procedures for heteroduplex formation were those of Godson (1973) and McConaughey et al. (1969) with modifications. Purified ^{3}H-wt RFI DNA was digested by Xho I as described under Enzyme Incubation

Conditions (section L). The resulting RFIII molecules were mixed with a five-fold excess of am3 viral strands from purified ϕX174 in a siliconized glass tube. (Clean glassware was siliconized by immersing it into 1% (v/v) dichlorodimethyl silane in dry benzene for 20 minutes, rinsing it, followed by sterilization.)

Formamide (99%) was added to the solution to make the final concentration 50% (v/v). The DNA was heated to 60°C for 20 minutes to denature the RFIII into their component strands and then the mixture was placed in dialysis tubing (size #8). Dialysis was at 25°C for four hours against 50% (v/v) formamide, 100 mM Tris (pH 8.5), 10 mM EDTA changing the buffer hourly. During this time complementary DNA strands reanneal. The reaction was stopped by dialysis against Tris-EDTA overnight at 5°C. The DNA from the dialysate was precipitated in one t nth volume 3 M sodium acetate (pH 5.6) and 3 volumes of isopropanol and collected by centrifugation for 90 minutes at 17300g in a Sorvall SS34 rotor. The DNA precipitate was dried in a vacuum dessicator and resuspended in 250 µl 20 mM Tris (pH 7.4), 0.5 mM EDTA and layered on a neutral isokinetic sucrose gradient as described under Centrifugation Techniques (section G). Fractions containing the heteroduplex DNA were pooled and precipitated from the solution by adding 3 volumes of Tris-EDTA, bovine serum albumin to a final concentration of 10 µg/ml, and 2.5 volumes isopropanol and cooling to -20°C overnight. The precipitate, collected by centrifugation for 120 minutes at 12,000g Sorvall SS34 rotor, was dried in a vacuum dessicator and dissolved in 20 mM Tris (pH 7.4), 0.5 mM EDTA and stored at -10°C.

L. Enzyme Incubation Conditions

(i) Pst I

Pst I, derived from Providencia stuartii, is a restriction endonuclease that is able to cleave the ϕX174 double-stranded genome at a single unique site, thereby producing RFIII molecules (Brown and Smith, 1976). This enzyme cleaves the ϕX sequence between nucleotides 5386 and 1, in the region of gene A (recognition sequence: CTGCA↓G).

Treatment with PstI followed published protocols (Brown and Smith, 1976; Smith et al., 1976). ^{3}H wt RFI DNA (.3 - 3.0 μg) was incubated with the enzyme for one to two hours at 37°C in 50 mM NaCl, 10 mM Tris (pH 7.4), 5 mM $MgSO_4$, 0.1 mM EDTA, 0.5 mM dithithreitol and 100 μg/ml gelatin, in a total reaction volume of 20 μl. The reactions were stopped by adding EDTA to 10 mM. Samples were analyzed on 1.4% agarose gels (see section M of Methods) and on isokinetic sucrose gradients.

(ii) Xho I

Xho I, derived from Xanthomonas holcicola, is a restriction endonuclease that is also able to cleave the ϕX174 genome at a single, unique site. This enzyme cleaves the ϕX174 double-stranded molecule between nucleotides 162 and 163, in the region of gene C (recognition site: C↓TCGAG).

Treatment with Xho I followed the protocol of Gingeras et al., (1978). ^{3}H wt RFI DNA was incubated with Xho I for one hour at 37°C in 150 mM NaCl, 6mM Tris HCl(pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg/ml BSA, in a total reaction volume of 20 μl. The reactions were stopped by adding EDTA to 10 mM. Samples were analyzed on 1.4% agarose gels.

(iii) T4 DNA Ligase

Ligation was completed following several published protocols (de Vries et al., 1976; Sgaramella, 1972; Raae and Kleppe, 1978; Sugino et al., 1976; Humayum, 1979). Wt/am3 molecules were incubated for two hours at 37°C in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM EDTA in a total reaction volume of 20 µl. Reactions were stopped by adding EDTA to 15 mM. Samples were analyzed on 1.4% agarose gels and 1.4% agarose gels containing 0.5µg/ml ethidium bromide.

(iv) Relaxing Enzyme

ϕX174 RFI molecules were exposed to various concentrations of relaxing enzyme following the protocol of Lebon et al. (1978). RFI molecules (.5 - 1 µg) were incubated for two hours with the enzyme at 37°C in 20 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.1 mM EDTA, and 100 µg/ml BSA in a total reaction volume of 20 µl. Reactions were stopped by adding EDTA to 10 mM. Samples were applied to 1.4% agarose gels containing 0.5 µg/ml ethidium bromide.

M. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed essentially as described by Johnson and Grossman (1977). Slabs of 1.4% (w/v) agarose and 1.4% (w/v) agarose with 0.5µg/ml ethidium bromide (Keller, 1975; Johnson and Grossman, 1977), 13.5 X 28 cm and 1.5 mm thick in 1X gel buffer were cast in a BioRad Dual Slab Gel electrophoresis cell, model 221. A teflon comb with 10, 15 or 20 teeth was imbedded in the agar to form the wells. The gel was cooled to approximately 5°C by circulating cold water through the core of the apparatus. After the gel had solidified, the comb was removed

from the gel and the wells were washed three times with 1X gel buffer before samples were applied.

Samples up to 30 µl in volume containing 0.3 to 3 µg of DNA, 10% glycerol and 0.025% bromphenol blue were placed in the wells. Electrophoresis was for 14 to 24 hours at a constant potential of 90-100 volts (3.29 v/cm) using a Heath-Schumberger regulated power supply (Model SP-17A).

After electrophoresis, the gels were stained in 1X gel buffer containing 0.5 µg/ml ethidium bromide and visualized by photography. Gels were placed on a Chromato-Vue Transilluminator (Model C-61, Ultraviolet Products Inc., Corp.) and photographed with a Polaroid CU-5 Industrial Camera equipped with 550 and 570 nm cut-off orange filters (Ealing Corporation, # 2643660 and 2643740) and Polaroid Type 665 positive/negative pack film. Exposures were from 30 sec to 4 min.

RESULTS

The relative effectiveness of gel electrophoresis and velocity ultracentrifugation in resolving the various forms of ϕX174 DNA is seen in figures 2 and 3. The two separation techniques were used analyze the products of reactions beween a ϕX174 RFI - RFII mixture, and the restriction enzyme Pst I. This enzyme cleaves the ϕX DNA molecule at only one site, converting both RFI and RFII into the double-stranded linear RFIII form. In figure 2 (a-c) 0, .01 and .05 units of Pst I, respectively, were incubated for 2 hours at 37°C with 1 μg of ϕX174 RFI and RFII mixture labeled with tritium. The product of each reaction was subsequently mixed with ^{14}C labeled RFI and RFII marker DNA and layered onto 4 ml isokinetic sucrose gradients. The RFI and RFII forms are adequately resolved by centrifugation, but RFIII, sedimenting only slightly slower than RFII, appears as a shoulder on the trailing edge of the RFII peak when both are present. As the enzyme concentration increases, more of the RFI and RFII molecules are converted to RFIII molecules until, at 0.05 units of Pst I, only linear RFIII molecules remain. By contrast, all three replicative form isomers separate completely on the 1.4% agarose gels (Fig. 3). Migration of the molecules is from top to bottom. (Only the relevant portion of the gel slab is shown.) It is quite evident that agarose gel electrophoresis provides much greater resolution of ϕX174 DNA isomers than ultracentrifugation.

When the restriction enzyme Xho I became commercially available in the later stages of this work, it was used to replace the much more

expensive Pst I. Xho I cleaves ϕX174 once between nucleotides 162 and 163 (while the Pst I cleavage is between 5386 and 1). In all other significant respects Xho I acts like Pst I in producing RFIII molecules from RFI and RFII. Figure 4 shows that the RFIII molecules produced by Xho I and Pst I migrate at the same rates in the gel.

The procedure for preparing ϕX174 heteroduplexes is outlined in the Introduction and is schematically presented in Figure 1. Xho I digested ^{3}H labeled wt molecules (RFIII) was mixed with a five-fold molar excess of viral ^{3}H am3 single-stranded DNA and sufficient formamide to produce a final concentration of 50% (v/v). The mixture was heated to 60°C in order to dissociate the two strands of the wt RFIII molecules. (The melting temperature of ϕX174 RF 86°C, is lowered by 0.71°C for each percent of formamide in the solution.) Renaturation was allowed to occur at room temperature for four hours in 50 percent formamide (see Methods, section K). Since there was a five-fold excess of am3 viral (or "+") strands over wt viral strands, the wt complementary (or "-") strands released from the RFIII are more likely to reassociate with the am3 viral (+) strands than with their original wt complementary (-) strand partners. Samples of the viral DNA, the RFIII and the mixture after annealing were analyzed by agarose gel electrophoresis (see Figure 5). The single-stranded viral DNA (lane a) exhibits two bands at the ssDNA position (compare with lane d) indicating the presence of both linear and closed circular viral DNA. The unit-length linear DNA was probable derived from disrupted virus particles in the phage preparation from which the DNA was extracted.

The annealed preparation (Figure 5, lane b) displays a band at the

top of the gel that appears in neither of the two DNA preparations which were used to make it (Figure 5, lanes a and c) The new band migrates at the position of RFII as would be expected for a newly formed heteroduplex (refer to Figure 1).

The wt/am3 heteroduplex migrated at the same rate as RFII molecules. Figure 5 also shows, the heteroduplex mixture (lane b) also contains single-stranded DNA molecules and RFIII. To separate the heteroduplex from these contaminants it was sedimented through an isokinetic sucrose gradient (see Methods, section G). The gradient separates molecules on the basis of mass and conformation. At high ionic strength, single-stranded DNA collapses into a compact particle and sediments much more rapidly than the other forms. RFIII sediments slightly slower than RFII (compare Figure 2) and is unlikely to be resolved from the RFII component by this procedure. Most of the unpurified heteroduplex preparation was applied to one isokinetic sucrose gradient while 20 µl was mixed with ^{14}C labeled RFI and RFII markers and applied to a duplicate gradient. Both were centrifuged and fractionated (see Methods, section G) identically. The gradient containing the markers was collected directly onto GF/B filters which were counted in the scintillation counter(see Figure 6). The gradient with the bulk of the heteroduplex was collected into vials and 10 µl samples were subsequently removed for scintillation counting. Using the profile of counts in the marker tube, the peak of RFII heteroduplex molecules in the companion gradient (not shown) was easily identified and pooled (fractions 26-31). To evaluate the purity of the heteroduplex RFII pool, samples of the pool were analyzed by electrophoresis on the 1.4% agarose gel shown in Figure 7. When the unpurified heteroduplex preparation (lane a) is

compared with the pool after ultracentrifugation (lanes d and e) it is clear that the circular and linear single-stranded DNA contaminants are efficiently removed. A significant amount of RFIII remains in the preparation, but its presence does not interfere with any of the subsequent steps.

The RFII wt/am3 heteroduplex molecules obtained from the previous step are expected to contain one interruption in the wt "-" strand (see Figure 1). However, since the am3 viral "+" strand preparation used to make the heteroduplex contained a substantial population of unit-length linear molecules (upper band in the doublet seen in Figure 5, lane a), it is likely that many of the heteroduplex RFII contain breaks in both strands. If the interruption in a strand is merely a broken phosphodiester bond, it can be closed with the enzyme T4 DNA ligase, thereby converting the heteroduplex into an RFI. If a molecule has a "gap", a site where one or more nucleotide units are missing from a strand, ligase will be ineffective and it will remain an RFII. Since the two strands of an RFI are topologically interlocked, they cannot be dissociated from each other by heat denaturation (see Figure 8, lanes b and c). An RFII molecule, by contrast, falls apart into its component strands under the same treatment (see Figure 8, lanes b and c). Therefore the progress of ligation can be monitored by the susceptibility of the heteroduplex to heat denaturation.

The gel shown in Figure 8 shows the effect of T4 DNA ligase on the RFII heteroduplex molecules. Before ligation (lanes d and e), heating and rapid cooling completely abolishes the heteroduplex RFII band and simultaneously causes the appearance of single-stranded DNA. After

ligation (lanes f and g), a significant fraction of the heteroduplex molecules resist denaturation while all of the RFIII species in the same sample are completely dissociated. Only 10 to 15 percent of the heteroduplex RFII were closed by T4 DNA ligase. Similar results were obtained with E. coli DNA ligase.

In order to verify that the band of denaturation-resistant heteroduplex was indeed relaxed RFI we compared it with relaxed RFI prepared from natural, superhelical RFI using the DNA relaxing enzyme (DNA topoisomerase) from Agrobacterium tumefaciens (see Methods, section M). The relaxing enzyme relieves the negative supercoils in natural RFI molecules by a nicking-closing mechanism. To distinguish the product of the reaction from RFII (molecules with a permanent break in one strand) it was analyzed by electrophoresis in 1.4 percent agarose gels with 0.5 μg/ml ethidium bromide. Under these conditions supercoiled RFI, relaxed RFI, RFII, RFIII and single-stranded DNA all migrate at different rates and can be readily identified with appropriate markers.

The effect of the relaxing enzyme on supercoiled ϕX174 RFI is shown in Figure 9. Increasing amounts of the enzyme progressively convert more of the supercoiled RFI to the relaxed form. The enzyme has no effect on RFII and RFIII which are already relaxed by virtue of their permanent single- or double-strand breaks. (The unlabeled lanes in Figure 9 show the same process with RFI from the mammalian virus SV40.) The relaxed, heteroduplex RFI produced by T4 DNA ligase migrates at the same relative position as the enzymatically relaxed molecules in agarose gel with ethidium bromide (see Figure 10).

In the course of this work we have noticed that natural RF

preparation isolated from cells sometimes exhibited minor bands representing species othere than those readily attributed to RFI, II and III. The relative mobilities and intensities of these minor bands strongly suggests that they are multiple-length RF molecules. Multiple-length rings have been observed by other workers to make up a few percent of the RF opoulation extracted from infected cells (Benbow et al., 1972; Gordon et al., 1970) and are presumed to be the results of replication errors. The most populous class of multiple-length rings under normal conditions, the circular dimers, are double-stranded circular molecules which have contour lengths exactly twice that of normal RF. Like the normal monomer RFI and RFII, the double-length rings also occur in supercoiled dimer RFI (DiI) and nicked circular dimer RFII (DiII) forms. Faint bands that can be attributed to these two species are often seen in our natural RF preparations. Figure 10 shows an electrophoretic analysis of the synthetic heteroduplex relaxed RFI (lanes a and b) described earlier. The heteroduplex molecules were compared with a natural RF preparation which displays faint bands just below and just above the natural RFII band (lane e) in the agarose gel with ethidium. That these are indeed dimer RFI and dimer RFII molecules is supported by the facts that the first is stable to heat denaturation while the second is completely dissociated.

DISCUSSION

The original aim of this work was to test the ability of S1 nuclease to cleave at a single base mismatch in an otherwise double-stranded moelcule. For this purpose we constructed a ϕX174 heteroduplex molecule consisting of an am3 viral (or "+") strand and a wt complementary (or "-") strand. The molecule would have a cytosine-adenine mispair at nucleotide 587 in the ϕX174 nucleotide sequence. This would provide a rigorous test of the ability of S1 to discriminate between double- and single-stranded DNA, especially in light of the fact that A-C base-pairing does occur to a limited degree (Lilley, 1981). If the enzyme had been successful in this trial, it would have provided a relatively simple and widely applicable method for generating site-specific mutations and deletions in DNA from many sources.

The relative ease of preparing a heteroduplex molecule containing a single base mismatch makes it an ideal starting point for the construction of site-specific mutations. The techniques we employed were well characterized. The preparation of ϕX174 ^{3}H am3 viral strand DNA and ^{3}H wt RFI molecules are standard methods in many laboratories. Cleavage of the RFI with restriction enzymes, heat denaturation and renaturation in formamide have been adequate described by others.

Denaturation of DNA is usually studied under reaction conditions where elevated temperatures sometime destroy the biological activity of the molecule by causing depurination and chain scission. A particularly useful method of denaturation and renaturation is with the use of formamide. With formamide both processes can proceed close to room

temperature thereby reducing the likelihood of damage. Each percentage of formamide in the solution lowers the melting point of the DNA by 0.71°C (McConaughey et al., 1969). Since the melting point of ϕX174 RF DNA is 85°C, 50 percent formamide lowers the melting point by 36.5°C to 48.5°C. Renaturation of complementary DNA strands occurs most readily at temperatures 20 to 25°C below the melting temperature. Small molecules like ϕX174 DNA are fully renatured in less than four hours under these conditions.

Separation of the excess single-stranded DNA from the open wt/am3 heteroduplexes after renaturation by sedimentation in an isokinetic sucrose gradient is a routine procedure. Since the heteroduplex has an RFII structure at this stage, it separates well from the faster-sedimenting single-stranded DNA, but poorly from the minor RFIII contaminant.

T4 DNA ligase was selected to close the heteroduplex. We compared the relative efficiencies of T4 DNA ligase and E. coli DNA ligase. There was no appreciable difference in their abilities to close the heteroduplex RFII. Both enzymes act only on those molecules that lack individual phosphodiester bonds, but not nucleotides. The T4 enzyme requires ATP as a cofactor while the E. coli ligase uses the somewhat more expensive DNA. We assume that the enzymes could ligate no more than 15 percent of the molecules because one or more nucleotides were missing from the am3 viral ("+") strand in many cases. This is suggested by the substantial population of nicked viral molecules in our starting preparation (Figure 5, lane a)

Isolation of the closed heteroduplex from the more numerous open

heteroduplex molecules was attempted using equilibrium buoyant density centrifugation in CsCl with propidium iodide. The technique relies upon the relative densities of the open and closed forms in the presence of propidium. This intercalating dye acts in a manner that is analogous to the ethidium bromide used in gel electrophoresis. It binds avidly to double-stranded molecules unwinding the double helix and consequently, thereby making them less dense. Because of the topological constraints to unwinding, closed circular molecules (like the heteroduplex RFI) resist the intercalation of propidium. Therefore, in the same concentration of free dye the open forms will have bound more dye, unwound to a greater degree and have a lower density than the closed form.

Though the bouyant density centrifugation appeared to proceed normall, we were unable to retrieve the closed heteroduplex from the gradient in spite of several attempts. We believe that the molecules were converted back to RFII during removal of propidium from the selected fractions by chromatography throu a Dowex 50-X column. We had previously observed this problem in the preparation of natural RFI isolated from infected cells. In some cases, more than half of the RFI was degraded to RFII in the column.

It is very likely that this difficulty can be eliminated in the future by avoiding direct contact between the closed heteroduplex and the Dowex. The fractions selected from the CsCl-propidium gradient could be placed in a dialysis bad in a slurry of the resin to remove the propidium. Another solution to the problem would be to replace the entire centrifugation step with electrophoresis in a preparative agarose

gel containing ethidium bromide. The relaxed heteroduplex RFI migrates to a unique position (see Figure 10, lane d) and could be excised easily. Techniques for eluting DNA from gel pieces and removing the ethidium have been described by other workers.

The analysis of nucleic acid structure by zone electrophoresis in agarose gels has become an increasingly important method in studies requiring the separation and identification of species differing only slightly in molecular weight or conformation. We have demonstrated the existence of several circular DNA forms that appear in cells infected with the bacteriophage ϕX174. In this Discussion, we first discuss the generalization concerning the electrophoretic behavior of DNA and secondly the possible role of heteroduplexes as intermediate in ϕX replication.

Several observations have emerged as results of this study. First, the electrophoretic mobilities of RFI, II and III change with the ionic species present in the buffer. Chloride ions in the gel and gel buffer alter the migration patterns of these three species. With the presence of Cl^+ ions, the RFI and RFIII bands switch positions. The reason for this change is not clear, but the conformation and hydrodynamic properties of superhelical RFI molecules are sensitive to small changes in the ionic strength.

Preliminary experimentation in the production of RFIII molecules by the cleavage of RFI molecules followed the protocol of Brown and Smith (1976) with a few modifications (see Methods, section L). The molecular species formed by incubation with restriction enzymes were analyzed using two different methods: isokinetic sucrose gradient centrifugation and gel electrophoresis. Isokinetic sucrose gradients

separated the two major ϕX replicative species RFI and II, the supercoiled form sedimenting at 21S, and RFII, the nicked circular duplex sedimenting at 16S. RFIII molecules, which are present at much lower concentrations were not completely resolved from the RFII peak. (RFIII molecules differ only slightly from the RFII molecules in conformation and the two would be expected to sediment very closely together.) Gel electrophoresis was able to distinguish other species in addition to the major components mentioned above: (i) the linear duplex RFII (ii) relaxed closed circular RFI (iii) the nicked circular dimer, DiII, and (iv) the supercoiled dimer, DiI. The closed circular and unit-length linear single-stranded viral DNA forms were also resolved.

In the standard Tris-acetate gel buffer at pH 8.2, the single-stranded DNA is the fastest molecular species, followed by RFI, RFIII, and RFII being the slowest. The migration of the molecular species depends upon the molecular weight as well as conformation. Since single-stranded DNA is the lowest molecular weight species, it will migrate the fastest. Since RFI, II and III each have the same molecular weight, their conformations determine their rates of migration. RFI, the supercoiled closed circular DNA is the most compact species of the three, and hence will migrate the fastest. RFII, the nicked circular DNA species is relaxed and less compact, thereby greatly reducing its speed in gels. RFIII DNA has been postulated to migrate end on end during gel electrophoresis (Fisher and Dingman, 1971; Aaij and Borst, 1972; Dingman et al., 1972), an orientation that would minimize its frictional resistance. The order of these three major molecular species does not seem to change in low concentrations of ethidium bromide. However, they are altered by

changes in pH, gel concentration or voltage (Johnson and Grossman, 1976).

The intercalating dye ethidium bromide offers further control over the conditions which can be manipulated to enhance separations of DNA topoisomers. The concentration of ethidium bromide added to the agarose gel and to the gel buffer directly influences the mobility of the topoisomers (Johnson and Grossman, 1976). Equally important, it provides a simple and rapid means for determining superhelical density and for distinquishing between relaxed closed circular forms (like relaxed RFI) and the corresponding nicked molecules (like RFII) which would migrate at the same rate in the absence of ethidium.

We have noticed a series of bands which run between the supercoiled RFI and relaxed RFI (Figure 9). These are closed circular RF molecules with degrees of supercoiling intermediate between natural RFI and the completely relaxed form. The molecules in the individual bands differ from each other by the number of superhelical turns.

We have also identified the positions of dimer RFI and dimer RFII on gels with ethidium with respect to the positions of monomeric RFI and RFII. As expected for their molecular weight, they migrate much slower than their monomeric counterparts. In two cases we have observed a very faint band migrating more slowly than dimer RFI or RFII, but which appears to be stable to heat denaturation (see Figure 10, lanes e and f). We have interpreted it as trimer RFI but additional characterization would be necessary to eliminate other possibilities.

One observation seems not to agree with our understanding of DNA migration in electrophoresis. The relaxed RFI species produced either by hybridization of am3 and wt complementary strands followed by ligation

or by enzymatic relaxation of natural ϕX174 RFI migrates more rapidly than natural supercoiled RFI in gels containing ethidium. Supercoiled RFI can be understood as an unwound segment of DNA that has been closed into a circle. The result of this unwinding is to cause the molecule to twist upon itself in supercoils. In the absence of ethidium it is expected to migrate faster than relaxed RFI. Our observations confirm that expectation (see Figure 8 a and b). In the presence of ethidium we would expect both molecules to move faster, but not to exchange positions as they do (Figure 9 and Figure 10, lanes c and d). The observation remains unexplained.

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Figure 1. Schematic of Heteroduplex Formation.
The actual enzymatic conditions and purification procedures as well as the rationale for each step are given in Materials and Methods.

SCHEMATIC OF HETERODUPLEX FORMATION

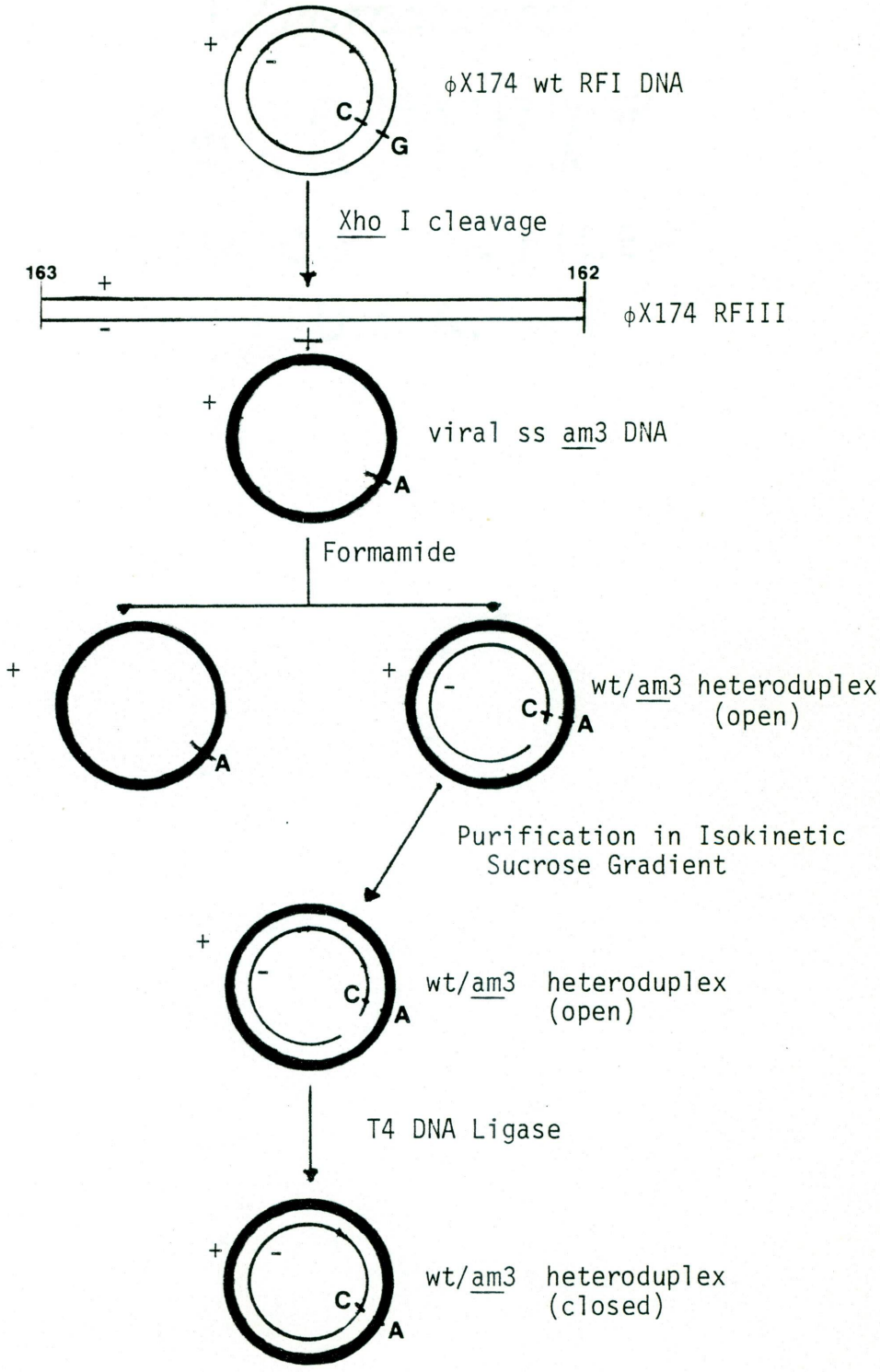
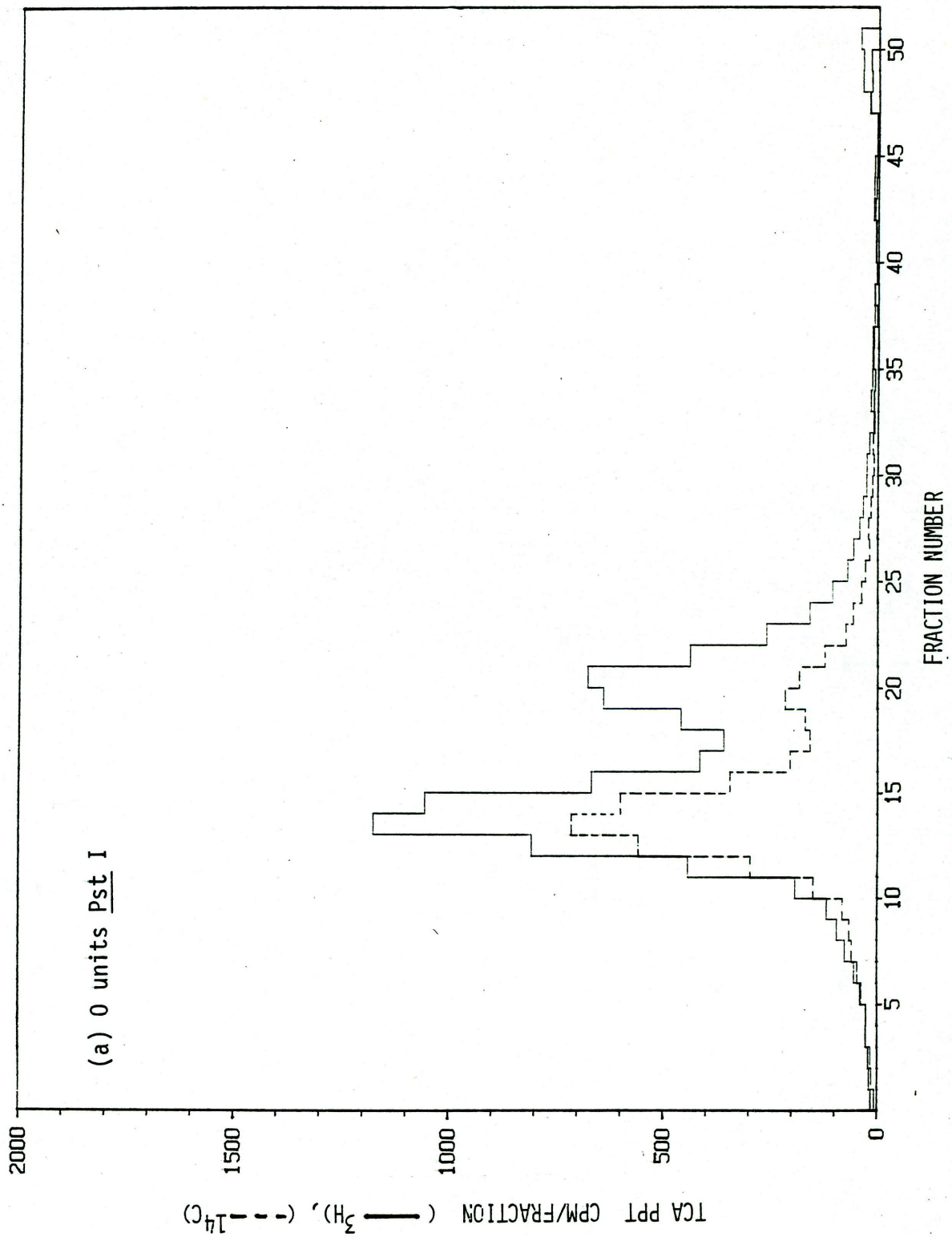
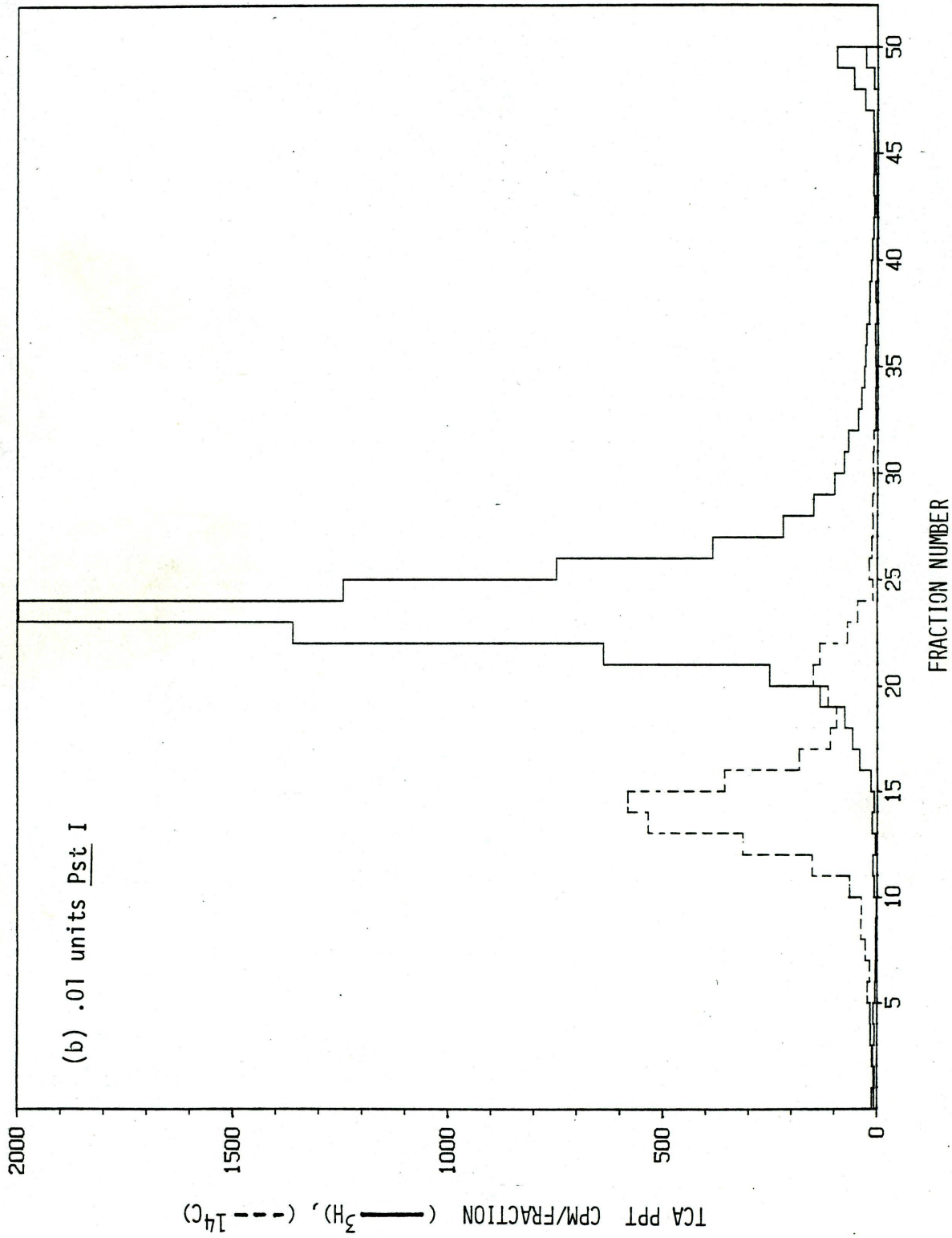


Figure 2. Separation of ϕX174 DNA molecules by Velocity Centrifugation. ϕX174 DNA forms, about 1 μg, was applied to a 4 ml isokinetic sucrose gradient in 50 mM Tris (pH 7.5), 0.95 M NaCl, 5 mM EDTA. Centrifugation was for 15 hours at 35,000 rev/min in a SW 56Ti rotor (see Methods, section G). The gradient was pierced at the bottom and 52 equal fractions were pumped out directly on GF/B filters, and were counted in the scintillation counter. (a) 1 μg ϕX RFI marker, (b) 1 μg RFI DNA incubated with 0.01 units of Pst I for 2 hours, (c) 1 μg RFI DNA incubated with 0.05 units of Pst I for 2 hours.


(a) 0 units Pst I
TCA ppt CPM/FRACTION (——— ^{3}H), (- - - ^{14}C)
2000
1500
1000
500
0
5
10
15
20
25
30
35
40
45
50
FRACTION NUMBER


(b) .01 units Pst I
TCA PPT CPM/FRACTION (——— ^{3}H), (- - - ^{14}C)
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1500
1000
500
0
5
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15
20
25
30
35
40
45
50
FRACTION NUMBER

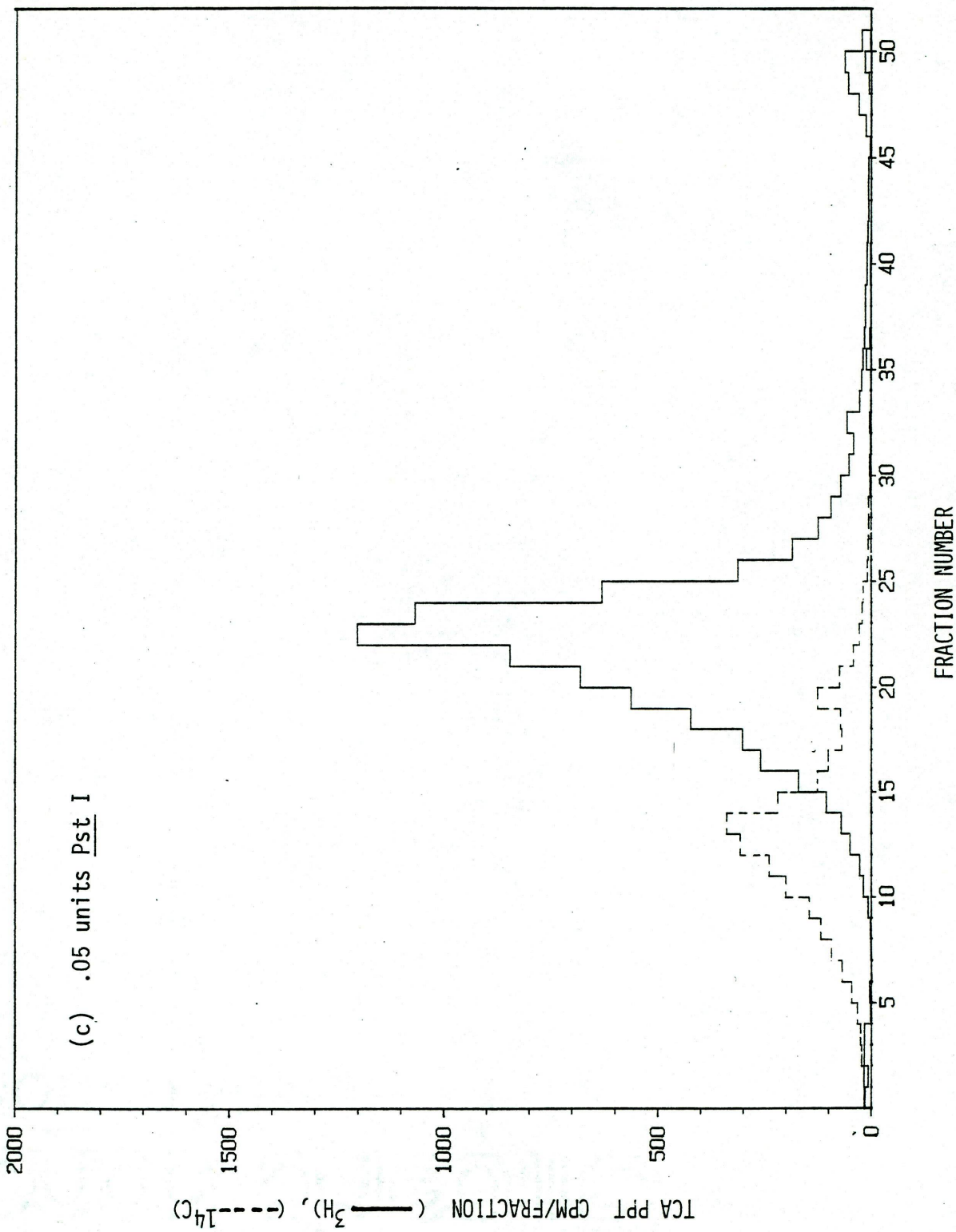

(c) .05 units Pst I
TCA ppt CPM/FRACTION (——³H), (----¹⁴C)
2000
1500
1000
500
0
5
10
15
20
25
30
35
40
45
50
FRACTION NUMBER

Figure 3. Separation of ϕX174 DNA Molecules by Gel Electrophoresis. ϕX174 DNA forms, about 1 μg, in 10% glycerol, 0.025% Bromphenol Blue, were applied in 20 μl to individual wells in a 13.5 X 28 cm X 1.5 mm 1.4% agarose gel. Electrophoresis was for 16 hours at 100 volts at 10°C. The gel was soaked in 0.1 μg/ml ethidium bromide for 1 hour and was photographed by transillumination with 300 nm UV light, using 1 min exposure, f/4.5, Polaroid Type 665 film, and a 570 nm filter on a CU-5 Industrial camera. (a) 1.0 μg of RF mixture; (b) 1.0 μg of RF mixture incubated with 0.1 units of Pst I for 30 min (c) 1.0 μg of RF mixture incubated with 0.1 units of Pst I for 1 hour (d) 1.0 μg of RF mixture incubated with 0.1 units of Pst I for 2 hours (e) 1.0 μg of RF mixture incubated with 0.1 units of Pst I for 3 hours.

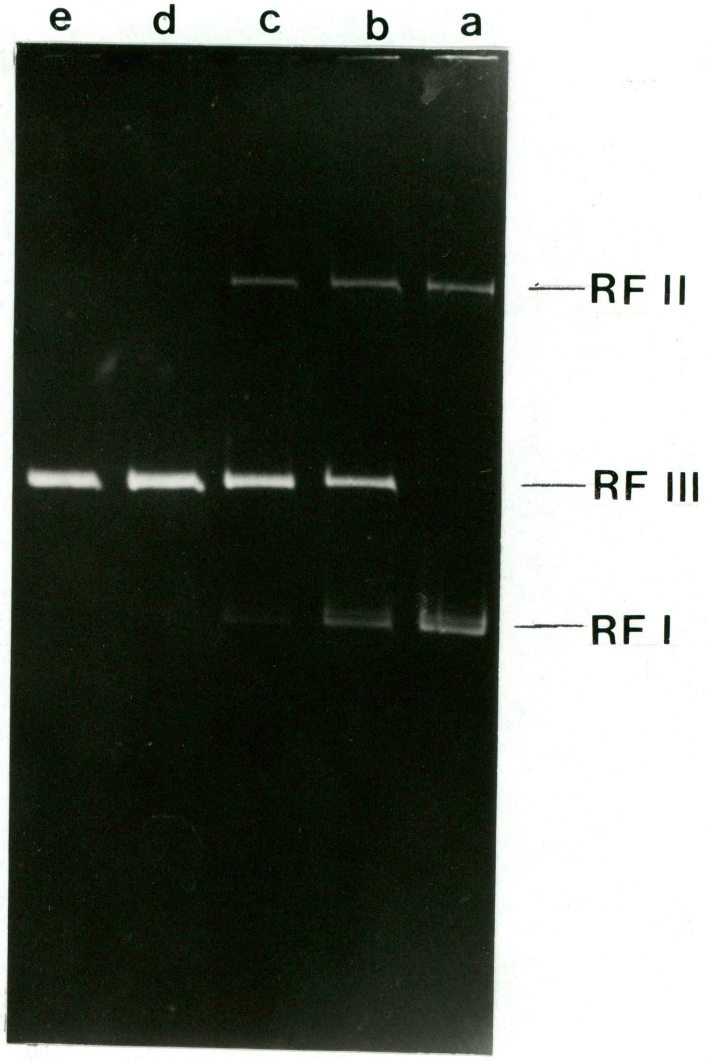

e
d
c
b
a
RF II
RF III
RF I

Figure 4. ϕX174 RFIII Generated by Xho I.
Gel electrophoresis of RF DNA treated with Xho I. Conditions of electrophoresis and visualization are under Figure 3. (a) Pst I digested RF mixture (see Methods, section L); (b) Xho I digested RF mixture; 1 μg of RF mixture incubated with 1 unit of Xho I; (c) 1 μg of RF mixture

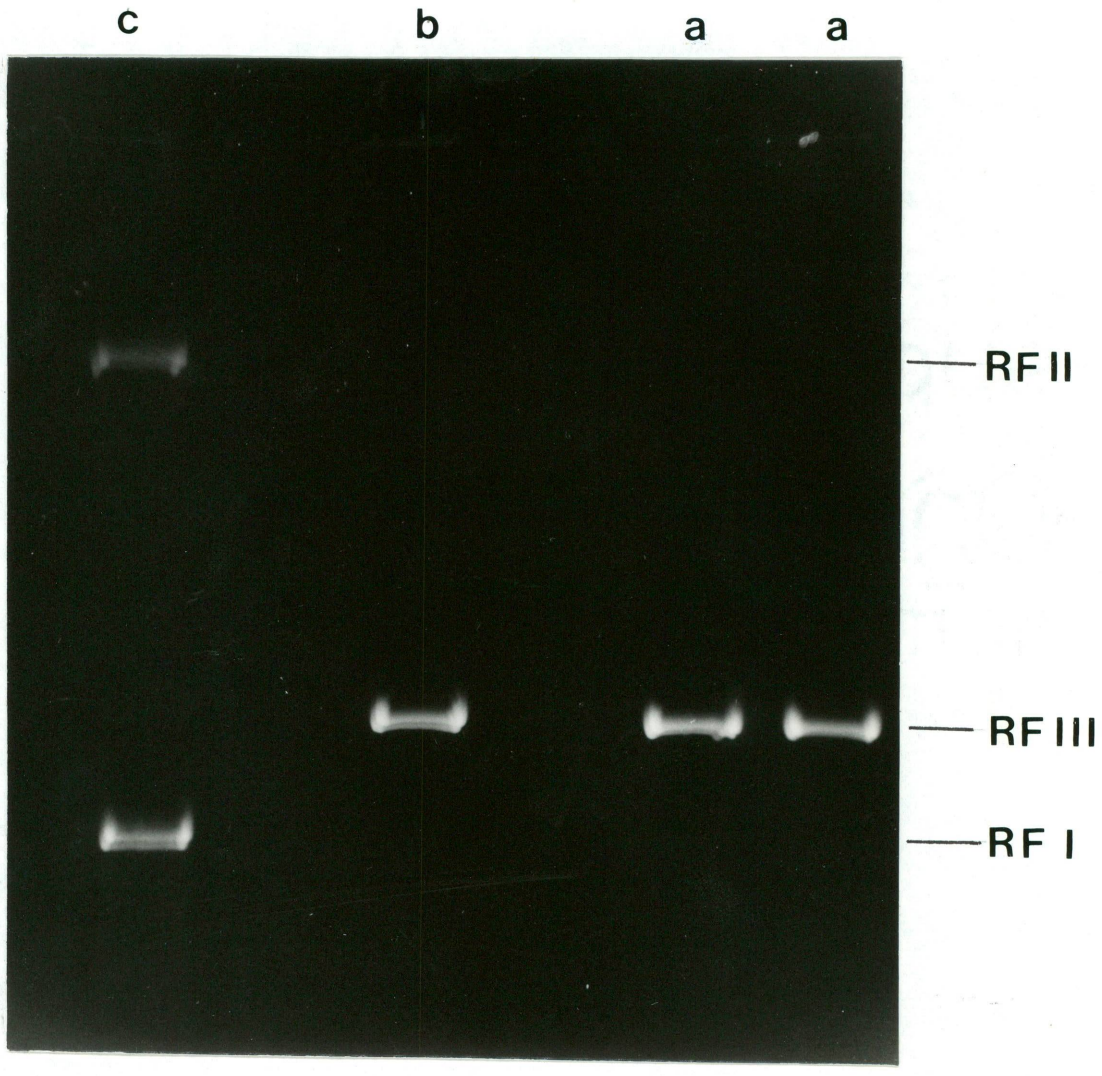

c
b
a
a
RF II
RF III
RF I

Figure 5. Formation of wt/am3 Heteroduplex RFIII.
Molecules used in the snythesis of the wt/am3 heteroduplex were analyzed on a 1.4% agarose gel. Conditions for electrophorsis are given in Figure 3. Electrophorsis was for 15 hours at 100 volts. (a) am3 SS viral DNA; (b) wt/am3 heteroduplex molecules; (c) wt RFIII DNA; (d) marder SS DNA.

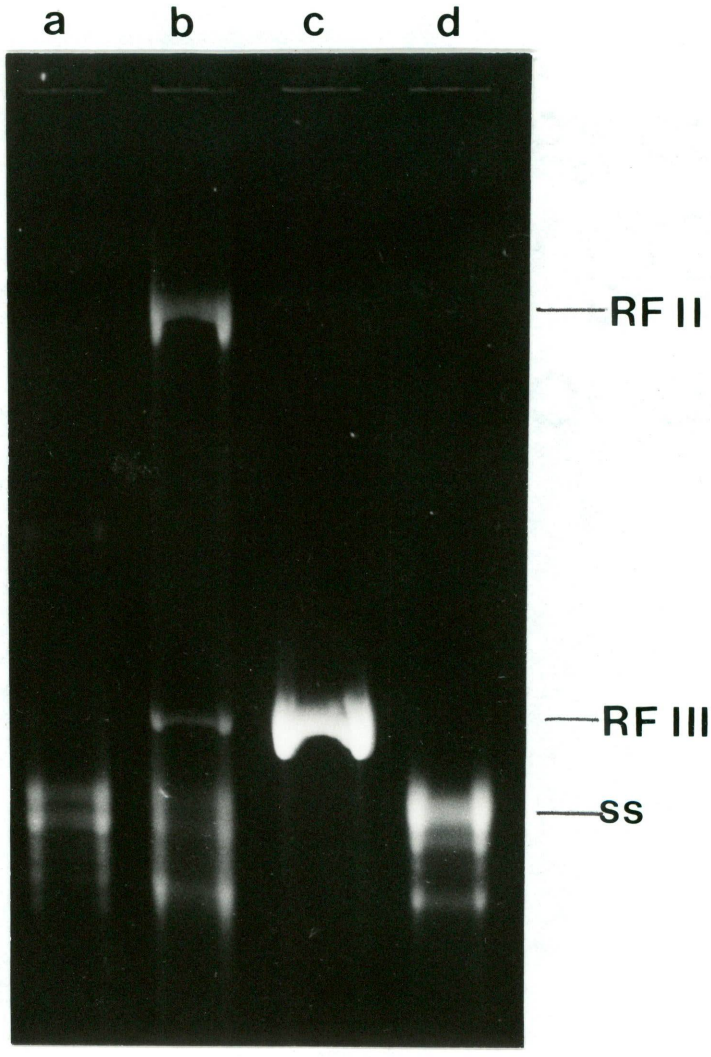

a
b
c
d
RF II
RF III
ss

Figure 6. Velocity Centrifugation of Heteroduplex Preparation.
20 μl of the heteroduplex preparation shown in Fig. 5, lane b, was applied to a 38 ml isokinetic sucrose gradient in 50 mM Tris (pH 7.5), 0.95 M NaCl, 5 mM EDTA, along with an appropriate ^{14}C RFI and RFII marker. Centrifugation was for 15 hours at 27,00 rev/min in a SW27 rotor (see Methods, section G). The gradient was pierced at the bottom and 45 equal fractions were pumped out directly onto GF/B filters. A duplicate gradient was run containing the bulk of the heteroduplex molecules. (————), ^{3}H wt/am3 heteroduplex; (--------), ^{14}C RFI and RFII marker.

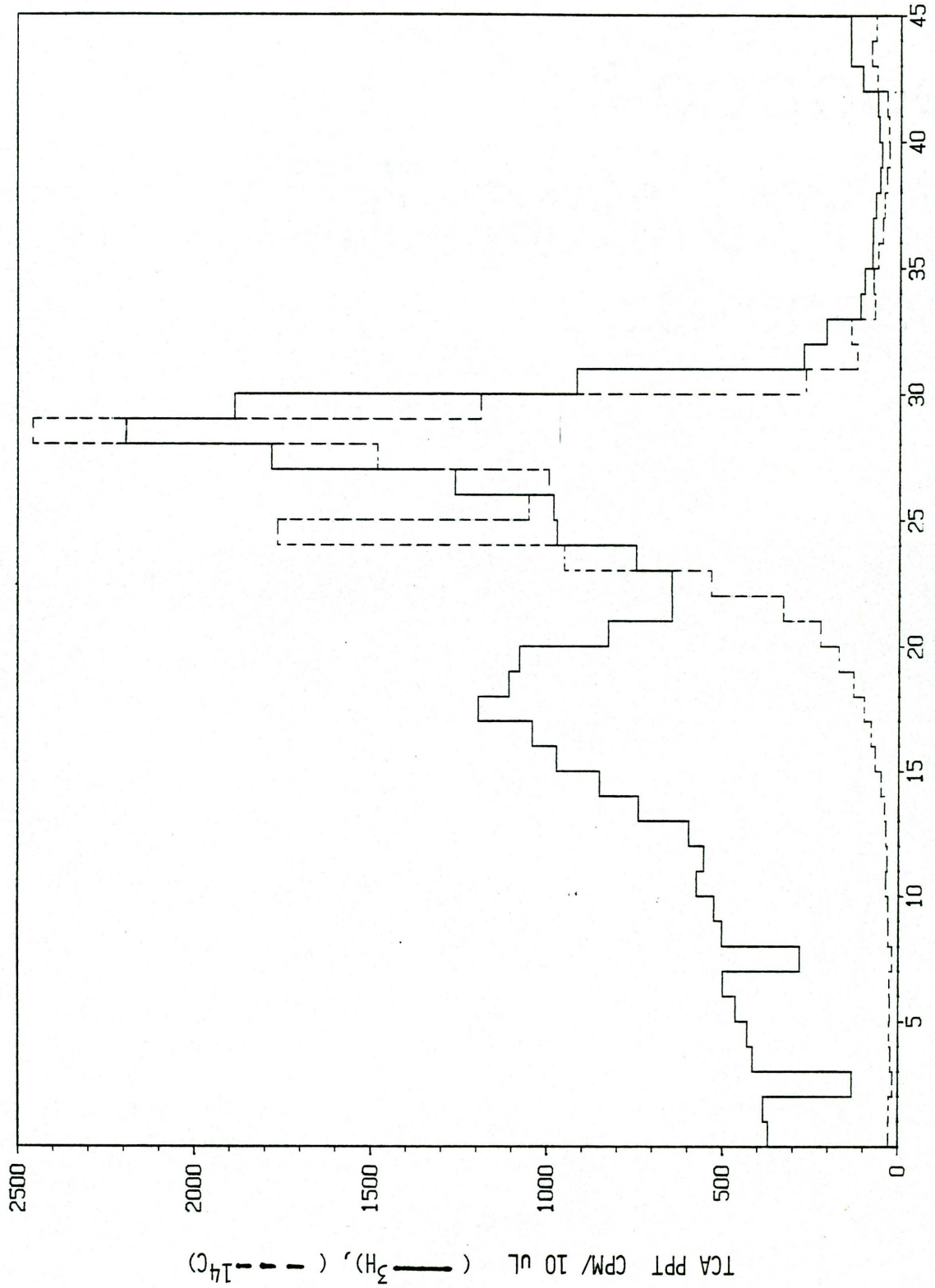

TCA PPT CPM/ 10 uL (——— ^{3}H), (- - - ^{14}C)
2500
2000
1500
1000
500
0
5
10
15
20
25
30
35
40
45
FRACTION NUMBER

Figure 7. Heteroduplex RFII after Sucrose Gradient Centrifugation. Purified wt/am3 heteroduplex obtained from the isokinetic sucrose gradient shown in Fig. 6 were analyzed by electrophoresis. The conditions of electrophoresis and visualization are described in Figure 3. (b) wt/am3 heteroduplex molecules before sedimentation; (c) RFIII marker; (d) SS DNA marker); (e) and (f) wt/am3 heteroduplex molecules after purification in the isokinetic sucrose gradient.

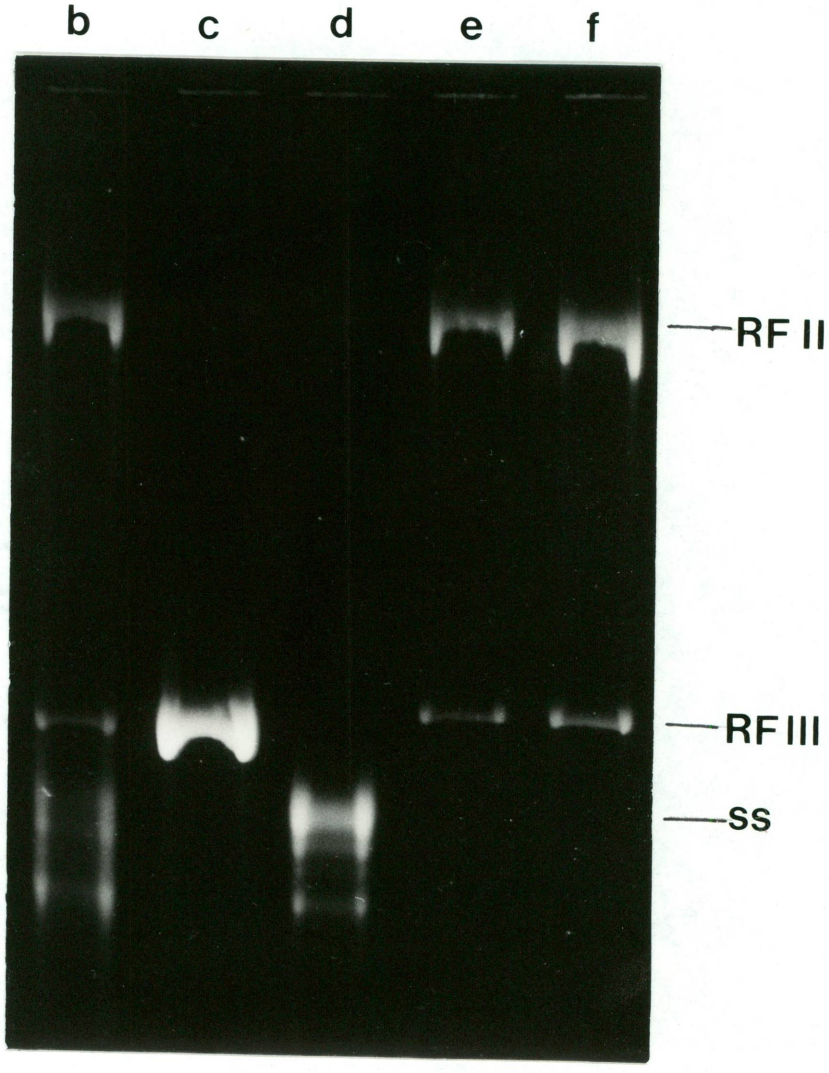

b
c
d
e
f
RF II
RF III
ss

Figure 8. Ligation of Heteroduplex RFII.
Purified wt/am3 heteroduplex molecules were closed with T4 DNA ligase (see Methods, section L). Analysis of the molecules after ligation is shown on this 1.4% agarose gel. Electrophoresis of the heteroduplex molecules followed conditions stated under Fig. 3. The gel was run at 100 volts for 20 hours at 10°C in 1X gel buffer.
(a) RFIII marker; (b) RFI and II markers, heated; (c) RFI and II marker, unheated; (d) wt/am3 heteroduplex molecules without ligation, heated; (e) wt/am3 heteroduplex molecules without ligation, unheated; (f) ligated wt/am3 molecules, heated; (g) ligated wt/am3 molecules, unheated. The approximate mass applied to each lane was 0.5 µg.

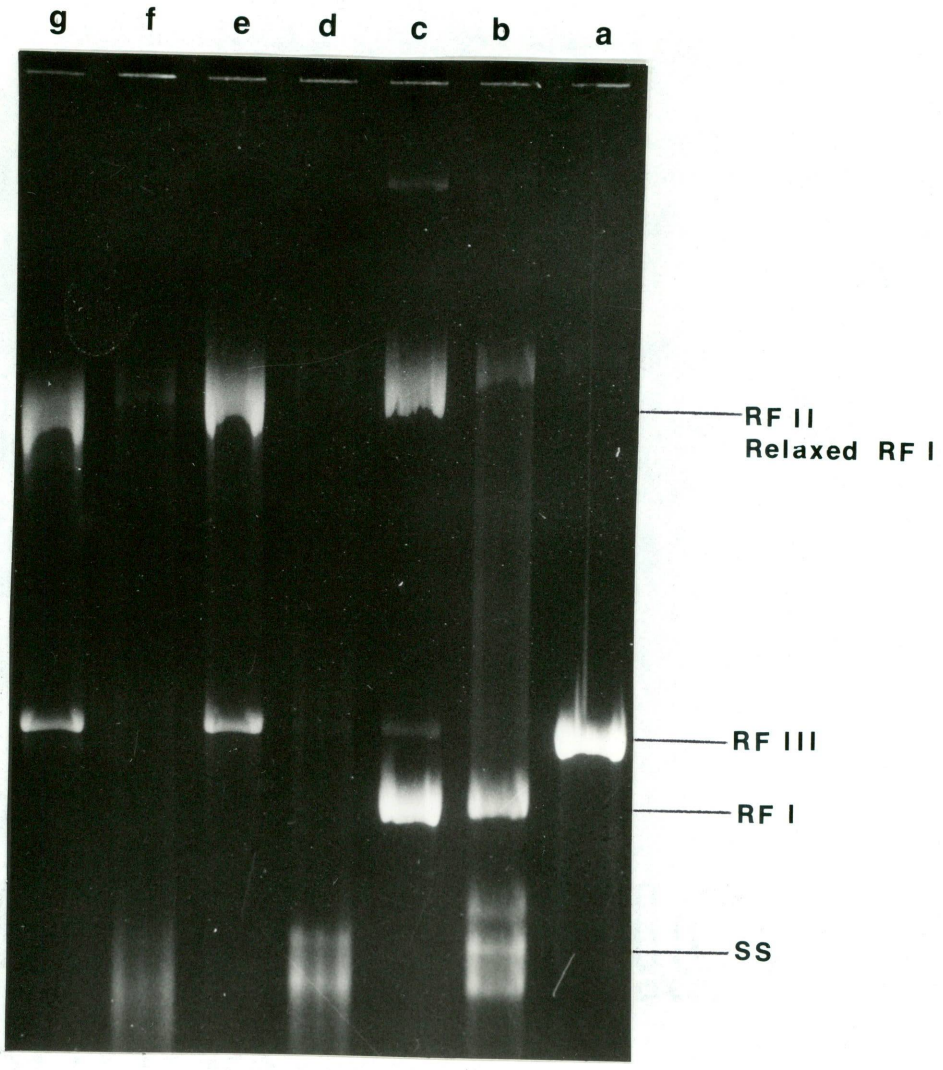

g
f
e
d
c
b
a
RF II
Relaxed RF I
RF III
RF I
SS

Figure 9. Action of Superhelical DNA Relaxing Enzyme.
ϕX174 RFI and II molecules were incubated with DNA relaxing enzyme (see Methods, section L). The relaxed molecules were analyzed on 1.4% agarose gels containing 0.5 µg/ml. Electrophoresis conditions are given under Figure 3. The gel was run for 24 hours at 100 v at 10°C. After the run, the gel was soaked in 1X gel buffer containing 0.5 µg/ml ethidium bromide for 30 min, then destained in 1X gel buffer for 45 min.
(a) 0.5 µg RFI and II mixture; (b) 0.5 µg RFI and RFII mixture incubated with 0.5 units of DNA relaxing enzyme; (c) 0.5 µg RFI and II mixture incubated with 1 unit of DNA relaxing enzyme; (d) 0.5 µg RFI and II mixture incubated with 2.5 units of DNA relaxing enzyme; (e) RFIII marker.

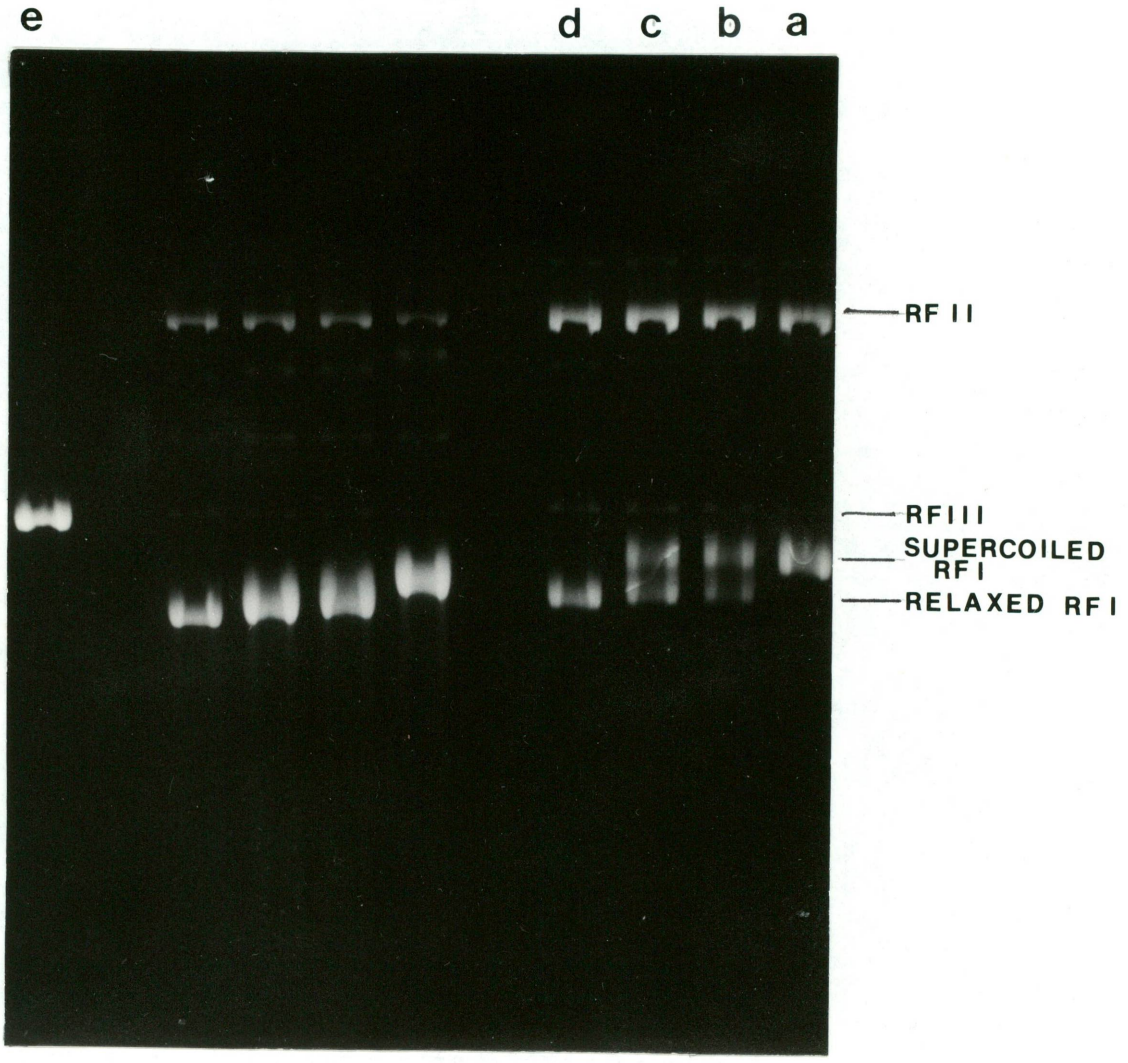
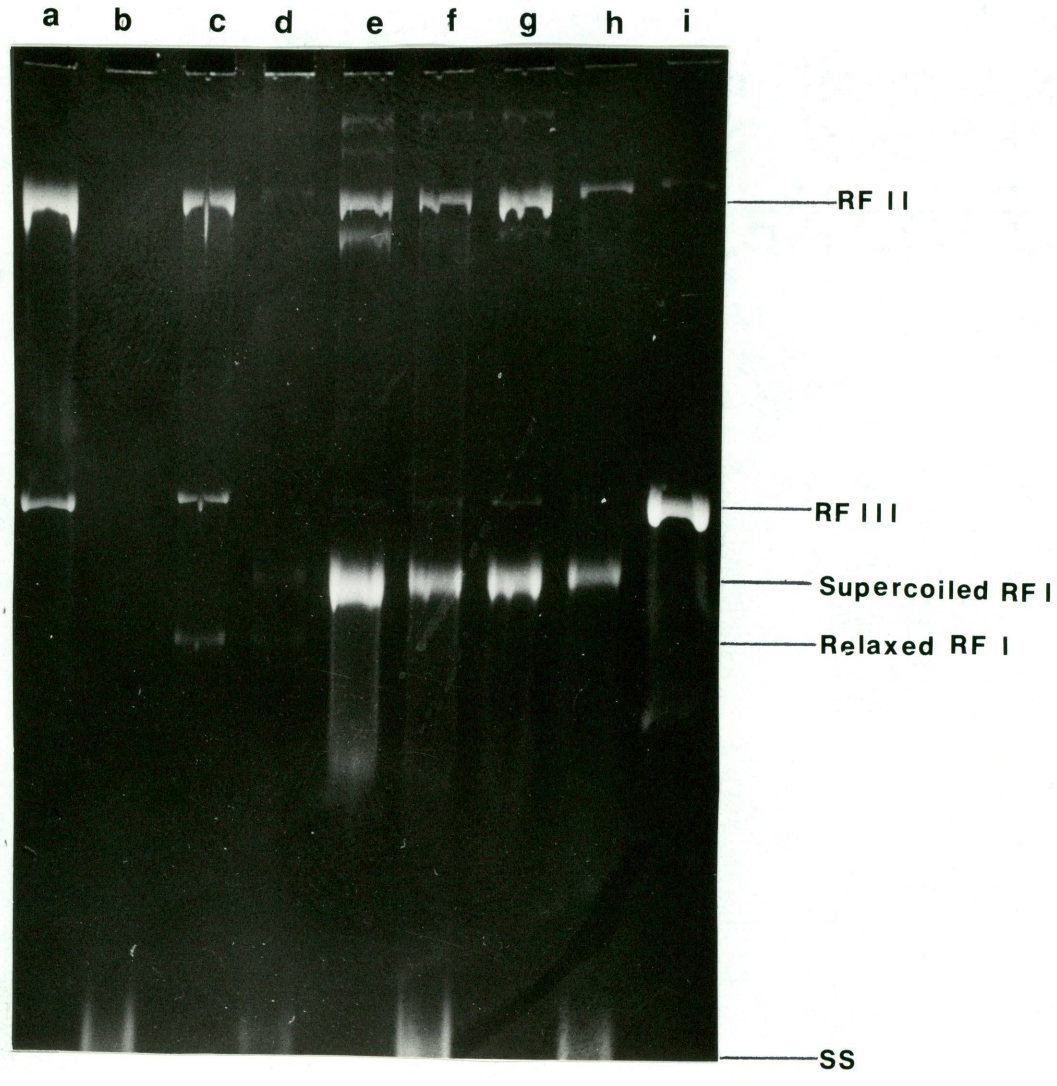

e
d
c
b
a
RF II
RF III
SUPERCOILED RF I
RELAXED RF I

Figure 10. Analysis of ϕX174 Isomers.
Various topoisomers of ϕX174 were analyzed on 1.4% agarose gels containing 0.5 μg/ml ethidium bromide. Electrophoresis conditions are given under Figure 3. The gel was run for 20 hours at 100 v at 10°C. (a) wt/am3 heteroduplex molecules (not ligated), unheated; (b) wt/am3 molecules (not ligated), heated; (c) wt/am3 molecules (ligase-treated), unheated; (d) wt/am3 molecules (ligase-treated), heated; (e) RFI and II mixture, unheated; (f) RFI and II mixture, heated; (g) RFI and II mixture, unheated; (h) RFI and II mixture, heated; (i) RFIII marker. The approximate mass of DNA layered on each lane was 1 μg.


a
b
c
d
e
f
g
h
i
RF II
RF III
Supercoiled RF I
Relaxed RF I
SS